Genetic Variability of *Hypothenemus hampei* (Ferrari) in Colombia and Development of Molecular Markers

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SUMMARY

The genetic variability of the coffee berry borer (*Hypothenemus hampei*) in Colombia was evaluated using the amplified fragment length polymorphism (AFLP) technique. Sixty *H. hampei* DNA samples from 16 coffee producing areas throughout Colombia were analyzed. We performed AFLP´s with these DNA samples using the two most polymorphic selective primers from a previous investigation. The results revealed very low genetic variation in the Colombian samples but enough polymorphism to perform a phylogenetic analysis. The findings indicated that there were multiple *H. hampei* introductions into Colombia probably from Peru, Ecuador, and Brazil. There was a main line of *H. hampei* present in every coffee-producing area in Colombia that spread throughout the country, perhaps due to coffee pickers moving within coffee regions and transporting infested coffee berries or adult *H. hampei* females. We also found that a line of *H. hampei* present in Costa Rica matched the fingerprint profile of this main line in Colombia, thus raising the possibility that the line of *H. hampei* in Costa Rica originated from Colombia. We designed sequence tagged sites STS markers from AFLP polymorphisms to answer some issues related to the biology and ecology of *H. hampei*. The design of co-dominant markers using Genome Walking and Single Stranded Conformational Polymorphism SSCP will be also of enormous relevance for a better understanding of the biology and ecology of *H. hampei.*
Key words: AFLP, SSCP, DNA Fingerprinting, broca del café, Coffee, insect-pest, molecular markers

INTRODUCTION

The beetle *Hypothenemus hampei* (Ferrarri) is the most destructive insect-pest of coffee and now threatens coffee crops worldwide (Le Pelley, 1968). This insect originated in Central Africa (Baker, 1999). *Hypothenemus hampei* increases production costs, decreases yield, and reduces commercial value of coffee beans (Benavides and Arévalo, 2002; Benavides et al., 2002).

Hypothenemus hampei was introduced to the American continent in 1913 imported in seeds from Congo or Java to Brazil (Bergamin, 1946). During the next half century, this insect spread to most coffee-producing countries in South America. It was introduced into Colombia in 1988, presumably from Ecuador (Bustillo et al., 1998). *Hypothenemus hampei* is currently present in all the regions where coffee is grown in Colombia, and annually infests up to 13%

of the total coffee production (Federacafé, personal comm. 1997). How this insect pest spread throughout Colombia is unclear. The presence of *H. hampei* was first documented at the Southwest of Colombia in 1988 (Bustillo, 1990). *Hypothenemus hampei* continued spreading from the south to the north in the following year, but in 1990 the insect was found at the middle of the country more than 400 miles away from the first reported location. It was hypothesized that *H. hampei* was introduced through the commercial trade of food from Ecuador, and then spread throughout the country on food or tools coming from infested areas (Bustillo, 1990).

Hypothenemus hampei disperses in the field by being physically transported (carried by wind or water, or being moved by human agencies) or by flying from one region to another (Baker, 1984; Sánchez, 1985; Bustillo et al., 1998; Castro et al., 1998; Moreno et al., 2001). Considering the geographical barriers and long distances between coffee producing areas in Colombia, dispersion by human agencies might be the main means of long-distance dispersal. Factors such as the illegal trade of agricultural products between coffee regions or between coffee producing countries might have contributed to this phenomenon since illegal trade bypasses normal agricultural inspection regulations and procedures.

Hypothenemus hampei completes most of its life cycle inside the berry, except for the short period of time that females fly in search of new berries to infest. Adult females emerge from infested berries and colonize new berries where oviposition takes place. Each female lays an average of 74 eggs. The total life cycle requires about 28 days (24.5 °C), and females live up to 150 days (Bergamin, 1943).

The reproductive behavior of *H. hampei* ensures a high degree of inbreeding. A biased sex ratio was estimated to be 10 females for each male (Bergamin, 1943). Females mate with their flightless male siblings while still inside the berry, so they leave the infested berry already fertilized. Although cytological examination of somatic cells in males proved that they were diploid, males failed to express paternally derived alleles and then transmitted only their maternally derived chromosomes, a condition termed functional haplo-diploidy (Brun et al., 1995).

Molecular techniques are currently available to establish relatedness among organisms and distinguish individuals within or between populations with genetic markers. Amplified Fragment Length Polymorphism (AFLP) can detect dominant loci throughout the genome. AFLP's can be used to establish relatedness between samples of genomic DNA, or to find genetic markers to identify phenotypic traits or genetic loci (Vos et al., 1995). AFLP polymorphic fragments often can be converted into co-dominant markers, which allow genetic characterization of individuals for a specific locus (Meksen et al., 2001).

After estimating the genetic variability and biogeography of a worldwide collection of *H. hampei* (Benavides et al., 2005), a new set of objectives were proposed in order to corroborate the multiple introduction nature of different lines into Colombia. The objectives of this experiment were to estimate the genetic variability of *H. hampei* in Colombia, to identify the relationship between Colombian lines and other lines found in Latin America, and to locate exclusive polymorphisms within Colombia that could be used in the development of molecular markers. Molecular markers could be used in further experiments aimed at tracing dispersion patterns in the field, determining reproductive behavior and the genetics of this insect. Consistent with our hypothesis, we found a low genetic variability of *H. hampei* in Colombia and evidences of multiple introductions into the country. AFLP also detected polymorphisms from isolated geographical regions, which were used to design site-specific molecular markers.

