Prevalence, Distribution and Identification of *Phytophthora* Species from Bleeding Canker on European Beech[1]

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Abstract

While bleeding canker of European beech trees (*Fagus sylvatica*) has long been recognized as a problem in Europe and North America, the cause in the northeastern United States has not been clear. To resolve this, we surveyed for disease prevalence on European beech, identified the pathogens involved, proved their pathogenicity, compared protocols for pathogen detection, and conducted a soil assay to determine pathogen presence in soil surrounding established trees in residential and commercial landscapes in New York, Pennsylvania, Maryland, Connecticut and Massachusetts. Approximately 40% of surveyed trees had bleeding cankers. While *Phytophthora cactorum*, *P. gonapodyides*, *P. cambivora* and two newly described species, *P. citricola* I and *P. plurivora*, were recovered from symptomatic tissue, *P. citricola* I and *P. cactorum* were most prevalent. All caused disease when artificially inoculated into European beech sapling stems, although *P. cambivora* and *P. gonapodyides* produced significantly smaller lesions. Recovery of the pathogen from symptomatic tissue using selective media, the preferred method of diagnosis, was significantly higher in the fall. ELISA detection was more successful and worked regardless of season, but did not allow identification to the species level. All five *Phytophthora* species were found in soil surveys; *P. cambivora* was most common, followed by *P. cactorum* and *P. citricola* I. These results provide a foundation for building management strategies to protect valuable specimens of European beech.

Index words: forest pathology, *Phytophthora ramorum*, etiology.

Species used in this study: European beech (*Fagus sylvatica*).

Significance to the Nursery Industry

Phytophthora species are recognized as important causes of disease in nurseries and landscapes. Recent studies have found *Phytophthora* thriving in irrigation water, soils and plant materials. In this study, we focus on the impact of *Phytophthora* species in the decline of European beech, a popular landscape tree. Elucidating the biology of these organisms and evaluating their disease potential are important steps in developing management strategies. Our research has shown that at least five different species of *Phytophthora* are capable of causing bleeding canker on European beech, these pathogens can be found in soil surrounding trees, and they are capable of causing disease when artificially inoculated into stems. In addition, a comparison of detection methods showed significant differences by time of year and method used, which can affect future diagnosis and survey efforts.

Introduction

With any plant disease, particularly one that is newly emerging or increasing in importance, it is essential to properly identify the causal organism. This information can guide researchers to information available in the literature, dictate which questions still need to be answered, and lead to appropriate management and containment strategies. Bleeding canker on European beech has been reported for 75 years. The lesions have been attributed to infection by *Phytophthora cambivora* in Europe (12) and *P. cactorum* in the United States (10). More recently, additional species of *Phytophthora* have been implicated in this disease (27, 33). In addition, it is now apparent that as the bleeding cankers expand on otherwise healthy trees, leaf wilt, branch dieback, decline and death of affected trees will eventually follow (27). Anecdotal reports from the general public and tree care professionals in New York State and our own field observations suggest that the number of trees with cankers and the number dying has increased markedly in the last decade. Because of the importance of European beech as a landscape tree in the northeastern United States, the decline of this species has caused concern.

A first step toward development of a management program is determination of the pathogen(s) involved. In this case, identification to the species level has been impeded by difficulty in isolating the pathogen, colonization of infected plant material with secondary opportunistic pathogens, and nonspecific diagnostic tools such as ELISA. Also, the recent discovery of *P. ramorum* and *P. kernoviae* causing bleeding canker on European beech in England has led to heightened concerns that one of these pathogens or another potentially invasive pathogen may have spread to the United States (6). However, numerous other species of *Phytophthora* that could be involved are already established in the northeastern United States (10, 12, 27, 33). The identity of the pathogen(s) involved could have a large impact on management strategies.

Identification of the pathogen can also lead to information regarding sources of inoculum and the pathogen life cycle. For example, many species of *Phytophthora*, particularly tree pathogens, are thought to be normal components of the soil biota and to attack susceptible host plants through the roots

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(34) . Alternatively, *P. ramorum* has been shown to spread aerially through wind and rain splash (22). Identification of the pathogen and an understanding of the disease etiology are the first steps towards a successful management program. The objectives of our research were to: (i) survey for symptomatic European beech in the northeastern United States to determine disease prevalence and distribution, (ii) compare methodologies for culturing or detecting the pathogen(s), (iii) identify the pathogen(s) involved with this disease to the species level, (iv) survey soil around European beech for the pathogen(s) and (v) confirm the identified organisms as the pathogen(s) through completion of Koch's postulates.

