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thesis entitled

Genetic Diversity of Prunus Serotinaand the
Evaluation of Other Wild Species for Breeding
Sour Cherry Resistance to Cherry Leaf Spot

presented by

Suzanne Lynn Downey

has been accepted towards fulfillment
of the requirements for

Masters of Science degree in Plant Breeding and Genetics
- Horticulture

Major professor

Date February 1, 1999

GENETIC DIVERSITY OF *PRUNUS SEROTINA* AND THE EVALUATION OF OTHER WILD SPECIES FOR BREEDING SOUR CHERRY RESISTANCE TO CHERRY LEAF SPOT

By

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A THESIS

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

MASTER OF SCIENCE

Plant Breeding and Genetics
Department of Horticulture

1999

Professor Amy F. Iezzoni

ABSTRACT

GENETIC DIVERSITY OF PRUNUS SEROTINA AND THE EVALUATION OF OTHER WILD SPECIES FOR BREEDING SOUR CHERRY RESISTANCE TO CHERRY LEAF SPOT

By

Suzanne Lynn Downey

Central to the cherry breeding program at MSU is the acquisition and screening of germplasm for the development of improved sour cherry cultivars. Breeding for disease resistance to cherry leaf spot caused by *Blumeriella jaapii* depends upon the identification of resistance. The following germplasm was screened to identify *B. jaapii* resistance: GI 148-2, GI 148-1, MxM2, MxM60 and seedlings of wild black cherry (*Prunus serotina*). MxM2, MxM60 and GI 148-2 were highly susceptible to *B. jaapii*. GI 148-1 was resistant, exhibiting no sporulation, chlorosis, or defoliation. This selection is currently being used as a donor of leaf spot resistance in a backcross breeding program. *P. serotina*, immune to *B. jaapii* in our study, is of particular interest because of the occurrence of large fruited types. To gain a baseline understanding of the genetic diversity within *P. serotina* from Michigan, Mexico and Ecuador, the following informative marker systems were utilized. Three chloroplast polymorphisms were identified with the most common fragment present in all three geographic groups. Putative allozymes were identified in each geographic group. Sequence tagged site markers revealed the most genetic diversity. Mexican *P. serotina* germplasm had the largest number of putative alleles. The data suggests that the Michigan and Ecuadorian germplasms are divergent, most likely due to ecological and geographic adaptation and domestication events.

DEDICATION

This work is dedicated to my family, whose constant love and support have made this all possible. Thank you for believing in me and constantly reminding me of those things that are truly important in life...all of you.

ACKNOWLEDGEMENTS

Without the constant support of my major professor, Dr. Amy Iezzoni, this accomplishment would not be possible. I sincerely thank her for understanding, support and never ending encouragement which helped me to develop my own research interests. Dr. Jim Hancock was not only instrumental in my education and research, but he is also one of the primary reasons why I pursued my MS at Michigan State University through the Plant Breeding and Genetics Program. A big thanks goes out to him, as well as the other members of my committee, Dr. Alan Jones and Dr. Bryan Epperson. Dr. Salvador Perez from the Universidad de Queretaro, Mexico, was essential to my research. He was key for the germplasm collection trip into central Mexico and remains the primary *P. serotina* collaborator for our lab. Dr. Albert Abbott and Graham King were our sweet cherry and peach derived primer pair sources and also deserve recognition. The successful completion of my MS would not have been possible without the love and support of the friends I have made during my stay at MSU, especially those people who are part of our extended laboratory and my extended 'family.' Thank you Renate Karle, Pete Callow, Dechun Wang, Erin Crowe, Christopher Owens, Rebecca Henry, Jaimie Houghton, Audrey Sebolt, Kirsten and Soren Ottosen, Roger Herr, Charlie Herman, Beth Faussey, the ladies of Alpha Omicron Pi, and Leslie Finical. You have made all the difference.

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CHAPTER ONE

EVALUATION OF WILD SPECIES FOR BREEDING SOUR CHERRIES (*PRUNUS CERASUS*) RESISTANT TO CHERRY LEAF SPOT CAUSED BY *BLUMERIELLA JAAPII*

ABSTRACT

Cherry leaf spot, caused by *Blumeriella jaapii* (Rhem) Arx., is a major fungal disease of sour cherry (*Prunus cerasus* L.) affecting production orchards worldwide. All principal sour cherry cultivars grown are susceptible to *B. jaapii* and numerous fungicide sprays are required to prevent this disease. One of the objectives of the sour cherry breeding program at MSU is to breed cultivars resistant to cherry leaf spot. To accomplish this goal, identification of suitable donor species of increased disease resistance is required. Previous work suggested that the best source(s) of resistance may be wild *Prunus* species and interspecific hybrids. Therefore, the following germplasm was obtained and screened for increased disease resistance: GI 148-1 and GI 148-2 (*P. cerasus* x *P. canescens*), MxM2 and MxM60 (*P. mahaleb* x *P. avium*), and open-pollinated seedlings of wild black cherry (*P. serotina*). Sour cherry seedlings were used as susceptible controls. Young leaves of each selection were inoculated with a fresh spore suspension of *B. jaapii* collected from naturally infected sour cherry leaves and disease progression was evaluated 14 days after inoculation. *P. serotina* was immune to leaf spot with no apparent infection. Sour cherry, MxM2, MxM60, and GI 148-2 were susceptible to *B. jaapii*, all exhibiting necrotic lesions, fungal sporulation, leaf chlorosis and defoliation. In contrast, GI 148-1 was resistant to *B. jaapii* infection exhibiting necrotic lesions that eventually excised, no fungal sporulation, and no leaf chlorosis or defoliation. GI 148-1 also had significantly fewer necrotic lesions per cm^2 than susceptible sour cherry controls. Currently, GI 148-1 is being used as a donor of leaf spot resistance in the MSU sour cherry breeding program.

INTRODUCTION AND LITERATURE REVIEW

The Disease

Blumeriella jaapii (Rhem) Arx. (syn. *Coccomyces hiemalis* Higgins) causes a major disease of sour cherry (*P. cerasus*) commonly referred to as cherry leaf spot or 'shot hole' disease. Unfortunately, the principal sour cherry variety grown within the United States, 'Montmorency,' is highly susceptible to this disease (Andersen, 1981). As a result, a major focus of the sour cherry breeding program at MSU is the development of cultivars resistant to cherry leaf spot disease.

The fungus causing cherry leaf spot was first reported in Europe during 1884 on *P. padus* (Karsten, 1884). Not until the work of Higgins (1914) was the life cycle of the fungus understood. Higgins (1914), after associating the sexual and asexual stages together and describing the life cycle, classified the fungus causing cherry leaf spot into three distinct species according to host range: *C. hiemalis* found on *P. avium*, *P. cerasus*, and *P. pennsylvanica*; *C. lutescens* found on *P. serotina*, *P. virginiana*, and *P. mahaleb*; and *C. prunophorae* found on plums (*P. americana*, *P. domestica*, and *P. insititia*). After several revisions of the taxonomy, the fungus was eventually named B. *jaapii* (Rhem) Arx., pooling all European and American species together as one (von Arx, 1961).

When not properly controlled, cherry leaf spot disease can cause petiole and leaf chlorosis and premature defoliation. In these instances, fruit that fail to ripen are poorly colored and have low soluble solids (Jones, 1976). Premature defoliation also contributes

to an overall reduction of tree vigor due to reduced flower bud and wood survival during winter months (Howell and Stackhouse, 1973).

The disease cycle begins each year in the spring when the overwintered fungus produces mature apothecia. Ascospores are released upon wetting and are disseminated from lingering leaf debris on the orchard floor to young cherry leaves primarily through wind and splashing rain (Higgins, 1914). When proper weather conditions exist, ascospore dispersal can continue from the end of cherry bloom until June (Keitt et al., 1937). Primary inoculation of cherry leaves begins when ascospores enter through open stomates on the abaxial leaf surface (Keitt et al., 1937; Jones et al., 1993; Garcia et al.,1993). Young unfolded leaves are the most susceptible to inoculation (Eisensmith et al. 1982). According to Eisensmith and Jones (1981), disrupted periods of wetness may be more favorable for severe primary infection on young cherry leaves than continuous wet periods. Once infection has occurred, fungal hyphae grow throughout the host leaf tissue causing the leaf to form necrotic lesions.

Disease severity progresses as the fungus advances throughout susceptible host leaf tissue. Severe disease symptoms include necrotic lesions which do not excise, considerable leaf and petiole chlorosis, and leaf abscission (Higgins, 1914; Jones 1976). A sign of disease progression is the eventual production of white acervuli on the abaxial leaf surface. Mature acervuli sporulate during periods of high humidity, releasing conidia which subsequently disseminate to new host tissue through water droplets and air currents (Higgins, 1914). Without control measures, secondary infection can occur readily throughout the cherry growing season, causing widespread orchard devastation. This secondary spread of the disease is dependent upon the amount of inoculum available and

the occurrence of favorable temperature and moisture conditions (Eisensmith and Jones, 1981). *B. jaapii* overwinters during its sexual phase in protective stroma-like structures which develop on infected cherry leaves on the orchard floor (Higgins, 1914).

Removal of infected leaf debris is critical to the reduction of primary inoculum and delaying disease infection in subsequent years (Keitt et al., 1937). Fungicide regiments of four to six sprays per year are the most common defense against the spread of *B. jaapii* in orchards (Jones and Ehret, 1980). Highly effective infection models for timing of fungicide applications have been developed to reduce unnecessary and ineffective sprays (Eisensmith, 1981). However, alternative control measures are needed since the fungicides used to control cherry leaf spot are costly and there is concern about the occurrence of fungicide resistance in *B. jaapii* (Jones and Ehret, 1980; Jones et al., 1993). Identification of natural disease resistance in closely related *Prunus* species is therefore a major part of the strategy to fight this disease.

Natural Disease Resistance in *Prunus*

The development of disease resistant varieties of sour cherry is a major priority of the Michigan State University sour cherry breeding program. The sour cherry variety 'Montmorency' is highly susceptible to cherry leaf spot disease. *P. cerasus* is a tetraploid species (2n = 4x = 32) believed to be the result of interspecific hybridization between sweet cherry (*P. avium* L.) and European ground cherry (*P. fruticosa* Pall.) (Olden and Nybom, 1968). All three species readily intermate and sour cherry exhibits traits from both parental species. This intermixing has created natural diversity from which to select individuals for superior fruit quality and adaptive traits (Iezzoni et al., 1990).

Initially, resistance was sought in closely related *Prunus* species. Sweet cherry, sour cherry, European ground cherry, and duke cherry (*P. gondouinii* Rehd.) and 'North Star' varieties which appeared to have field resistance to this disease were screened under laboratory conditions (Sjulin et al., 1989). If identified, resistance in these closely related species could readily be transferred into sour cherry. However, all screened selections were susceptible to *B. jaapii*. Sporulation and subsequent spread of the disease was reduced in some selections. As a result, breeding efforts are currently focused on identification of resistance in interspecific hybrids and wild *Prunus* species.

Existing interspecific hybridizations between sour cherry and selected *Prunus* species may be a good source of cherry leaf spot resistance. Commercialized dwarfing rootstocks for sweet cherry, GI 148-2 (Giesla 5) and GI 148-1 (Giesla 6), are German hybrids resulting from the cross between *P. cerasus* cv. Schattenmorelle and *P. canescens* (Schmidt and Gruppe, 1988). Since *P. canescens* is a diploid species, both hybrids are triploid. Schattenmorelle, the sour cherry parent, is highly susceptible to cherry leaf spot, whereas the *P. canescens* parent used to produce these hybrids has been observed to be immune to the disease (E. Gigadlo, pers. communication). It is possible that these full sibling hybrids may have inherited some disease resistance from their *P. canescens* parent.

Other commercially available cherry rootstocks (MxM2 and MxM60) are from presumed natural hybridizations originating in Oregon between *P. mahaleb* and *P. avium* (*P. mazzard*) (Cummins, 1979). These selections have been previously documented as exhibiting a variety of reactions to *B. jaapii* ranging from resistant to severely susceptible

(Westwood, 1976). Screening of this germplasm is necessary to evaluate these hybrids as donor species of increased disease resistance.

Another potential source of disease resistance to cherry leaf spot may be wild black cherry (*P. serotina* Ehrh). Commercially grown *P. serotina* within the United States have been documented to be susceptible to one specific race of *B. jaapii, C. lutescens* Higgins (Higgins, 1914; Heald, 1933; Davis, et al. 1942; Stanosz, 1992). *P. serotina* of the U.S., used for its high valued lumber, has been overlooked by cherry breeders as a source of resistance due to its production of extremely small fruit (aprox. 0.5 cm in diameter). However, large fruited (avg. fruit 2 cm in diameter) varieties of *P. serotina* called "Capulin" exist in Central Mexico and Ecuador (Popenoe and Pachano, 1922; Popenoe, 1924). Little information regarding the resistance of large fruited wild populations of *P. serotina* spp. *capuli* to *B. jaapii* is available. *P. serotina* is a tetraploid species (2n = 4x = 32, Stairs and Hauck, 1968) making it a good candidate for possible incorporation of disease resistance into sour cherry if superior resistance to *B. jaapii* is identified. Therefore, screening of Capulin selections with fruit characteristics more similar to the desired fruit size in commercial sour cherry cultivars is necessary investigate their potential as donors of leaf spot resistance in the MSU breeding program.