MATERIALS AND METHODS

Insect samples

We collected samples from several coffee producing areas in Colombia in order to determine the biodiversity and genetic relatedness of *H. hampei* within. Sixty-six samples were obtained from 16 geographical areas (departments) that grow coffee in Colombia (Table 1). Each sample consisted of all stages of *H. hampei* within single coffee beans and presumably represented the genotype of the offspring of a single female. Infested coffee beans were collected in the field and were dissected in the laboratory.

Table 1. Location of *Hypothenemus hampei* samples collected in Colombia.

Department	n	Latitude/Longitude
Antioquia	7	6.15N/75.60W
Cundinamarca	5	4.50N/74.20W
Caldas	6	4.98N/75.62W
Caqueta	1	1.61N/75.62W
Cauca	1	2.42N/76.61W
Cesar	8	10.05N/73.25W
Huila	4	2.21N/75.65W
Magdalena	2	11.26N/74.19W
Meta	3	4.15N/73.64W
Nariño	3	1.20N/78.80W
Norte de Santander	2	7.91N/72.51W
Quindio	1	4.53N/75.69W
Risaralda	9	4.88N/75.62W
Santander	5	7.13N/73.14W
Tolima	3	4.92N/75.08W
Valle	6	4.28N/75.93W

Genomic DNA was extracted from each sample using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA). DNA quality was then examined by agarose gel electrophoresis and DNA concentration was determined using a Hoefer Scientific TKO 100 Mini-fluorometer. Genomic DNA samples were maintained at –70 °C.

DNA Fingerprinting

AFLP analysis was performed using the AFLP Analysis System II for small genomes developed by Life Technologies (GIBCO Invitrogen Corporation, Carlsbad, CA) and ^{33}P-ATP end labeled primers as recommended by the manufacturer with minor modifications. Each sample of *H. hampei* genomic DNA (~125 ng) was completely digested with the restriction endonucleases *Mse*I and *Eco*RI. Double stranded DNA adapters provided in the kit were ligated to the ends of the DNA fragments. Preselective primers were complementary to the core of the adapter sequences. The new DNA fragments were then pre-amplified and selective amplification was performed using two primer combinations (E-AT combined with M-CTC and M-CTG). These two combinations previously proved to detect great numbers of polymorphisms (Benavides et al., 2005). Amplified fragments were separated by gel electrophoresis for 2 h at 50 V in 6% Long Ranger (FMC Bioproducts, Rockland, ME) denaturing polyacrylamide gels. After electrophoresis, the gels were dried and exposed to Biomax MR (Kodak Rochester, NY) X-ray film for 48 to 72 hours for autoradiography.

DNA polymorphic bands were visually detected and excised from the polyacrylamide gel then kept at –70ºC for further analysis.

Conversion of AFLP polymorphisms into STS markers

Amplified fragments of DNA were excised from the polyacrylamide gel. The DNA was eluted into approximately 50 µl of TE buffer (10 mM Tris-HCL, 1 mM EDTA). Then 5 µl of the eluted DNA was re-amplified using the selective primer combinations that originally produced the polymorphic band. The re-amplified fragments were cloned using the TOPO TA Cloning Kit for Sequencing (GIBCO Invitrogen Corporation, Carlsbad, CA) and pGEM®-T Easy vector System I (Promega, Madison, WI). Briefly, these techniques allow the direct insertion of PCR products into a plasmid vector for sequencing. The *Taq* polymerase in the PCR adds a single deoxyadenosine to the 3' ends of PCR fragments. The vector contains a single overhanging 3' deoxythydimidine residue that allows PCR inserts to ligate with the vector. The cloned plasmids were then transformed into *E. coli* cells in order to select recombinants. Cells were incubated overnight in pre-warmed LB plates with 50 µg/ml of kanamycin. About 10 colonies were selected and cultured overnight in LB medium containing 50 µg/ml of kanamycin. The selected *E. coli* colonies were placed in a plate and sent to the Genomic Center at Purdue University for the isolation and sequencing of the plasmids. Sequencing was performed through automated fluorescent DNA BigDye terminator using an ABI-3700 Automatic Sequencer (PE Applied Biosystems). BLASTX and BLASTN sequence analysis (Altschul et al., 1994) were used to compare the sequences with other sequences in the GenBank database.

These sequences were used to design site-specific primers to amplify genomic fragments from those samples in which polymorphisms were detected. Samples in which polymosphisms were present were used as positive controls, while samples in which polymorphisms were absent would amplify the alternative allele. PCR was performed on a PTC 100 thermocycler (MJ Research, Watertonwn, MA). DNA from populations with presence and absence of polymorphisms were used in a 25 µl PCR containing 15 pmol of each site-specific primer, 10 nM Tris-HCl pH 9.1, 50 nM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 2 mM of each dNTP and 1U of *Taq* polymerase. The PCR product was electrophoresed through a 1.0% agarose gel in 1X TBE.

Fragments that amplified both present and absent bands, were electrophoresed in an SSCP gel in order to reveal the polymorphisms. Those polymorphisms in which the nature of their genetics difference were not possible to observe using the above mentioned techniques, were used as template to design primers during a Genome Walking procedure (GenomeWalker®, Promega, Madison, WI). After getting larger fragments in both 5' and 3' ends, new primers were designed and polymorphic DNA samples tested again using the same principles and procedures as looking for STS´s.

Genetic analysis

The banding patterns associated with each selective primer combination and each sample were visually analyzed and converted into digital fingerprints. Each fragment position was scored as '1' if the fragment was present and as '0' if it was absent. To avoid artifacts, bands that were not recognized by 2 independent observers were eliminated from the analysis.

