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PHENETIC ANALYSIS OF *LEUCOSTOMA* FROM *PRUNUS* AND *MALUS*

By

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ABSTRACT

PHENETIC ANALYSIS OF *LEUCOSTOMA* FROM *MALUS* AND *PRUNUS* SPP.

by

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Isozyme patterns, morphology, virulence on peach and variation in rDNA were used to compare isolates of *Leucostoma cincta* and *L. persoonii* collected from *Malus* and *Prunus* spp. *Leucostoma persoonii* isolates formed three distinct phenetic groupings based on isozyme polymorphisms. The three groups were distinct from all phenetic groupings within *L. cincta*. Based on isozyme patterns of *L. cincta*, three phenetic groupings exist, a distinct group on *Malus* and two related groups on *Prunus*. Far more diversity was evident among isolates of *L. cincta* from different hosts than among isolates of *L. persoonii* from different hosts. Five of the six phenetic groups, two of *L. persoonii* and three of *L. cincta* were compared for virulence on 3-yr-old multistemmed peach seedlings. All five groups were virulent on inoculated peach including PG6, an *L. cincta* group found on *Malus* in nature. *L. persoonii* isolates in PG1 were most virulent while *L. cincta* isolates in PG5 were the least virulent. In culture, isolates of

the two taxa were differentiated on the basis of their pycnidial size and color and colony margin. Isolates of all five phenetic groups grew at 27^0C, but only isolates of phenetic groups PG1 and PG2 of *L. persoonii* showed growth at 33^0C. The phenetic groups were further compared by fragment length polymorphisms based on nuclear ribosomal genes. Digestion of the ITS-LrDNA with 40 restriction enzymes followed by electrophoresis revealed 9 enzymes that distinguished the isolates of *L. persoonii*. Several of the *L. persoonii* groups, defined by ITS-LrDNA phenotypes corresponded to those detected in isozyme studies and others corresponded to host and/or geographic distributions. Less variation in ITS-LrDNA was evident in *L. cincta* populations in contrast to isozyme studies. Most of the RFLP variation in the ITS-LrDNA was within the internal transcribed spacer rather than in the nuclear large rDNA. PCR amplification of the nuclear small rDNA revealed an insertion in *L. cincta* isolates from *Prunus* spp. that is absent in *L. cincta* isolates from *Malus* and in *L. persoonii* isolates.

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GENERAL INTRODUCTION AND LITERATURE REVIEW

Leucostoma canker, which is also called perennial canker, Cytospora canker and Valsa canker, seriously limits peach production in the northern portions of the region favorable for production of temperate fruits (Biggs, 1986; Dhanvantari, 1978; Hildebrand, 1947 & Kern, 1955). The disease is also a problem on plum and prune (*Prunus domestica* L.), sweet and sour cherry (*P. avium* L. and *P. cerasus* L., respectively), apricot (*P. armeniaca* L.), wild black cherries (*P. serotina Ehrh.*), ornamental quince (*Chaenomeles speciosa* (sweet) Nakai), and apple (*Malus domestica* Borkh.) among other mainly roseaceous hosts (Helton and Moisey, 1955 ; Helton & Konicek 1961, Proffer & Jones, 1989).

The disease is characterized by extensive perennial cankers on the limbs and branches, which results in branch dieback, progressive weakening of the tree and eventually the infected branch is girdled and killed (Biggs, 1986; Hampson & Sinclair, 1973; Tekauz & Patrick, 1974). Infections of small twigs appear as sunken discolored areas near winter killed buds or leaf scars. Symptoms on the main trunk, branch crotches, scaffold limbs and older branches begin with gum exudation. As cankers age, the gum becomes dark brown

to black, the infected bark dries out and cracks open exposing the blackened tissue beneath elliptical cankers along the length of the stem (Biggs, 1989). The disease is especially destructive in young orchards where it causes the premature death of orchard nursery stock. Fungicides have very little effect on the incidence of new infections or on the expansion of established cankers (Helton & Rohrbach, 1967 ; Palmiter & Hickey, 1970). Pruning and other cultural practices have had limited success in controlling leucostoma canker (Weaver, 1963).

This disease is caused by two closely related species of fungi, *Leucostoma cincta* (Pers. : Fr.) Hoehn. [anamorph = *Leucocytospora cincta* (Sacc.) Hoehn.] and *L. persoonii* (Nits.) Hoehn. [anamorph = *Leucocytospora leucostoma* (Pers.) Hoehn.]. Early literature in the nomenclature of the pathogen shows an obvious conflict. The two species were placed in the genus *Valsa* by some authors and in the genus *Leucostoma* by others (Von Hoehnel, 1917). In 1867, Nitschke divided the genus *Valsa* into five sub-genera based on the structure of the stromata and *Leucostoma* was included as one of these genera. In 1917, Von Hoehnel elevated the subgenus *Leucostoma* to an independent genus. He used the presence of a darkened conceptacle or marginal zone

delimiting the stromatic tissue as the distinguishing feature. *Leucostoma* and its corresponding imperfect state *Leucocytospora* exhibit the developed conceptacle, while *Valsa* and its anamorph *Eucytospora* do not. (Von Hoehnel, 1981 ; Linda Spielman, 1984)

A monographic treatment of *Leucostoma* was completed in 1958 by Urban in Czechoslovakia (Urban, 1958). Eight species were described, two from coniferous hosts, *L. kunzei* (Fr.) Munk and *L. curreyi* (Nit.) Defago; four with a broad host range of deciduous trees, *L. nivea* (Hoffm. : Fr.) Hoehn., *L. massariana* (DeNot.) Hoehn. *L. aureswaldi* (Nit.) Hoehn. and *L. translucens* (Ces. & DeNot.) Hoehn.; and two from *Prunus* species *L. cincta* (Fr.) Hoehn. and *L. persoonii* (Nit.) Hoehn. A third species on a conifer host, *L. sequoiae* Bonar (1928) was described in 1928. The species were differentiated primarily by the size of the stroma, number of spores per ascus, location of the pycnidium in relation to the stroma and the ascospore size.

In order to recognize the taxa in *Leucostoma,* a full range of criteria must be available. Those criteria pertaining to the sexual state are crucial. However, sexual states are rarely found in nature and identification of these species by plant pathologists

has been based on cultural characteristics described by Willison (1936 & 1937) and Hildebrand (1947). This has led to confusion in the identification of the two taxa due to the presence of a wide range of variation in cultural morphology (Adams, Hammer & Iezzoni, 1990). Kern (1955) suggested that typical isolates of *L. cincta* and *L. persoonii* might represent extremes and that intermediate forms are possible. Lukezic *et al* (1965) reported that monoascospore isolates from a single ascus of *L. persoonii* showed a range of cultural characteristics putatively typical to both species. Optimum growth temperature was suggested for differentiating the two species (Hildebrand, 1947) but this criterion is not used by most workers today (Luepschen *et al*, 1975 & Luepschen, 1981).

Defago (1934) conducted an extensive investigation of the pathogenicity of three *Leucostoma* spp. and two *Valsa* spp. on *Prunus*. He described nine *formae speciales* of *L. persoonii*. His concept of *formae speciales* was based on differences in the degree of virulence on each of 10 *Prunus* species rather than on host specificity on a specific *Prunus* species. The relative virulence of isolates of *L. cincta* and *L. persoonii* identified on cultural characteristics have been reported to differ greatly. *L. persoonii* has been reported to be of low

virulence as compared with *L. cincta* on peach. (Helton & Konicek, 1961 ; Willison, 1937). However, in other research *L. persoonii* was found to be more virulent in warmer weather and *L. cincta* more virulent in cool weather (Hildebrand, 1947 ; Wensley, 1964). Proffer and Jones (1989) identified Leucostoma canker caused by *L. cincta* as a new disease of apples in North America, specifically in Michigan. It is likely that in previous studies of apples *L. cincta* was either not identified or was identified as *L. persoonii* (Leonian, 1921). Thus no characteristic capable of reliably distinguishing cultures of these two species is currently available. This problem has significant consequences in current research on breeding peach trees for disease resistance. Breeding is an expensive and labor intensive process and release of new resistant germplasm faces the risk of being exposed to pre-existing races or biological species of the pathogen that might overcome such a resistance.

Although identification of a species is based on observable differences in morphology, species and populations may be genetically distinct even when they are not morphologically separable. In recent years, there has been an increasing interest in new approaches

to study genetic variation in fungi at several taxonomic levels using biochemical and molecular techniques. Electrophoretic analysis of isozymes has been extensively and successfully used for years to provide rapid and qualitative estimates of the variation within species of higher plants and animals (Gottleib, 1982 ; Hillis & Davis 1986). Enzymes which are coded by different alleles or separate genetic loci frequently possess different electrophoretic mobilities. Such differences are due to variations in the amino acid content of the molecule, which in turn is dependent on the sequence of nucleotides in the DNA. An analysis of isozyme variation by electrophoresis therefore approximates the analysis of gene variation and has been useful in the studies of population genetics. There are at least three major areas in which isozyme analysis has been useful in studies of fungi. These areas include ; 1. classification and delineation of fungal taxa, 2. identification of fungal cultures, and 3. fungal genetics

In classification and delineation of fungal taxa, the interpretation of isozyme banding patterns have proven to be very useful in solving taxonomic problems when few morphological characters are available or when the characters are plastic in nature. Bonde *et al*; (1984)

used isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildew of maize. Isozyme patterns of ten fungal cultures representing three species of the genus *Peronosclerospora* from Texas, southern India, Brazil, Taiwan and the Philippines were compared to aid species identification. Based on isozyme patterns the authors concluded that *P. sacchari* in Taiwan was the same pathogen as *P. philippines* in the Philippines. *P. sorghi* in Thailand was genetically different from *P. sorghi* in India, Brazil and the United States and was probably misidentified. They suggested that delineation of species needed further re-evaluation, which could be achieved by isozyme analysis of additional isolates of *Peronosclerospora*. Micales *et al*, (1987) used the same technique to prove that *Endothia eugeniae*, a pathogen of clove, and *Cryphonectria cubensis*, a pathogen of eucalyptus, were conspecific. The two species were indistinguishable by the analysis of their soluble proteins. In addition they shared alleles at 16 presumed loci as detected by isozyme analysis. Hanson and Wells (1991) characterized three species of *Tremella* based on their isozyme banding patterns. The three species were monomorphic at twelve enzyme loci but also

retained species specific mobilities. Similar studies were performed using isozyme analysis in *Colletotrichum* spp. (Bonde, Peterson and Mass, 1991), *Ustilago hordei* (Hellman and Christ, 1991) and in the *Acremonium/Epichloe* complex (Leuchtman and Clay, 1990). Interpretation of isozyme bands to compare allelic ratios expressed by fungal isolates has been used to study phylogenetic relationships among organisms. Stasz et al, (1989) used isozyme polymorphism to determine phylogenetic relationship in the genus *Trichoderma* by cladistic analysis. They evaluated five morphological species of *Trichoderma*. Their study determined that morphological species were not characterized by either specific alleles at single loci or by specific patterns of alleles at multiple loci. Oudemans and Coffey (1991) used isozyme analysis to study the systematics of twelve papillate *Phytophthora* species. Based on their studies they proposed that two species, *P. arecae* and *P. palmivora* were synonymous. Three species, *P. capsici, P. magekarya* and *P. citrophthora* demonstrated much higher levels of variation in isozyme pattern. They found no evidence to support the existence of distinct varieties in *P. parasitica*. In a related study on *Pythium,* Chen *et al.* (1991) reported the separation of seven homothallic species of *Pythium* on the basis of their

isozyme banding patterns.

In the identification of fungal cultures, isozyme analysis can be used to compare unknown isolates to previously identified cultures in order to determine their identity. Bonde *et al.* (1985) reported that 50% of the genetic loci of *Tilletia indica* were monomorphic. Tooley *et al.* (1985) detected 11 of the 24 loci tested to be monomorphic in *Phytophthora infestans*. It was suggested that these monomorphic loci be used when comparing a known isolate to an unknown. Burdon and Rolfs (1985) examined the effect of sexual and asexual modes of reproduction on the level of diversity of isozyme variation found within respective North American populations of *Puccinia graminis* f. sp. *tritici*. In their study they concluded that sexual populations of *P. graminis* were more diverse than asexual. Jeng *et al*, (1987) did a comparative study of electrophoretic characteristics of Eurasian and North American races of *Ophiostoma ulmi*. In the study they demonstrated additional differences between the two races. Their data also indicated that one of the isolates might be a hybrid between the two races. A comparative study of isolates of *Septoria* on citrus from Australia and United States was performed by the study

of their isozyme banding patterns (Bonde *et al*, 1991). The authors concluded that the isolates from both the countries belonged to the same species. In their study of isozyme comparison among worldwide sources of three morphologically distinct sources of *Phytophthora*, Oudemans and Coffey (1991) detected three enzyme loci that were found to be diagnostic of the three species. Studies like the preceding ones become extremely important when some crop materials are quarantined due to danger of entry of exotic plant pathogens. In such instances, isozyme analysis can facilitate identification when only a few morphological characters are available.

In fungal genetics , isozyme analysis has been an effective technique for studying the allelic variation in populations. Kerrigan and Ross (1989) used 18 field isolates of *Agaricus bisporus* to study genetic variation in the wild *Agaricus* population. Their study revealed the presence of new alleles supporting the belief that cultivars of *A. bisporus* escape from commercial cultivation and can reproduce under normal circumstances. It also revealed the potential of expanding the gene pool of commercial varieties. High variability was detected in the related species *Agaricus bitorquis* (Roux and Labarere, 1991). This

species has a phenotype comparable to *A. bisporus* and it was suggested that the high genetic variability compared to that of *A. bisporus* could provide a greater genetic base for cultivar expansion. Genetic variation was also reported to be high in *Crumenulopsis sororia* (Ennos and Swales , 1991). Zambino *et al* (1989) studied genetic variation between varieties of *Leptographium wagneri*. They reported one electrophoretic type to be abundant and broadly distributed within each variety. Gene diversity within each variety was observed to be low but genetic differentiation between varieties was high.

In other studies isozymes have been coupled with other markers for a comparative analysis. For example, Newton *et al* (1985) studied variation for isozyme and double-stranded RNA among isolates of *Puccinia striiformis*. These studies were aimed at using the two groups of molecules as markers for both the nucleus and the cytoplasm and thus allow the variability in each genome to be examined separately. No differences in isozyme banding patterns were observed among 29 diverse wheat-attacking isolates of *P. striiformis* (WYR). Isolates of the barley attacking form (BYR) of *P. hordei* and *P. recondita* showed similar uniformity. In

contrast, there were differences in the dsRNA phenotypes both among the three species and between WYR and BYR. Burdon *et al*, (1985) studied isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. They detected no correlation between isozyme and virulence diversity. In a related study on *Uromyces appendiculatus* Linde, *et al.* (1990) detected greater diversity in virulence patterns as compared to the isozyme patterns. Mills *et al.*(1991) used isozyme and mitochondrial DNA analyses to study intra- and interspecific variation in *Phytophthora cryptogea* and *P. drechsleri*. The majority of the isolates were subdivided into ten groups based on numerical analysis of 24 putative enzyme loci. Analysis of mitochondrial DNA restriction fragment length polymorphisms of selected isolates from each enzyme group supported their isozyme data. Vilgalys (1991) reported association between different intersterility groups of *Collybia dryophilla* with isozyme differences and postulated that fungal speciation evolves primarily by allopatry.

