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Canola Agronomic Research Program Project CA#2000-99-98-08 Final Report
May 31, 2001

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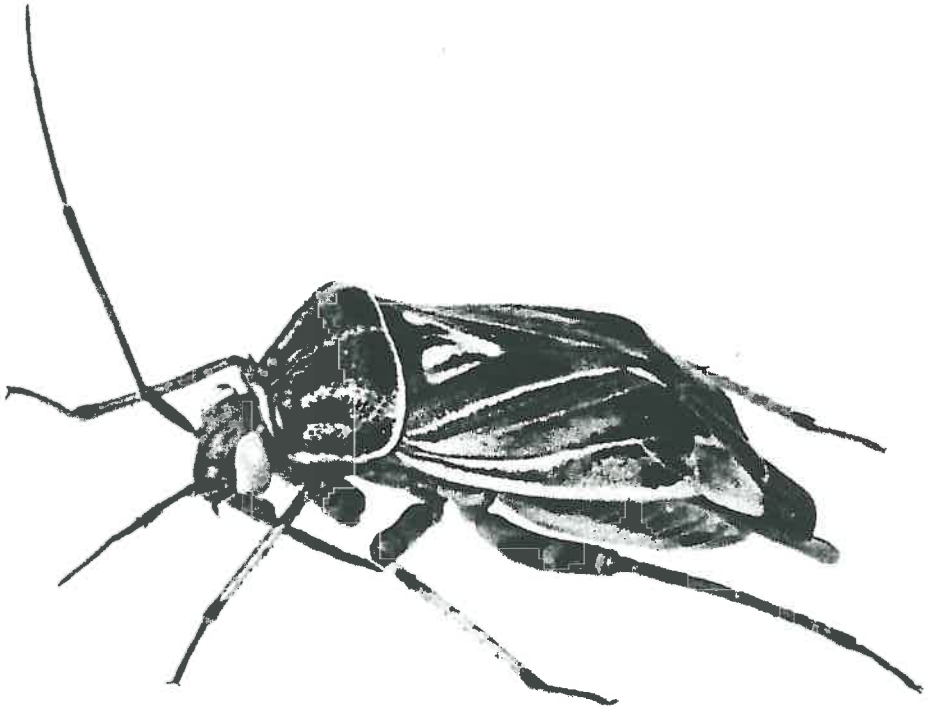

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Canada

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1.0 Abstract

Lygus bugs are increasingly important pests of canola in western Canada. Injury consists of lesions on the surfaces of stems, buds, flowers and pods which causes buds and flowers to abscise and seeds to collapse, reducing the weight of healthy seeds. Feeding activity of *Lygus* bugs can reduce seed yields by as much as 20%. In 1996 approximately 25,000 acres of canola were sprayed in the Vulcan area of Alberta. In 1997 very high numbers of *Lygus* nymphs were reported from a larger area in southern Alberta but also in the Meadow Lake and Spiritwood areas of Saskatchewan. Although damage is extensive chemical spraying is not being recommended because of the significant negative impact of insecticides on beneficial species, particularly insect pollinators essential for facilitating maximum canola yields. Thus, alternative control strategies are being pursued. Several species of wasps belonging to the genus *Peristenus* parasitize *Lygus* nymphs and are promising as biological control agents. To properly assess the impact of *Peristenus* species on *Lygus* populations it is necessary to identify the species but this is difficult because identifying the parasites requires rearing (a complex process requiring 1 year) to the adult stage. Developing a method to identify the immature stages of parasites would facilitate efficient and accurate assessment of parasitism in target *Lygus* populations.

To determine the nature of the recent *Lygus* spp. infestation in canola, season-long sampling of *Lygus* spp. in alfalfa, canola, and mustard fields was undertaken at two Saskatchewan locations for two years. *Lygus lineolaris* followed by *L. borealis* were the dominant species present in canola. Overwintered adult *Lygus* spp. migrated to budding canola where females laid eggs and produced a single generation. Parasitism of *Lygus* spp. in canola was negligible (<1%) because the parasitoid species (*Peristenus pallipes*) that attacks young

Lygus nymphs has a single yearly generation and occurs early in the season, before eggs are laid in canola. In contrast, in alfalfa, young *L. borealis* nymphs are present early in the season and parasitism is relatively high (up to 70%). Introduction of a parasitoid species that has two yearly generations or that occurs when *Lygus* nymphs are present in canola could reduce pest populations.

PCR and microsatellite-based molecular tools were developed to enable identification of genetically distinct populations of *Lygus* and identification of the immature stages of the *Peristenus* species that attack Lygus. Methods were developed for the generation of microsatellite fragments for *Lygus* spp. and *Peristenus* spp. and for the design of specific primers which involves the use of the flanking regions as well as the actual microsatellite tract. By maximizing the information detected in the flanking regions it is now possible to use these markers to detect within-species, population-level, genetic variation, as well as characterization of the same region across a number of species which results in an assessment of relationships among species (a phylogeny, or classification scheme). Application of the microsatellite techniques determined that there are at least two major groups of *Lygus lineolaris*. Further study will clarify the genetic diversity within species, identify specific populations, and facilitate development of a reliable model of the relationships among species.

A one-step PCR diagnostic technique based on species-specific primers was developed to distinguish nymphs of *L. borealis* and *L. lineolaris*. This technology can be used by any diagnostic laboratory versed in simple PCR techniques and provides an unambiguous test to distinguish the two species based on presence or absence of appropriate-sized PCR products. The flexibility of the technique and the DNA sequence analysis of additional *Lygus* spp. indicate that

the technique can be extended to include development of species-specific primers for other *Lygus* spp. commonly found in western Canada. A similar approach successfully developed a technique to distinguish three species of *Peristenus* parasitoids associated with *Lygus* populations in North America. The PCR-based techniques were tested on field collected *Lygus* spp. nymphs from alfalfa fields and proved to be a very useful tool to identify nymphs to species and to detect the presence of *Peristenus* parasitoids. Thus it is now possible to efficiently determine the species make up of field populations of *Lygus* populations and the parasitism rates by *Peristenus*.

The results of this research provide clarification that: *Lygus lineolaris* is the major pest species in canola, there are important seasonal and genetic differences between *Lygus* species and populations of a species, and there is a lack of natural enemy pressure to control *Lygus* in canola. The research developed molecular techniques to: differentiate species and populations within a species of *Lygus*, determine whether *Lygus* nymphs are parasitized, and differentiate species of the parasitoid. The information generated and the techniques developed provide the basis for developing integrated management that targets the specific populations of *Lygus* infesting canola and for introduction and augmentation of biological control agents to provide cost saving natural control of pest Lygus.

2.0 Introduction

In 1996 *Lygus* populations were so abundant in the Vulcan area of Alberta that insecticide spraying was implemented for the first time and approximately 25,000 acres of canola were sprayed (S. Meers, personal communication). In 1997 surveys by provincial agrologists in Alberta and Saskatchewan yielded samples containing up to 200 *Lygus* nymphs/10 sweeps at the

budding and early flowering stages. While chemical insecticides offer a partial solution to the problem there is a substantial risk to the insect species important as pollinators of canola during flowering. Alternative control methods include developing varieties of canola tolerant to *Lygus* attack and exploiting natural enemies as biological control agents. Because *Lygus* species attack a wide variety of plant species there is a need to establish the extent of genetic variability in populations attacking canola. Development of an effective integrated pest management strategy for *Lygus* bugs in canola is an important goal of researchers at Agriculture and Agri–Food Canada.

The plant bugs *Lygus lineolaris* (Palisot de Beauvois), *L. borealis* Kelton and *L. elisus* Van Duzee are pests of canola in western Canada. *L. borealis* and *L. elisus* are most abundant in Alberta and Saskatchewan (Schwartz & Foottit 1995) while *L. lineolaris* is most abundant in Manitoba (Gerber & Wise 1994, Schwartz & Foottit 1995). *Lygus* injury to canola consists of lesions on the surfaces of stems, buds, flowers and pods (Butts & Lamb 1990). Damage causes buds and flowers to abscise and seeds to collapse, reducing the weight of healthy seeds. Feeding activity of *Lygus* bugs reduced seed yields 20%. The highest yield losses were associated with a *Lygus* density of 52 per 10 sweeps (Butts & Lamb 1991). Gerber & Wise (1994) found that in Manitoba *Lygus* bug populations are highest after flowering when nymphs are present and pods are developing. These researchers recommended sampling soon after the end of flowering to best determine the need to control damaging infestations in canola. Wise & Lamb (1996) determined that in Manitoba the economic threshold (ET) for plant bugs in canola at the end of flowering or when upper pods form is 15 plant bugs per 10 sweeps. When pods are fully formed the threshold is 20 plant bugs per 10 sweeps.

Gerber (1996) found that *Lygus lineolaris* has different oviposition preferences on various lines of *B. napus* and that *B. juncea* is not a preferred host plant. Schwartz & Foottit (1992) concluded that there is a wide range of geographic variation within *Lygus* populations that may reflect adaptation to changing natural and agricultural environments. Identification of *Lygus* populations more prone to feed on canola than other populations will provide the technology to facilitate more precise targeting of tolerant crop variety development.

The use of biological control agents is an important alternative control strategy for regulation of pest insects. Successful implementation of these biological agents can provide the technology that will maintain population levels of the target (pest) species below economic levels (Debach & Rosen 1991). Input costs for producers will be reduced as a result. Several species of parasitic wasps belonging to the genus *Peristenus* attack and kill *Lygus* nymphs in North America and Europe. In western Canada *P. pallipes* is the only species found in *Lygus* populations (Craig & Loan 1987). In Europe there are three species, *P. digoneutis*, *P. stygicus* and *P. rubricollis* that attack *Lygus rugulipennis*, the most important pest species (Craig & Loan 1984). Of these species *P. digoneutis* has been successfully established in the eastern U.S. for control of *L. lineolaris* in alfalfa (Day *et al.* 1990). Introduction and establishment of *P. digoneutis* in western Canada would augment the levels of control by the existing *P. pallipes*. Because identifying the parasites requires rearing (a complex process requiring 1 year) to the adult stage, there is a need to develop a method for identifying the immature stages of parasites to facilitate accurate assessment of parasitism in target *Lygus* populations. (Soroka 1996). The ability to identify immature stages of the parasites would enable rapid and cost effective assessment of parasite effectiveness.

Over the past 25 years molecular techniques have been used to distinguish genetic differences between plant and animal species and populations within a species (Loxdale 1994). New molecular technologies such as automated DNA sequencing machines have the potential for producing routine finger prints of insect samples once informative gene sequences have been identified. This rapidly evolving field has seen numerous technological improvements that have enabled sharper focus on genetic differences with each new development. Molecular characters have proved useful to separate morphologically similar species and have the potential to define regional populations (Foottit & Schwartz 1996).

3.0 Objectives

The overall project objective was to develop a routine assay and diagnostic key to identify populations of *Lygus* in western Canada and immature stages of *Peristenus* species.

Specific objectives were:

1) examine the genetic composition of populations of *L. lineolaris* and *L. borealis* attacking different canola varieties in western Canada and develop unique molecular markers for these populations;

2) to develop unique molecular markers for species of *Peristenus* parasites attacking *Lygus* species;

3) to develop a protocol (technology) that can be used by non-specialists for documenting molecular patterns;

4) to develop a diagnostic key based on molecular characters for use by non- specialists to identify populations of *L. lineolaris* and *L. borealis*;

5) to develop a diagnostic key based on molecular characters for use by non specialists to identify immature stages of *Peristenus* species.

4.0 Host crop-Lygus-parasitoid associations

To clarify the phenology of *Lygus* spp. attacking canola and associated crops, and to determine which generations and *Lygus* spp. were attacked by parasitoids surveys were made. Field collections of overwintering and summer adults, and nymphs of *Lygus* spp. were made weekly at two locations in Saskatchewan from mid-April to late August in 1998 and 1999. Five sites were sampled, including alfalfa *Medicago sativa* L. (Leguminosae), mustard *Sinapis alba* L. (Brassicaceae), and canola *Brassica napus* L. (Brassicaceae) at Vonda, and alfalfa and canola at Saskatoon.

Collections were made at each of 10 locations per site through the top third of the foliage using a standard 38 cm diameter insect sweep net. Samples were collected at approximately the same location in the field every week, fanning out from the field edge, with 10 m between samples. Captured *Lygus* spp. were sorted into groups: nymphal instars L1-L3 and L4-L5, and adults, the latter identified to species according to Schwartz & Foottit (1992*b*). Randomly selected nymphs (L1-L3 or L4-L5) from each sample were dissected (to a maximum of 50/sample) to estimate parasitism. Each nymph was dissected under a microscope to determine the presence of a parasitoid. Plant development of canola and mustard was assessed using Harper & Berkenkamp (1975): seedling, stage 1; rosette, stage 2; bud, stage 3; flower, stage 4; and, pod ripening, stage 5. On each sampling date a growth stage score was recorded for each field on the basis of the predominant growth stage.

Data recorded included for each site and sampling date included: total number of *Lygus* spp. adults and nymphs, the ratios of parasitized nymphs per number of dissected nymphs (p/d), and the resulting estimated parasitism for L1-L3 and L4-L5 nymphs (Table 4.1). Weather station data from Saskatoon was used to calculate accumulated degree day units (base 10°C) at Saskatoon in 1998 and 1999.

4.1 *Lygus* species composition and seasonal occurrence

4.1.1 Alfalfa

In alfalfa, three main species, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae), *Lygus borealis* (Kelton), and *Lygus elisus* Van Duzee were found in samples collected in 1998 and 1999 (Table 4.1). At Vonda, in both years the adult population of the early, mid- and late season was composed predominately of *L. lineolaris*, *L. borealis*, and *L. elisus*, respectively (Fig. 4.1). In contrast to the warm spring conditions of 1998, the cool spring of 1999 (Fig. 4.6) delayed and extended the emergence of adult *Lygus* spp. from their overwintering sites. At Saskatoon, *Lygus* species composition was similar to that at Vonda although the seasonal occurrence differed slightly: in 1998, early season populations of *L. borealis* at Saskatoon were more numerous than *L. lineolaris* (Figs. 4.1a, 4.2a). The population phenology differed between the two years at Saskatoon because of the absence of a second adult population peak of *L. borealis* in 1999 (Fig. 4.2b), suggesting that first generation *L. borealis* adults entered reproductive diapause. This can be explained by the delayed occurrence of the population peak of first generation *L. borealis* in 1999 (30 June 1998 versus 13 July 1999). On to two generations of *Lygus* spp. can occur annually in Saskatchewan depending on location (Craig 1983) and

variability in the yearly temperature regime (this study Fig. 4.6).

The two peaks of *L. borealis* in alfalfa in Vonda in 1998 and Saskatoon in both years are consistent with the occurrence of two generations of *L. borealis* (Figs. 4.1a, 4.2a, 4.2b), although characterization of egg development in adult females is necessary to differentiate between overwintering and first generation adult populations (Gerber 1996). In both years at Saskatoon, and in 1998 at Vonda, two distinct peaks of *Lygus* spp. nymphs occurred, indicating that as observed by Gerber & Wise (1995) in Manitoba, *L. borealis* nymphal populations in Saskatchewan alfalfa do not overlap (Figs. 4.1a, 4.2a, 4.2b). The two smaller peaks of nymphs observed in Vonda alfalfa in 1999 support the occurrence of two generations (Fig. 4.1b). The reduced second peak of adult *L. borealis* in alfalfa at Vonda in 1999 (Fig. 4.1b) is likely due to high nymphal parasitism in late June (Table 4.1).

Lygus lineolaris was probably univoltine in alfalfa in and near Saskatoon in 1998 and 1999 based on our findings, although we were unable to determine if only one generation was completed, if adults left the alfalfa field, or if adults died. This contrasts with findings in southern Manitoba where two non-overlapping generations of *L. lineolaris* occurred on alfalfa at Winnipeg (Timlick *et al.* 1993; Gerber & Wise 1995). There; overwintering adults were present from early May until the last two weeks of July, when they were rapidly replaced by first-generation adults; second generation adults were present from the first half of August until mid-October (Gerber & Wise 1995). In eastern Ontario, the egg-laying period for first-generation *L. lineolaris* females was 6-7 weeks, from early July until late August (Painter 1929). Craig (1983) found that at Saskatoon, about 50% of first-generation adults become reproductively active without diapause and produce a second generation. In our study, the lack of a mid-season adult *L.*

lineolaris peak in alfalfa in 1998 and 1999 implied only one generation; Stewart & Gaylor (1991, 1994) and Gerber & Wise (1995) both noted that young parous *L. lineolaris* females readily disperse and are inclined to colonize new host plants.

There was no evidence to suggest that cutting the alfalfa stand resulted in emigration of *Lygus* from the field. Indeed, in 1998 at Vonda adult numbers increased after the second cut.

4.1.2 Canola

In canola, *L. lineolaris*, *L. borealis*, and *L. elisus* were the dominant species collected in 1998 and 1999 (Table 4.1). This corresponded to results from alfalfa fields, however the abundance of adults and nymphs in canola were considerably lower than in alfalfa at each location in each year. At Vonda, *L. lineolaris* was the predominant species in both years (Fig. 4.3). At Saskatoon, *L. borealis* dominated in 1998 and *L. lineolaris* in 1999 (Fig. 4.4).

Only one generation of *Lygus* spp. occurred in canola (Figs. 4.3, 4.4). An initial peak of immigrant adults was detected in late June or early July, coinciding with bud formation, the canola plant stage preferred by *Lygus* spp. (Butts & Lamb 1990; 1991a). Female *Lygus* spp. migrating to canola have fully developed eggs and oviposit immediately upon arrival (Gerber & Wise 1995; Otani 2000). At Vonda in 1998, the canola field was adjacent to the sampled alfalfa field and the decrease in adults in alfalfa observed between 30 June and 7 July (Fig. 4.1a) coincided with an increase in numbers of adults in canola for the same period. However, the species composition of adults collected was markedly different, the predominant species in canola was *L. lineolaris* whereas the adult population in alfalfa was *L. borealis*. The same phenomenon occurred at Vonda again in 1999 and at Saskatoon in 1998. Butts & Lamb (1991*a*) suggested that the most likely source of *L. lineolaris* populations invading canola is cruciferous

weeds.