Digital fingerprints were used to calculate the percentage polymorphism for insect populations within the country, as well as to infer population genetic structure and perform phylogenetic analyses. The percentage polymorphism for insect populations within the

country was determined as the proportion of the polymorphic bands to the total number of fragments generated by AFLP in all samples within the country.

Population genetic structure was inferred using an analysis of molecular variance (AMOVA, Arlequin v2.0) (Excoffier et al., 1992). The analysis used all the fingerprints generated with all the samples within the country. Locations were treated as populations under the null hypothesis of no population substructure. All locations were tested as one group. Geographical subdivision of a population is called population substructure and it is usually measured by the fixation index Φ_{ST} that quantifies the inbreeding effect of population substructure (Wright, 1921). Species that are spread over large geographical areas are usually divided into subpopulations (Hartl and Clark, 1997; Hanski, 1999). In general, mating between organisms within the same subpopulation would be more likely than mating between organisms in different subpopulations (Hartl and Clark, 1997). If we consider an Φ_{ST} =0 as no genetic divergence, we can follow the qualitative guidelines for the interpretation of population substructure given by Wright (Wright, 1978).

For the phylogenetic analysis, we performed a bootstrap using the *Phylogenetic Analysis Using Parsimony* software (PAUP 4.0b10 Altivec) (Swofford, 1998) under the Neighbor Joining algorithm. We used 2000 re-samplings and 10 000 replications with the total number of pair-wise character difference distance setting. No outgroup was used for this analysis. Furthermore, in order to determine the origin of *H. hampei* fingerprints that were introduced into Colombia, results from this experiment were phylogenetically compared to the fingerprints obtained in previous investigations that aimed to detect genetic diversity from *H. hampei* samples collected in most coffee producing countries in Latin America and the Caribbean islands (Benavides et al., 2005).

RESULTS AND DISCUSSION

Insect samples

A total of 66 samples of *H. hampei* that covered 16 coffee producing areas within Colombia were collected (Table 1). Genomic DNA was isolated from dissected *H. hampei* individuals preserved in absolute ethanol. We dissected an average of 29.3 ± 18.2 individuals per berry that yielded 4.3 ± 1.8 μg of genomic DNA per sample. We correlated the number of individuals of *H. hampei* dissected in each single bean and the amount of DNA obtained from these samples (Table 2). We found no statistical correlation between these 2 variables (*significance F* = 0.92 > 0.05). Although an average of 20 to 40 individuals yielded the most DNA, 1 to 20 individuals were sufficient to isolate enough DNA to perform genetic analysis.

Table 2. Isolation of genomic DNA from *Hypothenemus hampei* samples collected in Colombia.

H. hampei	DNA per sample (μg)	Standard deviation	Min.	Max.
1 – 20	3.9	1.5	1.0	6.6
21 – 40	4.4	1.8	1.5	8.2
41 – 60	5.7	2.3	4.0	9.3
61 – 80	3.7	1.5	2.0	5.0

DNA Fingerprinting

AFLP amplified 60 fragments of DNA when the 2 highest polymorphic primer combinations were used (Benavides et al., 2005). We found only 7 polymorphic bands (11.7% of the total bands generated by AFLP), which indicated a very low genetic variability within populations of *H. hampei* in Colombia (Figure 1). The band patterns observed in each of the 66 samples generated only 8 fingerprints (Table 3). Fingerprint COL01 comprised 76% of the total sample size and grouped all geographical areas that were tested within Colombia. These results are indicative of a main line distributed in all departments within Colombia; however, there were more lines present in specific geographical areas. We found 4 other fingerprints (COL02, COL03, COL04, and COL05) that were unique for 4 different regions and which suggested independent introductions into Cesar, Caldas, Valle, and Risaralda. The remaining 3 fingerprints, COL06, COL07, and COL08, comprised samples from 4 departments: Caldas, Risaralda, Antioquia and Nariño. These results were a possible indication of a shared line among these departments. The sharing of food trade and exchange of labor among coffee farms could explain the relationship among samples from Antioquia, Risaralda, and Caldas within these 3 neighboring departments. The relationship between samples from Nariño with other regions could be better explained by the exchange of labor that occurs along the country.

