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Genetic variation in eastern North American and putatively introduced populations of *Ceratocystis fimbriata* f. *platani*

C. J. B. ENGELBRECHT,* T. C. HARRINGTON,* J. STEIMEL* and P. CAPRETTI†
*Department of Plant Pathology, Iowa State University, 351 Bessey Hall, Ames, IA 50011, USA, †Dipartimento di Biotecnologie agrarie, Università di Firenze, Piazzale Cascine 28, 50144 Firenze, Italy

Abstract

The plant pathogenic fungus *Ceratocystis fimbriata* f. *platani* attacks *Platanus* species (London plane, oriental plane and American sycamore) and has killed tens of thousands of plantation trees and street trees in the eastern United States, southern Europe and Modesto, California. Nuclear and mitochondrial DNA fingerprints and alleles of eight polymorphic microsatellite markers of isolates of *C. fimbriata* from these regions delineated major differences in gene diversities. The 33 isolates from the eastern United States had a moderate degree of gene diversity, and unique genotypes were found at each of seven collection sites. Fingerprints of 27 isolates from 21 collection sites in southern Europe were identical with each other; microsatellite markers were monomorphic within the European population, except that three isolates differed at one locus each, due perhaps to recent mutations. The genetic variability of *C. fimbriata* f. *platani* in the eastern United States suggests that the fungus is indigenous to this region. The genetic homogeneity of the fungus in Europe suggests that this population has gone through a recent genetic bottleneck, perhaps from the introduction of a single genotype. This supports the hypothesis that the pathogen was introduced to Europe through Naples, Italy during World War II on infected crating material from the eastern United States. The Californian population may also have resulted from introduction of one or a few related genotypes because it, too, had a single nuclear and mitochondrial genotype and limited variation in microsatellite alleles.

Keywords: exotic pathogens, fungal pathogens, microsatellites, RFLP

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Introduction

Introduced plant pathogens have caused extensive damage to natural and urban forest ecosystems. Canker stain of plane tree, caused by the haploid ascomycete fungus *Ceratocystis fimbriata* (Ellis & Halsted) f. *platani* Walter, has killed tens of thousands of valuable street trees in the eastern United States (Walter *et al*. 1952), southern Europe (Panconesi 1999) and Modesto, California (Perry & McCain 1988), and the disease has been serious on plantation sycamores (*Platanus occidentalis*) in the southeastern United States (McCracken & Burkhardt 1977). Although *C. fimbriata* attacks a wide range of temperate and tropical plants (CABI 2001), the form *platani* is a monophyletic group specific to the genus *Platanus* (Baker *et al*. 2003) and should be recognized as a distinct species. Analysis of microsatellites (Barnes *et al*. 2001) and other markers (Santini & Capretti 2000; Baker *et al*. 2003) have shown that f. *platani* is distinct from *C. fimbriata* isolates from other hosts, and only isolates of f. *platani* are pathogenic to *Platanus* spp. (Baker *et al*. 2003). Hosts of *C. fimbriata* f. *platani* include *P. occidentalis* (American sycamore), *P. orientalis* (oriental plane) and their natural hybrid, *P. acerifolia* (London plane), which is particularly susceptible (Walter 1946), as well as *P. racemosa* (California sycamore) (Perry & McCain 1988). The native range of the pathogen is unknown, but seven of the 10 species of *Platanus* are native to southern North America (CABI 1965), and the close relatives of f. *platani* appear to be native to Latin America and the Caribbean region (Baker *et al*. 2003).

C. fimbriata f. *platani* is primarily a wound pathogen, infecting trees through pruning wounds or other injuries, although it also infects trees through roots (Vigouroux &

Correspondence: Dr Thomas C. Harrington. Fax: (515) 294 9420; E-mail: tcharrin@iastate.edu

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Stojadinovic 1990) and can spread to adjacent trees via root grafts (Mutto Accordi 1986). In urban plantings, the fungus is transmitted primarily on contaminated pruning equipment (Walter 1946). Once in the tree, the fungus moves through the xylem as a wilt pathogen and also causes cankers. Aleurioconidia of the fungus may survive several months or years in the wood of diseased trees (Grosclaude *et al*. 1995), and the fungus could thus survive in cut timber from infected trees.

Although canker stain has been found at low levels on American sycamore in natural forests of the eastern United States (Walter 1946; McCracken & Burkhardt 1977), most recorded instances of the disease have been in urban areas or in commercial plantations of *P. occidentalis* for fibre production. Canker stain was first noted on *Platanus* street trees in New Jersey in 1926 (Walter 1946) and in Pennsylvania in 1935 (Jackson & Sleeth 1935). Soon thereafter, the disease developed into an epidemic that caused devastating losses of London plane in the eastern United States, but establishment of sanitation practices, such as disinfecting pruning equipment, effectively reduced the level of the disease in the late 1940s (Walter *et al*. 1952). Sycamore plantations have been seriously affected in the southeastern United States, from Virginia and North Carolina to Arkansas and Louisiana (McCracken & Burkhardt 1977). In the 1980s, an outbreak of the disease was reported among *Platanus* street trees in Modesto, California (Perry & McCain 1988), where most of the planted *Platanus* trees have been killed.

