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Microsatellite loci and genetic structure of artificial populations of *Cotesia flavipes* (Hymenoptera, Braconidae)

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ABSTRACT. *Cotesia flavipes* (Cameron) is a parasitoid wasp used in the biological control of the sugarcane borer (*Diatraea saccharalis*) (Fabr., 1794). Studies on the genetic diversity of *C. flavipes* are hampered by the lack of highly polymorphic molecular markers. In this report, a set of 11 microsatellite loci were developed from an enriched library of *C. flavipes*. Four microsatellite loci were polymorphic and were screened in 212 *C. flavipes* individuals (183 females and 29 males) that were randomly sampled from seven rearing laboratory populations. The number of alleles ranged from two to three. The average inbreeding

coefficient (F_{IS}) among all laboratory populations was 0.120, indicating an excess of homozygotes. The average genetic diversity within the laboratory populations was 0.292, which is lower than the values reported for wild *Cotesia* spp populations. Genetic diversity was most pronounced within laboratory populations (70 to 90%). Most of the observed alleles were fixed or close to fixation. This low overall genetic diversity may have originated from a founder effect, i.e., the contribution of a small number of individuals (genes and alleles) to the formation of these populations. To our knowledge, this study is the first to provide microsatellite loci and an analysis of the genetic structure of *C. flavipes*. Our results suggest that new introductions of *C. flavipes* may increase genetic diversity and improve the efficiency of the biological control of *D. saccharalis*. In addition, population structure data could be used to estimate the minimum number of wasps to be imported.

Key words: Molecular marker; SSR; Rearing laboratories; Population genetics; Biological control

INTRODUCTION

Sugarcane is an important crop in Brazil, and this crop has social, economic and environmental impacts. Between the years 2012 and 2013, the area of sugarcane planted in Brazil was estimated at 9.8 million hectares (IBGE, 2013).

The sugarcane borer *Diatraea saccharalis* (Fabr.) is one of the major causes of sugarcane yield losses. The borer drills a hole at the stem and produces galleries, opening the door for the entry of microorganisms and pathogens that contaminate the broth used in the industry. *D. saccharalis* is widely distributed throughout the continent, being found in North, Central and South America (Cruz, 2007). The borer can be controlled using chemicals, which present a high cost and elevated environmental impact, or biological control agents, which present a lower cost and reduced environmental impact (Bueno, 2009). *Cotesia flavipes* (Cameron) (Hymenoptera - Braconidae), a parasitic wasp that originated from the Indo-Australian region (Polaszek and Walker, 1991), was introduced from India and Pakistan to Brazil in the 1970s as a biocontrol agent of the sugarcane borer (Botelho et al., 1983; Botelho, 1992). Since its introduction, *C. flavipes* has been produced on a large scale in commercial mills and rearing laboratories (RLs) and plays an important role in controlling this pest in sugarcane cultivation.

The process of mass production of *C. flavipes* is, briefly, as follows: borers are maintained and replicated in the RL, and some are selected as "prey" for the production of *C. flavipes*. Each borer is subjected to oviposition by only one *C. flavipes*. The eggs of *C. flavipes* develop into larvae, which consume the host insect and subsequently form pupae. The pupae form in a tangle of wires referred to as a mass. The pupal masses of borers are removed and placed in plastic cups for later release in the field when *C. flavipes* emerge. Each released plastic cup of *C. flavipes* contains approximately 1500 individuals on average (Bueno, 2009). The number of *C. flavipes* released in the field is decided based upon on a prior analysis identifying the borer index infestation per hectare and dispersion models (Dinardo-Miranda, 2008; Dinardo-Miranda et al., 2011). The *C. flavipes* released in the field find and parasitize the borer. The wasps that emerge from the borers can subsequently mate and parasitize other

borers; if the wasps are successful, then they repeat this cycle. Thus, field workers who monitor pests such as borers can occasionally find *C. flavipes* in fields that received wasps released up to a year previously. Although the wasps have adapted to Brazil, they have not adapted to the point of reproducing intensively. The number of wasps that remain in the field is very small, and new wasp masses produced in RLs need to be released. Despite the important role of the wasps, some Brazilian farmers have suggested that there has been a decrease in the efficiency of *C. flavipes* parasitism on *D. saccharalis* over the years (Dinardo-Miranda, personal communication). This hypothesis is supported by the finding that the activity radius of *C. flavipes* in the field has been decreasing over the years. This radius was reported to be 34 meters in 1980 (Botelho et al., 1980), 25 meters in 2009 (Volpe, 2009) and 15 meters in 2014 (Dinardo-Miranda et al., 2014). On the other hand, in an initial introduction of *C. flavipes* in maize fields in Kenya, Africa, larvae parasitized by *C. flavipes* were found at a distance of 64 meters from the release point (Sallam et al., 2001).