Objective

The objective of this study was to evaluate the following selections as sources of cherry leaf spot resistance: GI 148-1, GI 148-2, MxM2, MxM60, and wild black cherry selections from Mexico and Ecuador.

MATERIALS AND METHODS

Plant Material

Rooted cuttings of GI 148-1 were obtained form Hilltop Nurseries (Hartford, MI). Seeds of wild black cherry were collected in Central Mexico and Ecuadorian accessions were obtained from the Ecuadorian Germplasm Institute (Appendix A, B). The seeds were germinated and the resulting plants were grown in a MSU growth chambers and greenhouse. *P. serotina* seedlings, along with hybrid cuttings and sour cherry seedlings resulting from seed harvested in 1995, were transplanted and grown in a completely randomized design within a plastic greenhouse at the Michigan State University Clarksville Horticultural Research Station (CHES) in Clarksville, Michigan. In August of 1996, five GI 148-1 cuttings, five *P. serotina* seedlings from Mexico and Ecuador, and five sour cherry seedlings were randomly selected for inoculation. The experiment was repeated in 1997 with the addition of interspecific hybrids GI 148-2 , MxM2 and MxM60. Rooted cuttings of these selections were also obtained from Hilltop Nurseries (Hartford, MI).

Inoculation

Fresh inoculum was obtained for the 1996 and 1997 inoculations from naturally infected leaves of sour cherry collected from an unsprayed, infected sour cherry orchard (CHES). Infected leaf samples were placed in a moist plastic bag at 4°C until the time of inoculum preparation. To maximize spore germination and infection, overhead sprinklers were run in the plastic greenhouse for at least one hour prior to inoculation to increase humidity and ensure opening of the leaf stomates. Conidial suspensions of *B. jaapii* were

prepared by washing the infected leaves with water and carefully brushing off visible acervuli with a clean paint brush. After a cloudy inoculum slurry was obtained, the suspension was cleaned of debris by filtration through two layers of cheesecloth. The conidial inoculum was then transferred into a small clean spray bottle. A quantitative inoculator like that developed by Schein (1964) was not used since such equipment was not available and precise quantitation of inoculum was not deemed necessary for an initial disease assessment. In addition, identification of inoculum races was not performed. Seedlings were inoculated by 'uniformly' spraying the abaxial leaf surface of the second unfolded leaf of a vigorously growing shoot three times on a fine mist setting. The 1996 and 1997 inoculations were performed during the overcast, late afternoons of August 29 and August 20, respectively. The inoculum slurry was also sprayed onto potato dextrose agar (PDA) plates and subsequently examined under 200x magnification after 24 hours of incubation at room temperature to confirm germination of conidia.

Disease Evaluation

Fourteen days after initial inoculation, necrotic lesions were counted on five leaves of each inoculated plant. Observations of disease symptoms and progression were also periodically recorded for three weeks following initial inoculation. On day fourteen, leaf area for each evaluated leaf was determined. To be sure not to interfere with disease progression, inoculated leaves were not harvested from the selections. Instead, leaf outlines were traced on paper and used for calculating total leaf area with a leaf area meter (Li Cor, Lincoln, Nebraska). The number of lesions per cm^2 was calculated for each of the infected leaves as the number of lesions on day fourteen divided by the leaf area. The average of five replications for each selection was calculated. Statistical

comparisons among varieties were performed using Proc GLM in SAS (SAS Institute, 1989).

RESULTS

Conidial Germination

Conidial germination was defined as the stage at which the germ tube was twice as long as the conidia. In both 1996 and 1997, the spores used in inoculation experiments exhibited 80% germination.

Disease Susceptibility Evaluation

In 1996 and 1997, GI 148-1 exhibited 1.6 and 1.2 necrotic lesions per cm^2 respectively (Table 1). The numbers of necrotic lesions for GI 148-1 were significantly less than those exhibited by the susceptible sour cherry controls which averaged 19.4 and 5.4 necrotic lesions per cm^2 in 1996 and 1997, respectively ($P = 0.05$). GI 148-2 averaged 8.2 necrotic lesions per cm^2 in 1997. Although this result was not significantly different from the susceptible sour cherry controls, GI 148-2 had significantly more necrotic lesions per cm^2 than its full sibling, GI 148-1 ($P = 0.05$).

Sour cherry and GI 148-2 exhibited similar symptoms of susceptibility to cherry leaf spot (Table 2). Leaves from these selections became chlorotic while those of GI 148-1 failed to show symptoms of leaf or petiole chlorosis (Figure 1). In addition to producing relatively high lesion counts per cm^2, sour cherry controls and GI 148-2 also exhibited visible white acervuli containing conidia on the abaxial leaf surface and leaf abscission within 21 days of initial inoculation (Table 2, Fig. 2). In contrast, GI 148-1 exhibited no apparent sporulation, leaf chlorosis, or defoliation (Table 2, Fig. 2, Fig. 3, Fig. 4). Necrotic lesions found on GI 148-1 were small necrotic circles (~2mm diameter) which eventually excised (Fig. 4), producing characteristic 'shot hole' symptoms. When

inoculated leaves of GI 148-1 exhibiting necrotic lesions were removed from the trees and placed in optimal sporulation conditions (high humidity to force conidial release), no sporulation was observed.

MxM2 and MxM60 were susceptible to cherry leaf spot disease when inoculated with *B. jaapii* from naturally infected sour cherry leaves (Table 2). Necrotic lesion calculations were not taken for MxM2 and MxM60 selections because of the severity of disease symptoms exhibited by each hybrid.

P. serotina seedlings failed to exhibit any disease symptoms when inoculated with the spore suspension of *B. jaapii* isolated from infected *P. cerasus* (Table 2). Black cherry seedlings included in this study exhibited no signs of infection or symptoms of cherry leaf spot disease.

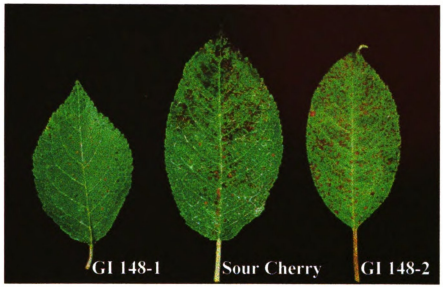
Table 1. Comparison of the mean number of necrotic lesions per cm^2 for sour cherry (*P. cerasus*), GI 148-1, and GI 148-2 fourteen days after artificial inoculation with *B. jaapii* conidia.

Year	Cherry selection	Average number of lesions per cm^2
1996	Sour cherry	19.4[y] a[z]
	GI 148-1	1.6 b
1997	Sour cherry	5.4 a
	GI 148-1	1.2 b
	GI 148-2	8.2 a

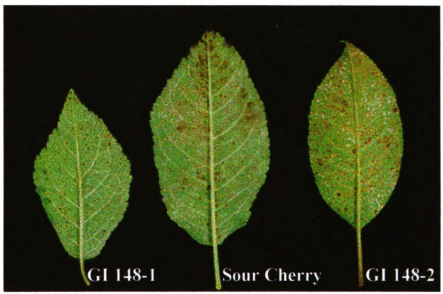
[y] Each value represents the mean of 5 replications.
[z] Within year, means followed by different letters are significantly different at P = 0.05

Table 2. A summary of disease symptoms exhibited by sour cherry, GI 148-1 and GI 148-2, MxM hybrids, and *P. serotina* selections following artificial inoculation with *B. jaapii* conidia.

Inoculated Selections	Necrotic Lesions	"Shot Hole" Symptoms	Leaf and Petiole Chlorosis	Acervuli Sporulation	Leaf Abscission and Disease Spread
Sour Cherry GI 148-2 MxM Hybrids	Present	Absent	Present	Present	Present
GI 148-1	Present	Present	Absent	Absent	Absent
P. serotina	Absent	Absent	Absent	Absent	Absent

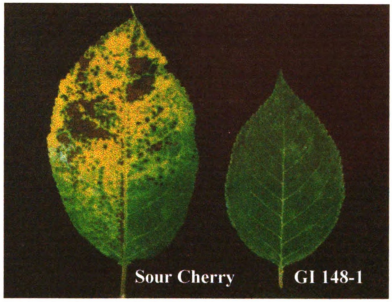


A



B

Figure 1. Cherry leaf spot disease on the (A) adaxial and (B) abaxial leaf surface of GI 148-1, GI 148-2, and sour cherry selections fourteen days after initial inoculation with *B. jaapii* conidia.



A

B

Figure 2. (A) Leaves of sour cherry and GI 148-1 selections infected with *B. jaapii* conidia. (B) German hybrid GI 148-1 exhibiting few necrotic lesions, no chlorosis or leaf abscission 21 days after initial inoculation with *B. jaapii* conidia.

DISCUSSION

GI 148-1 was identified as a source of resistance to cherry leaf spot since inoculation with *B. jaapii* resulted in small necrotic lesions that failed to sporulate. The characteristic shot hole symptoms exhibited by GI 148-1 prevented sporulation and further spread of *B. jaapii* to new host tissue. According to Parlevliet (1979), GI 148-1 exhibits a disease reaction defined as infection type zero (IT0), where necrotic symptoms fail to sporulate.

The sour cherry parent of GI 148-1, *P. cerasus* Schattenmorelle, is highly susceptible to cherry leaf spot whereas *P. canescens* has been observed to be immune to the disease (E. Gigadlo, pers. communication). Therefore, it is hypothesized that the gene(s) in GI 148-1 which confer this resistance are likely dominant and donated by the *P. canescens* parent. Disease reactions like that exhibited by GI 148-1 are typical of a resistance that is simply inherited or race specific (Parlevliet, 1979). The dramatically different reactions exhibited by triploid hybrid siblings GI 148-1 and GI 148-2 suggest that the resistance gene(s) within GI 148-1 were not homozygous in the *P. canescens* parent. Unfortunately the *P. canescens* parent used in the original cross in Germany has not been maintained and is therefore unavailable to test this hypothesis (H. Schmidt, per. communication). Due to its increased disease resistance, GI 148-1 is currently being used in a backcross breeding program to confer resistance into sour cherry.

CONCLUSIONS

Previous studies by Westwood et al. (1976) documented a spectrum of disease reactions of MxM selections to *B. jaapii* ranging from highly resistant to extremely susceptible. However, the two selections used in this study were susceptible to cherry leaf spot and therefore will not be used as parental species for crosses with sour cherry.

Although *P. serotina* has been documented as susceptible to certain races of *B. jaapii*, *C. lutescens* Higgins (Higgins, 1914, Keitt, 1918; Heald, 1933), in this study the *P. serotina* seedlings exhibited an immune response to cross infection of *B. jaapii* strains isolated from *P. cerasus* leaves. In accordance with Keitt's results (1918), the *P. serotina* spp. *capuli* seedlings from Mexico and Ecuador are immune to infection from strains of *B. jaapii*.

German hybrid, GI 148-1 exhibited resistance to infection by B. jaapii, in contrast to its full sibling, GI 148-2 which was highly susceptible to disease. Identification of an interspecific hybrid with increased disease resistance underscores the importance of screening wild species and interspecific hybrids for increased disease resistance.

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CHAPTER TWO

GENETIC DIVERSITY WITHIN AND AMONG BLACK CHERRY (*PRUNUS SEROTINA*) FROM MICHIGAN, MEXICO AND ECUADOR

ABSTRACT

Black cherry (*Prunus serotina* Ehrh) is a common secondary forest species with a wide endemic distribution, ranging from Nova Scotia into Mexico, Ecuador, and Peru. Although heavily planted for its valued lumber within the United States, *P. serotina* is essentially a wild species with small fruit (6-10 mm in diameter). In Mexico and Ecuador, however, domesticants and feral populations of this species called "Capulin," have much larger (2-2.5 cm), edible fruit. No information is available concerning the genetic diversity within *P. serotina* subsp. *capulin*, or between Capulin and *P. serotina* subsp. *serotina*, the common black cherry of the U.S. Our objective was to investigate the patterns of genetic diversity within and among black cherry germplasm collected from Michigan, Central Mexico, and Ecuador. The following molecular marker systems were informative: one chloroplast PCR product, two isozymes, and four sequence tagged sites (STS) primer pairs from *P. cerasus*, *P. avium*, and *P. persica*. Three chloroplast polymorphisms were identified with the most common fragment present in all three geographic groups. All of the putative allozymes were identified in each *P. serotina* population. STS markers identified the most genetic diversity. Mexican *P. serotina* germplasm had the largest number of putative alleles, many of which were common to the Michigan and Ecuadorian germplasm. Unique putative alleles were identified in the Michigan and Ecuadorian germplasm, however, these two populations had no common putative alleles that were not also present in the Mexican germplasm. This suggests that the Michigan and Ecuadorian germplasm are divergent, most likely due to ecological and geographic adaptation, selection and domestication events.

INTRODUCTION AND LITERATURE REVIEW

There are approximately 400 species within the genus *Prunus*, only twenty-five of which are native to North America (Maynard et al., 1991). Among these species endemic to the New World, the black cherry (*P. serotina*) is the only species commercially grown for its high valued hardwood within the United States. *P. serotina* is commonly found as a wild species in secondary forests (Maynard et al., 1991), thriving in recently disturbed areas such as logging sites. Although *P. serotina* has been heavily cultivated and studied by the forestry sector for decades, the small and non fleshy Northern *P. serotina* fruit (6-10 mm in diameter) has no commercial value (McVaugh, 1951). In Mexico and into the Andean Highlands, however, domesticants and feral populations of this species called "Capulin" have much larger edible fruit with large pits (Avg. 2-2.5 cm) (Popenoe and Pachano, 1922; Popenoe, 1924). It is generally accepted that the large fruited Capulin resulted from domestication by native peoples in Central America (Popenoe and Pachano, 1922).

