GENETIC VARIABILITY AND POPULATION STRUCTURE OF THE COFFEE BERRY BORER *Hypothenemus hampei* (Ferrari) IN BRAZIL, INFERRED BY AFLP MARKERS

Zulma Nancy Gil P.*, Pablo Benavides Machado*, Eric Hernández H.**,
Kátia Nogueira Pestana***, Daniela Fontes Gomide*, Eraldo Lima*

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The genetic variability of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) was evaluated in populations from Brazil using amplified fragment length polymorphism (AFLP). DNA samples from 15 coffee sites in Brazil were evaluated. Low genetic variability was observed, but enough polymorphisms to determine the population structure of *H. hampei* in Brazil. The results of this investigation allowed to comprise the entire *H. hampei* population from Brazil into three genetic branches, with a marginal value of directional gene flow of 6% between groups one and two and the absence of migrants from groups two and three. Of the total genetic variation of the coffee berry borer in Brazil, 96% was within populations. The total population differentiation value (F_{ST}= 0.1679) was high; however, heterogeneity was observed in the F_{ST} values when performing pairwise comparisons of populations. The average percentage of polymorphic loci was 16.89% among all of the samples, and the highest values were observed in the Uberlândia region (36.36%), which also harbored the highest number of unique AFLP fingerprints (seven). The results of this research allow to conclude that, contrary to old reports of the introduction of coffee berry borer in Brazil (Campinas), other coffee areas such as Uberlândia and Santa Cruz de Cabrália may have been the point of entrance of this insect pest into the country. The insect individuals from this region could serve in the development of molecular markers for further ecology and biology studies of the coffee berry borer in Brazil.

Keywords: Bayesian analysis, Coffee pest, Genetic structure, Molecular markers.

VARIABILIDAD GENÉTICA Y ESTRUCTURA DE POBLACIONES DE LA BROCA DEL CAFÉ *Hypothenemus hampei* (Ferrari) EN BRASIL, USANDO MARCADORES AFLP

La variabilidad genética de la broca del café *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) se evaluó en las poblaciones de Brasil mediante la técnica AFLP (Polimorfismos en la Longitud de Fragmentos Amplificados). Se evaluaron muestras de ADN de la broca del café procedentes de 15 sitios cafeteros en Brasil. Se observó baja variabilidad genética, pero suficientes polimorfismos para determinar la estructura de las poblaciones de la broca del café en Brasil. Los resultados de esta investigación permiten comprender toda la población *H. hampei* de Brasil en tres ramas genéticas, con un valor marginal del flujo de genes direccional de 6% entre los grupos uno y dos y la ausencia de emigrantes desde los grupos dos y tres. De la variación genética total de la broca del café en Brasil, el 96% estuvo dentro de las poblaciones. El valor total de diferenciación de la población (FST = 0.1679) fue alta; sin embargo, se observó heterogeneidad en los valores *FST* al realizar comparaciones por pares de poblaciones. El porcentaje medio de loci polimórficos fue 16,89% entre todas las muestras, y se observaron los valores más altos en la región de Uberlândia (36,36%), que también albergabó el mayor número de huellas de AFLP únicos (siete). Los resultados de esta investigación permiten concluir que, conrtario a los informes anteriores de la introducción de la broca del café en Brasil (Campinas), otras zonas cafeteras como Uberlândia y Santa Cruz de Cabrália pueden haber sido el punto de entrada de esta plaga en el país. Los individuos de insectos de estas regiones podrían servir en el desarrollo de marcadores moleculares para estudios de ecología y biología de la broca del café en Brasil.

Palabras clave: Análisis Bayesiano, plaga del café, estructura genética, marcadores moleculares

*Investigador Científico II e Investigador Científico III, respectivamente, Disciplina de Entomología, Centro Nacional de Investigaciones de Café, Cenicafé. Manizales, Caldas,Colombia.

**Candidato a Doctorado, Universidad Estatal Paulista (Brasil).

***Candidato a Post- Doctorado, Estudiante de pregrado y Profesor Ph.D., respectivamente. Universidad Federal de Viçosa, Minas Gerais, Brasil, CEP 36571-000.

The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is one of the most significant insect pests in coffee crops worldwide Baker *et al.* (3); Murphy and Moore (22). *H. hampei* causes severe losses in coffee production and quality by infesting developing berries, which provide a suitable habitat for the coffee berry borer to reproduce, rear offspring and seek protection from predators and adverse weather conditions Le Pelley (20).

