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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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

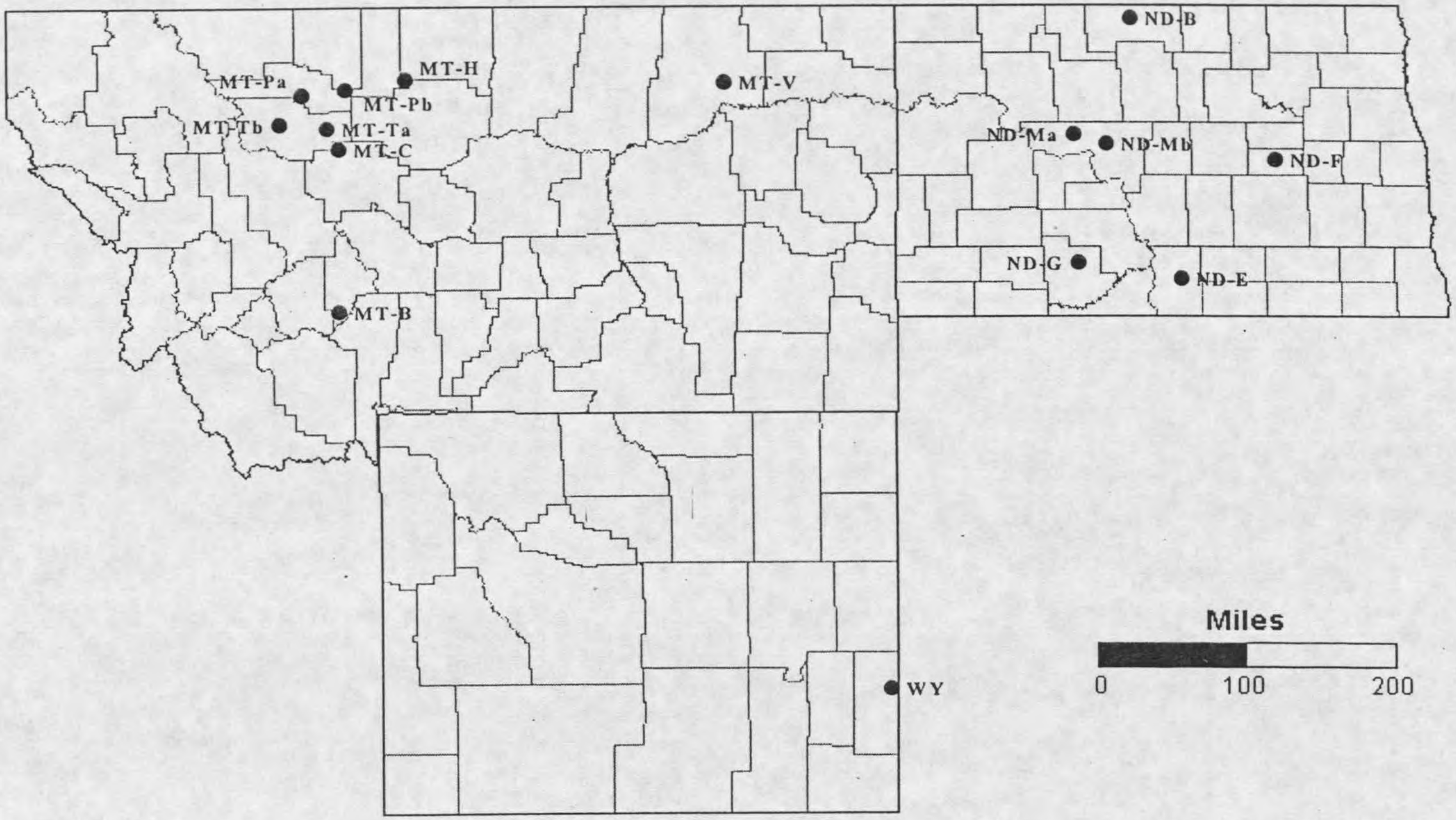

Figure 1. Collection sites of *C. cinctus* larvae sampled for RAPD-PCR studies in Montana, North Dakota and Wyoming in 1993 and 1994.

Table 1. Collection sites of wheat stubble containing overwintering *C. cinctus* larvae.

Year	State	County	Location	# of sawflies tested	Code
1993	MT	Broadwater	5N Three Forks	24	MT-B
	MT	Cascade	5S, 4E Powell	19	MT-C
	MT	Teton	1N, 4W Dutton	19	MT-Ta
1994	MT	Valley	8E Nashua	10	MT-V
	MT	Teton	Agawam (12N Choteau)	10	MT-Tb
	MT	Hill	9S, 2E Joplin	10	MT-H
	MT	Pondera	12W Conrad (A)	10	MT-Pa
	MT	Pondera	22E Conrad (B)	10	MT-Pb
	ND	Emmons	R 75W, T 135N, SECT. 21 SWSW	10	ND-E
	ND	Grant	R 86W, T 137N, SECT. 22 SWSW	10	ND-G
	ND	Mclean	R 82W, T 147N, SECT. 33 SSC	10	ND-Ma
	ND	Mclean	R 82W, T 146N, SECT. 3 NWNW	10	ND-Mb
	ND	Foster	R 67W, T 146N, SECT. 25 NNE	10	ND-F
	ND	Bottineau	R 81W, T 161N, SECT. 21 NWNN	10	ND-B
	WY	Goshen	Lingle area	10	WY

were incubated overnight at 50°C and gently extracted twice with buffered phenol, pH 8.0 (USB). DNA was precipitated by the addition of 0.2x volumes of 10M ammonium acetate and 2.0x volumes of ethanol at room temperature. Solutions were gently mixed and microfuged for 5 minutes to pellet the DNA. After removal of the supernatant, the pellets were air-dried for 5 minutes, resuspended in 20 μl of sterile distilled TE buffer, and stored at -20°C. DNA concentrations were determined at OD260 using a LKB Ultraspec 4050 spectrophotometer. Working solutions were prepared at a concentration of 10 ng/μl in sterile distilled H_2O and stored at 4°C.

Primers

Sixty-two decamer primers from Operon sets A, C, and E (plus ECO and BAM primers synthesized from sequences reported by Black et al. (1992) were screened for amplification potential. Each primer was resuspended at a concentration of 15 ng/ µl in sterile distilled H_2O and stored at -20°C. Of these primers, 31 produced well-amplified and reproducible electrophoretic bands (2 to 9 bands, 200-1900 bp in size), and 20 primers were polymorphic for the presence or absence of specific bands (Table 2). These 20 informative RAPD primers were used to analyze all *C. cinctus* individuals.

RAPD-PCR technique

Amplification reactions were performed according to a modification of the protocol recommended by Operon (1994). Since simple patterns containing two to four major bands and a minimum of minor bands were advantageous to compare bands, two annealing temperatures, 34°C and 37°C, were used. Each 25-µl reaction contained 1.5 µl $MgCl_2$, 2.5 µl buffer, 4.0 µl dNTPs, 1.0 µl primer, 2.0 µl genomic DNA, and 0.5 units of Taq DNA polymerase. Amplification was performed in a PTC-100 60-well thermocycler programmed for 1 cycle at 94°C for 4 minutes; 45 cycles at 94°C (denaturing) for 1 minute, 34°C or 37°C (annealing) for 1 minute, 72°C (extension) for 2 minutes; and 1 cycle at 72°C for 5 minutes (Ramp time from 37°C to 72°C was 30 seconds). Amplified DNA products were analysed by electrophoresis on 1.5% agarose gels in 1x Tris-borate-EDTA (TBE) buffer for 4.5 hours at 4 V/cm. Gels were stained with ethidium bromide and photographed on a ultraviolet

Table 2. Sequences of 31 primers used in RAPD analysis of sawfly genomic DNA and characteristics of their amplification products.

	Primer sequence(5'---3')	No. of bands	Size range	No. of polymorphic band
Annealing 37°C				
OPA03	AGTCAGCCAC	3	300-1900	0
OPA04	AATCGGGCTG	9	400-1500	6
OPA08	GTGACGTAGG	8	300-1500	2
OPA09	GGGTAACGCC	3	700-1100	2
OPA13	CAGCACCCAC	8	400-1800	1
OPA18	AGGTGACCGT	6	450-1700	5
OPA20	GTTGCGATCC	2	800-1000	1
OPC01	TTCGAGCCAG	4	700-1750	0
OPC02	GTGAGGCGTC	5	500-1000	1
OPC05	GATGACCGCC	6	500-1650	4
OPC06	GAACGGACTC	4	600-1700	0
OPC07	GTCCCGACGA	4	1000-1500	0
OPC09	CTCACCGTCC	4	700-1500	0
OPC12	TGTCATCCCC	5	300-1200	3
OPC14	TGCGTGCTTG	5	300-1850	0
OPC15	GACGGATCAG	7	300-1700	4
OPC16	CACACTCCAG	5	300-1700	0
OPE03	CCAGATGCAC	7	200-1900	0
OPE04	GTGACATGCC	8	400-1900	2
OPE07	AGATGCAGCC	6	300-1700	3
OPE15	ACGCACAACC	5	400-1760	1
OPE20	AACGGTGACC	5	400-1800	3
BAM	ATGGATCCGC	8	300-1600	1
ECO	ATGAATTCGC	4	500-1850	0
Annealing 34°C				
OPA01	CAGGCCCTTC	5	650-1600	0
OPA02	TGCCGAGCTG	5	500-1000	5
OPA10	GTTATCGCAG	5	600-800	3
OPC04	CCGCATCTAC	6	700-1600	6
OPC19	GTTGCCAGCC	5	350-1800	0
OPE18	GGACTGCAGA	4	300-1000	2
OPE19	ACGGCGTATG	5	800-1800	5

transilluminator. Sizes of amplified fragments were estimated by comparison with standard DNA marker pUC19 digested with PstI.

Data analysis

Using informative primers, 10-24 individuals from each collection site were analysed for DNA polymorphisms. Sixty DNA fragments from 20 polymorphic primers were scored for band presence (1) or absence (0). A data matrix was constructed in which each sawfly individual was coded by its collection site and a vector of 1s and 0s representing its RAPD multiband fingerprint. Relationships among individuals and collection sites were compared by three techniques.

Cluster analysis

From the data matrix described above, a pairwise distance matrix based on the measure 1-M was calculated, where $M = N_{ab}/N_T$, N_{ab} = total number of matches (i.e., both fragments present or absent) in individuals a and b, and N_T = total number of fragments scored. A value of M = 1 indicates that two individuals have identical fragment patterns, and a value of M = 0 indicates that two individuals do not share any fragments in common. Cluster analysis was performed using the unweighted pair group method algorithm (UPGMA) on the values of 1-M distance matrix (Felsenstein, 1994). The dissimilarity matrix calculated by RAPDPLOT was analysed with NEIGHBOR program of PHYLIP version 3.5C to construct a dendrogram using DRAWGRAM (Felsenstein, 1994). This method estimated both among-group distances (the distance among clusters) and the within-group distances (the distance between adjacent groups).

Principal coordinate analysis

To evaluate relationships among collection sites the data matrix was collapsed to a single vector per collection site representing band frequency within a collection site. Associations between the populations were visualized by principal coordinate analysis (PCOA), which was performed employing the NTSYS-pc package, version1.7 (Rohlf, 1992). The calculation was based on the band frequency across each population (Ballinger-Crabtree et al., 1992; Williams et al., 1994).

Statistical analysis by AMOVA (Analysis of Molecular Variance)

By treating each specific RAPD multiband profile as a distinct haplotype each *C. cinctus* individual was defined in terms of haplotype identity. Haplotype divergence was defined by the pairwise RAPD distances between two sawflies as estimated from the Euclidean distance of Excoffier et al. (1992), defined for RAPDs by Huff et al. (1993) as

$$E_{ij}=(\xi_{ij}^{2})=m\left(1-\frac{2m_{ij}}{2m}\right)$$

where m_{ij} was the number of bands shared by the two individuals i and j, and m was the total number of polymorphic RAPD bands. We used analysis of molecular variance (Excoffier et al., 1992) based on this matrix of Euclidean distances to partition the RAPD variation into among- and within-population components. Separate analyses were also undertaken for each region, by separately analysing the eight Montana populations and the seven North Dakota and Wyoming populations. A nonparametric permutational procedure computed the significance of variance components (Excoffier et al. 1992), using 250 permutations for all analyses.