Additional techniques would be helpful both in the identification of species in the absence of sexual states well as to detect intra- and interspecific variation. Recently, restriction fragment length

polymorphisms (RFLP), which reflect variation among homologous DNA sequences have provided more precise tools for detecting and quantifying genetic variation. RFLPs have been studied in many organisms including humans (Botstein *et al*, 1980), plants (Helentjaris *et al*, 1985) and animals (Hillis and Davis, 1986). These polymorphisms can be generated by loss or gain of restriction sites resulting from point mutations, or from rearrangement of DNA sequences. McDonald and Martinez (1990) used RFLP markers to measure the amount and distribution of genetic variation in *Septoria tritici*. Single and multilocus analysis of their RFLP data indicated that a high level of genetic variability was distributed in the population. RFLPs have also been used to study molecular variation in *Verticillium* (Carder & Barbara, 1991) and in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp in Australia (Braithwaite and Manners, 1990). Specht *et al.* (1984) used RFLPs to study strain specific differences in the nuclear ribosomal DNA (rDNA) of the fungus *Schizophyllum commune*. Restriction mapping of rDNA using four strains of the fungus revealed strain specific variation with repeat lengths of 9.2-9.6Kbp. These authors were also the first to report methylation of rDNA. RFLP markers

were used by Anderson *et al.* (1987) to compare biological species within the taxon *Armillaria mellea*. They identified significant variability and most of the variability occurred between rather than within biological species.

One of the difficulties in applying RFLPs to taxonomic problems is in selecting a DNA segment that can resolve at an appropriate taxonomic level, showing neither excessive and uninterpretable variation nor homogeneity at the taxonomic rank of interest. In fungi, rDNA is especially useful in taxonomic studies for the following reasons: 1)the rDNA repeat length is within a range that can be examined by RFLP analysis, 2) the rDNA repeat unit contains both slowly evolving regions (the 18S, 5.8S, and 28S rRNA genes) and more rapidly evolving regions (the transcribed and non transcribed spacers) so that information from various levels of evolutionary history can be recovered, and 4) the rDNA evolves in a concerted fashion. (Appels & Dvorak, 1982; Bruns, White & Taylor, 1991). The rDNA repeat unit has been widely used to study phylogenetic relationships and population genetics in fungi. Kohn *et al.* (1988) compared *Sclerotinia* species by the analysis of the RFLP patterns of nuclear and mitochondrial rDNA. They observed polymorphisms in the

rDNA between rather than within species. In addition extensive variation in RFLPs of mitochondrial DNA between species was also observed. Magee *et al.* (1987), used RFLPs in the nuclear rDNA to distinguish various isolates of *Candida albicans*. Six different classes were detected based on variations in the restriction pattern. Similar studies by Walsh *et al.* (1990) using a heterologous probe to detect RFLPs in *Entomophaga* rDNA sequences showed delineation of genera and species within *Entompthorales* but were not useful at lower taxonomic levels. William *et al.* (1988) compared RFLP patterns in the nuclear rDNA and mitochondrial (mt) DNA of three *Agaricus* species. Restriction patterns of one species (*A. brunescens*) were found to be identical, and the *A. bitorquis* and *A. campestris* isolates were subdivided into two. Vilgalys and Gonzalez (1990) detected variation both at the inter- and intragroup level of the 15 anastomosis groups of *Rhizoctonia solani* used in their study. In a related study on restriction analysis of rDNA of binucleate *Rhizoctonia* spp. Cubeta *et al.* (1991) reported the separation of 13 of 21 anastomosis groups of binucleate *Rhizoctonia* spp. into distinct groups. These groupings were found to be consistent with prior groupings based on hyphal

anastomosis. Egger, Danielson and Fortin (1991) analyzed the nuclear and mitochondrial ribosomal RNA genes to elucidate the species concept among the asexual E- strain mycorrhizal fungi and to examine their population structure. They found that most E- strain isolates could be assigned to the two sexual taxa *Wilcoxina mikolae* and *W. rehmii*, which have different habitat preferences. Analysis of the mitochondrial DNA revealed that within each species isolates could be differentiated based on host preference.

With the advent of enzymatic amplification, copies of selected regions of the rDNA repeat unit can be selectively amplified as non methylated copies from crude DNA preparations, greatly simplifying RFLP analysis of populations (Bruns, White & Taylor, 1991). Vilgalys and Hester (1990) used this technique for genetic identification and mapping of amplified rDNA of *Cryptococcus* species. Digestion and electrophoresis of the PCR products by using restriction enzymes produced restriction phenotypes that were unique for each strain or species. Hibbet & Vilgalys (1991) studied evolutionary relationships of *Lentinus* to the Polyporaceae by restriction analysis of enzymatically amplified rDNA. Their results suggested that *Lentinus tigrinus* was more closely related to the Polyporaceae

than to the Tricholomataceae

The aim of this project was to study the variability in the two closely related species of *Leucostoma*, *L. cincta* and *L. persoonii*. This research in particular aims at using markers that would be useful in revealing species and subspecific variation. The following three chapters describe the techniques used to achieve this goal. Chapter 1 describes the use of isozyme markers to clarify and delineate the two taxa. Chapter 2 describes the use of restriction fragment length polymorphisms to study the nuclear ribosomal DNA variation between various isolates of *Leucostoma*. In chapter 3, pycnidium size, temperature optimum and relative virulence among some of the genetically different phenetic groups within *L. cincta* and *L. persoonii* are compared. The concluding chapter stresses the importance of the research in providing a basis for understanding the relationship between the plant pathogenic isolates of *Leucostoma* and makes recommendations for future research.

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ISOZYME DETECTION AND VARIATION IN *LEUCOSTOMA* FROM *PRUNUS* AND *MALUS*.

ISOZYME DETECTION AND VARIATION IN *LEUCOSTOMA* SPECIES FROM *PRUNUS* AND *MALUS*

ABSTRACT

Isozyme analysis was used to study the relationship between two closely related taxa of *Leucostoma, L. cincta* and *L. persoonii*. Isolates were obtained from six *Prunus spp.* and *Malus domestica* Borkh. in North America. Monoascospore isolates collected from individual perithecia of *L. cincta* and *L. persoonii*, tissue isolates and isolates varying in vegetative compatibility groups of *L. persoonii* were examined. Thirty alleles were resolved at eight putative loci and the two species were found to be distinct at 48% similarity. In addition, this analysis separated the two species into six phenetic groups. *L. cincta* was separated into several closely related groups on *Prunus spp.* and one group on *Malus*. *L. persoonii* was separated into three distinct groups which were not correlated to hosts or geographic origins. Isozyme polymorphisms were not found to be associated with vegetative compatibility groups in *L. persoonii*. Far more diversity was evident among isolates of *L. cincta* from different hosts than among isolates of *L. persoonii* from one perithecium from different hosts.

Identification of a fungal species is based on observable differences in morphology, but species and populations may be genetically distinct even when they are not morphologically separable. Genetic and biochemical techniques such as isozyme analysis have been useful in combination with morphology to identify genetic variability between and among fungal species (Micales, Bonde & Peterson, 1986; Micales & Stipes, 1987; Hanson & Wells, 1991; Chen, Hoy & Schneider, 1991; Stasz *et al*, 1989) and to delineate taxa that are morphologically similar in culture (Bonde, Peterson & Mass, 1991). An alternative method to morphology is needed for differentiating the two closely related and important plant pathogens *Leucostoma cincta* (Fr.: Fr.) Hoehn. and *L. persoonii* Hoehn. that cause Cytospora canker of fruit trees. Morphological characteristics distinguishing the two species are present only in the teleomorph and the teleomorph is generally not found in nature associated with the disease symptoms. The *Leucocytospora* anamorphs, *Leucocytospora cincta* (Sacc.) Hoehn. and *L. persoonii* Hoehn., that are abundantly present in infected orchards are not separable morphologically.

Because the teleomorph generally is not found the practical delimitation of these two pathogens often has

been based on cultural characteristics. Current researchers have not been in agreement on the relevant cultural and physiological characteristics of the two species (Adams, Hammar & Proffer, 1990; Surve-Iyer, Adams & Iezzoni, 1992). Cultural characteristics and growth temperature responses described by Willison (1938), Hildebrand (1947) or Kastirr (1984) have been utilized as criteria for differentiating the two species in some studies (Adams, Hammer & Iezzoni, 1989, Proffer & Jones, 1989) but in others the criteria used for identification is not clear (Endert Kirkpatrick, 1986; Helton & Konicek, 1961, Regner & Johnson, 1990). Thus currently, the presence of the sexual fruiting body continues to be required for the definitive identification of these two species until a more thorough study of cultural characteristics of isolates can be correlated with specific sexual states. In this study we have begun the grouping of anamorphic isolates by comparing their similarity in cultural characteristics to those of isolates obtained from identifiable sexual states, assigning the sexual name to corresponding cultures and comparing their isozyme profiles. This approach provides the foundation of the isozyme study and it effectively clarifies our

understanding of the revealed genetic diversity. The usefulness of cultural characteristics alone, as criteria in distinguishing the species, is summarized and discussed elsewhere (Surve-Iyer, Adams & Iezzoni,1992).

Our objective is to identify and delineate the two taxa in the absence of the sexual state and to identify genetic variation in the *Leucocytospora* populations by using isozyme markers. Identifying the extent of genetic diversity in the two pathogens is necessary to construct an effective screening procedure in an ongoing peach breeding program selecting for canker resistance (Chang, Iezzoni & Adams, 1991; Chang *et al*, 1989). Breeding is an expensive and labor intensive procedure. Release of new resistant germplasm would face risks of being exposed to pre-existing races or biological species of the pathogen that might overcome such resistance if selection for resistance was based on a limited understanding of the genetic diversity of the pathogens.

The two *Leucostoma* species also cause annual or perennial cankers on nectarine, sweet, sour and black cherry, plum and prune, ornamental quince, apple, chokecherry, amelancheir, and wild roseaceous hosts. The genetic variation among isolates on various hosts has not been examined thoroughly in the past. Whether host

specific isolates or races might exist within the populations of the two *Leucostoma* is not known but in a study in 1934 Defago concluded that some isolates represented *formae speciales*. His concept of *forma speciales* was not based on host specificity rather his *formae speciales* isolates were virulent on all the hosts he tested and were comparatively more virulent on one host. Some believe the relative virulence of the two species differs greatly; *L. persoonii* has been reported to be of low virulence compared to *L. cincta* on peach (Helton & Konicek, 1961; Willison, 1989). However, others believe *L. persoonii* is more virulent in warm weather and *L. cincta* more virulent in cool weather (Hildebrand, 1947; Wensley, 1964). We attempt in this study to use isozymes to analyze the extent of genetic variability in diverse populations from numerous hosts. This approach has been effective in other studies of fungi (Hellman & Christ, 1991 ; Zambino & Harrington, 1989; Leuchtmann & Clay, 1990; Linde, Groth & Roelfs, 1990; Yoon, Gessner & Romano, 1990). Similarly we wish to examine whether specific populations might be traceable to specific geographic origins, as others have accomplished with isozyme analysis (Bonde, Peterson, Emmett & Menge, 1991; Leung & Williams, 1986; Oudemans &

Coffey, 1991). The extent of genetic variation revealed in this study and correlation of the diversity to host and geographic origins is discussed. Isolates representative of the genetic diversity revealed herein are examined in virulence studies elsewhere (Surve-Iyer, Adams & Iezzoni, 1992).

MATERIAL AND METHODS

The host, geographic origin and source of the isolates of *Leucocytospora* used in this study are given in Table 1. Isolates of *L. persoonii* from perithecia were collected from *Prunus persica* (L.) Batsch. and *P. serotina* Ehrh. and isolates of *L. cincta* from perithecia were collected on *P. armeniaca* L., *P. domestica* L., and *Malus domestica* Borkh. *Leucocytospora* isolates that corresponded in cultural characteristics to *L. persoonii* ascospore cultures were collected from diseased plant tissue from Michigan, North Carolina, California, and Oregon and Ontario, Canada. Similarly asexual isolates corresponding to *L. cincta* were collected from Michigan. Isolates of *L. persoonii* from 9 vegetative compatibility groups (Adams, Hammar & Proffer, 1990) were and 7 European *formae speciales* (Defago, 1935) were also compared in isozyme analysis.

