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Genetic structure of populations of *Pissodes castaneus* (De Geer) (Coleoptera, Curculionidae) using amplified fragment length polymorphism

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ABSTRACT. Genetic structure of populations of *Pissodes castaneus* (De Geer) (Coleoptera, Curculionidae) using amplified fragment length polymorphism**.** The objective of this study was to determine the genetic structure of populations of *Pissodes castaneus* from different areas and on different species of *Pinus* using the PCR-AFLP technique. Twenty samples were analyzed, representing 19 populations from Brazil and one from Florence, Italy, which is the region of origin of *P. castaneus*. The four combinations of primers generated a total of 367 fragments of DNA, and 100% of polymorphic loci, indicating high degree of molecular polymorphism. The dendrogram did not reveal trends for grouping the populations in relation to origin. The low genetic similarity (0.11 between the most distant groups) and genetic distances of 0.13 and 0.44 for 10 out of the 20 samples may indicate several founding events or multiple introductions of heterogeneous strains into Brazil. The allelic fixation index (Fst) was 0.3851, considered high, and the number of migrants (Nm) was 0.3991, indicating low gene flow among populations. The highest genetic distances were between the population from Irani, SC and Cambará do Sul, RS and Bituruna, PR, indicating an independent founding event or a particular allelic fixation in the former location. The high genetic diversity among populations points out that the populations are genetically heterogeneous with a diverse gene pool in the surveyed areas, what makes them to respond differently to control measures.

KEYWORDS. Banded pine weevil; Insecta; molecular marker; population genetics.

The banded pine weevil, *Pissodes castaneus* (De Geer, 1775) (Coleoptera, Curculionidae), is an important pest of *Pinus* (Pinaceae) in Europe. The female lays the eggs under the tree bark and the larvae feed in phloem of trunk and branches, interrupting the sap circulation and eventually causing death of young trees (Viedma 1961; Alauzet 1977; Ferreira & Ferreira 1989; Iede *et al.* 2007). Currently, this species is also found in Siberia, northern Africa, Madeira, Canary Island (Bichão *et al.* 2003; Grez *et al.* 2000), Turkey (Tozlu 2001). In South America, *P. castaneus* has been recorded in Uruguay and Argentina (Abgrall *et al.* 2000) and more recently, in 2001, it was detected on *Pinus taeda* L. in the county of São José dos Ausentes, in the southernmost Brazilian state, Rio Grande do Sul. Later it was detected in the states of Santa Catarina and Paraná (Iede *et al.* 2004). It seems that this weevil has moved north, dispersing about 600 km in eight years, or even new introductions occurred in new areas.

An important aspect of pest management is the knowledge of biological, behavioral and genetic population structure. In the case of invading species, population structure is largely dependent on the genetic characteristics and effective size of the founding population. Also, dispersal patterns are essential for monitoring population development and determining strategies for pest management. Genetic analysis of populations of *Pissodes strobi* (Peck, 1817), a native species from North America, using RAPD (Random Amplified Polymorphic DNA) indicated three groups of genetically distinct populations in Canada, where this species is an important pest of *Pinus* (Lewis *et al.* 2000, 2001). Later, Laffin *et al.* (2004) sequenced the mitochondrial COI gene of 130 individuals from 11 locations in Canada. These authors demonstrated the existence of four genetically distinct populations and concluded that the genetic variation is dependent on host and geographical origin of the populations studied. The description of the genetic patterns by sequencing of the mitochondrial COI region for the genus *Pissodes* by those authors evidenced high variation in its genome, which in turn may have significant implications for its management.

The objective of this study was to analyze the genetic structure of populations of *P. castaneus* collected in different growing areas of *Pinus* using the AFLP technique for further control measure decisions. The AFLP (Amplified Fragment Length Polymorphism) technique detects only one allele per locus (presence or absence of the dominant gene) and may have some repeatability drawbacks. Even though, it may be used in studies of genetic diversity and population structure of insects (Vos *et al.* 1995; Yan *et al.* 1999; Takami *et al.* 2004).

MATERIAL AND METHODS

Adults of *P. castaneus* were collected by both using log-traps and directly from infested trees. Three insect samples collected in Florence, Italy, were included to represent the population from the place of origin of this weevil species. In Brazil, the samples were collected from commercial producing areas and from spontaneous growing trees of *Pinus*, in 2007 and 2008 (Table I). Infested branches were taken to the laboratory and kept until adult emergence or dissected to remove the insects. These were placed in cryogenic tubes containing 1.5 mL absolute ethanol and stored at -18°C.