Similarly to coffee itself, *H. hampei* originated in Africa and has extended its range as cultivation has expanded; the coffee berry borer is now prevalent in more than 59 countries that produce this commodity, with the exceptions of Nepal and Papua New Guinea Vega *et al.* (32). The most recent introduction of *H. hampei* was documented in 2010 on several farms in the Kona region of Hawaii (García) (16).

The mating system of the coffee berry borer ensure high inbreeding such as to consider local mate competition (LMC); briefly, after colonization by only a founder female, the progeny is shaped by female-biased sex brood in which male siblings compete for mating opportunities within local groups. This reproductive system is aggravated by a combination between full sib-mating and pseudo-arrhenotoky, strongly suggesting that *H. hampei* reproduces in strict matrilineal lines. Thus, males do not fly outside of their ecological patch, and mating occurs as pre-dispersal events, which promotes homozygosity and causes low genotypic variability Borsa and Gingerich (8). This characteristic is accentuated by the potential mechanism of "functional haplodiploidy" in which both males and females are diploid, but later fail to express and transmit paternal chromosomes (Brun *et al.*) (9).

The genetics of *H. hampei* has not been studied extensively. Studies on the variability and genetic structure of this species were conducted by Borsa and Gingerich (8), who used allozymes to find variations in the coffee berry borer populations of New Caledonia and the Ivory Coast at two loci, Mdh-2 and Mpi, in 88 and 21 insects of seven and four populations, respectively. These authors reported a high level of inbreeding (F_{IS} = 0.298) and an average genetic heterozygosity of H = 0.080. These results were confirmed in Africa by Gauthier and Rasplus (17), who isolated seven polymorphic microsatellites from *H. hampe*i and evaluated them in coffee berry borer populations collected in Nairobi, Kenya and Jima, Ethiopia. A low genetic diversity per locus was observed, with between two and five alleles in 39 individual samples, a deficit of heterozygosity in the population averaging HO = 0.10/H_E = 0.50 and a high inbreeding coefficient (F_{IS} = 0.70-1.00).

Using the amplified fragment length polymorphism (AFLP) technique, Benavides *et al*. (4) studied the diversity and biogeography of *H. hampei* in coffee berry borer samples from 17 countries of Africa, Asia and America, and found a low genetic variability of this insect (10% average polymorphism per sample), confirming the findings of Borsa and Gingerich (8), Gingerich *et al*. (19), Andreev *et al*. (1) and Damon (12). In the same study, Benavides *et al*. (4) reported the introduction of three genetic lineages onto the American continent, either through independent introductions of several lineages or a single introduction of multiple lineages, and at least two lineages were first introduced to Brazil and subsequently dispersed throughout the Americas.

The results of Benavides *et al*. (4) are consistent with the colonization history of the pest in Brazil Bergamin (5) and support

the hypothesis that Brazil is the origin of this insect in the Americas. This model is substantiated by Gauthier (18), who analyzed microsatellite markers in 683 individuals collected from 37 locations in 18 countries and identified four genetic populations, K1 to K4. The samples from Brazil are the basis of the K3 (American) group, comprising Central American (except Jamaica) and South American countries (Colombia and Brazil). In group K3, two subpopulations were identified, in which all of the individuals in Brazil were assigned to subgroup K3a, and those belonging to other countries were assigned to subgroup K3b.

Previous reports support the conclusion that the introduction of *H. hampei* from Africa to the Americas occurred in Brazil and Jamaica and that Brazil is the origin of the American coffee berry borer (Benavides *et al*. (4), although coffee berry borer AFLP DNA fingerprints reported elsewhere in the Americas have not been found in Brazil, which may be due to the low number of samples tested. Therefore, including a larger number of samples from this country in further studies of the genetic diversity of the coffee berry borer will be useful in the search for polymorphisms for the development of markers as tools for molecular biology, ecology (studies of dispersion) and genetics research on this insect.

A range of techniques and markers has been developed to estimate genetic diversity, but there are no universal standards, and the choice of the technique depends on the type of study to be performed (Ulrich and Wolfenbarger) (31). Because the coffee berry borer is highly inbred and has very low genetic variability, techniques such as the analysis of AFLP markers, are adequate. This allows the evaluation or determination of the percentages of polymorphisms of dominant loci throughout the genome and is useful for establishing the relationships between genomic DNA samples and for finding genetic markers Vos *et al.* (34). When using dominant markers, such as AFLP, it is recommended to use several approaches to estimate population differentiation, thus providing greater confidence in data interpretation Bonin *et al*. (7).