In order to visualize the relationships among populations, another cluster analysis based on the genetic distance among populations was carried out. The pairwise RAPD distances between populations were computed from the following equation (Yeh et al., 1995): where $E_{ij(XY)}$ was the distance between individual i from population X and individual j from

$$\overline{E_{(ij)}} = \sum_{i=1}^{n_x} \sum_{j=1}^{n_y} \frac{E_{ij(xy)}}{n_x n_y}$$

population Y, as defined in the previous Equation, and n_X and n_Y were the number of individuals from population X and population Y, respectively. Pairwise comparisons of the 15 populations resulted in a matrix of average distance between populations. This matrix was used in cluster analysis with an option of UPGMA to construct a dendrogram that depicted the hierarchical structure of RAPD affinity among the populations. Significance of individual population pairwise comparisons was evaluated using Bartlett's test for homogeneity of RAPD variance (Yeh et al., 1995).

IV. RESULTS

DNA Microextraction

Extracting genomic DNA from individual *C. cinctus* by the method described above gave reproducible results. DNA was successfully extracted from fresh larvae and from larvae frozen in liquid nitrogen and stored at ultra-low temperatures. DNA was also extracted from *C. cinctus* adults although DNA concentrations were lower and more variable. Amplification results were routinely repeatable, even after the stock DNA was stored at -20°C for more than one year.

Preliminary Studies

In order to verify whether RAPD-PCR could distinguish different *C. cinctus* individuals, band patterns were compared between sawflies collected in Montana and Syria. Obvious band differences were evident within and among the two *C. cinctus* populations using primer OPE15 (Fig. 2). The Syrian population showed much more variation than the Montana population (Fig. 2). This result demonstrated that RAPD-PCR could be used to distinguish the differences among different geographical collections of wheat stem sawflies.

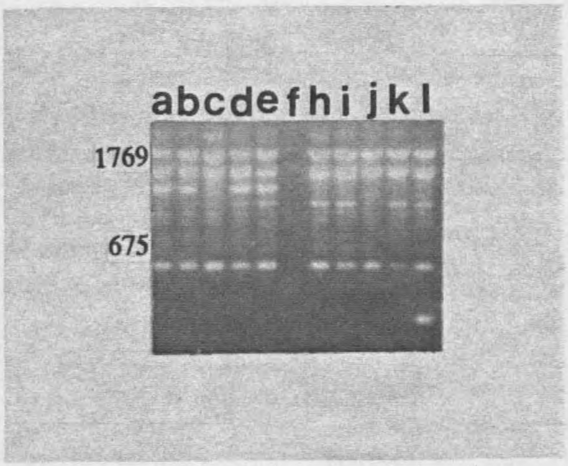

Figure 2. RAPD band profiles of *C. cinctus* amplified with primer OPE15 in Montana (lanes a - e) and Syrian (lanes f - j) populations.

Many of the primers screened produced identical DNA band patterns among all populations and were therefore not useful to differentiate populations. Because all populations were collected from a somewhat limited total geographic area, the populations were expected to produce similar banding patterns for many of the primers. Differences in DNA band profiles within populations were detected with many primers because of within-population polymorphism. Only clearly identifiable bands were included in the analysis.

Sixty-two primers were screened for potential use in sawfly DNA studies. Our results indicate large differences among primers for amplification. Thirty-one primers produce reproducible banding patterns at annealing temperature of 37°C or 34°C, while the other 31 primers did not amplify well. Among the 31 primers that amplified reproducible products, 20 (65%) showed polymorphic products. These 20 primers amplified 166 bands, 60 (58%) of which were polymorphic (Table 2). Primers produced from two to nine bands averaging 5.2

bands. Examples of RAPD profiles resulting from primers OPC05 are shown in Figure 3.

Preliminary studies based on 1993 collections of 10 larvae each from Broadwater (MT-B), Cascade (MT-C), and Teton (MT-Ta) counties of Montana indicated that no sex specific banding differences could be detected among male and female pupae collected from the same sites (Appendix Table 6). Subsequent studies did not classify individuals by gender. Another preliminary study indicated that a family of larval progeny from an unmated, caged sawfly female (haploid progeny) exhibited less genetic diversity as indicated by RAPD-PCR than the MT-B source population (Appendix Tables 7 and 8). These results, although expected and nonconclusive, indicated the technique has utility in estimation of genetic relationships.

RAPD Band Frequency

RAPD band frequencies in populations collected from each of the 15 collection sites are shown in Table 3. Of 60 bands which were scored only 8 (13%) were polymorphic in all populations. There was a single case where a rare band (A18-4) was restricted to a single population (ND-F). Several bands provided good discrimination among Montana and North Dakota populations, e.g. E04-1, E18-1, C05-1, C05-2, C15-3, and A08-2. Some bands were variable in one region but absent in the other, e.g. BAM-1, E18-2, C05-4, C04-5, C04-6. Still other bands were fixed or nearly fixed in single population or groups of geographically-related populations, e.g. A02-1 (MT-V), A04-1 (ND sites), A20-1 (ND sites), E18-1 (ND sites),

Table 3. RAPD band frequencies of wheat stem sawfly in 15 geographically-dispersed collection sites.

	RAPD band											
SITE*	A13-1	C02-1	E15-1	A09-1	A09-2	A02-1	A02-2	A02-3	A02-4	A02-5	A04-1	A04-2
MT-Tb	0.7	0.5	0.4	0.9	0.6	0.8	0.7	0.1	0	0	0.6	1
MT-Ta	0.53	0.47	0.47	0.95	0.47	0.74	0.79	0	0	0.11	0.58	1
MT-Pa	0.4	0.5	0.6	0.8	0.3	0.4	0.7	0	0	0	0.8	1
MT-H	0.6	0.5	0.7	1	0.1	0.7	0.9	0.1	0	0.1	0.6	1
MT-V	0.5	0.7	0.3	0.6	0.5	1	1	0	0	0	0.9	0.9
MT-C	0.74	0.58	0.74	0.84	0.37	0.47	0.84	0	0.16	0.16	0.37	0.79
MT-B	0.79	0.63	0.75	0.63	0.58	0.67	0.71	0	0	0.21	0.54	0.79
MT-Pb	0.5	0.7	0.7	1	0.4	0.5	0.8	0	0	0.1	0.6	1
ND-Ma	0.6	0.4	0.6	0.6	1	0.7	1	0	0	0.2	1	1
ND-Mb	0.4	0.8	0.7	0.6	1	0.7	0.9	0.1	0.1	0	1	1
ND-B	0.6	0.6	0.7	0.5	0.9	0.9	0.8	0	0	0.3	1	1
ND-E	0.7	0.6	0.7	0.5	0.7	0.9	0.7	0	0	0	1	1
ND-F	0.6	0.7	0.5	0.4	0.7	0.9	0.9	0.1	0.1	0.1	0.9	0.9
ND-G	0.8	0.5	0.7	0.5	0.8	0.7	0.9	0	0	0	0.9	0.9
WY	0.8	0.3	0.6	0.8	1	0.8	1	0	0	0	1	0.9

*: See Table1 for specific site descriptions.

Table 3. RAPD band frequencies of wheat stem sawfly in 15 geographically-dispersed collection sites (cont.)

	RAPD band											
SITE*	A04-3	A04-4	A04-5	A04-6	A10-1	A10-2	A10-3	BAM-1	E04-1	E04-2	A20-1	A18-1
MT-Tb	0	0.1	0.1	0.2	0.2	0.8	0	0	1	0.8	1	1
MT-Ta	0.05	0.16	0	0.05	0.79	0.32	0.84	0	1	0.84	0.47	0.95
MT-Pa	0.1	0.1	0.1	0.1	0.8	0.2	0.2	0	0.9	0.9	0.7	1
MT-H	0	0.1	0	0	0.5	0.5	0	0	1	1	1	1
MT-V	0	0.2	0.1	0	0.6	0.4	0	0	0.9	0.9	1	1
MT-C	0.21	0.11	0.16	0	0.21	0.74	0.42	0	0.95	0.84	0.16	0.89
MT-B	0.13	0.04	0.08	0.04	0.79	0.21	0.79	0	0.79	0.67	0.33	1
MT-Pb	0.1	0	0.1	0	0.7	0.4	0.1	0	1	0.9	0.7	1
ND-Ma	0	0	0	0	0.6	0.2	0	0	0.4	0.7	1	0.8
ND-Mb	0	0	0	0	0.6	0.4	0	0.1	0.5	0.9	1	0.9
ND-B	0	0	0	0	0.9	0.1	0.1	0	0.3	0.7	1	0.9
ND-E	0	0	0	0	0.9	0.1	0	0	0.2	0.8	1	1
ND-F	0.1	0.1	0.1	0.1	0.5	0.5	0	0.2	0.6	1	1	0.8
ND-G	0	0	0	0	0.7	0.3	0	0	0.2	0.8	1	1
WY	0	0	0.1	0	0.8	0.4	0	0.4	1	0.5	1	0.9

*: See Table1 for specific site descriptions.

Table 3. RAPD band frequencies of wheat stem sawfly in 15 geographically-dispersed collection sites (cont.)

	RAPD band											
SITE*	A18-2	A18-3	A18-4	A18-5	E18-1	E18-2	E20-1	E20-2	E20-3	C12-1	C12-2	C12-3
MT-Tb	0.4	0	0	0	0	0.1	0	0	0.1	0	0	0.2
MT-Ta	0.37	0	0	0.11	0	0.16	0	0.05	0.05	0	0.32	0.74
MT-Pa	0.3	0	0	0	0.2	0.1	0	0	0	0	0.4	1
MT-H	0	0	0	0	0	0	0	0	0.1	0	0.2	0.1
MT-V	0.1	0	0	0	0	0	0	0	0	0	0.2	0.2
MT-C	0.21	0	0	0.11	0	0.21	0	0	0.05	0	0.42	0.58
MT-B	0.33	0.04	0	0.25	0	0.29	0	0	0	0.04	0.25	0.54
MT-Pb	0.2	0	0	0	0	0.1	0.1	0	0	0.2	0.7	1
ND-Ma	0.3	0	0	0	1	0	0	0.4	0	0	0.8	0.1
ND-Mb	0.3	0	0	0	1	0	0	0.2	0	0	0.7	0.2
ND-B	0.1	0	0	0	1	0	0	0.5	0	0	0.4	0
ND-E	0.1	0	0	0	1	0	0.1	0.4	0	0	0.5	0
ND-F	0.4	0.1	0.1	0.1	1	0	0	0	0	0	0.6	0
ND-G	0.2	0	0	0	1	0	0	0.3	0.1	0	0.2	0.2
WY	0.4	0	0	0	0.6	0	0	0.3	0	0.1	0.4	0

*: See Table1 for specific site descriptions.

Table 3. RAPD band frequencies of wheat stem sawfly in 15 geographically-dispersed collection sites (cont.)

	RAPD band											
SITE*	C05-1	C05-2	C05-3	C05-4	C15-1	C15-2	C15-3	C15-4	E19-1	E19-2	E19-3	E19-4
MT-Tb	0	1	0.1	0	0	0.5	1	0	0.9	0.7	1	0.8
MT-Ta	0.42	0.68	0.11	0	0.05	0.37	1	0	0.79	0.74	0.21	0.32
MT-Pa	0.3	0.9	0	0	0	0.5	1	0	1	0.8	0.2	0.5
MT-H	0	1	0	0	0.1	0.3	1	0	0.9	0.9	0.8	0.9
MT-V	0	1	0.1	0	0	0.3	1	0	0.7	0.9	0.8	0.8
MT-C	0.26	0.63	0.32	0	0	0.32	1	0	0.68	0.84	0.21	0.53
MT-B	0.08	0.83	0.25	0	0	0.42	0.96	0.04	0.79	0.83	0.71	0.42
MT-Pb	0	0.7	0.2	0	0	0.5	1	0	0.8	0.8	0.2	0.3
ND-Ma	0.9	0	0	0.1	0.3	0	0.7	0.1	1	0.5	0.5	0.8
ND-Mb	0.9	0.3	0	0.3	0.4	0	0.4	0.1	0.9	0.6	0.4	0.8
ND-B	0.9	0	0	0	0.3	0.1	0.1	0	0.9	0.5	0.5	0.5
ND-E	1	0.1	0	0	0.1	0.1	0.4	0.2	0.9	0.6	0.5	0.6
ND-F	1	0	0	0.1	0.2	0	0.5	0.1	1	0.6	0.5	0.7
ND-G	0.9	0.2	0	0.1	0.5	0.1	0.4	0.2	1	0.5	0.5	0.5
WY	0.8	0.3	0.1	0	0.4	0.1	0.4	0	1	0.6	0.5	0.7

*: See Table1 for specific site descriptions.