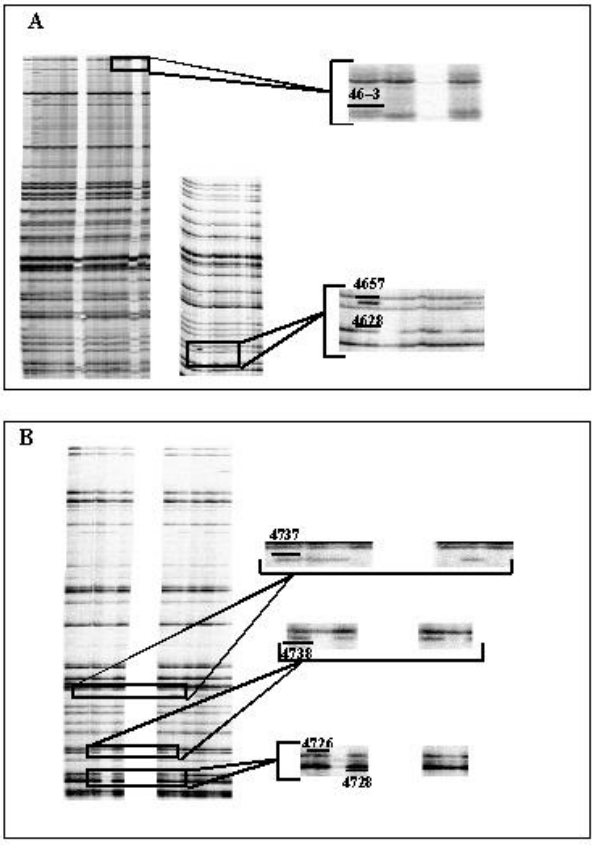

Figure 1. DNA fingerprints generated with primer combination E-AT/M-CTC (A) on samples Risaralda 08, Tolima 02, 03, Antioquia 01, 02,03, and Cesar 01, 02, 03, 04, 05, 07 (A1); and samples Risaralda 08, 05, 06, 07 08, 09, Caldas 06, Antioquia06, 07, Nariño02 and 03 (A2). DNA fingerprints with primer combination E-AT/M-CTG (B) on samples Risaralda08, Tolima02, 03, Antioquia02, 03, Cesar01, 02, 03, 04, 05 and 07. Two lanes in AFLP gel A1 did not amplified DNA fragments properly, one between samples Antioquia 03 and Cesar 01 and the other between samples Cesar 05 and 07. The 7 polymorphisms found within Colombian samples are indicated.

Table 3. AFLP Fingerprints generated with 2 primer combinations on 66 Colombian samples of *Hypothenemus hampei*.

Fingerprint	n	Sample	Equivalent Fingerprint in Latin America	Equivalent samples in Latin America
COL01	50	Antioquia01-05	FP02	CR02
		Cundinamarca01-05		
		Caldas01-04		
		Caqueta01		
		Cauca01		
		Cesar01-03,05,08		
		Huila01-04		
		Magdalena01-02		
		Meta01-03		
		Nariño01		
		Norte Santander01-02		
		Quindio01		
		Risaralda01,03-04		
		Santander01-05		
		Tolima01-03		
		Valle01-04,06		
COL02	2	Cesar04,07	FP27	Unique to Colombia
COL03	1	Caldas06	FP06	BR04,06 JA01, 02 EC01, 02, 04, 05 NI01, 03, 04 HO01, 04, 06, 07
COL04	1	Valle05	FP03	BR02, 07, 14
COL05	1	Risaralda07	FP05	Control. Unique to Colombia
COL06	2	Caldas05	FP28	Unique to Colombia
		Risaralda02		
COL07	5	Antioquia07	FP15	BR01, 03, 05
		Nariño02		PE01, 02, 05, 06, 10
		Nariño03		SA01
		Risaralda05		
		Risaralda06		
COL08	4	Antioquia06	FP12	PE03, 04, 07, 09
		Caldas07		
		Risaralda08		
		Risaralda09		
TOTAL	66			

Hypothenemus hampei was first detected inside Colombia in Nariño, which is located at the Southwest of the country at the border with Ecuador (Bustillo, 1990). Harvesters from Nariño travel yearly to the middle of the country where about 60% of the Colombian coffee is produced (Antioquia, Caldas, Risaralda, Quindio and Valle). These harvesters travel that long a distance (above 400 miles) motivated not only because salaries are higher in these regions but also because their main coffee harvest period is at a different time. More specifically, the main harvest has finished at the south by the time the harvest period starts in the middle of the country. We hypothesized that one line of *H. hampei* (COL07) might come to Risaralda and

Antiqouia carried by harvesters from the Southwest, which could also explain the presence of the line COL01 along the country. The exchange of labor is our strongest hypothesis to explain the dispersion of *H. hampei* within Colombia.

A phylogenetic analysis of the samples from Colombia revealed 3 clusters (Figure 2). Fingerprint COL01 formed the first isolated group. A second cluster indicated closer relatedness between fingerprints COL02 (Cesar) and COL06 (Caldas and Risaralda). A third cluster grouped Caldas, Risaralda, Valle, Antioquia and Nariño, which were contained in the remaining 5 fingerprints. Although this neighbor-joining analysis was exhibiting some relatedness among fingerprints, it was not relevant for revealing the origin of the introduced samples. The variation among samples was probably the result of multiple introductions and not the result of evolutionary events such as random mutations.