The head and thorax of a single specimen from each population of *P. castaneus* were removed and used for extracting the genomic DNA. The body parts were dried at room temperature and crushed manually with autoclaved polypropylene pestle in a microcentrifuge tube (1.5 mL) containing 180 µL of extraction buffer ATL, following the protocol of Qiagen® kit for animal tissue (QIAGEN DNeasy® Blood & Tissue Handbook – Spin-Column Protocol). Genomic DNA was stored at -20°C before the tests.

For the AFLP reactions, each sample at concentration of 1 µg of genomic DNA was digested with the restriction enzymes *Eco*R1 and *Mse*I and ligation with oligonucleotide adapters. Next step was the pre-selective amplification using pre-selective primers and Core Mix (kit) in Eppendorf® thermocycler. Subsequently, the pre-amplified DNA was diluted in 20 µL of 0.1 x TE and amplified using primers with three selective bases: FAM (ACT + CAT), FAM (ACT + CTG), NED (AGC + CTA) and JOE (AGG +CTT).

For the selective amplification, we mixed 1.5 ìL of the diluted pre-selective reaction + 7.5 µL of the Core Mix + 0.7 µL of the restriction enzymes *Eco*RI and *Mse*I in a thermocycler for denaturing, annealing and extension. After amplification, the fragments were separated by electrophoresis in a sequencer ABI Prism® 377. Each sample consisted of 0.5 µL of the amplified material and 0.5 mL of the ladder ROX-*Rox Size Standard* 1000, 0.25 µL of blue dextran/25 mM, and 1.25 µL of deonized formamide (Hi-Di™). It was denatured at 94°C for 2 min and applied on polyacrylamide gel in sequencer for five hours running.

The Genescan® Analysis software (Applied Biosystems) was used to determine PCR fragments sizes by comparison with internal size standards *Rox*. The detection and scoring of markers was performed using Genotyper (Applied Biosystems).

We established a standard for the lengths of the fragments, in which all fragments within a 0.5-bp range were considered the same size. The samples were genotyped for the presence (1) and absence (0) of each fragment, which was considered as a single locus. The percentage of polymorphism generated by each combination was determined based on the presence/absence matrix.

The analysis of genetic similarity was made with the NTSYS v.2 (Rohlf 1994) using the similarity coefficient of DICE. Cluster analysis was performed using UPGMA (Sneath & Sokal 1973). The degree of reliability that the dendrogram represents the similarity matrix was measured by the cophenetic correlation coefficient, and the significance of this correlation matrix was tested by Mantel's test (Mantel 1967). The indices of genetic identity and genetic distance were estimated for each locality and cluster analysis was performed using the UPGMA algorithm using the TFPGA program v.2 (Miller 2007).

Population parameters such as the expected heterozygosity (He), proportion of polymorphic loci (P), allelic fixation index (Fst) and genetic distance of Nei (Nei 1972) were calculated using the TFPGA Computer Application. The proportion of polymorphic loci was obtained through the formula: $P = x/m$, where x is the number of polymorphic loci and m is the total number of polymorphic loci of the population. Gene flow was measured with the parameter Nm (number of migrants), using the formula: $Nm = [0.25(1 – Fst)/Fst]$ (Slatkin 1987).

RESULTS AND DISCUSSION

The four combinations of primers generated a total of 367 polymorphic DNA fragments, being 100% polymorphic (Table II). These results show the efficiency of AFLP mark-

Table I. Origin of samples of *Pissodes castaneus* populations studied with AFLP markers.

Localities	Codes	Population number	Number of individuals	Stage	Latitude	Longitude	Host
Italy							
Florence	IT	1, 2, 3	3	IT-1 and 3: adults; IT-2: larvae.	43°43'09"N	11°09'57"E	*Pinus pinaster*
Brazil							
Cambará do Sul, RS	CS	4, 5, 6	3	CS-1: larvae, CS-2 and 3: adults	29°02'52"S	50°08'41"W	*Pinus taeda*
União da Vitória, PR	UV	7, 8	2	Adults	26°13'48"S	51°05'11"W	
Bituruna, PR	BT	9	1	Larvae	26°09'41"S	51°33'09"W	
Quedas do Iguaçu, PR	QI	10	1	Adult	25°26'59"S	52°54'29"W	
Curitibanos, SC	CT	11	1	Adult	27°16'58"S	50°35'04"W	
Campo Alegre, SC	CA	12	1	Adult	26°11'33"S	49°15'56"W	
Três Barras, SC	TB	13, 14, 15, 16, 17	5	Adults	26°06'23"S	50°19'20"W	
Major Vieira, SC	MV	18, 19	2	Adults	26°22'04"S	50°19'41"W	
Irani, SC	IR	20	1	Adult	27°01'29"S	51°54'06"W	