Canker stain was first noted outside the United States in the 1940s, in Naples, Italy (Panconesi 1999). Since then, the disease has spread throughout the Italian peninsula (Panconesi 1999) and into France (Ferrari & Pichenot 1979, 1974, 1976; Vigouroux 1986; Grosclaude *et al*. 1991) and Switzerland(Matasci & Gessler 1997). The disease is also believed to be in Spain and Armenia (Panconesi 1999). Canker stain continues to cause major damage to urban street trees in these countries. It has been suggested that the canker stain pathogen was introduced to Italy during World War II, perhaps from crating materials from the eastern United States (Panconesi 1972, 1973; Cristinzio *et al*. 1973).

The fungus has two mating types and is able to outcross, but one of the mating types (MAT-2) is self-fertile (homothallic) through unidirectional mating type switching (Witthuhn *et al*. 2000). Because of selfing, an introduced strain of the fungus could spread via both asexual and sexual spores in an essentially clonal manner. Analysis of the genetic variation of the respective pathogen populations could identify genetic bottlenecks and test the hypothesis that the fungus was introduced to Europe.

The aim of this study was to use DNA fingerprinting techniques and polymorphic microsatellite markers to compare variability in the nuclear and mitochondrial genomes of *C. fimbriata* f. *platani* populations from Europe, the eastern United States and California. It was hypothesized that the European and Californian populations would have little genetic diversity if they were the result of recent introductions, and the eastern United States population would have more genetic diversity if that population were indigenous.

Materials and methods

Fungal isolates

Sixty-seven *C. fimbriata* isolates were collected from dead or dying *P. occidentalis*, *P. orientalis* and *P. acerifolia* trees in Italy, France, Switzerland, California and the eastern United States (Table 1). The isolates from Europe and California were from individual dead and dying street trees. No current epidemics of the disease on street trees in the eastern United States could be identified, but isolates from individual trees in commercial plantations were obtained from Virginia, North Carolina and Kentucky. All the plantations were within the natural range of *P. occidentalis*.

Nuclear DNA fingerprinting

Mycelium was grown in 20 mL liquid broth (2% malt extract and 0.2% yeast extract) in 125 mL flasks at room temperature for 2 weeks, and DNA was extracted and analysed following the methods of DeScenzo & Harrington (1994). Extracted DNA was quantified using a Hoefer DyNAQuant 200 fluorometer (Hoefer Scientific Instruments).

Total genomic DNA was restricted with *Pst* I and electrophoresed for 1600 volt hours in 1% agarose gels (19.5 × 25.5 cm) (Bio-Rad Certified Molecular Biology agarose, Bio-Rad), with *Hin*dIII-restricted Lambda DNA standards (Promega) on the outer lanes of each gel. Gels were dried, hybridized with the oligonucleotide $(CAT)_5$ labelled with ^{32}P and washed (DeScenzo & Harrington 1994). Hybridized gels were exposed to phosphor screens for 4–6 days and then visualized by scanning the screens with a Storm PhosphorImager (Molecular Dynamics).

QUANTITY ONE quantification software (version 4.1.1.003, Bio-Rad) was used to estimate the molecular weight of each fingerprint band, based on comparison with the Lambda *Hin*dIII size standards. All DNA restrictions and fingerprints were repeated at least once for each isolate. Only bands that were consistently visible and unambiguously scored in all replicate gels were analysed.

Mitochondrial DNA fingerprinting

*Hae*III cuts GC-rich DNA and allows visualization of AT-rich mitochondrial DNA (Wingfield *et al*. 1996). Twenty-five μg of total genomic DNA was restricted overnight

Table 1 Locations, hosts, and genotypes of *Ceratocystis fimbriata* f. *platani* isolates

