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Short Communication

Genetic variability of *Mahanarva* sp (Hemiptera: Cercopidae) collected from different sites in Brazil

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ABSTRACT. Spittlebugs are the leading cause of damage to tall grasses. Annual losses are estimated to reach 2.1 billion dollars in sugarcane crops and grazing land throughout the world. Correct identification of these species is difficult due to similarities in color, body size and male genitalia. Molecular markers have been useful in the identification and assessment of genetic diversity of many species. We investigated the genetic diversity of the spittlebug species *Mahanarva fimbriolata*, *M. spectabilis* and *M. liturata* and looked for markers that could aid in their identification. DNA from 34 spittlebug specimens, collected from six different regions of Brazil (Brasília, Campo Grande, Valença, Presidente Prudente, Juiz de Fora, and Porto Alegre), was analyzed with 29 RAPD primers, generating 501 polymorphic markers. High genetic variability was found among individuals *M. fimbriolata* (0.37), *M. spectabilis* (0.18) and *M. liturata* (0.69). Species-specific molecular RAPD markers were identified for each of the three species; these could be used as auxiliary tools for their correct identification.

Key words: Molecular marker; RAPD; Spittlebugs

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It is estimated that spittlebugs cause annual losses of up to 2.1 billion dollars to sugarcane crops and grazing land throughout the world. In Brazil, cercopid species in the genus *Mahanarva* are the leading cause of damage to large grasses such as sugarcane and elephant grass, with the main offenders being *Mahanarva fimbriolata* (Stal, 1854), *M. spectabilis* (Distant, 1909) and *M. liturata* (Le Peletier and Serville, 1825).

It is often hard to identify the species of this genus because the main distinguishing characteristics are coloration and body size (subject to phenotypic plasticity) and morphology of the male genitalia. Molecular markers have been found very useful to identify species as well as to analyze the genetic distance and structure of populations (Castiglioni et al., 2005; Mikac and Clarke, 2006; de Oliveira et al., 2007; Castro et al., 2007). In less-studied species such as *Mahanarva*, the random amplified polymorphic DNA (RAPD) technique is a valuable molecular tool since it does not require previous DNA sequencing studies and since arbitrary primers are used (Ercan et al., 2004). However, the RAPD technique is sometimes criticized about its alleged lack of reproducibility and repeatability.

Nevertheless, these features can be avoided with improvement of laboratory techniques and scoring system, making RAPD markers highly stable in controlled experimental conditions (Skroch and Nienhuis, 1995; Weising et al., 1995; Parani et al., 1997; Bussell, 1999).

Populations of *Mahanarva spectabilis* show morphological differences depending on the regions in which they are found in Brazil. This would suggest a genetic isolation although there is little information on inter- and intraspecific genetic variations. The aim of this study was to use molecular markers to assess the genetic diversity of *M. fimbriolata*, *M. spectabilis* and *M. liturata* and to select specific markers to help in the correct identification of these species.

Specimens of *M. spectabilis* were captured in Brasília, Campo Grande, Valença, and Presidente Prudente, while individuals of *M. fimbriolata* and *M. liturata* were collected in Juiz de Fora and Porto Alegre (Figure 1). The spittlebugs *Deois* (Pandysia) *schach* (Fabricius, 1787) and *Notozulia entreriana* (Berg, 1879) were included as external groups. The sites were chosen because of the impact different spittlebug species have had on forage grasses.

Adults were collected with an insect net on *Brachiaria* and elephant grass, sorted and stored in 100% alcohol. Some of the specimens caught were submitted to taxonomic species identification according to the morphology of the male genitalia.

For the genetic studies, five specimens of *M. spectabilis* and *M. fimbriolata* and three specimens of *M. liturata* were sampled from each location, along with three specimens of each external control group. The appendages (legs and wings) and head were removed from each insect. DNA was extracted from the remaining insect parts with CTAB according to the protocol of Ferreira and Grattapaglia (1995) with modifications.

A specimen from each group was used in the amplification test with decamer primers to select the best ones, based on the sharpness and repeatability of the reaction. Of the 40 primers tested (obtained from Operon Technologies Inc.), 29 were finally used for amplification of all the individuals.

