Genetic variation in populations of the cacao wilt pathogen, Ceratocystis cacaofunesta

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Ceratocystis cacaofunesta (= Ceratocystis fimbriata) causes a lethal wilt disease of cacao (Theobroma cacao) in Latin America. Polymorphic microsatellite markers, (CAT), nuclear DNA fingerprints and Hae III mitochondrial DNA fingerprints were used to compare genetic diversity among isolates of C. cacaofunesta collected from populations in western Ecuador, Costa Rica, Colombia, and Rondônia and Bahia in Brazil. Microsatellite markers and nuclear DNA fingerprints separated Ecuadorian isolates from isolates of the other four populations, and these two major groups correspond to genetic lineages already identified from ITS-rDNA sequences and intersterility groupings. Mitochondrial DNA fingerprints also demonstrated substantial diversity and split the Ecuadorian isolates into two groups. All marker types showed limited variation in the Colombian, Costa Rican and Bahian populations, as might be expected for introduced populations that have gone through recent genetic bottlenecks. In contrast, the Rondonian and western Ecuadorian populations showed gene diversity values similar to natural populations of other Ceratocystis species. The Rondonian population was the only sampled population in the native range of T. cacao (the Upper Amazon), and the putatively introduced populations were more closely related to the Rondonian population than to the western Ecuadorian population. The Ecuadorian population is in an area with other native Theobroma species, which may serve as natural hosts.

Keywords: Ceratocystis fimbriata complex, fungal population genetics, genetic diversity, Herrania spp., Theobroma cacao

Introduction

Ceratocystis cacaofunesta is a serious pathogen of cacao (Theobroma cacao) and related Herrania spp., causing wilt and death of infected trees. The cacao pathogen is a member of the Latin American clade of the C. fimbriata species complex, which has substantial genetic variation and a wide range of hosts (Harrington, 2000; Barnes et al., 2001; Baker et al., 2003; Marin et al., 2003; Steimel et al., 2004; Engelbrecht & Harrington, 2005). The North American clade also contains several host-specialized species, but members of this clade are primarily wound colonizers that do not kill their hosts (Johnson et al., 2005). Ceratocystis cacaofunesta was recently differentiated from C. fimbriata based on minor morphological differences, pathogenicity to cacao, ITS-rDNA sequences and intersterility with other members of the Latin American clade, including isolates from T. cacao that proved to be non-pathogenic in inoculation studies (Baker et al., 2003; Engelbrecht & Harrington, 2005). The cacao pathogen has only been identified in Central and South America and may be indigenous to this region (Harrington, 2000; Baker et al., 2003; Engelbrecht & Harrington, 2005).

Ceratocystis cacaofunesta typically enters cacao plants through fresh wounds, such as pruning or pod harvesting wounds (Malaguti, 1952), and moves through the host in the secondary xylem. Ambrosia beetles of the genus Xyleborus often attack the wood of infected trees (Saunders, 1965), liberating frass that includes aleurioconidia and conidia (asexual spores) as the beetles excavate their galleries (Iton & Conway, 1961). This frass may be carried by wind or rainsplash to wounds on other trees (Iton, 1960). Machete blades are another efficient means of spreading the fungus (Malaguti, 1952). Like other members of the C. fimbriata complex, C. cacaofunesta is homothallic through unidirectional mating type switching (Witthuhn et al., 2000), and introduced populations could persist in an essentially clonal manner through asexual reproduction and production of sexual spores through selfing.

The genus Theobroma is indigenous from southern Mexico to the Amazon Basin (Purseglove, 1968), with many species on each side of the Andes (Baker, 1953). It is thought that the rising of the Andes may have facilitated speciation within the genus by separating previously
Ceratocystis wilt of cacao was first reported on cacao in western Ecuador in 1918 (Rorer, 1918). It caused extensive damage in Colombia after 1940, and in Venezuela in 1958 (Thorold, 1975). The disease caused severe losses in Costa Rica after 1958, though it may have been present as early as 1926 (Thorold, 1975). It also caused severe disease in Colombia in 1958, though it may have been present as early as 1926 (Thorold, 1975). The disease caused serious losses in Trinidad in the late 1950s (Spence & Moll, 1958). Reports of the disease stretch from Guatemala (Schieber et al., 1999), Ecuador, Colombia and Venezuela (Thorold, 1975). In Brazil, the disease was reported in the southwestern Amazon (Rondônia) in 1978 (Bastos & Evans, 1978) and more recently in Bahia (Bezerra, 1997), which is out of the native range of *T. cacao*.

