

RURALS: Review of Undergraduate Research in Agricultural and Life Sciences

Volume 2 *RURALS*, Volume 2 -- 2007
Issue 1 2007

Article 2

August 2007

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Recommended Citation

Pierson, Lanae M.; Serikawa, Rosana; Heng-Moss, Tiffany; and Foster, John E. (2007) "An Investigation of the Genetic Variation between *Blissus occiduus* Barber and *Blissus leucopterus leucopterus* (Say)," *RURALS: Review of Undergraduate Research in Agricultural and Life Sciences*: Vol. 2 : Iss. 1 , Article 2. Available at: <https://digitalcommons.unl.edu/rurals/vol2/iss1/2>

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Cover Page Footnote

The authors would like to thank the Undergraduate Creative Activities and Research Experiences (UCARE) program at the University of Nebraska-Lincoln for providing the funding to support this project. Lanae Pierson graduated from the University of Nebraska Insect Science program in 2006 and is currently a M.S. student in the Department of Entomology. The faculty sponsors for this project were Dr. John E. Foster, Professor, Department of Entomology and Dr. Tiffany Heng-Moss, Associate Professor, Department of Entomology. Review coordinated by Dr. Jeffrey T. Krumm, R&D scientist, Sygenta Crop Protection, Vero Beach, Florida.

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An Investigation of the Genetic Variation between *Blissus occiduus* Barber and *Blissus leucopterus leucopterus* (Say)*

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Abstract

In Nebraska, there are two chinch bug species that are of major economic importance: the common chinch bug, *Blissus leucopterus leucopterus* (Say) and the western chinch bug, *B. occiduus* Barber. The lack of key morphological characters to accurately differentiate between these two species in the immature stage and their extensive overlap of plant hosts and geographic distribution underscore the need to identify molecular markers to distinguish between these two chinch bugs. The objective of this research was to investigate the genetic diversity between *B. l. leucopterus* and *B. occiduus* using Amplified Fragment Length Polymorphism (AFLP). Five primer combinations were selected from 20 primer combinations to be used for testing 15 samples of each chinch bug species. The five primer combinations included a total of 151 AFLP markers. Of these, 148 AFLP markers (or 98.01%) were polymorphic between populations. Within *B. occiduus*, 133 AFLP markers (or 88.08%) were polymorphic and within *B. l. leucopterus*, 132 AFLP markers (or 87.42%) were polymorphic. Approximately 63% of the variation in the data set could be attributed to genetic variation within the populations according to the AMOVA analysis. Conversely, approximately 37% of the genetic variation occurred between populations. Several distinct molecular markers were identified that can be employed to distinguish between the two species when morphological characteristics show minimal, if any differences, during the immature stages. This research provides a genetic marker that can be used to differentiate between these two economically important chinch bug species. This new diagnostics tool will allow species-specific management options to be employed. In

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addition, this baseline data can advance future research on chinch bug genetics, including comparisons of additional species.

KEYWORDS: Amplified Fragment Length Polymorphism, chinch bugs, genetic diversity, molecular markers

1. Introduction

Chinch bug species in the genus *Blissus* (Hemiptera: Blissidae) are important pests of agricultural crops and turfgrasses throughout the central, eastern, and southern sections of the United States (Webster 1909, Horton and Satterthwait 1922, Luginbill 1922, Leonard 1966). This chinch bug complex is comprised of numerous species including the common chinch bug, *B. leucopterus leucopterus* (Say) and the western chinch bug, *B. occidentalis* Barber.

Blissus leucopterus leucopterus can be a serious pest of sorghum, corn, and several small grains. Additional grass hosts of *B. l. leucopterus* include bermudagrass, Kentucky bluegrass; perennial ryegrass, fescues, and zoysiagrass (Leonard 1966, Potter 1998). *Blissus leucopterus leucopterus* overwinters as an adult in clumps of bunch grasses or under plant debris in fields. The distribution of *B. l. leucopterus* ranges from the east coast to the western plains (Vittum et al. 1999). While the number of generations varies depending on the geographic location, there are typically two generations per year.

In recent years, *B. occidentalis* has emerged as an important pest of buffalograss. The reported distribution of *B. occidentalis* includes California, Colorado, Montana, Nebraska, and New Mexico in the United States, and Alberta, British Columbia, Manitoba, and Saskatchewan in Canada (Bird and Mitchener 1950, Slater 1964, Baxendale et al. 1999). *B. occidentalis* can be found on barley, sugarcane, wheat, corn, buffalograss, Kentucky bluegrass, perennial ryegrass, zoysiagrass, and other cool-and-warm season grasses (Baxendale et al. 1999, Ferris 1920, Parker 1920, Heng-Moss et al. 2002, Eickhoff et al. 2004).

Historically, insecticides have been employed as the principle method to control chinch bugs. However, growing concerns over the repeated use of chemicals and the potential negative side-effects have led to the development of integrated pest management tactics, including the use of chinch bug-resistant germplasm. Over the past several years, germplasm resistant to each of the two economically-important chinch bugs has been identified and integrated into pest management programs.

Because of the extensive geographical overlap of these two economically-important chinch bug species and their host plants, studies have been conducted to identify grasses with resistance to multiple chinch bug species. Anderson et al. (2006) conducted a comprehensive study to document the presence of resistance to multiple chinch bug species in selected warm- and cool-season grasses. The results of this research showed that *B. occidentalis*-resistant buffalograsses were moderately to highly susceptible to the other three chinch bug species tested. In contrast, *B. occidentalis* did not cause considerable damage to any of the turfgrasses

or sorghums evaluated, other than buffalograss, irrespective of whether or not they were resistant to another chinch bug species. The varying degrees of susceptibility and resistance exhibited by the grasses underscores the importance of accurately identifying *B. l. leucopterus* and *B. occiduus* to species.