Polymerase chain reaction cycling parameters consisted of an initial temperature of 94°C for 5 min, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, and 10 min at 72°C for a final extension (Applied Biosystems 9700 Thermocycler, Foster City, CA, USA). The total volume of each reaction was 25 µL, containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.4 µM primer, 0.1 mM of each dNTP, 1 U Taq DNA polymerase, and 45 ng genomic DNA. RAPD amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

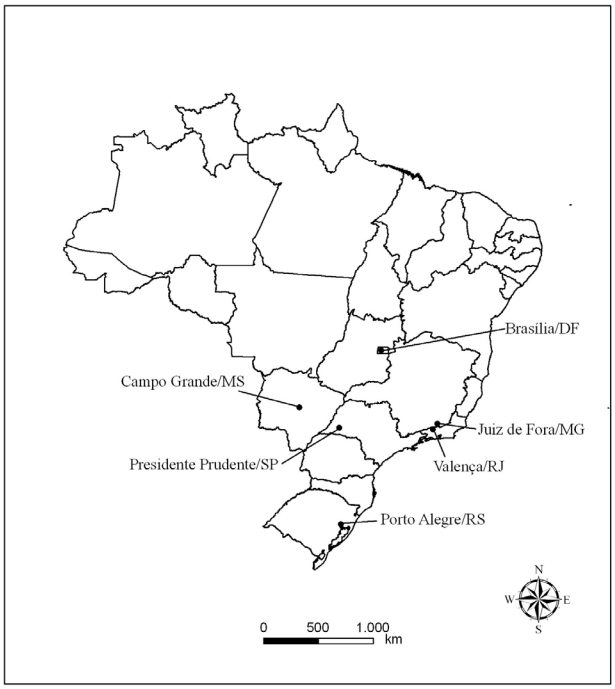

Figure 1. Geographical locations where spittlebugs were sampled in Brazil.

All reactions with selected primers were done in duplicate. Only the highly intense and reproducible fragments were included in the analysis, as recommended by Pérez et al. (1998). A binary matrix was constructed according to the presence (1) or absence (0) of bands among the specimens.

The relative genetic distance between each pair of individuals was estimated using the arithmetic complement of Dice's coefficient (Nei and Li, 1979). Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). The arithmetic means of the genetic distances found between all the individuals in question were calculated to analyze the intra- and intergroup mean genetic distances (MGDs).

Gene flow (Nm) between *M. spectabilis* groups was estimated from the Φst component of the Φ statistic introduced by Excoffier et al. (1992), using the following formula:

$$Nm = [(1 / \Phi st) - 1] / 4$$

This approach does not require previous knowledge of the allele frequencies or making specific assumptions (Excoffier et al., 1992), and thus provides an alternative to the use of dominant markers.

A total of 501 markers were analyzed. All of them were polymorphic among the 34 individuals studied, showing the high genetic variability of the specimens. The MGDs between individuals of *M. spectabilis* ranged from 0.15 to 0.22 within a single location and 0.16 to 0.33

among different locations, with an intraspecific MGD of 0.18 (Table 1). Among the *M. fimbriolata* and *M. liturata* individuals, the MGDs were 0.37 and 0.69, respectively, showing higher intraspecific variability compared to *M. spectabilis*. Cluster analysis clearly separated these three species, with MGDs of 0.67 between *M. spectabilis* and *M. fimbriolata*, 0.87 between *M. spectabilis* and *M. liturata* and 0.89 between *M. fimbriolata* and *M. liturata* (Table 1).

Table 1. Matrix of mean genetic distances (MGDs) among *Mahanarva* populations.

	M. spectabilis[1]	*M. spectabilis*[2]	*M. spectabilis*[3]	*M. spectabilis*[4]	*M. fimbriolata*	*M. liturata*	*Deois schach*	*Notozulia entreriana*
M. spectabilis[1]	0.22 (-)	- (0.93)	- (0.32)	- (0.35)	-	-	-	-
M. spectabilis[2]	0.27 (0.21)	0.2 (-)	- (0.39)	- (0.42)	-	-	-	-
M. spectabilis[3]	0.33 (0.44)	0.29 (0.39)	0.15 (-)	- (6.10)	-	-	-	-
M. spectabilis[4]	0.33 (0.42)	0.29 (0.37)	0.16 (0.04)	0.16 (-)	-	-	-	-
M. fimbriolata	0.65	0.66	0.68	0.67	0.37	-	-	-
M. liturata	0.88	0.87	0.87	0.87	0.89	0.69	-	-
Deois schach	0.95	0.93	0.93	0.94	0.93	0.94	0.43	-
Notozulia entreriana	0.94	0.93	0.92	0.92	0.95	0.94	0.91	0.3

Values of Φst generated by AMOVA (lower diagonal) and gene flow index (upper diagonal) are in parentheses. *M. spectabilis* sampled in the following locations: Valença (1), Campo Grande (2), Brasília (3), and Presidente Prudente (4).