Comparing genetic diversity of populations of *C. cacaofunesta* may help to distinguish introduced from indigenous populations, as was recently done with *C. platani* (Engelbrecht et al., 2004). The objective of this study was to evaluate genetic diversity in, and possible origins for, *C. cacaofunesta* in Ecuador, Costa Rica, Colombia and Brazil using polymorphic microsatellite markers and nuclear and mitochondrial DNA fingerprints.

Materials and methods

Fungal isolates

Isolates of *C. cacaofunesta* were collected from dead or dying *Theobroma cacao* trees. Two isolates were collected from *Herrania* spp., which are closely related to *Theobroma*. All isolates are listed in Table 1 and are held in the culture collection of TC Harrington, Iowa State University.

Mycelium was grown in 20 mL liquid broth (2% malt extract and 0.2% yeast extract) in 125 mL flasks at room temperature for two weeks, and the DNA was extracted and analyzed following the methods of DeScenzo & Harrington (1994).

Microsatellite markers

Sixteen PCR-based microsatellite markers (Steimel et al., 2004) were used to compare genetic variability. A fluorescently-labelled primer and an unlabelled primer flanking each of 16 three- or four-base pair repeat microsatellite regions were used in the PCR amplification reactions, as described in Steimel et al. (2004). PCR products were analyzed using an ABI Prism 377 DNA sequencer (Applied Biosystems Inc), with band size determination using standards, ABI GeneScan Analysis Software v3.1-2 and Genotyper 2.0 software (Applied Biosystems Inc.). Each unique product length (differing by more than 1 bp) was considered a different allele.

Nuclear DNA fingerprinting

To examine variability in nuclear DNA, total genomic DNA was restricted with *PstI* and electrophoresed on 1% agarose gels (19.5 × 25.5 cm) (Bio-Rad Certified Molecular Biology agarose, Bio-Rad) for 1600 volt hours, with *Hind*III-restricted Lambda DNA as size standards (Promega). Gels were dried, hybridized with the oligonucleotide (CAT), labelled with 32P and washed following the methods of DeScenzo & Harrington (1994). Labelled bands were visualized by exposing the gels to a phosphor screen for 4–6 days and then scanning the screens with a Storm PhosphorImager (Molecular Dynamics). Several representative isolates were extracted and fingerprinted two or more times to ensure consistency in results.

Quantity One quantitation software (Bio-Rad) was used to estimate the length (in bp) of each fingerprint band based on comparison with the Lambda *Hind*III size standards. The DNA restrictions and fingerprinting were repeated at least once for representative isolates. Only bands that were consistently visible and unambiguously scored in all replicate gels were analyzed.

Mitochondrial DNA fingerprinting

*Hae*III cuts GC-rich DNA and allows visualization of the AT-rich mitochondrial DNA (Wingfield et al., 1996). Twenty-five micrograms of extracted total genomic DNA was restricted overnight at 37°C with *Hae*III in a total volume of 500 µL (50 µL 10X buffer, 1 mM spermidine, 10 units µL–1 *Hae*III and 5 µL RNase). Restricted DNA was precipitated, dried, and washed as in Wingfield et al. (1996). Washed DNA was resuspended in 37.5 µL of 0–10 mM Tris HCl pH 8.0, and 10 µg of this resuspended DNA was elctrophoresed on a 1% agarose gel for 1600 volt hours, with *Hind*III-restricted Lambda DNA (Promega) as size standard. Separated bands of DNA were visualized using ethidium bromide and UV light. Several representative isolates were extracted and fingerprinted two or more times for comparison. Analyses were conducted as described above.

