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SPECIAL ISSUE: POPULATION STRUCTURE AND DYNAMICS OF INVASIVE SPECIES

Structure and genetic variation among populations of *Euschistus heros* from different geographic regions in Brazil

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Abstract

The Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), is a major Brazilian soybean pest. Aiming to provide relevant information to implement pest and insecticide resistance management, new microsatellite loci were developed for *E. heros* and used in a study of genetic diversity and population structure. The population analysis was performed using eight microsatellite loci from 17 samples (n = 243 individuals) collected in the major soybean-producing regions in Brazil (northeastern, midwestern, and southern regions). These microsatellite loci provided high genetic diversity values on the whole extension for the studied region (He = 0.895; total number of alleles = 400). Neotropical brown stink bug populations in general displayed low genetic structure levels among the samples (overall Φ_{ST} = 0.009). An exception was the sample from the northeastern region, which showed a significant genetic differentiation (pairwise Φ_{ST} = 0.031–0.063). Bayesian cluster analysis confirmed these results, did not show population subdivision, and indicated considerable levels of gene flow. Significant correlations between genetic differences and geographic distance were obtained. The lowest estimate of migration was found in the population from São Desidério, which was also the most distant from the remaining populations based on genetic distance. Some plausible hypotheses for the low genetic differentiation among these populations are the fast expansion of soybean production areas, the main food source of *E. heros*, polyvoltinism, and possible influence of anthropogenic dispersal. All these factors could have led to high population densities, a wide distribution that may contribute to reduced population differentiation, and increased genetic diversity.

Introduction

Stink bugs (Hemiptera: Pentatomidae) of the genus *Euschistus* are important pests of cotton, soybean, fruit crops, and vegetables. In the Nearctic region, the most economically important species are *Euschistus servus* (Say) and *Euschistus quadrator* Rolston, whereas in the Neotropical region, it is *Euschistus heros* (Fabricius), the Neotropical brown stink bug (Bundy & McPherson, 2000; Panizzi et al., 2000; Tillman & Mullinix, 2004; Hopkins et al., 2005).

Currently, the largest producer of soybean in the world is the USA, with 33.1 million ha sown in 2015/2016 (USDA, 2016). In Brazil, the second largest producer, the

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most important producing areas are located in the mid-western (45.5% of the total production) and southern regions (34.5%) (IBGE, 2016). In Brazil, *E. heros* is one of the major soybean pests, as its population density can reach 40–60 individuals m^{-1} of row by the end of the growing season. This high density hampers pest control, requiring extra insecticide applications at increasing doses, which has resulted in resistance of this species to organophosphate insecticides (Sosa-Gómez & Silva, 2010).

Despite the importance of this pest, studies related to the genetic structure of its populations have so far been neglected. To the best of our knowledge, only one study of dominant markers based on Random Amplified Polymorphic DNA (RAPD) has been published (Sosa-Gómez et al., 2004). Studies of genetic diversity and population structure of *E. heros* are relevant for adopting regional or widespread management strategies, as well as insecticide resistance management programs, as insecticide resistance has already been reported (Sosa-Gómez & Silva, 2010).

The advantages of using microsatellite markers to assess population structure in Hemiptera have been described by several authors (Pizarro et al., 2008; Sanchez et al., 2012). Due to the high mutation rate of Simple Sequence Repeat (SSR) compared to other markers, microsatellites allow the discrimination of significant differences between populations and genotypes and the detection of recent changes in the evolutionary history of the species (Oliveira et al., 2006). Therefore, the knowledge of the genetic composition of agronomically important insect species, including population structure and gene flow, is highly relevant and can be the foundation of studies of integrated pest management (IPM) (Pascual-Ruiz et al., 2014). Development of new microsatellite markers is useful because they are stable and easy to assay by polymerase chain reaction (PCR). Thus, this study aimed to develop new microsatellite markers in *E. heros* and to analyze the genetic diversity, structure, and gene flow of their populations in the main soybean-producing regions in Brazil.

Materials and methods

Development and characterization of eight microsatellite loci

Genomic DNA was extracted from *E. heros* specimens using a cetyltrimethylammonium bromide (CTAB)-based protocol following the method described by Rogers & Bendich (1988). Only the head and legs of each specimen were used to avoid possible contaminations with bacteria, protozoa, and nematodes that naturally occur in the hemolymph (Sosa-Gómez et al., 2005).

The isolation of microsatellites was performed by Genetic Marker Services (Brighton, UK; www.geneticmarkerservices.com). An enriched genomic library was constructed for *E. heros* following the protocol described by Edwards et al. (1996), with some modifications proposed by González et al. (2015) and Letelier et al. (2015). Genomic DNA (200 ng) was digested using RsaI and the fragments originated were ligated to a MluI adaptor and amplified by PCR (González et al., 2015). Fragments with microsatellite regions were selected using probes AG, AC, AAC, CCG, CTG, and AAT, and subsequently cloned. A total of 37 recombinant clones was selected and sequenced

Table 1 Geographic coordinates of *Euschistus heros* sampling sites, number of individuals per site, and collection dates

Code	n	Population	Geographic coordinates	Date of sampling
BeVi	15	Bela Vista do Paraíso, PR	22°54′12.8″S, 51°14′44.9″W	April 2013
Igu	13	Iguaraçú, PR	23°13′30.6″S, 51°48′18.8″W	May 2013
Ser	15	Sertaneja, PR	22°56′45.8″S, 50°56′30.9″W	April 2013
CaMo	15	Cândido Mota, SP	22°43′07.8″S, 50°18′56.3″W	April 2013
Ara13	14	Arapoti, PR, Point 2, 2013	24°11′55.8″S, 49°55′12.4″W	May 2013
StoAn	14	Santo Antônio da Platina, PR, Point 1	23°15′42.6″S, 50°06′12.9″W	June 2013
War	14	Londrina, Warta District, PR	23°12′15.7″S, 51°10′56.8″W	March 2013
Nan	14	Nantes, SP	22°36′37.1″S, 51°15′28.6″W	May 2013
PePal13	15	Pedrinhas Paulista, SP, 2013	22°49′09.1″S, 50°45′50.2″W	April 2013
PePal14	15	Pedrinhas Paulista, SP, 2014	22°48′11.4″S, 50°46′12.6″W	March 2014
SaHe	15	Santa Helena de Goiás, GO	17°50′07.3″S, 50°34′51.4″W	March 2014
Ara14	14	Arapoti, PR, 2014	24°11′58.0″S, 49°55′52.1″W	March 2014
Lon	15	Londrina, PR	23°17′53.0″S, 51°06′04.0″W	March 2014
Ita	15	Itaara, RS	29°35′31.7″S, 53°49′18.9″W	March 2014
Gua	15	Guarapuava, Entre Rios District, PR	25°33′09.9″S, 51°28′41.1″W	April 2014
SaDe	15	São Desidério, BA	12°46′23.7″S, 46°01′53.6″W	May 2015
Sor	10	Sorriso, MT	12°32′36.8″S, 55°41′15.9″W	April 2015

