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Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore

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Abstract: *Ceratocystis fimbriata* is a large, diverse complex of species that cause wilt-type diseases of many economically important plants. Previous studies have shown that isolates in three monophyletic lineages within the Latin American clade of *C. fimbriata* are host-specialized to cacao (*Theobroma cacao*), sweet potato (*Ipomoea batatas*) and sycamore (*Platanus* spp.), respectively. We paired testers of opposite mating type from isolates of these lineages to find intersterility groups. Two intersterility groups corresponded to the sweet potato and sycamore lineages, respectively. The cacao lineage contained two intersterility groups, corresponding to two genetic sublineages centered in western Ecuador and Brazil/Costa Rica/Colombia. Six isolates from cacao that were not members of the cacao lineage and were not pathogenic to cacao in an earlier study also were intersterile with members of the two cacao intersterility groups. Some pairings between testers from different lineages or sublineages yielded perithecia from which a few abnormal progeny could be recovered, typical of interspecific hybrids. These progeny showed abnormal segregation of the *MAT-2* gene and mycelial morphology, showing that they were indeed the result of crosses. Isolates of the sweet potato, cacao, and sycamore lineages were indistinguishable morphologically except for the presence or absence of a doliform (barrel-shaped) conidial state and minor differences in size of perithecial bases and necks and ascospores. *C. fimbriata* originally was described from sweet potato. We describe the cacao pathogen as a new species, *Ceratocystis cacaofunesta* and we raise the sycamore pathogen from a form to species *Ceratocystis platani*.

Key words: biological species, hybridization, partial interfertility

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INTRODUCTION

Ceratocystis fimbriata is a fungal plant pathogen that attacks a variety of temperate and tropical plants. Substantial genetic variation (Baker et al 2003, Barnes et al 2001, Marin et al 2003, Steimel et al 2004) and wide geographic and host ranges (CABI 2001, Webster and Butler 1967a) suggest that *C. fimbriata* contains many undescribed, cryptic species (Baker et al 2003, Harrington 2001). Phylogenetic analyses of rDNA-ITS and *MAT-2* mating type gene sequences reveal three geographic clades within *C. fimbriata,* centered in Asia, North America and Latin America, respectively (Harrington 2000). Within each geographic clade are several host-associated lineages that are likely cryptic species. Among the many lineages within the Latin American clade, isolates from cacao (*Theobroma cacao*), sweet potato (*Ipomoea batatas*) and sycamore (*Platanus* spp.) form three distinct ITS genotypes (Baker et al 2003). They differ in microsatellite alleles (Steimel et al 2004) and isolates in these lineages are strongly specialized to their respective hosts in inoculation studies (Baker et al 2003). Host specialization may be driving speciation.

We employ a species concept that defines species as ''. . . the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters'' (Harrington and Rizzo 1999). Intersterility may be a phenotypic character that can help to identify biological species. Perhaps more importantly, it helps to identify barriers to gene flow between species, which might identify distinct lineages. In addition to morphology, host specialization may be used as a diagnostic species character.

The aim of this study was to determine intersterility groups of representative *C. fimbriata* isolates from the sweet potato, cacao and sycamore lineages. Existence of reproductive barriers between these host-specialized lineages would support the hypothesis that these closely related lineages are distinct species. Furthermore, morphological characters were compared and two new species were recognized.

MATERIALS AND METHODS

Fungal isolates.—Isolates of *C. fimbriata* were obtained from cacao (or the closely-related *Herrania* sp.) or sycamore trees showing wilt symptoms, from decaying sweet potato storage

TABLE I. *Ceratocystis fimbriata* isolates used in intersterility experiments and morphological study

Isolate[a]	Other number[b]	Host plant	Location collected
C1593		cacao	Bahia, Brazil
C1587		cacao	Bahia, Brazil
C1597		cacao	Bahia, Brazil
C1983	CBS 115172	cacao	Rondônia, Brazil
C1984		cacao	Rondônia, Brazil
C2031		cacao	Rondônia, Brazil
C1935		cacao	Colombia
C1936		cacao	Colombia
C1937		cacao	Colombia
C1947		cacao	Colombia
C1547		cacao	Costa Rica
C1548	CBS 114722	cacao	Costa Rica
C1549		cacao	Costa Rica
C1634		cacao	Costa Rica
C1638		cacao	Costa Rica
C1642		*Herrania* sp.	Costa Rica
C940	CBS 152.62	cacao	Costa Rica (Ecuadorian lineage)
C1004	CBS 153.62	cacao	Ecuador
C1690		cacao	Ecuador
C1691		cacao	Ecuador
C1695	CBS 1151163	cacao	Ecuador
C1751		cacao	Ecuador
C1833	CBS115169	cacao	Ecuador
C1834		cacao	Ecuador
C1835		cacao	Ecuador
C1831		cacao (NC)[c]	Ecuador
C1696		cacao (NC)	Ecuador
C1832		cacao (NC)	Ecuador
C1836		cacao (NC)	Ecuador
C1584		cacao (NC)	Trinidad
C1750		cacao (NC)	Colombia
C1354		sweet potato	Japan
C1418		sweet potato	North Carolina, USA
C1476	ICMP 8579	sweet potato	Papua New Guinea
C1317	CBS 115152	sycamore	North Carolina, USA
C1339		sycamore	Virginia, USA
C1351		sycamore	Kentucky, USA

[a] Isolate numbers in the collection of T. C. Harrington.
[b] CBS = Centraalbureau voor Schimmelcultures; ICMP = Landcore Research New Zealand.
[c] NC = isolated from cacao but not of the cacao lineage and not pathogenic to cacao.

roots and from culture collections (TABLE I). All isolates are maintained in the culture collection of the junior author.

Intersterility experiments.—Most field isolates of *C. fimbriata* possess both idiomorphs (*MAT-1* and *MAT-2*) at the mating-type locus and are self-fertile due to unidirectional mating-type switching (Harrington and McNew 1997, Webster and Butler 1967b) through deletion of the *MAT-2* idiomorph (Witthuhn et al 2000b). Selfings of *C. fimbriata* typically produce both self-sterile (MAT-1) and self-fertile (MAT-2) progeny. For determining mating compatibility, MAT-1 and MAT-2 tester strains of several isolates were obtained from single ascospores produced by selfings as described in Harrington and McNew (1997). Ascospore masses were dissolved in a drop of Isopar M (a light sulfur oil, Exxon Corp.), which separates the spores and the dispersed spores were streaked onto malt-yeast extract agar (MYEA; 2% agar, 2% malt extract, 0.2% yeast extract). Young colonies from single ascospores then were transferred to fresh plates after ascospore germination. The MAT-1 strains were named by adding the suffix ''-ss'' (self-sterile) to the isolate number. Because most MAT-2 strains are self-fertile and therefore difficult to use in pairing studies, single-ascospore, MAT-2 progeny were transferred repeatedly to new plates and observed for self-sterile, mutant sectors. Generally, these mutant sectors lacked protoperithecia and could not, therefore, self or serve as females in crosses. Mutant, MAT-2 sectors were named by adding the suffix ''-sec'' (sector) to the isolate number.

