This is to certify that the

thesis entitled

LEUCOSTOMA SPECIES IN MICHIGAN PEACH ORCHARDS

presented by

Dechun Wang

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Horticulture
Plant Breeding & Genetics

Amy F. Iezzoni
Major professor

Date Aug. 2, 1994

LEUCOSTOMA SPECIES IN MICHIGAN PEACH ORCHARDS

By

Dechun Wang

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Horticulture
Plant Breeding and Genetics

1994

ABSTRACT

LEUCOSTOMA SPECIES IN MICHIGAN PEACH ORCHARDS

By

Dechun Wang

Perennial canker is the most important disease limiting peach production in the northern U.S. and Canada. The pathogens involved, *Leucostoma* spp., were characterized in Michigan peach orchards to: 1) identify which *Leucostoma* subgroups are most prevalent on the peach cultivars Loring and Redhaven; 2) determine if the canker infections on trunks and twigs are caused by similar *Leucostoma* profiles; and, 3) examine the number of maternal lineages present in one orchard. 403 isolates of *Leucostoma* were isolated from cankers on Redhaven and Loring cultivars in 3 southwest Michigan orchards. Based on colony margins and small nuclear rDNA size polymorphisms, 89% of the isolates were identified as *L. persoonii* group 1, 10% were *L. cincta*, and 1% were *L. persoonii* group 2. Significant differences in pathogen profiles of branch cankers were found between Redhaven and Loring. Significant differences were also found in the pathogen profiles of cankers on trunks and twigs. 95% of 1,020 pairs tested among isolates of *L. persoonii* group 1 were vegetatively incompatible. A minimum of 11 and a maximum of 17 maternal lines were identified among 69 isolates of *L. persoonii* group 1 from one orchard based upon mtDNA RFLPs. The frequency of incompatibility and the number of maternal lines indicate that wide genetic variations exist within the *Leucostoma* populations in Michigan peach orchards.

ACKNOWLEDGEMENTS

I wish to thank my major professor, Dr. Amy Iezzoni, for her financial and educational support, guidance, and editorial assistance.

I am grateful to my advisor and committee member, Dr. Gerry Adams, for his guidance, technical advice, and editorial assistance. I appreciated his permission to use his lab to conduct the whole project.

I would like to express my appreciation to my committee member, Dr. Jim Hancock, for his guidance and patience.

I would like to thank Dr. John W. Taylor and Dr. David Jacobson for providing mitochondrial DNA clones and for the use of Dr. Jacobson's lab facilities.

Special thanks to my wife Cuihua for her constant support, patience, and love.

TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION AND LITERATURE REVIEW

Leucostoma canker is one of the most important diseases limiting peach [*Prunus persica* (L.) Batsch] production in the northern United States and southern Canada (Biggs, 1986; Dhanvantari, 1978; Hildebrand, 1947; Kern, 1955; Layne, 1984). The disease is also called perennial canker, *Cytospora* canker, and *Valsa* canker. It limits peach production in Michigan, West Virginia, New Jersey, Pennsylvania, New York, Illinois, Colorado, Idaho, Ontario, and British Columbia (Biggs, 1989). A recent survey of the disease in 93 orchards in Ontario revealed that 98% of the 2,000 trees examined were infected and 90% of the trees had cankers on scaffold limbs (James and Davidson, 1971). Based on the survey, it was estimated that a loss of $68,000 per hectare could result from the disease alone (Biggs, 1989).

Leucostoma canker is characterized by extensive perennial cankers on limbs and branches (Biggs, 1986). The pathogens invade peach trees through wounds or dead tissue. Winter injury, a recurring problem of peach in Michigan, is one of the most common infection sites (Dhanvantari, 1978; Tekauz and Patrick, 1974; Willison, 1937). Cankers are first apparent in the spring at nodes, fruit pedicels, and oriental fruit moth injury sites at twig terminals (Dhanvantari, 1978). If left untreated, the cankers from the infected twigs rapidly invade scaffold limbs and large branches (Biggs, 1989). Extensive cankers result in dying of branches, girdling of trunks and scaffold limbs, and ultimately

a short orchard life for the infected trees (Biggs, 1986; Dhanvantari, 1978). Upon close examination, infections of small twigs appear as sunken, discolored areas near winter-killed buds or leaf scars. Cankers on trunks, branch crotches, scaffold limbs and older branches begin with excessive exudation of amber-colored gum. As cankers age, the gum becomes dark brown to black, the infected bark dries out, and cracks open, exposing blackened tissue beneath elliptical cankers along the length of the stem (Biggs, 1989). Histopathological investigations reveal that the disease causes xylem dysfunction which result in deficiencies of Ca, AI, B, Mg, Mn, P, and Zn in foliage distal to even nongirdling cankers (Hampson and Sinclair, 1973) and in noticeable reduction in water transport (Chang *et al.*, 1991).

The causal organisms of this disease are two closely related species of fungi, *Leucostoma cincta* (Pers. & Fr.) Höhn and *Leucostoma persoonii* (Nits.) Höhn. Either of the two species could be the prevalent pathogen in a particular growing region. *L. cincta* is the more common canker pathogen in some regions such as Ontario (Dhanvantari, 1982), while in some other regions such as Colorado (Luepschen, 1981), Utah (Treshow and Scholes, 1958; Treshow, Richards, and Scholes, 1959), New York (Hildebrand, 1947), and Michigan (Kern, 1955), *L. persoonii* is the prevalent pathogen. Variation between isolates of the same species was reported (Adams *et al.*, 1989; Konicek and Helton, 1962; Surve-Iyer, 1992; Wysong and Dickens, 1962). Based on isozyme polymorphisms, Surve-Iyer (1992) found that there were two distinct groups of *L. persoonii* (group 1 and group 2) and two distinct groups of *L. cincta* among the isolates from *Prunus* spp. hosts in Michigan, and another group of *L. cincta* among the

isolates from *Malus* hosts which also caused peach canker. A *L. persoonii* isolate from California was found to differ from both of the two *L. persoonii* groups found in Michigan.

Virulence of the pathogens was reported to vary among species and isolates of the same species. *L. persoonii* was reported by some investigators to be of low virulence on peach as compared with *L. cincta* (Helton and Konicek, 1961; Willison, 1937). Some other investigators found *L. persoonii* was more virulent in warm weather and *L. cincta* more virulent in cool weather (Hildebrand, 1947; Wensley, 1964). In Michigan, a wide range in virulence was found among isolates of *L. persoonii* and *L. cincta* (Adams *et al.*, 1989), and Group 1 of *L. persoonii* was found to be the most virulent and Group 6 of *L. cincta* the least virulent on peach among the 6 groups defined by Surve-Iyer (1992).

Leucostoma canker is difficult to control. Once a tree is infected, the disease can not be controlled with fungicides (Helton and Rohrbach, 1967). Current strategies for control of the disease are largely based on avoiding the factors that predispose the trees to this 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 (Biggs, 1989) but may not always be practical. The best long-term strategy is to develop cultivars with disease resistance (Biggs 1989).

Resistance to the disease, although not high or immune, has been found in the peach germplasm (Chang and Iezzoni, 1989; Chang *et al.* 1989; Luepschen, 1981; Scorza and Pusey, 1984). Cultivars Yennoh and Reliance were reported to be among the least susceptible cultivars, Redhaven among the intermediate, while Loring and Elberta

were among the most susceptible cultivars (Chang and Iezzoni, 1989; Chang *et al.* 1989; Luepschen, 1981; Scorza and Pusey, 1984). Heritability of the disease resistance was reported to be as high as 0.72 (Chang and Iezzoni, 1991) suggesting that it should be possible to develop resistant cultivars.

One of the constraints to the development of resistant cultivars is the lack of knowledge regarding pathogen variation (Biggs, 1989). Prior to extensive breeding efforts for host resistance to *Leucostoma* canker, the *Leucostoma* species present in orchards must be characterized. Differences in pathogen variation based upon infection site and host cultivar should also be considered. Genetic variation in the pathogen can be estimated by determining the frequency of inherited markers in *Leucostoma.* Vegetative compatibility groupings can serve as markers (Adams *et al.*, 1990), as can isozyme and DNA polymorphisms (Surve-Iyer, 1992). However, isozyme polymorphisms are not commonly encountered in *L. persoonii* in a local region (Surve-Iyer, 1992). Polymorphisms in mitochondrial DNA (mtDNA) are more common (Taylor *et al.*, 1986; Brooks *et al.*,1990). Mitochondrial DNA in fungi evolves approximately 5-8 times faster than nuclear DNA (Bruns and Szaro, 1992). Length mutations are the most prevalent source of variability in mtDNA of Ascomycetes like *Leucostoma* (Taylor *et al.*, 1986; Sanders *et al.*, 1977). Within isolates of *Neurospora crassa* the total size of mtDNA varies by 25% due to length mutations. Additionally, biases appear in the location of mtDNA mutations in fungi and specific regions are more susceptible to mutation, particularly the 8kb region encompassing the COXB-COX1 and a 10kb region of the Oli2-Oli1-COX2 genes (Taylor *et al.*, 1986). When an identifiable region of

mtDNA consistently shows a high level of mutation, specific probes can be chosen to hybridize to mtDNA fragments that encompass conserved regions with highly variable flanking regions. Length mutations in mtDNA has been used successfully to study variability among isolates within biological species (Taylor *et al.*, 1986; Brooks *et al.*, 1990) and permit determination of maternal inheritance among individuals in a local population.

