Genetic Heterogeneity of *Leucostoma* Species in Michigan Peach Orchards

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ABSTRACT

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The pathogens causing perennial canker of peach, *Leucostoma* spp., were characterized in Michigan orchards to identify which species and subgroups (cryptic species) were prevalent on a highly susceptible peach cultivar, Loring, and a less susceptible cultivar, Redhaven. Four hundred and three strains of *Leucostoma* were isolated from cankers in three south-west Michigan orchards where 'Loring' occurred adjacent to 'Redhaven' in side-by-side plots. Based on colony morphology and small nuclear rDNA size polymorphisms, three cryptic species were detected; 89% of the isolates were identified as *L. persoonii* phenetic group 1, 10% were *L. cincta* group 4, and 1% were *L. persoonii* group 2. Pathogen profiles differed significantly between cankers on small branches of 'Loring' and 'Redhaven', and between cankers on small branches and trunks. Of 1,232

random pairings among isolates of *L. persoonii* group 1, 95% were vegetatively incompatible. A minimum of 11 and a maximum of 17 maternal lines were identified based upon mitochondrial DNA restriction fragment length polymorphisms among 69 isolates of *L. persoonii* group 1 from one split-cultivar block of 72 trees. Vegetative compatibility loci were randomly associated with maternal lines. Evidence of clonality was absent in *L. persoonii* group 1, as no correlation occurred between these two sets of independent genetic markers and no genotype was over-represented. The number of cryptic species, the number of maternal lines, and the frequency of incompatibility within maternal lines indicate that considerable genetic variation exists within the *Leucostoma* populations in Michigan peach orchards, and that sexual recombination is common.

Additional keywords: *Cytospora*, pulse field electrophoresis, ribosomal DNA intron.

Perennial canker (synanamorph *Cytospora* canker, *Leucostoma* canker, and *Valsa* canker) is the most important disease limiting peach (*Prunus persica* (L.) Batsch) production in the northern United States and southern Canada (26). Infected orchards exhibit declining productivity and ultimately a reduced life span (7). The disease causes extensive perennial cankers that result in dieback of branches and scaffold limbs and girdling of trunks. The pathogens enter peach trees through wounds or senescing tissue, with cold-damaged tissue as one of the most important infection sites (13). While infection of cold-injured buds and shaded spurs is common on small branches, infection of trunks and major scaffolds occurs primarily on cold-injured sapwood.

Perennial canker is difficult to control. Once a tree is infected, the disease cannot be controlled with fungicides (21). Current strategies to control the disease are based largely on avoiding the factors that predispose trees to disease. A combination of cultural practices including removal of infected branches and prevention of insect, rodent, and winter injuries may reduce the incidence of the disease (8), but may not always be practical. Commercial cultivars differ in their susceptibility to canker; however, no resistant cultivars have been developed. The peach cultivar Redhaven is reported to be less susceptible than the peach cultivar Loring (11). Breeding for increasing canker resistance could contribute significantly to control.

One of the constraints to the development of resistant cultivars for Michigan is the lack of knowledge regarding the heterogeneity of the pathogen. The causal organisms of this disease are two related fungal taxa, *Leucostoma persoonii* Höhn. and *L. cincta* (Fr.:Fr.) Höhn. On peach, both are common, but perithecia of each

are rare (24). The two taxa have been found to consist of a minimum of five genetically different subgroups (36). The subgroups, called phenetic groups (PG), have been defined by isozyme analysis and likely represent genetically isolated cryptic species. Based on isozyme polymorphisms, Surve-Iyer et al. (36) identified two groups of *L. persoonii* (PG 1 and PG 2) and two groups of *L. cincta* (PG 4 and PG 5) among isolates from cankers on *Prunus* spp. in Michigan and another group (PG 6) among isolates from *Malus* that cause canker when used to inoculate peach. Some virulence differences have been associated with various groups (36); however, the genetic heterogeneity within the pathogen populations is not known. Additionally, it remains controversial whether ascospores or conidia are most responsible for infection and spread (3). Sexual recombination could significantly increase genetic variation.