Essential to the sour cherry breeding program at Michigan State University is the acquisition of germplasm that might be used as donor species for disease resistance. *P. serotina* was of particular interest because it is a tetraploid species (x = 8, 2n = 32) (Stairs and Hauck, 1968) like sour cherry (Olden and Nybom, 1968) and therefore may have the ability to produce fertile progeny when hybridized with sour cherry. For scion cherry breeding, the Capulin cherry may be the most useful *P. serotina* subspecies parent because of its large fruit size.

Termed one of the 'lost crops of the Inca's,' no information is available concerning the genetic diversity within Capulin, or between *P. serotina* subsp. *capulin* and the black cherry of the United States, *P. serotina* subsp. *serotina*. Molecular data which describes the genetic relationships within and among *P. serotina* populations would aid in the selection of parental *P. serotina* material, as well as reveal insights into its evolutionary history. Our goal is to characterize the natural genetic diversity within *P. serotina* by sampling selections from Michigan, Ecuador and Central Mexico using several molecular marker systems.

Background

P. serotina has a large endemic distribution ranging from Nova Scotia down the eastern U.S. coast, with disjunct populations also in west Texas, southern Arizona and New Mexico. This species is also endemic to Central Mexico and into Ecuador and Peru (McVaugh, 1951). It is generally accepted that the Spaniards introduced *P. serotina* from Mexico or Central America into the Andean region during Colonial times (Popenoe et al., 1989). Although not formally cultivated, the Capulin black cherry is domesticated, often harvested and sold in fresh fruit markets in the Andean region. *P. serotina* was subsequently introduced into Europe from the U.S. in the 17th century initially as decoration in gardens and parks. Due to its prolific characteristics in these areas, *P. serotina* is often referred to as a forest pest rather than a valued ornamental species today in Europe (van den Tweel and Eijsackers, 1987).

P. serotina of the U.S. and Canada differ from those in Mexico and Ecuador only by those horticultural traits that are easily manipulated by selective cultivation (Popenoe and Pachano, 1922). Mexican and Ecuadorian *P. serotina* trees usually grow as multiple-

stemmed trees of approximately 35 feet in height. In contrast, forestry planted trees in the U.S. are selected for and grown as straight, single trunked trees with heights of 120 feet (Maynard et al., 1991). The following descriptions of *P. serotina* are taken from McVaugh (1951):

> "The species as a whole is characterized as having alternate and simple oblong to lanceolate leaves with fine double toothed serrations. The bark has the characteristic horizontal lenticles of cherry species. The wood of black cherry is a valued hardwood in the U.S. commonly used to make prized home furnishings and interior paneling. Flowers of the black cherry are borne on loosely clustered racemes with approximately 20-30 flowers per inflorescence. When ripe, fruit are dark red to black. The name 'Capuli or Capulin' is used only to designate the larger fruited varieties."

Typically, the fruit of *P. serotina* in the U.S. and Canada averages 6-10 mm in diameter (McVaugh, 1951). Flesh of the black cherries are bitter in taste and typically surround large pits. Regardless, the fruit is consumed by many bird species and has been used in folk medicines and to flavor liquors. The larger, edible Capulins of Central Mexico Mexico have long been incorporated into local cultures. Ranging from 2 cm to the most famous "Gonzales Capulin" in Catiglata, Ecuador measuring 3.5 cm in diameter (Popenoe and Pachano, 1922) *P. serotina* continue to be domesticated. The largest of the Capulins in Ecuador are harvested and found frequently in fresh fruit and vegetable markets (Popenoe, 1924).

In Central Mexico, *P. serotina* is a common unmanicured species in home gardens and grows as a predominant species along roadsides and fence lines surrounding a variety of orchards within the mountains northeast of Mexico City (Appendix A). Although there is no formal cultivation, selection for the Capulin cherry is apparent by its overwhelming presence in these selected areas, protected and cherished by local people.

While many people of Mexico eat large fruited Capulins as fresh fruit, the smallest and darkest cherries are often used to make a brandy wine. The pits of the largest fruits are toasted and eaten like pistachios (S. Perez, Per. Comm., 1996). In some areas, the fruit is said to have a medicinal use as a tonic to relieve coughs (Appendix A).

Previous Research

Most formal research conducted on *P. serotina* has been through the forestry sector. Evaluation and phenotypic selection of seedlings for superior timber qualities and geographic distribution of naturally occurring *P. serotina* have dominated the literature (Pitcher and Dorn, 1972; Genys and Cech, 1974). Tissue culture manipulation as well as proper handling of seed for maximum germination have also been important research areas (Caponetti et al., 1971; Dills and Braham, 1988; Tricoli et al., 1985; Dradi and Biondi, 1991; Maynard et al., 1991; Forbes, 1972; Huntzinger, 1968). Little information is available regarding the genetic diversity in *P. serotina.*

Since no information is available regarding the use of molecular markers in *P. serotina*, molecular markers were chosen for this diversity study that had been shown to be polymorphic in the horticulturally important cherry species (*P. avium* and *P. cerasus*). Both chloroplast and nuclear markers were selected due to their different modes of inheritance and rate of mutation. Chloroplast DNA (cpDNA) is maternally inherited in cherry (Brettin, 1999) and it is especially useful for phylogenetic studies due to its high degree of base sequence conservation (Curtis and Clegg, 1984; Palmer, 1987). Although cpDNA is highly conserved within a species, non coding regions within the chloroplast genome have higher rates of mutation, including insertions/deletions. Tablerlet (1991) designed conserved primer sets in conserved regions flanking non-coding regions of the

chloroplast genome. Brettin et al (1999) used one of these sets, the 'AB' primer set, to amplify one of these variable chloroplast regions and search for polymorphisms in sour cherry. Within sour cherry seven length polymorphisms were found for the 'AB' fragment. To better define the insertion/deletion events resulting in these seven polymorphisms, approximately 300 bp of sequence was obtained for these 'AB' fragments (Brettin et al, 1999). The first 300 bp of sequence revealed two polymorphisms which differed for two insertion/deletions, one of 8 bp and another of 6 bp. Primers flanking these two insertions/deletions were designed and termed the nested AB primers, [AB], to facilitate the rapid screening of sour cherry germplasm of the 14 base pair length polymorphism. This nested cpDNA primer pair was chosen for use in this *P. serotina* study.

Two nuclear marker types were chosen; isozymes (enzymes with specific catalytic activity) and sequence tagged sites (STSs, i.e. PCR amplified fragments where the primers are designed from available sequence data). Both marker types are codominant which permits the identification of the heterozygous class. Although isozymes exhibit limited levels of polymorphism compared to other marker systems, they were included in this analysis since we have previous information on them and the data obtained represent actual plant proteins.

Isozymes have been used to study genetic diversity and polyploid inheritance in sour cherry (Beaver and Iezzoni, 1993). Two of the more polymorphic isozyme systems with inheritance data available were chosen for our study, 6-PGD and PGI. Both isozyme systems are dimeric and extensively studied in *Prunus* (Byrne and Littleton, 1988; Parfitt et al, 1985; Byrne, 1989, and Chaparro et al., 1987).

Microsatellites or simple sequence repeats (SSRs) are a group of sequence tagged sites (STSs) where primers of specific sequences are designed to flank hypervariable regions of di-, tri- or tetra-nucleotide repeats (Staub and Serquen, 1996; Queller et al., 1993). Amplifications of these specific non-coding regions is performed by PCR. These hypervariable non-coding regions of the nuclear genome are often highly polymorphic within species, producing fragment length patterns which can frequently identify specific progeny. At the time of this study, a limited number of SSR primer pairs were available from sweet cherry (G. King, pers. comm.) and sour cherry (A. Iezzoni, pers. comm.). The other available STS primer pairs were designed from cloned amplified fragment length polymorphism (AFLP) fragments in peach (A. Abbott, pers. comm.)

Objective

Our objective is to investigate the genetic relatedness of *P. serotina* selections from Michigan, Ecuador and Central Mexico using several molecular marker systems and provide baseline data from which future in depth studies of the evolution and domestication of *P. serotina* and its relationship with other *Prunus* species could begin.

MATERIALS AND METHODS

Plant Material

P. serotina seedlings used in this analysis were from three sources: Mexico, Ecuador and Michigan (Table 3). Accessions from Central Mexico were collected as open-pollinated seed from under Capulin trees August 12-17, 1996 (Appendix A). Open-pollinated seed was collected from twelve trees termed 12 collection 'families' (RG, P1, PH, TI, P3, P2, ARG, U2, U3, J1, J2, and J3). Three of these families (RG, TI and J3) were collected by collaborators of Sr. Perez prior to our arrival in Central Mexico (Appendix A). Ten Ecuadorian accessions were obtained from the Ecuadorian Germplasm Institute as open-pollinated seed presumably collected from five trees termed five collection 'families' (Ecu A 1-3, E 1, D 1-2, F 1-2, H 1-2) (Appendix B) [c/o Dr. Raul Castillo, Departamento Nacional de Recursos Fitogeneticos y Biotecholigia, Casilla 17-01-340, Quito, Ecuador]. Uneven progeny numbers from these five Ecuadorian families resulted from poor seed germination. Ten *P. serotina* subsp. *serotina* plants were obtained as young seedlings from the MSU Department of Forestry Extension Service. These seedlings were collected as bulk seed from the *P. serotina* collection at the Kellogg Biological Station. Selections of *P. avium* (Emperor Francis), *P. cerasus* seedlings ('Montmorency,' Erdi Botermo, Csengodi, and Rheinische Schattenmorelle), and *P. cerasus* x *P. canescens* hybrids (GI 148-1 and GI 148-2) were used as reference selections in the isozyme analysis. Plant material used for chloroplast fragment amplification, isozyme analysis, and STS fragment amplification are presented in Table 3.

CHLOROPLAST MARKER SYSTEM

DNA Isolation and Quantification

Procedures for extraction and quantification of total DNA from *P. serotina* seedlings were modified from Stockinger et al. (1996). After adding 10 ml of chloroform;isoamyl alcohol 24:1, each sample was homogenized by gentle shaking for at least five minutes to separate and extract proteins from the DNA. When pellets failed to dissolve in $T_{10}N_{700}E_{0.5}$, centrifugation time was decreased to 16 minutes and pellets were dissolved at 60°C after incubation at 4C overnight. For all samples, young leaves were harvested from seedlings and kept at -80°C overnight and subsequently lyophilized for 48 - 72 hours. Optimally, extraction yielded between 500ng - 1000ng/µl DNA per sample. Dilutions of 50 ng/µl were made and used as working stock samples for each accession.

Sample Amplification and Fragment Resolution

For each 25µl PCR reaction, the following reagents were used: 15.37µl autoclaved ddH_20, 2.5µl 10x PCR buffer, 1.0µl 25 mM $MgCl_2$, 2.0µl 10x dNTPs, 1.0µl forward primer, 1.0µl reverse primer, 1.0µl of 50ng/µl diluted DNA template and 0.13µl Taq polymerase. PCR parameters for the 'AB' amplification are listed in Table 4. Each of the 24 samples in total were amplified using the 'AB' primers from the chloroplast variable non-coding region (Tablerlet, 1991). PCR products from this reaction were diluted 1:100. Successive PCR reactions using the nested primer pair, [AB], used the diluted PCR product as template. The same PCR program was followed for the 'AB' and nested [AB] amplification (Table 4). Visualization of amplified products was resolved on 6% polyacrylamide gels run on 80v for 2 hours and stained using the Silver Sequence™

staining system (Promega, Madison, WI). Amplified fragments were scored against a 10bp ladder (Gibco, cat. #10821-015, Fredrick, MD).

NUCLEAR MARKER SYSTEMS

Isozyme Analysis

Starch gel electrophoresis was performed on protein extracts obtained from *P. serotina* seedlings and reference plants. Young leaves and eltiolated buds were collected and stored in eppendorf tubes at 4°C prior to enzyme extraction. All material was macerated using cooled mortar and pestles in a walk in cooler to ensure temperature stability and reduction of enzyme activity. Isozyme extraction procedures follow the procedures of Krebs and Hancock (1989) with slight modification. No nylon screens were used during the extraction process. In addition, extraction buffer for sample maceration was maintained at pH 7.5 instead of pH 8.0.

Two isozyme systems representing two loci each, 6-phosphogluconate dehydrogenase (6-PGD) and Phosphoglucose isomerase (PGI) were resolved on 12% potato starch gels with approximately six and a half hours of electrical current. 6-PGD was resolved on a morpholine-citrate pH 6.1 gel (Clayton and Tretiak, 1972) while PGI resolved best on a tris-citrate/lithium-borate pH 8.3 gel (Scandalios, 1969). For each gel system, an electrical current of approximately 50mA was maintained until wicks were removed after 30 minutes. Electrophoresis was continued for six hours without exceeding 300 V. Both stain recipes for PGI and 6-PGD were prepared as directed in Arulsekar and Parfitt (1986) at a volume of 50 ml per gel slice.

