SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Genetic Differentiation among Brazilian Populations of *Euschistus heros* (Fabricius) (Heteroptera: Pentatomidae) Based on RAPD Analysis

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Diferenciação Genética entre Populações de *Euschistus heros* (Fabricius) (Heteroptera: Pentatomidae) do Brasil Mediante Marcadores de RAPD

RESUMO - O percevejo marrom da soja, *Euschistus heros* (Fabricius), possui ampla distribuição na região sojicola do Brasil, sendo mais importante na região central do País. Tem sido o alvo principal das aplicações de inseticidas dirigidas contra o complexo de pentatomídeos praga. A variabilidade nos RAPD entre e dentro das populações do percevejo marrom foi determinada. Amostras da espécie foram coletadas nos campos de soja de Ubiratã (PR), Londrina (PR), Centenário do Sul (PR), Cândido Mota (SP), Ponta Porã (MS) e Sapezal (MT). O DNA genômico foi extraído da cabeça para minimizar a contaminação do DNA proveniente dos endoparasitóides e parasitas que ocorrem na hemocele. O DNA foi amplificado com iniciadores de 10 nucleotídeos. Quinze iniciadores produziram 246 bandas. A similaridade genética foi obtida com base na freqüência alélica dos RAPD utilizando-se a distância de Nei 1972. Os percevejos coletados da mesma região geográfica apresentaram a maior similaridade genética. As populações de Londrina e Centenário do Sul foram geneticamente mais próximas que as restantes. Também, percevejos coletados em Cândido Mota foram próximos das populações de Ponta Porã. A população geográfica de Sapezal foi a mais divergente das outras. As fêmeas e os machos agruparam-se em grupos diferentes dentro de cada população geográfica, o que significa que os RAPD possibilitam a diferenciação dos sexos. Não foram observados indivíduos de uma população agrupando-se com indivíduos de outra região. O número de loci polimórficos das diferentes populações variou entre 40,6% e 52,1%. Os índices de fluxo gênico de *E. heros* (*Nm* = 0,8307) foram menores que os observados anteriormente para *Anticarsia gemmatalis* Hübner e *Helicoverpa armigera* (Hübner) sugerindo que as populações do pentatomídeo apresentam maior isolamento geográfico que nos referidos noctuídeos, por exemplo.

PALAVRAS-CHAVE: RAPD, marcador molecular, estrutura genética, fluxo gênico

ABSTRACT - The Neotropical Brown Stink Bug (NBSB), *Euschistus heros* (Fabricius), has a wide distribution in Brazilian soybean fields, being more important in the central region of the country. The species is the main target of insecticide applications for stink bug pest control. We determined the variability among and within populations of the NBSB by random amplification of polymorphic DNA (RAPD) analysis. Samples of NBSB were collected in soybean fields from Ubiratã (PR), Londrina (PR), Centenário do Sul (PR), Cândido Mota (SP), Ponta Porã (MS) and Sapezal (MT). Genomic DNA was extracted from the head to minimize DNA contamination by endoparasites. DNA was amplified with 10 mer primers. Fifteen primers produced 246 bands. The genetic similarity matrix was obtained, based on RAPD allele frequencies, using Nei´s 1972 genetic distance. NSBS collected from the same geographical region clustered together. The populations from Londrina and Centenário do Sul were genetically closer than the others and stink bugs collected in Cândido Mota were closer to Ponta Porã population. The Sapezal geographical population was the most divergent from the others. Females and males clustered separately inside each geographical population, implying that RAPD permits gender discrimination. We did not observe individuals from one region clustering together with stink bugs from another region. The number of polymorphic loci from the different populations ranged between 40.6% and 52.1%. The gene flow indexes (overall *Nm* = 0.8307) were lower than that observed for *Anticarsia gemmatalis* Hübner and *Helicoverpa armigera* (Hübner) suggesting that in stink bug populations gene flow is lower than in the noctuid moths.

