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Source: Journal of Insect Science, 5(23) : 1-10

Published By: Entomological Society of America

URL: https://doi.org/10.1673/1536-2442(2005)5[1:PSOTBS]2.0.CO;2

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Population structure of the Brazilian southern green stink bug, *Nezara viridula*

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Abstract

The Southern Green Stink Bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), is a cosmopolitan and economically important pest to several crops. Studies on *N. viridula* migration and population structure have been neglected. We studied geographically distinct Brazilian *N. viridula* populations to assess their variability and to determine gene flow among them. DNA from specimens collected on soybean fields were subjected to RAPD analysis to determine genetic similarity and population structure parameters. All *N. viridula* populations studied were genetically distinct from the others. The maximum similarity occurred between populations from Londrina and Sertanópolis (Parana State). The Cruz Alta population was the most divergent from the others. Despite the short distance between Cambé and Londrina (*ca.* 29 km), and the absence of geographic barriers, both populations clustered in different groups and the estimated gene flow index (*Nm*) among them was 2.02, indicating relatively restricted migration. The estimated overall index, *Nm* was 1.41 suggesting that *N. viridula* is a better flier than the Neotropical Brown stink bug, *Euschistus heros* (*Nm* =0.83).

Cite this paper as:
Sosa-Gómez DR, da Silva JJ, Costa F, Binneck E, Marin SRR, Nepomuceno AL. 2005. Population structure of the Brazilian southern green stink bug, *Nezara viridula*. 10pp. *Journal of Insect Science* 5:23, available online: insectscience.org/5.23

Keywords: molecular markers, gene flow, population structure, insect, stink bugs
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Received: 27 October 2004 | Accepted: 14 March 2004 | Published: 10 October 2005
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Introduction

The Southern Green Stink Bug, *Nezara viridula* (L.), is a cosmopolitan pest with important economic effects on several crops, such as soybean, peas, cucumber, lettuce, tobacco, and several others, due to polyphagous behavior and potential to cause damage (Todd et al., 1980; Jackai et al., 1990). In Brazil, *N. viridula* populations are widely distributed in areas where soybean is cultivated, and their populations can reach high densities. At present it is found more frequently in southern Brazil, including the states of Paraná, Santa Catarina and Rio Grande do Sul (Corrêa-Ferreira et al., 1999; Panizzi, 2002). This species can cause yield reduction, affect soybean seed quality (Villas et al., 1990) and cause delayed maturity or foliar retention syndrome, in which the foliage remains green even at the soybean senescent stage, which interferes with harvest timing, due to a delay in bean water loss.

This species has been chemically controlled mainly with organophosphate insecticides such as methyl-parathion and monocrotophos for more than 40 years. *Nezara viridula* is one of the few cases of pentatomids that became resistant to insecticides (Georghiou et al., 1991). The evolution of resistance can be influenced by several factors, such as mutation, selection, genetic drift and gene flow (migration). Studies on *N. viridula* migration are relevant to determine strategies of resistance management, but these studies have been disregarded and little is known about gene flow and population structure of *N. viridula* . Intraspecific variability of enzymatic systems, that were used as biochemical markers, have been studied for *N. viridula* populations from Slovenia, France, and French West India by Meglic et al., (2001) but no relationships among geographical populations were established.

Population structure of species is important for planning preservation strategies, mainly on species that suffered habitat fragmentation (Lougheed et al., 2000). In the case of agricultural pests, the opposing view is also important, as crop expansion has created continuous habitat and food resources that increases the possibilities of gene flow, which results in the lowering of genetic differentiation. Studies on population structure allows a better understanding of applied aspects such as the effect of gene flow on insecticide resistance development among close or distant populations. Therefore, we report the intraspecific variation of geographically distinct *N. viridula* populations in order to assess the genetic variability of populations from Brazil and to determine gene flow among them.