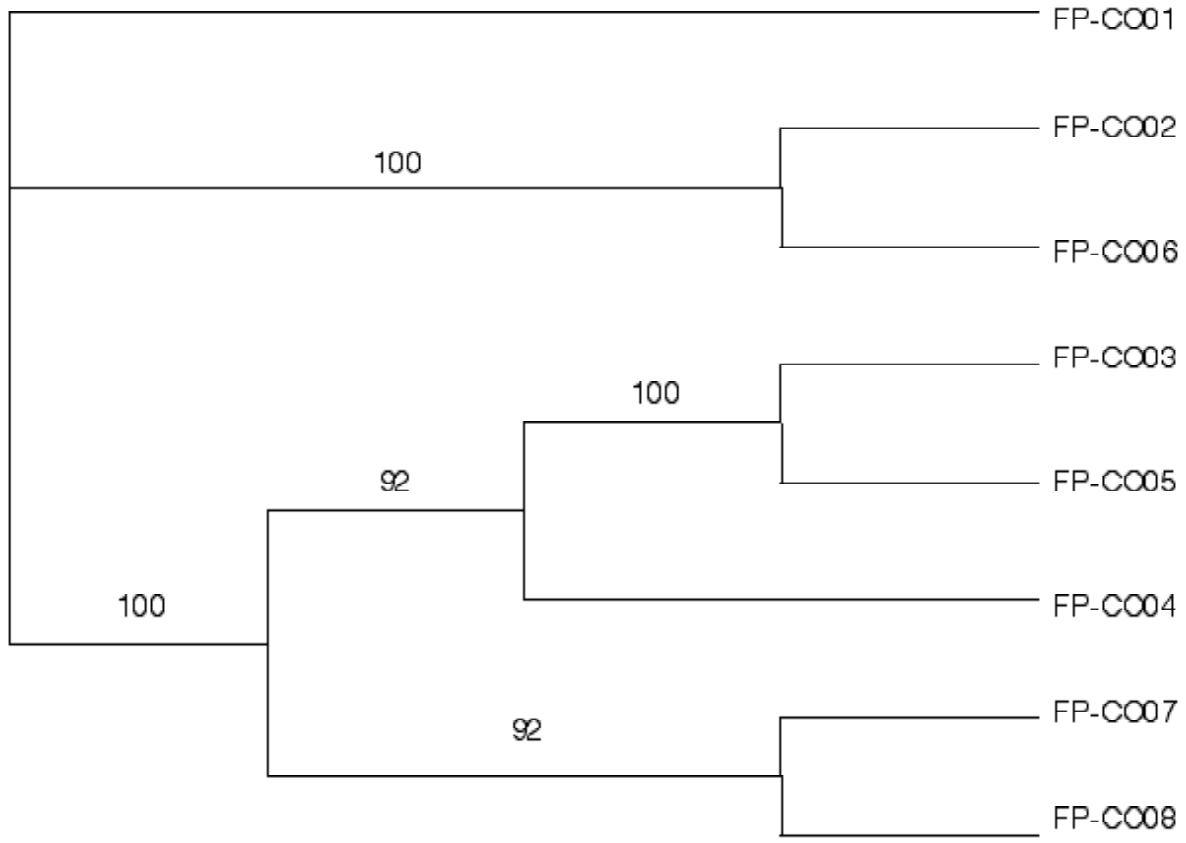

Figure 2. Bootstrap under Neighbor Joining Distance Analysis with the data obtained for the Colombian fingerprints generated by the primer combinations E-AT/M-CTC and E-AT/M-CTG. Fingerprint COL01 comprised 76% (50) of the total number of samples collected and was represented in all coffee producing departments in Colombia. Fingerprint COL02, COL03, COL04, and COL05 were exclusively found in the departments of Cesar, Caldas, Valle, and Risaralda respectively. Fingerprint COL06 contained Caldas and Risaralda; COL07 Antioquia, Nariño, and Risaralda, and COL08 Antioquia, Caldas and Risaralda.

In order to estimate population substructure on *H. hampei* in Colombia, we performed an analysis of molecular variance (Table 4). Samples Caqueta01, Cauca01 and Quindio01 were excluded from this analysis since only one sample was collected in each department and, therefore, variance within populations could have been altered. We found 92% of the variance due to difference among locations and 8% due to difference within locations, results expected due to inbreeding. According to Wright's Φ_{ST} parameters (Wright, 1978), a value between

0.05-0.15 is an indication of moderate genetic differentiation. Therefore, *H. hampei* in Colombia evidenced moderate genetic differentiation probably as a result of recent introductions from diverse populations.

Table 4. Hierarchical analysis of variance for 13 departments in Colombia with no grouping.

Variance		d.f.	Variance	% total	*p**	*Φ*-statistics
Among locations	σ_a^2	12	0.018	8.32	0.09	
Within locations	σ_c^2	50	0.202	91.68	0.00	Φ_{ST} = 0.083
Total		62	0.220			

Furthermore, in order to determine the origin of *H. hampei* fingerprints introduced into Colombia, we compared Colombian fingerprints with those previously found in all Latin American countries and the Caribbean islands (Benavides et al., 2005) (Table 3). An interesting finding related fingerprint COL01, which grouped the majority of the Colombian samples, with the fingerprint FP02 that was unique for Costa Rica. This result suggested that Colombia was probably the main source for the introduction of *H. hampei* into Costa Rica. We observed 3 fingerprints unique to Colombia and not related to any other country. We believe that these samples that generated unique fingerprints could have been present in other geographical areas, but were not sampled during this experiment. Fingerprint FP06 that was found in Brazil, Jamaica, Ecuador, Nicaragua, and Honduras, was also found in Caldas06; and fingerprint FP03, which was present in Brazil, was also present in Valle05. These results suggested that there may have been more than one single introduction of *H. hampei* into Colombia, and perhaps Colombia has been the source of later introductions into other Central American countries.

Fingerprint FP15 present in Brazil, Peru and El Salvador, and the Peruvian fingerprint FP12 were found in Colombia in the departments of Antioquia, Nariño, Caldas, and Risaralda. These results suggested that some lines were introduced from different origins and later dispersed throughout the country. Although the introduction of *H. hampei* was reported into Colombia directly from Ecuador to Nariño (Bustillo, 1990), the genetic analysis indicated no link between these 2 points. A clearer link was detected between samples from Antioquia, Risaralda, and Nariño and samples from Peru. The fact that *H. hampei* was reported first in Nariño is not a strong indication that this region was the initial source of this insect in Colombia. *Hypothenemus hampei* could have been introduced first into Antioquia from Peru and then transported to Nariño by the exchange of labor between coffee regions within the country, or even from Peru directly to Nariño and then carried to Antioquia by the same means. The presence of the Peruvian fingerprint FP12 into Colombia strengthens this hypothesis; therefore, *H. hampei* was more likely initially introduced from Peru into Colombia, and the mechanism of this introduction could be hypothetically explained by the trade of illegal drugs in the Americas (Figure 3).

An AMOVA with all Colombian and Latin American samples was performed (Table 5). We found similar results in this analysis as with those obtained when worldwide countries were analyzed. Great genetic differentiation was found in *H. hampei*.