The objective of this study was to determine the genetic variability and population structure of the coffee berry borer in Brazil. It is hypothesized that the lineages arrived in Brazil and then dispersed and colonized areas throughout the country and, therefore, should have the greatest genetic variability.

MATERIALS AND METHODS

Insect samples. *H. hampei* samples were obtained from 15 coffee regions in six states in Brazil (Figure 1). At each region, we collected 50 coffee beans infested by the coffee berry borer that only had a single hole. A fertile female was extracted from each berry to start a breeding stock on an artificial diet as described by Portilla (26), in order to obtain enough individuals for the extraction of genomic DNA.

METHODS

Isolation and quantification of genomic DNA. For this procedure, ten samples of *H. hampei* were taken from each location (n = 150), each sample was constituted by a pool of 30 individuals. Total genomic DNA was extracted from each sample using the DNeasy tissue kit (QIAGEN, Valencia, CA). The DNA concentration (ng nucleic acid/µl of solution) and quality (the 260/280 nm absorbance ratio of the DNA sample) were measured using a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, Delaware USA).

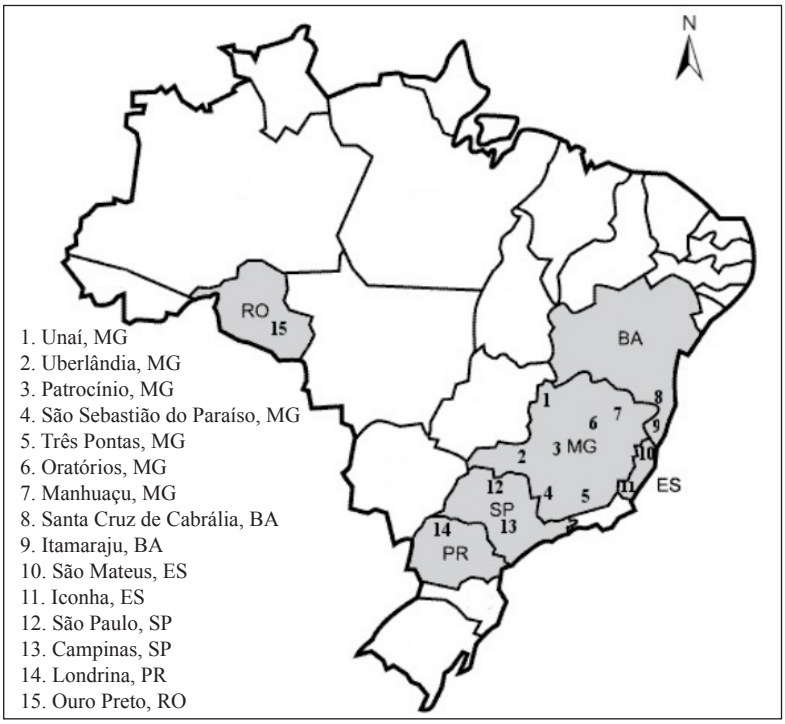

Figure 1. Map showing the locations of the 15 studied populations of *Hypothenemus hampei in* Brazil (Bahia, BA; Minas Gerais, MG; Espirito Santo, ES; São Paulo, SP; Paraná, PR; Rondônia, RO).

Methods for genetic analysis. A protocol for the AFLP technique Vos *et al.* (34) was used to assess the variability and genetic structure of the populations of *H. hampei.* High-quality DNA (50 ng/8 µl) from each sample was digested with a pair of restriction enzymes *(Eco*RI/*Mse*I) and ligated to double-stranded *Eco*RI/*Mse*I adaptors. The ligation was pre-amplified with nonselective primers, and selective amplification was subsequently performed using four primer combinations (E-CGC/M-ATG, E-CAG/M-AAC, E-CCG/M-AGT and E-CTC/M-AGT). The products were separated on 6% polyacrylamide gels in Owl vertical electrophoresis chambers at 90 W for 2.5 h. The gels were stained with a silver nitrate solution with formaldehyde, scanned and analyzed.

Data analysis. High levels of inbreeding have been observed in the coffee berry borer Borsa & Gingerich, (8). Therefore, the null allele frequency (the number of individuals without bands) must be used to estimate allele frequencies. Allele frequencies were estimated using the Bayesian method developed by Zhivotovsky (36) and implemented in AFLP-SURV 1.0 Vekemans *et al.* (33), which assumes a non-uniform distribution of allele frequencies. A value of F_{IS} = 1 was set, with 500 permutations of the F_{ST} to estimate the significance among populations. Using Genalex 6 (Peakall and Smouse) (23), an analysis of molecular variance (AMOVA) was performed to determine the variation between and within populations. A dissimilarity matrix was estimated using simple-matching coefficient (Sokal and Michener) (28) after loaded into MEGA software Tamura *et al.* (29) for Neighbor-joining tree construction; additionally, agglomerative

hierarchical clustering was implemented in order to corroborate Neighbor-joining clustering process.