Materials and Methods

Geographic distribution survey. Due to the scattered distribution of European beech in urban and suburban landscapes, we requested assistance from landscape managers, Cooperative Extension educators and other tree care professionals in the northeastern United States to find trees for this study (Table 1). Each location was surveyed in one of two ways. First, some sites were surveyed completely; every European beech on the site (e.g. campus, park, residential landscape, etc.) was checked for the presence of symptoms. These complete surveys allowed for an assessment of disease frequency. In other sites, the search was limited to selected European beech trees brought to our attention by the land owners. These limited surveys allowed us to address the concerns of residents, expand the geographic area of our survey, and to collect more isolates, but they were not included when calculating the frequency of diseased trees. The method of survey (complete versus limited) depended on the needs of the tree owners and on accessibility. A total of 18 locations were surveyed; 11 were surveyed completely and seven were limited to individually selected trees.

In the complete surveys, 321 trees were checked for the presence of dark, necrotic and/or oozing regions on the bark from the soil line to a height of 5 m. Data on leaf color (green- versus purple-leaf varieties) and canker aspect (cardinal and ordinal directions) were recorded to determine whether these factors were related to canker presence. Frequency of symptoms was analyzed using the chi square test of independence to determine whether canker presence was independent of host leaf color or trunk aspect. In the remaining seven locations, 24 trees were selected for examination in limited surveys.

Symptomatic trees were sampled by using a mallet and a disinfested chisel to excise one or more pieces of bark from canker margins. Differing trunk contours did not always allow for uniformity of sample size, but each piece was approximately 5 cm^2. Each sample was placed in a plastic bag in an insulated chest for transport back to the lab where it was then stored at 4C (40F) and processed within four days.

In the lab, bark slivers were cut from the inner surface of the sample at the margin of the symptomatic tissue with a sterile razor blade. These small (approximately 1 mm^3) pieces were placed on PARP semiselective medium (clarified V8 agar amended with pentachloronitrobenzene, ampicillin, rifampicin and pimaricin) (16). For 10 days thereafter, the plates were observed for growth of hyphae from the bark

Table 1. Locations for symptomatic trees for complete and limited surveys. The number of trees surveyed, number symptomatic, number sampled and isolates yielded is listed for each location, and the totals for the complete surveys are included.

County	No. of trees surveyed	No. of symptomatic trees	No. of trees sampled[z]	No. of trees yielding isolates	No. of *P. cactorum* isolates	No. of *P. citricola* I isolates
Complete surveys						
Tompkins, NY[x]	61	10	7	7[y]	1	6
Albany, NY	20	10	4	2	1	1
Rensselaer, NY	11	2	2	0	—	—
Greene, NY	8	2	1	0	—	—
Erie, NY	19	6	2	1	1	0
Monroe, NY[x]	57	26	12	12	6	5
Orange, NY	7	4	4	1	0	1
Dutchess, NY	11	7	7	4	2	2
Nassau, NY[x,u]	99	48	20	20	4	14
Montgomery, PA	8	4	3	1	0	1
Chester, PA	20	7	6	5	1	4
Totals	321	126	68	53	16	34
Limited surveys						
Kings, NY	n/a	n/a	1	1	0	1
Fairfield, CT	n/a	n/a	1	1	0	1
Anne Arundel, MD	n/a	n/a	2	2	0	2
Suffolk, NY	n/a	n/a	3	3	0	3
Bucks, PA	n/a	n/a	4	1	0	1
Centre, PA	n/a	n/a	3	3	0	3
Suffolk, MA[w]	n/a	n/a	10	8	1	6

[z]Some trees were not sampled due to access difficulty or at the request of the owner.

[y]One tree yielded two different species from two different cankers.

[x]*P. gonapodyides* isolated from trees in Nassau, Monroe and Tompkins counties, NY.

[w]*P. cambivora* isolated from one tree in Suffolk county, MA.

[u]*P. plurivora* from Tompkins county, NY.

into the medium. When visible hyphal growth was noted, the hyphae were transferred to clarified V8 agar (16). After 3 to 5 days, the subcultures were examined for reproductive and/or vegetative structures indicative of *Phytophthora*. If oogonia were observed or if hyphae remained sterile, then 5 to 7 days after the initial transfer, a 15-mm diameter agar plug was placed in a Petri dish (60 × 15 mm) and flooded with sterile distilled water. One day later, the plug was examined for sporangia.

