# DIVERSITY AND CONTROL OF *RALSTONIA SOLANACEARUM* IN THE SOUTHEASTERN UNITED STATES

By

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To my wife, Nina, and son, Aiden, for their love, encouragement, and bring joy in my life. We share this degree.

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# DIVERSITY AND CONTROL OF *RALSTONIA SOLANACEARUM* IN THE SOUTHEASTERN UNITED STATES

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*Ralstonia solanacearum*, the causal agent of bacterial wilt, is a soil-born phytobacterium native to the southeastern U.S. The pathogen can infect over 200 different plant species, including the agriculturally important crops for the southeastern U.S.; tomato, potato, and tobacco. Once the bacterium is established in the field and conditions are right, within 5 years growers may see 80% or more crop loss. *R. solanacearum* has been described as a species complex due the diversity that exist within the species. Although native stains have been a threat for growers, exotic strains have been known to infect new hosts, can be more aggressive, and deem current cultural practices to control the disease ineffective. The nonnative race 3 biovar 2 strains have been listed as a select agent under the Agricultural Bioterrorism Protection Act of 2002 due to the potential economic losses and effect it would have on international trade.

In order to identify if an exotic strains has become established, one must first know the characteristics of the native strains. This study gathered strains from different collections throughout the southeastern U.S. These strains were then characterized

based on classical and current methods for typing *R. solanacearum* strains. Using the current classification, phylogenetic analysis of the *egl*, the majority of strains isolated in the southeast belong to sequevar 7. It was determined that there were also strains belonging to sequevar 4, 5 and not yet defined sequevar. One of the strains from the undefined sequevar was further characterized by performing a pathogenicity test on triploid bananas and cucurbits and determining if the bacterium is able to produce a bacteriocin.

The preferred method identifying relatedness amongst organisms is by genotypic typing. Comparison of genome would give the greatest amount of information, however it is both timely and expensive. Microsatellites and microsatellite associated loci (MALs) have been used previously to show diversity between genotypes of the same species. MALs have been proven to be effective to show diversity with in human pathogenic bacterial species. This was the first study to use microsatellites and MALs in phytobacteria.

Methods for controlling bacterial wilt in the field are constrained to limiting the exposure of pathogen in the field or, when the field is infested, changing cultural methods to plant when the pathogen population is low. Previously reports of using Acibenzolar-*S*-methyl (ASM), a foliar spray, and thymol, as soil fumigant, as chemical controls were effective limiting disease symptoms and increasing yield production for tomatoes. This study wanted to determine if using a combination of thymol, ASM, and moderately resistant plants would elevate the level of efficacy to control bacterial wilt.

# CHAPTER 1
# LITERATURE REVIEW

## Introduction

*Ralstonia solanacearum*, the causal agent of bacterial wilt, is one of the most important phytopathogenic bacteria in the world. The bacterium is a soilborne pathogen that can cause major economic loss for growers worldwide. The pathogen complex is able to cause disease in over 200 different plant species, including important cash crops such as tobacco, tomato, potato, and banana (Hayward, 1995). Due to the economic impact caused by this pathogen, it has been studied extensively. *R. solanancearum* was one of the first plant pathogens to have its genome sequenced (Salanoubat et al., 2002). The bacterium's pathogenic cycle has been studied in great detail, and has served as a model system for host-pathogen interactions (Genin and Boucher, 2002).

*R. solanacearum* is an aerobic Gram-negative rod, 0.5-1.5 µm in length, and is motile by 1-4 polar flagella. The bacterium is grouped with non-fluorescent pseudomonads, and is catalase and oxidase positive and forms nitrite from nitrates (McCarter, 1991). Bacterial colonies on agar surfaces are initially smooth, shiny and opalescent, but become brown with age (Lelliott and Stead, 1987). The optimum temperature range for the bacterium is 30-37°C; however, previous tests have shown that the bacterium is unable to grow at 4 or 40°C (Denny, 2006).

## History of Bacterial Wilt

The first report describing the pathogen was by Burrill in 1890, when it was recovered from potato. He performed Koch's postulates to confirm that the disease was indeed caused by the pathogen. Six years later the bacterium was reported in other hosts. It was suspected that the bacterium was native to Florida soils due to reports of

frequent outbreaks of the disease on solanaceous crops grown on virgin land (Kelman, 1953).

The disease first gained attention in the United States in 1903, when losses in the tobacco growing region of in Granville County, North Carolina caused farmers to abandon their land. By 1907, all the farms in the county reported 25-100% crop losses. Buildings and lands were abandoned, and what were once prosperous cities became ghost towns. From the combined loss of income and the forced sale of farms, it was estimated that the county lost over $40 million (Kelman, 1953). As a result of the impact the disease had, it is sometimes referred to as Granville wilt when infecting tobacco. *R. solanacearum* can be problematic for communities that grow solanaceous plants in both tropical and temperate regions. The greatest economic damage has been reported on potatoes, tobacco and tomatoes in the southeastern United States, Indonesia, Brazil, Colombia, Taiwan, and South Africa. The Philippines reported average losses of 15% in tomatoes, 10% in eggplant and pepper, and 2-5% in tobacco (Zehr, 1969). Along the Amazon basin in Peru, rapid spread of the pathogen threatened to destroy half of the banana plantations (French and Sequeira, 1968). In Taiwan, incidence of bacterial wilt on tomato has been reported to range from 15-55% in the summer (Wang and Lin, 2005). The disease has affected most states of India, and in extreme incidences growers have reported up to 90% crop loss (Anuratha and Gnanamanickam, 1990). Extensive economic losses were due to outbreaks in potato in Israel (Volcani and Palti, 1960), and Greece (Zachos, 1957). Most of the regions of the world that are impacted by the pathogen are some of the poorest. Due to the demands of growing cash crops to provide money, growers plant the same crop year after year. This creates more

opportunities for the pathogen to become established in the field. Once the field is infested, farmers can no longer grow economically important crops (Smith et al., 1998).

Presently *R. solanacearum* is not only a concern pertaining to crop loss, but has become a matter of national security. The race 3 biovar 2 strains were placed on the top 10 list of potential plant pathogens that could be hazardous to U.S. crops, and this organism was included in the Agricultural Bioterrorism Act of 2002 (Hawks, 2002).

## Disease Cycle, Detection, and Epidemiology

In order for the bacterium to cause disease, it must first enter the host. Most of the time *R. solanacearum* strains enter through wounds on the host. Normal agricultural practices can result in wounding. The roots can also be injured by nematodes and during normal plant growth, as the roots expand and lateral roots are produced (Denny, 2006). The pathogen can be vectored by bees or other insects unintentionally as has been observed in bacterial wilt on banana and plantain (Buddenhagen and Kelman, 1964). The disease is favored by high temperature and moist soil. The disease develops rapidly when temperatures are greater than 20ºC. Other soil factors that affect the pathogen's ability to cause disease include soil depth, organic matter, host plant debris, and soil type (Denny, 2006). The pathogen has been reported to be present in virgin soil (Kelman, 1953). Once the bacterium has entered and colonized the host, it multiplies and moves systemically through the xylem.

Survival is one of the main obstacles a pathogen faces in the disease cycle. *R. solanacearum* is able to survive in soil in the absence of host plants for up to 2 years. Survival is dependent on soil temperature and soil moisture (McCarter, 1991). The bacterium has been reported to survive in pure water at 20-25°C for more than 40 years

(Denny, 2006). The bacterium can overwinter in irrigation water in close association with asymptomatic aquatic weed hosts, thus aiding in the spread of the pathogen throughout the waterways in Europe (Elphinstone et al., 1998; Janse et al., 1997). A similar phenomenon was observed in Florida (Hong et al., 2004).

Bacterial wilt can cause many symptoms and some are unique and differentiate bacterial wilt from other types of wilt. In Florida, symptoms have been reported mostly during summer tomato production compared to spring production. Symptoms of the disease in tomato include stunting, wilting of leaves and sometimes complete stem collapse. Advanced stages can include yellowing or browning and occasionally maceration of the pith. In the field, wilting can be observed in the afternoon in the terminal leaves or on one side of the plant. The following morning no sign of wilt is evident and the plant appears healthy. Depending on the temperature and humidity, complete wilting of the plant will occur between 5 and14 days after the first symptoms occur. Bacterial wilt can be distinguished from other wilt diseases by the leaf color. The leaves of infected plants will appear green, while other wilts, such as Fusarium wilt, will develop a yellowing of leaves.

Various methods have been used to detect the pathogen. A simple test to determine if a plant is infected with *R. solanacearum* is the ooze test. The test is performed by cutting the plant at the crown, and then squeezing the stem. A whitish ooze will be excreted from the surface of the cut. A variant of this is to place the cut surface of the stem in water and in a few minutes bacteria will stream out, resulting in visible milky strands emanating from the base of the stem. Recently, increased interest in methods of detection and identification of *R. solanacearum* has occurred as a result

of R3B2 being considered a threat to potato production in temperate environments (Caruso et al., 2005, Elphinstone et al., 2005, and Janse et al., 2005). Detection and identification assays have been designed to distinguish *R. solanacearum* from other species of phytobacteria. In some instances the bacterium may be present at concentrations below the limits of detection. In those instances, the population must be enriched to reach a detectable count. The semi-selective medium, SMSA, or the differential agar medium, Kelmans' TZC will enrich the population while selecting for *R. solanacearum* strains (Englebrecht, 1994 and French et al., 1995). Serological techniques such as ELISA or flow cytometry have been used for identification of *R. solanacearum* (Seal, 1998). Immunostrips also be used in the field for quick diagnosis (Agdia, Inc., Elkhart, Indiana). The use of PCR for identification is accurate and can detect strains at low concentrations, approximately 100 CFU/ml (Alvarez, 2005). Many primer sets have been developed for the identification of *R. solanacearum* (Gillings et al. 1993, Pastrik and Maiss, 2000, Seal et al. 1993, Opina et al., 1997. Two other methods for identification are fatty acid methyl ester (FAME) analysis and BIOLOG (MIDI, 2001, Li and Hayword, 1993).

Many virulence factors are associated with pathogenesis by the bacterial wilt pathogen. Extracellular polysaccharide (EPS1) production is a major virulence factor for the pathogen. A major cause of wilting is due to EPS which clogs the vascular tissue and prevents sufficient water from reaching the leaves (Buddenhagen and Kelman, 1964). As the bacterial population increases, an increased amount of EPS1 is produced (McGravey et al., 1999). The bacterium also produces 6 extracellular plant cell wall degrading enzymes, which are delivered through the type II secretion system (T2SS).

Inactivation of any one of these genes does not completely inhibit the disease (Lui et al., 2005). *R. solanacearum* has a type III secretion system (T3SS) common to Gram-negative plant pathogenic bacteria. Mutants lacking a functional T3SS are nonpathogenic on hosts (Schell, 2000). The T3SS is used to translocate effector proteins into the plant cell where they suppress basal defense or aid in nutrient release (Hueck, 1998). Plants have evolved to recognize effectors, thus triggering a rapid defense response called a hypersensitive response (HR). This phenomenon aids plant breeders in developing resistant cultivars.

## Classification

Classification of *R. solanacearum* has been in question for more than a century (Kelman, 1953). In the past 20 years the bacterium has been classified in 3 different genera (Kelman, 1953, Yabuuchi et al., 1992, and Yabuuchi et al, 1995). However, due to phenotypic differences, fatty acid composition, and genotypic traits, identified through rRNA-DNA hybridization and phylogenetic analysis of 16s rDNA , the bacterium was placed in the new genus *Ralstonia* (Yabuuchi et al, 1995). Although *R. solanacearum* strains share common traits, groups within the species have different characteristics such as: host range, optimal temperature for causing disease, and differential utilization of carbohydrates. These differences prompted many researchers to classify the bacterium to subspecies level. Hayward (1964), distinguished 5 different biovars (Table 1) based on the bacterium's ability to produce acid from several disaccharides and sugar alcohols. The bacterium has also been divided into subspecies based on the host range (Table 1).

Race 1 strains infect tobacco, tomato, many solanaceous weeds, non-solanaceous weeds, and diploid bananas (Buddenhagen et al., 1962). Additional hosts have

been added to include groundnut, potato, pepper, eggplant, olive, ginger, strawberry, geranium, and *Eucalyptus* (Denny, 2006).

Race 2 strains infect triploid bananas and members of the *Heliconia* species (Buddenhagen et al., 1962).

Race 3 strains were originally thought to be pathogenic on potato and tomato, but weakly pathogenic on other solanaceous crops. This race is considered to have a narrow host range and is able to survive in colder temperatures than other races (Buddenhagen et al., 1962).

Race 4 strains aggressively wilt ginger and can wit tomato, pepper, eggplant and some native weeds (Denny, 2006).

Race 5 strains are only found in China and cause wilt on mulberry trees (Denny, 2006).

Classical restriction fragment length polymorphism (RFLP) analysis was performed on 62 *R. solanacearum* strains representing 4 races and 5 biovars isolated in the Americas, Asia and Oceania (Cook and Sequeira, 1988). Southern blot analysis of restriction endonuclease-digested genomic DNAs probed with 9 DNA fragments cloned from *R. solanacearum* revealed that this bacterium can be divided into two distinct divisions (Cook and Sequeira, 1988).

Phylogenetic analysis of select genes is currently the preferred method for classifying *R. solanacearum* (Fegan & Prior, 2005). Phylotyping is based on performing a multiplex PCR, which includes using primers that are specific to 16S-23S internal transcribed spacer region (ITS region). *R. solanacearum* strains will display 1 of 4 possible bands in the gel; each band represents a different phylotype. A phylotype corresponds to a group of strains that originate from different regions of the world: phylotype 1, Asia; phylotype 2, the Americas; phylotype 3, Africa; and phylotype 4, Indonesia. The phylotypes are subdivided into sequevars. Sequevars are created by sequencing and phylogenetic analysis of the endoglucanase gene (*egl*). Three or more strains that group together in a clade form a sequevar.

## Disease Management

Control of bacterial wilt is difficult once the pathogen has been introduced into the field. Hence the best method for controlling the disease is for growers to use an integrated approach to lower the impact of bacterial wilt in their production. Control methods can be targeted to 3 components of a production cycle: field preparation, crop production and post-harvest practices (Wang and Lin, 2005).

The objective of the field preparation period is to limit introduction of the bacterium into the field, or if the field is infested, the goal is to reduce the pathogen population. In infested fields, resistant or moderately resistant cultivars should be chosen. Resistant genotypes provide the best method for control; however, it is extremely difficult with groundnut being the only host where dominant resistant genes have been identified (Boshou, 2005). Moderately resistant cultivars of tomato are commercially available; however, they tend to be limited in use to certain geographic regions (McCarter, 1991). Grafting on resistant rootstock has proven to be useful in controlling the disease, while providing the opportunity to harvest the desired fruit; usually from susceptible cultivars (Nakaho et al., 2000). If moderately resistant cultivars are not available, it is recommended to use pathogen free transplants. Seedlings can be symptomless carriers of the pathogen (Wang and Lin, 2005). It is also recommended to add soil amendments, such as compost and use a fumigant, such as thymol, when fields are infested with the bacterium (Saddler, 2005, Ji et al, 2007, Santos et al. 2006). It is advised to avoid planting in pathogen-infested soil, irrigate with pathogen free water, and use proper sanitation practices to exclude or reduce the pathogen (Pradhanang et al., 2005). One of the simplest methods for avoiding disease is to plant in cooler and/or drier conditions that would be less favorable for disease development (Denny, 2006).

The use of non-hosts as cover crops for crop rotation has helped to reduce the incidence of bacterial wilt in the field. These non-host crops include sorghum-sudan, rye, and other grass crops (Denny, 2006). High soil moisture can increase the bacterium's population, thus it is suggested to plant in well-drained fields.

The goal for controlling disease during production is to maintain low pathogen populations and to minimize spread of the organism. Contamination can be reduced by applying strict sanitation practices such as pathogen free irrigation water, transplants, machinery, and stakes. Excessive irrigation will increase pathogen population; thus irrigation should be minimized and based on water requirements. (Wang and Lin, 2005).

Following harvest the intention is to reduce bacterial populations for the next season. The crop residue should be plowed under or destroyed immediately. Infected plants and fruit sometimes are left in the field, thus allowing the pathogen to survive and provide inoculum for the next year. Rotation with cover crops should begin after harvest to minimize weeds that may aid in the survival of the pathogen.

## Project goals and objectives

The objectives of this study were to:

Determine the effectiveness of using the combination of thymol, acibenzolar-*S*-methyl and moderately resistant plants for control of bacterial wilt.

Characterize *R. solanacearum* strains in the southeastern U.S to determine diversity and to identify exotic strains.

Investigate the effectiveness of microsatellites for determining infrasubspecies groups, and determine their effectiveness for identifying exotic strains in the southeastern U.S.

Table 1.1 Characteristics of races and their relationship to other sbdivisions of *R. solanacearum*

| Race | Host Range | Geographical Distribution | Biovar | RFLP Division[a] | Optimum Temperature |
|---|---|---|---|---|---|
| 1 | wide | Asia, Australia, Americas | 3,4<br>1 | I<br>II | 30-37°C |
| 2 | banana other *Musa* spp | Caribbean, Brazil, Philippines | 1 | II | 30-37°C |
| 3 | primarily potato | Worldwide[b] | 2[c] | II | 25-37°C |
| 4 | ginger | Asia | 3,4 | I | 30-37°C |
| 5[d] | mulberry | China | 5 | I | 30-37°C |

[a] Based on restriction fragment length polymorphism (RFLP) analysis
[b] Originating in the Andes, but disseminated worldwide on latently infected potato tubers
[c] Typical race 3 strain are sometimes referred to as biovar 2A. Strains from the Amazon basin have been placed in a new biovar, designated by various authors as 2T or N2
[d] Although originally designated as race 4, the prior designation of the ginger strains as race 4 takes precedence

# CHAPTER 2
# MANAGEMENT OF BACTERIAL WILT IN TOMATOES WITH THYMOL AND ACIBENZOLAR-*S*-METHYL

## Introduction

Bacterial wilt, caused by the soilborne pathogen *Ralstonia solanacearum*, occurs worldwide in tropical and subtropical regions of the world (Yabuuchi et al., 1995). The bacterium can cause disease symptoms in over 200 different plant species (Buddenhagen and Kelman, 1964; Hayward, 1991). In the southeastern United States, economic losses for important solanaceous crops including tomato, tobacco, and eggplant can be attributed to bacterial wilt. The bacterium enters the plant through the root and colonizes the vascular tissue in the stem. In field conditions signs of the disease usually appear in mature tomato plants. Leaves will wilt during the day and recover at night or the early hours of the morning. If the weather is favorable for the disease, with high humidity and high temperatures, complete wilting of the plant will occur and eventually death. The leaves of wilted plants remain green and the vascular tissue usually turns a brownish yellow in the advanced stages of wilt. In the field the disease occurs mostly in areas where water accumulates; however, plants showing signs of the disease can be found sporadically throughout. Plants affected by *R. solanacearum* can also be stunted, due to the lack of water and poor uptake of nutrients.

Current integrated management strategies include the use of resistant cultivars, pathogen free transplants, and crop rotation with non-host cover crops (Pradhanang et al., 2005). However, these strategies have proven to be limited due to the complex nature of soilborne pathogens. Resistant cultivars have been developed for fresh market production in the U.S.; however, growers have only adopted moderately

resistant cultivars (Scott et al., 1995). Resistant cultivars are limited to locations, climate, and strains of the pathogen (Saddler, 2004). Transplants limit the spread of the bacterium, yet due to it being a soilborne pathogen, most plants in the field can be infected. Cover crops or crop rotation can be difficult due to the diverse host range of *R. solanacearum* strains, and the fact that pathogen is able to survive or colonize various weeds that surround the field (Hayward, 1991). With the limited control measures and the gravity of bacterial wilt on important economical crops, investigating other methods for controlling the disease has become critical.

Plants are able to activate a protective mechanism after contact by a pathogen, their metabolites, or by a diverse group of structurally unrelated organic and inorganic compounds. This phenomenon has been dubbed as systemic acquired resistance (SAR) (Kuc, 2001). SAR inducers are ideal for controlling diseases because they trigger a response that may protect the plant from fungal, bacterial and viral pathogens, if the product is applied at the correct time. Acibenzolar-*S*-methyl (ASM; Actigard 50 WG, Syngenta, Basel, Switzerland) is a chemical compound that triggers SAR when applied to plants (Oostendrop et al., 2001). ASM has been used to reduce the incidence of fire blight in pear and apple, bacterial spot and speck in tomato and pepper, and common bunt in wheat seedlings (Louws et al., 2001; Lu et al., 2006; Norelli et al., 2003; Obradovic et al., 2005). Previously it was reported that ASM enhanced host resistance in moderately resistant tomato cultivars against bacterial wilt (Pradhanang et al**.,** 2005).

Thymol (2-isopropyl-5-methylphenol) is a monoterpene phenol derivative of thyme (Aeschbach et al., 1994). Essential oils have been used in the past for flavoring and preserving food, for their antioxidant power, and for their antimicrobial activity (Lambert

et al., 2001; Rojano et al., 2008; Scheie, 1989). Both medical and food sciences have shown that thymol is able to inhibit both Gram-positive and Gram-negative bacteria (Cailet and Lacroix, 2006; Evans and Martin, 2000; Lambert et al., 2001; Shapira and Mimran, 2007; Walsh et al., 2003). Previously thymol applied as a biofumigant was reported to be effective to control bacterial wilt. Thymol applications in the field on susceptible tomato cultivars were able to reduce the incidence of bacterial wilt and increase yield (Ji et al., 2005).

In previous studies bacterial wilt was reduced by applying ASM in combination with moderately resistant tomato cultivars (Pradhanang et al., 2005), or by applying thymol and using susceptible tomato plants (Ji et al., 2005). In this study, we wanted to determine if using a combination of thymol, ASM, and moderately resistant plants would elevate the level of efficacy in controlling bacterial wilt. This would be the first time that the two products had been applied together on moderately resistant tomato cultivars in a field trial. It was unknown if the chemicals would work synergistically or would have little to no effect in enhancing disease control. Success with both of the chemicals in controlling the disease would provide another tool in a small arsenal to control bacterial wilt.

## Materials and Methods

### Bacterial Culture and Inoculum Preparation

*R. solanacearum* strain RS5 isolated from tomato in Quincy, Florida, was used in this study (Pradhanang and Momol**,** 2001). Pathogenicity was determined by performing Koch's postulates by inoculating tomato plants and re-isolating RS5. Bacteria were plated on modified semi-selective agar, SMSA (Engelbrecht, 1994), and casamino acid peptone glucose agar, CPG (Schaad et al. 2001). Plates were stored at 28°C. The

inoculum contained bacteria grown on CPG for 24 h and suspended in sterile deionized water. The bacterial suspension was adjusted to $10^7$ CFU/ml using sterile deionized water. Inoculum concentration was estimated using a spectrophotometer (Sigma-Aldrich Co., Milwaukee, WI) at 600 nm. The actual bacterial concentration (cfu/ml) was determined by performing 10-fold dilutions of the inoculum suspension and plating on CPG. Where each tomato plant was to be transplanted, 15 cm holes were created in the soil and 50 ml of the bacterial suspension was poured into each hole (Ji et al, 2005). The holes were covered with tape prior to the thymol fumigation.

**Application of Thymol and ASM**

Thymol was applied as a soil-fumigant 24 h after the field was inoculated. The field was aerated 7 days post thymol application by removal of the tape. Thymol was applied at 9.42 kg per ha, in a solution consisting of water, 70% ethanol and detergent. ASM was applied as a foliar spray at a volume of 10ml of ASM solution (25µg/ml) per plant. The ASM solution was applied 6 times: 1 week before the seedlings were transplanted, 1 day after transplanting, followed by 2 treatments that were applied once a week, and then 2 treatments that were applied biweekly.

**Tomato Plants and Experimental Design**

In the 2006 trial tomato cultivars ‘Phoenix’, ‘FL7514’, and ‘BHN669’ were used in the field experiment, the first being susceptible and the last 2 moderately resistant to bacterial wilt. For the 2008 trial, only ‘Phoenix’ and ‘FL7514’ were used. Tomato plants were grown in Terra-Lite agricultural mix (Scott Sierra Horticultural Products Co., Marysville, OH) in expanded polystyrene flats with 3.5 × 3.5 cm cells. For each experiment 5-week-old tomato seedlings were transplanted 1 week after the thymol application.

The experiment for both years was conducted in experimental fields at the University of Florida North Florida Research and Education Center located in Quincy. Previously, the fields were used for growing tomatoes. The beds were fumigated with methyl bromide (67%) and chloropicrin (33%) at a broadcast equivalent rate of 392 kg a.i./ha for control of weeds and other soilborne pathogens, fertilized with 218-31-181kg/ha of N-P-K, and covered with polyethylene mulch. The plots consisted of 4 rows, 5 m long with the raised beds, 10 cm high by 91 cm wide and centered 1.8 m apart. Tomato plants were treated with standard foliar sprays for insecticides and fungicides at weekly intervals until harvest. Over time the plants were tied and staked. Experimental plots were comprised of 2 rows 10 to 12 m long with 14 tomato seedlings transplanted per block in 2006 and 18 in 2008. Thus each treatment consisted of 84 plants per cultivar in 2006 and 72 plants per cultivar in 2008. In the 2006 experiment each block of plants received one of the following treatments: thymol, the combination of thymol and ASM, or neither thymol nor ASM which was the untreated control (UTC). The treatments for the 2008 experiment consisted of thymol, ASM, both thymol and ASM, or the UTC. In between each block was a 2 m buffer where no tomato seedlings were planted. A randomized complete block design was used including 6 blocks in 2006 and 4 in 2008. Each block constituted a replication.