While a trained eye can distinguish between morphological characteristics of chinch bug species in the adult stage, there is a lack of distinguishing features to use to differentiate between species in immature stages. Molecular diagnostics is playing an increasingly important role in species identification. Genetic markers have the ability to unambiguously identify and differentiate species. The goal of this research was to identify molecular markers for distinguishing between the species *B. occiduus* and *B. l. leucopterus*. To accomplish this, extracted DNA from chinch bugs was run through a polyacrylamide gel. The gel was analyzed using software to detect a presence or absence of bands at various loci.

2. Materials and Methods

Insects. *Blissus leucopterus leucopterus* specimens were collected from sorghum at the University of Nebraska-Lincoln Agricultural Research and Development Center near Mead, Nebraska. They were also collected from sorghum plants maintained under greenhouse conditions. *Blissus occiduus* specimens were vacuumed from buffalograss lawns at the University of Nebraska East Campus, Lincoln, Nebraska. A mechanical aspirator was used to separate the chinch bugs from the plant material and debris. Chinch bugs were aged according to Baxendale et al. (1999) and immediately frozen at -80°C.

DNA Isolation. DNA was isolated from the chinch bugs using a modified CTAB extraction protocol (Black and Duteau 1997). The gut was removed from each chinch bug before placing it in distilled water for rinsing. A single chinch bug was then placed in an eppendorf tube where it was homogenized in 500 μ L CTAB buffer (100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 0.02M EDTA, 2% CTAB [Hexa decyl tri methyl ammonium bromide], and 0.2% β -mercaptoethanol) using disposable micro pestles. A 10 μ L quantity of proteinase K (200 μ g/mL extraction buffer) was added to each sample. The tubes were then heated for one hour at 65°C, and they were mixed every 20 min by gently inverting each tube. Twenty μ L RNase A (50 mg/mL) was then pipetted into each tube. The samples were heated for three hours at 37°C, and were again mixed every 20 min by gently inverting the tubes. The tubes were centrifuged at 14,000 rpm for five min at room temperature. The supernatant was transferred to a new eppendorf tube. A

500 μL quantity of chloroform (Isoamyl alcohol 24:1) was added to each tube before centrifuging at 12,000 rpm for 20 min at room temperature. The aqueous phase was then transferred to a new tube and the chloroform/centrifugation steps were repeated. After centrifugation, the aqueous phase was transferred to a new tube and 400 μL chilled isopropanol was added. The samples were incubated at 4°C for at least two hours. The tubes were centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was discarded from each tube, leaving the DNA pellet remaining. The pellets were washed in 500 μL absolute ethanol, then centrifuged for five min at 4°C. The supernatant was discarded, and the pellets were washed in chilled 70% ethanol before centrifuging for five min at 4°C. The ethanol was removed, and the pellets were allowed to air dry for at least 30 min. Each pellet was dissolved in approximately 25 μL 1X TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). DNA concentration and quality of each sample was determined by a 0.8% TAE (Tris-Acetate-EDTA) agarose gel using a known λ concentration standard (22.2 ng/ μL) (New England BioLabs, Beverly, MA). TE buffer (1X) was added to samples not having a genomic DNA concentration of 22.2 ng/ μL . The gels were visualized with Genomics Solutions software.

AFLP-PCR. A modified protocol of Vos et al. (1995) was used incorporating fluorophore (IRD-700) labeled *EcoRI* primers in PCR (LI-COR, Lincoln, NE).

Template Preparation. Restriction Digestion. Approximately 1 μg of genomic DNA in a 7 μL volume was incubated with 1.25 μL 10x One-Phor-All buffer (Amersham Pharmacia Biotech Inc., Piscataway, NJ), 0.125 μL BSA (New England BioLabs, Beverly, MA), 3.938 μL ddH₂O, and restriction endonucleases *EcoRI* and *MseI* (New England Biolabs, Beverly, MA) in quantities of 0.0625 μL and 0.125 μL , respectively. Restriction digestion was performed in a DNA thermal cycler 2700 (Applied Biosystems, Foster city, CA) with the following profile: 2.5 h at 37°C, 15 min at 70°C to deactivate the restriction enzymes, and soak at 4°C.

Adapter Ligation. A 5 μL mixture was added to each tube from the preceding step, which included 0.5 μL 10x T4 DNA ligase buffer (New England Biolabs, Beverly, MA), 0.5 μL *EcoRI* adapter, 0.5 μL *MseI* adapter, 0.15 μL T4 DNA ligase (New England Biolabs, Beverly, MA), and 3.35 μL ddH₂O. The tubes were placed in the thermal cycler, which was set at 25°C and ran for eight to ten h. Afterwards, 135 μL 1X TE buffer (10 mM Tris-Cl, 0.1 mM EDTA [pH 8.0]) was added to each tube in order to dilute the mixtures. The solutions were mixed and stored at -20°C.