Isolates were maintained on Leonian's media (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 water) (Leonian, 1923). Five 1 cm plugs of mycelia grown on Leonian's agar medium were added to 100 ml of liquid Leonian's media in a 500 ml Erlenmeyer flask. The isolates were grown at room temperature in still cultures. Mycelial mats were collected from each flask after 10-14 days incubation by vacuum filtration onto Miracloth and blotted dry on sterile paper towels. The mats were either used immediately or stored at -70°C. Mycelium was ground in liquid nitrogen and 3 ml of extraction buffer (50 mM Tris HCl, pH 7-7.5, 10% glycerol and 0.1% beta mercaptoethanol) and sand in a cold mortar and pestle. The mixture was centrifuged in a microfuge at 12,700 g for 20 min at 4°C, and 4 ul of the supernatant was applied to thin layer cellulose acetate plates ("Titan III" [94mm 76mm], Helena Laboratories, Beaumont, Texas) and electrophoresed at 12-20 mA for 20 min at 4°C. Excess supernatant was stored at -70°C and reused till the enzyme showed signs of degradation, after which it was discarded. Each isolate was tested three times and each gel was run with two standard isolates (11.3 and ATCC 62910). Five buffer systems were used in electrophoresis (Richardson, 1987); buffer

A, 10 mM Na_2HPO_4, 2.5 mM citric acid, pH 6.4; buffer B, 11.6 mM Na_2PO_4, 8.4 mM NaH_2PO_4, pH 7.0; buffer C, 50 mM Tris, 20 mM maleic acid, pH 7.8; buffer D, 15 mM Tris, 10 mM $MgCl_2$, 5 mM Na_2EDTA, pH 7.8; and buffer I, 25 M Tris, 192 mM glycine, pH 8.5). Enzyme stains were from Richardson (1987) and were scaled to approximately 2 ml total volume and applied to plates as overlays mixed 1:1 with 1.5% Bacto agar in distilled water. For each enzyme assay, mobility of the enzyme reaction was noted for each isolate and similar mobilities were compared in adjacent lanes in subsequent gels. Each band was considered as an allele of a specific locus. The most anodally migrating band was designated "a" and the alleles of the same locus were assigned "b", "c", etc., according to their mobility relative to the "a" allele. The genetic basis of the allelic patterns could not be unequivocally determined because it was not possible to induce formation of sexual states. Therefore we assumed the mobility differences or electromorphs were allelic variants within a given locus. Nomenclature for designating enzymes, loci and alleles followed the convention of Hanson & Wells (1991). Enzymes were identified by a short, upper-case abbreviation, e.g., IDH (isocitrate dehydrogenase). Genetic loci were

capitalized and italicized, e.g., *IDH*. When more than one allele was present an alphabet identifier was added: *IDH-a* or *IDH-b*

The data as binary codes was analyzed using two computer assisted programs and their subprograms; SIMQUAL, SAHN, COPH & MXCOMP in the NTSYS version 1.7 (Rohlf, 1992) and BOOT program in PHYLIP (Felsenstein, 1992). The SIMQUAL program was used to calculate similarity coefficients, Simple Matching Coefficient, which emphasizes both the positive and negative matches and the Jaccard's Coefficient, which emphasizes the positive matches over the negative matches. The similarity coefficients were analyzed with UPGMA (Unweighted pair group analysis using arithmetic means) and clustered using the SAHN subprogram. To estimate the accuracy of the phenograms produced, each similarity matrix was converted to a matrix of cophenetic values, using COPH in NTSYS. Matrix correlations between the original similarity coefficients and the cophenetic values were assessed by MXCOMP in NTSYS. When the cophenetic correlation was greater than 0.9, the fit of the tree to the similarity coefficient matrix was considered very a good fit. The BOOT program of PHYLIP was used to generate the most parsimonious tree by bootstraping using Wagner's assumptions with global

options. In addition genotypic diversity was calculated for each species or subgroup using the procedure of Selander *et al.* (1986). Isolates were assigned to electrophoretic types (ETs) each representing a group of isolates with identical phenotypes for all loci scored. The genotypic measure considers the number of distinct ETs found in a species or subgroup and does not assume random mating. Isozyme phenotypes for the ETs of each isolate are given in Table 3.

RESULTS

Table 2 lists the enzymes that were either undetected, poorly resolved (bands were either faint or did not stain for all isolates) and well resolved on cellulose acetate gels. Thirty alleles were resolved at eight putative loci among the 56 isolates that stained for the eight enzymes. All the eight enzymes stained for a single locus (Table 4). Significantly greater variability was seen among isolates of *L. cincta* than among *L. persoonii*. No variability was detected among the representative isolates of the 9 vegetative compatibility groups. The *L. persoonii formae speciales*, from Europe showed unique alleles that were different from all North American *L. persoonii* isolates tested.

Californian *L. persoonii* from nectarine also showed unique polymorphisms at two loci.

Genetic Distances and Identities

The phenogram in Fig. 1 provides a visual summary of the pattern of genetic differentiation between the two *Leucostoma* taxa and among several subgroups. The UPGMA phenogram constructed for the Jaccard's and the Simple Matching coefficients had identical topology however, the Jaccard's phenogram had longer branch lengths at major divisions and showed no (0%) similarity between the two *Leucostoma* taxa. The phenogram of the Simple Matching coefficient assigned closer relationships to the isolates than Jaccard's coefficient. The phenogram in Fig. 2 is the most parsimonious Wagner unrooted tree calculated from 1000 bootstrap replications (13 hours of 386-33 MHZ PC-computer time) and shows confidence limits calculated for each branch, represented as the percent of the bootstrap estimates. The two taxa, *L cincta* and *L. persoonii* were distinctly different at 48.4% similarity and 99.6% confidence. Isolates of *L. cincta* were separated into two closely related phenetic groups on *Prunus* species (77.4% similarity and 47.3% confidence) and one distinct phenetic group on *Malus* (64.5% similarity and 99.6% confidence). *L. persoonii*

was separated into three distinct groups each sharing 74.2% similarity, one widespread group on *Prunus* spp. (PG1) (100% confidence), a second on Michigan peach and black cherry (PG2) (100% confidence) and a third group of on nectarine in California (PG3) (42.8% confidence). Genotypic diversity in the two taxa was higher in *L. cincta* at .84%, the *L. persoonii* at 50% (Table 4). Most of the diversity in *L. cincta* was within the phenetic group occurring on *Prunus* spp (66%) for which two alleles occurred in each of the PGI, PGM, IDH, G6PD, ME, MAN, , GDH, and PGD loci (Table 2 & 3).

DISCUSSION

Isozyme analysis has proved to be an useful technique to study the relationship between the isolates of *Leucostoma*. Plant pathologists in the past have routinely substituted vague cultural characteristics in absence of sexual states to identify the two species. The results presented here clearly show the extent of differentiation between *L. cincta* and *L. persoonii* with respect to the electrophoretic banding patterns for eight different soluble enzymes. The eight could be used as diagnostic loci to identify the two species. The patterns of the stained enzyme loci obtained were clear, easy to interpret and readily obtained for

isolates from North America. However, several of the *L. persoonii formae speciales* isolates from Europe did not produce any bands when stained for a particular enzyme. These enzymes may be produced at extremely low levels or below the level of detection of the staining procedure. In addition, this technique has also demonstrated the separation of these two species into six phenetic groups.

The phenogram generated by NTSYS-pc (Rohlf, 1987) using the SIMQUAL sub-program and the UPGMA option (NTSYS) resolved the genetic distances between 56 of the isolates and separated them into six phenetic groups. Isolates of *L. persoonii* were separated into three phenetic groups (PG1, PG2 and PG3); those of *L. cincta* were subdivided into at least three phenetic groups, (PG4, PG5, PG6).

The *L. cincta* isolates from *Malus* appeared to be ecologically host specialized (although found exclusively on *Malus* this group is found to be virulent on inoculated peach seedlings). Proffer & Jones (1989) were the first to report the presence of *L. cincta* on *Malus* in North America. The reasons for the absence of these strains on *Prunus* in nature remains unknown. In addition to their distinct electrophoretic

patterns, these isolates also showed differences in cultural characteristics when compared to other *L. cincta* isolates. They released a reddish brown pigment in culture which was not detected in other isolates. These isolates were also slow growing compared to the rest of the isolates used in this study (Proffer & Jones, 1989). Screening of additional *L. cincta* isolates on *Malus* to study their isozyme banding patterns will be required to further elucidate the ecological host specialization of this group

Unlike the distinct phenetic group found on *Malus*, the other two phenetic groups consisting of ascospore progeny of *L. cincta* on *Prunus* show substantial amounts of diversity both within and between the phenetic groups. This diversity might be due to sexual outcrossing through meiotic recombination and perhaps these groups are a single diverse group found on *Prunus* species. In nature *L. cincta* teleomorphs have been more abundant than *L. persoonii* teleomorphs. Genetic diversity has been reported as being higher in populations of *Crumenulopsis soraria* (Ennos & Swales, 1991) where reproduction of this population is through sexual outcrossing than in asexual populations. Similarly high isozyme diversity has been reported in sexually outcrossing fungi such as *Magnaporthe grisea*

(Leung & Williams, 1986), *Epichloe* (Leuchtmann & Clay, 1989), and natural populations of *Agaricus bitorquis* (Roux & Labarere, 1991) when compared to related asexual species and populations. However it should be noted that isozyme variability might depend on the number of isolates used and the particular enzymes tested.

The first phenetic group (PG1) of *L. persoonii* was geographically widespread and diverse in host range in nature. PG1 consisted of ascospores, isolates representing five vegetative compatibility groups, and tissue isolates of from four *Prunus* spp. Isolates within this group had identical banding patterns regardless of their origins. The PG2 isolates of *L. persoonii* have been found only from a peach seedling planting and two canker isolations from native black cherry in Michigan. Isolates in this group were not from sexual states, and sexual fruiting bodies have not been identified with this group so far. Perhaps this group represents a native infection source and hence it's presence on seedlings rather than on imported nursery stock. Nursery stock might be often shipped infected (Wensley, 1964). The third group, PG3 occurred on nectarine from California. The PG3 isolates were not similar to isolates from California on plum (PG1), but

climatic factors vary greatly between the California locations of the plum and nectarine orchards. Therefore, whether the genetic isolations between the groups is due to geographic origins, climatic origins or host preference is speculative. Additional sampling in PG3 will be necessary before further speculations can be made about this group.

Several factors may be responsible for the low genetic diversity seen in the phenetic group 1 of *L. persoonii*. Lack of isozyme variability could occur because of founder effect (Ennos & Swales, 1991). Individual populations could have been established from founder isolates and it is possible that the introduced pathogen represented only part of the genetic variability that existed in the parent species, especially if the species is native to Europe or Asia. Absence of isozyme variability could be through genetic drift if the population size was dependent on transatlantic importation. Population bottlenecks also could naturally occur through seasonal environmental factors. Though sexual states have been found in this species and the fungus is reported to be heterothallic (Adams, Hammar & Proffer, 1990), lack of polymorphism could be due to the rarity of the sexual cycle or effective lack of a sexual cycle. It is also possible

that all the allelic variability is not detected by electrophoresis and the amount of genetic variability and genetic differentiation between groups may be underestimated (Ayala *et al*, 1973; Bonde, Peterson & Dowler, 1988). Low levels of isozyme variability have been reported in other plant pathogenic fungi including some species of *Phytophthora* (Oudemans & Coffey, 1991), *Ustilago hordei* (Hellman & Christ, 1991) , *Tremella* (Hanson & Wells, 1991) and also in highly biotrophic fungi in *Puccinia Styriformis* (Newton, Caten & Johnson, 1985). There are no comparative studies of genetic variation in fungi having biotrophic, necrotrophic or saprotrophic nutrition to make general assumptions on their genetic variability.

These results provide considerable information on the relationship between *L. cincta* and *L. persoonii*. Based on isozyme polymorphism we have demonstrated the presence of two closely related sympatric species, *L. cincta* and *L. persoonii*. Both species and several phenetic groups are known to occur in the same orchard in our study. The amount of variation at the level of local phenetic groups was high both between the two species and within *L. cincta*. Such patterns have been documented for *Drosophila* (Ayala *et al*, 1973) and

Neurospora (Speith, 1975). According to Ayala (1973) the genetic distances between closely related species depends in part on the speed with which reproductive isolation mechanisms have developed and their efficacy during the speciation process. Polymorphism can occur if temporal variation is coupled with spatial heterogeneity (Gillispie and Langley, 1974). This could be the case in *L. persoonii* whose origin might be traced to Europe or perhaps Asia, having later being transported to the United States on susceptible hosts. Over time *L. persoonii* may have become three reproductively isolated groups.

The large genetic distances between *L. cincta* and *L. persoonii* suggests that reproductive isolation between the two may have been quite rapid. In his review of genetic variation in natural populations, Nevo concluded that genetic polymorphism and heterozygosity are correlated with ecological heterogeneity (Nevo, 1978). Perhaps selection pressure due to adaptation to the many host species in the wild or to nursery stocks derived from selective breeding may have contributed to the above process and to the development of ecologically host specific races, as seen in isolates of *L. cincta* from *Malus*. This could also lead to formation of localized populations where peaches have been cultivated

in the same orchard for decades. This could stabilize certain geographical populations. Perhaps this has occurred in phenetic group 2 of *L. persoonii* on black cherry, a tree which is native in mixed deciduous woodlands of Michigan. In inoculation experiments, Defago (1934) recognized forms of *L. persoonii* uniquely more virulent on one *Prunus* species than on others although all isolates had been found on *P. persica*. Though the concept of *forma specialis* was incorrectly used by Defago we have not detected in Michigan the type of host specialized isolates of *L. persoonii* he observed (Surve-Iyer, Adams & Iezzoni, 1992). However, the phenomenon of ecological host specialization in *L. cincta* was detected in our study.

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Table 1. *Leucostoma* isolates used in the isozyme studies

Species isolate number & code *	Sexual or asexual stromal	Origin	Host	Host	Group
L. cincta					
(1) A2	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(2) A8	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(3) A9	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(4) A12	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(5) A43	a	Michigan	*M. domestica*	Proffer & Jones	PG6
(6) A45	a	Michigan	*M. domestica*	Proffer & Jones	PG6
(7) A48	a	Michigan	*M. domestica*	Proffer & Jones	PG6
(8) A46	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(9) A79	s	Michigan	*M. domestica*	Proffer & Jones	PG6
(10) A32	a	Michigan	*M. domestica*	Proffer & Jones	PG6
(11) 5P4	s	Michigan	*P. domestica*	Proffer & Jones	PG5
(12) 2P2	s	Michigan	*P. domestica*	Proffer & Jones	PG5
(13) 4P1	s	Michigan	*P. domestica*	Proffer & Jones	PG5
(14) 1P1	s	Michigan	*P. domestica*	Proffer & Jones	PG4
(15) LP62	a	Michigan	*P. domestica*	Proffer & Jones	PG4
(16) LP63	a	Michigan	*P. domestica*	Proffer & Jones	PG4
(17) LP66	a	Michigan	*P. domestica*	Proffer & Jones	PG4
(18) LP70	a	Michigan	*P. domestica*	Proffer & Jones	PG4
(19) LP73	a	Michigan	*P. armeniaca*	Proffer & Jones	PG5
(20) LP40	a	Michigan	*P. armeniaca*	Proffer & Jones	PG5
(21) LP39	a	Michigan	*P. armeniaca*	Proffer & Jones	PG4
(22) F1h	s	Michigan	*P. armeniaca*	Adams	PG4
(23) LP47	s	Michigan	*P. armeniaca*	Adams	PG5
(24) LP49	s	Michigan	*P. armeniaca*	Adams	PG4
(38) ATCC 62910	a	Ontario	*P. persica*	ATCC (Biggs)	PG4
F1u	s	Michigan	*P. armeniaca*	Adams	
F1g	s	Michigan	*P. armeniaca*	Adams	
F1r	s	Michigan	*P. armeniaca*	Adams	
F1b	s	Michigan	*P. armeniaca*	Adams	
F1s	s	Michigan	*P. armeniaca*	Adams	
F1f	s	Michigan	*P. armeniaca*	Adams	

Table 1. contd.