**Disease and Yield Assessment and Statistical Analysis**

Completely wilted tomato plants were removed from the field weekly and a few of the plants were tested for presence of the bacterium. *R. solanacearum* was confirmed by performing a bacterial ooze test and either isolation on SMSA and confirmation by gas chromatographic profiling of whole-cell fatty acid methyl esters (FAME) (MIDI, Newark, DE), as described previously (Pradhanang et al., 2003; Stead, 1992), or by

using specific immunoassay strips (Agdia, Inc., Elkhart, IN). RS5 was used as positive control for each test. In both 2006 and 2008 completely wilted plants were counted weekly after transplanting. Bacterial wilt incidence was recorded at weekly intervals and was quantified as the percentage of plants wilted. Percentage of plants wilted was calculated by dividing the number of completely wilted plants by total number of transplanted plants. Two harvests were conducted for each crop. The total marketable and unmarketable yield was determined for both years according to the USDA standards by using a fruit and vegetable processing machine (Model No. 1650 Roller, TEW Manufacturing Corp., Penfield, NY). Marketable fruit size was categorized as extra large, large and medium (Stavisky et al., 2002; USDA, 1976). The average fruit size and average fruit number was calculated for each size, cultivar and treatment. The variance of the treatments' effects on bacterial wilt incidence and tomato yield was analyzed by using a general linear model (GLM) conducted in Statistical Analysis System version 9.1 (SAS Institute, Cary, NC). To determine the significance of interaction of the treatments, the differences between means of the disease incidence and yield were contrasted using least significant difference (lsd) test. The results were tested for normality.

In the 2008 experiment, a week after transplanting, Hurricane Fay descended on the Florida panhandle and did not move for 72 hours. During that time the experimental station received 45 cm of rain. In a normal year for the month of August the station receives on average 19 cm of rain. The water collected at the north end of the field and many of the plants were submerged. Some replications of the trial were destroyed, but data was collected from at least 2 of the 4 replications.

## Results

### Field Experiment 2006

Typical bacterial wilt symptoms were observed as early as 1 week post transplanting. Wilted plants were sampled for *R. solanacearum* by performing a bacterial ooze test, FAME, or using the immunoassay strips. All the plants that were sampled were positive for the presence of the bacterium. In all the replications the susceptible cultivar, Phoenix, was affected the most by the pathogen; by the end of the experiment the Phoenix plants in the UTC produced the least amount of fruit compared to the 2 resistant cultivars, BHN669 and FL7514 (Figure 2.1). Phoenix plants that received the thymol or thymol and ASM treatments had an over 200-fold increase of fruit production, and a 3-fold decrease of plants wilting for thymol and an almost 5-fold for thymol and ASM compared to the UTC. By the end of the experiment 94% of the UTC Phoenix plants were completely wilted, while 30% of the thymol treated plants wilted and 19% of the thymol and ASM plants wilted (Table 2.1). The plants treated with thymol and ASM resulted over 70% increase in marketable fruit yield and a 30% reduction of disease incidence for all three cultivars. In addition all three cultivars treated with thymol or with thymol and ASM were significant statistical different than the UTC when comparing disease incidence (Table 2.1). A significant statistical difference was also observed when FL5714 was treated with thymol and ASM compared to thymol alone (Table 2.1).

### Field Experiment 2008

Typical bacterial wilt symptoms were observed as early as week 1, and all wilted plants tested by the bacterial ooze test and with the immunoassay strips assay were positive for *R. solanacearum*. In this experiment the Phoenix cultivar survived better

than FL7514, the moderately resistant cultivar, which might be due to the amount of rain received from the hurricane. Regardless of the differences between the 2 cultivars, the thymol, ASM, and thymol and ASM treated plants resulted in a greater yield and had fewer plants wilt than the untreated controls for both cultivars (Figure 2.2). Even in unfavorable weather conditions, significant statistical difference were observed with the yield for the susceptible plants and both the disease incidence and yield for the moderately resistant plants treated with thymol and ASM when contrasted with the UTC (Table 2.2). The treatments alone for both cultivars were not statistically significant when contrasted with the UTC. The difference in bacterial wilt incidence between thymol and ASM and the UTC, thymol, or ASM treatments for FL7514 was significant (Figure 2.2).

## Discussion

Controlling bacterial wilt in field conditions has been studied for decades (Kelman, 1953), and to date a single strategy proven to be effective to reduce the incidence of disease or severity of bacterial wilt does not exist (Denny, 2006). Factors such as the pathogen's ability to colonize alternative host (Hong et al., 2008), the longevity of the bacterium in fallow soil and water (Hayward, 1991), and its ability to persist in infested plant debris (Granada and Sequeira, 1983), have made it difficult to control the disease once it has established itself in the field.

Good cultural practices also referred to as Integrated Disease Management (IDM) encompasses multiple strategies for controlling the disease. Included in IDM is avoiding planting in pathogen infested soil with pathogen free crops, irrigating with pathogen free water, and proper sanitation practices of operation tools, which are all important to exclude or reduce the pathogen (Anith et al., 2004; Champoiseau et al.,

2009; Hong et al., 2008; Denny, 2006). Complete resistance is only found in groundnut, but semi-resistant cultivars are available, however resistance is limited to geographical location (Denny, 2006).

With the decreased use of methyl bromide, alternatives to control soil pathogens have been increasingly studied (Martin, 2003; Noling and Becker, 1994; Santos et al., 2006). Thymol has proven to be effective in controlling pests such as fungi, nematodes, insects, and bacteria (Delespaul, 2000; Ji et al., 2005; Lee, 1997; Šegvić et al., 2006). Acibenzolar-*S*-methyl too has been proven to be effective against soilborne fungi, nematodes, and bacteria (Benhamou and Bélanger, 1998; Chinnasri et al., 2003; Pradhanang et al, 2005). Further studies to determine the effectiveness of thymol alone, in a non-pretreated field could aid in determining its use as an alternative to methyl bromide.

Previously, we determined that the use of thymol and acibenzolar-*S*-methyl in field conditions was able to decrease disease incidence and increase fruit yield (Ji et al., 2005; Pradhanang et al., 2005). This study was the first time the application of thymol and acibenzolar-*S*-methyl was used together in field conditions to control bacterial wilt on moderately resistant tomato cultivars. We report that the use of both products will not have a negative affect on the tomato production. The combination of both products numerically increased the fruit yield and decreased the disease incidence for the susceptible cultivar. In both trials the moderately resistant plants that received the thymol, acibenzolar-*S*-methyl, the combination of both chemicals increased fruit yield and the lower disease incidence when compared to the UTC. In both studies the combination of thymol and acibenzolar-*S*-methyl was significantly statistically different

from the UTC, thymol or acibenzolar-*S*-methyl alone, when focusing on disease incidence for the different treatments on moderately resistant cultivars. Thus correlating to what Pradhanang et al. (2005) reported, in which a greater difference in disease incidence was shown with resistant plants than susceptible plants when both cultivars were treated with acibenzolar-*S*-methyl. Susceptible tomato cultivars treated with acibenzolar-*S*-methyl were resistant to the pathogen only when the bacterial populations were low, $10^5$-$10^6$; acibenzolar-*S*-methyl was determined ineffective in increasing resistance when the pathogen populations were $10^7$ or higher (Anith et al., 2004; Pradhanang et al., 2005).

Again, it is recommend using moderately resistant cultivars to lower disease incidence and for maximum yield. We showed that if a grower were to use both chemicals, neither would be detritus to yield production. As shown in before mentioned studies, both products are effective at decreasing the incidence of different plant diseases. Thus, the combination of both products could offer a wider protection against multiple biological inhibitory factors. Further studies would include determining the minimum inhibitory concentration (MIC) in field conditions for the most effective and economical benefit for the growers. In conjunction with determining the MIC, further studied of the effect of the combination of thymol and acibenzolar-*S*-methyl would have on other plant pathogens or on multiple diseases. Further research would also need to be conducted to determine the plant's responses to the chemicals and grafting, as grafting could be a new method for controlling the disease (Rivard and Louws, 2008).

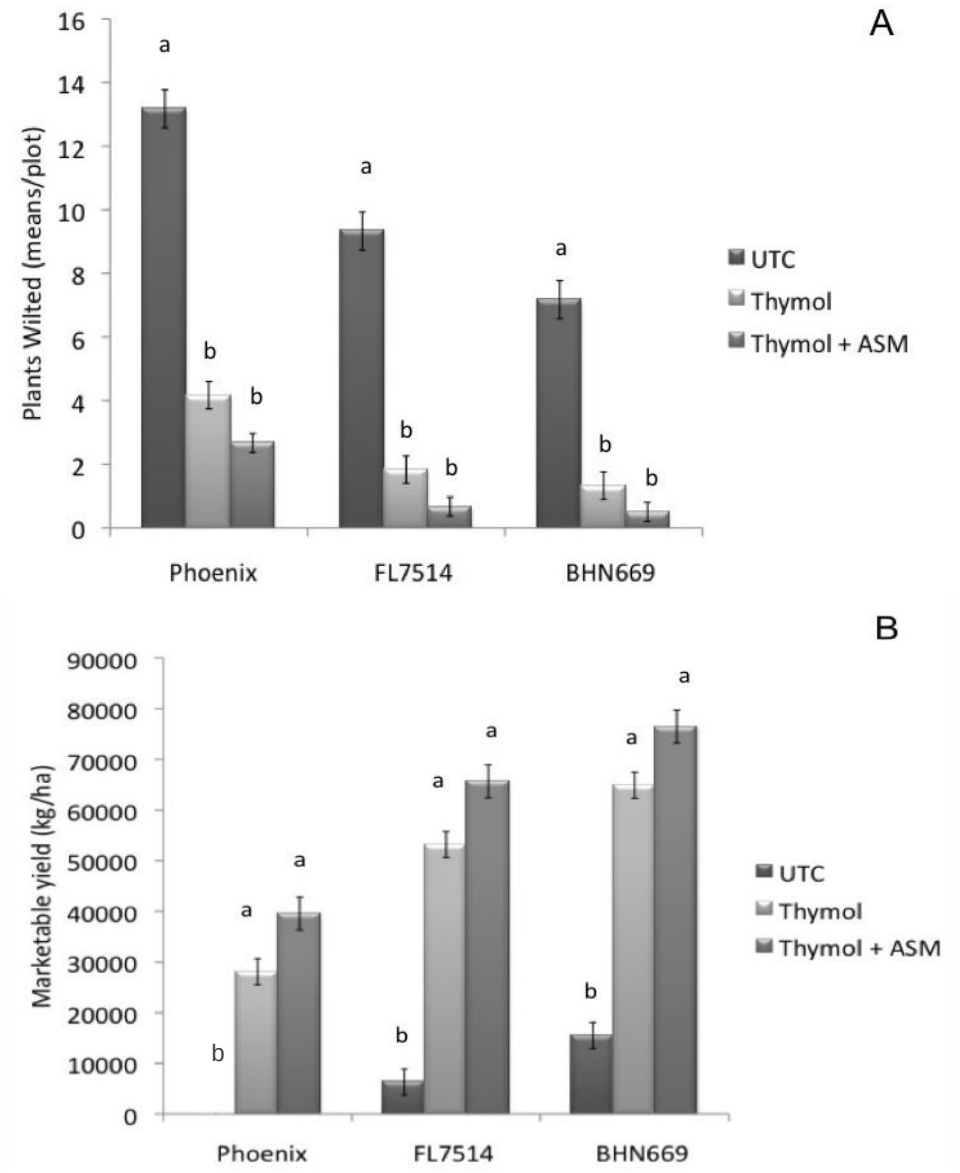


Figure 2.1 Effect of thymol and the combination of thymol and ASM on the number of plants wilted (Graph A) and marketable fruit yield (Graph B) when applied to susceptible and moderately resistant tomato cultivars in a bacterial wilt field experiment (fall 2006, Quincy, FL). Means and SE (standard error of the mean) were the results of 6 replications, and a total of 16 plants per plot. Treatments for each cultivar were tested for significance. Same letter over each bar indicates no significant difference according to Duncan's multiple range test at $P = 0.05$. Untreated control (UTC).

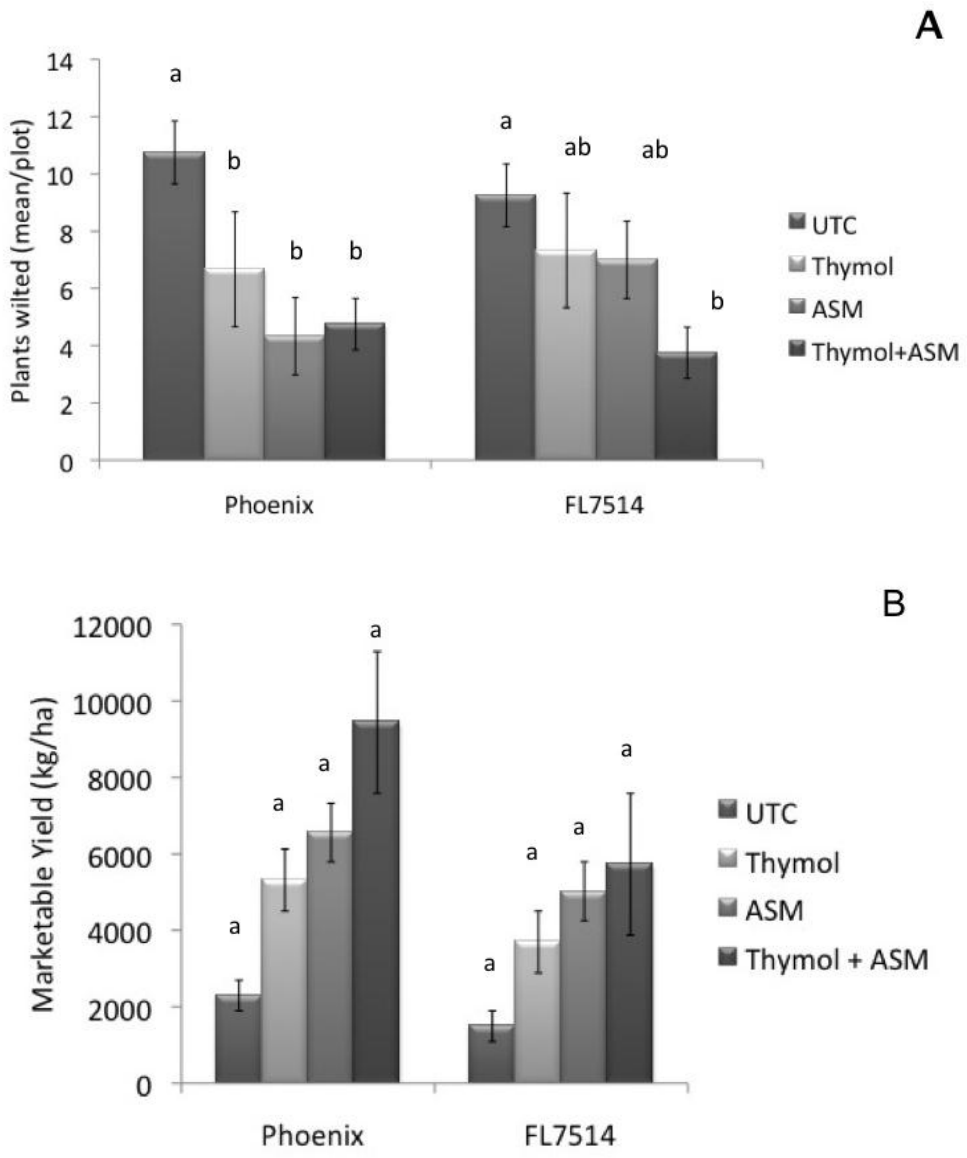


Figure 2.2 Effect of thymol, ASM, and the combination of thymol and ASM on the number of plants wilted (Graph A) and marketable fruit yield (Graph B) when applied to susceptible and moderately resistant tomato cultivars in a bacterial wilt field experiment (fall 2008, Quincy, FL). Means and SE (standard error of the mean) were the results of 4 replications, and a total of 18 plants per plot. Treatments for each cultivar were tested for significance. Same letter over each bar indicates no significant difference according to Duncan's multiple range test at $P = 0.05$. Untreated control (UTC).

Table 2.1 Effect thymol and the combination of thymol and ASM on bacterial wilt incidence and marketable yield for tomato cultivars Phoenix, BH669, and FL7514 (fall 2006, Quincy, FL).

| Cultivar [v] | Treatment [w] | Disease incidence (%)[x] | | | Marketable yield (kg/ha)[x] | | |
|---|---|---|---|---|---|---|---|
| Phoenix | UTC | 94.3 | | | ID[y] | | |
| | Thymol | 30.0 | | | 28,100.9 | | |
| | Thymol + ASM | 19.3 | | | 39,569.4 | | |
| BH669 | UTC | 51.4 | | | 15,519.3 | | |
| | Thymol | 9.3 | | | 64,915.4 | | |
| | Thymol + ASM | 3.6 | | | 76,452.0 | | |
| FL7514 | UTC | 66.4 | | | 6,294.6 | | |
| | Thymol | 12.9 | | | 53,225.7 | | |
| | Thymol + ASM | 5.0 | | | 65,699.6 | | |
| | Contrast [z] | df | F | P>F | df | F | P>F |
| Phoenix | Thymol vs. UTC | 1 | 177.8 | 0.0001 | 1 | 49.1 | 0.0001 |
| | Thymol + ASM vs. UTC | 1 | 204.6 | 0.0001 | 1 | 26.7 | 0.0004 |
| | Thymol + ASM vs Thymol | 1 | 2.79 | 0.1256 | 1 | 1.8 | 0.2130 |
| BH669 | Thymol vs. UTC | 1 | 46.1 | 0.0001 | 1 | 38.6 | 0.0001 |
| | Thymol + ASM vs UTC | 1 | 65.6 | 0.0001 | 1 | 73.7 | 0.0001 |
| | Thymol + ASM vs Thymol | 1 | 2.1 | 0.1556 | 1 | 2.0 | 0.1895 |
| FL7514 | Thymol vs. UTC | 1 | 69.8 | 0.0001 | 1 | 31 | 0.0002 |
| | Thymol + ASM vs. UTC | 1 | 91.4 | 0.0001 | 1 | 48.2 | 0.0001 |
| | Thymol + ASM vs Thymol | 1 | 6.6 | 0.0277 | 1 | 1.8 | 0.2066 |

[v] BHN669 and FL7514 are moderately resistant cultivars, and Phoenix is a susceptible cultivar to bacterial wilt.

[w] Thymol was applied once before transplanting. ASM was applied by foliar spray 6 times: once before transplanting and 5 times afterwards.

[x] Disease incidence was the final percentage of wilted plants. Disease incidence and yield values were means from 6 replications.

[y] Insignificant Data.

[z] Contrast determined by using a GLM (general linear model) and the means of disease incidence and yield treatments for each cultivar were compared using least significant difference (lsd).

Table 2.2 Effect of soil fumigation with thymol, foliar application of ASM, and the combination thymol and ASM on tomato plants in bacterial wilt field experiment on disease incidence of the tomato plants and marketable fruit yield in 2008 (fall, Quincy, FL).

| Cultivar [w] | Treatment [x] | Disease incidence (%)[y] | | | Marketable yield (kg/ha)[y] | | |
|---|---|---|---|---|---|---|---|
| Phoenix | UTC | 38.9 | | | 3,061.0 | | |
| | Thymol | 37.2 | | | 5,316.9 | | |
| | ASM | 23.9 | | | 6,553.8 | | |
| | Thymol + ASM | 26.7 | | | 9,440.4 | | |
| FL7514 | UTC | 51.7 | | | 1,494.3 | | |
| | Thymol | 40.6 | | | 2,804.5 | | |
| | ASM | 38.9 | | | 5,018.7 | | |
| | Thymol + ASM | 21.1 | | | 5,727.8 | | |
| | Contrast [z] | df | F | P>F | df | F | P>F |
| Phoenix | Thymol vs. UTC | 1 | 0.3 | 0.6519 | 1 | 1.4 | 0.3251 |
| | ASM vs. UTC | 1 | 2.1 | 0.2241 | 1 | 5.5 | 0.0785 |
| | Thymol + ASM vs UTC | 1 | 1.3 | 0.3018 | 1 | 7.1 | 0.0376 |
| | Thymol + ASM vs Thymol | 1 | 0.6 | 0.4923 | 1 | 1.2 | 0.3242 |
| | Thymol + ASM vs ASM | 1 | 0.1 | 0.8498 | 1 | 0.1 | 0.7678 |
| | Thymol vs. ASM | 1 | 0.1 | 0.7406 | 1 | 0.1 | 0.8338 |
| FL7514 | Thymol vs. UTC | 1 | 0.6 | 0.4609 | 1 | 1.0 | 0.3671 |
| | ASM vs. UTC | 1 | 1.6 | 0.2571 | 1 | 10.47 | 0.0178 |
| | Thymol + ASM vs UTC | 1 | 15.5 | 0.0077 | 1 | 6.0 | 0.0499 |
| | Thymol + ASM vs. Thymol | 1 | 2.8 | 0.1556 | 1 | 0.6 | 0.4910 |

Table 2.2 Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| Thymol + ASM vs ASM | 1 | 4.4 | 0.0805 | 1 | 0.2 | 0.6972 |
| Thymol vs. ASM | 1 | 0.1 | 0.9001 | 1 | 0.35 | 0.5783 |

[w] BHN669 and FL7514 are moderately resistant cultivars, and Phoenix is a susceptible cultivar to bacterial wilt.
[x] Thymol was applied once before transplanting. ASM was applied by foliar spray 6 times: once before transplanting and 5 times afterwards.
[y] Disease incidence was the final percentage of wilted plants. Disease incidence and yield values were means from 4 replications.
[z] Contrast determined by using a GLM (general linear model) and the means of disease incidence and yield treatments for each cultivar were compared using least significant difference (lsd).

# CHAPTER 3
# DIVERSITY AMONG *RALSTONIA SOLANACEARUM* STRAINS FROM THE SOUTHEASTERN UNITED STATES

## Introduction

Bacterial wilt, caused by the bacterial pathogen *Ralstonia solanacearum* (Yabuuchi et al., 1995), is one of the most destructive bacterial plant diseases in the tropical, sub-tropical, and temperate regions of the world. *R. solanacearum*, as a group, is known to infect over 200 different plant species, but more importantly can cause serious yield losses on important agricultural crops such as tomato, potato, pepper, eggplant, tobacco, banana and geranium (*Pelargonium*) (Hayward, 1991). Once the bacterium is established in the field and conditions are right, within 5 years growers may experience 80% or more crop loss. As a result of the fear of the potential economic impact and yield loss the bacterium could cause in potato production in the temperate regions of the U.S., *R. solanacearum* race 3 biovar 2 is listed as a select agent in the U.S. under the Agricultural Bioterrorism Protection Act of 2002 (Hawks, 2002).

Classification of *R. solanacearum* has become a highly debated topic for more than a century. In the past 20 years the bacterium has been classified in 3 different genera. Previously the bacterium was placed in the genus *Burkholderia*, but due to phenotypic and genotypic differences, it was transferred to the new genus, *Ralstonia* (Yabuuchi et al., 1995). Although *R.solanacearum* strains share common traits, groups within the species have different characteristics such as host range, optimal temperature for causing disease symptoms, and differential utilization of an array of carbohydrates. These differences prompted many researchers to classify bacterial strains to subspecies level based on biovar determination (Hayward, 1964), host range

(Buddenhagen & Kelman, 1964), restriction fragment length polymorphism (RFLP) analysis (Cook and Sequeira, 1989) and phylogenetic analysis of specific genes (Fegan & Prior, 2005).

Phylogenetic analysis has made it possible to determine the diversity of strains quickly and with a degree of accuracy. Previously, various techniques could take up to a month for definitive results (i.e. host tests or biovar test), or the tests were difficult to analyze (i.e. amplified fragment length polymorphisms (AFLP), RFLP, or pulsed-field gel electrophoresis). Within the past few years many studies have been published using phylogenetic analyses to determine the diversity of *Ralstonia* strains (Xu et al., 2009, Liu et al., 2009, Jeong et al., 2007, Lewis Ivey et al., 2007, Sanchez Perez et al., 2008, Cardozo et al., 2009, Toukam et al., 2009, Ji et al., 2007 and Hong et al., 2008).

Most *R. solanacearum* strains isolated from the southeast have been characterized as race 1 biovar 1 (Martin et al. 1982; McLaughilin and Sequeria, 1989; Robertson et al., 2001; Ji et al., 2008), Previous reports had not demonstrated diversity within these strains. Robertson et al. (2004) determined that strains from the Carolinas contained a truncated non-functional avirulence gene, compared to the Georgia and Florida strains, which had the full length functional avirulence gene. Strains with the truncated avirulence gene also had a broader host range, including tobacco, whereas strains with the functional avirulence gene, *avrA*, caused a hypersensitive reaction (HR) on tobacco.

Exotic *R.solanacearum* strains recently have been identified in northern, central, and southern Florida (Ji et al., 2007, Hong et al., 2008 and Norman et al., 2009). These strains may have originated from Asia or the Caribbean and have a wider host range

than the native Florida strains. It is believed that many of the bacterial wilt outbreaks may have been due to these exotic strains instead of the native strains (Norman et al., 2009). Plant species previously considered non-hosts of *R. solanacearum*, were reported to develop bacterial wilt symptoms by strains of Asiatic origin (Ji et al., 2007).