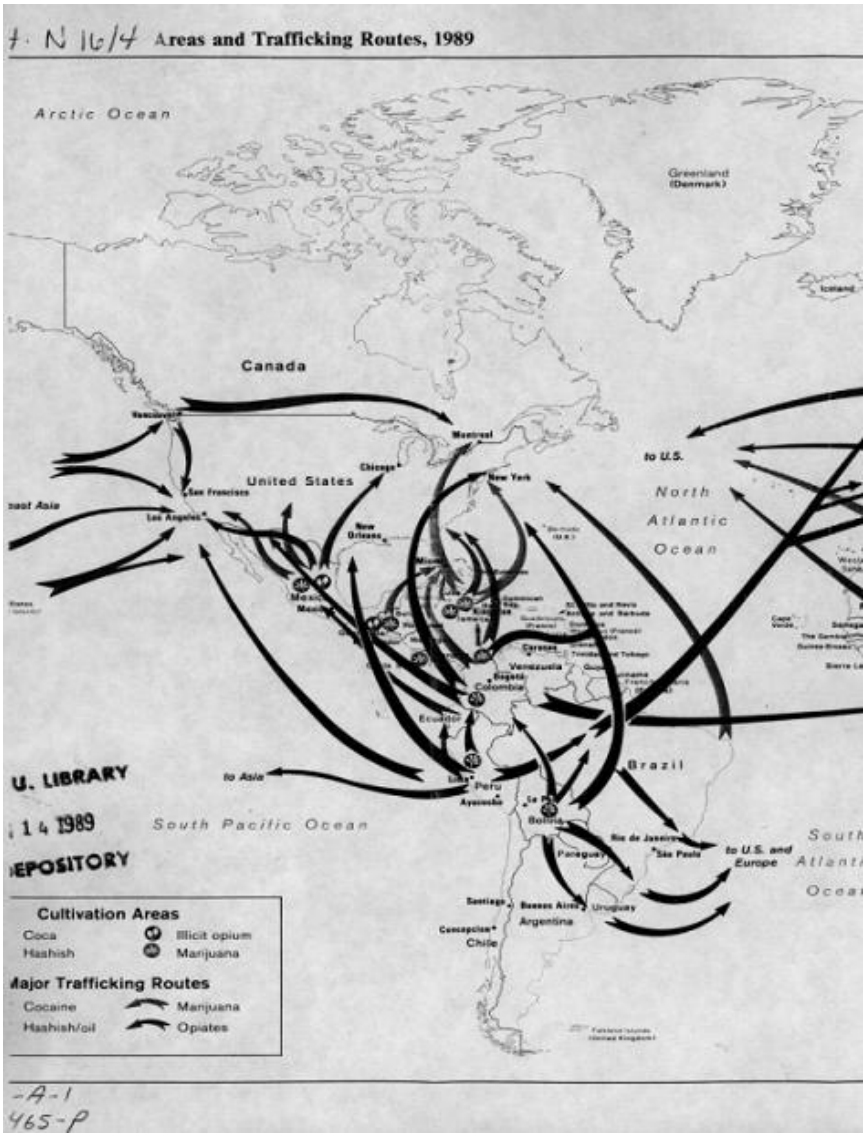

Figure 3. Areas and trafficking routes of illegal crops (C.I.A. 1989). Observe clear routes coming from Peru straight to the south portion of Colombia and to Ecuador. There is also a route to Jamaica from Colombia.

Table 5. Hierarchical analysis of variance for Colombian and Latin American samples with no grouping.

Variance		d.f.	Variance	% total	p*	Φ-statistics
Among locations	σ_a^2	18	0.178	44.12	0.00	
Within locations	σ_c^2	96	0.225	55.88	0.00	$\Phi_{ST} = 0.4412$
Total		114				

Conversion of AFLP polymorphisms into STS markers

In order to design molecular markers, AFLP polymorphisms were converted into site-specific primers. Ten polymorphic bands were excised from the AFLP acrylamide gels; therefore, those polymorphisms were cloned and sequenced. We obtained AFLP fragments between 60 and 578 total bases (Figure 5). Site-specific primers were designed with these sequences and PCR was performed on samples where AFLP polymorphisms were absent and present (Figure

4). Site-specific primers 4742, 4753, 4754, and 4628 showed amplification on AFLP present samples and partial amplification on AFLP absent samples. We believe that this partial amplification is due to the nature of the mutation detected in these samples with these primer combinations. A single mutation in the *Mse*I or *Eco*RI restriction site would allow partial amplification when using the site-specific primers. Only the site-specific primer 46-3 revealed presence and complete absence patterns in present and absent samples. These results suggested the lack of one of the restriction sites sequence in the AFLP absent sample, or perhaps an inversion of a large fragment that could have not been amplified by PCR. Complete amplification of DNA fragments in absent samples was observed when site-specific primers 4756, 4737, 46-2, 46-6, and 4657 were used. Primers 46-2 and 46-6 were present in the AFLP of all Colombian samples, except for a few samples where their presence was not clear. The results obtained after this experiment allowed us to corroborate the presence of these 2 fragments in all Colombian samples. The amplification of fragments in AFLP absent samples when using primers 4756, 4737, and 4657 can also be explained as the presence of a single point mutation in the sequence of one of the restriction sites, and the successful amplification through PCR.