Leucostoma spp. have been distinguished based upon DNA sequence and isozyme and DNA polymorphisms (1,4,35,36). Isozyme polymorphisms have revealed cryptic speciation, but polymorphisms have not been commonly encountered within a phenetic group of *Leucostoma* (36). Indeed, the over-representation of some isozyme phenotypes has suggested clonal reproduction in other fungal species (39). However, genetic diversity within a phenetic group of *L. persoonii* has been revealed by the occurrence of numerous vegetative compatibility groupings (VCG) in a restricted locale (3). The genetic system regulating VCG is encoded by nuclear genes in certain fungal species (31). Vegetative compatibility occurs only between closely related genotypes, and numerous groupings imply numerous genetically different individuals (5).

To determine if genetic diversity is the result of sexual recombination, markers that correspond to maternal inheritance would be useful. Fungal mitochondrial (mt) DNA is uniparentally inherited in ascomycetous fungi like *Leucostoma* (29). Variability in mtDNA has been used successfully to study relationships of population structure and genetics within a fungal species (19) and *forma specialis* (23). It has permitted determination of maternal

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inheritance among individuals in a local fungal population (32), in a race (18), and in a single VCG (20). The nonrandom association of mtDNA haplotypes with vegetative compatibility loci and nuclear haplotypes has been used to demonstrate that *Fusarium oxysporum* is a clonal fungus (6).

The objectives of this study were to (i) identify which *Leucostoma* species and subgroups were most prevalent on the cultivar Loring compared with 'Redhaven' in Michigan orchards; (ii) determine if a similar profile of *Leucostoma* species occurs in cankers on trunks as on small branches; (iii) examine the genetic variability and the number of maternal lineages present in an orchard; (iv) ascertain whether the distribution of genotypes provides evidence of clonal or sexual reproduction; and (v) determine whether ascomycetes are produced on and near peach.

MATERIALS AND METHODS

Canker sampling. Diseased tissues were collected from cankers on trunks and large scaffolds and from cankers on smaller canopy branches of each tree (approximately 12 years old) of two peach cultivars, Redhaven and Loring, grown in three orchards, GO, BA, and KE, located in southwest Michigan. The sampled 'Redhaven' and 'Loring' plots were adjacent to each other in each orchard. Thirty-six trees of each cultivar were sampled from each orchard in a block of six consecutive rows (with six consecutive trees in each row) right next to the plot of the other cultivar. A total of 432 cankers were sampled.

Isolation and identification of *Leucostoma*. Diseased tissue excised from the margins of cankers was surface-sterilized by soaking in 5% sodium hypochlorite for 3 min and then blotting dry between sterile paper towels. The tissue was pushed into the flesh of firm apples (*Malus domestica* L., cv. Granny Smith) that were then incubated at room temperature (20 to 24°C) for 7 to 10 days. Small pieces of apple flesh from the margins of each diseased lesion were then placed on Leonian's medium (1.2 g of KH_2PO_4 , 0.6 g of MgSO_4 , 6.25 g of maltose, 6.25 g of malt extract in 1 liter of distilled H_2O) solidified with 15 g of agar, in separate petri plates under sterile conditions. The fungi were cultured in the petri plates at room temperature for 7 to 10 days and then stored at 4°C.

All isolates were grouped phenotypically according to morphological criteria described by Surve-Iyer et al. (36). *L. persoonii* group 1 (PG 1) was distinguished from the other isolates by characteristic lobate colony margins. Based on this criterion, all isolates of *Leucostoma* other than group 1 were screened for size polymorphisms in the nuclear small subunit of the ribosomal DNA repeat (nu-SSU-rDNA). The nu-SSU-rDNAs of *L. cincta* group 4 (PG 4) and group 5 (PG 5) have an insert of about 450 base pairs (bp) in the fragment amplified between the annealing sites of two primers (NS21/NS22), while the nu-SSU-rDNAs of *L. cincta* group 6 (PG 6) and *L. persoonii* do not have the insert (35). The relative molecular weights of the polymerase chain reaction (PCR) products following electrophoretic separation on agarose gels identified PG 4 and differentiated it from the morphologically similar PG 2. PG 5, a tentative subgroup (36), was included as part of the PG 4 population.

Vegetative compatibility test. Vegetative compatibilities were initially examined among PG 1 isolates from cankers of adjacent trees using the protocol described by Adams et al. (3). When the isolates were subdivided into maternal lines based on mtDNA restriction fragment length polymorphisms (RFLPs), vegetative compatibilities were tested among isolates within maternal lines. The pairing experiments were designed in such a way that each isolate was paired at least once with all other isolates within the maternal line.