AFLP Assay. Pre-selective PCR Amplification. A 1.25 μL quantity of the diluted DNA solution was added to a mixture containing 10 μL Pre-Amp Primer Mix II (containing two oligonucleotide primers to correspond to *EcoRI* adapted ends and *MseI* adapted ends, from Invitrogen, Carlsbad, CA), 1.25 μL 10X PCR buffer (Applied Biosystems, Foster City, CA), 0.75 μL mM MgCl_2 (Applied Biosystems, Foster City, CA), and 0.25 μL of 5U/ μL *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA). The tubes of solution were put in the thermal cycler for 20 cycles of the following profile sequence: 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min. The mixtures were then diluted by adding 190 μL ddH₂O and stored at -20°C.

Selective PCR Amplification. A 2.0 μL quantity of the diluted pre-amp DNA product was added to a solution of 4.32 μL ddH₂O, 1.2 μL 10X PCR buffer, 0.72 μL 15 mM MgCl_2 , 0.08 μL of 5U/ μL *AmpliTaq* DNA polymerase, 2.0 μL *MseI* primer (LI-COR, Lincoln, NE), and 0.3 μL *EcoRI* primer (LI-COR, Lincoln, NE). The samples were placed into the thermal cycler using a “Touchdown” program with the following profile: 1 cycle of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min; 12 cycles of 94°C for 30 sec and 72°C for 1 min; and 23 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min with a soak at 4°C.

Reactions were stopped by adding 2.5 μL blue stop solution (LI-COR, Lincoln, NE). The tubes were placed in the thermal cycler for 3 min at 94°C for denaturing. They were soaked at 4°C.

Samples were flash cooled on ice prior to electrophoresis. One μL of each sample along with 1 μL IRD-labeled 50-700 bp size marker (LI-COR, Lincoln, NE) were electrophoresed through KB^{Plus} 6.5% ready-to-use gel matrix (LI-COR, Lincoln, NE). Infrared fluorescent bands were detected by the laser scanning system (LI-COR Model 4200S-2).

Scoring AFLP Data. The presence or absence of fragments was detected using a computer program called SAGA MX (LI-COR Inc. Version 3.2, 2004, Krumm 2005). In addition, the program estimated the sizes of the fragments by comparing them with the IRD-700 labeled 50-700 bp ladder. The program converted the presence or absence of bands into numerical data. A “1” was used to indicate presence of a band of a particular size, a “0” was used to indicate absence of a fragment, and a “?” was used to indicate places where there was no data available. Of the twenty different primer combinations evaluated, five primer combinations were selected to be analyzed based on differences in banding patterns between the two chinch bug species.

Coefficient of Variation Analysis. Another computer program, DBOOT (Coelho Version 1.1, 2001, Krumm 2005) was used to analyze the data from the output of the SAGA program in order to assess the number of polymorphic loci required for acceptable precision in genetic studies. The bootstrapping analysis was replicated 1000 times, and the simple matching coefficient (SM) was used to assess the strength of the molecular markers. The number of markers scored was plotted on a graph against the coefficient of variation.

Genetic Diversity and Gene Flow of Chinch Bugs. POPGENE version 1.32 (Yeh and Boyle 1997, Krumm 2005) was then used to analyze data to genetically compare the two chinch bug species. A dominant marker data set was used assuming Hardy-Weinberg equilibrium. Analyses within and among the species included the percent polymorphism, genetic diversity (h), and gene flow estimation (Nm).

A total of 151 markers were selected to estimate genetic diversity using POPGENE in order to determine the amount of polymorphism within a chinch bug species. Each species was analyzed for genetic diversity using Nei's (1973) gene diversity. Loci were considered polymorphic only if the frequency of the most common allele fell below a threshold of 0.99. This rule was used in order to avoid a positive correlation between P and the sample size.

Analysis of Molecular Variance. The computer program ARLEQUIN (AMOVA version 2.0; Excoffier et al. 1992, Krumm 2005) was used for population genetic analysis. Total variance for the AFLP data set was separated into two items: (1) variance among populations and (2) variance within populations. The program partitions the variation into correlating genotypes rather than gene frequencies because of the dominant expression of the AFLP tool. The variance was tested using 1000 permutations.

Tree Analysis. A dendrogram of the two chinch bug species was constructed using the NT-SYSPC software version 2.11T (Rhoif 2002, Krumm 2005). A similarity coefficient matrix was constructed using the SIMQUAL program. A dendrogram was then generated with the SAHN program using the unweighted pair-group method with arithmetic mean (UPGMA). SAHN performs clustering analysis based on Sequential, Agglomerative, Hierarchical, and Nested methods (Sneath and Sokal 1973). BOOD version 3.0 (Coelho 2001) was used to conduct bootstrap analysis using 1000 permutations to test cluster strength. Cluster analysis was performed on 15 samples of *B. occiduus* and 15 samples of *B. l. leucop-terus* to yield a dendrogram.

Table 1. Selective primers used for AFLP analysis and their associated AFLP markers and range of fragment sizes for *B. occiduus* and *B. leucopterus leucopterus*.

Primer Pair + Selective Ext.	No. of AFLP markers	Fragment size range (bp)
<i>EcoRI</i> + ACC/ <i>MseI</i> + CAA	23	63-431
<i>EcoRI</i> + AGC/ <i>MseI</i> + CAA	35	44-455
<i>EcoRI</i> + AGG/ <i>MseI</i> + CAT	33	70-490
<i>EcoRI</i> + AAG/ <i>MseI</i> + CTC	29	38-488
<i>EcoRI</i> + AAG/ <i>MseI</i> + CTT	31	53-428
Total	151	38-490
Average/Primer Pair	≈30	

3. Results

Number and fragment lengths of AFLP markers observed. Five primer combinations were selected from 20 primer combinations to be used for testing 15 samples of each chinch bug species. Each combination of primers averaged approximately 30 AFLP markers ranging from 38 to 490 bp in fragment length (Table 1). The five primer combinations included a total of 151 AFLP markers. Of these, 148 AFLP markers (or 98.01%) were polymorphic among all populations. Within *B. occiduus*, 133 AFLP markers (or 88.08%) were polymorphic and within *B. l. leucopterus*, 132 AFLP markers (or 87.42%) were polymorphic.