In 2008 we reported on an exotic strain that was found in the waterways in northern Florida (Hong et al., 2008). Based on previous research, phylogenetic analysis of the *egl* of this strain was 100% identical to new emerging strains reported in Martinique (Wicker et al., 2007). In both papers, it was reported that this strain was more aggressive when compared to the typical native strain. Furthermore the new strains found in Martinique, although not race 2, was able to colonize and move systemically in triploid banana, without causing symptoms. The bacterial strain also had a larger host range than the typical strains. During the sampling that encompassed over 2 years, haplotypes of RS5, the typical northern Florida *R. solanacearum* strain, were never detected. We determined that this exotic strain not only colonized triploid banana, but also was deleterious to the growth of the banana.

In this study an extensive analysis was performed on strains isolated from the southeastern U.S. Strains were characterized based on biovar, pathogenicity tests, HR on tobacco, and phylogenetic analyses of *egl* sequences.

## Materials and Methods

### Bacterial Culture, Biovar and Inoculum Preparation

All the *R. solanacearum* strains used in this study are listed in Table 1. Strains were renamed to reflect the origin of labs from where they came. Upon receiving bacterial cultures, strains were streaked on modified semi-selective medium (SMSA;

Englebrecht, 1994), were transferred to casamino acid peptone glucose (CPG) agar (Kelman, 1954) and then stored at -80°C in 30% glycerol solution.

Biovar was determined based on the bacterium's ability to utilize various carbon sources as described by Hayward (Hayward, 1991). RS5, race 1 biovar 1, GMI1000, race 1 biovar 3, and UW447, race 3 biovar 2T, were used as standards and positive controls for the biovar tests. A bacterial culture grown on CPG for 12 hr was suspended in sterile tap water and adjusted to approximately$10^8$ cfu/ml with a spectrophotometer (Sigma-Aldrich Co., Milwaukee, WI). The suspension was added to plates containing the panel of carbon sources.

For all pathogenicity tests, each strain was grown on CPG agar for 24 hr and then suspended in sterile de-ionized water. Suspensions were adjusted to $10^8$ cfu/ml. A few inoculated plants from each experiment were selected on which to perform Koch's postulates. Plants were sampled at the stem and were soaked in 70% ethanol and then flame sterilized. The plant material was crushed using a mortar and pestle. The residue was suspended in 1ml of de-ionized water and the suspension was streaked on SMSA. The plates were stored at 28°C. *R. solanacearum* colonies were confirmed by PCR using the *R. solanacearum* specific primers, 759/760 (Opina et al., 1997). The specific details for the PCR amplification are described later in the document.

The bacteriocin test consisted of growing a loopful of the producing strain in the center of the plate for 24 hr. The indicator strain adjusted to $10^8$ cfu/ml was sprayed on the plate and the plates were incubated for 48 hr at 28°C and checked every 12 hr for a zone of inhibition. Three different types of media, King B, Luria-Bertani and CPG, were tested to determine which would produce the clearest zone. The indicator strain was

grown in CPG broth for 24 hr prior to spraying onto the agar surface. To determine if the agent causing the zone of inhibition was a bacteriocin or a lytic bacteriophage, the zone of inhibition was cut out of the agar and washed in 1ml of sterile de-ionized water. The sample was then vortexed and centrifuged at 10,000 rpm and the supernatant was removed and added to CPG broth with the indicator bacterial strain. The suspension was incubated at 28°C on a shaker, set at 300 rpm, for 24 hr. The suspension was then centrifuged at 10,000 rpm and the supernatant was then sterilized using a low protein binding Microcon filter (Amicon, Beverly, MA) with a 0.22 µm pore size. Then 1 ml of the filtered product was added to CPG agar. A 10-fold-series dilution of the susceptible strain was prepared and 0.1 ml was added to the amended CPG agar. Plates were then observed for formation of plaques.

**Pathogenicity Tests on Tomato, Pepper and Banana and HR test on Tobacco.**

A pathogenicity test was performed using tomato (c.v. Bonny Best) and pepper (c.v. Aruba). Strains were inoculated on 3 to 4 week-old plants in 10-cm diameter pots. Root inoculation consisted of wounding the roots and then pouring 30 ml of a bacterial suspension on the soil surrounding in close proximity to the crown of the plant. The inoculated plants were transferred to a growth room with 12 hr light/dark cycle and the air temperature was maintained at 30°C. Each trial consisted of 3 plants per strain, and was repeated twice.

The *Musa* pathogenicity test was performed on 3 different *Musa* genotypes, ‘Dwarf Nam wa’ (ABB), ‘French Horn’ (AAB), and ‘Gran Nain’ (AAA). The plants were inoculated with RS5, RS37, and the race 2 strains UW2, UW70, UW170, and sterile de-ionized water. For the first experiment, 8 plants of each genotype were inoculated by root inoculation or by syringe. The plants were at the 3 full leaf stage when inoculated.

The root inoculation was performed as described previously. For syringe inoculation, 1 ml of the inoculum was injected into the plant tissue at the corm. Further *Musa* pathogenicity tests consisted of only root inoculation of 'Dwarf Nam wa' (ABB) plants. Thirty days post inoculation (dpi) plant roots were rinsed, air dried overnight and then weighed. For each experiment the root masses were analyzed by Tukey's range test in conjunction with an analysis of variance (ANOVA) by the SAS System for Windows program (release 9.1; SAS, Cary, NC).

For the tobacco HR assay, *R. solanacearum* suspensions were infiltrated into the intercellular space of fully expanded tobacco leaves. Each strain was inoculated twice on the same leaf and inoculations were repeated on 3 other plants. Inoculated plants were placed in a growth chamber with conditions previously described. Infiltrated areas were assessed for HR 24 hr after infiltration.

**PCR Amplification and Phylogenetic Analysis of the *egl***

Each PCR amplification reaction contained 1 unit of *Taq* DNA polymerase (GoTaq® Flexi DNA Polymerase (Promega Corp., Madison, WI)) with 5.0 µl of 5x buffer, 1.5 µl of $MgCl_2$, 4.0 µl dNTP, each primer at 10 pmol, and 100 ng of DNA. The total volume was adjusted to 25 µl with sterile deionized water. The amount of water was adjusted if the protocol required more than two primers or a greater amount of DNA. A hot start at 95°C for 5 min followed by 30 cycles at 95°C for 45 sec, 68°C for 30 sec and 72°C for 60 sec, and finished with a 10 min extension at 72°C was used for the amplification of a DNA sequence in a thermocycler (BioRad, Hercules, CA). The annealing temperature was adjusted according to the composition of the oligonucleotide sequence.

Each strain was tested to determine phylotype and to ensure it was not race 3 biovar 2. Phylotype classification, as outlined by Fegan & Prior (2005), consisted of doing PCR using the primers; Nmult21:1F, Nmult21:2F, Nmult23:AF, Nmult22:InF, and Nmult22:RR. The PCR products were visualized on a gel, and according to the band length the phylotype was assigned. Primer pair 630/631, specific for race 3 biovar 2 strains, was used to amplify DNA from each strain. (Fegan et al., 1998). DNA from UW551, a race 3 biovar 2 strain, was used as a positive control.

The *egl* gene was amplified by PCR and the product was sequenced and phylogenetically analyzed to determine the relationship of each strain to each other and strains previously described (Table 2). The primer pair JHFegl: GACGATGCATGCCGCTGGTCGC and JHRegl: CACGAACACCACGTTGCTCGCATTGG were designed based on the *egl* sequences of GMI1000 (AL646053.1), UW551 (DQ657596.1), and Molk2 (CU694393.1) accessed from Genbank. Closely related *R. syzygii* strains, blood disease bacterium R223 (DQ011538.1) and *R. syzygii* strain R058 (DQ011543.1) served as outgroups. Amplicons were sequenced in both the forward and reverse directions to create a consensus sequence. The sequences were aligned using Muscle (Edgar, 2004) via http://www.ebi.ac.uk/Tools/muscle/index.html. Se-Al version 2.0a11 was used for manual manipulation of the sequences. The program is available at http://tree.bio.ed.ac.uk/software/seal/ (Rambaut, 1996). Phylogenetic analysis using maximum parsimony (MP) and maximum likelihood (ML) were run on PAUP* version 4.0b10 (Sworfford, 2003). The MP and ML dendrograms were created by performing tree bisection/reconnection (TBR) branch swapping and heuristic searches with random

stepwise addition. Modeltest 3.7 was used to select a nucleotide model that best fit the data (Posada and Crandall, 1998). ML settings were adjusted to the TIM+I+G nucleotide substitution model; the best-fit model suggested by Akaike's information criterion. Branch support for both the MP and ML trees was estimated by nonparametric bootstrapping (n=1,000 replicates for MP and n=200 replicates for ML) by TBR swapping (n=1,000 replicates for MP and n=100 replicates for ML; Felsenstein, 1985). RAxML-VI-HPC was used to run additional ML and ML bootstrap analysis, which was performed on the Fisher Cluster at The University of Florida Genetics Institute Gainesville, FL (Stamatakis et al., 2005). The settings in RAxML could not be adjusted to the TIM+I+G model, thus a GTR+G model was used. For bootstrap analysis, 5000 runs were used to generate 5000 trees. Treefig was used to create and visualize the consensus tree (Rambaut, 2006). MrBayes 3.1 was used to perform Baysesian analysis (Huelsenbeck et al., 2001). The settings were adjusted to resemble TIM+I+G and were set for 1,000,000 generations being sampled at every 500 generations. The burn-in was placed at 88,500, and the consensus tree of the posterior probabilities was created by PAUP* 4.0b10.

Previously RS37 was reported to be identical to nonpathogenic banana strains from Martinique (Hong et al., 2008). To determine if RS37 was a *Musa* strain, the *Musa* multiplex PCR protocol as described by Prior and Fegan was used (2005). The following primers were used: Mus20-F, Mus20-R, Mus35-F, Mus35-R, Mus06-F, Mus06-R, Si28-F and Si28-R. RS5 was used as a negative control and 527, a race 2 strain, was used as a positive control.

## Results

### Bacterial Strains

All *R. solanacearum* strains received from the various libraries or isolated and collected within the lab were first streaked on semi-selective agar SMSA. The colonies that did not appear as typical *R. solanacearum* colonies were tested with specific *R. solanacearum* immunoassay strips. All strains that were negative for the immunoassay strips (Agdia, Inc., Elkhart, IN) were tested a second time with the strips, and if these failed they were discarded. Multiplex PCR, as described by Fegan and Prior, was performed for the remaining strains. All of these strains produced a 281 bp product, which is considered positive for the Opina 759/760 universal *R. solanacearum* primers. All strains, except for 3 isolated from Florida, had a 372 bp band that corresponds to phylotype II. The 3 Florida strains, isolated on pepper, were identified as phylotype I due to the 144 bp band they produced. The 630/631 primers, specific for race 3 biovar 2 strains, were tested on all the collected strains. None of the strains produced a band with these primers.

Strains were classified based on their ability to oxidize a panel of different carbon sources. The three pepper strains were identified as biovar 3. These three strains were able to acidify mannitol, sorbitol, dulcitol, trehalose, and maltose. The remainder of the strains corresponded to biovar 1, which only acidified trehalose.

### Pathogenicity Tests

After plating the strains on SMSA it was discovered that some appeared dry and not shiny, which is typical of non-pathogenic *R. solanacearum* strains (Table 1). All strains wilted ‘Bonny Best’ tomato plants except for the 6 non-mucoid strains. Wilt

symptoms were observed 7-12 dpi for all 3 replicates. Of the 142 strains tested, 136 caused wilt symptoms in the susceptible cultivar. A few plants from each trial were sampled and bacterial colonies were isolated from the plants, and then streaked for individual colonies on SMSA. The bacterial colonies were confirmed as *R. solanacearum* by immunoassay using immunostrips.

A pathogenicity test on pepper, cultivar 'Aruba', was performed for all the strains. This variety previously was reported to be susceptible to phylotype I strains (Ji et al., 2007). All three of the phylotype I strains wilted pepper within 7-10 dpi. Symptoms were observed on the three replicates. Besides the three phylotype I strains five other strains from Florida and three strains from Georgia also wilted pepper, but were biovar 1 and phylotype II. A few plants from each trial were sampled for *R. solanacearum*, and the bacterium was confirmed using Immunostrips.

An HR test on tobacco was performed because it was previously reported that Florida strains produce an HR while Carolina strains do not. HR was observed 24 hr after inoculation. An HR was observed in 9.7% of the Carolina strains, 46.2% of the Georgia strains, and 56.2% of the Florida strains. Even though 38 strains were isolated in Florida, these strains were isolated from plants that originated from the Caribbean (Norman et al. 2009). Phylogenetic analysis of these Caribbean-like strains grouped them with strains originating from the Caribbean. The total number of strains originating in Florida was 67. Thus, with the adjusted total, 88.1% of the Florida strains gave a positive HR on tobacco.

**Phylogenetic Analysis of the *egl***

The trees created by the three different analyses (Figures 3.1-3.2): MP, ML and Bayesian, were similar in respect to the cladist placement of the representative strains

from our library and strains acquired from GenBank (Table 3.2). Strains acquired from GenBank represent all of the *R. solanacearum egl* sequences submitted to Genbank. These strains originated from different regions of the world and were isolated from multiple hosts. The phylogenetic trees confirm the phylotype determination based on multiplex PCR in that all the strains are phylotype II, except for strain 232, which will be discussed in Chapter 4. Furthermore, phylogenetic analysis of the *egl* revealed that the strains isolated from Florida were more diverse than strains isolated from the Carolinas and Georgia. The majority of the strains in our collection grouped with the sequevar 7 strains. All the Carolina and Georgia strains were found in this clade. The strains isolated in Florida, including those with exotic origins, grouped with sequevar 4, 5/6, and 7, and an undefined sequevar. The undefined clade did not contain any previously reported strains. The likelihood score using RAxML and a GTR+G model was –lnL 2575.71. The likelihood score using Paup*4.0b10 with a TIM+I+G model was -lnL 2597.05.

**Bacteriocin Test Between RS37 and U.S. Southeastern Race 1 Biovar 1 Strains**

RS37 was tested to determine if it produced a bacteriocin that would inhibit the growth of other race 1 biovar 1 strains. In the first test RS37 was used as the producing strain and RS5 as the indicator. A zone of inhibition was detected when RS37 was first spotted on the agar and RS5 was sprayed on it 24 hr later. A zone was not detected when RS5 was spotted and RS37 was sprayed, nor when RS5 was tested against itself, or when RS37 was tested against itself (Figure 3.3). The largest zone of inhibition was noted on King's Medium B agar when compared to nutrient agar, CPG agar, or LB agar. The test to determine if the causal agent was a bacteriocin or a lytic phage confirmed it to be a bacteriocin. Attempts were made to isolate the bacteriocin. RS37 was grown in

nutrient broth and the cells were spun down and the supernatant was extracted and filtered. The supernatant was poured in wells on King's B agar and RS5 suspension was sprayed on it immediately, 8 or 24 hr later. However, the bacteriocin was not detected when the supernatant was run through a series of different size filters. RS37 did not have activity against 3 other *R. solanacearum* strains: AW1, P553, and V-170 representing Alabama, an older Florida strain, collected in 1997, and a typical Carolina strain, respectfully.

**Musa Multiplex PCR and Triploid Musa Pathogenicity Test**

Based on our previous study (Hong et al., 2008) it was assumed that RS37 belonged to sequevar 4NPB. The *Musa* multiplex PCR, a diagnostic test for identifying race 2 strains (Prior and Fegan, 2005), was performed on RS37 with 527, a race 2 strain used as a positive control, and RS5, a race 1 strain used as a negative control. Strain 527 produced a 220 bp band, thus corresponding to sequevar 6. RS5 did not produce a band. RS37 produced a 167 bp band (Figure 3.4). As reported, a strain that produces a 167 bp band also produces a band at 400 bp belongs to sequevar 4. However, RS37 never produced a 400 bp band. Sequevar based on phylogenetic analysis of the partial *egl* placed RS37 in an undefined clade. It was below sequevars 5 and 6 and above sequevar 19-23 (Figure 3.1). The other strains collected at the same location over a 2-year period belonged to the same clade as RS37 and also produced a 167 bp band for the *Musa* multiplex.

Due to the presence of a positive but unique band in RS37 with the *Musa* specific primers, a triploid banana pathogenicity test was performed. Two different experiments were done. In the first experiment, three different *Musa* genotypes, 'Dwarf Nam wa' (ABB), 'French Horn' (AAB), and 'Gran Nain' (AAA) were root inoculated or the bacterial

suspension was injected into the corm with a hypodermic needle and syringe. Plants were kept in greenhouse conditions for 80 dpi, and sampled every 10 days. The first symptoms for the race 2 corm inoculated plants were observed 7-10 dpi, while root inoculation symptoms were observed 15-20 dpi. For sampling, plants were divided in 3 parts; roots, corm to the first leaf, and from the first leaf to the top of the plant with the unfolded leaves removed. The bacterium was recovered mostly in the roots and corm section for the race 2 strains and RS37 for the root inoculated plants. In plants inoculated with RS5, the bacterium was recovered close to the source of inoculation or sometimes not at all. However, in plants inoculated by the syringe injection technique, RS5 and the other strains were detected in all 3 parts of the plants.

The root mass of the 3-*Musa* genotypes inoculated with the race 2 strains, RS5, and RS37 were weighed. The plants inoculated by the root wounding method had less root mass than those inoculated by syringe injection (figure not shown). Plants inoculated with UW2 had the lowest mass. RS37 reduced the root mass in all 3 genotypes, however symptoms were more severe in 'Dwarf Nam wa' (figure not shown). Statistical comparison of the 'Dwarf Nam wa' root masses revealed that the root masses of the plants inoculated with RS37 were more similar to the root mass of the plants inoculated with race 2 strains than the root masses of the plants inoculated with either water inoculated or RS5 (Figures 3.5 and 3.6).

**Pathogenicity Test on Cucurbits**

Wicker et al. (2007) indicated that a new emerging *R. solanacearum* strain was identified in Martinique. These strains had a wider host range than typical biovar 1 strains found on the island. These strains were pathogenic on cucurbits, solanaceous crops, and ornamental plants. RS37 was inoculated on a panel of cucurbit plants to

determine if it could cause wilt symptoms. This panel of plants included watermelon cultivars (i.e., ‘sugar baby’, ‘crimson sweet’, and ‘madera’); squash cultivars (i.e., ‘taybelle’, ‘senator’, and ‘goldfinger’); cucumber cultivar, ‘marketmore 76’; and cantaloupe cultivar, ‘summet’. No symptoms were observed on any of the plants after 30 dpi for either RS5 or RS37.

## Discussion

In the continental U.S. *R. solanacearum* is primarily found in the southeast. All the native strains are classified as race 1 biovar 1 and belong to phylotype II. Although these strains are similar, differences were noted in HR, host range tests, and phylogenetic analysis of the *egl*. We determined that the greatest diversity was found in Florida with strains isolated belonging to two different phylotypes and at least two different sequevars. One of the exotic strains isolated in FL, RS37 was further characterized. This strain was able to cause symptoms on tomato plants, like the native strains, but it also was deleterious to growth of triploid banana.

The greatest diversity of *R. solanacearum* strains was found predominantly in Florida. The strains from the Carolinas were all identified as the same haplotype. Furthermore, all the Carolina strains reacted similarly to all the tests and analyses performed in this study. The diversity of haplotypes found in Florida could be due to several factors. The most plausible explanation is that Florida is a sub-tropical region where many exotic crops are imported. The importation of these plants offers an avenue for introduction of exotic strains of the pathogen.

Phylogenetic analysis to determine diversity can be a useful tool; however, if not done correctly or interpreted incorrectly it can result in incorrect conclusions. One of the examples of using phylogenetics incorrectly was the sequevar placement of RS37 strain

in previous studies. RS37 was isolated in northern Florida and was identical to a Caribbean strain based on *egl* sequence. Previous reports placed this strain and other clones of it in sequevar 5 and 4NPB (Ji et al. 2007; Hong et al., 2008). It appears that those studies incorrectly assigned sequevar due to a lack of taxa used. Hence, phylogenetic trees in other papers may not be correct due to their lack of representative strains or the analysis used is based on old and outdated techniques. Using the current methods for phylogenetic analysis, the trees created are reproducible. However support for many sequevar clades was low, being less than 75% for bootstrap and less than 95 for posterior probability.

The phylogenetic trees created in this study indicate that strains isolated from Florida were more diverse than those isolated in Georgia and the Carolinas. Yet, phylogenetic analysis was not able to differentiate between strains which caused wilt on pepper or the results of an HR test. The majority of strains from Florida grouped with sequevar 7 strains; however, a few strains grouped with sequevar 4 and 5/6. Previously it was reported that strains belonging to sequevar 4 can infect triploid bananas and can be detected throughout the plant. Some of the sequevar 4 strains are true race 2 strains and cause wilt in triploid banana, while others may not cause disease (Wicker et al., 2007). Most of the strains that grouped within sequevar 4 originated in pothos and anthurium cuttings. A M*usa* pathogenicity test was conducted, using 'Dwarf Cavendish' (AAA) with these strains, and they did not cause symptoms (Norman et al., 2009). In this study, we observed that the severity of symptoms caused by RS37 depended on the genotype of banana plant. Hence *Musa* pathogenicity tests using a variety of

genotypes and a *Musa* multiplex PCR should be performed with all strains that grouped with sequevar 4.

As stated previously, it was assumed that RS37 was similar to Caribbean strains that infected triploid bananas. *Musa* multiplex PCR was performed to determine if it belonged to a race 2 sequevar. Unlike the race 1 which produced no bands, RS37 produced a single 167bp band. For some sequevar 4 strains this band is amplified, but it is never amplified for the other race 2 sequevars. However, all the seqeuvar 4 strains produced a single 400bp band or one band at 400 bp and one at 167 bp. Based on the results for the PCR test, RS37 was inoculated on a panel of *Musa* genotypes to determine if could cause symptoms The bacterium was detected systemically on all three genotypes. The observed symptoms caused by RS37, less root mass and reduced plant growth, were more severe on the ABB genotype than the other genotypes. We also determined that the preferred technique employed to infect *Musa* plants was by root inoculation. The race 1 strains were periodically detected in the banana leaves. We believe that when the bacterium was injected into the corm, it was readily transported as the leaves grew, thus indicating a false movement. Race 2 strains only infect triploid banana. RS37 is unique in that it can infect and wilt tomato and also move systemically through a banana plant. Further research will need to be done to determine the differences and similarities between this strain and typical race 1 and 2 strains. Hopefully, further research using RS37 could indicate which genes are responsible for determining host specificity.

In our previous study it was noted that only haplotypes of RS37 were found and not the native sequevar 7 strains (Hong et al. 2008). This led us to investigate the

potential reasons for RS37 to outcompete RS5. Bacteria that compete for the same resources commonly produce bacteriocins that inhibit other bacteria from growing in close proximity (Bradley, 1967). The putative bacteriocin from RS37 did not inhibit growth of all representative strains from the collection. This could be attributed to some bacteriocins being specific to the strains they target. Further research could be done to characterize the bacteriocin for possible use as a control agent in the field. Creating and characterizing a collection of *R. solanacearum* strains from the southeast provides a reference for future comparison. Future researchers can now determine if a population shift has taken place in the Carolinas or if a new strains have been introduced.