Total fungal DNA preparations. Fungal isolates were cultured in 100 ml liquid Leonian's medium at room temperature for 10 to 14 days. Mycelia were harvested by vacuum filtration through Miracloth (Calbiochem Corp, San Diego, CA), lyophilized, and

stored at -20°C. Total genomic DNA was extracted from lyophilized hyphae by the method of Lee et al. (27).

PCR amplifications. A specific fragment of the nu-SSU-rDNA was amplified by PCR using NS21 (5'-GAATAATAGATAGGACG-3') and NS22 (5'-AATTAAGCAGACAACTC-3') as the primer pair (17). PCR reactions were performed with Amplitaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) as described by White et al. (41). Thirty PCR cycles were performed on a Perkin-Elmer-Cetus thermocycler (Perkin-Elmer Corp.) with the following parameters: 94°C denaturation for 1 min, 48°C annealing for 1 min, 72°C extension for 1 min plus 4-s extension after each cycle, and with a final extension at 72°C for 10 min. PCR products (8 µl) were separated by electrophoresis on agarose minigels and viewed under ultraviolet light (33).

mtDNA preparations. To determine whether the two mtDNA probes used in the Southern analysis hybridize only to mtDNA, intact mtDNA of PG 1 isolate MI11.10 was separated from intact chromosomes in 0.8% agarose gels by pulse field electrophoresis (34). Electrophoresis, using the CHEF-DRII system (Bio-Rad Laboratories, Melville, NY), was for 18 h at 10°C and 200 V with a pulse of 60 s in 0.5× Tris-borate EDTA (TBE). Intact chromosomes were distinguished from cytoplasmic DNA by the relative distance of migration in the agarose gel, which is correlated to the sizes of the molecules. The intact mitochondrial and chromosomal DNA sample was prepared as follows: 23 500-ml Erlenmeyer flasks containing 50 ml of 1.3× Leonian's medium were seeded with 3 ml of *L. persoonii* conidia (10^7 conidia per ml) and incubated on a reciprocal shaker (300 rpm) for 36 h at room temperature (20 to 24°C). Mycelium was harvested on Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) and rinsed with sterile water and cold (4°C) 0.45 M EDTA. Mycelium was scraped into 5 ml of protoplasting medium (0.1 M NaPO_4 buffer, pH 5.8; 1 M mannitol; 0.1% NovoZym 234 [Novo BioLabs, Danbury, CT]; 0.05% chitinase from *Serratia* [Sigma Chemical Co., St. Louis]; and 1% low-melt agarose) in a 50- by 15-mm petri plate and incubated overnight at 33°C with an overlay of 10 ml of EDTA-mannitol solution (0.45 M EDTA, pH 9.0; 1 M mannitol; and 9 mM cysteine-HCl), and then overnight at 50°C with an overlay of 10 ml of proteinase-SLS solution (0.2% proteinase XIV [Sigma Chemical Co.]; 1% *N*-lauroylsarcosine sodium salt; 0.45 M EDTA, pH 9.0; and 10 mM Tris-HCl). Agarose plugs containing the treated hyphae were desalted and equilibrated by soaking for 4 h at 4°C in 0.5× TBE buffer, pH 8.0, and then directly loaded into the wells of 0.8% agarose gel and sealed in with warm 0.8% agarose. The *L. persoonii* samples alongside chromosome DNA size standards of *Saccharomyces cerevisiae* strain YNN295 and *Schizosaccharomyces pombe* strain 972 h- (Bio-Rad Laboratories) were separated on the CHEF-DRII pulse field electrophoresis system.

Southern hybridizations with mtDNA probes. Two mtDNA probes were used to determine the maternal lineage of the PG 1 isolates from the BA orchard, B3 (38) and A13 (22). B3 is an 8.8-kilobase pair (kbp) *EcoRI* restriction fragment of *Neurospora crassa* 74A mtDNA and was provided by Taylor and Smolich (38). A13 is a 5.3-kbp *PstI* mtDNA restriction fragment of *F. oxysporum* f. sp. *melonis* strain P-2 and was provided by Jacobson and Gordon (22).