To confirm that these MAT-2 testers retained the *MAT-2* gene, genomic DNA was extracted using the methods of DeScenzo and Harrington (1994), and PCR was used to amplify a portion of the *MAT-2* gene. We used the primers CFM2-1 (5′-GCTACATTTTGTATCGCAAAGAC-3′) and CFM2-2 (5′-TAGTGGGGATATGTCAACATG-3′), which amplify a portion of the high mobility group (HMG) box of the *MAT-2* gene of this species (Witthuhn et al 2000b). The PCR reactions had a total volume of 100 μL, containing 2.5 units *Taq* DNA polymerase (Promega Inc., Madison, Wisconsin), 1× PCR reaction buffer, 4 mM MgCl2, 200 μm DNTPs, 5% (V/V) DMSO, and 0.25 μm of each primer. PCR cycling conditions were an initial denaturation 94 C for 95 s followed by 35 cycles of denaturation (94 C) for 35 s, annealing 58 C for 60 s, and extension 72 C for 60 s. Final extension occurred at 72 C for 15 min. Several mutant sectors did not retain the *MAT-2* gene (there was apparently a spontaneous loss of the *MAT-2* idiomorph), but those that did retain the *MAT-2* idiomorph were used in pairings. We obtained MAT-2 tester strains from two sweet potato, three sycamore and five cacao isolates; MAT-1 strains were obtained from three sweet potato isolates, three sycamore isolates and 23 cacao isolates.

Procedures for intersterility tests were similar to those of Harrington and McNew (1998). Tester strains were grown on MYEA at room temperature (about 22–24 C). Seven days after transfer, each spermatizing plate was flooded with 10 mL of sterile deionized water, the mycelium was scraped, and 1 mL of the resulting spore/mycelium suspension was applied to the mycelium on the recipient plate. All tester strains were used as both spermatizers and recipients, and all possible pairings of strains were performed at least twice using freshly grown cultures. Spermatized plates were incubated at room temperature and checked periodically (up to 30 d) for production of perithecia and ascospores. Ascospores were observed under 500× magnification, and ascospores were streaked on MYEA plates and incubated at room temperature to assess viability.

A second pairing experiment compared intersterility among several cacao isolates, including some cacao isolates that did not fall within either cacao pathogen sublineage and were not pathogenic to cacao (Baker et al 2003). Five MAT-2 mutant strains were obtained from self-fertile cacao isolates as above. Field isolate C1004 from Ecuador was not self-fertile, but it contained the *MAT-2* gene and functioned as a MAT-2 in test pairings, so a single-conidium strain of this isolate was obtained and named C1004-sec1. These six MAT-2 tester strains were paired against 23 MAT-1 strains and against themselves using the methods described above. Analysis of mitochondrial DNA fingerprints of progeny from some of the earlier crosses using the methods of Wingfield et al (1996b) showed that even when a MAT-1 tester was used as the spermatizing strain, the MAT-1 strain was always the female in crosses, so reciprocal pairings were not performed in the later cacao pairings.

Progeny analysis.—Single-ascospore progeny from representative crosses were collected using the methods described above. Progeny sets were isolated from a single perithecium when possible (within-lineage pairings), but pairings between host groups and between the two cacao sublineages yielded so few viable progeny that ascospores from several perithecia were combined to obtain sufficient numbers for analyses. We observed mycelial morphology on MYEA of all progeny and compared them to the parents to determine parental and nonparental morphotypes. Genomic DNA was extracted from progeny using the methods of DeScenzo and Harrington (1994) and tested for the presence of the *MAT-2* gene as described above.

Morphological studies.—Cultures were grown concurrently at room temperature (about 22–24 C) on MYEA. Three sweet potato and three sycamore isolates were included in the morphological study, as were the following cacao isolates: C1547, C1548, C1638 (Costa Rica population); C1587, C1593, C1597 (Bahia, Brazil population); C940, C1690, C1691, C1695, C1751, C1833, C1834, C1835 (Ecuador population); C1935, C1936, C1937, C1947 (Colombia population); and C1983, C1984, and C2031 (Rondônia, Brazil population). Spores and mycelium were stained in lactophenol on microscope slides and observed using an Olympus BHS compound microscope (Olympus America Inc., Melville, New York). The microscope was connected to a Kodak Digital Science DC120 Zoom Digital Camera and OpenLab 2.2.5 imaging software (Improvision Ltd., Coventry, England) was used to take measurements. Dimensions of ascospores, endoconidia, and aleurioconidia were measured using the 40× objective lens, while dimensions of conidiophores were measured using the 20× objective lens. Since perithecia are too large to be measured easily by this method, perithecia measurements were taken at 250× magnification using an ocular micrometer. Measurements were taken from ten representative spores or structures of each isolate. Analysis of variance was used to compare variation in morphological measurements among populations and among isolates within populations. In instances with significant ($P < 0.05$) variation among populations, Duncan's multiple range test was used to compare means. All statistics were performed using SAS version 8.2 statistical software (SAS Institute, Cary, North Carolina).

RESULTS

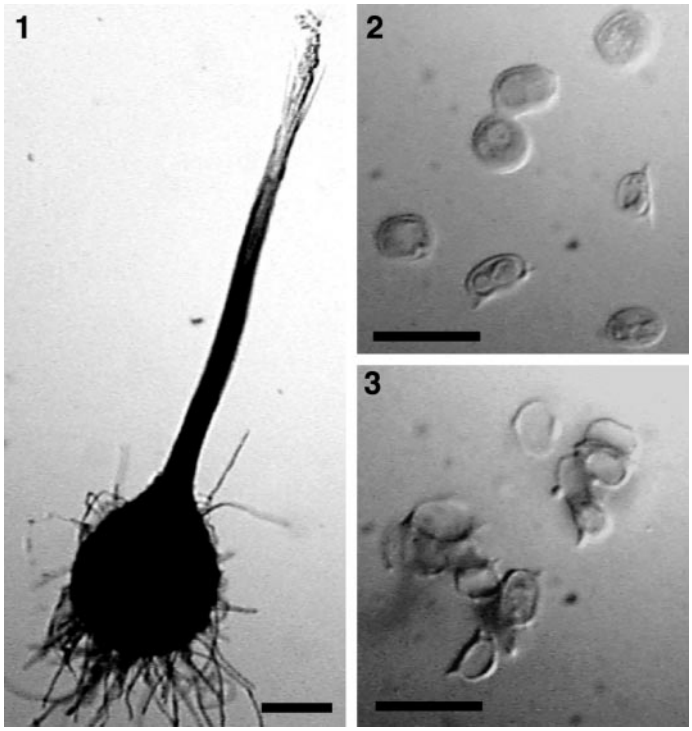
Intersterility.—Most pairings between sweet potato testers of opposite mating type and between sycamore testers of opposite mating type resulted in abundant perithecia within 2 wk; ascospore masses were large, viscous and opaque, and ascospore production and viability were similar to those of a selfing (TABLE II). Ascospores from these crosses appeared normal under high magnification (FIGS. 1, 2).