4.1.3 Mustard

In mustard, *L. lineolaris* and *L. borealis* were found in fields sampled in 1998; *L. elisus* was also found in 1999 (Table 4.1). Only one *Lygus* generation occurred at the Saskatchewan sites sampled in 1998 and 1999, and numbers of both nymphs and adults collected were considerably lower than in alfalfa. As in canola, there was no indication of an overwintering population of *Lygus* spp. in mustard but, rather, incoming *Lygus* spp. adults entered the crop when it was at four-leaf to bud stage (Fig. 4.5). One generation of nymphs occurred as indicated by the single peaks for L1-L3 and L4-L5 in 1998 (mid-July) and 1999(early August) (Fig. 4.5).

4.2 Parasitism of *Lygus* nymphs

In alfalfa, parasitoids were first found in nymphs collected one (at Vonda) to two (at Saskatoon) weeks after the appearance of *Lygus* spp. nymphs in 1998 (Figs. 4.1a, 4.2a). At Vonda, parasitized nymphs were found only in samples collected until 14 July 1998, despite the presence of a second nymph population beginning in early August (Fig. 4.1a). Parasites were recovered from *Lygus* spp. nymphs collected in Saskatoon from 17 June to 11 August, with peak collections on 30 June and 21 July (24% and 36% parasitized, respectively) (Fig. 4.2a). In 1999, parasitized nymphs were collected from alfalfa fields at both Vonda and Saskatoon from 22 June until 3 August. Parasitism reached a maximum of 70% at Vonda on 30 June (Fig. 4.1b); parasitism in Saskatoon was similar to that in 1998 (28% on 6 July) (Fig. 4.2b).

In canola and mustard, the low parasitism of nymphs (Table 4.1), even in those located immediately adjacent to alfalfa, where parasitism was high, is conspicuous.

Adult female parasitoids were present only in early summer (late May to early June) and parasitize early instar nymphs that are present in high numbers in alfalfa (Figs. 4.1, 4.2) but have not yet appeared in canola and mustard (Figs. 4.3, 4.4, 4.5). This suggests the presence of a univoltine parasitoid such as *P. pallipes*, previously reported by Loan & Craig (1976). Because the parasitoid occurs only early in the season, estimated parasitism expressed as a total over the season (last column in Table 4.1) severely underestimates the actual impact of the parasitoid.

Because the majority of first generation adults collected in alfalfa are *L. borealis*, it is likely that the majority of nymphs parasitized are *L. borealis.* Further, because our results suggest that a single generation of *L. lineolaris* occurred in 1998-1999, that overwintered *Lygus* spp. oviposit over a prolonged time period (Kelton 1975), and that parous *L. lineolaris* females readily disperse to new host plants (Stewart & Gaylor 1991, 1994; Gerber & Wise 1995), overwintering adult *L. lineolaris* probably moved into budding canola in late June to complete oviposition. Thus, adult *P. pallipes* are not present when suitable nymphs (L1-L3) of *L. lineolaris* are available in canola. The introduction of a bivoltine parasitoid, (*e.g.*, the European *Peristenus digoneutis* Loan and/or *P. stygicus* Loan), or a late season (summer) species, (*e.g.*, the eastern *Peristenus pseudopallipes* Loan), to parasitize second generation nymphs could possibly increase overall parasitism of *Lygus* spp. nymphs in canola.

4.3 Discussion

Lygus lineolaris is most likely responsible for economically damaging populations in canola in Saskatchewan. Parasitism of *Lygus* spp. nymphs in alfalfa ranged up to 70%, whereas parasitism in canola and mustard fields was negligible (<1%). Additionally, parasitism in alfalfa

was associated only with the occurrence of the nymph population in mid-June nymphal, indicating that a univoltine nymphal parasitoid was responsible for observed parasitism. An important next step is clarifying whether low *Lygus* parasitism in canola is the result of host-parasitoid temporal asynchrony or caused by specific host-parasitoid incompatibility.

Table 4.1. Total numbers of nymph and adult *Lygus* spp., and estimated % parasitism of *Lygus* spp. nymphs, collected by sweep netting three crops in Saskatchewan in 1998 and 1999.

			Adults				Nymphs						
							L1 - L3			L4 - L5			
Crop	Year	Site	*L. lineolaris*	*L. borealis*	*L. elisus*	other spp.	No. collected (n)	No. dissected (n)	parasitism (%)	No. collected (n)	No. dissected (n)	parasitism (%)	Total % parasitism
Alfalfa	1998	Vonda 'Beaver' 52°18'N, 106°8'W	966	924	3	2	277	57	9	1068	377	12	12
	1999	Vonda 'Beaver' 52°18'N, 106°8'W	1491	561	6	0	301	160	38	863	430	24	28
	1998	Saskatoon 'AC Graceland' 52°9'N, 106°34'W	246	528	8	0	100	39	10	395	246	15	14
	1999	Saskatoon 'Beaver' 52°9'N, 106°35'W	871	559	69	0	466	182	7	1124	443	10	9
Canola	1998	Vonda '46A73' 52°19'N, 106°8'W	46	8	2	0	18	17	0	69	69	0	0
	1999	Vonda '46A73' 52°18'N, 106°8'W	345	23	1	0	64	53	0	268	179	1	1
	1998	Saskatoon 'AC Excel' 52°9'N, 106°34'W	73	122	65	1	46	36	0	227	124	0	0
	1999	Saskatoon 'AC Excel' 52°9'N, 106°34'W	62	20	2	0	66	55	0	87	77	0	0
Mustard	1998	Vonda 'AC Pennant' 52°18'N, 106°3'W	10	8	0	0	26	26	0	48	47	0	0
	1999	Vonda 'AC Pennant' 52°18'N, 106°4'W	34	8	2	0	22	22	0	23	23	0	0

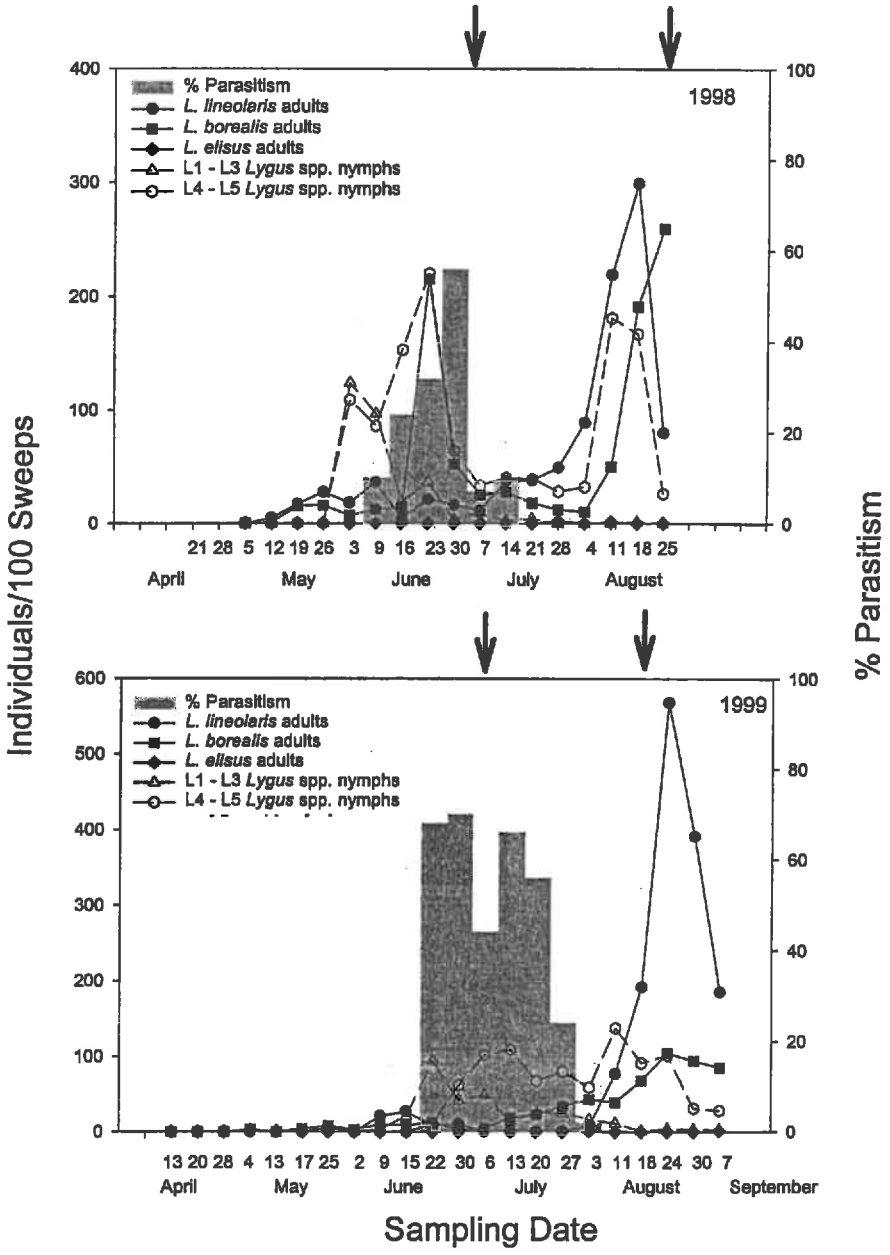

Figure 4.1. The total number adults and nymphs (10 sweeps/10 locations), and estimated parasitism (%) of *Lygus* spp. collected in alfalfa (*Medicago sativa*) at Vonda, Saskatchewan in 1998 and 1999. Arrows indicate crop harvest dates.

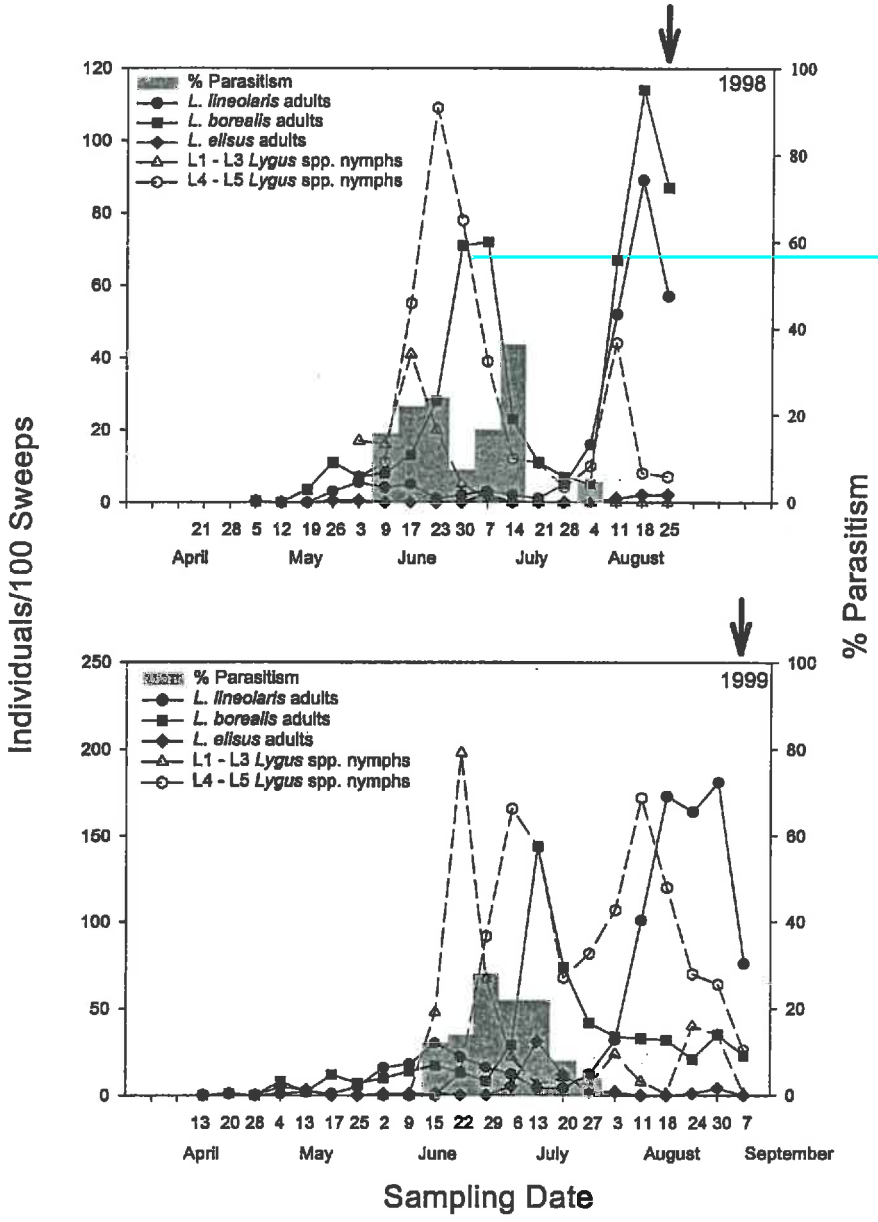

Figure 4.2. The total number adults and nymphs (10 sweeps/10 locations), and estimated parasitism (%) of *Lygus* spp. collected in alfalfa (*Medicago sativa*) at Saskatoon, Saskatchewan in 1998 and 1999. Arrows indicate crop harvest dates.

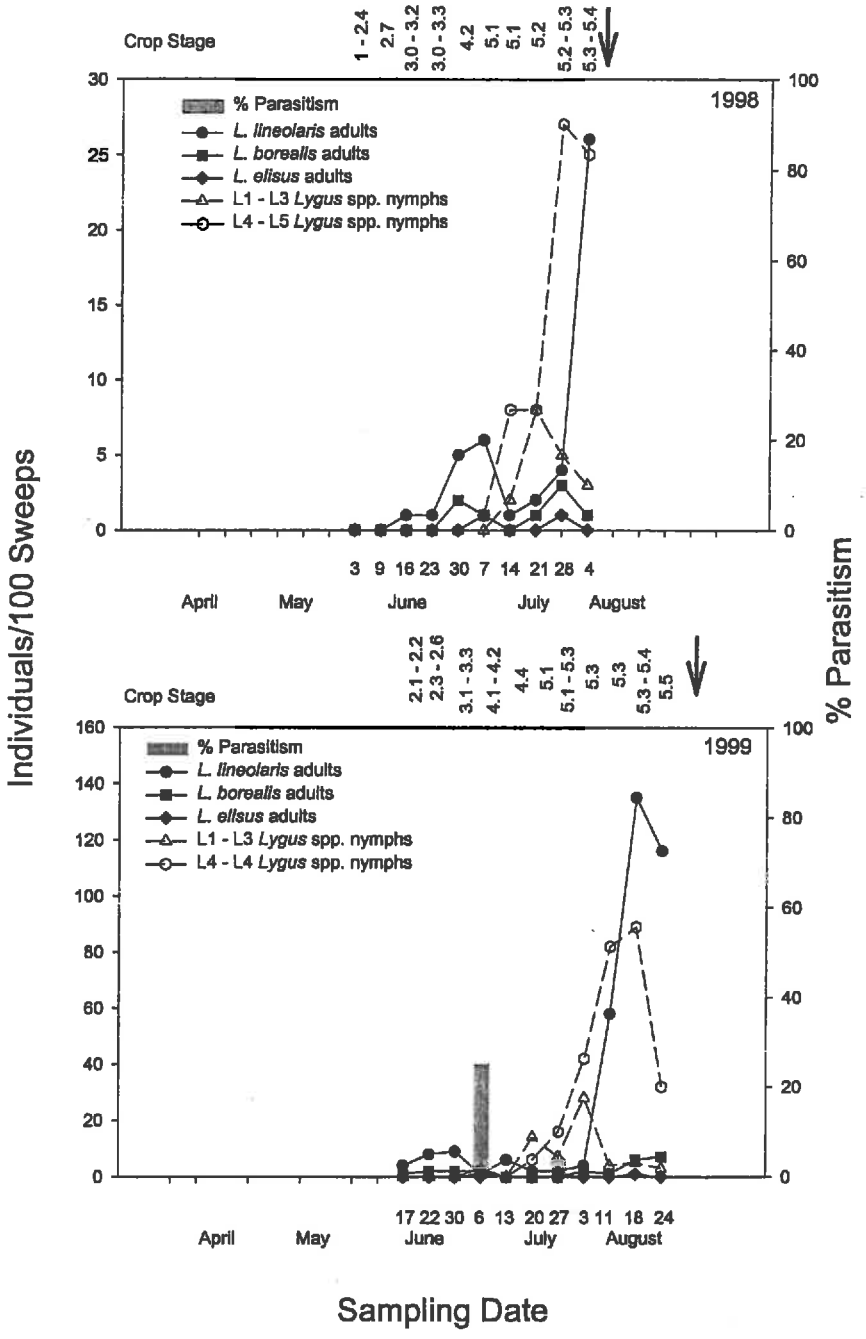

Figure 4.3. The total number adults and nymphs (10 sweeps/10 locations), and estimated parasitism (%) of *Lygus* spp. collected in canola (*Brassica napus*) at Vonda, Saskatchewan in 1998 and 1999. Arrows indicate crop harvest dates. Crop stage on top axis according to Harper and Berkenkamp (1975).