Recombinant plasmids pBR325 with B3 fragment and pUC119 with A13 fragment were isolated from overnight cultures of *Escherichia coli* strains HB101 and MV1139, respectively, using Wizard Minipreps (Promega Corp., Madison, WI). The probes were labeled with digoxigenin using the Genius 1 DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

One to three micrograms of genomic DNA from each isolate were digested with restriction enzymes *HpaII* and *HaeIII* separately, and the products were transferred to nylon membranes for Southern hybridizations (33). Fluorescent hybridization signals were detected as described in the User's Guide of the Genius System (Boehringer Mannheim Biochemicals).

Data analysis. Percentage data were arcsine transformed (i.e., arcsine of the square root of the value of percentage data) and analyzed as a split-plot design with cultivars as the main plot factor, canker sites and pathogens as subplot factors, and orchards as replicates. The data was further analyzed considering each pathogen separately. All analyses were done using MSTAT-C computer software (15).

Occurrence of ascocarps. Branches of peach trees were inoculated in replicated trials using 56 isolates of *Leucostoma*, as described with isolate information in Surve-Iyer et al. (36). An aluminum tag inscribed with the isolate number was attached to each branch at the time of inoculation. Following the analysis of the inoculation trial described in Surve-Iyer et al. (36), the branches were pruned to approximately 50 cm in length and laid on the ground in May. Branches were collected 2 years later in September. Bark showing signs of fungal fruiting bodies was excised and placed in labeled envelopes for later microscopic observation. Bark containing the stromatic ascocarps of *Leucostoma* was placed in inner envelopes. These voucher specimens were stored in the *Valsa* herbarium of G. Adams.

Native wild black cherry, *Prunus serotina* Ehrh., in Michigan woodlands were examined for *Leucostoma* ascocarps. Wind-thrown branches below established trees were identified based on bark and bud characteristics, collected, and examined for mature ascocarps. Exposed saplings from the forest edge were examined directly for cankers and ascocarps. Voucher specimens of ascocarps were stored in the *Valsa* herbarium.

RESULTS

Four hundred and three isolates of *Leucostoma* were recovered from the 432 cankers sampled, and each isolate was recovered from a different canker. Of the 403 isolates, 124, 135, and 144 were recovered from cankers sampled from the BA, GO, and KE orchards, respectively. Three cryptic species of *Leucostoma* canker fungi, PG 4, PG 1, and PG 2, were detected among these 403 isolates. PG 1 was the most common, representing 357 of the 403 isolates. PG 4 and PG 2 represented 41 and 5 of the 403 isolates, respectively.

There was a significant interaction between position of the canker on the tree and species of *Leucostoma* (*F* was significant at *P* = 0.01). Therefore, the data were analyzed individually for each *Leucostoma* species. For both PG 1 and PG 4, position of the canker on the tree had a significant effect (Tables 1 and 2, respectively). A significantly larger percent of cankers on tree trunks yielded PG 1 than did cankers on small branches (Table 3). On the

TABLE 1. Analysis of variance for the occurrence of *Leucostoma persoonii* group 1

Source	df	Mean square	F	P value
Orchard	2	69.14	5.17	0.16
Cultivar	1	122.88	9.2	0.09
Error (a)	2	13.36		
Canker site	1	421.03	21.02	0.01
Cultivar by canker site	1	27.18	1.36	0.31
Error (b)	4	20.03		

TABLE 2. Analysis of variance for the occurrence of *Leucostoma cincta* group 4

Source	df	Mean square	F	P value
Orchard	2	80.79	8.68	0.10
Cultivar	1	48.36	5.19	0.15
Error (a)	2	9.31		
Canker site	1	450.07	30.74	0.01
Cultivar by canker site	1	34.92	2.38	0.20
Error (b)	4	14.64		

other hand, a significantly larger proportion of cankers on branches yielded PG 4 than did cankers on tree trunks.

Significant differences were observed in the composition of *Leucostoma* spp. recovered from the different peach cultivars (*F* was significant at *P* = 0.04). The interaction of cultivars $\times$ *Leucostoma* species was also significant (*F* was significant at *P* = 0.02). When the data were separated between small branch and trunk infections, differences were found only on the small branches. PG 1 was recovered from significantly more cankers than was PG 4 in 'Redhaven' versus 'Loring' (Table 4). PG 2 was only isolated from cankers on 'Loring'.