Analysis

Different fragment sizes of microsatellite markers were considered different alleles. Each fingerprint band was considered a locus with two possible alleles, present or absent. The dataset was corrected for clonal reproduction.
by removing isolates from each collection site that were genetically identical except where noted. In analyses concerning the Amazonian population (all populations except C940 and the Ecuadorian isolates), the dataset was clone-corrected by removing duplicate isolates that were genetically identical within a population rather than within a site.

Genotypic diversity within the five populations was calculated as the number of genotypes found using each marker type individually and using all three marker types together. Nei's gene diversity for each marker type and for all marker types together for each population was calculated with clone-corrected data using PopGen 1·32 software (Yeh & Boyle, 1997).

Weir and Cockerham’s theta (θ), which is comparable to Wright’s $F_{ST}$ (Weir & Cockerham, 1984), was calculated among pairs of populations using the program Multilocus (Mac version 1·21, Department of Biology, Imperial College at Silwood Park, UK). Gene flow between pairs of populations ($N_{m}$) was estimated based on the average coefficient of gene differentiation ($G_{ST}$) across all loci using PopGen 1·32. For both of these measures, values

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Table 1 Origin and genotypes of isolates of *Ceratocystis cacaofunesta* used for genetic analyses

<table>
<thead>
<tr>
<th>Population</th>
<th>Location collected</th>
<th>Isolate numbers</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecuador (unknown location)</td>
<td></td>
<td>C1984</td>
<td>VIIIa, VIIIb</td>
</tr>
<tr>
<td>Rondônia, Brazil</td>
<td>Rondônia, Brazil (site 1)</td>
<td>C1983 (= CBS 115172)</td>
<td>IXa</td>
</tr>
<tr>
<td>Rondônia, Brazil (site 2)</td>
<td></td>
<td>C2031</td>
<td>IXb</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>La Lola, Matine, Costa Rica</td>
<td>C1548 (= CBS 114722)</td>
<td>X</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>La Lola, Matine, Costa Rica</td>
<td>C1547</td>
<td>XI</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Bri Bri, Costa Rica</td>
<td>C1639</td>
<td>XII</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Cahuita, Costa Rica</td>
<td>C1640</td>
<td>XIII</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Turrialba, Costa Rica</td>
<td>C1778, C1779, C1776, C1549, C1775, C1550, C1634, C1635, C1636, C1638, C1642, C1777, C1637, C1638</td>
<td>XIV, XV, XVI, XVII, XVIII</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Costa Rica (unknown location)</td>
<td>C040 (= CBS 152·62)</td>
<td>XIX</td>
</tr>
<tr>
<td>Colombia</td>
<td>Palestina, Caldas, Colombia</td>
<td>C1935, C1947, C1937, C1936, C1937</td>
<td>XX, XXI, XXII, XXIII</td>
</tr>
<tr>
<td>Bahia, Brazil</td>
<td>Novo Oriente, Bahia, Brazil</td>
<td>C1595, C1593, C1594, C1596</td>
<td>XXIV, XXV, XXVI</td>
</tr>
<tr>
<td>Uruçuca, Bahia, Brazil</td>
<td>site 1</td>
<td>C1719, C1720, C1723, C1718, C1722</td>
<td>XXVI, XXVII, XXVIII, XXIX</td>
</tr>
<tr>
<td>Uruçuca, Bahia, Brazil</td>
<td>site 2</td>
<td>C1728, C1729, C1733, C1735, C1737, C1736, C1585, C1586, C1587</td>
<td>XXVI, XVI, XXX</td>
</tr>
<tr>
<td>Camacã, Bahia, Brazil</td>
<td>site 1</td>
<td>C1725, C1727, C1724, C1725, C1726, C1727</td>
<td>XXVI, XXVII, XXVIII, XXIX</td>
</tr>
<tr>
<td>Camacã, Bahia, Brazil</td>
<td>site 3</td>
<td>C1730, C1732, C1731, C1599, C1601, C1597, C1600</td>
<td>XXVI, XXVIII, XXXIII</td>
</tr>
<tr>
<td>Itabuna, Bahia, Brazil</td>
<td>site 1</td>
<td>C1598</td>
<td>XXIV</td>
</tr>
<tr>
<td>Itabuna, Bahia, Brazil</td>
<td>site 2</td>
<td>C1585, C1586, C1587, C1598</td>
<td>XXXIII</td>
</tr>
<tr>
<td>Itabuna, Bahia, Brazil</td>
<td>site 3</td>
<td>Ubaitaba, Bahia, Brazil</td>
<td>C1738–C1749</td>
</tr>
</tbody>
</table>

*a* C1642 and C1778 were collected from *Herrania* spp.