This decrease in efficiency may be due to the low genetic variability of *C. flavipes*. This wasp is not native to Brazil, and its introduction occurred over 40 years ago. Although there are no reports indicating the number of mating pairs of *C. flavipes* that were imported (Botelho, 1992), the number is suspected to be small. This situation contrasts with the high biological diversity of the sugarcane borer (Lopes et al., 2014), which is native and is widely distributed throughout the continent. The low diversity of *C. flavipes* could affect behaviors related to the decreased efficiency of parasitism.

The relationship between the efficiency of biological control and genetic variability remains unclear. Baker et al. (2003) suggested that the low genetic variability associated with founder effects may affect the potential for biological control in studies on wasps from the same family as *C. flavipes*.

One practice that is recommended to avoid stagnation and decreases in wasp diversity is periodically exchanging some *C. flavipes* individuals among the RL populations. Additionally, reintroduction of *C. flavipes* individuals collected in the field to RLs some days after their release may be beneficial. However, this process is not widely accepted by RLs because it can introduce wasps that may have acquired contamination or disease in the field.

A number of reports have described the biology and behavior of *C. flavipes* in Brazil (Campos-Farinha et al., 2000; Dinardo-Miranda et al., 2014; Volpe et al., 2014). Despite its economical importance and use in applied biological control, little is known about the genetic diversity of *C. flavipes*. Allozyme analysis has revealed 15-36% polymorphism among populations of *C. flavipes* from Africa (Omwega and Overholt, 1996; Kimani-Njogu et al., 1998), and mitochondrial DNA analysis of 21 worldwide populations belonging to the *C. flavipes* complex have indicated that geography and recent biological control introductions have had important roles in population structuring, although mtDNA provided little power to differentiate recent biological events (Muirhead et al., 2006). DNA markers have been widely adopted for analyzing the dynamics of plant pest/pathogen populations because of their high levels of precision and accuracy (Milgroom and Peever, 2003). Simple sequence repeats (SSRs) or microsatellite sequences are abundant and evenly distributed in eukaryote genomes (Weising et al., 1995). Where polymorphic loci are available, they can be very useful for genetic diversity and population genetic studies; therefore, the search and development of such genetic markers is warranted. Microsatellite loci are available for several parasitoids, including those in *Cotesia*, such as *C. congregata* (Say) (Jensen et al., 2002), *C. melitaearum* (Wilkinson) (Kankare et al., 2004) and *C. sesamiae* (Cameron) (Abercrombie et al., 2009),

which were successfully tested against their natural populations. Recently, the cross-species transferability of nine SSRs was demonstrated for *Cotesia* spp., including *C. flavipes* (Abercrombie et al., 2009); however, to our knowledge, there has been no report of microsatellites developed for the *C. flavipes* genome.

One of the rare study about phylogenic analysis of *C. flavipes* complex, comparing 86 samples from 15 countries, using molecular markers (CO1 and 16S ribosomal RNA gene), grouped *C. nonagriae* com *C. flavipes* in a single branch. Furthermore, clades presented in this study reflect the genetic origin of the various introductions in different countries. Moreover, mtDNA and nDNA data show no obvious relationship of *Cotesia* spp. with their different hosts (Muirhead et al., 2012).

SSRs are usually abundant, polymorphic and neutral. As a co-dominant marker, microsatellite markers are a powerful tool for elucidating the genetic diversity and structure of insect populations. Using SSR markers, the present study aimed to characterize the genetic diversity of seven populations of *C. flavipes* from commercial RLs and gather information about their genetic structure. This information could be used to devise new management strategies to be applied in RLs to increase genetic diversity levels in wasp populations, which can then be efficiently employed in the biological control of the sugarcane borer. Consequently, the efficiency of parasitism might be improved in an attempt to conduct a type of "breeding" in these parasitoids.

MATERIAL AND METHODS

SSR-enriched library construction and loci identification

Because *C. flavipes* is haplodiploid, only females were used in this part of the study. Total DNA was extracted from a pool of 20 wasps (Aljanabi et al., 1999) that were collected from a RL. The Simple Sequence Repeats (SSRs) loci-enriched library was developed using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles (Creste et al., 2006). Briefly, 5 mg of genomic DNA was digested with *Rsa*I, and a sample of 600 ng DNA was ligated to adaptors (*Rsa*21: 5'CTCTTGCTTACGCGTGGACTA3'; *Rsa*25 5'TAGTCCACGCGTAAGCAAGAGCACA3'). The ligated fragments were then amplified by polymerase chain reaction (PCR), and the products were allowed to hybridize to biotinylated I5(CT)8 and I5(GT)8 probes, which were later recovered by Streptavidin MagneSphere Paramagnetic Particles (Promega - Madison, WI, USA).