Table 3. RAPD band frequencies of wheat stem sawfly in 15 geographically-dispersed collection sites (cont.)

SITE*	RAPD band E19-5	C04-1	C04-2	C04-3	C04-4	C04-5	C04-6	E07-1	E07-2	E07-3	A08-1	A08-2
MT-Tb	0.8	0.2	0	0.9	0.8	0	0	0.1	0.1	0	0.3	1
MT-Ta	0.32	0.58	0.21	0.37	1	0.05	0.16	0.21	0.53	0	0	0.63
MT-Pa	0.5	0.2	0.3	0.6	0.9	0	0	0.4	0.9	0	0	1
MT-H	0.8	0.4	0	1	0.9	0	0	0.5	0	0	0.2	1
MT-V	0.7	0	0.1	1	0.4	0	0	0.5	0.2	0	0	1
MT-C	0.21	0.05	0.47	0.32	0.58	0	0.11	0.11	0.32	0	0	0.84
MT-B	0.38	0.08	0.17	0.33	0.54	0.13	0	0.13	0.33	0	0	0.75
MT-Pb	0	0.3	0	0.5	1	0.1	0	0.3	0.8	0.1	0	1
ND-Ma	0.4	0.4	0	0.7	0.5	0	0	0.2	0.3	0.1	0.2	0.2
ND-Mb	0.5	0.5	0.1	0.9	0.5	0	0	0.4	0.5	0	0.2	0.1
ND-B	0.5	0.2	0.3	0.8	0.5	0	0	0.4	0.2	0	0	0
ND-E	0.5	0.7	0.1	0.6	0.9	0	0	0.2	0.6	0	0.2	0.3
ND-F	0.6	0.4	0.1	0.8	0.5	0	0	0.2	0.5	0.1	0.3	0.3
ND-G	0.5	0.5	0	0.4	0.5	0	0	0.4	0.2	0.1	0.2	0.2
WY	0.5	0.3	0.2	0.8	0.4	0	0	0.1	0.7	0	0.2	0.3

*: See Table1 for specific site descriptions.

C12-3 (MT-Pa and MT-Pb), C15-3 (MT sites), E19-3 (MT-Tb). Because relatively few bands exhibited fixed differences among regions and among populations within regions, RAPD divergence among natural populations of *C. cinctus* in Montana and North Dakota is based, in large part, on frequency differences of variable RAPD markers.

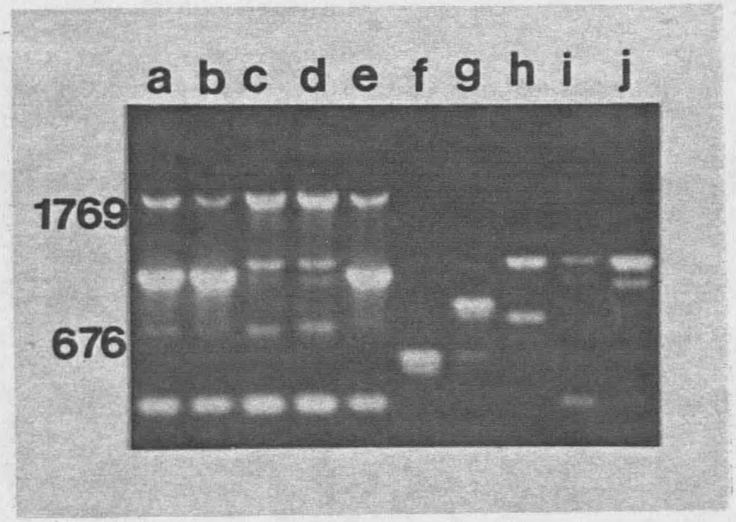

Figure 3. PCR amplification of DNA from 10 individual *C. cinctus* larvae collected from two geographically-separated collection sites using random primer C05. Lanes a - e: ND-E population; lane f: negative control, PCR reaction with all reagents except *C. cinctus* DNA; lanes h - l: MT-B population. Numbers at left margin denote approximate fragment size (bp).

Cluster Analysis

A dendrogram was constructed based on the similarity matrix using UPGMA (Fig. 4) (for more detailed relationships among individuals, see text file, Appendix Table 9). This dendrogram showed a high degree of within-site variability among *C. cinctus* individuals using RAPD-PCR since all individuals from common collection sites did not group together. Variability was observed among *C. cinctus* collection regions. All sawflies collected in

Figure 4. UPGMA cluster analysis of 186 *C. cinctus* individuals collected from 15 sites based on genetic polymorphism (1-M) estimated from 60 RAPD loci from 20 primers. Arrow denotes their separation. The 70 individuals below the arrow were collected from six North Dakota sites and one Wyoming site; the others were collected from eight sites in Montana.

Montana clustered separately from those collected in North Dakota and Wyoming (Fig. 4).

In addition to the combined data set, data subsets from the Montana and North Dakota (including Wyoming) collection sites were analysed separately (Fig. 5 and Fig. 6) (see also text files for MT populations, Appendix Tables 10). The results were similar to the combined data set. Because of within-population variation, not all individuals from the same site clustered into the same group.

Although the cluster analysis using RAPDPLOT demonstrated relatively large within-site RAPD diversity and good evidence of genetic divergence of wheat stem sawfly in the Montana and North Dakota regions, the technique provided no statistical differentiation of among- and within-population RAPD variation and relatively little information regarding the population structure of *C. cinctus*.

Principal Coordinate Analysis of RAPD Data

Relationships between populations based on band frequencies were also visualized by principal coordinate analysis (Fig. 7). Variation among populations was expressed in three dimensions. The greater the distance between two populations in the X, Y, or Z planes, the more variation they represent. On the basis of this analysis, the fifteen populations examined could be clearly subdivided into three separate groups, similar to results from the cluster analysis. Montana populations formed two distinct groups based on the X, Y plane, one grouping containing Agawam (MT-Tb), Nashua (MT-V), and Joplin (MT-H) populations and the other the Three Forks (MT-B), Power (MT-C), Dutton (MT-Ta), and two Conrad (MT-

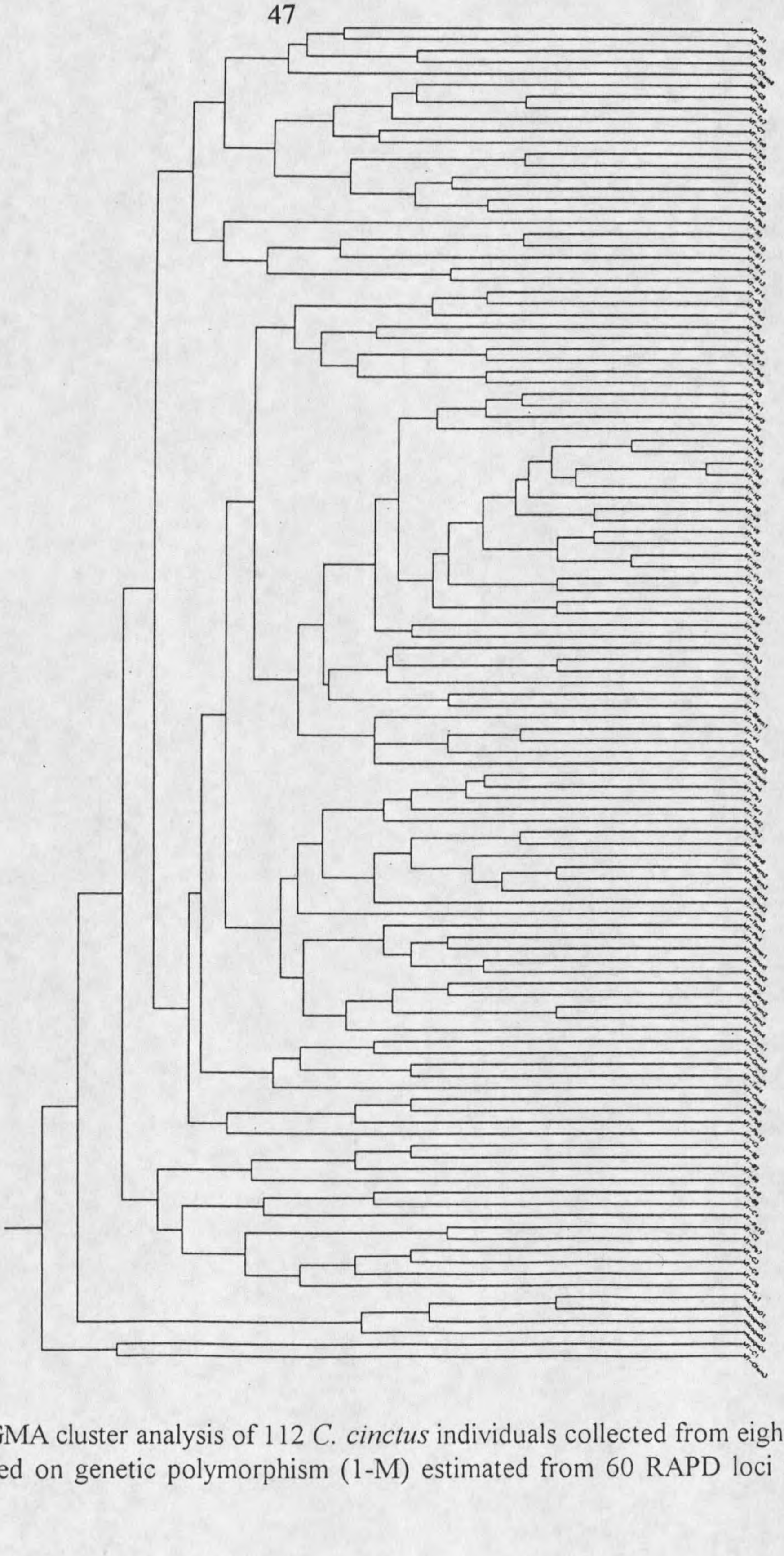

Figure 5. UPGMA cluster analysis of 112 *C. cinctus* individuals collected from eight sites in Montana based on genetic polymorphism (1-M) estimated from 60 RAPD loci from 20 primers.

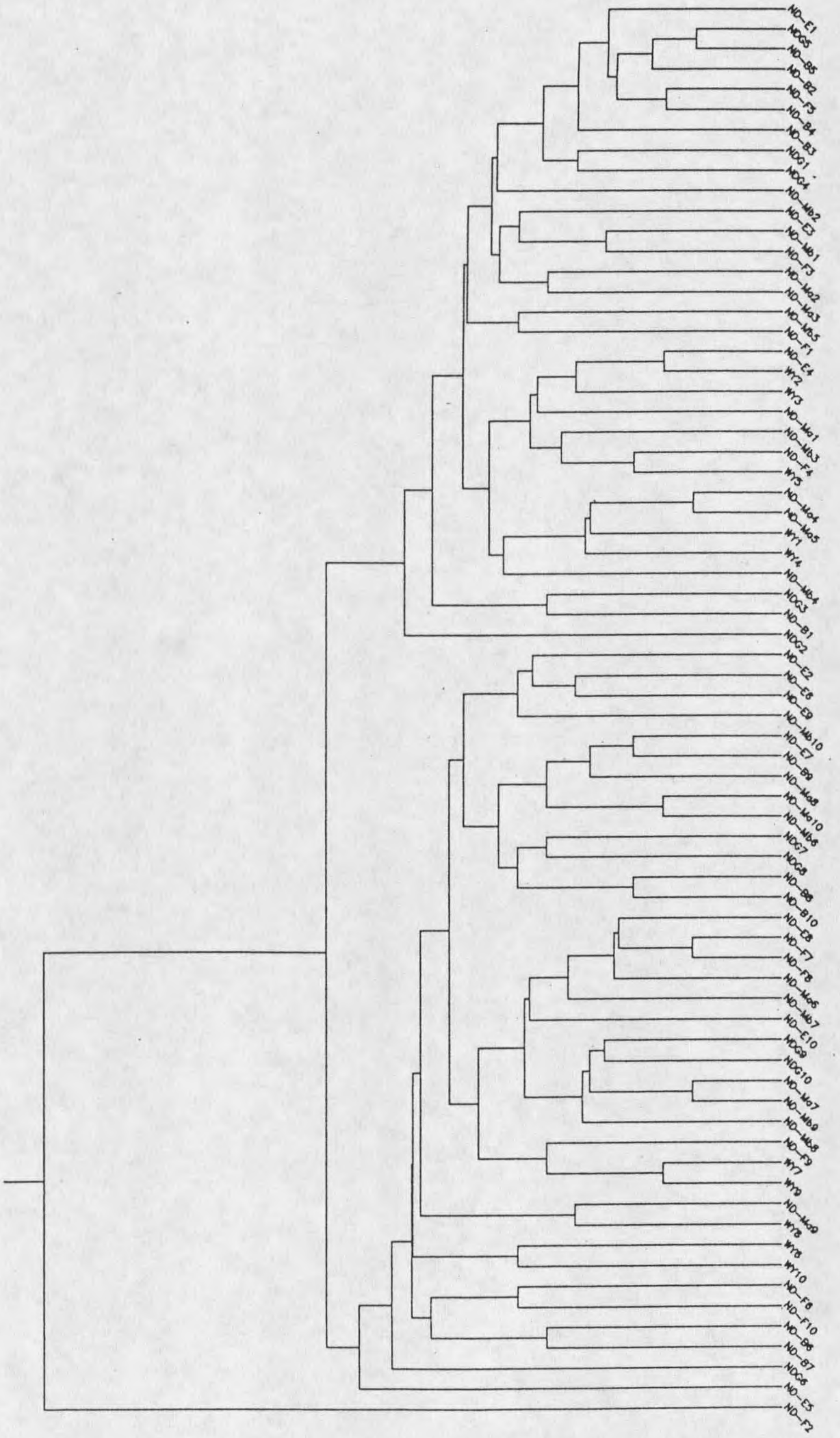

Figure 6. UPGMA cluster analysis of 70 *C. cinctus* individuals collected from six sites in North Dakota and one site in Wyoming based on genetic polymorphism (1-M) estimated from 60 RAPD loci from 20 primer.