STRUCTURE 2.2 was used to determine the probability that each sample collected belonged to a different population Pritchard *et al.* (25), using a model of recessive alleles for dominant markers and assuming the presence of an ancestral mix among populations and correlated alleles Falush *et al.* (15), i.e., the allele frequencies of the different populations are likely to be similar due to migration or a degree of shared ancestry, indicating that each individual has a certain fraction of each of the *K* assumed populations in its genome. STRUCTURE assumes that within a population, loci are in Hardy-Weinberg equilibrium (HWE) and in linkage equilibrium and assigns individuals to separate populations to eliminate violations of these assumptions.

With the above analysis, estimates of population and individual mix were obtained, and stratification analyses of the populations were performed using the Bayesian algorithm implemented in STRUCTURE. Simulations were performed with different *K* ancestral groups *(K*= 1 to *K* = 15 (total number of populations analyzed)). These simulations were performed with the specific parameters of STRUCTURE: BL = 10,000 and CL = 10,000, and 10 algorithm runs were performed for each *K*.

The Δ*K* statistical parameter Evanno *et al.* (14) was used to define the most probable number of populations *(K)* in the data. The modal value of the distribution of Δ*K* was obtained with STRUCTURE Harvester v0.6.1 Earl, (13). The approximate values of gene flow among the *(K)* clusters were estimated with BAPS (Tang *et al.*) (30).

RESULTS

Population structure and total polymorphisms. The total number of fragments or loci evaluated was 88, with an average of 54.1 per individual, and 36 of these fragments segregated (the fragments were present in certain individuals and absent in others), which accounted for 40.9% of all bands. The percentage of polymorphic loci averaged 16.89% among populations, ranging between 0 % and 36.36% (Table 1); the populations of São Mateus, Oratórios and Três Pontas showed no polymorphisms.

Samples from Patrocínio and Uberlândia were the only ones that presented unique or specific alleles, with one and two bands, respectively. Fewer than 25% of the populations shared rare alleles, specifically Patrocínio, Uberlândia, Santa Cruz de Cabrália, Manhuaçu and Campinas.

Using AMOVA, we found that the variation was within populations, with a 96% contribution to the total genetic variation observed. A total of 37 AFLP DNA fingerprints (F) were observed in all of the populations; the most frequent was band pattern 2 (F-2), with frequencies above 0.5 in most populations, except for Santa Cruz de Cabrália and Uberlândia , where a frequency of 0.3 was observed. F-2 is the only DNA pattern present in São Mateus, Oratórios and Três Pontas (Figure 2). In addition to the pattern F-2, the population of Londrina harbored F-4 as a specific or unique DNA fingerprint highly related with F-2 and his cluster (distance 0,011) (Figure 3). Similarly, F-23 in Patrocínio population (frequency = 0.2) was related directly to F-2 (Figure 3). The population in Uberlândia (2) was more polymorphic and exhibited a higher number of specific fingerprints (seven): F-10, F-16, F-22, F-25, F-27, F-33 and F-35.

Table 1. Genetic diversity (percentage of polymorphic loci) of coffee berry borer populations in Brazil.

Identification map	Population	n	No. Polymnrphic bands	% polymorphism
10	São Mateus, ES	10	0	0
6	Oratórios, MG	10	0	0
5	Três Pontas, MG	10	0	0
9	Itamarajú, BA	9	1	1,14
14	Londrina, PR	10	1	1,14
4	São Sebastião do Paraíso, MG	10	7	7,95
12	São Paulo, SP	10	18	20,45
11	Iconha, ES	10	19	21,59
15	Ouro Preto, RO	10	19	21,59
3	Patrocínio, MG	10	21	23,86
13	Campinas, SP	10	23	26,14
1	Unaí, MG	10	23	26,14
7	Manhuaçu, MG	10	29	32,95
8	Santa Cruz de Cabrália, BA	9	30	34,09
2	Uberlândia, MG	10	32	36,36
	Average			16.89