The objectives of this study are to: 1) identify which *Leucostoma* species and subgroups are most prevalent on the peach cultivars Loring and Redhaven in Michigan orchards; 2) determine if the canker infections on trunks and twigs of Redhaven and Loring are caused by similar *Leucostoma* profiles; and, 3) examine the genetic variability and the number of maternal lineages present in one orchard.

LIST OF REFERENCES

Adams, G.C., S.A. Hammar, and A. Iezzoni. 1989. Optimum sample size for detecting virulence differences in *Leucostoma* isolates from peach. Plant Dis. 73:754-759.

Biggs, A.R. 1986. Comparative anatomy and host response of two peach cultivars inoculated with *Leucostoma cincta* and *Leucostoma persoonii*. Phytopathology 76:905-912.

Biggs, A.R. 1989. Integrated approach to controlling *Leucostoma* canker of peach in Ontario. Plant Dis. 73:869-874.

Brooks, S., T. D'Souza, and G.C. Adams. 1990. Relationship of three distinct populations of *Fusarium graminearum* examined by allozyme and ribosomal DNA polymorphisms. Phytopathology 80:963.

Bruns, T.D., and T.M. Szaro. 1992. Rate and mode differences between nuclear and mitochondrial small-subunit rRNA genes in mushrooms. Mol. Biol. Evol. 9:836-855.

Chang, L.S. and A.F. Iezzoni. 1989. Excised-shoot assay for tolerance of peach to *Leucostoma persoonii*. HortScience 24:1011-1012.

Chang, L.S., A.F. Iezzoni, G.C. Adams, and G.S. Howell. 1989. *Leucostoma persoonii* tolerance and cold hardiness among diverse peach genotypes. J. Amer. Soc. Hort. Sci. 114:482-485.

Chang, L.S., A.F. Iezzoni, G.C. Adams, and F.W. Ewers. 1991. Hydraulic conductance in susceptible versus tolerant peach seedlings infected with *Leucostoma persoonii*. J. Amer. Soc. Hort. Sci. 116:831-834.

Chang, L.S. and A.F. Iezzoni. 1991. Heritability of *Leucostoma persoonii* canker resistance among diverse peach genotypes. HortScience 26:60-62.

Dhanvantari, B.N. 1978. Cold predisposition of dormant peach twigs to nodal cankers caused by *Leucostoma* spp. Phytopathology 68:1779-1783.

Dhanvantari, B.N. 1982. Relative importance of *Leucostoma cincta* and *L. persoonii* in perennial canker of peach. Can. J. Plant Pathol. 4:221-225

Hampson, M.C., and W.A. Sinclair. 1973. Xylem disfunction in peach caused by *Cytospora leucostoma*. Phytopathology 63:676-681.

Helton, A.W., and D.E. Konicek. 1961. Effect of selected *Cytospora* isolates from stone fruits on certain stone fruit variaties. Phytopathology 51:152-157.

Helton, A.W., and K.G. Rohrbach. 1967. Chemotherapy of *Cytospora* canker disease in peach trees. Phytopathology 57:442-446.

Hildebrand, E.G. 1947. Perennial canker and tree canker complex in New York, with methods of control. Cornell Univ. Agr. Sta. Mem. 276:3-61.

James, W.C., and T.R. Davidson. 1971. Survey of peach canker in the Niagara peninsula during 1969 and 1970. Can. Plant Dis. Surv. 51:148-153.

Kern, H. 1955. Taxonomic studies in the genus *Leucostoma*. Pap. Mich. Acad. Sci. Arts and Letters 40:9-22.

Konicek, D.E., and A.W. Helton. 1962. An optimum environment for the culturing of *Cytospora* isolates from stone fruits. IV. Hydrogen-ion concentration. Mycopath. Mycol. Appl. 16:243-248.

Layne, R.E.C. 1984. Breeding peaches in North America for cold hardiness and perennial canker (*Leucostoma* spp.) resistance - review and outlook. Fruit Var. J. 38:130-136.

Luepschen, N.S. 1981. Criteria for determining peach variety suceptibility to *Cytospora* canker. Fruit Var. J. 35:139-140.

Sanders, J.P.M., C. Heyting, M.P. Verbeet, F.C. Melijlink, and P. Borst. 1977. The organization of genes in yeast mitochondrial DNA. III. Comparisom of the physical maps of the mitochondrial DNAs from three wild-type *Saccharomyces* strains. Mol. Gen. Genet. 157:239-261.

Scorza, R., and L. Pusey. 1984. A wound-freezing inoculation technique for evaluating resistance to *Cytospora leucostoma* in young peach trees. Phytopathology 74:569-572.

Surve-Iyer, R.S. 1992. Phenetic analysis of *Leucostoma* from *Prunus* and *Malus*. PhD dissertation. Michigan State University. 112pp.

Taylor, J.M., B.D. Smolich, and G. May. 1986. Evolution and mitochondrial DNA in *Neurospora crassa*. Evolution 40:716-739.

Tekauz, A., and Z.A. Patrick. 1974. The role of twig infections on incidence of perennial canker of peach. Phytopathology 64:683-688.

Treshow, M., B.L. Richards, and J.F. Scholes. 1959. The relation of temperature to pathogenicity of *Cytospora rubescens* (abs.). Phytopathology 49:114.

Treshow, M., and J.F. Scholes. 1958. The taxonomy of some species of *Cytospora* found in Utah. Utah Acad. Proc. 35:49-51.

Wensley, R.N. 1964. Occurrence and pathogenicity of *Valsa* species and other fungi associated with peach canker in southern Ontario. Can. J. Bot. 42:841-857.

Willison, R.S. 1937. Peach canker investigations III. Further notes on incidence, contribution factors, and related phenomena. Can. J. Res. 15:324-339.

Wysong, D.S., and L.E. Dickens. 1962. Variation in virulence of *Valsa leucostoma*. Plant Dis. Rep. 46:274-276.

MATERIALS AND METHODS

The flow chart in Figure 1 outlines the research approach followed in the investigations discussed below.

Canker sampling

Diseased tissues were collected from cankers on trunks and large scaffolds, and cankers on smaller canopy branches of each tree (approximately 12 years old) of two varieties, 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 variety 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 variety. A total of 432 cankers were sampled.

Leucostoma isolation

Diseased tissue excised from the margins of cankers was surface sterilized by soaking in 5% sodium hypochlorite for 3 minutes and then blotting dry between sterile paper towels. The tissue was pushed into the flesh of firm apples ('Granny smith') and the pathogenic fungi allowed to grow at room temperature (20-24°C) for 7 - 10 days. Small pieces of apple flesh from the margins of each diseased lesion were then placed on agar-solidified Leonian's medium (1.2 g KH_2PO_4, 0.6 g $MgSO_4$, 6.25 g maltose, 6.25 g malt extract in 1 L of distilled H_2O) (Leonian, 1923) in separate Petri dishes under sterile conditions. The fungi were cultured in the Petri dishes at room temperature for 7 - 10 days and then stored at 4°C.