using the Big Dye Terminator kit v.3.1 (Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using the BioEdit v.7.0.0 (Hall, 1999) and the primers were drawn for 17 loci using Primer 3 (Rozen & Skaletsky, 2000). The AutoDimer software was used to check for specificity and possible primer dimer or hairpin loop (Vallone & Butler, 2004).

The selected loci were tested under different amplification conditions and then underwent individual genotyping in an automatic sequencer, following the protocol described by Schuelke (2000) and Giangarelli et al. (2015). To perform the reading in the automatic sequencer, fluorescent-labeling required an additional forward primer with an M13 universal sequence (5′-TGTAAAACGACGGCCAGT-3′) added to the 5′ end of each locus (Schuelke, 2000). The amplification reactions were carried out at a final volume of 10 µl, containing 2.3 µl water, 1X GoTaq Colorless Master Mix (Promega, Fitchburg, WI, USA), 10 ng DNA sample, 1% glycerol, 0.125 µM M13 fluorescent labeled primer (FAM, HEX, NED, or PET; Applied Biosystems, Carlsbad, CA, USA), 0.125 µM reverse primer, and 0.0125 µM forward primer. Polymerase chain reactions were performed in a thermal cycler ProFlex 3 × 32-well PCR System (Thermo Fisher Scientific, Waltham, MA, USA) programed as follows: initial denaturation at 94 °C for 5 min, followed by 10 cycles at 94 °C for 30 s, 48 °C, 54 °C, 58 °C, or 60 °C for 1 min, 72 °C for 1 min, 25 cycles at 89 °C for 30 s, 48 °C, 54 °C, 58 °C, or 60 °C for 1 min, 72 °C for 1 min, and the final extension at 72 °C for 30 min (Giangarelli et al., 2015). Electrophoresis of amplification products was performed in ABI PRISM 3500-XL automated sequencer (Applied Biosystems), using the GeneScan 600 Liz (Applied Biosystems) molecular weight marker. Fragment length genotyping was performed using GeneMarker v.1.85 software (Soft Genetics, State College, PA, USA).

Among the 17 loci tested, eight (Eus10, Eus14, Eus23, Eus25, Eus56, Eus57, Eus58, and Eus77) exhibited good amplification conditions and high levels of polymorphism. Therefore, their applicability in population studies was assessed analyzing 30 individuals collected in soybean crops across Brazil (Balsas, MA; Rio Coco, MA; Brasília, DF; Dourados, MS; Pedrinhas Paulista, SP; Cândido Mota, SP; Warta, PR; and Campo Mourão, PR).

Structure and genetic diversity of *Euschistus heros* populations

Sampling. Populations of *E. heros* were collected manually during the soybean seasons of 2012/2013 and 2013/2014, at 17 sampling points within the major soybean-producing regions in Brazil, mainly targeting the states of São Paulo and Paraná. Insects were collected by hand in an area of 100 m^2 to minimize the collection of individuals from the same parents. Most of the time, the infestation in the fields sampled was above the economic threshold. The geographic coordinates of the sampling sites, number of individuals per site, and collection dates are shown in Table 1 and Figure 1.

Extraction and quantification of DNA. Genomic DNA was obtained from 243 stink bugs, collected at the sampling points shown in Table 1. DNA integrity was checked using electrophoresis, run in 1% agarose gel in 1X TBE buffer at 120 V cm^{-1} for 1 h, and stained with 10 mg ml^{-1} ethidium bromide. DNA was visualized and the images were digitalized using a transluminator, image

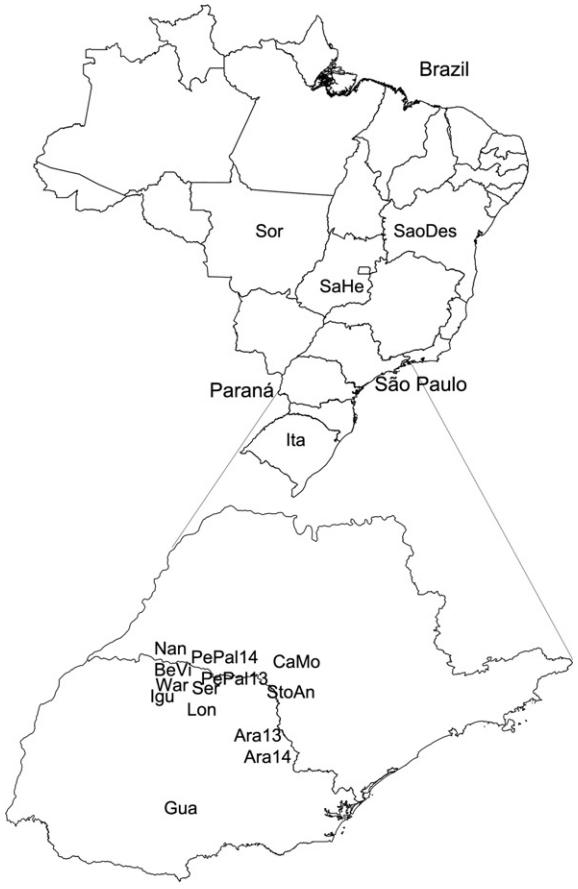

Figure 1 Sampling points of *Euschistus heros*: Arapoti, PR, 2013 (Ara13), 2014 (Ara14); Bela Vista do Paraiso, PR (BeVi); Cândido Mota, SP (CaMo); Guarapuava, Entre Rios District, PR (Gua); Iguaraçú, PR (Igu); Itaara, RS (Ita); Londrina, PR (Lon); Nantes, SP (Nan); Pedrinhas Paulista, SP, 2013 (PePal13), 2014 (PePal14); Santa Helena de Goiás, GO (SaHe); São Desidério, BA (SaoDes); Sertaneja, PR (Ser); Sorriso, MT (Sor); Santo Antônio da Platina, PR (StoAn); Londrina, Warta District, PR (War).