RS: Rio Grande do Sul; PR: Paraná e SC: Santa Catarina.

ers in the detection of high genetic variability in the populations of *P. castaneus* studied herein.

The number of fragments generated by primer combinations ranged from 50 to 131, with an average of 91 fragments (Table II). The combination with the highest number of fragments was the FAM (ACT + CTG), with 131 fragments, while the combination NED (AGC + CTA) showed the lowest number of fragments. A relatively high proportion of rare alleles was detected using these combinations (Table II).

Table II. Polymorphism obtained with oligonucleotide adapters and primers used for AFLP analysis. PL, number of polymorphic loci.

Fluorescent dye	Primer combinations				Total
	FAM	FAM	NED	JOE	
Eco RI	ACT	ACT	AGC	AGC	
Mse I	CAT	CTG	CTA	CTT	
Rare alleles	62	59	27	43	
PL	116	131	50	70	367

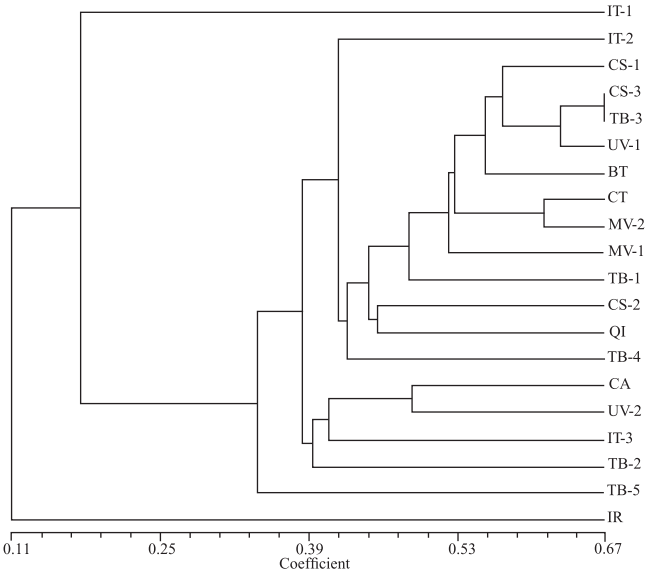

Fig. 1. Dendrogram of genetic similarity determined by the Dice coefficient using UPGMA, based on data from PCR-AFLP, for 20 samples of *Pissodes castaneus* collected in *Pinus* spp. from 10 localities in Brazil and Italy. See Table I for codes of localities.

The dendrogram based on genetic similarity among 20 individuals of *P. castaneus* from 10 localities showed that the genetic similarity was low among the populations studied (Fig. 1). Analyzing the dendrogram, one can verify the formation of two groups with genetic similarity of 0.11, with the first group, consisting of 19 individuals, formed by two subgroups with similarity of 0.18. The first subgroup is represented solely by one sample from Italy (IT-1) with similarity of 0.18. The second subgroup, with similarity of 0.3433 consisted of all remaining individuals. This subgroup was formed by the other two samples from Italy (IT-2 and IT-3) and samples from Cambará do Sul (CS-1, CS-2 e CS-3), Três Barras (TB-1, TB-2, TB-3, TB-4 e TB-5), União da Vitória (UV-1 e UV-2), Bituruna (BT), Curitibanos (CT), Major Vieira (MV-1 e MV-2), Quedas do Iguaçu (IQ), and Campo Alegre (CA), except from Irani (IR). Apparently, there is no grouping based on place of origin or pine species. The cophenetic correlation coefficient (r) was 0.9226, indicating that there is a good fit between the similarity matrix and the dendrogram obtained.