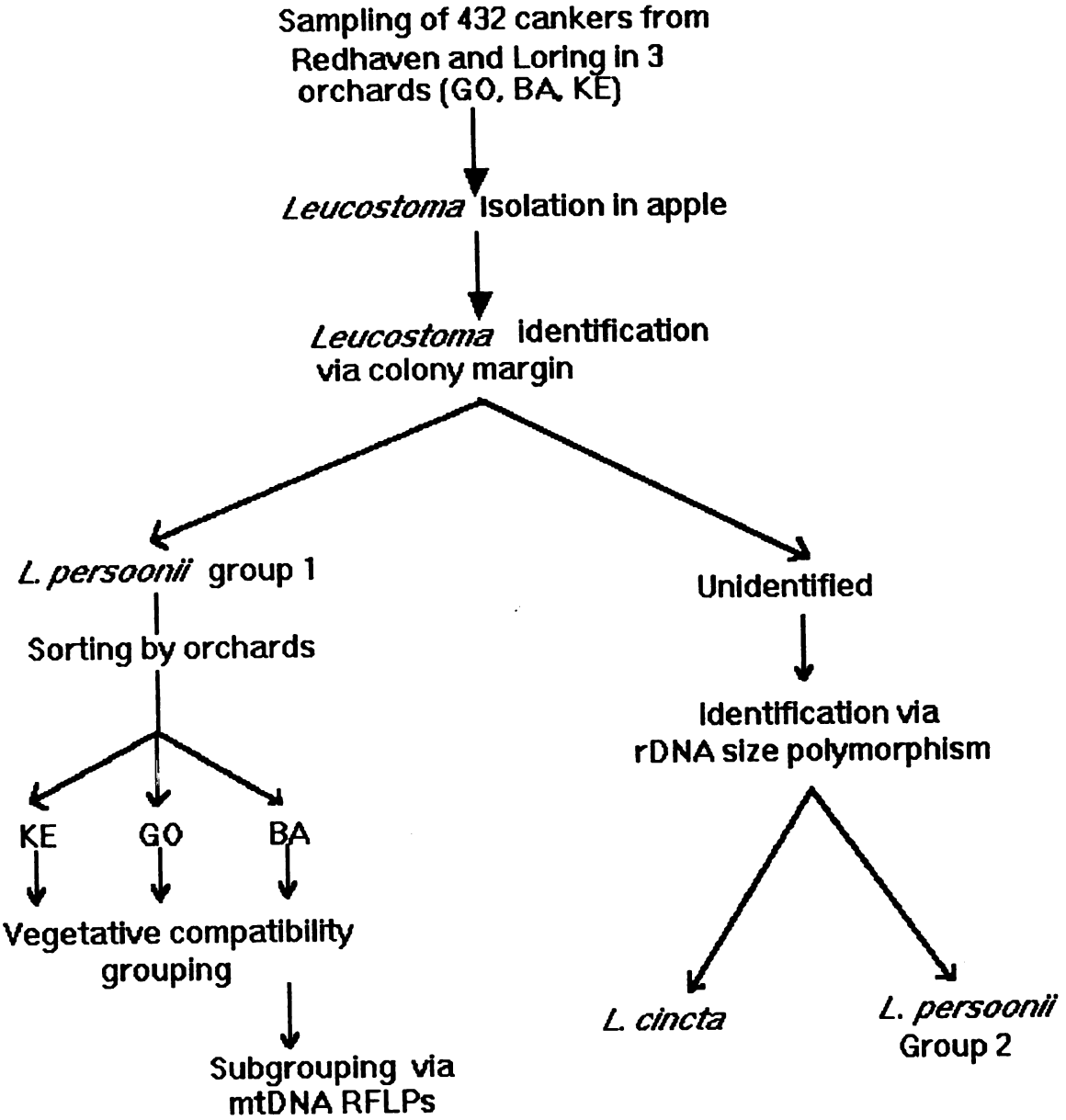

Figure 1. Flow chart of *Leucostoma* investigation

Leucostoma identification

The isolates were phenotypically grouped according to the characters of colony margins of the isolates on agar-solidified Leonian's medium. *L. persoonii* group 1 (PG1) had lobate colony margins which clearly distinguished this group from others (Surve-Iyer, 1992). Based on this criterion, all isolates of *Leucostoma* were grouped into two preliminary groups, "L.p group 1" and "unidentified".

Isolates belonging to *L. cincta* were distinguished from other isolates in the "unidentified" group based on the characteristic presence of a unique insertion in the ribosomal DNA (rDNA). A specific fragment of the nuclear rDNA was amplified by polymerase chain reaction (PCR) (see methods below) using NS21 (5'-GAATAATAGAATAGGACG-3') and NS22 (5'-AATTAAGCAGACAAACTC-3') as a pair of primers (Gargas and Taylor, 1992). The nuclear small rDNA of *L. cincta* isolated from *Prunus* has an insert of about 450 base pairs in the fragment amplified using these two primers while the nuclear small rDNA of *L. persoonii* does not have the insert (Surve-Iyer, 1992). The relative molecular weights of the PCR products following electrophoretic separation were used directly to distinguish *L. cincta* from *L. persoonii* isolates in the "unidentified" group.

The group identity of the rest of the "unidentified" *L. persoonii* isolates was determined phenotypically by colony color, pycnidial size, and temperature maxima, as described by Surve-Iyer (1992).

Examination of vegetative compatibility

The most prevalent phenetic group among the *Leucostoma* isolates was further

examined for the number of vegetative compatibility groups among isolates. The isolates were grown on agar-solidified Leonian's medium for 4 days at room temperature. Plugs (4 mm in diameter) from colony margins of the culture were paired on clarified oatmeal agar medium (Adams *et al.*,1990). Plugs were placed 1.5 cm apart from each other in 150 x 15 mm Petri plates such that 61 plugs were on each plate. The plates were then incubated in the dark at room temperature (20-24°C) for 2-3 weeks. Compatibility or incompatibility between a pair of isolates were determined based on the absence or presence of a barrage zone between the pair (Adams *et al.*, 1990).

Subgrouping of the most prevalent group of *Leucostoma* isolates

The most prevalent phenetic group of the *Leucostoma* isolates was further grouped using two mitochondrial DNA probes, B3 (Taylor and Smolich, 1985) and A13 (Jacobson and Gordon, 1990). B3 is a 8.8 kb *Eco*RI restriction fragment of *Neurospora crassa* 74A mtDNA and was provided by Taylor and Smolich (1985). A13 is a 5.3 kb *Pst*I mtDNA restriction fragment of *Fusarium oxysporum* f. sp. *melonis* strain P-2 and was provided by Jacobson and Gordon (1990). The genomic DNA of each isolate was digested with two restriction endonucleases, *Hpa*II and *Hae*III. The digested DNA was analyzed by Southern Blotting (Southern, 1975). Isolates were grouped according to the banding patterns revealed by the two mitochondrial DNA probes.

Plasmid preparation of mitochondrial DNA clones

Recombinant plasmids pBR325 with B3 fragment and pUC119 with A13 fragment were isolated from overnight cultures of *E. coli* strains HB101 and MV1139 (Taylor and

Smolich, 1985; Jacobson and Gordon, 1990), respectively, using Wizard™ Minipreps (Promega, Madison, WI). The *E. coli* strains were cultured in LB medium (Maniatis *et al.*, 1982) containing 60μg/ml Ampicillin (for strain MV1139) or 60μg/ml Ampicillin and 50μg/ml tetracycline (for strain HB101) overnight at 37°C with shaking (250 rpm). The cells were harvested from 3ml of culture by centrifugation at 13,000g for 2 minutes and resuspended in 200μl of cell resuspension solution (50mM Tris-HCl, pH7.5, 10mM EDTA, 100μg/ml RNase A) in a 1.5ml eppendorf tube. 200μl of cell lysis solution (0.2M NaOH, 1% SDS) was added to the tube and mixed by inverting the tube several times. 200μl of neutralization solution (1.32M Potassium acetate, pH 7.5) was then added to the tube and centrifuged at 13,000g for 5 minutes. The clear supernatant was transferred to a new 1.5ml eppendorf tube. 1ml of the Wizard™ Minipreps DNA purification resin (Promega, Madison, WI) was added to the supernatant and mixed by inverting the tube several times. The resin/DNA mixture was transferred to a syringe barrel attached with a Wizard™ Minicolumn (Promega, Madison, WI) and pulled through the Minicolumn by applying a vacuum. The Minicolumn was washed by drawing 2ml of column wash solution (83mM NaCl, 8.3mM Tris-HCl, pH7.5, 2mM EDTA, 55% ethanol) through the Minicolumn with vacuum and then transferred to a 1.5ml eppendorf tube and centrifuged at 13,000g for 20 seconds to remove any residual column wash solution. The Minicolumn was then transferred to a new 1.5ml eppendorf tube and 50μl of preheated (65°C) TE buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA) was applied to the Minicolumn. The plasmid DNA was eluted from the Minicolumn by centrifugation at 13,000g for 20 seconds and collected in the attached eppendorf tube and stored in the

eppendorf tube at -20°C untill use.

DNA isolation

The isolates were cultured in 100 ml of liquid Leonian's medium at room temperature for 10-14 days. The mycelia were then harvested by vacuum filtration through miracloth (Calbiochem Corp, San Diego, CA), lyophilized and stored at -20°C. DNA was extracted from lyophilized hyphae by the method of Lee *et al.* (1988). Lyophilized mycelia was ground in a 1.5 ml eppendorf tube with 750 μl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol) and incubated at 65°C for 1 hour. 700 μl of phenol:chloroform (1:1) was added and centrifuged at 12,000g for 10 minutes. The aqueous phase was removed to a new 1.5 ml eppendorf and another 700 μl of phenol:chloroform (1:1) was added and centrifuged at 12,000g for 10 minutes. The aqueous phase was collected and mixed with 700 μl of chloroform:isoamyl alcohol (24:1) in a new eppendorf tube and the mixture was centrifuged at 12,000g for 5 minutes. The top phase was removed to a new tube and DNA was precipitated with 0.1 volume of 3 M NaOAC (pH 8.0) and 600 μl ice cold isopropanol. The DNA pellet was washed with 1 ml of 70% ethanol and dried overnight at room temperature. The extracted DNA was stored in TE buffer at -20°C.

