Origin of Ceratocystis platani on Native Platanus orientalis in Greece and Its Impact on Natural Forests

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ABSTRACT


Canker stain of plane tree recently was reported in a small area of southwestern Greece on natural populations of the important riparian species, oriental plane tree, Platanus orientalis. The fungus Ceratocystis platani (= C. fimbriata f. platani) was successfully isolated from infected, stained wood of 15 dead or dying trees on the Peloponnese Peninsula. Genetic analyses of these 15 isolates from Greece, using nuclear and mitochondrial DNA fingerprints, showed the fungus to be identical to the genotype reported from Italy, France, and Switzerland. A polymerase chain reaction-based microsatellite analysis of eight polymorphic loci discovered a new microsatellite allele in one of the isolates from Greece, but this may be due to a mutation after introduction of a single strain. Earlier studies indicated that the most common European genotype had been introduced from eastern North America to Italy during World War II. The recent introduction to Greece appears to have originated from Italy, France, or Switzerland, rather than from eastern North America, where the fungus is native. The pathogen is having a dramatic impact on the natural population of P. orientalis in southwestern Greece, and containment measures should be imposed before it spreads throughout the natural range of this ecologically and historically important host.

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attacks by woodboring ambrosia beetles (Coleoptera: Curculionidae, subfamily Platypodinae) were seen on the trunk of many of the dead and dying oriental plane. Adults of the ambrosial beetle species Platypus cylindrus F. were excavated from galleries in the wood of two trees. We collected ambrosia beetle frass that had been expelled from three diseased trees. The frass was collected in plastic sterile vials and the carrot disc method (17) was used to isolate C. platani by placing a small quantity of frass between two slices (5 mm thick) of carrot. The carrot "sandwiches" were placed into plastic petri dishes with moistened filter paper and incubated for 1 week at 23 to 24°C. The carrot discs were examined under a dissecting microscope for the presence of perithecia and ascospores, and the fungus was isolated from the ascospore masses as described above.

**DNA extraction.** Genetic markers of 15 isolates from Greece; an isolate from Florence, Italy; and an isolate from Avignon, France were compared with those of the C. platani isolates that had been studied earlier (7). The 17 new isolates were grown on malt yeast extract agar (MYEA; 2% malt extract, 0.2% yeast extract, and 2% agar) for 7 days for conidial production. Plates were flooded with 10 ml of sterile water, and conidia, conidiophores, and mycelium were scraped from the plates and placed in 500 ml of broth medium (2% malt extract and 1% yeast extract in 2-liter flasks). The flasks were incubated at room temperature with shaking for 24 to 48 h. Genomic DNA was extracted from the germlings following the method of DeScenzo and Harrington (5).

**Nuclear DNA fingerprinting.** A total of 15 µg of extracted DNA was restricted with PsI following the procedure of Engelbrecht et al. (7). The digested DNA was electrophoresed for 20 h at 80 V on a 1.2% agarose gel (19.5 by 25.5 cm) (Bio-Rad Certified Molecular Biology agarose, Bio-Rad). Gels were dried, hybridized with the 32P-labeled (CAT) 1200 oligonucleotide, and washed (5). Hybridized gels were exposed to a phosphor screen (Molecular Dynamics) for 7 days.

**Mitochondrial DNA fingerprinting.** Approximately 30 µg of total genomic DNA was restricted using HaeIII, which selectively digests GC-rich nuclear DNA and leaves relatively large fragments of multicopy, AT-rich mitochondrial DNA (30). The restriction products were electrophoresed as described above. Lambda DNA/HindIII marker (Promega) was used as the standard on the outer lanes. Gels were stained with ethidium bromide for 30 min and visualized with ultraviolet (UV) light.

**Microsatellite analysis.** Alleles (bands of differing size) from 8 of 16 polymorphic, PCR-based microsatellite markers (23) were generated and compared with those from the earlier study (7). Only eight loci were used because those were the ones that showed polymorphism within the North American population of C. platani. For each primer pair, one of the primers was fluorescently labeled. The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc.). Earlier studies (7,23) used polyacrylamide gel electrophoresis, and there were some differences in the band sizes (±1 bp) with some of the alleles using the capillary system. All reported allele designations (approximate product size in base pairs) correspond to the allele names given in the earlier studies (7,23).