L. persoonii

(25) 11.3	a	Michigan	*P. persica*	Adams	PG1
(26) VSS4	s	Ontario	*P. persica*	Biggs	PG1
(27) VSS5	s	Ontario	*P. persica*	Biggs	PG1
(28) VSS3	s	Ontario	*P. persica*	Biggs	PG1
(29) LP8	s	Michigan	*P. serotina*	Proffer	PG1
(30) LP10	s	Michigan	*P. serotina*	Proffer	PG1
(31) LP16	a	Michigan	*P. domestica*	Proffer	PG1
(32) LP34	a	Michigan	*P. armeniaca*	Proffer	PG1
(33) T28.1	a	Michigan	*P. persica*	Adams & Surve	PG2
(34) T20.3	a	Michigan	*P. persica*	Adams & Surve	PG2
(35) T4.7	a	Michigan	*P. persica*	Adams & Surve	PG2
(36) T7.4	a	Michigan	*P. persica*	Adams & Surve	PG2
(37) T8.6	a	Michigan	*P. persica*	Adams & Surve	PG2
(39) Cy2	a	Oregon	*P. avium*	Spotts	PG1
(40) Cy3	a	Oregon	*P. avium*	Spotts	PG1
(41) Cy4	a	Oregon	*P. avium*	Spotts	PG1
(42) Cy5	a	Oregon	*P. avium*	Spotts	PG1
(43) T28.1	a	Michigan	*P. persica*	Adams & Surve	PG2
(44) Lp12	a	Michigan	*P. cerasus*	Proffer	PG2
(45) TN	a	California	*P. persica* var. *nucercisa*	Michailides	PG3
(46) LCC	a	California	*P. persica* var. *nucercisa*	Michailides	PG1
(47) LCN	a	California	*P. persica* var. *nucercisa*	Michailides	PG3
(48) T26.3	a	Michigan	*P. persica*	Adams & Surve	PG2
(49) NC17	a	North Carolina	*P. persica*	Endert-Kirkpatrick	PG1
(50) NC49	a	North Carolina	*P. persica*	Endert-Kirkpatrick	PG1
(51) T16.7	a	Michigan	*P. persica*	Adams & Surve	PG2
(52) NC9.2	a	North Carolina	*P. persica*	Endert-Kirkpatrick	PG1
(53) NC8.2	a	North Carolina	*P. persica*	Endert-Kirkpatrick	PG1
(54) T18.1	a	Michigan	*P. persica*	Adams & Surve	PG2
(55) R5T10	a	California	*P. domestica*	Adams	PG1
(56) R3T16	a	California	*P. domestica*	Adams	PG1
f.sp *mahaleb*	a	Switzerland	*P. persica*	CBS(264.34)	
f. sp *persica*	a	Switzerland	*P. persica*	CBS(266.34)	
f. sp *oeconomica*	a	Switzerland	*P. persica*	CBS(265.34)	
f.sp *armeniaca*	a	Switzerland	*P. persica*	CBS(260.34)	
f. sp *avium*	a	Switzerland	*P. persica*	CBS(261.34)	

Table 1. contd.

f. sp *cerasi*	a	Switzerland	*P. persica*	CBS(262.34)
R5T15	a	Michigan	*P. persica*	Adams
R6T5	a	Michigan	*P. persica*	Adams
R5T12	a	Michigan	*P. persica*	Adams
10.14	a	Michigan	*P. persica*	Adams
11.12	a	Michigan	*P. persica*	Adams
58	a	Michigan	*P. persica*	Adams
R1T12	a	Michigan	*P. persica*	Adams
10.1	a	Michigan	*P. persica*	Adams
11.2	a	Michigan	*P. persica*	Adams
R4T14	a	Michigan	*P. persica*	Adams
R1T4	a	Michigan	*P. persica*	Adams
R1T16	a	Michigan	*P. persica*	Adams
105	a	Michigan	*P. persica*	Adams
R6T14	a	Michigan	*P. persica*	Adams
R5T11	a	Michigan	*P. persica*	Adams

*represents the number on the phenogram in Fig. 1
@ "s" represents the sexual state and "a " represents asexual state

Table 2. Enzyme stains tested with *Leucostoma*

Enzyme (EC number)	Buffer*
Undetected enzymes	
Aconitate hydratase (EC 4.2.1.3)	A, B, C
Alcohol dehydrogenase (EC 1.1.1.1.)	B, C, D
Adenylate kinase (EC 2.7.4.3)	A, B, C
Aldehyde oxidase (EC 2.7.1.40)	I
Fructose diphosphatase (EC 3.1.3.11)	B, D
Glyceraldehyde-3-phosphate dehyrogenase (EC 1.2.1.12)	C, D
Glutathione reductase (EC 1.6.4.2)	C, D
B- Hydroxybutarate dehydrogenase (1.1.1.30)	B, C, D
Lactate dehydrogenase (EC 1.1.1.27)	A, C, D
Pyruvate kinase (EC 1.2.3.1)	A, B
Shikimic dehydrogenase (EC 1.1.1.25)	D, B
Poorly resolved enzymes	
Acid phosphatase (EC 3.1.3.2)	A, C
Alkaline phosphatase (EC 3.1.3.1)	C, D
Diaphorase (EC 1.6.4.3)	A, D,
Glucose dehydrogenase (EC 1.1.1.47)	B, D, I
Hexokinase (EC 2.7.1.1)	B, C, D
Mannose- phosphate isomerase (EC 5.3.1.8)	A, B
Well-resolved enzymes	
Esterase (EC 3.1.1.1)	A, C, D
Glutamate dehydrogenase (EC 1.4.1.3)	D, B
Glucose-phosphate-dehydrogenase (EC 1.1.1.49)	D
Glucose-phosphate-isomerase (EC 5.3.1.9)	B
Isocitrate dehydrogenase(EC 1.1.1.42)	A
Malate Dehydrogenase (EC 1.1.1.37)	B, D
Mannitol dehydrogenase (EC 2.4.2.1)	B
Phosphogluconate dehydrogenase (EC 2.7.5.1)	D
Phophoglucomutase (EC 2.7.5.1)	A

*Buffer A= 10 mM Na_2HPO_4 and 2.5 mM Citric acid, pH 6.4
*Buffer B = 11.6 mM Na_2HPO_4 and 8.4 mM NaH_2PO_4, pH 7.0
*Buffer C= 50 mM Tris and 20 mM Maleic acid, pH7.8
*Buffer D= 15 mM Tris, 5 mM Na_2EDTA, 10 mM $MgCl_2$, pH 7.8
*Buffer I= 25 mM Tris and 192 mM Glycine

Table 3. Alleles scored at eight loci in *Leucostoma*.

Isolate number*	Locus							
	ME	PGI	PGM	IDH	G6PD	MAN	GDH	PGD
	Alleles							
1	a	a	b	b	a	b	b	a
2	a	a	b	b	a	b	b	a
3	a	a	b	a	a	b	b	a
4	a	a	b	a	a	b	b	a
5	a	a	b	a	a	b	b	a
6	a	a	b	a	a	b	b	a
7	a	a	b	a	a	b	b	a
8	a	a	b	a	a	b	b	a
9	a	a	b	a	a	b	b	a
10	a	a	b	a	a	b	b	a
11	b	a	a	b	b	a	a	b
12	b	b	a	b	b	a	a	b
13	b	a	a	b	b	a	a	b
14	b	b	b	a	a	a	a	a
15	b	a	a	b	b	a	a	b
16	b	b	b	b	a	a	a	b
17	b	a	b	b	a	a	a	b
18	b	b	b	a	b	a	a	b
19	b	b	b	b	b	a	a	b
20	b	b	b	b	b	a	a	b
21	b	b	a	a	a	a	a	b
22	b	b	b	b	a	b	b	b
23	b	b	b	b	a	b	b	b
24	a	a	b	b	a	b	b	b
25	c	c	c	c	c	c	c	c
26	c	c	c	c	c	c	c	c
27	c	c	c	c	c	c	c	c
28	c	c	c	c	c	c	c	c
29	c	c	c	c	c	c	c	c
30	c	c	c	c	c	c	c	c
31	c	c	c	c	c	c	c	c
32	c	c	c	c	c	c	c	c
33	d	d	d	c	d	c	c	d
34	d	d	d	c	d	c	c	d
35	d	d	d	c	d	c	c	d
36	d	d	d	c	d	c	c	d
37	d	d	d	c	d	c	c	d
38	b	b	b	b	b	b	b	b
39	c	c	c	c	c	c	c	c
40	c	c	c	c	c	c	c	c
41	c	c	c	c	c	c	c	c

Table 3. contd.

42	c	c	c	c	c	c	c	c
43	d	d	d	c	d	c	c	d
44	d	d	d	c	d	c	c	d
45	e	e	c	c	c	c	c	c
46	c	c	c	c	c	c	c	c
47	e	e	c	c	c	c	c	c
48	c	c	c	c	c	c	c	c
49	c	c	c	c	c	c	c	c
50	c	c	c	c	c	c	c	c
51	d	d	d	c	d	c	c	d
52	c	c	c	c	c	c	c	c
53	c	c	c	c	c	c	c	c
54	d	d	d	c	d	c	c	d
55	c	c	c	c	c	c	c	c
56	c	c	c	c	c	c	c	c

*Isolate number corresponds to that on the phenogram in Fig. 1

Table 4. Summary of genotypic diversities for species and subgroups in *Leucostoma*.

Species/ subgroup	Number of isolates	Number of loci	Number of ETs	Genotypic diversity
L. cincta	25	8	10	0.84
L. cincta on *Malus*	10	8	2	0.28
L. cincta on *Prunus*	15	8	8	0.66
L. persoonii	31	8	3	0.50

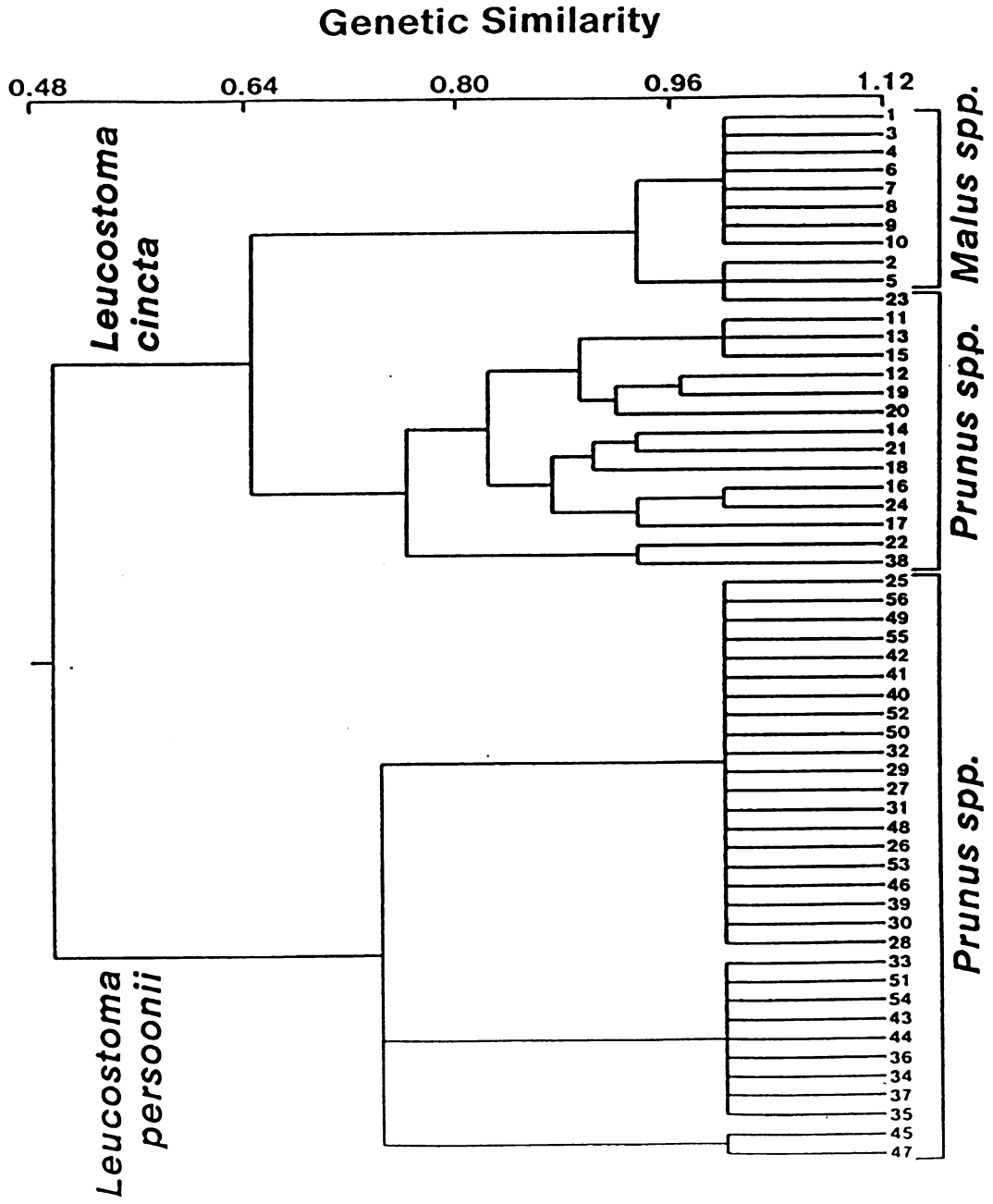

Fig. 1. Phenogram based on isozyme analysis showing the groupings of isolates of *L. cincta* and *L. persoonii*. The phenogram was constructed with the NTSYS program using the unweighted pair-group method with arithmetic averaging (UPGMA) from Simple Matching Coefficient values. The numbers in parentheses correspond to isolate numbers (Table 1).

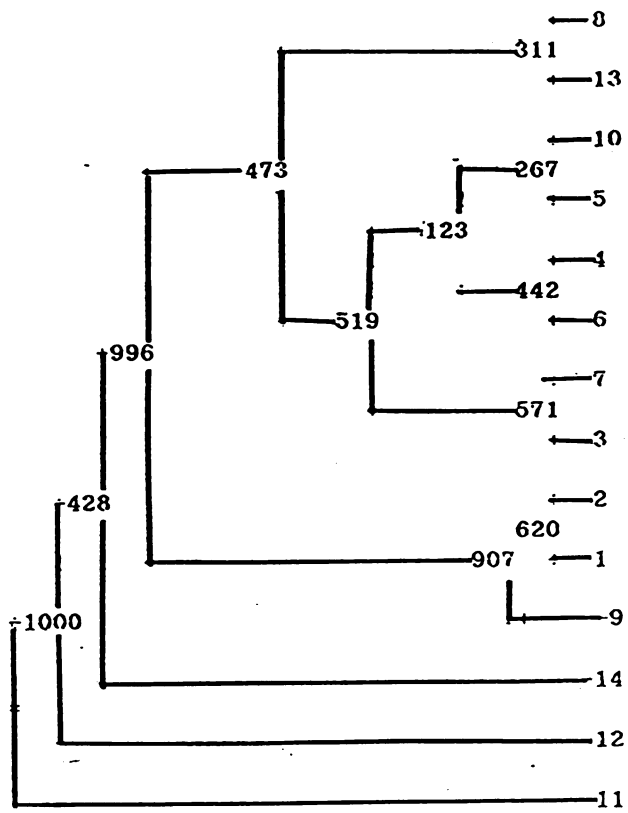

Fig. 2 Most parsimonious tree generated using the BOOT program of PHYLIP. The numbers on the branches correspond to the confidence limit on each branch. Those on the end of the tree correspond to the isolate numbers in Table 1.