Table 3.1 List of strains used in this study

| Strains[a] | Origin | Host Origin | Other Name | Year | Biovar[b] | Phylotype[c] | Pathogenicity Test[d] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Tomato | Pepper | HR[e] |
| RS5 | FL | Tomato | | 1999 | 1 | II | + | + | + |
| 102 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 103 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 104 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 105 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 106 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 107 | FL | *Polygonum pennsylvanicum* | | 2004 | 1 | II | + | - | - |
| 108 | FL | *P. pennsylvanicum* | | 2004 | 1 | II | + | - | - |
| 109 | FL | *P. pennsylvanicum* | | 2004 | 1 | II | + | - | - |
| 110 | FL | *Hydrocotyle ranunculoides* | | 2004 | 1 | II | + | - | - |
| 111 | FL | *H. ranunculoides* | | 2004 | 1 | II | + | - | - |
| 112 | FL | *H. ranunculoides* | | 2004 | 1 | II | + | - | - |
| 113 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 114 | FL | Pond water | | 2004 | 1 | II | + | + | - |
| 118 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 119 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 120 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 125 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 128 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 130 | FL | Pond water | | 2004 | 1 | II | + | - | - |
| 201 | FL | Pothos | P487 | 1996 | 1 | II | + | - | + |
| 202 | FL | Tomato | P503 | NA[g] | 1 | II | + | - | - |
| 203 | FL | Tomato | P504 | NA[g] | 1 | II | + | - | + |
| 204 | FL | Tomato | P505 | NA[g] | 1 | II | - | - | + |
| 205 | FL | Tomato | P507 | 1996 | 1 | II | + | - | + |
| 206 | FL | Tomato | P530 | 1997 | 1 | II | + | - | + |
| 207[f] | FL | Tomato | P532 | 1997 | 1 | II | - | - | + |

Table 3.1 Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 208 | FL | Tomato | P533 | 1997 | 1 | II | + | - | + |
| 209 | FL | Tomato | P534 | 1997 | 1 | II | + | - | + |
| 210[f] | FL | Tomato | P535 | 1997 | 1 | II | - | - | + |
| 211 | FL | Tomato | P536 | 1997 | 1 | II | + | - | + |
| 212[f] | FL | Potato | P541 | 1997 | 1 | II | - | - | + |
| 213 | FL | Potato | P543 | 1997 | 1 | II | + | - | + |
| 214 | FL | Potato | P550 | 1997 | 1 | II | + | - | + |
| 215 | FL | Potato | P553 | 1997 | 1 | II | + | - | + |
| 216 | FL | NA[g] | P557 | 1997 | 1 | II | + | - | - |
| 217 | FL | Pothos | P564 | 1996 | 1 | II | + | + | + |
| 218 | FL | Pothos | P573 | 1999 | 1 | II | + | - | + |
| 219 | FL | Tomato | P576 | 1999 | 1 | II | + | - | + |
| 220 | FL | Tomato | P594 | 2000 | 1 | II | + | - | - |
| 221 | FL | NA[g] | 136 | NA[g] | 1 | II | + | - | + |
| 222 | FL | Heliconia | 158 | NA[g] | 1 | II | + | - | + |
| 223 | FL | Pothos | 485 | NA[g] | 1 | II | + | + | + |
| 224 | NA[g] | Pothos | | NA[g] | 1 | II | + | + | + |
| 225 | NA[g] | Pothos | 517 | NA[g] | 1 | II | + | + | + |
| 226 | NA[g] | Pothos | 521 | NA[g] | 1 | II | + | + | + |
| 227 | NA[g] | | 528 | NA[g] | 1 | I | + | - | + |
| 228 | FL | Potato | 544 | 1994 | 1 | II | + | - | + |
| 229 | NA[g] | Pothos | 545 | NA[g] | 1 | II | + | + | + |
| 230 | NA[g] | Pothos | 548 | NA[g] | 1 | II | + | - | + |
| 231 | NA[g] | Tomato | | NA[g] | 1 | II | + | - | + |
| 232 | Guadeloupe | Tomato | 506 | 1985 | 1 | II | + | - | + |
| 233 | Martinique | Tomato | 609 | 1986 | 1 | I | + | - | - |
| 234 | Martinique | Tomato | 610 | 1987 | 1 | II | + | - | + |
| 235 | NA[g] | Anthurium | 618 | NA[g] | 1 | II | + | - | + |
| 236 | NA[g] | Anthurium | 621 | NA[g] | 1 | II | + | - | + |
| 237 | USA | Tomato | 660 | NA[g] | 1 | II | + | - | + |
| 238 | NA[g] | Pothos | 673 | NA[g] | 1 | II | + | - | - |
| 239 | NC | | 688 | NA[g] | 1 | II | + | - | + |

Table 3.1 Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 240 | NC | Potato | 689 | NA[g] | 1 | II | + | - | + |
| 241 | NC | Tomato | 690 | NA[g] | 1 | II | + | - | + |
| 242 | NC | Tomato | 691 | NA[g] | 1 | II | + | - | + |
| 301[f] | FL | Soil | RS14 | 2001 | 1 | II | - | - | + |
| 302[f] | FL | Tomato | RS18 | 1991 | 1 | II | - | - | + |
| 303 | FL | Geranium | RS37 | 2001 | 1 | II | + | - | - |
| 304 | FL | Geranium | RS38 | 2001 | 1 | II | + | + | - |
| 305 | FL | Geranium | RS39 | 2001 | 1 | II | + | - | - |
| 306 | FL | Geranium | RS40 | 2001 | 1 | II | + | - | - |
| 307 | FL | Pond Water | RS51 | 2001 | 1 | II | + | - | - |
| 308 | FL | *Bidens mitis* | RS55 | 2002 | 1 | II | + | - | - |
| 309 | FL | *P. pennsylvanicum* | RS56 | 2002 | 1 | II | + | - | - |
| 310 | FL | *P. pennsylvanicum* | RS57 | 2002 | 1 | II | + | - | - |
| 311 | FL | *H. ranunculoides* | RS58 | 2002 | 1 | II | + | - | - |
| 312 | FL | *H. ranunculoides* | RS59 | 2002 | 1 | II | + | - | - |
| 313 | FL | *H. ranunculoides* | RS60 | 2002 | 1 | II | + | - | - |
| 314 | FL | *H. ranunculoides* | RS61 | 2002 | 1 | II | + | - | - |
| 315 | FL | Pond Water | RS62 | 2002 | 1 | II | + | - | - |
| 316 | FL | Pond Water | RS65 | 2002 | 1 | II | + | - | - |
| 317 | FL | *B. mitis* | RS66 | 2002 | 1 | II | + | - | - |
| 318 | FL | Pond Water | RS67 | 2002 | 1 | II | + | - | - |
| 319 | FL | *P. pennsylvanicum* | RS70 | 2002 | 1 | II | + | - | - |
| 320 | FL | Pond Water | RS74 | 2003 | 1 | II | + | - | - |
| 321 | FL | Pond Water | RS77 | 2003 | 1 | II | + | - | + |
| 322 | FL | Pond Water | RS79 | 2003 | 1 | II | + | - | - |
| 323 | FL | Pond Water | RS85 | 2003 | 1 | II | + | - | + |
| 324 | FL | Pond Water | RS89 | 2003 | 1 | II | + | - | - |
| 325 | FL | Pond Water | RS90 | 2003 | 1 | II | + | - | + |
| 326 | FL | Pond Water | RS101 | 2004 | 1 | II | + | - | - |
| 327 | FL | Pond Water | RS109 | 2004 | 1 | II | + | - | + |
| 328 | FL | Hydrangea | RS116 | 2005 | 1 | II | + | - | + |
| 329 | FL | Hydrangea | RS118 | 2005 | 1 | II | + | - | + |

Table 3.1 Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 330 | FL | Pepper | RS121 | 2005 | 3 | I | + | + | - |
| 331 | FL | Pepper | RS122 | 2005 | 3 | I | + | + | - |
| 332 | FL | Pepper | RS123 | 2005 | 3 | I | + | + | + |
| 333 | FL | Hydrangea | RS124 | 2005 | 1 | II | + | - | - |
| 334 | FL | Geranium | RS125 | 2005 | 1 | II | + | - | + |
| 335 | FL | Geranium | RS126 | 2005 | 1 | II | + | - | + |
| 336 | FL | Geranium | RS127 | 2005 | 1 | II | + | - | - |
| 337 | FL | Geranium | RS128 | 2005 | 1 | II | + | - | + |
| 338 | FL | Pond Water | RS129 | 2005 | 1 | II | + | - | + |
| 339 | FL | Pond Water | RS130 | 2005 | 1 | II | + | - | - |
| 340 | FL | Tomato | RS133 | 2005 | 1 | II | + | - | - |
| 341 | FL | Tomato | RS134 | 2005 | 1 | II | + | - | - |
| 342 | FL | Tomato | RS135 | 2005 | 1 | II | + | - | + |
| 343 | FL | Tomato | RS136 | 2005 | 1 | II | + | - | + |
| 344 | FL | Tomato | RS137 | 2005 | 1 | II | + | - | + |
| 348 | NC | Tobacco | TD294 | NA[g] | 1 | II | + | - | + |
| 352 | GA | Tomato | TD674 | NA[g] | 1 | II | + | - | - |
| 353 | FL | Tomato | RS73 | 2006 | 1 | II | + | - | + |
| 355 | FL | Tomato | RS1 | 1999 | 1 | II | + | - | + |
| 356[f] | GA | Tomato | RS3 | 1999 | 1 | II | - | - | - |
| 357 | GA | Tomato | UW26 | 1954 | 1 | II | + | - | - |
| 359 | NC | Tomato | UW123 | NA[g] | 1 | II | - | - | - |
| 362 | NC | Tobacco | UW203 | 1969 | 1 | II | + | - | - |
| 363 | NC | Tobacco | UW209 | 1969 | 1 | II | + | - | - |
| 368 | FL | Tomato | | NA[g] | 1 | II | + | - | + |
| 369 | FL | Tomato | | NA[g] | 1 | II | + | - | + |
| 370 | NC | Tobacco | | NA[g] | 1 | II | + | - | - |
| 373 | GA | Tomato | | NA[g] | 1 | II | - | - | - |
| 374 | GA | Tomato | 286 | NA[g] | 1 | II | - | - | - |
| 375 | FL | Tomato | | NA[g] | 1 | II | - | - | - |
| 401 | GA | Tomato | Rso81-5 | 1981 | 1 | II | + | - | - |
| 402 | GA | Tobacco | Rso96-41 | 1996 | 1 | II | + | + | - |

Table 3.1 Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 403 | GA | Tomato | Rso81-2 | 1981 | 1 | 2 | + | - | - |
| 404 | GA | Tomato | Rso86-2 | 1986 | 1 | 2 | + | - | - |
| 405 | GA | Coffee Weed | Rso80-1 | 1980 | 1 | 2 | + | + | - |
| 406 | GA | Tomato | Rso87-105 | 1987 | 1 | 2 | + | - | - |
| 407 | GA | Potato | Rso84-1 | 1984 | 1 | 2 | + | + | - |
| 501 | NC | Tobacco | K60 | NA[g] | 1 | 2 | + | - | - |
| 502 | NC | Tobacco | NC116 | NA[g] | 1 | 2 | + | - | - |
| 503 | GA | Tobacco | GA122 | NA[g] | 1 | 2 | + | - | - |
| 504 | GA | Tobacco | GA142 | NA[g] | 1 | 2 | + | - | - |
| 505 | SC | Tobacco | SC06 | NA[g] | 1 | 2 | + | - | - |
| 506 | SC | Tobacco | SC121 | NA[g] | 1 | 2 | + | - | - |
| 507 | SC | Tobacco | SC108 | NA[g] | 1 | 2 | + | - | - |
| 508 | NC | Tobacco | L-17 | NA[g] | 1 | 2 | + | - | - |
| 509 | NC | Tobacco | L-18 | NA[g] | 1 | 2 | + | - | - |
| 510 | NC | Tobacco | L-19 | NA[g] | 1 | 2 | + | - | - |
| 511 | NC | Tobacco | J-12a | NA[g] | 1 | 2 | + | - | + |
| 512 | NC | Tobacco | T-24 | NA[g] | 1 | 2 | + | - | + |
| 513 | NC | Tobacco | T-30 | NA[g] | 1 | 2 | + | - | - |
| 514 | NC | Tobacco | T-32 | NA[g] | 1 | 2 | + | - | - |
| 515 | NC | Tobacco | T-33 | NA[g] | 1 | 2 | + | - | - |
| 516 | NC | Tobacco | V-167a | NA[g] | 1 | 2 | + | - | - |
| 517 | NC | Tobacco | V-167b | NA[g] | 1 | 2 | + | - | - |
| 518 | NC | Tobacco | V-168 | NA[g] | 1 | 2 | + | - | - |
| 519 | NC | Tobacco | V-170 | NA[g] | 1 | 2 | - | - | - |
| 520 | NC | Tobacco | 40a | NA[g] | 1 | 2 | + | - | - |
| 521 | NC | Tobacco | K66 | NA[g] | 1 | 2 | + | - | - |
| 522 | NC | Tobacco | K68 | NA[g] | 1 | 2 | + | - | - |
| 523 | NC | Tobacco | K69 | NA[g] | 1 | 2 | + | - | - |
| 524 | NC | Tobacco | K71 | NA[g] | 1 | 2 | + | - | - |
| 525 | NC | Tomato | K74 | NA[g] | 1 | 2 | + | - | - |
| 526 | NC | Tomato | K136 | NA[g] | 1 | 2 | + | - | - |
| 527 | HI | Helicona | A3908 | NA[g] | 1 | 2 | - | - | - |

Table 3.1 Continued

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| UW2 | NA[g] | NA[g] | NA[g] | 1 | 2 | NA | NA | NA |
| UW70 | Colombia | Plantain | NA[g] | 1 | 2 | NA | NA | NA |
| UW170 | Colombia | Heliconia | NA[g] | 1 | 2 | NA | NA | NA |
| AW1 | AL | Tomato | NA[g] | 1 | 2 | + | - | + |

[a] Strains in the 100 series were part of Jason Hong's collection, 200 series came from Dave Norman's collection, 300 from Tim Momol 400 from Ron Gitaitis' , 500 from Dan Kluepfel and Asimina Mila's except for 527 which is part of Anne Alvarez' collection, UW from Caitlyn Allen's collection, AW from Mark Schell's collection.

[b] Biovar was determined as outlined by Hayward, 1991.

[c] Phylotype was determined by multiplex PCR and confirmed by phylogenetic analysis of the *egl* as outlined by Fegan and Prior, 2005.

[d] Two week old plants were root inoculated with a bacterial suspension at $10^8$ cfu/ml, three plants per trial each trial repeated twice. Only when all 3 plants for both trials were considered positive. Plants were stored in growth room conditions at 28ºC.

[e] Only fully expanded leaves were inoculated with a bacterial suspension at $10^8$ cfu/ml. The HR test was repeated 3 times. Plants were stored in growth room conditions at 28ºC.

[f] Strains lost pathogenicity and appear dry and circular on agar.

[g] NA=information was not available.

Table 3.2 List of strains obtained from GenBank[a] used for phylogenetic analysis

| Strain[b] | Origin[b] | Host[b] | Other No | Biovar[b] | Phylotype[b] | GenBank acc. no. |
|---|---|---|---|---|---|---|
| A3909 | USA | Heliconia | | 1 | II/6 | EF371812.1 |
| ACH0732 | Australia | Tomato | UW433 | 2 | IV/7 | GQ907150.1 |
| ANT307 | FWI | Anthurium | CFBP6784 | 1 | II/4 | DQ657648.1 |
| Aoyu | Australia | Potato | | 2 | II/1 | FJ561083.1 |
| B1 | China | Sweet potato | | 4 | I/15 | FJ561159.1 |
| Blooddisease R233 | ND | ND | | | IV | DQ011542.1 |
| CFBP734 | Madagascar | Potato | JS767 | | III/19 to 23 | AF295274.1 |
| CFBP765 | Japan | Tomato | JS771 | 4 | I/ND | EF371810.1 |
| CFBP1183 | Costa Rica | Heliconia | JS793 | 1 | II/3 | EF371805.1 |
| CFBP1409 | Honduras | *Musa* sp. | K135, JS77 | 1 | II/3 | EF371808.1 |
| CFBP2047 | USA | Tomato | | 1 | II/7 | AF295262.1 |
| CFBP2957 | FWI | Tomato | MT5 | 1 | II/5 | EF371807.1 |
| CFBP2958 | FWI | Tomato | GT4 | 1 | II/5 | AF295266.1 |
| CFBP2968 | Guadeloupe | Eggplant | RUN58 | | I/13 to 18 | EF371806.1 |
| CFBP2972 | Martinique | Tomato | RUN27 | 1 | II/5 | EF371809.1 |
| CFBP3059 | Burkina Faso | Eggplant | JCG.AU28 | 1 | III/23 | AF295270.1 |
| CFBP3858 | Netherlands | Potato | JS907 | 1 | II/1 | AF295259.1 |
| CFBP6786 | Martinique | Tomato | SPV98-1537 | | II/4 | EF371823.1 |
| CIP301 | Peru | Potato | R311 | 1 | II/5 | GU295003.1 |
| CIP309 | Colombia | Potato | UW80, S206 | 2 | II/2 | EF647735.1 |
| CIP418 | Indonesia | Peanut | MOH6 | 1 | II/3 | GU295005.1 |
| Col8 | Colombia | *Desmodium* | | | II/4PB | EU795341.1 |
| Col41 | Colombia | *Musa* sp. | | | II/4PB | EU795348.1 |
| DAR64836 | Australia | *Musa* sp. | | 1 | II/6 | DQ011551.1 |
| E2 | China | Eggplant | | 4 | I/15 | FJ561157.1 |
| E69 | China | Eggplant | | 3 | I/34 | FJ561092.1 |
| EU2 | China | Eucalyptus | | 3 | I/44 | FJ561152.1 |
| GMI1000 | F-Guyana | Tomato | JS771, RUN54 | 3 | I/12 or 18 | EF192968.1 |
| ICMP6782 | Brazil | *Musa* sp. | | | II/6 | DQ011553.1 |
| ICMP7963 | Kenya | Potato | RUN55 | | II/7 | AF295263.1 |
| ICMP9600 | Brazil | *Musa* sp. | | | II/6 | DQ011554.1 |
| IPO1609 | Netherlands | Potato | RUN1 | | II/1 and 2 | EF371814.1 |
| ISBSF1900 | Brazil | *Musa* sp. | RUN301 | | II/6 | EF371839.1 |
| J25 | Kenya | Potato | | N2 | III/22 | AF295279.1 |
| JT516 | Reunion Is. | Potato | RUN160 | | II/1 and 2 | AF295258.1 |
| JT523 | Reunion Is. | Potato | RUN333 | | I/13 to 18 | AF295252.1 |

Table 3.2 Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| JT525 | Reunion Is. | *Pelargonium asperum* | RUN60 | | III/19 to 23 | DQ657650.1 |
| JT528 | Reunion Is. | Potato | | 1 | III/19 | AF295273.1 |
| K60 | USA | Tomato | UW25 | 1 | II/7 | DQ657614.1 |
| M2 | China | Mulberry | | 5 | I/48 | FJ561067.1 |
| M3 | China | Mulberry | | 5 | I/44 | FJ561106.1 |
| M4 | China | Mulberry | | 5 | I/12 | FJ561107.1 |
| M6 | China | Mulberry | | 3 | I/48 | FJ561109.1 |
| M7 | China | Mulberry | | 5 | I/12 | FJ561110.1 |
| MAFF301558 | Japan | Potato | RUN71 | | IV/8 or 10 | DQ657634.1 |
| MOLK2 | Philippines | *Musa* sp. | | | II/3 | EF371841.1 |
| NCPPB332 | Zimbabwe | Potato | RUN75 | | III/19 to 23 | DQ657649.1 |
| NCPPB3190 | Malaysia | Tomato | RUN78 | | I/13 to 18 | AF295253.1 |
| NCPPB3987 | Brazil | Potato | RUN81 | | II/ND | AF295261.1 |
| O3 | China | Olive tree | | 3 | I/44 | FJ561069.1 |
| P11 | China | Peanut | | 3 | I/17 | FJ561068.1 |
| P16 | China | Peanut | | 3 | I/18 | FJ561114.1 |
| Pe1 | China | Pepper | | 3 | I/14 | FJ561154.1 |
| Pe5 | China | Pepper | | 3 | I/34 | FJ561091.1 |
| Po2 | China | Potato | | 2 | II/1 | FJ561158.1 |
| Po14 | China | Potato | | 2 | I/13 | FJ561162.1 |
| Po82 | Mexico | Potato | | 1 | II/4 | FJ561070.1 |
| Po152 | Mexico | Potato | | 3 | I/18 | FJ561148.1 |
| Po276 | Australia | Potato | | 2 | II/1 | FJ561082.1 |
| PSI7 | Indonesia | Tomato | RUN83 | 2 | IV/8 or 10 | EF371804.1 |
| PSS81 | Taiwan | Tomato | | 3 | I/14 | FJ561066.1 |
| PSS219 | Taiwan | Tomato | | 3 | I/34 | FJ561167.1 |
| PSS358 | Taiwan | Tomato | | 3 | I/15 | FJ561065.1 |
| R230 | Indonesia | Banana | | BDB | IV/10 | AF295280.1 |
| R288 | China | Mulberry | HT659 | 5 | I/18 | GQ907153.1 |
| R292 | China | Mulberry | RUN91 | | I/12 | DQ657635.1 |
| R058 | ND | ND | | ND | ND | DQ011543.1 |
| Tb3 | China | Tobacco | | 3 | I/17 | FJ561128.1 |
| Tm2 | China | Tomato | | 3 | I/14 | FJ561134.1 |
| Tm3 | China | Tomato | | 3 | I/18 | FJ561135.1 |
| Tm11 | China | Tomato | | 3 | I/13 | FJ561150.1 |
| Tm13 | China | Tomato | | 3 | I/17 | FJ561133.1 |
| Tm82 | China | Tomato | | 4 | I/16 | FJ561094.1 |
| UW9 | Costa Rica | Heliconia | JT644 | | II/3 | AF295257.1 |
| UW21 | Honduras | Banana | R371, CIP21 | 1 | II/6 | DQ011546.1 |
| UW129 | Peru | Plantain | | 1 | II/4 | EF371811.1 |
| UW160 | Peru | Plantain | R282 | 1 | II/4 | GU295051.1 |
| UW162 | Peru | *Musa* sp. | JT648 | | II/4 | AF295256.1 |
| UW163 | Peru | Plantain | | 1 | II/4 | GU295052.1 |

Table 3.2 Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| UW167 | Costa Rica | Banana | R283, CIP125 | 1 | II/3 | DQ011545.1 |
| UW175 | Colombia | Plantain | | 1 | II/4 | DQ011547.1 |
| UW181 | Venezuela | Plantain | JT649, K261 | 1 | II/6 | GU295053.1 |
| UW477 | Peru | Potato | RUN110 | | II/ND | DQ657604.1 |
| UW551 | Kenya | Geranium | | 3 | II/1 | DQ657596.1 |
| Z7 | China | Ginger | | 4 | II/16 | FJ561142.1 |
| Zo4 | Philippines | Ginger | | 4 | I/14 | FJ561156.1 |

[a] http://www.ncbi.nlm.nih.gov/genbank/

[b] The phylotype, sequevar, biovar, host, and origin information for the were obtained from previously published literature ( Poussier et al., 2000; Fegan and Prior, 2005; Fegan and Prior, 2006; Castillo and Greenberg, 2007; Wicker et al., 2007; Cardozo et al., 2009; Xu et al., 2009;, or if unknown= ND.

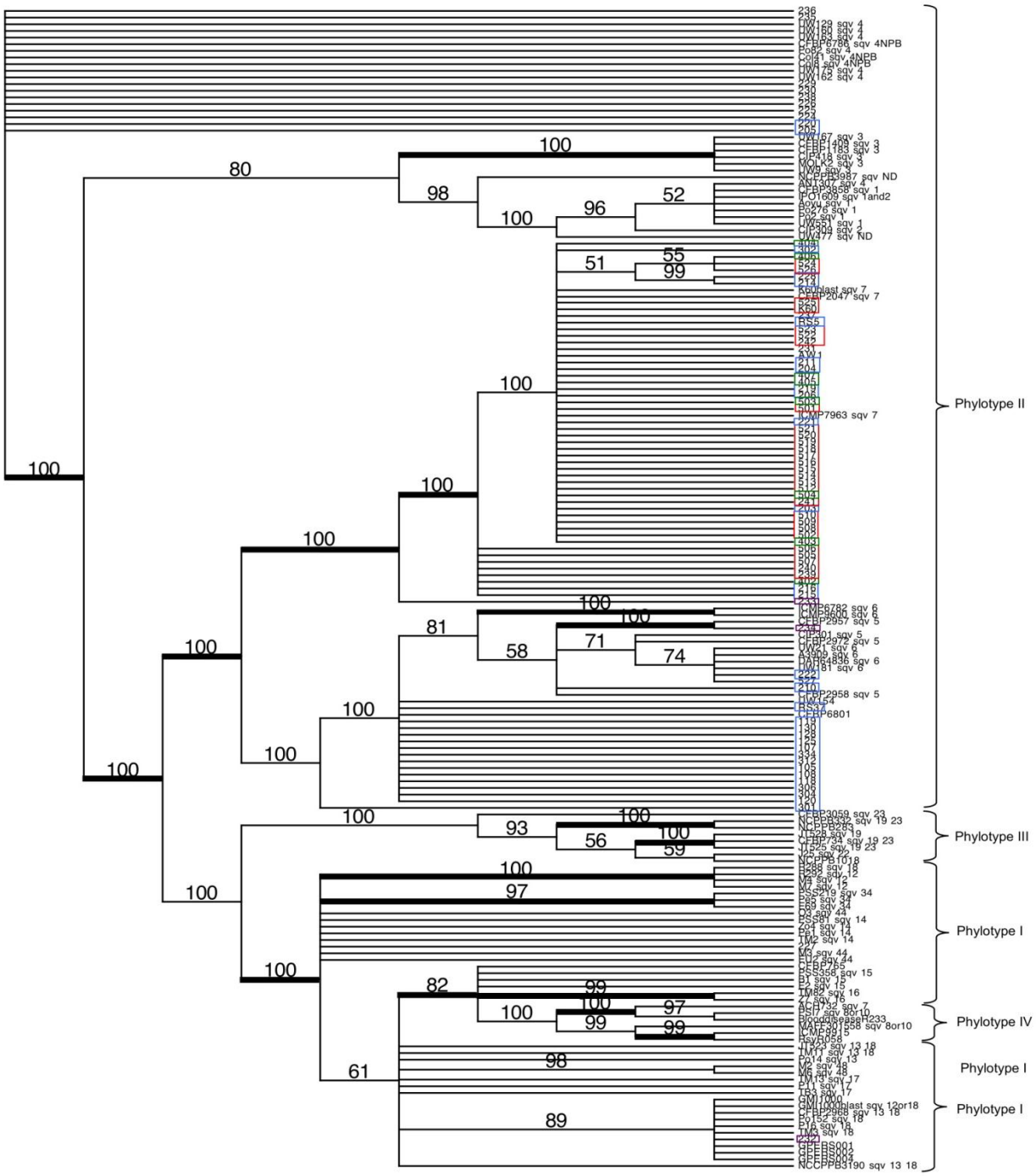


Figure 3.1 A 50% majority rule consensus rooted tree created by Bayesian analysis of the *egl*. Weighted line indicates posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black.