Most pairings between testers from different host groups resulted in few or no perithecia or perithecia without ascospore masses. Some pairings between testers from different host groups resulted in production of a few perithecia with ascospore masses that were transparent or milky in appearance. There were few ascospores in these masses with many empty of

TABLE II. Pairings between MAT-1 and MAT-2 testers of islates of *Ceratocystis fimbriata* from three hosts

	Recipient strains															
	MAT-1									*MAT-2*						
	cacao			sweet potato			sycamore			cacao		sweet potato		sycamore		
Spermatizing strains	C940 ss	C1548 ss	C1587 ss	C1354 ss	C1418 ss	C1476 ss	C1317 ss	C1339 ss	C1351 ss	C940 sec5	C1587 sec2	C1354 sec1	C1418 sec1	C1317 sec1	C1339 sec1	C1351 sec1
C940-ss	–[a]	–	–	–	–	–	–	–	–	C	H	H	P	P	P	P
C1548-ss	–	–	–	–	–	–	–	–	–	–	C	H	P	P	P	P
C1587-ss	–	–	–	–	–	–	–	–	–	–	C	P	P	H	–	P
C1354-ss	–	–	–	–	–	–	–	–	–	H	S	C	C	–	–	P
C1418-ss	–	–	–	–	–	–	–	–	–	H	H	C	C	H	–	–
C1476-ss	–	–	–	–	–	–	–	–	–	–	S	C	–	H	–	–
C1317-ss	–	–	–	–	–	–	–	–	–	P	S	H	H	C	C	C
C1339-ss	–	–	–	–	–	–	–	–	–	H	H	H	–	C	C	C
C1351-ss	–	–	–	–	–	–	–	–	–	P	S	–	–	C	C	C
C940-sec5	C	–	H	P	H	P	H	H	H	–	–	–	–	–	–	P
C1587-sec2	H	C	C	H	P	H	H	H	H	S	S	S	S	S	S	S
C1354-sec1	H	P	H	C	C	C	H	H	–	P	S	–	P	P	–	–
C1418-sec1	P	H	P	C	C	C	H	H	H	–	S	P	–	P	–	P
C1317-sec1	P	H	–	H	H	H	C	C	C	–	S	–	–	–	–	H
C1339-sec1	H	H	–	H	H	–	C	C	C	–	S	–	–	–	–	P
C1351-sec1	P	P	H	–	H	–	C	C	C	–	S	–	–	P	–	P

[a] No perithecia (−), sterile perithecia (P), perithecia with few viable ascospores, typical of an interspecific hybridization (H), perithecia with many viable ascospores, typical of an interfertile cross (C) or induced selfing (S).



FIGS. 1–3. Perithecium and progeny from pairings of testers from the *Ceratocystis fimbriata* complex. 1. Perithecium from C1418-ss × C1354-sec1, an interfertile cross between two sweet potato testers. 2. Normal ascospores from intra-specific cross C1418-ss × C1354-sec1. 3. Deformed ascospores from C1418-ss × C1317-sec1, a hybridization between testers from sweet potato and sycamore. Scale bars: 1 = 100 μm; 2, 3 = 10 μm.

cytoplasm or misshapen when viewed microscopically (FIG. 3). If ascospore progeny were recovered, the colonies generally were abnormal, fluffy (abundant aerial mycelium) and showed restricted growth. Such progeny are typical of interspecific hybridizations in other *Ceratocystis* species (Harrington and McNew 1998).

Several pairings between MAT-2 testers and between MAT-1 and MAT-2 testers resulted in formation of perithecia without viable ascospores (TABLE II). Pairings between MAT-1 testers produced no perithecia. One pairing between two MAT-2 testers (C1351-sec1 × C1317-sec1) yielded perithecia and ascospores typical of an interspecific hybridization. Several pairings of C1587-sec2 with other MAT-2 testers yielded perithecia without necks, and the recovered progeny resembled C1587-sec2 morphologically, indicating the ascospores were from induced selfings. This was the only MAT-2 tester found to self in that experiment, although C1642-sec1 also selfed in the later crosses of cacao isolates.

Some pairings between cacao testers were interfertile, while others appeared to be interspecific hybridizations (TABLE II). Because cacao isolates were not consistently interfertile with each other, we performed a second pairing study using only cacao testers. There are two genetic sublineages within the cacao lineage, one centered in Ecuador and the other containing isolates from Brazil, Costa Rica and Colombia (Baker et al 2003). A Brazilian MAT-2 tester, C1587-sec2 and a Costa Rican tester, C1642-sec1, were interfertile with most Brazilian, Colombian, and Costa Rican MAT-1 testers (TABLE III). The Ecuadorian MAT-2 testers (C1004-sec1, C1691-sec1 and C1834-sec1) and C940-sec5 were interfertile with most of the cacao testers from Ecuador. Isolate C940 purportedly was collected from Costa Rica but is similar genetically to the Ecuadorian isolates (Baker et al 2003). Pairings between testers of the Ecuadorian and the other cacao lineage resulted in no perithecia; or the perithecia and ascospores produced appeared to be from induced selfings. Six cacao isolates that did not fall within the cacao lineage and were not pathogenic to cacao (Baker et al 2003) were not interfertile with testers of either cacao group. Some pairings between testers of the two cacao lineages resulted in a few perithecia with few ascospores. The authors were surprised by the difficulty in obtaining hybrid progeny from pairings between testers of the two cacao sublineages, despite repeated attempts. Viable progeny from a hybrid of C940-ss and C1587-sec2 were obtained after several attempts.