Coefficient of variation compared to the number of AFLP markers explored. The DBOOT program was used to analyze the correlation between the coefficient of variation and the number of molecular markers examined in order to indicate the robustness of the data collected (Figure 1). From this data, one can determine that all except for 7.3% of the variation in the population can be explained using these markers. The high quantity of markers used decreased the coefficient of variation, meaning that 151 markers were sufficient to further analyze genetic variability and genetic structure.

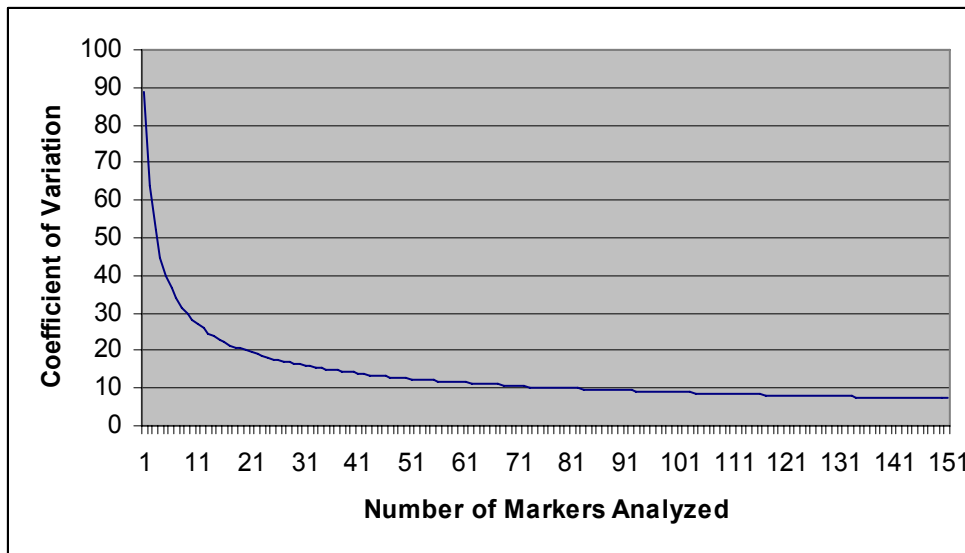


Figure 1. DBOOT was used to assess the coefficient of variation of the AFLP markers used for two chinch bug species.

Genetic Similarity Between *B. occiduus* and *B. leucopterus leucopterus*.

Based on visual analysis of the gels in the SAGA program, fifteen AFLP markers from the five gels run showed a distinct banding difference between the two species (Table 2). This visual analysis took into account error that the SAGA program might not have, such as erroneous samples that may have shown a presence of bands at all basepair sizes or an absence of bands throughout (Figures 2-6). Gel pictures are shown from 0 basepairs to a basepair size at which there is still relevant data. This basepair size varies between the gels. Gel pictures also have tabs to the side indicating various markers at which data was collected.