The eluted fragments were re-amplified using the *Rsa*21 primer and cloned into the pGEM-T Easy vector (Promega - Madison, WI, USA). Plasmids were transformed into *Escherichia coli* XL1-Blue. Positive clones were sequenced using the M13 primer and the BigDye terminator Cycle Sequencing Kit (Applied Biosystems - Foster City, CA, USA) in an ABIPRISM® 3100 DNA Analyzer (Applied Biosystems - Foster City, CA, USA).

The sequence analysis for microsatellite (SSR) identification loci was performed using the WEBSAT program (Martins et al., 2009). The search parameters were mono- and di-nucleotides with at least eight repetitions and tri-, tetra-, penta- and hexa-nucleotides with at least four repetitions.

Based on these parameters, primers were designed for 11 microsatellite loci using the program Primer3Plus (Untergasser et al., 2007), and their quality was evaluated using NETPRIMER software (http://www.premierbiosoft.com/netprimer).

DNA extraction, PCR conditions and polymorphic loci selection

An additional 20 *C. flavipes* female wasps were random sampled from seven RLs (named A to G) located in São Paulo State, Brazil. The seven RLs are from different cities, of which the closest ones are 25 km apart and the most distant are 258 km apart.

DNA extraction from unique wasps was achieved as described previously (Lima et al., 2002) with modifications. For each individual sample, 60 µL of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, proteinase K 60 mg/mL) was added. The material was ground with a pestle, and the volume was adjusted to 80 µL and incubated at 65°C for 20 min followed by 95°C for 10 min and then immediately frozen at -20°C until use.

The eleven primers designed for microsatellite loci, as described above, were tested. The PCR reactions were performed in a final volume of 20 µL containing 1X PCR buffer KCl (Fermentas® - Waltham, MA, USA), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, 0.2 U of Taq polymerase and 15 ng of DNA. Amplifications were carried out in a MyCycler thermocycler (Biorad) programmed with a touchdown cycle with an initial step of 95°C for 5 min followed by 10 cycles of 95°C for 40 s, 65°C for 30 s (decreasing 0.5°C per cycle), and 72°C for 30 s; followed by 30 cycles of 95°C for 40 s, 60°C for 30 s, and 72°C for 30 s; followed by one cycle at 72°C for 5 min.

The PCR products were resolved by gel electrophoresis on a 5% denaturing polyacrylamide gel slab and stained with silver nitrate (Creste et al., 2001).

The heterozygosity and the polymorphic information content (PIC) from the polymorphic loci were performed as described in Nagy et al. (2012).

Molecular and diversity analyses

A total of 183 female *C. flavipes* were sampled from seven RLs. Each female was removed from an independent pupal mass; i.e., each wasp represents a sample that emerged from a borer that received the oviposition of only one *C. flavipes*.

In addition to the females, 29 males were sampled from the same RLs. Seven of these males were matched with seven females from the same oviposition generation; therefore, these males and females are full siblings (Table 1).

Table 1. Sample numbers of female wasps, males and pairs of full-siblings from the seven sampled rearing laboratories.

	Rearing Laboratories							Total
	A	B	C	D	E	F	G	
Female	19	27	27	27	29	27	27	183
Male	4	4	5	4	4	4	4	29
Full-sibling pairs	1	1	2	0	1	1	1	7

DNA extraction was performed as in Lima et al. (2002) with the modifications and amplification conditions described above.

Individual wasps were genotyped to determine the alleles at each of the four SSR polymorphic loci tested (Cot 1, Cot 10, Cot 12 and Cot 17).

Population genetic structure was estimated through Nei's genetic diversity partitioning (Nei, 1973) where H_T is the total genetic diversity; H_S is the genetic diversity within populations; and G_{ST} is the coefficient of genetic divergence between populations. These parameters were

estimated using the software FSTAT (Goudet, 2002). The presence of null alleles was estimated with the software Micro-Checker (version 2.2.3) (Oosterhout et al., 2004). The G_{ST} estimation was standardized according to Nei (1973) and was calculated as follows:

$$G_{ST} = \frac{H_T - H_S}{H_T}$$

We also estimated F_{IS}, the average coefficient of inbreeding within populations (Wright, 1951), and its equivalent $G_{IS=1-H_{O/H_S}}$ (Nei, 1987). In addition, we obtained estimates of the expected heterozygosity (H_E), which is equivalent to H_S. Where the data of both sexes were combined, we used weighted averages, with the total number of alleles of each class taken as weights.