Progeny analysis.—Mycelial morphology of recovered progeny was compared with that of the parents to

TABLE III. Pairings between *Ceratocystis fimbriata* testers from cacao

Recipient *MAT-1* strains	Origin	Spermatizing *MAT-2* strains: C1587-sec2 Br	C1642-sec1 CR	C940-sec5 CR	C1004-sec1 Ec	C1691-sec1 Ec	C1834-sec1 Ec
C1587-ss	Bahia, Brazil	C[a]	C	–	–	–	–
C1593-ss	Bahia, Brazil	C	C	–	–	–	–
C1597-ss	Bahia, Brazil	C	C	–	P	–	–
C1983-ss	Rondônia, Brazil	C	C	–	–	–	–
C2031-ss	Rondônia, Brazil	C	C	–	–	–	–
C1935-ss	Colombia	C	C	–	–	–	–
C1936-ss	Colombia	C	C	–	–	–	–
C1937-ss	Colombia	C	C	–	–	–	–
C1947-ss	Colombia	C	C	–	–	–	–
C1548-ss	Costa Rica	C	C	–	–	–	–
C1549-ss	Costa Rica	C	C	–	–	–	–
C1634-ss	Costa Rica	C	C	–	–	–	–
C1638-ss	Costa Rica	H	C	–	–	–	–
C1642-ss	Costa Rica	C	C	–	–	–	–
C940-ss	Costa Rica	H	H	C	C	C	H
C1691-ss	Ecuador	S	S	C	C	C	H
C1834-ss	Ecuador	–	S	C	C	C	C
C1750-ss (NC)[b]	Colombia	H	H	H	H	H	H
C1696-ss (NC)	Ecuador	H	H	H	H	H	H
C1831-ss (NC)	Ecuador	–	H	–	H	–	–
C1832-ss (NC)	Ecuador	H	H	–	H	–	–
C1836-ss (NC)	Ecuador	S	H	–	H	H	–
C1584-ss (NC)	Trinidad	H	H	H	H	H	–

[a] No perithecia (–), sterile perithecia (P), perithecia with few viable ascospores, typical of a hybridization (H), perithecia with many viable ascospores, typical of an interfertile cross (C), or induced selfing (S).

[b] NC = isolated from cacao, but not of the cacao lineage and not pathogenic to cacao.

determine if there had been a cross, that is, if the morphology of both parents could be found among the progeny (TABLE IV). Based on these observations, a pairing between C940-ss and C1354-sec1 was apparently an induced selfing of C940-ss, but progeny from the other pairings showed segregation of male-parental, female-parental and nonparental morphotypes, indicating that crossing had occurred. Some pairings produced progeny with no nonparental phenotypes, while others had high proportions of nonparental types.

The *MAT-2* gene tended to have a skewed segregation among progeny, but not consistently in one direction (TABLE IV). The progeny from the sweet potato × sweet potato and sycamore × sycamore pairings were mostly MAT-2. Progeny from between-group pairings had abnormal distribution of parental and nonparental mycelial types, and there were generally more MAT-1 (the genotype of the female parent) than MAT-2 progeny. The progeny from a cacao × cacao pairing (C1548-ss × C1587-sec2) showed identical results.

Morphology.—No diagnostic difference occurred among isolates from different populations for most of the characters examined, although significant differences ($P < 0.05$) existed between the different groups of isolates in several measurements of perithecia and ascospores (TABLE V). The ANOVAs indicated: Ascospore length ($F = 13.21$, $P < 0.0001$), width ($F = 14.40$, $P < 0.0001$) and height ($F = 5.07$, $P < 0.0001$), perithecial base width ($F = 9.98$, $P < 0.0001$) and height ($F = 17.14$, $P < 0.0001$) and neck length ($F = 58.11$, $P < 0.0001$) differed significantly among the isolates from sweet potato, sycamore and the five populations from cacao. Isolates from the Ecuadorian cacao sublineage had longer ascospores and average perithecia neck lengths longer and more variable than those of the other cacao isolates. Sycamore isolates had significantly wider perithecial bases and narrower ascospores than isolates from the other groups.

Sycamore isolates produced abundant doliform (barrel-shaped) conidia from wide-mouthed phialides and narrower, cylindrical endoconidia from ta-

TABLE IV. Segregation for mycelial phenotype and presence or absence of the *MAT-2* gene among progeny from pairings of testers within and between lineages of *Ceratocystis fimbriata*

			MAT-2 absent[b]			*MAT-2* present[b]		
Female (MAT-1)	Male (MAT-2)	Total progeny recovered	Female mycelia phenotype	Male mycelia phenotype	Nonparental mycelial phenotypes	Female mycelia phenotype	Male mycelial phenotype	Nonparental mycelial phenotype
C1418-ss SP[a]	C1354-sec1 SP	36	4	1	6	0	3	22
C1339-ss SY	C1351-sec1 SY	17	1	2	0	7	7	0
C1548-ss CA	C1587-sec2 CA	37	14	8	0	4	9	2
C940-ss CA	C1587-sec2 CA	20	5	0	4	3	3	5
C940-ss CA	C1339-sec1 SY	14	2	3	2	0	4	3
C1418-ss SP	C1351-sec1 SY	17	11	0	4	1	0	1
C1317-ss SY	C1354-sec1 SP	7	0	0	7	0	0	0
C1317-ss SY	C1418-sec1 SP	17	9	6	0	2	0	0
C1339-ss SY	C1354-sec1 SP	16	3	0	4	4	0	5
C1339-ss SY	C1418-sec1 SP	36	1	8	18	0	6	3
C940-ss CA	C1354-sec1 SP	19	19	0	0	0	0	0

[a] CA = cacao, SP = sweet potato, SY = sycamore.

[b] Presence or absence of the MAT-2 idiomorph based on PCR testing.

pered phialides. Sweet potato isolates produced only the narrower, cylindrical endoconidia from tapered phialides. Few or no doliform conidia were observed in isolates of the two cacao sublineages.

Because each pathogen forms distinct genetic lineages, has a unique host range and is intersterile with *C. fimbriata* from sweet potato, we describe the cacao pathogen as a new species and elevate the sycamore pathogen, *Ceratocystis fimbriata* f. *platani,* to the level of species. The type of *C. fimbriata* was obtained from sweet potato, so the sweet potato pathogen remains *C. fimbriata* sensu stricto.

TAXONOMY

Ceratocystis fimbriata sensu stricto Ellis et Halsted, New Jersey Agricultural College Experiment Station Bulletin 76, 1890, p. 14. FIGS. 1, 2

Description. Perithecia superficial or embedded in the substrate, bases dark brown to black, globose, 110–250 μm wide, 120–250 μm tall. *Necks* dark brown to black, straight, 440–770 μm long, 28–40 μm wide at the base and 16–24 μm wide at the tip. *Ostiolar hyphae* divergent, light brown to hyaline, non-septate, smooth-walled, 20–120 μm long. *Asci* not seen. *Ascospores* hyaline, one-celled, galeate, 5.0–7.5 μm long × 3.5–5.0 μm wide, 3.0–4.5 μm tall, spores accumulating in a cream colored mass at the tip of the neck.

Endoconidiophores arising laterally from vegetative hyphae, scattered or arising in clusters, 1–8 septate, 55–120 μm long, 3.0–8.0 μm wide at the base. *Phialides* lageniform, 27–60 μm long, 4.0–8.5 μm wide at the middle, 3.0–6.0 μm wide at the tip, hyaline to pale brown. *Endoconidia* unicellular, hyaline to light brown, smooth, cylindrical with flattened ends, straight, biguttulate, 9–33 × 3.0–5.0 μm, borne in chains of variable length. Doliform endoconidia absent. *Aleurioconidiophores* arising laterally from the mycelium, with 0–5 septa, 9–98 × 4.5–6.5 μm. *Aleurioconidia* brown, globose to pyriform, 11–16 × 6.5–12 μm, occurring singly or in short chains.