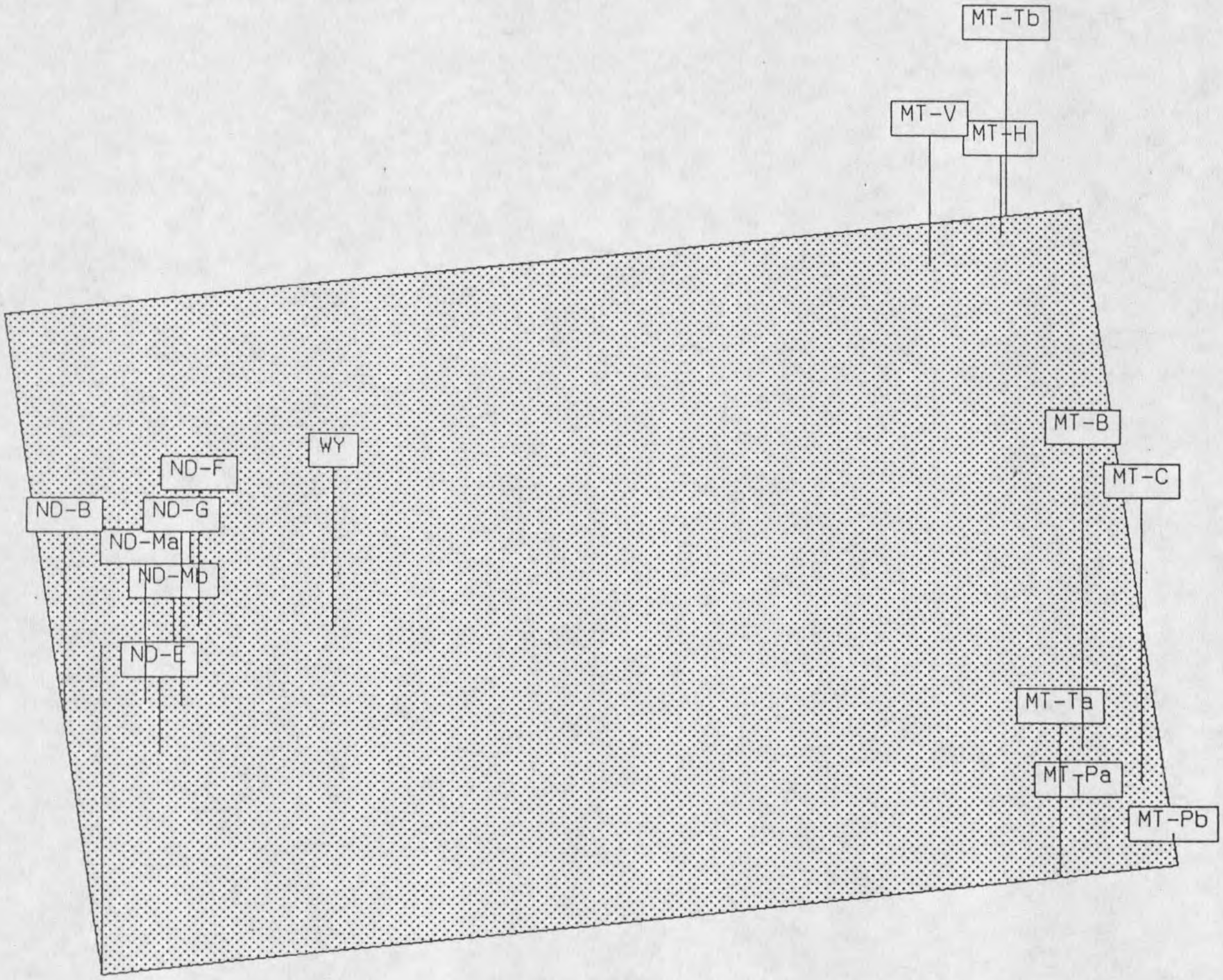

Figure 7. Three-dimensional model based on a principal coordinate analysis of RAPD band frequency within 15 geographically-dispersed populations of *C. cinctus.*

Pa and MT-Pb) populations. The latter group exhibited diversity in the Z dimension with the Conrad populations being distinctly different from the Power (MT-C) and Three Forks (MT-B) populations. All North Dakota populations were tightly grouped and distinctly different from Montana populations. The Wyoming population was intermediate, but much closer to the North Dakota cluster, suggesting a closer relationship to North Dakota populations.

Analysis of Molecular Variance (AMOVA)

Among 16471 (182 * 181 / 2) pairwise comparisons of the multiband phenotypes from 182 wheat stem sawflies, none of them shared the same unique 60-band phenotype. Therefore all 182 multiband phenotypes were treated as haplotypes in the AMOVA analysis.

The results of AMOVA partitioning of variance indicated significant differences ($P < 0.01$) between the Montana and North Dakota regions, among populations within regions, and among individuals within populations (Table 4). The within-population component accounted for 71.58% of the variation and was significantly different from zero at the 1% probability level. The among-population and among-region components accounted for 6.29% and 22.13% of the total variation, respectively.

For within-region analyses, most of the genetic variation was partitioned into the within-population component, 87.73% for Montana and 99.35% for North Dakota. In Montana, the among-population component accounted for 12.27% of the total variation and was significantly different from zero. In North Dakota the among-population component accounted for less than 1% of the variation and was not significantly different from zero.

Table 4. Analysis of Molecular Variance (AMOVA) for 182 individuals sampled from 15 populations of *C. cinctus*, using 60 RAPD markers. The combined data set contains individuals from two regions, Montana and North Dakota, which had eight and seven populations, respectively. AMOVA were also performed for each region. Statistics include sums of squared deviations (SSDs), mean squared deviations (MSDs), variance component estimates, the percentages of the total variance (%Total) contributed by each component, and the probability (P) of obtaining a more extreme component estimate by chance alone.

Source of variation	df	SSD	MSD	Variance component	%Total	P-value
Combined analysis						
Regions (MT vs. ND)	1	198.92	198.92	2.14	22.13	<0.01
Populations/regions	13	184.86	14.22	0.61	6.29	<0.01
Individuals/populations	167	1156.55	6.95	6.93	71.58	<0.01
Montana						
Populations/Montana	7	140.04	20.01	0.96	12.27	<0.01
Individuals/populations	104	713.35	6.86	6.86	87.73	<0.01
North Dakota						
Populations/North Dakota	6	45.00	7.50	0.05	0.65	0.26
Individuals/populations	63	443.30	7.04	7.04	99.35	<0.01

Bartlett's test (based on AMOVA) for homogeneity of RAPD variance among pairwise population comparisons indicated significant divergence among 81 of the 105 (76%) population pairs (Table 5). Among these comparisons, all Montana populations differed from Wyoming and North Dakota populations. There were no significant differences among any of the population comparisons from North Dakota, and the Wyoming population was significantly different from only a few North Dakota populations. With three exceptions, all Montana populations differed from one another. There was no significant difference between the west Conrad and east Conrad populations, the Dutton and Nashua populations, or the Joplin and Nashua populations.

Pairwise comparisons of individuals between populations resulted in a matrix of average RAPD distances between the populations (below diagonal of Table 5). Cluster analysis of this distance matrix, based on an UPGMA algorithm, produced a dendrogram that depicted the RAPD affinity among populations (Fig. 8). The dendrogram clearly showed that all populations from Montana were clustered into one group and populations from North Dakota and the population from Wyoming were clustered into another group. In Montana the MT-V, MT-H, and MT-Tb populations were closely related as were the MT-Pa and MT-Pb populations, while the populations collected from MT-Ta, MT-B, MT-C were more distantly related. In the North Dakota group, populations collected from ND-E and ND-B were most closely related while the population collected in Wyoming was most divergent. These population groupings derived by Euclidean distance-based cluster analysis were similar to population groupings derived by RAPD band frequency based Principal Coordinate Analysis.

Table 5. Euclidean distance between populations[a] of *C. cinctus* Nort. (below diagonal) given as E/m, where E is Euclidean distance and m (m = 60) is number of polymorphic bands detected.

	MTB	MTC	MTa	MPa	MPb	MTb	MTH	MTV	NDE	NDG	NMA	NMB	NDF	NDB	WY
MTB	0	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MTC	0.28	0	***	***	***	***	***	***	***	***	***	***	***	***	***
MTa	0.272	0.278	0	***	***	***	***	***	***	***	***	***	***	***	***
MPa	0.268	0.26	0.245	0	NS	***	***	***	***	***	***	***	***	***	***
MPb	0.271	0.273	0.248	0.214	0	***	***	***	***	***	***	***	***	***	***
MTb	0.27	0.274	0.275	0.254	0.251	0	*	NS	***	***	***	***	***	***	***
MTH	0.262	0.264	0.258	0.232	0.229	0.171	0	NS	***	***	***	***	***	***	***
MTV	0.257	0.271	0.269	0.241	0.233	0.184	0.167	0	***	***	***	***	***	***	***
NDE	0.332	0.35	0.311	0.309	0.296	0.307	0.28	0.282	0	NS	NS	NS	NS	NS	***
NDG	0.336	0.35	0.327	0.328	0.315	0.306	0.289	0.289	0.224	0	NS	NS	NS	NS	NS
NMA	0.337	0.344	0.323	0.317	0.31	0.301	0.286	0.28	0.219	0.232	0	NS	NS	NS	NS
NMB	0.348	0.351	0.329	0.318	0.307	0.305	0.286	0.28	0.229	0.242	0.226	0	NS	NS	NS
NDF	0.353	0.351	0.338	0.334	0.321	0.303	0.292	0.285	0.24	0.256	0.239	0.243	0	NS	NS
NDB	0.336	0.356	0.33	0.335	0.319	0.316	0.295	0.282	0.21	0.223	0.217	0.227	0.244	0	***
WY	0.324	0.333	0.312	0.307	0.297	0.279	0.273	0.265	0.245	0.256	0.239	0.248	0.257	0.24	0

Significance of Bartlett's test of homogeneity of RAPD variance among populations is above the diagonal (*:P <= 0.05; **: P <= 0.01; and ***: P <= 0.001).

[a] MTB = MT-Three Forks, MTC = MT-Power, MTA = MT-Dutton, MPa = MT-East Conrad, MPb = MT-West Conrad, MTb= MT-Agawam, MTH = MT-Joplin, MTV = MT-Nashua, NDE = ND-Emmer, NDG = ND-Grantco, NMA = ND-Mcla, NDB = ND-Mclb, NDF = ND-Fost, NDB= ND-Bott, WY = Wyoming

Figure 8. UPGMA cluster analysis of 15 populations of *C. cinctus* based on Euclidean distance estimated from 60 RAPD loci from 20 primers.

