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Genetic Variation in Field and Laboratory Populations of the Spined Soldier Bug, *Podisus maculiventris*

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Abstract

The predatory spined soldier bug, *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae), is an economically important and highly valued biological control agent. There is substantial information on the biology, ecology, behavior, and rearing of this stink bug. However, virtually nothing is known of its genetic variation, in natural or domesticated populations. To address this lacuna, we used amplified fragment length polymorphism (AFLP) to assess the genetic variability of field and laboratory populations. Four AFLP universal primer combinations yielded a total of 209 usable loci. The AFLP results showed greater genetic variability between populations from Missouri and Mississippi (both USA), and relatively low variability within Missouri populations. We infer little genetic isolation among Missouri field populations and within laboratory populations but a significant genetic isolation between Missouri and Mississippi populations.

Keywords: AFLP, predatory bug, Pentatomidae, genetic similarity, Heteroptera, stink bug

Introduction

The spined soldier bug, *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae), is a free-living, Nearctic terrestrial generalist predator associated with agricultural and forest environments, where it feeds on eggs and larvae of over 100 species of Coleoptera and Lepidoptera (McPherson, 1980). It is economically important and a high-value biological control agent because of its voracious feeding habits, high reproductive capacity, and mass-rearing potential (Hough-Goldstein, 1988; Hough-Goldstein & McPherson, 1996). All life stages are predaceous, which supports the use of immatures for augmentative control (Richman & Whitcomb, 1978). The biological control potential of *P. maculiventris* for several important pests has been demonstrated and includes the Colorado potato beetle (Aldrich & Cantelo, 1999), the tomato looper (De Clercq et al., 1998), and the viburnum leaf beetle (Desurmont & Weston, 2008). In addition, *P. maculiventris* manifested no negative developmental effects following the consumption of natural prey containing the *Bacillus thuringiensis* Berliner Cry1Ac toxin. This shows that *P. maculiventris* is complimentary with pest control strategies in transgenic crops (Torres & Ruberson, 2007).

Much is known of *P. maculiventris* basic biology (De Clercq & Degheele, 1997; Wittmeyer et al., 2001), nutrition and life history parameters (De Clercq & Degheele, 1992;Wittmeyer & Coudron, 2001; Coudron et al., 2002), effect of nutrition and fecundity on gene expression (Coudron et al., 2006), response to cold storage (Coudron et al., 2007), and pheromone production (Aldrich, 1988). Because individuals manifest variation in these parameters, we posed the hypothesis of substantial genetic variability in *P. maculiventris*. The extent of genetic variation has not been documented within wild or laboratory populations—laboratory populations are presumably domesticated after being reared for several generations under laboratory conditions. Inbreeding depression has been reported within laboratory populations of many other insect species, including laboratory and wild populations of *Drosophila melanogaster* Meigen (Latter & Mulley, 1995). Laboratory populations can also diverge genetically from wild populations after many generations in captivity, as was shown for *Anopheles albimanus* Wiedemann (Arias et al., 2005). These genetic events are of fundamental interest in biology; however, they have not been considered in programs dedicated to producing large numbers of insects for application in biological control. Changes in the genetic composition of insect species in mass-rearing programs could have serious financial consequences in terms of the costs of producing the insects and the possible crop losses resulting from deploying ineffective biological control agents. Consequently, it is important to understand the genetics of both wild and laboratory populations of insects used in mass-rearing programs for biological control purposes.

For this study we posed the hypothesis that wild and laboratory spined soldier bug populations express significant genetic variation. DNA from wild and laboratory populations of spined soldier bug was analyzed to test our hypothesis using amplified fragment length polymorphism (AFLP). AFLP is a reliable tool to study genetic diversity in insects at the population level without a priori knowledge of genome structure or sequence (Vos et al., 1995; Reineke et al., 1999; Bensch & Akesson, 2005). For example, AFLP was used to differentiate strains of screwworms *Cochliomyia hominivorax* (Coquerel) (Alamalakala et al., 2009), elucidate genetic differences among ecotypes of *Ostrinia nubilalis* (Hübner) (Krumm

et al., 2008), and to assess gene flow and genetic structure in populations of *Spodoptera frugiperda* (JE Smith) across a large geographic region (Clark et al., 2007).