Detection method evaluation. From the initial complete surveys, a subset of 234 trees at four locations in New York (Albany, Tompkins, Monroe and Nassau Counties) were selected to determine whether frequency of *Phytophthora* detection was independent of assay method (isolation on an agar-based medium versus ELISA) and seasonality. Presence or absence of cankers were noted from each tree, and a 2.5 cm^2 (1 in^2) sample was collected from the margin of each canker for isolation and ELISA tests at each of four sampling dates (July 2005, October 2005, January 2006, and May 2006). Isolations of *Phytophthora* spp. were attempted by plating bark chips from each sample on PARP as described above. In addition, 0.1 g of finely-ground, dry bark from each sample was used in a *Phytophthora* ELISA test kit (Agdia, Elkhart, IN) for the detection of *Phytophthora* spp. The differences between ELISA and selective media were analyzed using a 2 × 2 contingency table chi square test of independence. Contingency tables were also used to determine whether detection was independent of location and sample date for each detection method separately.

Identification of pathogens. Identity of each isolate was determined to the species level using morphological characteristics and confirmed by DNA sequencing. Features including oogonium size, oospore size, sporangium size, sporangium shape, persistence of sporangia on the sporangiophore and colony morphology on PDA (potato dextrose agar) were recorded. At least 30 measurements of each microscopic structure were recorded from each isolate. Characteristics were compared to descriptions in Erwin and Ribiero (16), Gallegly and Hong (17) and in Stamps et al. (37) to identify each isolate to species.

Isolates were also grown in pea broth for 7 to 10 days (19) and DNA extracted from the resulting mycelium following published protocol (36). Resulting DNA was used as a template for polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) regions following published protocol (11). Successful amplification was confirmed by running 3 µl of the product on a 0.8% agarose gel at 80V for 30 minutes and visualizing the product using ethidium bromide. The PCR product was purified using the Qiaquick® PCR purification kit (Qiagen Sciences, MD) with the alternative, higher yield protocol and then sequenced using primers ITS4 and ITS6 (11, 42) at the Cornell Life Sciences Core Laboratories Center (Ithaca, NY). ITS sequences were compared to sequences published by the National Center for Biotechnology Information using BLAST (1). Identity based on ITS sequence data was used to confirm the morphological identification.

Survey for pathogens in the soil. Five trees growing on the Cornell University campus in Ithaca, NY, and 10 trees growing on the grounds of Planting Fields Arboretum, Oyster Bay (Long Island), NY, were selected for a survey of *Phytophthora* populations in soil (Table 2). Eight of the trees had cankers from which *P. cactorum*, *P. citricola* I or *P. gonapodyides* had been isolated in the geographic distribution survey. The remainder of the trees were either symptomatic, but had not tested positive for the pathogen, or were asymptomatic and apparently disease-free. One tree (site 5) was an American beech (*Fagus grandifolia*) from a forested area (Ithaca, NY); the other 14 trees were in landscape settings. Soil was sampled in a qualitative assay using published protocol (4) in which 500 ml of soil to a depth of 10 cm (3.9 in) was collected from four points around each tree after removing the overlaying organic material. Soil from all four points for each tree was combined in a plastic bag for transport back to the lab. Each soil sample was thoroughly mixed, and a 300-ml subsample was placed into a 2-liter plastic container and flooded with distilled water to at least 3 cm (1.2 in) above the soil line. Water and soil were mixed, soil allowed to settle for 24 to 48 hrs, and all floating organic material removed. Flooded soil was maintained between 16 and 20C (60–68F).

To bait the flooded soil for *Phytophthora*, newly emerged English oak (*Quercus robur*) leaves were used (4). Leaves were grown in the greenhouse from acorns collected in Ithaca, NY. Oak seedlings were periodically pruned back to encourage growth of new leaves. Leaves were removed from the oak seedlings and immediately placed on the water surface of the soil that had been flooded two days before, 5 to 7 leaves per soil sample.

Over the next 10 days, leaves were monitored for the development of discolored lesions that were then checked microscopically for sporangia. When sporangia were observed, the leaf was rinsed with distilled water, blotted dry, cut into small (5 mm^2) pieces and plated onto PARPH selective media (PARP with hymexazol added to deter growth of *Pythium* species commonly found in soil samples) (16). Culture dishes were monitored for growth for 7 days, and all cultures with characteristics of *Phytophthora* were transferred to cV8 agar where morphological traits were used to make species identifications. In addition, following methods described above, DNA from the resulting cultures was extracted and the ITS regions were sequenced to support identification.