Country	Location	Host	Genotype	Isolate numbers†
USA	Bertie, North Carolina (NC-1)	*P. occidentalis*	I	C1329
			II	C1330, C1331
			III	C1332, C1333, C1335–C1337
			IV	C1334
			V	C1338
			VI	C1342
	Coastal, North Carolina (NC-2)	*P. occidentalis*	VII	C1115, C1117
			VIII	C1116
	George, North Carolina (NC-3)	*P. occidentalis*	IX	C1340
	Halifax County, North Carolina (NC-4)	*P. occidentalis*	X	C1316
			XI	C1317 (CBS115162), C1320–C1324
			XII	C1318
			XIII	C1319, C1325
			XIV	C1326
			XV	C1327
			XVI	C1328
	Hickman, Kentucky (KY)	*P. occidentalis*	XVII	C1351
			XVIII	C1352
	Isle of White, Virginia (VA-1)	*P. occidentalis*	XIX	C1343, C1344
	Sussex, Virginia (VA-2)	*P. occidentalis*	XX	C1341
	Modesto, California (CA)	*Platanus* spp.	XXI	C858
			XXII	C859
			XXIII	C860, C1830
			XXIV	C1818–C1820
Italy	Unknown	*Platanus* sp.	XXV	C867
	Firenze	*P. acerifolia*	XXV	C1266 (CF 26)
	Brozzi, Firenze	*P. acerifolia*	XXV	C811 (CF 12), C1262 (CF 13)
	Camaiore, Lucca	*P. acerifolia*	XXV	C808 (CF 2), C1259 (CF 1)
	Versiliana, Lucca	*P. acerifolia*	XXV	C1275 (CF 44)
	Torre Annunziata, Naples	*P. acerifolia*	XXV	C1265 (CF 21)
	Padova	*P. acerifolia*	XXV	C1268 (CF 32)
	Crosone di Presano, Padova	*P. acerifolia*	XXV	C1270 (CF 37), C1271 (CF 38)
	Marina di Pisa, Pisa	*P. acerifolia*	XXV	C810 (CF 7), C1260 (CF 8)
	S. Rossore, Pisa	*P. acerifolia*	XXV	C809 (CF 5), C1261 (CF 11)
			XXVI	C1276 (CF 4)
	Aventino, Roma	*P. acerifolia*	XXVII	C807 (CF 15)
	Grillo, Roma	*P. acerifolia*	XXV	C812 (CF 16)
	Nomentano, Roma	*P. acerifolia*	XXV	C1264 (CF 17)
	Pantalica, Siracusa	*P. orientalis*	XXV	C1267 (CF 30)
	Tiziano, Roma	*P. acerifolia*	XXV	C1277 (CF 18)
	Castelfranco Veneto, Treviso	*P. acerifolia*	XXV	C1274 (CF 41)
	Vicenza	*P. acerifolia*	XXV	C1273 (CF 20)
	Legnago, Vicenza	*P. acerifolia*	XXVII	C1272 (CF 39)
	S. Bonifacio, Vicenza	*P. acerifolia*	XXV	C1269 (CF 34)
France	Unknown	*Platanus* sp.	XXV	C868 (CMW 2220)
Switzerland	Unknown	*Platanus* sp.	XXV	C866 (CMW 1895)

†Numbers are from the culture collection of T.C. Harrington; numbers in parentheses are those of the culture collection of P. Capretti (CF), M. Wingfield (CMW) or Centraalbureau voor Schimmelcultures (CBS).

at 37 °C with *Hae*III in a total volume of 500 μL (50 μL 10× buffer, 1 mM spermidine, 5 units/μg *Hae*III and 5 μL RNAse). Restricted DNA was precipitated, dried and washed (Wingfield *et al*. 1996). Washed DNA was resuspended in 37.5 μL of 10 mM Tris HCl pH 8.0, and 10 μg of this resuspended DNA was electrophoresed as described above. Separated bands of DNA were visualized using ethidium bromide and ultraviolet (UV) light. Analyses were conducted as described above. Some bands, such as the 2.28 kb band, were twice as intense in some isolates as the corresponding band in other isolates and were scored as either double or single bands.

Microsatellite markers

Sixteen polymerase chain reaction (PCR)-based microsatellite markers (Steimel *et al*. 2004) were used to compare genetic diversity of the three populations. A fluorescently labelled primer and an unlabelled primer flanking each of 16 microsatellite regions of three- or four-base repeats were used in the PCR amplification reactions as described in Steimel *et al*. (2004). The labelled PCR products were electrophoresed using an ABI Prism 377 DNA sequencer (Applied Biosystems Inc.), with verification of the size standard and matrix files using ABI GENESCAN analysis software version 3.1.2 (Steimel *et al*. 2004). The length of the PCR products was determined using GENOTYPER 2.0 software (Applied Biosystems Inc.), and each product length [rounded to the nearest base pair (bp)] was considered a different allele.

Analysis

Each fingerprint band was considered a locus with two possible alleles, present or absent. Different fragment sizes of microsatellite markers were considered different alleles. In all statistical analyses, the data set was clone-corrected by removing data from duplicate isolates from each collection site that were genetically identical.

Diversity within the three populations was calculated as the number of genotypes found using each marker type 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 using POPGEN 1.32 software (Yeh & Boyle 1997).

Four statistics were used to test for similarity and differentiation among the populations. Nei's genetic identity (I) was used to measure genetic similarity between populations (Nei 1972). Gene flow between pairs of populations (Nm) was estimated based on the average coefficient of gene differentiation (G_{ST}) across all loci. All three of these values were calculated using POPGEN 1.32. Weir & 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).

To test for random mating within the eastern United States population, the clone-corrected data set for this population was divided into seven subpopulations by site, and linkage disequilibrium was analysed using the index of association (I_A) statistic in MULTILOCUS. We used randomization procedures (1000 replications, without replacement) to test the significance of the observed I_A 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 2001).

Results

Fingerprinting of the nuclear genome of the *C. fimbriata* f. *platani* isolates using $(CAT)_5$ resolved a total of 30 consistently scorable bands ranging in size from 2.1 to 16.9 kb. Bands that were inconsistently scorable between the same isolates on different gels, such as bands at 17 and 17.2 kb in Fig. 1, were not scored. Nineteen of the scorable bands were polymorphic (Fig. 1), and there were 19 distinct nuclear genotypes (unique combinations of bands) delineated by $(CAT)_5$ (Table 2). Seventeen of the 19 genotypes were found among the 33 isolates from the eastern United States (Table 3). One unique genotype was found in California and one genotype was found among the 27 isolates from Europe (Table 2).