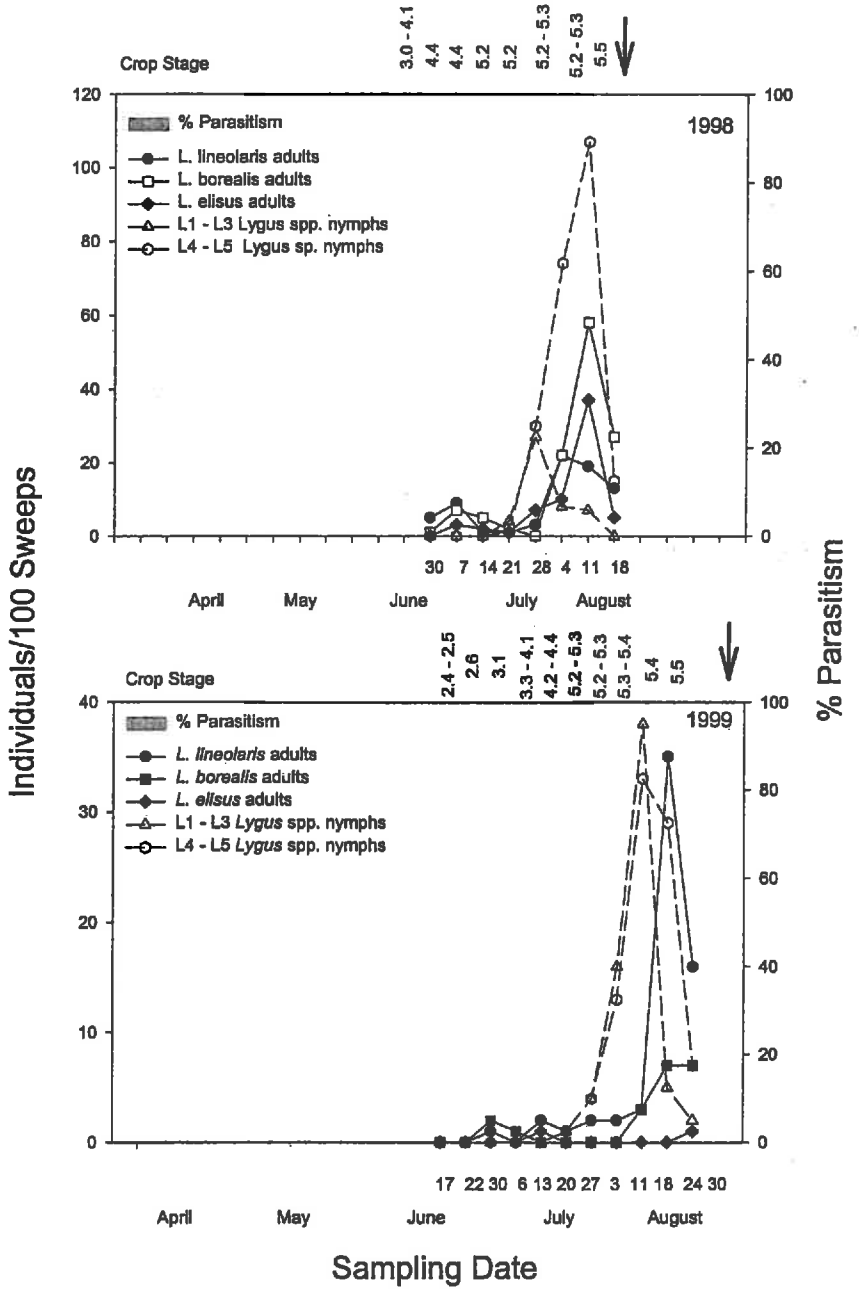

Figure 4.4. The total number adults and nymphs (10 sweeps/10 locations), and estimated parasitism (%) of *Lygus* spp. collected in canola (*Brassica napus*) at Saskatoon, Saskatchewan in 1998 and 1999. Arrows indicate crop harvest dates. Crop stage on top axis according to Harper and Berkenkamp (1975).

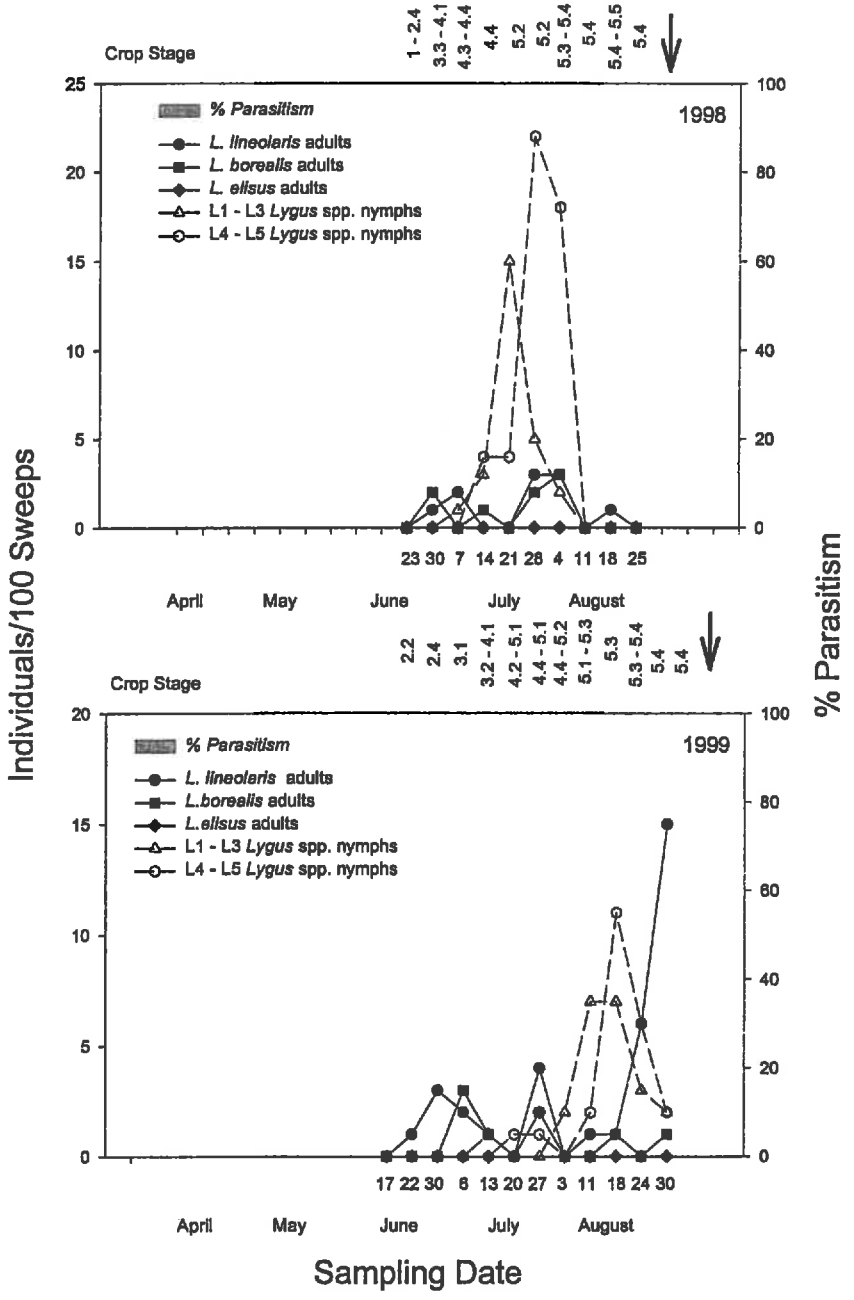

Figure 4.5. The total number adults and nymphs (10 sweeps/10 locations), and estimated parasitism (%) of *Lygus* spp. collected in mustard (*Sinapis alba*) at Vonda, Saskatchewan in 1998 and 1999. Arrows indicate crop harvest dates. Crop stage on top axis according to Harper and Berkenkamp (1975).

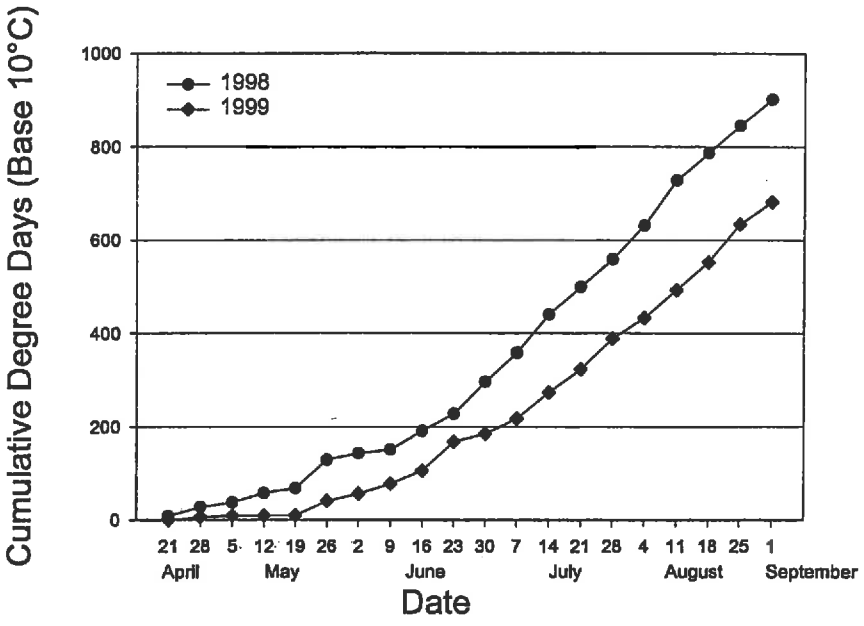

Figure 4.6. Accumulation of degree day units (Lygus developmental threshold 10°C) at Saskatoon, 1998 and 1999.

5.0 Genetic composition of *L. lineolaris* and *L. borealis* populations

5.1 Protocol for microsatellite locus identification and primer development

5.1.1 DNA isolation

Total nucleic acids sufficiently pure for library construction was obtained using a CTAB (hexadecyltrimethyl ammonium bromide: Sigma) extraction procedure. Two to four hundred mg of insect material was partitioned into six 1.5ml Eppendorph microcentrifuge tubes. Tissues were first homogenized in 100 µl of 2X CTAB extraction buffer (55 mM CTAB, 1.4 M NaCl, 50 mM Tris pH 7.5, 20 mM EDTA, 0.2% v/v beta-mercaptoethanol) using sterile disposable plastic mortars (Fisher Scientific). The final volume of each tube was adjusted to 800 µl with 2X CTAB buffer and then incubated for two hours at 65°C. Six hundred µl chloroform was added to each tissue lysate, tubes were gently pulse vortexed for one to two seconds, then centrifuged at 14000 RPM @ 4°C for 10 minutes using an Eppendorph microcentrifuge. The upper aqueous phase was transferred to a new 1.5 ml tube containing 600 µl phenol/chloroform 1:1, tubes were briefly mixed by hand then incubated for 20 minutes on a rocker platform. Samples were centrifuged for 10 minutes at 14000 RPM @ 4°C, the upper aqueous phase decanted and re-extracted with 600 µl chloroform. Eight hundred µl isopropanol was added to the final aqueous phase, tubes were mixed again by gentle hand agitation, then incubated at -20°C for one hour. Total nucleic acids were pelleted by centrifugation at 9000RPM @ 4°C for 15 minutes, then washed three times in 1 ml 70% ethanol, centrifuging at 9000RPM @ 4°C for 5 minutes for each wash. Nucleic acids were dried under vacuum, then dissolved overnight at 4°C in 50 µl low TE (1 mM Tris pH 7.5, 0.1 mM EDTA) containing one µl 1mg/ml RNase (Boehringer).

5.1.2 Library construction

Twenty mg (10 µl of extraction) total DNA was digested for 3 h at the recommended temperature in a 40 µl final reaction volume containing 15 units *Tsp509 I* or *EcoRI* restriction enzyme and 1X digest buffer 1 (New England Biolabs). The restriction digest was then extracted by the addition of 200 µl water and 200 µl phenol/chloroform 1:1, incubated on a rocker platform for 10 minutes and centrifuged for 10 minutes at 14000 RPM @ 4°C. The upper aqueous phase was decanted and extracted twice with 300 µl chloroform, centrifuging for 5 minutes at 14000 RPM @ 4°C for each chloroform extraction. DNA digest products were precipitated from the final aqueous phase by the addition of 20 µl 4 M sodium chloride and 600 µl absolute ethanol and incubating at -20°C for 4 hours. Precipitated DNA was pelleted by a 10 minute centrifugation at 14000 RPM @ 4°C and washed three times with 1ml 70% ethanol, centrifuging for 5 minutes at 14000RPM @ 4C for each wash. DNA was dried under vacuum, then dissolved overnight at 4°C in 20 µl low TE. The concentration of DNA was quantified on a spectrophotometer and diluted accordingly in low TE to a final working concentration of 100 ng/ µl.

Microsatellite libraries were generated using the Lambda ZAP II / EcoRI / CIAP-Treated Vector cloning system (Stratagene). One hundred ng of restriction-digested target DNA was ligated to 1 µg ZAP-II phage arms in a 10 µl final reaction containing 400 units T4 DNA ligase (NEB) and 1X final concentration ligation buffer. Ligation reactions were incubated at 12°C for 12 to 16 hours then stored on ice until packaging. Four µl of ligation reaction was added to one tube Gigapack III Gold packaging extract, tube contents mixed by very gentle pipetting, then incubated for 2 hours at 22°C. The packaging reaction was terminated by the addition of 500 µl

SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris pH 7.5, 0.01% w/v gelatin) and 25 µl chloroform followed by a mild pulse vortexing. XL1-Blue MRF' bacterial cell line (Stratagene) was used as host for phage growth. Host cells were prepared for phage infection in accordance to manufacturer's recommendations. A single colony of XL1-Blue MRF'cells was inoculated into 12 µl LB containing 10mM magnesium chloride and 0.2% maltose and grown for 14 hours at 37°C in a Brunswick shaker incubator at 250 RPM. Cells were then pelleted by a 10 minute centrifugation at 6000 G and resuspended in 12 ml TM (50 mM Tris pH 7.5, 10 mM $MgSO_4$) buffer, re-incubated at 37°C for an additional 30 minutes then stored at 4°C until use. Immediately prior to phage infection, XL1-Blue MRF' bacterial host cells were diluted to an OD_{600nm} of 0.5 to 0.6 with TM buffer. The library titre was quantified by infecting 300 µl diluted XL1-Blue MRF' cells with 100 µl of a 10-fold to 1000-fold dilution series of the SM packaging reaction in 15 mm X 100 mm glass culture tubes. After a 15 minute incubation at 37°C, 3 µl top NZY agar pre-warmed to 50°C was added to the infection culture tube, the contents gently mixed, then poured onto 100 X 15 mm NZY plates. Plates were incubated for 14 hours at 37°C.

The titre of the library was determined and the necessary volume to plate 50,000 plaque forming units (pfu) calculated and used to infect six culture tubes containing 600 µl freshly diluted (OD_{600nm} 0.5 to 0.6) XL1-Blue-MRF' cells. Following infection for 15 minutes at 37°C, 8 ml NZY top agarose was added to the tube, the contents gently mixed, then poured onto 150 X 15 mm NZY plates. Plates were incubated at 37°C for 14 hours, then cooled to 4°C for one hour. Plaque lifts were performed in duplicate using Hybond N+ (Amersham/Pharmacia) nylon membrane, the first lift transferred for 1 minute and the replicate membrane transferred for three minutes. Membranes denatured for 5 minutes on Whatman 3MM filter paper pre-soaked in

denaturing solution (0.5 N NaOH, 1.5 M NaCl), neutralized twice for 3 minutes Whatman 3MM paper pre-soaked in neutralizing solution (1.0M Tris pH 8.0, 1.5 M NaCl) and finally washed for 2 minutes on Whatman 3MM paper pre-soaked in 2X SSC solution (300 mm NaCl, 30 mm sodium citrate pH 7.0). Membranes were dried at 70°C for 30 minutes then U.V. cross-linked in a UVP CL-1000 Ultraviolet Crosslinker preset to 120 000 microjoules/cm^2. Membranes were prehybridized at 30°C for three hours in prehybridization buffer (5X SSPE, 5X Denhardt's, 0.1% SDS), then hybridized overnight at 30°C following the addition of labelled oligonucleotide probes (probe preparation described below). Membranes were washed twice at room temperature for 15 minutes in 2X SSC/ 0.1% SDS, briefly blot dried with Whatman filter paper, wrapped in Saran wrap and autoradiographs generated by exposing Fugi-NIF Rx X-ray film for 48 hours at -80°C using Dupont Cronex Hi-Plus intensifying screens. Plaques containing microsatellites target inserts were pulled (4mm diameter plugs) from the plates and transferred to 1.5 ml Eppendorph tubes containing 500 µl SM buffer and 25 µl chloroform.

Cloned target inserts were excised from the purified phage in pBluescript SK- phagemid using the SOLR excision plating strain as described by Stratagene. Fifty µl secondary purified stock plug elution was added to 200 µl freshly grown and diluted XL1-Blue MRF' cells (OD_{600nm} of 1.0) containing 1 µl ExAssist helper phage and infected for 15 minutes at 37°C. Three µl LB was added and tubes were incubated for 3 hours at 37C in a New Brunswick shaker- incubator at 250 RPM, then heated to 68°C for 20 minutes in a water bath and placed on ice until centrifugation. Tubes were centrifuged at 6000 G for 10 minutes and the supernatant decanted into Falcon 2059 centrifuge tubes and stored at 4°C. Five µl phage lysate was used to infect 200 µl SOLR cells (OD600nm 1.0) for 15 minutes at 37°C, 300 µl NZY media was added followed

by 45 minutes of growth at 37°C in a shaker-incubator. Five µl of each phagemid excision was plated onto LB ampicillin plates and grown at 37°C for 14 hours. Plates were stored at 4°C until liquid culturing of bacterial colonies. One colony from each excision was used to innoculate 3 ml LB-ampicillin (100 µg per ml) media and grown for 14 hours at 37°C in a shaker incubator at 250 RPM. Glycerol stocks for long term -80°C storage of phagemid material were prepared by aliquoting one ml liquid culture into 2ml screw cap cryovials containing 500 µl sterile glycerol. The balance of the liquid culture was poured into 1.5 ml Eppendorph tubes, cells pelleted at 14000 RPM for 1 minute, supernatant decanted and the cells suspended in 200 µl buffer (50 mm glucose, 25 mm Tris pH 7.5, 10 mm EDTA). Four hundred µl cracking solution (0.2N NaOH, 1.0% SDS) was added, tubes incubated on ice for 20 minutes, 300 µl 7.5 M ammonium acetate added, mixed and again incubated on ice for 20 minutes. Samples were then centrifuged at 14000 RPM for 10 minutes, the supernatant transferred and extracted once with 300 µl phenol chloroform 1:1 and once with 500 µl chloroform. Phagemid DNA was precipitated from the upper aqueous phase by the addition of 700 µl isopropanol and incubation at -20°C for 2 hours. DNA was pelleted at 14000RPM for 10 minutes at 4°C and washed three times in 1ml 70% ethanol. Pellets were dried in a vacuum desiccator then dissolved overnight at 4°C in 50 µl low TE and 1 µl 1mg/ml RNase.