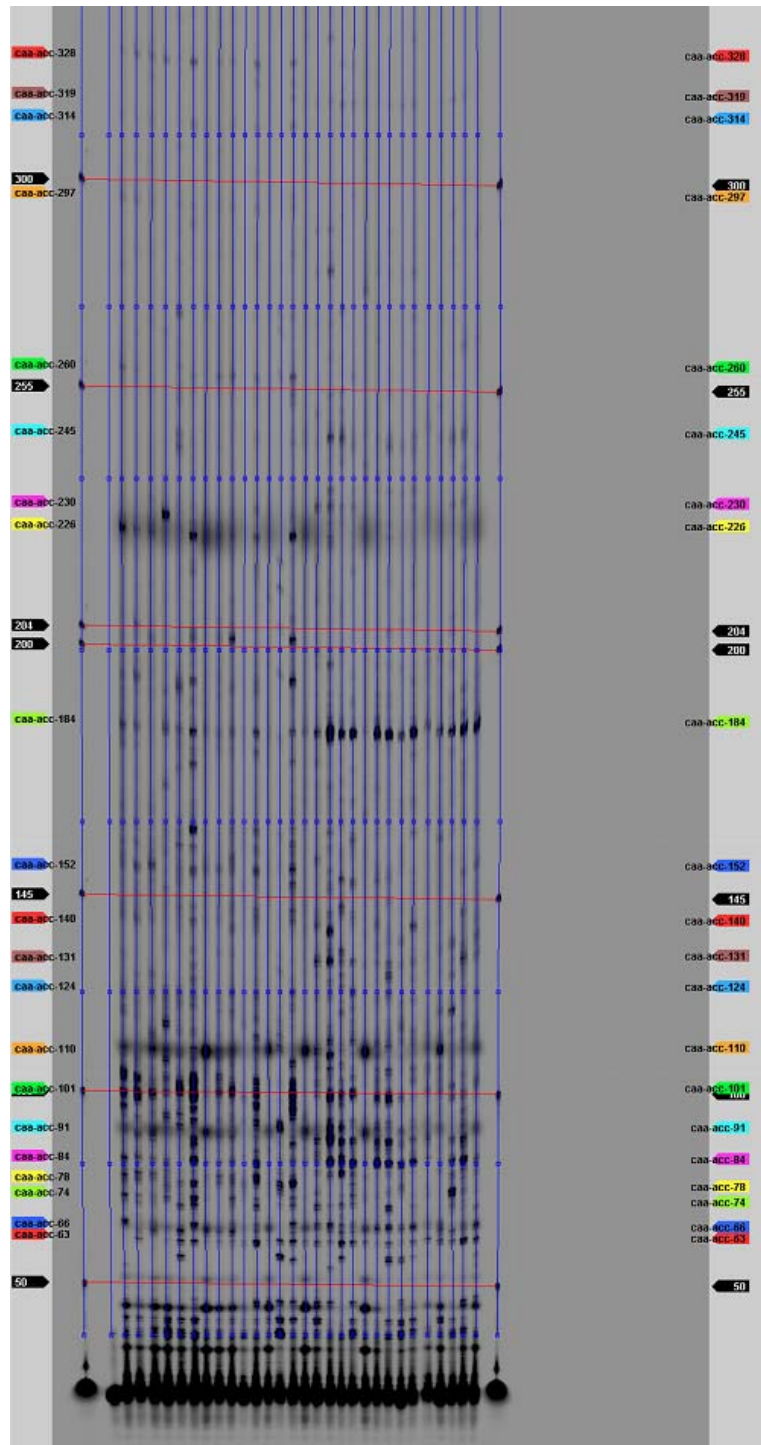


Figure 2. Gel picture with primers *EcoRI* + ACC and *MseI* + CAA.

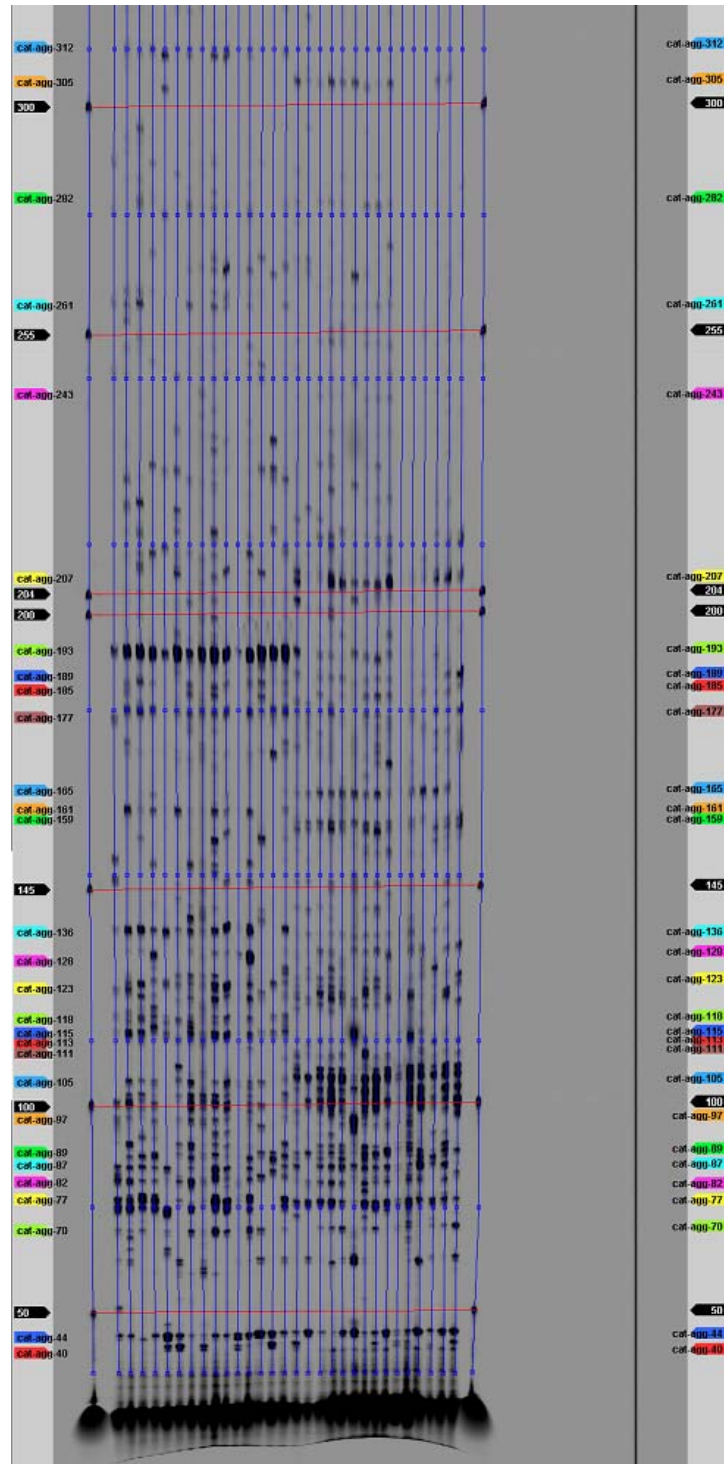


Figure 3. Gel picture with primers *EcoRI* + AGG and *MseI* + CAT.