Following pulse field electrophoresis, two dense bands of fungal DNA were evident on the ethidium bromide-stained agarose gels. One band of chromosomal DNA was greater than 1.6 megabases, which is in the common range of chromosome length (1.3 to 6.2 megabases) for ascomycete fungi (30). The other band of DNA, the putative mtDNA, occurred at less than 0.6 megabases. When the separated DNA was probed with the two mtDNA probes, the probes hybridized strongly to the DNA smaller than 0.6 megabases (Figs. 1 and 2). Figure 1 demonstrates that B3 is specific for mtDNA in *L. persoonii*. In Figure 2, probe A13 does show some hybridization to the DNA just below the sample well. The strength of the A13 hybridization in well 1 compared with well 3 is most likely due to mtDNA tangled with the chromosomal DNA, a common situation in pulse field electrophoresis due to the size and concentration of the unsheared DNA (9). We treat A13 as specific for mtDNA in *L. persoonii* based on its origin from mtDNA and on its stronger hybridization to the lower molecular weight DNA.

Sixty-nine isolates of PG 1 from the BA orchard were analyzed by Southern hybridization with B3 and A13 mtDNA probes. Considerable mtDNA variation was found among the isolates (Figs. 3 and 4). Eleven maternal lineages, defined as two or more isolates having the same banding pattern, were identified within the split-cultivar block from the BA orchard (Table 5). Two major lineages, 1 and 2a, comprised 55% of the 69 isolates investigated. Lineages 3a, 3b, 4b, 4c, and 5a were only isolated from cankers on cultivar Redhaven, and lineages 2b and 6 were only isolated from cankers

TABLE 3. Percentage of cankers on branches and trunks caused by different cryptic species of *Leucostoma*^a

Canker location	Percent cankers for each causal pathogen		
	<i>L. persoonii</i> group 1 ^b	<i>L. cincta</i> group 4	<i>L. persoonii</i> group 2
Branch	82.55	16.00* ^c	1.15 ns
Trunk	94.50**	4.50	0.17

^a The data are summed over three orchards and two cultivars. *Leucostoma* spp. were isolated from 403 cankers.

^b "group" refers to the phenetic group based on isozyme polymorphisms (36), and herein were identified based upon cultural morphology and rDNA polymorphism.

^c * = the means in the column are significantly different at the *P* < 0.05; ** = the means in the column are significantly different at the *P* < 0.01; ns = the means in the column are not significantly different.

TABLE 4. Percentage of cankers on branches of 'Redhaven' and 'Loring' caused by different cryptic species of *Leucostoma*^a

Cultivar	Percent cankers for each causal pathogen		
	<i>L. persoonii</i> group 1 ^b	<i>L. cincta</i> group 4	<i>L. persoonii</i> group 2
Redhaven	88.77* ^c	11.23	0.0
Loring	76.33	20.77*	2.9 ns

^a The data are summed over three orchards and two cultivars.

^b "group" refers to the phenetic group based on isozyme polymorphisms (36), and herein were identified based on cultural morphology and rDNA polymorphism.

^c * = the means in the column are significantly different at *P* ≤ 0.05; ns = the means in the column are not significantly different.

on ‘Loring’ (Table 5). In addition to the 11 lineages, six isolates each with a unique banding pattern (single-occurrence maternal lines) were found on cultivar Redhaven (Fig. 4).

One hundred eighty-two isolates of PG 1 from cankers on adjacent trees of all three orchards were paired on agar solidified medium to examine vegetative compatibility. Of a total of 1,232 pairs examined, 95% of the pairs were incompatible with each other. No compatible isolates were found within five of the six maternal lines examined (Table 6). Additionally, no isolates representing different maternal lines were compatible. Such a high incompatibility among isolates made it impractical to further divide PG 1 into vegetative compatibility subgroups.

In September of the second year, excised branches having intact labels for 43 of the isolates were collected. Of nine isolates of PG 1, six showed ascocarps. The isolate that produced the most abundant ascocarps was Lp10, voucher 335. Of 10 isolates of PG 2, 5 showed ascocarps. The most prolific isolate was T7.4, voucher 347. Of 16 isolates of PG 4, 7 showed ascocarps. The most prolific isolate was F1H, voucher 367. Of eight isolates of PG 6, seven showed ascocarps. The most prolific isolate was A45, voucher 377. Ascocarps of PG 1 were common on branches of wild black cherry. Compiled collections separated by location were stored as vouchers 378 to 383.