Due to the haplodiploid nature of *C. flavipes*, some analysis were performed only in females (SSR loci characterization and Nei's genetic distance), whereas other analyses (allele frequency, diversity of gene loci per population, genetic diversity partitioning) were performed in both sexes.

Although the FSTAT program was developed for diploid individuals, this program allowed us to analyze data from haploid individuals because they are treated as homozygous diploids (Goudet, 1994). In this study, each RL was considered to be a population.

Nei's genetic distance (Nei, 1972) was estimated among the populations using TFPGA software (Miller, 1997) and was visualized using a dendrogram obtained via the UPGMA method using NTSYS software (Rohlf, 2000).

RESULTS

Microsatellite loci characterization

From the 96 clones sequenced, 62 (64%) exhibited SSR sequences. Among these sequences, the dinucleotide (CA)n appeared in 66% of the sequences, followed by (GA)n in 13% and (GC)n in 10% of the sequences. The frequencies of the different tri-, tetra- and hexa-nucleotides were each present at 2 to 3% in the microsatellite sequences.

From the eleven primers tested, nine were successfully amplified, and four (Cot 1, Cot 10, Cot 12 and Cot 17) were polymorphic in a sample of 20 individuals. The microsatellite loci data are described in Table 2. One primer did not amplify, and another produced unspecific fragments even after several tests (Table 2). The number of alleles per locus was two for both Cot 10 and Cot 12 and three for both Cot 1 and Cot 17. The PIC value ranged from 0.3648 to 0.4514 between the four polymorphic loci (Table 3).

The identity of the sequence homology of the *C. flavipes* microsatellite locus developed in this study was assessed using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990). Cot 8 and Cot 12 showed 81 and 90% of identity, respectively, to the *Glyptapanteles indiensis* sequence. The sequence of the locus Cot 15 exhibited 93% identity to *C. sesamiae* and 86% to *Anastrepha fraterculus.* The locus Cot 16 presented 85% identify to *C. congregata.* The other sequences associated with the loci Cot 1, Cot 7, Cot 9, Cot 10, Cot 13 and Cot 17 were unique and did not align to other sequences.

Table 2. Description of microsatellite loci of *Cotesia flavipes*: locus ID, repeat motif, primer sequence, expected fragment size (EFS) (bp-base pair), microsatellite sequence GenBank accession number, and PCR result.

Locus ID	Repeat motif	Primer Sequence (5'-3')	EFS (bp)	GenBank No.	PCR result
Cot 1	(CT)13	F: AAAGGTCCCGATTGGAGAAT R: TTGCCTTGTCAAACCACTCA	176	KJ124563	Polymorphic
Cot 5	(TG)29	F: TGAAGGTCACAGAAAACTTACAT R: TATCGGAATCGGTCAAAAT	213	KJ124564	No fragment
Cot 7	(GT)18	F: TGACTACCCCCGCAAGTTT R: TTGAAATCTATCCCTCTGAATCG	188	KJ124565	Monomorphic
Cot 8	(AC)22 (CG)8	F: TTGTCCAAATCGGTTCAAAA R: CGAGGGTTCCTGAGAGAGTG	212	KJ124566	Monomorphic
Cot 9	(GCAT)4	F: TGTGTATCTGTGGGGAGGTG R: CGGACCGTTACATCGTTAGC	209	KJ124567	Monomorphic
Cot 10	(ATA)6 (A)10	F: GTGACACCCTCCGCTAAAAA R: AGGTTTTGTGTTCTTGCTTGC	244	KJ124568	Polymorphic
Cot 12	(GT)14	F: CTCAGTCCCAACTCGTATGC R: TGATGTCACTGGTTGTCCTTTC	196	KJ124569	Polymorphic
Cot 13	(GAC)5	F: ACTTGCACACACCCACACAC R: GCTGACGGGCAGACTATTTT	227	KJ124570	Monomorphic
Cot 15	(GT)22	F: CTTCGGAGAGTGAGGTGGAG R: CTGTTATCGGGAGCAGAGGT	186	KJ124571	Monomorphic
Cot 16	(AC)29	F: TTTGTAAAGCCCTTGCGAAT R: TGGTTTCACCCTGTTTTTCG	241	KJ124572	Unspecific fragment(s)
Cot 17	(TTTTAT)4	F: TCCGGCAAATGTCTATCGTA R: TTGCCTCATTTATGCACCAC	238	KJ124563	Polymorphic

Table 3. Characterization of microsatellite loci of *Cotesia flavipes*: locus ID, size range observed (SRO) (bp-base pair), observed number of alleles (N), heterozygosity (H), and polymorphic information content (PIC).