After 10 days, excess water was removed from the soil and it was allowed to dry for 10 weeks. The soil was then reflooded and the process repeated. The 15 soil sampling sites were sampled on five different dates: December 2005, April 2006, June 2006, October 2006, and March 2007. Some sites were not sampled at each of the dates, but each site was sampled at least twice.

All soil samples were baited as described above, in a first baiting attempt (baiting 1). Portions of the soil samples that were not flooded were stored at 4C (40F). In September of 2007, another 300 ml from each soil sample was rebaited (baiting 2). The same protocol was used, except that after the soil had been baited, drained and dried, it was remoistened for 2 days and then reflooded (S.N. Jeffers, personal communication). In addition, the pH of each soil sample was measured. The pH values were analyzed using a 2 sample t-test for significant differences between soil samples testing positive for *Phytophthora* and those testing negative, and for correspondence between pH level and species isolated (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

Table 2. Sites included in soil sample survey listed by location, and description of symptoms of the nearby tree. Baiting results are listed for each sampling date and divided into the first and second baiting attempt.

Site no.[z]	Tree disease description	Dec 05		Apr 06		Jun 06		Oct 06		Mar 07	
		baiting 1	baiting 2	baiting 1	baiting 2	baiting 1	baiting 2	baiting 1	baiting 2	baiting 1	baiting 2
1	*P. citricola* I/ *P. gonapodyides*	—[y]	—	—	—	—	—	—	—	*P. cactorum*	*P. citricola* I
2	Asymptomatic	*P. cactorum*, *P. citricola* I	—	—	—	—	—	—	—	*P. cactorum*	*P. citricola* I
3	*P. cactorum*	*P. cambivora*	*P. citricola* I	*P. cambivora*	*P. citricola* I	*P. citricola* I	*P. citrophthora*	*P. cambivora*	—	—	*P. citricola* I, *P. cambivora*
4	*P. citricola* I	—	—	*P. cactorum*	*P. citricola* I	—	—	—	—	—	—
5	Asymptomatic	—	—	—	—	—	—	X[x]	X	—	—
6	symptomatic, no isolate	—	*P. cambivora*, *P. citricola* I	X	X	—	*P. cambivora*	—	—	—	—
7	*P. citricola* I	—	—	X	X	*P. cactorum*	—	—	—	*P. gonapodyides*	—
8	*P. citricola* I	—	—	X	X	—	—	*P. cactorum*	—	—	—
9	*P. citricola* I	X	X	X	X	*P. citricola* I	*P. cambivora*	*P. cambivora*	—	*P. cambivora*	*P. citricola* I, *P. cambivora*
10	*P. cactorum*	X	X	X	X	—	*P. cambivora*	—	*P. cambivora*	—	*P. cambivora*
11	symptomatic, no isolate	*P. plurivora*	—	X	X	*P. cactorum*, *P. gonapodyides*	—	X	X	X	X
12	Asymptomatic	X	X	X	X	—	*P. cactorum*	—	—	X	X
13	*P. gonapodyides*	X	X	X	X	*P. cactorum*	—	*P. cactorum*, *P. citricola* I	—	X	X
14	Asymptomatic	X	X	X	X	*P. cactorum*	*P. cambivora*, *P. cactorum*	*P. cactorum*	*P. cactorum*, *P. cambivora*	X	X
15	symptomatic, no isolate	X	X	X	X	—	—	—	*P. cambivora*	X	X

[z]Sites 1 to 5 are located in Ithaca, NY, while sites 6 to 15 are in Oyster Bay, NY. All sites contained a European beech (Fagus sylvatica) with the exception of site 5, which had an American beech (Fagus grandifolia).

[y]— indicates no Phytophthora was recovered.

[x]X indicates no sample was taken on that date.

Table 3. Isolates and results for stem inoculations of European beech saplings.

Isolate	Species	% inoculations with lesion development (n = 6 per isolate)	Average severity rating
NYfs20	*P. cactorum*	100	2.833ab[z]
NYfs24	*P. cactorum*	100	3.666a
NYfs11	*P. citricola* I	100	3.833a
MDfs1	*P. citricola* I	100	3.833a
NYfs18	*P. citricola* I	100	4a
NYas1	*P. plurivora*	100	4a
NYfs9	*P. plurivora*	100	4a
Pcam	*P. cambivora*	33	1.833b
Pgon	*P. gonapodyides*	100	2.166b
***P. cactorum* combined[y]**		**100**	**3.25a**
***P. citricola* I combined[y]**		**100**	**3.889a**
***P. plurivora* combined[y]**		**100**	**4a**

[z]Average severity ratings (based on the 1 to 4 rating system) followed by different letters were significantly different at p = 0.05 according to the Kruskal-Wallis test.