DISCUSSION

Three cryptic species of *Leucostoma* were present in the three southwest Michigan peach orchards, PG 1, PG 2, and PG 4. PG 1 was the predominant *Leucostoma* sp. PG 2 and *L. cincta* occurred

only rarely in these orchards. This result differs from that of Dhanvantari (14) and Wensley (40), who identified *L. cincta* as the primary causal agent of perennial canker in Ontario peach orchards.

Although PG 1 predominated in the isolations from both canker locations on the tree, it was significantly less frequent in the isolations from small branch cankers than from trunk cankers. Dhanvantari (13) also found isolate differences between canker sites. *L. cincta* was predominantly isolated from cold-injured buds, while *L. persoonii* was predominatly isolated from perennial cankers. These observations may be caused by differing availabi-

Fig. 2. *Leucostoma persoonii* DNA hybridized to *Fusarium oxysporum* mitochondrial DNA probe A13 following separation from intact chromosomes by pulse field electrophoresis. **A**, Ethidium bromide-stained gel viewed under ultraviolet light. **B**, Autoradiograph after hybridization of probe A13. Lanes 1 and 3, *L. persoonii* MI11.10 phenetic group 1; and lane 2, yeast chromosome DNA size standard, *Saccharomyces cerevisiae* strain YNN295.

Fig. 1. *Leucostoma persoonii* DNA hybridized to *Neurospora crassa* mitochondrial DNA probe B3 following separation from intact chromosomes by pulse field electrophoresis. **A**, Ethidium bromide-stained gel viewed under ultraviolet light. **B**, Autoradiograph after hybridization of probe B3. Lanes 1 and 2, *L. persoonii* MI11.10 phenetic group 1; and lane 3, yeast chromosome DNA size standard, *Saccharomyces cerevisiae* strain YNN295.

Probe/ Enzyme	Maternal lineage group											DNA size Marker (kb)
	1	2a	2b	3a	3b	4a	4b	4c	5a	5b	6	
												21.23
												5.15
												4.97
												4.27
												3.53
B3/ HaeIII												2.03
												1.90
												1.58
												1.38
												5.15
												4.97
												4.27
												3.53
B3/ HpaIII												2.03
												1.90
												21.23
												5.15
												4.97
												4.27
												3.53

Fig. 3. Representative diagram of the mitochondrial DNA restriction fragment length polymorphism banding patterns distinguishing 11 maternal lineages of *Leucostoma persoonii* phenetic group 1 from the BA peach orchard.

lities and relative virulence of different *Leucostoma* cryptic species at the time when the different canker sites are susceptible to the disease. *L. cincta* has been reported to be more virulent in cool weather and *L. persoonii* more virulent in warm weather (12). Leaf scars and cold-injured buds predispose small branches to infection in late fall and early spring when *L. cincta* could be relatively more virulent. Any injuries to the scaffolds and tree trunks during summer, such as sunburns, injury from boring insects, or mechanical injuries caused by field equipment, might predispose the trees to the disease when *L. persoonii* inoculum is most abundant (28) and more virulent.

L. cincta was isolated from significantly more cankers on the 'Loring' branches compared with the 'Redhaven' branches. These differences might be related to the cultivar difference in canker tolerance and virulence differences between the pathogens. 'Redhaven' was reported to be relatively tolerant to *Leucostoma* spp. (13) and 'Loring' highly susceptible (11). Field tests showed that PG 1 was more virulent than *L. cincta*, and *L. cincta* isolated from *Prunus* spp. was more virulent than PG 2 in Michigan on peach (36). *Leucostoma* spp. of relatively low virulence may be more successful on a more susceptible cultivar than on a more tolerant cultivar.

Seventeen mtDNA RFLP patterns were identified among 69 PG 1 isolates from the BA orchard. Intraspecific variation of mtDNA has been reported for several fungal species (16,37). However, intraspecific variation of mtDNA is rare in populations of *Ceratocystis fagacearum* and other species (25). In *Neurospora* spp., length mutations are the most important source of variability in mtDNA, although nucleotide substitutions are also common (37). Apparently, 50 to 80% of fungal mtDNA sequences lack coding function and it has been hypothesized that deletions of large blocks of non-coding sequences occur (10).