Amplification of DNA fragments using PCR

The DNA fragment in the nuclear small rDNA used for the identification of *L. cincta* was amplified by polymerase chain reaction. The PCR reactions were set up with Amplitaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) in 25-μl volume using

buffer conditions recommended by the manufacturer. The primer pair used was NS21 and NS22. Thirty PCR cycles were performed on a Perkin-Elmer-Cetus thermocycler with the following parameters, 94°C denaturation for 1 minute, 48°C annealing for 1 minute, 72°C extension for 1 minute plus 4 seconds extension after each cycle, and with a final extension at 72°C for 10 minutes. 8 μl of each PCR products were separated according to molecular weight by electrophoresis on agarose minigels (1% Nusieve + 2% Seakem agarose in 1X TAE, FMC Bioproducts, Rockland, ME) along with 1 kb DNA weight marker (Boehringer Mannheim Biochemicals, Indianapolis, IN) and viewed under ultraviolet light.

Southern Blotting analysis using Genius nonradioactive system

The most prevalent phenetic group of the *Leucostoma* isolates was further analyzed with Southern hybridization. 1-3 μg of genomic DNA of each isolate was digested with 10 units of restriction enzyme (*Hpa*II or *Hae*III) at 37°C for 2 hours. The products of digestion were separated by electrophoresis in 0.9% agarose gels in TAE buffer with Digoxigenin-labeled DNA weight marker III (Boehringer Mannheim Biochemicals, Indianapolis, IN) as a standard. The electrophoresis was carried out at 80 volt for 7 hours at 4°C. Prior to Southern transfer, the gel containing the separated DNA fragments was submerged, in order, in the following solutions: 0.25 M HCl (5 minutes), 0.5 M NaOH plus 1.5 M NaCl (45 minutes), and 1.0 M Tris-HCl (pH 8.0) plus 1.5 M NaCl (45 minutes). The DNA was then transferred from the gel to Boehringer Mannheim's positively charged Nylon membranes (Boehringer Mannheim, Indianapolis, IN) by capillary blotting overnight, using 10X SSC (1.5 M NaCl, 0.15 M sodium citrate;

pH 7.0) as transfer buffer. The DNA was fixed to the membrane by U.V. crosslinking for 1 minute at 120,000 microjoules in a U.V. crosslinker (Stratagen model 2400, La Jolla, CA). The membrane was incubated in prehybridization solution (5X SSC, 2.0% blocking reagent for nucleic acid hybridization, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate, 50% formamide) at 42°C for 2 hours and then incubated in hybridization solution (prehybridization solution plus 10-20 ng/ml digoxigenin-labeled probe) at 42°C overnight. After hybridization, the membrane was washed twice (5 minutes each) in 2X wash solution (2X SSC, 0.1% SDS) at room temperature and twice (15 minutes each) in 0.5X wash solution (0.5X SSC, 0.1% SDS) at 42°C. After wash, the membrane was immediately treated for the detection of hybridization. The detection procedure was the same as described in the User's Guide of the Genius System (Boehringer Mannheim, Indianapolis, IN) with slight modification. The membrane was briefly (1 minute) soaked in filtered Genius buffer 1 (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) and then soaked in Genius buffer 2 (2% blocking reagent for nucleic acid hybridization dissolved in Genius buffer 1) for 45 minutes. The membrane was then incubated in anti-digoxigenin-alkaline phosphatase solution (0.1 unit/ml polyclonal sheep anti-digoxigenin Fab-fragments in Genius buffer 2) for 30 minutes and washed twice (15 minutes each) in filtered Genius buffer 1. The washed membrane was equilibrated in Genius buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$; pH 9.5) for two minutes and then placed between the two sheets of a plastic page protector. While the membrane was still wet, Lumi-Phos 530 (Boehringer Mannheim, Indianapolis, IN), in the amount of 0.2 ml/100 cm^2 of membrane, was quickly dropped and evenly spread on the membrane.

The membrane was immediately exposed to X-ray film (Kodak XAR) for 1-2 hours at room temperature to detect hybridization.

The probes used in the hybridization were labeled with digoxigenin using the Genius 1 DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). A random primed labeling reaction in the volume of 40 μl was set up according to the instructions in the Genius System User's Guide. The labeling reaction was carried out at 37°C overnight. The yield of the labeling reaction was estimated according to the instructions in the User's Guide.

Separation of mitochondrial DNA from chromosomal DNA

In order to examine whether the two mitochondrial DNA probes used in the Southern analysis hybridize only to mitochondrial DNA of *Leucostoma*, intact mitochondrial DNA of *L. persoonii* was separated from intact chromosomes in 8% agarose gels by pulse field electrophoresis (Skelly and Malexzka, 1989). Electrophoresis, using the CHEF-DRII system (BIO-RAD Laboratory, Melville, NY), was for 18 hours at 10°C at 200 volt with a pulse of 60 seconds in 0.5X TBE. Intact chromosomes were distinquished 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 500ml-Erlenmeyer flasks containing 50ml of 1.3X Leonian's medium were inoculated with 3ml of *L. persoonii* conidia (10^7 conidia/ml) and incubated on a reciprocal shaker (300rpm) for 36 hours at room temperature (20-24°C). The hyphae were harvested on miracloth and rinsed with sterile H_2O and cold (4°C) 0.45mM EDTA. The hyphae were then scrapped into 5ml

of protoplasting medium (0.1 M $NaPO_4$ buffer, pH 5.8; 1 M mannitol; 0.1% NovoZym 234, Novo BioLabs, Danbury, Conn.; 0.05% chitinase from *Serratia*, Sigma Chemical Company, St. Louis, MO; and 1% low-melt agarose) in a 50 x 15 mm Petri plate and incubated overnight at 33°C with an overlay of 10ml of EDTA-mannitol solution (0.45 M EDTA, pH 9.0; 1 M mannitol; 9 mM Cysteine-HCl) and then overnight at 50°C with an overlay of 10ml of proteinase-SLS solution (0.2% proteinase XIV, Sigma Chemical Company, St. Louis, MO; 1% N-lauroylsarcosine sodium salt; 0.45 M EDTA, pH 9.0; 10 mM Tris-HCl) (McCluskey *et al.*, 1990). Agarose plugs containing the treated hyphae were desalted and equilibrated by soaking for 4 hours at 4°C in 0.5X TBE buffer, pH 8, then directly loaded into the wells of 1% agarose gel prepared for CHEF DRII pulse field electrophoresis system and sealed in with warm 1% agarose. The *L. persoonii* samples were electrophoresed alongside yeast chromosome DNA size standards of *Saccharomyces cerevisiae*, strain YNN295, and *Schizosaccharomyces pombe*, strain 972 h- (Bio-Rad Laboratories, Melville, NY).

Data analysis

Percentage data were arcsine transformed, i.e. arcsine of the square root of the value of percentage data, and analyzed as split-plot design with cultivars as main plot factor and canker sites and pathogens as subplot factors. The data were analyzed using MSTAT-C computer software (Freed *et al.*, 1988).

RESULTS

403 isolates of *Leucostoma* were isolated from the 432 cankers sampled. The 403 isolates were phenotypically placed in 2 groups (*L. persoonii* group 1 and "unidentified") according to the characteristics of the colony margins. *L. persoonii* group 1 has lobate colony margins and therefore could be easily distinquished from the other *Leucostoma* isolates (Figure 2).

The unidentified isolates were determined to be *L. persoonii* group 2 or *L. cincta* based upon rDNA size differences. The nuclear small rDNA of *L. cincta* from *Prunus* has an insertion of approximately 450 bp between the annealing sites of the 2 primers NS21/NS22, while the nuclear small rDNA of *L. persoonii* does not have the insertion (Figure 3).

The most common pathogens of Leucostoma canker in Michigan

Loring and Redhaven trees in the 3 southwest Michigan peach orchards were found to be infected by 3 *Leucostoma* canker fungi (Table 1). *L. persoonii* group 1 was the most common causal organism isolated from the cankers sampled. Of the 403 cankers sampled from three orchards, 88.6% were caused by *L. persoonii* group 1. *L. cincta* was the second most common pathogen causing 10.2% of the cankers. *L. persoonii* group 2 was the least common pathogen causing only 1.2% of the cankers sampled.

Pathogen profiles of cankers on Redhaven and Loring cultivars

Table 2 presents the percentage of cankers on the branches of the two cultivars caused by each pathogen. Significant differences between cultivars were observed in the

percentage of cankers caused by *L. persoonii* group 1 and *L. cincta*. *L. persoonii* group 1 was responsible for significantly more cankers in Redhaven versus Loring. *L. persoonii* group 2 was only isolated from cankers on Loring.

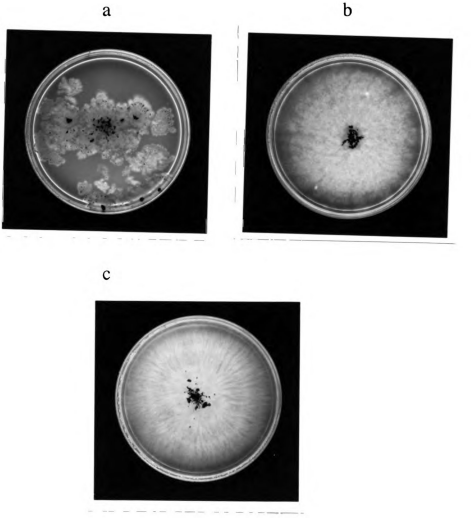

Figure 2. Colony characteristics representative of the three *Leucostoma* subgroups; a) *L. persoonii* group 1 with a lobate colony margin, b) *L. persoonii* group 2, and c) *L. cincta*, with uniform radial colony margins.