Materials and Methods

Sites of insect collection

N. viridula were obtained from Sertanópolis, Warta (Londrina), Cambé, Curitiba (Paraná State), Platina and Palmital (São Paulo), Chapecó (Santa Catarina), Passo Fundo, Cruz Alta (Rio Grande do Sul), and Planaltina (GO), Brazil. All the adult specimens of *N. viridula* f. *smaragdula* (n = 270) were stored at -20° C until DNA extraction. Stingkbugs were collected during March, April and May of 2001 (Table 1).

Table 1. Data of the *Nezara viridula* adult samples collected in soybean fields.

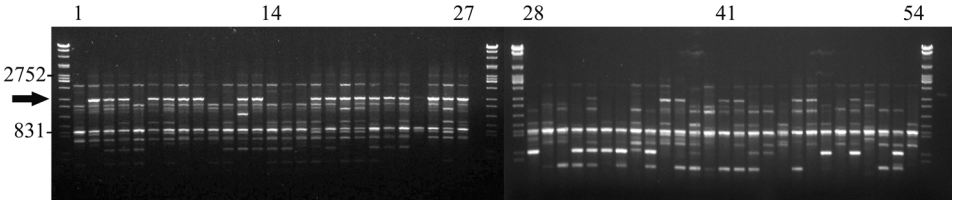
Origin	Collection date
Planaltina, Goiás	March 27, 2001
Platina, São Paulo	April 4, 2001
Sertanópolis, Paraná	April 3, 2001
Cambé, Paraná	April 30, 2001
Curitiba, Paraná	March 20, 2001
Londrina, Paraná	March 20, 2001 & May 23, 2001
Palmital, Paraná	March 23 & 29, 2001
Chapecó, Santa Catarina	March 22, 2001
Passo Fundo, Rio Grande do Sul	March 21, 2001
Cruz Alta, Rio Grande do Sul	March 20, 2001

Genomic DNA extraction and RAPD analysis

DNA was extracted individually from the antennae, head or legs to avoid DNA contamination from parasitoids that could be present inside the body. We used a protocol based on CTAB method with minor modifications (Sosa-Gómez et al., 2004). DNA samples were subjected to PCR-RAPD analyses using twelve 10-mer primers (OPA-01, OPA-09, OPA-16, OPA-17, OPB-01, OPB-05, OPB-10, OPB-20, OPC-09, OPC-15, OPF-03 and OPG-13) from Operon Technologies (www.operon.com). Amplification reactions were performed in a total volume of 25 µl using 10-15 ng of template DNA. Control reactions were conducted without genomic DNA for each primer. A 20 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 as the molecular weight marker. Gels were stained with 4.5 µl ethidium bromide 10 mg/ml and digitally documented with a Kodak Digital DC 290 imaging system.The methods used were the same as those of

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Figure 1. RAPD amplification products obtained with Operon-A17 primer and DNA template from *Nezara viridula* populations. A) Lane 1 to 14: females from Curitiba, PR. Lane 15 to 27 males from Curitiba, last lane control: amplification performed without template DNA. B) Lane 28 to 41 females from Passo Fundo, 42 to 54 males from Passo Fundo. MW: λDNA cut with *Eco*R I, *Hin*d III and *Bam*H I enzymes.



Sosa-Gómez et al., (2004), except that the RAPD-PCR was performed using a PTC 200 thermal cycler (MJ Research, www.mjr.com/) with the following thermal program: 45 cycles at 94° C for 15 sec, 39° C for 30 sec, and 72° C for 1 min; and a final extension at 72° C for 7 minutes. Although RAPD is not exempt of technical limitations (Black, 1993), care was taken to minimize errors. RAPD reproducibility was checked performing two replicates of PCR amplifications. Due to that we analyzed intraspecific variability, bands that comigrated were assumed to represent homologous loci and null bands were used to estimate the frequency of recessive allele. Presence or absence of RAPD products were codified in a binary matrix. The genetic similarity matrix based on RAPD allele frequencies was obtained using Nei's (Nei, 1972) genetic distance, with the NTSYS-pc software (Rohlf, 1993). Cophenetic Correlation Analysis to test the consistence of the cluster analysis was done with Bionumerics software (Applied Maths..., 2000). Population structure studies (percentage of polymorphic loci, *Gst* and *Nm* indices) were performed with the help of Popgene Genetic Software (Yeh et al., 1997). Loci were considered polymorphic regardless of allele frequencies. Unbiased heterozygosities from each population were estimated using Nei (Nei, 1978) proposal, under the assumption that the populations were at Hardy-Weinberg equilibrium. Distances among the sampling points were determined with the SPRING software from the Brazilian National institute for Space Research, INPE (http://www.dpi.inpe.br/spring/) .