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN THE NUCLEAR RIBOSOMAL DNA OF *LEUCOSTOMA CINCTA* AND *L. PERSOONII*

RESTRICTION FRAGMENT LENGTH POLYMOPTHISMS IN THE NUCLEAR RIBOSOMAL DNA OF *LEUCOSTOMA CINCTA* AND *L. PERSOONII*.

ABSTRACT

Genetic variation within and between groups of two *Leucostoma* species was examined by analysis of restriction fragment length polymorphisms (RFLPs) of nuclear ribosomal DNA (rDNA). The small nuclear rDNA and the internal transcribed spacers plus a portion of the adjacent large rDNA (ITS-LrDNA, 1600 base pairs) were separately amplified by polymerase chain reaction (PCR). rDNA pattern variation appeared in digests with 10 of 40 restriction enzymes. Phenetic analysis revealed seven groups among various isolates of *L. persoonii*. Several ITS-LrDNA groups corresponded to those previously detected in isozyme studies and others corresponded to host and/or geographic distributions. Less variation in ITS-LrDNA was evident in *L. cincta* groups in contrast to isozyme analysis. Most of the RFLP variation in the ITS-LrDNA was within the internal transcribed spacer rather than in the large rDNA. PCR amplification of the nuclear small rDNA revealed an insertion in *L. cincta* isolates from *Prunus* spp. that is absent both in *L. cincta* isolates from *Malus* and in *L. persoonii* isolates.

Leucostoma persoonii Hoehn. and *Leucostoma cincta* (Fr.: Fr.) Hoehn. are the pathogens that cause Cytospora canker on cultivated peach, nectarine, apricot, plum, prune, sweet and sour cherry, native black cherry, chokecherry, amelanchier and ornamental quince. The most destructive diseases are those on peach, sweet cherry and plum where the canker is perennial. Taxonomy of the two closely related pathogens has been complicated by the lack of availability of species specific characteristics (Kern, 1955), particularly the frequent absence of the sexual state. Kern's (1955), criteria in literature for identifying the two taxa in the absence of the sexual state have been perceived as inadequate or create confusion judging from frequent current use of incorrect species epithets (Surve-Iyer, 1992). Misidentification and a lack of understanding of genetic diversity in the taxa present a cogent problem to breeding programs screening for canker resistance in perennial fruit trees (Adams, Hammer, Iezzoni, 1989). Although identification of a fungal taxon is based on observable differences in morphology, biological species and populations may be genetically distinct even when they are not morphologically separable. Recently Surve-Iyer (1992) used isozyme analysis to separate the two *Leucostoma* species at eight

polymorphic loci. In addition to the separation of these two species, isozyme polymorphisms revealed variation within the two species. Three phenetic groups were detected within *L. persoonii* and three closely related groups were found within *L. cincta*. Molecular genetic techniques such as restriction fragment length polymorphisms (RFLPs) can identify genetic variability between and within fungal species. RFLP analysis of a region of DNA sequence that evolves at a tempo roughly corresponding to species divergence could reveal whether isozyme phenetic groups correspond to populations or to biological species.

RFLPs have been useful genetic markers in organisms such as humans (Botstein *et al*, 1980), crop plants (Helentjaris *et al*), and animals (Hillis & Davis, 1986). Such polymorphisms can be generated by the loss or gain of restriction endonuclease sites by point mutations or by rearrangement of DNA sequences. In fungi, RFLPs have been used both to resolve taxonomic groups (Kohn *et al*, 1988; Cubeta *et al*, 1991) and in population genetic studies (Hulbert *et al*, 1985; McDonald and Martinez, 1990; Michelmore & Hulbert,1987). The ribosomal repeat unit (rDNA) has been used extensively for restriction enzyme studies in fungi.

Polymorphisms in fungal rDNAs have been used to demonstrate both species and strain specific differences (Kohn *et al*, 1988; Specht, Novotony & Ullrich, 1984). In most eukaryotes, the nuclear rDNA exists as a tandemly repeated array of three rRNA genes (18S, 5.8S and 28S) separated by internal transcribed (ITS) and non transcribed spacers (IGS). The various rRNAs and their respective DNA coding regions are known for their value as evolutionary markers, since they contain regions of both high and low sequence variability (Apples & Dvorak, 1982). Among fungi, analysis of this region has been applied to numerous genera including *Neurospora* (Verma & Dutta, 1987), *Candida* (Magee, D'Souza & Magee, 1987), *Schizophyllum* (Spect, Novotony & Ullrich, 1984), *Coprinus* (Wu, Cassidy & Pukkila, 1983), *Armillaria* (Anderson, Petsche & Smith, 1987) and *Lentinus* (Hibbet & Vilgalys, 1991).

With the advent of enzymatic amplification, copies of selected regions of the rDNA repeat unit can be selectively amplified as non methylated copies from crude DNA preparations, greatly simplifying RFLP analysis of populations (Bruns, White & Taylor, 1991). This technique has been used by Vilgalys and Hester (1990) for genetic identification and mapping of *Cryptococcus* species and by Hibbet and Vilgalys (1991)

to study evolutionary relationship of *Lentinus* to the Polyporaceae.

In studies presented here we examined variation in portions of the nuclear rDNA repeat of isolates of *L. persoonii* and *L. cincta* by analysis of the restriction endonuclease fragment patterns. The small nuclear rDNA (18s), and the internal transcribed spacers plus a portion of the adjacent large rDNA (28S), were enzymatically amplified using polymerase chain reaction (PCR) and universal primers for fungal rDNA (White, Arnheim & Erlich, 1989; White *et al*, 1990). Forty restriction enzymes were used to study the rDNA variation between and within the two species. The data was analyzed by phenetic methods and the results are diagrammed in an unrooted tree. The results were compared to those obtained by isozyme analysis and the various groupings by the two methods are discussed in this paper.

MATERIALS AND METHODS

DNA preparation

The fungal isolates used in this study are listed in Table 6. *Formae speciales* cultures of *L. persoonii* obtained from Centraalbureau voor Schimmecultures,

Netherlands (CBS) were also used in this study. The fungi were grown for 10-12 days in 100 ml of Leonian's medium (1.2g KH_2PO_4, 0.6g $MgSo_4$, 6.25g maltose, 6.25g malt extract in 1 L of distilled water). Mycelia were harvested by vacuum filtration through Miracloth (Calbiochem Corp, San Diego, U. S. A), lyophilized and stored at -20^{o}C until the DNA was extracted. DNA was extracted by the method of Lee & Taylor, (1990). Lyophilized mycelium was ground in a mortar and pestle in 750 ul of lysis buffer and incubated at 60^{o} C for 20 minutes. 700 ul of phenol:chloroform (1:1) was added followed by microcentrifugation for 15 minutes. 700 ul of SEVAG was added to the top aqueous phase, the mixture was centrifuged for 10-15 minutes. The aqueous phase was removed and 10ul of 3M NaoAC pH 8 and 0.54 (600 ul) volume of isopropanol was added. DNA ropes were seen precipitating at this stage. The DNA was washed with 1.5 ml of 70% ethanol and the pellet was allowed to dry in a vacuum oven at 50^{o}C for 15 minutes. The extracted DNA was stored in TE buffer at 4^{o}C.

Enzymatic amplification of DNA with the use of PCR

The rRNA coding regions were amplified from genomic DNA by use of the polymerase chain reaction with oligonucleotides complementary to 5′ and 3′ end of the

nuclear coding region (White *et al.*, 1990, Fig. 4). The PCR reactions were set up with Amplitaq DNA polymerase (U.S Biochemicals) in either 50- or 25-ul volumes (White *et al.*, 1990) using buffer conditions recommended by the manufacturer. The following primer pairs were used, ITS5/ TW14 (ITS5: ggaagtaaaagtcgtaacaagg, TW14: gctatcctgagggaaacttc) Ctb6/TW13 (Ctb6: gcatatcaataagcggagg, TW13: ggtccgtgtttcaagacg), Ctb6/TW14 and Ctw13/TW14 (Ctw13: cgtcttgaaacacggacc) (Fig. 4). Restriction patterns of fragments amplified by primers ITS5/TW14 were compared while the remaining primer pairs were used to locate restriction sites or length mutations. For each amplification, one negative control (excluding the DNA template) was used and all the amplification reactions were performed with a positive displacement pipetmen in a PCR-product-free room. Thirty PCR cycles were performed on an automated thermocycler device (Perkin-Elmer-Cetus), with the following parameters, 94^{o}C denaturation (1 min), 48^{o}C annealing (1 min), 72^{o}C extension (45 sec + 4 sec/cycle) and with a final extension at 72^{o}C for 10 minutes. The PCR products were checked by running 3-4 ul of each reaction mixture on 3% agarose minigels.

Previously, a large insert was discovered in the nuclear small rDNA of *L. cincta* isolate LP59 located

between primers NS21/NS22 (NS21: gaataatagaataggacg, NS22: aattaagcagacaaactc) that was absent in *L. persoonii* isolate LP8 (Mary Berbee, personal communication). A portion of the nuclear small rDNA between the primer pair NS21/NS22 was amplified to examine the presence of an insert in the isolates of *L. cincta* and *L. persoonii*.

Restriction analysis of PCR products

After removal of the mineral oil overlay from the PCR reactions, the amplified products were directly used for restriction analysis. 5 ul of the PCR product was digested in a 20 ul volume containing 13 ul of H_2O, 2 ul of the 10X buffer and 0.1 ul of the restriction enzyme under incubation conditions supplied by the manufacturer. Forty restriction enzymes (Boehringer Mannheim, U.S.A, New England Biochemicals, U. S. A & Strategene, U. S. A) were used in this study (Table 7). The products of the restriction reactions were separated by electrophoresis in 1%+2% agarose gels in TAE, pH 8.1 (1% Nuseive +2% Seakem, FMC Bioproducts, U.S.A) using 1Kb lambda and 123bp ladders as molecular weight standards (Boehringer Mannheim) and non-digested controls. After staining with ethidium bromide, the gels were photographed over an UV transilluminator.

Nine enzymes (AluI, RsaI, MboI, TaqI, BSTNI, MseI, BSTUI, HpaII, and ScaI) were further used to study the intraspecific variation within *L. persoonii*. To identify the areas of variability in the region of ITS-LrDNA, parts of the 28S rDNA were also amplified.

Data analysis

Digested rDNAs were run side by side on agarose gels and the restriction patterns were compared. Fragments which migrated the same distance during agarose gel electrophoresis as compared to the lambda ladders were considered to be fragments in common. The digested DNA fragments were compared with each other on the gels, their position and molecular weight recorded. To preclude errors in estimates of genetic divergence fragment patterns for each enzyme were coded as different allelic forms of a given locus (Bruns, White and Taylor, 1991). The data marix was entered into a computer file of the Numerical Taxonomy Multivariate System program, version 1.7 (Rohlf, 1987) and the discrete data were converted to Simple Matching coefficient using the SIMQUAL program. The coefficient emphasizes both the positive and negative matches Cluster analysis was performed using the program SAHN by unweighted pair group method using arithmetic averages

(UPGMA). For preliminary studies, one isolate from each phenetic group separated by isozyme analysis (Surve-Iyer, 1992) was used for restriction digestion. Similarities were deduced from the pairwise comparison of isolates by using their ITS-LrDNA profiles.

To detect length mutations, the following procedures were performed: 1. For each gel a standard graph was constructed by plotting the molecular weights of the lambda ladders against the distance of migration of the fragments. For every restriction pattern the molecular weight of each fragment was calculated from these standard graphs and were compared with each other. 2. 8% nondenaturing polyacrylamide gels (12 x 16 cm) were run loaded with uncut PCR amplified fragments between primer pairs ITS1/ITS4 & Ctb6/TW14 to obtain relatively precise length comparisons.

RESULTS

PCR amplification of the nuclear small rDNA between NS21/NS22 revealed the presence of the insert in all of the 16 isolates of *L. cincta* from *Prunus* spp. (Fig. 6) (isolate LP66, 65P3, LP49, LP59, LP62, LP47, 2P3, BS5, ATCC72910, F1h, T33.6, 5p4, 4p1, LP40, BS1, 1P1). The insert was absent in all of the 13 isolates of *L. cincta* from *Malus* (isolate A15, A32, A9, A70, A8, A52, A48, A2,

A77, A45, LP39, BS3, BS2) and did not occur in any isolate of *L. persoonii* (Fig. 6). The nuclear small rDNA of *L. cincta* from *Malus* and *L. persoonii* were similar in size.

Among the 40 restriction enzymes used, 19 recognized sites in the ITS-LrDNA and 10 enzymes showed polymorphisms. The restriction profile of only one enzyme, Hinf I differentiated the isolates of *L. cincta* and *L. persoonii* in ITS-LrDNA region. A 268bp band which was detected in all the isolates of *L. persoonii* was absent in the isolates of *L. cincta* (Fig. 5). Low variability was seen between the two taxa, but a high level of variability was detected among the isolates of *L. persoonii* in the region of ITS-LrDNA. Restriction length polymorphism was detected with nine restriction enzymes (AluI, RsaI, MboI, TaqI, BSTNI, MseI, BSTUI, HpaII, and ScaI). For each of these restriction enzymes, two or three patterns of restriction profiles were observed. These patterns were also shared by isolates of *L. cincta* from both *Malus* and *Prunus* and the *formae speciales* cultures of *L. persoonii* from CBS.

The phenogram generated by the SIMQUAL program using Simple Matching coefficient (Fig. 3) summarizes the relationship between the isolates of *L. persoonii*. *L.*

persoonii was separated into seven clusters. The first cluster consisted of two isolates (LP6 and 11.3) from peach. Isolates on cherry formed a second cluster, three of these (LP8, LP9, and LP10) were on black cherry, one (LP13) was on sour cherry (all the four isolates were from Michigan) and one (CHR) from Oregon near the Hood river. Five isolates on peach (T18.1, T26.1, T4.7, T16.1, T7.4) collected from a single orchard in Michigan and one isolate (LCN) on nectarine from California clustered together. Two isolates, one from Michigan (LP21) and one from North Carolina (NC9.7) were clustered together. The fifth cluster consisted of two isolates, one on prune (LP2) and the other on peach (NC9.7). Two North Carolina isolates on peach (NC14.1 and NC8.2) formed a sixth cluster. Two California isolates (R3T16 and R5T10) from plum were grouped together to form the seventh cluster.

Length mutations appeared to be evident when the restriction patterns were compared by standard graphs. However, comparison of the restriction profiles of ITS-LrDNA and portions of the 28SrDNA did not reveal difference in size prior to digestion. Comparison of restriction profiles between ITS4/ITS5 and Ctb6/Tw14 showed maximum variability in the region between primers ITS5 and ITS4 (Fig. 4)

DISCUSSION

Analysis of the restriction patterns of two separate regions of the rDNA revealed information on the relationship between the isolates of *Leucostoma*. We were able to detect enzymes that showed restriction length polymorphisms by using 40 restriction enzymes with four to eight base recognition sequences.