Specimen examined. USA. NEW JERSEY: Swedesboro, from *Ipomoea batatas,* 12 April 1891, col. *B. D. Halsted* (NEOTYPE; BPI 595863).

Cultures examined. USA. NORTH CAROLINA: Harrelsville, from *Ipomoea batatas,* December 1998, col. *M. Cubeta* (C1418); JAPAN. KOGOSHIMA PREF.: Chiran-machi, from *Ipomoea batatas,* September 1998, col. *Y. Kajitani* (C1354); PAPUA NEW GUINEA. KOMEA: Upper Mendi, from *Ipomoea batatas,* March 1984, col. *E.H.C. McKenzie* (C1476 = ICMP 8579).

Commentary. Ceratocystis fimbriata originally was named by Ellis and Halsted (Halsted 1890), but a more detailed description was given by Halsted and Fairchild (1891). A specimen (BPI 595869) deposited by Halsted in 1890 should serve as the holotype. However, this specimen is now crumbled powder and useless as a type specimen. Halsted deposited another sweet potato specimen in 1891 (BPI 595863) with pictures from the Halsted and Fairchild (1891) description. This specimen consists of infected sweet potato shoots and includes mature perithecia and is designated here as the neotype. It fits into the morphological description of *C. fimbriata* sensu stricto given above.

As reviewed by Baker et al (2003), only isolates of *C. fimbriata* from sweet potato have been found to be pathogenic to sweet potato in inoculation studies. Fourteen isolates and specimens from diseased sweet potato plants in China, Japan, Papua New Guinea, New Zealand, St. Vincent and the USA have nearly identical ITS-rDNA sequences (AY157956,

TABLE V. Mean and standard deviations of dimensions (in μm) of perithecia and ascospores of isolates of *Ceratocystis fimbriata* from sweet potato, sycamore, and five populations of cacao[a]

Group	Number of isolates	Perithecia base width	Perithecia base height	Perithecia neck length	Ascospore length	Ascospore width	Ascospore height
sweet potato	3	192.2 ± 31.3 b	177.5 ± 32.5 c	628.6 ± 73.6 b	6.0 ± 0.5 a	4.4 ± 0.4 bc	3.5 ± 0.3 a
sycamore	3	238.3 ± 32.5 a	229.6 ± 28.0 a	657.1 ± 75.4 ab	5.6 ± 0.6 bc	3.9 ± 0.4 d	3.4 ± 0.3 bc
cacao, Ecuador	8	199.3 ± 43.3 b	183.3 ± 35.9 c	664.8 ± 141.8 a	5.8 ± 0.3 b	4.4 ± 0.3 ab	3.5 ± 0.3 a
cacao, Rondônia	3	192.8 ± 27.3 b	175.6 ± 24.3 c	551.8 ± 102.6 c	5.5 ± 0.3 cd	4.5 ± 0.3 ab	3.2 ± 0.2 c
cacao, Bahia	3	202.5 ± 24.0 b	203.3 ± 33.5 b	531.3 ± 64.8 c	5.4 ± 0.3 d	4.5 ± 0.2 a	3.6 ± 0.2 a
cacao, Costa Rica	3	198.4 ± 35.9 b	199.6 ± 39.1 b	484.5 ± 72.7 d	5.4 ± 0.4 d	4.2 ± 0.3 c	3.5 ± 0.3 ab
cacao, Colombia	4	196.9 ± 27.6 b	186.8 ± 28.0 c	463.1 ± 56.3 d	5.5 ± 0.3 cd	4.4 ± 0.3 abc	3.5 ± 0.3 ab

[a] Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test.

AY157957). This sequence has differed from those of more than 280 isolates and specimens of *C. fimbriata* from other hosts (Baker et a 2003, Barnes et al 2001, Barnes et al 2001, unpublished data), including those of Colombian coffee isolates (Marin et al 2003). Microsatellite analyses (Barnes et al 2001, Steimel et al 2004) also have shown *C. fimbriata* from sweet potato to be nearly uniform and distinct from isolates derived from sycamore, cacao and other hosts.

Ceratocystis cacaofunesta Engelbrecht et Harrington, sp. nov. FIGS. 4–9

Differt a *Ceratocystis fimbriata* morbo efficienti in ligno Theobromatis cacaonis et raro producenti endoconidiis doliiformibus.

Differs from *Ceratocystis fimbriata* in pathogenicity to *Theobroma cacao* and rare production of doliform endoconidia.

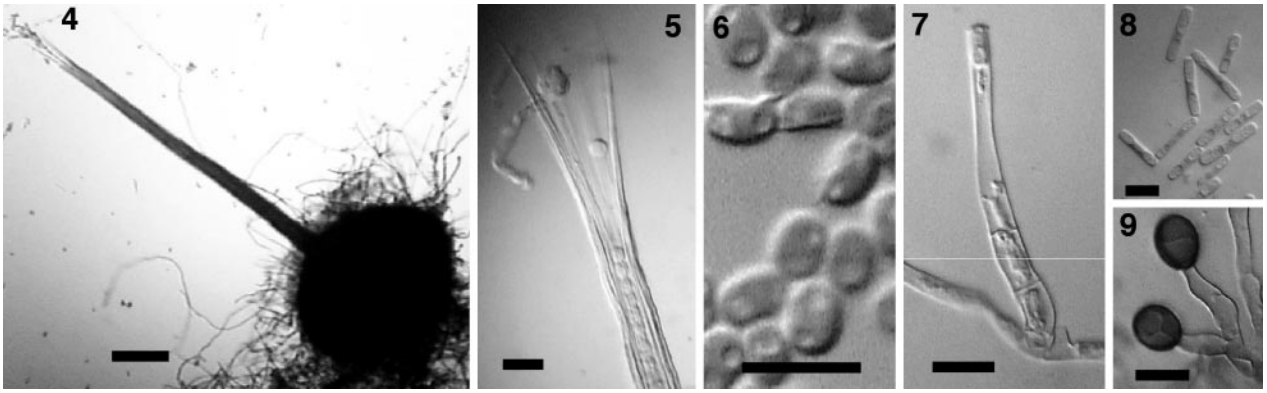
Description. *Perithecia* superficial or embedded in the substrate, bases dark brown to black, globose, 95–305 μm wide, 100–275 μm tall. *Neck* dark brown to black, straight, 310–1010 μm long, 20–45 μm wide at the base and 12–25 μm wide at the tip. *Ostiolar hyphae* divergent, light brown to hyaline, non-septate, smooth-walled, 30–125 μm long. *Asci* not seen. *Ascospores* hyaline, one-celled, galeate, 4.5–6.5 μm long × 3.5–5.5 μm wide, 3.0–4.0 μm tall. Spores accumulate in a cream-colored mass at the tip of the neck.