The gels were scored following the general procedures of Beaver et al. (1995). Band mobility and allelic designations of *P. serotina* isozyme samples were compared to out group controls as reported by Beaver et al. (1995).

SEQUENCE TAGGED SITE AMPLIFICATION

DNA Isolation and Quantification

Procedures for extraction and quantification of total DNA from black cherry seedlings were as reported above.

Sample Amplification and Fragment Resolution

In total, eight Sequence Tagged Sites (STSs) primer pairs were used to identify informative markers. Optimization of PCR conditions for each primer pair was done by using a temperature gradient on a Robocycler (Stratagene, La Jolla, CA). PCR conditions for each primer pair and STS are listed in Table 5. For each 25µl PCR reaction, the following reagents were used: 15.37µl autoclaved ddH_20, 2.5µl 10x PCR buffer, 1.0µl 25 mM $MgCl_2$, 2.0µl 10x dNTPs, 1.0µl forward primer, 1.0µl reverse primer, 1.0µl of 50ng/µl diluted DNA template and 0.13µl Taq polymerase. Three SSR amplifications were attempted using one primer pair from sour cherry, GA34, and primer pairs from sweet cherry, PS12AO2 and PS08E08 (Appendix C). Primer pairs B3D5, B10B9, B6B1, B10H3, and B4G3 of peach origin (Appendix C) were also used.

Visualization of amplified products was resolved on 6% polyacrylamide gels run on 80v for 2 hours and stained using the Silver Sequence™ staining system (Promega, Madison, WI). Fragment length patterns were scored against a 10 bp ladder (Gibco, cat. #10821-015, Fredrick, MD).

RESULTS

Chloroplast Amplification

Amplification of the chloroplast nested [AB] fragment between the non-coding region between the trnT and trnL genes in *Prunus* was successful in *P. serotina*, indicating that this region within the chloroplast genome is conserved across cherry species. As expected, only one fragment was amplified in each reaction (Table 5, Figure 3). Amplification of the chloroplast non-coding region identified fragments of three different lengths, 274 bp, 280 bp, and 250 bp. All Michigan selections (MI 1-10), Mexican selections (PH, TI, P2 and P3) and Ecuador *P. serotina* selections (Ecu A, E, F, and H) had the 274 bp fragment (Table 5, Figure 3). Two of the three samples from Mexican accession RG had one fragment of 250 bp. The third sample from the RG collection family exhibited a fragment of 280 bp. One Ecuadorian accession, Ecu D, also had the 280 bp fragment.

The 280 bp fragment exhibited by the Ecuadorian accession Ecu D is also not unexpected because Ecuadorian accessions were probably derived from multiple maternal material as well (Appendix B). Morphologically, the RG and Ecu D families were indistinguishable from other collection families and subsequent assays with isozymes and STSs show no further deviation of these accessions from the rest of the Capulin gene pool. Therefore, cpDNA divergence is most likely due to collection of open pollinated seed from multiple maternal trees. Resolution at 280 bp for both Ecu D and RG supports the theory that Mexican and Ecuadorian *P. serotina* still share much of their genetic background with one another, despite geographic isolation between the two.

Amplification of the 274 bp fragment suggests that collection families from Michigan, Mexico and Ecuador share a common gene pool, despite the limited geographic sampling from the Northern *P. serotina*. Of course, to get a more accurate view of the true diversity within this species, a larger sampling of Northern black cherry from various geographic locations is necessary.

Isozyme Analysis

Two loci were resolved well and exhibited good activity for phosphoglutose isomerase, *Pgi*-1 and *Pgi*-2 (Figure 4). The *Pgi*-1 locus was monomorphic for the putative allele *Pgi*-1^{105} for all *P. serotina* progeny tested. *Pgi*-2 locus exhibited three different alleles in the *P. serotina* selections tested, *Pgi*-2^{100}, *Pgi*-2^{80} , and *Pgi*-2^{70} . *Pgi*-1^{105} and *Pgi*-2^{80} were found in both the *P. serotina* selections as well as the reference selections *P. avium* cv. Emperor Francis, *P. cerasus* cvs. Csengodi, 'Montmorency' and Rheinische Schattenmorelle. For *Pgi*-2, the *P. serotina* selections either exhibited one or two putative alleles per locus (Figure 5): (1) *Pgi*-2^{100} and *Pgi*-2^{80} and the associated heterodimer (Pattern 1), (2) *Pgi*-2^{100} and *Pgi*-2^{70} and the associated heterodimer (Pattern 2) or (3) only the *Pgi*-2^{100} allele (Pattern 3). All three allelic patterns encoded at the Pgi-2 locus were exhibited by selections of *P. serotina* from all three of the geographic collections, Michigan, Mexico and Ecuador (Figures 4 and 5).

Two loci for 6-phosphoglutonate dehydrogenase, 6-*Pgd*-1 and 6-*Pgd*-2 resolved clearly in Mexican and Ecuadorian *P. serotina* progeny (Figures 6 and 7). Unfortunately, Michigan progeny assayed for 6-PGD repeatedly failed to resolve bands with clarity and therefore scoring the bands was not possible. For the *P. serotina* selections assayed, two putative alleles at each locus were identified, 6-*Pgd*-1^{105} and 6-*Pgd*-1^{94}, and 6-*Pgd*-2^{88}

and 6-*Pgd*-2^{66} (Figure 7). None of these putative alleles were found to be in common with reference *P. cerasus*, *P. avium*, and *P. cerasus* x *P. canescens* selections. Banding patterns of 6-PGD could differentiate enzymes extracted from *P. serotina* from those extracted from sweet and sour cherry. All P. *serotina* progeny tested exhibited the 6-*Pgd*-1^{105} and 6-*Pgd*-2^{66} putative alleles. However, some selections were heterozygous at both loci (Pattern 1, Figure 7), heterozygous at 6-Pgd-1 (Pattern 2, Figure 7) or heterozygous at 6-Pgd-2 (Pattern 3, Figure 7). Progeny from both Mexico and Ecuador exhibited all three patterns for 6-PGD. Diagnostic alleles were only found in one non-*P. serotina* reference group. In this case, GI 148-1 can be distinguished from its half sibling, GI 148-2 (Figure 7).

STS Analysis

A summary of informative and non-informative primer pairs from sweet and sour cherry and peach on *P. serotina* selections can be found in Table 6. Of the eight sequence tagged site (STS) marker systems attempted, two primer sets failed to amplify fragments in *P. serotina*. Both peach derived primer sets B3D5 and B10B9 repeatedly failed to yield fragments. Two primer pairs, one derived from peach (B4G3) and one derived from sweet cherry (PS08E08) exhibited monomorphic banding patterns across *P. serotina* from Michigan, Mexico and Ecuador. Primer set B4G3 yielded a 166 bp fragment and PS08E08 exhibited a 138 bp fragment for all *P. serotina* selections. Due to the lack of diversity in fragment sizes for these two primer pairs, they were deemed uninformative.

Four of the eight primer pairs tested were found to identify polymorphisms, one derived from sweet cherry (PS12A02), one derived from sour cherry (GA 34) and two derived from peach (B6B1 and B10H3). GA34 and PS12A02 amplify SSR containing

sequences, while B6B1 and B10H3 amplify AFLP-identified regions of the genome. Together, these four primer pairs resolved 54 putative alleles for the 66 *P. serotina* selections assayed (Table 6).

SSR Amplification with Sweet Cherry Derived Primers

Amplification with sweet and sour cherry derived primers was successful. These hypervariable regions (GA34 and PS12A02) are conserved between *P. avium* L. and *P. serotina*. Sour cherry derived primer pair GA34 amplified a total of fourteen fragments ranging from 140-174 bp in length (Appendix C and Table 6). A maximum of two fragments were amplified per individual sampled. Of the fourteen putative alleles, one (140 bp) was specific to Ecuador, two (150 and 160 bp) were specific to Mexico, and two fragments were identified only in Michigan *P. serotina* selections (170 and 174 bp) (Figure 10 and 11). Two fragments (146 and 162 bp) were shared between Michigan and Mexico gene pools only. Fragments 144, 154, 158, 160, 164, and 168 bp were shared among the Mexican and Ecuadorian accessions only. One fragment, 148 bp, was found in all three gene pools. No putative alleles were shared among the Michigan and Ecuador germplasm without also being present within the Mexican gene pool (Figures 8 and 9).

Primer pair PS12A02 was also highly informative, producing twelve putative alleles with a maximum of four fragments amplified per accession (Table 6). For this primer set, only four fragments were specific to any one particular gene pool: 164, 172, 176, and 178 bp all identified only Michigan selections. Four fragments were shared between Mexico and Michigan (150, 156, 162, and 168 bp) while three fragments were shared between Mexico and Ecuador (154, 160, and 170 bp) (Figures 8 and 10). Only fragment 148 bp was shared among all three gene pools. Again, no putative alleles were

shared between Ecuador and Michigan selections without also being present within the Mexico gene pool.

STS Amplification with Peach Derived Primers

Peach primer set B6B1 resolved the most putative alleles with nineteen in total, ranging from 170 to 230 bp in length (Table 6, Figures 11 and 12). A maximum of four fragments were amplified for all *P. serotina* progeny assayed. None of the fragments amplified were specific to Michigan or Ecuador gene pools. However, five alleles were unique to Mexican accessions (176, 196, 214, 226, and 228 bp). Two alleles were shared between Mexico and Ecuador *P. serotina* (202 and 222 bp), while five alleles (186, 188, 192, 210 and 230 bp) were shared between Mexico and Michigan *P. serotina* selections. All together, seven putative alleles were shared among all three gene pools: 170, 180, 190, 200, 216, 220, and 224 bp. Once again, no putative alleles were exclusive to the Michigan and Ecuador selections without also being present within the Mexican *P. serotina* gene pool.

Peach derived primer set B10H3 also successfully amplified fragments from all *P. serotina* selections from Michigan, Mexico and Ecuador. Nine putative alleles were resolved using this primer set, with a maximum of two fragments per sample (Table 6, Figures 11 and 13). Using this primer set, three putative alleles identified specific gene pools: 130 bp (Michigan), 150 bp (Mexico) and 132 bp (Ecuador). No fragments were shared among all three gene pools. Four alleles were shared between Mexican and Ecuador *P. serotina* selections (138, 140, 142, and 144 bp). Only two fragments were exclusively shared between Mexico and Michigan *P. serotina* selection, 146 and 152 bp. As with the previous STS markers, no putative alleles were found to be exclusively

shared between the Michigan and Ecuador *P. serotina* selections without also being present within the Mexican germplasm.

A total of nine STS fragments were shared among all three geographically distinct groups. Thirteen fragments were shared among the Michigan and Mexican groups, while fifteen were shared exclusively between the Mexican and Ecuadorian groups. Of all the informative STS fragments, none of the putative alleles were shared between the Michigan and Ecuadorian *P. serotina* families without also being present within the Mexican families. Each geographically distinct group exhibited putative alleles which were diagnostic to their group (7 Michigan, 8 Mexican, and 2 Ecuadorian fragments). Although all three geographically distinct groups of P. serotina share a large number of putative alleles, amplified fragments which identified progeny to Mexican, Michigan, or Ecuadorian groupings were common in the STS markers. However, none of the putative alleles assayed could be used to identify progeny trees to their respective collection families such as RG or Ecu E.

Table 3. Number of open-pollinated *P. serotina* seedlings per family assayed for cpDNA fragments, 6-PGD, PGI, and sequence tagged sites (STSs).

P. serotina **Family Origin**	**Family**	**cpDNA Fragment**	**6-PGD**	**PGI**	**STSs**
MEXICO	RG	3	5	5	10
	PH	3	5	5	10
	P1	-	5	5	-
	P2	3	5	5	10
	P3	3	5	5	10
	TI	3	5	5	10
	ARG	-	5	5	-
	U2	-	5	5	-
	U3	-	5	5	-
	J1	-	5	5	-
	J3	-	5	5	-
ECUADOR	Ecu A	1	3	3	3
	Ecu D	1	2	2	2
	Ecu E	1	1	1	1
	Ecu F	1	2	2	2
	Ecu H	1	2	2	2
MICHIGAN	MI	3	-	10	5
	Beal	1	-	-	1

Table 4. Optimized PCR conditions for each primer pair used to amplify a chloroplast non-coding region and nuclear sequence tagged sites (STSs) in *P. serotina* from Michigan, Mexico, and Ecuador.

Primer Pair ID	Pre Cycle Denature Temp/Time	CYCLE Denature Temp/Time	CYCLE Annealing Temp/Time	CYCLE Extension Temp/Time	Number of Cycles	Post Cycle Extension Temp/Time
AB [AB]	94°/ 5 min	94°/ 1 min	56°/ 1 min	72°/ 1min	25	72°/ 5 min
GA34 PS08E08 B6B1 B10H3	94°/ 5 min	94°/ 1 min	60°/ 1 min	72°/ 1.5 min	25	72°/ 5 min
B4G3	94°/ 5 min	94°/ 1 min	58°/ 1 min	72°/ 1.5 min	25	72°/ 5 min
PS12AO2 B3D5 B10B9	94°/ 5 min	94°/ 1 min	56°/ 1min	72°/ 1.5 min	25	72°/ 5 min

Table 5. Fragments amplified from successive PCR reactions on a chloroplast non-coding region [AB] from *P. serotina* selections from Michigan, Mexico and Ecuador.

