Biogeography and biosystematics of plum curculio, *Conotrachelus nenuphar* (Herbst)/*Wolbachia* interactions

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Abstract

This research focused on the reproductive incompatibility and genetic differences between the two strains of plum curculio, *Conotrachelus nenuphar* (Herbst). Two molecular markers served as the basis for the strain distribution analysis of plum curculio and *Wolbachia* symbiont. One marker is the partial mitochondrial cytochrome oxidase gene subunit I (mtCOI) of plum curculio. Another marker is the *Wolbachia* Surface Protein (*wsp*) gene of *Wolbachia* associated with plum curculio. First, the reproductive compatibility of cross-populations mating in plum curculio was studied during the summers of 2004 and 2006. The results confirmed the reproductive incompatibility among plum curculio geographic populations. A unidirectional incompatibility was revealed in an approximate north and south transect of the range of plum curculio (4 x 4 two factorial design: NY, VA, FL, and WV): there was a significant low fertility in WV males mated with NY (40%) and VA (29%) females. The Florida population showed a different pattern: FL males have a significantly lower fertility with VA (46%) and WV (37%) females while FL females were compatible with all males from the four populations. The results of experiment 2 indicated that within the northern geographic area populations (3 x 3 two factorial design: NY, MA, and NJ) were compatible with each other. An opposite unidirectional reproductive incompatibility was revealed in the combination of NJ males with FL females, which showed a significant low fertility (47%). A bi-directional incompatibility occurred between FL and WV reciprocal cross mating. FL males mated with WV females and WV males mated with FL females, but with reduced fertility (26% and 21%, respectively) compared to fertility of within their population matings.

The genetic diversity among plum curculio populations from different geographic locations

was investigated using the partial mtCOI gene. A total of 50 samples from 10 populations were sequenced. PCR products were 863 bp in length. A total of 23 unique sequence haplotypes were found in the 50 samples tested. Haplotype G (n = 5), L (n = 12) and T (n = 13) comprised 60% of 50 samples. The nucleotide distances between those haplotypes ranged from 0.12% to 4.87%. Genetic distances between northern and southern group plum curculios range from 4.17% to 4.87%. Two distinct major clades were found, using three different phylogenetic analyses: 1) neighbor joining (NJ), 2) maximum-parsimony (MP), and 3) maximum-likelihood (ML). 100% bootstraps support the northern clade and the southern clade was strongly supported (100/100/86, NJ/MP/ML) as well. The mid-southern subclade within the southern clade was also strongly supported (70/82/71, NJ/MP/ML) and the far-southern subclade was supported in NJ tree (81%) but was not resolved in MP and ML trees. The mid-southern subclade included haplotypes from two NJ, Washington, VA (Ra), Blacksburg, VA (BL) and 50% of WV populations and the far-southern subclade included haplotypes from FL, GA, Whitethorne, VA (Ke), Troutville, VA (Bo) and another 50% of WV populations. The results suggested that the northern and the southern clade could correspond with the northern and southern strains, respectively, of plum curculio. In this study, the mtCOI sequence was highly informative as a molecular marker in that it was useful to distinguish *C. nenuphar* from northern and from southern geographic locations in the eastern United States. However, the number of generations per year of several geographic populations within the southern clade still needs to be determined.

The distribution of *Wolbachia* infection associated with plum curculio strains was investigated. 91 of 93 samples were infected by *Wolbachia.* Three unique *Wolbachia* strains were identified. The strains wCne1 and wCne2 (593 bp) were 97% identical, and their sequences were both 84% identical with wCne3 (590 bp). Degree of similarity with sequences in other *Wolbachia* strains is discussed. PCR - Restriction Fragment Length Polymorphism (RFLP) was used for superinfection detection. Of 93 samples, 15 (16.1%), 21 (22.6%), 19 (20.4%), 36 (38.7%) samples were infected by wCne1, wCne2, wCne1 plus wCne2, and wCne3, respectively. Only two (2.2%) samples had no infection. The wCne3 strain was always present as a single infection.

Therefore, current results suggest that *Wolbachia* strains approximate the distribution of plum curculio strains: the northern strain is infected with wCne1 and wCne2 strains in supergroup B, the southern strain is infected with wCne3 strain in supergroup A and the mid-Atlantic region is the convergence area. Compared with the haplotype distribution of plum curculio mtCOI gene, there was a closer relation of the mid-southern PC clade to the far-southern clade than to the northern clade. However, *Wolbachia* symbionts in mid-southern PC are more closely related to those in northern PC than to those in far-southern PC. The relationship of *Wolbachia* infection with reproductive incompatibility between plum curculio populations is also discussed.

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Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction and Literature Review

Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is a major pest of the fruit in eastern North America. There is a wide host range: apple, peach, nectarine, plum, cherry, apricot, pear and blueberry, all major fruit in eastern North America. So far, no effective monitoring strategy exists. The insect can cause almost all fruit to be injured if no control actions are taken.

There are two strains of plum curculio. Normally, the northern strain has an obligate winter diapause, and is a univoltine strain. The southern strain does not display the obligate diapause. It has two or three generations annually. Chapman (1938) made a distribution description of the two strains but it shows only an approximate range of the strains. In his map, the mid-Atlantic region is the convergence area of the two strains and Virginia contains both strains. Presence of a second generation may cause the imposition of a trade barrier by other states or other countries because larvae may be present in the fruit at harvest. In addition, the differing number of generations complicates the control of the pest in the area.

Currently, there are many studies focusing on the endosymbiotic bacterium *Wolbachia* because it may play a role in reproductive isolation and speciation. We suspect *Wolbachia* infection may cause the reproductive incompatibility known to exist between the two strains of plum curculio. However, the role of *Wolbachia* in plum curculio reproduction is still unknown.

In my research, I tried to refine a molecular method to distinguish the two strains of plum curculio. Two molecular markers were used in my research: partial mitochondrial cytochrome oxidase I gene (mtCOI) of plum curculio DNA and PCR analysis for *Wolbachia* Surface Protein (*wsp*) gene, which serve as the basis for a strain distribution analysis for plum curculio and *Wolbachia*. The analysis will aid in plum curculio management in Virginia fruit production and

the rest of the mid-Atlantic region. The trade barrier may be adjusted if the multivoltine strain does not exist in fruit-producing counties and monitoring will be increased if the multivoltine strain does exist in those areas. At the same time, *Wolbachia* analysis will enrich our knowledge of population ecology and biosystematics of plum curculio and may help for future control research.

Literature Review

Life history

The plum curculio is a native insect of North America, which occurs wherever fruit are grown east of the Rocky Mountains, especially apple and peach, the most important fruit produced in eastern North America. Many researchers have reported their findings about the life history of plum curculio because of its injurious nature. Chapman (1938) reviewed the plum curculio as an apple pest throughout eastern North America. The life history of plum curculio in different states and in different fruit were described, including on peaches in North Carolina (Leiby and Gill 1923) and Georgia (Snapp 1930), on apples in Virginia (Woodside 1935, Bobb 1952), Maine (Lathrop 1949), Ontario, Canada (Armstrong 1958), and Quebec, Canada (Racette et al. 1992), on plum in Ohio (Neiswander 1948), and on blueberry in North Carolina (Mampe and Neunzig 1967).

The egg is pearly white and normally elliptical in shape. It is about 0.4 mm wide and 0.6 mm long (Chapman 1938). There are four larval instars. When fully grown, the larva is a whitish, naked, legless grub, 6-9 mm in length (Chapman 1938). The pupa is cream-colored and is 4-7 mm long (Bobb 1952). The adult is 4-6 mm long and has a proboscis one-fourth the body length. It is brown-black in color and has patches and bands of short white and orange hairs (Bobb 1952).

Normally, the overwintered adults enter the orchard in early spring. The immigration time varies with the climate of the different areas. Plum curculio is highly responsive to climate fluctuations (Bobb 1952). The adults can be caught in early April in Virginia (Woodside 1935). The adults emerge from hibernation in mid-May in Quebec, Canada (Racette et al. 1992). When immature fruit are available, the weevils will start to oviposit in the fruit. Bobb (1952) reported the incubation period ranged from 3-12 days in Virginia, depending on temperature. The larvae feed in the fruit for approximately 15 days, and then leave. The mature larvae spend 1-3 weeks preparing a pupal chamber in the soil, and then pass their 10-day pupal period in this cell before emerging from the soil as adults. The adults can live a relatively long time. Bobb's data (1952) showed that adult curculios live an average of about 10 months and the longest duration was 22

months. The adults have a diapause in the winter. Chapman (1938) showed that adults overwinter under leaves, grass roots, dried grass, trash, honey suckle growth, pruning piles near or in the orchard, woodlots, brush land, fence rows and stone walls. Snapp (1930) and Bobb (1952) stated that hibernation occurs in the first 2.5 cm and 5 to 7.5 cm of the soil layer, respectively.

Importance

Plum curculio causes both early season and late season injury. Normally, early season injury is the most import injury. There are two types of injury in early season (Coli 1983): feeding injury and oviposition injury. Feeding injury consists of small round holes and usually is seen in association with oviposition injury. During oviposition, the female uses her snout to cut a crescent-shaped slit just below the oviposition site after inserting an egg. Oviposition scars appear on fruit as crescent or D-shaped corky areas at harvest if eggs fail to hatch or larvae die. Early season feeding injury appears on harvested fruit as raised, rounded, corky blemishes on the skin (Coli 1983). Plum curculio can cause 100% fruit damage if uncontrolled. On peach, plum curculio feeding and egg laying injury can induce fungal infection. Much of the loss to peaches from brown rot is indirectly due to plum curculio injury (Bobb 1952).

Plum curculio has a broad host range, including plants in the Rosaceae (Quaintance and Jenne 1912, Maier 1990) and in the Ericaceae (Stearns 1931, Mampe and Neunzig 1967, Polavarapu et al. 2004). The insect species originally attacks the fruit of wild plum (*Prunus* spp.), crab apple (*Malus angustifolia* (Aiton)), hawthorn (*Crataegus* spp.), but with introduction of large acreages of cultivated fruit, particularly apple (*Malus domestica* Borkh.) and peach (*Prunus persica* L.), the plum curculio became a major pest of these fruit (Bobb 1952). Armstrong (1958) reported that plum curculio attacks apricot (*Prunus armeniaca* L.), nectarine (*Prunus persica* variety *nectarina* L.), plum (*Prunus americana* Marshall), peach, cherry (*Prunus avium* (L.) L.), apple, pear (*Pyrus communis* L.) and gooseberry (*Ribes* spp.) in the Niagara Peninsula. Maier (1990) compared the host range of three apple-infesting weevil species. This study indicated that plum curculio had the

broadest host range. It infested 19 of 24 species of native and exotic rosaceous hosts. Hallman and Gould (2004) reported that plum curculio adults caused feeding damage to the majority of the fruit but they only oviposited in members of the Rosaceae. In this study, they exposed 22 fruit from 16 families to females of the plum curculio in Florida. However, they evaluated mostly subtropical and tropical fruit except major rosaceous fruit: apple, peach, plum and loquat. Jenkins et al. (2006) reported the first record of plum curculio in muscadine grapes (*Vitis rotundifolia* L.) in the plant family Vitaceae and in deerberry (Vaccinium stamineum Michaux) in the plant family Ericaceae in central Georgia. They also mentioned that the ability to adopt new host plants is a key to the success of the genus Conotrachelus and is an important consideration when developing holistic strategies of pest management for the bivoltine southern strain of plum curculio.

Apple and peach are two of the most important fruit crops in Virginia fruit production. For example, Virginia ranks sixth in the United Sates in apple production. There are 280 million pounds of apple yields in Virginia in 2005 (National Agricultural Statistics Service 2006).

Management

Currently, effective control of plum curculio is provided by organophosphate (OP), neonicotinoids, and oxidiazine insecticides. However, following Environmental Protection agency (EPA) review of many OP insecticides, many uses of OPs are being severely restricted, i.e. removed uses, longer restricted entry intervals, lower application rates, and longer preharvest intervals (McClanan 2002). Several OP insecticides are no longer registered for use on apples and peaches. A potential alternative control in peach is kaolin particle film spray. Lalancette et al. (2005) evaluated kaolin particle films for arthropod and disease management in peach of New Jersey. The results showed that kaolin provided similar or better control of oriental fruit moth, Grapholita molesta (Busck), plum curculio and Japanese beetles, Popillia japonica Newman, compared with the standard pesticide program. Other likely alternative controls may employ ecological and behavioral methods. However, we need more information to develop such methods (Racette et al. 1992). Research on the behavior and ecology of plum curculio is difficult because of its cryptic coloration, nocturnal behavior, death-feigning (thanatosis), poorly known

pheromones, limited visual and olfactory response (Racette et al. 1992). Racette et al. (1992) mentioned a study in which the use of radioisotope 66Zn can offer more new information about activities of plum curculio within a tree. This knowledge may provide some new control approaches.

Racette et al. (1992) and Vincent et al. (1999) both mentioned that there are only a few predatory and parasitic species attacking plum curculio, and these are unable to provide an effective alternative to chemical control in commercial orchards. But they mentioned several experiments to test entomogenous nematodes for the control of plum curculio larvae. In laboratory studies, the highest larval mortality (95.1%) was caused by *Steinernema carpocapsae* Weiser (Olthof and Hagley 1993). Shapiro-Ilan et al. (2004) measured field efficacy of two *Steinernema* nematode species (*S. feltiae* (Filipjev) and *S. riobrave* (Cabanillas Poinar & Raulston)) to suppress plum curculio larvae in two peach orchards in Georgia and Florida, respectively. Their results indicate that *S. riobrave* applications resulted in greater than 97% plum curculio control in 3 of 4 trials. To achieve a high level of efficacy, timing applications in accordance with pest phenology and environmental conditions could be critical (Shapiro-Ilan et al. 2004). Although nematode treatments applied to soil would not prevent damage to fruit, it could lower populations of the pest for the subsequent growing season (Vincent et al. 1999).

So far, there is no effective monitoring system. However, there are many studies to develop a trap-based monitoring system for use in commercial orchards. Grandisoic acid (GA) was isolated and described as a male-produced aggregation pheromone (Eller and Bartelt 1996). Several host plant volatiles were tested to attract the weevils (Butkewich and Prokopy 1993, Prokopy et al. 1995, Butkewich and Prokopy 1997). These findings suggested that both sexes should respond equally well to traps baited with plant volatile lures, regardless of the orientation of the trap. Prokopy et al. (2000) reported that, out of 30 odor components from unripe apple and plum fruit, eight components showed evidence of attractiveness, and were evaluated in field tests. Leskey et al. (2001) tested linalool, 2-hexanone and 3-hydroxy-2-butanone, and found significant attractiveness in laboratory tests, but only ethyl isovalerate and limonene were attractive in the

field. Piñero et al. (2001) evaluated the response of plum curculio to odor-baited traps in apple orchard in Massachusetts. They found that synthetic fruit volatiles, benzaldehyde (BEN), combined with aggregation pheromone, grandisoic acid (GA), were significantly more attractive to plum curculio. Moreover, Piñero and Prokopy (2003) evaluated the attractive effects using different release rates of benzaldehyde and GA combinations in field. The results indicated that BEN was the only host volatile that synergized the response of plum curculio to GA. Leskey et al. (2005) tested synthetic nonfruiting host plant volatile blends for the plum curculio. The results showed that volatiles released by foliar and woody tissues of plum were at least as attractive as the single fruit-based attractant, benzaldehyde, and there is more competitive attractive when it was combined with benzaldehyde and GA.

To develop an effective monitoring trap, Leskey and Wright (2004) tested the influence of host tree proximity on monitoring traps. Significantly more weevils were recaptured in traps baited in an open field compared with those in apple orchards. The results indicated that plum curculio response to traps was influenced by presence of apple trees. They concluded that "an effective trap-based monitoring system for plum curculio will require better odor baits, trapping mechanisms, and/or development strategies that can overcome visual and olfactory interference presented by host apple trees." Prokopy et al. (2004) proposed a field application using odor baited trap trees as sentinels to monitor plum curculio in apple orchards in Massachusetts. In this study, they developed a threshold (1 freshly injured fruit per 50 fruit sampled on a trap tree) to trigger one insecticide application for peripheral-row trees and two to prevent orchard-wide damage from exceeding an EIL of 1% (Prokopy et al. 2004). The only other published study to develop an economic threshold for plum curculio was by Johnson et al. (2002), who proposed a threshold (0.045 plum curculio adults per pyramid trap per day equated to 1% new fruit damage) by using odor-baited pyramid trap to capture the adults in peach orchards in Arkansas and Oklahoma.

Lan et al. (2004) developed a degree-day model to predict emergence of the summer generation of plum curculio. They developed linear regression models to describe the

relationships between developmental rate and temperature for larval and pupal development stages and estimated degree-day requirements for completing development from these models. In their model validation trials, the degree-day model was accurate when the insects were exposed to temperatures within the linear range. However, when temperatures were close to the lower thresholds, the predictions were less accurate. Another study was conducted by Hoffmann et al. (2004), who examined the relationship between degree-day accumulation and female reproductive development by measuring mating status, oocyte size and number of oocytes for both northern and southern strains of plum curculio. The results could be used to determine field-caught female reproductive status and then assist in determining the potential for plum curculio damage and for making control decisions.

Strains

There are two strains of plum curculio: a northern univoltine strain and a southern multivoltine strain (Chapman 1938). The multivoltine strain has been reported in many states: Virginia, North Carolina, Georgia, Missouri, and Illinois (Leiby and Gill 1923, Snapp 1930, Chandler 1932, Bobb 1952, Sarai 1969). The two strains are identical morphologically. The northern strain has an obligatory diapause (Bobb 1952). The southern strain has at least two generations per year. Diapause is facultative for the multivoltine strain (Bobb 1952). However, McClanan (2002) stated a multivoltine strain is found in New Jersey. This strain may have been brought from other areas by human activity and began to spread to other places in the state. In addition, an isolated univoltine population was found in northern Utah in the early 1980's. In Utah and the western U.S., PC is a quarantine insect and can prohibit or reduce the export of fruit to certain states and countries (Alston and Stark 2000). In another hand, there is some dispute as to whether southern strain males have an obligatory diapause (McGiffen et al. 1987).

Unlike the first generation, which attacks the fruit early, the second generation may be present as larvae in the fruit at harvest. A fruit export concern exists if the second generation larvae occur in harvested fruit. Other states and foreign countries that do not have plum curculio have refused to import fruit from those areas where the multivoltine strain of plum curculio exists. Chapman

(1938) presented an approximate distribution map of the two strains. The northernmost point of the weevil range is about 50^{o} north latitude. The southern line appears to be about 28^{o} north latitude. The western boundary falls near the 100^{th} meridian from Texas to Nebraska and along the 105^{th} meridian from there northward; the eastern boundary is the Atlantic Ocean. According to Chapman (1938), the mid-Atlantic region is the convergence area of the two strains, and Virginia has both strains. The distribution line is approximately the Appalachian Mountains (Chapman 1938). The multivoltine strain not only causes a trade barrier to be imposed by other states or countries but also a late brood would complicate pest control. Therefore, a method to distinguish the two strains and clarification of the distribution of plum curculio strains is necessary for IPM decision-making in eastern North America.

Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) and Mitochondrial DNA

RAPD-PCR has been successfully used to genetically analyze populations of many insect species. RAPD-PCR uses single short primers to bind to homologous sites of the genome. If the primers bind at opposing sites at least 3000 bases pairs apart with the 3' ends oriented toward each other, amplification occurs (Loxdale and Lushai 1998). Amplification will not occur if there are mutations in the binding sites between individuals or between strains of a species. The separation patterns of amplified DNA produced by small primers of generally arbitrary sequence can be analyzed (Loxdale and Lushai 1998). Several studies have used RAPD-PCR to analyze insect populations: alfalfa weevil, *Hypera postica* (Gyllenhal) (Erney et al. 1996); a weevil pest of sugar beet, *Aubeonymus mariaefranciscae* Roudier (Taberner et al. 1997); whiteflies (Gawell and Bartlett 1993); the alfalfa leafcutting bee, *Megachile rotundata* (Fabricius) (Lu and Rank 1996); orange wheat blossom midge, *Sitodiplosis mosellana* (Gehin) (He et al. 2001) and a parasitic wasp (Hymenoptera: Encyrtidae), *Ageniaspis citricola* Heppner (Alvarez and Ma 2002). McClanan (2004a) analyzed the difference between a univoltine strain and a multivoltine strain of plum curculio using a RAPD-PCR assay. The study has shown four primers of 15 primers can yield consistent banding patterns. The four primers: OPE-01, OPE-03, OPE-04 and OPE-07 can

be used to distinguish the two strains of plum curculio from two states (Massachusetts and Georgia).

Another genetic tool is the polymorphic randomly amplified microsatellite (RAMS). The strategy can avoid genomic library construction and screening, which can be very time-consuming. Microsatellite or simple sequence repeats (SSRs) have been consistently gaining importance as a single-locus DNA marker in population genetics and behavioral ecology (Ender et al. 1996). Ender et al. (1996) used PCR-based techniques for finding microsatellite loci in anonymous genomes in waterfleas (*Daphnia*). In all their cases, simple sequence repeats were detected. Thirteen positive RAPD fragments from three *Daphnia* species and two hybrid 'species' were cloned and sequenced. Seven perfect repeat loci were characterized (Ender et al. 1996).