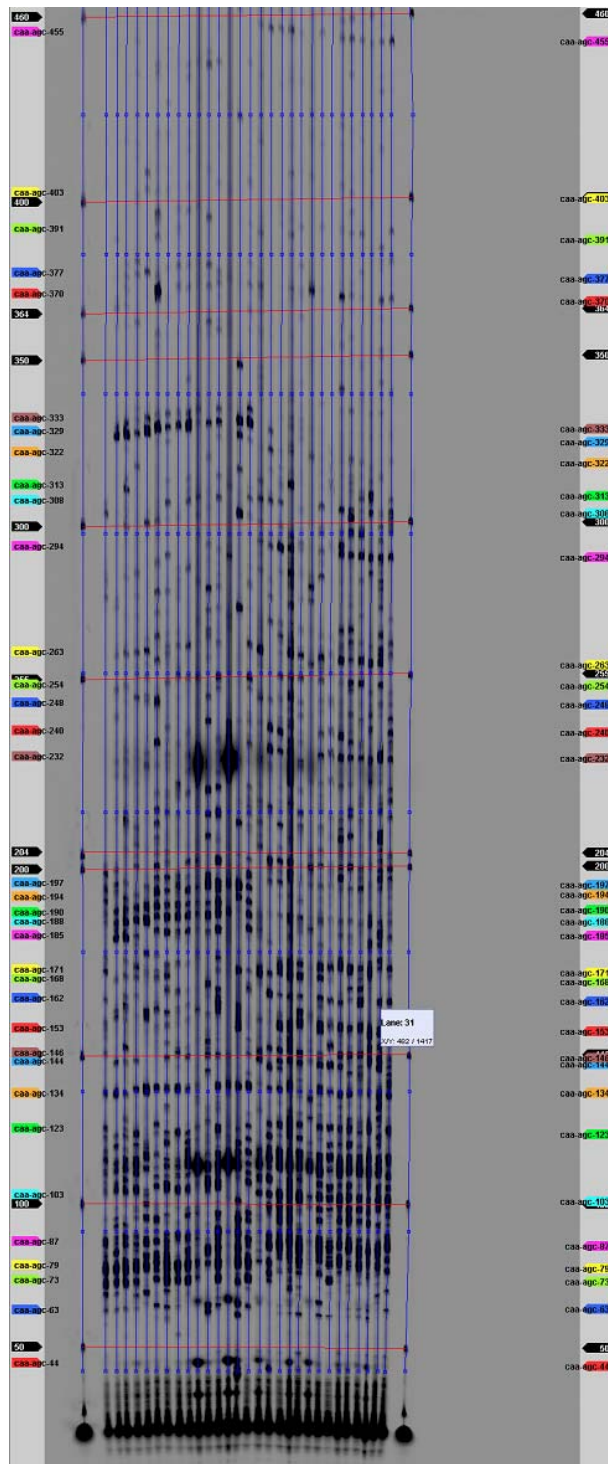


Figure 4. Gel picture with primers *EcoRI* + AGC and *MseI* + CAA.