Based on RFLPs, the isolates of *L. persoonii* appear to be composed of several genetically heterogeneous groups. Seven clusters were observed, four clusters were separated on the basis of host adaptation, one cluster consisted of isolates from a single orchard in Michigan and isolates from cherry and nectarine. The remaining two clusters were separated by geographical location. Previous studies (Surve-Iyer, 1992) using isozyme analysis have shown the separation of *L. persoonii* into three groups. These three groups did not reveal significant variation within themselves. One of these groups was widespread, a second group consisted of specimens isolated from one peach orchard in Michigan and one canker isolation on black cherry, and a third group consisted of two specimens on nectarine from California.

RFLPs have revealed greater intraspecific genetic variation than isozyme analysis, perhaps this may be due to due to genetic divergence among *L. persoonii* caused by geographhical isolation. Intraspecific variation as seen here in the groups of *L. persoonii* has also been observed using *Drosophila* (Williams *et al*, 1987). However in similar studies of rDNA variation in fungi less intraspecific variability was detected (Anderson, Petsche & Smith, 1987; Kohn *et al.*, 1988; Braithwaite, Iwrin & Manners, 1990; Egger, Danielson & Fortin, 1991). Sequence analyis of the nuclear encoded rDNA has also shown higher interspecific than intraspecific variability in *Laccaria* (Gardes *et al.*, 1990) and *Gibberella* spp. (Peterson and Logrieco, 1991). Recently, there has been one report by Vilgalys & Gonzalez (1991) in which high variability was detected in the nuclear encoded rDNA repeat unit of *Rhizoctonia solani* both among and within the different anastomosis groups. McDonald and Martinez (1990) have reported a high level of genetic variation in the local population of *Septoria tritici* using restriction analysis of the nuclear DNA. This form of variation within the species may provide an important method of investigating the identification, origin and spread of certain isolates.

Isozyme analysis (Surve-Iyer, 1992) has proven to be more informative with respect to the separation of the two taxa of *Leucostoma* than RFLP analysis. Isozyme analysis separated the two species *L. cincta* and *L. persoonii* at each and every loci tested. RFLPs of the rDNA did not show distinct separation of *L. cincta* on *Malus* (PG6) from the isolates of *L. persoonii*. *L. cincta* on *Malus* is likely to represent a genetically isolated biological species in the *L. cincta* species complex. It differs in isozyme loci and morphological markers and the lack the insertion in the nuclear small rDNA (Surve-Iyer, 1992; Proffer & Jones, 1989). PG6 was virulent on 3-yr-old inoculated peach seedlings (Surve-Iyer, 1992), however, in nature PG6 is absent on *Prunus* spp. This ecological host specialization of PG6 may have led to the genetic isolation of *L. cincta* on *Malus* and *L. cincta* on *Prunus* spp.

This study revealed that an insert in the 18S region of the rDNA was present in all specimens of *L. cincta* from *Prunus* but absent in *L. cincta* on *Malus* and *L. persoonii*. Inserts in the nuclear small rDNA have not been detected in plants or animals, however, such inserts have been noted in lichens (De Priest and A. Gargas, personal communications) and Chytrids (B. Bowman, personal communication). Furthermore, the PCR

amplification of isolates of *L. cincta* containing this insert often showed two amplified products rather than one. This phenomenon apparently occurs commonly during amplification of DNA containing large inserts (Bruns, personal communication). The collections of *L. cincta* are limited (inserts have been detected in 16 isolates and absent in 13 isolates) and examination of more widespread collections are needed to confirm that the insert is unique and indicative of *L. cincta* on *Prunus*.

Calculations of the molecular weights of digest fragments indicated that length mutations were present in the ITS-LrDNA regions. However comparing smaller uncut PCR amplified products separately for the ITS (ITS5/ITS4) and the LrDNA (Ctb6/Tw14) did not reveal length mutations. Quantitative analysis of RFLP pattern comparison can be prone to error if length mutations are present. This can cause overestimations of divergence when multiple enzymes are used. We believe that the variation we have detected in the ITS-LrDNA regions is due to site changes rather than length mutations. Further support for our argument is that of the forty restriction enzymes used, we detected differences with ten enzymes, but nine other enzymes that cut the ITS-LrDNA (ClaI, AvaI, DdeI, HaeI, NciI, SstI, MnI, EcoRI

and Fnu4H) did not reveal variation among diverse isolates. However there is still a possibility that we may have not detected minor length mutations due to comigration of nonhomologous fragments of identical molecular weights.

This study has been useful in identifying variation within *L. persoonii*. In a study on *Rana* (Hillis and Davis, 1986), allozyme data provided extensive information on the relationship between closely related groups in a species complex. However their RFLP analysis identified several unresolved or poorly resolved portions of the phylogeny at the intergroup level much like our studies of *Leucostoma*. It may be early to assign evolutionary relationship in *Leucostoma* based solely on RFLP banding patterns, however this study might be phylogenetically informative when the molecular basis of the variation is known. Detailed sequence data would be useful in understanding the variation in this region. If the variability detected in the groups of *L. persoonii* precisely reflects the genetic variability present in this species, than this variation should be taken into consideration when screening for resistant peach cultivars. Comparison of levels of variation from isozyme marker and RFLP markers to levels of variation in the mitochondrial DNA

and the rDNA intergenic spacer region combined with anastomosis grouping might provide a basis for detailed population genetic and epidemiological studies of the two taxa that would benefit in selecting resistance to perennial canker in peach varieties.

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Table 5. *Leucostoma* isolates used in the RFLP studies.

Species & Isolate	Origin	Host
L. persoonii		
LP6	Michigan	*Prunus persica*
11.3	Michigan	*Prunus persica*
LP10	Michigan	*Prunus serotina*
LP9	Micigan	*Prunus serotina*
LP13	Michigan	*Prunus serotina*
LP8	Michigan	*Prunus serotina*
CHR	Oregon	*Prunus avium*
Cy5	Oregon	*Prunus avium*
T18.1	Michigan	*Prunus persica*
T26.1	Michigan	*Prunus persica*
T4.7	Michigan	*Prunus persica*
LCN	California	*Prunus persica* var. nucercisa
T7.4	Michigan	*Prunus persica*
T16.1	Michigan	*Prunus persica*
LP21	Michigan	*Prunus domestica*
NC9.7	North Carolina	*Prunus persica*
LP2	Michigan	*Prunus domestica*
NC17	North Carolina	*Prunus persica*
NC14.1	North Carolina	*Prunus persica*
NC8.2	North Carolina	*Prunus persica*
R3T16	California	*Prunus domestica*
R3T10	California	*Prunus domestica*
f.sp mahaleb	Switzerland	*Prunus persica*
L. cincta		
LP66	Michigan	*Prunus domestica*
ATCC 62910	Ontario	*Prunus persica*
5P4	Michigan	*Prunus domestica*
A48	Michigan	*Malus domestica*
A45	Michigan	*Malus domestica*
A9	Michigan	*Malus domestica*

Table 6. Restriction enzymes used in the digestion of PCR products of the ITS-LrDNA* fragment in *L. cincta* and *L. persoonii*

AluI	ClaI	HinfI	PvuII
AvaI	EaeI	HpaII	RmaI
AvaII	DdeI	HpaII	RsaI
BamHI	EcoNI	KpnI	SalI
BclI	EcoRI	MboI	ScaI
BglII	EcoRV	MnI	SstI
BglIII	Fnu4H	MluI	StuI
BstEII	HaeIII	MseI	TaqI
BstNI	HhaI	NruI	XbaI
BstUI	HindIII	PstI	XhoI

*ITS-LrDNA= ITS1, ITS2, 5.8SrDNA and portion of the 28SrDNA

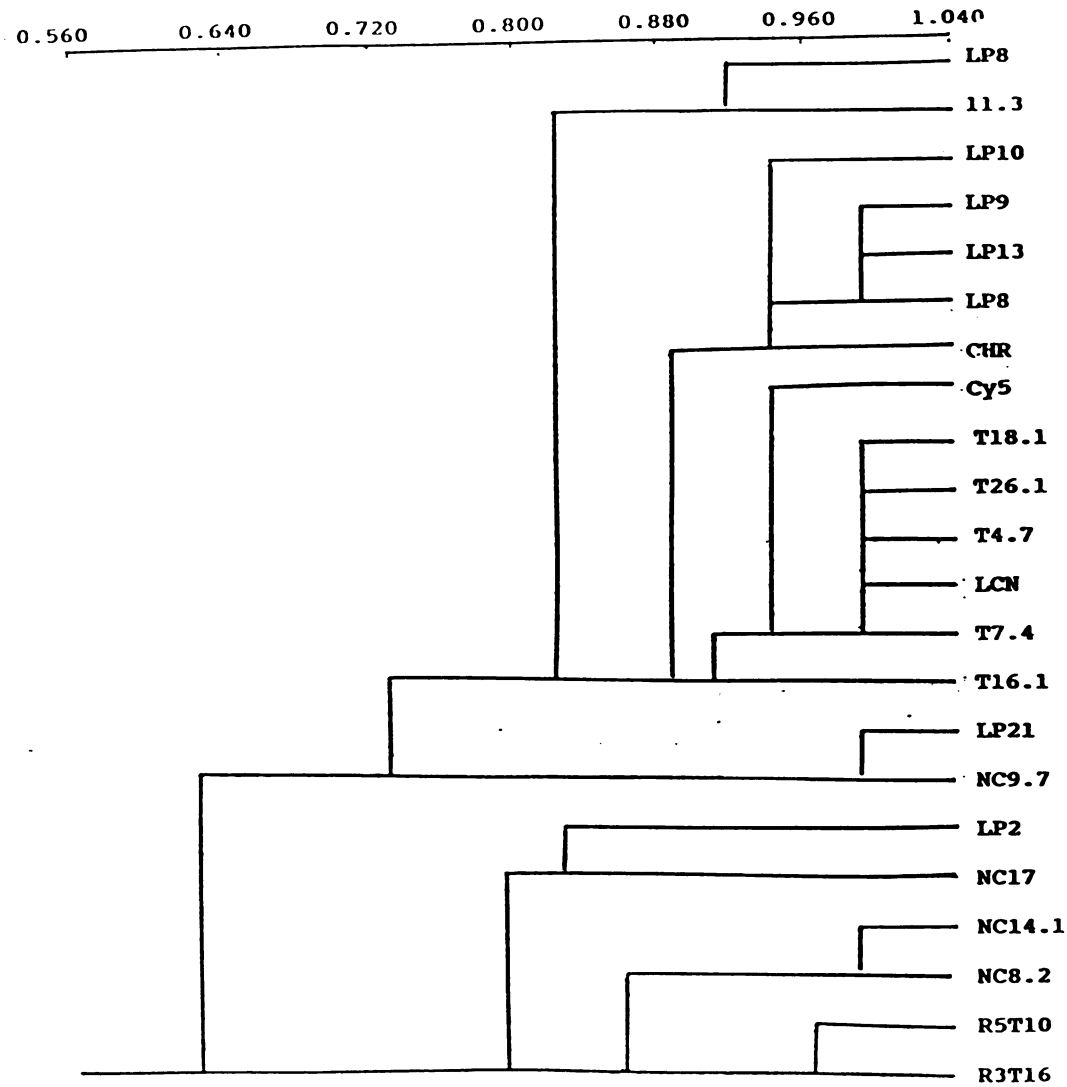

Fig. 3 Phenogram of *Leucostoma persoonii* isolates based on the UPGMA cluster analysis of simple matching coefficient. Similarity generated from restriction fragment length polymorphisms in the portion of the nuclear rDNA containing ITS1, ITS2, 5.8S rDNA and part of the 28S rDNA.

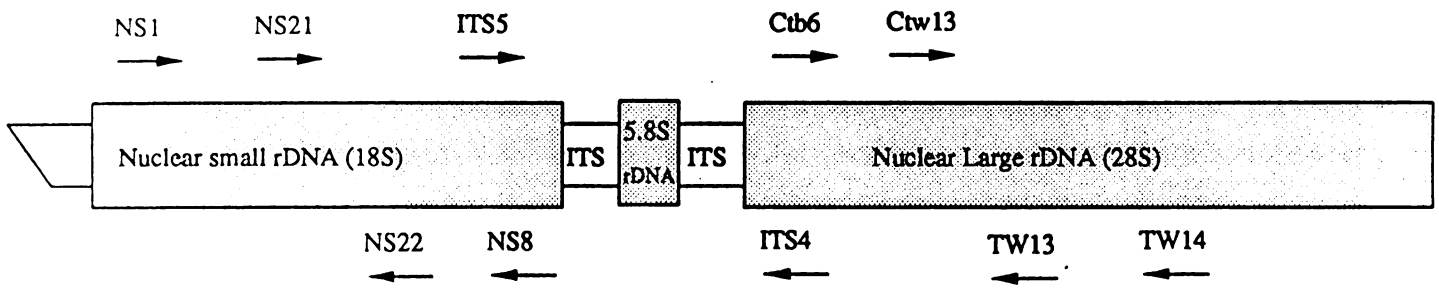

Fig. 4 Map of a portion of rDNA repeat showing the location of oligonucleotide primer sites used to amplify rDNA from *Leucostoma* spp.

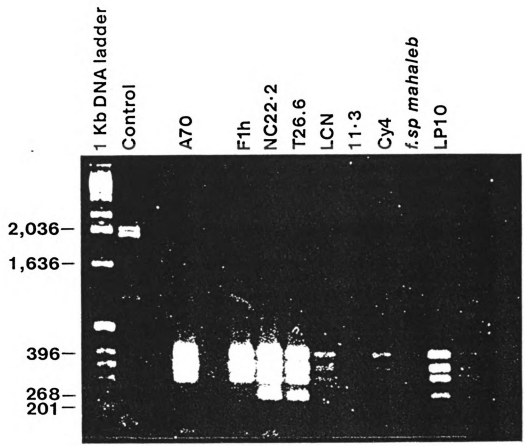

Fig. 5 Restriction profiles of PCR product of the fragment containing the two ITS regions, 5.8S rDNA and a portion of 28S rDNA after digestion with Hinf I. A70 & F1h are isolates of *L. cincta* and NC22.2, T26.6 LCN, 11.3, Cy4, *f. sp. mahaleb* and LP10 are isolates of *L. persoonii*. Primers used: ITS5/TW14.