Mitochondria are found in most eukaryotic cells. Their primary function is to convert organic materials into energy in the form of ATP via the process of oxidative phosphorylation. Mitochondria have their own DNA and are thought to have originated from once free-living bacteria that were closely related to α-proteobacteria (Boussau et al. 2004). Ballard and Rand (2005) reviewed the wide use of mitochondrial DNA (mtDNA) sequence data to reconstruct evolutionary trees for a wide array of species. They also argued that a fuller understanding of the biology of mitochondria is essential for the rigorous application of mtDNA to make inferences about the phylogeny of species or populations (Ballard and Rand 2005). Sequence data of mtDNA have become a proven standard for many phylogenetic studies (Caterino et al. 2000). Many mitochondrial genes have been studied, and subunit I of the cytochrome oxidase gene (COI) has become a standard for phylogenetic inference of many insect groups (Lunt et al. 1996). The mtCOI gene sequences were utilized as a marker to construct a phylogenetic tree for different geographic populations. Those applications include different taxonomic levels, i.e. species level (Caterino and Sperling 1999) in Lepidoptera, subgenus level (Koulianos 1999) in Hymenoptera and genus level (Clark et al. 2001) in Coleoptera. Moreover, there are many studies on the intraspecific level in different species. Most of these intraspecific studies were phylogeographical analysis: Frohlich et al. (1999) and Berry et al. (2004) studied sweet potato whitefly (*Bemisia*

tabaci Gennadius) in different geographic areas, Szalanski and Owens (2003) studied southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber), Shufran et al. (2004) worked on *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Aphidiidae), Soucy and Danforth (2002) worked on a sweat bee (*Halictus rubicundus* Christ), Artiss (2004) worked on a dragonfly (*Libellula quadrimaculata* L.) and Torres et al. (2006) analyzed the molecular systematics of the Philippine malaria vector *Anopheles flavirostris* (Ludlow). Additionally, Cognato et al. (2003) analyzed genetic diversity among Pinyon pine beetle (*Ips confuses* LeConte) populations in different hosts. According to their analyses, the mtCOI gene seemed a reliable molecular marker of intraspecific polymorphism of those insects. However, only a few studies have found the mtCOI gene to be uninformative, with low genetic variation in some species, e.g. a malaria vector complex *Anopheles messeae* Falleroni (Luca et al. 2004) and a Chinese shrimp (*Penaeus chinensis* Osbeck) (Quan et al. 2001).

Wolbachia and Reproductive Incompatibility

Wolbachia is a very common cytoplasmic symbiont of insects, crustaceans, mites, and filarial nematodes, and has been intensively studied since the 1990's. This intracellular α-proteobacterium was reported for the first time in 1924 by Hertig and Wolbach. Hertig considered this a new rickettsia found in the mosquito *Culex pipiens* L., and named it as *Wolbachia pipientis* in honor of his collaborator Wolbach (Stouthamer et al. 1999). Since Yen and Barr (1971) discovered that *Wolbachia pipientis* could cause cytoplasmic incompatibility, *Wolbachia* has become a frequent study topic. *Wolbachia* research is progressing rapidly by using molecular biological techniques e.g. PCR. Surveys have shown *Wolbachia* to be present in at least 16% of sampled species (Werren et al. 1995a). Jeyaprakash and Hoy (2000) examined 63 arthropod species, and 76% of them were infected by this bacterium. Kikuchi and Fukatsu (2003) investigated 134 heteropteran species from 19 families. Of these, 47 species from 13 families were infected by *Wolbachia*. Tagami and Miura (2004) surveyed the samples of lepidopteran insects from Japan. Their results indicated that 45% of 49 species and 7 of 9 families were found to be infected by *Wolbachia*.

Originally, the phylogeny of *Wolbachia* was based on 16S rDNA gene sequence. Stouthamer et al. (1993) divided the genus *Wolbachia* into two clades: supergroup I and supergroup II based on the 16S rDNA sequence analysis. However, the 16S rDNA gene is highly conserved across all eubacteria, making definitive phylogenetic analyses of species difficult. There is 3% difference in the 16s rDNA of several *Wolbachia* strains (Stouthamer et al. 1993). Werren et al. (1995b) used the cell-cycle (fts Z) gene to classify the *Wolbachia* strains into two clades: supergroups A and B. The fts Z gene evolves faster than the 16S rDNA gene (Werren et al. 1995b). A maximum sequence variation of 15% was reported between groups A and B but was still relatively low within group A (3%) (Werren et al. 1995b). Faster evolution of a gene allows for a more definitive phylogenetic analysis within the clades when gene sequences are analyzed. Zhou et al. (1998) used the *wsp* (*Wolbachia* surface protein) gene sequence to classify strains of *Wolbachia*. The *wsp* gene evolves more rapidly than either the 16S rDNA or the fts Z genes previously used. Based on the *wsp* gene sequences, the genus *Wolbachia* is divided into two supergroups (A and B) for insects, one supergroup for mites (C) and one supergroup for nematodes (D). Within the clades the *wsp* gene sequence analysis allows for classification of *Wolbachia* by reference group and strain. Since Zhou's 1998 work, there have been many studies using the *wsp* gene as a marker to perform the phylogenetic analysis of *Wolbachia* infections in different insect taxonomic levels. Van Meer et al. (1999) studied phylogeny of *Wolbachia* in arthropods based on the *wsp* gene, including 19 insect and 1 isopod host species, and combined with the data set of Zhou et al. (1998) to make a phylogenetic analysis. Shoemaker et al. (2002) analyzed the distribution of *Wolbachia* in fig wasps (Hymenoptera: Agaonidae) and compared the correlations with their host phylogeny, ecology and population structure. Kyei-Poku et al. (2005) detected and made a phylogenetic analysis of *Wolbachia* in lice (Phthiraptera). In those three studies, there was no relationship between host phenotype and *Wolbachia* phylogenetic position, and the horizontal *Wolbachia* transfer was possible. At the species level, Egyed et al. (2002), Behbahani et al. (2005), Hoy and Jeyaprakash (2005) analyzed the phylogeny of *Wolbachia* in *Aedes scutellaris* (Walker) group, *Onchocerca lupi* (Nematoda: Filarioidea) and predatory mite, *Metaseiulus occidentalis* (Nesbitt)

and its prey *Tetranychus urticae* Koch. The *wsp* gene is a common marker used in those studies and fts Z gene were also used in some studies, e.g. Malloch, et al. (2000), Egyed et al. (2002), Jamnongluk et al. (2002), Ruang-areerate et al. (2003). Additionally, only one study used transposable element (sequences of DNA that can move around to different positions within the genome of a single cell) of *Wolbachia* to detect polymorphism, superinfection and recombination in the mosquito, *C. pipiens* (Duron et al. 2005). This is a new way to investigate evolution of *Wolbachia* populations and CI dynamics. Wu et al. (2004) completed the whole sequence of genome of *Wolbachia pipientis* *w*Mel, the intracellular bacteria of *Drosophila melanogaster* Meigen. They found *w*Mel containing very high levels of repetitive DNA and mobile DNA elements.

According to those *Wolbachia* studies, superinfection (more than one *Wolbachia* strain infection in a single host) is a common phenomenon in *Wolbachia* infections. There are many studies relating to the superinfection, e.g. Fukatsu and Nikou (1998), Malloch, et al. (2000), Dobson et al. (2001), Egyed et al. (2002), Jamnongluk et al. (2002), Riegler and Stauffer (2002), Reuter and Keller (2003), Ruang-areerate et al. (2003), Keller et al. (2004), Mercot and Charlat (2004). Specially, Jamnongluk et al. (2002) and Reuter and Keller (2003) detected up to five *Wolbachia* strains infecting a tephritid fruit fly, *Bactrocera ascita* (Hardy) and the ant, *Formica exsecta* Nylander. Restriction fragment length polymorphism (RFLP) typing is usually used to detect the superinfection (Fukatsu and Nikou 1998, Malloch et al. 2000, Jamnongluk et al. 2002, Reuter and Keller 2003). Strain specific primers were designed for some known double infection species to differentiate two *Wolbachia* strains (Riegler and Stauffer 2002, Keller et al. 2004).

Wolbachia can cause several reproductive alterations in host species, including cytoplasmic incompatibility (CI), parthenogenesis (Stouthamer et al. 1990, Rousset et al. 1992, Bandi et al. 2001, Pannebakker et al. 2004), increased fecundity (Vavre et al. 1999), and killing of male embryos (Hurst et al. 1999). Most studies deal with CI because it is thought that CI may play a key role in the speciation of arthropods. Werren (1998), Stouthamer et al. (1999), Rokas (2000), Wade (2001), Bandi et al. (2001) and Zimmer (2001) reviewed the role of *Wolbachia* in

speciation. However, Mandel et al. (2001) studied this issue in field cricket hybrids and found that *Wolbachia* is unlikely to be involved in reproductive isolation between cricket species. Therefore, several scientists doubt the hypothesis, and it remians a controversial problem.

In diploid hosts, cytoplasmic incompatibility can decrease offspring viability. These anomalies occur when an infected male mates with an uninfected female or a female that is infected with an incompatible strain of *Wolbachia* (Stouthamer et al. 1999). Werren (1997) offered a possible mechanism for CI: the *Wolbachia* strain carried by the male leaves a molecular imprint on the sperm. Only if the female is infected with the same or a compatible strain of *Wolbachia* is the egg able to "recognize" the imprint and save the paternal genetic material. Otherwise, during embryonic meiosis the paternal genetic material is lost, and the resulting embryo is haploid. In diploid species the embryo dies, reducing the number of viable offspring produced from a mating. In species with haploid males, the embryo develops as a male, biasing the sex ratio among offspring toward males (Stouthamer et al. 1999).

There are a few studies of cytoplasmic incompatibility that have examined unidirectional and bi-directional CI. Strong unidirectional CI has been shown in *Ephestia cautella* (Walker), *H. postica* and *Laodelphax striatellus* (Fallen) (O'Neill 1997). Laven (1959, 1967) developed the idea that bi-directional CI could lead to speciation. *Wolbachia* was proposed as an agent of speciation if the CI was unidirectional and coupled with another pre-zygotic or post-zygotic barrier as in *Drosophila recens* (Wheeler) and *D. subquinaria* Spencer (Shoemaker et al. 1999).

Bordenstein et al. (2001) reported that *Wolbachia*-induced incompatibility has preceded other pre-zygotic and post-zygotic barriers in *Nasonia giraulti* (Darling) and *N. longicornis* (Darling). The cytoplasmic incompatibility between the two species of *Nasonia* was bi-directional and occurred at high levels since each species of wasp is infected with two strains of *Wolbachia*. The wasps are typically infected with a strain of *Wolbachia* from supergroup A and supergroup B, respectively. When the wasps were treated with antibiotics, crosses between the species resulted in viable offspring (F_1 generation), which were in turn capable of producing viable offspring (F_2 generation). Dobson et al. (2001) reported that both single and superinfection (more than one

Wolbachia strains infection in a single organism) induced high levels of CI of *Aedes albopitus* (Skuse). Similar results were reported by Riegler and Stauffer (2002) and Keller et al. (2004). They studied the European cherry fruit fly (*Rhagoletis cerasi* L.) and a neotropical beetle species (*Chelymorpha alternans* Boheman), respectively. There is CI among different geographic populations that were infected by single or superinfection *Wolbachia* strains. Merçot and Charlat (2004) reviewed *Wolbachia* polymorphism and levels of CI in *D. melanogaster* and *D. simulans*. Five *Wolbachia* strains have been detected in *D. simulans* natural populations but just one *Wolbachia* strain was found in *D. melanogaster*. *D. melanogaster* has just partial or no CI expressed, but CI was fully expressed in *D. simulans.*

Reproductive incompatibility between plum curculio strains has been studied since the 1960's. Stevenson and Smith (1961) reported reproductive incompatibility in plum curculio from crosses of southern females and northern males. Padula and Smith (1971) reported that crossing a northern (univoltine) female plum curculio with a southern (multivoltine) strain male resulted in significantly fewer eggs laid and fewer larvae hatched from the eggs. They observed similar reductions in oviposition and egg hatch in this crossing of the multivoltine female with the univoltine male. The results conflicted with the results of Stevenson and Smith (1961), who found no significant drop in fertility in crosses between multivoltine females and univoltine males. Furthermore, sections of spermathecae showed some pathological changes: nuclei of the spermathecal gland cells became enlarged and vacuolated areas appeared; the gland lost its ovoid shape, appeared to contain only degenerating sperm and began to collapse. The reproductive incompatibility is similar to that CI associated with *Wolbachia* infection of reproductive tissue reported from other arthropods. McClanan (2004b) analyzed three strains of *Wolbachia* associated with Massachusetts (MA), Georgia (GA) and Florida (FL) plum curculio populations, respectively. The results of McClanan (2004b) study suggested that univoltine and multivoltine weevils carry different strains of *Wolbachia*. *Wolbachia* associated with MA weevils was placed in supergroup B. At the nucleotide level, the strain of *Wolbachia* carried by the MA weevils had 91.48% similarity with *w*Ten-B1. Therefore this strain was named as a new strain: "*w*Nen" and a

new reference group "Nen" according to Zhou et al. (1998). *Wolbachia* associated with FL and GA weevils were in supergroup A. *Wolbachia* from GA curculios was unique and *Wolbachia* from FL curculios was 99.6% identical to that associated with *Drosophila destillatoria*. They belong in the reference group "Des" (McClanan 2004b). The reproductive incompatibility between the two plum curculio strains could be due to the different *Wolbachia* strain infection.

Wolbachia may not only play a role in rapid speciation in insects, but also could be used as a biological control tool and for genetic manipulation of pests and disease vectors (Beard et al. 1993, O'Neill et al. 1997). First, there are three studies that reported DNA recombination in *Wolbachia* between different strains. Jiggins et al. (2001) showed recombination between two genes of *Wolbachia*. Werren and Bartos (2001) described that recombination occurred between *Wolbachia* that infect a parasitoid and its host at the *wsp* gene. Reuter and Keller (2003) detected an unexpectedly high rate of recombination with three of the five *Wolbachia* strains by using *wsp* gene sequence analysis in the ant, *F. exsecta*. Jiggins (2002) estimated the rate of recombination in *Wolbachia* using a mathematic analysis for *wsp* and fts Z genes. Recombination means that novel beneficial mutations could be able to spread across different genetic backgrounds, and different bacteria strains may be able to change the phenotypic effect on their hosts rapidly (Hurst et al. 2002). The alternative possibility of genetic manipulation is increased because of horizontal transfer of the relevant genetic machinery by recombination (Werren and Bartos 2001). Recently, two studies primarily applied *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. Zabalou et al. (2004) reported that they transferred the *Wolbachia* strain from a naturally-infected cherry fruit fly species, *R. cerasi*, to the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), which is not infected naturally with *Wolbachia*. *Wolbachia* induced complete CI when they mated with uninfected medflies. Populations of laboratory caged medflies were completely suppressed by a single release of infected males. Xi et al. (2005) established stable infections of *w*AlbB *Wolbachia* in *Aedes aegypti* L., a vector of yellow fever and other diseases. There is no natural *Wolbachia* infection in this species. The infection caused complete CI as well. Laboratory cage tests indicated that *w*AlbB was able to

spread into an *A. aegypti* population after seeding of an uninfected population with infected females, reaching infection fixation within seven generations (Xi et al. 2005). Those studies suggested *Wolbachia*-induced CI could be used as a novel, environmentally friendly control strategy to suppress or modify natural populations of the pest and vector species.

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Figure 1-1. Delineation of the range of plum curculio as determined by Chapman (1938). The blue lines indicate the northern-most boundary (Maine) of plum curculio's range in the United States, and the southern-most boundary (tip of Florida). The black line is the western-most boundary of the plum curculio's range. The red line is the line set by Chapman (1938) delineating the ranges of the univoltine strain (above the red line) and the multivoltine strain (below the red line)

Chapter 2 Evaluation of the success of cross-mating among plum curculio populations

Abstract

The success of cross-strain mating in plum curculio, *Conotrachelus nenuphar* (Herbst), was studied during the summers of 2004 and 2006. The reproductive incompatibility among plum curculio populations from different geographic locations was confirmed. Experiment 1 evaluated the reproductive compatibility of cross-matings among a rough north to south transect of the range of plum curculio (4 x 4 two factorial design: NY, VA, FL, and WV). A unidirectional incompatibility was revealed. There was significant low fertility in WV males mated with NY (40%) and VA (29%) females. The Florida population showed a different pattern of reproductive compatibility: FL males have a significant lower fertility with VA (46%) and WV (37%) females and FL females were compatible with males from all four populations. Experiment 2 evaluated fertility of cross-matings among populations within the northern geographic area (3 x 3 two factorial design: NY, MA, and NJ). The results suggested that within the northern geographic area, populations are compatible with each other in all cross-mating combinations. Experiment 3 was included to evaluate feritility of cross-matings between FL and NJ populations and to repeat mating success between FL and WV populations (2 x 2 two factorial design: FL and NJ or FL and WV). They were reciprocal crosses. A unidirectional reproductive incompatibility was revealed in the combination of NJ males with FL females, which showed a significant low fertility (47%). A bi-directional incompatibility occurred between FL and WV reciprocal cross mating. FL males mated with WV females and WV males mated with FL females, but with reduced fertility (26% and 21%, respectively) compared to fertility of within their population matings.

Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is a major pest of fruit in eastern North America. The insect may cause almost all of the fruit to be injured if no control actions are taken. There are two strains of plum curculio. Normally, the northern strain has an obligate winter diapause, and is a univoltine strain. The southern strain does not display the obligate diapause and has two or three generations annually (Bobb 1952). Chapman (1938) gave an approximate distribution description of the two strains. In his map, the mid-Atlantic region is the convergence area of the two strains and Virginia contains both strains.

Reproductive incompatibility between plum curculio strains was studied in the 1960's. Stevenson and Smith (1961) reported a unidirectional reproductive incompatibility in plum curculio from crosses of southern females and northern males. They observed reductions in oviposition and egg hatch in this crossing of the multivoltine female with the univoltine male. Padula and Smith (1971) reported that crossing the northern (univoltine) female plum curculio with the southern (multivoltine) strain males resulted in significantly fewer eggs and fewer larvae hatched from the eggs. Padula and Smith's results showed another directional incompatibility compared with the results of Stevenson and Smith (1961), who found no significant drop in fertility in crosses between univoltine female and multivoltine male. Furthermore, sections of spermathecae exhibited some pathological changes: nuclei of the spermathecal gland cells became enlarged and vacuolated areas appeared; the gland lost its ovoid shape, appeared to contain only degenerating sperm and began to collapse. The reproductive incompatibility is similar to that reported for other studies on arthropods associated with infection of reproductive tissues by *Wolbachia*.

In this study, I implemented several cross-mating experiments to evaluate the success of mating among various plum curculio geographic populations. These experiments were intened to provide new infromation on the role of *Wolbachia* infection on reproductive incompatibility of plum curculio. The pattern of the reproductive incompatibility among the weevil's populations

could enrich our knowledge of population ecology and biosystematics of plum curculio and may guide future control research.

Materials & Methods

Insect - plum curculio

To ensure virgin adults were used in cross-mating trials, plum curculio colonies from different geographic populations were set up separately in our laboratory. Adults were collected in orchards by other contributors or me, using beating trays or baited pyramidal traps. To acquire plum curculio larvae, infected fruit were collected in orchards. Plum curculios from different geographic populations were maintained separately.

The larvae were collected from the infested fruit using the following procedure: infested fruit were placed in 60 cm x 60 cm wooden-frame racks with screen (6 mm x 6 mm grid) floors (Figure 2-1), and a collection tray (Figure 2-2) to contain the dropping plum curculio larvae. Larvae were then placed in one pint (473 ml) glass mason jars containing moistened 50:50 potting soil: vermiculite (Figure 2-3). Each jar received around 100 larvae, and soil moisture (150 ml water added in 850 ml mixed soil) was maintained throughout the pupation period (approximately 3 weeks). The newly emerged adults were sexed according to morphological characteristics described by Thomson (1932), and then maintained separately by gender and geographical locations.

The adults from field collection were caged, allowed to mate and establish new colonies in lab conditions using the procedures of Leskey (unpublished). Adults were placed into a plastic box (34 x 26 x 8.5cm) with organza covered ventilated top (Figure 2-4). About 25 green thinning apples (around 3 cm in diameter) were used as food and oviposition sites. The plastic box was held in an incubator chamber at 25^{o}C and 60 – 80% RH. Photoperiod was controlled at 14 L : 10D per day. The thinning apples were replaced every week for colony maintenance and the apples taken from the adult rearing boxes were placed in the larvae collection trays. The same procedure was used for larvae collection from infected fruit.

For northern strain plum curculio populations, an obligate diapause was necessary to obtain the reproductive adults. Males and females were separately sustained on green thinning apples throughout the summer and early fall. From late fall to early spring, northern plum curculio adults were placed in one quart (946 ml) glass mason jars (around 100 adults per jar) containing moistened potting soil, paper towel and thinning apple, and dry dropped leaves, then put into soil covered by dropped leaves and a wood cover in Kentland Farm (Whitethorne, VA) separated by their geographic origin in single-sex groups. At the first sign of plum curculio activity in jars in spring, the adults were brought into the laboratory and removed from containers. This process provided virgin adults that had experienced natural overwintering environmental conditions for cross-mating experiments. Southern plum curculio adults were allowed to continue their life circle under lab conditions. Green thinning apples and water were provided during this period.

In this experiment, plum curculio colonies were set up from the following geographic sites: a. overwintered (or diapaused) populations: Amherst, Massachusetts (MA), Geneva, New York (NY), Bridgeton, New Jersey (NJ), Blacksburg, Virginia (VA); b. continuing colonies: Kearneysville, West Virginia (WV) and Quincy, Florida (FL).

Experimental procedures

Experimental design

Experiment 1: To evaluate reproductive compatibility of cross-matings among a rough north to south transect of the range of plum curculio. This was a 4 x 4 factorial (maternal origin and paternal origin) design. The four levels within each factor were extreme north (NY), lower north (WV), upper south (VA), and extreme south (FL). Twenty pairs within each cell were used.

Experiment 2: To evaluate reproductive compatibility of cross-matings among populations of plum curculio from the northern geographic area. This was a 3 x 3 factorial (maternal origin and paternal origin) design. The three levels within each factor were NY, MA,

and NJ. Twenty pairs within each cell were used (due to insufficient MA females, sixteen pairs were used).

Experiment 3a: To evaluate reproductive compatibility of cross-matings between FL and NJ populations. This involved a reciprocal cross mating design. Ten pairs for each combination were used.

Experiment 3b: To evaluate reproductive compatibility of cross-matings between FL and WV populations. This involved a reciprocal cross mating design. Ten pairs for each combination were used.