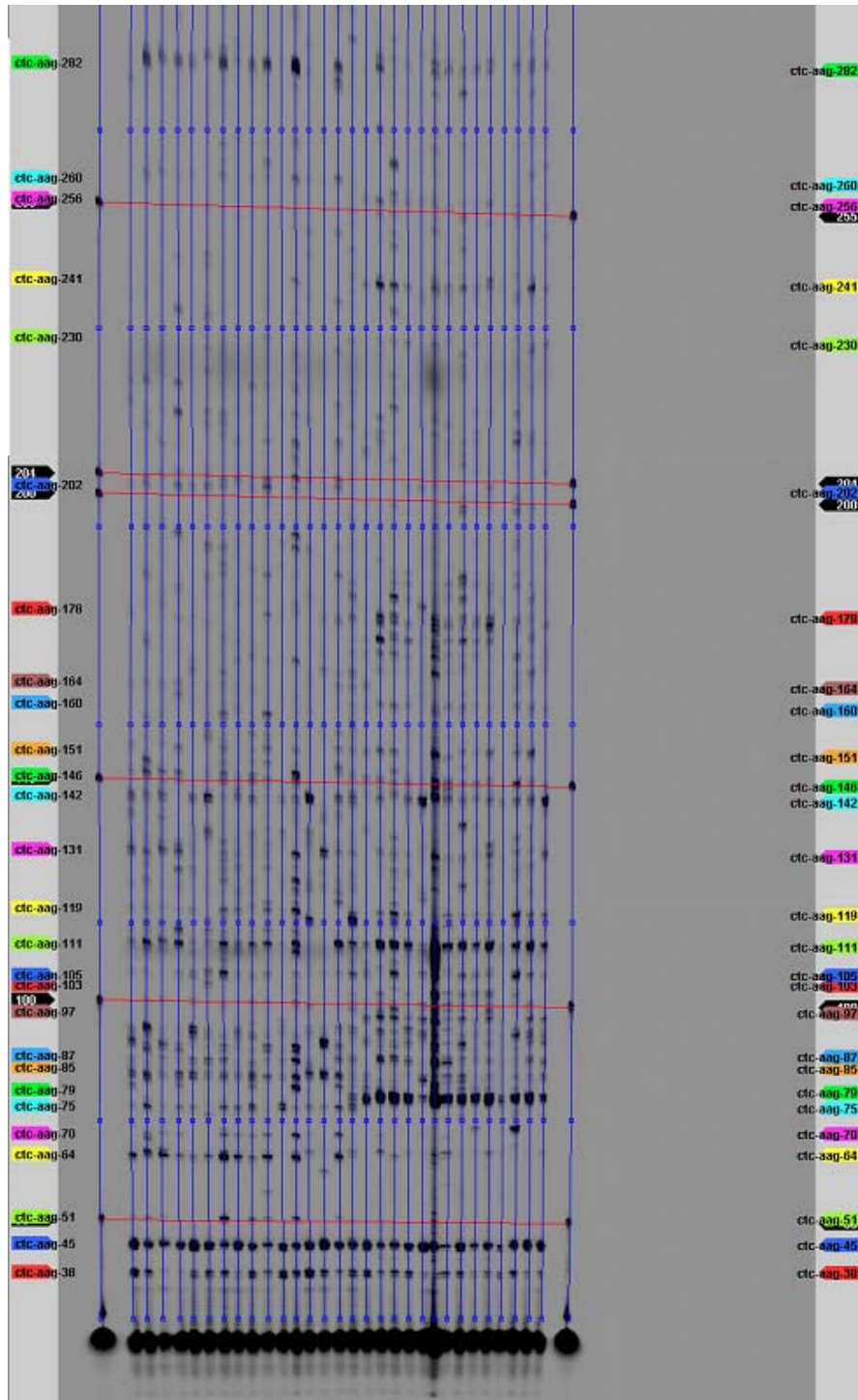


Figure 5. Gel picture with primers *EcoRI* + AAG and *MseI* + CTC.

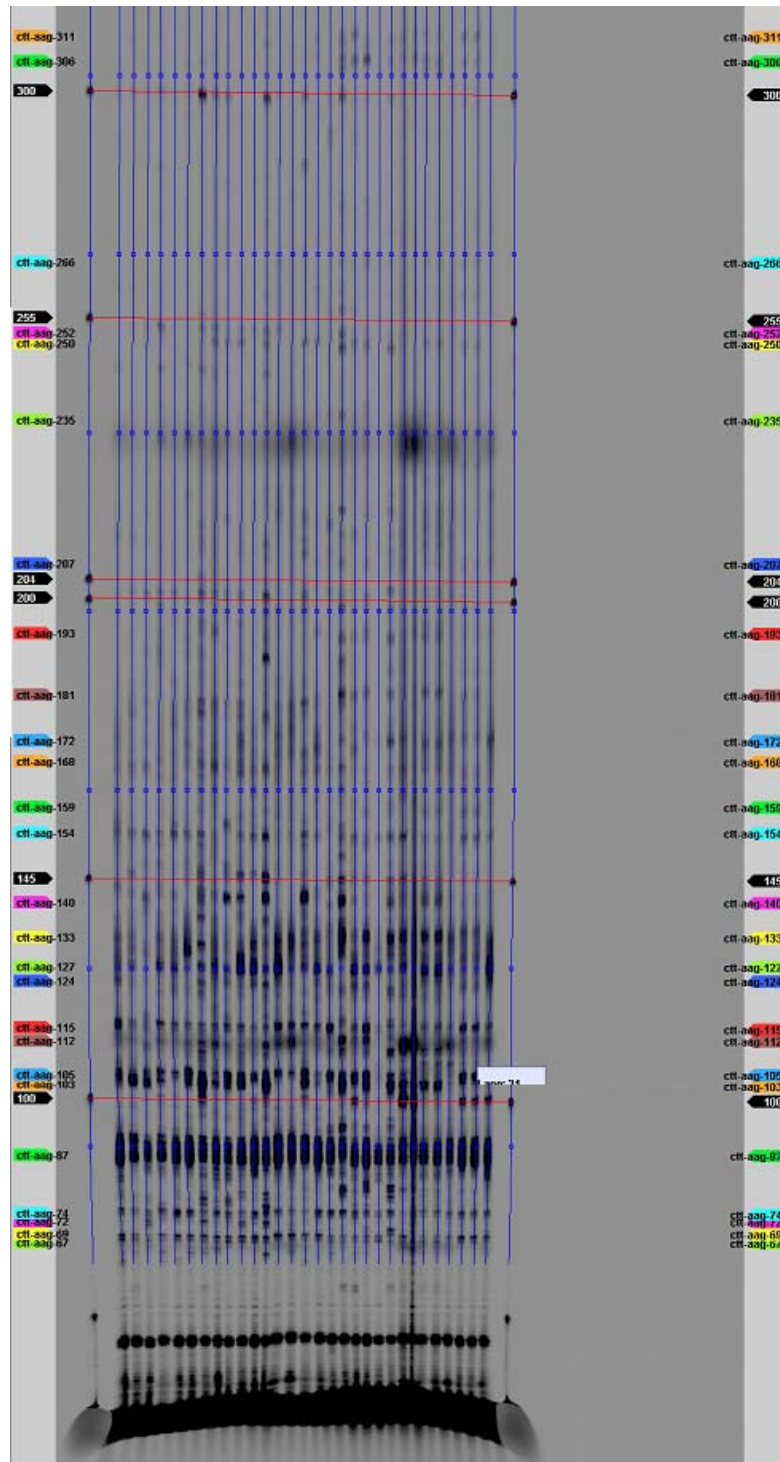


Figure 6. Gel picture with primers *EcoRI* + AAG and *MseI* + CTT.

Table 2. Selective primers used for AFLP analysis and basepair sizes at which a distinct difference could be seen between *Blissus occiduus* and *B. leucopterus leucopterus* (One species showed a complete presence of bands while the other species showed a complete absence of bands at that particular basepair size).