[y]Data pooled for all inoculations with all isolates of that species.

Completion of Koch's postulates. European beech saplings were inoculated with a representative sample of *Phytophthora* isolates to complete Koch's postulates. Three isolates of *P. citricola* I, two isolates of *P. plurivora*, two isolates of *P. cactorum*, one isolate of *P. gonapodyides*, and one isolate of *P. cambivora* (Table 3) were grown on cV8 agar for 7 to 10 days and a 5 mm plug removed from the margin of each colony. Using a 5-mm cork borer, bark was removed from the main stem of 2-year-old European beech saplings and the agar plug, mycelium side facing the tree, was placed in the hole. For a control, non-colonized V8 agar plugs were used. Parafilm was wrapped around each branch to cover the wound. After 2 weeks, the parafilm was removed and each plant checked visually for typical necrotic symptoms associated with diseased trees. If necrosis was present, lesions were measured in the vertical direction. The presence or absence of a lesion was recorded, and lesion severity was rated on a scale of 1 to 4 (1 = no lesion, 2 = up to 10 mm, 3 = 11 to 20 mm, 4 = 20 + mm) as described by Linderman et al. (31). Necrotic bark on a subset of plants was removed in order to confirm that the external canker corresponded with the area of necrosis within the bark. In addition, bark samples were plated on PARP to reisolate the pathogen. Two trials were conducted; each trial had three replicates of each host-pathogen combination. Severity ratings for each isolate and each species were compared using the Kruskal-Wallis test (Minitab Statistical Software, release 15.1; Minitab Inc., State College, PA).

Results and Discussion

Geographic distribution survey. Symptomatic trees were located at all sampling locations (Table 1 and Fig. 1). Approximately 40% of European beech surveyed had bleeding cankers, with incidence ranging from 16% in Tompkins County, NY, to 64% in Dutchess County, NY. Presence of cankers was independent of leaf coloration (n = 205, p = 0.146) and trunk aspect (n = 107, p = 0.639). Of the 92 symptomatic trees sampled in both the complete and limited surveys, 78% yielded cultures of *Phytophthora* spp. at least once during the course of the survey (Table 1).

Detection method evaluation. Regardless of the season or tree location, detection by isolation on PARP was less successful than by ELISA (Table 4). Chi-square analyses indicated detection was not independent of method ($p < 0.001$) but was independent of location for each method (isolation $p = 0.257$ and ELISA $p = 0.083$). Comparison of four different sample dates revealed variation by season; isolation success was highest in fall and least in summer (Table 5); however, the frequency of *Phytophthora* detection by ELISA remained relatively stable across each of the four sampling dates. Chi-square analyses indicated detection was not independent of sampling date for isolation ($p < 0.001$) but was independent of sampling date for ELISA ($p = 0.414$).

Identification of pathogens. All but four isolates produced oogonia in single spore culture, indicating homothallism. Antheridia were paragynous. Sporangial characteristics were more valuable for species identification, inasmuch as isolates with variable shaped, semipapillate sporangia were tentatively identified as *P. citricola*, and isolates with sympodially arranged, caducous, papillate sporangia as *P. cactorum* (16, 17, 37). Most isolates were categorized into one of these two groups. BLAST searches of sequenced ITS regions from all of these isolates indicated that each isolate matched either published *P. cactorum* or *P. citricola* sequences, confirming the identification made using sporangial morphology (1). Additional analysis indicated that the isolates originally classified as *P. citricola* were better classified as *P. citricola* I and *P. plurivora*, based on comparison of sequence and SSCP data (17, 29).