Cloned inserts were released from the pBluescript phagmid vector by *EcoRI* restriction digest of 2 µl plasmid DNA and fragments separated on 1% agarose gels. Microsatellite inserts were confirmed by Unblot hybridizations as follows: gels were denatured for 30 minutes in denaturing solution (0.5 N NaOH, 1.5 M NaCl) and neutralized for 30 minutes in neutralizing solution (1.0M Tris pH 8.0, 1.5 M NaCl), rinsed in water and dried onto Whatman 3MM filter

paper for 2 hours at 62°C using a model 583 gel drying unit (Bio-Rad). The dried gels were removed from the filter paper by soaking the gel/filter paper in water for 2 minutes and pealing away the filter paper. The gel was washed thoroughly to remove residual filter paper and placed immediately in 25 µl hybridization solution containing 5X SSPE/ 0.1% SDS and ^{32}P end-labelled oligonucleotide probe. Gels were hybridized for 14 hours at 30°C, washed twice for 5 minutes in 3X SSC/ 0.1% SDS, blot dried on Whatman filter paper, wrapped in saran wrap and exposed to Fuji NIF-Rx film for 3 hours at room temperature. Clones containing microsatellite inserts ranging in size from 0.7 kilobase pairs (Kb) to 2Kb were set aside for sequencing. Microsatellite-containing inserts greater then 2Kb were digested with a variety of restriction enzymes and subsequently unblotted to confirm the release of the microsatellite loci into fragments within the 0.7Kb to 2Kb size range. Two µg of the phagemid preparation was digested with 20 units of the appropriate restriction enzyme or combination of restriction enzymes for 3 hours at the temperature and buffer conditions recommended by the manufacturer. Restriction enzyme(s) were heat inactivated at 80°C for 10 minutes and cooled on ice for 2 minutes. Restriction products were blunt ended by addition of 1 µl 2mM dNTP's (Boehringer), 0.5 µl 10X buffer 1 (NEB), 3 µl distilled water, 0.5 µl (2.5 units) DNA polymerase I, Large (Klenow) fragment (NEB) and incubating at 25°C for 15 minutes. Samples were immediately loaded onto 1% agarose gels and electrophoresed at 80 volts for 2.5 hours in 1X TBE buffer. Target bands were excised from the gel and electroeluted from the agarose gel matrix as follows: gel slice was inserted into a 3 cm strip of Spectra/Por dialysis membrane (MWCO 12-14,000: 6.4 mm diameter: Spectrum Scientific), 400 µl 0.5X TBE added followed by electrophoresis for 2 hours at 170 volts in 0.5X TBE. The fluid from the dialysis tubing was transferred to a 1.5ml

eppendorph tube containing 30 µl 4M NaCl, 0.5 µl 20mg/ml glycogen (Boehringer) and 1ml absolute ethanol, mixed and incubated overnight at -20°C. DNA was pelleted by a 10 minute centrifugation at 14,000 RPM, then washed three times in 70% ethanol. Pellets were dried under vacuum to near dryness and dissolved overnight in 20 µl low TE. The concentration of DNA was determined by electrophoresing 4 µl of this purified target DNA against a low molecular weight mass ladder (Gibco-BRL). Three to 7 µl electroeluted DNA was added to 100 ng EcoRV LITMUS-28 (NEB) plasmid vector treated with calf intestinal phosphatase, 1 µl 10X NEB ligase buffer, 0.8 µl T4 ligase enzyme (NEB) and water added to yield a final volume of 10 µl. Ligation reactions were incubated at 16°C for 14 hours. Three µl of the ligation reaction was added to 50 µl TOP-10 electrocompetent cells (Invitrogen), incubated on ice for 5 minutes then electroporated in 1 mm gap electroporation cuvettes (Molecular BioProducts) at 1.4kv and 129 ohms resistance. One ml LB was added to the cuvette, the contents transferred to a 16 X 100 mm sterile culture tube and incubated at 37°C in a shaker-incubator for 45 minutes. Fifty µl, 250 µl and 600 µl LB transformation was spread onto LB + ampicillin + IPTG + x-gal plates, and grown for 14 hours at 37°C. Six white colonies from each transformation experiment were grown up in 3ml LB + ampicillin cultures, glycerol stocks made and plasmid DNA isolated as previously described. Cloned inserts were excised from the Litmus 28 vector by Xho-I / Xba-I restriction digest and confirmed to be microsatellite containing sequences by Unblot hybridization.

5.1.3 Oligonucleotide probe preparation

Two oligonucleotide sequences (5' CACACACACACACAC 3' and 5' GAGAGAGAGAGAGAG 3') synthesized by Gibco BRL were diluted in HPLC grade water (Caledon) to a working concentration of 10 pmoles/µl. Oligonucleotides were end-labelled with

^{32}P using a polynucleotide kinase (PNK) reaction. Ten pmoles of each oligonucleotide were aliquoted into a 1.5 ml eppendorph tube containing 1.5 µl 10X PNK buffer (NEB), 10 units T4 polynucleotide kinase (NEB), 5 µl gamma P32 ATP at 6000 µCi/mmol (Dupont) and 5.5 µl water. Reactions were incubated for 3 hours at 37°C then heated to 100°C for 5 minutes, cooled on ice for 2 minutes and added directly to the hybridization mix without purification of unincorporated label.

5.1.3 Primer design and testing

Plasmid preparations containing microsatellite tracks were sequenced by a commercial service (Canadian Molecular Research Services, Ottawa) and the sequence information used to design primers using Lasergene PrimerSelect software (DNAStar Inc.). Initial primer sets were designed to cross the microsatellite track. Subsequently, additional PCR primers were designed to specifically examine sequence variability in the regions flanking the microsatellite. Sequencing reactions generally proceed more efficiently if the sequencing primers are situated away from the ends of the PCR product. Hence, additional nested primers were developed to be used in sequencing reaction.

Primer sets were then tested for specificity and product yield. Approximately 100 ng of template DNA was added to 25 µl reaction mix (2.5 µl 10x reaction buffer [Boehringer Mannheim], 1.5 to 2 units Taq polymerase [Boehringer Mannheim], 2.5 mm $MgCl_2$, 40 µM each primer, 400 µM each dNTP), and amplified in a Thermolyne Amplitron II thermocycler under the following conditions: 27 cycles of denature at 94°C for 1 minute, primer annealing at 56°C for 1.5 minutes, product extension at 72°C for 1.5 minutes, followed by 8 cycles of the same conditions but with an extension time of 10 minutes. Ten µl of PCR product were loaded

on a 1% agarose gel, and following electrophoresis the gel dried and probed for the presence of microsatellite-containing PCR products of the expected size. Products which hybridized were rerun on an agarose gel, the desired band excised from the gel and DNA extracted by 'freeze-squeeze' elution (gel slice frozen, then thawed and the liquid physically expressed by squeezing between sheets of parafin film). The eluted DNA was sequenced manually using ^{33}P-labelled terminator chemistry (Amersham/Pharmacia) to verify that the expected product had been obtained. PCR conditions were then tuned to maximize specificity and yield.

Assays of suitability of PCR primers for *Lygus* were performed using CTAB extractions of DNA from 6 pooled individuals of *L. lineolaris* from each of 5 sites (London ON, Durham Co. ON, Waco NE, Portage-la-Prairie MB, and Echo Bay ON) and a pooled sample of 6 individuals of *Lygus elisus* from Twin Falls ID.

5.2 Geographic and phylogenetic surveys

5.2.1 Methods

DNA was extracted from the abdomen of individual bugs using a modified CTAB extraction method, and the remainder of the body retained as a voucher.

PCR amplifications were performed using Qiagen Taq PCR Master Mix in a Techne Genius thermocycler under the conditions specified in the following locus specific discussions.

Sequencing of survey samples was done using one of 4 methods: 1) direct manual sequencing of PCR products using ^{33}P labelled terminator chemistry (Amersham/Pharmacia); 2) PCR product cloned using Topo-TA cloning (Invitrogen) and sequenced on a LI-COR system using dye-labelled M13 primers; or 3) direct sequencing of PCR product on a LI-COR/NEN

Global IR^2 automated sequencing system with DYE-namic Sequencing Kit (Amersham/Pharmacia) using labelled custom primers. The first two methods were used for initial assays to determine if sufficient variation was present to warrant further investigation. Cloning was also used for PCR templates which could not be successfully sequenced directly.

Cycle-sequencing reactions for LI-COR were done on a Techne Genius thermocycler with a program of 30 cycles of 95° for 30 sec + 52° for 15 sec + 70° for 40 sec unless otherwise specified.

5.2.2 Material

Extensive collections were made by R. Foottit, E. Maw and M. Schwartz throughout southern Ontario in June 1999 (43 samples), September 1999 (extreme southeastern Ontario; 12 samples), June 2000 (Ottawa-Carleton, Renfrew and Lanark Counties of Ontario, and in Gatineau and Pontiac regions of Québec; 22 samples); Continental transects (Pennsylvania to Colorado, and British Columbia to northern Ontario; 50 samples) in July, 1999; 80 samples collected in 1995 throughout the agricultural areas of the Prairie provinces; 11 collections in 1995 from northern New Brunswick and the Gaspé. Localities in eastern Ontario sampled in 1999 were recollected in 2000.

Additional samples of *L. lineolaris* and *L. keltoni* from Alberta and Saskatchewan were provided by P.G. Mason in 1997.

Most specimens were frozen in the field in a liquid nitrogen dry-shipper and subsequently stored at -75°C. Specimens from a few collections (those from Gaspé and New Brunswick and the eastern Ontario collections made in June and July 2000) were collected into 95% ethanol and DNA extracted soon thereafter.

Individual extractions are identified by collection number plus individual number separated by a period (e.g. 00em23.3 represents individual number 3 from collection 00em23). For compactness, collections for which only one individual have currently been examined, the individual indicator is omitted (assume '.1').

6.0 Molecular diagnoses of pest *Lygus* species and populations using microsatellite markers

6.1 Microsatellite flanking region LL13F

6.1.1 Reference sequence

Table 6.1 provides a reference sequence derived from a majority consensus of cloned library sequences for *Lygus lineolaris*.

All following discussion of this locus is in terms of variance from this reference sequence. Available sequence for *L. hesperus*, *L. shulli*, *L. keltoni*, *L. borealis* (except LB7), *L. vanduzeei* and *L. lineolaris* (except 99em125.2) spans region between primers LL13F-3F and LL13F-3R. Sequence data bracketed by LL13F-4F and LL13F-4R has been confirmed bidirectionally; sequence outside this region has not been so confirmed. The remaining species were sequenced (bidirectionally) between primers LL13F-4F and LL13F-5R only.

6.1.2 Interspecific comparison

Table 6.2 indicates states of sites which showed variation among species. Positions which vary within a species but which are in general otherwise constant across species are not included in this table.

Most species are currently represented by a single sample and the indicated sequence differences therefore may not in general be taken as diagnostic.

Specimens assigned to *Lygus lineolaris* fall into two distinct sequence patterns ('group A' and 'group B', see below) and are treated separately in Table 6.2.

The most striking feature of Table 6.2 is the consistent heterozygosity (dominated by G/A transitions) shared among several species at 19 positions. Because the apparent heterozygosity is present in sequence primed in both forward and reverse directions, and because sequence from amplifications of cloned PCR product shows no variance at these sites, it is believed that this is a real phenomenon, not a PCR artefact, but this remains to be confirmed. Relative signal strength of the contributing allelic states is usually not equal, but, within a taxon, the inequality is generally relatively consistent (Table 6.3). *L. keltoni*, *L. shulli* and *L. hesperus* show a similar trend at a significant subset of these sites. Since a 1:1 ratio would be expected for a diploid individual heterozygous for a single-copy gene, it is suggest that the observed patterns may be explained by assuming that there are additional copies of the gene either through gene duplication within a haplotype or by polyploidy. Within the degree of error of interpreting of chromatogram peak intensities, the observed frequencies may be reasonably interpreted as a 4-copy system.

All of the species demonstrating this pattern are western montane, except *L. solidaginis* (northern Great Plains) and *L. shulli* (primarily western montane, but extending across the content in the boreal zone).

6.1.3 Variation within *L. lineolaris*

6.1.3.1 Primary grouping

Two distinct groups of *L. lineolaris* may be defined based on differences at 14 positions (Table 6.2). Eight of these (201,352,430, 473, 526, 551, 662, 663) are unique to, and constant

within, group A; two (457, 476) are unique to, and constant within, group B, three (346, 373, 388) are shared among group A and *L. humeralis*, *L. rugulipennis* (including *L. perplexus*), and as allelic forms within the 'heterozygous' species discussed in the previous section; and one (260) is unique to group A but with one individual showing the common state.

Group A predominates in southwestern Ontario from London westward, and also occurs along the shore of Lake Ontario, in extreme eastern Ontario near the confluence of the Ottawa and St. Lawrence Rivers, and at several localities between Indiana and eastern Nebraska (maps Figure 6.1). Group B is present throughout the sampled areas of the continent.

There is no evidence of interbreeding between the two groups (no extensive heterozygosity at the relevant positions).

6.1.3.2 Variation within *L. lineolaris* group A

Most of the sequence variation among individuals of group A (Table 6.4) is unique to a single sample. However, variants of a G/A transition are shared among several collections. The various states at this position show no clear geographic correlation, except that all samples of group A from extreme eastern Ontario are heterozgous at this position. Minor examples of shared variation occurs also at positions 179 (G/T transversion, T appearing only as heterozygote K), 250 (G/C transversion, C mainly as heterozygote S) and 551 (G/T transversion, T both homozygous and as heterozygous K). However, again no geographic pattern is exhibited.

6.1.3.3 Variation within *L. lineolaris* group B

Variation with group B is described in Tables 6.5 and 6.6. Of the 166 individuals sequenced, 54, geographically distributed throughout the sample range, were identical. Variation at six positions (Table 6.5) is shared among a significant portion of individuals; four of these are

in the segment confirmed bidirectionally, the remainder between primers LL13F-3R and LL13F-4R. Seventy-four individuals are identical for these 6 positions.

One of these changes is a 3 base insertion between positions 580 and 581 of the reference sequence. A number of individuals heterozygous for this insertion showed bias in favour of one or the other, such that the sequence was readable, but with a shadow sequence offset by 3 bases, while others were unreadable due to the overlapping equal intensity sequences. It is not yet clear whether this is an artefact of amplification, or reflects polyploidy or a multiple-copy gene, as suggested for the patterns described above for interspecific variation. Geographic distribution of the insertion is mapped in Figure 6.2. No clear geographic correlations are exhibited. The possession of a T-G combination at positions 599 and 615 is associated with homozygosity of the insertion (present in 7 of 9 individuals homozygous for the insertion, but present only in 1 individual homozygous for no insertion, and absent or state unknown in all individuals heterozygous for the insertion). No other clear indications of linkage among the various positions is apparent.

The possession of a G (or S) in place of C at position 65 is primarily restricted to the Pontiac area of Quebec and the adjacent Matawa Valley area of Ontario. Among the minor variants (Table 6.1), a G to C transversion at position 616 is found in 5 samples, of which 4 were also from the Pontiac/Mattawa area (the fifth from north of Winnipeg). No other clear indications of geographic segregation seen.

Although no geographic patterns were observed among individual characters, it is apparent that the Pontiac/Mattawa valley area shows considerably more variation than any other comparable area, both within and among collection localities. In particular, samples from the

Gatineau Valley of Quebec (separated from the Pontiac samples by the Gatineau Hills), from extreme eastern Ontario, and from areas south of Ottawa largely conform to the most common sequence. It may be significant that most of the samples from the Pontiac/Mattawa area were from roadside vegetation, somewhat removed from extensive agriculture, while the other samples were dominated by collections from alfalfa fields. The common form was also the dominant form elsewhere on both alfalfa and on *Brassica* crops and Brassicaceous agricultural weeds. This suggests that populations found in agricultural situations are genetically less diverse than those from more varied habitats, and is not a reflection of differences in host preference. More extensive sampling is required to determine the validity of this assertion.

6.1.4 Variation with *L. borealis*

Seven individuals of *L. borealis*, collected from southern Ontario to western Saskatchewan have been examined (one of these, LB7, over the region from LL13F-4F to LL13F-5R only). All the samples differ consistently from all other species at 5 positions (Table 6.2) (except LB7, state unknown for 3 of these positions). Six of the 7 agree at a sixth position (A replacing the G found in all samples of all other species, the exception being heterozygous A/G at this site). Remaining differences (four positions) are each unique to an individual (Table 6.7).

6.2 Microsatellite flanking region LL42F

6.2.1 Reference sequence

The majority consensus of all species sequences is given in Table 6.1. All following discussion of this locus is in terms of variance from this reference sequence.