Materials and methods

Insects

Laboratory colony

The *P. maculiventris* laboratory colony originated from adults collected in alfalfa near a dairy farm 10 km northeast (52°19′31″N, 113°51′37″W) of Columbia, Missouri, USA, in the spring of 2004, using a WHY Trap (Sterling International, Inc., Spokane, Washington, USA) equipped with a lure impregnated with the male pheromone (containing trans-2-hexenal, α terpineol, benzyl alcohol, linalool, terpinen-4-ol and S-1-isopropyl-4-methyl-3-cyclohexen-1-ol; Sigma-Aldrich, St. Louis, Missouri) (Aldrich, 1988). The laboratory colony had been maintained in continuous culture at a level of 30 mated pairs since 2004 for more than 100 generations in half pint (i.e., ca. 250 ml) paper cups with a Petri dish lid and continuously fed live and coddled fourth instars of *Trichoplusia ni* (Hübner).Water was provided *ad libitum* via a dental wick, and the insects were held at 26°C, L16:D8 photoperiod, and 70% r.h. in a walk-in chamber (Coudron et al., 2002).

Wild populations

Wild adults from the Missouri sites, collected in the spring of 2010 using the trapping method described above had overwintered from the previous summer. Missouri collection sites included fescue near the dairy farm 10 km northeast (52°20′39″N, 113°50′30″W) of Columbia (Dairy Farm), an alfalfa field ca. 16 km southeast (38°53′58″N, 92°12′34″W) of the dairy farm (Alfalfa), and Powell Gardens, ca. 250 km west (38°52′10″N, 94°2′21″W) of Columbia (Garden). Adults from Mississippi were collected in mid-summer 2010 using sweep nets and were under continuous development when collected. Headlands surrounding cotton fields near Greenville, Mississippi (33°26′1″N, 90°54′55″W) served as the collection site of the "Mississippi" colony. Adults field collected in 2010 from the Missouri and Mississippi sites were maintained in 250-ml paper cups with a Petri dish lid and continuously fed live and coddled fourth instar *T. ni* as described above. Subsequent generations, labeled $G_{1–100}$, were maintained in a similar manner.

DNA extraction and quantification

Individual adult specimens were provided only water for 3 days to clear the alimentary canal and then placed in 1.5-ml microtubes, weighed, and the specimens preserved by treating three times with fresh 95% ethanol at 4°C for 24 h, decanting the ethanol after each treatment. The DNA from each individual was analyzed separately, with 20, 20, 12, 8, and 58 individuals analyzed from the Laboratory, Dairy Farm, Alfalfa, Garden, and Mississippi populations, respectively. An equal number of males and females were analyzed except in the Mississippi population which contained 43 females and 15 males. DNA was extracted using the CTAB (hexadecyl-trimethyl-ammonium bromide) protocol modified from Doyle & Doyle (1987). The thorax of each individual sample was homogenized in 500 ml of CTAB

extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB, 0.2% β-mercaptoethanol). Proteinase K was added (10 μl of 0.02 g ml^{-1}) and samples were incubated for 100 min at 65°C; RNase A (15 μl of 0.05 g ml^{-1}) was added, followed by incubation for 2 h at 37°C. Samples were mixed throughout the incubation period by inverting tubes every 20 min. After incubation the samples were centrifuged at 14.8 *g* at 20°C for 10 min, the supernatant was removed and put in a new 1.5-ml microcentrifuge tube and the tissue was discarded. Supernatant was mixed with 500 μl chloroform: isoamyl alcohol (24:1) then centrifuged at 14.8 *g* and 20°C for 20 min. The top aqueous phase was transferred to a new 1.5-ml tube and the chloroform: isoamyl alcohol step was repeated. DNA was precipitated from the resulting top aqueous phase with cold (–20°C) 100% isopropanol and stored overnight at 4°C.

Samples were centrifuged for 30 min at 14.8 *g* and 4°C to form a pellet of DNA. The isopropanol was poured off and the pellet was washed with 400 μl cold absolute ethanol, and centrifuged for 5 min at 14.8 *g* and 4°C. The wash was repeated with 70% cold ethanol. The ethanol was removed and samples were air dried until remaining ethanol had evaporated (ca. 30 min). DNA was resuspended in 50 μl 1x TE buffer (10 mM Tris-HCl and 0.1 mM EDTA) and stored at 4°C.

Samples were analyzed on a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for quality and quantity of DNA and also run on a 1% (wt⁄vol) agarose gel for additional verification of DNA quality.