Locus ID	SRO (bp)	N	H	PIC
Cot 1	180 - 186	3	0.5064	0.4514
Cot 10	248 - 252	2	0.4888	0.3693
Cot 12	200 - 204	2	0.4800	0.3648
Cot 17	240 - 246	3	0.5288	0.4475

Allele frequency

The frequency of alleles at the four polymorphic loci analyzed in the seven studied RLs is described in Table 4. In females, two loci (Cot 1 and Cot 17) showed the existence of fixed alleles and/or alleles close to fixation in some RLs. In RLs B and F, the frequency of the Cot 1.1 allele was 1.0, and the same frequency was observed in RL E for the Cot 17.1 allele. In contrast, some alleles, such as Cot 17.2, showed a low frequency in many RL populations, including those from laboratories E, F and G. The Cot 1.2 allele was observed only in populations from RLs E and G; the Cot 1.3 allele was only found in populations from RLs A, C and D; and Cot 17.3 was detected only in populations from RLs B and G. Among the males, the four loci tested in 29 individuals exhibited only two alleles, and the Cot 1.2 and Cot 17.3 alleles were not found in any of the samples analyzed.

Partitioning of genetic diversity

In the 183 females tested, the expected heterozygosity ranged from 0.145 to 0.415 (mean 0.292), while the observed heterozygosity ranged from 0.053 to 0.421 (mean 0.257) (Table 5).

The average inbreeding coefficient (F_{IS}) was 0.120, which indicates homozygous excess. An analysis using the program Micro-Checker identified the possible presence of a null allele at the Cot 1 locus.

Table 4. Allele frequencies observed among the seven tested rearing laboratories for female and male wasps. N - number of wasps with scorable alleles.

	Rearing Laboratories						
	A	B	C	D	E	F	G
Females							
N	19	27	27	27	27	27	27
Cot 1.1	0.842	1.000	0.963	0.852	0.889	1.000	0.889
Cot 1.2	0	0	0	0	0.111	0	0.111
Cot 1.3	0.158	0	0.037	0.148	0	0	0
N	18	27	25	21	25	26	25
Cot 10.1	0.861	0.833	0.780	0.786	0.180	0.442	0.540
Cot 10.2	0.139	0.167	0.220	0.214	0.820	0.558	0.460
N	19	27	27	27	29	27	27
Cot 12.1	0.842	0.556	0.759	0.796	0.672	0.556	0.630
Cot 12.2	0.158	0.444	0.241	0.204	0.328	0.444	0.370
N	19	27	27	27	29	27	26
Cot 17.1	0.789	0.815	0.815	0.704	1.000	0.963	0.865
Cot 17.2	0.211	0.111	0.185	0.296	0	0.037	0.058
Cot 17.3	0	0.074	0	0	0	0	0.077
Males							
N	4	4	5	4	4	4	4
Cot 1.1	1.000	1.000	1.000	0.750	1.000	1.000	0.750
Cot 1.2	0	0	0	0	0	0	0
Cot 1.3	0	0	0	0.250	0	0	0.250
N	3	4	2	3	3	4	4
Cot 10.1	0.667	0.500	1.000	1.000	0	0	0.500
Cot 10.2	0.333	0.500	0	0	1.000	1.000	0.500
N	3	3	3	2	2	2	2
Cot 12.1	0.667	0.667	1.000	1.000	1.000	0	0.500
Cot 12.2	0.333	0.333	0	0	0	1.000	0.500
N	3	2	4	3	4	4	3
Cot 17.1	1.000	1.000	1.000	0.333	1.000	1.000	0.333
Cot 17.2	0	0	0	0.667	0	0	0.667
Cot 17.3	0	0	0	0	0	0	0

Table 5. Characterization of microsatellite loci of *Cotesia flavipes*: loci ID, repeat motif, size range (bp-base pair), observed number of alleles (N), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F_{IS}). Data are from females only.

Loci ID	Repeat motif	Size range (bp)	N	H_O	H_E	F_{IS}
Cot 1	$(CT)_{13}$	180-186	3	0.053	0.145	0.636
Cot 10	$(ATA)_6$ $(A)_{10}$	248-252	2	0.333	0.363	0.082
Cot 12	$(GT)_{14}$	200- 204	2	0.421	0.415	-0.015
Cot 17	$(TTTTAT)_4$	240- 246	3	0.221	0.246	0.100

The total average genetic diversity (H_T) in the 29 tested males was 0.354, and the average genetic diversity within an RL (H_S) was 0.245. The G_{ST} value was 0.308. Comparative data between females and males are shown in Table 6.