V. DISCUSSION

Results of this study show that RAPD-PCR is a useful and effective technique in detecting genomic variation among and within *C. cinctus* populations. One limitation to the RAPD approach is the phenomenon of convergence, such that bands of similar electrophoretic mobility in RAPD profiles of different individuals may correspond to nonhomologous DNA sequences. Another potential limitation is that RAPD alleles are mostly dominant/recessive (presence or absence of a specific DNA band) and heterozygotes cannot be differentiated from homozygous dominant genotypes. Black et al. (1992) detected the presence of parasitoids in aphids which could be an undetectable sample contaminant, although the common parasitoids of *C. cinctus* in Montana do not live within the host. In addition, many RAPD generated amplification products may correspond to repetitive sequences, which are regarded as likely sources of error in phylogenetic studies. It would therefore be desirable to determine the presence or absence of homology between shared bands, as well as the repetitive status of the sequences from which they are derived. Currently there is no practical way to resolve these problems.

Overall results of the current study show highly correspondent results by different analyses. Montana populations showed much more variation among populations than North Dakota populations.

A historical perspective of *C. cinctus* indicates the species has rapidly extended its adaptation from native grasses to spring wheat, and more recently to winter wheat. Based on historical records, Morrill and Kushnak (1996) concluded that *C. cinctus* wasp are emerging 20 days earlier than the past, and oviposition activity currently coincides with susceptible growth stages of winter wheat. They postulated that the changes in host availability could have influenced this change. Wheat production in Montana was primarily spring wheat until the mid-1950's. Since that time acreages of spring and winter wheat have been similar while North Dakota has remained primarily a spring wheat monoculture system. Increased availability of winter wheat (early maturity) and decreased availability of spring wheat could provide selection pressure for earlier activity of *C. cinctus*. Close synchronization of wasp emergence and the susceptible growth stage of the wheat host plant is essential due to the wasp's limited dispersal potential and short life span. Because the *C. cinctus* adult can only survive about one week (Criddle, 1923), the time of emergence is very important. Wasps that emerge before plant stems have developed are unable to find suitable hosts. Late-emerging wasps would oviposit in hosts that could desiccate before larval development is completed (Davis, 1955). *C. cinctus* was first reported in wild grasses, and in some areas remains predominantly in grasses even when grown in proximity to wheat (Morrill and Kushnak, 1996). If *C. cinctus* wasps have adapted to wheat by emerging earlier, differences in emergence dates of populations from wheat and grass should exist. Morrill and Kushnak (1996) found that wasp emergence in wild grass was consistently several weeks later than *C. cinctus* populations in a nearby wheat field.

The adaptation of *C. cinctus* to its local environment may in large part be associated with host availability and possibly to variation in host availability. Wheat culture in Montana with 40-50% winter wheat and 50-60% spring wheat is much more variable than the near spring wheat monoculture characteristic of North Dakota. Since winter wheat physiologically matures before spring wheat, a mixed culture system would provide greater diversity in host availability, and provide a mechanism for adaptations toward earlier wasp emergence. If such a hypothesis was valid, MT populations would be expected to show more variations than the ND populations.

Although the exact mechanism by which *C. cinctus* has evolved towards earlier emergence is not known, larval cannibalism would provide strong selection pressure for early season activity in *C. cinctus* (Morrill and Kushnak, 1996). We hypothesize that cannibalism of eggs and late-developing larvae by earlier-developed larvae within the stem, in a synchronous crop like wheat, could provide selection pressure for wheat stem sawfly to evolve towards earlier emergence and oviposition. Tarpley et al. (1993) also reported that cannibalism may play an important role in controlling the geographic genetic variation of corn borer, *Diatraea grandiosella*. If true, this hypothesis could explain the apparent increased virulence of wheat stem sawfly on winter wheat in Montana. Furthermore, the hypothesis would suggest that continued evolution of sawfly toward earlier maturity would result in winter wheat becoming a preferred host crop relative to spring wheat. Finally, if this hypothesis is valid it would also be expected that sawfly populations adapted to developmentally-synchronous wheat would differ genetically from sawfly populations collected from nonsynchronous native grasses, which presumably underwent little selection pressure for earlier maturity.

Like most other Hymenoptera species, the basic mode of sex determination in *C. cinctus* is arrheniotoky; all normal males are haploid and females are diploid (Farstad, 1938; Mackay, 1955). Askew (1968) reported that all haplo-diploid groups should have very significantly reduced stores of genetic variability compared with bisexual species with both sexes diploid, due to the inevitable exposure of all loci to selection in a hemizygous condition. Male haploidy would also decrease equilibrium frequencies of deleterious recessive alleles (Crozier, 1977). In addition, the weak dispersal potential and short life span of sawfly would be expected to enhance reproductive isolation and limit gene migration which would further reduce genetic variation within populations. However, Crozier (1970) reported that Hymenoptera could have significant stores of genetic variability based on rapid response to selection in many species and abundant polymorphic isozyme loci. So and Takafuji (1992) studied one non-hymenopterous haplo-diploid species, the phytophagous mite (*Tetranychus urticae*) and reported a slow rate of fixation of specific genes/traits and the resultant in the coexistence of a large number of different genotypes/phenotypes within populations. This genetic variation is highly favorable for colonizing species, because it provides the genetic raw material for selection and enables rapid response to the process. Genetic variation and rapid response to selection are particularly important to species like *T. urticae* and *C. cinctus* because of the passiveness of long-distance dispersal. Like wheat stem sawfly, successful colonization of a new habitat by *T. urticae* depends on the ability of the migrants to adapt to new environments rather than their ability to find a suitable one. Our results are consistent with results of other studies of haplo-diploid species showing relatively high levels of genetic polymorphism.

No information regarding the extent and distribution of genetic variation in *C. cinctus* had been reported. The diversity in wheat stem sawfly from Montana and North Dakota revealed by amplification of total cellular DNA with RAPD primers is extensive (Tables 3-5; Figs. 4-8). Each of 182 *C. cinctus* individuals had a distinct multiband phenotype. Large genetic differences were apparent between populations collected from Montana and North Dakota. There was considerable variation within each of the 15 populations, and every individual was genetically unique. In spite of substantial internal variation there was measurable divergence among Montana populations but not the North Dakota populations.

Relatedness of Montana populations showed little association with proximity of collection sites or geographical location. The lack of correspondence between geographical distance and genetic distance suggests establishment of diverse populations through adaptation to local environmental conditions and/or host differences. This also provides evidence for limited gene flow between *C. cinctus* populations due to reproductive isolation and limited dispersal potential. The random nature of population structuring does not support a hypothesis that a single virulent sawfly biotype is expanding across the state. The limited sample size in each population (most populations have 10 individuals) may have influenced the results. Huff et al. (1993) suggested use of as many as 25 individuals within each population to characterize population to population variation within a region.

An understanding of gene flow mechanisms in *C. cinctus* is important because it influences the distribution of genetic variation within natural populations. Understanding how the environment affects the development and adaptation of *C. cinctus* and how *C. cinctus* reacts to changing conditions could help us to understand genetic changes in local populations and its speciation. Gene flow and the mating system are important determinants of the genetic structure of *C. cinctus* populations. Methods of estimating gene flow and mating system are dependent on the ability to detect heterozygotes (Clegg, 1980). Genetic polymorphism detected with RAPD reveals one allele per locus which corresponds to the amplification product visualized. RAPDs do not identify heterozygous loci. In this context, RAPD is limited.

VI. CONCLUSIONS AND PERSPECTIVE

A historical perspective indicates that *C. cinctus* has extended its adaptation from native grasses to spring wheat, and from spring wheat to winter wheat. As resistant solid-stem wheat varieties were developed, it also adapted to some extent to the solid-stem varieties. This ready response to changing host availability is consistent with a high degree of genetic diversity within the species. Based on the history of *C. cinctus* in Montana, development of a virulent biotype capable of overcoming currently available solid stem resistance is possible. Determining different biotypes requires differential genotypes and considerable colony maintenance and time (Puterka and Peters, 1988). Because of morphological similarity and no resistance differentials, there is no available conventional method to distinguish whether the two sawflies represent different biotypes or not.

In this study, sixty-two primers were used. Different primers provided different information regarding the sawfly genetic diversity (Chalmers et al., 1992), so it is obvious that resolution would be improved by using more informative primers (Chapco et al., 1992). There is no standard criteria for reference. Different studies use different numbers and different sets of primers.

There are many different analysis methods used in published genetic diversity papers, but the basis is the same. A genetic distance between individuals is generated and all subsequent analyses are based on this genetic distance. In our study we used three analyses to deal with our data, they produced similar results.

The result of AMOVA in this study showed significant genomic variation of wheat stem sawflies among Montana and North Dakota (including Wyoming) populations, with the majority of the variation attributable to within population diversity. Within Montana, there were significant difference among populations. Within North Dakota, there was no significant difference among populations. All three cluster analyses based on different genetic distances showed a high level of concordance, grouping Montana populations into one group and North Dakota (including Wyoming) populations into another group.

Sawfly biotypes identified in this study could be categorized as geographic races or possibly host races as defined by Diehl and Bush (1984). At this moment, we are not sure whether this genomic diversity indicates differences in sawfly virulence, preference, behavioral or developmental pattern, and/or morphology. Some evidence is reported for diversity in sawfly emergence date. Insect bioassays must be developed to further characterize these populations. The present results do not support the assumption that a new virulent sawfly biotype has evolved in Montana and is spreading from one location across the entire state. These results do not invalidate the hypothesis that *C. cinctus* in some locations has evolved towards earlier emergence and flight activity which better coincided with the vulnerable jointing stage of winter wheat. Future research in this area using PCR markers could compare *C. cinctus* collected early and late during emergence at the same locality, as well as native grass populations of *C. cinctus* to wheat populations collected in the same locality (Morrill and Kushnak, 1996). In addition, more detailed evaluation of variation in diapause and emergence date as affected by host and geographic location is warranted.

Whether genomic diversity observed among sawfly populations at geographically and ecologically diverse collection sites using this technique indicates diversity in insect virulence and/or differentiation of biotypes based on virulence is not known. If RAPD-PCR molecular markers do identify differences in wheat stem sawfly virulence, then the technique could be used to assess insect variability over the sawfly region and target resistance screening nurseries to sites where new and/or more virulent biotypes are present, which would be an valuable tool for plant breeding and insect management.

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APPENDIX

Tables 6 - 10

Table 6. The results of RAPD-PCR from 186 wheat stem sawflies.