The low genetic similarities and relatively high genetic distance in samples of populations of *P. castaneus* from 10 localities are evidence of several founding events, or multiple introductions of this species in Brazil with heterogeneous strains. However, because *P. castaneus* is an invasive species and has been introduced so recently, it is not possible to establish if there was enough time for drift to cause such differentiation within it. Thus, the simplest explanation for the low similarity detected is the founder effect, meaning that the differences are related to the individuals that colonized each given area. The first record of *P. castaneus* in Brazil was in Rio Grande do Sul (Iede *et al.* 2004) and it is believed that the initial population was introduced from Uruguay and Argentina (Abgrall *et al.* 2000). Unfortunately, for our study, it was not possible to collect samples from those countries to confirm this hypothesis. We consider that, in Brazil, this insect is spreading from Rio Grande do Sul north towards Santa Catarina and Paraná, and the populations analyzed may reveal new colonizing events.

The identity of Nei was 0.7840, ranging from 0.6392 to 0.8665, and the genetic distance 0.2457, ranging from 0.1303 to 0.4475 (Table III), considering 20 samples from 10 localities. The lowest genetic identities and consequent larger genetic distances were 0.6392 and 0.4475 between Cambará do Sul (CS) and Irani (IR) and 0.6477 and 0.4343 between Bituruna (BT) and Irani (IR). These populations presented higher genetic distance than most Brazilian populations compared to the samples from Italy, which are more distant geographically. Despite the relatively high genetic distance detected among 20 samples of 10 locations, the cluster analysis based on Nei's genetic distance separated the populations only in three groups (Fig 2). Cambará do Sul (CS), Irani (IR) and Bituruna (BT) were again the locations with the highest genetic distances.

The allelic fixation index (Fst) is the most common method to quantify genetic differentiation between populations, being inversely proportional to gene flow, i.e., the lower the Fst the higher the gene flow among populations (Freeland 2007). The coefficient of gene differentiation can be interpreted according to Wright's (1978) criteria for Fst: the range 0 to 0.05 may be considered as little, 0.05 to 0.15 as moderate, 0.15 to 0.25 as great and values above 0.25 as very great differentiation. Similarly, gene flow (Nm) values of less than one can indicate little or no gene flow (Crow & Aoki 1984). A Fst value of 0.3851 was considered as a high allelic fixation index and the Nm of 0.3991 indicates a low migration rate.

Table III. Genetic identity (above diagonal) and Nei's genetic distance (Nei 1972) (below diagonal) for *Pissodes castaneus* populations, considering samples from 20 localities determined by PCR-AFLP. Code information is given in Table I.