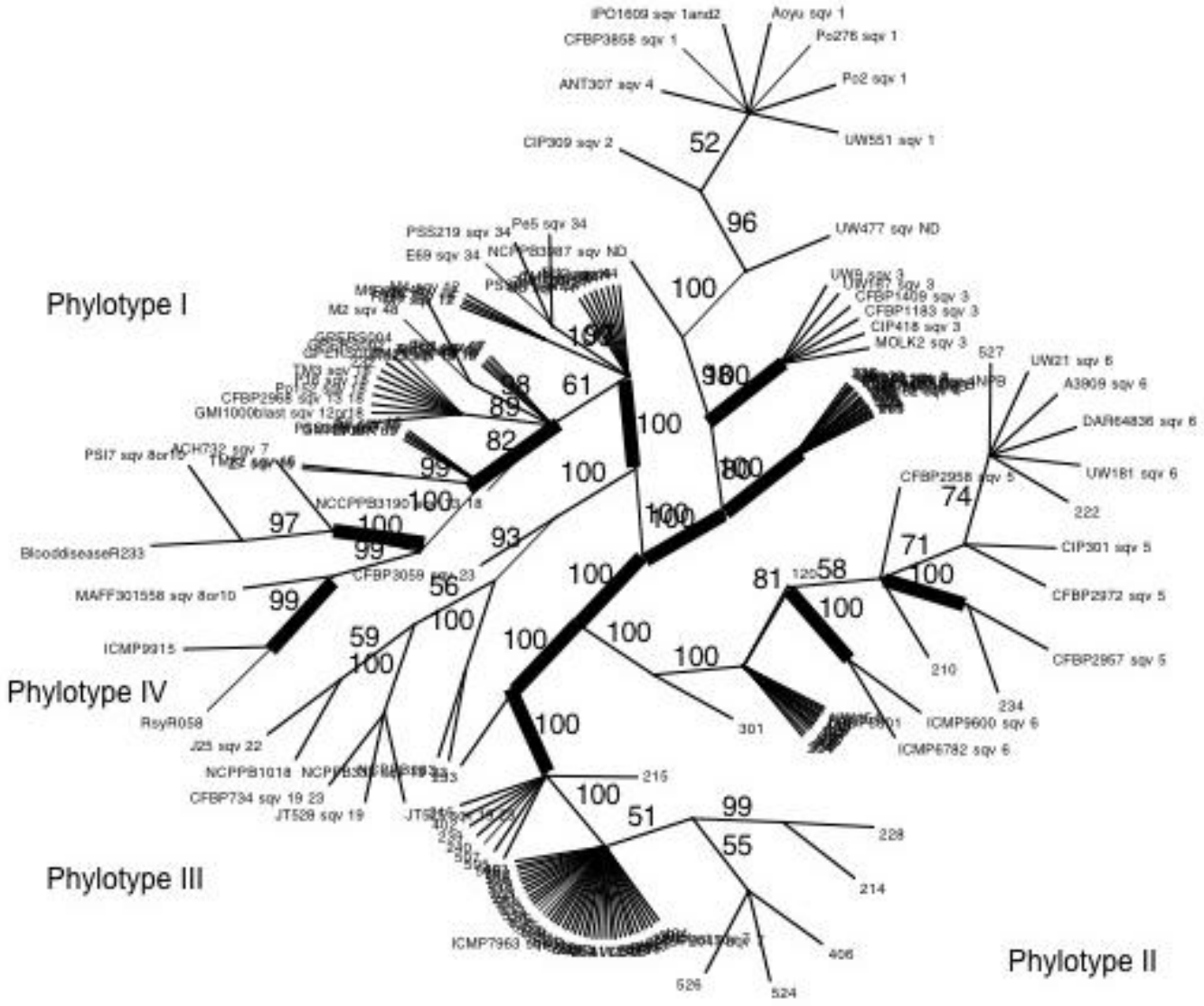


Figure 3.2 A 50% majority rule consensus unrooted tree created by Bayesian analysis of the *egl*. Weighted line indicates posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood.


Figure 3.3 The zone of inhibition, indicated by the red circle, produced by *Ralstonia solanacearum* strains RS 37 and RS 5. The lawn strain was sprayed 24hr after the center strain was streaked on King's B agar. Photos were taken 48hr after the lawn strain was sprayed.

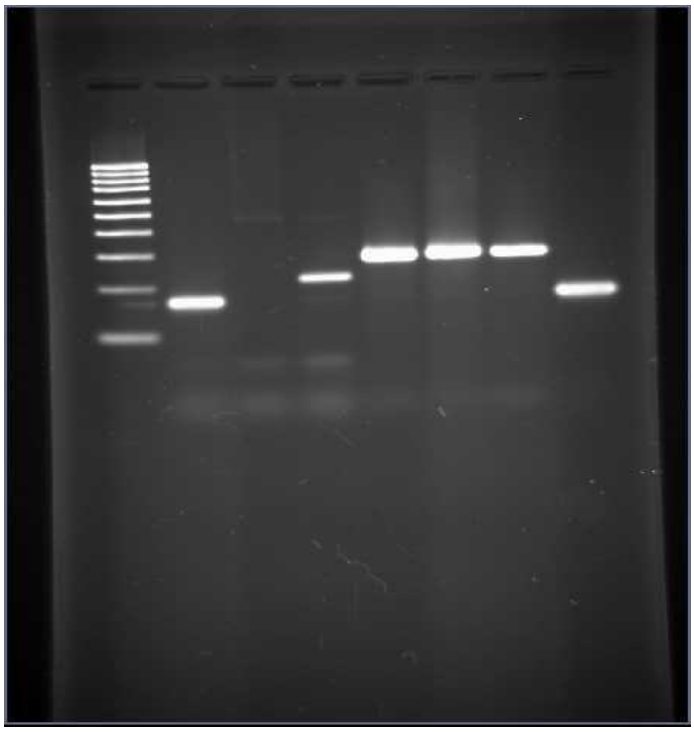

Figure 3.4 Gel displaying bands created by using primers for multiplex Musa, lanes 2-4, 759/760 *Ralstonia* specific primers, lanes 5-7, and one primer set from the Musa multiplex PCR Musa06, lane 8. Lane 1 contains Ladder 10 100 bp markers. Strain RS37 was used in lanes 2, 5 and 8, RS5 in lanes 3 and 6, and 527 in lanes 4 and 7.

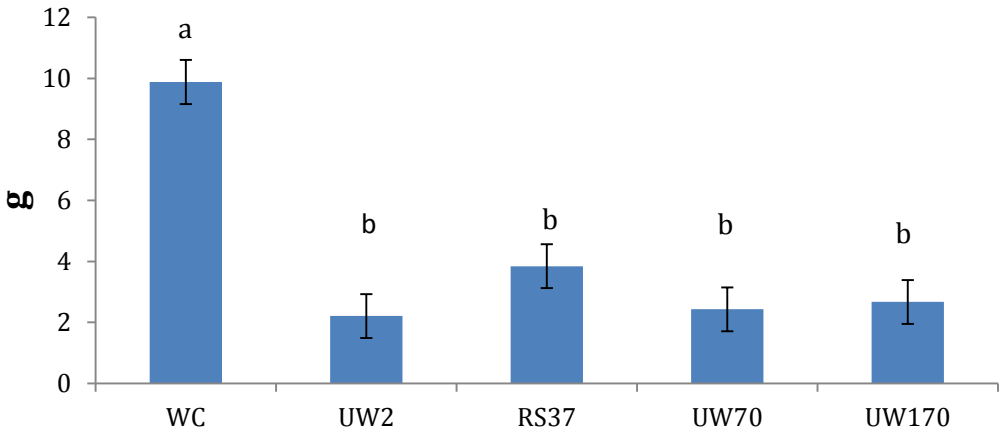


Figure 3.5 Comparison of dried *Musa* 'Dwarf Nam wa' root mass 30 days post inoculation of water control (WC), RS37 and Race 2 strains; UW2, UW70, and UW170. Same letter over each bar indicates no significant difference according to Tukey's range test.

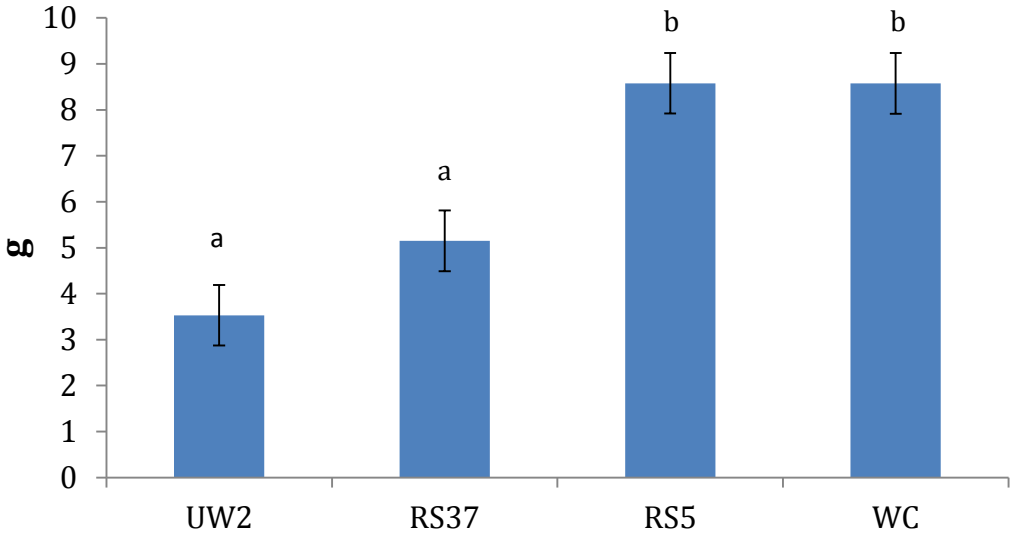


Figure 3.6 Comparison of *Musa* 'Dwarf Nam wa' root mass 30 days post inoculating plants with RS 5 (Race 1), UW2 (Race 2), and RS 37. Same letter over each bar indicates no significant difference according to Tukey's range test.

# CHAPTER 4
# MULTIPLE LOCI VARIABLE NUMBER TANDEM REPEAT ANALYSIS AMONG *RALSTONIA SOLANACEARUM* STRAINS FROM THE SOUTHEASTERN UNITED STATES

## Introduction

Bacterial taxonomy has been an arduous task. Aspects of the current taxonomy for all life can be traced to Aristotle. Carl Linnaeus in 1753 published *Systema Naturae*, in which he described the binomial system of nomenclature used today. Over a century later Ferdinand Cohn, with the aid of the compound microscope, published the first bacterial classification system (Palleroni, 2003). Bacteria were classified based on phenotypic characteristics, which included morphology and their biochemical and physiological characteristics. This was the standard until the late 1960's when DNA-DNA hybridization first was used to estimate relatedness among animals (Hoyer et al., 1964) and then among bacteria (Johnson and Ordal, 1968). This permitted bacterial classification to be based loosely on genotype, which provided some clarity over the relative confusion inherent in phenotypic classification. Soon thereafter, bacterial diversity was studied using conserved regions of the bacterial genome such as rRNA (Pace and Campbell, 1971), which led to the pervasive sequencing of the 16s rRNA to infer phylogenetic relationships (Woese, 1987). The use of 16s rRNA brought additional order to bacterial classification. Bacterial nomenclature, like other biological systems, consists of a Latin binomial (genus and species). Bacterial species are defined by members with 70% or greater similarity DNA-DNA hybridization and sharing phenotypic characteristics (Konstantinidis et al., 2006; Staley, 2006). Phenotypic characteristics presently used in defining a bacterial species include: biochemical reactions, chemical composition, cellular structures, and immunological features (Tortora et al., 1995).

“Identifying a species and determining its limits presents the most challenging aspect of biological classification for any type of organism” (Holt et al., 1994).

Currently, one of the main approaches to show intraspecies relatedness among eukaryotes, bacteria, or archaea is genetic typing (Bickford et al. 2007, Jacobs et al. 2004, Konstantinos et al, 2006, Li et al, 2009). Complete genome sequencing might be ideal for intraspecies comparisons, but the process can be costly and time consuming, especially if surveying many individuals. Researchers searched for a few molecular markers that would be cost effective and discriminative, but simple and reproducible. Multilocus sequence typing (MLST) consists of sampling a panel of multiple housekeeping genes and analyzing them phylogenetically. Multilocus phylogenetic analyses have been shown to increase phylogenetic accuracy (Maiden, et al., 1998) and have become the “gold standard” used to assess the variation of bacterial genomes (Hommais et al., 2005). Although useful in determining possible ancient relationships questions in bacteria, MLSTs are often unable to differentiate closely related bacterial strains (Li et al. 2009).

Simple sequence repeats (SSRs), also called microsatellites, have been used in multi-cellular organisms to differentiate among populations of the same species. These repeating units of DNA have been used as molecular markers for various applications, including analysis of kinship and population structure. Previously, it was shown that the use of SSRs in unicellular organisms would be inconsistent due to the high mutation rate and horizontal gene transfer in *Escherichia coli* (Metzgar et al., 2001). Metzgar et al. (2001) showed inconsistency when comparing phylogenetic trees constructed with 25 long microsatellites to trees created with multilocus enzyme electrophoresis.

However, Diamant et al. (2004) showed that combining four SSRs for phylogenetic analysis created a tree that was in agreement with those constructed with the standard multilocus enzyme electrophoresis. They also noted that the flanking regions of the microsatellite were just as informative as the microsatellite itself. Over the years multi-locus SSR analysis has been increasingly recognized as the method of choice for genotyping a number of human pathogens including, *Bacillus anthracis* (Keim et al., 1999), *Borrelia* species (Farlow et al., 2002), *Salmonella enterica* (Lindstedt, 2005), *Enterococus faecium* (Titze-deAlmeida et al., 2004), *E. faecalis* (Top et al., 2004), *E. coli* (Keys et al., 2005 and van Belkum et al., 1998), *Staphylococcus aureus* (Francois et al., 2005), *Vibrio cholera* (Danin- Poleg et al., 2007) and *V. vulnificus* (Broza et al., 2007). Smith et al. (2007) showed the clades in the phylogeny of *Corynespora cassiicola* created by the analysis of SSRs correlated with the geographic region from which the pathogen was isolated.

The taxonomic history of the bacterium known to cause bacterial wilt followed the same pattern of classification as previously described. It was first assigned to a taxonomic group based on phenotype and then later based on genotype. At first, phenotypic characters were used to place the bacterium in the genus *Bacillus* because it was rod-shaped. Further investigation revealed that the bacterium contained polar flagella indicating that the bacterium was motile; therefore it was placed in the genus *Pseudomonas*. It was given the specific epithet "*P. solanacearum*" to highlight its association with solanaceous plants (Kelman, 1953). The bacterium was classified further to subspecies based on the differential ability of strains to produce acid from several disaccharides and sugar alcohols (Hayward, 1964), as well as by host range

(Buddenhagen et al., 1962). In the 1970's, when nucleic acid hybridization became the standard, pseudomonads were grouped as either fluorescent or non-fluorescent based on genotypic characteristics (Palleroni, 2003). The pseudomonads were officially separated into different genera with the genus *Burkholderia* created to contain *B. solanacearum* among other species (Yabuuchi et al., 1992). Based on phenotypic characteristics, phylogenetic analysis of 16s rDNA and rRNA-DNA hybridization, the pathogen was transferred to a new genus, *Ralstonia,* where it resides today (Yabuuchi et al., 1995). However, DNA-DNA hybridization revealed that *R. solanacearum* strains often have less than 70% homology, which is the threshold level expected within a bacterial species (Palleroni and Doudoroff, 1971, Fegan and Prior, 2005). Restriction fragment length polymorphism (RFLP) analysis of 62 strains determined that 28 unique groups exist within *R. solanacearum*, and a similarity coefficient matrix showed that these groups formed two distinct major groups (Cook, 1989). Other DNA profiling methods used to group strains to the subspecies level included pulse-field gel electrophoresis (Jaunet and Wang, 1999), amplified fragment length polymorphsim (AFLP) (Poussier et al., 2000), random amplification of polymorphic DNA (RAPD) (Jaunet and Wang, 1999), and PCR amplification of repetitive genetic elements, such as REP, ERIC, and BOX PCR (Thwaites et al., 2002, Norman et al., 2009).

Phylogenetic analysis of several genes has revealed that the bacterium has diverged into four different groups with distinct geographic origins. These four groups have been labeled phylotypes 1 through 4 (Table 1.1). The phylotype groups were confirmed using the following genes: 16s rRNA (Poussier et al., 2000), internal transcribed spacer region (ITS) (Fegan et al., 1989), *egl* (Fegan, 1989), *hrpB* (Poussier

et al., 2000), *mutS* (Guidot et al., 2007, Wicker et al., 2007), and cytochrome *b561* (Norman et al., 2009). Phylogenetic analysis of *egl* has been used to determine infrasubspecific groups, referred to as sequevars, which are similar in pathogenicity or originate from a common geographic region (Fegan and Prior, 2005). However, sequevar clustering differs depending on the gene used when tested with worldwide representatives (Villa et al., 2005). A panel of five housekeeping genes and three virulence-related genes were used in an MLST analysis of a worldwide sample of 58 *R. solanacearum* strains, which confirmed the existence of four phylotypes and further subdivision in phylotype II (Castillo and Greenberg, 2006).

Genomic comparison of six different strains representing the four phylotypes and the two divisions within phylotype II, confirmed the diversity that exists amid the *Ralstonia* complex (Remenant et al., 2010). *R. solanacearum* could be reclassified into at least three distinct taxonomic groups based on genomic differences that exist between phylotypes. Grouping based on average nucleotide identity values (ANI) revealed that phylotypes I and III should be considered one species and phylotypes II and IV should each be a distinct species. Topology of the phylogenetic tree computed from ANI values was consistent with trees previously computed with comparative genomic hybridization (CGH) microarray data and the *mutS* and *hrpB* sequences.

To develop markers that could discriminate among closely related strains, microsatellites were identified in the *R. solanacearum* strain GMI1000, and PCR primers were developed that encompassed not only the SSR, but also the potentially variable flanking regions on either side of the SSR. The DNA sequences were phylogenetically analyzed using parsimony, maximum likelihood and Bayesian analysis. These trees

were compared to each other using SSRs alone, SSRs with the flanking regions also called microsatellite associated loci (MAL) and combining microsatellite associated loci (MALs.) These trees were then compared to trees created by *egl* and to previously created trees in other studies. The goal of this work was to determine whether the trees created by SSRs were similar to the *egl* trees, and whether these markers would be informative when comparing strains isolated in the southeastern U.S.

## Materials and Methods

### Bacterial Cultures

Strains were collected from researchers across the southeastern U.S. (Table 1). Dave Norman's collection (which was given ID numbers in the 200s) included strains isolated from Florida and the Caribbean. Strains from Tim Momol's (IDs in the 300s) strains originated from north Florida. Strains from Ron Gitaitis' collection (IDs in the 400s) were isolated in Georgia, and the 500 series of strains included those isolated from the Carolinas, which were from the collections of Dan Kluepfel and Asimina Mila. Strain AW1, isolated in Alabama, came from Mark Schell's collection, whereas Caitlyn Allen provided strains UW2, UW70, and UW170, and Anne Alvarez provided strain A3908. To confirm that the strains were indeed *R. solanacearum,* they were streaked on semi-selective-SMSA (Englebrecht, 1994), then PCR-amplified using the 759/760 *Ralstonia*-specific primers (Opina et al., 1997).

### Genomic DNA Extraction and Multiplex PCR

Genomic DNA was extracted and collected following methods outlined by Sambrook *et al*. (1989). To confirm the presence of DNA, each DNA extract was tested by PCR using the 759/760 primers. The phylotype of all strains was identified using multiplex PCR as outlined by Fegan & Prior (2005). The PCR products were visualized

on a gel, and phylotype was assigned according to band length. In each reaction RS5 phylotype II served as a positive control.

**Determining SSRs**

I used the two web-driven software packages, http://insilico.ehu.es/microsatellites (Bikandi et al., 2004) and http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php (Liolios et al., 2008), to find novel SSRs in the GMI1000 genome. A homology search was performed using NCBI-BLAST to determine if the SSRs were present in four additional genomes (or partial genomes) of *R. solanacearum* strains (i.e., UW551 race 3 biovar 2, IPO1609 race 3 biovar 2, and MolK race 2 biovar 3). Because previous studies showed that SSR flanking regions were phylogenetically informative (Diamant et al, 2004), I located not only the microsatellites themselves, but also flanking regions outside the SSR that totaled 800-1,200 base pairs. These amplified DNA sequences were aligned manually using Se-Al (Rambaut, 2002). Primers were designed based on the conserved regions of the available *R. solanacearum* strains. PCR amplification using these primers was first tested using genomic DNA from GMI1000. The SSRs were then tested on a panel of *R. solanacearum* strains; GMI1000 race 1 biovar 3, RS5 race1 biovar1, and A3908 race2.

**PCR, Sequencing and Phylogenetic Analysis**

Three SSRs (including their flanking regions) and the *egl* gene were amplified using PCR for each strain collected. The PCR reactions consisted of GoTaq® Flexi DNA Polymerase (Promega Corp., Madison, WI) with 5.0µl of 5x buffer, 1.5µl of $MgCl_2$, 4.0µl dNTP, each primer at 10pmol, 1 unit of Taq DNA polymerase, and 100ng of DNA. Sterile deionized water was used to adjust the total volume to 25µl. DNA cycle sequence amplification was conducted in a thermocycler (Bio-Rad Laboratories,

Hercules, CA) using the following program: hot start at 95°C for 5 min followed by 30 cycles of 95°C for 45 sec, 68°C for 30 sec and 72°C for 60 sec, and finished with a 10 min extension at 72°C. The annealing temperature was adjusted according to the composition of the oligonucleotide sequences.

For *egl* amplification the primer pair JHFegl: GACGATGCATGCCGCTGGTCGC and JHRegl: CACGAACACCACGTTGCTCGCATTGG were designed based on the *egl* sequences of GMI1000 (AL646053.1), UW551 (DQ657596.1), and Molk2 (CU694393.1) accessed from NCBI-BLAST. Sequevar was determined by phylogenetically analyzing the *egl* with the strains from this study with strains previously described (Table 3.2). The DNA sequences of the *egl* gene were used to select a panel of representative strains that underwent PCR and sequencing for the three SSRs and their flanking regions (Table 4.1). Closely related *R. syzygii* strains, blood disease bacterium R223 (DQ011538.1) and *R. syzygii* strain R058 (DQ011543.1) served as the outgroups for the *egl* tree. The SSRs and flanking regions were amplified using the primers in Table 4.2. *R. pickettii* was used as the outgroup for the analysis performed with the SSRs. GenBank accession numbers are listed in Table 4.3. PCR products were submitted to Interdisciplinary Center for Biotechnology Research (ICBR) sequencing facility located at the University of Florida's campus in Gainesville, FL for PCR cleaning and sequencing. Amplicons were sequenced in both the forward and reverse directions to create a consensus sequence that was used for phylogenetic analysis.

Phylogenetic analysis was performed for the *egl*, single SSRs, individual MALs, and the combination of 3 MALs. Sequences were aligned using Muscle (Edgar, 2004), via http://www.ebi.ac.uk/Tools/muscle/index.html. Se-Al version 2.0a11 was used for

manual sequence manipulation; the program was available at http://tree.bio.ed.ac.uk/software/seal/ (Rambaut, 1996). Phylogenetic analysis was performed using maximum parsimony, maximum likelihood and Bayesian methods. Maximum parsimony was performed using PAUP* version 4.0b10 (Sworfford, 2003) and performed on the Fisher Cluster at The University of Florida Genetics Institute Gainesville, FL. The parsimony dendrograms were created by performing tree bisection/reconnection (TBR) branch swapping and heuristic searches with random stepwise addition. Branch support for the parsimony trees was estimated by nonparametric bootstrapping (n=1,000 replicates) using TBR swapping (n=1,000 replicates; Felsenstein, 1985). RAxML-VI-HPC was used to run maximum likelihood and maximum likelihood bootstrap analysis (Stamatakis et al., 2005). RAxML-VI-HPC was also performed on the Fisher Cluster. For the bootstrap analysis, 5,000 runs were used to generate 5,000 trees. Treefig was used to create the consensus trees (Rambaut et al., 2006). MrBayes 3.1 was used to perform Bayesian analysis (Huelsenbeck et al., 2001) on the Fisher cluster. The likelihood settings in Bayesian analysis were adjusted for each data set to the model that was suggested by Akaike's information criterion (AIC). Modeltest 3.7 provided the AIC model selection strategy, and the best-fit model for each data set is found in Table 4.4 (Posada and Crandall, 1998). The model selected for *egl* was described in Chapter 3. The settings were adjusted for 1,000,000 generations being sampled at every 500 generations. The burn-in values for each analysis are listed in Table 4.4, and the consensus tree of the posterior probabilities was created by PAUP* 4.0b10.

## Results

### Determining the SSRs

*R. solanacearum* SSRs were determined by using the completely sequenced genome of GMI1000 and the algorithms found at http://insilico.ehu.es/microsatellites (Bikandi et al., 2004) and http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php (Liolios et al., 2008). The first website identified 13 SSRs and the second 63. The results from each website were compared to each other and based on length and 0% mismatch, 11 SSRs were selected for further development. The websites suggest primers for the 11 microsatellites, which were tested on GMI1000. Of the 11 primer sets only six produced PCR products. The SSRs that corresponded to the six primer pairs were compared to complete or partially sequenced genomes. More specific primers were designed based on homologous regions when comparing the genomes. Only four of the six SSRs consistently produced the correct PCR product when amplified from GMI1000, RS5 and A3908. When sequenced, one of the SSRs produced sequences that differed dramatically in the forward and reverse sequencing direction. This could represent the amplification of two radically different alleles or non-specific binding during PCR. Consequently, this SSR was discarded from further development. Three SSRs and their flanking regions, designated as SSR 1, SSR 3, and SSR 9,were used for phylogenetic analysis.