Origin of Selections	Selection ID	No. Samples Assayed	No. Bands Amplified Per Lane	No. Bands Resolved at 250bp	274bp	280bp
MEXICO	RG	3	1	2	-	1
	PH	3	1	-	3	-
	TI	3	1	-	3	-
	P2	3	1	-	3	-
	P3	3	1	-	3	-
ECUADOR	Ecu A	1	1	-	1	-
	Ecu D	1	1	-	-	1
	Ecu E	1	1	-	1	-
	Ecu F	1	1	-	1	-
	Ecu H	1	1	-	1	-
MICHIGAN	MI	3	1	-	3	-
	Beal	1	1	-	1	-

Table 6. Summary of informative and non-informative primer pairs derived from sweet cherry and peach on *P. serotina* selections from Michigan, Mexico and Ecuador.

Primer Pair Plant Source	Primer Name F or R	Lab Code	Amplification Temperature	Maximum No. Bands per Sample	Total No. Bands For Each Primer Pair
SWEET CHERRY	GA34 F	AI 20	60°	2	14
	GA34 R	AI 19	60°		
	PS12AO2 F	AI 24	56°	4	12
	PS12AO2 R	AI 25	56°		
	PS08E08 F	AI 32	60°	1	1
	PS08E08 R	AI 33	60°		
PEACH	B3D5 F	AI 38	56°	0	0
	B3D5 R	AI 39	56°		
	B10B9 F	AI 40	56°	0	0
	B10B9 R	AI 41	56°		
	B6B1 F	AI 42	60°	4	19
	B6B1 R	AI 43	60°		
	B10H3 F	AI 44	60°	2	9
	B10H3 R	AI 45	60°		
	B4G3 F	AI 46	58°	1	1
	B4G3 R	AI 47	58°		

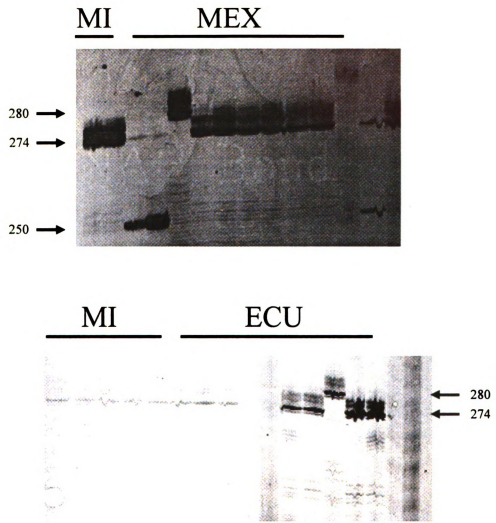

Figure 3. Amplified non-coding regions of *P. serotina* chloroplast genome resolved on 6% polyacrylamide gels and stained with Silver Sequence™ Stain. Gels are oriented with loading origin at the top of each photograph.

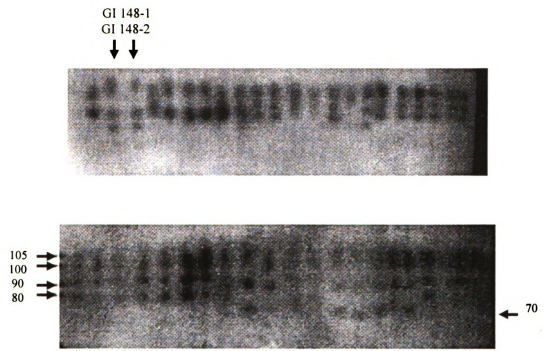

Figure 4. *Pgi*-1 and *Pgi*-2 loci patterns in *P. serotina* samples from Michigan Mexico, and Ecuador. Gels are oriented with the sample loading origin at the bottom of each photograph. Selected loci assigned alleles and out groups GI 148-1 and GI 148-2 are labeled with arrows.

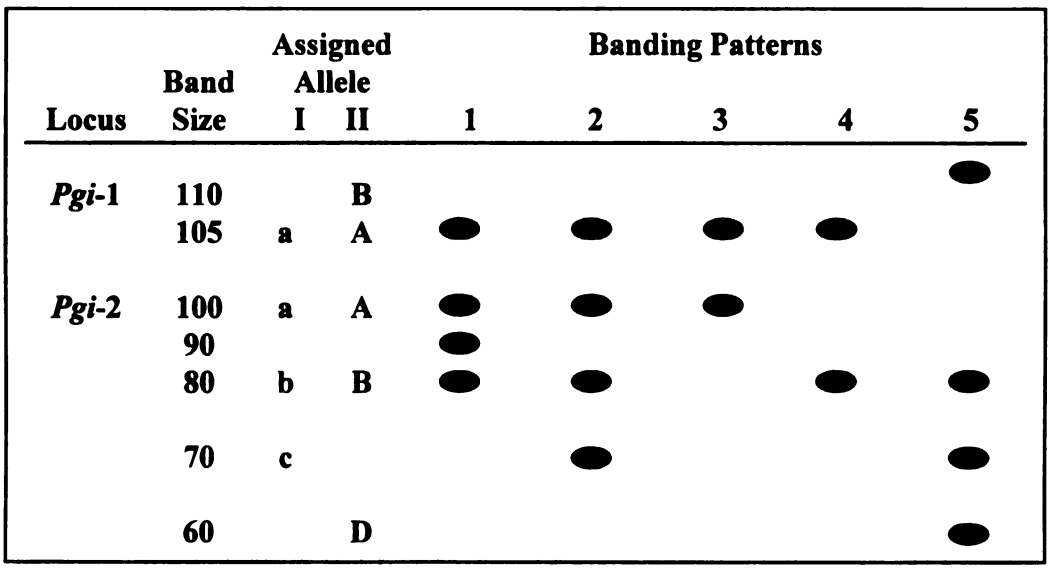

Figure 5. Zymogram representing patterns exhibited by *P. serotina* selections from Michigan, Mexico and Ecuador and out group controls assayed for Phosphoglucose isomerase (PGI). Alleles I represent putative alleles for *P. serotina*. Alleles II represent putative alleles for out group controls. Pattern 1: Michigan (MI), Mexican (U2, ARG, P3, RG, U3, J1, P1, TI, PH, and J3), and Ecuadorian (Ecu A-H) *P. serotina* and EB (Erdi Botermo). Pattern 2: Michigan (MI), Mexican (P2, J1, P1, TI and U3), and Ecuadorian *P. serotina* (Ecu A-H). Pattern 3: Michigan (MI), Mexican (U3 and RG) and Ecuadorian *P. serotina* (Ecu A-H). Pattern 4: Emperor Francis (EF), Csengodi, Montmorency, and Rheinische Schattenmorelle (RS). Pattern 5 was only exhibited by GI 148-1 and GI 148-2.

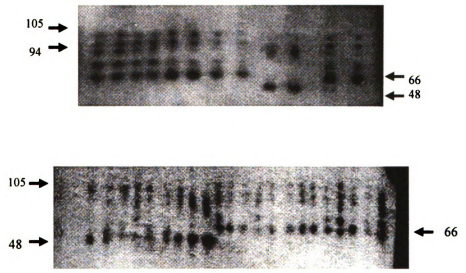

Figure 6. 6-*Pgd*-1 and 6-*Pgd*-2 loci patterns in *P. serotina* samples from Michigan Mexico, and Ecuador. Gels are oriented with the sample loading origin at the bottom of each photograph. Selected loci assigned alleles are labeled with arrows.

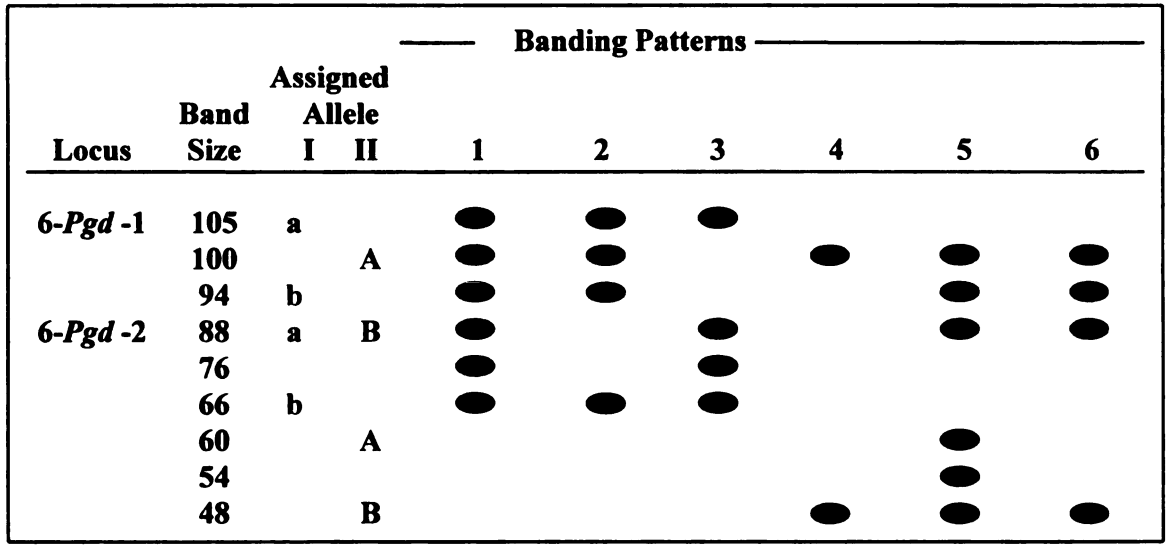

Figure 7. Zymogram representing banding patterns exhibited by *P. serotina* selections from Mexico and Ecuador and out group controls assayed for 6-phosphogluconate dehydrogenase (6-PGD). Alleles I represent putative alleles for *P. serotina*. Alleles II represent putative alleles for out group controls. Banding pattern 1: Mexican (TI, P2, P1, P3, U2, U3, ARG, J3, PH, RG, and J1) and Ecuadorian (Ecu A-H) *P. serotina* accessions. Pattern 2: Mexican (P1, ARG, J3 and J1) and Ecuadorian (Ecu A-H) accessions. Pattern 3: Mexican (P3 and P1) and Ecuadorian (Ecu A-H) accessions. Pattern 4: Emperor Francis (EF). Pattern 5: Csengodi and GI 148-2. Pattern 6: GI 148-1, Montmorency, Rheinische Schattenmorelle (RS) and Erdi Botermo (EB).

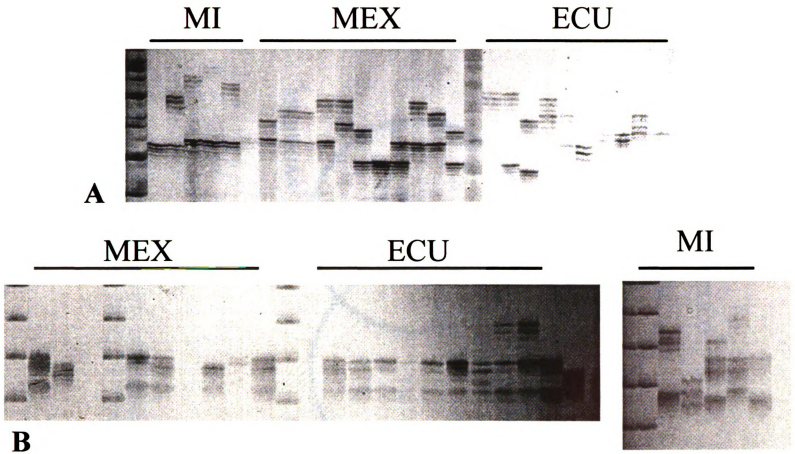

Figure 8. Photographs of 6% polyacrylamide gels resolving informative STS markers from sour and sweet cherry. Putative alleles for Michigan, Mexico and Ecuador *P. serotina* accessions resulting from amplification with primer pair GA 34 (A) and primer pair PS12A02 (B).

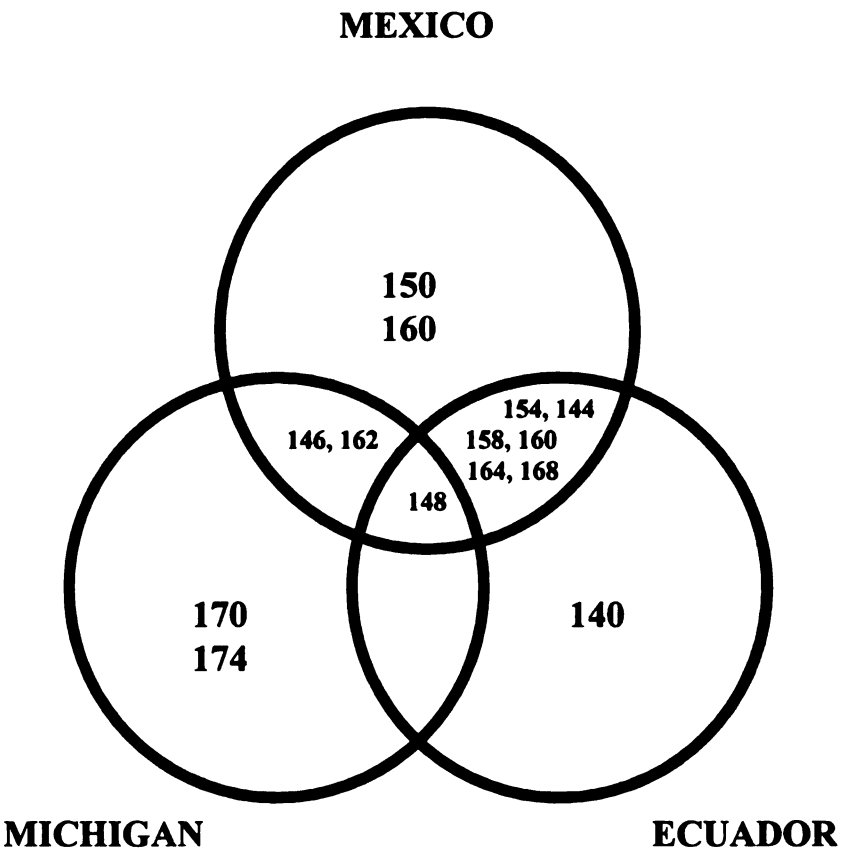

Figure 9. Putative alleles identified by STS GA34 for Michigan, Mexico and Ecuador *P. serotina* selections. Putative allele numbers correspond to amplified band sizes (bp).