6.2.2 Interspecific comparison

A matrix of parsimony-informative characters is given in Table 6.9. Within-species variation for this fragment has not yet been assessed.. Hence, conclusions based on groupings defined by one or a few base changes are not warranted. However, given this limitation, certain patterns are suggestive. The segregation of species now placed in *Nonlygus* and *Henrilygus* from *Lygus*, proposed by Schwartz and Foottit (1998) based on morphological characters, is amply supported by this fragment, with 29 consistent base changes. The following relationships are also suggested: (((*borealis*, *unctuosus*), (*rubroclarus* , remainder of *Lygus*)) based on positions 110, 196, 316, 319 and 331; four of these characters are shared with *Nonlygus* and *Henrilygus* suggesting that these species are primitive with respect to the remainder of *Lygus*. An association of these species with *L. lineolaris*, *L. rufidorsus*, *L. vanduzeei* and *L. pratensis* is weakly suggested by position 317. Most of these species occur near the base of the phylogeny proposed by Schwartz and Foottit (1998); the major disagreement is the basal position of *L. borealis*.

Note also the heterozygosity present in *L. plagiatus* at 5 sites, indicating that this species may be a hybrid, or of hybrid origin. Positions 337 and 420 would suggest *L. lineolaris* and *L. unctuosus* as the parent species. However, this specimen presented some problems in obtaining clean sequence data, and the observed results may be an artefact.

6.2.3 Variation within *L. lineolaris*

Sequence data for *L. lineolaris* geographic samples is not yet available.

6.3 Microsatellite flanking region LL56F

6.3.1 Reference sequence

Table 10 gives the majority consensus, including gaps introduced to accommodate alignment. All following discussion of this locus is in terms of variance from this reference sequence.

6.3.2 Interspecific comparison

A matrix of parsimony-informative characters is given in Table 6.11. *Henrilygus nubilus* has a number of unique differences from the remaining species; data for *Nonlygus nubilatus* is not yet available. Several groupings are suggested by consistent shared changes: *L. elisus* + *L. humeralis* (positions 609, 635, 637, 644) and *L. oregonae* + *L. abroniae* (positions 357, 669, 703), *L. solidaginis* + *L. atriflavus* + *L. robustus* + *L. columbiensis* (= *L. punctatus*) (positions 66 and 641), and *L. 'perplexus'* + *L. hesperus* + *L. rubrosignatus* (positions 583, 586, and a pairing of the first two based on position 590). Note, however, that *L. perplexus* was considered a synonym of *L. rugulipennis* by Schwartz and Foottit (1998) A partitioning unsupported by other characters is suggested by position 703.

6.3.3 Variation with *Lygus lineolaris*

Analysis of geographic variation within *L. lineolaris* is currently under way. Preliminary observed variation is summarized in Table 6.12 and mapped in Figure 6.3.

Although not as dramatic as seen with fragment LL13, there is a distinction between samples from southwestern Ontario and those from eastern Ontario based on an A/T transition at position 734, and for samples near Lake Erie (and one sample from near the shore of eastern Lake Ontario), an additional A/G transversion at position 732. Several samples from central Ontario share a T at position 207 with the southwestern group, but are otherwise more similar to the eastern group.

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TGACACGTTA GACTGGAATC GTCAAGTGGC GGAAATATGT AAGAAGGTGT ATGGTAGCCT  60
GTATCAGTTG CGTAGAATTG CCTTCGATTT TCCAAAGCAT GTCAGGACGC AATTGGCTCA 120
GGCCCTCCTG GTGCCTTACT TTGAATATGC GCCTTTGGCT TTCTGCGATT TGAATAACGA 180
GCAAATGGGC AGACTACAAA AAACGCTCAA CTGTGTTGTG CGTTTTGTAT GTCGTCTAAG 240
GCTGGATGCG CATGTTACGC CGGCGTACTT GGAGCTTGGG TGGCTTAAAA TGGAGGAGAG 300
GAAAAGGTTG GCGGTTGGAG CAATGTTGTT CAAAATTCTC AAATTCAGGA AACCTCAGTA 360
CTTATATAAC CAATTTAGGT ATCTTTCGTC TGTGCATACG GTTTCAACGC GCAAAGCGGC 420
TACTACACTC CAGATACCGA AGCACAATAC GGTTTTATTC AGCAGATCGT TCATAATGCA 480
AGCCATTGAA ATGTATAACT CTAATGCAGA GATTTTTGAT TTAAGCACAA GTGTTACAGC 540
TTTCCGAAAC TCGCTTAAAA CATCGTTATT GGAGAGGTAT*ATGTAGTAGG ATGAGTTGAT 600
AAGTTAAACC CACAAGTGCA GCGTTTTCCG ACCATGTACT GCTGCTCGCT GTTATATAAT 660
TGCGGGCGGT GGT                                                     673
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Table 6.1. Reference sequence based on majority consensus of states found in *Lygus lineolaris*. A sterisk represents location of 3-base insertion found in many individuals of *L. lineolaris*.

C. saundersi (1)	??TACCGGAGACAGGGGAGCATACAGTTGGGACAGTGGTGATA????????????????????
H. nubilus (1)	??CATCAGAAACAGGCAAGCACGTAGCTTAATCGTTAGTCATT????????????????????
H. ultranubilus(1)	??CATCAGAAACAGGCAAGCACATAGCTAAAT-------CATT????????????????????
N. nubilatus (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
abroniae (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
oregonae (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
striatus (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
'nigropallidus' (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
robustus (1)	??CACCRGRRACRRGGGRRTATRCWSYYRGGACRYYKGWGATA????????????????????
'columbiensis' (1)	??CACCRGRRACRRGGGRRTATRCWSYYRGGACRYYKGWGATA????????????????????
solidaginis (1)	??CACCRGRRACRRGGGRRYATRCWSYYRGGACRYYKGWGATA????????????????????
affinis (2)	??CACCGGR$^{G}_{R}$ACRGGGGRRYATRCTSY$^{Y}_{T}$RGGACRYYKGWGATA????????????????????
potentillae (1)	??CACCRGRGACRGGGGRRYATRCTSYYRGGACGYYKGWGATA????????????????????
shulli (1)	TCCAC?GGAGACAGGGGRAYATRCAGYYAGGACRTTTGWGATACCYTTTAGTGTTTATGGA?
keltoni (14)	cCCACC$^{A}_{R}$GR$^{R}_{G}$ACrGGGGga$^{Y}_{C}$AT$^{R}_{G}$CA$^{S}_{G}$CC$^{R}_{A}$GGACatTTG$^{W}_{A}$GATACC$^{Y}_{T}$T$^{c}_{Y}$$^{C}_{T}AGT^{A}_{g}a^{G}_{K}at^{A}_{W}G^{C}_{g}$$^{A}_{G}$$^{G}_{T}$
hesperus (2)	?CCACG$^{G}_{A}$GA$^{G}_{R}$AC$^{A}_{G}$GGGGA$^{R}_{A}$YATRCAGY$^{T}_{Y}$$^{R}_{K}$GGACA$^{C}_{T}TTG^{W}_{A}$GATACCCTTTAGTATTTATGGGT
elisus A (1)	??CACCGGAGACAGGGGAGCATACWSTTGGGACGCTGGTGATA????????????????????
elisus B (1)	TCCACCGGAGACAKGGGGRCATACAGTTAGGACGYTTGTGATA?C??TTAGTRTTTAT????
plagiatus (1)	??CACCGGAGACAGGGGAGCATACAGTTGGGAGGCTGGTGATA????????????????????
rubroclarus (1)	??CACCGGAGACAGGGGAGCATACAGTTGGGACGCTGGTGATA????????????????????
unctuosus (1)	??CACCGGAGACAGGGGAGCATACAGTTGGGACGCTGRTGATA????????????????????
adspersus (1)	??CACCGGAGACAGGGGAGCATACAGTTGGGACGYTGATGAAA????????????????????

Table 6.2. [part 1]

pratensis (1)	??CACCGGAGACAGGGGAGCATACAGTCAGGACGTTGGTGATA????????????????????
borealis (7)	TTCTCCGAAGACAGaGGAGCATACAGTTGGGACGCTGGTGATAGCCTTTGGGG$^{T}_{G}$TTATGGAT
vanduzeei (4)	TTCAC$^{G}_{C}$GGAGACAGGGGA$^{G}_{R}$CATACAGTT$^{R}_{G}$GGACGCTGGTGATAGCCTTTAGTGTTTATGGAT
lineolaris B (165)	TC$^{T}_{C}$AC$^{G}_{C}$GGAGACAGGGGAGCATACAGTTGGGACGCTGATGAAAGCCTT$^{A}_{T}$A$^{A}_{\underline{G}}$TGTTtaTGGAT
lineolaris A (22)	TCCACCGGAGG$^{G}_{\underline{S}}$AGGGGAGTGTACTCTTGGGAAGCTGGTGGTAGTCG$^{T}_{A}$TAGTGTTTGAGGAT
rubrosignatus	??CACCGGAGACAGGGGAGTATACTCTTGGGACGCTGGTGGTA??????????????????
mexicanus (1)	??CACCGGARACAGGGGAGTATACTCTTGGGACGCTGGTGATA??????????????????
humeralis (1)	??CACCGGAGACAGGGGAGTATACTCTTGGGACGCCGGTGATA??????????????????
ceanothi (1)	??CACCGGAGACAGGGGAGTATACTCTTGGGACGCCGGTGATA??????????????????
'perplexus' (1)	??CACCGGAGACAGGGGAGTATACTCTTGGGACGCYGGTGGTA??????????????????
rugulipennis (1)	??CACCGGAGACAGGGGAGTATACTCTTGGGACGCCGGTGATA??????????????????

Table 6.2[concluded]. Summary of variance among *Lygus* species, *Henrylygus nubilatus* and *Nonlygus nubilatus*. Matrix of states corresponding to positions 1, 4, 55, 56, 65, 97, 115, 177, 188, 201, 260, 289, 306, 314, 322, 328, 346, 352, 369, 373, 388, 391, 397, 400, 412, 430, 439, 445, 448, 451, 457, 459, 473, 476, 506, 526, 544, 551, 599, 606, 615, 643, 651, 653, 655, 657, 659, 660, 664, 666, 668, and 673 of reference sequence (see Table 1). Number of individuals sequenced for each species indicated in parentheses following name. IUPAC ambiguity codes represent heterozygosity within sampled individuals. For sites showing variation among individuals within a group, if less than 20% of individuals sampled show variance from the majority, the majority form is shown in lower case; if greater than 20% of individuals vary from majority, the two most common (or only) forms are shown in small font, the majority above; underlined codes indicate that a small proportion of individuals showing a third compatible state (*e.g.* $^{T}_{\underline{Y}}$ means at least 1 individual is homozygous for C at this site, $^{T}_{\underline{C}}$ means most individuals homozygous but at least 1 individual is heterozygous at this site [Y] present).

position	97	177	188	289	306	322	328	346	369	373	388	391	397	400	439	445	448	451	459
base 1	G	A	G	A	G	A	G	T	A	T	C	T	T	G	G	C	C	G	T
base 2	A	G	A	G	A	G	A	C	G	A	G	C	C	A	A	T	T	T	A
N. nubilatus	5	7	6	5	9	7	5	6	5	4	5	5	7	4	5	5	5	6	x
abroniae	5	5	6	4	5	4	4	4	6	4	5	4	5	5	5	5	4	5	2
oregonae	2	3	3	1	2	1	2	2	2	2	3	2	2	2	2	2	2	2	2
striatus	4	7	5	4	4	5	5	5	5	5	4	5	6	5	5	5	2	5	4
'nigropallidus'	3	5	5	2	3	3	3	4	3	4	4	4	5	4	4	4	4	5	3
robustus	6	8	8	8	8	8	7	7	7	8	6	8	8	8	7	7	7	9	-
'columbiensis'	3	2	2	2	5	2	2	8	3	2	4	2	2	2	3	3	3	2	2
solidaginis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
potentillae	6	7	8*	5	x	5	8	8	5	8*	7	7	8	8	6*	6	5	8	8
elisus A	10	10	10	10	3*	10	10	2*	10	3	4	10	10	10	10	10	0	10	10
elisus B	10	10	10	10	x	0	x	0	10	0	0	10	10	0	10	x	0	0	10
'perplexus'	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	3	10	10
shulli	10	10	10	10	10	x	0	x	x	0	0	x	x	10	x	0	0	0	x
hesperus	x	10	10	x	10	x	x	x	x	0	0	x	x	x	0	x	0	0	x
keltoni	x	x	x	x	10	0+	0+	x	x	0	x	0	0	x	0+	x	0	0	x

+ one heterozygote.

Table 6.3. Approximate proportions of components at positions heterozygous in several species. Value in body of table indicates 10 x approximate relative proportion of base 1 to total peak height for that position (e.g. for *L. oregonae* at position 114, value of 2 indicates apparent mix is 20% G and 80% A) (signal corrected for relative lane intensity). 'x' represents heterozygous position for which relative intensity has not been determined. The component selected as 'base 1' is that matching the reference sequence.

subgrp	#	state values	collections
		7 37 52 65 90 109 147 152 179 250 290 338 405 430 551 557 600 602 607 632	
1	1	GGTCTGTCGGACCAGATAAC	99em60, 99em62, 99em150
	2	GGTCTGTCGGTCCAGATAAC	99em67
	3	GGTCYGTCGGACCAGATAAC	99em80
2	1	GRTCTGTCGGACCAGATAAC	99em30, 99em750, 99em753
	2	GRTCTGTCKGACCAGATAAC	99em141
	3	GRTCTGTCKSACCAGATAAC	99em746
	4	GRTCTGTCGSACCCGATAAC	99em126
	5	GRTCTGTCGCATCAGGTAAT	99em52
3	1	GATCTGTCGGACCAGATAAC	99em53, 99em65, 99em66, 99em81
	2	GATCTGTCGGACCAGAWWCC	99em57
	3	GAYCTGTCGGACCMGATAAC	99em54
	4	SATCTGTCGGACCAGATAAC	99em64
	5	GATCTSYMGSACYAGATAAS	99em77
	6	GATCTGTCGGACCAKATAAC	99em146
	7	GATGTGTCGGACCATATAAC	00em11a.1

Table 6.4. Variation within *L. lineolaris* group A. States corresponding to 7, 37, 52, 65, 90, 109, 147, 152, 179, 250, 290, 338, 405, 430, 551, 557, 600, 602, 607, 632 of reference sequence (see Table 6.1).

49-412	indel	599, 615	sample
GTCC	----	AA	95em277.1, -281a.1, -282.1, -302a.1, -303a.2, -306a.5, -349a.2, -385.3, -386a.1, -387a.2, -392.4, -395.2, -395.4, -397.1, -399.1, -399.5, -402a.5, -406a.4, -409a.5, -413, -414.3, -417.5, -418a.3, -431a.1, -468a.1, -469a.4, -470a.5, 99em28, -35, -36, -37, -38, -44, -47, -49, -61, -68, -69, -72, -78, -82, -84, -123, -125.1, -148, -151, -149, -593, -689, -723, -734, -735, -738, -747, -748, -749, -751, -752, 00em4.3, -4.4, -4.5, -12a.5, -13.4, -13.5, -14.3, -14.1, -14.2, -14.5, -15.3, -15.4, -15.5, -16.1, -16.2, -17.4, -23.4
		WA	00em15.1, 99em50, pgm97-7.1
		TA	99em178, -697, 00em13.3, -14.4
		WR	99em51
		AR	99em55
	---+	AA	95em326a.4, -409a.6, pgm97-8.1, 99em63, -732, 00em5.1, -06a.1
		WA	99em70
		TA	pgm97-8.3, 00em15.2
	--++	??	99em744, 00em04.2, -5.4, -11a.2, -12a.1, -12a.3
	?	??	99em743, 00em164.1, F2000-B.4
GYCC	----	AA	95em403.1
GTGC	----	AA	95em303a.1, 00em22.2, -22.5, -23.1, -23.3
	---+	AA	00em21.4
	--++	??	95em254a.5
	?	??	00em11a.5, -10.1
GT?C	----	AA	00em16.5
GTSC	----	AA	00em10.5
GTCY	-+	AA	00em05.5
	--++	??	95em385.5, 99em121, -79, -124
GTGY	-++	??	00em23.2
GTCT	-++	??	99em76, pgm97-7.2
GYCY	++++	TG	99em717
GCKY	-++	??	95em254a.1
GCCC	----	TG	00em11a.3
	---+	TA	00em12a.4
	--++	??	99em691
GCGC	-++	??	00em08a.2, -10.4
G?CC	-++	??	00em13.1
KTCT	-++	??	00em17.5
KTCC	----	AA	95em402a.1
	---+	AA	95em401.5, 00em23.5
		WA	95em406a.1
	--++	??	00em21.3
KTCY	-+	TA	99em130
KCCC	-++	??	99em757
KYCY	-++	??	99em701
TYAC	-++	??	00em17.3
TCCC	-++	??	95em396a.5
TTCC	-++	??	00em21.2
TTCY	-++	??	95em429a.1
TYCY	-+	TA	99em40
	++++	TG	99em692
TCCY	--++	??	95em297.5, 00em20.1
	++++	TG	99em27, 99em690, 99em695
TCCT	--++	??	95em398a.5, 99em71, -73, -745
	++++	TG	99em45, 00em21.1
		TA	95em473a.4, 99em48
TCKT	-++	??	guelph28.1
TCGY	-++	??	95em324a.5
TCGC	?	??	00em08a.3, 00em10.2
TC?Y	-++	??	00em22.3
K?CT	-++	??	95em397.4
T?CT	-++	??	00em04.1
?CCY	?	??	99em125.2

Table 6.5. Principal variants of Group B, based on states corresponding to positions 49, 55, 65, 412, insertion

between 580 and 581, 599 and 615 of reference sequence (Table 1). Codes for indel are '++++': homoplasmic for insertion; '----' : homoplasmic for deletion (or insertion sequence very weak); '--++' : codominant heteroplasmy for indel (sequence beyond unreadable); '---+': deletion dominant; '?': sequence not available across indel site. Other variants within Group B (mostly unique changes) are specified in Table 6.6. Sample numbers abbreviated for compactness.