KEY WORDS: RAPD, molecular marker, genetic structure, gene flow

The Neotropical Brown Stink Bug (NBSB), *Euschistus heros* (Fabricius), has a wide distribution in Brazilian soybean fields, being prevalent in Mato Grosso, Paraná, Goiás and Mato Grossso do Sul. According to Panizzi & Corrêa-Ferreira (1997) this pentatomid is the most abundant species in Brazilian latitudes below 23ºS. Although the potential of NBSB to cause damage and delay maturity is lower than for other species of the pentatomid complex (Sosa-Gomez & Moscardi 1995), it is more widely distributed. According to Cividanes & Parra (1994) the number of generations may reach five to seven in warmer regions. Most insecticide applications are due to the prevalence of this pest. Studies on biological control, nutritional ecology, and the use of pheromones have been pursued but almost nothing is known about the differentiation among geographical populations, biotypes or genotypes and their distribution. The first case of insecticide resistance in a soybean related pest in Brazil was recently reported for the NBSB (Sosa-Gomez *et al*. 2001). Therefore the characterization of geographical populations from sites with or without suspicion of insecticide resistance will be useful to evaluate if gene flow occurs among these populations. Although dispersion studies on stink bugs have been scarce, Toscano & Stern (1976) reported that stink bugs winter in the neighborhood of the attacked crops, whereas Aldrich (1990) reported dispersal over long distances by strong winds. The determination of molecular markers associated with resistance genes will be useful to evaluate if certain populations are resistant or not, thereby avoiding unnecessary qualitative bioassay methods. Knowledge about dispersion and distribution patterns may also aid resistance management programs.

For this purpose we determined the genetic diversity among and within NBSB geographical populations from Brazil using Random Amplified Polymorphic DNA.

Material and Methods

Insect Samples. Data on the *E. heros* adult samples are shown in Table 1 and the geographical distribution of localities sampled in Fig. 1. To minimize possible contamination of DNA from endoparasites, we extracted DNA from the head without antennae and mouthparts. Samples were maintained at -18ºC until DNA extraction. Samples from Sapezal were kept in ethanol at -15ºC, during 25-30 days before extraction. Although sample preservation conditions for DNA from bugs collected at Sapezal was poorer than for the other locations we obtained satisfactory random amplification of polymorphic DNA (RAPD) products as pointed out by Oliveira *et al*. (2002).

DNA Extraction. DNA was extracted from the head without antenna and mouthparts, according to Rogers & Bendich (1988) with minor modifications. The head was selected due to the presence of high concentration of several microorganisms as bacteria, protozoa and nematodes occurring naturally in the hemolymph (Fuxa *et al*. 2000, Sosa-Gomez *et al*. 2002).

For each head we used 400 µl of extraction buffer, at a final concentration of 200 mM Tris-HCl (pH 8.0), 70 mM EDTA, 2 M NaCl and 1% of β-mercaptoethanol, crushed inside a microcentrifuge tube with a homogenizer. After addition of 160 µl of 5% CTAB, the sample was kept at 65ºC for 5 min. following homogenization. An additional incubation at 65ºC for 60 min. was performed after adding 5.6 µl of 10 mg/ml proteinase K. After that, samples were kept at room temperature and centrifuged at 14,000 rpm for 15 min. The aqueous phase was recovered (400 µl) and transferred to a new microcentrifuge tube, to which the same volume of chloroform / isoamyl alcohol (24:1) was added. After gentle homogenization samples were centrifuged at 14,000 rpm for 15 min.; and the aqueous phase recovered (300 µl) and transferred to another tube, where the nucleic acids were precipitated with two volumes of cold 100% isopropanol plus 45% of the volume with 10 M NH_4OAc. These tubes were kept overnight at 4ºC or for 2h at -20ºC. Samples were centrifuged at 14,000 rpm for 15 min., the supernatant discarded and the pellet washed with 300 µl of 70% ethanol. The pellets were dried, with the tubes inverted on the bench at room temperature for 1h. The pellets were then resuspended with 100 µl TE buffer with RNAse at final concentration of 10 µg/ml and kept at 37ºC for 30 min. DNA aliquots kept at –15°C were thawed when necessary to perform DNA amplification.