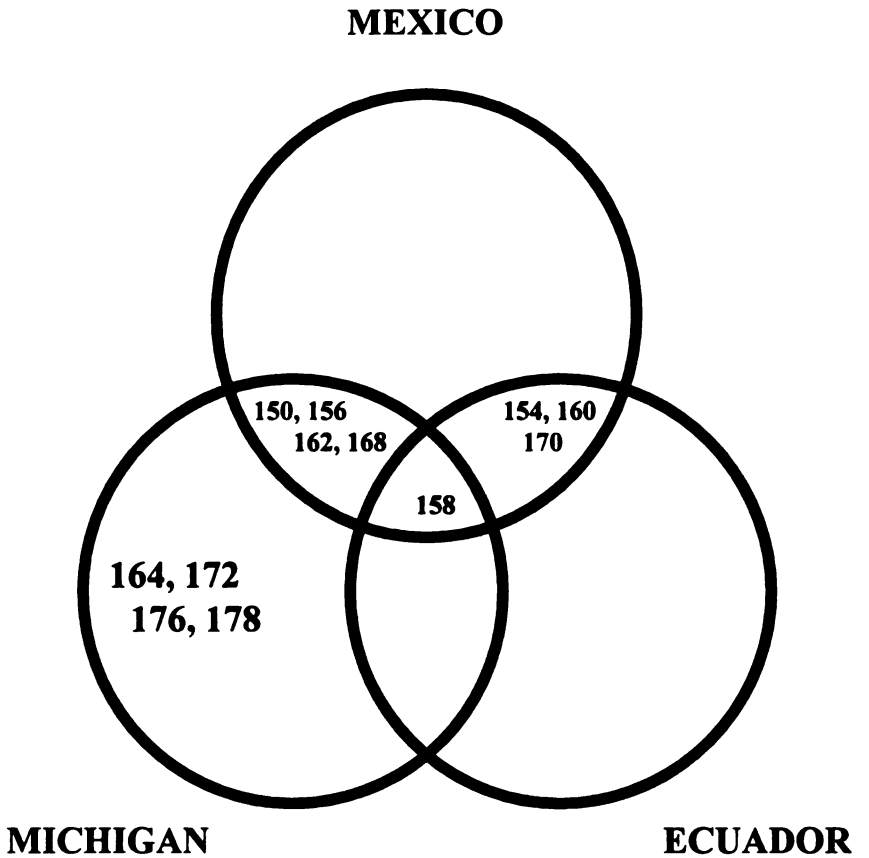

Figure 10. Putative alleles identified by STS PS12A02 for Michigan, Mexico and Ecuador *P. serotina* selections. Putative allele numbers correspond to amplified band sizes (bp).

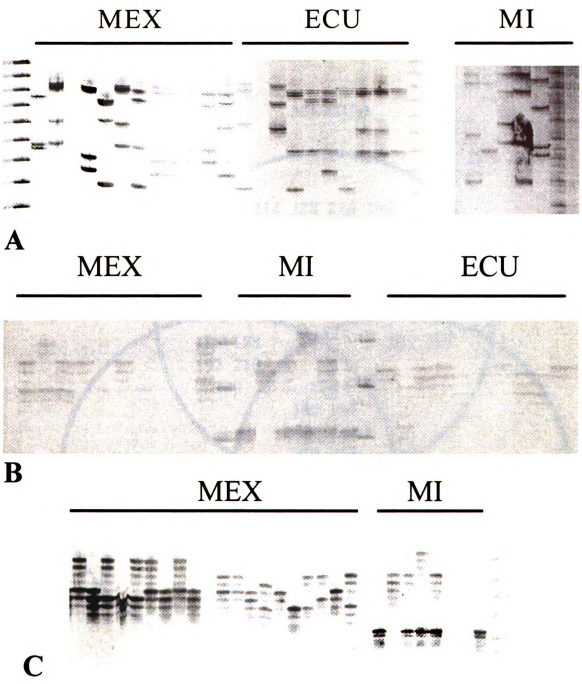

Figure 11. Photographs of informative STS marker systems from peach resolved on 6% polyacrylamide gels. Putative alleles for Michigan, Mexico and Ecuador *P. serotina* accessions resulting from amplification with primer pair B6B1 (A) and B10H3 (B&C).

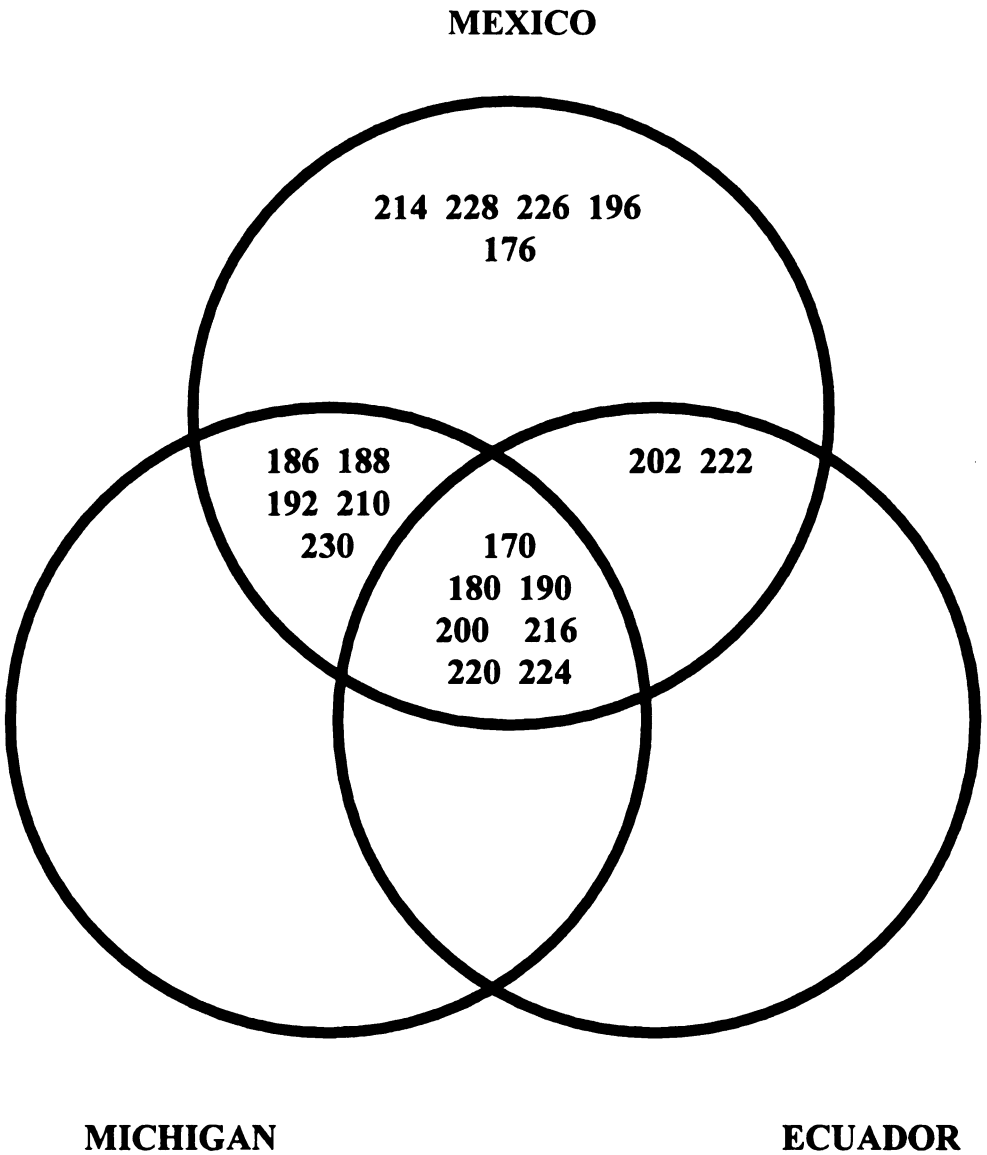

Figure 12. Putative alleles identified by STS B6B1 for Michigan, Mexico and Ecuador *P. serotina* selections. Putative allele numbers correspond to amplified band sizes (bp).

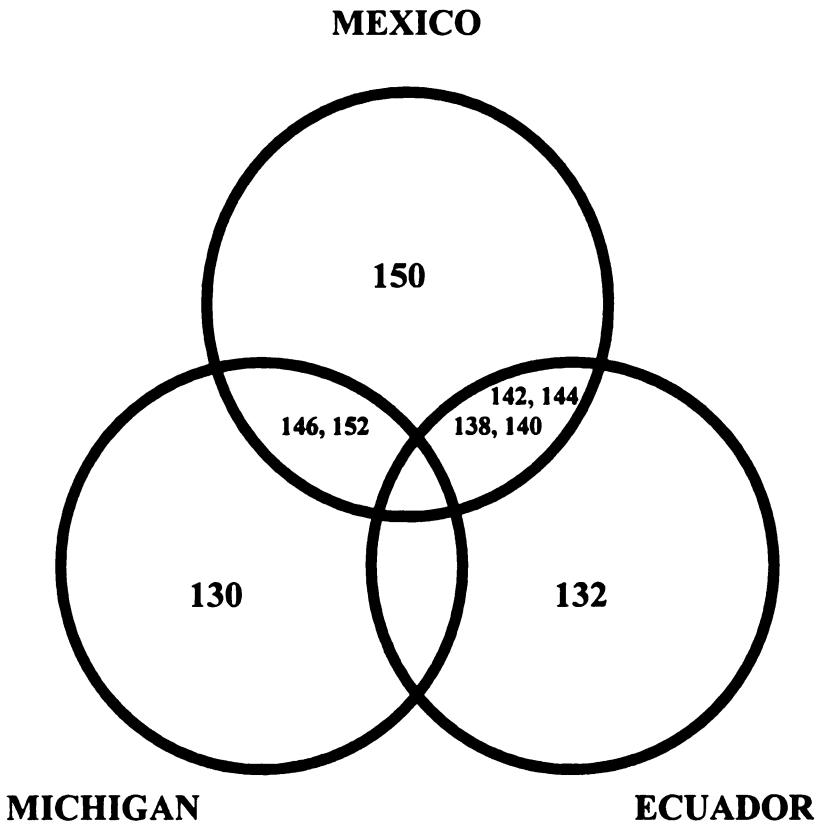

Figure 13. Putative alleles identified by STS B10H3 for Michigan, Mexico and Ecuador *P. serotina* selections. Putative allele numbers correspond to amplified band sizes (bp).

DISCUSSION

Evidence of shared gene pool among the Mexican, Michigan, and Ecuadorian germplasm is evident, based upon the similar patterns and putative alleles identified by the chloroplast and nuclear markers, respectively. Even with limited sampling from Ecuador and Michigan, there is evidence that these geographically distinct populations, while sharing a common gene pool, exhibit divergence which likely has resulted from geographic and ecological adaptation, selection pressures and domestication events in Central America.

Since cpDNA is maternally inherited in *Prunus*, it was expected that all the open-pollinated seedlings from one family would have the same chloroplast fragment. However, seedlings from the Mexican family RG exhibited different fragment sizes (250 bp and 280 bp). Two different fragment sizes exhibited by Mexican family RG can be explained by field collection procedures. For the RG family, open-pollinated seed was collected prior to my arrival in Mexico (Appendix A). Until cpDNA results, it was assumed that the open-pollinated seed was collected from only one maternal parent tree. However, the cpDNA data suggests that multiple maternal parents were sampled for this family.

The 280 bp fragment exhibited by the Ecuadorian accession, Ecu D, may have resulted because Ecuadorian accessions were probably also derived from multiple maternal parents (Appendix B). Morphologically, the RG and Ecu D families were indistinguishable from other collection families and subsequent assay with isozymes and STSs show no further deviation of these accessions from the rest of the Capulin gene

pool. Therefore, cpDNA divergence is most likely due to collection of open-pollinated seed form multiple maternal trees and not due to sampling of other *Prunus* species.