*b* C1749 was collected from soil under a diseased cacao tree.
obtained from microsatellite markers and nuclear DNA fingerprints were nearly identical and were averaged to obtain an overall nuclear DNA value.

To test for random mating within the Ecuadorian, Bahian and Amazonian (all populations except Ecuador combined) populations, linkage disequilibrium was analyzed using the index of association (IA) statistic in Multilocus. Randomization procedures were used (1000 replications, without replacement) to test the significance of the observed IA value.

Relationships among isolates were also examined using genetic distance matrices and UPGMA (unweighted pair group method with arithmetic mean) trees generated using PAUP* (Swofford, 1998). Nei’s genetic distance between populations and UPGMA dendrograms comparing populations were calculated using PopGen 1.32. Bootstrap values for the population trees were calculated from 100 bootstrap replicates of 100 UPGMA trees using SEQBOOT in PHYLIP version 3.6 (Felsenstein 1989, 1993).

Results

All 16 microsatellite markers were polymorphic for the isolates tested. Each locus had between two and 10 alleles (Table 2), and together they resolved 20 microsatellite genotypes among the 77 isolates tested from the five populations (Table 3). No microsatellite genotype was found in more than one population.

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Nuclear (CAT)5 fingerprinting resolved 34 consistently scorable bands (Fig. 1a, Table 2). Thirty-one of these bands were polymorphic, and bands of sizes of 6.5, 7.3 and 11.2 kb were present in all isolates. Other bands that could not be consistently scored between gel runs of the same isolates were excluded from the analysis. Fourteen different nuclear
fingerprint patterns were found. Isolates from Colombia had an identical (CAT)$_5$ fingerprint (Table 3), which was the same as that of 15 of the 17 isolates from Costa Rica. 

HaeIII mitochondrial DNA fingerprints yielded 30 consistently scorable bands (Fig. 1b). Bands of sizes of 2·3, 2·4, 2·5 and 4·0 kb were monomorphic, and the remaining bands were polymorphic among the isolates tested (Table 2). A total of 24 mitochondrial fingerprint patterns were found, and none were shared between populations. Bands were not as clearly separated as with the (CAT)$_5$ fingerprinting, so it is possible that some co-migrating bands that were scored as identical in different populations were actually of different sizes and were not homologous.

All three combined marker types resolved 34 total genotypes among the 77 isolates tested (Table 2). None of the multilocus genotypes were shared among populations. However, only seven unique genotypes were found among the nine isolates from Ecuador, nine unique genotypes were found among the 17 isolates tested from Costa Rica, and 19 unique genotypes were found among the 44 isolates from Bahia (Table 3). Further analyses of populations used clone-corrected data, i.e. only unique genotypes from a particular population were used.

Table 3 Number of genotypes identified in populations of *Ceratocystis cacaofunesta*

<table>
<thead>
<tr>
<th>Population</th>
<th>No. of isolates</th>
<th>No. of collection sites</th>
<th>No. of genotypes</th>
<th>Isolates after clone correction$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecuador</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Rondônia</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Colombia</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>17</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Bahia</td>
<td>44</td>
<td>9</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>all non- Ecuadorian populations</td>
<td>68</td>
<td>16</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>all populations</td>
<td>77</td>
<td>17</td>
<td>20</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$Data were clone-corrected by removing duplicate isolates within a site that had the identical genotype.