DNA Quantification and RAPD. The amount of DNA was estimated with a spectrophotometer and its integrity was assessed by 0.8% agarose gel electrophoresis, stained with 10 mg/ml ethidium bromide. The electrophoresis was performed with 1X TBE buffer at 120 V. The visualization was done with a UV transiluminator and the gel was photographed using Polaroid film. The amplification reactions were conducted in a volume of 25 µl containing approximately 9 ηg of template DNA, an appropriate amount of Milli Q sterile water, 10X buffer, 0.4 µM primer, 2.4 mM $MgCl_2$, 0.1 mM of each dNTP and Taq polimerase enzyme (Gibco BRE) (1U) per PCR tube amplification.

Table 1. Data of the *E. heros* adult samples.

Origin	Number of females	Number of males	Collection date	Host
Londrina, Paraná	11	16	January 4, 2000	Sunflower
Centenário do Sul, Paraná	6	9	March 27, 2000	Soybean
Ubiratã, Paraná	20	7	March 7, 2000	Soybean
Cândido Mota, Sâo Paulo	11	4	March 17, 2000	Soybean
Ponta Porã, Mato Grosso do Sul	12	15	January 7, 2000	Soybean
Sapezal, Mato Grosso	14	1	March 6, 2000	Soybean

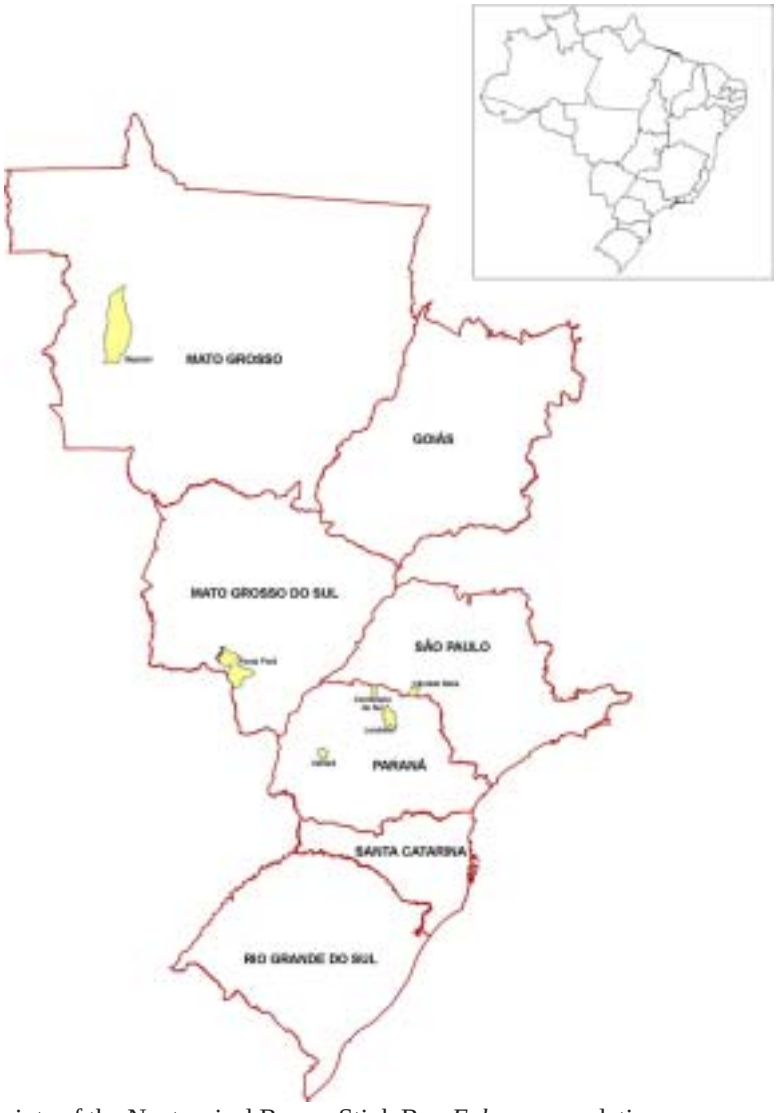

Figure 1. Sampling points of the Neotropical Brown Stink Bug *E. heros* populations.