capture systems L-PIX -ST and L-PIX IMAGE 7.1M Pixel, and the software L-PIX IMAGE v.1.0.1 (Loccus Biotecnologia, São Paulo, SP, Brazil). DNA concentration was determined using a NanoDrop 8000 Spectrophotometer v.2.0 (Thermo Fisher Scientific, Wilmington, DE, USA) and the samples were diluted to 10 ng total DNA μl^{-1}.

Data analysis

Genetic diversity of *E. heros* populations was estimated using the following software: MicroChecker v.2.2.1 (Van Oosterhout et al., 2004) to check the presence of null alleles, drop-out alleles, stutter peaks, and other genotyping errors; GenAlEx v.6.41 (Peakall & Smouse, 2006) to calculate total number of alleles (A), number of private alleles (N_P), average number of alleles per locus (N_A), number of effective alleles per locus (N_E), observed heterozygosity (H_O), and expected heterozygosity (H_E); FSTAT v.2.9.3 (Goudet, 2002) to calculate allelic richness (AR); Arlequin v.3.5 (Excoffier & Lischer, 2010) to estimate the inbreeding coefficient (F_{IS}) per locus and per population, whereas the polymorphism information content (PIC) was estimated using Cervus v.3.0 software (Marshall et al., 1998). The probabilities of genetic identity (I) (Paetkau et al., 1995) and paternity exclusion (Q) (Weir, 1996) were estimated using Identity v.1.0 (Wagner & Sefc, 1999).

Possible deviations from Hardy–Weinberg equilibrium (HWE) and the presence of linkage disequilibrium were evaluated using the Genepop v.1.2 software (Raymond & Rousset, 1995), with α values of probability adjusted using the sequential Bonferroni correction (Rice, 1989).

Possible signs of recent population bottlenecks were assessed using two approaches of the software Bottleneck v.1.2.02 (Piry et al., 1999). In the first one, the mode shift test was applied to verify signs of genetic bottleneck based on changes in allele frequency distributions (Luikart et al., 1998). In the second one, the Wilcoxon sign-rank test was used to indicate signs of genetic bottleneck based on significant excess heterozygosity values, using different evolutionary models such as infinite allele model (IAM), stepwise mutation model (SMM), and two-phase model (TPM: 70% SMM and 30% IAM) (Cornuet & Luikart, 1996).

For the analysis of genetic structure, the following software packages were used: Arlequin v.3.5 for the analysis of molecular variance (AMOVA), which describes the partitioning of genetic variation within and among populations, as well as for estimating the index of pairwise population differentiation (Φ_{ST}), based on 10 000 permutations, used to estimate significance; SMOGD v.1.2.5 (Crawford, 2010) to assess population structure using Jost's genetic differentiation estimator (D_{Jost}) (Jost, 2008); STRUCTURE v.2.3.3 (Pritchard et al., 2000, 2010) to check the genetic structure among populations using the Bayesian inference and the admixture ancestry model with correlated alleles. The estimates of the number of populations (K) ranged from 1 to 20 (Evanno et al., 2005), reproducing 20 runs for each value of K, with 50 000

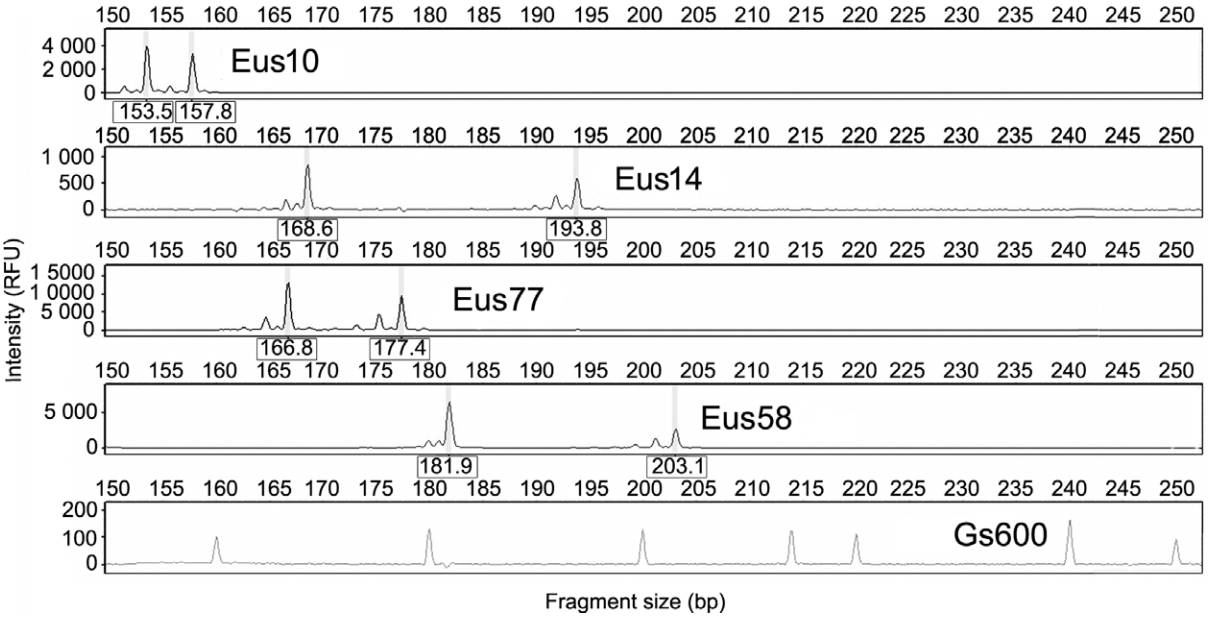

Figure 2 Genotype data profiles of microsatellite loci from *Euschistus heros*.