Figure 1 (a) Representative (CAT)$_5$ nuclear DNA fingerprints and (b) HaeIII mitochondrial DNA fingerprints of *Ceratocystis cacaofunesta* isolates from populations in Ecuador, Costa Rica, Colombia and Brazil (Rondônia and Bahia). Arrowheads indicate polymorphic bands. Sizes (kb) of standards are given on the right.
Based on Nei’s gene diversity from clone-corrected populations (Table 4), the populations from Ecuador and Rondônia had the highest gene diversity values, while those from Costa Rica, Colombia and Bahia had lower diversity values. The non-Ecuadorian population (all isolates except those from Ecuador and isolate C940) had a high gene diversity value. Table 4 also lists the gene diversity values of other homothallic *Ceratocystis* species based on comparable markers.

Weir and Cockerham’s theta (θ) was used to estimate the degree of differentiation between populations, using combined nuclear DNA markers (Table 5) from the clone-corrected dataset. Theta varies from zero (no differentiation among populations) to one (populations completely differentiated). Based on nuclear DNA markers, the Costa Rican and Colombian populations were the most similar (θ = 0.94), while the Bahian and Ecuadorian populations were the most differentiated (θ = 0.94). Other than the comparison between Costa Rican and Colombian populations, all θ values were above 0.7, indicating differentiation among the populations. The estimate of gene flow (Nm) was also used to make pairwise comparisons of populations with the two nuclear markers together using the clone-corrected dataset (Table 5). The value of Nm ranges from zero (no gene flow) to infinity (complete gene flow between populations). Analysis of nuclear DNA markers revealed that the Costa Rican and Colombian populations had the highest amount of probable gene flow (Nm = 4.96), while the Costa Rican and Bahian population comparison had the least (Nm = 0.07). Most other values of Nm were also relatively low, not exceeding 0.34.

The UPGMA trees constructed using microsatellite and (CAT)5 fingerprint data separated the isolates into two distinct groups, one containing all Ecuadorian isolates and the other containing isolates from Costa Rica, Brazil and Colombia (Fig. 2a,b). In both cases, the branches separating the Ecuadorian and non-Ecuadorian isolates had strong bootstrap support. With both nuclear marker types, there was moderate support of branches grouping the Bahian isolates, and the Colombian and Costa Rican isolates also grouped together. The three Rondonian isolates were divergent, but the nuclear DNA fingerprints of the Rondonian isolates grouped them with the Bahian isolates. A UPGMA tree constructed from *Hae*III mitochondrial DNA fingerprints showed similar trends, with the Bahian isolates grouping together and the Costa Rican and Colombian isolates grouping together, but the Ecuadorian isolates splitting into two groups (Fig. 2c). However, bootstrap support for the branches was generally low.

The UPGMA trees constructed using populations instead of isolates showed trends similar to those found when analyzing by isolate, but there was little bootstrap support for branches connecting populations (Fig. 3). Nuclear and mitochondrial markers showed slightly different
Figure 2 Dendrograms generated by UPGMA (unweighted pair group method, arithmetic mean) based on Nei's genetic distance among *Ceratocystis cacaofunesta* genotypes from populations in Ecuador, Costa Rica, Colombia and Brazil (Rondônia and Bahia), generated from (a) microsatellite data, (b) (CAT)$_5$ nuclear DNA fingerprint data and (c) *Haell*III mitochondrial DNA fingerprint data. Bootstrap values greater than 50 are shown. Roman numerals in (c) correspond to genotypes in Table 2.
trends. In each analysis, isolate C940 from Costa Rica was excluded because this isolate is genetically similar to the Ecuadorian isolates in phylogenetic analyses (Baker et al., 2003) and intersterility studies (Engelbrecht & Harrington, 2005). The other Costa Rican isolates and the Colombian population grouped together strongly based on the microsatellite and nuclear DNA fingerprint data, and the Bahian and Rondonian populations were strongly linked based on nuclear DNA fingerprints. There was moderate support for the branch connecting the Costa Rican and Colombian populations in the mitochondrial DNA analysis.