Results

The twelve 10-mer primers amplified a total of 178 RAPD products (Table 2) that were enough to discriminate the geographical populations of *N. viridula* . The molecular weight of the products ranged from approximately 300 bp (primer OPB-01) to 2600 bp (primer OPA-17) (Fig. 1).

Table 2. Ten-mer primers which produced reliable amplification products after RAPD reaction using DNA template of different *Nezara viridula* populations.

Operon primers	Band number
OPA-01	11
OPA-09	18
OPA-16	19
OPA-17	21
OPB-01	16
OPB-05	9
OPB-10	17
OPB-20	12
OPC-09	15
OPC-15	13
OPF-03	16
OPG-13	11

The populations clustered into 11 major groups, which corresponded to the geographic origin of the populations (Fig. 2 and Fig. 3). Group 1 comprised individuals from Warta, (Londrina) group 2 individuals from Sertanópolis, group 3 from Platina, and Planaltina, group 4 from Curitiba, group 5 from Palmital, group 6 males from Planaltina, group 7 females from Platina, group 8 from Cambé, group 9 from Chapecó, group 10 from Passo Fundo and group 11 from Cruz Alta (Fig. 3).

The highest degree of genetic similarity, among individuals, occurred between two females collected in Curitiba and two females from Planaltina, but none of them were genetically identical. The Cambé, Platina and Chapecó populations were the most similar to each other (Nei 72 coefficient =0.029) (Table 4).

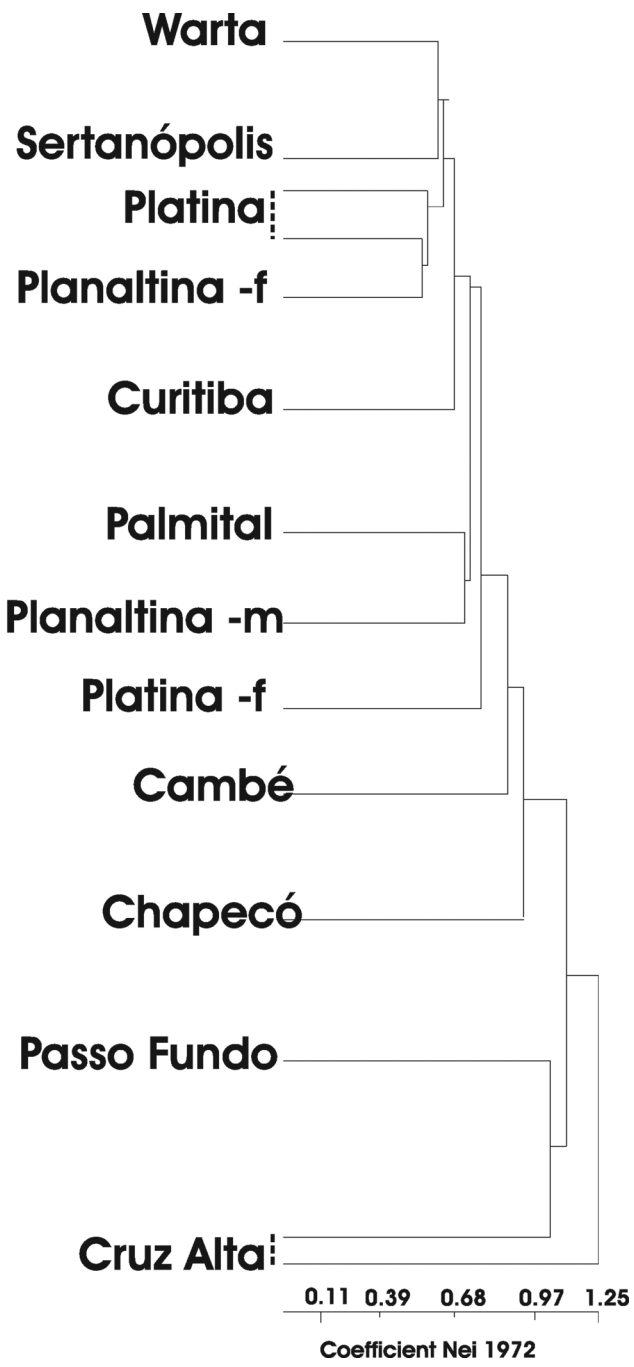
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Figure 2. Geographic location of *Nezara viridula* sampling sites.