Immediately after adding the reaction mixture, DNA was amplified. Independent control reactions were conducted without genomic DNA for each primer. Primers that produced spurious products were not considered in the analysis. PCR was performed using a GeneAmp-9600 thermal cycler (Perkin Elmer) with the following thermal program: 45 cycles at 94°C for 15 sec., 35°C for 30 sec., and 72°C for 1 min.; and a final extension at 72°C for 7 min. A 25 µl volume of each RAPD product was electrophoresed in agarose (0.6%)-synergel (1.0 %) with TBE 1x buffer at 120 volts. Lambda DNA cut with *Eco*RI, *Hind*III, and *Bam*HI was used to produce molecular weight markers. Gels were stained with 4.5 µl ethidium bromide 10 mg/ml and photographed under UV light using a black and white, Polaroid 667 ISO 3000 film.

Data analysis. RAPD products were analyzed to quantify the genetic variability within a population and among populations. Photographs of gels generated from the RAPD analysis were scanned. Only distinct bands were included in the binary matrix. Each individual was scored for the presence (1) or the absence (0) of distinct bands. Genetic distance matrices were computed with SIMGEND program and the dendrograms were generated with the SAHN program using the unweighted pair-group method with arithmetic mean (UPGMA) of the NTSYS-pc software (Rholf, 1993). Also, a principal coordinate analysis was conducted based on dissimilarity measures using the SIMGEND, DCENTER and EIGEN procedures of NTSYS-pc software in order to determine which component accounted for most of the variation in all variables. Bootstrap analysis was performed with Winboot program (Yap & Nelson 1996)

POPGENE genetic software (Yeh & Boyle 1997) was used to determine the percentage of polymorphic loci, heterozigosity, *Gst* and *Nm* indices taking into account the same restrictions considered by Ayres *et al.* (2002). Distances among the populations from different sample points were calculated with SPRING software version 3.06.02 (Instituto Nacional de Pesquisas Espaciais, 2002).

Table 2. 10 mer primers that produced reliable products after RAPD reaction using DNA templates from different *E. heros* populations.

Operon primers	Band number
OPA-01	11
OPC-06	14
OPC-09	16
OPC-15	17
OPC-16	17
OPG-18	22
OPK-02	8
OPK-17	21
OPL-13	18
OPN-01	21
OPN-02	16
OPO-15	18
OPY-20	21
OPW-14	16
OPZ-14	10

Results

The primers that gave reliable amplification products are mentioned in Table 2. Each individual was genetically unique and the RAPD amplification produced between eight and 22 bands (Table 2). For example, amplifications with the OPK-02 primer produced eight bands, of which 750 bp band was common to all the populations, but the 600 bp band was shared by Londrina, Cândido Mota, Ponta Porã and Sapezal populations but was not shared by individuals from Centenário do Sul and Ubiratã (all data not shown) (Fig. 2). NBSB collected from the same geographical area clustered together (Fig. 3). Populations from Londrina and Centenário do Sul were genetically closer than the others. Similarly, stink bugs collected in Cândido Mota were closer to Ponta Porã population. Ubiratã stink bugs were closer to the group formed by Londrina and Centenário do Sul populations than the other geographical populations. The Sapezal geographical population was the most divergent from the others. Females and males clustered separately inside each geographical population, which means that RAPD discriminates between genders (Fig. 3). We did not observe individuals from one region clustering together with stink bugs from another region.

The principal component analysis results were similar to that obtained with the cluster analysis. Up to 68.1% of the total variation was explained by the first three axes, which accounted for 31.4%, 22.7% and 14.0% of the observed variation (Fig. 4). The Sapezal group appeared well separated from the others populations. Sapezal population appeared in the right quadrant, Londrina population appeared in the left upper quadrant, with Centenário do Sul close to it.

Nei's gene diversity of each NBSB population is shown in Table 3. Polymorphism among different populations ranged from 41% to 52% (Table 3). The percentage of polymorphic loci was lowest in the specimens obtained in Centenário do Sul and Sapezal; both populations were also less diverse than the others.

When the Sapezal population was included in the pairwise analysis with the remaining populations, the total gene diversity (H_T) ranged from 0.1703 to 0.1926, but the diversity within populations (H_S) was lower ($0.1183 > H_S < 0.1297$) (Table 4).