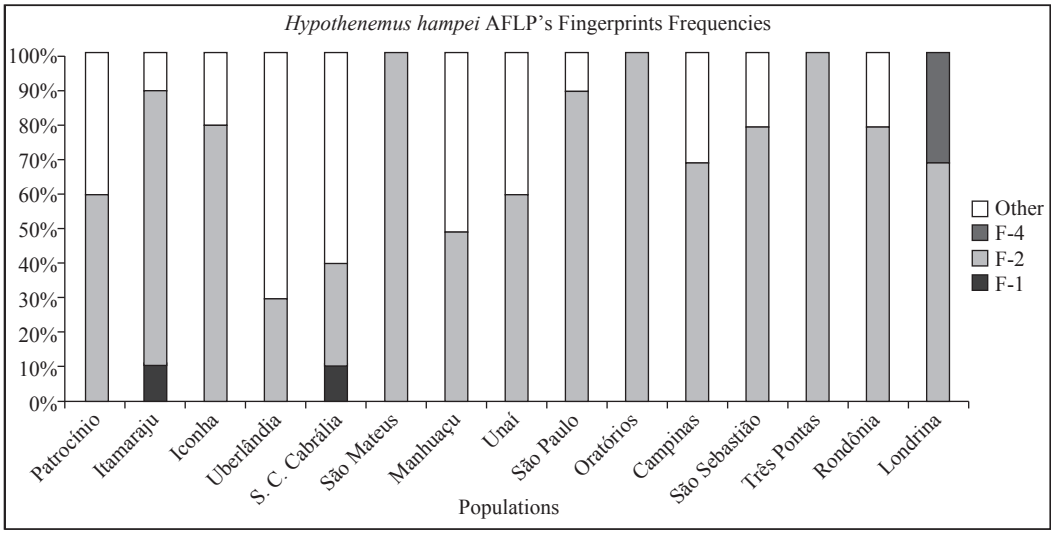

Figure 2. DNA fingerprints frequencies in coffee berry borer populations in Brazil determined by Haplotype Analysis 1.05.

Genetic differentiation. In the analyses of paired populations, the total population genetic differentiation was high (F_{ST} = 0.167) (Wright) (35). However, when performing a pairwise comparison of populations, heterogeneity was observed in F_{ST} values (Figure 4).

The greatest genetic differences were found among monomorphic and Uberlândia populations (F_{ST} = 0.22) followed by Santa Cruz de Cabrália (F_{ST} = 0.17); likewise, genetic differentiation was observed between Itamaraju and Uberlândia (F_{ST} = 0.17) and

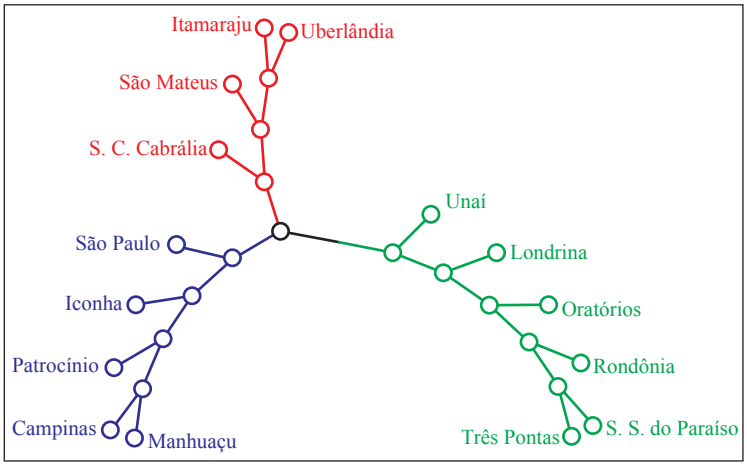

Figure 3. *H. hampei* AFLP fingerprints (A) and neighbor-joining analysis (B). The distribution of fingerprints among the samples as listed in table 1. Fingerprints and localities are color coded according to groups (B). Three major groups were identified. Bootstrap values is 95% or higher for all interior branches.