Sample	position/state
guelph28.1	78/C, 320/T
95em254a.5*	44/G
95em277.1	72/T
95em297.5	41/C, 144/R, 535/G, 541/K
95em306a.2	415/W
95em306a.5	67/R
95em326a.4**	44/R, 500/W
95em385.3	379/R, 388/S
95em387a.2	115/K, 616-618/CAT
95em399.5, 95em468a .1	590/T
95em413*	44/R
95em418a.3	618/T, 641/C
95em473a.4**	235/Y, 535/A
pgm98-8.3*	44/G, 597/A
99em36	592/K, 601/R
99em40+	535/W
99em55	221/M, 619/A
99em63, 99em732	584/A
99em61	after ?/insert 29 bases
99em68	415/T, 560/R
99em70	511/R
99em72	319/R
99em73**	535/C
99em82	596/G
99em84	72/K,
99em124**	535/W
99em125.1**	535/W
99em125.2	379/R

Sample	position/state
99em130	527/M
99em593	27/Y
99em689	631/M
99em695	528/M
99em701*	44/R, 316/Y, 385/K
99em735	388/K
99em743	184/R, 313-321/del (heterozygous), 400/A, 462/T
99em745	173/R, 235/Y, 358/R
99em747	633/G
00em4.1*	44/C, 338/R
00em4.2**	535/W
00em4.5	31/S, 67/A
00em5.4	33/R
00em5.5	537/G
00em6a.1	28/T
00em8a.2	533/C
00em10.4	533/S
00em10.5	616/C, 623/A
00em12a.4	616/C, 666/C
00em13.1	82/G
00em13.5	584/W
00em16.2	63/W
00em17.3	92/Y, 115/K, 157/K
00em21.3, 00em23.5	616/C
00em21.4	500/Y, 533/S
00em22.5	623/T
00em23.2	533/K
00em22.3	531/A

Table 6.6. Minor variants (present in fewer than 5% of samples) within *L. lineolaris* Group B. Note that samples heterozygous for insertion at position 581 (coded '---++' in Table 5) are unreadable beyond position 581 and variation for these samples in this region is thus unknown. The majority of these states are found in a single sample, but changes at positions 44 (marked *), 535 (marked **) and 616 (in bold face) are shared among several samples.

?CA??	LB7
TCAGT	99em41, 95em219, 99em696
TCRRT	99em686
TTAGT	95em435.3
CCAGG	95em435.4

Table 6.7. Variation within *L. borealis*. States corresponding to positions 2, 58, 314, 636, and 653 of reference sequence (see table 6.1).

```
TTCATCGTGC ATGAAGGAAA GGAGATTGTC CCGTGTCGTC AGTGCCACCT CTGAAGCCGA  60
TAAGCAAGTT GAACTATTGA ATGTTATTGA GGAGATAAGG GAGGAAATTA GGAGTTCTAA 120
TCGGCAACTT CAAGAGGAGA TTGAACGCCA GGCCGGTGAG ATTAATGATC TTAAAGTCCA 180
GCTCAGTACT TACTCAGATT ATATAGAATC TAACAAACAA TCGTTGGATC GTGTGGACTC 240
TTCTTTGAAG GCTCTCGCCG ATAAAGTGGA CAATGTGATG GACTGTCAGA AGGGATATGA 300
TAAGAAGCTC GAAGAGATGA CTGAAATGAT AAACAATGTT GATCAACAAG CTCGTGAGTC 360
GTCAGTGGAA ATTACTGGAT ATCCAGAAAC GGAAAATGAG AATGTTCTTG AGATCGTAAG 420
AAAGATCGGC GATGCAGTAA AGTTTCCTAT TTCTGAGCAA ATGCTGGATG ACTGCTATCG 480
AATTAAACCC AGGAATCCAC GACCAGGTTT                                  510
```

Table 6.8. Majority consensus sequence for locus LL42F.

	40	58	77	82	91	106	110	118	130	163	169	172	181	196	202	211	229	247	268	278	280	289	316	317	319	331	337	352	362	367	391	420	442	446	451	466	469	472	479	481	503
N. nubilatus	C	A	T	A	A	T	C	G	C	A	C	C	G	C	C	C	C	A	A	G	A	G	G	T	A	T	T	C	A	T	T	A	C	G	A	A	C	T	R	G	A
H. nubilus	C	A	C	A	A	T	C	G	C	A	C	C	G	M	C	C	C	A	A	G	A	G	G	T	A	T	T	C	A	T	T	A	C	G	A	A	C	T	A	G	A
borealis	C	C	C	T	G	A	C	Y	T	T	T	T	A	C	T	T	T	G	G	A	G	G	T	C	A	T	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
unctuosus	C	C	C	T	G	A	C	T	T	T	T	T	G	C	T	T	T	G	K	A	G	G	T	C	A	T	T	T	T	G	G	A	G	C	T	G	T	C	C	A	C
plagiatus	C	C	C	T	G	A	M	T	T	T	T	T	G	M	T	T	T	G	G	A	G	G	?	C	A	W	Y	T	T	G	G	R	G	C	T	G	T	C	C	A	C
rubroclarus	C	C	C	T	G	A	C	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	C	G	T	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
vanduzeei	C	C	C	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	C	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
lineolaris	A	C	C	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	C	G	A	C	T	T	G	G	G	G	C	T	G	T	C	C	A	C
rufidorsus	A	C	C	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	C	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
ceanothi	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
convexicollis	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
pratensis	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	C	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
shulli	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
elisus (2)	C	C	C/T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T/Y	T	T	G	G	G	G	C	T	G	T	C	C	A	C
humeralis	C	C	C	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
oregonae	C	C	C	T	G	A	A	T	T	T	T	T	R	A	T	T	T	G	G	A	G	A	G	C	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
abroniae	C	C	C	T	G	A	A	T	T	T	T	T	A	A	T	T	T	G	G	A	G	R	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
'nigropallidus'	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
striatus	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
rugulipennis	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
'perplexus'	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
hesperus (2)	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
rubrosignatus	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
potentillae	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
solidaginis	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
atriflavus	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
robustus	C	C	Y	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	R	M	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C
'columbiensis'	C	C	T	T	G	A	A	T	T	T	T	T	G	A	T	T	T	G	G	A	G	G	G	A	G	A	T	T	T	G	G	G	G	C	T	G	T	C	C	A	C

Table 6.9. Informative positions for locus LL42. *Nonlygus nubilatus* is based on consensus of 4 clones derived from PCR product, *Lygus robustus* from 2 clones, remaining species from direct sequencing of PCR product. Position relative to reference sequence (Table 8).

```
GGTTGATTAG GCTTATCACC CAATCCTGCG TTGGGTGGCA TGAGAATCGG AGTAGAAGGC 60
TCGGGAGTGG GAAAGCGGAG ACAAGCCATA CTTAACTACG GCTCCCTCAT TGGAAATCTC 120
CCTGCATAGT GATGCAAATA CGGGCGAGAC TGTTAAGGCT TCACTAGGCT CATTATGCTC 180
TAAATAATTA ATGAAGGCAT GCATAATAGA TCCGCCATGC ACCACTGTTA TGCTGTAATC 240
CGTTCATTAC GAGCGATCGT GGCGGGTAAA AGGAGCTCGA GTGGATAATG GTAGATGACT 300
TCACACCGAC CTCCCTCTCA TACAGCTCGT GGTTTCTGCC CTATATACAA CCTTCCAAGG 360
CATCTAATTT ATGGCCCACC CGCATCCTAT ACCTAAGTAG AAGCGGCGTC TTATCTATCC 420
TGACGTCCTT TTGCAATAAA TGAGGAAACC GCGGGTACCG CTGGTGCCTC GCAGCTTGGG 480
ACCCCT-CAT TTTCCATCAT TCACCTTATG ATATTCAACA C-CATGGTTC ATAAACACTT 540
GGGCGGCGCG CAAGTTTTCC CCAACATGAA CTTATCTCTG GGTGTGGTAT AT----ACCA 600
TGCCAAGTAT A---GGTATG TGTTCATTGT CATCAGCTCG TAA-GTGCTC GTATTTGCAT 660
GTTCATTTTC TCAATCAACA GGTTCCATCT GGAAATCATT AGTCGTACCT AATGCTCTTT 720
TCTGCTACGA AATTATTATC AATTCTTTTG ATGATCTCGA TTTCGTAA              768
```

Table 6.10. Consensus sequence for locus LL56.

	34	66	357	407	544	583	586	590	609	635	637	638	641	644	669	703
H. nubilus	A	A	A	C	C	T	G	T	A	A	C	A	T	-	T	T
borealis	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	T
unctuosus	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	T
plagiatus	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	?
rubroclarus	G	A	A	T	C	T	G	T	A	A	C	T	T	-	C	T
lineolaris	G	A	A	T	C	T	G	T	A	A	C	T	T	-	C	T
rufidorsus	T	A	A	C	C	T	G	T	A	A	C	T	T	-	C	T
ceanothi	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	T
convexicollis	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	T
elisus	G	A	A	C	C	T	G	T	G	G	T	T	T	T	C	T
humeralis	G	A	A	C	C	T	G	T	G	G	T	T	T	T	C	T
oregonae	G	A	G	C	C	T	G	T	A	A	C	T	T	-	T	G
abroniae	G	A	G	C	C	T	G	T	A	A	C	T	T	-	T	G
'nigropallidus'	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	G
striatus	T	A	A	C	C	T	G	T	A	A	C	T	T	-	C	G
rugulipennis	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	G
'perplexus'	G	A	A	C	-	G	A	G	A	A	C	T	T	-	C	G
hesperus	G	A	A	C	-	G	A	G	A	A	C	T	T	-	C	T
rubrosignatus	G	A	A	C	-	G	A	T	A	A	C	T	T	-	C	K
potentillae	G	A	A	C	C	T	G	T	A	A	C	T	T	-	C	G
solidaginis	G	G	A	C	C	T	G	T	A	A	C	T	C	-	C	G
atriflavus 1	G	G	A	C	C	T	G	T	A	A	C	T	C	-	C	K
atriflavus 2	G	G	A	C	C	T	G	T	A	A	C	W	C	-	C	G
robustus	G	G	A	C	C	T	G	T	A	A	C	A	C	-	C	G
'columbiensis'	G	G	A	C	C	T	G	T	A	A	C	T	C	-	C	G

Table 6.11. Informative positions for locus LL56. Position relative to reference sequence (Table 6.10). *Lygus humeralis* based on consensus of 4 clones from PCR product, remaining species from direct sequencing of PCR product.

	179	560	561	659	675	729	732	734	737	
1	C	C	C	A	T	T	A	A	A	99EM-34, -35, -36, -37, -38, -79, -84
2	C	C	C	A	T	K	A	A	C	99EM-28
0.1	C	C	C	A	T	G	A	A	T	99EM-44, -45, -48, -49, -69, -70, -75, -76
3b	C	C	C	R	T	G	A	A	T	99EM-71
369	Y	C	C	A	T	G	A	A	Y	99EM-77
25										
4	T	C	C	A	T	G	A	A	T	99EM-40, -72,- 73,- 78
0.2	Y	C	C	A	G	G	A	W	T	99EM-50
5	T	C	C	A	G	G	A	T	T	99EM-51
6	T	C	C	A	T	G	A	T	T	99EM-54, -62, -63, -64, -81
7	T	C	C	A	T	G	G	T	T	99EM-55, -68
0.3	T	M	C	G	T	G	R	T	T	99EM-65
8b	T	M	Y	G	T	G	G	T	T	99EM-57
0.4	T	C	T	G	T	G	G	T	T	99EM-52, -53, -61, -66, -67
9b	T	C	T	R	T	G	G	T	T	99EM-80

Table 6.12. *Lygus lineolaris*. Preliminary state matrix of variant sites for fragment LL56 (single base changes occurring a single sample ignored). Sites within Ontario are indicated on map (Figure 6.3). Numbers to right of sequence data identify observed motifs; lower case letters identify sequences differing only at heterozygous sites; motif identifiers with slash indicate possible hybrids between indicated constituent motifs. Position relative to reference sequence (Table 6.10).

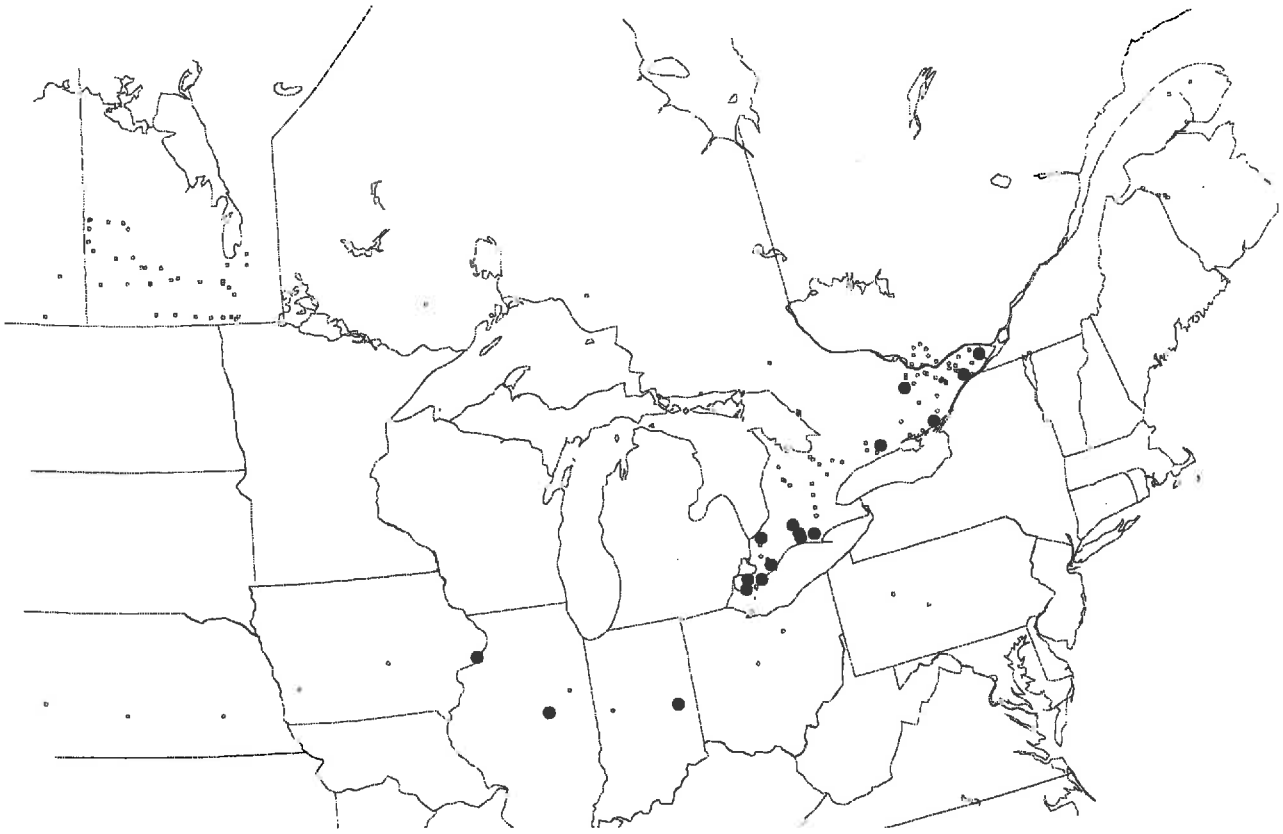

Figure 1. Distribution of *L. lineolaris* group A, filled circles; collections of group B represented by small open circles.

Figure 2. Geographic distribution of insertion. Symbols corresponding state notation in Table 5: open = ---; light shading = --++; dark shading = ---+; black ----.

7.0 Molecular diagnosis of pest *Lygus* spp. and associated *Peristenus* spp. parasitoids using species-specific PCR primer sets.

To properly undertake ecological studies and assess the population dynamics of *Lygus* spp. and their associated *Peristenus* species it is essential to have efficient methods to identify all life stages of both insects to the species level. The identification of *Lygus* spp. nymphs is not possible based on morphological characters until at least the last instar and then only by highly skilled personnel. Similarly, the reduced morphology of larvae Hymenopteran parasitic insects such as *Peristenus* limits the usefulness of physical appearance for the practical identification to species or even to the genus level (Loan and Shaw 1987; Carignan *et al.* 1995). Currently species identification is a difficult and time consuming task and the positive identification of the parasites requires rearing to the adult stage, a complex process taking up to one year due the obligate diapause of the pupal stage (Tilmon *et al.* 2000). Developing a method for identifying the immature stages of *Lygus* spp. and their associated *Peristenus* parasites would facilitate efficient and accurate assessment of parasitism in target *Lygus* populations and be useful in identifying *Lygus* nymphs to species in order to under take ecological interaction studies and to assess their economic damage potential in alfalfa and canola. One of the strategies previously used for the timely identification of insects is the development of molecular markers based on species-specific primers based on polymerase chain reaction (PCR) amplification of DNA sequences from variable regions of conserved genes (Hoy 1994). Recently, Tilmon *et al.* (2000) were able to quantify parasitism rates and identify *Peristenus* parasites to species at the immature stage within *Lygus* spp. hosts using a two-step molecular marker approach. This method used PCR primers specific to the mitochondrial protein-coding gene cytochrome oxidase I (COI) from

the *Peristenus* genus to amplify DNA extracted from potentially parasitized *Lygus* spp. nymphs. Restriction endonuclease (REN) digestion of the PCR product was used to identify *Peristenus* immatures to species based on species-specific REN fragment profiles. These authors found this system to be very sensitive and theoretically capable of detecting even newly laid parasitoid eggs within parasitized *Lygus* spp. nymphs in which the parasitoid would constitute only 0.01% of host tissue. Here we describe the development of a one step PCR-based screening technique for identifying both *Lygus* and *Peristenus* species occurring in western Canada.