### Comparison of SSRs

All of the microsatellites consisted of repeats that were 6 nucleotides or longer in length and were located on the chromosome (Figures 4.1– 4.3). SSR 1 of GMI1000 consists of TTGCGA repeated 6 times and is part of a probable dioxygenase related to 2-nitropropane dioxygenase and probable hemagglutinin-related autotransporter

protein. The SSR 3 of GMI1000 consisted of GATCACCAG repeated three times and was part of a putative sugar transporter transmembrane protein. SSR 9 of GMI1000 was CAGCTGGAG repeated 3.5 times and was part of a probable formate dehydrogenase iron-sulfur subunit. SSR 1 of GMI1000 were not homologous to the SSR 1 amplified from UW551, IPO1609, and Molk2, and majority of the microsatillite is missing from these strains (Figure 4.1) SSR9 of GMI100 appears to be homologous to the SSR 9 amplified from UW551, IPO1609, and Molk2 (Figure 4.3). SSR 3 from GMI1000 was 100% identical to UW551 and IPO1609 (Figure 4.2). All of the SSRs of UW551 and IPO1609 were 100% identical to each other. Molk2 was identical to UW551 and IPO1609 for SSR 1 and 9, but appears to be missing the first 9 nucleotides in SSR 3. Comparing the flanking regions of the microsatellites, SSR 1 and 3 from the 4 strains appear to be homologous, while the flanking regions of SSR 9 are almost 100% identical. Using the primers that were designed from the homologous flanking regions, the SSRs of strains listed in Table 4.1 were amplified and sequenced. The flanking regions of each microsatellite were removed before analysis of the SSRs alone. Not all of the strains produced bands strong enough to be amplified and sequenced. SSR 9 contained the greatest sequence diversity followed by SSR 1 (Figures A.1-A.2). The SSR 3 was identical for all the strains except for Molk2 (not shown).

**Phylogenetic Analysis of the SSRs**

Maximum parsimony, maximum likelihood, and Bayesian methods were used to phylogenetically analyze the SSRs with and without their flanking regions (Figures 4.4-4.7, A.3 and A.4). The most informative microsatellites were SSR 1, which grouped most of the phylotype I strains separate from the phylotype II (Figures 4.4-4.5). SSR 9 was able to group most of the phylotype I strains together except for 227, 346, 364, and

367. However, SSR 3 was uninformative (Figures A.3 and A.4). Strain 232, isolated from tomato in Guadeloupe, was classified as phylotype II by multiplex PCR; however, it grouped with phylotype I strains in both SSR 1 and SSR 9 trees (with or without their flanking regions; Figures. 4.4-4.8 and 4.12). Included in this clade were other strains, which were isolated from tomato in Guadeloupe that were classified as phylotype I based on multiplex PCR and clustering on the *egl* trees. Phylogenetic analysis of the *egl* also placed strain 232 in a group with other phylotype I strains. Multiplex PCR was performed twice and each time this strain displayed bands that were consistent with those of phylotype II (not shown).

**Analysis of MALs**

The flanking regions of microsatellites from bacteria were reported to be as informative as the SSR by itself when phylogenetically analyzed (Diamant et al., 2004). Thus, the primers designed for sequencing the SSRs included up to 420bp on either side of the SSR. The MALs were analyzed in the same manner as the SSRs alone. Strains whose genomes were recently sequenced (i.e. CFBP2957, PSI07, and CMR15) were included with these analyses (Figures 4.8-4.13).

The MALs were considerably more informative than the SSRs alone, especially for SSR 3. For all three MALs, the phylotype I strains grouped together, and within phylotype II, the two sequevar 1 strains formed a clade. CMR15, phylotype III, was more closely related to phylotype I, than phylotype II or IV were to phylotype I for trees created by MALs 1 and 3 (Figures 4.9 and 4.11). Remenant et al. (2010) suggested that the close relationship of phylotype I and III based on DNA homology is sufficient to classifify them in a single, distinct genus; and that phylotype II and IV should each be placed in a distinct genus (2010).

Trees created using MAL 9 were less informative than those created with SSR 1 and 3 (Figures 4.12-4.13 and A.5-A.6). MAL 9 grouped the sequevar 1 strains in one clade and some of the phylotype II strains formed another clade. The lack of support for the tree could be due to the diversity of the microsatellite that makes up MAL 9. The product size of MAL 9 is smaller than the other MALs due to the lack of homology in the flanking regions. Comparing the trees created from the different methods, strains CFBP2957, phylotype IIA with CMR15, phylotype III, and PSI07, phylotype IV, grouped with some of the Carolina strains (Figures A.5-A.6). The maximum parsimony tree placed the UW551, whose sequence came from GenBank, with the other sequevar 1 strains. However the UW551 MAL 9 sequenced in the lab did not group with any group. Both of the UW551 sequences were identical (Figure A.6).

**Combination of the Three MALs**

The three SSRs and their flanking regions were combined and phylogenetically analyzed using the same methods as before. Combining all 3 MALs appears to give the trees greater resolution because the posterior probability and bootstrap values were greater than those from trees generated by single SSRs with or without their flanking regions (Figures 4.14-4.15). The phylotype I and sequevar 1 strains grouped together as they did previously with the *egl*, SSRs, and MALs. The phylotype III strain was more closely related to the phylotype I strains and phylotype IV was more closely related to phylotype II, as mentioned above. Phylotype II grouped into 4 distinct groups designated A-D (Figure 4.15). Group A contained strains isolated from the southeastern U.S. Group B contained the sequevar 1 and race 2 strains. Group C contained strains that originated from the Caribbean, including RS37, which was described in the

previous chapter. Lastly, group D contained strains isolated in Florida from pothos and anthurium cuttings that originated from Costa Rica (Norman et al., 2009).

The trees created using the 3 MALs were very similar to trees generated with the *egl* gene. The 4 groups (A-D) noted in the combined MALs tree were also found in the *egl* trees. Strains belonging to group A strains are the same as sequevar 7, group B strains are similar to sequevar 1, group C strains belong to sequevar 5/6 and an undefined sequevar, and group D were found within sequevar 4.

## Discussion

We have demonstrated that sequenced loci containing SSRs can be used to determine infrasubspecific groups. This is the first time that SSRs have been used to distinguish subspecies groups in plant pathogenic bacteria. The flanking regions of the SSRs were more informative than the SSRs by themselves. This study corroborates the importance of combining multiple SSRs for phylogenetic analysis (Diamant et al., 2004). In this study, the MALs phylogenetic trees showed greater resolution and higher confidence values than trees generated with the microsatellites alone. To reach consensus among phylogenetic hypotheses it is useful to employ multiple types of phylogenetic analysis. Here we used maximum parsimony, maximum likelihood, and Bayesian analysis. Similar to the trees created by *egl* and the other genes used for phylogenetic analysis, SSRs were able to group the strains based on geographic region. However, like the previously published trees, trees created by SSRs were unable to group strains based on host.

All strains that originated from southeastern U.S. were found in group A or sequevar 7. Two genotypes of *R. solanacearum* were identified in the southeastern U.S. that differed by a truncated non-functional avirulence gene and the wild type gene

(Robertson et al., 2004). The Carolina strains, which had the truncated gene, have a broader host range and can cause wilt in tobacco, whereas the Georgia and Florida strains cause a hypersensitive reaction (HR) in tobacco. Tobacco is grown more frequently in the Carolinas than Florida, thus these strains might be under different selection pressures. A geographic division among these strains was not detected using either the *egl* or the SSR trees. Even though the SSRs used in this study confirmed the clades created by *egl*, other microsatellites could be more informative for resolving geographic variation than the SSRs used in this study. These microsatellites were discovered using GMI1000 as a reference; hence, it is possible that microsatellites identified in another strain could exhibit more diversity within a sequevar. Microsatellites are believed to give organisms the ability to adapt rapidly to new environments (Li et al. 2004). Highly variable SSRs are correlated with the high frequency appearance of new alleles within species and populations (Weber and Wong, 1993). It is therefore possible that the SSRs would differ among populations; the Carolina strains, for example, experiencing selection pressures that differ from the Florida strains.

Using SSRs we demonstrated which strains were native to the U.S. and identified exotic strains that were isolated in the U.S. According to this study and the previous chapter, Florida appears to have multiple populations of exotic strains. Based on phylogenetic analysis some of these exotic strains appear to have originated in the Caribbean or Latin America, whereas others, those of phylotype I, likely came from Asia (Hong et al., 2008, Norman et al., 2009, Ji et al., 2007). Two predominant groups of exotic strains were isolated in Florida, group C/sequevar 5/6 and an undefined sequevar and group D/sequevar 4. Strains belonging to group C were isolated in the waterways,

greenhouses, and fields in northern Florida (Hong et al., 2008). Group D strains were isolated from pothos and anthurium cuttings originating from Costa Rica (Norman et al., 2009).

Microsatellites proved to be useful in separating populations within the *Ralstonia* species complex, and could be used to confirm sequevar status as well. The accepted model to determine sequevar is sequencing and analysis the *egl*. Yet, confirmation of sequevar has not been determined when comparing trees created by other genes. A few of these genes have shown promise in the preliminary stages, but upon using strains representing different regions of the world the sequevar grouping was inconsistent with the topology of the *egl* trees (Villa et al., 2005). Clades created by cytochrome *b561* or *mutS* were similar to those produced by *egl*, however a worldwide survey still needs to be performed to certify these genes' effectiveness (Norman et al., 2009, and Wicker et al, 2007). Careful consideration should be made when the phylogeny of an organism is based on a single gene. DeLong and Pace (2001) stated that "these extrapolations will vary in accuracy," when compared to other genes. Palleroni (2003) goes so far as to say, "… phylogeny based on 16s rDNA may reflect little more than the phylogeny of the 16s rDNA." In other words, phylogeny based on one gene traces the evolution of the gene and not the organism. Comparison of the complete genome would be the most accurate way to distinguish among closely related bacteria. However, due to time and costs, this method currently is not feasible for a large survey of related organisms. The analysis of microsatellites along with their flanking regions provides a survey of different areas of the chromosome (Table 4.2.), while being less expensive than complete genome sequencing.

The use of MALs could provide support for dividing *R. solanacearum* into three distinct species. If MALs are effective in determining phylotype and sequevar with strains originating worldwide, then a large survey could provide support for the division of the genus. Multiplex PCR could assign phylotype to multiple strains more quickly than phylogenetic analysis. However, in this study multiplex PCR assigned strain 232 to phylotype II, while it was classified as phylotype I based on phylogenetic analysis of the *egl* and the SSRs. Using phylogenetic analysis strain 232 clustered other strains isolated from the same host from the same geographic region

The methods used in this study for phylogenetic analysis are the current recommended methods. Many previously published papers regarding the phylogeny of *R. solanacearum* used weaker methods of phylogenetic inference such as Neighbor Joining and UPGMA. Therefore, their results could be drawn into question. These weaker methods are often used because they produce a single tree and give the researchers quick trees for publication. These methods were shown to be less effective and sometimes positively misleading under certain realistic conditions (Felsenstein, 1978; Kuhner, and Felsenstein, 1994; Hillis, et al., 1994).. This could explain the inconsistencies found when comparing trees previously published for *R. solanacearum* (Taghavi et al., 1996, Poussier et al., 2000, Villa et al., 2005, and Li et al., 2009). Discrepancies between current and past phylogenetic reconstructions (e.g., their ability to tease apart fine-scale differences among populations) could be due to either insufficient data or incomplete taxon sampling. Unknown diversity or lack of diversity could be seen as a result of unintentionally biased sampling. One way to overcome this error is to include multiple taxa and to compare analyses from different parts of the

genome. Phylogenetic analysis using maximum parsimony, maximum likelihood and Bayesian methods are currently used to reconstruct consensus phylogenetic trees that are reproducible. In this study we found small inconsistencies with a tree created using the parsimony method when creating the trees for MAL 9 (A.6). However being able to compare the topology with the other 2 methods and relying on the confidence values, the placement of this strain was determined to be incorrect based on maximum parsimony (Figure 4.9).

This study showed that MALs could be used for phylogenetic analysis to demonstrate diversity within a bacterial species. Additional studies should be done to determine whether methods used in this study are applicable to other phytobacteria. Further studies are needed to determine if these markers can be used to show diversity in *R. solanacearum* strains originating from different parts of the world. This would indicate that MALs can determine the phylotype divisions and confirm sequevar groupings as created by *egl*. Plants resistant to bacterial wilt are geographically constrained. Hopefully with the use of MALs, local populations can be identified and aid in our understanding of how the bacterium is able to overcome resistance. Further research is also needed to determine the frequency with which these microsatellites mutate, and how consistent those mutations are over time.

In studies investigating the diversity of eukaryotes, both molecular and morphological data have been combined for phylogenetic analysis (Bybee et al., 2008; Goloboff et al., 2009; Ogden and Whiting, 2003). It could be possible to combine phenotypic characteristics along with molecular data to show a greater diversity in the infrasubspecific groups. Classical methods for grouping subdivisions of *R.*

*solanacearum*, such as HR on tobacco, pathogenicity tests, and utilization of carbohydrates could be used as characters when comparing strains. Simultaneous analysis of both molecular and phenotypic data could possibly reveal where or when divergence took place for host specialization. Currently, bacterial species are defined as "bacteria from coherent genomic clusters that are characterized by distinctive phenotypic properties" (Konstantinidis et al., 2006). Thus, it might be possible to combine these characteristics in a phylogenetic analysis to determine clear divisions between closely related bacterial populations.

Table 4.1 Strains sequenced and used in this study

| Strains[a] | Origin | Host Origin | Other Name | Year | Biovar[b] | Phylotype[c] | SSR sequenced[d] |
|---|---|---|---|---|---|---|---|
| 203 | FL | Tomato | P504 | NA[e] | 1 | 2 | 1 |
| 204 | FL | Tomato | P505 | NA[e] | 1 | 2 | 1 |
| 205 | FL | Tomato | P507 | 1996 | 1 | 2 | 1 |
| 206 | FL | Tomato | P530 | 1997 | 1 | 2 | 1 |
| 208 | FL | Tomato | P533 | 1997 | 1 | 2 | 1, 9 |
| 209 | FL | Tomato | P534 | 1997 | 1 | 2 | 1 |
| 210 | FL | Tomato | P535 | 1997 | 1 | 2 | 1 |
| 211 | FL | Tomato | P536 | 1997 | 1 | 2 | 1 |
| 212 | FL | Potato | P541 | 1997 | 1 | 2 | 1, 9 |
| 213 | FL | Potato | P543 | 1997 | 1 | 2 | 1, 9 |
| 214 | FL | Potato | P550 | 1997 | 1 | 2 | 1, 9 |
| 215 | FL | Potato | P553 | 1997 | 1 | 2 | 1, 9 |
| 216 | FL | NA[e] | P557 | 1997 | 1 | 2 | 1, 9 |
| 217 | FL | Pothos | P564 | 1996 | 1 | 2 | 1, 9 |
| 218 | FL | Pothos | P573 | 1999 | 1 | 2 | 1, 9 |
| 219 | FL | Tomato | P576 | 1999 | 1 | 2 | 1, 9 |
| 220 | FL | Tomato | P594 | 2000 | 1 | 2 | 1, 9 |
| 221 | FL | NA[e] | 136 | NA[e] | 1 | 2 | 3, 9 |
| 222 | FL | Heliconia | 158 | NA[e] | 1 | 2 | 1, 3, 9 |
| 223 | FL | Pothos | 485 | NA[e] | 1 | 2 | 9 |
| 224 | NA[e] | Pothos | NA | NA[e] | 1 | 2 | 1, 3, 9 |
| 225 | NA[e] | Pothos | 517 | NA[e] | 1 | 2 | 1, 3, 9 |
| 226 | NA[e] | Pothos | 521 | NA[e] | 1 | 2 | 1, 3, 9 |
| 227 | NA[e] | NA | 528 | NA[e] | 1 | 1 | 1, 3, 9 |
| 228 | FL | Potato | 544 | 1994 | 1 | 2 | 1, 3, 9 |
| 229 | NA[e] | Pothos | 545 | NA[e] | 1 | 2 | 1, 3, 9 |
| 230 | NA[e] | Pothos | 548 | NA[e] | 1 | 2 | 1, 3, 9 |
| 231 | NA[e] | Tomato | NA[e] | NA[e] | 1 | 2 | 1, 3 |

Table 4.1 Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 232 | Guadeloupe | Tomato | 506 | 1985 | 1 | 2 | 1, 9 |
| 234 | Martinique | Tomato | 610 | 1987 | 1 | 2 | 1, 3, 9 |
| 235 | NA[e] | Anthurium | 618 | NA[e] | 1 | 2 | 1, 3, 9 |
| 236 | NA[e] | Anthurium | 621 | NA[e] | 1 | 2 | 1, 3, 9 |
| 237 | USA | Tomato | 660 | NA[e] | 1 | 2 | 3, 9 |
| 238 | NA | Pothos | 673 | NA[e] | 1 | 2 | 1, 3, 9 |
| 239 | NC | NA[e] | 688 | NA[e] | 1 | 2 | 1, 3, 9 |
| 240 | NC | Potato | 689 | NA[e] | 1 | 2 | 1, 3, 9 |
| 241 | NC | Tomato | 690 | NA[e] | 1 | 2 | 1, 3, 9 |
| 242 | NC | Tomato | 691 | NA[e] | 1 | 2 | 1 |
| 301 | FL | Soil | RS14 | 2001 | 1 | 2 | 1 |
| 302 | FL | Tomato | RS18 | 1991 | 1 | 2 | 1 |
| 304 | FL | Geranium | RS38 | 2001 | 1 | 2 | 1 |
| 305 | FL | Geranium | RS39 | 2001 | 1 | 2 | 1 |
| 306 | FL | Geranium | RS40 | 2001 | 1 | 2 | 1 |
| 307 | FL | Pond Water | RS51 | 2001 | 1 | 2 | 1 |
| 329 | FL | Hydrangea | RS118 | 2005 | 1 | 2 | 9 |
| 330 | FL | Pepper | RS121 | 2005 | 3 | 1 | 3 |
| 331 | FL | Pepper | RS122 | 2005 | 3 | 1 | 9 |
| 346 | Peru | Tomato | TD406 | NA[e] | 3 | 1 | 9 |
| 364 | Costa Rica | Pepper | UW255 | 1972 | 3 | 1 | 9 |
| 366 | China | Peanut | UW368 | NA[e] | 1 | 1 | 9 |
| 367 | China | Pepper | UW371 | NA[e] | 3 | 1 | 9 |
| 401 | GA | Tomato | Rso81-5 | 1981 | 1 | 2 | 1, 3 |
| 402 | GA | Tobacco | Rso96-41 | 1996 | 1 | 2 | 1 |
| 403 | GA | Tomato | Rso81-2 | 1981 | 1 | 2 | 1 |
| 404 | GA | Tomato | Rso86-2 | 1986 | 1 | 2 | 1 |
| 405 | GA | Coffee Weed | Rso80-1 | 1980 | 1 | 2 | 1 |
| 406 | GA | Tomato | Rso87-105 | 1987 | 1 | 2 | 1 |
| 407 | GA | Potato | Rso84-1 | 1984 | 1 | 2 | 1 |

Table 4.1 Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 501 | NC | Tobacco | K60 | NA[e] | 1 | 2 | 1, 3, 9 |
| 502 | NC | Tobacco | NC116 | NA[e] | 1 | 2 | 1, 3, 9 |
| 503 | GA | Tobacco | GA122 | NA[e] | 1 | 2 | 1, 3, 9 |
| 504 | GA | Tobacco | GA142 | NA[e] | 1 | 2 | 1, 3, 9 |
| 505 | SC | Tobacco | SC06 | NA[e] | 1 | 2 | 1, 3, 9 |
| 506 | SC | Tobacco | SC121 | NA[e] | 1 | 2 | 1, 3, 9 |
| 507 | SC | Tobacco | SC108 | NA[e] | 1 | 2 | 1, 3, 9 |
| 508 | NC | Tobacco | L-17 | NA[e] | 1 | 2 | 1, 3, 9 |
| 509 | NC | Tobacco | L-18 | NA[e] | 1 | 2 | 1, 9 |
| 510 | NC | Tobacco | L-19 | NA[e] | 1 | 2 | 1, 9 |
| 511 | NC | Tobacco | J-12a | NA[e] | 1 | 2 | 9 |
| 512 | NC | Tobacco | T-24 | NA[e] | 1 | 2 | 1, 9 |
| 513 | NC | Tobacco | T-30 | NA[e] | 1 | 2 | 1, 3, 9 |
| 514 | NC | Tobacco | T-32 | NA[e] | 1 | 2 | 1, 3, 9 |
| 515 | NC | Tobacco | T-33 | NA[e] | 1 | 2 | 1, 3, 9 |
| 516 | NC | Tobacco | V-167a | NA[e] | 1 | 2 | 1, 3, 9 |
| 517 | NC | Tobacco | V-167b | NA[e] | 1 | 2 | 1, 3, 9 |
| 518 | NC | Tobacco | V-168 | NA[e] | 1 | 2 | 1, 3, 9 |
| 519 | NC | Tobacco | V-170 | NA[e] | 1 | 2 | 1, 3, 9 |
| 520 | NC | Tobacco | 40a | NA[e] | 1 | 2 | 1, 3, 9 |
| 521 | NC | Tobacco | K66 | NA[e] | 1 | 2 | 1, 3, 9 |
| 522 | NC | Tobacco | K68 | NA[e] | 1 | 2 | 1, 3, 9 |
| 523 | NC | Tobacco | K69 | NA[e] | 1 | 2 | 1, 3, 9 |
| 524 | NC | Tobacco | K71 | NA[e] | 1 | 2 | 1, 3, 9 |
| 525 | NC | Tomato | K74 | NA[e] | 1 | 2 | 1, 3, 9 |
| 526 | NC | Tomato | K136 | NA[e] | 1 | 2 | 1, 3, 9 |
| 527 | HI | Helicona | A3908 | NA[e] | 1 | 2 | 1, 3 |
| RS5 | FL | Tomato | NA[e] | 1999 | 1 | 2 | 1, 3, 9 |
| RS37 | FL | Geranium | NA[e] | 2001 | 1 | 2 | 1, 3, 9 |
| GPERS001 | Guadeloupe | Tomato | NA[e] | 2009 | 1 | I | 1, 9 |
| GPERS002 | Guadeloupe | Tomato | NA[e] | 2009 | 1 | I | 1, 9 |
| GPERS004 | Guadeloupe | Tomato | NA[e] | 2009 | 1 | I | 1, 9 |

Table 4.1 Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AW1 | AL | Tomato | NA[e] | NA[e] | 1 | II | 1, 9 |
| UW2 | NA[e] | NA[e] | NA[e] | NA[e] | 1 | II | 1 |
| UW70 | Colombia | Plantain | NA[e] | NA[e] | 1 | II | 1 |
| UW170 | Colombia | Heliconia | NA[e] | NA[e] | 1 | II | 1 |
| UW551 | Kenya | Geranium | NA[e] | 2003 | 3 | II | 1, 3, 9 |
| GMI1000 | French Guyana | Tomato | JS771, RUN54 | NA[e] | 3 | I | 1, 3, 9 |

[a] Strains in the series 200 series came from Dave Norman's collection, 300 from Tim Momol, including RS5 and RS37, 400 from Ron Gitaitis' , 500 from Dan Kluepfel and Asimina Mila's except for 527 which is part of Anne Alvarez' collection, UW from Caitlyn Allen's collection, AW from Mark Schell's collection, and GPERS were isolated by Patrice Champoiseau.
[b] Biovar was determined as outlined by Hayward, 1991.
[c] Phylotype was determined by multiplex PCR and confirmed by phylogenetic analysis of the *egl* as outlined by Fegan and Prior, 2005.
[d] Three different simple sequence repeats (SSRs) were identified using GMI1000 and http://insilico.ehu.es/microsatellites and http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php. All of the SSRs were sequenced.
[e] NA=information was not available.

Table 4.2 List of strains obtained from GenBank[a] used for phylogenetic study[b]

| Strain | Origin | Host | Other No | Biovar | Phylotype | GenBank acc. no. | Starting nucleotide on the chromosome[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | SSR1 | SSR3 | SSR9 |
| GMI1000 | French Guyana | Tomato | JS771, RUN54 | 3 | I | AL646052.1 | 132,470 | 590,335 | 2,573,692 |
| UW551 | Kenya | Geranium | | 3 | IIB | GQ907150.1 | 97,625 | 89066 | 92,556 |
| IPO1609 | Netherlands | Potato | RUN1 | 3 | IIB | CU914168.1 | 197,514 | 637,913 | 894,776 |
| CFBP2957[d] | French West Indies | Tomato | | ND | IIA | FP885897.1 | 3,292,177 | 2,868,703 | 1,086,444 |
| CMR15 [d] | Cameroon | Tomato | | ND | III | FP885895.1 | 3,464,998 | 3,006,982 | 1,061,305 |
| PSI07 [d] | Indonesia | Tomato | | ND | IV | FP885906.2 | 3,397,158 | 2,936,982 | 1,105,420 |
| Molk2 | Philippines | *Musa* sp. | | 1 | IIB | CU861906.1 | 165,121 | 602,150 | 13,516 |
| *Ralstonia pickettii* 12J [d] | Michigan | Water | | ND | ND | CP001068.1 | 3,809,633 | 470,824 | 2,692,895 |

[a] http://www.ncbi.nlm.nih.gov/genbank/

[b] Phylotype, origin and date for the strains other than those characterized in this study are from the literature. ND = not determined

[c] Start and stop were determined when the simple sequence repeat (SSR) was compared and aligned to the given strain.

[d] These strains were added at the end of the study thus not included in the SSR by itself analysis.