Nested [AB] chloroplast primers designed based upon *P. cerasus* sequence identified three different length fragments in *P. serotina*. The most common fragment had a length of 274 bp. This may be the most "ancestral fragment," and the other polymorphisms of 250 bp and 280 bp may have resulted from a 24 bp deletion even(s) and a 6 bp addition event(s), respectively. Such insertion and deletion polymorphisms have been reported to account for a substantial fraction of intraspecific cpDNA variation (Zurawski and Clegg, 1987). Interestingly, two of the three fragments amplified in *P. serotina* (274 and 250 bp) are similar in length to those identified in French sweet cherry selection (FPA 276bp) and in sweet, ground and sour cherry selections (249 bp) respectively (Brettin, et al., 1999). Sequence data for the *P. serotina* fragments would be necessary for comparison with sour cherry.

The common 280 bp chloroplast fragment for both Ecu D and RG supports the theory that Mexican and Ecuadorian *P. serotina* still share a similar genetic background wit one another, despite their geographic isolation. Amplification of the 274 bp fragment in the families from Michigan, Mexico and Ecuador suggest that germplasm from these regions share a common gene pool. Of course, to get a more accurate view of the true diversity within this species, a larger sampling of Northern *P. serotina* from various geographic and ecological locations is necessary.

Nuclear markers also identified unique putative alleles among the collection families form Michigan, Mexico and Ecuador. As expected, isozymes exhibited less diversity than the non-coding hypervariable regions of the nuclear genome amplified by

the PCR based STS marker systems. Putative isozyme alleles identified were conserved across all three geographical *P. serotina* groupings. Isozyme data supports the theory that *P. serotina* from Michigan, Mexico and Ecuador remain genetically similar on the protein level, despite their morphological differences.

Successful amplification of nuclear regions using primer sets derived from distantly related *Prunus* species has been demonstrated indicating that these primer sequences are conserved. However, not all primer sets were successful. For example, two of the six peach primer sets failed to amplify and fragment, two others produced only monomorphic fragments, and the remaining primer sets, B6B1 and B10H3 were the only informative primer pairs. None the less, our results suggest that useful *P. serotina* STS primer pairs can be selected from those available for peach and cherry.

STS marker systems were the most informative for this diversity analysis due to the relatively large number of putative alleles identified among the *P. serotina* selections. This was expected since non-coding DNA regions, particularly short repeat regions, have a higher occurrence of insertion and deletion events. Only STS markers were unique for the different geographic groupings. No more than four putative alleles per progeny assayed were amplified by the STS markers. A maximum of four alleles would be expected since *P. serotina* is a tetraploid and four alleles would represent the maximum number of alleles at two duplicate loci.

CONCLUSIONS

This is a preliminary study of the genetic diversity found within *P. serotina* from Michigan, Mexico and Ecuador. Its primary application is to derive baseline molecular data and identify primer sets that may be useful for more extensive and comprehensive inquiries in the future. The conclusions made are based upon an extremely limited sample sizes taken from specific regions of Michigan, Mexico, and Ecuador. No broad-based conclusions should be made from this data without further investigation.

Of the marker systems assayed in this study, the STS nuclear markers provided the most diversity of putative alleles within the *P. serotina* selections. The Mexican *P. serotina* germplasm was the most diverse geographical grouping, sharing many common alleles with the Michigan and Ecuadorian germplasm. Despite the similar gene pools, however, there does appear to be some genetic divergence between Michigan and Ecuadorian germplasm, most likely due to ecological and geographic adaptation, selection and domestication events. This is supported by chloroplast and STS marker data. Although this diversity study is preliminary due to the small amount of germplasm, the data does suggest that he molecular markers identified would provide a useful starting point to investigate the evolution and domestication of the large fruited Capulin cherries.

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LITERATURE CITED

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APPENDICES

APPENDIX A

COLLECTION INFORMATION FOR MEXICAN *P. SEROTINA* SELECTIONS

The following tables are a summary of information collected on *P. serotina* subsp. *capulin* species collected in Mexico during August 12-17, 1996. Collection information, physical data, as well as cultural notes are included. Twelve accessions were collected as open-pollinated seed from *P. serotina* trees growing in various areas northeast of Mexico City. Collected seeds were equally divided between the Michigan State University sour cherry breeding program and the collaborating lab at the Universidad de Queretaro, directed by Dr. Salvador Perez. At the end of this appendix are listed the *P. serotina* contacts in Mexico.

Table 7. Collection information for Mexican *P. serotina* selections.

Family ID	Collection Location	Date	Community	Cultivation Status	Soil	Elevation in meters	Aprox. Age (yr)	Height in feet
J1	Jerahuaro	8/12/96	Fence line	Fence line	Sand	2,400	17	30
J2	Jerahuaro	8/12/96	Fence line	Fence line	Sand	2,400	20	35
J3 [a]	Jerahuaro	8/12/96	Plum Orchard	Fence line	Sand Loam	2,400	Unknown	NA
P1	Los Pablos Peach Orchard	8/13/96	Along Fence line	Wild	Clay Loam	2,000	70	43
P2	Los Pablos Peach Orchard	8/13/96	Edge of Corn field	Wild	Clay Loam	2,000	80	50
P3	Los Pablos Peach Orchard	8/13/96	Along Peach Orchard	Wild	Clay Loam	2,000	50	35
TI [a]	La Esperanza	8/13/96	Peach Orchard	Wild	Clay Loam	2,200	Unknown	NA
U2	Uruapan	8/14/96	Backyard Garden	Home Cultivation	Clay Loam	1,690	30	33
U3	Uruapan	8/14/96	Backyard Garden	Home Cultivation	Clay Loam	1,690	15	13
ARG	Uruapan	8/14/98	Fence line	Wild	Clay Loam	1,700	20	30
RG [a]	Raul Garcia's Plum Orchard in Jerahuaro	8/14/98	Plum Orchard	GRAFTED MATERIAL	Sand Loam	2,400	Unknown	NA
PH	Puebla Huejotzingo	8/15/96	Fence line	Wild	Sand Loam	2,400	5	9

[a] Selections were collected by colleagues of Salvador Perez prior to my arrival in Mexico.

Table 7 (Continued). Collection information for Mexican *P. serotina* selections.

Family ID	Bloom Date	Harvest Date	Avgerage Summer Temp °C	Maximum High/Low Temp °C		Frost Free Days	Avg. Pit Length (cm)	Avg. Pit Width (cm)	Cultural Notes Including Local Uses of Capulin
J1	Feb.-March	June-July	18	27	-5	220	0.90	0.8	Dark fruit used to make brandy wine
J2	Feb.-March	June-July	18	27	-5	220	1.0	1.0	
J3 [a]	Feb.-March	June-July	18	27	-5	220	1.1	0.9	
P1	Mid Feb.	May-June	24	30	-3	320	1.0	0.8	Pits roasted like pistachios
P2	Mid Feb.	May-June	24	30	-3	320	1.0	0.8	
P3	Mid Feb.	May-June	24	30	-3	320	0.9	0.65	
TI [a]	Mid Feb.	May-June	26	30	-2	340	0.8	0.7	
U2	Early Jan.	Mid May	28	33	5	365	1.0	0.8	Used as a tonic to relieve coughs
U3	Early Jan.	Mid May	28	33	5	365	1.0	0.8	Tree given by parents, planted in backyard
ARG	Early Jan.	Mid May	28	32	2	350	0.9	0.7	Nursery across street from tree didn't know of the species.
RG [a]	Feb.-March	June-July	18	27	-5	210	1.0	0.9	GRAFTED MATERIAL
PH	Mid Feb.	June-July	25	29	-6	220	1.2	1.1	

[a] Selections were collected by colleagues of Salvador Perez prior to my arrival in Mexico.

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Salvador Arteaga
RE: propagated capulins
via: Salvador Perez

APPENDIX B

ECUADORIAN *P. SEROTINA* GERMPLASM INFORMATION

The following appendix contains information regarding the identification of Ecuadorian *P. serotina* Ehrh. Selections obtained from the Ecuadorian Germplasm Institute. Ten Ecuadorian accessions were obtained as open-pollinated seed, presumably collected from five trees, termed five collection 'families' (Ecu A 1-3, D 1-2, E 1, F 1-2, and H 1-2) [c/o Dr. Raul Castillo,, Departamento Nacional de Recursos Fitogeneticos y Biotecholigia, Casilla 17-01-340, Quito, Ecuador.] Uneven progeny from these five Ecuadorian families resulted from poor seed germination. Identification of accessions with the codes used at the Ecuadorian Germplasm Institute, as well as information regarding seed germination are included.

Table 8. Ecuadorian *P. serotina* germplasm identification and germination information.

Accession ID	Germplasm Institute Identification	No. Seeds Germinated
Ecu A	DPRU 2188	3 / 5
Ecu D	DPRU 2191	2 / 5
Ecu E	DPRU 2192	1 / 5
Ecu F	DPRU 2193	2 / 5
Ecu H	DPRU 2195	2 / 5

APPENDIX C

PRIMER PAIR SOURCES AND SEQUENCES

The following data table is a summary of information regarding the primer sets used in the chloroplast fragment amplifications and the sequence tagged site (STS) amplification of *P. serotina* selections for this thesis. Plant and laboratory sources of each primer set, including sequence information are included for each primer pair set.

Table 9. Primer pair sources and sequences.

Primer Pair Plant Source	Primer Name	Lab Code	Lab Source	Nuclear or cp	Sequence 5'.....3'
cp variable region	AB F	AI 16	Pierre	cp	CAT TAC AAA TGC GAT GCT CT
	AB R	AI 17	Taberlet	cp	TCT ACC GAT TTC GCC ATA TC
cp variable region	[AB] F	AI 22	Amy	cp	GCT GGA ACC GTT GAA TTC A
	[AB] R	AI 23	Iezzoni	cp	GGG GCA TAT CTA AGT ATA A
SWEET CHERRY	GA 34 F	AI 20	Amy	Nuclear	GAA CAT GTG GTG TGC TGG TT
	GA 34 R	AI 19	Iezzoni	Nuclear	TCC ACT AGG AGG TGC AAA TG
	PS12A02 F	AI 24	Graham	Nuclear	GCC ACC AAT GGT TCT TCC
	PS12A02 R	AI 25	King	Nuclear	AGC ACC AGA TGC ACC TGA
	PS08E08 F	AI 32	Graham	Nuclear	CCC AAT GAA CAA CTG CAT
	PS08E08 R	AI 33	King	Nuclear	CAT ATC AAT CAC TGG GAT G
PEACH	B3D5 F	AI 38	Albert	Nuclear	GGA TCA TTG AAC TAC GTC AAT CCT C
	B3D5 R	AI 39	Abbott	Nuclear	GGT TCA CTC TCA CAT ACA CTC GGA G
	B10B9 F	AI 40	Albert	Nuclear	CCA GTA GAT TTC AAC GTC ATC TAC A
	B10B9 R	AI 41	Abbott	Nuclear	GGT TCA CTC TCA CAT ACA CTC GGA G
	B6B1 F	AI 42	Albert	Nuclear	ACG CTA TGT CCG TAC CAT CTC CAT G
	B6B1 R	AI 43	Abbott	Nuclear	CAA CCT GTG ATT GCT CCT ATT AAA C
	B10H3 F	AI 44	Albert	Nuclear	GTC AAT GAG TTC AGT GTC TAC ACT C
	B10H3 R	AI 45	Abbott	Nuclear	AAT CAT AAC ATC ATT CAG CCA CTG C
	B4G3 F	AI 46	Albert	Nuclear	CAT TGT TCA TGG GAG GAA TT
	B4G3 R	AI 47	Abbott	Nuclear	AGA ACA TTC CTA AAG GAG CA

APPENDIX D

RAW SEQUENCE TAGGED SITE (STS) DATA

The raw sequence tagged site (STS) data of four primer pairs producing 54 putative alleles for 66 *P. serotina* selections follows. The matrix is separated into four primer pair selections.