7.1 Protocol for documenting molecular markers based on species-specific PCR

7.1.1 Insects

Voucher adult samples of *L. lineolaris*, *L. borealis* and *L. elisus* and parasitoids *Peristenus digoneutis* Loan, *P. stygicus* Loan and *P. pallipes* (Curtis) were obtained from P. Mason and H. Goulet (Systematic Entomology, AAFC, ECORC, Ottawa, Canada). In addition, field collections of overwintering and summer adults, and nymphs of *Lygus* spp. were made weekly in alfalfa fields at two locations in Saskatchewan from mid-April to August in 1998 and 1999 (Braun *et al.*, 2001).

7.1.2 Insect DNA extraction

Insect DNA extraction procedure was as follows: insects were frozen in liquid nitrogen then individually homogenized in 350 µl of Lifton buffer (0.2M sucrose, 50mM EDTA, 100mM Tris-HCL pH=7.5, and 0.5% SDS) in 1.5 ml centrifuge tubes using disposable pestles (Mandel Scientific). The sample was incubated on ice for 1 h following the addition of 1/10th volume of 8M potassium acetate and centrifuged at 12,000xg for 20 min in a bench-top microfuge. The supernatant was extracted once with an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1) and once with an equal volume of chloroform. The DNA in the aqueous fraction was

then precipitated with 2 volumes of ice-cold ethanol at -20oC for 30 min and pelleted by centrifugation at 14,000xg for 30 min. The DNA pellet washed with ice-cold 70% ethanol and then air-dried. The DNA pellet was resuspended in 50 (*Peristenus*) to 100 (*Lygus*) µl of sterile dH_2O plus 1 µl of 10mg/ml of RNase A.

7.1.3 PCR primers and cloning

To develop molecular markers to differentiate *Lygus* and *Peristenus* spp. a number of sets of oligonucleotide primers targeted to semi-conserved regions of nuclear and mitochondrial insect genes were tested for their ability to amplify DNA from voucher insect samples. The oligonucleotide primers were obtained in kit form, Insect mt-DNA set & Insect n-DNA set, from the Nucleic Acid-Protein Service at the University of British Columbia, Vancouver, BC (www.biotech.ubc.ca/services/naps/). Standard PCR conditions were used as follows: approximately 100 ng of DNA template was added to 25 µl reaction mix (2.5 µl 10X reaction buffer [Promega], 1 to 2 units of Taq polymerase [Promega], 2.5mM $MgCl_2$, 40 µM of each primer, and 400 µM each dNTP) and amplified in a thermocylcer. The thermocylcer conditions for *Lygus* spp. samples was as follows: 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 62°C for 30 sec, and product extension at 72°C for 1 min, followed by a final extension at 72°C for 5min. The *Peristenus* samples were run under similar conditions except that the annealing temperature was 55°C.

PCR products were electrophoresed on 1% agarose gels containing 0.4 ug/ml ethidium bromide at 80 volts for 60-90 min with appropriate molecular weight DNA markers and the gels photographed. Those PCR products to be sequenced were excised from the gel, purified and concentrated using Gene Clean™ and cloned into pGemT-easy vector (Promega) using conditions recommended by the supplier. The cloned PCR products were sequenced at the DNA

Services Lab, Plant Biotechnology Institute (NRC, Saskatoon, SK) using universal forward and reverse primers to generate sequence from both ends of the cloned insert.

7.1.4 Species-specific primer design

DNA sequence data was complied and aligned using GeneDOC Software. Consensus sequence for each species was compared using GCG of the software. The sequence information was used to design primers using Lasergene PrimerSelect Software (DNAStar, Inc.). Species-specific primers were configured based on sequence differences between species.

7.2 Molecular diagnosis of pest *Lygus* spp. using PCR primers

The primers mtDNA32 (CCGGTCTGAACTCAGATCACGT) and mtDNA34 (CGCCTGTTTAACAAAAACAT) amplified a 550 bp region of the mitochondrial small rRNA subunit gene for all *Lygus* species tested namely, *L. lineolaris*, *L. borealis* and *L. elisus* (Fig. 7.1a). The 550 bp PCR DNA products for each *Lygus* species were excised from gels, purified and cloned. Two to four independent clones derived from each of the voucher species were sequenced. The DNA sequence alignment for consensus sequence for each species are shown in Figure 7.2. Several single nucleotide differences or allelic variants were detected between clones for each species, these are shown as either **R** or **W** in the DNA sequence, for example nucleotide 200 in *L. elisus* (Fig. 7.2). There was a high degree of sequence conservation in the 550 bp region amplified by the mtDNA32/34 primer set from the three *Lygus* species with only six single nucleotide differences scattered throughout the 550 bp region and one two nucleotide difference at bp 333 and 334 of the 550 bp region which distinguish *L. borealis* from *L. lineolaris* DNA sequence (Fig. 7.2). This two nucleotide difference was exploited to produce two 19 oligonucleotide "forward" primers with specific 2 nucleotide differences at the 3' end and

specific for either *L. borealis* or *L. lineolaris* (Figure 7.2). In conjunction with the mtDNA34 primer these *bor/linF1* primers would be predicted to produce a 230 bp PCR product with the respective species. Similarly, a set of two 18 oligonucleotide "reverse" primers with specific 2 nucleotide differences at the 3' end and specific for either *L. borealis* or *L. lineolaris* were developed and these along with the mtDNA32 primer would be predicted to produce a 350 bp PCR product with the respective species. Indeed, when tested against DNA extracted from either *L. borealis* or *L. lineolaris* the designed primers *borR1* and *linR1* amplified a 350 bp PRC product with the respective species (Fig. 7.1b). Similarly, *borF1* and *linF1* amplified a 230 bp PRC product with the respective species (Fig. 7.1b). Significant sequence differences in the 550 bp mtDNA32-34 primer amplification products from *L. elisus* and *L. lineolaris* were not detected (Fig. 7.2) and thus primers to distinguish these species were not developed for the current study.

7.3 Molecular diagnosis of *Peristenus* spp. using PCR primers

The mtDNA32-34 primer set did not amplify a PCR product from any of the voucher *Peristenus* species. The PCR primer set inDNA44 (TCCTCCGCTTATTGATATGC) and inDNA45 (GGAAGTAAAAGTCGTAACAAGG) did however amplify a 1600 bp PRC product representing the internal transcribed spacer region between the 5S and 18S nuclear rRNA genes (ITS2) from voucher sample DNAs from each of the *Peristenus* species, namely *P. digoneutis*, *P. stygicus* and *P. pallipes* (Fig. 7.3a). The 1600 bp PCR product from each *Peristenus* species was excised from agarose gels, purified and cloned into pGemT-easy as described above. Two to four independent clones for each species were sequenced from both ends of the cloned insert as described above and an alignment of the consensus sequence is shown in Figure 7.4. There was a

much higher degree of sequence divergence among the *Peristenus* species for the ITS2 region than was detected for the mitochondrial small rRNA subunit gene among the *Lygus* species tested. Species-specific primers, F1 and F2, were designed for two regions of the ITS2 region that, in conjunction with the inDNA45 primer, would distinguish between the three *Peristenus* species tested (Fig. 7.5) and would be predicted to amplify PCR products of 1320-1370 and 1210- 1230 bp respectively depending on the species of DNA template. Indeed, when the species-specific F2 primers, *digF2*, *palF2*, and *styF2*, were used in conjunction with the inDNA45 primer in PCR reaction with DNA samples from voucher samples of respective *Peristenus* species appropriate size PCR products were detected for each species with the respective species-specific primers (Fig. 7.3b). Given the higher degree of sequence divergence observed in the ITS2 region an attempt was made to amplify a PCR product from *Lygus* DNA using the inDNA44-45 primer set; however, no amplification products were detected.

7.4 Validation of the PCR primer diagnoses using field material

Once the utility of species-specific primers was established using voucher specimens of respective *Lygus* and *Peristenus* species their use with individual field collected insects was tested. The *borF1* and *linF1* primers in conjunction with the mt-DNA34 primers were used to test a series of individual *Lygus* spp. nymphs collected during June and July in 1998 and 1999 in the course of a larger field study (Braun *et al.*, 2001). These nymphs were confirmed, by microscopic dissection, to contain a parasitoid larva and the tissues of the remnant *Lygus* nymph was frozen, homogenized and its DNA extracted. Figure 7.5 shows a series of 10 nymphs tested with either the *linF1*+ mt-DNA34 primer set (Fig. 7.5a) or the *borF1*+ mt-DNA34 primer set (Fig. 7.5b) and demonstrates that nine of the ten nymphs were *L. borealis* based on the

amplification of a 240 bp PCR product with the *borF1*+ mt-DNA34 primer set. Each of the primer sets produced PCR amplification from an exclusive nymph set indicating no cross amplification between species. A series of 50 nymphs from each collection year were subjected to similar PCR analysis and the results are shown in Table 7.1. The vast majority of nymphs, 41 and 36 out of 50 for 1998 and 1999 respectively, were determined to be *L. borealis* with the remainder being identified as *L. lineolaris*. A small minority, 4-6%, could not be identified as either species. As a secondary check, those nymphs for which DNA samples did not show amplification with species-specific primers were tested with the mtDNA32-34 primer set. The mtDNA32-34 primer set did not amplify a PCR product from these indicating that the DNA samples extracted from these nymphs were not good templates for the reaction either because of the quality of the DNA or the nymphs were indeed not from the genus *Lygus*. The former is the most probable explanation for failure to generate the correct PCR product.

In a similar fashion, the *palF2* + inDNA45 primers were used with the DNA extracted from parasitoid tissue dissected from the field-collected nymphs. Between 50 and 60% of the parasitized nymphs showed amplified PCR products of the appropriate size with the *P. pallipes* specific primers (Table 7.1). Those parasitized samples which did not amplify with the *P. pallipes* specific primers were then tested with the inDNA44-45 primer set which was positive for all members of the *Peristenus* genus tested in our study. In all cases those parasitoid samples that failed to amplify with the *P. pallipes* specific primers were also negative with inDNA44-45 primer set. These negative samples were from early in the season and upon dissection were identified as likely being early-first-instar *Peristenus* larvae. Thus these samples either contained too little DNA to be detectable using our PCR conditions after the extended storage time (up to 16 months) or were of a different parasite genus. The other important outcome of this

preliminary analysis of field collected samples was that *P. pallipes* parasitism was identified in nymphs of both *L. borealis* and *L. lineolaris*. For example *P. pallipes* parasitism was confirmed in 23 out 41 *L. borealis* and 6 out of 7 *L. lineolaris* nymphs tested in 1998 (Table 7.1). The sample sizes were too small to determine with any precision whether one *Lygus* species was more susceptible than the other to parasitism. In addition, temporal distributions of the two *Lygus* species were some what different with *L. lineolaris* occurring later in the summer season and this may play a role in a differing exposure of each *Lygus* species to the univoltine parasite, *P. pallipes*.

7.5 Discussion

The one step PCR technique developed here can be used to identify *Lygus* spp. nymphs to species for at least several of the more important species of *Lygus* bugs in western Canada. We were able to identify a primer set, mt-DNA32 + 34, that amplified a 550 bp region of the mitochondrial small rRNA subunit gene from the three *Lygus* species tested. Subsequently based on DNA sequence analysis of the above PCR product several primer sets including *linF1* + mt-DNA34 and *borF1* + mt-DNA34 were developed (Fig. 7.2) and used to distinguish between *L. borealis* and *L. lineolaris*. Although the *linF1*+ mt-DNA34 gave identical results with both *L. lineolaris* and *L. elisus*, this was not a significant problem in the current context because among the field collections examined in this study *L. elisus* constituted a very small proportion (< 1%) of the *Lygus* populations as determined by subsequent adult population counts (Braun *et al.*, 2001). The flexibility of this technique is clear in that additional primers could be designed on the basis of the single nucleotide differences in DNA sequence among the three *Lygus* species tested (Fig. 7.2) in order to distinguish between *L. lineolaris* and *L. elisus*. The vast majority,

72-82%, of the field collected nymphs examined from 1998 and 1999 were shown to be *L. borealis* with the remainder being identified as *L. lineolaris*. A small minority, 4-6%, however could not identified as either species. Although the population samples studied here were only those *Lygus* spp. nymphs shown to be parasitized, based upon microscopic examination of dissected nymphs, the population makeup does reflect the subsequent adult population recorded from the same field collection sites (Braun *et al.*, 2001). Thus the species-specific PCR primer technology developed here is a useful tool for species identification of field collected nymphs and will be invaluable in any ecological study of the populations structure of *Lygus* spp. when examined at the nymphal stage.

A one step PCR method was also developed for the identification of *Peristenus* species based on the ITS2 region between the 5S and 18S nuclear rRNA genes. However, only 50- 60% of the nymphs known to be parasitized by microscopic dissection actually showed amplified PCR products of the appropriate size with the *P. pallipes* specific primers. None of the samples amplified appropriate sized products with *P. digoneutis* or *P. stygicus* specific primers. This is in agreement with earlier studies that found *P. pallipes* to be the only *Peristenus* species associated with *Lygus* spp. on the Canadian prairies (Loan and Craig 1976). The nymphs showing no positive reaction with the *P. pallipes* specific primers were subsequently tested with the inDNA44-45 primer set which gives a 1600 bp with all *Peristenus* species tested. These PCR results were also negative suggesting that the parasites were either of some other genus or else insufficient DNA was present to give good PCR amplification. The latter is likely the explanation as parasite and *Lygus* spp. nymphal tissue had been stored at -70°C for up to 18 months prior to be analyzed and older samples have been shown to amplify much less efficiently than fresh samples. Thus our method appeared to be less sensitive than that developed by Tilmon

et al. (2000) based on a two-step method including genus-specific PCR amplification and subsequent REN analysis of the PCR product. These authors were able to detect as little as 0.01% *Peristenus* DNA mixed with *Lygus* DNA although the absolute concentration of DNA was not given. Thus Tilmon *et al.* (2000) suggest their system will easily detect newly laid eggs of *Peristenus* parasitoids in *Lygus* spp. nymph tissues. The one-step PCR system described here has not yet been optimized in terms of primer and DNA template concentrations and the sensitivity of the system can be increased substantially. The system described here also has the benefit of potentially being able to identify to species *Lygus* nymphs and their associated *Peristenus* parasites to species with the use of multiple primers in single PCR run with field collected nymphs. Follow-up studies will test the system developed here for multiple primer runs and its ability to estimate parasitism rate in field collected *Lygus* spp. nymphal populations.

Table 7.1. Species determination of field collected *Lygus* spp. nymphs and associated parasitoid species using species-specific PCR primers.

	Number of parasitized nymphs (N=50/year) determined to species (*Lygus*) and confirmed to be parasitized by *Peristenus pallipes* using PCR test			
Year	*Lygus borealis*	*Lygus lineolaris*	Negative PCR	*Peristenus pallipes*
1998	41 (23)	7 (6)	2 (1)	30
1999	36 (19)	9 (6)	3 (0)	26

Note: Numbers in parentheses are the number of *P. pallipes* confirmed by PCR in each species of *Lygus*.

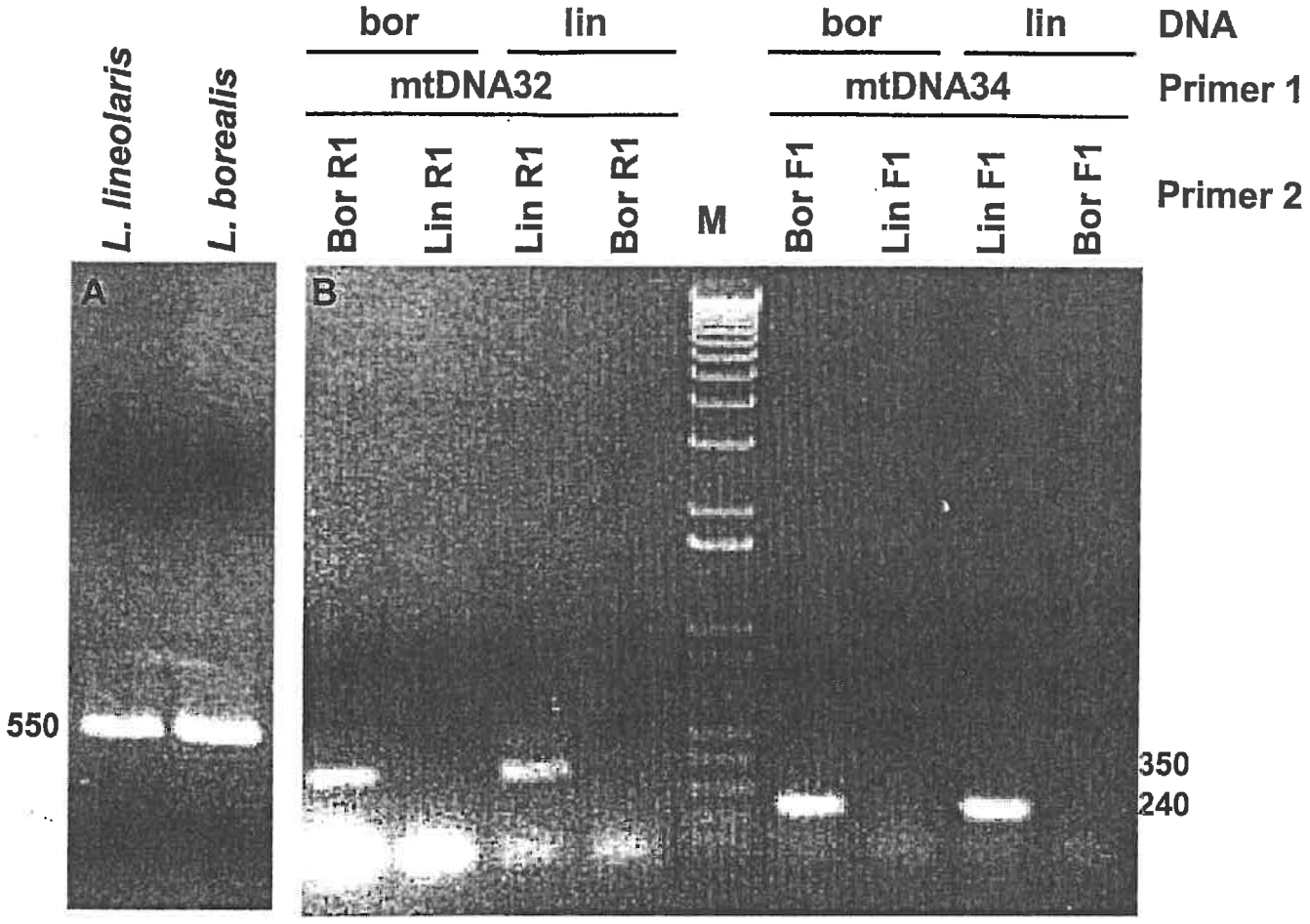

Figure 7.1 Diagnostic PCR for *Lygus* species.
PCR products were electrophoresed on 1.0% agarose gels containing ethidium bromide. Panel A shows the common 550 bp PCR product produced from all *Lygus* species tested using the mtDNA32-34 primer set. Panel B shows the 350 and 240 bp PCR amplification products resulting from the mtDNA32+species specific R1 and mtDNA34+species specific F1 primer sets, respectively. Note that appropriate PCR amplification products are produced only for the species specific primers with the species of voucher specimen DNA for which they were designed.