Table 2 Description of eight polymorphic microsatellite loci isolated from *Euschistus heros* and results for each of the flanking primers. Analysis performed with 30 individuals from different localities

Locus	Primer (5′–3′)	Motif	T_A (°C)	A	Allele size range (bp)	H_O	H_E	Q	I	PIC	F_{IS}	GenBank
Eus10	F: AAAGTTAGGTCGGAAATTGTTAG R: ATGCAAGCACACACGAACAC	$(TG)_{14}$	58	12	149–171	0.767	0.851	0.712	0.037	0.837	0.116	MF084763
Eus14	F:GTACAGTTTATTTTTAAAAGTAATTA R: TGTGCCCGAAGACCTTATCT	$(AC)_{21}$	54	25	168–254	0.500*	0.950	0.902	0.005	0.947	0.487#	MF084764
Eus23	F: TTCCGGTAGAACCAACAAGG R: ATCGCTGAGGGAAAAACAAA	$(AC)_{24}$	58	31	114–232	0.750*	0.957	0.916	0.003	0.956	0.234#	MF084765
Eus25	F: GAGTCGTCTTTACCAAGGGAGA R: TCCAATTTCTTTTTGCTCCA	$(AC)_{36}$	54	25	107–201	0.414*	0.942	0.888	0.006	0.939	0.573#	MF084766
Eus56	F: TCAGAATGTAGGCGAACCTG R: CCGGTGTTAATAGGCGAACT	$(CT)_{10}$ TT$(CT)_7$	58	13	263–315	0.481*	0.811	0.697	0.040	0.795	0.422#	MF084767
Eus57	F: TGAATGCTGGAACTATGGAA R: GGTAATCTTCCAACTGTTTTCA	$(TG)_{19}$	45	32	203–347	0.750*	0.963	0.877	0.007	0.961	0.241#	MF084768
Eus58	F: ACCGATAGCGAAGTAAATTTGT R: CCAAAAACAATCAGCCTAACC	$(AC)_{26}$	58	22	172–240	0.800*	0.931	0.861	0.009	0.927	0.157#	MF084769
Eus77	F: TGCAGTCTTATGCTGAACACC R: GGAGGTTTCAATTATCCATTCG	$(TG)_{37}$	54	36	168–240	0.900	0.966	0.930	0.002	0.964	0.085#	MF084770
Mean				25		0.670	0.921	0.999	1.74×10^{-17}	0.916	0.289#	

*Significant deviations from Hardy–Weinberg equilibrium ($P<0.05$).

#Significant values for the inbreeding coefficient (F_{IS}).

T_A, annealing temperature; A, total number of alleles; bp, allele size in base pairs; H_O, observed heterozygosity; H_E, expected heterozygosity. Parameters estimated for 30 individuals: Q, probability of paternity exclusion; I, probability of genetic identity; PIC, polymorphism information content; F_{IS}, inbreeding coefficient. GenBank accession numbers.

interactions in the burn-in and 500 000 interactions in the Markov Chain Monte Carlo (MCMC) method; and STRUCTURE HARVESTER v.0.6.94 (Earl & vonHoldt, 2012) to estimate the value of K that best adjusts to our data based on the criteria proposed by Evanno et al. (2005) (ΔK) and Pritchard et al. (2000) [likelihood ln (K)]; CLUMPP v.1.1.2 (Jakobsson & Rosenberg, 2007) to obtain the best K from independent STRUCTURE runs, using the 'greedy' algorithm; and DISTRUCT v.1.1 (Rosenberg, 2004) to generate the graph of genetic structuration.

The analysis of gene flow of *E. heros* samples was carried out using the software Migrate-n v.3.6.4 (Beerli & Palczewski, 2010) to estimate the migration rate (M) and the effective population size (Θ). For this, the maximum likelihood estimate, calculated from MCMC chains, was used, including 10 short chains with 1 000 genealogies, followed by three long chains with 10 000 genealogies, and a burn-in of 10 000. The estimate of immigrants per generation was obtained multiplying M by Θ.

Distances among sampling points were estimated with latitude/longitude spherical geodesy tools (http://www.movable-type.co.uk/scripts/latlong.html). In addition, a Mantel test was carried out using TFPGA v.1.3 (Miller, 1997) to assess the correlation between genetic differentiation and geographic distance obtained by comparing linearized Φ_{ST} values and geographic distance.

Results

Development and characterization of eight SSR loci in *Euschistus heros*

Eight microsatellite polymorphic loci were successfully amplified for *E. heros* (Eus10, Eus14, Eus23, Eus25, Eus56, Eus57, Eus58, and Eus77), showing clearly defined peaks in the electropherogram and good applicability in population studies (Figure 2). The remaining loci that produced monomorphic, faint, and stutter peaks were not used in the analysis. A total of 196 alleles resulted from the 30 individuals employed in the characterization of loci, with an average of 24.5 alleles per locus. H_O and H_E ranged from 0.414 (Eus25) to 0.900 (Eus77) and from 0.811 (Eus56) to 0.966 (Eus 77), respectively (Table 2).

No loci combinations showed linkage disequilibrium after sequential Bonferroni correction; however, of the eight loci analyzed, six (Eus14, Eus23, Eus25, Eus56, Eus57, and Eus58) had significant deviations from HWE. MicroChecker indicated null alleles in seven loci.