Table 6. Diversity measures in the population studied: H_T (total diversity), H_S (diversity within populations), D_{ST} (diversity among populations), G_{ST} (coefficient of genetic divergence between populations), G_{ST}' (G_{ST} corrected for sample size), F_{IS} (inbreeding coefficient).

Group	H_T	H_S	D_{ST}	G_{ST}	G_{ST}'	F_{IS}
Female	0.328	0.292	0.035	0.108	0.124	0.120
Male	0.354	0.245	0.109	0.308	0.342	-
Weighted average	0.330	0.289	0.040	0.122	0.140	-

Gene diversity

The genetic diversity per locus per RL in the male and female populations was analyzed. The highest average diversity was found in laboratory G, followed by D, A, B and C (B and C showed equal levels of diversity), F and finally, E (Table 7). The dendrogram based on Nei's genetic distance separated the populations into two clusters (Figure 1).

Table 7. Diversity in gene loci per population of female and male wasps. Averages of the seven sampled rearing laboratories are presented.

	Loci	Rearing Laboratories						
		A	B	C	D	E	F	G
Females N = 183	Cot 1	0.281	0	0.074	0.262	0.202	0	0.201
	Cot 10	0.248	0.282	0.352	0.345	0.302	0.503	0.508
	Cot 12	0.275	0.499	0.373	0.330	0.447	0.506	0.476
	Cot 17	0.342	0.325	0.308	0.427	0	0.073	0.246
	Average	0.287	0.277	0.277	0.341	0.238	0.271	0.358
	Standard deviation	0.040	0.207	0.138	0.068	0.188	0.272	0.157
Males N = 29	Average	0.333	0.333	0	0.292	0	0	0.708

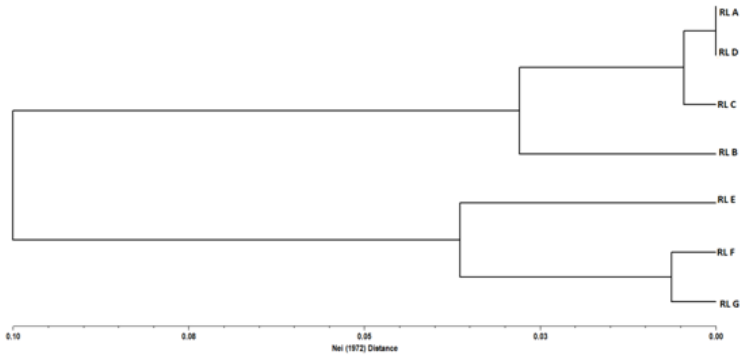

Figure 1. Dendrogram based on Nei's distance obtained from microsatellite markers screened among seven *C. flavipes* rearing laboratory (RL) populations (A to G).

The first cluster included the populations derived from RLs A, D, C and B (Figure 1), and the second cluster comprised the populations derived from RLs F, G and E. The highest identity was observed between RLs A and D (0.9954), and the lowest identity was observed between RLs A and E (0.8136) (Table 8).

Table 8. Nei's genetic distances (1972) (below the diagonal) and identity (above the diagonal) among female wasps from the seven rearing laboratories studied.

Rearing Laboratories	A	B	C	D	E	F	G
A	****	0.9601	0.9901	0.9954	0.8136	0.8930	0.9339
B	0.0407	****	0.9824	0.9621	0.8400	0.9421	0.9628
C	0.0099	0.0177	****	0.9916	0.8628	0.9390	0.9667
D	0.0047	0.0386	0.0084	****	0.8319	0.9066	0.9429
E	0.2063	0.1743	0.1476	0.1840	****	0.9682	0.9520
F	0.1132	0.0596	0.0630	0.0981	0.0323	****	0.9892
G	0.0684	0.0379	0.0339	0.0587	0.0492	0.0109	****

The clustering analysis suggested that the exchange of wasps was most intensive between RLs A and D, followed by RLs C and B. On the other branch, the greatest number of wasps may have been exchanged between RLs F and G, followed by RL E.

The Cot 1.3 allele is present only on the branch comprising RLs A, D and C, although this allele was also observed in one male from each of RLs D and G (Figure 1 and Table 4). Cot 17.2 occurred at a low frequency on the branch comprised of RLs E, F and G, in fact, this allele was not detected in RL E (Figure 1 and Table 4).