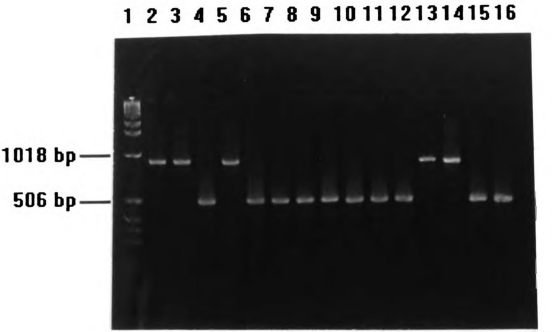

Figure 3. Electrophoretic separation of PCR products of nuclear small rDNA (primers NS21/NS22) on 1% agarose gel stained with ethidium bromide and viewed under ultraviolet light. An insertion of about 450bp is present in the isolates of *L. cincta* (lanes 2, 3, 5, 13, 14) as compared to *L. persoonii* (lanes 4, 6-12, 15, 16). Lane 1 is a 1kb lambda DNA molecular weight marker.

Table 1. Number and percentage of cankers caused by each of the pathogens isolated from 3 Michigan orchards.

Pathogen[1]	No. of cankers caused	% of the total cankers sampled
L. persoonii group 1	357	88.6 a[2]
L. persoonii group 2	5	1.2 c
L. cincta	41	10.2 b
Total	403	100

[1]Pathogens were grouped based upon characteristics of colony margins and rDNA polymorphism.
[2]Different letters indicate the means are significantly different at $P < 0.01$ (Appendix B1).

Table 2. Percentage of cankers on branches of Redhaven and Loring caused by different pathogens. The data is summed over 3 orchards.

	Percent cankers for each causal pathogen[1]		
Cultivar	*L. persoonii* group 1	*L. cincta*	*L. persoonii* group 2
Redhaven	88.77 a[2]	11.23 b[2]	0
Loring	76.33 b	20.77 a	2.9

[1]Pathogens were grouped based upon characteristics of colony margin and rDNA polymorphism.
[2]Different letters indicate the means in columns are significantly different at $P \leq 0.05$ (Appendix B2, Appendix B3).

Pathogen profiles of cankers on tree trunks and branches

Cankers on tree trunks and branches were found to be caused by different pathogen profiles (Table 3). A significantly larger percent of cankers on tree trunks were caused by *L. persoonii* group 1, than were cankers on branches. On the other hand, a significantly larger proportion of cankers on branches were caused by *L. cincta*, than were cankers on tree trunks.

Significant differences were found between the two cultivars, Redhaven and Loring, in the pathogen profile of cankers on branches, as shown in Table 2. However, if only cankers on tree trunks were compared, no significant difference was found between the two cultivars, except that no isolates of *L. persoonii* group 2 were found on Redhaven (Table 4).

Vegetative compatibility among isolates of *L. persoonii* group 1

One hundred eighty two isolates of *L. persoonii* group 1 from cankers of adjacent trees were paired on agar solidified medium to examine vegetative compatibility. Of a total of 1,020 pairs examined, 95% of the pairs were incompatible with each other (Table 5, Figure 4). Such a high incompatibility among isolates made it impractical to divide *L. persoonii* group 1 into vegetative compatibility subgroups.

Table 3. Percentage of cankers on branches and trunks caused by different pathogens. The data is summed over 3 orchards and 2 cultivars.[1]

	Percent cankers for each causal pathogen[2]		
Canker location	*L. persoonii* group 1	*L. cincta*	*L. persoonii* group 2
Branch	82.55 b[3]	16.00 a[4]	1.15
Trunk	94.50 a	4.50 b	0.17

[1]*Leucostoma* was isolated from 403 cankers.
[2]Pathogens were grouped based upon characteristics of colony margin and rDNA polymorphism.
[3]Different letters indicate the means in column are significantly different at the $P<0.01$ (Appendix B4).
[4]Different letters indicate the means in column are significantly different at the $P<0.05$ (Appendix B5).

Table 4. Percentage of cankers on tree trunks of Redhaven and Loring caused by different pathogens. The data is summed over 3 orchards.[1]

	Percent cankers for each causal pathogen[2]		
Cultivar	*L. persoonii* group 1	*L. cincta*	*L. persoonii* group 2
Redhaven	94.9	5.1	0
Loring	94.1	3.9	2.0

[1]*Leucostoma* was isolated from 403 cankers.
[2]Pathogens were grouped based upon characteristics of colony margin and rDNA polymorphism.

Table 5. Percentage of vegetatively incompatible pairs among 182 isolates of *L. persoonii* Group 1

Test[1]	Total No. of pairs	No. & % of incompatible pairs	
		No.	%
1	69	62	90
2	88	88	100
3	88	87	99
4	88	81	92
5	107	99	93
6	88	88	100
7	107	104	97
8	81	73	90
9	107	99	93
10	62	60	97
11	84	78	93
12	51	48	94
Total	1020	967	95

[1]The number represent the order in time of different pairing tests.

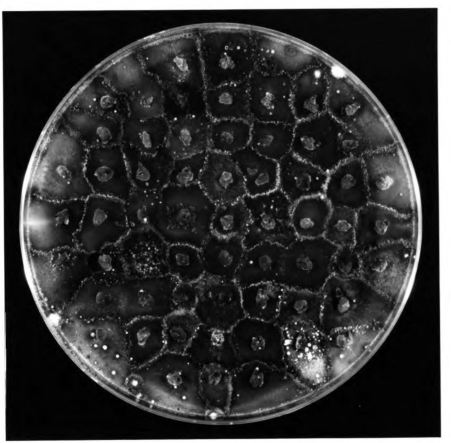

Figure 4. Pairing of isolates of *L. persoonii* group 1 on clarified oatmeal agar medium. Barrage zones formed between vegetatively incompatible isolates.

Target DNA hybridized by the two mitochondrial DNA probes

In order to investigate whether the two mitochondrial DNA probes, B3 and A13, which were used in subgrouping of *L. persoonii* group 1, also hybridized to nuclear DNA, hyphae in agarose were protoplasted and lysed *in agaro* and intact chromosomes were separated from intact mitochondrial DNA by pulse field electrophoresis. Following electrophoresis, two dense bands of DNA were evident on the ethidium bromide-stained agarose gels. One band of chromosomal DNA occurred that was greater than 1.6 megabase, which is in the common range of chromosome length (1.3 to 6.2 megabase) for ascomycete fungi (Mills and McCluskey, 1990). The other band of DNA, the putative mitochondrial DNA, occurred at less than 0.6 megabases. The separated DNA was probed with the two mitochondrial DNA probes. Neither probe hybridized to the intact chromosomal DNA nor elswhere on the gel, except to the DNA smaller than 0.6 megabases (Figure 5 and Figure 6).

Subgrouping of *L. persoonii* group 1

Sixty nine isolates of *L. persoonii* group 1 from one orchard, BA orchard, were analyzed by Southern hybridization with the two mitochondrial DNA probes. Considerable mitochondrial DNA variation was found among the isolates (Figure 7). Eleven subgroups, defined as 2 or more isolates having the same banding pattern, were identified within this group isolated from BA orchard. The banding patterns of the 11 subgroups is shown in Figure 8. The number and percentage of isolates belonging to each subgroup is shown in Table 6. Two major subgroups, 1 and 2a, comprised 55% of the 69 isolates investigated. Subgroup 3a, 3b, 4b, 4c, 5a were only isolated from

cankers on cultivar Redhaven and subgroup 2b and 6 were only isolated from cankers on Loring (Table 6). In addition to the 11 subgroups, 6 single isolates, each with a unique banding pattern (Figure 9), were found on cultivar Redhaven.

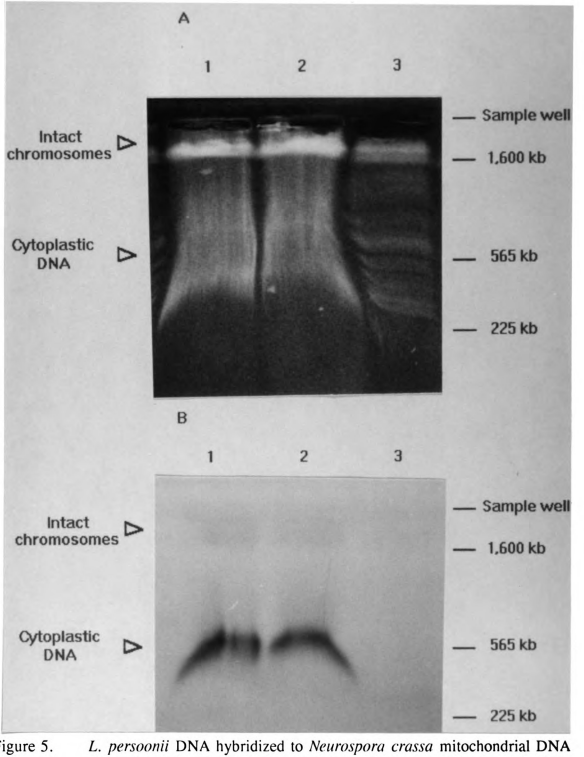

Figure 5. *L. persoonii* DNA hybridized to *Neurospora crassa* mitochondrial DNA probe B3. A. Ethidium bromide stained gel viewed under ultraviolet light; B. Autoradiograph after hybridization of probe B3. 1 and 2, *L. persoonii*; 3, yeast chromosome DNA size standard, *Saccharomyces cerevisiae*, strain YNN295.