Fingerprinting of the mitochondrial genome using *Hae*III yielded 27 consistently scorable bands of 2.0–7.1 kb, of which 17 were polymorphic (Fig. 2). Bands that were not consistently scorable or varied in relative intensity among gels, such as bands visible in Fig. 2 at approximately 5.0 and 8.0 kb, were not scored. Such bands were probably incompletely digested mtDNA (Wingfield *et al*. 1996). Some bands in Fig. 2 (such as at 2.2 kb) were monomorphic among the isolates shown but polymorphic for other isolates. A total of 16 different mitochondrial genotypes were detected, 14 of which were found among isolates from the eastern United States (Tables 2 and 3). As with the nuclear fingerprints, the 27 European isolates shared a unique mitochondrial genotype, and the seven Californian isolates also shared a unique mitochondrial fingerprint pattern.

Eight of the microsatellite loci were monomorphic for all isolates tested, while eight were polymorphic. Microsatellite markers delimited a large amount of variation in the eastern United States population and limited variation in California and Europe (Table 2). Eleven different genotypes were found among the eastern USA isolates using microsatellite markers. All European isolates had identical alleles at each microsatellite locus, except that two isolates (C807 from Aventino, Rome and C1272 from Legnago, Vicenza) differed from the rest at locus CAG15 (3 bp size difference) and one other isolate (C1261 from San Rossore, Pisa) differed at locus GACA6K (5 bp size difference). Only loci CAG15 and CAA80 were polymorphic among the Californian isolates.

The three marker types showed different levels of gene diversity in the three populations, with microsatellite loci showing the most and nuclear fingerprints showing the least variation (Table 3, Fig. 3). Combining all three marker types, there were 20 genotypes in the eastern United States, three in Europe and four in California (Table 3, Fig. 4). The UPGMA analyses showed that the European and Californian isolates each formed tight clusters, distinct from each other but nested within the wide diversity of the eastern United States population (Fig. 4).

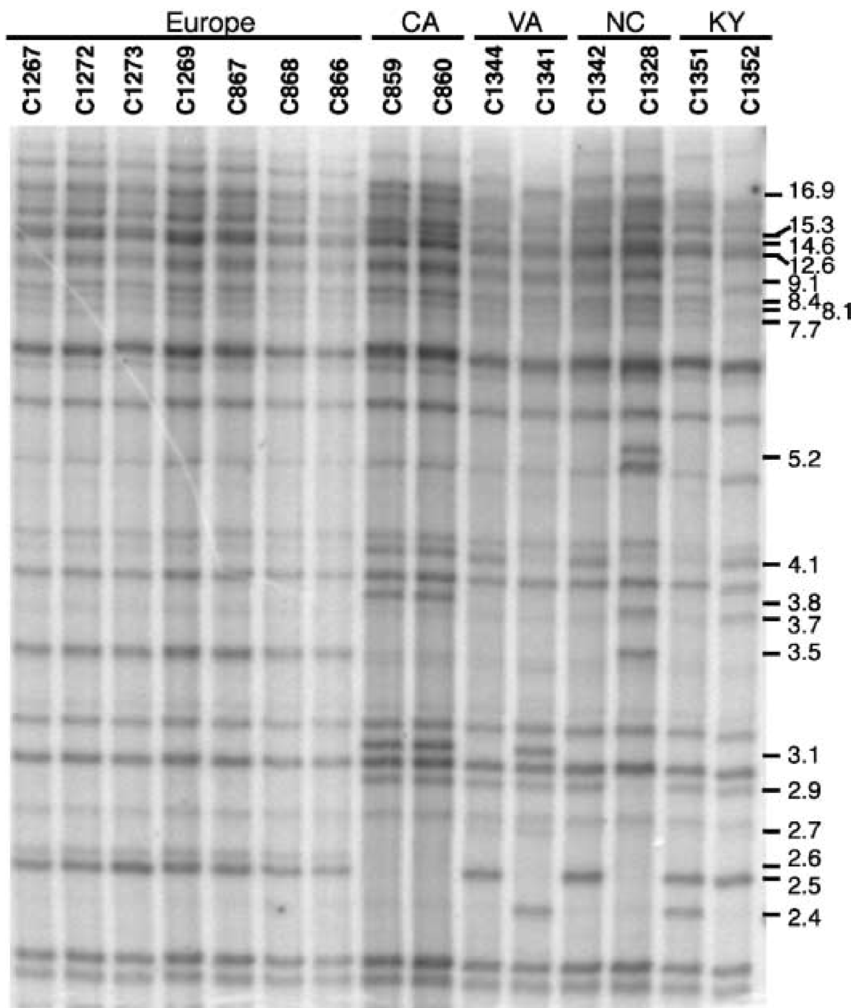

Fig. 1 Nuclear fingerprints using the probe $(CAT)_5$ of representative isolates of *Ceratocystis fimbriata* f. *platani* in Italy, California, Virginia, North Carolina and Kentucky. Sizes (in kb) of the scorable polymorphic bands are indicated on the right.