Fertility examination protocol

Each pair of curculios was placed into a 4 oz (120 ml) plastic cup with a green thinning apple as a food and oviposition site and about 2.5 cm soaked cotton dental wick (Absorbal, Wheat Ridge, CO) as the water source (Figure 2-5). The cups were held at uncontrolled room temperature (68 – 85 ^{o}F), uncontrolled humidity (45 – 70% RH), and photoperiod at 14L : 10D. Males were removed if ovipostion scars were found in the apples. Mated females were placed alone with uninfested thinning apples. The apples were removed every two days if the oviposition scars were visible (Figure 2-6) and held in a plastic box with screen tops at same temperature condition. The number of eggs laid and number of hatched eggs were determined after the apples were held for a minimum 5 days. A dissection needle was used to peel the apple skin to uncover the oviposition site under the dissecting microscope. A small hole appeared if the egg hatched successfully (Figure 2-7) while infertile eggs remained in the oviposition sites (Figure 2-8).

Statistical analyses

The reproductive compatibility of cross mating was analyzed separately for each experiment. The fertility, being a percentage, was subjected to arcsin transformation for analysis. First, the maternal origin and paternal origin treatment factors were analyzed by two-way ANOVA using SAS (SAS Institute 2001). Then, for each level in each factor, separate one-way

ANOVA of each factor if the interaction effect between the two factors was significant. All analyses of variance were followed by Tukey's honestly significant difference (HSD) test to separate treatment means (Zar 1998), and results were evaluated for significance at $P \leq 0.05$.

Results

Experiment 1

There were 16 treatment combinations (n = 20) in total in experiment 1. Table 2-1 shows the mean fertility (% ± SEM) for each treatment combination. Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. After two-way ANOVA, maternal factor (F = 11.89, df = 3, 237, P < 0.0001), paternal factor (F = 26.29, df = 3, 237, P < 0.0001), and interaction between maternal and paternal factors (F = 6.98, df = 9, 237, P < 0.0001) were significant. The factor-level means were not meaningful, therefore, for each level in each factor, separate one-way ANOVA of each factor were employed and followed by Tukey's HSD test to separate treatment means. The results of multiple comparisons were shown in Figure 2-9 to Figure 2-16.

Within the maternal factor, the first multiple comparison (NY males; first row in Table 2-1) was NY males mated with NY, VA, FL, and WV females, respectively. The fertility between NY males and females (95.66 ± 2.35) was significantly higher than NY males mated with WV females (77.12 ± 6.54) (F = 4.5342, df = 3, 62, P = 0.0061, Figure 2-9); there was no significant difference between NY males mated with VA (90.29 ± 5.40) and FL (92.76 ± 2.18) females. In the second multiple comparison (VA males; second row in Table 2-1), VA males mated with NY (91.72 ± 2.53) and VA (97.03 ± 1.71) females have significantly higher fertility than when mated with WV females (80.23 ± 3.46) (F = 10.9191, df = 3, 66, P < 0.0001, Figure 2-10). Fertility was divided into two groups in the third multiple comparison (FL males; third row in Table 2-1): fertility was significantly higher in FL (99.25 ± 0.37) and NY (88.96 ± 5.66) females mated with FL males than VA (35.12 ± 17.22) and WV (37.43 ± 10.16)

females mated with FL males (F = 11.7982, df = 3, 47, P < 0.0001, Figure 2-11). In the last multiple comparison of maternal factor (WV males; fourth row in Table 2-1), cross of WV males with NY (40.25 ± 9.87) and VA (31.32 ± 9.88) females has significantly lower fertility than the cross of WV males and FL females (78.14 ± 7.94) (F = 4.5060, df = 3, 62, P = 0.0063, Figure 2-12).

Within the paternal factor, the first multiple comparison (NY females; first column in Table 2-1) indicated that WV males mated with NY females (40.25 ± 9.87) had a significantly lower fertility than NY (95.66 ± 2.35), VA (91.72 ± 2.53), and FL males (88.96 ± 5.65) mated with NY females (F = 20.3508, df = 3, 72, P < 0.0001, Figure 2-13). In the second column comparison (VA females; second column in Table 2-1), the fertilities of VA females crossed with NY (90.29 ± 5.40) and VA (97.03 ± 1.71) males were significantly higher than when crossed with FL (35.12 ± 17.22) and WV (31.32 ± 9.88) males (F = 15.5251, df = 3, 53, P < 0.0001, Figure 2-14). There was no significant difference in the third comparison within the paternal factor (FL females; second column in Table 2-1) (F = 2.4425, df = 3, 47, P = 0.0758, Figure 2-15), the four treatments exhibited high fertility. A significantly low fertility of FL males crossed with WV females (37.43 ± 10.16) was shown in the last comparison (WV females; fourth column, Table 2-1) (F = 5.1256, df = 3, 67, P = 0.0030, Figure 2-16) and a low fertility was shown within WV population mating (59.24 ± 9.92).

Experiment 2

There were 9 treatment combinations (n = 20) in experiment 2. Table 2-2 shows the mean fertility (% ± SEM) for each treatment combination. Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. After two-way ANOVA, the paternal factor (F = 0.12, df = 2, 146, P = 0.8847), and the interaction between maternal and paternal (F =1.01, df = 4, 146, P = 0.4060) were not significant. The significant effect was shown in maternal factor (F = 4.44, df = 2, 146, P = 0.0135). Therefore, one-way ANOVA of maternal factor was employed and followed by Tukey's HSD test to the maternal factor means. Fertility of NJ females (85.02 ±3.97) was significantly lower than fertilities of NY females (95.51 ± 1.70)

($F = 4.4308$, $df = 2, 152$, $P = 0.0135$, Figure 2-17).

Experiment 3a

There were 4 treatment combinations in experiment 3a. Table 2-3 shows the mean fertility (% ± SEM) for each treatment combination. Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. In two-way ANOVA, the maternal factor ($F = 8.81$, $df = 1, 36$, $P = 0.0053$), and interaction effects between maternal and paternal factors ($F = 8.75$, $df = 1, 36$, $P = 0.0054$) were significant. The paternal factor effect was not significant ($F = 2.34$, $df = 1, 36$, $P = 0.1348$). Therefore, a one-way ANOVA of the four treatments was employed and followed by Tukey's HSD test to separate treatment means. In this multiple comparison, fertility of NJ males crossed FL females (47.22 ± 16.19) was significantly lower than the other three treatments ($F = 5.7951$, $df = 3, 36$, $P = 0.0024$, Figure 2-18).

Experiment 3b

There were 4 treatment combinations in experiment 3b. Table 2-4 shows the mean fertility (% ± SEM) for each treatment combination. Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. In two-way ANOVA, the effects of the maternal factor ($F = 0.01$, $df = 1, 31$, $P = 0.9180$) and the paternal factor ($F = 0.42$, $df = 1, 31$, $P = 0.5224$) were not significant. But the interaction effect between maternal and paternal factors was significant ($F = 43.41$, $df = 1, 31$, $P < 0.0001$). Therefore, a one-way ANOVA of the four treatments was employed and followed by Tukey's HSD test to separate treatment means. In this multiple comparison, fertility of FL males crossed with WV females (25.88 ± 8.82) and the reciprocal cross, WV males and FL females (20.63 ± 9.60), were significantly lower than when they mated within their own populations ($F = 15.4290$, $df = 3, 31$, $P < 0.0001$, Figure 2-19). Part of experiment 1 contains the same treatment combinations as the experiment 3b. Here, I employed the one-way ANOVA followed by Tukey's HSD for the reciprocal cross between FL and WV populations that was used in experiment 1 in order to compare the results with experiment 3b. In this analysis, fertility of FL males and WV females (37.35 ± 10.15) was also significantly lower ($F = 6.4028$, $df = 3, 54$, $P = 0.0009$, Figure 2-20) than when they mated within

their own populations, but the reciprocal cross, WV males with FL females had a significantly high fertility (78.19 ± 7.94) even higher than mating within the WV population (59.24 ± 9.92).

Discussion

The reproductive incompatibility among plum curculio geographic populations was confirmed in this study. The lowest mean fertility level was 31.32% between WV males and VA females in experiment 1 and 20.61% between WV males and FL females in experiment 3. The result is consistent with a previous study by Stevenson and Smith (1961), who showed 27% fertility between northern females and southern males. In other reproductive incompatibility studies, highly incompatible mating (0 – 0.28% fertility) was found between single *Wolabachia* infection or uninfected females and doubly infected males in the mosquito, *Aedes albopictus* (Skuse) (Dobson et al. 2001). Riegler and Stauffer (2002) mentioned an incompatible cross (1.5% fertility) between different *Wolbachia*-infected females and males in the European cherry fruit fly, *Rhagoletis cerasi* L., citing Boller and Bush (1974). Moreover, Keller et al. (2004) studied reproductive effects in the neotropical beetle, *Chelymorpha alternans* Boheman, ranging from weakly incompatible (~ 80% fertility) to moderately incompatible (~ 10 – 30% fertility) crossing among different geographic populations. In my study, weak to moderate (~ 20% - 60% fertility) reproductive incompatibility was found in different combinations among the plum curculio geographic populations.

From cross-mating experiment 1, a unidirectional incompatibility was revealed. There was significant low fertility in WV males mated with NY (40%) and VA (31%) females. Although WV females have significantly lower fertility mated with NY (77%) and VA (80%) males, they were still higher than within WV population (59%). The Florida population showed a different pattern of compatibility; males have a significantly lower fertility with VA (35%) and WV (37%) females while females were compatible with all males from the four populations. Stevenson and Smith

(1961) reported incompatibility between northern females (New York) and southern males (North Carolina). The result is similar with WV males crossed with NY or VA females. In this experiment, an unusually low fertility was shown within WV matings. It suggests that reproductive incompatibility could occur within this population. Of 20 mated WV plum curculio females, four females showed fertility less than 7%, but eight females showed more than 94% fertility. The sample size was very small. A large-scale mating experiment within this population could clarify the compatibility among the individuals from WV. Another possible explanation is the physiological status of those individuals. The development of the reproductive system could strongly influence their fertility. In 2006, from the same WV lab colony showed a normal fertility (81%). However, it is hard to explain why WV females have even higher fertility mated with NY and VA males, and WV males mated with FL females also have higher fertility than within WV population. Another problem lies in the Florida population. In both 2004 and 2006, FL weevils used in mating trials were the first generation progeny from the summer generation collected from the field. Of 40 pairs of FL weevils, only 50% (20 pairs) have laid eggs. The phenomenon is similar for the univoltine weevils, a diapause may be needed before reproductive development. According to Hoffmann et al. (2004), all sampled southern strain females mated by day 13 after adult eclosion. The FL adults were reared at least three weeks before they were used in the mating experiments described here. More intensive phenological study of Florida curculios needs to be done in the future.

Experiment 2 showed no significant differences in the effects of the paternal factor and interaction between maternal and paternal factors in mating among NY, MA, and NJ geographic populations. Only one significant difference was shown, where NJ females have a lower fertility (85%) than other the two populations of females. Therefore, within the northern geographic area, populations appear to be compatible with each other. The lower fertility of NJ females may due to their reproductive physiological conditions or a fairly weak incompatibility.

In experiment 3, I assessed the fertility of cross-matings between a southern population (Florida) and two mid-Atlantic populations (West Virginia and New Jersey). FL vs. NJ was a new

combination and FL vs. WV was a repeat of part of experiment 1. In FL vs. NJ reciprocal crosses, a unidirectional reproductive incompatibility was revealed. The combination of NJ males with FL females showed a significantly lower fertility (47%). This was a moderate incompatibility and the results were not consistent with the FL compatibility in experiment 1, which showed that FL females were compatible with all males from the four populations.

In experiment 3b, a bi-directional incompatibility was shown between FL and WV cross mating. FL males mated with WV females (26%) and WV males mated with FL females (21%) both had low fertility. However, the reciprocal cross between the two populations showed a unidirectional incompatibility in experiment 1. Figures 2-19 and 2-20 showed the different pattern of the same cross in 2006 and 2004, respectively. The result of experiment 1, where FL females mated with WV males had even higher fertility than mated within WV population was unusual. Again, fertility from a small sample size experiment could be influenced dramatically by population compositions of different individuals.

In summary, the results indicated a fairly complicated reproductive incompatibility pattern. More discussion will be included in Chapter 4, which will include the results of the *Wolbachia* strain analysis.

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Table 2-1. Mean fertility (%) ± SEM in experiment 1, evaluating fertility of cross-matings in a rough north to south transect along the range of plum curculio, using a 4 x 4 factorial (maternal origin and paternal origin) design. The four levels within each factor were extreme north (NY), lower north (WV), upper south (VA), and extreme south (FL). Twenty pairs within each cell were used (n = 20). Number of eggs laid and hatched was checked after holding the apples for 5 days. All values are expressed as percentage of fertility ± SEM in each treatment.

Male (paternal)	Female (maternal)			
	NY	VA	FL	WV
NY	95.66 ± 2.35	90.29 ± 5.40	92.76 ± 2.18	77.12 ± 6.54
VA	91.72 ± 2.53	97.03 ± 1.71	87.05 ± 2.50	80.23 ± 3.46
FL	88.96 ± 5.65	35.12 ± 17.22	99.25 ± 0.37	37.43 ± 10.16
WV	40.25 ± 9.87	31.32 ± 9.88	78.14 ± 7.94	59.24 ± 9.92

Table 2-2. Mean fertility (%) ± SEM in experiment 2, evaluating fertility of cross-matings among populations within the northern geographic area, using a 3 x 3 factorial (maternal origin and paternal origin) design. The three levels within each factor were NY, MA, and NJ. Twenty pairs within each cell were used (n = 20). Number of eggs laid and hatched was checked after holding the apples for 5 days. All values are expressed as percentage of fertility ± SEM in each treatment. Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with different letter were significantly different.

Male (paternal)	Female (maternal)			
	NY	MA	NJ	Mean paternal
NY	92.82 ± 4.53	91.29 ± 6.65	90.00 ± 3.96	91.36 ± 2.81
MA	96.26 ± 2.61	99.27 ± 0.32	79.06 ± 8.79	91.12 ± 3.37
NJ	97.16 ± 1.28	97.13 ± 1.04	86.00 ± 7.08	92.99 ± 2.74
Mean maternal	95.51 ± 1.70 a	95.97 ± 2.16 ab	85.02 ± 3.97 b	

Table 2-3. Mean fertility (%) ± SEM in experiment 3a, evaluating fertility of cross-matings between FL and NJ populations, using a reciprocal cross mating design. Ten pairs within each cell were used (n = 10). Number of eggs laid and hatched was checked after holding the apples for 5 days. All values are expressed as percentage of fertility ± SEM in each treatment.

Male (paternal)	Female (maternal)	
	FL	NJ
FL	84.22 ± 8.26	86.42 ± 5.24
NJ	47.22 ± 16.19	97.00 ± 1.13

Table 2-4. Mean fertility (%) ± SEM in experiment 3b, evaluating fertility of cross-matings between FL and WV populations, using a reciprocal cross mating design. Ten pairs within each cell were used (n = 10). Number of eggs laid and hatched was checked after holding the apples for 5 days. All values are expressed as percentage of fertility ± SEM in each treatment.

Male (paternal)	Female (maternal)	
	FL	WV
FL	84.22 ± 8.26	25.92 ± 8.78
WV	20.61 ± 9.61	81.52 ± 8.72

Figure 2-1. Wooden-frame racks (60 cm x 60 cm) with screen (6 mm x 6 mm grid) floors to hold the fruit containing plum curculio larvae.

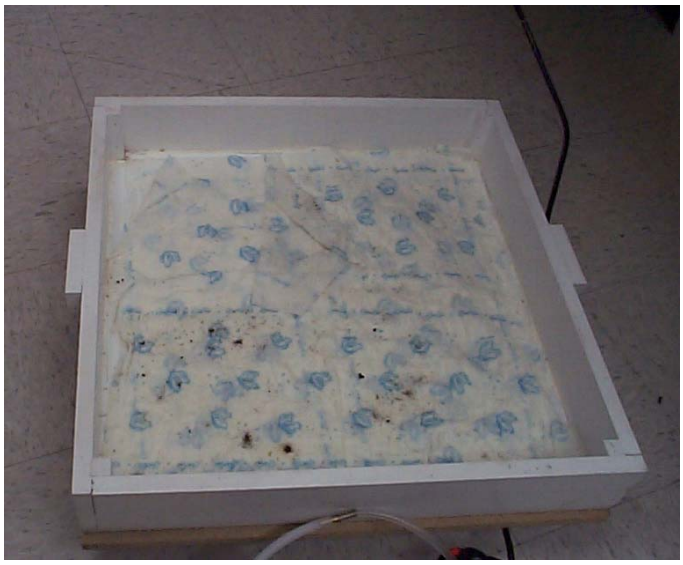

Figure 2-2. Collection tray put under the wooden rack to collect the dropped full grown plum curculio larvae. Wet paper towels were placed in the bottom to maintain moisture.

Figure 2-3. Pupation jars. Pint glass mason jars containing moistened 50:50 potting soil: vermiculite to allow larvae to pupate and adults to emerge.

Figure 2-4. Rearing box. Adults were placed into the plastic box (34 x 26 x 8.5cm) with organza covered ventilated top. About 25 green thinning apples were used as food and oviposition sites. About 100 adults per box.

Figure 2-5. Cross-mating experimental setup. Each single curculio pair was placed into a 4 oz (120 ml) plastic cup with a green thinning apple as a food and oviposition site and one inch (2.5 cm) soaked cotton dental wick (Absorbal, Wheat Ridge, CO) as water source.

Figure 2-6. A typical crescent-shaped oviposition scar.

Figure 2-7. Black arrow indicates a small hole. The hole indicates that the egg hatched successfully. A newly hatched larva made the hole and burrowed into the apple.

Figure 2-8. Unhatched egg. After holding for 5 days, the egg did not hatch and it was counted as an unhatched egg.

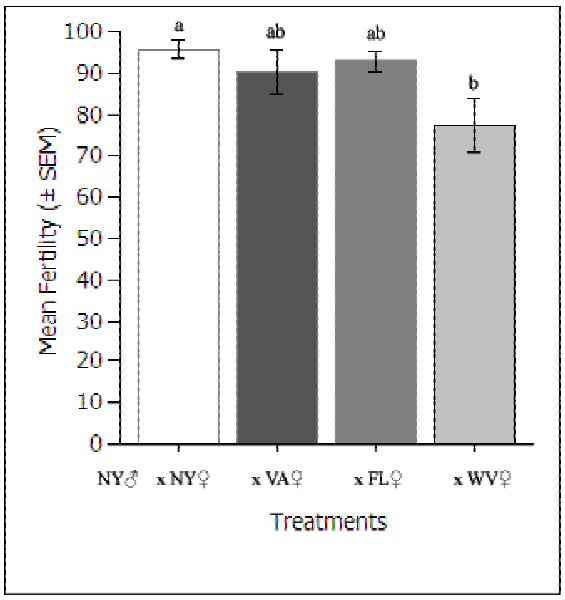

Figure 2-9. Mean fertility (± SEM) of NY males mated with NY, VA, FL, and WV females, respectively. Values represent percentage of eggs hatch (fertility) (*n* = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

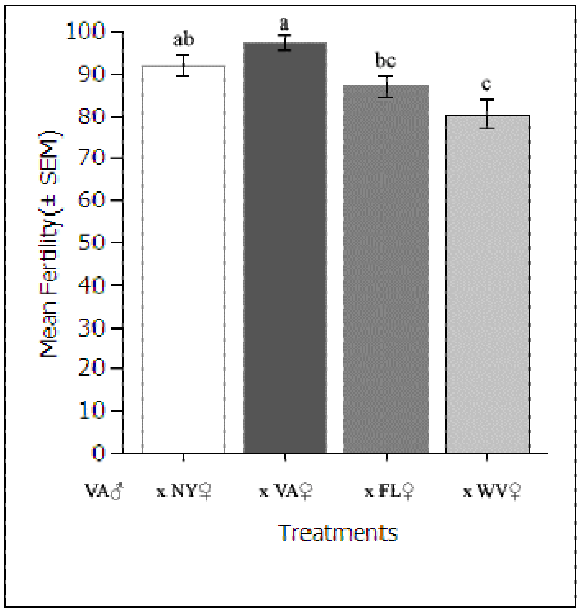

Figure 2-10. Mean fertility (± SEM) of VA males mated with NY, VA, FL, and WV females, respectively. Values represent percentage of eggs hatch (fertility) (*n* = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05)

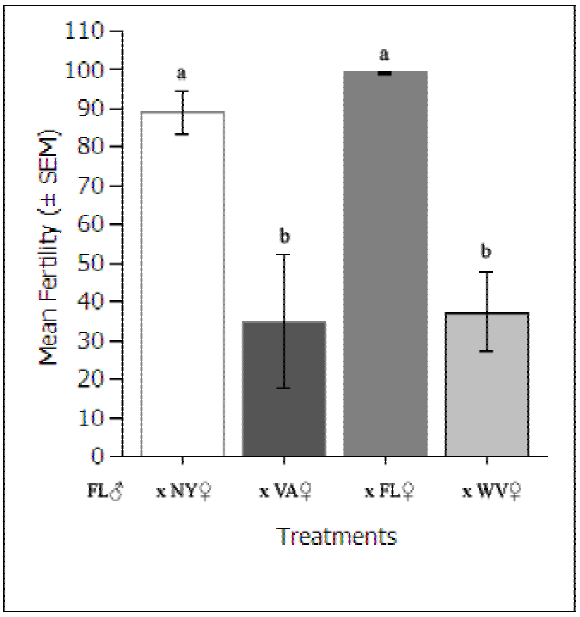

Figure 2-11. Mean fertility (± SEM) of FL males mated with NY, VA, FL, and WV females, respectively. Values represent percentage of eggs hatch (fertility) (n = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