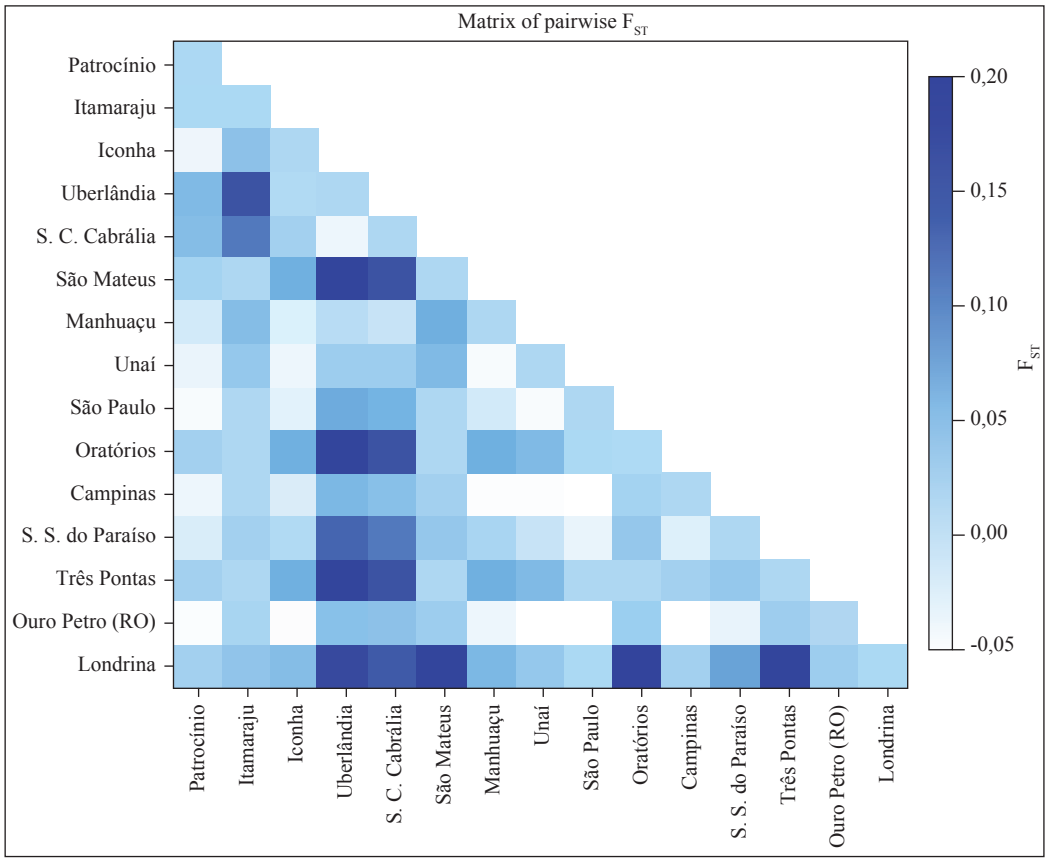

Figure 4. Indicator matrix of F_{ST} values for population pairs according to the analysis of genetic distances between fingerprints. Color intensity indicates F_{ST} values between population pairs. S.C. Cabrália, Santa Cruz de Cabrália; S.S. do Paraíso, São Sebastião do Paraíso.

between Londrina and the populations of Uberlândia (0.20), Santa Cruz de Cabrália (0.16), São Mateus (0.22), Oratórios (0.22) and Três Pontas (0.22). The population of Santa Cruz de Cabrália exhibited moderate differentiation from the population of Itamaraju (0.11).

Bayesian analysis of the structure of coffee berry borer populations in Brazil. Model simulations using the Bayesian method implemented in STRUCTURE showed a population structure of $K = 2$ and $K = 3$. However, the modal value of the distribution of ΔK indicated that the data clustered into three groups (i.e., $K = 3$) (Figure 5), i.e., that the populations of *H. hampei* in Brazil form three genetic groups.

The populations of Santa Cruz de Cabrália and, to a lesser extent, the Itamaraju samples exhibited genetic mixing of the three groups. Londrina, Patrocínio, Iconha, Uberlândia, Manhuaçu, Unaí, São Paulo, Campinas, São Sebastião do Paraíso and Ouro Preto (RO) exhibited a mixture of two groups, and the populations of São Mateus, Oratórios and Três Pontas were unmixed, suggesting that a founder effect of the F-2 band pattern and geographic isolation maintains the homogeneity of the coffee berry borer in these regions (Figure 6).

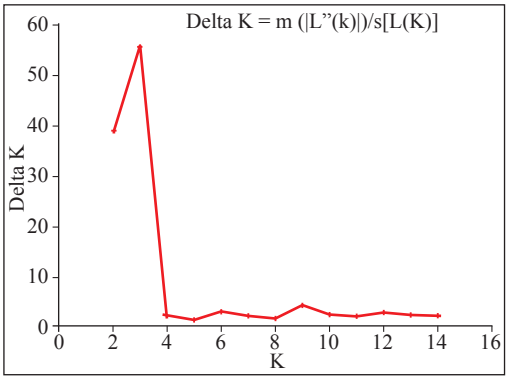

Figure 5. Modal value of the ΔK distribution (Evanno *et al.*) (14). The peak in the ΔK value confirms the grouping of data into three different populations.