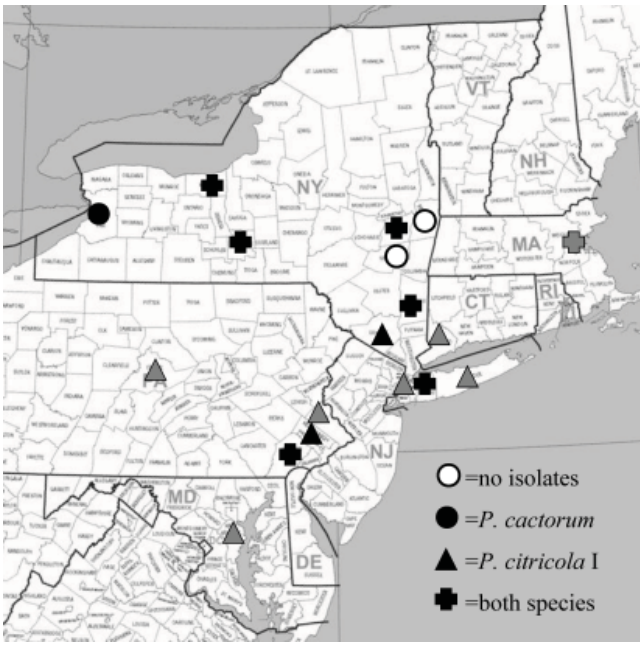

Fig. 1. Location of surveyed counties and distribution of species recovered. White circles indicate no isolates were recovered, darkened circles are *P. cactorum*, triangles are *P. citricola* I, and crosses are both species. Gray shapes indicate limited survey locations, black shapes are complete survey locations.

Table 4. Culture and ELISA results for trees surveyed from July 2005 to May 2006, listed by location.

Location	No. trees surveyed	No. trees cankered (% of surveyed)	No. trees yielding cultures (% of cankered)[z]	No. trees ELISA positive (% of cankered)[z]
Albany	20	10 (50%)	1 (10%)	6 (60%)
Monroe	55	23 (42%)	8 (35%)	22 (96%)
Tompkins	61	10 (16%)	4 (40%)	7 (70%)
Nassau	98	46 (47%)	20 (43%)	36 (78%)
Total	234	89 (38%)	33 (37%)	71 (80%)

[z]Sample dates and seasonality reported for each culture method in Table 5.

Chi-square analyses indicated detection frequency was not independent of method ($p < 0.001$), but was independent of location ($p = 0.257$ for isolation, $p = 0.083$ for ELISA).

By combining morphological and molecular identification methods, we identified 51 isolates of *P. citricola* I and 17 isolates of *P. cactorum*. These two species were found in 15 and eight sites respectively, with seven sites yielding isolates of both species (Fig. 1). In most cases, trees yielded only one species of *Phytophthora*, although one tree (Tompkins County, NY) did yield an isolate of *P. citricola* I and *P. gonapodyides* (see below) from two separate cankers. In one symptomatic tree in Greene County, NY, and two more trees in Rensselaer County, NY, we were unable to isolate *Phytophthora*, although two trees (one in each location) were ELISA positive (Table 1). Some trees were sampled on more than one occasion, which may have increased the chances of *Phytophthora* recovery. In addition to *P. cactorum* and *P. citricola* I, we identified one isolate of *P. plurivora*, one isolate of *P. cambivora*, and three isolates of *P. gonapodyides* using the same methods.

Survey for pathogens in the soil. Of the 15 soil sampling sites, only one (site 5) failed to yield any *Phytophthora* isolate on any of the sample dates (Table 2). This site was occupied by an asymptomatic American beech in a forest setting; very different from the landscaped and managed areas that the other soil samples were taken from. The remaining 14 sites (three of which had asymptomatic trees) yielded *Phytophthora* spp. from the soil, and three yielded the same *Phytophthora* sp. as the species isolated from the cankered tree, all of which were infected with *P. citricola* I.

Phytophthora species recovered from each site varied depending on sample date. Of all sampling sites, only two sites (10 and 14) yielded the same species on all sampling dates. Twelve sampling sites yielded different species across sampling dates or failed to yield any *Phytophthora* species depending on the sampling date. Of the five sample dates, June 2006 and March 2007 had the highest successful baiting rate, defined as the number of samples yielding at least one isolate of *Phytophthora*. Success rates varied from 40 to 60% of the samples collected on each date. Of the 52 soil samples, 24 never yielded *Phytophthora* , but 15 yielded more than one species of *Phytophthora*. *Phytophthora cambivora*, the most frequently isolated species, was found in 16 samples. *Phytophthora cactorum* was found in 14 samples, *P. citricola* I in 12 samples, and *P. gonapodyides* in 2 samples while *P. citrophthora* and *P. plurivora* were found in one each.

The first baiting, conducted immediately after collection of the soil in December 2005, April 2006, June 2006, October 2006 and March 2007, had different results than the second baiting, conducted in September 2007. Although success rates (20/52 and 18/52) were similar, detection of *P. citricola* I and *P. cambivora* increased in the second baiting (from 4 to 8 and 5 to 11 respectively) while that of *P. cactorum* and *P. gonapodyides* decreased (from 11 to 3 and 2 to 0 respectively).