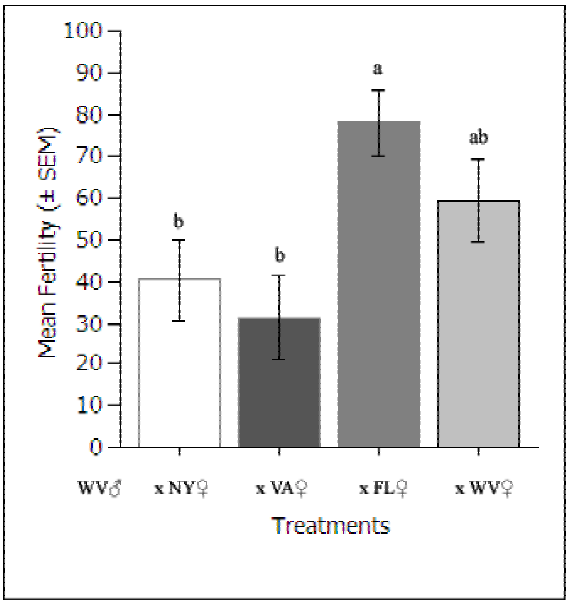

Figure 2-12. Mean fertility (± SEM) of WV males mated with NY, VA, FL, and WV females, respectively. Values represent percentage of eggs hatch (fertility) (n = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

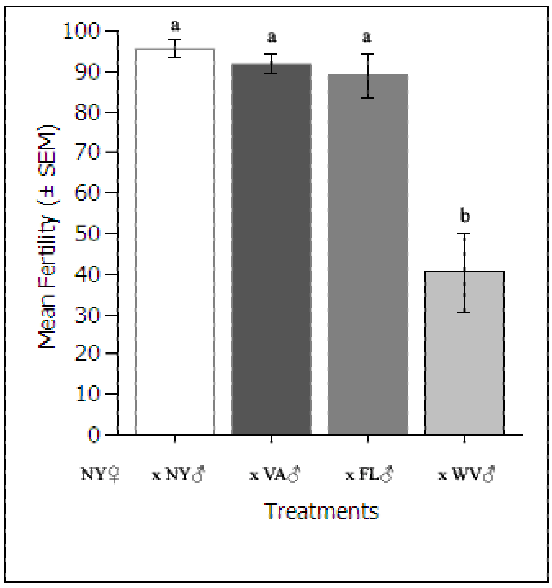

Figure 2-13. Mean fertility (± SEM) of NY females mated with NY, VA, FL, and WV males, respectively. Values represent percentage of eggs hatch (fertility) (n = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

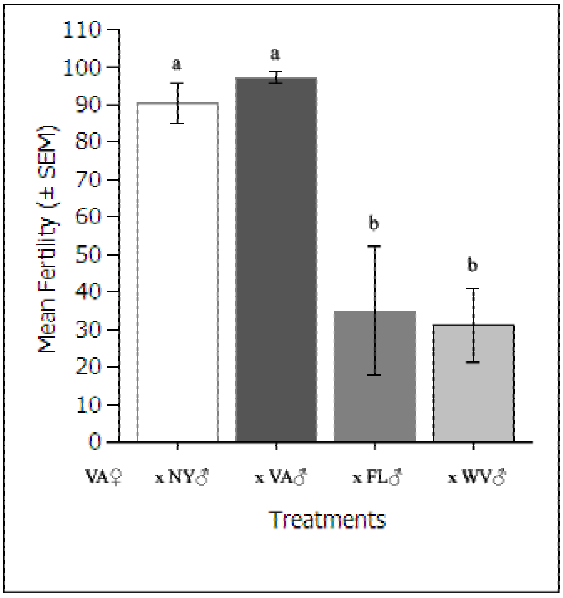

Figure 2-14. Mean fertility (± SEM) of VA females mated with NY, VA, FL, and WV males, respectively. Values represent percentage of eggs hatch (fertility) (n = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

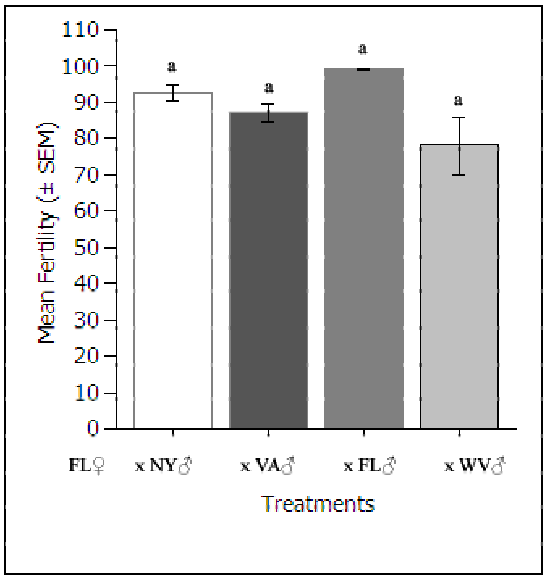

Figure 2-15. Mean fertility (± SEM) of FL females mated with NY, VA, FL, and WV males, respectively. Values represent percentage of eggs hatch (fertility) (*n* = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

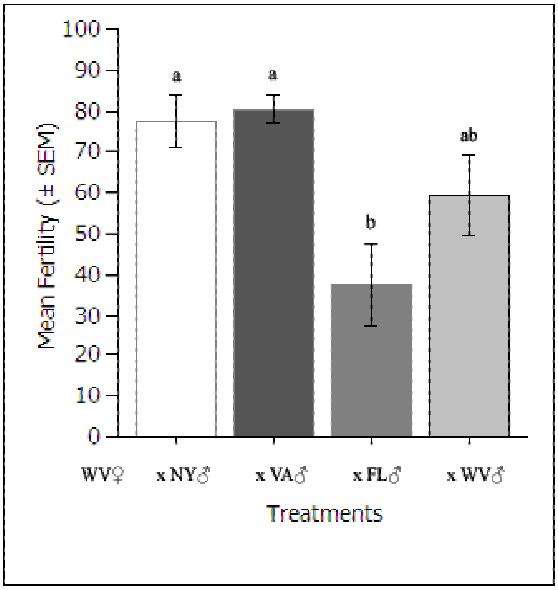

Figure 2-16. Mean fertility (± SEM) of WV females mated with NY, VA, FL, and WV males, respectively. Values represent percentage of eggs hatch (fertility) (*n* = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

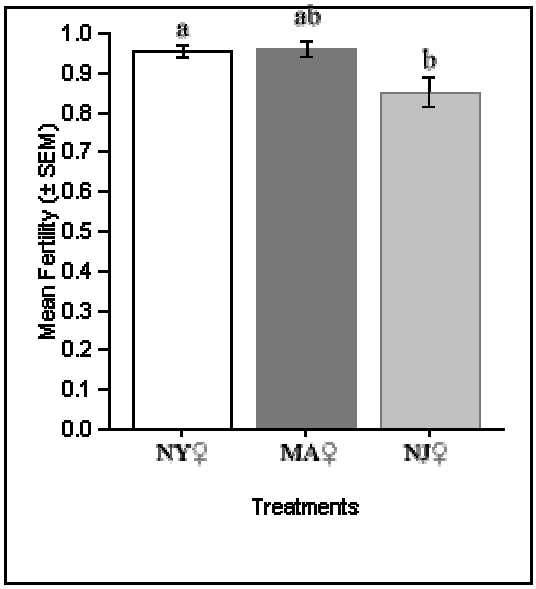

Figure 2-17. Mean fertility (± SEM) of NY, MA, and NJ females, respectively. Values represent percentage of eggs hatch (fertility) (n = 16). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

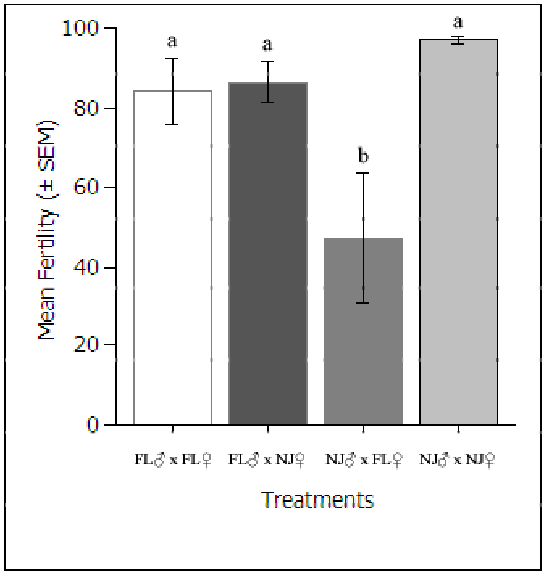

Figure 2-18. Figure 2-18 Mean fertility (± SEM) of FL and NJ reciprocal cross. Values represent percentage of eggs hatch (fertility) (n = 10). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

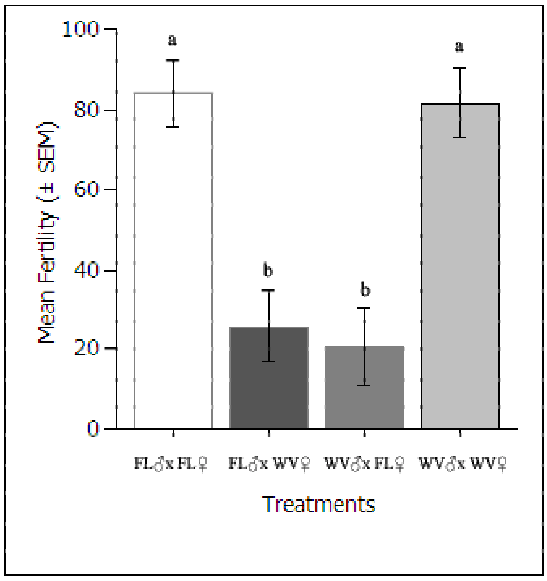

Figure 2-19. Mean fertility (± SEM) of FL and WV reciprocal cross in 2006. Values represent percentage of eggs hatch (fertility) (n = 10). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05).

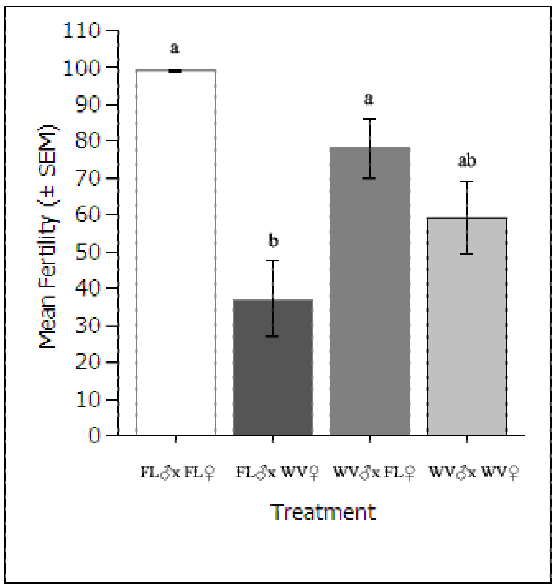

Figure 2-20. Mean fertility (± SEM) of FL and WV reciprocal cross in 2004. Values represent percentage of eggs hatch (fertility) (n = 20). Actual data are shown. Data were transformed by arcsin transformation before statistical analysis. Means with the same letter were not significantly different (P > 0.05)

Chapter 3 Phylogeographical analysis of *Conotrachelus nenuphar* populations based on mitochondrial DNA markers

Abstract

I investigated the genetic diversity among plum curculio, *Conotrachelus nenuphar* (Herbst) populations from different geographic locations using the partial mtCOI gene. A total of 50 samples were sequenced for the partial mtCOI gene. PCR products were 863 bp in length. A total of 23 unique sequence haplotypes were found in the 50 samples tested from 10 populations. Haplotypes G (n = 5), L (n = 12) and T (n = 13) comprised 60% of 50 samples. The nucleotide distances between those haplotypes ranged from 0.12% to 4.87%. Distances between northern and southern group plum curculios ranged from 4.17% to 4.87%. Two distinct major clades were found. In all three kinds of phylogenetic analyses: 1) neighbor joining (NJ), 2) maximum-parsimony (MP), and 3) maximum-likelihood (ML). 100% of the bootstraps support the northern clade, and the southern clade was supported strongly (100/100/86, NJ/MP/ML) as well. The mid-southern subclade within the southern clade was also strongly supported (70/82/71, NJ/MP/ML) and the far-southern subclade was supported in NJ tree (81%) but was not resolved in MP and ML trees. The mid-southern subclade included haplotypes from two NJ, Washington, VA (Ra), Blacksburg, VA (BL) and 50% of WV populations and the far-southern subclade included haplotypes from FL, GA, Whitethorne, VA (Ke), Troutville, VA (Bo) and another 50% of WV populations. The results suggested that the northern and the southern clades corresponded with northern strain and southern strain of plum curculio. In this study, the mtCOI sequence was highly informative as a molecular marker in that it was useful to distinguish *C. nenuphar* from northern and from southern geographic locations in eastern United States. However, the number of generations per year of several geographic populations within southern clade still needs to be determined.

Introduction

There are two strains of *Conotrachelus nenuphar* (Herbst): a northern univoltine strain and a southern multivoltine strain (Chapman 1938). The strains are morphologically indistinguishable; they only differ phenologically. The northern univoltine strain has an obligatory diapause and the southern multivoltine strain commonly has at least two generations per year (Bobb 1952). The multivoltine strain has been reported in many states: Virginia, North Carolina, Georgia, Missouri, Illinois (Leiby and Gill 1923, Snapp 1930, Chandler 1932, Bobb 1952, Sarai 1969). However, these strains may have been brought from other areas by human activity and spread to other places over time. An isolated univoltine population was found in northern Utah in the early 1980's (Alston and Stark 2000).

Unlike the first generation, which attacks the fruit early, the second generation may be present as larvae in the fruit at harvest. A fruit export concern exists if second generation larvae occur in harvested fruit. Other states and foreign countries that do not have plum curculio have refused to import fruit from those areas where the multivoltine strain of plum curculio exists. Chapman (1938) presented an approximate distribution map of the two strains in which the mid-Atlantic region is the convergence area of the two strains, with Virginia having both strains. The distribution line is approximately the Appalachian Mountains (Chapman 1938). The multivoltine strain not only causes a trade barrier to be imposed by other states or countries but also a late brood would complicate pest control. Therefore, a method to distinguish the two strains and clarification of the distribution of plum curculio strains is necessary for IPM decision-making in eastern North America.

Only one previous work has studied molecular markers for plum curculio strain analysis. McClanan (2004) analyzed the difference between a univoltine strain and a multivoltine strain of plum curculio using a RAPD-PCR assay. That study showed that four primers (OPE-01, OPE-03, OPE-04 and OPE-07) of 15 primers tested could be used to distinguish the two strains of plum curculio from two states (Massachusetts and Georgia).

Mitochondria are found in most eukaryotic cells. Mitochondria have their own DNA and are thought to have originated from free-living bacteria, which were closely related to α-proteobacteria (Boussau et al. 2004). Sequence data of mitochondrial DNA (mtDNA) have become a proven standard for many phylogenetic studies (Caterino et al. 2000). Many mitochondrial genes have been studied, and subunit I of the cytochrome oxidase gene (COI) has become a standard for phylogenetic inference of many insect groups (Lunt et al. 1996). The mtCOI gene sequences were utilized as a marker to construct a phylogenetic tree for different geographic populations (Frohlich et al. 1999, Soucy and Danforth 2002, Cognato et al. 2003 Szalanski and Owens 2003, Artiss 2004, Berry et al. 2004, Shufran et al. 2004, and Torres et al. 2006). According to those analyses, the mtCOI gene seems a reliable molecular marker of intraspecific polymorphism of those insects. Therefore, we examined genetic diversity among plum curculio populations of different geographic locations using the partial mtCOI gene.

Materials and Methods

Insect - plum curculio

All plum curculios used in this study were adults. Both adults and larvae were collected in orchards. Plum curculio larvae from these geographical populations were maintained separately. The larvae were collected from infested fruit using the following procedure: Infested fruit were placed in 60 cm x 60 cm wooden-frame racks with screen (6 mm x 6 mm grid) floors and fitted with a collection tray to catch the dropping PC larvae under the floors. Larvae were placed in 450 ml glass jars containing moistened 50:50 potting soil: vermiculite. Each jar received around 100 larvae, and soil moisture was maintained throughout the pupation period (approximately 3 weeks). The newly emerged adults were collected and maintained separately.

Plum curculio adults were placed in 300 µl of TE (Tris-HCl, EDTA) buffer and then frozen at -80°C until processed. Plum curculios were collected from different geographic locations that included the northern and southern parts of their distribution range in the eastern United States. Collection sites, host plants, and sample sizes are listed in Table 3-1.

DNA extraction

The DNA extraction protocol was modified from a protocol developed for *Drosophila melanogaster* Meigen by Ashburner (1989). Each plum curculio adult was placed in individual 1.5 ml microcentrifuge tubes and ground with Kontés pestles in 300 µl of homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 10 mM EDTA with 5% sucrose) and homogenized. Then a volume of 300 µl of lysis buffer (300 mM Tris-HCl, pH 7.5), 100 mM EDTA, 0.625% sucrose, 1% DEPC) was added and the samples incubated for 15 min at 70°C. When cooled to room temperature, 90 µl of 8 M potassium acetate was added and the samples were incubated for 30 min in ice. Samples were centrifuged at 20,800 g at 4°C for 10 min and then the supernatant was removed to a fresh microcentrifuge tube without disturbing the surface lipid or pellet. The supernatant was extracted twice with an equal volume of 1:1 phenol/chloroform and spun at 20,800 g at 4°C for 5 min. Another extraction was performed with chloroform only to remove residual phenol in the supernatant. DNA was precipitated by adding 2 volumes of absolute ethanol and incubated at room temperature for 5 min. Samples were centrifuged at 20,800 g at room temperature for 5 min and then the supernatant was discarded. The pellet was washed with 400 µl of 70% ethanol and allowed to dry. The dried pellet was resuspended in 50 µl of TE (Tris-HCl EDTA) buffer. RNA was removed by adding 1.5 µl of RNAse Cocktail A (Ambion, Austin) to each sample and incubated for 1 hour at 37°C (or overnight at 4°C) to degrade the RNA. The samples were column-purified (Microcon® Centrifugal Filter Devices, Millipore Corporation, Bedford, MA) to remove RNA fragments. Aliquots of 10 µl of each sample were electrophoresed on a 0.8% agarose gel containing ethidium bromide to visually assess the quality of the DNA. Samples were also tested using a GeneQuant DNA Calculator (Pharmacia Biotech, Piscataway, NJ) spectrophotometer to estimate the concentration of DNA calculated from absorbance at 260 µm.

Polymerase Chain Reaction (PCR)

PCR was carried out in a total volume of 20 µl containing 600 nM mtCOI primers,

C1-J-2195 MTD-10 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and L2-N-3014 MTD-12 (5' TCCAATGCACTAATCTGCCATATTA-3') (Simon et al. 1994), 1 µl of weevil DNA, 2.5 mM $MgCl_2$, 150 µM dNTP mix (PE Applied Biosystems), 2 µl 10x PCR buffer, 0.75 unit AmpliTaq™ Gold (PE Applied Biosystems) DNA polymerase, and 12.7 µl distilled water. Amplification was completed with the following cycling profile on a Eppendorf® Mastercycler® (Eppendorf North America): 95°C for 2 min (denaturation), then 30 cycles of 95°C for 1 min (denaturation), 52°C for 1 min (primer annealing), 72°C for 1 min (amplification), final extension 72°C for 5 min and held at 4°C. A total of 10 µl PCR product was run on a 0.8% agarose gel containing ethidium bromide to estimate the size of the amplified DNA fragment. Images of stained gels were photographed and stored electronically using a gel photodocumentation system.

Cloning and DNA Sequencing

Fragments amplified with mtCOI gene primers were prepared for cloning as follows using a TA-cloning kit (TOPO-TA® Cloning, Invitrogen): 1-2 µl of the PCR products were directly ligated into pCR®2.1-TOPO® plasmid vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The vector was transformed into bacterial cells (Top 10 component cell, Invitrogen, Carlsbad). The cells tested positive after the transformation, were cultured overnight in 3 ml LB medium containing 100 mg/ml ampicillin. Plasmids containing the PCR product were extracted from bacterial transformants by using Promega plasmid mini-prep kit (Wizard® Plus SV Minipreps DNA Purification System, Promega). The purified plasmids were sent to Davis Sequencing (Davis, CA) or Virginia Bioinformatics Institute (VBI) (Blacksburg, VA). DNA sequencing was carried out using an ABI 3730 automated sequencer in both locations. T7 or M13 Reverse or both sequencing primers were used to sequence those plasmids. Two to nine individuals for the mtCOI gene were sequenced from each population. All sequence data were analyzed using Chromas 1.45 (Conor McCarthy, School of Health Science, Griffith University, Southport, Queensland, AU). Sequences were used in the phylogenetic analysis.

Phylogenetic Analysis

Sequences were chosen for phylogenetic analysis by comparing all individuals from each geographical location and each different sequence represented was included in the analysis. The exactly identical sequences from different individuals were combined to one sequence to represent a haplotype (Table 3-1). Sequences were aligned using CLUSTALW (Thompson et al. 1994). Pairwise nucleotide distances were estimated for mtCOI sequences using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 b10 software package (Swofford 2002).

Phylogenetic tree construction was performed using distance, maximum parsimony (MP) and maximum likelihood (ML) algorithms using PAUP 4.0 b10. Two mtCOI sequences available species *Curculio hilgendorfi* (Harold) (AB177150) and *Drosophila melanogaster* (AJ400907) were used as outgroups to root the trees.

Phylogenetic analyses were conducted by using neighbor joining distance (using simple addition and Uncorrected p distances; NJ), maximum-parsimony (equally weighted; MP) and maximum-likelihood (ML) methods. To generate NJ trees, 1000 bootstrapping replications were performed at a 50% majority-rule consensus tree limit. For parsimony analysis, bootstrapping was performed with PAUP using the heuristic search option for 1,000 replications at a 50% confidence limit with a single step-wise addition and tree-bisection-reconnection (TBR) branch sweeping. For likelihood analysis, phylogenetic trees were constructed using ML optimality criterion with default configuration. Bootstrapping was performed using heuristic search option for 500 replications with a single step-wise addition and TBR branch sweeping and without a molecular clock assumption. The Rogers-Swofford approximation limit was set to 5%. Gaps were treated as missing data.