Table 3 Parameters of genetic diversity of *Euschistus heros* obtained from eight microsatellite markers

		Microsatellite								
Sample	n	A	N_p	AR	N_A	N_E	H_O	H_E	F_{IS}	HWE^{Eus**}
BeVi	15	126	2	11.304	15.750	12.625	0.678	0.895	0.213*	$5^{Eus\ 14.\ 23.\ 25.\ 56.\ 57}$
Igu	13	114	4	10.918	14.250	11.660	0.658	0.900	0.079*	$4^{Eus\ 14.\ 25.\ 56.\ 57}$
Ser	15	126	5	11.096	15.750	12.365	0.675	0.905	0.124*	$4^{Eus\ 25.\ 56.\ 57.\ 77}$
CaMo	15	131	5	11.179	16.375	12.262	0.637	0.911	0.179*	$4^{Eus\ 14.\ 25.\ 56.\ 58}$
Ara13	14	122	7	11.221	15.250	12.254	0.639	0.905	0.289*	$6^{Eus\ 14.\ 23.\ 25.\ 56.\ 57.\ 58}$
StoAn	14	113	6	10.604	14.125	11.331	0.634	0.893	0.196*	$4^{Eus\ 14.\ 25.\ 56.\ 57}$
War	14	121	7	11.014	15.125	11.579	0.722	0.905	0.105*	$3^{Eus\ 14.\ 56.\ 57}$
Nan	14	120	3	11.055	15.000	11.603	0.585	0.896	0.273*	$5^{Eus\ 14.\ 25.\ 56.\ 57.\ 77}$
PePal13	15	138	4	11.632	17.250	14.000	0.687	0.916	0.211*	$4^{Eus\ 25.\ 56.\ 57.\ 58}$
PePal14	15	135	5	11.130	16.875	11.755	0.689	0.889	0.215*	$3^{Eus\ 14.\ 25.\ 57}$
SaHe	15	127	3	10.901	15.875	12.459	0.616	0.893	0.292*	$8^{Eus\ 10.\ 14.\ 23.\ 25.\ 56.\ 57.\ 58.\ 77}$
Ara14	14	118	3	10.801	14.750	11.159	0.679	0.897	0.134*	$5^{Eus\ 14.\ 23.\ 25.\ 57.\ 58}$
Lon	15	131	5	11.242	16.375	12.787	0.662	0.906	0.258*	$5^{Eus\ 14.\ 25.\ 56.\ 57.\ 58}$
Ita	15	129	11	10.982	16.125	11.924	0.640	0.907	0.197*	$5^{Eus\ 14.\ 25.\ 56.\ 57.\ 77}$
Gua	15	135	7	11.254	16.875	12.975	0.619	0.904	0.242*	$7^{Eus\ 14.\ 23.\ 25.\ 56.\ 57.\ 58.\ 77}$
SaDe	15	90	5	8.424	11.250	7.843	0.667	0.831	0.295*	$5^{Eus\ 10.\ 23.\ 25.\ 57.\ 77}$
Sor	10	89	2	10.125	11.125	8.955	0.602	0.862	0.331*	$6^{Eus\ 14.\ 23.\ 25.\ 56.\ 57.\ 77}$
Mean		122	5	10.875	15.184	11.737	0.652	0.895		

*Significant values for the inbreeding coefficient (F_{IS}).

**Number of loci with deviation from Hardy–Weinberg equilibrium (HWE).

n, number of individuals analyzed; A, total number of alleles; N_P, number of private alleles; AR, allelic richness; N_A, average number of alleles; N_E, number of effective alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient.

Significant F_{IS} values were observed in almost all loci, except Eus10. A high combined Q (0.999) and a low combined I (1.74×10^{-17}) were obtained, showing that the loci set has good applicability to studies of parentage and population differentiation. Polymorphism information content confirmed that the eight loci selected are highly informative, with values ≥0.795 (Table 2) (Botstein et al., 1980).

Population study of *Euschistus heros* in soybean crops in Brazil

Genetic diversity and bottleneck. Automatic genotyping of 243 individuals of *E. heros*, collected within the major soybean-producing regions in Brazil (Figure 1), resulted in an average of 122 alleles per sample (Table 3). A per sample ranged from 89 (So.MT) to 138 (PePal13), with an average number of alleles per locus (N_A) and N_E ranging from 11.1 to 17.2 and from 8.9 to 14.0, respectively (Table 3).

The variations found for the following parameters of genetic diversity were: AR ranged from 8.424 (SaDe) to 11.632 (PePal2013); H_O ranged from 0.585 (Nan) to 0.722 (War); H_E ranged from 0.831 (SaDe) to 0.916 (PePal13); and N_P ranged from 2 (BeVi and Sor) to 11 (Ita). All the populations had significant F_{IS}. In addition, significant deviations from HWE were found for all samples, ranging from three (War and PePal14) to eight loci (SaHe) per sample.

Table 5 Analysis of molecular variance (AMOVA) for 17 populations of *Euschistus heros* based on eight microsatellite loci

Source of variation	% variation
Among individuals within populations	99.09
Among populations	0.91
Fixation index	Φ_{ST} = 0.009*

Index Φ_{ST} = genetic differentiation among populations.
*Significance test using 10 000 permutations (P<0.05).

The bottleneck program indicated an absence of recent bottleneck signs in the mode-shift test. All samples had typical L-shaped distribution (non-bottleneck) in the allele frequency. On the other hand, Wilcoxon sign-rank test revealed significant excess heterozygosity values (bottleneck sign) in 10 samples (Ser, Ara13, StoAn, PePal13, SaHe, Ara14, Lon, Ita, Gua, and SaDe) using the IAM model, in seven samples (Igu, Ser, Ara13, StoAn, War, PePal13, and Lon) using the TPM model, and in two samples (Igu and Ara13) using the SMM model (Table 4). According to Piry et al. (1999), the Wilcoxon test is more robust than the Mode shift test to detect bottleneck signs in natural populations.