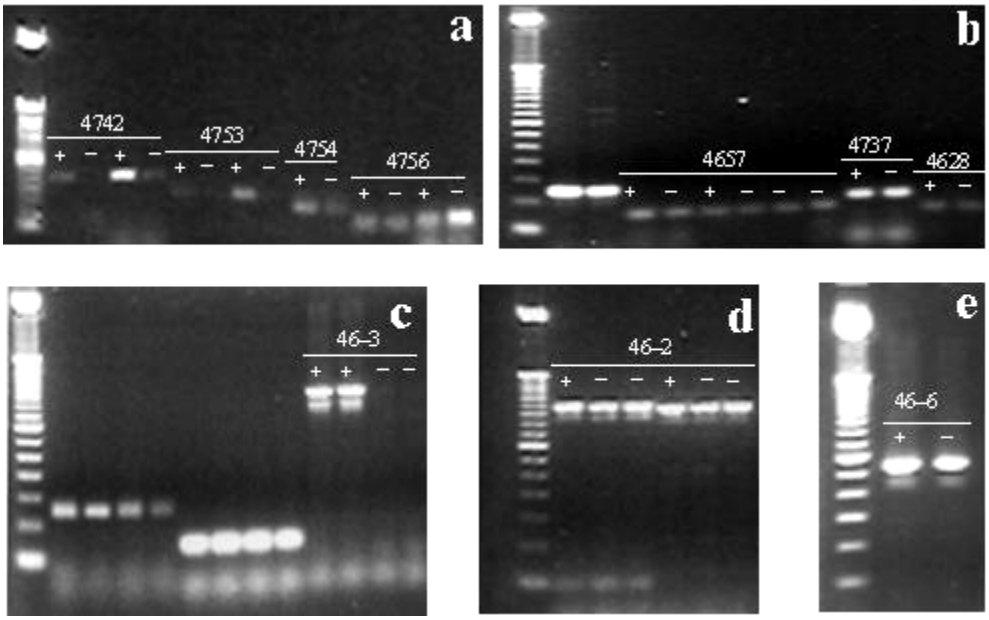

Figure 4. PCR using site-specific markers. First lanes indicate a 50 bp marker. We obtained partial amplification of negative samples when primers 4742, 4753, 4754, and 4628 were used. Complete absence on the alternative allele with primer 46-3 and complete amplification of negative samples with primers 4756, 46-2, 46-6, 4657, and 4737.

Even though we did not find a marker that revealed co-dominance, the site-specific primer 46-3, unique for only 2 samples within Colombia and located in the geographical area of Cesar, showed dominance and can be used as a genetic marker in field experiments aimed to trace dispersion patterns in the field. To corroborate this information, the primer combinations designed to amplify fragment 46-3 was used in a PCR with all Colombian populations (Figure 5). The results showed the amplification of this particular fragment in the 2 initially amplified populations, and complete absence in the rest of the samples from Colombia. Unfortunately, this polymorphic band was not possible to be recovered from the original population once in Colombia due perhaps to genetic drift events, the presumed nature of the polymorphism as being a transposon, or the presence of genomic contamination from insect symbionts.

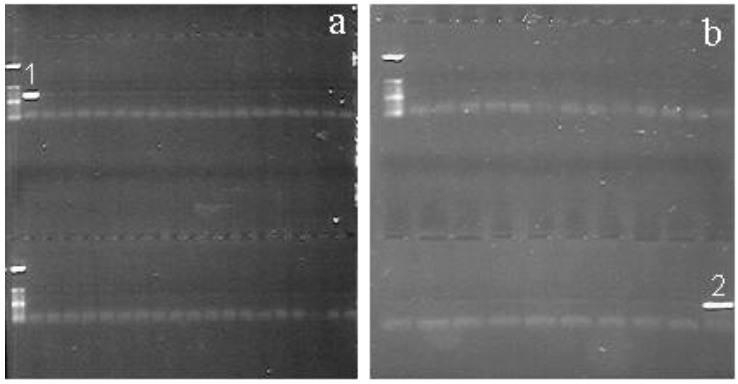

Figure 5. PCR with primers designed to amplify allele 46-3. Two positive Colombian samples, 1 in gel a and 2 in gel b (1:Cesar04 and 2:Cesar07) were used with 59 other Colombian samples (38 samples in figure a and 23 samples in figure b). amplification occurred only in the 2 original samples.

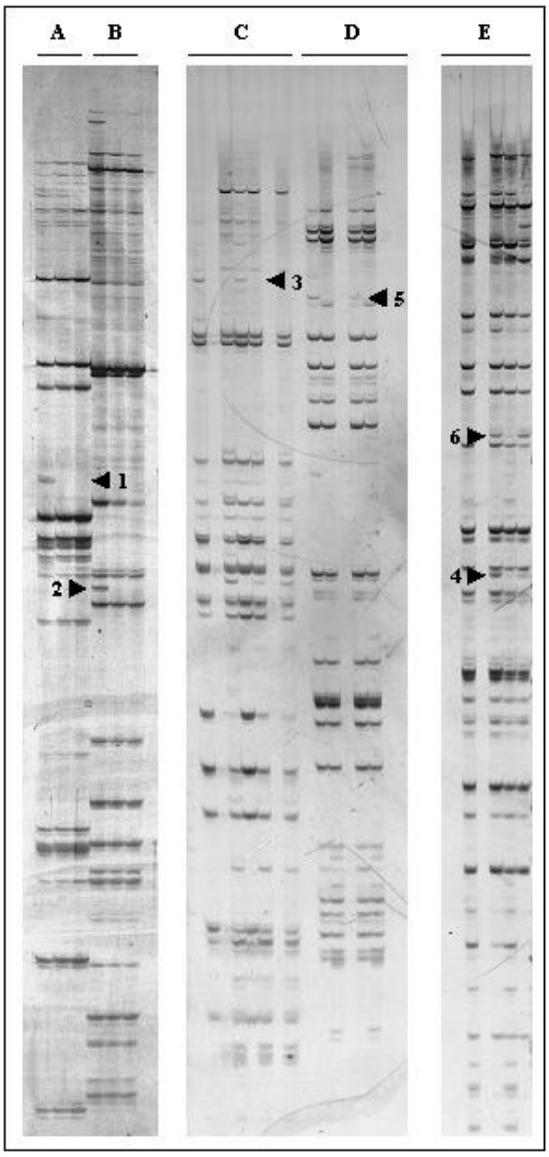

Figure 6. New AFLP polymorphisms detected in field Colombian *H. hampei* populations. Five new sets of primer combinations (A to E) were selected using the four most polymorphic *H. hampei* Colombian samples.