Nei's genetic identity showed greatest similarity between the eastern United States and European populations and considerably less similarity between the Californian and European populations (Table 4). Identity values were similar with the two fingerprinting methods and slightly lower using microsatellites. The coefficient of gene differentiation (G_{ST}) estimates the degree to which populations are genetically differentiated from each other, relative to the entire population as a whole. The value of G_{ST} ranges from zero (populations not differentiated) to one (completely differentiated). The G_{ST} values found here show that the European and Californian populations are well differentiated from each other but not from the eastern United States population (Table 4). The interrelated estimate of gene flow (*Nm*) indicates the degree to which gene flow may have occurred or may be occurring between populations; it is calculated based on G_{ST} and ranges from zero (no gene flow) to infinity (high gene flow). This measure also indicated that there has been little gene flow between the European and Californian populations, but there has been more gene flow between each of these populations and the eastern United States population.

Weir & Cockerham's theta is similar to Wright's F_{ST} but compensates for differences in sample sizes. Values of theta calculated from all marker types combined together varied from 0.4409 for the Californian and eastern United States population comparison to 0.9816 for the Californian and European population comparison (Table 4). Each comparison was significantly different from zero ($P < 0.001$ in each case) as determined using 1000 randomizations.

Linkage disequilibrium analysis of the eastern United States population yielded index of association (I_A) values of 0.9385 ($P < 0.001$) for nuclear fingerprints, 0.9671 ($P < 0.001$) for microsatellites and 1.953 ($P < 0.001$) for both nuclear markers combined. In a randomly mating population without selfing or asexual reproduction, there should be no association of alleles at unlinked nuclear loci, and I_A has an expected value of zero. High linkage equilibrium values would be expected for the mitochondrially inherited markers, and the value for mitochondrial fingerprints was 2.5434 ($P = 0.005$).

Table 2 Nuclear and mitochondrial fingerprint bands and microsatellite alleles for the 27 genotypes identified in populations of *C. fimbriata* f. *platani*

Genotype	No. of isolates	$(CAT)_5$ fingerprint†	*Hae*-III fingerprint†	Microsatellite loci‡							
				CAA9	CAA15	CAA38	CAA80	CAG15	CAT3K	CAT1200	GACA650
I	1	0100100001100111101	01110001111100001	410	288	157	300	359	326	409	259
II	2	0101100001011011101	01110001011100001	410	288	157	300	333	323	391	300
III	5	0100100001011011101	01110001011100001	410	288	157	300	333	323	391	300
IV	1	0100100001101111101	01110001111100001	410	288	157	300	359	326	409	259
V	1	0101100001011111101	01110001011100001	410	288	157	300	333	323	391	300
VI	1	0100100001011111001	01110011011100001	410	288	157	300	333	323	391	300
VII	2	0000001011111110101	00101000100010001	410	288	157	300	365	326	391	235
VIII	1	0100001011101110101	11001100100010001	400	288	157	300	356	323	391	235
IX	1	1100100000011011001	01101100100000011	386	317	157	300	365	323	391	328
X	1	0000011010111111101	11110000100010001	410	288	157	300	365	323	391	235
XI	6	0000110001011011001	01101000100000011	368	288	134	300	359	323	391	300
XII	1	0000110001011011001	01110000100010001	368	288	134	300	359	323	391	300
XIII	2	0000110001011011101	01101000100000011	368	288	134	300	359	323	391	300
XIV	1	0000110001011011001	01101000100010011	368	288	134	300	359	323	391	300
XV	1	0000110001011011101	01101000100010011	368	288	134	300	359	323	391	300
XVI	1	0000001011111111001	00101000100110001	410	288	157	300	365	326	391	235
XVII	1	1100100001011111100	01110000111110001	312	317	157	294	317	326	391	256
XVIII	1	0100100011001101100	00101000100011001	270	317	157	268	399	326	391	276
XIX	2	0100100001011111101	01110001011100001	410	288	157	300	333	323	391	300
XX	1	1001110001011111010	11110001011100100	410	317	157	300	337	329	391	300
XXI	1	0000110101001111110	11110001011100001	276	317	134	291	353	329	391	295
XXII	1	0000110101001111110	11110001011100001	276	317	134	291	346	329	391	295
XXIII	2	0000110101001111110	11110001011100001	276	317	134	297	346	329	391	295
XXIV	2	0000110101001111110	11110001011100001	276	317	134	291	343	329	391	295
XXV	24	0110001001011111101	00110000100010001	294	317	157	300	340	326	394	290
XXVI	1	0110001001011111101	00110000100010001	294	317	157	300	340	326	394	285
XXVII	2	0110001001011111101	00110000100010001	294	317	157	300	343	326	394	290

†Polymorphic bands are listed smallest to largest; band sizes as in Figs 1 and 2, present = 1, absent = 0.
‡Estimated band sizes in bp based on GeneScan analysis.

Table 3 Genotype diversity of the three *C. fimbriata* f. *platani* populations from 29 collection sites

Population	No. of collection sites	No. of isolates	No. of genotypes: Nuclear fingerprints	Mitochondrial fingerprints	Microsatellites	All markers	No. of isolates after clone correction†	Nei's gene diversity (H, clone corrected): Nuclear fingerprints	Mitochondrial fingerprints	Microsatellites	All markers
Eastern USA	7	33	17	13	11	20	20	0.1683	0.2006	0.2178	0.1911
Europe	21	27	1	1	3	3	24	0	0	0.0191	0.0042
California	1	7	1	1	4	4	4	0	0	0.0625	0.0137
All	29	67	19	15	18	27	48	0.1498	0.1540	0.2591	0.1753

†Clone correction removed isolates that had genotypes identical to other isolates from the same site.