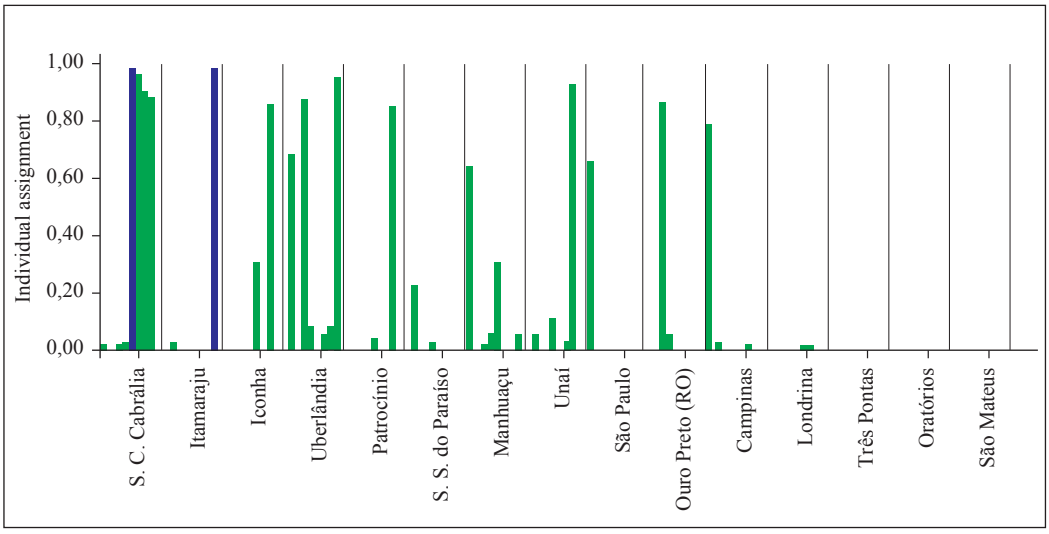

Figure 6. Stratification of Brazilian populations of *H. hampei* for $K = 3$. Each sample is represented by a vertical line divided into colors according to the proportion of a sample genome derived from an ancestral population.

With the assignment of samples to each of the groups identified as ancestral populations, a directional gene flow from the K1 group to the K2 group of approximately 6% and an absence of migrants from the K2 and K3 groups were observed (Figure 7).

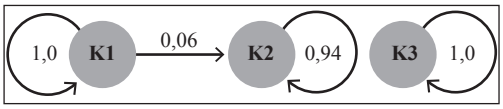

Figure 7. Gene flow between the Brazilian population groups that were evaluated *(K* = 3).

DISCUSSION

This study provides the first indication of the variability and genetic structure of populations of the coffee berry borer in Brazil and complements historical records (Bergamin) (5, 6) on the appearance of the pest in this country.

The four combinations of primers revealed low genetic variability but enough polymorphisms to determine the genetic structure of *H. hampei* populations in Brazil. The low genetic diversity of the coffee berry borer in this country confirms the results of Borsa and Gingerich (8) (allozymes), Gingerich *et al*. (19), Andreev *et al*. (1), Damon (12), Gauthier *et al*. (18) (microsatellites) and Benavides *et al*. (4) (AFLP) who found that the pest has a high degree of homozygosity and low genetic variability. However, these results diverge from those of Gauthier (18), who found considerable variation between groups of *H. hampei* when using microsatellite markers and other approaches. Deviation from HWE was observed at all loci, with a significant heterozygote deficiency and a very high F_{IS} ($\geq$ 0.74).

Benavides *et al*. (4) found that the genetic diversity of *H. hampei* is greater in Brazil than in other Latin American countries. This observation supports the hypothesis that the coffee berry borer was introduced to the American continent through Brazil as a result of seed importation, becoming the origin of the American coffee berry borer. The above results are supported by Gauthier (18), who used Bayesian analysis and worldwide phylogenetic reconstructions and found five morphocryptic evolutionary units within *H. hampei* that grouped into four genetic populations: K1 (Ethiopia), K2 (Kenya), K3 (the American group with the exception of Jamaica and comprising Brazil, Colombia, the Dominican Republic, El Salvador, Nicaragua, Mexico, Guatemala and Costa Rica) and K4 (Jamaica, Togo, Cameroon, the Ivory Coast, India, New Caledonia and Java). In the American group, samples from Brazil formed the basis of the low genetic variability found in populations of this group, explaining the lack of heterozygosity and the high degree of consanguinity of this insect throughout the Americas. Additionally, the genetic variability of the Brazilian coffee berry borer is not dissimilar to that of Colombian populations, i.e., the introduction of a few founder lineages in Brazil and the subsequent dispersion of these lineages throughout the Americas explains the very low genetic variability on this continent Benavides *et al.* (4); Gauthier (18).