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Figure 3. Dendrogram based on UPGMA clustering method and Nei 1972 genetic distance of *Nezara viridula* populations. f = females and m = males.



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Table 3. Genetic variability indices (polymorphism and gene diversity) of *Nezara viridula* populations.

Geographic Origin	Number of polymorphic loci (%)	Heterozigosity
Planaltina, Goiás	92 (51.69)	0.14
Platina, São Paulo	92 (51.69)	0.14
Sertanópolis, Paraná	79 (44.38)	0.11
Cambé, Paraná	90 (50.56)	0.13
Curitiba, Paraná	75 (42.13)	0.12
Warta, Paraná	82 (46.07)	0.12
Palmital, Paraná	69 (38.76)	0.10
Chapecó, Santa Catarina	103 (57.87)	0.14
Passo Fundo, Rio Grande do Sul	81 (45.51)	0.11
Cruz Alta, Rio Grande do Sul	112 (62.69)	0.14
Entire population	178 (100.00)	0.16

Nei's (1973) gene diversity

The highest dissimilarity was found within Cruz Alta stinkbugs, in this case the percentage of polymorphic loci (*ca.* 63%) was the highest compared to the other populations (Table 3). Specimens from Warta (Londrina) were collected on two different dates (Table 1), almost two months apart, but the individuals did not group into different clusters (Fig. 3).

The *N. viridula* population from Cruz Alta and Passo Fundo were the most divergent relative to the other groups with a Nei distance coefficient of 1.10 (Fig. 3). The genetic similarity among individuals from different geographic regions was low. Cluster separation by gender using the RAPD technique has been observed in other species (Borges et al., 2000; Sosa-Gómez et al., 2004). Statistically, groupings were supported by cophenetic correlation values ranging from 65 to 83, for Sertanópolis and Curitiba, respectively (Bionumerics, 1000 replicates).

Relationships among populations were visualized in the two dimensional graph (Fig. 4) that shows eight populations subdivided in four main groups, being Passo Fundo, Cruz Alta and Chapecó divergent from Planaltina, Palmital, Curitiba, Platina and Cambe. This is in agreement with the dendogram, where populations from the Brazilian central region (groups 1 to 8) have genetic affinities and are distant from the most southern populations, such as Santa Catarina and Rio Grande do Sul populations (groups 9, 10 and 11).

The highest proportion of polymorphic loci within each population was observed in the Cruz Alta and Chapecó populations and the smallest percentage of polymorphic loci was detected in the Palmital population. The percentages of polymorphic loci varied between 38.76 and 62.92 for the Palmital and Cruz Alta populations respectively (Table 3).