Table 4 Nei's genetic identity (I), coefficient of gene differentiation (G_{ST}), estimate of gene flow (Nm), and Weir and Cockerham's theta (θ) of comparisons between populations of *C. fimbriata* f. *platani*

	I				G_{ST}†				Nm‡				θ§			
Populations	nuc¶	mito¶	micro¶	all	nuc	mito	micro	all	nuc	mito	micro	all	nuc	mito	micro	all
Europe vs. California	0.7000	0.7037	0.5879	0.6776	1.000	1.000	0.8289	0.9470	0.000	0.000	0.1032	0.0280	1.000	1.000	0.9349	0.9816
Eastern USA vs. Europe	0.8882	0.9051	0.7278	0.8601	0.3860	0.3108	0.5074	0.4005	0.7953	1.1085	0.4854	0.7485	0.5603	0.4741	0.6757	0.5746
Eastern USA vs. California	0.8370	0.8761	0.6861	0.8197	0.4753	0.3672	0.4928	0.4468	0.5519	0.8616	0.5146	0.6190	0.4584	0.3281	0.5350	0.4409
All populations	—	—	—	—	0.6886	0.6258	0.6700	0.6625	0.2261	0.2990	0.2463	0.2547	0.6425	0.5681	0.6990	0.6382

†G_{ST} ranges from 0 (no differentiation between populations) to 1 (complete differentiation of populations).
‡Nm ranges from 0 (no gene flow) to infinity (complete gene flow).
§θ ranges from 0 (no differentiation between populations) to 1 (complete differentiation of populations).
¶nuc = nuclear DNA fingerprints; mito = mitochondrial DNA fingerprints; micro = microsatellite markers.

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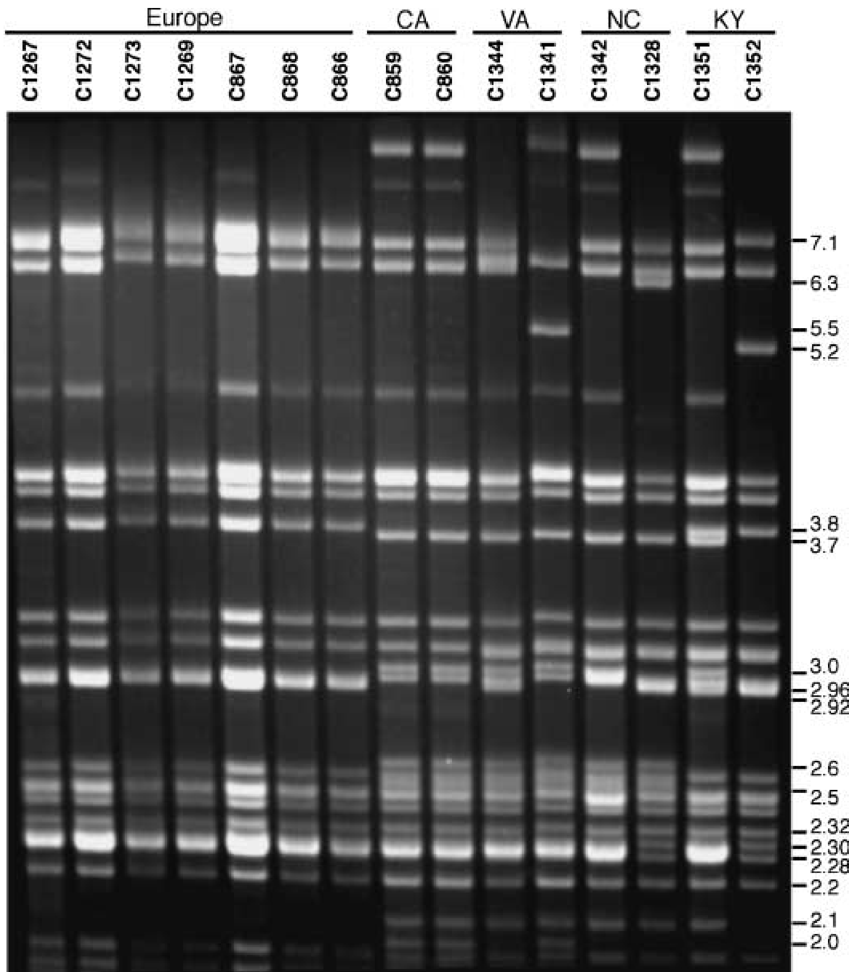

Fig. 2 Mitochondrial fingerprints of total genomic DNA restricted with *Hae*III and visualized using ethidium bromide staining. The gel shows representative isolates of *Ceratocystis fimbriata* f. *platani* from Italy, California, Virginia, North Carolina and Kentucky. Sizes (in kb) of the scorable polymorphic bands are indicated on the right.