The pH of the soil samples ranged from 5.21 to 7.74. No significant differences were found between the pH of soil samples testing positive for *Phytophthora* and those testing negative ($p = 0.19$) or based on species isolated.

Completion of Koch's postulates. Inoculation of European beech saplings resulted in necrosis developing around the point of inoculation for all isolates and all *Phytophthora* species (Table 3). Control inoculations never developed necrosis. Excavation into the diseased bark indicated that when necrosis occurred externally, it also extended through the cambium, and internal and external symptoms covered the same area. Disease incidence and severity differed among isolates and pathogen species. The Kruskal-Wallis test revealed that, based on the 1 to 4 rating scale, the severity of lesions caused by *P. cactorum*, *P. citricola* I and *P. plurivora* was significantly greater ($p < 0.01$) than the severity of the lesions caused by *P. cambivora* and *P. gonapodyides*. Average severity ratings for each isolate and each species that are significantly different are followed by different letters in Table 3. In addition, *P. cambivora* and *P. gonapodyides*

Table 5. Number of trees producing cultures or positive ELISA results by method, sampling date, and location.

	Culture				ELISA			
Location	Jul 2005	Oct 2005	Jan 2006	May 2006	Jul 2005	Oct 2005	Jan 2006	May 2006
Albany	0	1	0	0	5	2	2	5
Monroe	0	8	0	1	12	16	11	9
Tompkins	1	4	3	0	6	7	6	7
Nassau	2	14	8	8	22	27	28	34
Total	3	27	11	9	45	52	47	55

Chi-square analyses indicated detection frequency was not independent of sampling date for isolation ($p < 0.001$) but was independent of sampling date for ELISA ($p = 0.414$).

did not cause girdling cankers. All sampling from necrotic lesions resulted in successful reisolation of the respective pathogen.

Our survey shows that *Phytophthora*-incited bleeding canker on European beech is common in the northeastern United States, affecting approximately 40% of mature trees regardless of leaf color or growth habit. All trees that had cankers were of large diameter (> 40 cm), but this may be partially due to sampling artifact. Larger trees were easier to find and were more likely to be brought to our attention by concerned tree owners. However, mature trees may be more susceptible to bleeding canker development or many years may be required for cankers to become visible.

For detecting the presence of *Phytophthora* in cankers throughout the year, ELISA tests had a higher success rate than isolation on selective media, which was most successful in October. However, pathogen isolation from symptomatic tissue has the advantage of yielding a culture that can be used for subsequent analyses including species identification and pathogenicity trials. Diagnosticians and researchers will therefore need to weigh the advantages of each method, taking into consideration the needs of the tree owner and tree care provider, the time of year, the availability of the expertise required to speciate *Phytophthora* cultures, and the effect that species identification will have on any subsequent treatment or research conducted. For our purposes, pathogen isolation was preferred.

Fluctuation in isolation rates by time of year may be due to temporal differences in growth rates of *Phytopthora* species. *Phytophthora* species do have seasonal fluctuations in ability to colonize plant tissue (15, 18, 23, 32). Our isolation results suggest that these pathogens may be more actively growing during certain times of year, making them easier to isolate from symptomatic tissue.

Identification of isolates using both morphological and molecular techniques is important because of the difficulty in identifying members of the genus *Phytophthora* to the species level (11). Morphological characteristics often overlap, making identification difficult by these criteria alone (11, 16, 17, 37), while nucleotide sequences from different regions of the genome may produce conflicting results when used for species identification (30). Morphological characteristics, particularly sporangial morphology, were helpful in identifying isolates of *P. cactorum*, *P. gonapodyides*, and *P. cambivora* in this study, but the diversity seen among isolates tentatively classified as *P. citricola* and previously reported as either *P. citricola* or *P. inflata* (28) warranted further investigation. Subsequent morphological and molecular comparison with newly described species indicated a new identification for these isolates as *P. citricola* I and *P. plurivora*. This underscores the importance of careful identification using multiple methods of comparison.

In contrast to results from European surveys, where three species of *Phytophthora* (*P. cactorum*, *P cambivora*, and *P. citricola*) are usually associated with bleeding canker of European beech (28), we consistently found only two species in the northeastern United States: *P. cactorum* and *P. citricola* I. The presence of *P. cactorum* on approximately one-third of the surveyed trees in our study confirms the observation initially made by Caroselli in 1953 (10) that identified *P. cactorum* as the cause of bleeding canker on numerous tree species in the northeastern United States. *Phytophthora cambivora* was isolated only once during our survey and was not widely encountered as might be suggested by reports from Europe (12, 28).