The overall gene differentiation among the six populations presented *Gst* equal to 0.3757 and the estimate of gene flow (*Nm*) was 0.8307. The highest genetic diversity indices were obtained in those pairwise populations in which the Sapezal samples were included in the analysis (Table 5). Gene flow indexes were highest between Cândido Mota and Ponta Porã, Centenário do Sul and Londrina, and Centenário do Sul and Cândido Mota. The lowest gene flow values were obtained when the Sapezal population was included in the analysis. Sapezal county is the most distant from the other sites, the closest sampling site to Sapezal was Ponta Porã located at 1,008 km away (Table 6). Bootstrap analysis of the binary matrix resolved one cluster for each geographical population. The bootstrap P values at each node were: 99.8, 97.6, 99.2, 98.8, 98.8 and 100 for Ubiratã, Ponta Porã, Cândido Mota, Centenário do Sul, Londrina and Sapezal populations, respectively. That means that the group of genotypes within each cluster occurred more than 97.6% of times in 500 tree replicates produced during the bootstrap analysis.

Discussion

The heterogeneity among individuals collected from the

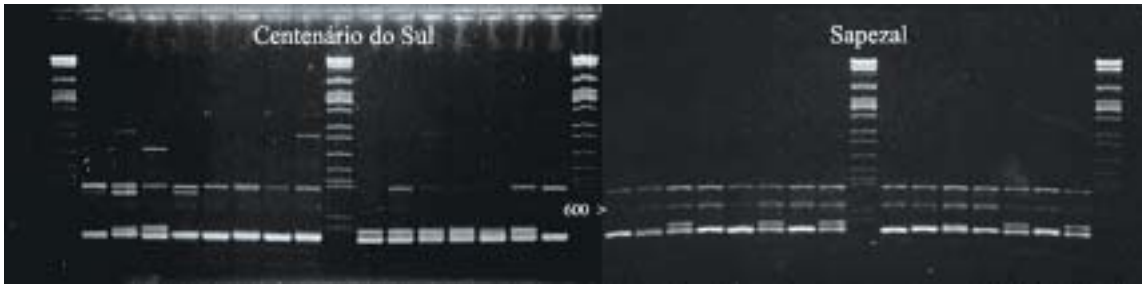

Figure 2. RAPD amplification products obtained with Operon-K02 primer and DNA template from *E. heros* specimens from the following geographical regions: CS = Centenário do Sul, and SP = Sapezal. (Molecular weight markers: ëDNA cut with *Eco*RI, *Hind* III and *Bam*HI enzymes).