Total gene diversity (HT) ranged from 0.1000 to 0.1364, and the diversity within populations (Hs) ranged from 0.1261 to 0.1582. The overall gene differentiation among the ten populations presented a *Gst* equal to 0.2618 and ranged from 0.0858 (comparison between Chapecó and Platina) to 0.2814 (comparison between Passo Fundo and Londrina) and the calculated gene flow (*Nm*) among all populations was 1.4098 (Table 5). Gene flow indices ranged from 1.28, between the Londrina and Passo Fundo populations to 5.33, between populations of Platina and Chapecó.

Discussion

RAPD partitioned *N. viridula* populations from different geographical origins. Even samples taken from areas of geographical proximity, such as Cambé and Warta (Londrina) populations (Fig. 2 and Fig. 3) were discriminated. As in the *E. heros* case (Sosa-Gómez et al., 2004) discrete clusters of each *N. viridula* geographic population did not include specimens from other localities.

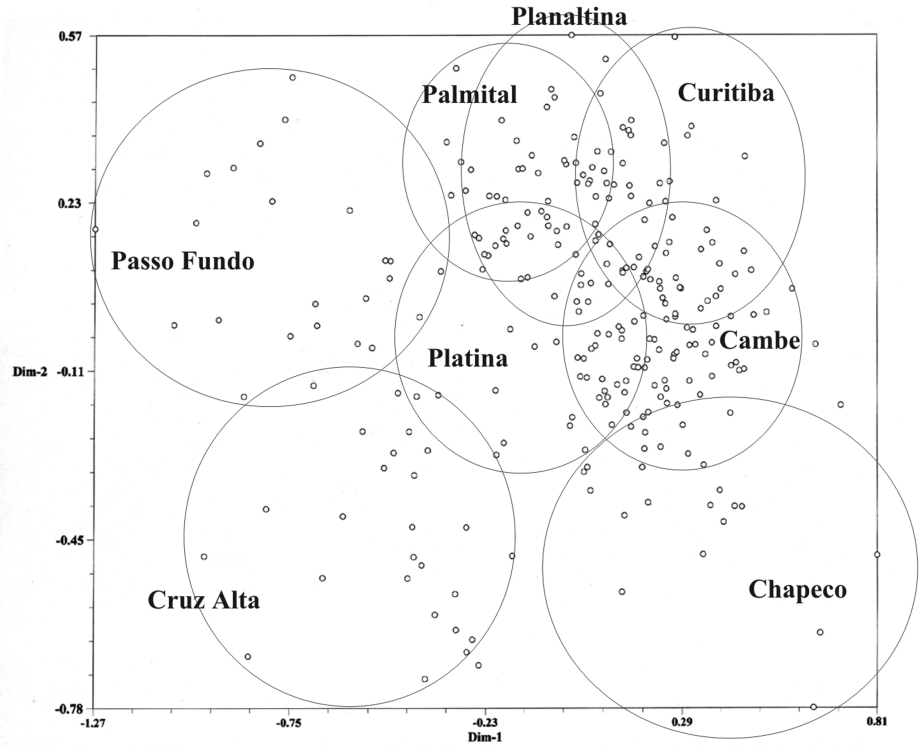
The percentage of polymorphic loci were slightly higher than that observed on *E. heros* populations

Table 4. Genetic distancecoefficient (Nei, 1972) between populations of *Nezara viridula*.

	Planaltina, GO	Platina, SP	Sertanópolis, PR	Cambé, PR	Curitiba, PR	Warta, PR	Palmital, PR	Chapecó, SC	Passo Fundo, RS	Cruz Alta, RS
Planaltina, GO	0									
Platina, SP	0.0394	0								
Sertanópolis, PR	0.0495	0.0388	0							
Cambé, PR	0.0478	0.0289	0.0405	0						
Curitiba, PR	0.0563	0.0414	0.0440	0.0492	0					
Warta, PR	0.0657	0.0608	0.0504	0.0684	0.0647	0				
Palmital, PR	0.0570	0.0543	0.0531	0.0486	0.0633	0.0756	0			
Chapecó, SC	0.0541	0.0299	0.0470	0.0288	0.0530	0.0559	0.0592	0		
Passo Fundo, RS	0.0523	0.0505	0.0632	0.0465	0.0782	0.1006	0.0597	0.0537	0	
Cruz Alta, RS	0.0662	0.0477	0.0687	0.0498	0.0803	0.0838	0.0640	0.0480	0.0338	0

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Figure 4. Principal coordinate analysis plot of *Nezara viridula* populations based on the first two principal components.