The index of association ($I_A$) was used to test for random mating in the Ecuadorian, Bahian and Amazonian (non-Ecuadorian) clone-corrected populations. The value of $I_A$ is expected to be zero in a randomly mating population, and 1,000 randomized replicates were performed to assess whether the value obtained was significantly different from zero. Nuclear markers in the Ecuadorian population showed index of association values consistent with an outcrossing population ($I_A = -0.08333$, $P = 0.595$ for microsatellites, and $I_A = 0.3741$, $P = 0.168$ for nuclear DNA fingerprints). Analysis of the group of populations other than the Ecuadorian population showed evidence of clonal reproduction (microsatellite $I_A = 2.1337$, $P < 0.001$; nuclear DNA fingerprint $I_A = 7.6492$, $P < 0.001$). The Bahian population showed evidence of clonal reproduction using microsatellites ($I_A = 0.6372$, $P = 0.04$). Analysis of nuclear DNA fingerprints of the Bahian population showed no evidence of clonal reproduction ($I_A = -0.1111$, $P = 1.000$), but this lack of resolution may be due to the small number of polymorphic loci (three) in the Bahian population.

Discussion

Despite the limitations of small sample sizes due to the rarity of the disease in some locations, the gene diversity values for the Rondonian and Ecuadorian populations of *C. cacaofunesta* are similar to those of indigenous populations of other homothallic *Ceratocystis* species analyzed using the same markers. Although the data may be considered preliminary, the diversity values found in Rondónia and Ecuador are similar to those found in a native population of *C. platani* from the eastern USA (Engelbrecht et al., 2004). They are also comparable to values obtained from a native population of *C. albopunctatus* in South Africa (Roux et al., 2001) and those of a native population of *C. virescens* in eastern North America (Harrington et al., 1998). All of these *Ceratocystis* species can produce perithecia and ascospores through selfing via unidirectional mating type switching (Harrington & McNew, 1997). Thus, even natural populations would be expected to have relatively low genetic diversity (Harrington et al., 1998), and introduced populations are expected to be essentially clonal and have very limited genetic diversity (Engelbrecht & Harrington, 2004).

Gene diversity values of two introduced populations of *C. platani* in Modesto, California and southern Europe (Engelbrecht & Harrington, 2004) were similar to those found in the *C. cacaofunesta* populations in Costa Rica, Colombia and Bahia, Brazil, suggesting that these three populations on cacao are the result of introductions. The Costa Rican and Colombian isolates share nuclear DNA fingerprints and group closely together in UPGMA analyses using all the markers, and they may have been derived from one interbreeding population based on analyses of nuclear markers. Except for the Costa Rica and Colombia populations, the samples of populations of *C. cacaofunesta* are highly differentiated from each other as shown by the theta values based on nuclear markers. This further supports the conclusion that the Costa Rican and Colombian isolates are from the same original population, perhaps on cuttings of cacao from the Upper Amazon, and it is possible that the Colombian population of *C. cacaofunesta* was introduced from Costa Rica.

The Bahian, Costa Rican and Colombian populations are likely to be introduced as all three locations are beyond the native range of cacao. Infested cuttings of *T. cacao* appear to be the means of introduction of *C. cacaofunesta* (Harrington, 2000). The disease has only been known in Bahia since 1997 (Bezerra, 1997), when the fungus was recognized in grafted cuttings in an experiment station.
The source of those cuttings is not known, but it is possible that they originated from the cacao experiment station in Rondônia where two isolates of *C. cacaofunesta* were collected for this study. The low level of gene flow between Bahia and the Costa Rican/Colombian populations suggests that the Bahian population was the result of a separate, independent introduction of a limited number of genotypes.

Although only three isolates were obtained from Rondônia, they were genetically unique, and the Rondonian population had gene diversity values similar to native populations of selfing *Ceratocystis* species. These *C. cacaofunesta* isolates were collected from within the native range of cacao and near its centre of genetic diversity (Laurent *et al*., 1993, 1994a,b; Figueira *et al*., 1994; N’goran *et al*., 1994; Ronning & Schnell, 1994; Warren, 1994; Whitkus *et al*., 1998; Marita *et al*., 2001). It is possible that these isolates represent an indigenous population of *C. cacaofunesta* on *T. cacao*. The populations in Costa Rica, Colombia and Bahia were more similar to the population in Rondônia than to the population in Ecuador, so it is likely that Rondônia or elsewhere in the Upper Amazon is the source of the introduced populations. Unfortunately, only three isolates of *C. cacaofunesta* from the Upper Amazon were collected, two closely related isolates from the experimental plantation in Ouro Preto do Oeste, and another from a small farm less than 50 km west, near the Bolivian border.