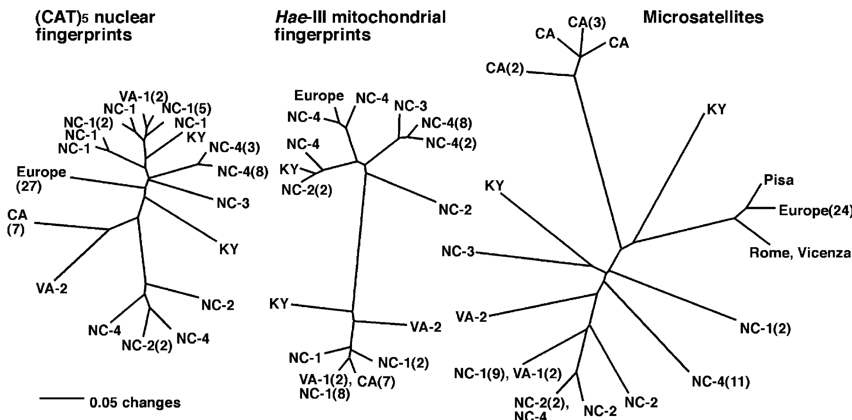

Fig. 3 UPGMA dendrograms showing relationships among *C. fimbriata* f. *platani* isolates using nuclear DNA fingerprints, mitochondrial DNA fingerprints and microsatellites. Letters indicate locations where the isolates were collected (California, Kentucky, North Carolina, Virginia or Europe). If more than one isolate from a location had the same genotype, then the number of isolates is given within parentheses.

Discussion

In contrast to the European and Californian populations, the eastern United States population of *C. fimbriata* f. *platani* was very diverse. As many as seven genotypes of *C. fimbriata* f. *platani* were found at individual sites in the eastern United States, and each of the 20 genotypes found there was found at only one site. Multiple isolates of a single genotype were found at some of the sites, probably due to local, clonal spread through conidia or sexual spores (ascospores), which are produced primarily through selfing (Witthuhn *et al*. 2000).

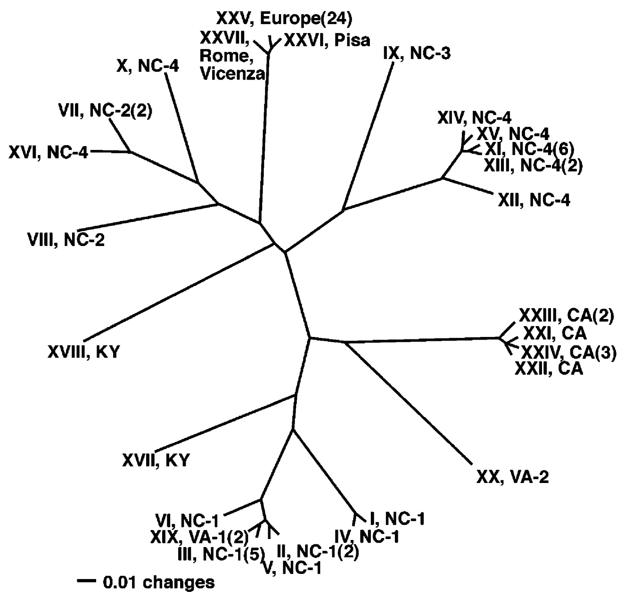

Fig. 4 UPGMA dendrogram showing relations among *C. fimbriata* f. *platani* isolates combining all data types (nuclear DNA fingerprints, mitochondrial DNA fingerprints and microsatellites). Roman numerals indicate genotypes and letters designate location from which isolates were collected (California, Kentucky, North Carolina, Virginia or Europe). If more than one isolate from a location had the same genotype, then the number of isolates is given within parentheses.

The relatively high level of diversity in the eastern United States population is similar to that of other putatively natural populations of homothallic *Ceratocystis* species (Harrington *et al*. 1998; Roux *et al*. 2001), suggesting that *C. fimbriata* f. *platani* is indigenous to the eastern United States. The levels of gene diversity found in the eastern United States using fingerprints (nuclear $H = 0.1683$, mitochondrial $H = 0.2006$) were slightly lower than those of a population of *C. albofundus* in South Africa analysed using the same markers (nuclear $H = 0.2137$, mitochondrial $H = 0.2490$) (Roux *et al*. 2001) but slightly higher than those of a population of *C. virescens* in eastern North America (nuclear $H = 0.0935$, mitochondrial $H = 0.0928$) (Harrington *et al*. 1998). All three of these *Ceratocystis* species can produce perithecia and ascospores through selfing via unidirectional mating type switching (Harrington & McNew 1997). In contrast, comparable values for nuclear and mitochondrial markers in a strictly heterothallic species (*C. eucalypti*) were $H = 0.3747$ and $H = 0.1115$, respectively (Harrington *et al*. 1998). Index of association values in the eastern United States population of *C. fimbriata* f. *platani* suggest that reproduction via selfing and/or mitotic reproduction is common, and selfing is probably the main means of reproduction, with rare outcrossing.

It is noteworthy that significant mortality caused by *C. fimbriata* f. *platani* has been reported almost exclusively on trees in environments that are highly modified by humans. The scattered nature of individual sycamores in natural forests, limited root grafting and the relative rarity of wounds (infection courts) may account for the low levels of disease found in natural stands (Walter 1946). However, the disease does occur in natural stands of *P. occidentalis* in the eastern United States (Walter 1946; McCracken & Burkhardt 1977), and Walter (1946) suggested that the fungus was native to this region. *Platanus occidentalis* is native in all the states in eastern North America in which canker stain had been reported up until the 1970s. Individual trees of *P. occidentalis* vary in susceptibility to *C. fimbriata* f. *platani* (El Modafar *et al*. 1995), and it is reasonable to suggest that this host-specialized pathogen evolved in North America. The high gene diversity and relatively low level of differentiation (as shown by G_{ST} and theta values) found in the eastern United States population of *Ceratocystis fimbriata* f. *platani* supports the hypothesis that the fungus is indigenous to that region.