(Sosa-Gómez et al., 2004), but lower than reported for better fliers, such as lepidoptera (Pashley et al., 1985; Sosa-Gómez, 2004)

Gene diversity was lower (HT = 0.16) than that observed for *E. heros* (HT = 0.20) using a similar technique (Sosa-Gómez et al., 2004). Species that suffered bottlenecks presented very low heterozigosity index. The overall gene differentiation (Gst = 0.2618) was lower than the observed for *E. heros* (Gst = 0.3757) by (Sosa-Gómez et al., 2004) meaning that *N. viridula* populations present lower allele fixation. The calculated Gst value when all the populations were analyzed indicates that most of the genetic variation was present within population rather than among populations. Thus, on average the variability inside each geographical population was higher than in the case of the neotropical stink bug, *E. heros* (37%) and lower that in the case of the velvetbean caterpillar, *Anticarsia gemmatalis* (14%) (Sosa-Gómez, 2004; Sosa-Gómez et al., 2004).

The *Nm* index can be defined as the number of migrants between populations in each generation (McDermott et al., 1993). Comparing populations of *E. heros* and *N. viridula* with approximately the same distance between them (Table 6), it can be observed that the *E. heros Nm* was 1.5639 for populations separated by 1,008 km (between Sapezal and Ponta Porã), while *N. viridula Nm* was higher, 2.2558, for populations separated by 968 km [between Warta (Londrina) and Planaltina]. For that reason, *N. viridula* may be a better flier than *E. heros* . This could also explain the higher *Nm* indexes observed among *N. viridula* populations compared to *E. heros* ; the overall gene flow among *E. heros* populations was 0.8307 (Sosa-Gómez et al., 2004).

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Table 5. Pairwise comparisons of Nei`s coefficient of gene differentiation (Gst) (below diagonal) values between *Nezara viridula* populations and estimates of gene flow (Nm) (above diagonal).

	Nm									
Gst	**Planaltina, GO**	**Platina, SP**	**Sertanópolis, PR**	**Cambé, PR**	**Curitiba, PR**	**Warta, PR**	**Palmital, PR**	**Chapecó, SC**	**Passo Fundo, RS**	**Cruz Alta, RS**
Planaltina, Go	0	4.0889	2.8576	3.1120	2.6398	2.2558	2.3733	2.9257	2.2040	2.3969
Platina, SP	0.1090	0	3.6927	5.1847	3.6264	2.4741	2.5360	5.3280	2.7497	3.5337
Sertanópolis, PR	0.1489	0.1193	0	3.2415	2.9707	2.5828	2.2398	2.9804	1.9115	2.0305
Cambé, PR	0.1384	0.0880	0.1336	0	2.8080	2.0277	2.5773	5.0814	2.7215	2.9179
Curitiba, PR	0.1592	0.1212	0.1441	0.1511	0	2.1269	1.9847	2.7825	1.6379	1.8315
Warta, PR	0.1814	0.1681	0.1622	0.1982	0.1903	0	1.6591	2.6202	1.2765	1.7428
Palmital, PR	0.1740	0.1647	0.1825	0.1625	0.2012	0.2316	0	2.2711	1.9188	2.0706
Chapecó, SC	0.1460	0.0858	0.1437	0.0896	0.1523	0.1602	0.1804	0	2.5249	3.2190
Passo Fundo, SC	0.1849	0.1539	0.2073	0.1552	0.2339	0.2814	0.2067	0.1653	0	3.9065
Cruz Alta, RS	0.1742	0.1240	0.1976	0.1463	0.2145	0.2229	0.1945	0.1344	0.1135	0

No systematic migration studies of *N. viridula* have been reported. But it is known, that this species is capable of flying short distances when disturbed. Costa and Link (Costa et al., 1982) reported that the *N. viridula* can move 100 to 120 m in 28 days, in the presence of soybean plants. Long distance adult dispersion by hurricane has been documented (Aldrich, 1990). Consequently, studies on predominant winds and geographic barriers are necessary to explain differences in dispersion patterns.