Genetic relationships among the isolates of C. platani from Greece and the isolates from the United States and Europe were determined with the microsatellite alleles using the unweighted pairgroup method with arithmetic means (UPGMA) algorithm in PAUP (24).

### RESULTS

**Disease occurrence and symptoms.** The most obvious symptom of the disease on oriental plane was the sudden death of a portion of the crown. A branch or the entire tree may have failed to produce leaves in the spring, or the emerging leaves suddenly withered and died. In most of the diseased trees, the outer bark of the trunk was thick and roughened, so cankers were not evident without cutting through the bark and observing necrosis in the inner bark and the bluish-black to reddish-brown discoloration of the sapwood. Also, most infections of streamside trees appeared to occur through the roots, and stem cankers were not evident. In cross section, the stained sectors in the outer sapwood rings often were lens shaped, and the discoloration tapered toward the center of the stem. Successful isolations of C. platani were made from stained wood of 1 London plane and 14 oriental planes at 13 localities.

Canker stain of oriental plane appeared to be confined to a region of approximately 400 km² in Messinia Prefecture (latitudes 37° to 37°30'), in the southwestern region of the Peloponnesian Peninsula, southwest of Athens. Ornamental plane trees of different ages and sizes in residential areas and recreational sites apparently have died from infection; some of the dead trees were large and centuries old. However, the impact has been greater in natural stands. Hundreds of dead and dying oriental planes were found along streams and rivers, where it appeared that the pathogen had spread tree-to-tree through root contact or through running water. Patches of up to 15 to 20 dead and dying trees were often evident along streams. Examination of root systems suggested that some of the spread to adjacent trees was through root grafting. Also, dead logs and pieces of branches from killed trees were observed to be carried by water downstream, and such infested material may serve as an important source of inoculum.

Some newly infected trees were observed that had been wounded by cutting tools during road maintenance, and infection appeared to have taken place through these pruning wounds, probably from contaminated saws. The northernmost focus of infection was along a highway where overhanging branches had been cut, and the cut surfaces were heavily colonized by the fungus. In a few cases, it appeared that the pathogen had spread into new areas with excavation machinery, and infections may have originated in wounded roots.

Many of the recently dead and dying oriental planes were found infested by wood-boring ambrosia beetles, and attacking adults of P. cylindrus F. were identified from two trees. Excavated frass on the outer bark was collected from three trees, and C. platani was successfully isolated from the frass collected from two of these trees.

**Nuclear DNA fingerprinting.** A total of 17 scorable (CAT) 3 bands resulted from

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Table 1. Microsatellite alleles found in the United States and European populations of Ceratocystis platani

<table>
<thead>
<tr>
<th>Locus</th>
<th>Eastern United States (n = 33)</th>
<th>California (n = 6)</th>
<th>Italy (n = 26)</th>
<th>Switzerland (n = 1)</th>
<th>France (n = 2)</th>
<th>Greece (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAA9</td>
<td>270, 312, 368, 386, 400, 410</td>
<td>276, 294</td>
<td>294</td>
<td>294</td>
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<td>294</td>
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<tr>
<td>CAA15</td>
<td>288, 317</td>
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<td>317</td>
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<td>317</td>
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<tr>
<td>CAA38</td>
<td>134, 157</td>
<td>134</td>
<td>157</td>
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<tr>
<td>CAA80</td>
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<td>291, 297</td>
<td>300</td>
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<td>300</td>
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<tr>
<td>CAT13K</td>
<td>323, 326, 329</td>
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<td>326</td>
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<td>CAT1200</td>
<td>391, 409</td>
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</tr>
<tr>
<td>GACA650</td>
<td>235, 256, 259, 276, 300, 328</td>
<td>295</td>
<td>285, 290</td>
<td>290</td>
<td>290</td>
<td>290, 319</td>
</tr>
</tbody>
</table>

*All data on isolates from eastern United States, California, Italy, Switzerland, and France are from Engelbrecht et al. (2004), except for new data on one isolate from Italy and one isolate from France; n = number of isolates tested.*
the PstI nuclear DNA restriction of the 15 isolates from Greece and the new isolate from Italy. The nuclear fingerprinting band pattern of the 15 isolates from Greece and the new Italian isolate was identical to that of the other isolates from Europe (7).