Results of our soil surveys reveal a difference between *Phytophthora* populations in cankers and in the European beech rhizosphere. In soil, the most frequently isolated species was *P. cambivora* (16 out of 52 soil samples). However, *P. cambivora* was found at only 6 of the 15 sites, indicating it may not be widespread but simply easier to detect through baiting with English oak leaves when present. None of the sites sampled contained trees found to be infected with *P. cambivora*. It is unclear why this pathogen, which has been documented to cause bleeding canker on European beech (12, 28), is not widely association with this disease in the northeastern United States. *Phytophthora plurivora* was only recovered from soil at one site on one sample date. The disease survey found few trees infected with *P. plurivora*, and therefore trees infected with *P. plurivora* were not included in the soil survey, which may have effected these results.

Successful isolation of both *P. citricola* I and *P. cactorum* from 12 soil samples each suggests that baiting with *Q. robur* leaves is an effective method of detecting these pathogens in soil. The selective medium used in this study, PARPH, contains hymexazol, which has been reported to inhibit growth of *P. cactorum* (20, 24). However, our results indicate that it is able to grow on this media, in accordance with Tay et al. (38) and Jung et al. (27) . Differential sensitivity to hymexazol among isolates of *P. cactorum* may have led us to underestimate the prevalence of this species.

While there is not a correlation between soil pathogen populations and occurrence of diseased trees, others have reported significant associations between soil populations of *Phytophthora* and tree health (2, 3, 4). Also, the changes observed in *Phytophthora* isolation frequency between sample dates correspond with previous studies that found seasonal fluctuations in soil populations (4, 9, 35, 39, 41) . Because this soil assay did not measure the quantity of inoculum in the soil, it is not possible to determine if fluctuations in population size also occurred.

Almost half of the soil samples (24 out of 52) did not yield any species of *Phytophthora*, possibly due to limitations in the sampling methods. Baiting may not be very efficient at recovering small *Phytophthora* populations, particularly if there are many other competitors which limit *Phytophthora* colonization of the oak leaf bait. Bait type could also have biased results in terms of number of positive soil samples and species recovered. In addition, the volume of soil sampled may have been insufficient to recover all *Phytophthora* species associated with the rhizosphere. However, the pooling of four samples combined with the thorough mixing of the samples from each tree may have helped to limit error caused by this factor. Baiting is the most common method for surveying soils for *Phytophthora* because of the method's ability to eliminate faster growing saprobic fungi found in soil (2, 3, 4, 5, 8, 9, 21, 25, 26, 27, 39, 40). This specific protocol has been used by Balci et al. (4) with a success rate similar to ours. Baiting also has advantages because the resulting culture can be identified using morphological traits and multiple gene sequences, and then used in pathogenicity assays.

Isolates of *P. gonapodyides* and *P. cambivora* were significantly less aggressive than the other *Phytophthora* species tested, as measured by canker size. This was surprising because *P. cambivora* is recognized as the causal agent of bleeding canker on European beech (12, 13, 14, 28). Isolates

used in this study were recovered from soil, and therefore they might not be as aggressive as isolates obtained from bleeding cankers.

In addition to *P. cactorum*, *P. cambivora*, *P. gonapodyides*, *P. plurivora* and *P. citricola* I, European forest surveys have reported *P. citricola*, *P. syringae*, *P. pseudosyringae*, *P. ramorum* and *P. kernoviae* causing bleeding canker on European beech (6, 7, 28, 33) . In view of the perceived threat of the latter two species to North American forests (6, 7), understanding the current causes and characteristics of this disease is essential. Continued surveying for symptomatic European beech along with identification to the species level is needed. The sampling and detection methodologies described in this study will be important in these efforts.

This study has revealed the identity and the distribution of the pathogens involved in bleeding canker of European beech in the northeastern United States. Five different species of *Phytophthora* were shown to be involved through their presence in symptomatic plant tissue, in soil surrounding European beech, and their ability to cause disease on artificially inoculated trees. This study provides a baseline to which future surveys can be compared. In addition, understanding the possible reservoirs and sources of pathogen inoculum helps to build a disease development model as we seek to disrupt the disease cycle. Future researchers can use this information to explore the effectiveness of promoting tree health and deterring pathogen growth through chemical treatments and cultural methods. The need for these management strategies will increase as the impact of *Phytophthora* species on European beech is better understood.

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