Table 4.3 Simple sequence repeats (SSRs) primers designed from the conserved regions of GMI1000, UW551, Molk2, and IPO1690.

| SSR | Primer | Sequence (5' to 3') | Product Size |
|---|---|---|---|
| 1 | GMMS1F1 | GCCAAGGTCCGGCACTGGCTCGACAAGG | 832bp |
| | GMMS1R3 | TCCAGCGTCGATTCCGACACCGACA | |
| 3 | GMMS3F1 | GGTGGCGAATCAGGGACYCG | 815bp |
| | GMMS3R6 | CCTTGAACAGCGGGAAGTAGGTC | |
| 9 | GMMS9F | TCGTATCCATGGCTAGTACGC | 210bp |
| | GMMS9R | GATGTCATTGCCGACATCCT | |

Table 4.4 Parameters adjusted for maximum likelihood settings for Bayesian method for phylogenetic analysis and forming posterior probability trees of simple sequence repeats (SSRs).

| SSR | Model[a] | Burnin[b] |
|---|---|---|
| 1 | JC | 15,000 |
| 3 | GTR+I+G | 20,000 |
| 9 | TIM | 13,000 |
| SSR with flanking regions | | |
| 1 | GTR+I+G | 30,000 |
| 3 | GTR+I+G | 23,500 |
| 9 | TVM+I+G | 43,500 |
| 1+3+9 | GTR+I+G | 19,000 |

[a] Models were selected by Akaike's information criterion using Modeltest 3.7.
[b] Burnin was calculated by plotting the number of generation versus the –Lnl values and determining when the graph becomes stationary.

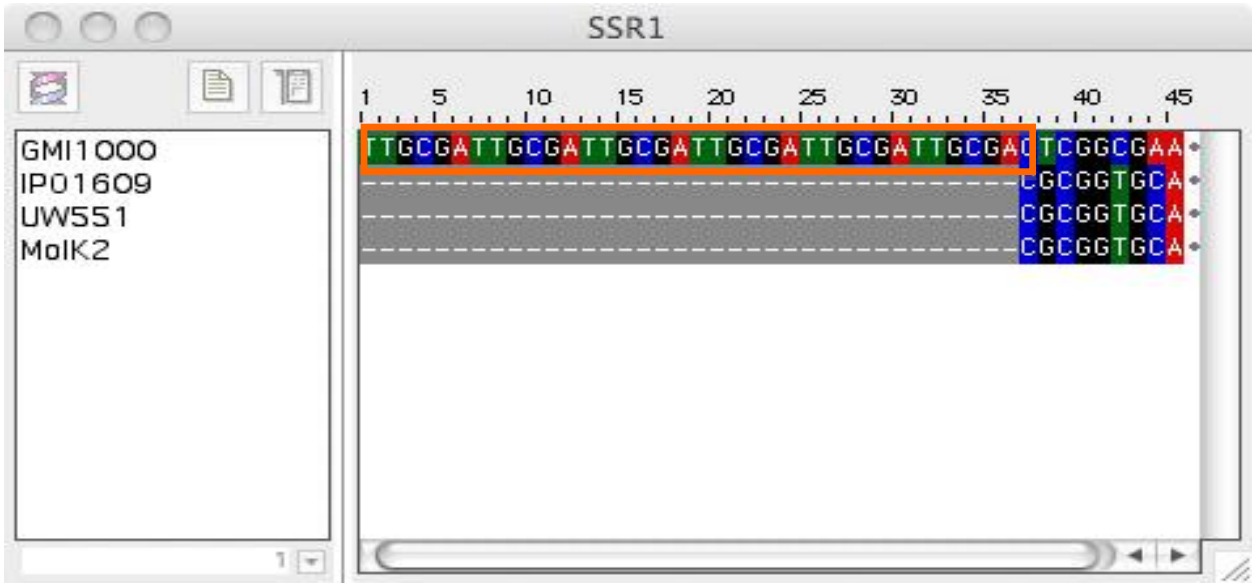


Figure 4.1 Simple sequence repeat 1 as found on *Ralstonia solanacearum* strain GMI1000. All *R. solanacearum* strains were obtained from NCBI-BLAST. The repeat was highlighted in orange by the author.

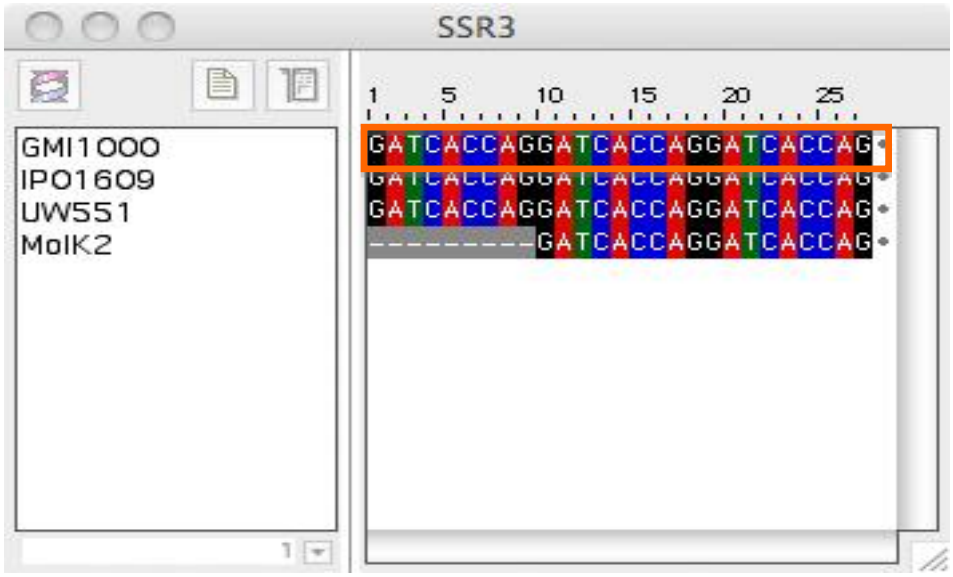


Figure 4.2 Simple sequence repeat 3 as found on *Ralstonia solanacearum* strain GMI1000. All *R. solanacearum* strains were obtained from NCBI-BLAST. The repeat was highlighted in orange by the author.

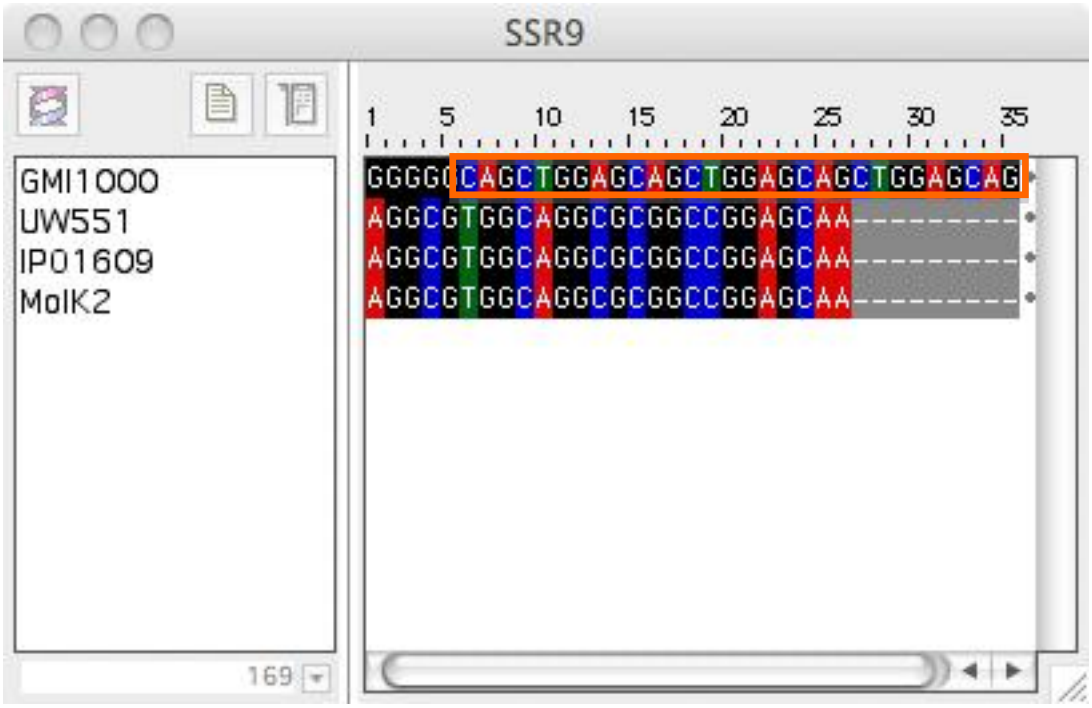


Figure 4.3 Simple sequence repeat 9 as found on *Ralstonia solanacearum* strain GMI1000. All *R. solanacearum* strains were obtained from NCBI-BLAST. The repeat was highlighted in orange by the author.

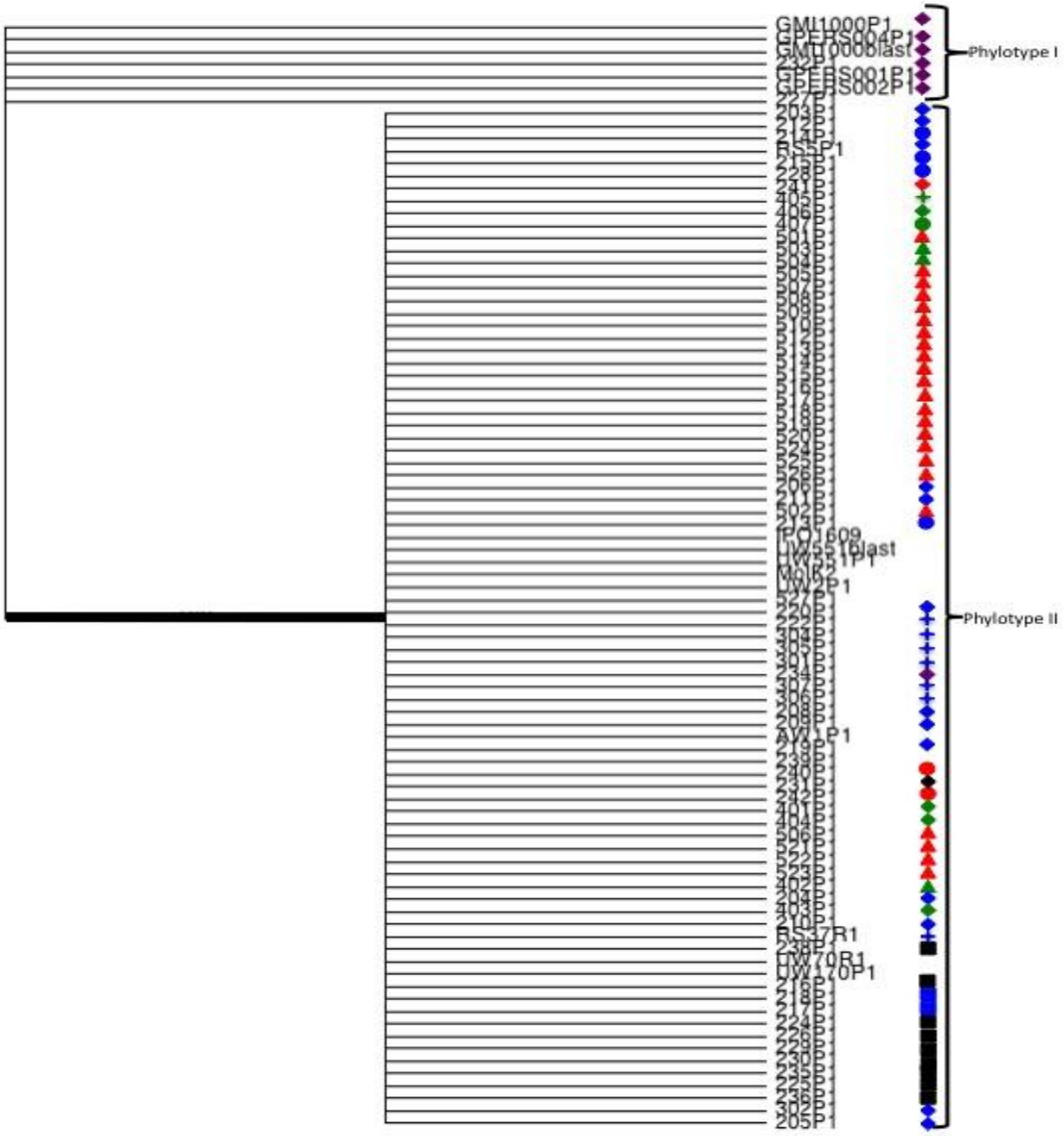


Figure 4.4 A majority rule consensus rooted tree created by Bayesian analysis of SSR 1. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word "blast" after strain's name indicates the sequence was acquired from GenBank.

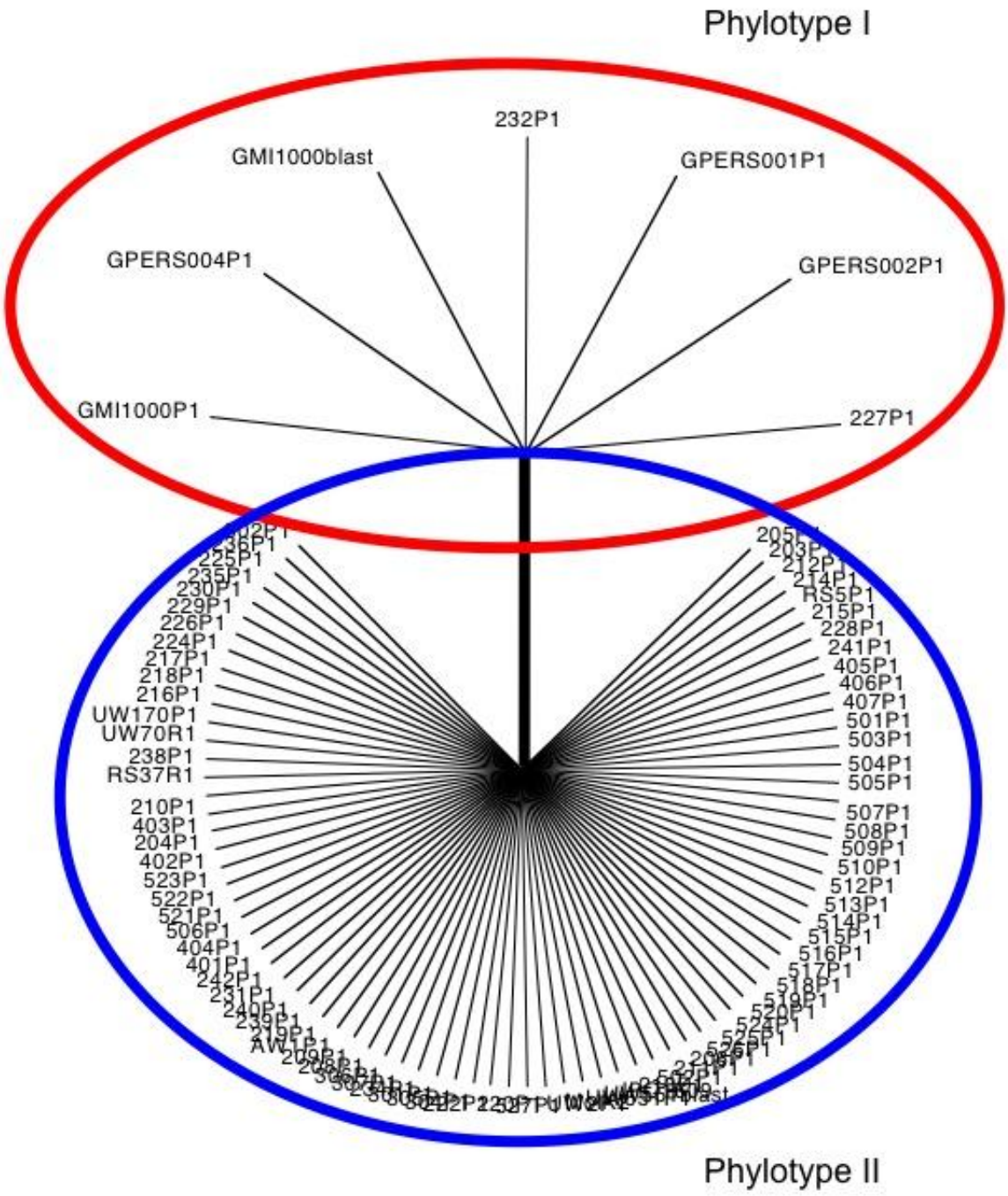


Figure 4.5 An unrooted majority rule consensus tree created by Bayesian analysis of SSR 1. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

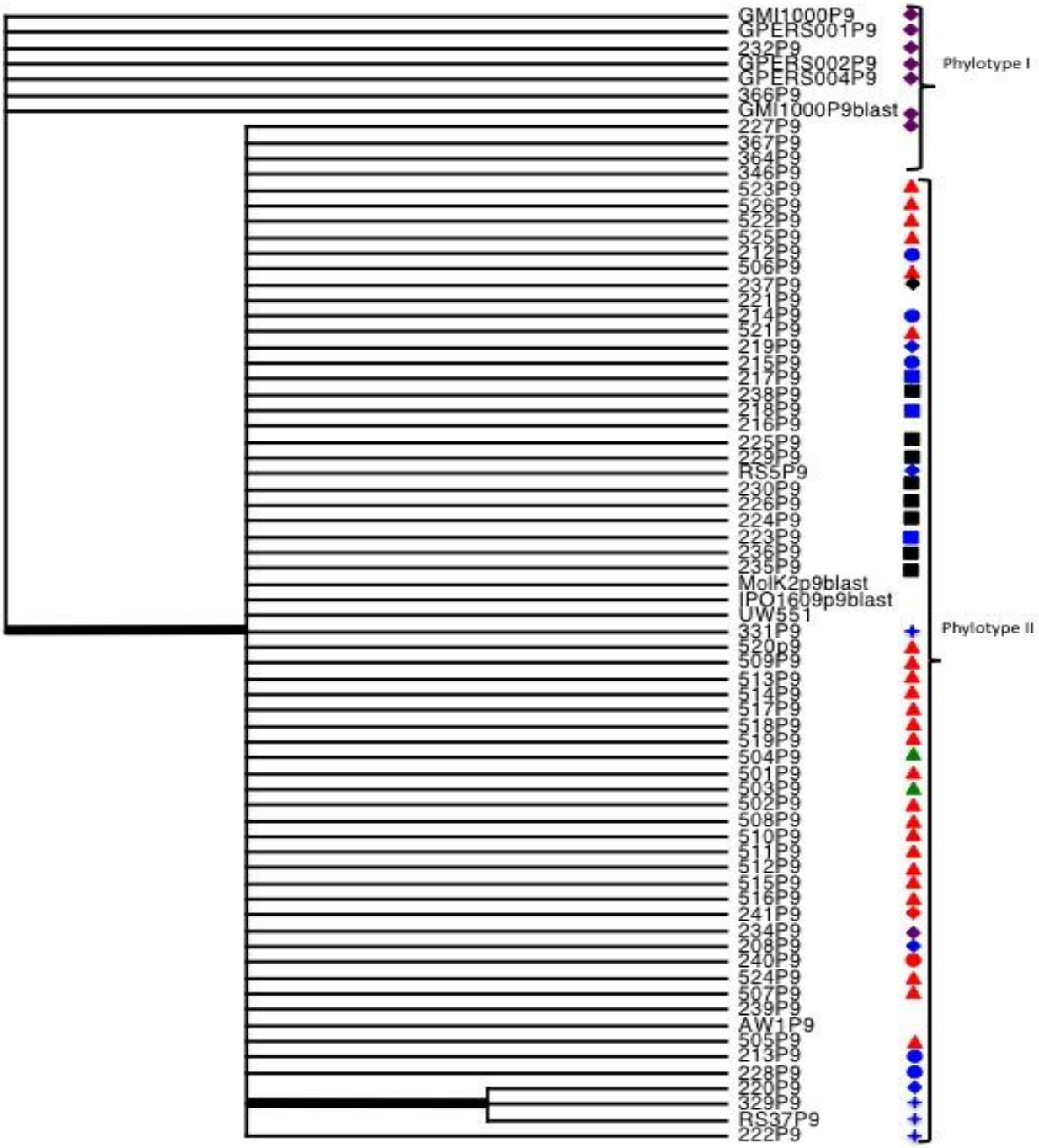


Figure 4.6 A majority rule consensus rooted tree created by Bayesian analysis of SSR 9. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

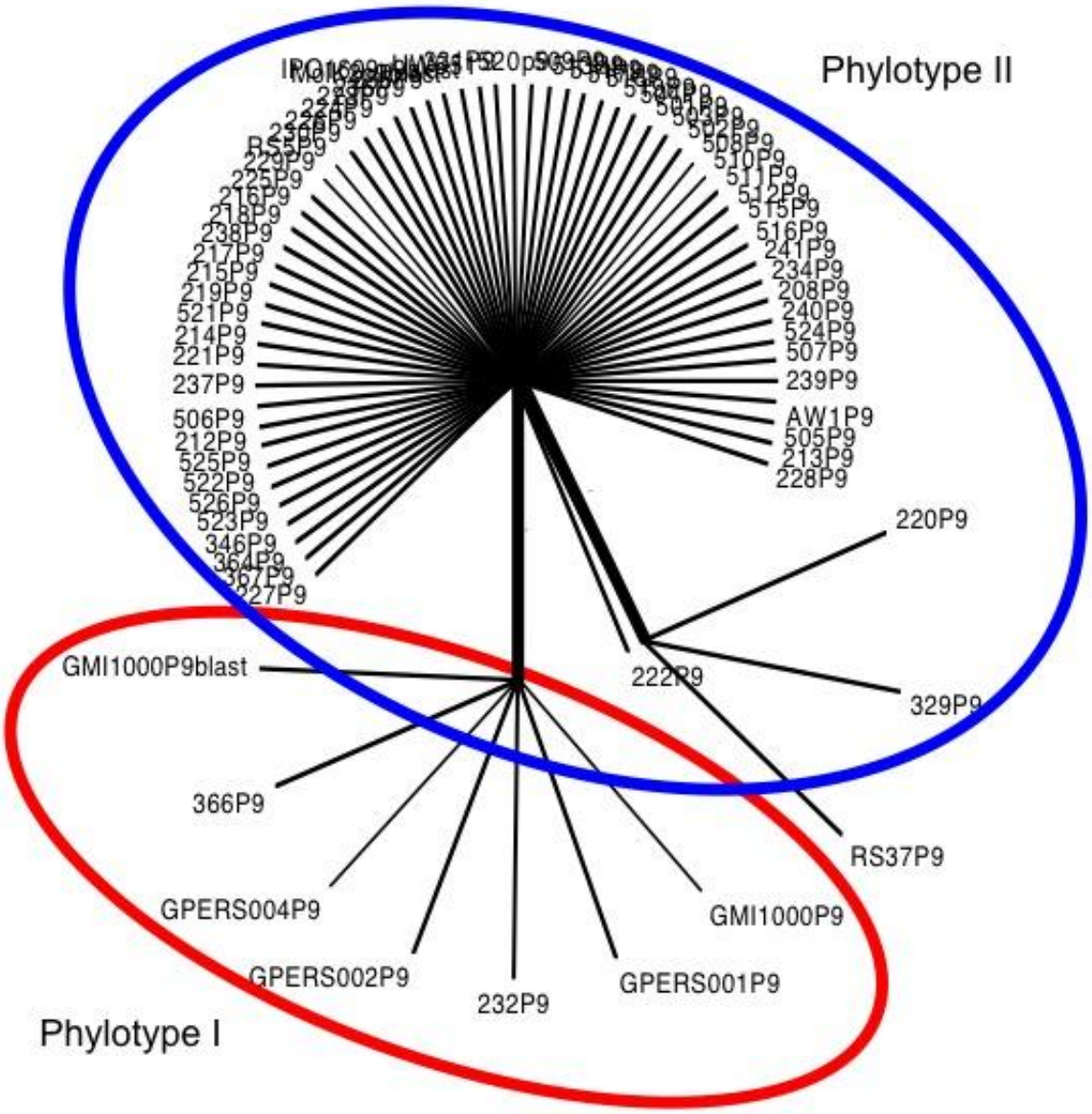


Figure 4.7 An unrooted majority rule consensus tree created by Bayesian analysis of SSR 9. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. The word "blast" after strain's name indicates the sequence was acquired from GenBank.