There was similarity between and within populations of *M. spectabilis* denoting a tendency for clustering individuals from Presidente Prudente and Brasília. The distances between these populations were greater than the distances between populations from Campo Grande and Valença. These findings corroborate the identification done using morphological traits based on the individuals' coloration of the tegmen, head and scutellum. It is important to point out that the samples from Presidente Prudente and Brasília had wings with four color patterns, while the populations from Valença and Campo Grande had wings with only one color pattern. However, the MGD between the populations with greater wing morphological differences was smaller (0.16) than those with a single color pattern wing (0.27), indicating that there is no correlation between genetic distance and the number of morphological differences in the specimens. The absence of a correspondence between morphological and RAPD data has been reported elsewhere in the literature (Semagn, 2002).

Morphological differences between populations of the same species dispersed over latitude and elevation gradients have been reported for various insect groups (Hawkins and Lawton, 1995; Smith et al., 2000) and are generally associated with temperature, humidity and photoperiod conditions (Tauber et al., 1986).

The cluster analysis carried out based on genetic distances showed the genetic separation between the species *M. fimbriolata*, *M. spectabilis* and *M. liturata*, as well as a strong separation from the control group with an MGD of 0.94.

Six markers were exclusively identified for *M. spectabilis* individuals. Likewise, eight exclusive markers for *M. fimbriolata* and four exclusive markers for *M. liturata* (Table 2) were identified. These markers can be used as auxiliary tools in the correct identification of these species, currently carried out by taxonomists using external morphological traits.

Table 2. Oligonucleotide primers (Operon Technologies), nucleotide sequence, number of distinct fragments analyzed, and the exclusive markers identified for the *Mahanarva* species and their respective fragment sizes.

Primer	Nucleotide sequence	Fragments analyzed	Exclusive marker	
			Species	Fragment size (bp)
OPA-13	CAGCACCCAC	16	*M. spectabilis*	575
OPA-16	AGCCAGCGAA	10	*M. fimbriolata*	981
OPB-01	GTTTCGCTCC	10	-	-
OPB-06	TGCTCTGCCC	9	-	-
OPB-07	GGTGACGCAG	17	*M. spectabilis*	723
OPB-20	GGACCCTTAC	10	*M. spectabilis*	764
			M. fimbriolata	1595
OPC-13	AAGCCTCGTC	12	*M. spectabilis*	888
OPC-18	TGAGTGGGTG	17	*M. liturata*	428
OPC-19	GTTGCCAGCC	22	-	-
OPD-08	GTGTGCCCCA	15	*M. liturata*	303
OPE-04	GTGACATGCC	18	*M. fimbriolata*	313
OPE-11	GAGTCTCAGG	15	-	-
OPE-18	GGACTGCAGA	13	*M. liturata*	392, 183
OPF-01	ACGGATCCTG	15	-	-
OPF-06	GGGAATTCGG	10	-	-
OPF-09	CCAAGCTTCC	11	*M. spectabilis*	667
OPF-10	GGAAGCTTGG	24	-	-
OPG-08	TCACGTCCAC	16	*M. fimbriolata*	420
OPG-10	AGGGCCGTCT	22	-	-
OPG-11	TGCCCGTCGT	15	-	-
OPG-17	ACGACCGACA	26	*M. fimbriolata*	642
OPG-18	GGCTCATGTG	8	*M. fimbriolata*	738
OPH-03	AGACGTCCAC	19	-	-
OPH-05	AGTCGTCCCC	26	-	-
OPH-08	GAAACACCCC	35	-	-
OPH-12	ACGCGCATGT	39	*M. spectabilis*	480
			M. fimbriolata	762
OPH-19	CTGACCAGCC	22	-	-
OPI-04	CCGCCTAGTC	10	*M. fimbriolata*	401
OPJ-04	CCGAACACGG	19	-	-

An exclusive marker was also found for the individuals of *M. spectabilis* collected in Presidente Prudente and Brasília (primer OPH-08, 757-bp marker). Oliveira et al. (2004), using RAPD markers to distinguish subspecies of *Tetragonisca angustula* Latreille, found specific bands for each subspecies. Waldschmidt et al. (2000) also identified RAPD markers present in one subspecies of *Melipona* and absent in another.

Among the *M. spectabilis* populations, the fixation index ranged from 0.04 to 0.44, and the estimated gene flow index ranged from 0.32 to 6.10. The lowest fixation indices and highest flow indices were found between the populations from Presidente Prudente and Brasília (Table 1). These findings indicate the possible existence of subspeciation within the species. However, a more informative sampling will be necessary to confirm this conjecture.

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