Table 10. Data matrix for primer pair GA 34 containing 14 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	140	144	146	148	150	154	156	158	160	162	164	168	170	174
BEAL	0	0	0	1	0	0	0	0	0	0	0	0	0	0
MI1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
MI2	0	0	1	0	0	0	0	0	0	1	0	0	0	0
MI3	0	0	0	1	0	0	0	0	0	0	0	0	1	0
MI4	0	0	0	1	0	0	0	0	0	0	0	0	0	1
MI5	0	0	0	1	0	0	0	0	0	0	0	1	0	0
RG 1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
RG 2	0	0	0	0	0	0	0	0	1	0	0	0	0	0
RG3	0	1	0	0	0	0	0	0	0	0	0	0	0	0
RG4	0	1	0	0	0	0	0	0	0	0	0	0	0	0
RG5	0	0	0	0	0	0	0	0	1	0	0	0	0	0
RG6	0	0	0	0	0	0	0	0	1	0	0	0	0	0
RG7	0	0	0	0	0	1	0	0	0	0	0	0	0	0
RG8	0	1	0	0	0	0	0	0	1	0	0	0	0	0
RG9	0	0	0	0	1	0	0	0	1	0	0	0	0	0
RG10	0	0	0	0	1	0	0	0	1	0	0	0	0	0
PH1	0	0	0	1	0	1	0	0	0	0	0	0	0	0
PH2	0	0	0	1	0	0	0	1	0	0	0	0	0	0
PH3	0	0	0	1	0	0	0	0	0	1	0	0	0	0
PH4	0	0	0	0	0	1	0	0	0	1	0	0	0	0
PH5	0	1	0	0	0	1	0	0	0	0	0	0	0	0
PH6	0	1	0	0	0	0	0	0	0	0	0	0	0	0
PH7	0	1	0	1	0	0	0	0	0	0	0	0	0	0
PH8	0	0	0	1	0	0	0	0	0	1	0	0	0	0
PH9	0	0	0	1	0	0	0	1	0	0	0	0	0	0
PH10	0	1	0	0	0	1	0	0	0	0	0	0	0	0
P2 1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
P22	0	0	0	0	0	1	0	0	0	0	0	0	0	0
P23	0	0	0	0	0	0	0	0	0	0	1	0	0	0
P24	0	1	0	0	0	0	0	0	1	0	0	0	0	0
P25	0	1	0	0	0	0	0	0	1	0	0	0	0	0
P26	0	1	0	0	0	0	0	1	0	0	0	0	0	0
P27	0	1	0	0	0	0	0	0	0	0	0	0	0	0
P28	0	0	0	0	0	0	0	0	1	0	0	0	0	0
P29	0	1	0	0	0	0	0	0	0	0	0	0	0	0
P210	0	0	0	0	0	0	0	1	0	0	0	0	0	0
TI1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
TI2	0	0	0	0	0	1	0	0	0	0	0	0	0	0
TI3	0	0	0	1	0	0	0	0	1	0	0	0	0	0
TI4	0	0	0	0	0	1	0	0	0	0	0	0	0	0
TI5	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Table 10 (Continued). Data matrix for primer pair GA 34 containing 14 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	140	144	146	148	150	154	156	158	160	162	164	168	170	174
TI6	0	0	1	0	0	1	0	0	0	0	0	0	0	0
TI7	0	0	1	0	0	0	0	0	0	0	0	0	0	0
TI8	0	0	0	0	0	0	0	0	1	0	0	0	0	0
TI9	0	1	0	0	0	0	0	0	0	0	0	0	0	0
TI10	0	1	0	0	0	1	0	0	0	0	0	0	0	0
P31	0	0	0	0	0	0	1	0	0	0	0	0	0	0
P32	0	0	0	0	0	0	0	1	0	0	0	0	0	0
P33	0	0	0	0	0	0	0	0	1	0	0	0	0	0
P34	0	0	0	0	0	0	0	0	1	0	0	0	0	0
P35	0	0	0	0	0	0	0	1	0	0	0	0	0	0
P36	0	0	0	0	0	0	0	1	0	0	0	0	0	0
P37	0	0	0	0	0	0	0	0	1	0	0	0	0	0
P38	0	1	0	0	0	0	0	1	0	0	0	0	0	0
P39	0	0	0	0	0	0	0	1	0	0	0	0	0	0
P310	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ECUA1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
ECUA2	0	1	0	0	0	0	0	0	0	0	0	1	0	0
ECUA3	1	0	0	0	0	0	0	1	0	0	0	0	0	0
ECUE1	0	0	0	0	0	0	0	0	1	0	1	0	0	0
ECUD1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
ECUD2	0	0	0	0	0	1	0	0	0	0	0	0	0	0
ECUF1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
ECUF2	0	0	0	1	0	0	0	0	0	0	0	0	0	0
ECUH1	0	0	0	0	0	1	0	0	1	0	0	0	0	0
ECUH2	0	0	0	0	0	1	0	0	0	0	0	0	0	0

Table 11. Data matrix for primer pair PS12A02 containing 12 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	150	154	156	158	160	162	164	168	170	172	176	178
BEAL	1	0	1	0	0	0	0	0	0	0	0	0
MI1	0	0	0	1	0	0	0	0	0	0	1	0
MI2	0	0	0	1	0	1	0	0	0	0	0	0
MI3	0	0	1	0	0	0	1	1	0	1	0	0
MI4	0	0	0	1	0	0	1	1	0	0	0	1
MI5	0	0	1	0	0	0	0	1	0	0	0	0
RG 1	1	1	0	0	0	0	0	1	0	0	0	0
RG 2	1	0	0	1	1	0	0	0	0	0	0	0
RG3	1	0	1	1	0	0	0	0	0	0	0	0
RG4	1	0	0	1	1	0	0	0	0	0	0	0
RG5	1	0	1	0	0	0	0	0	0	0	0	0
RG6	0	1	0	0	0	0	0	0	0	0	0	1
RG7	0	1	1	1	0	0	0	0	0	0	0	0
RG8	0	0	1	0	0	0	0	0	0	0	0	0
RG9	1	0	1	0	0	0	0	0	0	0	0	0
RG10	0	0	1	0	0	0	0	0	0	0	0	0
PH1	0	1	0	0	1	0	0	0	0	0	0	0
PH2	0	1	0	0	1	0	0	0	0	0	0	0
PH3	0	1	0	0	1	0	0	0	0	0	0	0
PH4	0	1	0	0	1	0	0	0	0	0	0	0
PH5	0	0	0	1	0	0	0	0	0	0	0	0
PH6	0	1	0	0	1	0	0	0	0	0	0	0
PH7	0	1	0	1	0	0	0	0	0	0	0	0
PH8	1	0	1	1	0	0	0	0	0	0	0	0
PH9	0	1	0	1	0	0	0	0	0	0	0	0
PH10	0	0	0	1	0	0	0	0	0	0	0	0
P2 1	0	1	0	0	0	1	0	0	1	0	0	0
P22	0	0	1	0	0	1	0	0	1	0	0	0
P23	0	0	0	0	0	1	0	0	1	0	0	0
P24	0	0	0	0	1	0	0	0	1	0	0	0
P25	0	1	1	0	0	1	0	0	1	0	0	0
P26	0	1	0	0	1	0	0	0	1	0	0	0
P27	0	1	0	0	1	0	0	0	1	0	0	0
P28	0	0	0	0	0	1	0	1	0	0	0	0
P29	0	0	0	0	0	1	0	0	0	0	0	0
P210	0	0	0	0	1	0	0	0	0	0	0	0
TI1	0	0	1	1	0	0	0	0	0	0	0	0
TI2	0	0	1	1	0	0	0	0	0	0	0	0
TI3	0	1	0	1	0	0	0	0	1	0	0	0
TI4	0	1	1	1	0	0	0	0	0	0	0	0
TI5	0	1	1	1	0	0	0	0	0	0	0	0

Table 11 (Continued). Data matrix for primer pair PS12A02 containing 12 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	150	154	156	158	160	162	164	168	170	172	176	178
TI6	0	1	1	1	0	0	0	0	0	0	0	0
TI7	1	0	1	0	0	0	0	0	0	0	0	0
TI8	0	1	1	1	0	0	0	0	0	0	0	0
TI9	1	0	1	0	0	0	0	0	0	0	0	0
TI10	1	0	1	0	0	0	0	0	0	0	0	0
P31	0	1	0	1	0	0	0	0	0	0	0	0
P32	0	0	0	1	0	0	0	0	0	0	0	0
P33	0	0	0	1	0	0	0	0	0	0	0	0
P34	0	1	0	1	0	0	0	0	0	0	0	0
P35	0	1	0	1	0	0	0	0	1	0	0	0
P36	0	0	1	1	1	0	0	0	0	0	0	0
P37	0	0	1	1	0	0	0	0	0	0	0	0
P38	0	0	1	1	0	0	0	0	0	0	0	0
P39	0	0	1	1	0	0	0	0	0	0	0	0
P310	0	1	1	1	0	0	0	0	0	0	0	0
ECUA1	0	1	0	1	1	0	0	0	0	0	0	0
ECUA2	0	1	0	1	1	0	0	0	0	0	0	0
ECUA3	0	1	0	1	1	0	0	0	0	0	0	0
ECUE1	0	1	0	0	1	0	0	0	0	0	0	0
ECUD1	0	1	0	1	1	0	0	0	0	0	0	0
ECUD2	0	1	0	1	1	0	0	0	0	0	0	0
ECUF1	0	0	0	0	1	0	0	0	0	0	0	0
ECUF2	0	1	0	1	1	0	0	0	0	0	0	0
ECUH1	0	1	0	0	1	0	0	0	1	0	0	0
ECUH2	0	1	0	0	1	0	0	0	1	0	0	0

Table 12. Data matrix for primer pair B6B1 containing 19 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	170	176	180	186	188	190	192	196	200	202	210	214	216	220	222	224	226	228	230
BEAL	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
MI1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1
MI2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
MI3	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1
MI4	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
MI5	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
RG 1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0
RG 2	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
RG3	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
RG4	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
RG5	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1
RG6	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0
RG7	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1
RG8	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0
RG9	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
RG10	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
PH1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
PH2	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
PH3	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
PH4	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
PH5	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
PH6	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
PH7	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
PH8	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
PH9	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0
PH10	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0

Table 12 (Continued). Data matrix for primer pair B6B1 containing 19 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	170	176	180	186	188	190	192	196	200	202	210	214	216	220	222	224	226	228	230
P2 1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
P22	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
P23	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
P24	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
P25	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
P26	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
P27	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
P28	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
P29	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
P210	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
TI1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
TI2	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0
TI3	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1
TI4	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0
TI5	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
TI6	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
TI7	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0
TI8	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
TI9	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
TI10	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
P31	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
P32	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
P33	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
P34	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
P35	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
P36	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0

Table 12 (Continued). Data matrix for primer pair B6B1 containing 19 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	170	176	180	186	188	190	192	196	200	202	210	214	216	220	222	224	226	228	230
P37	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
P38	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
P39	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
P310	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0
ECUA1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
ECUA2	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
ECUA3	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0
ECUE1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
ECUD1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ECUD2	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
ECUF1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
ECUF2	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
ECUH1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0
ECUH2	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0

Table 13. Data matrix for primer pair B10H3 containing 9 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	130	132	138	140	142	144	146	150	152
BEAL	1	0	0	0	0	0	0	0	0
MI1	1	0	0	0	0	0	0	0	0
MI2	0	0	0	0	0	0	1	0	0
MI3	1	0	0	0	0	0	1	0	0
MI4	1	0	0	0	0	0	0	0	1
MI5	1	0	0	0	0	0	1	0	0
RG 1	0	0	0	0	0	1	0	0	0
RG 2	0	0	0	0	1	0	1	0	0
RG3	0	0	0	1	0	0	1	0	0
RG4	0	0	0	0	1	0	1	0	0
RG5	0	0	0	0	1	0	0	0	0
RG6	0	0	0	0	1	0	1	0	0
RG7	0	0	0	0	1	0	1	0	0
RG8	0	0	0	1	0	0	0	1	0
RG9	0	0	0	0	1	0	0	0	0
RG10	0	0	0	1	0	0	0	1	0
PH1	0	0	0	0	1	0	1	0	0
PH2	0	0	0	0	1	0	1	0	0
PH3	0	0	0	0	1	0	0	0	0
PH4	0	0	1	0	0	1	0	0	0
PH5	0	0	0	0	1	0	0	0	0
PH6	0	0	1	0	0	0	0	0	0
PH7	0	0	1	0	0	0	1	0	0
PH8	0	0	0	1	0	0	1	0	0
PH9	0	0	0	0	1	0	0	0	0
PH10	0	0	1	0	0	0	1	0	0
P2 1	0	0	0	1	0	0	1	0	0
P22	0	0	0	1	0	0	0	1	0
P23	0	0	0	1	0	0	1	0	0
P24	0	0	0	0	0	0	1	0	0
P25	0	0	0	1	0	0	0	1	0
P26	0	0	0	0	0	0	1	0	0
P27	0	0	0	1	0	0	0	1	0
P28	0	0	0	1	0	0	0	0	0
P29	0	0	0	0	0	0	1	0	0
P210	0	0	0	0	1	0	0	1	0
TI1	0	0	0	1	0	0	0	0	1
TI2	0	0	0	1	0	0	0	0	0
TI3	0	0	0	0	1	0	0	1	0
TI4	0	0	0	1	0	0	0	1	0
TI5	0	0	0	0	1	0	0	0	0

Table 13 (Continued). Data matrix for primer pair B10H3 containing 9 putative alleles numbered according to their corresponding fragment lengths for each of the 66 *P. serotina* selections assayed.

ID	130	132	138	140	142	144	146	150	152
TI6	0	0	0	1	0	0	0	0	1
TI7	0	0	0	1	0	0	0	0	0
TI8	0	0	1	0	0	0	1	0	0
TI9	0	0	0	0	1	0	0	1	0
TI10	0	0	0	0	1	0	0	1	0
P31	0	0	0	0	1	0	0	0	0
P32	0	0	0	0	1	0	0	1	0
P33	0	0	0	0	1	0	0	0	0
P34	0	0	0	1	0	0	0	1	0
P35	0	0	0	1	0	0	0	0	0
P36	0	0	0	1	0	0	0	1	0
P37	0	0	0	0	1	0	0	1	0
P38	0	0	0	0	1	0	0	0	0
P39	0	0	0	0	1	0	0	1	0
P310	0	0	0	0	1	0	0	0	0
ECUA1	0	0	0	0	0	1	0	0	0
ECUA2	0	1	0	1	0	0	0	0	0
ECUA3	0	0	0	0	0	1	0	0	0
ECUE1	0	0	0	0	0	1	0	0	0
ECUD1	0	0	0	0	1	0	0	0	0
ECUD2	0	0	0	0	1	0	0	0	0
ECUF1	0	0	0	0	0	1	0	0	0
ECUF2	0	0	1	0	1	0	0	0	0
ECUH1	0	0	0	0	0	1	0	0	0
ECUH2	0	0	0	0	0	1	0	0	0