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NUMBER OF INDIVIDUALS: 186
TITLE: C. cinctus DATASET
NUMBER OF FRAGMENTS: 60
(4(12(1X,A5)/), 12(1X,A5))
A13-1 C02-1 E15-1 A09-1 A09-2 A02-1 A02-2 A02-3 A02-4 A02-5 A04-1 A04-2
A04-3 A04-4 A04-5 A04-6 A10-1 A10-2 A10-3 BAM-1 E04-1 E04-2 A20-1 A18-1
A18-2 A18-3 A18-4 A18-5 E18-1 E18-2 E20-1 E20-2 E20-3 C12-1 C12-2 C12-3
C05-1 C05-2 C05-3 C05-4 C15-1 C15-2 C15-3 C15-4 E19-1 E19-2 E19-3 E19-4
E19-5 C04-1 C04-2 C04-3 C04-4 C04-5 C04-6 E07-1 E07-2 E07-3 A08-1 A08-2
(A10,1X,60A1)
MT-B1      101010100011000010001101100101000001010000101111001010000001
MT-B2      1110111000100000001001001000101000001011001100110000110000001
MT-B3      1110101001000000010101001000000000000010100001011111100010000001
MT-B4      1010110001101000101001010000000000001010001101110000100000001
MT-B5      100010100101000010101001100100000000100100110111110000000000001
MT-B6      1110110000101000010001011001000000110010001001100000010000001
MT-B7      11101010011000100100100110010000001101000001011100000110000001
MT-B8      111010100111000010101001100000000011010001011011000011000001
MT-B9      11011110000100000010001011000000000000000001101010000011000001
MT-B10     111111000011000010100101000001000010010000101110101100000001
MT-B11     111111100001000010101001100001000000010000101110101100000001
MT-B12     111110100011010010100001000100000111000001101110101100000001
MT-B13     111111100001000010101101100001000010010001100111100000000001
MT-B14     101111100001100010101111010000000000011001100111100000000001
MT-Bfem1 0011010000110010101011110000000000001010000101101100110010000
MT-Bfem2 1101011000010001101011110000000000000110000101100000110011000
MT-Bfem3 1001001000010000101011110000000000000010000101100000010001001
MT-Bfem4 1101001000010000101011010000000000000010000101100010010000000
MT-Bfem5 0111011000110000101010010000000000001011000101101010010001000
MT-Bmal1 00110100001100001010110100000100000010100001010000000000001001
MT-Bmal2 1111010000110000101011110000000000000010001101101100000001001
MT-Bmal3 0101011000010000101011110000000000000010000101111000011001000
MT-Bmal4 001001100011000001101111000001000000010001100110000000011001
MT-Bmal5 1011010000010000101011110000000000001111000101010100000001000
MT-C1      1110001000001000100011000000000001011111001101111001000000001
MT-C2      001101100011000001001101000100000011110001100101100010010001
MT-C3      0111001010000000010010010000000000011101000101101000000000001
MT-C4      10011110001100000010011010000000000000001001101001101010000001
MT-C5      1101001011010000101011010000000000011101000101000100000000001
MT-C6      10111110000000000010011010000000000011001001101100000010000001
```

Table 6. (cont.)

MT-C7	01011110010100001000110000000000000011000000101001000110000001
MT-C8	01010110001000001000100100000000000011000000101110000010000001
MT-C9	10110010110100000100100100010000000111000000101110000110000001
MT-Cfem1	11110100000110000110110110000000000001010000100101001000011000
MT-Cfem2	11110100001101000110110100000000000000011000100101000010001000
MT-Cfem3	1100001000011010011011010000010000000010001100101001100100001
MT-Cfem4	011101100011001000101111100001000000010000100100001010000001
MT-Cfem5	1011001000110000011011110000000000000101000010010110000000001
MT-Cfem6	11111010000101000110110100000000000001010000101110000010000001
MT-Cmal1	1111101000010010010001011000000000000010000101100001100101000
MT-Cmal2	101000100001000001001111100001000000010000101100011010001001
MT-Cmal3	101110100011000001001101000000000000010001101101001100001001
MT-Cmal4	101111000001100001101101000001000000010000101100001110001001
MT-Ta1	001100100011000001001001100000001011101001101100011010001001
MT-Ta2	1001111000110000101011011000000000000100001101000011110000001
MT-Ta3	111110100001000010101101100000000011000001101001111010000001
MT-Ta4	1111101000110000101011011000000000000110001101000000110000001
MT-Ta5	010111100001000010101100000001000001110000101101110011010001
MT-Ta6	1001111001010000101011010000000000011100001101100010010000001
MT-Ta7	1101010001010000101011010000010000111000001011000000010000001
MT-Ta8	1101111000010000101011010001010000111000011011111001100000001
MT-Ta9	0101101000110000010010010000000000001111010100110010010000001
MT-Taf1	0111011000110000111011110000000000001010000101100110010111000
MT-Taf2	1011011000110100101011010001000000001010000101100010110111001
MT-Taf3	0001010000110000010011110000000000000010000101100011110000001
MT-Taf4	1011110000110000111011111000000000000010000101000000110001000
MT-Tam1	0000101000110000101010110000000000000010000100100010010111000
MT-Tam2	0111011000011100101011111000000000001010000101101010010001001
MT-Tam3	0111010000110001101011110000000000001010000100001100010001000
MT-Tam4	1011011000010100101011111000000000001010000100101000010001000
MT-Tam5	000101100001000001101111000000010001010001101110000110001000
MT-Tam6	100101100011000010101111000000000011010000101110100010001000
MT-Pa1	01110100001100000100111100000000000001011000101100000110000001
MT-Pa2	1111101001010000101011010000000000001010001101001010110001001
MT-Pa3	011100100001000010001111100000100011010001100001000010001001
MT-Pa4	0111111000110000110011110000000000001010000100100010010001001
MT-Pa5	0001001000110000100010010000000000011010000101100000110010001
MT-Pa6	1111001000110010100011110000000000011011001101100000111001001
MT-Pa7	111101100011000001001111100001000011000001101101010010011001
MT-Pa8	1011111000011000100011110000000000011000000101100000010011001
MT-Pa9	1101001000110000100011110000000000111010000101110000010001001

Table 6. (cont.)

MT-Pa10	0001110000010000010011010000000001110000011011100001100011O1
MT-Pb1	1111010000110001100011110000000000001010000101100010110011001
MT-Pb2	0011001000010000100010110000100000010100001011000101100010O1
MT-Pb3	1101001000110000101011010000000000001010001101101100110001001
MT-Pb4	0001010000110110100011110000000000001010000101001101000001001
MT-Pb5	0101001000111000010011111000000000001010000101000000110001001
MT-Pb6	0101011000110000010011110000000000001010001101101000010001001
MT-Pb7	1011001000010000100001011000000000111100011011011000100100O1
MT-Pb8	0110110000110000100011110000010000111000001011000011100110O1
MT-Pb9	1011101000110000101011011000000000110100011011111000100010O1
MT-Pb10	0010101000110000100011110000100000111100011011101011100110O1
MT-Tb1	1011111000110000010011110000000000001010000101010000000001011
MT-Tb2	0001010000110001010011110000000000000100011001101001100100O1
MT-Tb3	0111111000010100100011110000010000000100011010110001100000O11
MT-Tb4	1001001100010000100011110000000000001010001101011100110000011
MT-Tb5	1101111000110000010011111000000000000100011011111001100000O1
MT-Tb6	0001010000110010010010110000000010000100001011111001100000O1
MT-Tb7	1111101000110000010011111000000000000100001011111001100000O1
MT-Tb8	1111111000010000010011110000000000000100001011111101100000O1
MT-Tb9	1101010000110000010010111000000000000100001011111001000000O1
MT-Tb10	1000111000010001010011111000000000000110011011111101100000O1
MT-H1	1011001000110000100011110000000010000100011001110001100100O11
MT-H2	1011001000110000010011110000000000000100011011111101100000O1
MT-H3	1101001000110000010011110000000000100100001010100001100100O1
MT-H4	1011010000010000010011110000000000000100001011011001100100O1
MT-H5	1001011000010000100011110000000000110100001011111001100000O11
MT-H6	0011011000110000100011110000000000000100001011111101100100O1
MT-H7	0111011100010000100011110000000000000100001011111001000000O1
MT-H8	0111011000010000010011110000000000000100001011111101100000O1
MT-H9	1101011000110100010011110000000000000100101011111001100000O1
MT-H10	0111111001110000100011110000000000000100011011011101100100O1
MT-V1	1001111000110010100011110000000000110100001011110001100100O1
MT-V2	1101011000110000100000111000000000100100011011100001100100O1
MT-V3	1101111000010100100011110000000000001010000100010001100010O01
MT-V4	0011011000110000010011110000000000000100011001011001000010O1
MT-V5	0100011000110100100011110000000000000100011001011001000000O1
MT-V6	1000111000110000010011110000000000000110001011111001100000O1
MT-V7	0100011000110000100011110000000000000100001011111001100100O1
MT-V8	1110111000100000010011110000000000000100001011111001000000O1
MT-V9	0111111000110000010011110000000000000100001011111001000110O1
MT-V10	0101011000110000100011110000000000000100001011111001000000O1

Table 6. (cont.)

mtcage01	100111100011001000101111000000000111001000101110000110011000
mtcage02	011111100011001010100111000000000011001000101110000110011000
mtcage03	100111110010001000100111000000000011111000101110010010011000
mtcage04	111111101110001010111111000000000011111000101110010110011100
ND-E1	101101000011000010000111000010000000100000001001100000000000
ND-E2	110011100011000010000111000010010000100000011000110010001000
ND-E3	111111100011000010000111000010000001010000110100100111001000 0
ND-E4	101111100011000000100101100001000000010000000100110001000000 0
ND-E5	110000100011000010000111000010100010100000000111110010001010
ND-E6	010111000011000010000111000010010000110000101110110110001000
ND-E7	101011100011000010000011000010010010100000101110010110001001
ND-E8	011011100011000010000111100010000010100010101111010110011001
ND-E9	111111000011000010000111000010010000100000001100010110000010
ND-E10	001001100011000010001111000010000010100000011111010110001001
ND-G1	010110100011000001000111000010000000100010001001100000010010
ND-G2	101101000011000001000111000010000000100010101000100000011010
ND-G3	100011100011000010001011000010001000100100111000100100010000
ND-G4	111010100011000010000111000010000000100011011001100000010000
ND-G5	101010100000000001000011100001000000010000000100110010000000 0
ND-G6	101011100011000001000111000010010001110010101111010110001101
ND-G7	101111100011000010000111000010010001010000001110010010000000
ND-G8	111101100011000010000111000010010000100010101110010010000001
ND-G9	110011100011000010000111100010000010100000001110010110000000
ND-G10	011111100011000010001011100010000010100000001111010010000000
ND-Ma1	011110100011000000000101100001000001000000010100110010000010 0
ND-Ma2	100110100111000001000110000010000010100000101001100100010010
ND-Ma3	001110100111000000000111000010000010100000001001000100001000
ND-Ma4	110111100011000010001011000010000010100010101001100000000001
ND-Ma5	001111100011000010001011000010000010100000101001100000000001
ND-Ma6	110011100011000010001111100010010010100000101111010110011010
ND-Ma7	111011100011000010000111000010000001100000001111010110000000
ND-Ma8	000011100011000010000111000010010010100010011110010010000000
ND-Ma9	101111100011000010000111100010010000100100101111000110001000
ND-Ma10	101011100011000001000110100010010010100010101110010110000000
ND-Mb1	110110100011000000000110000010000010000010101001100100011000
ND-Mb2	011010100011000001000111000010000010100000000001100000001001
ND-Mb3	011111101011000001000111000010000010100100101001100100001000
ND-Mb4	001110110011000010001011000010000010100000001111100100000010
ND-Mb5	100111100011000010010111000010000010100110001001101100001000
ND-Mb6	010011100011000001000111100010010011100010101110010110000000
ND-Mb7	111011100011000011001111100010000010110010101111010110010010

Table 6. (cont.)

ND-Mb8	0111111000110000100011111000100000001100000011010101100010000
ND-Mb9	0111111000110000100011110000100000011000000011110101100100000
ND-Mb10	1110110000110000100011110000100100001101000111000101100100000
ND-F1	110111100011000010000111000010000010100100101111100100000100
ND-F2	111111111000111101001110011110000010100010001001101100000011
ND-F3	011011100011000010000111000010000010100010101001100100011000
ND-F4	001011100011000001011111000010000010100000101001100100001000
ND-F5	011110100111000010000111000010000000100000001001100000000010
ND-F6	101101100011000001001110000010000000100000001110010110010000
ND-F7	110011100011000001001111100010000010100000101111010110001001
ND-F8	010001100011000010001111100010000010100000101111010110001001
ND-F9	110011100011000010011111100010000000100000011110100110001010
ND-F10	100001000011000001000111100010000000100000001100010010000000
ND-B1	001011000011000010001011000010000000100010101001101100010000
ND-B2	010011000011000010000111000010000000100000001001100100000000
ND-B3	010111100011000010100111000010000000100001001001100100010000
ND-B4	111111100111000010000111000010000000100000001001100100001000
ND-B5	111010100011000010000111000010000000100000001001100100000000
ND-B6	101111100011000010001011000010010010100010001110001010000000
ND-B7	000011100011000001001011100010010000100000000110001010000000
ND-B8	111101100111000010000111000010010010000000001110000110011000
ND-B9	101011100011000010000110000010010010100000001110010110000000
ND-B10	111111100111000010000111000010010010100010001110010110010000
WY1	000110100011000010001011000010000010100010001001100000011000
WY2	111111100011000001001011000010000000100000001001100100001000
WY3	101111100011000010001011000010000000000010001101100100001000
WY4	001110100010000010001011000010000110110010001001100000000001
WY5	101111100011000001011011000010000010110001101001100100000000
WY6	100111100011000010011110000010000000100000001110001110001001
WY7	111011100011000011011111100000010000100000101110010110001010
WY8	101111100011001010001111100000010000001000101111000100001000
WY9	110011100011000011011111100000010000100000001111010110000010
WY10	100111100011000010001111100000000010110010101110011110001001

Table 7. The (1-match) index among individuals.

	cage1	cage2	cage3	cage4
cage1	.0000			
cage2	.1000	.0000		
cage3	.1333	.1667	.0000	
cage4	.2000	.1667	.1667	.0000

Table 8. The (1-match) index among individuals* in Broadwater, Montana.

	tf01	tf02	tf03	tf04	tf05	tf06	tf07	tf08	tf09	tf10	tf11	tf12	tf13	tf14
tf01	.0000													
tf02	.2167	.0000												
tf03	.1833	.2333	.0000											
tf04	.2500	.2333	.2000	.0000										
tf05	.1833	.2667	.1667	.2333	.0000									
tf06	.2500	.1667	.3000	.2333	.3000	.0000								
tf07	.2000	.1500	.1833	.2500	.2500	.1833	.0000							
tf08	.2167	.3000	.1667	.2667	.2000	.3333	.2167	.0000						
tf09	.2833	.2667	.3000	.3000	.2667	.2333	.2833	.2667	.0000					
tf10	.2333	.2833	.2500	.1833	.3500	.2833	.3000	.3167	.2833	.0000				
tf11	.2000	.2500	.1833	.2500	.2500	.3500	.2667	.2833	.2500	.1000	.0000			
tf12	.2667	.2833	.2500	.2500	.2500	.3167	.2667	.2833	.3167	.1667	.2000	.0000		
tf13	.2167	.2667	.2000	.2667	.2333	.3000	.3167	.2333	.2333	.1500	.1167	.2500	.0000	
tf14	.2833	.3000	.2333	.2333	.2333	.3333	.4167	.3333	.3000	.2500	.2167	.3167	.1333	.0000

*: tf01-tf14 represents 14 individuals of *C. cinctus* from MT-B

Table 9. Results of UPGMA in text file of the combined data set using (1-M) index.