Primer Pair + Selective Ext.	Fragment sizes (bp) with distinct differences
<i>EcoRI</i> + ACC/ <i>MseI</i> + CAA	131, 319
<i>EcoRI</i> + AGC/ <i>MseI</i> + CAA	190, 194, 294, 329
<i>EcoRI</i> + AGG/ <i>MseI</i> + CAT	111, 159, 165
<i>EcoRI</i> + AAG/ <i>MseI</i> + CTC	79, 97, 256, 260
<i>EcoRI</i> + AAG/ <i>MseI</i> + CTT	306, 311

A dendrogram with similarity coefficients ranging from 48% to 86% for the two chinch bug species was constructed based on consensus values (Figure 7). Bootstrap values ranged from 7.9% to 100%, with a majority of the values above 30%. The dendrogram showed a distinct difference between the two species with varying degrees of similarity within each species.

Genetic diversity. Genetic diversity values, also known as population heterozygosity (h), were calculated by POPGENE version 1.32. Genetic diversity was considered high for each species, with a value of 0.3222 (± 0.1661) for *B. occiduus* and a value of 0.2912 (± 0.1671) for *B. l. leucopterus* (Table 3). Genetic diversity for the combined species was higher at a value of 0.4099 (± 0.1158).

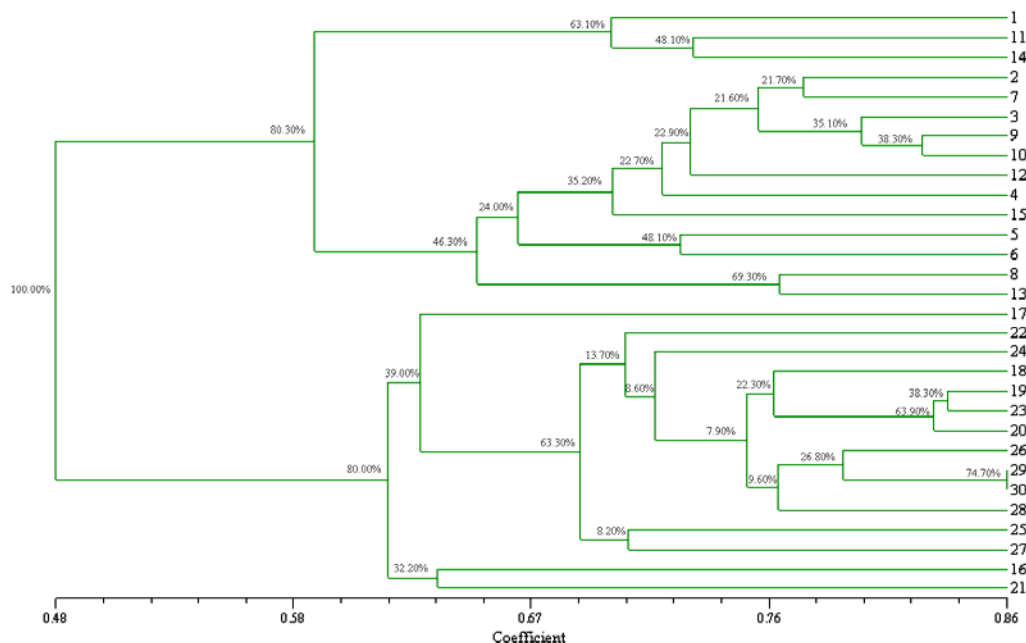


Figure 7. Dendrogram showing genetic relationships within and between *Blissus occiduus* and *B. leucopterus leucopterus*. Numbers to the right of the dendrogram represent individual samples. Samples 1-15 are *B. occiduus* and samples 16-30 are *B. l. leucopterus*. Numbers within the dendrogram represent bootstrap values. Results are based on data from five primer pair combinations. Calculations are based on the Jaccard coefficient and UPGMA clustering.

Table 3. Gene diversity and degree of polymorphism (%) for *Blissus occiduus* and *B. leucopterus leucopterus*.

Species	Polymorphic Bands	Polymorphism (%)	Gene Diversity
<i>Blissus occiduus</i>	133	88.08	0.3222+/-0.1661
<i>Blissus leucopterus leucopterus</i>	132	87.42	0.2912+/-0.1671
Average	132.5	87.75	0.3067+/-0.1666
Both Species	148	98.01	0.4099+/-0.1158

Analysis of Molecular Variance (AMOVA). Approximately 63% of the variation in the data set could be attributed to genetic variation within the populations according to the AMOVA analysis (Table 4). Conversely, approximately 37% of the genetic variation occurred between the populations.

Table 4. Hierarchical analysis of molecular variance (AMOVA) for *Blissus occiduus* and *B. leucopterus leucopterus*. Significance values obtained from 1,000 permutation tests.

Source of Variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among Species	1	209.167	12.51905	36.93
Within Species	28	598.667	21.38095	63.07
Total	29	807.833	33.90000	

4. Discussion

This research represents the first report on the genetic variation between *B. l. leucopterus* and *B. occiduus*. From these results, we can conclude that the two species are genetically very similar, with more genetic variation between individuals than between species. Several distinct molecular markers were identified that can be employed to distinguish between the two species when morphological characteristics show minimal, if any differences, during the immature stages. The ability to differentiate between the two species of chinch bugs in the immature stage using molecular markers will allow species-specific management approaches to be implemented.

This research provides baseline data for comparing the genetic diversity of chinch bugs within a specific region as well as among regions. In addition, other chinch bugs species can now be studied at the molecular level with the methods developed in this research to identify additional molecular markers for differentiating among chinch bug species and for studying genetic diversity within the *Blissus* complex.

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