Genetic structure and gene flow. Analysis of molecular variance indicated that most of the variance took place

Table 4 Test of recent genetic bottleneck for 17 populations of *Euschistus heros*, Wilcoxon sign-rank test for heterozygosity excess, and mode shift test for allele frequency distribution patterns

		IAM		TPM		SMM	
Sample	n	H_E/H_D	P	H_E/H_D	P	H_E/H_D	P
BeVi	15	6/2	0.23	6/2	0.13	6/2	0.16
Igu	13	6/2	0.19	6/2	0.010	6/2	0.010
Ser	15	6/2	0.014	6/2	0.020	5/3	0.27
CaMo	15	5/3	0.23	4/4	0.42	3/5	0.63
Ara13	14	6/1	0.008	6/1	0.027	6/1	0.039
StoAn	14	7/1	0.010	6/2	0.013	6/2	0.13
War	14	6/2	0.13	6/2	0.037	4/4	0.58
Nan	14	5/3	0.16	6/2	0.27	5/3	0.53
PePal13	15	6/1	0.008	6/1	0.012	6/1	0.12
PePal14	15	3/5	0.73	4/4	0.84	3/5	0.98
SaHe	15	6/2	0.027	6/2	0.13	5/3	0.27
Ara14	14	7/1	0.014	6/2	0.16	5/3	0.42
Lon	15	7/1	0.027	7/1	0.006	5/3	0.23
Ita	15	7/1	0.006	4/4	0.23	3/5	0.73
Gua	15	6/2	0.010	6/2	0.13	2/6	0.73
SaDe	15	6/2	0.020	4/4	0.16	4/4	0.77
Sor	10	6/2	0.23	6/2	0.13	6/2	0.23

P values are based on the Wilcoxon sign-rank test; P<0.05 indicates heterozygosity excess. IAM, infinite allele model; TPM, two-phase model (70% SSM); SMM, stepwise mutation model; n, number of individuals analyzed; H_E, number of loci presenting heterozygosity excess; H_D, number of loci presenting heterozygosity deficiency.

Table 6 Pairwise genetic differentiation for microsatellite data obtained for samples of *Euschistus heros* within the major soybean-producing regions in Brazil. Below diagonal, pairwise Φ_{ST}, parameter based on Wright's F-statistics. Above diagonal, Jost's genetic differentiation estimator (D_{Jost})

Sample	BeVi	Igu	Ser	CaMo	Ara13	StoAn	War	Nan	PePal13	PePal14	SaHe	Ara14	Lon	Ita	Gua	SaDe	BeVi
BeVi		0.153	0.024	0.018	0.04	0.053	0.235*	−0.108	−0.037	−0.085	0.031	−0.034	−0.045	0.142	−0.126	0.242*	0.074
Igu	0		−0.051	−0.118	−0.074	−0.061	0.015	0.101	−0.006	0.072	0.03	−0.025	0.043	−0.039	−0.063	0.317*	0.153
Ser	0	0		−0.133	0.013	−0.059	−0.106	−0.05	−0.024	0.128	−0.134	−0.102	−0.015	−0.064	−0.144	0.245*	0.045
CaMo	0.003	0	0		−0.127	−0.064	−0.055	−0.015	−0.07	0.077	−0.031	−0.028	0.041	0.076	−0.068	0.320*	0.038
Ara13	0.004	0.001	0.006	0.001		0.106	0.052	−0.123	−0.269	−0.028	0.01	0.012	−0.01	−0.102	−0.068	0.312*	0.008
StoAn	0.002	0	0	0.004	0.004		0.301*	0.128	0.072	0.078	0.049	−0.038	0.073	−0.032	−0.01	0.275*	0.257
War	0.003	0.002	0.000	0.001	0	0.007		0.132	−0.03	0.287*	0.164	0.101	0.129	0.014	0.18	0.433*	0.147
Nan	0.002	0.005	0.009	0.007	0.000	0.014	0.005		−0.016	−0.102	0.061	−0.004	−0.124	0.041	−0.172	0.356*	0.06
PePal13	0	0	0	0	0	0.001	0	0.004		0.053	−0.024	−0.027	−0.108	−0.056	−0.175	0.276*	−0.025
PePal14	0.003	0	0.012	0.012	0.003	0.014	0.010	0	0.001		0.024	0.06	0.063	0.163*	0.035	0.346*	0.174*
SaHe	0.009	0	0.002	0.005	0.005	0.009	0.009	0.021	0	0.013		0.047	0.082	0.044	−0.02	0.119*	0.003
Ara14	0.006	0.001	0.001	0.007	0.010	0.007	0.007	0.015	0.005	0.011	0.007		0.042	−0.021	−0.016	0.278*	0.152
Lon	0	0	0.000	0.011	0.005	0.006	0.009	0.000	0.005	0.006	0.015	0.011		0.073	0.001	0.342*	−0.004
Ita	0.008	0.000	0.002	0.009	0.005	0.009	0.003	0.007	0.000	0.014	0.007	0.009	0.014		−0.058	0.282*	0.139
Gua	0	0	0.000	0.000	0.007	0.008	0.009	0.009	0	0.011	0.008	0.001	0.011	0.003		0.217*	−0.044
SaDe	0.039*	0.038*	0.031*	0.045*	0.038*	0.042*	0.054*	0.063*	0.033*	0.058*	0.019	0.033*	0.049*	0.038*	0.035*		0.231*
Sor	0.011	0	0.005	0.006	0.008	0.007	0.014	0.018	0.004	0.016	0.008	0.007	0.010	0.015	0.007	0.039*	

*Significance test using 1 023 permutations ($P<0.01$).

within the populations (99.09%), whereas only 0.91% occurred among them (Table 5). The estimates of pairwise Φ_{ST} indicated lack of genetic structure, except for sample SaDe, which showed significant genetic structuration in relation to most of the other samples studied, presenting Φ_{ST} values ranging from low (0.031) to moderate (0.063) (Wright, 1965). Similarly, most of pairwise D_{Jost} estimates were not significant. However, some significant estimates were obtained and showed values higher than pairwise Φ_{ST} values, ranging from 0.163 (PePal14 × Ita) to 0.433 (SaDe × War) (Table 6).

Bayesian clustering analysis performed using STRUCTURE indicated that the most probable value of K (number of clusters), calculated from the mean likelihood ln(K) and ΔK, was K = 2 (Figure 3A, B). The graph representation of this analysis showed the non-existence of well-defined groups among the individuals analyzed and that the estimated ancestry seems to be distributed similarly (Figure 3C).