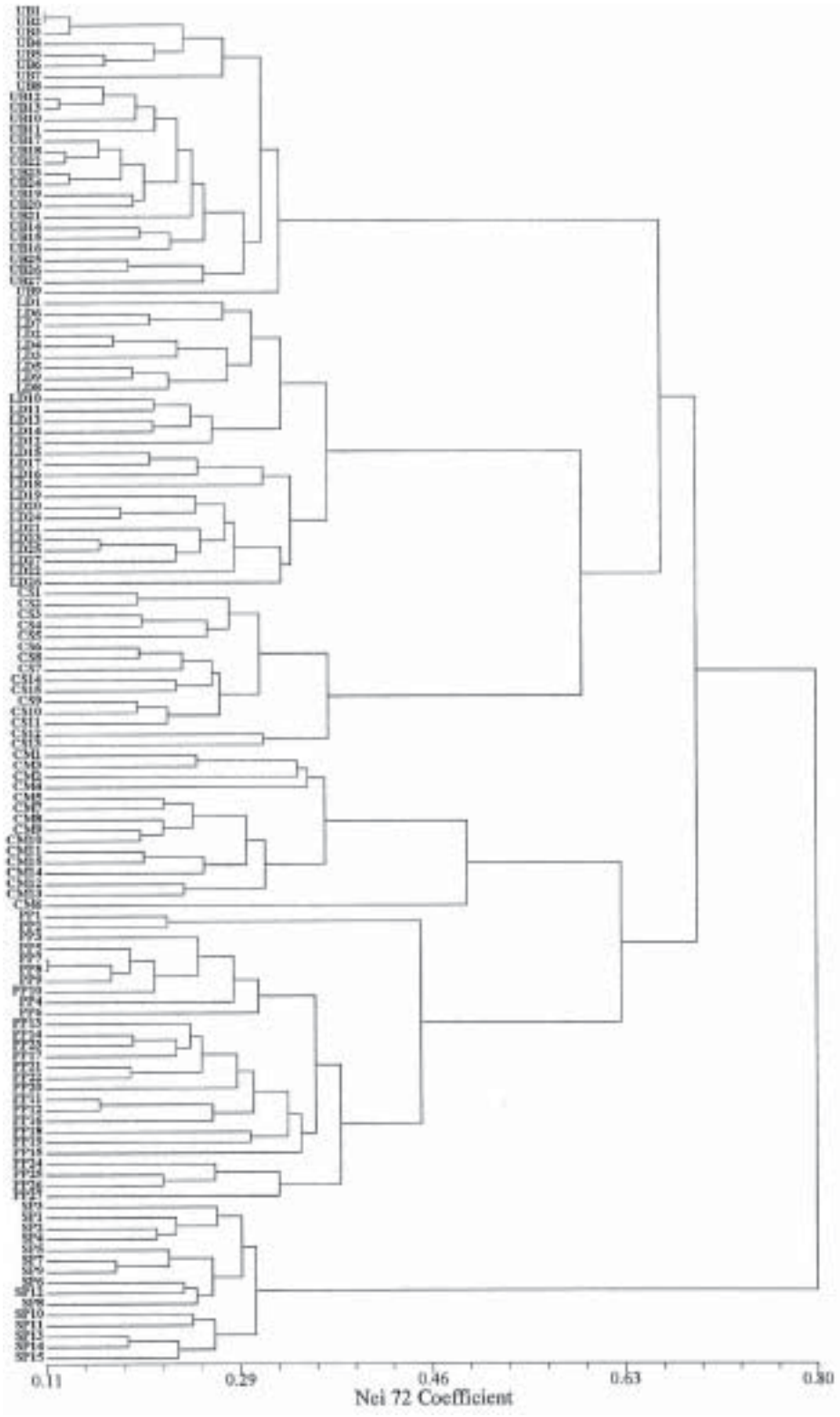

Figure 3. Dendrogram based on Nei (1972) genetic distance of six populations of *E. heros*. The letters indicate geographical origin: UB: Ubiratã, LD: Londrina, CS: Centenário do Sul, CM: Cândido Mota, PP: Ponta Porã, SP: Sapezal. Numbers were assigned to each individual. Females: UB1-UB7, LD1-LD11, CS1-CS6, CM5-CM15, PP1-PP12, SP1-SP14. Males: UB8-UB27, LD13-LD27, CS07-CS15, CM1-CM4, PP13-PP27, SP15.