Mitochondrial DNA fingerprinting. The mitochondrial DNA fingerprint pattern of the 15 isolates from Greece was identical to that of the isolates from the Italian, French, and Swiss populations of C. platani (7). In all, 27 scorable bands were found in the U.S. populations based on the previous study (7), and 17 of these bands were polymorphic. The Greek isolates had 19 scorable bands, the same bands found in the European population (7).

Microsatellite analysis. For the eight polymorphic microsatellite markers, 14 of the 15 Greek isolates of C. platani, a new isolate from Italy, and a new isolate from Avignon, France had the same microsatellite alleles as the most common genotype from Italy, France, and Switzerland. Alleles for each locus for the C. platani isolates from Greece as well as for the isolates previously studied (7) are shown in Table 1. One isolate from the Greek population, isolate C2177, had a unique allele at locus GACA650. The allele was not found in the U.S. population studied earlier (7).

Using the microsatellite markers, the Greek isolates of C. platani were placed together with the other isolates from Europe using UPGMA (Fig. 1).

DISCUSSION
The isolates of C. platani from Greece proved to be identical to the isolates collected in other regions in Europe based on both (CAT)5 nuclear and HaeIII mitochondrial fingerprinting. In addition to the 25 isolates from Italy used in the previous study (7), we analyzed a new isolate from Florence, Italy that proved to be the same genotype as the other European isolates.

A new microsatellite allele was found in one C. platani isolate from Greece. The PCR product for locus GACA650 was of a larger size (319 bp) than the product found in other European isolates. Microsatellite regions are prone to mutations because of the numerous, tandemly repeated sequences (9). This hypervariability could be an explanation for the presence of this new allele in the Greek population. Two unique alleles were found earlier in isolates from Italy, and those were thought to be the result of mutations after the introduction of a single genotype into Naples (7).

Aside from possible mutations at three microsatellite loci, the European population of C. platani is a single clone, and it appears that a single genotype of C. platani was introduced to Europe (7). Most Ceratocystis spp. have a sexual state but, like many Ceratocystis spp., C. platani can self via unidirectional mating type switching (10,31). Selfing is likely more common than outcrossing in such Ceratocystis spp. (11) and, thus, the genotype of a single, introduced strain could be maintained clonally.

The population in Italy, France, and Switzerland is thought to be the result of a single introduction of the pathogen through Italy during World War II (7,18). The Greek population likely is derived from this Italian introduction. One possibility is that the source for the Greek introduction was infected rooted-cuttings from Italy. Movement of such nursery stock is common among European Union countries, and London plane is produced and shipped from Italy (19), where canker stain is widespread. However, we cannot rule out the possibility that the fungus was introduced on wood used for packaging, the probable pathway of the fungus from the United States to Italy (7,18).

Ambrosia beetle attacks were common on most of the dead and dying oriental planes in Greece. The two adult beetles that we excavated from the wood of diseased trees were identified as Platypus cingulatus. C. platani was isolated from the frass emanating from the galleries on two separate trees. The frass from ambros-
sia beetles is known to contain viable propagules of other Ceratocystis spp. (13), and aerially dispersed frass is infectious. This is known to be a mechanism of dispersal of other members of the Latin American clade of *C. fimbriata*, specifically *C. cacaofunesta* (12) and *C. fimbriata sensu stricto* (21). Ambrosia beetle attacks on diseased plane trees have not been noted in European countries outside the natural range of orientalis; therefore, the association with ambrosia beetles in Greece suggests that the pathogen will spread more quickly there than it has in Italy, France, and Switzerland.

Movement of *P. platani* through water is an important dispersal factor in France, Switzerland, and Italy (8). Because *Platanus orientalis* is an important dispersal factor in France, introduction of the disease in these countries is relatively limited. Aside from other introductions, where the pathogens could be driven quickly to extinction. Protection would need to be taken soon.

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**LITERATURE CITED**