In the estimates of Migrate-n, the largest M estimate per generation (estimate M × Θ) was obtained for localities <200 km apart. On the other hand, the smallest gene flow estimates were obtained from São Desidério to the other localities, with distances ranging from 745 to 2 035 km (Table 7). Analysis of isolation by distance using Mantel tests indicated significant correlations between genetic and geographical distances from pairwise Φ_{ST} (r = 0.620, P = 0.002) and D_{Jost} (r = 0.455, P = 0.022).

Discussion

This paper reports new microsatellite markers developed for the assessment of genetic diversity in *E. heros*. The eight loci obtained and used here were highly informative (Botstein et al., 1980), revealing high combined Q and low combined I. Moreover, PCR amplification patterns showed well-defined alleles and lack of linkage disequilibrium. Therefore, the great potential of these markers for the analysis of genetic structure and diversity of *E. heros* is evident. They can provide fundamental information to understand the adaptive and evolutionary potential of this species (Frankham et al., 2010), including aspects of integrated population management.

The results obtained in this study revealed high genetic diversity and low structuration among the populations of *E. heros* studied. Considering the pairwise Φ_{ST}, the population collected in São Desidério was the only exception to this pattern of low genetic differentiation among the

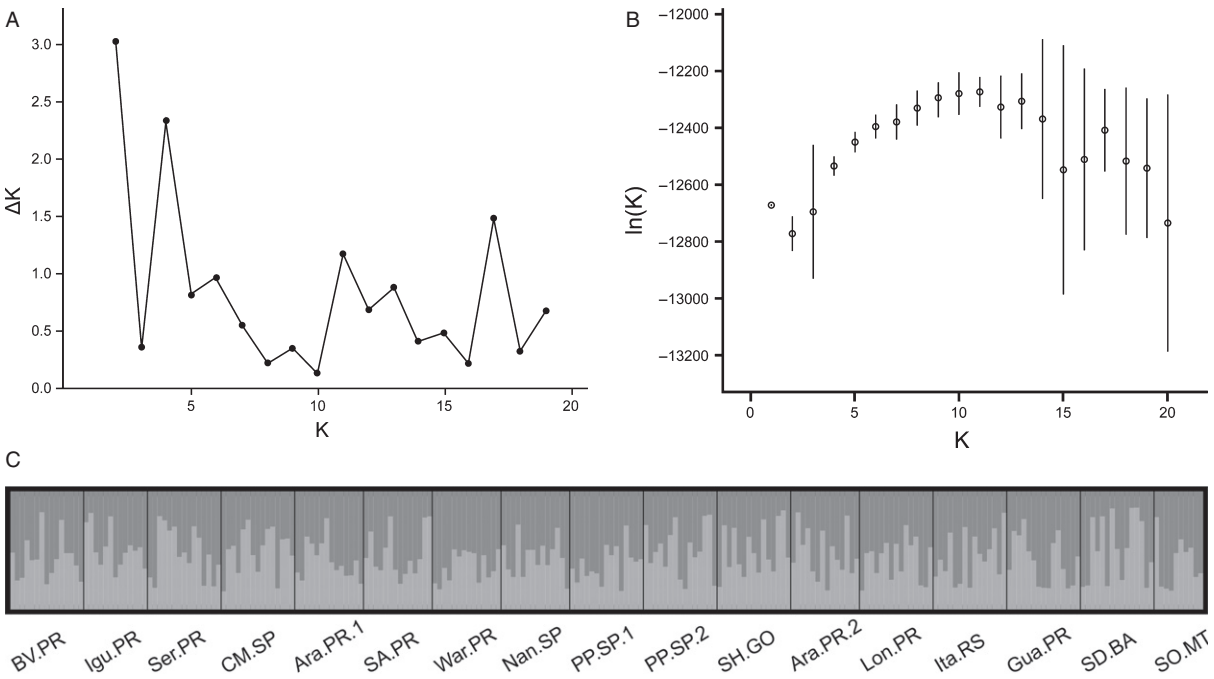

Figure 3 Estimates of K (i.e., number of clusters) (A) from ΔK (Evanno et al., 2005), and (B) based on the mean likelihood ln(K). (C) Structure bar representing the results of the Bayesian analysis of 17 populations of *Euschistus heros* within the major soybean-producing regions in Brazil (K = 2); each column represents one individual and the shades (dark vs. light gray) indicate the relative proportion of each individual genome that belongs to either of the clusters (K).

Table 7 Effective population size (Θ) of *Euschistus heros* and migration estimation based on the migration rate M (= m/μ) × Θ