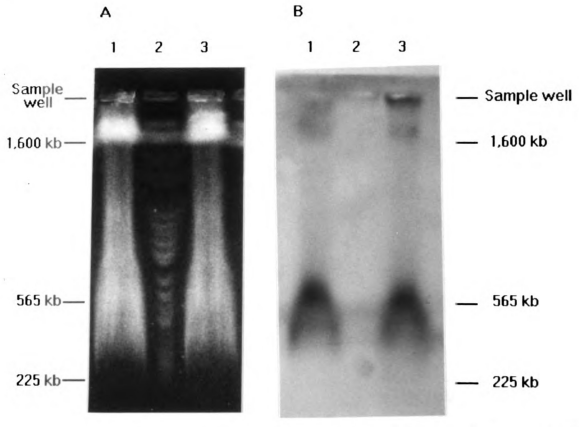

Figure 6. *L. persoonii* DNA hybridized to *Fusarium oxysporum* mitochondrial DNA probe A13. A. Ethidium bromide stained gel viewed under ultraviolet light; B. Autograph after hybridization of probe A13. 1 and 3, *L. persoonii*; 2, yeast chromosome DNA size standard, *Saccharomyces cerevisiae*, strain YNN295.

Table 6. Number and percentage of isolates belonging to each subgroup of *L. persoonii* group 1 based upon mitochondrial DNA polymorphism[1]

	Host			
Subgroups	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[2]	6	0	6	8.7

[1]The isolates were isolated from cankers on two cultivars at the BA orchard.
[2]Each isolate had its unique banding pattern which only appeared once in the 69 isolates examined.

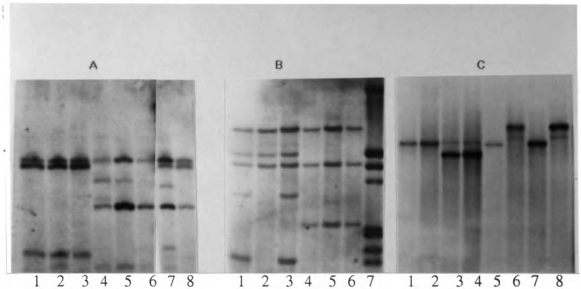

Figure 7. Autoradiographs of Southern blotting using mitochondrial DNA as probes. A. *Hpa*II digested genomic DNA hybridized with labeled B3, lanes 1-8, *L. persoonii* group 1; B. *Hae*III digested genomic DNA hybridized with labeled B3, lanes 1-6, *L. persoonii* group 1; lane 7, DNA size standard; C. *Hae*III digested genomic DNA hybridized with labeled A13, lanes 1-8, *L. persoonii* group 1.