Samples	IT-1	IT-2	IT-3	CS-1	CS-2	CS-3	UV-1	UV-2	BT	QI	CT	CA	TB-1	TB-2	TB-3	TB-4	TB-5	MV-1	MV-2	IR
1		0.6989	0.6903	0.6676	0.7131	0.6676	0.6932	0.7301	0.6761	0.7301	0.6932	0.7330	0.6733	0.6790	0.6903	0.6932	0.7102	0.6761	0.6648	0.6761
2	0.3583		0.7926	0.7756	0.7813	0.7642	0.8182	0.7926	0.7557	0.7813	0.7727	0.8068	0.7756	0.7585	0.8097	0.8011	0.7557	0.8068	0.7784	0.6818
3	0.3706	0.2324		0.7557	0.7670	0.7727	0.8040	0.8580	0.7528	0.7841	0.7983	0.8381	0.8239	0.8068	0.8182	0.8210	0.7983	0.8381	0.7813	0.7244
4	0.4040	0.2542	0.2801		0.7841	0.8182	0.8324	0.7784	0.8040	0.7898	0.7869	0.7983	0.7898	0.7727	0.8068	0.7813	0.7415	0.8097	0.7869	0.6392
5	0.3382	0.2469	0.2652	0.2432		0.8068	0.8267	0.8068	0.7869	0.8068	0.7869	0.8097	0.8125	0.7898	0.8068	0.7983	0.7813	0.7813	0.7926	0.6847
6	0.4040	0.2689	0.2578	0.2007	0.2147		0.8665	0.8068	0.8494	0.7898	0.8153	0.8324	0.8295	0.7841	0.8580	0.8097	0.7813	0.8210	0.8210	0.6619
7	0.3665	0.2007	0.2182	0.1835	0.1903	0.1433		0.8267	0.8239	0.8210	0.8125	0.8295	0.8324	0.8097	0.8608	0.8239	0.7727	0.8580	0.8295	0.6761
8	0.3146	0.2324	0.1532	0.2505	0.2147	0.2147	0.1903		0.7926	0.8239	0.8040	0.8778	0.8636	0.8352	0.8352	0.8210	0.8438	0.8324	0.7926	0.7472
9	0.3914	0.2801	0.2839	0.2182	0.2396	0.1632	0.1938	0.2324		0.7813	0.7841	0.8011	0.8153	0.7699	0.8040	0.7784	0.7670	0.7955	0.7955	0.6477
10	0.3146	0.2469	0.2432	0.2360	0.2147	0.2360	0.1972	0.1938	0.2469		0.7926	0.8324	0.8011	0.7727	0.7841	0.7869	0.7926	0.8153	0.7699	0.6903
11	0.3665	0.2578	0.2253	0.2396	0.2396	0.2041	0.2076	0.2182	0.2432	0.2324		0.8239	0.8153	0.7642	0.8040	0.8182	0.7841	0.8182	0.8352	0.6875
12	0.3107	0.2147	0.1767	0.2253	0.2111	0.1835	0.1869	0.1303	0.2217	0.1835	0.1938		0.8551	0.8324	0.8438	0.8295	0.8466	0.8182	0.8125	0.7500
13	0.3956	0.2542	0.1938	0.2360	0.2076	0.1869	0.1835	0.1466	0.2041	0.2217	0.2041	0.1565		0.8295	0.8466	0.8267	0.8267	0.8438	0.8267	0.7131
14	0.3872	0.2764	0.2147	0.2578	0.2360	0.2432	0.2111	0.1801	0.2615	0.2578	0.2689	0.1835	0.1869		0.7955	0.7869	0.7926	0.8040	0.7699	0.6790
15	0.3706	0.2111	0.2007	0.2147	0.2147	0.1532	0.1499	0.1801	0.2182	0.2432	0.2182	0.1699	0.1665	0.2288		0.8097	0.7983	0.8381	0.8551	0.7074
16	0.3665	0.2217	0.1972	0.2469	0.2253	0.2111	0.1938	0.1972	0.2505	0.2396	0.2007	0.1869	0.1903	0.2396	0.2111		0.7841	0.8182	0.8125	0.7273
17	0.3422	0.2801	0.2253	0.2991	0.2469	0.2469	0.2578	0.1699	0.2652	0.2324	0.2432	0.1665	0.1903	0.2324	0.2253	0.2432		0.7841	0.7841	0.7216
18	0.3914	0.2147	0.1767	0.2111	0.2469	0.1972	0.1532	0.1835	0.2288	0.2041	0.2007	0.2007	0.1699	0.2182	0.1767	0.2007	0.2432		0.8239	0.6932
19	0.4083	0.2505	0.2469	0.2396	0.2324	0.1972	0.1869	0.2324	0.2288	0.2615	0.1801	0.2007	0.1903	0.2615	0.1565	0.2076	0.2432	0.1938		0.6648
20	0.3914	0.3830	0.3224	0.4475	0.3788	0.4126	0.3914	0.2915	0.4343	0.3706	0.3747	0.2877	0.3382	0.3872	0.3462	0.3185	0.3263	0.3665	0.4083	

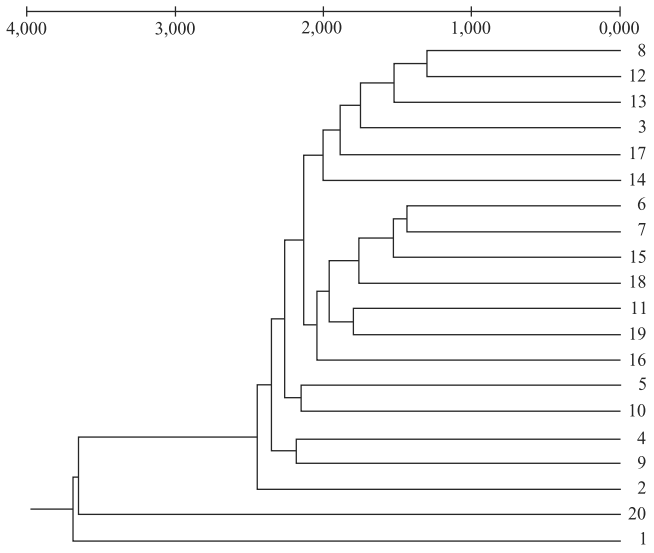

Fig. 2. Dendrogram based on Nei's genetic distance between population samples of *Pissodes castaneus* from different localities, by UPGMA. Code information is given in Table I.