Apparently, the most susceptible cultivars of *T. cacao* planted in this region have died and the disease is rare in the surviving cultivars, making it very difficult to obtain isolates there. The initial plantings in the 1970s of ‘Theobahia’ genotypes in Ouro Preto do Oeste died quickly (Luiz Carlos Almeida, personal communication). *Theobahia* genotypes proved to be highly susceptible to *Ceratocystis* wilt in Bahia after introduction of *C. cacaofunesta* in 1997.

Like the Rondonian population, the Ecuadorian isolates had gene diversity values similar to indigenous populations of homothallic *Ceratocystis* species, even though they were collected outside *T. cacao*’s native range. It is possible that they represent an indigenous population that is pathogenic to another member of the genus *Theobroma*, or to a related genus, native to Ecuador west of the Andes (Baker, 1953; Purseglove, 1968). All the Ecuadorian isolates were collected from plantings of cacao in an experimental station in western Ecuador, where seven genotypes were identified among the nine isolates, suggesting a relatively high level of genetic diversity there. Attempts to locate the disease in commercial plantings in Ecuador failed, perhaps because commercial cultivars in the region are resistant to the disease. Cacao wilt was first described from this cacao-growing region (Rorer, 1918) and was initially devastating, so growers are likely to use resistant cacao cultivars. Isolate C940, obtained from a culture collection, was reportedly collected in Costa Rica, but it groups genetically with the Ecuadorian population (Baker *et al*., 2003; Engelbrecht & Harrington, 2005), and it may represent an introduction from Ecuador.

In addition to the nuclear and mitochondrial markers used here, the western Ecuadorian and Upper Amazon genotypes are distinguished by ITS-rDNA sequences (Baker *et al*., 2003), sequence analyses of mating type genes (unpublished), and intersterility (Engelbrecht & Harrington, 2005). These two sublineages might be considered different species, but they overlap in morphological characters (Engelbrecht & Harrington, 2005) and do not differ in pathogenicity to cacao seedlings (Baker *et al*., 2003). Using the phylogenetic species concept of Harrington & Rizzo (1999), these sublineages are not recognized as distinct species because they cannot be distinguished by diagnostic phenotypic characters. The sublineages may have arisen from diversification of an original population that was divided by the Andean geographical barrier.

Genetic markers such as DNA fingerprints and microsatellite markers can help to distinguish indigenous populations from introduced ones, as has been done with *C. platani* populations in the eastern USA and Europe (Engelbrecht *et al*., 2004). The data here support the hypotheses that the Costa Rican, Colombian and Bahian populations of *C. cacaofunesta* resulted from introductions, probably on cuttings, while the Rondonian and Ecuadorian populations may be indigenous. The Rondonian population is within the natural range of *T. cacao*, and the Upper Amazon was the source of *T. cacao* germplasm used elsewhere in Latin America and the Caribbean (Baker, 1953). The experiment station in Rondônia was not in existence when ceratocystis wilt first appeared in Costa Rica and Colombia, so the source of *C. cacaofunesta* in those countries was likely to be elsewhere in the Upper Amazon. However, the Bahian population of *C. cacaofunesta* is genetically closely related to the Rondonian population, and the disease in Bahia appeared in an experiment station (Bezerra, 1997) operated by the same federal agency that operates the Rondonian experiment station where the isolates were collected (Bastos & Evans, 1978). Great care should be taken in moving cuttings from the Upper Amazon or other locations in Latin America where *C. cacaofunesta* is present. The disease has caused dramatic losses in Bahia, and similar losses could develop if it was moved to cacao-growing regions of western Africa and Southeast Asia.

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Genetic diversity of *Ceratocystis cacaofunesta*