The uniformity of the nuclear and mitochondrial DNA fingerprints of the Modesto, California population of *C. fimbriata* f. *platani* suggests that this population has experienced a recent genetic bottleneck. *Ceratocystis fimbriata* forms three different asexual states and can produce fruiting bodies (perithecia) and sexual spores (ascospores) through selfing (Harrington & McNew 1997; Witthuhn *et al*. 2000). Thus, if a single genotype had been introduced to Modesto, the fungus could persist and spread as an essentially clonal population. The slight variation in microsatellite markers in the Californian population may reflect recent mutations after introduction of a single genotype, because highly polymorphic microsatellite markers can undergo rapid change (Wostemeyer & Kreibich 2002).

Aside from the Modesto, California population, reports of *C. fimbriata* f. *platani* in North America are only from the eastern United States. Perry & McCain (1988) speculated that the fungus had been present in Modesto since the 1960s. Fortunately, the pathogen has not spread to neighbouring communities or nearby stands of native California sycamore (*P. racemosa*), a susceptible host, nor to neighbouring communities in California (Perry & McCain 1988). Because most of the *Platanus* trees in Modesto have died, there are now very few trees to sample for isolates. The seven isolates obtained show diversity values similar to those of the European population.

The genetic uniformity of the European population is also typical of a pathogen that has undergone a recent genetic bottleneck (Nei *et al*. 1975). The European and Californian populations are highly differentiated from each other, but their genotypes clearly fall within the diversity of the eastern United States population. Gene flow estimates suggest that both the European and Californian populations were derived independently from different populations in the eastern United States. Other studies

have verified that the European and United States populations have identical rDNA-internal transcribed spacer (ITS) sequences (Baker *et al*. 2003), are morphologically identical (Engelbrecht & Harrington 2005) and represent a single species.

Although the European population was sampled from 21 sites, both from *P. acerifolia* and *P. orientalis*, the only genetic variation found was in two highly polymorphic microsatellite loci, and these new alleles could have arisen through mutation from a single introduced genotype. Barnes *et al*. (2001) reported variation between French isolates of *C. fimbriata* f. *platani* using a microsatellite marker, but we did not find any variation among our European isolates using that same marker (unpublished data). Santini & Capretti (2000) found no diversity within the European population using random amplified polymorphic DNA marker (RAPD) and minisatellite (M13) markers, but they did not test those markers on North American isolates of *C. fimbriata* f. *platani*.

A large number of shipments of equipment and supplies was transported to the Mediterranean area during World War II from the eastern United States, particularly from the Philadelphia area, where canker stain was epidemic in the 1930s and 1940s (Walter *et al*. 1952). Dead trees in this area were probably used for crating material, and the fungus survives well in wood (Grosclaude *et al*. 1995) and sporulates heavily on cut surfaces. The perithecia and ascospores are well adapted for insect dispersal, and nitidulid beetles (Moller & DeVay 1968) or other insects (Hinds 1972) in Europe could have served as vectors. Alternatively, *C. fimbriata* may be dispersed in the insect frass of ambrosia beetles (Iton 1960; Rossetto & Medeiros 1967), which may have begun tunnelling in infected *Platanus* wood in the United States. The likelihood of transmission and successful establishment of the pathogen by such means is very low, but the large volume of such material brought into Europe during World War II may have allowed a single genotype of the pathogen to become established. Chief among the receiving ports during World War II was the Naples–Caserta area, believed to be the initial focus of the current European epidemic (Cristinzio *et al*. 1973; Panconesi 1999).

Genetic markers such as $(CAT)_5$ and *Hae*III fingerprinting bands and microsatellite markers are proving to be powerful tools in tracing the movements by humans of forest pathogens (Milgroom *et al*. 1996; Et-touil *et al*. 1999; Coetzee *et al*. 2001). The expansion of international trade will probably result in increased movement of pathogens between continents (Harrington & Wingfield 1998), and it is important to identify the means of such introductions. The rapidity with which *C. fimbriata* f. *platani* has spread through southern Europe, and the degree of damage it has caused there, emphasize the need for increased attention to developing and enforcing phytosanitary measures. The recent concern for movement of pests in solid wood packing materials is justified by the purported shipment of the Dutch elm disease fungus to the eastern United States and elsewhere (Brasier 1990) and now *C. fimbriata* f. *platani* to Italy.

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This work was from the Ph.D. Thesis of Christine Engelbrecht, who completed her Ph.D. with Dr. Harrington at Iowa State University (ISU) in 2004. She continues at Iowa State University as an Extension Plant Pathologist. Thomas Harrington is a Professor of Plant Pathology at ISU, specializing in forest pathology and mycology. Joe Steimel works in the laboratory of Dr. Harrington at ISU. Paolo Capretti is an Associate Professor of Forest Pathology at the University of Florence, specializing in fungal epidemiology, population biology, insect-fungus interactions and biological control.