Endoconidiophores arising laterally from vegetative hyphae, scattered or arising in clusters, 0–12 septate, 40–295 μm long including the basal cells, 2.0–8.0 μm wide at the base. *Phialides* of two forms, the more common lageniform, 12–85 μm long, 2.0–9.0 μm wide in the middle, 2.0–6.5 μm wide at the tip, hyaline to light brown. *Cylindrical endoconidia* unicellular, hyaline to light brown, smooth, mainly cylindrical with flattened ends, straight, biguttulate, 8–40 × 2.5–5.0 μm, borne in chains of variable length. *Wide-mouthed phialides* rare or absent, borne near the base of perithecia, hyaline to light brown. *Doliform endoconidia* produced from wide-mouthed phialides, often remaining in chains, hyaline to light brown. *Aleurioconidiophores* arising laterally from the mycelium, with 0–13 septa, 5–255 × 3.5–7.0 μm. *Aleurioconidia* brown, globose to pyriform, 10–20 (37) × 3.5–11.5 μm, occurring singly or in short chains,

HOLOTYPE. BRAZIL. RONDÔNIA: Ouro Preto do Oeste, from diseased *Theobroma cacao,* October 2002, *T. Harrington,* BPI 843740, from isolate C1983 (= CBS 115172).

Etymology. the specific epithet means ''cacao-killing'', referring to its unique pathogenicity to cacao.

Cultures examined. BRAZIL. BAHIA: Camacao, from *Theobroma cacao,* December 1999, col. *T. C. Harrington* (C1587); Ubatan, from *T. cacao,* December 1999, col. *T. C. Harrington* (C1597); Urucuca,



FIGS. 4–9. *Ceratocystis cacaofunesta* isolate C1983. 4. Perithecium. 5. Ostiolar hyphae. 6. Ascospores. 7. Cylindrical endoconidiophore. 8. Cylindrical endoconidia. 9. Aleurioconidiophore and aleurioconidia. Scale bars: 4 = 100 μm; 5–9 = 10 μm.

from *T. cacao,* December 1999, col. *T. C. Harrington* (C1593); RONDÔNIA: Ouro Preto do Oeste, from *T. cacao,* October 2002, col. *T. C. Harrington* (C1983 = CBS 115172 = BPI 843740); from *T. cacao,* October 2002, col. *T. C. Harrington* (C1984); from *T. cacao,* October 2002, col. *T. C. Harrington* (C2031); COLOMBIA. CALDAS: Palestina, from *T. cacao,* June 2002, col. *E. Alvarez* (C1935); from *T. cacao,* June 2002, col. *E. Alvarez* (C1936); from *T. cacao,* June 2002, col. *E. Alvarez* (C1937); from *T. cacao,* col. *E. Alvarez* (C1947); COSTA RICA. Atlantic side, from *T. cacao,* May 1962, col. *A. J. Hansen* (C940 = CBS 152.62); La Lola Experiment Station, from *T. cacao,* July 1999, col. *A. Paulin,* (C1547); from *T. cacao,* July 1999, col. *A. Paulin* (C1548 = CBS 114722 = BPI 843730); Turrialba, from *T. cacao,* March 2000, col. *T. C. Harrington* (C1638); ECUADOR. Pichilingue, from *T. cacao,* July 2000, col. *T. C. Harrington* (C1690); from *T. cacao,* July 2000, col. *T. C. Harrington* (C1691); from *T. cacao,* July 2000, col. *T. C. Harrington* (C1695 = CBS 115163 = BPI 843731); from *T. cacao,* March 2001, col. *C. Suarez* and *C. Belazaca* (C1751); from *T. cacao,* August 2001, col. *C. Suarez* and *C. Belazaca* (C1833 = CBS 115169 = BPI 843736); from *T. cacao,* August 2001, col. *C. Suarez* and *C. Belazaca* (C1834); from *T. cacao,* August 2001, col. *C. Suarez* and *C. Belazaca* (C1835).

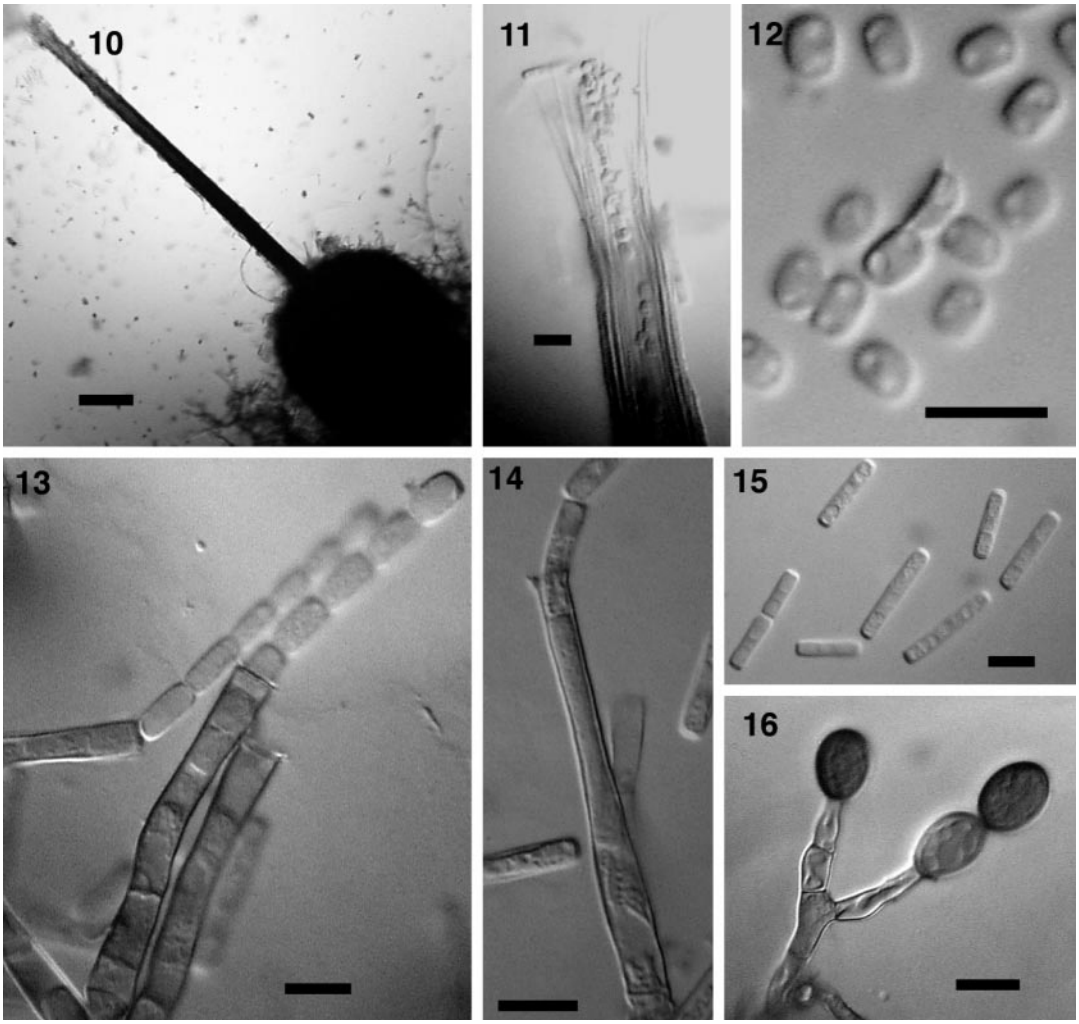
Commentary. Two closely-related sublineages exist within this species, one centered in western Ecuador and the other containing isolates from Brazil, Colombia and Costa Rica. The two sublineages differ little in morphology, but they are intersterile and have unique microsatellite markers (Steimel et al 2004) and ITS-rDNA sequences (Baker et al 2003). Their ITS sequences differ from all other tested isolates from the *C. fimbriata* complex. Representative ITS-rDNA sequences of the Ecuadorian and Brazilian sublineages have been deposited in GenBank (AY157950 and AY157951–157953, respectively). Cacao is native to the Upper Amazon region, including the Brazilian state of Rondônia, the origin of the type specimen for *C. cacaofunesta.*