Sample	Θ	BeVi	Igu	Ser	CaMo	Ara13	StoAn	War	Nan	PePal13	PePal14	SaHe	Ara14	Lon	Ita	Gua	SaDe	Sor
BeVi	0.86		8.43	14.29	5.21	8.89	12.18	16.21	11.47	19.15	24.63	8.30	20.42	13.73	8.58	14.25	6.35	14.72
Igu	1.12	9.96		14.88	13.71	13.40	19.02	16.95	9.69	12.62	15.04	17.38	17.07	30.66	9.61	13.52	8.55	7.34
Ser	0.51	9.80	7.22		6.81	11.50	4.45	17.15	11.85	7.20	11.06	12.81	8.53	7.18	4.98	9.23	5.35	7.35
CaMo	1.03	7.73	16.18	13.61		10.19	8.70	19.60	14.35	9.45	11.71	11.12	8.00	8.78	11.04	11.19	3.46	10.15
Ara13	1.52	20.71	17.85	42.48	14.72		21.45	40.36	21.66	16.90	26.11	26.06	16.47	31.62	16.26	25.69	6.41	22.83
StoAn	0.85	7.62	11.61	5.79	8.39	12.44		9.51	6.59	7.36	9.39	7.62	10.22	9.66	5.91	11.55	3.46	5.35
War	1.11	20.68	15.20	38.75	19.92	36.69	16.43		20.85	31.63	27.67	27.04	13.39	20.22	13.18	12.30	10.19	18.45
Nan	0.98	15.44	10.13	18.47	12.45	12.18	9.39	16.38		21.16	21.50	10.63	10.90	18.84	13.09	11.27	4.88	20.52
PePal13	0.82	22.22	11.08	10.50	7.94	6.94	9.53	18.47	20.85		13.12	8.86	10.32	10.87	9.47	11.66	4.81	7.70
PePal14	0.85	24.11	14.70	17.41	13.16	12.69	9.31	27.39	20.16	16.67		24.14	17.44	19.35	12.12	10.25	9.58	8.87
SaHe	0.93	10.08	15.66	19.25	8.90	19.72	6.26	22.06	7.58	13.09	20.30		10.76	14.21	9.88	13.77	11.67	11.54
Ara14	0.30	10.45	5.65	9.33	2.54	5.79	4.27	3.18	3.31	3.91	5.56	2.78		2.57	3.22	5.58	2.68	3.11
Lon	0.45	8.85	12.08	7.65	4.90	10.95	3.69	9.44	11.89	6.53	8.78	7.39	4.43		10.58	11.16	3.15	3.80
Ita	0.73	4.74	8.38	8.84	6.98	10.13	4.85	13.41	7.75	9.39	8.79	10.29	4.60	14.95		6.54	2.66	7.04
Gua	0.40	5.22	3.79	10.69	4.97	7.30	5.05	6.42	7.33	4.42	7.25	6.57	7.22	8.43	3.44		6.09	4.52
SaDe	0.07	0.45	1.22	0.68	0.38	0.87	0.55	0.50	0.62	0.46	1.20	1.01	0.75	0.74	0.59	1.02		0.77
Sor	1.38	18.25	7.29	18.51	13.60	18.29	6.18	10.36	28.65	9.34	19.36	10.36	17.06	9.68	10.66	12.24	4.35	

samples analyzed. This population was among those that had the lowest levels of genetic diversity, presenting the lowest values of AR, H_E, and N_E. Samples of this population were collected in areas very distant from the remaining populations studied.

Euschistus heros is originally from the Neotropical region (Panizzi & Slansky, 1985), and its populations became a serious problem in Brazilian soybean areas, in southern states, in the late 1980s and early 1990s. As a consequence of the expansion of soybean production to lower latitudes (midwestern and northeastern regions), this pest has become more prevalent. Additionally, the rapid and intensive increase in soybean-planted area in the last decades may be related to the genetic bottleneck signs observed in great part of the samples analyzed (significant values using the Wilcoxon sign-rank test). Given that the bottleneck analysis (Wilcoxon sign-rank test) responds to signs of recent decreases in Θ (Cornuet & Luikart, 1996) and that the expansion of soybean-planted area is still ongoing (Flaskerud, 2003; IBGE, 2016), our results suggest the influence of a dissemination pattern to new areas as small groups, that is, it could be the occurrence of the founder effect (Sun et al., 2012).

In our study, gene flow and lack of genetic differentiation were observed among most of the sampled populations, except for individuals from São Desidério. These findings agree with the Bayesian cluster analysis and the small molecular variation (<1%) found among populations in AMOVA. The low genetic structure and gene flow observed could be partly attributed to anthropogenic dispersion caused by the expansion of soybean production areas and active transport of seeds and soybean grains among Brazilian regions. In addition, the Neotropical brown stink bug population can reach high densities (40–60 individuals m^{-1} of row at the end of the growing season), mainly at the end of the season, in March and April, in areas where they remain in the field even after most of the soybean has been harvested. At this time, *E. heros* may disperse, looking for shelter to remain in oligopause during part of the fall and winter. High levels of gene flow and large populations, as suggested for *E. heros*, are determinants of the maintenance of high levels of genetic diversity and low population differentiation (Freeland, 2005).

Genetic studies using RAPD markers of individuals of this species collected during 2000 (Sosa-Gómez et al., 2004) revealed genetic differentiation among *E. heros* geographic populations. However, some aspects should be considered to explain differences between these findings and the results of the present study. In the previous study, the samples were collected 13–14 years before those of this study, and several changes in the scenario have occurred. Among them, the increase in soybean-planted area, from 13.6 million ha in 2000 to 30.2 million ha in 2014 (Conab, 2017), could provide conditions for insect outbreaks, spreading from local epicenters to cover large areas. Also, the attractiveness of soybean seeds and the high population density during harvest have favored active truck transport of stink bugs, together with great volumes of soybean, across the country. In addition, climatic changes such as global warming (Kiritani, 2006; Musolin, 2007) may create favorable conditions for the development of this insect pest. All these factors, together with its polyvoltinism (Chevarria et al., 2013), could have led to high population densities and a wide distribution of *E. heros*, which could have contributed to reduced population differentiation and increased genetic diversity.

It is interesting to point out that, in spite of the low levels of differentiation among samples, our results suggest a trend of differentiation due to isolation by distance (IBD). Significant positive correlations were found between the geographic and genetic distances using both estimators of genetic differentiation (Φ_{ST} and D_{Jost}). Isolation by distance patterns are common in species that display a limited capacity of dispersion (Slatkin, 1993). Nonetheless, even in these species, gene flow may occur between different areas of distribution following the stepping-stone dispersal model. In this scenario, dispersion takes place preferably between neighboring demes, and is considered rare between more distant demes. Therefore, gene exchange between neighboring demes allows the distribution of different alleles step by step along the different demes (Kimura & Weiss, 1964). In fact, although the present results suggest the influence of various factors on the levels of genetic structuration of *E. heros*, including anthropogenic dispersion, current expansion of planted area, and intensive use of insecticides, geographic distances between planted areas also seem to be determinant factors, mainly when taking into consideration the large and uninterrupted soybean areas planted in Brazil.

The study of *E. heros* samples collected within the major soybean-producing regions in Brazil revealed high levels of genetic diversity and occurrence of gene flow among localities. These factors are probably essential for the adaptation of this species to the constant changes that have been taking place in contemporary agroecosystems, which together with the broad geographic distribution did not provide conditions for genetic structuration. These findings brought to light the genetic distribution of *E. heros* in vast extensions of the Neotropical region. This information may contribute to a better understanding of the species and to plan future actions for implementing IPM programs.

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