DISCUSSION

In studies on *C. flavipes* using allozymes, the frequency of monomorphic loci has been reported to be high, at 85 or 64% (Omwega and Overholt, 1996; and Kimani-Njogu et al., 1998). Both studies were performed using wasps collected in Africa, a continent where *C. flavipes* was introduced several times in different countries for biological control. In this study, we observed a frequency of monomorphic loci of 55% for microsatellite markers developed from an enriched *C. flavipes* genomic library in samples from RLs, suggesting low genetic diversity in this artificial population. Note that microsatellites tend to be more variable than are allozymes.

C. flavipes is not originally from the Americas but the wasps were introduced in Brazil in the 1970s from India and Pakistan for biological control of the sugarcane borer (Botelho, 1992). No reintroductions of *C. flavipes* have been made since the original introduction in the 1970s. This species has been mass-reared and released for decades as it is highly successful in controlling the sugarcane borer, but there are no reports of the establishment of this species in the field. Thus, the individuals sampled in this study were produced for use in the field and may not be representative of a natural or native population in the countries where *C. flavipes* originated. Thus, loci that were monomorphic in the sample analyzed may yield different results if tested in larger populations or in native or sister species samples, which are heterogeneous populations.

In the RL populations, only two alleles were observed in males, and a maximum of three alleles were observed in females. In addition to the 29 male wasps, the results distributed among the RLs also indicated a low number of alleles (Table 4). Furthermore, despite the difference in the number of females and males tested, some alleles were found only in females (Cot 1.2 and Cot 17.3), suggesting the possibility of the existence of exclusive alleles in this sex in *C. flavipes* (Table 4).

In a study examining natural populations of six species of *Cotesia* spp., the number of alleles was found to range from 1 to 25 (Kankare et al., 2004), whereas it ranged from 1 to 14 in *C. sesamiae* (Abercrombie et al., 2009). We do not know whether the low number of alleles is a natural feature of *C. flavipes*, a characteristic of the wasps produced in this country, or is due to differences in the applied methodologies between the present report and previous studies.

In this study, the observed heterozygosity in *C. flavipes* ranged from 0.053 to 0.421 (mean 0.257), consistent with the mean levels found in *C. congregata* (0.312), *C. melitaearum* (0.30) and *C. sesamiae* (0.39) (Jensen et al., 2002; Kankare et al., 2004; Abercrombie et al., 2009), respectively. Thus, in studies on *Cotesia* spp, the average observed heterozygosity is near these values. However, the genetic diversity within the *C. flavipes* RLs was lower on average (0.292 for females) than the values of 0.458, 0.6 and 0.5 reported in the literature for *C. congregata*, *C. melitaearum* and *C. sesamiae* (Jensen et al., 2002; Kankare et al., 2004; Abercrombie et al., 2009),

respectively.

According to Nei's genetic diversity partitioning, the proportion of the total genetic diversity distributed in females among the laboratory populations was 10.8% (G_{ST} = 0.108), while within the laboratory populations, it was 89.2%. In males, the diversity between the laboratory populations was 30.8% (G_{ST} = 0.308), and within the laboratory populations, it was 69.2%. To our knowledge, information on the genetic population structure of *C. flavipes*, including its natural populations, is lacking, which makes comparison of our results difficult. However, the obtained G_{ST} value for females and males, which is a measure of divergence among populations, was higher than the analogous F_{ST} value of 0.032 reported for natural populations of *C. glomerata* (Elias et al., 2010), suggesting that the artificial wasp populations from different laboratories are becoming divergent and that little or no exchange is being conducted among these laboratories. Nevertheless, the G_{ST} value was lower than that reported for natural populations of *Diaeretiella rapae* (another parasitoid wasp), which was evaluated in four countries from different continents (Baker et al., 2003).

The high frequency of fixed alleles, rare alleles and alleles at low frequencies, together with the low number of alleles observed per locus, reinforces the conclusion that the exchange of wasps among RLs is lacking or is not sufficiently efficient to maintain the genetic diversity of the artificially produced wasps in laboratories. Additionally, a founder effect (i.e., the establishment of a new population by a small number of individuals) may have contributed to the low overall diversity observed here. In fact, according to personal communications, the first introductions of *C. flavipes* in Brazil were performed using a very small number of wasp breeding pairs. According to Omwega and Overholt (1996), 1000 breeding females of *C. flavipes* are necessary to prevent the rapid decay of heterozygosity in the wasp colonies, and this number is greater than the estimated number of individuals introduced to begin the large-scale production of *C. flavipes* in Brazil.

Moreover, according to Botelho (1992), the first introduced *C. flavipes* (formerly *Apanteles flavipes*) showed the greatest adaptation to Brazilian conditions and overcame native parasitoid populations to control the sugarcane borer, even in areas without a mass release of *C. flavipes*. It is possible that over the years, the laboratories may have unintentionally selected less adaptive and less competitive wasps.