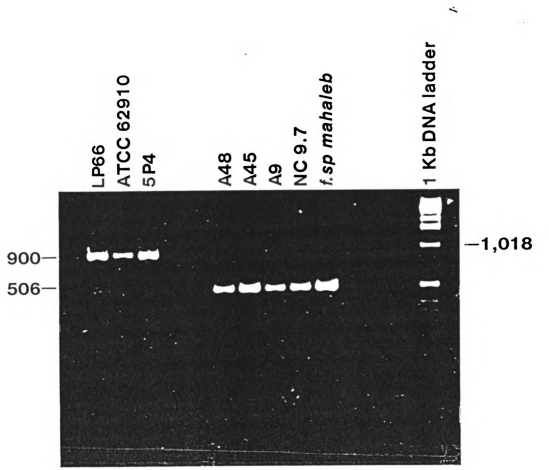

Fig. 6 PCR products from enzymatic amplification of nuclear small rDNA using primers NS21/NS22. An insertion of about 400bp present in the isolates of *L. cincta* on *Prunus* (LP66, ATCC 62910, & 5P4) is missing in isolates of *L. cincta* on *Malus* (A48,A45,A9) & *L. persoonii* (NC9.7, f.sp. *mahaleb*)

COMPARISON OF FIVE PHENETIC GROUPS OF *LEUCOSTOMA* USING MORPHOLOGY AND VIRULENCE ON PEACH

Comparison of Five Phenetic Groups of *Leucostoma* Using Morphology and Virulence on Peach.

ABSTRACT

Phenetic groups separated by isozyme analysis, two groups of *Leucostoma persoonii* (PG1 and PG2) and three of *L. cincta* (PG4, PG5 and PG6) were compared using pycnidium size, color, relative growth at 27 C and 33 C, and virulence markers. Five representative isolates from each group were used and each group was assigned a standard isolate. Each group was inoculated on 3-yr-old multistemmed peach (*Prunus persica*) seedling trees with replications on eight trees for a total of 25 isolates on 40 trees. All five groups were virulent on inoculated peach including PG6, a *L. cincta* group found on *Malus* in Michigan. Isolates of *L. persoonii* in PG1 were the most virulent while isolates of *L. cincta* in PG6 were the least virulent. *L. cincta* isolates in PG3 and PG4 were more virulent than *L. persoonii* isolates in PG2. The color of *L. persoonii* isolates in culture ranged from black and dark olivaceous to dark mouse grey whereas those of *L. cincta* ranged from vinaceous buff to honey. Phenetic group five *L. cincta* was unique by it's reddish brown color in culture. Isolates of PG1 had restricted colony growth and lobate colony margins which

clearly distinguished this group from others. Pycnidia of *L. cincta* were larger in size than those of *L. persoonii*, regardless of the phenetic grouping. Isolates of all five phenetic groups showed growth at 27 C, but only isolates of phenetic groups PG1 and PG2 of *L. persoonii* showed growth at 33 C.

Perennial or Cytospora canker is a major limiting factor in peach production in the northern range of it's cultivation (Biggs, 1986; Dhanvantari, 1978; Hildebrand, 1947). Cytospora canker also affects plum and prune (*P. domestica* L.), sweet and sour cherry (*P. avium* L. and *P. cerasus* L., respectively), apricot (*P. armeniaca* L.), black cherry (*P. serotina* Ehrh.) and apple (*Malus domestica* Borkh.) among other mainly roseaceous hosts (Helton & Moisey, 1955; Hildebrand, 1947;). The disease is characterized by extensive perennial cankers on trunks, scaffold limbs and branches (Hampson & Sinclar, 1973; Tekauz & Patrick, 1984). Common infection sites include pruning wounds, leaf scars, and winter injury (Biggs, 1989). Willison, 1937 reported that approximately one third of all new infections were associated with pruning wounds. Winter injury which results in dead and dying tissue predisposes the tree to *Leucostoma* invasion (Bertrand & English). *Leucostoma*

infected trees are commonly observed to be more susceptible to winter injury than healthy trees (Dhanvantarî, 1978; Helton & Konicek, 1961). The disease is caused by two closely related fungi, *Leucostoma cincta* (Pers. : Fr.) Hoehn. [anamorph = *Leucocytospora cincta* (Sacc.) Hoehn.] and *L. persoonii* (Nits.) Hoehn. [anamorph = *L. leucostoma* (Pers.) Hoehn.]. The morphological differences between the two species as they occur in nature are often small, indistinct or overlap greatly (Kern, 1955). Because of this the two species generally have been separated by plant pathologists on the basis of cultural characters following the criteria defined by Willison, 1937. According to Willison (1937) and Kastirr (1984) the two species can be separated on the basis of color, pycnidial size and colony margin in culture. Hildebrand (1947) reported that the two species had different tolerance to growth at high temperature, for *L. cincta* growth maxima was 33 C and for *L. persoonii*, 37 C . Due to significant variation seen in cultural characters of the two taxa, the validity of using these to distinguish the two is questioned by many authors (Adams, Hammar & Proffer, 1989; Kern, 1955). Lukezic *et al* (1981), reported that monoascospore isolates from a single ascus

of *L. persoonii* showed a range of cultural characteristics putatively typical to both species. Currently, plant pathologists in the United States appear to be incorrectly assigning the name *L. cincta* to most virulent *Cytospora* isolates from stone fruit trees (Endert- Kirkpatrick, 1987; Regner & Johnson, 1990 & Spotts *et al.*, 1990).

The relative virulence of isolates of *L. cincta* and *L. persoonii* have been reported to differ greatly. *L. persoonii* has been reported to be of low virulence as compared to *L. cincta* on peach (Helton & Moisey, 1955; Willison, 1937). However in other research *L. persoonii* was found to be more virulent in warmer weather and *L. cincta* more virulent in cooler weather (Hildebrand, 1947; Leonian, 1921; Wensley, 1964). Several researchers have evaluated the virulence of isolates on peach trees, generally inoculating few trees but several branches per tree with an isolate (Bertrand & English, 1976; Helton & Konicek, 1926; Lukezic, 1981; Wensley, 1964). In efforts to improve statistical comparisons of virulence of isolates, Adams *et al* (1989) reported that increasing the number of branches inoculated with an isolate per tree had an insignificant effect on reducing the sample variance, while increasing the number of trees inoculated up to six (one branch per tree)

greatly reduced the sample variance. Precise comparisons of the virulence of isolates can have significant consequences in current research on breeding peach trees for disease resistance (Chang *et al.*, 1989; Chang *et al*, 1991). Breeding is an expensive and labor intensive procedure. Without thorough knowledge of the genetic variability in the virulence of the pathogen the release of new resistant germplasm would face the threat of exposure to pre-existing races or biological species of the pathogen that might overcome such a resistance.

Recently, using isozyme analysis, Surve-Iyer (1992) separated isolates of the two *Leucostoma* species into six phenetic groups (PG). PG1 isolates of *L. persoonii* were widespread and occurred on a wide range of *Prunus* spp. PG2 consisted of isolates of *L. persoonii* from Michigan on *P. persica* and *P. serotina*. PG4 and PG5 consisted of isolates of *L. cincta* on species of *Prunus* and PG6 consisted of isolates of *L. cincta* on *Malus*. The sixth phenetic group was of *L. persoonii* isolates from nectarine in California and could not be used in this study in Michigan for inoculations in the field. The aim of this research was to compare cultures of different phenetic groups using morphological markers

such as pycnidium size and color as well as in the biological characteristics of high temperature tolerance and relative virulence on peach. The genetic diversity in virulence among the 25 strains was compared for selection of isolates for screening peach germplasm in an ongoing program on breeding for canker resistance.

MATERIAL AND METHODS

Cultural characteristics and temperature response. Colonies for each isolate were initiated by inverting a 4-mm-diameter mycelial plug onto a petri plate containing Leonian's medium (1921). After 2 weeks of incubation at room temperature under cool-white fluorescent light, the cultural characteristics were compared. Characteristics that were examined included (i) whether the colony margin was lobate with a restricted growth, or uniformly radial, (ii) whether pycnidia were small (< 1 mm) or large (1-3 mm) (Adams, Hammer & Iezzoni, 1988), (iii) the colony color using Rayner's colour chart (Rayner, 1970), and (iv) the ability to grow at 33 C. The experiment was replicated three times.

Virulence tests on 3-yr-old peach seedlings. Twenty five isolates were used in this study, 15 of *L. cincta* and 10 of *L. persoonii*. The host of origin, the isozyme

phenetic group, high temperature tolerance, pycnidial size, colony color, colony margin and mean canker length are listed in Table 8. One isolate from each of the five isozyme phenetic groups was chosen as a standard for the group. Isolates were grown on Leonian's agar for 5 days at 25 C prior to use in inoculation experiments. 3-yr-old peach multistemmed seedling trees (*P. persica* 'Loring' X 'Harrow Beauty' & 'Loring' X 'Polly') were inoculated with isolates from one group along with five standard isolates and a control, one inoculation per branch (stem). A caliper was used as a gauge for choosing portions of branches with uniform diameter of 17 mm and 2-yr-old wood for inoculation. The inoculation site was cleaned with a gauze soaked in 95% ethanol, wounded with an empty hand held stapling gun, and then frozen with a 5-second from a can of aerosol cryogen (100% dichlorofluoromethane, Chemtronics, Inc., Hauppage, NY) (Scorza & Pusey, 1984) at a distance of approximately 15 cm. A 5-mm-diameter mycelial plug was placed on the wounded bark and wrapped with parafilm. A stem on each tree was wounded in an identical manner except a plug of sterile Leonian's agar was substituted for the mycelial plug.

Inoculations were carried out in mid-October of 1989 and on a second set of trees in mid-March of 1990. Branches were removed, bark was shaved off with a potato peeler and canker length was measured on the wood (length of necrotic area distal to inoculation point) in mid-May of 1990. Statistical analysis of data using the computer program MSTAT C (Michigan State University, 1988) included analysis of variance (ANOVA) for the randomized block design with trees as blocks (replicates). Isolates were ranked according to average canker length using LSD at P = 0.05 level. Because no tree could contain all 25 isolates, the experiment was analyzed in the following manner. Firstly, to determine the relative virulence of specific isolates within one phenetic group, the entire experiment was analyzed as five separate sub-experiments. Each sub-experiment consisted of eight replicate trees inoculated with five isolates of one phenetic group, the five standards and a control (10 isolates/tree). Secondly, to determine the relative virulence of the five standards and the control, the entire experiment was analyzed as one experiment of 40 trees inoculated with five isolates (5 isolates plus control per tree). Thirdly, the error terms from the two preceding analyses were used to determine the relative virulence of the standard

isolates and the rest of the isolates of each phenetic group.

RESULTS

Cultural characteristics and temperature response. Colony color of the *L. persoonii* isolates varied from dark mouse grey to dark olivaceous and black. Only colonies of isolates belonging to PG1 of *L. persoonii* exhibited lobate margins. The color of *L. cincta* isolates ranged from hazel to vinaceous buff (Table 8). Isolates of *L. cincta* from apple, PG6 were unique in producing reddish brown hyphae in the culture medium not seen in other *Leucostoma* isolates. Pycnidial size ranged from 1-2 mm for isolates of *L. cincta* and 0.5 -1 mm for isolates of *L. persoonii*. The pycnidia of PG2 were smaller than the pycnidia of PG1. Isolates of *L. persoonii* PG1 and PG2 but not of *L. cincta* PG4, PG5 and PG6 were able to grow at 33 C, The growth of isolates of *L. persoonii* PG1 and PG2 was slower at 33 C than at 27 C. Isolates of all phenetic groups showed good growth at 27 C also shared the cultural and temperature characteristics of the phenetic groups reported here. Isolates used elsewhere in isozyme studies (Surve-Iyer, 1992). However, occasionally isolates of PG1 will not

have a lobate colony margin and occasionally isolates of PG2 will not have small pycnidia.

Virulence on 3-yr-old peach seedlings. Inoculations of trees in mid-October resulted in cankers formation except the control. Cankers were evident as extensive discoloration occurring in patterns of apparently diurnal lines under the bark. The mean canker length varied from 4.9-11.5 cm (Table 8). All the isolates used in this study were virulent on the peach seedlings including isolates of *L. cincta* that originated from apple. Significant difference in tree susceptibility was observed when the five standard isolates were analyzed as one experiment of 40 trees and in the eight tree test of isolates of PG2. Analysis of variance (ANOVA) tests with each of the five phenetic groups showed a significant difference in virulence among and within these five phenetic groups (Table 9-10). In particular the two phenetic groups of *L. persoonii* differed in mean virulence. Isolates of PG1 from various species of *Prunus* were significantly more virulent than PG2 strains which were isolated from peach. Within *L. cincta*, PG4 and PG5 from *Prunus* spp. were moderate in virulence and relatively similar in virulence but PG6 from apple was the least virulent among the five phenetic groups. Within each phenetic

group the isolates showed significant difference in virulence. In the entire experiment the most virulent isolate was T18.1 (PG2) and least virulent A43 (PG6). The species of *L. cincta* and *L. persoonii* and *L. cincta* were not more or less virulent than one another. *L. persoonii* PG1 and *L. cincta* PG4 were almost similar in virulence, likewise *L. persoonii* PG2 and *L. cincta* PG5. Significant differences in tree susceptibility were evident in some but not all of the replicated experiments. The presence of cankers was sporadic in the trees, inoculated in mid-March so this data could not be analyzed.

DISCUSSION

Significant differences in morphology and virulence among the phenetic groups of the two closely related taxa of *Leucostoma* were evident. Comparison of *L. cincta* and *L. persoonii* for color, agreed with previous studies by Willison (1937). Also, *L. cincta* PG6 was readily distinguished by the reddish brown colorin culture previously described by Proffer and Jones (1989). The lack of high temperature tolerance for *L. cincta* isolates was also similar to previous reports by Hildebrand (1947) and Togashi (1930) but not to that of

Helton & Konicek (1961). The lobate margin reported to be characteristic of *L. persoonii* isolates (Kastirr & Ehrig, 1984; Willison 1937) was observed only in PG1 of *L. persoonii*, but colony color and high temperature clearly distinguished other *L. persoonii* from *L. cincta*. Phenetic groups PG1, PG4, PG5 and PG6 include many isolates originating from identifiable sexual states and these isolates in culture were readily identified to species using the cultural characteristics described by Willison (1937) and Hildebrand (1947). Many isolates belonging to *L. persoonii* PG1 or PG2 were previously identified as *L. cincta* (Adams *et al*, 1990; Endert-Kirkpatrick, 1986; Regner & Johnson 1990; Spotts *et al*. 1990). This has been a problem particularly in studies when the two culturally distinct *L. persoonii* (PG1 and PG2) occurred in an orchard. We believe that with practice one can readily recognize the two taxa in culture using the cultural characteristics discussed here. Many isolates of PG1 can be distinguished readily from other *L. persoonii*, and all PG6 from other *L. cincta*, however PG2 and PG3 are not visually distinguishable nor are PG4 and PG5.

Although little variation was evident in isozyme banding patterns within each phenetic group (Surve-Iyer, 1992), significant variation in virulence was present.