```
186 Populations
Neighbor-Joining/UPGMA method version 3.55c
UPGMA method
Negative branch lengths allowed

          +--MT-B1
        +-127
        ! +--MT-B5
      +-144
      ! ! +-MT-B3
    +-150 +-88
    ! !   +-MT-Cfem6
    ! !
    ! +--MT-B8
    !
    !         +-MT-C5
    !       +-82
    !       ! ! +MT-Ta6
  +-170      +-117  +-25
  ! !     ! !   +MT-Ta7
  ! !   +-124   !
  ! !   ! ! +--MT-Ta8
  ! ! +-152   !
  ! ! ! ! +--MT-C7
  ! ! ! !
  ! ! ! +--MT-Ta5
  ! +-159
  !   !   +MT-Ta2
  !   ! +-24
  !   ! ! +MT-Ta4
+-175     ! !
! !   +-125     +-MT-Ta3
! !     ! +-59
! !     ! ! +-MT-Pb9
! !     +-83
! !       ! +-MT-Pa2
! !       +-46
! !         +-MT-Pb3
! !
! ! +---MT-B4
```

Table 9. (cont.)

```
! ! !
! ! !     +MT-B10
! +-168    +-35
!   ! +-131  +MT-B11
!   ! ! !
!   +-158  +--MT-B12
!     !
!     ! +-MT-B13
!     +-69
!       +-MT-B14
!
!       +--MT-B9
!     +-119
!     ! ! +-MT-C4
!     ! +-81
!   +-165    +-MT-C6
!   ! !
!   ! ! +-MT-Pa10
!   ! +-115
!   !   +-MT-Tamal5
!   !
!   !         +-MT-C2
!   !       +-84
!   !       ! +-MT-Pb7
!   !     +-157
!   !     ! ! +--MT-Pa7
!   !     ! +-128
!   !     !   +--MT-Pa8
!   !   +-164
!   !   ! !     +-MT-C8
!   !   ! !   +-45
!   !   ! ! +-105   +-MT-Pa5
!   !   ! ! ! !
!   ! +-169  +-148   +-MT-V2
!   ! ! !   !
!   ! ! !   +--MT-Pa6
!   ! ! !
!   ! ! ! +-MT-Pb8
!   ! ! +-85
!   ! !   +-MT-Pb10
!   ! !
```

Table 9. (cont.)

```
  !   ! !            +MT-Pa1
  !   ! !          +-26
  !   ! !          ! +MT-Tafem3
  ! +-176 !         +-76
  ! ! ! !        ! ! +-MT-Pa4
  ! ! ! !        ! +-47
+-180 ! ! !       +-112    +-MT-Pb6
! ! ! ! !       ! !
! ! ! ! !       ! ! +-MT-Pb5
! ! ! ! !     +-126 +-61
! ! ! ! !     ! !   +-MT-H3
! ! ! ! !     ! !
! ! ! ! !     ! +--MT-Tb1
! ! ! ! !     !
! ! ! ! !     !      +-MT-Tb2
! ! ! ! !     !    +-60
! ! ! ! !     !    ! +-MT-Tb6
! ! ! ! !     !    !
! ! ! ! !     !    !            +MT-Tb5
! ! ! ! !     !    !          +--2
! ! ! ! !     !    !        +-22  +MT-Tb7
! ! ! ! !     !    !        ! !
! ! ! ! !     !    !      +-23  +MT-Tb9
! ! ! ! !     !    !      ! !
! ! ! ! !     !    !    +-39  +MT-H9
! ! ! ! !     !    !    ! !
! ! ! ! !     !    !    ! ! +MT-V8
! ! ! ! !     !  +-109    ! +-14
! ! ! ! !     !   ! !   !   +MT-V9
! ! ! ! !     !   ! ! +-43
! ! ! ! !   +-145  ! !  ! !   +MT-Tb8
! ! ! ! !   ! !   ! !   ! !  +--1
! ! ! ! !   ! !   ! !   ! ! +-11 +MT-H8
! ! ! +-173    ! !   ! !  ! ! ! !
! ! !   !   ! !   ! ! +-70 +-36 +MT-H2
! ! !   !   ! !   ! ! ! !   !
! ! !   !   ! !   ! ! ! !   +-MT-H4
! ! !   !   ! !   ! ! ! !
! ! !   !   ! !   ! ! ! ! +MT-Tb10
! ! !   !   ! !   ! ! ! +-13
! ! !   !   ! !   ! +-79    +MT-V6
```

Table 9. (cont.)

```
  ! ! !   !   ! ! +-123    !
  ! ! !   !   ! ! ! !   !     +MT-H6
  ! ! !   !   ! ! ! !   !   +--5
  ! ! !   !   ! ! ! !   !   ! +MT-V7
  ! ! !   !   ! ! ! !   ! +-21
  ! ! !   !   ! ! ! !   ! ! ! +MT-H7
  ! ! !   !   ! ! ! !   +-54  +--3
  ! ! !   !   ! ! ! !     !   +MT-V10
  ! ! !   ! +-161 ! ! !       !
  ! ! !   ! ! ! ! ! !     +-MT-H10
  ! ! !   ! ! ! ! ! !
  ! ! !   ! ! ! +-141 !     +-MT-V4
  ! ! !   ! ! !   ! !   +-48
  ! ! !   ! ! !   ! ! +-75  +-MT-V5
  ! ! !   ! ! !   ! ! ! !
  ! ! !   ! ! !   ! +-101  +-MT-Bmal2
  ! ! !   ! ! !   !   !
  ! ! !   ! ! !   !   +-MT-Cfem5
  ! ! !   ! ! !   !
  ! ! !   ! ! !   !     +MT-Tb4
  ! ! !   ! ! !   !   +-12
  ! ! !   ! ! !   ! +-74  +MT-H5
  ! +-177    ! ! !   ! ! !
  !   !   ! ! !   +-107  +-MT-V1
  !   !   ! ! !     !
  !   !   ! ! !     +-MT-H1
  !   !   ! ! !
  !   !   ! ! ! +--MT-Tb3
  !   !   ! ! +-129
  !   !   ! !   +--MT-V3
  !   !   ! !
  !   !   ! !       +-MT-Pa3
  !   !   ! !     +-114
+-182   !   ! !     ! ! +MT-Tamal2
! !   !  +-171    ! +-27
! !   !     !    !   +MT-Tamal4
! !   !     !    !
! !   !     !    !    +MT-Pa9
! !   !     !    !   +-28
! !   !     !   +-146   ! +MT-Tamal6
! !   !     !   ! !   !
```

Table 9. (cont.)

```
  ! !   !     !   ! ! +-100   +-MT-Bfem2
  ! !   !     !   ! ! ! ! +-57
  ! !   !     !   ! ! ! ! ! ! +MT-Bfem3
  ! !   !     !   ! ! ! +-58 +-15
  ! !   !     !   ! ! !   !  +MT-Bfem4
  ! !   !     !   ! ! !   !
  ! !   !     !   ! +-142   +-MT-Bmal3
  ! !   !     !   !   !
  ! !   !     !   !   !     +-MT-Pb1
  ! !   !     !   !   !   +-63
  ! !   !     !   !   ! +-111  +-MT-Tafem2
  ! !   !     ! +-154    ! ! !
  ! !   !     ! ! !   ! ! +-MT-Pb2
  ! !   !     ! ! !   +-138
  ! !   !     ! ! !     !   +-MT-Bfem1
  ! !   !     ! ! !     ! +-64
  ! !   !     ! ! !     ! ! +-MT-Tamal3
  ! !   !     ! ! !     +-102
  ! !   !     ! ! !       ! +-MT-Bfem5
  ! !   !     +-167 !        +-62
  ! !   !       ! !          +-MT-Tafem1
  ! !   !       ! !
  ! !   !       ! !   +-MT-Pb4
  ! !   !       ! ! +-86
  ! !   !       ! +-120 +-MT-Bmal1
  ! !   !       !   !
  ! !   !       !   +--MT-Bmal5
  ! !   !       !
  ! !   !       +---MT-Tamal1
  ! !   !
  ! !   !       +--MT-Bmal4
+-183 !   !      +-130
! ! !    !  +-135 +--MT-Cfem4
! ! !    !   ! !
! ! !    !   ! +--MT-Cmal2
! ! !    !   !
! ! !    ! +-166   +-MT-Cfem1
! ! !    ! ! ! +-87
! ! !    ! ! ! ! +-MT-Cfem2
! ! !    ! ! ! !
! ! !    ! ! +-156   +-MT-Cmal1
```

Table 9. (cont.)

```
! ! !   ! !   ! +-89
! ! !   +-174   ! ! +-MT-Cmal3
! ! !     !   +-137
! ! !     !     ! +-MT-Cmal4
! ! !     !     +-90
! ! !     !       +-MT-Tafem4
! ! !     !
! ! !     +---MT-Cfem3
! ! !
! ! !       +-MT-B2
! ! !     +-80
! ! !   +-118 +-MT-B7
! ! !   ! !
! ! !   ! +--MT-B6
! ! !   !
! ! ! +-178       +-MT-C3
! ! ! ! !   +-113
! ! ! ! ! +-155 +-MT-C9
! ! ! ! ! ! !
! ! +-181 +-163 +--MT-Ta1
! !   !   !
! !   !   +--MT-Ta9
! !   !
! !   +---MT-C1
! !
! !     +mttfcage01
! !   +-29
! ! +-91 +mttfcage02
! ! ! !
! +-122 +-mttfcage03
!   !
!   +--mttfcage04
!
!               +-ND-E1
!               !
!             +-38   +ND-G5
!             ! ! +--4
!             ! ! ! +ND-B5
!             ! +-33
!           +-53   ! +ND-F5
!           ! !   +-18
```

Table 9. (cont.)

```
  !             ! !     +ND-B4
  !             ! !
  !           +-78  ! +ND-B2
  !           ! ! +-17
  !           ! !   +ND-B3
  !           ! !
  !           ! ! +-ND-G1
  !         +-116  +-65
  !         ! !   +-ND-G4
  !         ! !
  !         ! !   +-ND-Ma3
  !         ! ! +-67
  !         ! ! ! +-ND-Mb2
  !         ! +-72
  !         !   !   +ND-Mb3
  !         !   ! +-32
+-184           !  +-51  +ND-F4
! !       +-132       !
! !       ! !     +-ND-F3
! !       ! !
! !       ! !       +ND-E4
! !       ! !     +--9
! !       ! !   +-41  +WY2
! !       ! !   ! !
! !       ! ! +-73  +-WY3
! !       ! ! ! !
! !       ! ! ! +-WY5
! !       ! ! !
! !       ! +-108    +-ND-Ma1
! !     +-140    ! +-93
! !     ! !   ! ! +-ND-Mb4
! !     ! !   ! !
! !     ! !   +-104      +ND-Ma4
! !     ! !     !   +--6
! !     ! !     ! +-42  +ND-Ma5
! !     ! !     ! ! !
! !     ! !     +-44  +-WY4
! !     ! !       !
! !   +-147 !        +-WY1
! !   ! ! !
! !   ! ! ! +--ND-E3
```

Table 9. (cont.)