Results

COI sequence variation

A total of 51 samples were sequenced for partial mtCOI gene (Table 3-1). PCR products for the COI gene were 863 bp in length. Only one sample (SC4) has no amplicon (Figure 3-1) and this sample was excluded from this study. A total of 23 unique sequence haplotypes were in the

50 tested samples from 10 populations (Table 3-1). Haplotype G included 5 samples from 2 populations: 3 from NY (population acronyms are listed in Table 3-1) and 2 from MA; Haplotype L (n = 12) were from 5 different populations: 2 from Bo, 6 from FL, 2 from GA, 1 from Ke and 1 from WV; Haplotype T had the most samples (n = 13) from 4 populations: 5 from NJa, 2 from NJb, 2 from Ra and 4 from WV. The G, L, T three haplotypes included 10%, 24% and 26% individuals, respectively, in 50 samples. The three haplotypes in total were 60% of 50 individuals. The 20 remaining individuals represented one haplotype in each. Although the number of samples in each population was uneven, the three haplotypes appeared most frequently in most populations (8 of 10 populations). BL was the only one having no G, L, or T appearing in the population. Five of six (83.3%) samples in NJa population were haplotype T and 83.3% was the biggest frequency of one haplotype in one population among all populations. The average number of haplotype of a geographic population was 3.1. However, population Bo was represented by only one haplotype and five haplotypes were represented in population WV (Table 3-1). The average of nucleotide frequencies were A = 0.31604; C = 0.17386; G = 0.14756; T = 0.36254. Therefore, the nucleotide frequencies were biased toward A + T, averaging 67.86%. The bias was consistent with other insect mitochondrial genes (Simon et al. 1994, Frati et al. 1997, Artiss 2004). No significant differences were detected between taxa in terms of base composition in the base homogeneity Chi – Squire test using PAUP ($\chi^2 = 1.289$ ($df = 66$), $P = 1.000$).

Nucleotide distances

Pairwise uncorrected p distance between 23 plum curculio COI haplotypes were performed using PAUP 4.0. The results are listed in Table 3-2. From this table, the nucleotide distances between those haplotypes was from 0.12% to 4.87%. The most different (4.87%) pairs were between haplotypes NY3 (D) vs. FLF2 (I), WVF1 (P), (L) or FL4 (H), respectively. Distances between the geographical population groups (northern group: NY and MA; southern group: other 8 populations) ranged from 4.17% to 4.87%. Within northern group distances ranged from 0.12% to 0.58% and within southern group distances ranged from 0.12% to 1.04% (Table 3-2). Within southern group, distances within haplotype 1 – 10 (Table 3-2) ranged from 0.12% to

0.35% and distances within haplotype 11 – 16 (Table 3-2) ranged from 0.12% to 0.46%. Distances between the two subgroups ranged from 0.46% to 1.04%. Distances between the most common haplotypes were compared: G vs. L = 4.06%; G vs. T = 4.52%; L vs. T = 0.70%.

Phylogenetic analysis

Bootstrap values of 70% or greater were considered as strong, 50 to 70% as moderate, and below 50% as weak in describing trees (Hillis and Bull 1993). For neighbor joining (NJ) distance analysis, a NJ tree was constructed with most strongly supported nodes (Figure 3-2). NJ phylogenetic analysis revealed that there were two major geographical clades in the ten plum curculio populations (Figure 3-2). Eleven samples of seven haplotypes (A - G) were from New York and Massachusetts populations, which were placed into the northern clade; the bootstrapping support equaled 100%. Within this clade, there was just moderate resolution: 62% bootstraps for a subclade including haplotypes A and B; 63% bootstraps for a subclade including haplotypes C, D, and E. Haplotypes F and G were in the third subclade. I did not mark these three subclades in the NJ tree because they were not strongly supported and not consistent in other analyses. Another 39 samples of 16 haplotypes from eight other populations were placed into the southern group and also have 100% bootstrapping support. Therefore, the two major clades were distinct. They had $\approx$ 5% divergence from each other (describing in nucleotide distances section). Within the southern clade, there were two major clades: a mid-southern group (bootstrap = 70%), including 18 samples of six haplotypes (R - W) (9 samples from the two NJ populations, 4 from WV, 3 from VA-Ra, and 2 from VA-BL) and a far-southern group (bootstrap = 81%), including 21 samples of ten haplotypes (H - Q) (2 from VA-Bo, 2 from VA-Ke, 4 from WV, 4 from GA and 9 from FL). Haplotype W (BLF2) appeared separated from other five haplotypes but still belonged to the mid-southern subclade.

In maximum parsimony (MP) analysis, 598 nucleotides were constant in 868 characters spanned after the sequences aligned using 23 haplotypes and two outgroups. MP analysis indicated that 180 sites were uninformative and 90 sites were parsimony informative. Equally weighted parsimony analysis of the data set produced a single tree of 327 steps (Figure 3-3). The

MP tree received similar strong support at most of the same nodes as the NJ tree. Clearly, both 100% bootstraps supported the two major clades: northern and southern. Within the southern clade, strong support (82%) indicated that mid-southern clade was a separated subclade. And also, strong (94%) support showed that haplotypes R, S, T, U, V separated with haplotype W within the mid-southern subclade. The far-southern subclade was not resolved in MP tree. But those 10 haplotypes, from H to Q, were still separated with the mid-southern subclade (Figure 3-3).

For maximum likelihood (ML) analysis, nucleotide empirical frequencies were: A = 0.31680; C = 0.17087; G = 0.14700; T = 0.36533. A tree (Figure 3-4) that was indentical to the MP tree was constructed using the ML method, but bootstrap support differed somewhat. The two distinct major clades were 100% (northern clade) and 86% (southern clade). The mid-southern subclade within the southern clade was strongly supported (71%). The same structure shown by using MP and ML analyses within the mid-southern group was strong supported (89%). Same result with MP tree, the far-southern subclade was not resolved in ML tree. But haplotypes H to Q were still separated with the mid-southern subclade (Figure 3-4).

The two major clades and the major subclades within the southern clade received strong bootstrap support in all analyses (NJ, MP, ML). Therefore, I assigned the 50 samples of 23 haplotypes into three groups according to their major members' geographic locations (described in NJ tree results). Figure 3-5 is a summary map of the plum cuculio mtCOI haplotype distribution in the eastern United States.

Discussion

Molecular methods have been used to differentiate genetic variants based on genetic polymorphisms in many insects. The mtCOI sequence used as a molecular marker reveals moderate to high degrees of divergence among phylogeographic populations and is an informative molecular marker for discerning genetic differences between haplotypes of many insects, e. g. a sweat bee (*H. rubicundus*) (Soucy and Danforth 2002), Pinyon pine beetle (*I.*

confusus) (Cognato et al. 2003), a migratory dragonfly (*L. quadrimaculata*) (Artiss 2004), whitefly (*B. tabaci*) (Frohlich et al. 1999, Berry et al. 2004), and *L. testaceipes* (Shufran et al. 2004).

There has been no previous work on mitochondrial molecular markers in plum curculio. In this study, the mtCOI sequence was highly informative as a molecular marker in that it distinguished *C. nenuphar* from northern and from southern geographic locations in the eastern United States. The mtCOI sequence analyses carried out for the first time for plum curculio populations suggested that two distinct major clades were found. In all three kinds of phylogenetic analyses, 100% bootstraps support the northern clade (Figure 3-2) and the southern clade was strongly supported (100/100/86, NJ/MP/ML) as well (Figure 3-2). The individuals from NY and MA populations were of the univoltine biotype and corresponded with the northern clade in phylogenetic analyses. The individuals from GA and FL were clearly of the multivoltine biotype and were within the southern clade. Therefore, the results of phylogenetic analyses suggest that the northern clade and the southern clade corresponded with the northern strain and southern strain of plum curculio. However, the status of voltinism in several populations is unclear. In the Mid-Atlantic area, two NJ populations, Ra, BL and partial of WV populations belonged to a mid-southern subclade. FL, GA, Ke, Bo and partial WV populations belonged to a far-southern subclade (Figure 3-5). Of eight samples from WV population, 50% of the individuals were haplotype T and were placed in the far-southern group; another 50% have L, M, P, and Q haplotypes and were placed in the mid-southern group. T haplotype individuals can continuously reproduce in lab conditions (Leskey, unpublished data), i.e. they represent a multivoltine strain. Another 4 individuals are still unclear to date. From my lab observation, the summer generation of NJ, Ke, Bo, and BL populations can reproduce in lab conditions and after feeding for various periods. There is no evidence for a second generation in the Ra population because of limited samples. Therefore, I believe that NJ, Ke, Bo, BL populations have the reproductive potential for a summer generation, but more lab observation and field work are required to confirm their reproductive situations.

Moreover, the sequence divergence within the southern clade was low (0.46% to 1.04% between the two subclades). In 863 bp, 4 to 9 nucleotides (nt) differed, but within the far-southern group only 1 to 3 different nts differed and 1 to 4 nts differed within the mid-southern group. The low diversity was not surprising, compared with other studies. Sequence difference among haplotypes ranged from 0.25 to 1.6% in pinyon pine beetle (Cognato et al. 2003). However, there were up to 17.1% pairwise divergences in whitefly (Frohlich et al. 1999). The sequence divergences are dependent on the length of COI gene fragment selected and species. According to phylogenetic analyses, the mid-southern subclade was supported strongly (70/82/71, NJ/MP/ML) in the three methods. However, the far-southern subclade was only strongly supported by NJ tree. This subclade was not resolved in MP and ML trees may due to their very close genetic distances within the southern clade. To fully resovle this subclade, combining COI and COII gene data to enlarge the informative sites is a possible method.

The sample size was still small. So far, 50 individuals were tested for mtCOI gene analysis, with only two samples each from 3 populations. The uneven sample sizes may have caused a bias in the frequency of haplotype. According to the results, samples from northern strain plum curculio should be increased because only two northern geographic populations were included in this study. The status of subclades within the northern clade is still ambiguous. More samples could clarify the haplotype distribution within the northern clade.

In this study, only one sample (SC4) had no PCR product using mtCOI primers. This is the only sample using ethanol preservation. All other samples were alive until just before they were put into a –80 °C freezer. The DNA extraction protocol may have to change somewhat to adapt the different preservation conditions because it is not easy to keep specimens alive in most situations.

To date, there are few molecular marker studies in species closely related to plum curculio. Using a BLAST search (Altschul et al. 1997) in the website of the National Center of Biotechnology Information (NCBI, http:// www.ncbi.nlm.nih.gov/BLAST/), the available mtCOI sequence is *Curculio hilgendorfi,* which is in the same family as plum curculio but not in the

same genus. The available information of these closely related species may help in studies of the evolutionary relationships for plum curculio.

Plum curculio is a key pest in many tree fruits in North America. Knowledge of the strain distribution status is essential to the management of this pest. The phylogenetic analysis based on mtCOI gene sequence could serve as a potential means to differentiate the northern and the southern plum curculio strains. This study would enrich our phylogeographical information about the pest from a molecular view and could provide an ecological and economic benefit to fruit production in the mid-Atlantic area. In future studies, increasing sample size, especially more northern strain weevils may need to be tested; and combine with COII gene or other molecular markers to enlarge the informative sites to more fully understand the evolution of plum curculio strains.

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Table 3-1. Population, geographic source, host plant and haplotype for plum curculio mitochondrial cytochrome oxidase I (mtCOI) gene sequences.

Population	Location	Host Plant	Acronym	Haplotype(# of samples)
Massachusetts*	Amherst, MA	Apple	MA	A(1), B(1), E(1), G(2)
New York*	Geneva, NY	Apple	NY	C(1), D(1), F(1), G(2)
New Jersey A*	Bridgeton, NJ	Nectarine	NJ or NJa	T(5), U(1)
New Jersey B*	Chatsworth, NJ	Blueberry	NJb	R(1), T(2)
West Virginia*	Kearneysville, WV	Peach, plum	WV	L(1), M(1), P(1), Q(1), T(4)
Rappahannock*	Washington, VA	Apple	Ra	S(1), T(2)
Botetourt	Troutville, VA	Apple	Bo	L(2)
Blacksburg	Blacksburg, VA	Apple, plum	BL	V(1), W(1)
Kentland	Whitethorne, VA	Apple	Ke	L(1), N(1)
South Carolina*	Clemson, SC	Peach	SC	-
Georgia*	Byron, GA	Peach, plum	GA	K(1), L(2), O(1)
Florida*	Quincy, FL	Peach	FL	H(1), I(1), J(1), L(6)

* Plum curculio contributors: Massachusetts: Jaime Piñero, Ronald Prokopy; New York: Arthur Agnello; New Jersey A: Peter Shearer; New Jersey B: Sridhar Polavarapu; West Virginia: Tracy Leskey; Rappahannock, VA: Kenner Love; South Carolina, Georgia: David Jenkins, Dan Horton; Florida: Russell Mizell.

Table 3-2. Uncorrected ("p") pairwise distance matrix for cytochrome oxidase I (COI) sequences between 23 plum curculio haplotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 GAF2 (K)	-																						
2 GAP1 (O)	0.00232	-																					
3 FLF2 (I)	0.00348	0.00348	-																				
4 WVF1 (P)	0.00348	0.00348	0.00463	-																			
5 (L)	0.00116	0.00116	0.00232	0.00232	-																		
6 FL4 (H)	0.00232	0.00232	0.00348	0.00348	0.00116	-																	
7 WVF3 (M)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	-																
8 FLF1 (J)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	-															
9 KeF1 (N)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	0.00232	-														
10 WVM1 (Q)	0.00232	0.00232	0.00348	0.00348	0.00116	0.00232	0.00232	0.00232	0.00232	-													
11 BLF1 (V)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	-												
12 NJb1 (R)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	-											
13 Ra3 (S)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	0.00232	-										
14 NJF3 (U)	0.00927	0.00927	0.01043	0.01043	0.00811	0.00927	0.00927	0.00927	0.00927	0.00927	0.00232	0.00232	0.00232	-									
15 (T)	0.00811	0.00811	0.00927	0.00927	0.00695	0.00811	0.00811	0.00811	0.00811	0.00811	0.00116	0.00116	0.00116	0.00116	-								
16 BLF2 (W)	0.00463	0.00463	0.00579	0.00579	0.00348	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00463	0.00348	-							
17 MAF1 (A)	0.04519	0.04519	0.04635	0.04635	0.04403	0.04519	0.04519	0.04519	0.04519	0.04287	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	-						
18 MA5 (B)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04519	0.04519	0.04519	0.04519	0.04403	0.04056	0.00232	-					
19 NYM1 (C)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	-				
20 MAF2 (E)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	0.00232	-			
21 NY2 (F)	0.04287	0.04287	0.04403	0.04403	0.04171	0.04287	0.04287	0.04287	0.04287	0.04056	0.04751	0.04751	0.04751	0.04751	0.04635	0.04287	0.00463	0.00232	0.00232	0.00232	-		
22 NY3 (D)	0.04403	0.04403	0.04519	0.04519	0.04287	0.04403	0.04403	0.04403	0.04403	0.04171	0.04867	0.04867	0.04867	0.04867	0.04751	0.04403	0.00579	0.00348	0.00348	0.00348	0.00348	-	
23 (G)	0.04171	0.04171	0.04287	0.04287	0.04056	0.04171	0.04171	0.04171	0.04171	0.0394	0.04635	0.04635	0.04635	0.04635	0.04519	0.04171	0.00348	0.00116	0.00116	0.00116	0.00116	0.00232	-

Different haplotypes are labeled by sample name and name of haplotype included in parentheses. Haplotype G includes samples NYF1, NYF2, NYM2, MA4 and MA31; Haplotype L includes samples Bo3, Bo4, FL1, FL2, FL3, FLF4, FLM1, FLM2, GAM1, GAu1, KeF2, WVM3; Haplotype T includes samples NJb11, NJb12, NJa5, NJF2, NJF4, NJM1, NJM4, Ra4, Ra5, WVp1, WVP2, WVu1, WVu3.

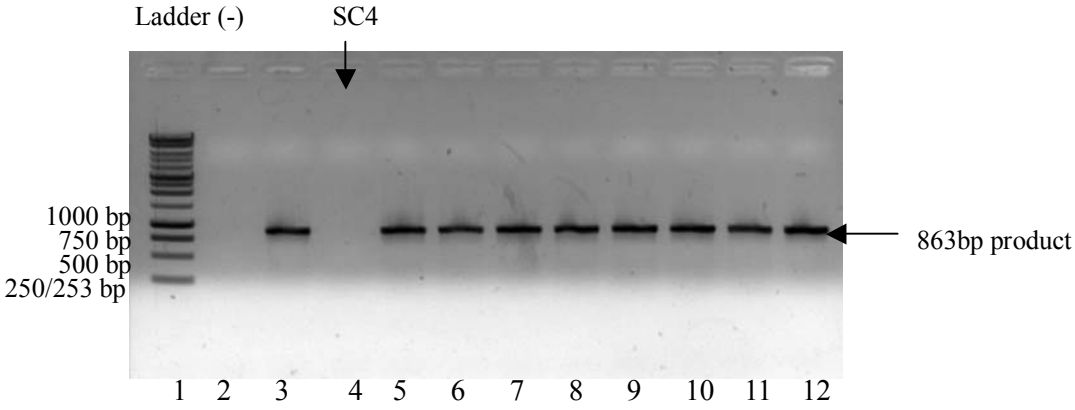

Figure 3-1. Mitochondrial cytochrome oxidase I (mtCOI) PCR results. 10 µl PCR products were run on a 0.8% agarose gel containing ethidium bromide. Lane 1 is 1Kb DNA ladder (Promega); Lane 2 is negative control; Lane 3-12 are samples: Bo3, SC4, NY2, NY3, MA4, MA5, WVu1, WVp1, FL1, FL2, respectively. There was no PCR product in lane 4 (SC4).

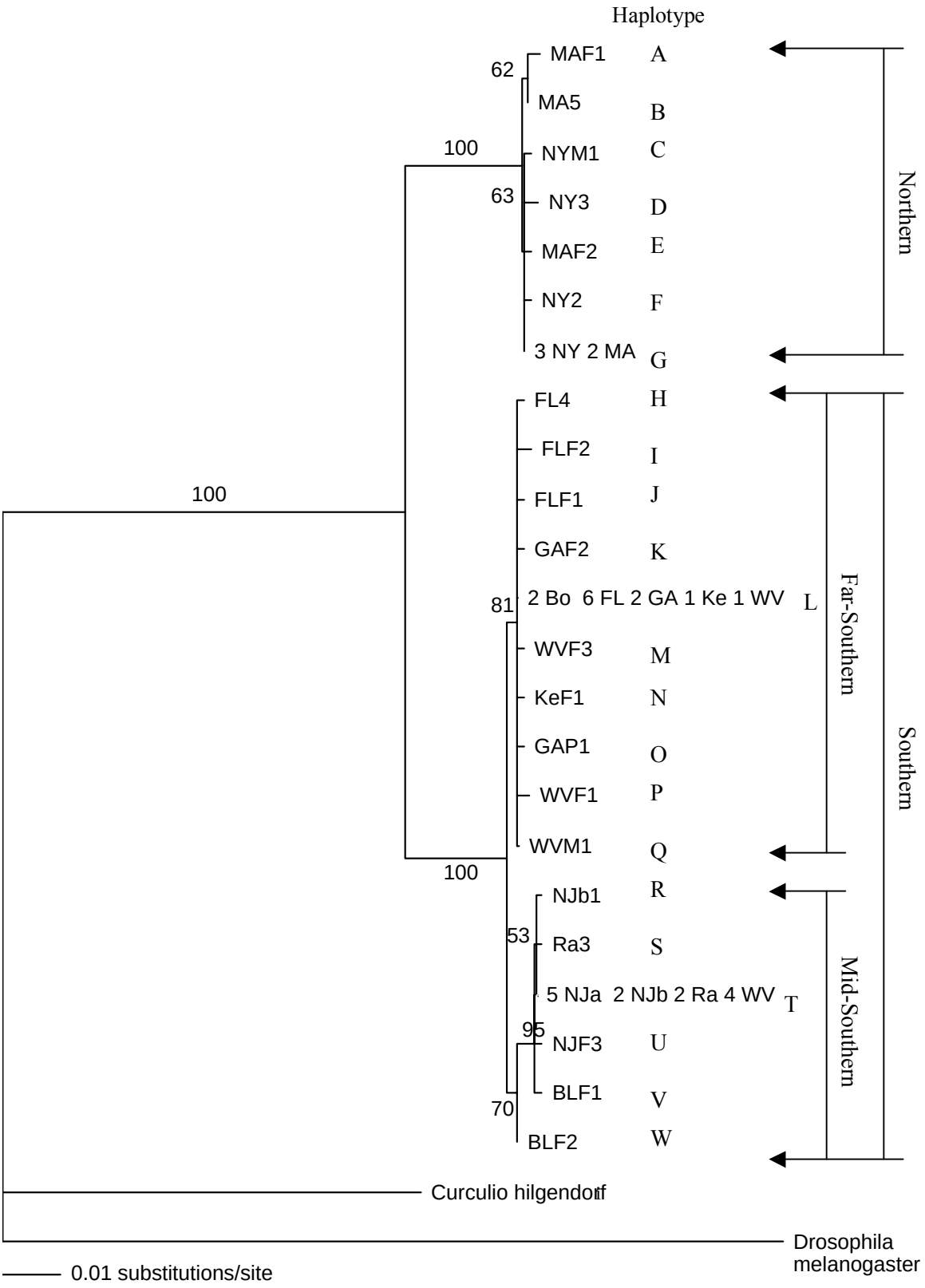

Figure 3-2. Neighbor Joining tree based on mtCOI sequences of plum curculio. Bootstrap percentages of 1000 replications are shown around the branches. Branch lengths are proportional to inferred substations/site. The individual names refer to locations. Outgroups are *Curculio hilgendorfi* and *Drosophila melanogaster*.