Given the frequency of F-2 in all of the populations evaluated, we can infer that this variant was the first DNA fingerprint initially introduced into Brazil in 1913 and subsequently created new patterns or was introduced together with other less frequent DNA patterns; the dissemination of this pattern is consistent with the history of colonization of the pest. According to Bergamin (5), after the first official record in 1924, the coffee berry borer colonized all of the bean-producing areas within a few years, and 33 years later,

it was documented throughout the country, which explains the widespread presence of this fingerprint in all of the samples.

The large number of patterns found in the populations of Uberlândia and Santa Cruz de Cabrália suggests that because of their relatively high genetic polymorphisms, these populations may be the oldest, or were subjected to higher rates of migration; however, according to records of the presence of the coffee berry borer in Brazil, the oldest populations should correspond to the locations of Campinas and São Paulo, where the infestation originated (Bergamin) (6). This finding is giving more arguments to consider other areas inside Brazil as the origin of the colonization of the coffee berry borer. The Uberlândia population was the most polymorphic (36.36%), with the highest number of unique band patterns (seven). These band patterns could be used to develop molecular markers to be used in ecological and biological studies of the coffee berry borer in Brazil.

These results differ from the historical record of the presence and colonization of the coffee berry borer in Brazil (Bergamin) (6). This difference may be due to introductions into sites outside of Campinas before 1913 that were not recorded because of factors such as unfamiliarity with the taxonomy of this species, which created confusion in identification and prevented the timely recognition of its introduction, or because a lack of plant health monitoring made it impossible to establish the correct date of arrival of the coffee berry borer in Brazil. Prior to 1922, Brazil unofficially imported whatever was needed, and seeds for planting different crops were brought in directly by the interested parties (Piza Junior) (24).

Bergamin (5) argued that both seed introductions by officers of the Board of Agriculture before 1913 and direct imports by producers were undoubtedly important factors in the introduction of the pest, leaving open the possibility of introductions before this date at sites other than Campinas. Bergamin (5) considered this region to be the site of introduction because in 1913, the State Agricultural Institute (Campinas) received coffee seeds from Java and Congo infested with the coffee berry borer; however, coffee planters in São Paulo imported seeds prior to 1913 from West Java, an island infested by the pest in 1909. Additionally, between 1901 and 1902, Brazil imported seeds of 88 varieties of coffee from around the world. It is not possible to confirm that these samples were carriers of the coffee berry borer, but it is also not possible to state that they were free of the pest; by this date, the coffee berry borer was already present in Congo (Africa) (Bergamin) (5).

Bayesian cluster analysis showed that *H. hampei* populations in Brazil formed three homogeneous genetic groups (i.e., $K = 3$). First, this finding appears to be consistent with Bergamin (5) records regarding the two possible introductions of this insect in seeds from Indonesia (Java) and Congo or both regions simultaneously and an additional group mediated by dispersal and gene flow between populations of the insect. This result also agrees with Benavides *et al*. (4), who found a large number of lineages in samples from two crops from Brazil, showing possible gene flow events between populations in these localities. Second, this introduction may have been a single introduction of multiple lineages, with the groups found today being defined by subsequent gene flow events between populations Benavides *et al*. (4) and a founder effect with genetic drift.

The dispersion of the pest is another important factor in population structure; *H. hampei* is dispersed in the field by human activities, wind and, on a larger scale, domestic and international trade Baker (2); Sánchez (27); Bustillo *et al.* (10); Castro *et al.* (11); Moreno *et al.* (21). In Brazil, due to geographical barriers and distances between coffee-producing areas, anthropogenic dispersal may be the primary cause; when the coffee berry borer arrived in Brazil, the coffee was harvested manually, and collectors moved among different production regions, becoming active vehicles for the movement of the insect between different locations. This factor could favor gene flow or migration among populations of the coffee berry borer and its effect depends on differences in the allele frequencies of the original populations.

The results of this research allow to conclude that, contrary to old reports of the introduction of coffee berry borer in Brazil (Campinas), other coffee areas such as Uberlândia and Santa Cruz de Cabrália may have been the point of entrance of this insect pest into the country, or were subjected to higher rates of migration. The insect individuals from this region could serve in the development of molecular markers for further ecology and biology studies of the coffee berry borer in Brazil.

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