A similar phenomenon has been documented in *Uromyces appendiculatus* (Linde *et al*, 1990) and in asexually reproducing populations of *Puccinia graminis* , *P. recondita* on wheat and *Magnaporthe grisea* (Burdon & Roelfs, 1985; Leung & Williams, 1986). If the high degree of variation in virulence is an indication of the diversity present in *Leucostoma* populations in Michigan then this population may have the potential to change rapidly and overcome resistance in newly introduced cultivars. High levels in virulence diversity might have come about through the selection pressure placed on the pathogen by the continuous introduction of new peach cultivars. Such selection pressure may have been responsible for the development of new virulent phenotypes. Alternatively, perhaps the less virulent phenotypes on peach are better adapted to parasitizing other native or domesticated trees.

Presence of *L. cincta* on apple in Michigan was first reported in 1989 by Proffer and Jones (1990) these isolates, our PG6, were virulent on peach seedlings in this study, and on a peach cultivar in an earlier study (Proffer & Jones, 1989). This pathogenicity was surprising since we have not found PG6 on *Prunus*, however PG6 was the least virulent group. Perhaps

bark chemistry might play a significant role in the exclusion of this group from *Prunus* (Rohrbach & Luepschen, 1968) in nature. The asci and ascospores of *L. cincta* on *Malus* mature at different times of a year than asci and ascospores of *L. cincta* on *Prunus* (Proffer & Jones, 1989), the former being fully mature in March and April, whereas the latter are immature at that time. Perhaps the relative susceptibilities of the tissues of *Malus* and *Prunus* differ in early spring. We also found it surprising that *L. persoonii* PG2 was less virulent on peach seedlings than PG1, PG5 and PG6. PG2 has been most prevalent naturally occurring phenetic group causing cankers in a similar nearby seedling planting even though PG1 and PG4 occurred at very low frequencies in the planting. We speculate at this time from our observations that perhaps PG2 preferentially infects spurs that have senesced from shading while PG1 preferentially infects cold injured bark.

We found that 3-yr-old peach seedlings were ideal for testing the virulence of a large number of *Leucostoma* isolates, because at this age the seedlings have multiple stems of uniform diameter and wood age permitting a large number of isolates to be tested concurrently on the same tree. In addition, few naturally occurring *Leucostoma* cankers are present after

3 years. Generally trees from grafted nursery stock are severely infected with cankers by the time they have sufficient branches for extensive inoculation tests, in Michigan. Surprisingly, the greater genetic variability in susceptibility to the inoculations that would be expected among outcrossed seedlings was not evident in these trials. In previous inoculation trials at the same location on grafted orchard stock, greater tree to tree variability was measured. We were not able to analyze the data of our replication in mid-March 1990, despite a seven day period following inoculation with minimum temperatures above freezing (ca. 5 C) and maximum daily temperatures averaging 13 C. Perhaps the defense mechanisms in the tree could have been initiated at this time, because a significant number of the inoculations succeeded and showed minute cankers. Prior to this trial, it was hypothesized that tree defenses were mobilized on resumption of active growth, because spring and early summer inoculations caused only minute resinous cankers Scorza & Pusey, 1984).

It would be interesting to determine the frequency of each PG in a particular orchard or location. Such experiments will be useful in epidemiological studies in *Leucostoma*.

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Table 7. High temparature growth tolerance, culture color, pycnidial size and mean canker length of Michigan isolates of *Leucostoma**.

Isolate	Host of Origin	Species	Phenetic group	Colony Margin	High temparature tolerance 33 C	Pycnidial size[a]	Color [a]	Mean canker length[c] (cm)
11.	peach	*L. persoonii*	PG1	lobate	+	S	Dark mouse grey	8.9 ab
LPSS.S2P2.1	peach	*L. persoonii*	PG1	lobate	+	S	Dark mouse grey	9.6 a
LPBP.S27.7	peach	*L. persoonii*	PG1	lobate	+	S	Dark mouse grey	8.3 abc
VSS3	peach	*L. persoonii*	PG1	lobate	+	S	Dark mouse grey	8.8 ab
14.1	peach	*L. persoonii*	PG1	lobate	+	S	Dark mouse grey	6.7 bc
T28.1	peach	*L. persoonii*	PG2	entire	+	S	Black	6.5 cd
T16.7	peach	*L. persoonii*	PG2	entire	+	S	Dark mouse grey	5.6 d
T18.1	peach	*L. persoonii*	PG2	entire	+	S	Dark mouse grey	11.5 a
T4.1	peach	*L. persoonii*	PG2	entire	+	S	Black	7.2 bcd
T7.4	peach	*L. persoonii*	PG2	entire	+	S	Black	10.9 ab
P1h	apricot	*L. cincta*	PG4	entire	-	L	Vinaceous buff	7.2 bc
LP45	apricot	*L. cincta*	PG4	entire	-	L	Vinaceous buff	7.9 ab
LP39	apricot	*L. cincta*	PG4	entire	-	L	Vinaceous buff	7.2 abc
LP37	apricot	*L. cincta*	PG4	entire	-	L	Vinaceous buff	7.6 ab
LP49	apricot	*L. cincta*	PG4	entire	-	L	Hazel	4.8 c
LP66	prune	*L. cincta*	PG5	entire	-	L	Vinaceous buff	6.9 bc
LP62	prune	*L. cincta*	PG5	entire	-	L	Vinaceous buff	9.4 a
LP63	prune	*L. cincta*	PG5	entire	-	L	Vinaceous buff	9.5 a
LP70	prune	*L. cincta*	PG5	entire	-	L	Hazel	7.2 ab
LP59	prune	*L. cincta*	PG5	entire	-	L	Hazel	8.2 ab
A3	apple	*L. cincta*	PG6	entire	-	L	buff	5.9 d
A43	apple	*L. cincta*	PG6	entire	-	L	Honey	4.9 d
A46	apple	*L. cincta*	PG6	entire	-	L	Honey	5.0 d
A48	apple	*L. cincta*	PG6	entire	-	L	Honey	5.9 d
A77	apple	*L. cincta*	PG6	entire	-	L	Buff	6.5 cd

*growth after 14 days of incubation on Leonian's agar medium. [a] L=large (1- 2mm) and S=small (0.5-1.0 mm)
[b] Color key used: Rayner's colour chart(23) [c] Length of the canker distal to inoculation point. Means followed by the same letter are not significantly different by the least significant difference test, LSD, P = 0.05.

Table 8. Summary of analysis of variance of virulence on peach of the five phenetic groups in Leucostoma.

		PG1		PG2		PG4		PG5		PG6	
Source of variation	df	Mean square	F	Mean square	F	Mean square	F	Mean square	F	Mean square	F
Tree	7	24.089	4.01	63.949	4.23*	4.391	0.35	3.927	0.50	7.667	1.58
Isolates	10	53.021	8.83*	74.473	4.92*	49.321	3.95*	47.936	6.08*	27.728	5.73*
Error	69	6.003		15.129		12.310		7.881		4.842	

*= significant at P = 0.05 level

TABLE 9. Virulence of standard isolates of the five phenetic groups of *Leucostoma* on 3-yr-old peach multistemmed seedlings ranked in order of decreasing canker length.

Standard isolates (cm)	*Leucostoma* species	Phenetic group	Mean canker length [a]	
11.3	*L. persoonii*	PG1	8.9	a
LP59	*L. cincta*	PG5	8.2	ab
F1h	*L. cincta*	PG4	7.2	bc
T28.1	*L. persoonii*	PG2	6.5	cd
A3	*L. cincta*	PG6	5.9	d

[a] Length of the canker distal to inoculation point. Means of forty replicates followed by the same letter are not significantly different by the least significant difference test, LSD, P = 0.05.

CONCLUSIONS AND PROSPECTIVES FOR FUTURE STUDY

CONCLUSIONS AND PROSPECTIVES FOR FUTURE STUDY

This research has provided the foundation of an understanding of the variation in two closely related taxa of *Leucostoma, L. cincta* and *L. persoonii*. Isozyme analysis has been very useful in separating the two species. Isozymes can be used as a diagnostic tool to identify the two taxonomic species. In addition, isozyme analysis has identified six distinct phenetic groups in *Leucostoma*, three of which were found within *L. persoonii*, PG1, PG2 and PG3, and three within *L.cincta*, PG4, PG5 and PG6. A higher level of genetic diversity was detected in the populations of *L. cincta* than *L. persoonii*, which may indicate that the primary mode of reproduction in *L. cincta* is sexual outcrossing. In contrast the low level of diversity seen in the populations of *L. persoonii* may indicate that the primary mode of it's reproduction is asexual or that this population may have arrived in North America as a founder population.

Virulence experiments on three year old peach seedlings were also indicative of the diversity present in *Leucostoma*. The virulence experiments showed that *L. cincta* isolates from *Malus* could also be virulent on peach. The two species, *L. cincta* and *L. persoonii*

could not be distinguished on the basis of their virulence on peach. Cultural morphology also differentiated the phenetic groups of the two species. In culture *L. cincta* and *L. persoonii* could be differentiated by their color and pycnidial size. In addition, phenetic group 2 of *L. persoonii* was differentiated from phenetic group 1 on the basis of it's color in culture and the absence of the distinct lobate margin typical of PG1 cultures. Phenetic group 6 of *L. cincta* was readily differentiated from PG4 and PG5 by formation of a reddish pigmentation in culture. Studies on restriction fragment length polymorphisms revealed populations within isolates of *L. persoonii*. Seven populations were revealed, several corresponded to those detected in isozyme studies and others corresponded to hosts and or geographic distributions. RFLPs also revealed two populations within *L. cincta*. The *L. cincta* isolates on *Prunus* spp. were differentiated from *L. cincta* isolates on *Malus* and *L. persoonii* by the presence of an insert in the 18S region of the nuclear rDNA.

The new information from this research has opened the way to future approaches to the study of speciation, population biology and molecular evolution in

Leucostoma. Several directions in research, rich in prospectives are listed and described here.

1. Anastomosis studies can be used to separate the two taxa in terms of their anastomosis groups and correlate these groupings with isozyme analysis.

2. Sequencing of the ITS region will provide information on the type of variation seen in the rDNA, whether it is due to site changes or length mutation.

3. Additional isolates of *L. cincta* on *Prunus* should be screened to verify that the insert in the 18S rDNA region is unique to this group. Sequencing will then aid in the identification of the insert and perhaps reveal information on it's role in evolution of *Leucostoma*.

4. Fungal mtDNA has been estimated to evolve approximately 5-8X faster than nuclear DNA. Variation in the mtDNA could be used to study maternal inheritance among individuals in local populations or geographically distinct groups within the two species.

5. Collection of single ascospores from perithecia of *L. cincta* isolates from various orchards and studying their isozyme patterns will reveal information on the genetic diversity in the population of *L. cincta*.

Such future experimental possibilities will provide

valuable information on the population genetics and phylogenetic studies in *Leucostoma*.

APPENDIX

Table 11. Alleles scored in *Leucostoma* study.

Isolate	Locus									
	ME	PGI	PGM	IDH	G6PD	MPI	EST	DIA	MDH	EST
	Alleles									
NC 9.2	c	c	c	c	c	-	a	-	ab	-
NC10.2	c	c	c	c	c	-	a	-	ab	-
NC22.2	c	c	c	c	c	-	a	-	ab	-
NC4A	c	c	c	c	c	-	a	-	ab	-
11.1	c	c	c	c	c	-	a	-	ab	-
NC23	c	c	c	c	c	-	a	-	ab	-
NC8.2	c	c	c	c	c	-	a	-	ab	-
NC17	c	c	c	c	c	-	a	-	ab	-
NC49	c	c	c	c	c	-	a	-	ab	-
T36.1	d	d	d	d	-	c	-	-	-	-
T9.3	d	d	d	d	-	c	-	-	-	-
T4.1	d	d	d	d	-	c	-	-	-	-
T32.2	d	d	d	d	-	c	-	-	-	-
T28.1	d	d	d	d	-	c	-	-	-	-
T10.6	d	d	d	d	-	c	-	-	-	-
T26.6	d	d	d	d	-	c	-	-	-	-
T18.1	d	d	d	d	-	c	-	-	-	-
T16.7	d	d	d	d	-	c	-	-	-	-
T3.6	d	d	d	d	-	c	-	-	-	-
R5T15	c	c	c	c	c	-	ac	c	ac	-
10.14	c	c	c	c	c	-	ac	c	ac	c
11.2	c	c	c	c	c	-	ac	c	ac	c
58	c	c	c	c	c	-	ac	c	ac	c
R1T12	c	c	c	c	c	-	ac	c	ac	c
10.1	c	c	c	c	c	-	ac	c	ac	c
11.12	c	c	c	c	c	-	ac	c	ac	c
R4T14	c	c	c	c	c	-	ac	c	ac	c
R1T4	c	c	c	c	c	-	ac	c	ac	c
RT16	c	c	c	c	c	-	ac	c	ac	c
105	c	c	c	c	c	-	ac	c	ac	c
R6T14	c	c	c	c	c	-	ac	c	ac	c
R4T11	c	c	c	c	c	-	ac	c	ac	c

Table 12. Restriction fragment patterns of Leucostoma persoonii

Isolate	Restriction enzyme								
	AluI	MboI	MseI	BstUI	TaqI	BstEII	HpaII	ScaI	RsaI
11.3	1 0 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	1 0 0	0 1	0 1
LP8	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	0 1
LP10	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	0 1
LP9	0 1 0	0 1 0	0 0 0 1	0 0 1	0 1	1 0	1 0 0	1 0	1 0
LP13	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	1 0
T4.7	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
T7.4	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
CHR	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	1 0
Cy5	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	1 0
T18.1	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
LCN	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
LP21	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	1 0 0	1 0	1 0
T26.1	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
NC9.7	0 1 0	1 0 0	0 1 0 0	0 0 1	0 1	0 1	1 0 0	1 0	0 1
LP2	1 0 0	1 0 0	0 0 1 0	0 0 1	1 0	1 0	1 0 0	1 0	0 1
NC17	1 0 0	1 0 0	0 1 0 0	1 0 0	1 0	1 0	1 0 0	1 0	1 0
NC14.1	1 0 0	0 0 1	1 0 0 0	0 1 0	0 1	1 0	1 0 0	1 0	0 1
NC8.2	1 0 0	0 0 1	1 0 0 0	0 1 0	0 1	0 1	1 0 0	0 1	1 0
R5T10	1 0 0	1 0 0	1 0 0 0	1 0 0	1 0	1 0	1 0 0	1 0	0 1
T16.1	0 1 0	1 0 0	0 1 0 0	0 0 1	1 0	0 1	0 0 1	0 1	0 1
LP8	0 1 0	0 1 0	0 1 0 0	0 0 1	1 0	1 0	0 0 1	0 1	0 1
R3T16	1 0 0	1 0 0	1 0 0 0	1 0 0	1 0	1 0	1 0 0	1 0	0 1