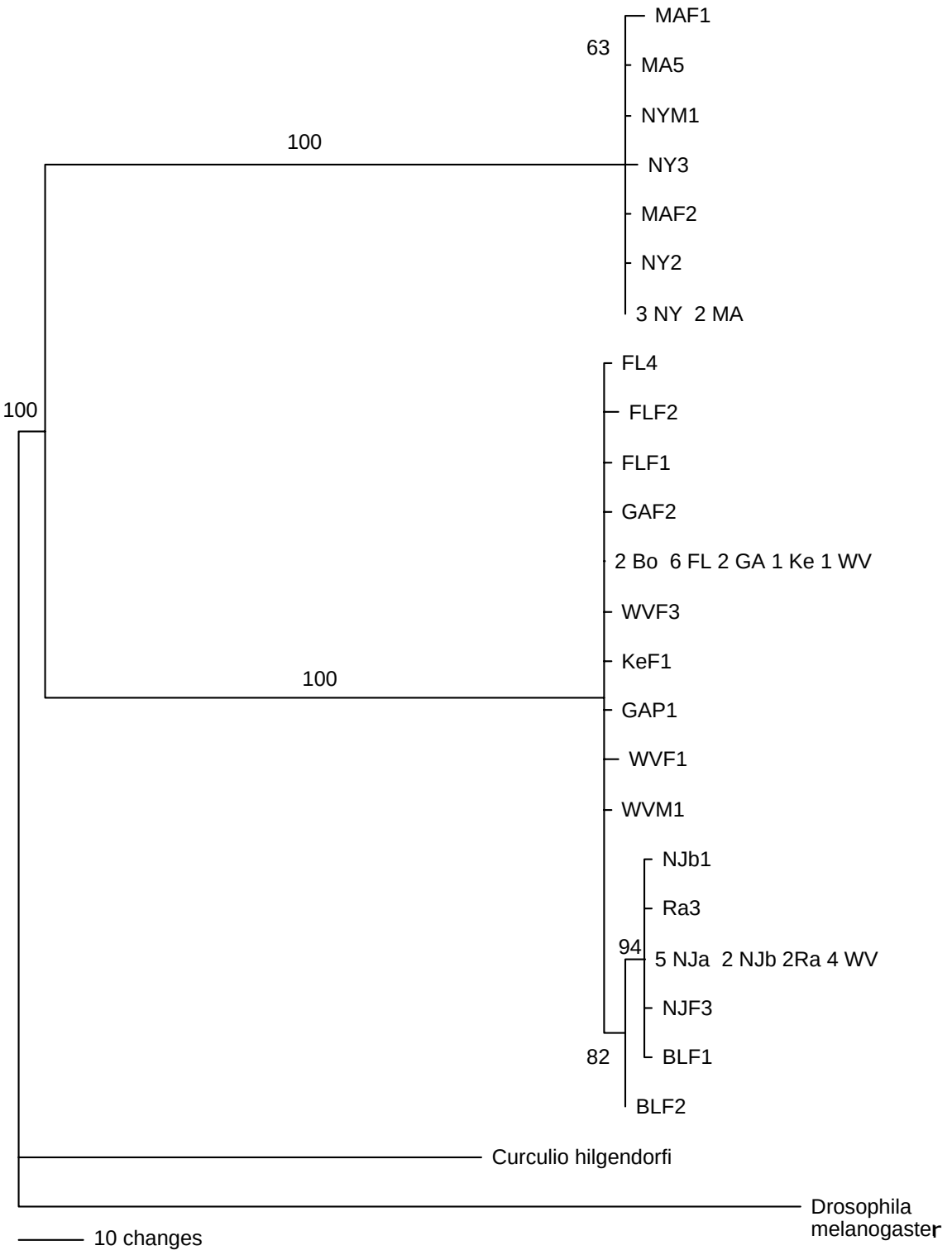

Figure 3-3. Maximum parsimony tree based on mtCOI sequences of plum curculio. Bootstrap percentages of 1000 replications are shown around the branches. Branch lengths are proportional to inferred nucleotide changes. The individual names refer to locations. Outgroups are *Curculio hilgendorfi* and *Drosophila melanogaster*.

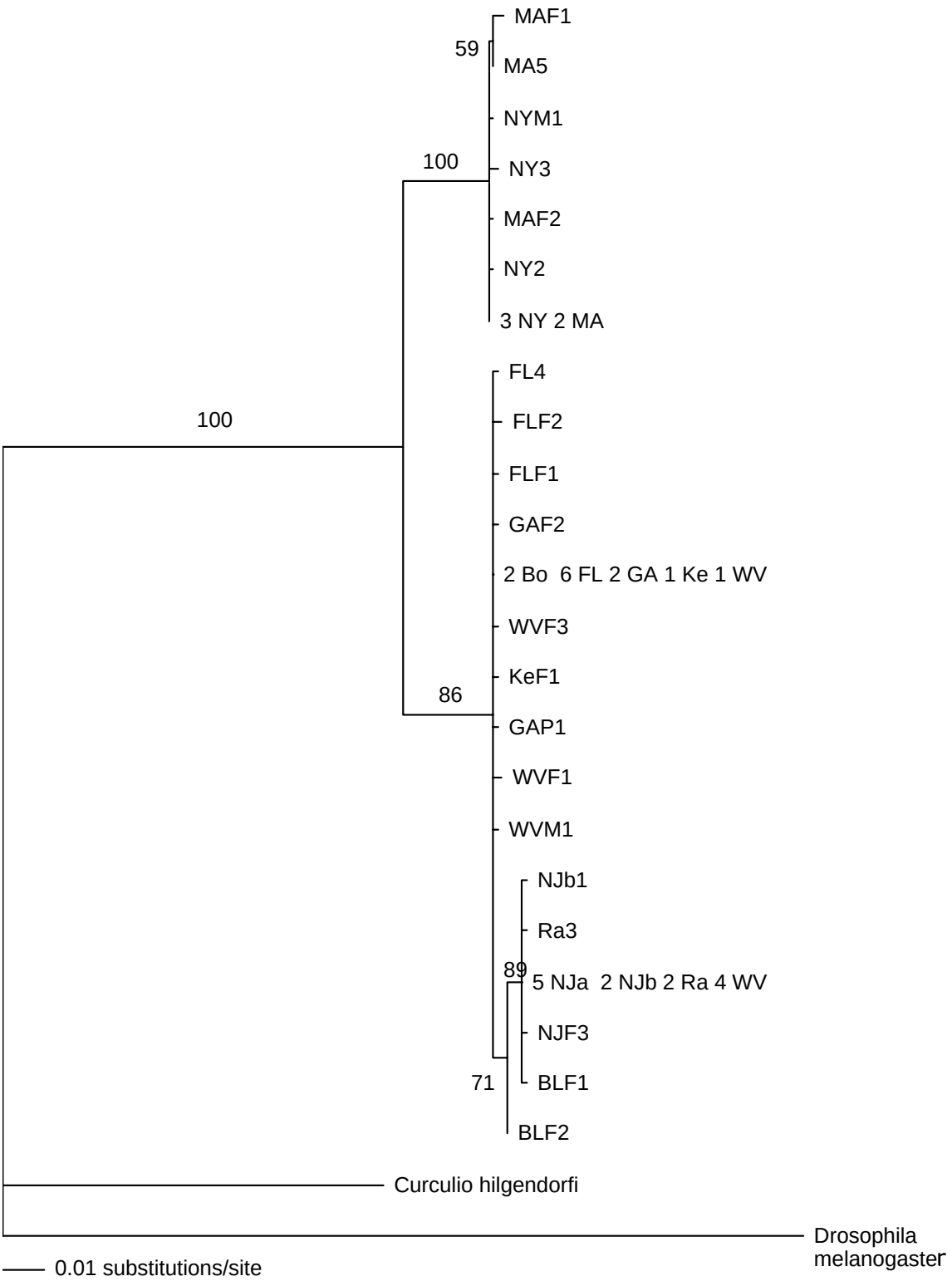

Figure 3-4. Maximum likelihood tree based on mtCOI sequences of plum curculio. Bootstrap percentages of 500 replications are shown around the branches. Branch lengths are proportional to inferred nucleotide substitutions/site. The individual names refer to locations. Outgroups are *Curculio hilgendorfi* and *Drosophila melanogaster*.

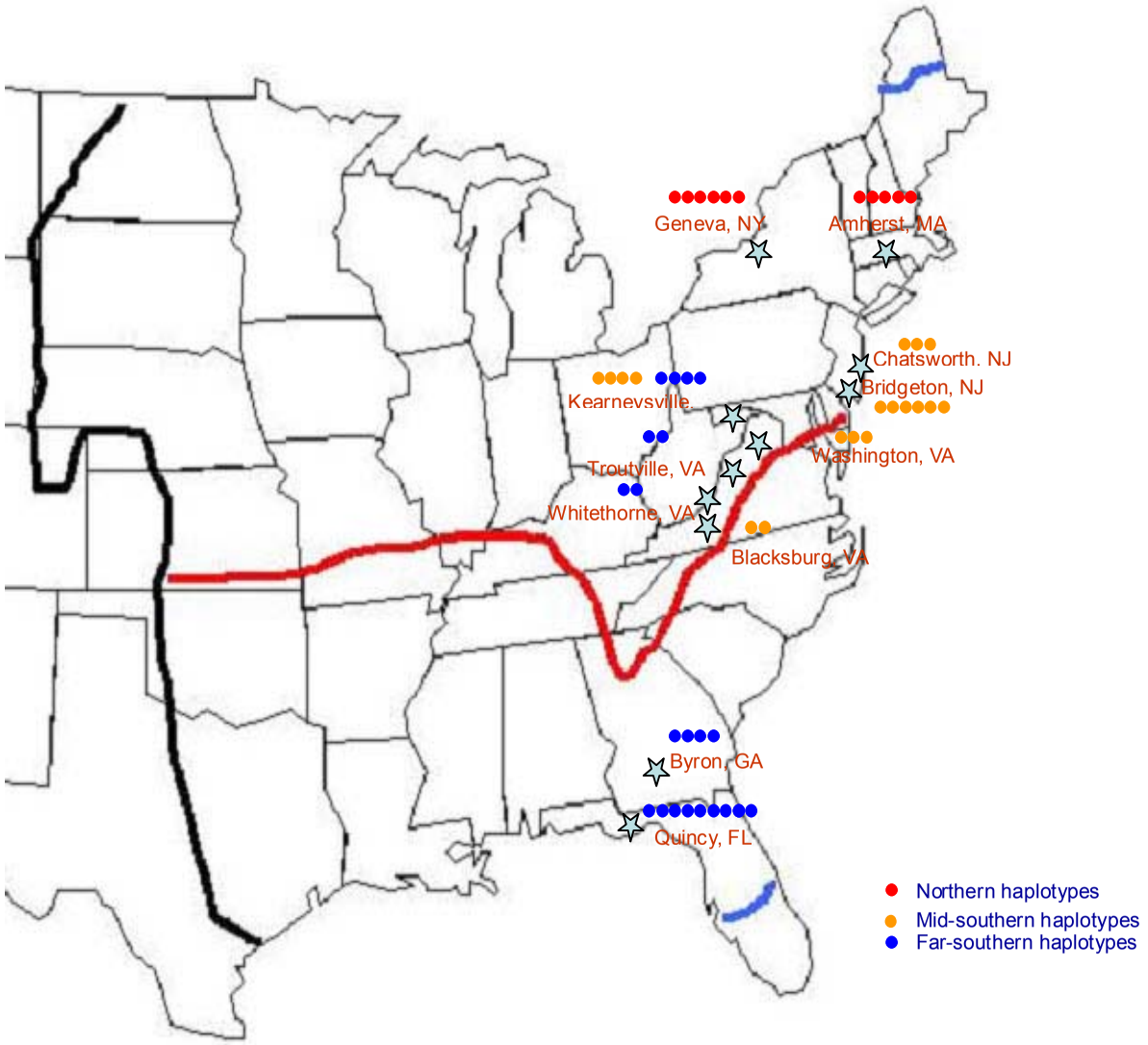

Figure 3-5. A distribution map of mtCOI haplotype of plum curculio geographical populations in eastern United States. Colored lines indicate plum curculio distribution range and red line is an approximate line between two plum curculio strains (Chapman 1938). Colored dots indicate different haplotype group. Stars indicate geographic locations (positions indicated approximately)

Chapter 4 Analysis of *Wolbachia* strains associated with plum curculio in the eastern North America

Abstract

I investigated the distribution patterns of *Wolbachia* infection associated with plum curculio strains. 91 of 93 samples (97.8%) were infected by *Wolbachia*. Three distinct *Wolbachia* strains were identified. The strains wCne1 (593 bp) and wCne2 (593 bp) were 97% identical, and their sequences were both 84% identical with wCne3 (590 bp). BLASTN searches through GenBank (Altschul et al. 1997) revealed strong similarities between the *wsp* sequences of the three strains compared with *Wolbachia* sequenced from other hosts. Degree of similarity with sequences in other *Wolbachia* strains is discussed. PCR - Restriction Fragment Length Polymorphism (RFLP) was used for superinfection detection. Of 93 samples, 15 (16.1%), 21 (22.6%), 19 (20.4%), 36 (38.7%) samples were infected by wCne1, wCne2, wCne1+2, and wCne3, respectively. Only two (2.2%) samples had no infection. The wCne3 strain was always present as a single infection. *Wolbachia* strains approximate the distribution of PC strains: northern strain infected with wCne1 and wCne2 strains in supergroup B, southern strain infected with wCne3 strain in supergroup A, with the mid-Atlantic region as the convergence area. Based on haplotype distribution of plum curculio mitochondrial cytochrome oxidase I (mtCOI), there was a closer relation of the mid-southern PC clade to the far-southern clade than to the northern clade. However, *Wolbachia* symbionts in mid-southern PC are more closely related to those in northern PC than to those in far-southern PC. The relationship of *Wolbachia* infection with reproductive incompatibility between plum curculio populations was also discussed.

Introduction

Plum curculio (PC), *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is a

key pest of pome and stone fruit in the eastern North America. The insect can cause almost all of the fruit to be injured if no control actions are taken. There are two strains of plum curculio. Chapman (1938) gave an approximate distribution description of the two strains. In this map, the mid-Atlantic region is the convergence area of the two strains and Virginia contains both strains.

Wolbachia is a very common cytoplasmic symbiont of insects, crustaceans, mites, and filarial nematodes, and has been intensively studied since the 1990's (Werren et al. 1995a, Jeyaprakash and Hoy 2000, Kikuchi and Fukatsu 2003, Tagami and Miura 2004). *Wolbachia pipientis* could cause cytoplasmic incompatibility in their mosquito host (Yen and Barr 1971). Both unidirectional and bi-directional CI had been examined (Laven 1959, 1967, O'Neill 1997, Dobson et al. 2001, Riegler and Stauffer 2002, Keller et al. 2004, and Merçot and Charlat 2004). *Wolbachia* was proposed as an agent of speciation (Shoemaker et al. 1999 and Bordenstein et al. 2001).

Reproductive incompatibility between plum curculio strains was reported since the 1960's (Stevenson and Smith 1961 and Padula and Smith 1971). This reproductive incompatibility is similar to that reported from other arthropods associated with infection of reproductive tissues by *Wolbachia*. To date, there is no direct evidence linking *Wolbachia* with CI in plum curculio. McClanan et al. (2004) analyzed three strains *Wolbachia* associated with Massachusetts (MA), Georgia (GA) and Florida (FL) plum curculio populations, respectively. Their results suggested that univoltine and multivoltine weevils carry different strains of *Wolbachia*. The reproductive incompatibility between the two plum curculio strains could be due to the different *Wolbachia* strain infection.

Expanding on McClanan's previous work, I investigated *Wolbachia* single or multiple infection status in ten geographical populations of plum curculio. This study was conducted to classify *Wolbachia* infecting strain(s) infecting various populations of each of the two plum curculio strains in the eastern United States. The distribution of *Wolbachia* strains associated with plum curculio was analyzed by using the *Wolbachia* surface protein (*wsp*) gene.

Materials and Methods

Insect - plum curculio

All plum curculios used in *Wolbachia* analyses were adults. Both adults and larvae were collected in orchards (Table 4-1). Plum curculio larvae from these geographical populations were maintained separately. The larvae were collected from infested fruit using the procedure described in the materials and methods section of Chapter 3.

Plum curculio adults were placed in 300 µl of TE (Tris-HCl, EDTA) buffer and frozen at –80°C until processed. All 50 samples tested in Chapter 3 were included in the *Wolbachia* strain analysis. Collection sites, host plants, and number of samples tested are listed in Table 4-1.

DNA Extraction

The DNA extraction protocol was modified from a protocol developed for *D. melanogaster* by Ashburner (1989) and was described in the materials and methods section of Chapter 3.

Polymerase Chain Reaction (PCR) Amplification

wsp gene

PCR was carried out in a total volume of 20 µl containing 300 nM *wsp* primers, 81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') and 691R (5'-AAA AAT TAA ACG CTA CTC CA-3') (Zhou et al. 1998), 5-10 ng of weevil DNA, 1.5 mM $MgCl_2$, 200 µM dNTP mix (PE Applied Biosystems), 2 µl 10x PCR buffer, 0.5 unit AmpliTaq™ Gold (PE Applied Biosystems), and 13.5 µl distilled water. Amplification was completed with the following cycling profile on an Eppendorf® Mastercycler® (Eppendorf North America): 94°C for 10 min, then 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension 72°C for 7 min and held at 4°C. A total of 10 µl PCR product was run on a 0.8% agarose gel containing ethidium bromide to estimate the size of the amplified DNA fragment. Images of stained gels were photographed and stored electronically using a gel photodocumentation system. The DNA quality of sample was checked

by amplifying mitochondrial cytochrome oxidase (mtCOI) gene using two conserved primers C1-J-2195 (MTD-10) and L2-N-3014 (MTD-12) (Berry et al. 2004).

fts Z gene

To confirm *wsp* gene amplification, *fts*Z gene PCR was also carried out in a total volume of 20ul containing 300 nM *ftsZ* primers, *fts*ZF (5'-GTA TGC CGA TTG CAG AGC TTG -3') and *fts*ZR (5'-GCC ATG AGT ATT CAC TTG GCT-3') (Kikuchi and Fukatsu 2003), 1-2 µl of weevil DNA, 1.5 mM $MgCl_2$, 200 µM dNTP mix (PE Applied Biosystems), 2 µl 10x PCR buffer, 0.5 unit AmpliTaq^{TM} Gold (PE Applied Biosystems), and 13.5 µl distilled water. Amplification was completed with the following cycling profile on a Eppendorf® Mastercycler® (Eppendorf North America): 94°C for 10 min, then 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, final extension 72°C for 4 min and held at 4°C. A total of 10 µl was run on a 0.8% agarose gel containing ethidium bromide and was visualized using UV light to estimate the size of the amplified DNA fragment. Images of stained gels were photographed and stored electronically using a gel photodocumentation system.

Cloning and DNA Sequencing

Fragments amplified with *wsp* gene primers were prepared for cloning as follows using a TA-cloning kit (TOPO-TA®, Invitrogen): 1-2 µl of the PCR reaction was directly ligated into pCR®2.1-TOPO® plasmid vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The vector was transformed into bacterial cells (Top 10 component cell, Invitrogen, Carlsbad, CA). The cells which tested positive after the transformation were cultured overnight in 3 ml LB medium containing 100 mg/ml ampicillin. Plasmids containing the PCR product were extracted from bacterial transformants by using Promega plasmid mini-prep kit (Wizard® Plus SV Minipreps DNA Purification System, Promega). The purified plasmids were sent to Davis Sequencing (Davis, CA) or Virginia Bioinformatics Institute (VBI) (Blacksburg, VA). DNA sequencing was carried out using an ABI 3730 automated sequencer in both locations. T7 or M13 reverse or both sequencing primers were used to sequence those plasmids. Six to fourteen

individuals for the *wsp* gene were sequenced from each population (Table 4-1).

DNA Sequences Analysis

All sequence data were entered and analyzed using Chromas 1.45 (Conor McCarthy, School of Health Science, Griffith University, Southport, Queensland, AU). The *wsp* sequences were aligned using CLUSTAL W (Thompson et al. 1994) in Biology Workbench (http://workbench.sdsc.edu). Sequences were compared with published *wsp* sequences by running a BLAST (Altschul et al. 1997) search in website of National Center of Biotechnology Information (NCBI, http:// www.ncbi.nlm.nih.gov/BLAST/).

PCR-Restriction Fragment Length Polymorphism (RFLP) Typing

After PCR products were sequenced, specific restriction enzymes were used to digest known *Wolbachia* strains. Three restriction endonucleases, *Bcc*I, *Bst*F5I and *Aci*I (New England Biolabs Inc., Beverley, MA) were used to digest those PCR products; 10 µl of PCR products were incubated with 1 µl of either one of the three enzymes in 25 µl reaction volume at 37°C for 3 hours (65°C for 3h for *Bst*F5I). If an uncut band was shown in the first digestion, the uncut PCR products were digested by other enzymes to detect superinfection. Another enzyme—*Hind*III (Promega, Madison, WI) was used to confirm the results, using the following reaction: 10 µl of PCR products were incubated with 1 µl of *Hind*III enzyme in 25 µl reaction volume at 37°C for 3 hours. The restriction digests were run on a 2.0% agarose gel containing ethidium bromide and was visualized using UV light. Images of stained gels were photographed and stored electronically using a gel photodocumentation system. Sequencing multiple clones for a single PCR product has a higher possibility to find the *Wolbachia* superinfcetion. Therefore, three to six clones were sequenced for five individuals (NY2, NY3, GAp1, Bo1 and FL2) to confirm the superinfection.