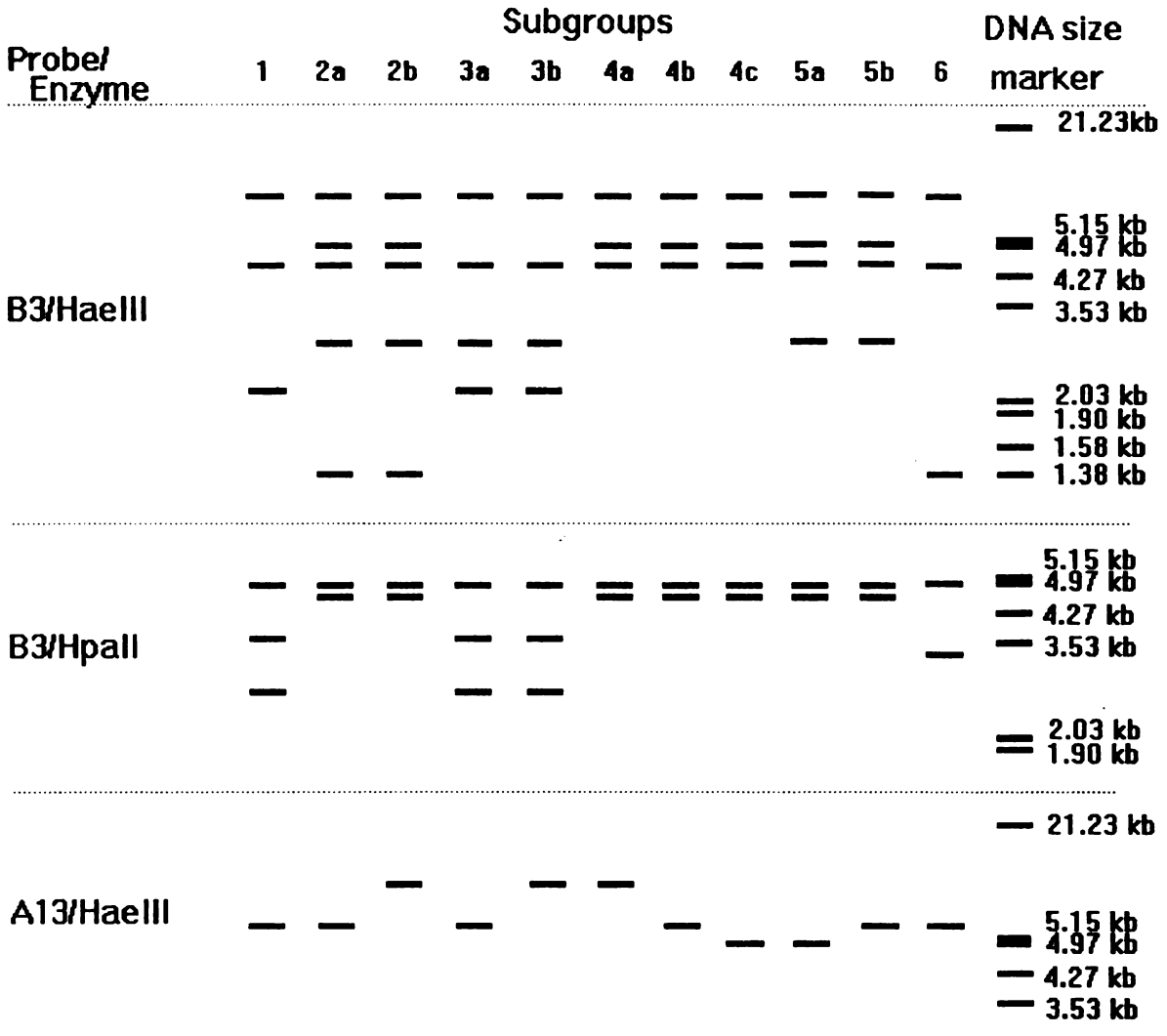

Figure 8. Banding patterns of subgroups of *L. persoonii* group 1

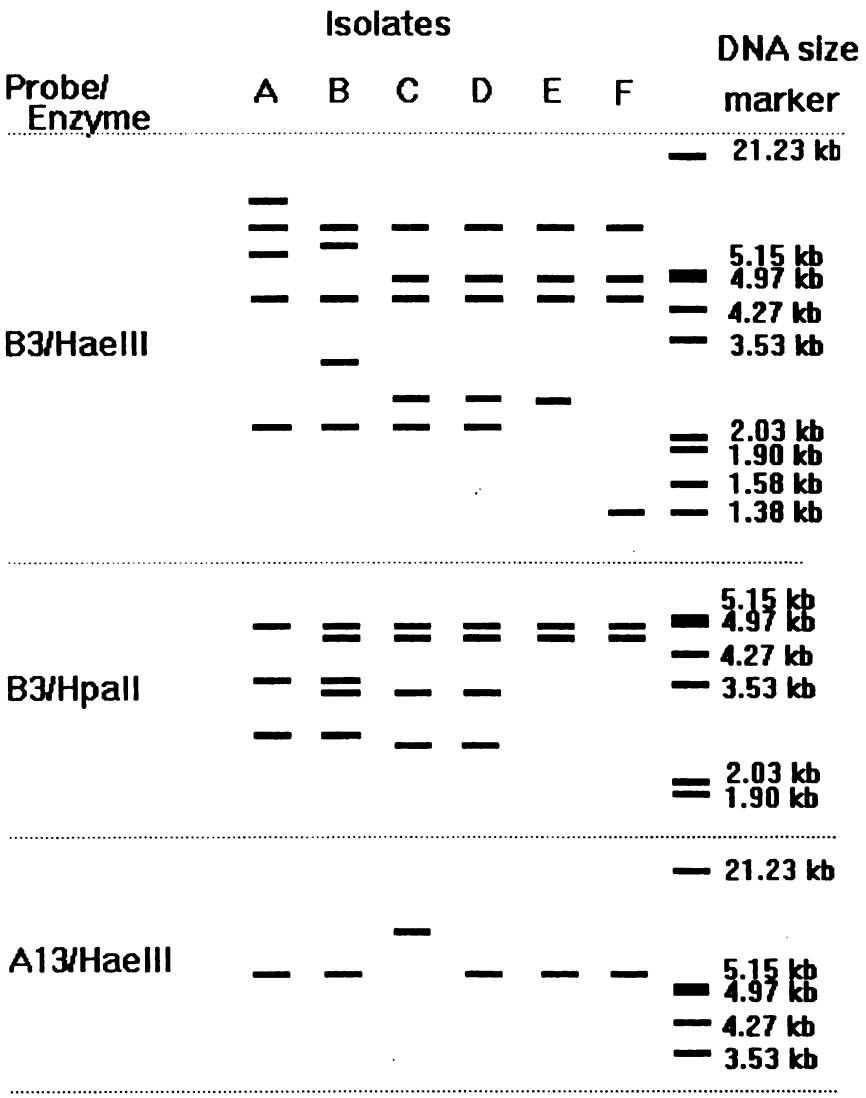

Figure 9. Unique banding patterns of *L. persoonii* group 1

DISCUSSION

L. persoonii group 1 is the predominant subgroup of *Leucostoma* in Michigan peach orchards. *L. cincta* has been reported to be a primary pathogen in some northern extremes of the peach production area (Dhanvantari, 1982; Northover, 1976; Tekauz and Patrick, 1974; Willison, 1936), however, it is second to *L. persoonii* group 1 in importance in Michigan.

Differences in the isolation frequencies of *L. persoonii* and *L. cincta* from different canker sites on peach trees were reported by Dhanvantari (1982). In his study, *L. cincta* predominated in the isolations from node cankers while *L. persoonii* predominated in the isolations from perennial and fruit pedicel cankers. In our study, differences were also observed in the isolation frequencies of *L. persoonii* group 1 and *L. cincta* from the two different canker sites, tree trunk and branches. Although *L. persoonii* group 1 predominated in the isolations from both canker sites, it was significantly less frequent in the isolations from branch cankers than from trunk cankers. On the other hand, *L. cincta* was significantly more frequent in the isolations from branch cankers than from trunk cankers (Table 3). The epidemiological factors that result in these differences still need to be determined. Possible factors may include availability and relative virulence of different *Leucostoma* subgroups 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 (Dhanvantari, 1969; Hildebrand, 1947; Wensley, 1964). Leaf scars and winter injuries 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, mechanical injuries caused by field equipment and harvesting might predispose the trees to the disease when *L. persoonii* inoculum is most abundant (Luepschen and Rohrbach, 1969) and more virulent.

Significant differences in the profiles of branch canker pathogens on the cultivars Redhaven versus Loring are observed in this study. The difference might be related to the cultivar difference in canker tolerance. Redhaven was reported to be relatively tolerant to canker (Dhanvantari, 1982) and Loring very susceptible to the disease (Chang *et al.*, 1989). Pathogens of low virulence may have a better chance to invade and establish cankers on a more susceptible cultivar than on a tolerant cultivar and this appeared to be the case in our study. Field tests showed that *L. persoonii* group 1 was more virulent than *L. cincta*, and *L. cincta* isolated from *Prunus* spp. was more virulent than *L. persoonii* group 2 in Michigan on peach (Surve-Iyer, 1992). *L. cincta* caused a significantly larger percentage of cankers on the branches of Loring than on the branches of Redhaven in our investigation. *L. persoonii* group 2 was only isolated from cankers on Loring.

The commonly observed vegetative incompatibility among isolates from cankers of adjacent trees in this study is consistent with the findings by Adams *et al.* (1990). Each incompatible reaction indicates that the two isolates are different individuals. The pairings herein demonstrate that many individuals are parasitizing the trees in a small orchard area. This supports the suggestion that clonal spread might be less frequent than spread by sexual recombinants, and thus ascospores are important propagules in the

dissemination of *L. persoonii*. The frequency of incompatible reactions and the number of maternal lines are a demonstration of the amount of genetic variation among individuals of *L. persoonii* in the orchards. However, isozyme polymorphisms are not commonly encountered in *L. persoonii* in a local region (Surve-Iyer, 1992).

The DNA restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA were used to identify maternal lines among *L. persoonii* group 1 from one split-cultivar block containing 72 trees. As shown in Figure 5 and Figure 6, the two probes used hybridized only to the putative mitochondrial DNA (DNA smaller than 600kb). The size of the intact DNA molecules detected by the two probes suggested their mitochondrial identity. And the probes themselves are restriction fragments of mitochondrial DNA from related species. Maternal inheritance has been demonstrated to be the principal mode of mitochondrial DNA inheritance in fungal species (Mannella *et al.*, 1979; May and Taylor, 1989; Milgroom and Lipari, 1993; Mitchell and Mitchell, 1952; Taylor, 1986b). Each grouping of isolates of *L. persoonii* group 1 with distinct RFLP patterns of mitochondrial DNA in this study could be considered as a maternal line, of which all the isolates had a common maternal ancestor. At least 11 maternal lines of *L. persoonii* group 1 were found in the BA orchard. Lines 3a, 3b, 4b, 4c, and 5a were only isolated from cultivar Redhaven and lines 2b and 6 were only isolated from Loring. Whether differences in host specificity, virulence or local dissemination exist among the maternal lines needs to be further investigated.

Significant variation in mitochondrial DNA of *Leucostoma* was revealed by RFLPs in this study. A total of 17 different RFLP patterns were detected with only three

probe-enzyme combinations among 69 isolates of *L. persoonii* group 1 from a 72 tree block. Intraspecific variation of mitochondrial DNA was reported for fungal species *Armillaria mellea* (Smith and Anderson, 1986), *Cochliobolas heterostrophus* (Garber and Yoda, 1984), *Cryphonectria parasitica* (Milgroom and Lipari, 1993), *Fusarium oxysporum* (Taylor, 1986b), and *Neurospora crassa* (Collins and Lambowitz, 1983; Taylor, 1986a). In *Neurospora* species, length mutations were found to be the most important source of variability in mitochondrial DNA (Collins and Lambowitz, 1983). Nucleotide substitutions are also common in *Neurospora* mitochondrial DNA (Taylor, 1986a). Recombinations of mitochondrial DNA during sexual reproduction have been reported for some fungal species (Baptista-Ferreria *et al.*, 1983; Borst and Grivell, 1978; Economou *et al.*, 1987). It would be interesting to determine which sources contributed to the variability in *Leucostoma* mitochondrial DNA.

LIST OF REFERENCES

Adams, G.C., S.A. Hammar, and T. Proffer. 1990. Vegetative compatibility in *Leucostoma persoonii*. Phytopathology 80:287-291.

Baptisa-Ferreria, J.L.C., A. Economou, L.A. Casselton. 1983. Mitochondrial genetics of *Coprinus*: recombination of mitochondrial genomes. Curr. Genet. 7:405-407.

Borst, P., and L.A. Grivell. 1978. The mitochondrial genome of yeast. Cell 15:705-723.

Chang, L.S., A.F. Iezzoni, G.C. Adams, and G.S. Howell. 1989. *Leucostoma persoonii* tolerance and cold hardiness among diverse peach genotypes. J. Amer. Soc. Hort. Sci. 114:482-485.

Collins, R.A., and A.M. Lambowitz. 1983. Structural variations and optional introns in the mitochondrial DNAs of *Neurospora* strains isolated from nature. Plasmid 9:53-70.

Dhanvantari, B.N. 1969. Comparative physiology and pathogenicity of *Leucostoma* spp. on peach. Phytopathology 59:1023.

Dhanvantari, B.N. 1982. Relative importance of *Leucostoma cincta* and *L. persoonii* in perennial canker of peach. Can. J. Plant Pathol. 4:221-225.

Economou, A., V. Lees, P.J. Pukkila, M.E. Zolan, L.A. Casselton. 1987. Biased inheritance of optional insertions following mitochondrial genome recombination in the basidiomycete fungus *Coprinus cinereus*. Curr. Genet. 11:513-519.

Freed, R., S.P. Eisensmith, S. Goetz, D. Reicosky, V.W. Smail, and P. Wolberg. 1988. MSTAT-C a software program for design, management, and analysis of agronomic research experiments. Michigan State University, East Lansing, MI.

Garber, R.C., and O.C. Yoder. 1984. Mitochondrial DNA of filamentous ascomycete *Cochliobolus heterostrophus*. Curr. Genet. 8:621-628.

Gargas, A., and J.W. Taylor. 1992. Polymerase chain reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. Mycologia 84:589-592.

Hildebrand, E.G. 1947. Perennial canker and three canker complex in New York, with methods of control. Cornell Univ. Agr. Sta. Mem. 276:3-61.