C. flavipes exhibits genetic variation in association with different hosts and geographical barriers (Muirhead et al., 2006). As such, *C. flavipes* may exhibit different behavior when confronted with *D. saccharalis* used for reproduction at the RLs versus *D. saccharalis* subjected to control in the field. In addition, the RLs can serve as geographical barriers if there is no exchange of *C. flavipes* among them, subsequently decreasing genetic diversity within RLs.

The presence of infertile diploid males in the Hymenoptera is related to a decrease in the diversity of the group. *C. flavipes* diploid males are observed more frequently in populations showing a rapid loss of heterozygosity in comparison with those displaying a slower loss of heterozygosity (Omwega and Overholt, 1996). In contrast, the presence of fertile diploid *C. glomerata* males (Elias et al., 2010) reduces the negative impact of inbreeding. In this study, no diploid males were identified, although the number of males analyzed was lower (29) than the number of females (183).

Some RLs have adopted different management strategies regarding feeding, the choice of reproductive wasps or other aspects for the reproduction of *C. flavipes* (Veiga et al., 2013). Such strategies may be related to morphological differences, the sex ratio, developmental stages

and longevity. These patterns are likely associated with the genetic differences in the wasps from each RL, and these strategies may also be linked to the maintenance of certain alleles.

In addition to the differences in wasp management among the laboratories (Veiga et al., 2013), wasps collected in the field days after their release should be incorporated into the RL populations and used in the mating of subsequent generations. This practice appears to not to be occurring efficiently. Perhaps the RL populations showing the greatest diversity are those that apply these rules more often than others, although there is no detailed information about mass production in these RLs.

The diversity studies that have been carried out on *C. flavipes* wasp RL populations in Brazil have been criticized. However, high inbreeding can cause low diversity, and the data presented here suggest the existence of low genetic diversity levels in the laboratory populations as a whole. However, among the seven evaluated RLs, the laboratory populations that showed greater diversity likely correspond to those that are exchanging and reintroducing wasps more frequently than the other laboratories. This management strategy can decrease the rate of inbreeding and increase genetic diversity, reflecting an improvement of the efficiency of the control of *D. saccharalis*. Importantly, genetic drift and selection are more intensified in small populations and can lead to the fixation of both favorable and unfavorable alleles.

São Paulo State, in the southeast region, is the major sugarcane-producing state in Brazil. The agro-industry, including the RLs of *C. flavipes* production, is also highly developed in this state. The *C. flavipes* RLs located in São Paulo State export wasps for sugar fields in other Brazilian federal states and regions. Although this represents a broad area of *C. flavipes* control activity, it is unknown whether wasp parasitism efficiency varies among Brazilian states or regions.

One suggested strategy to improve the efficiency of biological control in our country is the import of new *C. flavipes* mating pairs to increase the diversity and parasitism capacity of the wasp populations. We can use the information on the existing population structure to consider the introduction of new germplasm and to determine which countries are best to import from and the minimum number of wasps to be imported.

To our knowledge, this study is the first to describe the genetic structure of artificial populations of *C. flavipes* derived from RLs. Our initial findings confirmed the hypothesis of low overall genetic diversity, with most of the genetic diversity (70 to 90%) existing within the RLs.

Consideration of the allozyme and SSR information gives rise to the hypothesis that *C. flavipes* is a species that naturally exhibits low levels of diversity. However, *C. flavipes* reproduces naturally, indicating that it is adaptable despite its low diversity. This fact requires that extra attention be paid to isolated populations of *C. flavipes*, such as the populations in RLs.

In general, *C. flavipes* is described as a low-diversity species, presenting many fixed alleles when studied using allozymes (Omwega and Overholt, 1996; Kimani-Njogu et al., 1998). This low diversity may affect the capacity of the wasps to parasitize the sugarcane borer, as this ability has diminished since the introduction of *C. flavipes* into Brazil. Thus, the findings and discussion presented in this report suggest the reintroduction of new mating pairs of *C. flavipes* from countries where these insects are native (origin center) as a strategy to increase diversity and improve biological control. In addition, attention must be given by RLs to compliance with management strategies, including the exchange of wasp matrices and reintroduction of wasps collected in the field to maintain adequate genetic diversity levels among the wasps that are produced.

The importance of the present study lies in the economic and environmental impact

of borer control by sugarcane farmers. This form of biological control leads to improvements with a low cost and low environmental impact, guaranteeing the sustainability of the sugar and ethanol industry in the country.

Conflicts of interest

The authors declare no conflict of interest.

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