Phylogenetic Analysis

The data set contained 31 sequences in total, and consisted of 3 *wsp* sequences from this

study and combined with 28 additional *wsp* sequences retrieved from GenBank, including 11 sequences (GenBank access number: AF020058, AF020061, AF020063, AF020071, AF020075, AF020077, AF020081, AF020082, AF020083, AF020084, AF020085) from Zhou et al. (1998), four sequences (AF071910, AF071911, AF071912, AF071915) from Van Meer et al. (1998), two sequences (AF237884, AF237886) from Ono et al. (2001), two sequences (AF295344, AF295349) from Jamnongluk et al. (2002), three sequences (AF317478, AF317489, AF317491) from Ruang-Areerate et al. (2003), one sequence (AJ130716) from Hurst et al. (1999), one sequence (AF124859) from Vavre et al. (1999), one sequence (AB046721) from Noda et al. (2001), one sequence (AB038339) from Kondo et al. (2000a), one sequence (AB094368) from Miura and Tagami (2002, unpublished), and one sequence (AJ291372) from Nirgianaki et al. (2003). Most of the 28 *wsp* sequences were selected from previous studies which clearly assigned them into the two supergroups and several sequences were selected from BLASTN searches, which find available *wsp* gene sequences from GenBank. All *wsp* sequences were aligned by using the CLUSTAL W (Thompson et al. 1994) in Biology Workbench. The data set was analyzed by using the neighbor joining algorithm from the Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 b10 software package (Swofford 2002). Bootstrap analysis was done with 1000 replications. The phylogenetic trees were midpoint rooted because no suitable outgroup was available.

Results

Detection of Wolbachia infection in plum curculio

In total, 94 individuals representing 11 different geographic populations were screened for *Wolbachia* infection by PCR assay, using *Wolbachia*-specific *wsp* gene and *fts*Z gene primers (Figure 4-1 and 4-2). According to DNA quality checking by amplifying mtCOI gene (PCR protocol was described in Chapter 3), one sample from Clemson, SC (SC4) has no PCR product (Figure 3-1). This sample was eliminated because its DNA quality was insufficient to employ PCR assay. Of 93 samples, 91 samples tested were determined to be infected by *Wolbachia*, using *wsp* primers and confirmed by *fts*Z gene amplification. Two samples, NJM3 and NJb11, did not

yield PCR products in both *wsp* and *ftsZ* amplifications (Table 4-1). The two samples were treated as non-infected.

Three unique sequences were identified from 91 sequences using *wsp* gene. According to the nomenclature convention of Zhou et al. (1998), they were referred to wCne1 (593bp), wCne2 (593bp), and wCne3 (590bp), respectively. Pairwise comparison of their DNA sequences, wCne1 and wCne2 were 97% identical and they were both 84% identical to wCne3. Figure 4-3 shows the alignment result of the *wsp* sequence of the three strains. Blastn searches through GenBank (Altschul et al. 1997) revealed similarities between the *wsp* sequences of the three strains with *Wolbachia* sequenced from other hosts. The *wsp* sequence of wCne1 was 99% identical to *Wolbachia* sequenced from the tortoise beetle, *C. alternans* (wCalt2, AY566421, Keller et al. 2004). The wCne2 sequence was 98.5% identical to the flower bug, *Orius nagaii* Yasunaga (wNag1, AB094368, Miura and Tagami, unpublished). The wCne3 sequence was 100% identical to *Wolbachia* sequenced from the tephritid fruit fly, *Dacus destillatoria* (wDes, AF295344, Jamnongluk et al. 2000) and also from the ant, *Formica exsecta* Nylander (wFex1, AY101196, Reuter and Keller 2003).

To confirm the *Wolbachia* infection in plum curculio, *fts*Z gene amplicons from three individuals (NYM1, MAF2, WVF1, Figure 4-2) were sequenced and they identified infection solely by wCne1, wCne2, and wCne3, respectively, by using the *wsp* gene sequence. Three *fts*Z gene sequences were referred as *fts*Z1, *fts*Z2 and *fts*Z3 in those samples. The *fts*Z1 and *fts*Z2 sequences were 758 bp and the *fts*Z3 sequence was 769 bp in length. Pairwise comparison of their DNA sequences showed *fts*Z1 and *fts*Z2 to be 97% identical and they were both 87% identical to *fts*Z3. According to Blastn searches, the sequence of *fts*Z1 was 99% identical to the *Wolbachia* *fts*Z gene sequenced from the gall wasp, *Diplolepis rosae* L. (WPU83893, Schilthuizen and Stouthamer 1998); the sequence of *fts*Z2 was 99% identical to the *Wolbachia* sequenced from the predatory thrips, *Franklinothrips vespiformis* Crawford (wVes, AB045315, Arakaki et al. 2000, unpublished); the sequence of *fts*Z3 was 98% identical to *Callosobruchus chinensis* L. (AB080665, Kondo et al. 2002b). Therefore, the *fts*Z gene analysis also confirmed the *Wolbachia*

infection in plum curculio.

Superinfection examination

PCR-RFLP was employed to detect superinfection by different *Wolbachia* strains in a single plum curculio individual. Partial results are shown in Figures 4-4, 4-5, and 4-6. The complete results are shown in Table 4-1. Two PCR-RFLP enzyme sets were used to test the superinfection and they confirmed each other. In the first method, the wCne1 strain was digested by *Bcc*I to produce two fragments (550bp + 40bp, Figure 4-4); the wCne2 strain was digested by *Bst*F5I to produce two fragments (410bp + 180bp, Figure 4-4); the wCne3 strain was digested by *Aci*I to produce two fragments (440bp + 150bp, Figure 4-5). In the second method, all PCR products were digested by *Hind*III. The RFLP band pattern of *Hind*III digestion was different from the first method. The wCne1 had no break caused by *Hind*III (Figure 4-6). The wCne2 produced two fragments (450bp + 140bp, Figure 4-6) and the wCne3 produced two fragments (350bp + 240bp, Figure 4-6) when digested by *Hind*III. The *Hind*III digestion results were consistent with the former three enzyme digestion results. Another method was employed: multiple clones from a single PCR product were sequenced to confirm the superinfection. The results are shown in Table 4-2. NY2 was doubly infected by wCne1 and wCne2, NY3 was singly infected by wCne2, and GAp1, Bo1 and FL2 were singly infected by wCne3. The results were consistent with PCR-RFLP analysis.

The results of PCR-RFLP indicated that the *Wolbachia* strains wCne1 and wCne2 were either singly or doubly infected in a single individual, but wCne3 was always present as a single infection in an individual. Of 91 positive samples, 15 (16.1%) were singly infected by wCne1 strain, 21 (22.6%) were singly infected by wCne2 strain, 19 (20.4%) were doubly infected by wCne1 and wCne2, and 36 (38.7%) were singly infected by wCne3 (Table 4-1). Figure 4-8 is a summary map of *Wolbachia* strain distribution in the eastern United States.

Phylogenetic Analysis

A phylogenetic tree of *Wolbachia*, based on the *wsp* sequences, was constructed by Neighbor

Joining algorithm using the Kimura distance and midpoint rooted. *Wolbachia* strains were named by host insect species and the GenBank access numbers were listed as well (Figure 4-7). According to phylogenetic analysis, *Wolbachia* strains wCne1 and wCne2 belong to supergroup B, but wCne3 strain is in supergroup A (Figure 4-7).

Discussion

Population infection frequency and strain identification

In the *Wolbachia* analysis, almost all individuals, 97.8% (91 of 93) were infected by *Wolbachia*. There was only one negative sample in each of two New Jersey populations. The result is consistent with previous studies, e.g. 99.7% infection rate in the European cherry fruit fly, *R. cerasi* (Riegler and Stauffer 2002) and 99.2% infection rate in the tortoise beetle, *C. alternans* (Keller et al. 2004). Here, the infection frequency of plum curculio is slightly lower than in those two studies. The reason could be due to the sample size of this study, which was still small. One individual could cause a larger percentage difference in the infection rate.

There are three distinct *Wolbachia* strains identified by using *wsp* gene sequences. The sequences described by McClanan et al. (2004) were very close to my results. The *Wolbachia* strain carried by MA weevils is wCne1 and the strain associated with FL curculios is wCne3 in this study. There are some differences between our works in GA strain identification: it is same as wCne3 in this study, but it was unique in McClanan's study. The divergence could be due to sequencing error. There were several unclear signals of nucleotide in McClanan's previous sequencing results. Therefore, the consensus sequence based on those sequences may produce the divergence. A new unique *Wolbachia* strain, wCne2 was found in MA weevils in my study. There is ~ 3% nucleotide distance in sequence between wCne1 and wCne2. Therefore, they could be placed into two different subgroups (reference groups) according to Zhou et al. (1998).

There is very strong similarity between *Wolbachia* strains associated with plum curculio and other insect host species. The *wsp* sequence of wCne1 was 99% identical to wClat2 (from the

neotropical beetle, *C. alternans*, Keller et al. 2004). The wCne2 sequence was 98.5% identical to wNag1 (from the flower bug, *Orius nagaii* Yasunaga, (Miura and Tagami, unpublished)). Moreover, the wCne3 sequence was 100% identical to wDes (from the tephritid fruit fly, *Dacus destillatoria*, Jamnongluk et al. 2000) or wFex1 (from the ant, *Formica exsecta*, Reuter and Keller 2003). The result suggested that the horizontal transmission of *Wolbachia* strain might have occurred between those species, which were infected by similar *Wolbachia* strains.

The *fts*Z gene was used to classify the *Wolbachia* strains into two clades: supergroups A and B (Werren et al. 1995b). A maximum sequence variation of 15% was reported between groups A and B but was still relatively low within group A (3%) (Werren et al. 1995b). The *wsp* gene is more commonly used as a phylogenetic analysis marker for *Wolbachia* strains since Zhou et al. (1998) and Van Meer et al. (1998) found that *wsp* gene sequence variation is much higher than the *fts*Z gene. In this study, there was 13% variation between *fts*Z3 sequence and *fts*Z1 or *fts*Z2 sequence. The results are consistent with the previous studies. My goal in using the *fts*Z gene was to confirm *Wolbachia* infection. Therefore, I only sequenced three individuals, which were each singly infected by a *Wolbachia* strain by using *wsp* gene. The *fts*Z gene sequencing results agrees with the *wsp* gene results. Therefore, the *fts*Z gene PCR results reliably confirmed *Wolbachia* infection. A similar method was used in a *Wolbachia* diversity study of heteropteran bugs (Kikuchi and Fukatsu 2003).

Phylogenetic analysis

According to phylogenetic analysis, *Wolbachia* strains wCne1 and wCne2 were in supergroup B and wCne3 was in supergroup A. This agrees with McClanan et al. 2004. In that study, MA weevils carried supergroup B *Wolbachia* strain and FL and GA curculios were infected by A supergroup *Wolbachia* strains. Compared with previous studies, those *wsp* sequences used in their phylogenetic analysis have the similar position in my phylogenetic analysis, including 11 sequences from Zhou et al. (1998), 4 sequences from Van Meer et al. (1998), 2 sequences from Jamnongluk et al. (2002), and 3 sequences from Ruang-Areerate et al. (2003).

Superinfection and strain distribution

Superinfection refers to infection by more than one *Wolbachia* strain in a single individual. According to previous studies, infections with two or three *Wolbachia* strains are common in some insect hosts (e.g. Merçot et al. 1995, Vavre et al. 1999, Dobson et al. 2001, Kondo et al. 2002a, Riegler and Stauffer 2002, Kikuchi and Fukatsu 2003, Keller et al. 2004). Moreover, several studies reported very high levels of multiple infections. Jamnongluk et al. (2002) detected five strains in the fruit fly, *Bactrocera ascita* Hardy, four strains infecting *Acromyrmex octospinosus* (Reich) (Van Borm 2003), and Reuter and Keller (2003) found four or five strains in *F. exsecta*. In this study, PCR-RFLP revealed a double infection in some plum curculio individuals. The *Wolbachia* strains wCne1 and wCne2 doubly infected 19 of 93 individuals (20.7%). wCne1 and wCne2 were also presented in single infections in 15 (16.1%) and 21 (22.6%) individuals, respectively. The wCne3 strain was unique and was always present as single infections. The wCne3 infection accounts for the highest numbers of infections (36, 38.7%).

There was a distinct geographic pattern to the distribution of the *Wolbachia* infection types (Figure 4-8). Populations in the northern and middle parts of eastern United States (New York, Massachusetts, New Jersey, West Virginia and Virginia) were mainly either wCne1 or wCne2 as single infections, or wCne1 and wCne2 as double infections. Populations in the southern part of the eastern United States (Georgia and Florida, and part of Virginia) always contained single infections by wCne3.

In summary, current results suggest that *Wolbachia* strains approximate the distribution of plum curculio strains: northern strain plum curculios are infected with wCne1 and wCne2 strains in *Wolbachia* supergroup B, southern strain plum curculios are infected with wCne3 strain in *Wolbachia* supergroup A and the mid-Atlantic region is the convergence area. In supergroup B infected populations, only one population (NJb) was singly infected by wCne2 or was not infected. Other populations were always infected by the wCne1 and wCne2 together. A possible explanation is that there is an ongoing sweep of a double infection of wCne1 and wCne2 that is

replacing some populations of the pre-existing single infection populations in the northern area. Some populations or some individuals were left with mixed or single infections. So far, the superinfection ratio is fairly low (20.7%). The fitness consequences of double infections relative to single infections in this weevil are still unknown.

Mitochondrial Oxidase I (mtCOI) gene polymorphism and Wolbachia infection

In Chapter 3, I investigated the genetic structure of plum curculio geographic populations by using the partial mtCOI gene. The 50 samples from ten geographic populations were placed into two major distinct clades: a northern clade (NY and MA populations), and a southern clade including the other 8 populations (NJ, WV, Ra (Washington, VA), Bo (Troutville, VA), BL (Blacksburg, VA), Ke (Whitethorne, VA), GA, and FL). Furthermore, there are two major subclades within the southern group: a mid-southern subclade including 18 samples and a far-southern subclade including 21 samples. From Figure 4-9, the two NJ populations, Rappahannock, Blacksburg populations and 4 WV individuals belong to mid-southern subclade and they were mostly infected by strains wCne1 or wCne2 or wCne1 + wCne2. There were two samples from NJ with no infection. While geographically in the mid-Atlantic area, Kentland (Ke) and Botetourt (Bo) populations and 4 WV individuals were infected by wCne3 and therefore similar to GA and FL populations of far-southern subclade. Therefore, current results suggest that the northern clade of PC is infected by *Wolbachia* strains *w*Cne1 and *w*Cne2, far-southern subclade is infected with the *w*Cne3 strain and the mid-Atlantic region is the convergence area. Mitochondrial DNA analysis indicated a closer relation of the mid-southern PC subclade to the far-southern subclade than to the northern clade. However, *Wolbachia* symbionts in the mid-southern PC are more closely related to those in the northern PC than to those in far-southern PC.

In my case, WV, Blacksburg, and Kentland populations have interesting diversity in mtCOI and *Wolbachia* analysis. First, 4 of 8 WV individuals were mid-southern haplotype and they carried supergroup B *Wolbachia* strains, but another 4 WV individuals carried supergroup A strain

and belong to far-southern subclade in mtCOI haplotype. Both groups were separately collected from a single location (Kearneysville, WV). They could come from two separate colonies. The mid-southern haplotype colony might be from a cross-mating between northern and southern strains, keeping southern mtCOI haplotypes and carrying northern *Wolbachia* strains. A similar phenomenon could have occurred between Blacksburg and Kentland populations. The two locations are close to each other (about 10 km). The results suggest that the mid-southern subclade coule be a transitional type between northern and southern PC strains.

It is hard to explain why there are four samples infected by wCne1 or wCne1+2 in Florida populations. They distinctly belong to far-southern mtCOI haplotype. A possible explanation is several northern strain PCs may have been moved by human activity, the wCne1 and wCne2 *Wolbachia* strains having been introduced into the Florida population. More extensive research needs to be completed to explain this apparent discrepancy.

Reproductive incompatibility and Wolbachia infection

In Chapter 2, a complicated incompatibility pattern was shown in the cross-mating experiments among several different plum curculio geographic populations. Tables 4-3, 4-4, 4-5 show the reproductive incompatibility and *Wolbachia* infection status in experiment 1 and experiment 3. The results of *Wolbachia* strains carried by different plum curculio populations were shown in Table 4-1. A roughly directional incompatibility was suggested by the results of experiment 1. In experiment 1, females infected by *Wolbachia* strains w1, 2 or 1+2 are incompatible with males infected by *Wolbachia* strain w3 in most cases. However, males infected by *Wolbachia* strains w1, 2 or 1+2 are compatible with females infected by *Wolbachia* strain w3 (Table 4-3). From this experiment, *Wolbachia* strain wCne2 may contribute more incompatibility with wCne3 because Virginia (Blacksburg) population was infected by almost all w2 strain and VA females were incompatible with both WV and FL males.

In experiment 2, NY, MA, and NJ carried similar *Wolbachia* strains (w1+2, w1 and w2). Therefore, they were compatible when they mated with each other. The lower fertility of NJ females maybe due to the physiological condition in their reproductive development.

From experiment 3a, an opposite directional incompatibility with experiment 1 was shown. Males infected by *Wolbachia* strains w1, 2 or 1+2 are incompatible with females infected by *Wolbachia* strain w3 (Table 4-4). In experiment 3b, a bi-directional incompatibility was shown in cross-mating between FL and WV populations but they carried the similar *Wolbachia* strains (w3 in most individuals).

According to my results, different *Wolbachia* strain infections cause reproductive incompatibility between different geographic plum curculio populations. However, the clear incompatibility pattern between those *Wolbachia* strains needs more intensive studies. A possible method is to set up a *Wolbachia*-free colony. Then, cross-mating between the artifical *Wolbachia* strain infection from *Wolbachia*-free populations may help us better understand the function of *Wolbachia* infections in reproductive incompatibility among plum curculio populations.

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Table 4-1. Plum curculio populations surveyed for *Wolbachia* infection.

Population	Location	Host Plant	Acronym	*n* individuals	Samples (*Wolbachia* strains)	Infection rate
Massachusetts*	Amherst, MA	Apple	MA	8	MA5, MA32 (w1)**; MA31, MAM1, MAM2 (w2)**; MA4, MAF1, MAF2 (w1+2)**	100%
New York*	Geneva, NY	Apple	NY	10	NY15, NYM1 (w1); NY3 (w2); NY2, NY14, NY36, NY37, NYM2, NYF1, NYF2 (w1+2)	100%
New Jersey A*	Bridgeton, NJ	Nectarine	NJ or NJa	11	NJM1, NJF1, NJF2, NJF3 (w1); NJF3 (w2); NJa1, NJa2, NJa5, NJM2, NJM4 (w1+2); NJM3 (-)	90.9%
New Jersey B*	Chatsworth, NJ	Blueberry	NJb	6	NJb1, NJb2, NJb12, NJb13, NJb14 (w2); NJb11 (-)	83.3%
West Virginia*	Kearneysville, WV	Peach, plum	WV	14	WVp2 (w1); WVu3, WVp1 (w2); WVu1, WVu2, WVu4 (w1+2); WVM1, WVM2, WVM3, WVM4, WVF1, WVF2, WVF3, WVF4 (w3)	100%
Rappahannock*	Washington, VA	Apple	Ra	6	Ra3, Ra4 (w1); Ra5, Ra6, Ra7, Ra22 (w2)	100%
Botetourt	Troutville, VA	Apple	Bo	6	Bo1, Bo2, Bo3, Bo4, Bo5, Bo6 (w3)**	100%
Blacksburg	Blacksburg, VA	Apple, plum	BL	6	BLM2 (w1); BL1, BL22, BLM1, BLF1, BLF2 (w2)	100%
Kentland	Whitethorne, VA	Apple	Ke	6	Ke1, Ke22, KeM1, KeM2, KeF1, KeF2 (w3)	100%
South Carolina*	Clemson, SC	Peach	SC	1	-	-
Georgia*	Byron, GA	Peach, plum	GA	8	GAp1, GAp2, GAu1, GAu3, GAM1, GAM2, GAF1, GAF2 (w3)	100%
Florida*	Quincy, FL	Peach	FL	12	FLM1, FLM4, FLF1 (w1); FL1 (w1+2); FL2, FL3, FL4, FLM2, FLM3, FLF2, FLF3, FLF4 (w3)	100%
			Total:	94	w1: 15 (16.1%); w2: 21 (22.6%); w1+2: 19 (20.4%); w3: 36 (38.7%); No infection: 2 (2.2%)	97.8%

* Plum curculio contributors: Massachusetts: Jaime Piñero, Ronald Prokopy; New York: Arthur Agnello; New Jersey A: Peter Shearer; New Jersey B: Sridhar Polavarapu; West Virginia: Tracy Leskey; Rappahannock, VA: Kenner Love; South Carolina, Georgia: David Jenkins, Dan Horton; Florida: Russell Mizell.

** w1 = wCne1; w2 = wCne2, w1+2 = wCne1+2; w3 = wCne3

Table 4-2. Multiple clones sequenced to confirm *Wolbachia* superinfection in plum curculio.

Sample	*n* clone	*n* sequence (strain)	Superinfection
NY2	6	4 (wCne1), 2 (wCne2)	Yes
NY3	5	5 (wCne2)	No
GAp1	4	4 (wCne3)	No
Bo1	3	3 (wCne3)	No
FL2	5	5 (wCne3)	No

Table 4-3. Reproductive incompatibility and *Wolbachia* infection. Experiment 1, evaluating reproductive compatibility among a rough north and south transect along the range of plum curculio. This was a 4 x 4 factorial (maternal origin and paternal origin) design. The four levels within each factor were extreme north (NY), lower north (WV), upper south (VA), and extreme south (FL).

	Females			
Males	NY w1+2, w1, w2	VA w2, w1	FL w3, w1	WV w3
NY w1+2, w1, w2	--*	--	--	--
VA w2, w1	--	--	--	--
FL w3, w1	--	X	--	X
WV w3	X**	X	Weak X	Weak X

* --: indicates the compatible combinations;

** X: indicates the incompatible combinations.

Table 4-4. Reproductive incompatibility and *Wolbachia* infection. Experiment 3a, evaluating reproductive compatibility between FL and NJ populations. It was a reciprocal cross mating design.