Furthermore, we developed six new STS markers from field *H. hampei* samples (Figure 6). After recovering the polymorphisms from the populations used in AFLP, we designed a new set of primers based on the AFLP fragment sequences. The fragments were further amplified on the present and absent populations used during the AFLP technique (Figure 7). Those *H. hampei* samples that amplified the fragment in both the present and the absent populations, were electrophoresed in an SSCP gel and allowed us to identify the nature of the polymorphic STS *Hha*STS2 and *Hha*STS5 (Figure 8).

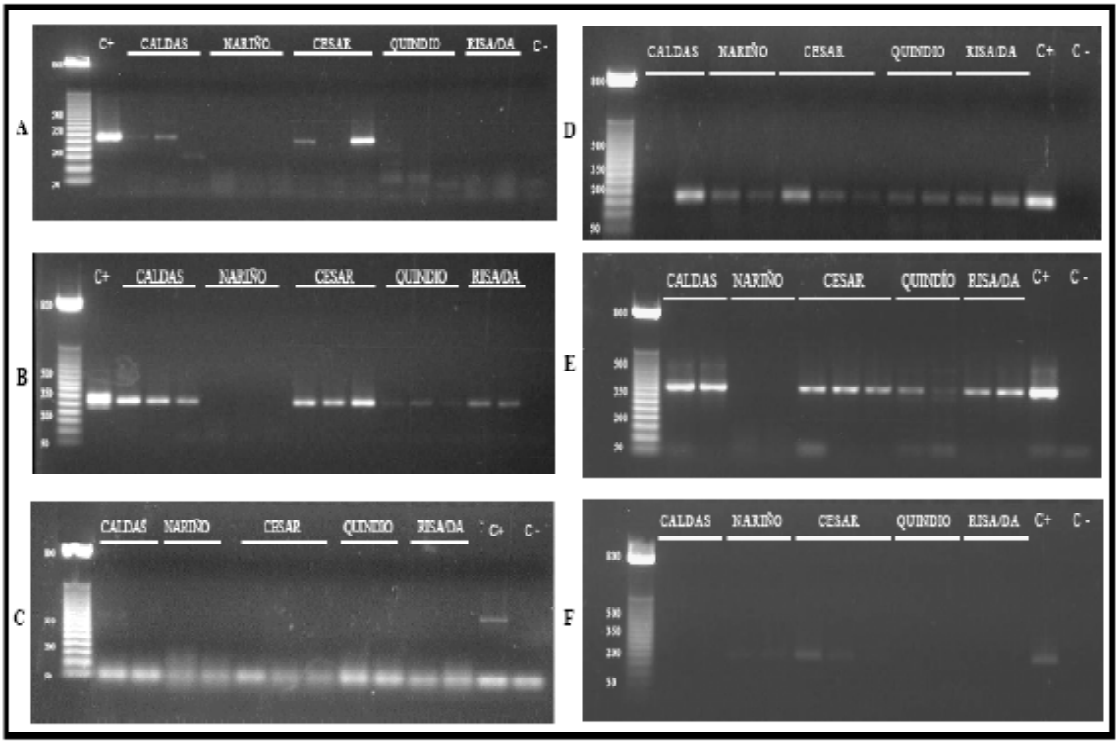

Figure 7. PCR using site-specific markers. First lanes indicate a 50 bp marker. We obtained amplification of negative samples when primers *Hha*STS2 (B), *Hha*STS4 (D) and *Hha*STS5 (E) were used. Partial amplification on the alternative allele with primer *Hha*STS1 (A) and *Hha*STS6 (F); and no amplification with *Hha*STS3 (C). C^+ and C^- were the positive and negative controls for the PCR respectively.

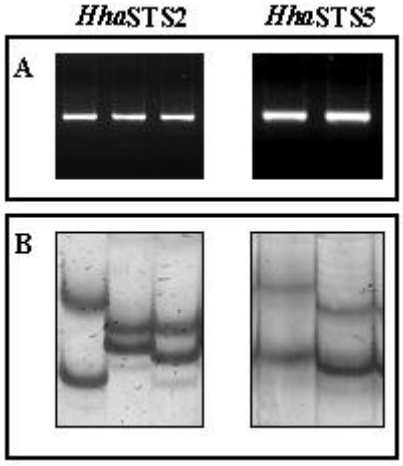

Figure 8. Electrophoresis on 2% TBE agarose gel (A) and SSCP (B) of present and absent *H. hampei* populations SSCP. SSCP allowed us to detect the different alleles with the STS *Hha*STS2 and *Hha*STS5.

*Hha*STS2 polymorhic alleles were further sequenced and aligned. This alignment showed a highly polymorphic region of four nucleotides and a deletion of 39 bases into one allele. The nature of this polymorphism was clearly revealed. We are currently working on the STS *Hha*STS5 in order to find the sequence of this new polymorphism.

We believe that these new co-dominant molecular markers can be used now as tools for studying the inheritance of genetic traits in *H. hampei*, as well as the dispersal patterns used by this insect in the field.

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