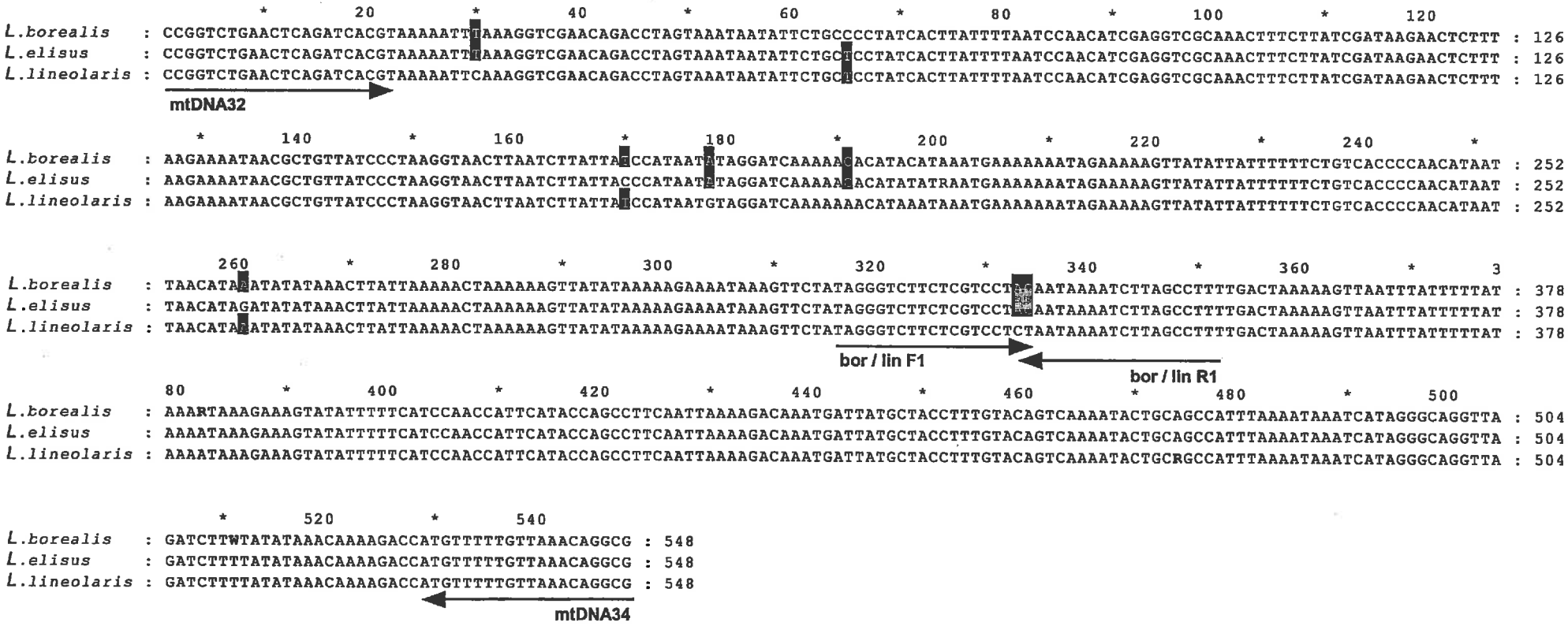

Figure 7.2 Consensus sequence for the mtDNA32-34 primer set PCR amplification products for the three *Lygus* species, *L. borealis*, *L. elisus*, and *L. lineolaris*. The position of the mtDNA 32 and 34 primers and the species specific F1 and R1 primers designed from the sequence are indicated with arrows below the appropriate line of sequence data. Nucleotide positions which vary between the three species are shown in black blocks.

Figure 7.3 Diagnostic PCR for *Peristenus* species.
PCR products were electrophoresed on 1.0% agarose gels containing ethidium bromide. Panel A shows the common ~1600 bp PCR product produced from all *Peristenus* species tested using the inDNA 44-45 primer set. Panel B shows the ~1200 bp PCR amplification products resulting from the inDNA45+species specific F2 primer sets. Note that appropriate PCR amplification products are produced only for the species specific primers with the species of voucher specimen DNA for which they were designed.

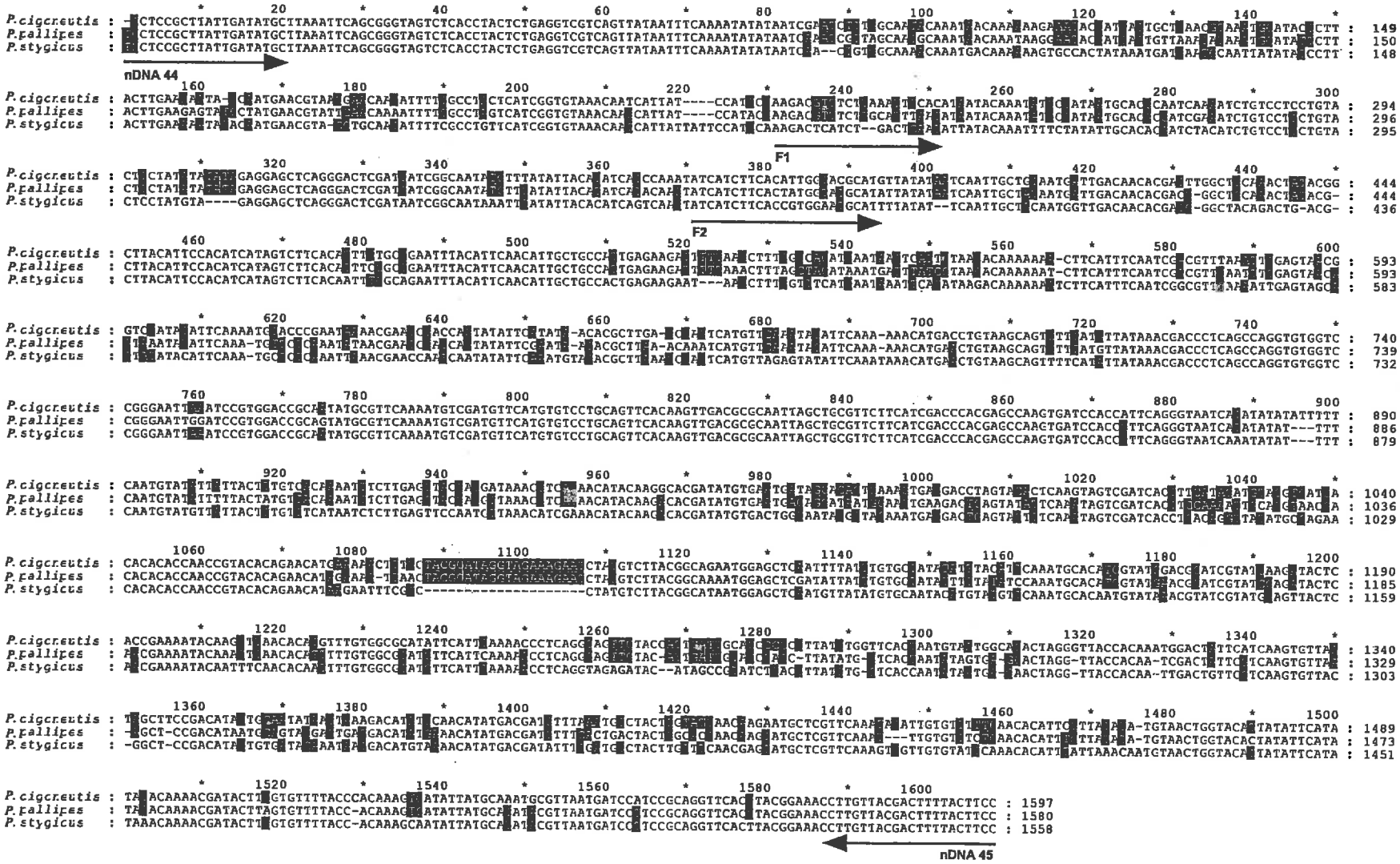

Figure 7.4 Consensus sequence for the inDNA44-45 primer set PCR amplification products for the three *Peristenus* species, *P. digoneutis*, *P. pallipes*, and *P. stygicus*. The position of the inDNA 44 and 45 primers and the species specific F1 and F2 primers designed from the sequence are indicated with arrows below the appropriate line of sequence data. Nucleotides positions which vary between the three species are shown in black blocks.

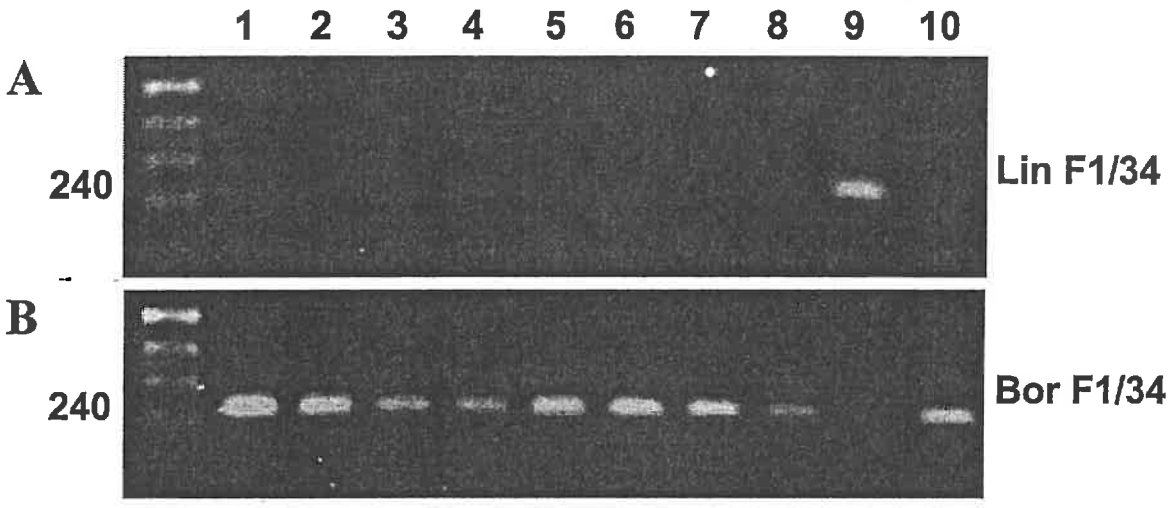

Figure 7.5 Diagnostic PCR for *Lygus* species.
PCR products were electrophoresed on 1.0% agarose gels containing ethidium bromide. Panel A shows PCR product (240 bp) produced from DNA extracted from *Lygus* spp nymphs (#1-10) and using the mtDNA34+*linF1* primer set specific for *L. lineolaris*. Note that only nymph #9 gives a positive PCR product. Panel B shows PCR product (240 bp) produced from DNA extracted from *Lygus* spp nymphs (#1-10) and using the mtDNA34+*borF1* primer set specific for *L. borealis*. Note that all nymphs except #9 result in a positive PCR product.

8.0 General Conclusions

Based on the survey results, the species of *Lygus* most likely to be responsible for economically damaging populations in canola in Saskatchewan is *L. lineolaris*. There were two generations of *L. borealis* and one of *L. lineolaris* in alfalfa whereas there was evidence of only one generation of *L. lineolaris* in canola. Populations of *L. borealis* in alfalfa were high in mid-summer, but there was no evidence that they moved into canola. Rather, *L. lineolaris* moved in from alternative overwintering sources to oviposit in canola during the bud stage. Populations of *Lygus* spp. were low in mustard and did not reach economic thresholds. A univoltine nymphal parasitoid attacked *Lygus* spp. nymphs in alfalfa, resulting in up to 70% parasitism, whereas parasitism in canola and mustard fields was negligible.

The strategy developed here provides the flexibility to address both microsatellite size variation and sequence variation with the same locus. The flanking region sequences also provide independent confirmation of the homology of the PCR products obtained among populations and species. Standard microsatellite applications generally infer homology based on assumed primer specificity. Microsatellites are useful markers for identification and characterization of populations of *Lygus* and *Peristenus*. This work has shown size variation within the microsatellite tract as well as variation in sequence of flanking regions. There are also indications that there is recognizable geographic and genetic structure within species, as shown for *L. lineolaris*. The existence of geographic variation in *Lygus* populations shown here demonstrated the usefulness of primers for some loci across species, potentially providing additional phylogenetic information on relationships among species. The microsatellite marker techniques developed are also applicable to other organisms: this strategy has been successfully applied to *Lygus* bugs and *Peristenus* parasitoids and to aphid species in the genera *Myzus* and

Aphis.

A one-step molecular marker system for identifying *Lygus* nymphs to species was developed based on species-specific PCR primers. The species-specific PCR primers were developed from DNA sequence data generated from clones of a 550 bp region of the mitochondrial small rRNA subunit gene for all *Lygus* species tested namely, *L. lineolaris*, *L. borealis* and *L. elisus*. The utility of these species-specific PCR primers to identify *Lygus* species was confirmed with voucher specimens. In addition, their utility to identify to the species level field-collected-nymphs of the two major species of *Lygus* was confirmed by analysis of DNA samples from 100 nymphs. We were able to unambiguously identify nymphs to species well before they reached a life stage at which they could be identified based on morphological differences.

In a similar fashion, a one-step molecular system for identifying the immature stages of *Peristenus* parasites to species level was developed based on species-specific PCR primers. The species-specific PCR primers were developed from DNA sequence data generated from clones of a 1600 bp region of the internal transcribed spacer region between the 5S and 18S nuclear rRNA genes (ITS2) of the *Peristenus* species, *P. digoneutis*, *P. stygicus* and *P. pallipes*. The utility of these species-specific PCR primers to identify *Peristenus* species was confirmed with voucher specimens. In addition, their utility to identify to species level parasites dissected from field-collected *Lygus* nymphs was confirmed by analysis of DNA samples from 100 parasite samples. . We were able to unambiguously identify the parasites as being *P. pallipes* in 50-60% of the parasitized nymphs without the need of the tedious and time consuming rearing procedure required to produce parasite adults which can be identified on the bases of physical characteristics. The sensitivity of the PCR identification system for *Peristenus* parasitoids needs

to be enhanced and re-tested with field collected material to reduce the number of false negative identifications.

9.0 Publications

Braun, L., M. Erlandson, D. Baldwin, J. Soroka, P. Mason, R. Foottit, and D. Hegedus. 2001. Seasonal occurrence, species composition, and parasitism of *Lygus* spp. (Hemiptera: Miridae) in alfalfa, canola, and mustard in Saskatchewan. The Canadian Entomologist 133:

M Erlandson, L Braun, D Baldwin, J Soroka, and D Hegedus. Development of diagnostic molecular markers for *Lygus* spp. (Hemiptera: Miridae) and associated *Peristenus* spp. (Hymenoptera: Braconidae) parasitoids. (In preparation).

10.0 Recommendations

Further work is needed to:

1) confirm the number of generations of *Lygus* spp. in south, central and northern agricultural areas of Saskatchewan and Alberta;

2) determine the non-crop plants that overwintered *Lygus* spp. feed on early in the season (e.g. cruciferous weeds);

3) confirm that overwintered *Lygus lineolaris* populations are the group invading canola;

4) fully clarify the host-parasitoid relationships of *Lygus lineolaris*, *L. borealis*, and *L. elisus* to establish whether *Peristenus pallipes* (*Lygus* group) parasitizes each species and if all are equally susceptible to parasitism;

5) enhance the sensitivity of the PCR identification system for *Peristenus* parasitoids by optimizing ratios of PCR primers and DNA templates and re-test with field collected material;

6) further validate this technology for ecological studies of *Lygus* and associated parasitoid populations in various crop systems and compare with results of a parallel study using traditional

rearing and dissection techniques to estimate parasitism rates in the pest *Lygus* populations;

7) generate reliable microsatellite markers to extensively characterize the genetic structure of the economic species of *Lygus* (*L. borealis*, *L. elisus*, *L. keltoni*, *L. lineolaris*) and species of *Peristenus* used for biological control, and refine the geographic and genetic structure within species to provide a genetic framework for consideration of factors (such as the potential for reservoir populations on non-crop hosts, regional differences in the pests which may adapt to local conditions of climate and variety of crop, and for the tracking of the movements of introduced *Peristenus* biological control agents) important for planning of pest management strategies;

8) determine if distinct molecular markers exist for *Lygus lineolaris* populations feeding on weeds versus canola;

9) using microsatellite markers, refine knowledge of the relationships between species to develop a reliable classification scheme of *Lygus* spp. (and related Mirid genera) to aid in the search for optimal parasitoid species and to develop a reliable model of relationships among *Peristenus* species to aid in the development of a thorough revision and description of the species in this genus;

10) develop and introduce a cold-tolerant parasitoid species that will attack 2nd generation *Lygus* nymphs;

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