DNA analysis

DNA samples were each diluted to 20 ng μl^{-1}. A restriction endonuclease digestion mixture was prepared using 7 μl of the diluted DNA sample, 0.125 μl of a 10 U μl^{-1} *Mse*I enzyme (1.25 U per reaction), 0.0625 μl of a 20 μl *Eco*RI restriction enzyme (1.25 U per reaction) (Applied Biosystems), 0.125 μl of a 10 ng ml^{-1} Bovine Serum Albumen (New England Biolabs, Ipswich, Massachusetts, USA), 1.25 μl 10x NEB buffer (New England Biolabs), and Nanopure H_2O for a total volume of 12.5 μl. The mixture was dispensed into 0.2 ml PCR tubes and incubated on a PTC-200 thermal cycler (MJ Research, GMI, Ramsey, Minnesota, USA) at 37°C for 2 h, with a final cycle of 70°C for 15 min and a 4°C soak. An adapter ligation mix was prepared (0.5 μl EcoRI adapter, 0.5 μl *Mse*I adapter, 0.15 μl T4 DNA ligase, 0.5 μl T4 DNA ligase buffer, 3.35 μl Nanopure H_2O) and 5 μl of the mix was added to each sample. Samples were incubated at 25°C for 8 h on the PTC-200 thermal cycler and left overnight at 4°C.

Samples were diluted (1:10) by adding 135 μl 1x TE buffer to each. A preamplification mix was prepared containing 10 μl preamplification mix II, 1.25 μl 10x PCR buffer, 0.75 μl 15 mM $MgCl_2$, 0.25 μl of 5 U μl^{-1} Ampli-*Taq* DNA polymerase (1.25 U per reaction) (Applied Biosystems), transferred in 12.25 μl aliquots to new PCR tubes, and 1.25 μl of the diluted ligation reactions were added to each tube. This preamplification mix was run for 20 cycles at the following preselected PCR amplification: 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min followed by storage at 4°C. Four primers were selected in four primer pair combinations for selective amplification (Table 1). The preamplification product was diluted 1:20 with nanopureH_2O and a selective amplification PCR mix was prepared (1.2 μl 10× PCR buffer, 0.72 μl 15mM $MgCl_2$, 0.08 μl 5 U μl^{-1} Ampli-*Taq* polymerase, 4.1 μl dH_2O,

and the primers M-CAA and E-ACG (IDT; http://www.idtdna.com). Then 8.6 μl selective amplification mix was added to 2.0 μl of diluted preamplification product in new PCR tubes and ran on the thermal cycler for 1 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by 12 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Reactions were stopped by adding 2.5 μl stop solution (LI-COR, Lincoln, Nebraska, USA) to each tube, then run on a thermal cycler at 94°C for 1 min for denaturation. Subsequently, 1 μl of the samples was electrophoresed on KB^{Plus} 6.5% polyacrylamide gel (LI-COR) for 2 h to separate the DNA, and the band images were saved for scoring and analysis. The entire process was repeated using the primer pairs M-CTA/E-ACA, M-CTG⁄E-ACT, M-CTA⁄E-ACT, and M-CTG⁄E-ACA.

Table 1. Primers and adapters used in the AFLP process. Sequences taken from Vos et al. (1995)

Oligonucleotide	Purpose	Sequence (5′–3′)
*Eco*RI-forward adapter	Adapter ligation	CTCGTAGACTGCGTACC
*Eco*RI-reverse adapter	Adapter ligation	AATTGGTACGCAGTCTAC
*Mse*I-forward adapter	Adapter ligation	GACGATGAGTCCTGAG
*Mse*I-reverse adapter	Adapter ligation	TACTCAGGACTCAT
*Eco*RI primer	Preamplification	GACTGCGTACCAATTC
*Mse*I primer	Preamplification	GATGAGTCCTGAGTAA
E-ACA	Selective amplification	GACTGCGTACCAATTC + ACA
E-ACT	Selective amplification	GACTGCGTACCAATTC + ACT
M-CTA	Selective amplification	GATGAGTCCTGAGTAA + CTA
M-CTG	Selective amplification	GATGAGTCCTGAGTAA + CTG

Statistical analysis

The AFLP bands were detected on a Gene Read IR 4200 sequencer (LI-COR), evaluated using IRD-700 labeled 50–700 bp ladder as a reference, and scored using the SAGA Generation 2 Software Version 3.2 (LI-COR). Bootstrap analysis [using the DBOOT software (Departmento de Biologia Geral ICB, UFG, C. P. Goiânia, Goiás, Brazil)] of the coefficient of variation (Coelho, 2001) was used to assess the amount of genetic diversity present in the markers used. Nei's genetic diversity, number of polymorphic loci, and genetic diversity of subpopulations relative to total values (GST) were calculated using Popgene v. 1.32 (Yeh et al., 1997). Genetic diversity (heterozygosity, H), G_{ST}, and gene flow estimations (N_m) were then assessed within and between all populations. Arlequin v. 3.5 (Excoffier et al., 2005) was used to perform the analysis of molecular variance (AMOVA) as well as pairwise Fst (Fixation index) values. Popgene v. 1.32 (Yeh et al., 1997) was used to construct an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram.