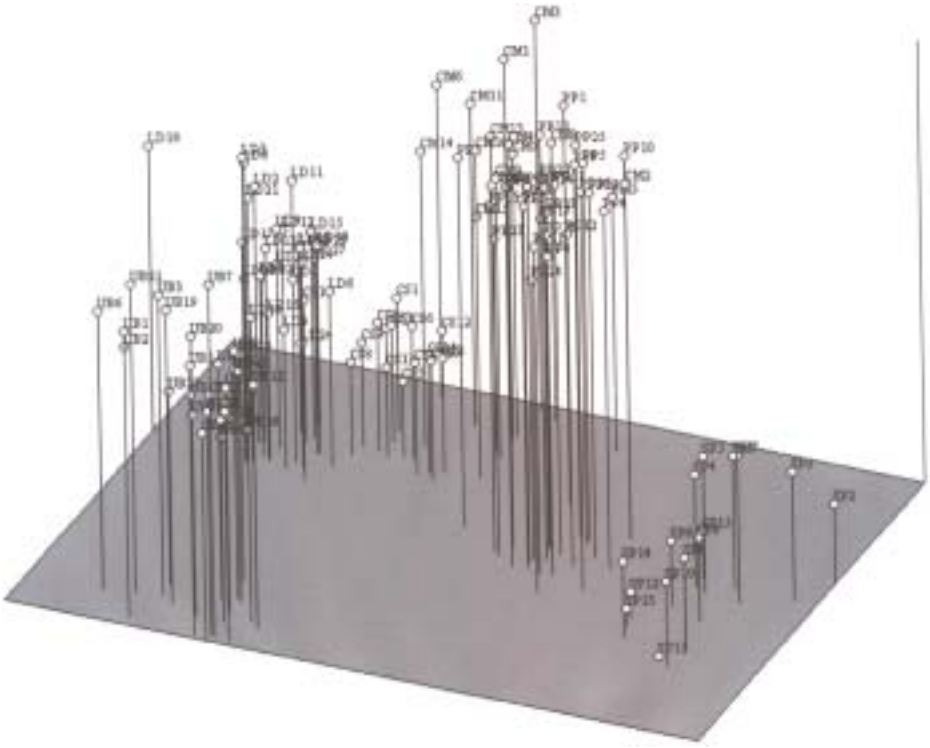

Figure 4. Principal coordinate analysis diagram of *E. heros* populations based on RAPD genetic distances inferred from Nei (1972) index. The letters indicate geographical origin: UB: Ubiratã, LD: Londrina, CS: Centenário do Sul, CM: Cândido Mota, PP: Ponta Porã, SP: Sapezal. Numbers were assigned to each individual.

Table 3. Genetic variability indices (polymorphism and gene diversity) of *E. heros* populations.

Geographic origin	Number of polymorphic loci (%)	Heterozigosity[1]
Londrina	128 (52)	0.1287
Centenário do Sul	100 (41)	0.1159
Ubiratã	126 (51)	0.1246
Cândido Mota	112 (46)	0.1387
Ponta Porã	124 (50)	0.1374
Sapezal	107 (44)	0.1207
Entire population	246 (100)	0.2014

[1]Nei's (1973) gene diversity

same geographic area was lower than that observed for noctuid moths; e.g. *Anticarsia gemmatalis* Hübner (D.R. Sosa-Gomez, unpub.). Clusters for each NBSB population did not include individuals from different geographical origins. Furthermore, the overall $Nm = 0.8307$ indicates that in the NBSB populations the gene flow occurs to a lesser extent than in *A. gemmatalis* populations ($Nm = 3.055$) (D.R. Sosa-Gomez, unpub.), and *Helicoverpa armigera* (Hübner) (*Nm* ranging from 12.1 to 66.9) (Zhou *et al.* 2000). This could be explained by a higher dispersal ability of these noctuids. The low immigration rates could be favorable to local differentiation of NBSB populations. Although, values of $Nm > 1$, as observed for the populations of the NBSB (Table 5), are considered sufficient to make gene flow overcome genetic drift (Wright 1931). The overall *Gst* value among all populations was 0.3757, which means that 37% of the variation in the RAPD markers was observed among populations compared to 63% within populations. This contrast with the *Gst* value of *A. gemmatalis* (0.1406) that showed greater variation within geographical populations (D.R. Sosa-Gomez, unpub.). The similar sampling size used for both species, *E. heros* and *A. gemmatalis*, by the first author of this paper, minimize the bias in genetic differentiation values (Wu *et al.* 1999).

The Sapezal population was the most divergent, possibly due to the isolation provided by the great distances from the other populations. The similarity between Londrina and Centenário do Sul populations may due to the short distance between these sites, approximately 52 km.

Population from Sapezal, the most distant locality from the other sites, showed the greatest divergence. Considering the low flight capabilities, the genetic differences among populations in this case could be due to the overriding effect of distance. The highest migrating *Nm* values were observed between Cândido Mota and Ponta Porã and Centenário do Sul and Londrina.

The percentage of polymorphic loci ranged from 41 in Centenário do Sul population to 52 for the population from Londrina. The most heterogeneous populations were collected from Cândido Mota and Ponta Porã.