Uniparental maternal inheritance has been demonstrated to be the principal mode of mtDNA inheritance in species of ascomycetous fungi (29). Each grouping of isolates of PG 1 with distinct

RFLP patterns of mtDNA in this study was considered to be a maternal line, of which all isolates have a common maternal ancestor. The different patterns, therefore, represent a conservative estimate of the number of distinct maternal lines in the sampled orchard. Within and between the maternal lineages, isolates were generally vegetatively incompatible. Vegetative incompatibility was the rule among isolates from cankers on adjacent trees in this study, which is consistent with the findings in northcentral Michigan orchards (3). These results demonstrate that many genetically different individuals are parasitizing the trees in the sampled orchard and that these individuals are progeny of a minimum of 17 sexual outcrossing events. Sexual fruiting bodies of PG 1 are seldom found in peach orchards, even when we carefully search for them. Also, conidia are immensely abundant in orchards and are virulent propagules in inoculations of wounds (2). For these reasons, it has been controversial to suggest that clonal spread is less frequent than spread by sexual recombinants and that ascospores are important propagules in the dissemination of *L. persoonii*. In Michigan, we commonly find *L. persoonii* ascocarps on fallen dead branches of the wild black cherry throughout the deciduous forests. We have demonstrated herein that ascocarps of *L. persoonii* and *L. cincta* form on dead branches of peach on the ground. Additionally, *L. cincta* ascocarps are common on cultivated *Prunus* spp. other than peach (36).

The two fundamental consequences of sexual outcrossing are segregation and recombination. Unfortunately, it is difficult to demonstrate Hardy-Weinberg equilibrium in natural populations of haploid ascomycetes, and it is also difficult to prove clonality. Before outcrossing in a natural population can be studied, cryptic speciation must be identified so that such species are eliminated from the analysis; otherwise, they appear as clonal lineages. We accomplished this previously by isozyme analysis (36). Tibayrenc et al. (39) have proposed several criteria of population genetics for demonstrating if eucaryotic microorganisms are sexual or clonal. Several of the proposed criteria are not useful for haploid organisms such as demonstrating fixed heterozygosity or absence of segregation, but we were able to utilize some of the criteria that are inde-

Fig. 4. Representative diagram of the mitochondrial DNA restriction fragment length polymorphism banding patterns distinguishing six single-occurrence maternal lines of *Leucostoma persoonii* phenetic group 1 from the BA peach orchard.

TABLE 5. Number and percentage of isolates, from the BA orchard, belonging to each maternal lineage of *Leucostoma persoonii* phenetic group 1 based upon mitochondrial DNA polymorphism^a

Maternal lineage	Number of times recorded on host			
	'Redhaven'	'Loring'	Total	Percent
1	11	15	26	37.7
2a	5	7	12	17.4
2b	0	4	4	5.8
3a	4	0	4	5.8
3b	2	0	2	2.9
4a	1	2	3	4.4
4b	2	0	2	2.9
4c	3	0	3	4.4
5a	3	0	3	4.4
5b	1	1	2	2.9
6	0	2	2	2.9
Other ^b	6	0	6	8.7

^a Polymorphisms were observed by Southern hybridization with labeled probes.
^b Each isolate had its unique maternal line (banding pattern) that only appeared once among the 69 isolates examined.

TABLE 6. Vegetative compatibility among isolates within maternal lines of *Leucostoma persoonii* phenetic group 1

Maternal line	Number of isolates tested	Number of compatible pairs
1	9	0
2a	10	0
2b	3	3
4a	2	0
5b	2	0
6	2	0

pendent of ploidy. For example, lack of sexual recombination would be evident if any one genotype, composed of both a mitochondrial and nuclear marker, was over-represented in the population. We recorded no over-represented genotype. Additionally, we showed segregation of the nuclear marker in PG 1 in previous work (3). Lack of recombination also would be evident if there was correlation (joint transmission) between the mitochondrial marker and the nuclear marker. We were not able to disprove joint transmission because of the large number of different VCG present, but the association between the VCG loci and the mtDNA markers appears random. Even though a clonal population structure does not imply total absence of sexual recombination, the evidence does not support the conclusion that clonal propagation could be the predominant mode of reproduction in PG 1 in Michigan. The frequency of incompatible reactions and the number of maternal lines are a demonstration of abundant genetic variation among individuals of PG 1 in the orchards. The limited number of markers we surveyed makes it probable that much additional variability exists within each genotype we observed. Larger population samples and more loci will be required to settle the issue of population structure in PG 1. Further work will be needed to demonstrate that observed frequencies of multilocus genotypes are close to the expected ones under the assumption of free recombination.

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