Results

After screening several primer combinations, four combinations were selected for AFLP analysis (Table 1). Four primers in four combinations were used to screen the DNA from five populations of P. maculiventris. Two kinds of parameters were considered for this

study: (1) the presence or absence of diagnostic bands; and (2) the existence of a characteristic, reproducible pattern of AFLP-PCR products, revealing a potential diagnostic profile for a particular geographical population. The four primer combinations used generated many fragments that depended on the type of selective nucleotides identified and provided a total of 209 usable loci. The coefficient of variation demonstrated that the 209 loci encompassed >95% of the genetic variation (Figure 1), sufficient for statistical analysis (Bonin et al., 2007).

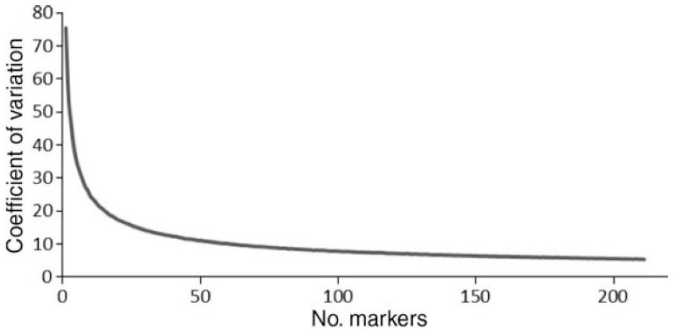

Figure 1. Bootstrap analysis: the coefficient of variation was used to ensure the 209 markers used in this study were sufficient for statistical significance. Here the coefficient of variation becomes less than 5% with use of about 150 markers.

Heterozygosity values between populations were measured using Nei's gene diversity index (Table 2). The Dairy Farm population had the most polymorphic loci at 85%, whereas Mississippi had the least at 69%. Nei's gene diversity index was highest in the Alfalfa population (H = 0.3093) and lowest in the Mississippi population (H = 0.2680). The laboratory population had 74% polymorphic loci and a genetic variation of H = 0.2692; lower than natural populations from Missouri but slightly higher than the genetic diversity found in the Mississippi samples. This indicates that the laboratory population did not have (much) less genetic variation than wild populations.

Table 2. Single population statistics for the one Mississippi and four Missouri populations of *Podisus maculiventris*

Location	Nei's gene diversity (H)	% polymorphic loci
Laboratory	0.2692	74
Mississippi	0.2680	69
Dairy farm	0.2707	85
Garden	0.3067	83
Alfalfa	0.3093	83

Heterozygosity values (Nei, 1973) were calculated to determine the degree of polymorphism within and between populations (Table 3). Populations were divided into three groups. The laboratory and Mississippi locations were each placed into their own group whereas the three field locations from Missouri were grouped together. Analysis of these

groups resulted in a G_{ST} value of 0.2695 and a G_{CS} value of 0.0658 (Table 3). The total G_{ST} for all locations without groups was 0.3176.

Table 3. Multiple population statistics calculated in Popgene. (A) Hierarchical analysis of GST with three groups of *Podisus maculiventris* (laboratory, Mississippi, and the three field locations from Missouri combined). (B) No groups; total G_{ST} for all locations.

	H_T	H_C	H_S	G_{ST}	G_{CS}
A	0.4173	0.2848	0.3048	0.2695	0.0658
B	0.4173		0.2848	0.3176	

H, heterozygosity of total (H_T), groups (H_C), and populations (H_S).

G, genetic diversity of populations, relative to total (G_{ST}), and relative to groups (G_{CS}).

With the populations arranged in three groups, AMOVA revealed that a slight majority of genetic variation occurred within populations (Table 4). The data show that 52% of the genetic variation was found within populations, 38% was among population groups, and 9% was among populations within groups. There is significant genetic variation within populations, but there is almost as much variation between population groups. Pairwise Fst values reveal how these locations are related to one another (Table 5). The three locations from Missouri all have low pairwise Fst values, indicating a moderate amount of gene flow.

Table 4. Analysis of molecular variation (AMOVA) results and fixation indices. Significance tested with 1023 permutations.