Interestingly, despite the short distance (*ca.* 29 km) and the absence of geographic barriers between Cambé and Warta, Londrina, both populations clustered in different groups and the estimated gene flow index between them was equal to 2.02, indicating that migration may be relatively restricted, in this particular case.

Sosa-Gómez *et al.* (2004) observed that gene flow between *E. heros* populations from Warta (Londrina) and Centenário do Sul (50 km apart) was 0.2065, suggesting null or restricted migration between these populations. The estimated overall index of gene flow for *N. viridula* was 1.41. According to Wright (1931), gene flow above one is considered enough to overcome genetic drift and delay or stop genetic differentiation. Species with low potential for dispersion, such as *Afgekia sericea* Craib (Leguminosae) that is a perennial plant with large and heavy seeds, have an average value of *Nm* = 0.35 (Prathepha et al., 1999), while gene flow estimates (*Nm*) for a good flier such as the velvetbean caterpillar can be as high as 15.26 (Sosa-Gómez, 2004).

Therefore, considering only isolation among the various others factors influencing resistance, the possibilities of developing resistance to insecticides in relatively isolated local populations are higher than in species that show higher immigration rate (Georghiou et al., 1986). In fact, *N. viridula* was one of the early reports of pentatomid species selected for insecticide resistance in the field, in Australia (Georghiou et al., 1991). But resistance monitoring of *N. viridula* populations in Brazil carried out by Sosa-Gómez D.R. and Silva J.J (unpublished) did not show an increase of LD50 for organophosphates insecticides until recently.

Comparative studies with more mobile species such as *P. guildinii* (Panizzi et al., 1980; Costa et al., 1982) should be performed to confirm suspicions of insecticide resistant cases in *P. guildinii* populations from Santa Fé, Argentina (Juan C. Gamundi, pers. com.) and Uruguay (Enrique R. Castiglioni, pers. com.).

The RAPD technique provides useful information of intraspecific variation. It is inexpensive and large number of samples can be processed. In addition, sequence characterized amplified regions (SCAR) studies of fragments generated by RAPD can be

Table 6. Distances in straight line (Km) amog the different sampling points of *Nezara viridula* population.

	Planaltina, GO	**Platina, GO**	**Palmital, PR**	**Sertanópolis, PR**	**Warta, PR**	**Cambé, PR**	**Curitiba, PR**	**Chapecó, SC**	**Passo Fundo, RS**	**Cruz Alta, RS**
Planaltina, GO	0									
Platina, SP	852	0								
Palmital, PR	863	19	0							
Sertanópolis, PR	937	101	89	0						
Warta, PR	968	124	114	30	0					
Cambé, PR	952	123	123	25	29	0				
Curitiba, PR	1145	328	314	323	300	330	0			
Chapecó, SC	1410	557	545	482	445	470	385	0		
Passo Fundo, RS	1520	602	656	591	567	570	447	128	0	
Cruz Alta, RS	1602	750	745	677	642	658	560	196	130	0

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helpful to establish boundaries between populations.

Acknowledgements

We thank Dr. George G. Brown, José de Barros França Neto and Antonio R. Panizzi for critically reviewing the manuscript and Dr. Luiz Amilton Foerster, Dr. Edison Sujii, Jovenil J. da Silva, Oriverto Tonon, Wilson Pozenato and José Francisco for help with the collection of *N. viridula* samples. This work received financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Embrapa Soja. Approved for publication by the editorial board of Embrapa Soja as manuscript 163/2004.

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