Males	Females	
	FL w3, w1	NJ w1, w1+2, w2
FL w3, w1	--	--
NJ w1, w1+2, w2	X	--

Table 4-5. Reproductive incompatibility and *Wolbachia* infection. Experiment 3b, evaluating reproductive compatibility between FL and WV populations. It was a reciprocal cross mating design.

Males	Females	
	FL w3, w1	WV w3
FL w3, w1	--	X
WV w3	X	--

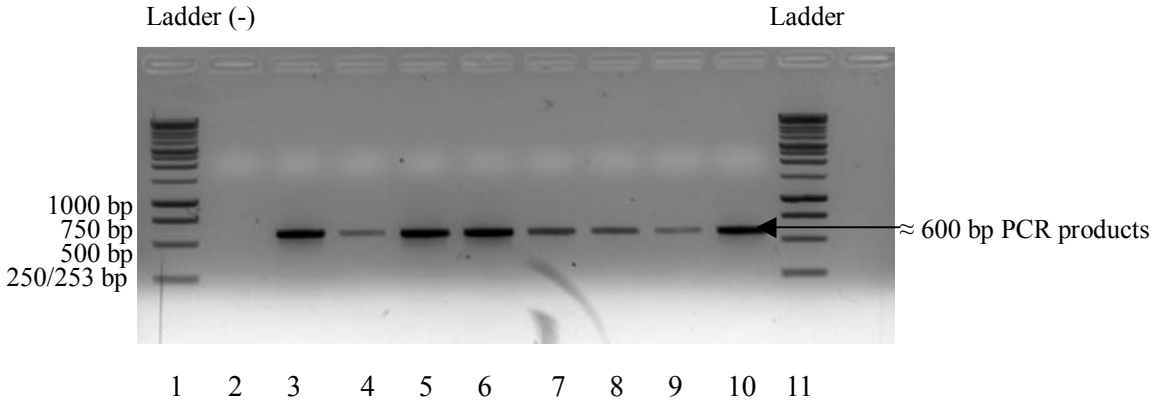

Figure 4-1. *Wolbachia* surface protein (*wsp*) gene PCR results. 10 µl PCR products were run on a 0.8% agarose gel containing ethidium bromide. Lanes 1 and 11 are 1Kb DNA ladder (Promega); Lane 2 is a negative control; Lanes 3-10 are samples: WVF4, FLM4, FLF3, FLF4, NJb13, NJb14, BLM2, and BLF1 respectively. Those PCR products are 590 bp or 593bp in size.

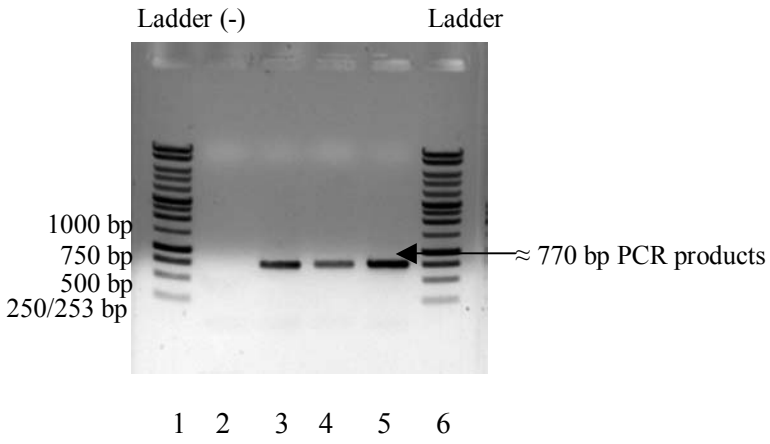

Figure 4-2. *Wolbachia ftsZ* gene PCR results. 10 µl PCR products were run on a 0.8% agarose gel containing ethidium bromide. Lanes 1 and 6 are 1Kb DNA ladder (Promega); Lane 2 is a negative control; Lanes 3-5 are samples: NYM1 (758 bp), MAF2 (758 bp), and WVF1 (769 bp) respectively.

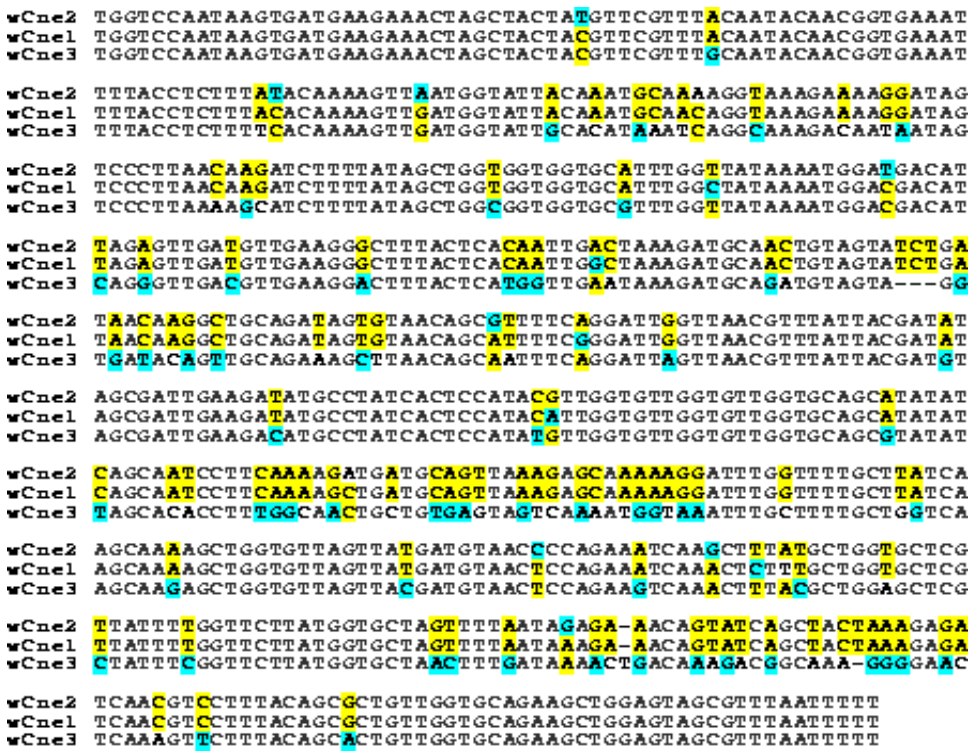

Figure 4-3. The alignment result of the three *Wolbachia wsp* gene sequences. The colors highlight nucleotides different among the three sequences.

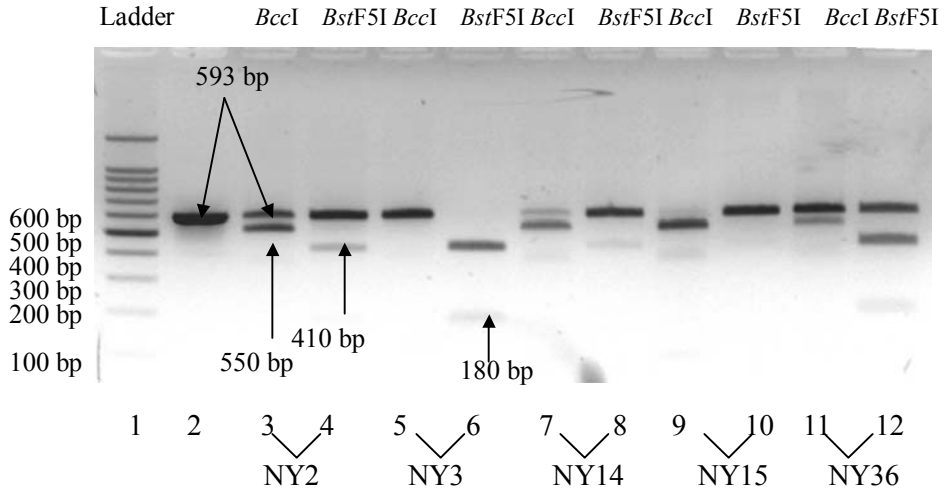

Figure 4-4. PCR-RFLP results. PCR products were digested by *Bcc*I or *Bst*F5I. The reaction was: 10 µl of PCR products were incubated with 1 µl of *Bcc*I or *Bst*F5I enzymes in 25 µl reaction volume at 37°C (*Bcc*I) or 65°C (*Bst*F5I) for 3 hours. All restriction digests were run on a 2.0% agarose gel containing ethidium bromide. Lane 1 is 100 bp DNA ladder (Promega); Lane 2 is undigested PCR product (593 bp); Lane 3, 4: NY2; Lane 5, 6: NY3; Lane 7, 8: NY14; Lane 9, 10: NY15; Lane 11, 12: NY36. The wCne1 produced two fragments (550bp + 40bp) after digestion by *Bcc*I. The wCne2 produced two fragments (410bp + 180bp) after digestion by *Bst*F5I. The digestion results indicated that NY2, NY14, NY36 were doubly infected by wCne1+2, NY3 was singly infected by wCne2, and NY15 was singly infected by wCne1.

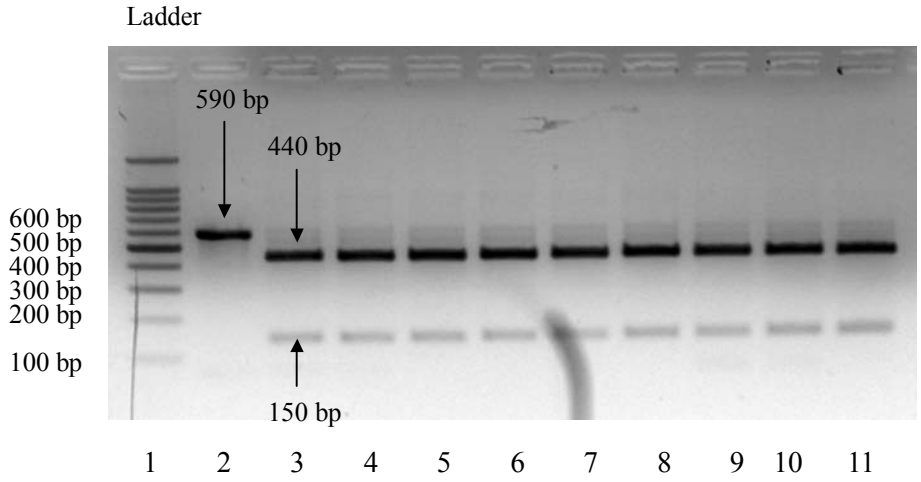

Figure 4-5. PCR-RFLP results. PCR products were digested by *Aci*I. The reaction was: 10 µl of PCR products were incubated with 1 µl of *Aci*I enzyme in 25 µl reaction volume at 37°C for 3 hours. All restriction digests were run on a 2.0% agarose gel containing ethidium bromide. Lane 1 is 100 bp DNA ladder (Promega); Lane 2 is undigested PCR product (590 bp); Lanes 3 – 11 were samples: Ke1, Ke22, GAp1, GAp2, GAu1, GAu3, Bo1, Bo2, and FL2, respectively. The wCne3 produced two fragments (440bp + 150bp) after digestion by *Aci*I. The digestion result indicated that all these nine samples were singly infected by wCne3.

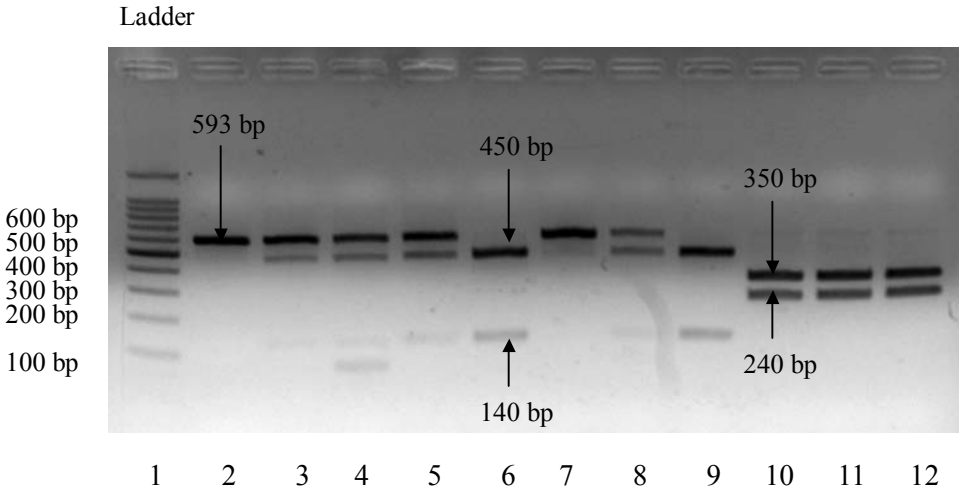

Figure 4-6. PCR-RFLP results. PCR products were digested by *Hind*III. The reaction was: 10 µl of PCR products were incubated with 1 µl of *Hind*III enzyme in 25 µl reaction volume at 37°C for 3 hours. All restriction digests were run on a 2.0% agarose gel containing ethidium bromide. Lane 1 is 100 bp DNA ladder (Promega); Lanes 2 – 12 were samples: NYM1, NYM2, NYF1, NYF2, MAM1, MAM2, MAF1, MAF2, WVM1, WVM2, and WVM3, respectively. The wCne1 has no cut digested by *Hind*III. The wCne2 produced two fragments (450bp + 140bp) and the wCne3 produced two fragments (350bp + 240bp) after digestion by *Hind*III. The digestion results indicated that NYM1 and MAF1 were singly infected by wCne1; MAM2 and MAF2 were singly infected by wCne2; NYM2, NYF1, NYF2, MAF2 were doubly infected by wCne1+2; WVM1, WVM2, and WVM3 were singly infected by wCne3.

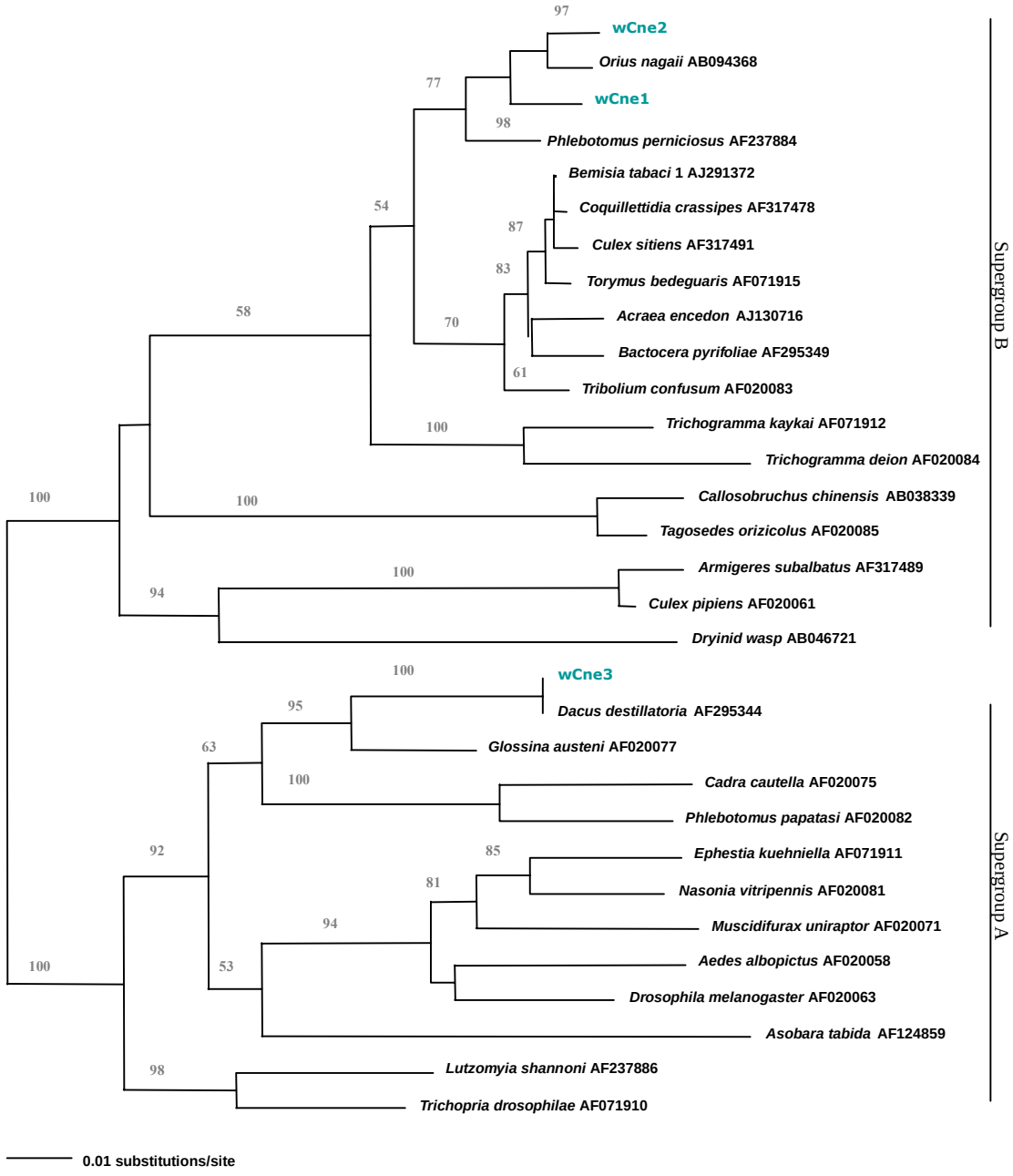

Figure 4-7. Phylogenetic tree of *Wolbachia* based on the *wsp* sequences. *Wolbachia* strains are named by host insect species. The tree has been constructed by the Neighbor Joining algorithm using the Kimura distance and midpoint rooted. Numbers on the nodes indicate percentage of 1000 bootstrap replications. The strains wCne1 and wCne2 belong to supergroup B and wCne3 strain belongs to supergroup A.

Figure 4-8. The distribution map of *Wolbachia* strains associated with plum curculio geographical populations in eastern United States. Colored lines indicate plum curculio distribution range and red line is an approximate line between two plum curculio strains (Chapman 1938). Colored dots indicate different *Wolbachia* strains and one dot represents one plum curculio individual. Stars indicate geographic locations (positions were labeled approximately)

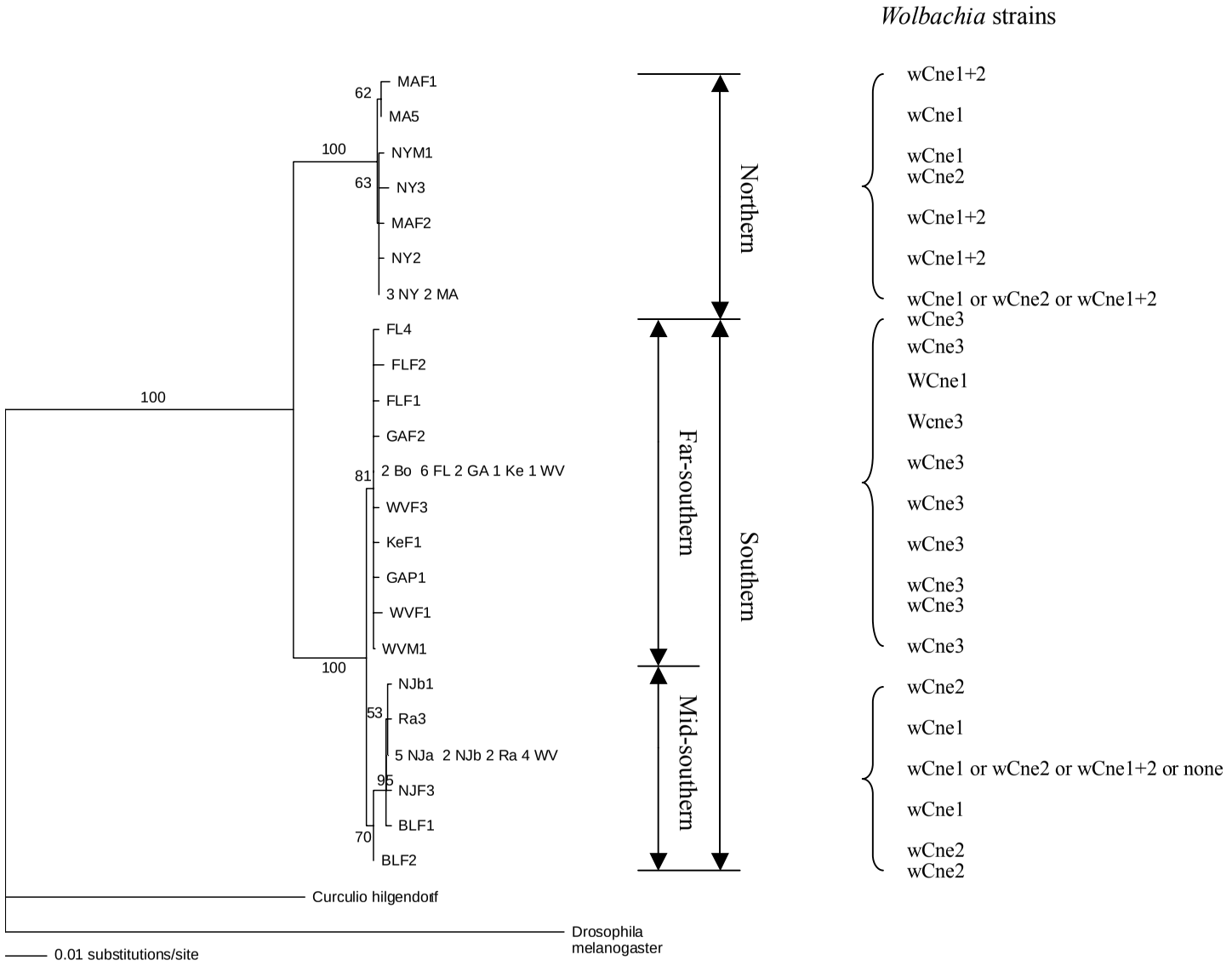

Figure 4-9. Relationship between mtCOI and *Wolbachia* strains for plum curculio populations. Neighbor Joining tree based on mtCOI sequences of plum curculio. Bootstrap percentages of 1000 replications are shown around the branches. Branch lengths are proportional to inferred substations/site. The individual names refer to sample.