Source	d.f.	SS	Variance components	% variation
Among groups	2	1480.673	17.1759 Va	38.4
Among populations within groups	3	148.875	4.1146 Vb	9.2
Within populations	108	2529.010	23.41676 Vc	52.4
Total	112	4158.558	44.7073	
Fixation indices	Significance			
Fsc	0.1495	Vb and Fsc	0.000 ± 0.000	
Fst	0.4762	Vc and Fst	0.000 ± 0.000	
Fct	0.3842	Va and Fct	0.104 ± 0.012	

Table 5. Pairwise Fst values calculated in Arlequin v. 3.5 for the one Mississippi and four Missouri populations of *Podisus maculiventris*

	Laboratory	Mississippi	Dairy farm	Garden	Alfalfa
Laboratory	0				
Mississippi	0.4539	0			
Dairy farm	0.3712	0.5959	0		
Garden	0.3673	0.5799	0.1319	0	
Alfalfa	0.2804	0.5308	0.0687	0.0962	0

A UPGMA consensus cluster analysis was used to illustrate genetic similarity/difference in a dendrogram, confirming the results of our pairwise Fst analyses (Figure 2). All the Missouri locations, including the laboratory population, cluster into a single clade. However, there is a moderate amount of genetic isolation between the laboratory and the field populations. Notably, the Mississippi location is significantly isolated from all other locations.

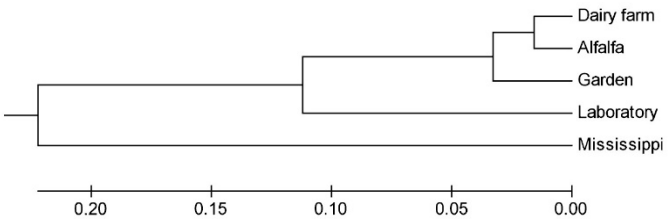

Figure 2. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram depicting relationships among the one Mississippi and four Missouri locations of *Podisus maculiventris*. UPGMA tree constructed using genetic distances calculated using pairwise genetic distance according to Nei (1973).

Discussion

The results presented in this article support our hypothesis of substantial genetic variation between spined soldier bug populations. Our data document the genetic variability within and among populations and assess the impact of continuous rearing on genetic variability. AFLP analysis generated polymorphic molecular markers suitable for assessing genetic variation in wild and laboratory populations of *P. maculiventris*.

Our results document significant genetic isolation between *P. maculiventris* populations in Missouri and Mississippi. We speculate that this is because of climatic differences that led to genetic divergence. In particular, *P. maculiventris* overwinters in northern regions but continually develops in southern regions (Richman & Whitcomb, 1978). In addition, the exposure to pesticide use may be greater in Mississippi than in Missouri, and the crop diversity may be lower. The influence of pesticides on adaptation in insect populations is thoroughly documented (Metcalf, 1989) and is not treated herein. The wide range of agricultural diversity in Missouri (http://www.nass.usda.gov/Statistics_by_State/Missouri/Publications/Current_Estimates/index.asp) potentially attracts a broad range of prey species and adaptation to a wider range of prey may influence genetic variability in soldier bug populations. Our results also support additional studies to assess the extent of gene variation in *P. maculiventris* within the continental USA. This information could prove important for selecting wild populations with desired traits that are retained when used in mass production of the spined soldier bug for augmentative biological control applications.

The genetic variation between Missouri field and laboratory populations was less pronounced. In particular, we infer that the populations within Missouri are functionally a

single population with considerable gene flow among locations. To the extent that performance of a biological control agent is gene related and gene diversity dependent, we reason that the retention of most genetic diversity in the laboratory colony indicates the predator fitness has likely been retained after 100+ generations of laboratory rearing. In this case, fitness retention most probably was due, in part, to maintaining a large colony size of 30 mated pairs. Yet, we recognize, that the high differentiation observed between the laboratory and Dairy Farm populations shows some rearing effect, the significance of which is unknown.

Questions about the quality of wild insects used for starting colonies and fitness of domesticated insect populations for mass production in biological control programs are long-term concerns. Studying the genetic variation within a population helps understand the influence of genetic processes, such as mutation and selection, on insect biology (Roderick, 1996). Beyond understanding, however, knowledge of genetic variation in populations of beneficial insects is a crucial foundation for work designed to improve the quality of biological control agents via artificial selection protocols. For example, global use of beneficial insects is expanding and at the same time limited by relatively high costs of producing large numbers of insects at the specific times needed. The high costs could, in principle, be reduced by continuous, rather than episodic production. Lines of beneficials within species artificially selected for long-term cold storage can reduce production costs. Such selection protocols depend on substantial genetic diversity within populations.

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