```
 ! !   ! ! +-121
 ! !   ! !  ! +-ND-Mb5
 ! !   ! !   +-94
 ! ! +-151 !     +-ND-F1
 ! ! ! ! !
 ! ! ! ! ! +-ND-G3
 ! ! ! ! +-96
 ! ! ! !   +-ND-B1
 ! ! ! !
 ! ! ! ! +--ND-G2
 ! ! ! +-136
 ! ! !   ! +-ND-Ma2
 ! ! !   +-49
 ! ! !     +-ND-Mb1
 ! ! !
 ! ! !                +-ND-E2
 ! ! !              +-92
 ! ! !              ! +-ND-E6
 ! ! !            +-103
 ! ! !            ! ! +-ND-E9
 ! ! !            ! +-50
 ! ! !            !   +-ND-Mb10
 ! ! !            !
 ! ! !            !          +ND-E7
 ! ! !            !        +-19
 ! ! !            !      +-37  +ND-B9
 ! ! !          +-134      ! !
 ! ! !          ! !   +-56  +-ND-G9
 ! ! !          ! !   ! !
 ! ! !          ! ! +-71  +-ND-Ma8
 ! ! !          ! ! ! !
 ! ! !          ! ! ! ! +ND-Ma10
-185 ! !          ! ! ! +-16
 ! ! !          ! ! !   +ND-Mb6
 ! ! !          ! +-110
 ! ! !          !   !      +-ND-G7
 ! ! !          !   !    +-66
 ! ! !          !   ! +-97  +-ND-G8
 ! ! !          !   ! ! !
 ! ! !          !   +-99  +-ND-B6
 ! ! !          !      !
```

Table 9. (cont.)

```
! ! !         !     ! +ND-B8
! +-179          !    +-20
!   !      +-143         +ND-B10
!   !       ! !
!   !       ! !        +ND-E8
!   !       ! !      +-34
!   !       ! !      ! ! +ND-F7
!   !       ! !    +-55 +--8
!   !       ! !    ! !  +ND-F8
!   !       ! !    ! !
!   !       ! !  +-77 +-ND-E10
!   !       ! !  ! !
!   !       ! !  ! ! +ND-Ma6
!   !       ! !  ! +-30
!   !       ! ! +-106  +ND-Mb7
!   !       ! ! ! !
!   !       ! ! ! !  +ND-G10
!   !     +-153 ! ! ! +-31
!   !     ! ! ! ! ! ! +ND-Mb8
!   !     ! ! +-133 +-40
!   !     ! !   !  ! +ND-Ma7
!   !     ! !   !  +--7
!   !     ! !   !   +ND-Mb9
!   !     ! !   !
!   !     ! !   ! +-ND-F9
!   !     ! !  +-68
!   !     ! !    ! +WY7
!   !     ! !    +-10
!   !   +-160 !     +WY9
!   !   ! ! !
!   !   ! ! !  +-ND-Ma9
!   !   ! ! ! +-52
!   !   ! ! ! ! +-WY8
!   !   ! ! +-149
!   !   ! !   ! +-WY6
!   !   ! !   +-98
!   ! +-162 !     +-WY10
!   ! ! ! !
!   ! ! ! !    +-ND-F6
!   ! ! ! ! +-95
!   ! ! ! +-139 +-ND-F10
```

Table 9. (cont.)

```
!   +-172  !   !
!      ! !  +--ND-B7
!      ! !
!      ! +--ND-G6
!      !
!      +---ND-E5
!
+------ND-F2
```

Table 10. Results of UPGMA in text file of the MT region.

```
116 Populations
Neighbor-Joining/UPGMA method version 3.55c
UPGMA method
Negative branch lengths allowed


          +--MT-B1
        +-79
        ! +--MT-B5
      +-87
      ! ! +-MT-B3
    +-93  +-54
    ! !   +-MT-Cfem6
    ! !
    ! +--MT-B8
    !
    !       +-MT-C5
    !     +-49
    !     ! ! +-MT-Ta6
  +-105 +-60 +-18
  ! !   ! !   +-MT-Ta7
  ! !   ! !
  ! ! +-81 +--MT-C7
  ! ! ! !
  ! ! ! ! +--MT-Ta5
  ! ! ! +-68
  ! ! !   +--MT-Ta8
  ! +-96
  !   !   +-MT-Ta2
  !   ! +-17
  !   ! ! +-MT-Ta4
+-108     ! !
! !   +-78    +-MT-Ta3
! !     ! +-36
! !     ! ! +-MT-Pb9
! !     +-50
! !       ! +-MT-Pa2
! !       +-26
! !         +-MT-Pb3
! !
```

Table 10. (cont.)

```
! ! +---MT-B4
! ! !
! ! !     +-MT-B10
! +-104    +-16
!   ! +-80 +-MT-B11
!   ! ! !
!   +-98 +--MT-B12
!     !
!     ! +-MT-B13
!     +-34
!       +-MT-B14
!
!            +-MT-C8
!          +-24
!        +-44 +-MT-Pa5
!        ! !
!        ! +-MT-V2
!        !
!      +-92    +--MT-Pa3
!      ! ! +-69
!      ! ! ! +--MT-Pa6
!      ! ! !
!      ! +-85  +-MT-Pa7
!      !   ! +-27
!      !   ! ! +-MT-Pb6
!      !   +-76
!      !     ! +-MT-Pb5
!      !     +-28
!      !       +-MT-H3
!      !
!      !             +-MT-Pa1
!      !           +-20
!      !         +-29 +-MT-Taf3
!      !         ! !
!      !       +-42 +-MT-H4
!      !       ! !
!      !       ! +-MT-Tb2
!      !       !
!      !       !           +MT-Tb5
!      !       !         +--2
!      !       !         ! +MT-Tb7
```

Table 10. (cont.)

```
  !       !       !        +-14
  !       !       !        ! !   +MT-Tb8
  !       !       !        ! ! +--1
  !       !       !      +-15  +--6 +MT-H8
  !       !       !      ! !   !
  !       !       !      ! !   +MT-H2
  !       !       !    +-22 !
  !     +-99     +-57      ! ! +-MT-H9
  !     ! !     ! !     ! !
  !     ! !     ! !     ! ! +MT-V8
  !     ! !     ! !     ! +--5
  !     ! !     ! !   +-31   +MT-V9
  !     ! !     ! !   ! !
  !     ! !     ! !   ! !   +MT-H6
  !     ! !     ! !   ! ! +--4
  !     ! !     ! !   ! ! ! +MT-V7
  !     ! !     ! ! +-40 +-13
  !     ! !     ! ! ! !   ! +MT-H7
  !     ! !   +-64 ! ! !   +--3
  !     ! !   ! ! ! ! !    +MT-V10
  !     ! !   ! ! ! ! !
  !     ! !   ! ! +-43 ! +MT-Tb6
+-111      ! !   ! !   ! +--7
! !     ! !   ! !   !   +MT-Tb9
! !     ! !   ! !   !
! !     ! !   ! !   ! +MT-Tb10
! !     ! !   ! !   +--9
! !     ! !   ! !     +MT-V6
! !     ! ! +-83 !
! !     ! ! ! ! ! +-MT-H10
! !     ! ! ! ! +-52
! !     ! ! ! !   +-MT-V5
! !     ! ! ! !
! !     ! ! ! !     +--MT-Tb3
! !     ! ! ! !   +-59
! !     ! ! ! !   ! ! +MT-Tb4
! !     ! ! ! ! +-61 +--8
! !     ! ! ! ! ! !   +MT-H5
! !     ! +-90 ! ! !
! !     !   ! +-82 +--MT-H1
! !     !   !   !
```

Table 10. (cont.)

```
  ! !     !   !   ! +-MT-V1
  ! !     !   !   +-37
  ! !   +-103    !     +-MT-Bfem1
  ! !   ! !   !
  ! !   ! !   ! +--MT-Tb1
  ! !   ! !   ! !
  ! !   ! !   ! !    +-MT-V4
  ! !   ! !   +-70   +-19
  ! !   ! !     ! +-41 +-MT-Cfem5
  ! !   ! !     ! ! !
  ! !   ! !     +-65 +-MT-Cmal3
  ! !   ! !       !
  ! !   ! !       +--MT-Bmal4
  ! !   ! !
  ! !   ! !            +-MT-Pa4
  ! !   ! !          +-25
  ! !   ! !        +-33  +-MT-Pb1
  ! !   ! !        ! !
  ! !   ! !      +-46 +-MT-Taf1
  ! !   ! !      ! !
  ! !   ! !     +-62 +-MT-Taf2
  ! !   ! !     ! !
  ! !   ! !     ! +--MT-Pb2
  ! !   ! !     !
  ! !   ! !     !    +-MT-Pa9
  ! !   ! !   +-84   +-21
  ! !   ! !   ! !   ! +-MT-Tam6
  ! !   ! !   ! ! +-56
  ! !   ! !   ! ! ! ! +-MT-Bfem2
  ! !   ! !   ! ! ! ! !
  ! !   ! !   ! ! ! +-32   +MT-Bfem3
  ! !   ! ! +-91  +-66    ! +-10
  ! !   ! ! ! !   !   +-23 +MT-Bfem4
  ! ! +-106  ! ! !   !     !
+-112  ! ! ! ! ! !   !     +-MT-Bmal3
! ! ! ! ! ! ! !   !
! ! ! ! ! ! ! !   +--MT-Tam5
! ! ! ! ! ! ! !
! ! ! ! ! ! ! +--MT-Tam1
! ! ! ! ! +-94
! ! ! ! !   !   +--MT-Pb4
```

Table 10. (cont.)

```
   ! ! ! ! !   !   !
   ! ! ! ! !   ! +-63   +-MT-Bmal1
   ! ! ! ! !   ! ! ! +-38
   ! ! ! ! !   ! ! ! ! +-MT-Bmal5
   ! ! ! ! !   ! ! +-53
   ! ! ! ! !   ! !   ! +-MT-Bmal2
   ! ! ! ! !   ! !   +-30
   ! ! ! ! !  +-86      +-MT-Tam3
   ! ! ! ! !    !
   ! ! ! ! !    !     +-MT-Bfem5
   ! ! ! ! !    !   +-39
   ! ! ! ! !    !   ! +-MT-Cfem2
   ! ! +-107  !    ! +-58
   ! !   ! !    ! ! ! +MT-Tam2
   ! !   ! !    +-77 +-11
   ! !   ! !      !  +MT-Tam4
   ! !   ! !      !
   ! !   ! !      +--MT-Cfem1
   ! !   ! !
   ! !   ! !    +--MT-Cfem4
   ! !   ! !  +-71
   ! !   ! !  ! +--MT-Cmal2
   ! !   ! ! +-89
   ! !   ! ! ! ! +-MT-Cmal4
   ! !   ! +-95  +-55
   ! !   !   !  +-MT-Taf4
   ! !   !   !
   ! !   !   +--MT-Cmal1
   ! !   !
   ! !   !     +-MT-Pa8
+-114  !   !   +-51
! ! !   ! +-75 +-MT-Pb8
! ! !   ! ! !
! ! !  +-102 +--MT-Pb10
! ! !     !
! ! !     +---MT-V3
! ! !
! ! !       +-MT-B2
! ! !     +-47
! ! !   +-73 +-MT-B7
! ! !   ! !
```

Table 10. (cont.)

```
 ! ! ! +-100 +--MT-B6
 ! ! ! ! !
 ! ! ! ! +---MT-Ta9
 ! ! ! !
 ! ! ! !     +--MT-B9
 ! ! +-110   +-67
 ! !   ! +-97 +--MT-C4
 ! !   ! ! !
 ! !   ! ! +--MT-Pa10
 ! !   ! !
-115 !   +-109   +-MT-C2
 ! !     ! +-35
 ! !     ! ! +-MT-Pb7
 ! !     ! !
 ! !     +-101     +-MT-C3
 ! !       !   +-48
 ! !       ! +-74 +-MT-C6
 ! !       ! ! !
 ! !       +-88 +--MT-C9
 ! !         !
 ! !         +--MT-Ta1
 ! !
 ! !     +mtcage01
 ! !   +-12
 ! ! +-45 +mtcage02
 ! ! ! !
 ! +-72 +-mtcage03
 !   !
 !   +--mtcage04
 !
 ! +---MT-C1
 +-113
   +---MT-Cfem3
```