The new species is distinguished from *C. fimbriata* sensu stricto by its pathogenicity to cacao and the rare production of doliform conidia from wide-mouthed phialides, which have not been observed in *C. fimbriata.* In addition to the 14 isolates of *C. cacaofunesta* tested by Baker et al (2003), four cacao isolates from Colombia and three from Rondônia, Brazil (including the ex holotype isolate) have proven highly pathogenic to cacao seedlings in inoculation studies (unpublished). Other isolates from cacao are morphologically similar to *C. cacaofunesta* but were non-pathogenic in inoculation studies, were intersterile with testers of *C. cacaofunesta,* and had different ITS-rDNA sequences (Baker et al 2003). *Ceratocystis cacaofunesta* can be distinguished from *C. pirilliformis* (Barnes et al 2003) by its globose (rather than pyriform) perithecial base. *Ceratocystis cacaofunesta* can be distinguished from *C. albofundus* (Wingfield et al 1996a) by its dark brown to black perithecial base, while that of *C. albofundus* is yellowish brown.

Ceratocystis platani (Walter) Engelbrecht et Harrington comb. et stat. nov. FIGS. 10–16

≡ *Endoconidiophora fimbriata* (Ellis & Halsted) Davidson f. *platani* Walter, Phytopathology 42:236, 1952.

Description. Perithecia superficial or embedded in the substrate, bases dark brown to black, globose, 175–290 μm wide, 175–290 μm high. *Neck* dark brown to black, straight, 535–835 μm long, 20–45 μm wide at the base and 15–35 μm wide at the tip. *Ostiolar hyphae* divergent, light brown to hyaline, non-septate, smooth-walled, 20–90 μm long. *Asci* not



FIGS. 10–16. *Ceratocystis platani* isolate C1317. 10. Perithecium. 11. Ostiolar hyphae. 12. Ascospores. 13. Endoconidiophores with doliform conidia. 14. Endoconidiophorewith cylindrical conidia. 15. Cylindrical endoconidia. 16. Aleurioconidiophores and aleurioconidia. Scale bars: 10 = 100 μm; 11–16 = 10 μm.

seen. *Ascospores* hyaline, one-celled, galeate, 4.0–6.5 μm long × 3.0–4.5 μm wide, 3.0–4.5 μm tall. Spores accumulate in a cream colored mass at the tip of the neck.

Endoconidiophores arising laterally from vegetative hyphae, scattered or arising in clusters, 1–4 septate, 55–165 μm long including the basal cells, 3.5–7.5 μm wide at the base. *Phialides* of two forms, the more common lageniform, 24–90 μm long, 3.5–9.0 μm wide in the middle, 2.5–7.5 μm wide at the tip, hyaline to pale brown. *Cylindrical endoconidia* unicellular, hyaline to light brown, smooth, mainly cylindrical with flattened ends, straight, biguttulate, 11–22 × 3.0–5.0 μm, borne in chains of variable length. *Wide-mouthed phialides* common, 35–50 μm long, 4.5–5.0 μm wide at the base, 5.5–6.5 μm wide at the tip, borne near the base of perithecia. *Doliform endoconidia* produced from wide-mouthed phialides, often remaining in chains, hyaline to light brown, 6.0–10.0 μm × 3.5–5.0 μm. *Aleurioconidiophores* arising laterally from the mycelium, with 0–14 septa, 10–285 × 4.0–7.0 μm. *Aleurioconidia* brown, globose to pyriform, 10–20 × 6.0–12.0 μm, occurring singly or in short chains.

HOLOTYPE. USA. WASHINGTON, D.C., from stained wood of *Platanus occidentalis,* August 1951, col. *R. Day* and *H. Wester* (BPI 595622).

Cultures examined: USA. KENTUCKY, from *Platanus occidentalis,* September 1998, col. *T. C. Harrington* (C1351); NORTH CAROLINA: Janet Day Plantation, from *Platanus occidentalis,* July 1998, col. *T. C. Harrington* (C1317 = CBS 115162); VIRGINIA: Holmeville, from *Platanus occidentalis,* August 1998, col. *K. Britton* (C1339).

Commentary. Ceratocystis platani can be distinguished from *C. fimbriata* and *C. cacaofunesta* by its

pathogenicity to *Platanus* spp. and frequent production of doliform endoconidia from wide-mouthed phialides. *Ceratocystis platani* is distinguished from *C. pirilliformis* (Barnes et al 2003) by its globose (rather than pyriform) perithecial base. *Ceratocystis platani* can be distinguished from *C. albofundus* (Wingfield et al 1996a) by its dark brown to black perithecial base, while that of *C. albofundus* is yellowish brown.

Baker et al (2003) found that 11 isolates from American sycamore and plane tree had identical ITS-rDNA sequences (AY157961), which differs from those of all other members of the *C. fimbriata* complex. Microsatellite markers of *C. platani* isolates differ from those of *C. fimbriata* sensu stricto, *C. cacaofunesta* and other members of the *C. fimbriata* complex (Barnes et al 2001, Steimel et al 2004). Analyses of microsatellite markers and other polymorphic nuclear and mitochondrial markers suggest that *C. platani* is native to the eastern USA and was introduced to Modesto, California and southern Europe (Engelbrecht et al 2004).