Jacobson, D.J., and T.R. Gordon. 1990. Variability of mitochondrial DNA as an

indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol. Res. 94:734-744.

Lee, S.B., M.G. Milgroom, and J.W. Taylor. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Gen. Newsl. 35:23-24.

Leonian, L.H. 1923. The physiology of perithecial and pycnidial formation in *Valsa leucostoma*. Phytopathology 13:257-272.

Luepschen, N.S., and K.G. Rohrbach. 1969. *Cytospora* canker of peach trees: Spore availability and wound susceptibility. Plant Dis. Rep. 53:869-872.

Maniatis, T.E., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p 545.

Mannella, C.A., T.H. Pittenger, and A.M. Lambowitz. 1979. Transmission of mitochondrial deoxyribonucleic acid in *Neurospora crassa* sexual crosses. J. Bacteriol. 137:1449-1451.

May, G., and J.W. Taylor. 1989. Independent transfer of mitochondrial plasmids in *Neurospora crassa*. Nature 339:320-322.

McCluskey, K., B.W. Russell, and D. Mills. 1990. Electrophoretic karyotyping without the need for generating protoplasts. Curr. Genet. 18:385-386.

Milgroom, M.G., S.E. Lipari. 1993. Maternal inheritance and diversity of mitochondrial DNA in the Chestnut Blight fungus, *Cryphonectria parasitica*. Phytopathology 83:563-567.

Mills, D., and K. McCluskey. 1990. Electrophoretic karyotypes of fungi: The new Cytology. Mol. Plant-Microbe Interact. 3:351-357.

Mitchell, M.B., and H.K. Mitchell. 1952. A case of "maternal" inheritance in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 38:442-449.

Northover, J. 1976. Protection of peach shoots against species of *Leucostoma* with benomyl and captafol. Phytopathology 66:1125-1128.

Skelly, P.J., and R. Malexzka. 1989. Isolation of mitochondrial DNA using pulsed field gel electrophoresis. Nucleic Acids Res. 17:7537.

Smith, M., and J.B. Anderson. 1986. variation in mitochondrial DNAs of the North

American biological species of *Armillaria*. Mycol. Soc. Amer. Newslett. 37:43.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Surve-Iyer, R.S. 1992. Phenetic analysis of *Leucostoma* from *Prunus* and *Malus*. PhD dissertation 112 pp. Michigan State University, East Lansing, MI.

Taylor, J.W., and B.D. Smolich. 1985. Molecular cloning and physical mapping of *Neurospora crassa* 74-OR23-1A mitochondrial genome. Current Genetics 9:597-603.

Taylor, J.W. 1986a. Mitochondrial DNA and evolution in *Neurospora* species. Fungal Genet. Newslett. 33:14.

Taylor, J.W. 1986b. Topical review: fungal evolutionary biology and mitochondrial DNA. Exp. Mycol. 10:259-269.

Tekauz, A., and Z.A. Patrick. 1974. The role of twig infections on incidence of perennial canker of peach. Phytopathology 64:683-688.

Wensley, R.N. 1964. Occurrence and pathogenicity of *Valsa* species and other fungi associated with peach canker in southern Ontario. Can. J. Bot. 42:841-857.

Willison, R.S. 1936. Peach canker investigations II. Infection studies. Can. J. Res. 14:27-44.

APPENDICES

Appendix A

Table 7. Percentage of cankers caused by *Leucostoma persoonii* group 1, group 2, and *L. cincta* isolates from trunks and branches of Redhaven and Loring from 3 Michigan orchards.

Orchard	Cultivar	Canker Location	No. (%) of *L.persoonii* Group 1	Group2	No. (%) of *L. cincta*
GO	Redhaven	Trunk	30 (90.9)	0 (0.0)	3 (9.1)
		Branch	28 (87.5)	0 (0.0)	4 (12.5)
	Loring	Trunk	34 (94.4)	0 (0.0)	2 (5.6)
		Branch	23 (67.6)	2 (5.9)	9 (26.5)
BA	Redhaven	Trunk	30 (93.8)	0 (0.0)	2 (6.2)
		Branch	27 (87.1)	0 (0.0)	4 (12.9)
	Loring	Trunk	29 (93.5)	1 (3.3)	1 (3.3)
		Branch	25 (78.1)	0 (0.0)	7 (21.9)
KE	Redhaven	Trunk	34 (100.0)	0 (0.0)	0 (0.0)
		Branch	33 (91.7)	0 (0.0)	3 (8.3)
	Loring	Trunk	34 (94.4)	1 (2.8)	1 (2.8)
		Branch	30 (83.3)	1 (2.8)	5 (13.9)

Appendix B

Output of ANOVA analysis using MSTAT-C software. Percentage data were arcsine transformed as described in Materials and Methods before analysis.

Appendix B1

Output of ANOVA analysis using MSTAT-C software. Cultivars, canker sites, and pathogens were considered as experimental factors and analyzed together as described below.

Function: FACTOR

Experiment Model Number 11:
Randomized Complete Block Design for Factor A, with
Factors B and C as Split Plots on A

Factorial ANOVA for the factors:
Replication (orchard) with values from 1 to 3
Factor A (cultivar) with values from 1 to 2
Factor B (canker site) with values from 1 to 2
Factor C (pathogen) with values from 1 to 3

Variable: arcsine transformed percentage data

A N A L Y S I S O F V A R I A N C E T A B L E

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Replication	2	1.96	0.98	1.00	0.50
Factor A	1	24.14	24.14	24.64	0.04
Error	2	1.96	0.98		
Factor B	1	1.00	1.00	0.03	
AB	1	1.00	1.00	0.03	
Factor C	2	31197.93	15598.97	479.23	0.00
AC	2	306.90	153.45	4.71	0.02
BC	2	871.17	435.59	13.38	0.00
ABC	2	62.17	31.09	0.96	
Error	20	651.00	32.55		
Total	35	33119.23			

Appendix B2

Output of ANOVA analysis using MSTAT-C software. Arcsine transformed percentage data of *L. persoonii* group 1 on branches of Redhaven and Loring were analyzed as described below.

Function: ANOVA-2

Two-way Analysis of Variance over
variable 1 (orchard) with values from 1 to 3 and over
variable 2 (cultivar) with values from 1 to 2.

Variable: arcsine transformed percentage data

A N A L Y S I S O F V A R I A N C E T A B L E

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Orchard	2	53.08	26.54	3.34	0.23
Cultivar	1	132.82	132.82	16.74	0.05
Error	2	15.87	7.94		
Non-additivity	1	9.98	9.98	1.69	
Residual	1	5.89	5.89		
Total	5	201.77			

Appendix B3

Output of ANOVA analysis using MSTAT-C software. Arcsine transformed percentage data of *L. cincta* on branches of Redhaven and Loring are analyzed as described below.

Function: ANOVA-2

Two-way Analysis of Variance over
variable 1 (orchard) with values from 1 to 3 and over
variable 2 (cultivar) with values from 1 to 2.

Variable: arcsine transformed percentage data

A N A L Y S I S O F V A R I A N C E T A B L E

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Orchard	2	47.38	23.69	6.94	0.13
Cultivar	1	82.73	82.73	24.23	0.04
Error	2	6.83	3.41		
Non-additivity	1	5.16	5.17	3.10	
Residual	1	1.66	1.66		
Total	5	136.94			

Appendix B4

Output of ANOVA analysis using MSTAT-C software. Arcsine transformed percentage data of *L. persoonii* group 1 on cultivars and canker sites were analyzed as described below.

Function: FACTOR

Experiment Model Number 9:
Randomized Complete Block Design for Factor A, with
Factor B a Split Plot on A

Factorial ANOVA for the factors:
Replication (orchard) with values from 1 to 3
Factor A (cultivar) with values from 1 to 2
Factor B (canker site) with values from 1 to 2

Variable: arcsine transformed percentage data

ANALYSIS OF VARIANCE TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Replication	2	138.28	69.14	5.17	0.16
Factor A	1	122.88	122.88	9.20	0.09
Error	2	26.72	13.36		
Factor B	1	421.03	421.03	21.02	0.01
AB	1	27.18	27.18	1.36	0.31
Error	4	80.11	20.03		
Total	11	816.20			

Appendix B5

Output of ANOVA analysis using MSTAT-C software. Arcsine transformed percentage data of *L. cincta* on cultivars and canker sites were analyzed as described below.

Function: FACTOR

Experiment Model Number 9:
Randomized Complete Block Design for Factor A, with
Factor B a Split Plot on A

Factorial ANOVA for the factors:
Replication (orchard) with values from 1 to 3
Factor A (cultivar) with values from 1 to 2
Factor B (canker site) with values from 1 to 2

Variable: arcsine transformed percentage data

ANALYSIS OF VARIANCE TABLE

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
Replication	2	161.58	80.79	8.68	0.10
Factor A	1	48.36	48.36	5.19	0.15
Error	2	18.63	9.31		
Factor B	1	450.07	450.07	30.74	0.01
AB	1	34.92	34.92	2.38	0.20
Error	4	58.57	14.64		
Total	11	772.12			