The separation of cluster by gender using RAPD has been observed in *Triatoma brasiliensis* Neiva (Heteroptera: Reduviidae) and *A. gemmatalis* populations (Borges *et al.* 2000, D.R. Sosa-Gomez, unpub.).

Table 4. Measures of genic variation in *E. heros* populations from Brazil, H_T = total gene diversity estimates (lower part of table) and H_S = within populations gene diversity (upper part of table), according to Nei (1973).

	Londrina	Centenário do Sul	Ubiratã	Candido Mota	Ponta Porã	Sapezal
Londrina	X	0.1223	0.1266	0.1337	0.1331	0.1247
Centenário do Sul	0.1541	X	0.1202	0.1273	0.1267	0.1183
Ubiratã	0.1681	0.1676	X	0.1316	0.1310	0.1226
Cândido Mota	0.1713	0.1615	0.1848	X	0.1381	0.1297
Ponta Porã	0.1703	0.1672	0.1715	0.1730	X	0.1290
Sapezal	0.1878	0.1781	0.1883	0.1926	0.1703	X

Table 5. Pairwise comparisons of Nei's coefficient of gene differentiation (*Gst*) (lower part of table) values between *E. heros* populations and estimates of gene flow (*Nm*) (upper part of table).

			Nm			
Gst	Londrina	Centenário do Sul	Ubiratã	Cândido Mota	Ponta Porã	Sapezal
Londrina	--	1.9217	1.5274	1.7787	1.7885	0.9878
Centenário do Sul	0.2065	--	1.2696	1.8626	1.5624	0.9892
Ubiratã	0.2466	0.2825	--	1.2385	1.6147	0.9332
Cândido Mota	0.2194	0.2116	0.2876	--	1.9750	1.0312
Ponta Porã	0.2185	0.2424	0.2364	0.2020	--	1.5639
Sapezal	0.3361	0.3357	0.3489	0.3265	0.2423	--

Table 6. Distances in straight line (km) among the different sampling points of *E. heros* populations.

	Londrina	Centenário do Sul	Ubiratã	Cândido Mota	Ponta Porã	Sapezal
Londrina	--					
Centenário do Sul	52	--				
Ubiratã	225	225	--			
Cândido Mota	80	108	306	--		
Ponta Porã	443	411	356	526	--	
Sapezal	1317	1262	1360	1334	1008	--

The flight behavior could explain why geographical populations are discrete (more homogeneous genetically) and can be easily differentiated compared to *A. gemmatalis* (D.R. Sosa-Gomez, unpub.). Although the dispersion of stink bugs is not well documented, it seems that only rarely are these bugs transported more than a few kilometers by means of unusual events, such as hurricanes (Aldrich 1990). Among stink bugs of the pentatomid soybean complex in Brazil, *E. heros* seems to be the species with the lowest flight capability compared to other species [e.g. *Piezodorus guildinii* (Westwood) and *Nezara viridula* (L.)], and adults of the latter species exhibited low dispersion moving only 100 m or 120 m in 28 days, during the soybean season (Costa & Link 1982). *N. viridula* and *P. guildinii* can fly 40 m to 70 m when disturbed during warm weather (32-35°C) (D.R. Sosa-Gomez, pers. observ.). In contrast, even without food, *Euschistus conspersus* Uhler remain in the same area for overwintering (Toscano & Stern 1976). In Brazil, *E. heros* can be found under the mulch of different plants during the winter (Panizzi & Niva 1994, Panizzi & Vivan 1997).

The differentiation of NBSB as well as other economically important stinkbugs using the present technique will be useful to generate strain-specific markers to study gene flow, and molecular markers related to resistant specimens. Dispersion capabilities could have important implications on the rate of resistance evolution as evidenced by modeling; insects with high dispersion potential tend to show delayed evolution of resistance rates (Guse *et al.* 2002). Although studies with molecular markers are useful, ecological observations and studies of flight dispersal also should be performed to validate these data.

No data are available on the population structure of stink bugs. In our study we have demonstrated that RAPD is useful to discriminate genotypes within and among populations. Regardless of the fact that RAPD has limitations, this technique is very effective in partitioning geographically discrete populations and genetic variation within subpopulations, additionally is fast and can be applied to a large number of samples.

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