DISCUSSION

Intersterility of cacao, sweet potato and sycamore isolates correlates with genetic lineages as shown by ITS sequences (Baker et al 2003) and microsatellite markers (Steimel et al 2004). Members of these intersterility groups also are highly specialized to their respective hosts in inoculation studies (Baker et al 2003). The ability of isolates of the three host-specialized groups to infrequently produce hybrid sexual progeny when paired with testers of the other groups suggests that these species are closely related and probably have diverged recently, such that intersterility barriers are not complete. A limited amount of genetic exchange might occur between populations of these species where they coexist in the same area, as do the sweet potato and sycamore pathogens in the southeast USA, although there is no evidence that this has occurred.

The slight but significant morphological differences found among the sweet potato, sycamore and cacao pathogens further emphasize the divergence of these lineages. The absence of the doliform conidial state in the sweet potato isolates and rarity in the cacao isolates may be ecologically significant, but all isolates abundantly produce narrow endoconidia from tapered phialides. While probably not ecologically important, minor morphological differences might be the result of genetic drift of populations that are not interbreeding, either because of intersterility barriers, geographic separation, or occurrence on different hosts.

The pairings within the sweet potato and sycamore groups resulted in more progeny with the *MAT-2* gene than progeny without it (MAT-1 phenotype). This is consistent with results from crosses of other *C. fimbriata* isolates, where self-fertile progeny (MAT-2) generally are recovered more frequently than self-sterile progeny (MAT-1) from crosses or selfings of *C. fimbriata* (Olson 1949, Webster and Butler 1967b). All of the progeny from the sycamore cross were morphologically like one or the other parent, while many of the progeny from the sweet potato cross were unlike either parent. Mycelial morphology is likely a polygenic trait, and greater genetic differences between the two sweet potato testers might explain the high number of recombinant phenotypes.

The pairing between the Costa Rican and Brazilian testers C1548-ss and C1587-sec2 was unusual because the MAT-1 strain was the spermatizing strain and the MAT-2 strain the recipient. Yet analysis of maternally-inherited mitochondrial DNA (Wingfield et al 1996b) showed that the MAT-1 strain served as the female. All the progeny had the mitochondrial DNA profile of the spermatizing strain. In the mitochondrial DNA analyses of other pairings (data not shown), the MAT-1 tester consistently served as the female, regardless of whether it was the recipient or spermatizing strain. The high proportion of MAT-1 progeny from the C1548-ss × C1587-sec2 cross also was unexpected.

The difficulty in obtaining hybrid progeny from pairings between the two cacao sublineages is surprising. The existence of two intersterility groups in the cacao lineage suggests possible selection for the development of intersterility barriers between these groups, or these two populations might have been separated long enough to lose interfertility by genetic drift. Cacao is probably indigenous to the Upper Amazon region (Cheesman 1944), the center of diversity of this species (N'goran et al 2000), and it is probable that the Brazilian/Costa Rican/Colombian population of *C. cacaofunesta* is native to the Upper Amazon. The Ecuadorian population may be native to Ecuador on another species of *Theobroma* or a different member of the Sterculiaceae family. Alternatively, since all our Ecuadorian isolates came from one experimental station (Pichilingue, near Quevedo), it is possible that the fungus was introduced there on cacao cuttings or whole plants (Baker et al 2003). Isolate C940 purportedly was collected from Costa Rica but grouped with the Ecuadorian isolates genetically (Baker et al 2003) and was interfertile with Ecuadorian testers. This might represent an introduction of the Ecuadorian sublineage into Costa Rica.

The two cacao sublineages showed some differences in morphology. The average perithecia neck length was significantly shorter among isolates from

the Brazil/Colombia/Costa Rica lineage than those of the Ecuador lineage. Ascospores of Ecuadorian isolates also were slightly longer. The two sublineages are equally pathogenic to cacao (Baker et al 2003), and morphological differences were trivial and not consistent, so the phenotypic differences are not considered sufficient at this time to describe the Ecuadorian sublineage as a separate species.

Intersterility within the *C. fimbriata* complex has not been examined thoroughly before, but our results mesh with those of earlier studies. Feazell and Martin (1950) paired a sweet potato isolate with a sycamore isolate and found that only three of 255 ascospores recovered were viable. In a cross between a sweet potato isolate and a rubber tree (*Hevea brasiliensis*) isolate from Mexico (Olson and Martin 1949), only two of 300 ascospores germinated. Webster and Butler (1967a) paired *C. fimbriata* isolates from several hosts and found that pairings between isolates from different host groups generally yielded perithecia with few ascospores. They interpreted this partial interfertility as evidence that the isolates tested represented a single biological species. However, we suggest that the few progeny recovered were a result of interspecific hybridizations. Other studies examining intersterility between *Ceratocystis* species demonstrated similar hybridizations between closely-related species (Harrington and McNew 1998, Harrington et al 2002).

Specialization to different hosts and selfing may have allowed populations of a *C. fimbriata*-like ancestor to undergo allopatric speciation. Cacao, sweet potato and sycamore are native to different regions of the Americas, the proposed center of origin of the Latin American clade of *C. fimbriata* (Harrington 2000). The sweet potato pathogen has very little genetic diversity (Steimel et al 2004) and may be native to northern South America, the purported origin of the cultivated sweet potato (Austin 1978, Jarret and Austin 1994). However, we have no sweet potato isolates from South America. *Ceratocystis platani* probably is indigenous to the southeastern USA and perhaps Mexico (Engelbrecht et al 2004). *Theobroma cacao* is believed indigenous to the Amazon Basin and northern South America (Cheesman 1944). We (unpubl) have found the greatest genetic diversity of *C. cacaofunesta* in the Upper Amazon, and thus have selected a specimen from Rondônia as the holotype.

Host specialization may be a major factor driving speciation within the genus *Ceratocystis*. *Ceratocystis polonica* and *C. laricicola,* for instance, are morphologically indistinguishable but intersterile and specialized to *Picea* and *Larix,* respectively (Harrington et al 2002). Lineages on maple and oak have been identified within *C. virescens* (Harrington et al 1998, Witthuhn et al 2000a). The host-specialized lineages on sweet potato, sycamore and cacao also differ only slightly in morphology, suggesting that the evolution and divergence of these species has been driven primarily by host specialization. Because *C. fimbriata* and relatives are able to reproduce by selfing, intersterility barriers may not be necessary to maintain uniquely adapted populations, but intersterility barriers are clearly evident between the newly-recognized species. Although these species might have evolved allopatrically, adaptation of an isolated population to a novel host might exert selection pressure to develop intersterility barriers with sympatric populations on other hosts.

The wide genetic, geographic and host variation found in the Latin American clade of the *C. fimbriata* complex suggests more cryptic species awaiting description (Baker et al 2003, Barnes et al 2001, Harrington 2000, Marin et al 2003). Taken in the strictest sense, *C. fimbriata* is based solely on a narrowly defined lineage pathogenic to sweet potato. Controlled inoculation studies and detailed morphological comparisons will be needed to identify diagnostic phenotypic characters for delineation of new species (Harrington and Rizzo 1999).

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