The chances of this insect to fly directly from Italy to Brazil or to another country in South America are very remote or even impossible. However, individuals could have been brought in inside some wood or plant material. The Nm probably will have the most meaning among populations where dispersion is by flight, contributing to the gene flow. It would be expected that the Fst would be lower and Nm higher when only samples from Brazil were considered. Thus, *P. castaneus* present low gene flow among populations, which contributes to reproductive isolation and the establishment of independent evolutionary lineages (Slatkin 1994) or extinction of a given population by inbreeding (Freeland 2007).

In our study, we found the patterns of population structure by AFLP markers for *P. castaneus* similar to those described for *P. strobi* by Lewis *et al.* (2000, 2001) based on RAPD and isozyme markers. Lewis *et al.* (2000) reported a Fst value of 0.084, based on allozyme data, and our study showed an overall Fst value of 0.385. Using the formula to determine the number of migrants per generation, the value from our study was 0.399, whereas 0.452 was the value in Lewis *et al.* (2001). Laffin *et al.* (2004), based on mitochondrial DNA (mtDNA) COI gene, determined three patterns of genetic structuring based on gene flow with Fst value of 0.50 and Nm of 0.284. Interpopulation mtDNA differences detected between populations of *P. strobi* across Canada may reflect different selective pressures acting on relatively isolated populations (Namkoong *et al.* 1979).

Similar results were also obtained by Zhang *et al.* (2007) for *Pissodes yunnanensis* Langor & Zhang. They found an allelic fixation index of 0.879, with a high level of differentiation among populations. Another weevil species, *Hylobius abietis* L., studied by Conord *et al.* (2006) using also amplified fragment length polymorphism, showed low level of al-

lelic fixation (0.07), indicating very low genetic differentiation and high rate of migration between populations of this important pest of *Pinus* in Europe.

The heterozygosity (He) between the populations ranged from 0.0718 to 0.1397. The samples collected in Italy showed the highest value, He = 0.1397, indicating that there is higher genetic diversity in the population in the region of origin than in populations from Brazil. In Italy the populations of *P. castaneus* continue to serve as a reservoir gene pool of this pest species in that region. The relatively high genetic diversity among populations of *P. castaneus* indicates a diverse gene pool in the regions studied. As a result, the response of different populations to control strategies, chemical, biological or silvicultural, may vary according to the heterogeneity of the populations (Conord *et al.* 2006; Planter 2007).

Despite the results of the AFLP analysis, including low genetic similarity, the results are not sufficient to prove the origin and dispersion of *P. castaneus*. However, they do provide consistent evidence about the diversified genetic composition of the populations of *P. castaneus*. The fact that the population from Irani (IR) has been separated from all others may be because it is a new introduction, probably by the transport of infested trees or logs or even better climate conditions of the planting areas. However, further studies including a larger number of samples are needed to prove the causes of population differentiation of *P. castaneus* in Brazil.

Apparently, the Brazilian populations of *P. castaneus* still present high genetic identity with the population of their place of origin. The lack of a trend for the population groupings may be a strong indication that the founder populations established in southern Brazil are in the process of establishment (high polymorphism and low genetic similarity). This founder effect would not have been expressing themselves in function of the mass dispersion of the species, i.e., many individuals within populations migrating from one region to another, with low gene flow among populations (Planter 2007), but still not differentiated enough to be evidenced in the dendrograms. The high dispersion with a low gene flow can be explained by founder effect that shows the difference between populations.

Our research represents the first study about the genetic structure of populations of *P. castaneus* from Italy and Brazil. The following can be concluded from the data: 1) The populations present high degree of polymorphism and low genetic similarity; 2) It is not possible to determine grouping tendencies for the populations; 3) Heterozygosity is larger in the population from Italy than in the populations introduced into Brazil; 4) The population samples present high index of allelic fixation and low genetic flow; and finally 5) The relatively high genetic distance of one of the populations may indicate an independent and recent introduction of *P. castaneus*. These results have important implications for decisions about population management because tactics such as chemical control and pheromone mating disruption are affected by insect dispersal and gene flow among populations and need to be evaluated for each population.

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