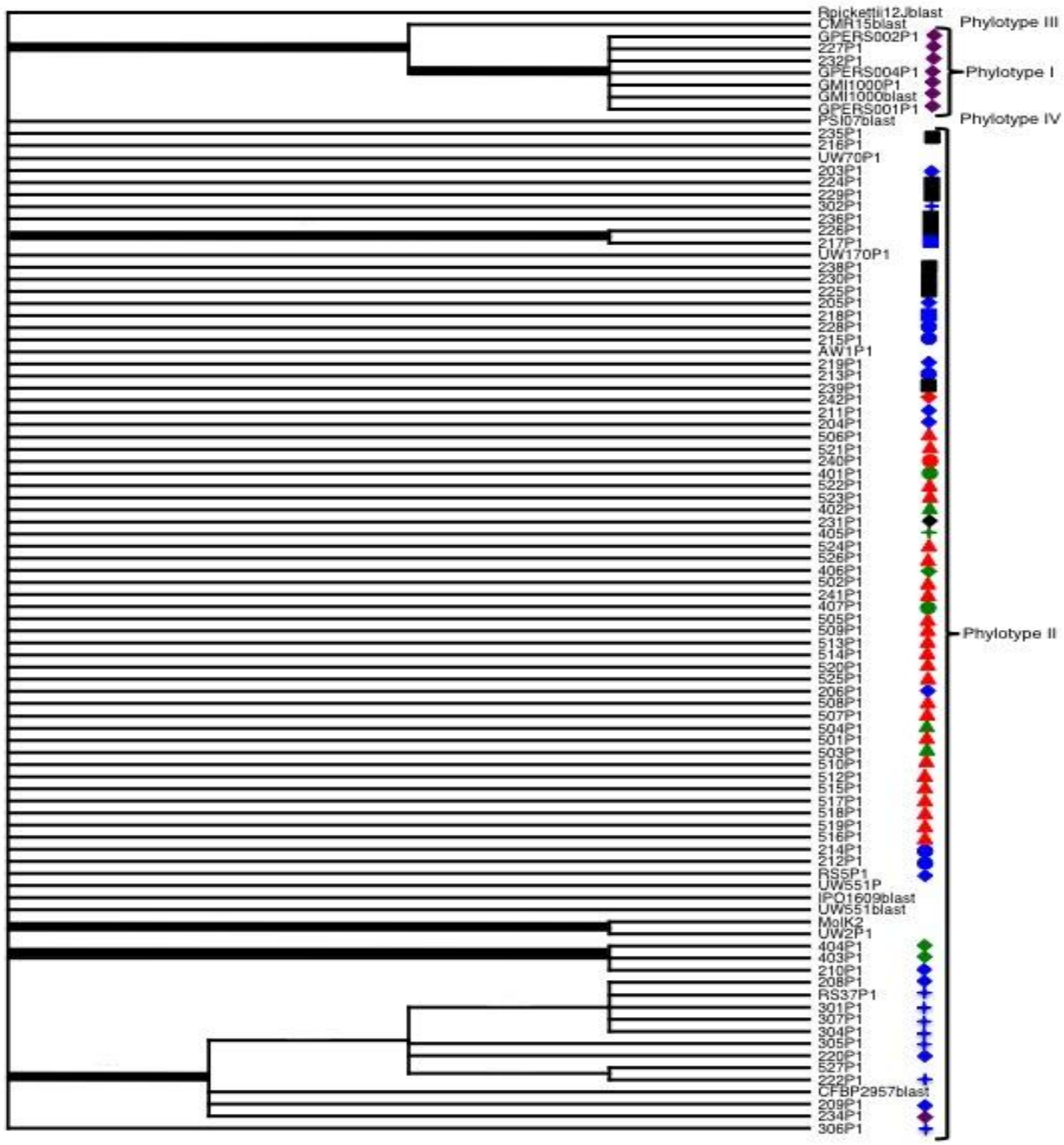


Figure 4.8 A majority rule consensus rooted tree created by Bayesian analysis of MAL 1. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

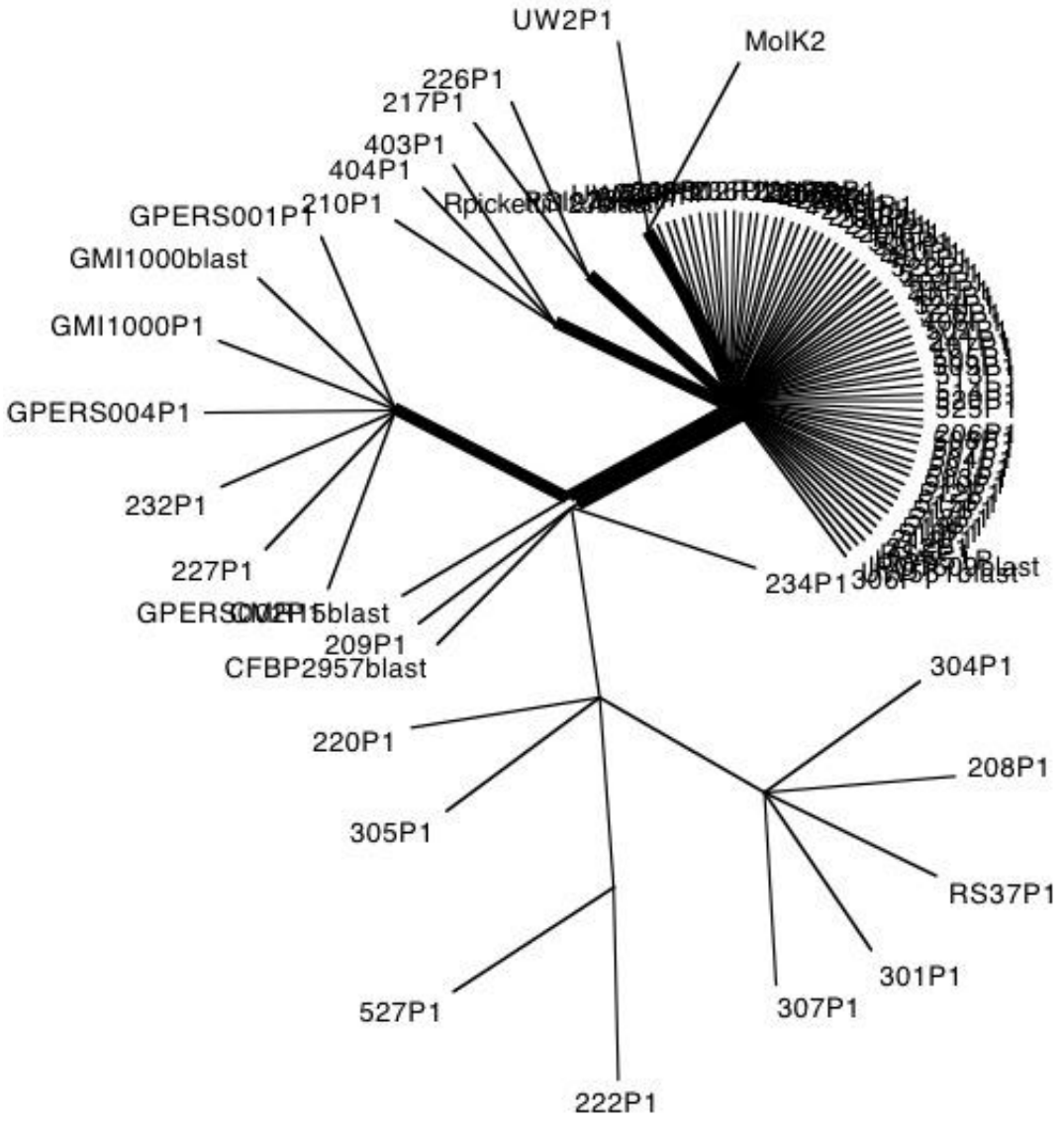


Figure 4.9 An unrooted majority rule consensus tree created by Bayesian analysis of MAL 1. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. The word "blast" after strain's name indicates the sequence was acquired from GenBank.

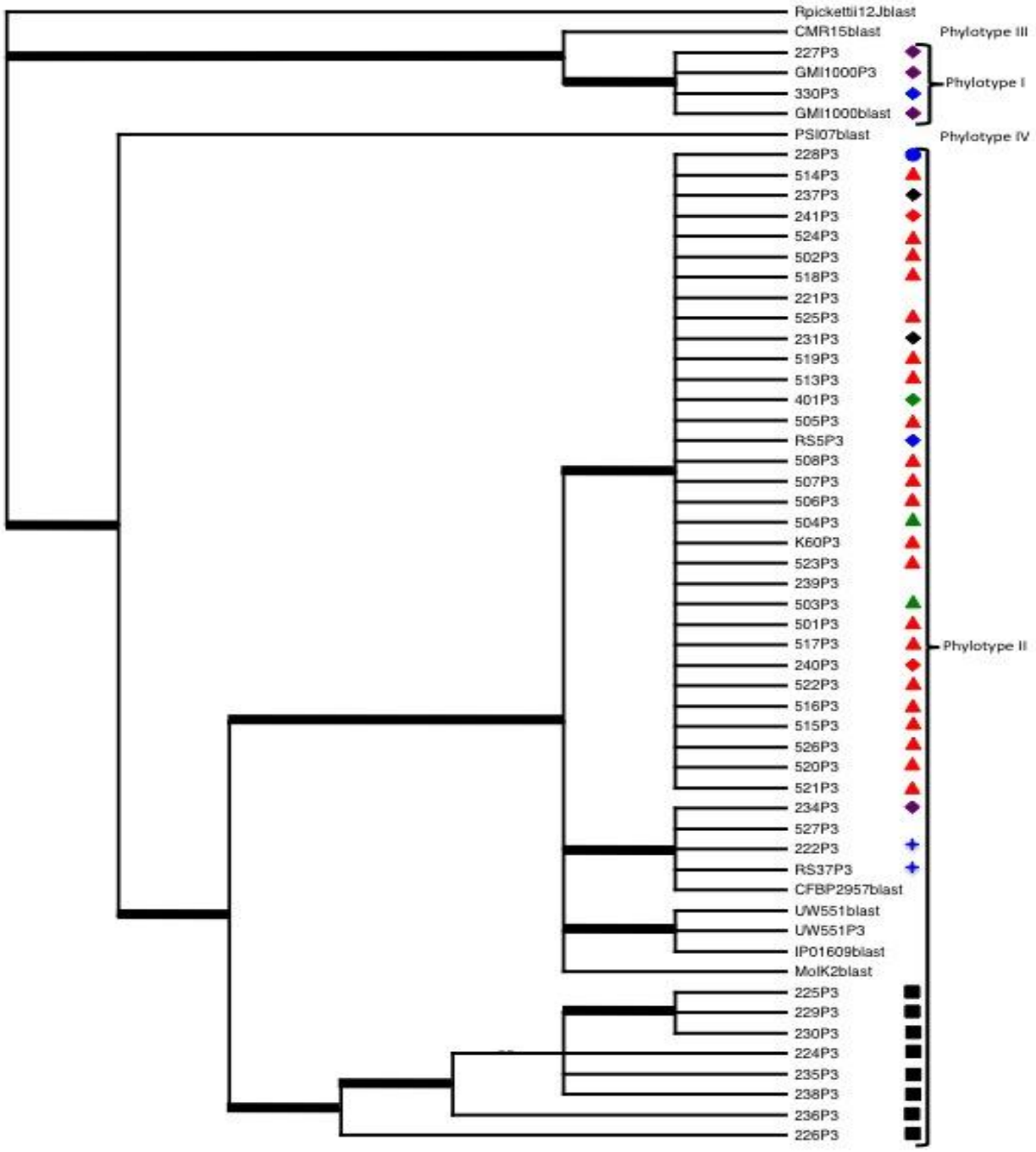


Figure 4.10 A majority rule consensus rooted tree created by Bayesian analysis of MAL 3. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

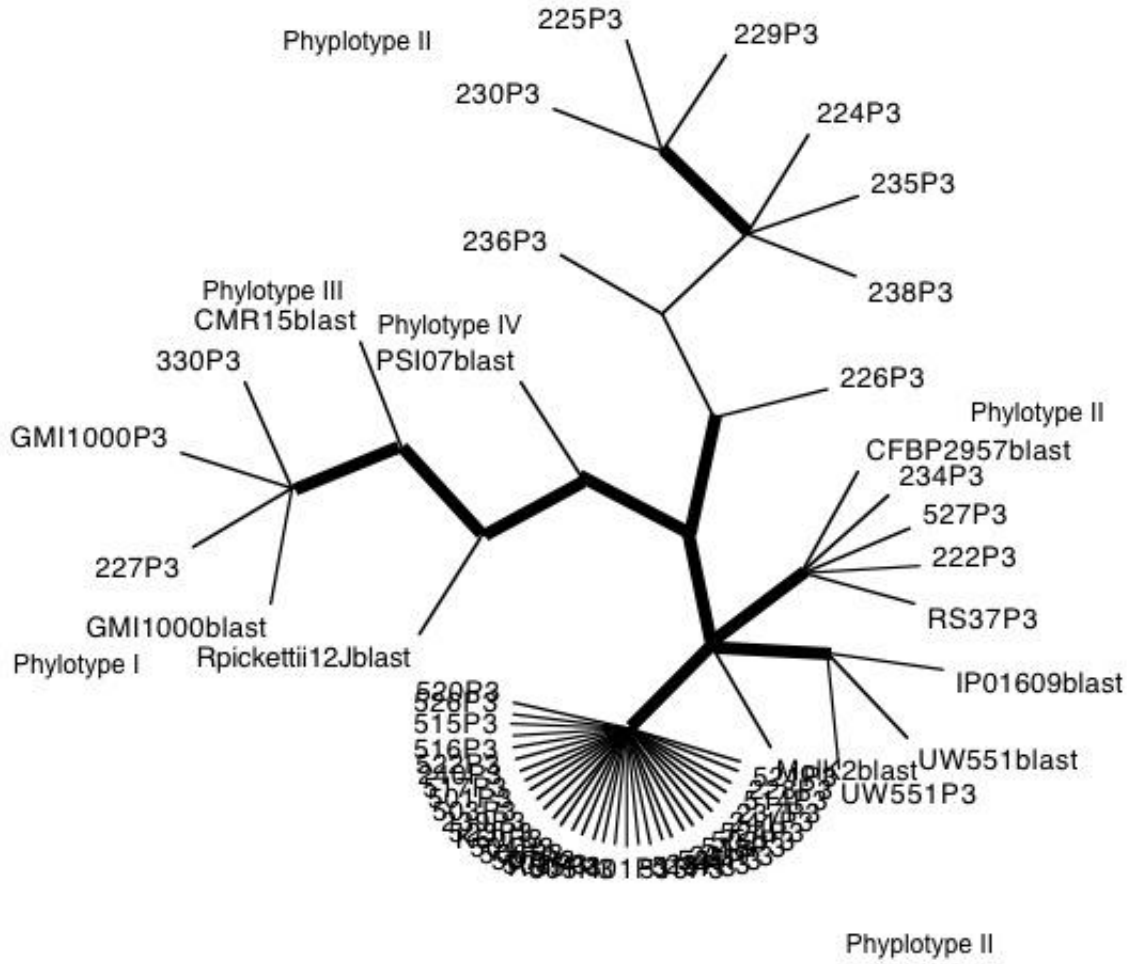


Figure 4.11 An unrooted majority rule consensus tree created by Bayesian analysis of MAL 3. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

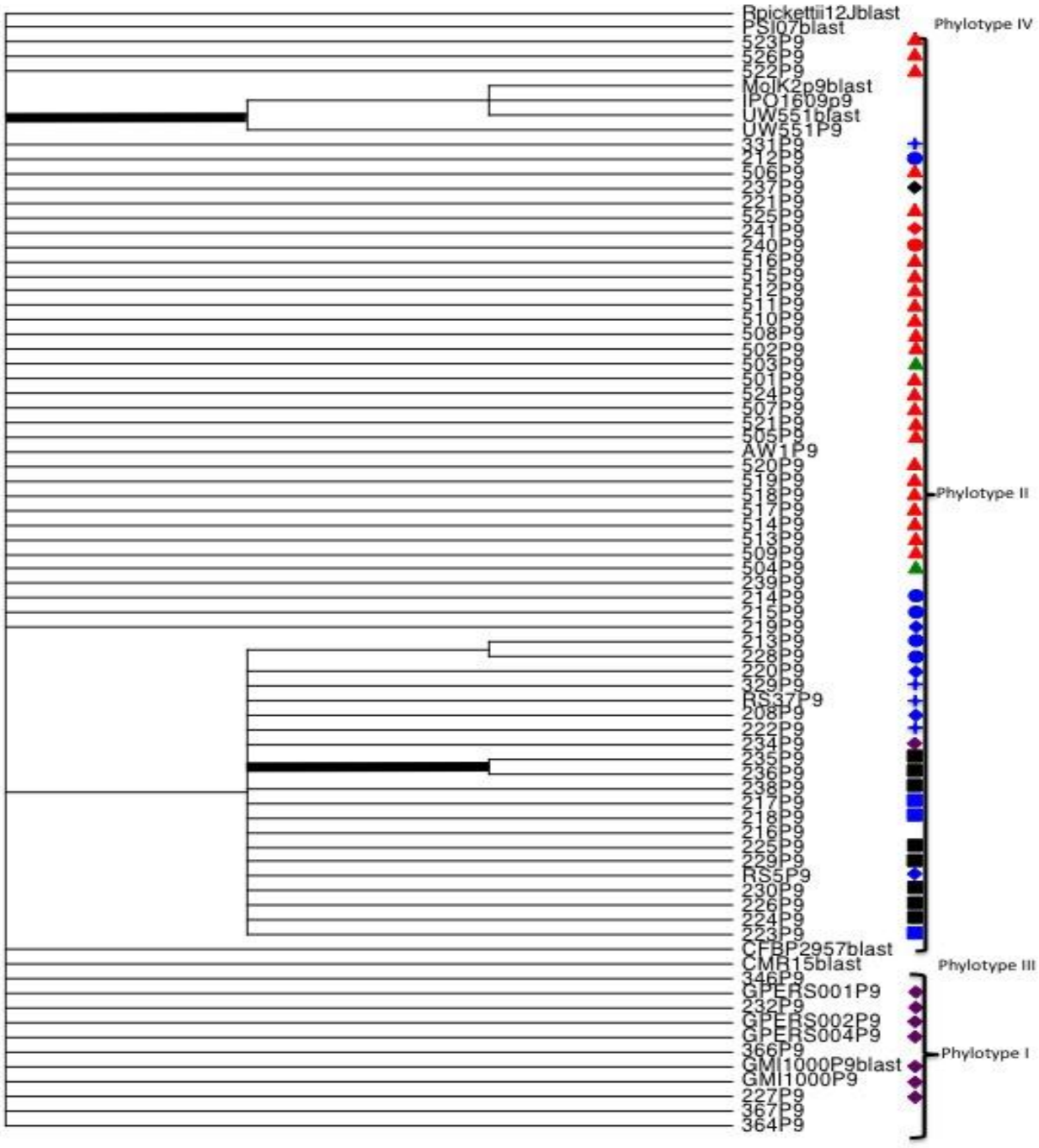


Figure 4.12 A majority rule consensus rooted tree created by Bayesian analysis of MAL 9. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

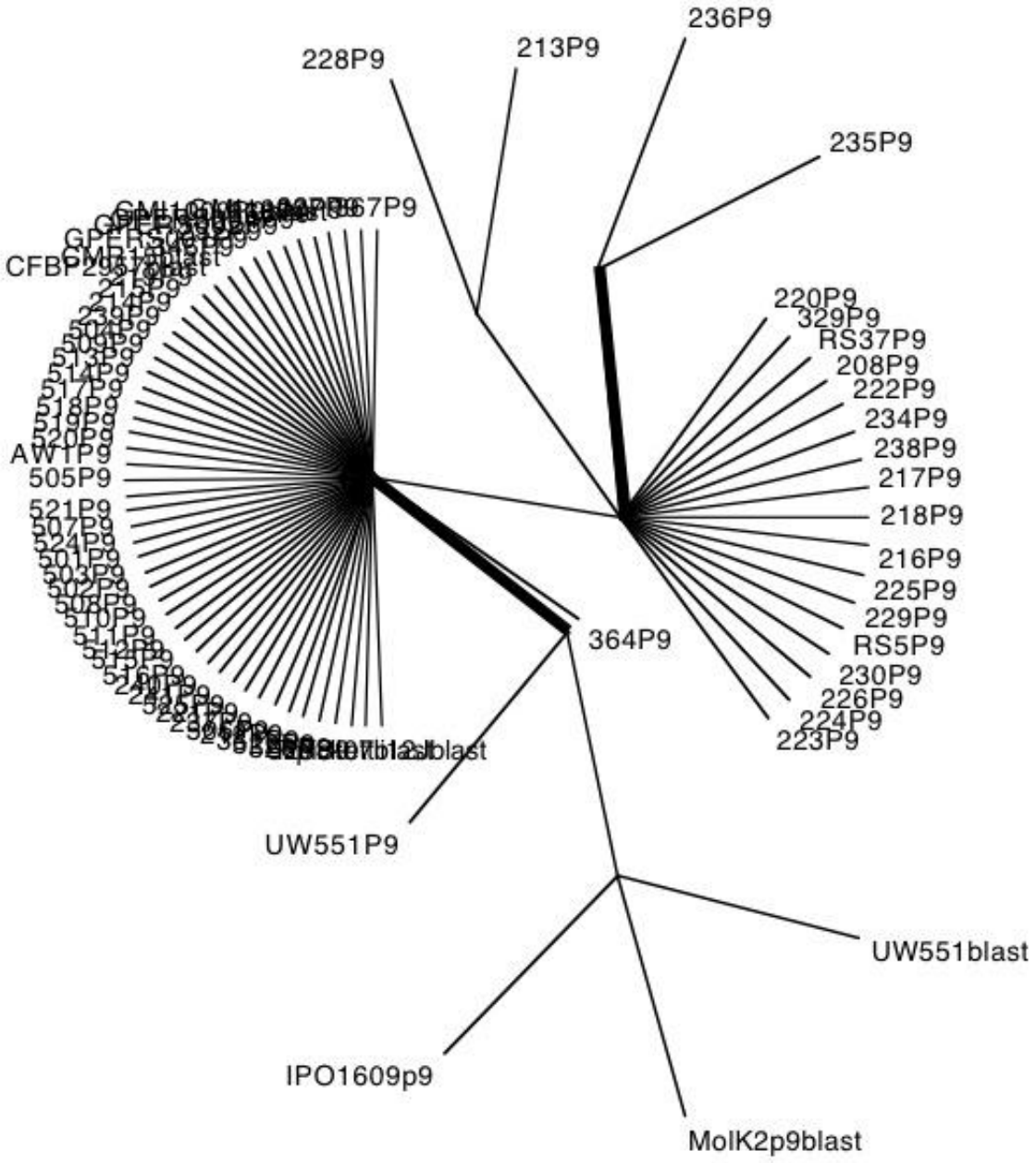


Figure 4.13 An unrooted majority rule consensus tree created by Bayesian analysis of MAL 9. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. The word "blast" after strain's name indicates the sequence was acquired from GenBank.

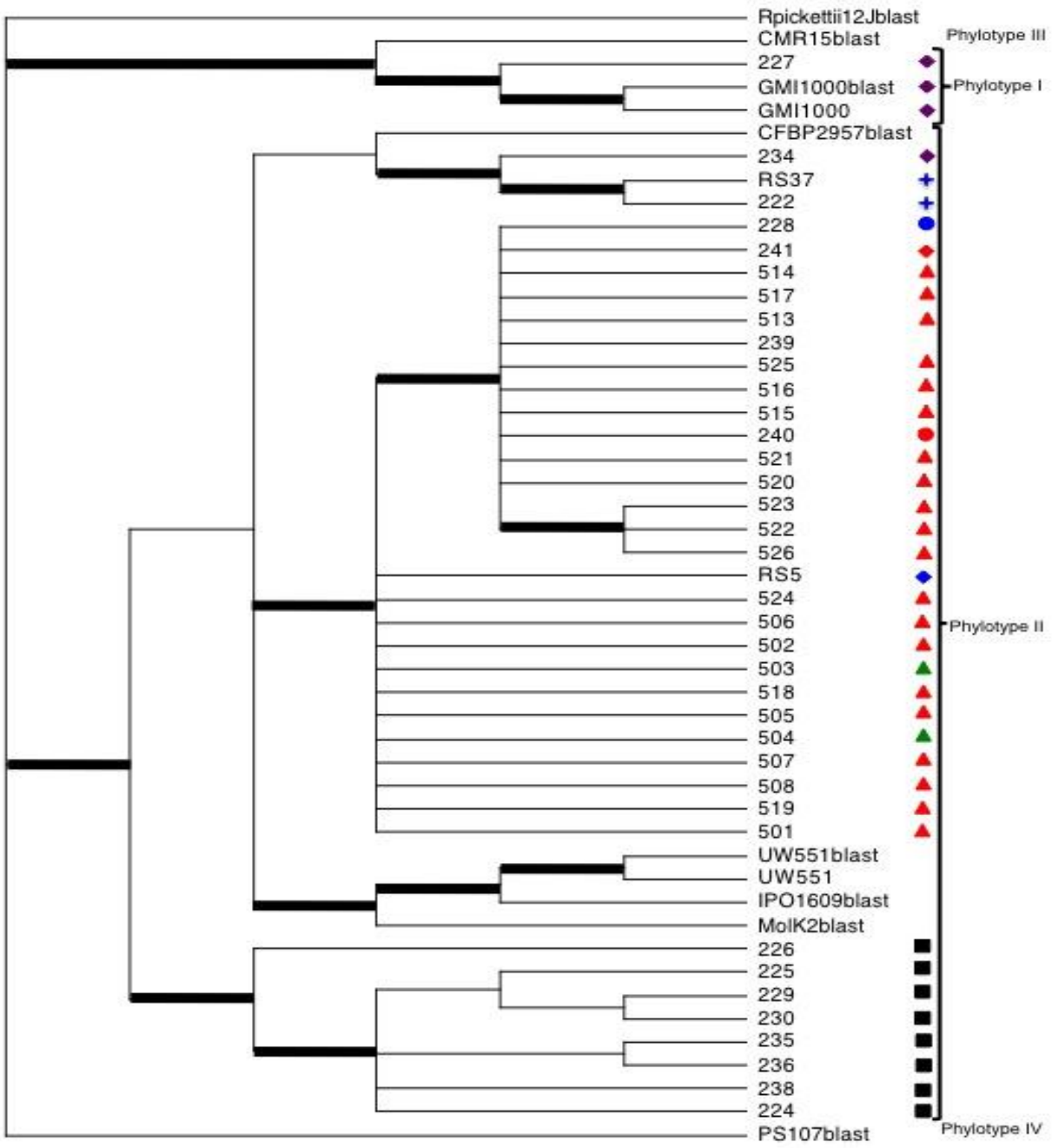


Figure 4.14 A majority rule consensus rooted tree created by Bayesian analysis of all three MALs. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Strains from Carolina are red, Georgia green, Florida blue, Caribbean purple, and unknown location black. *R. solanacearum* was isolated from potato (circle), tobacco (triangle), tomato (diamond), pothos/anthurium (square), and diverse location (star). Strains were not marked if location and host were unknown. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

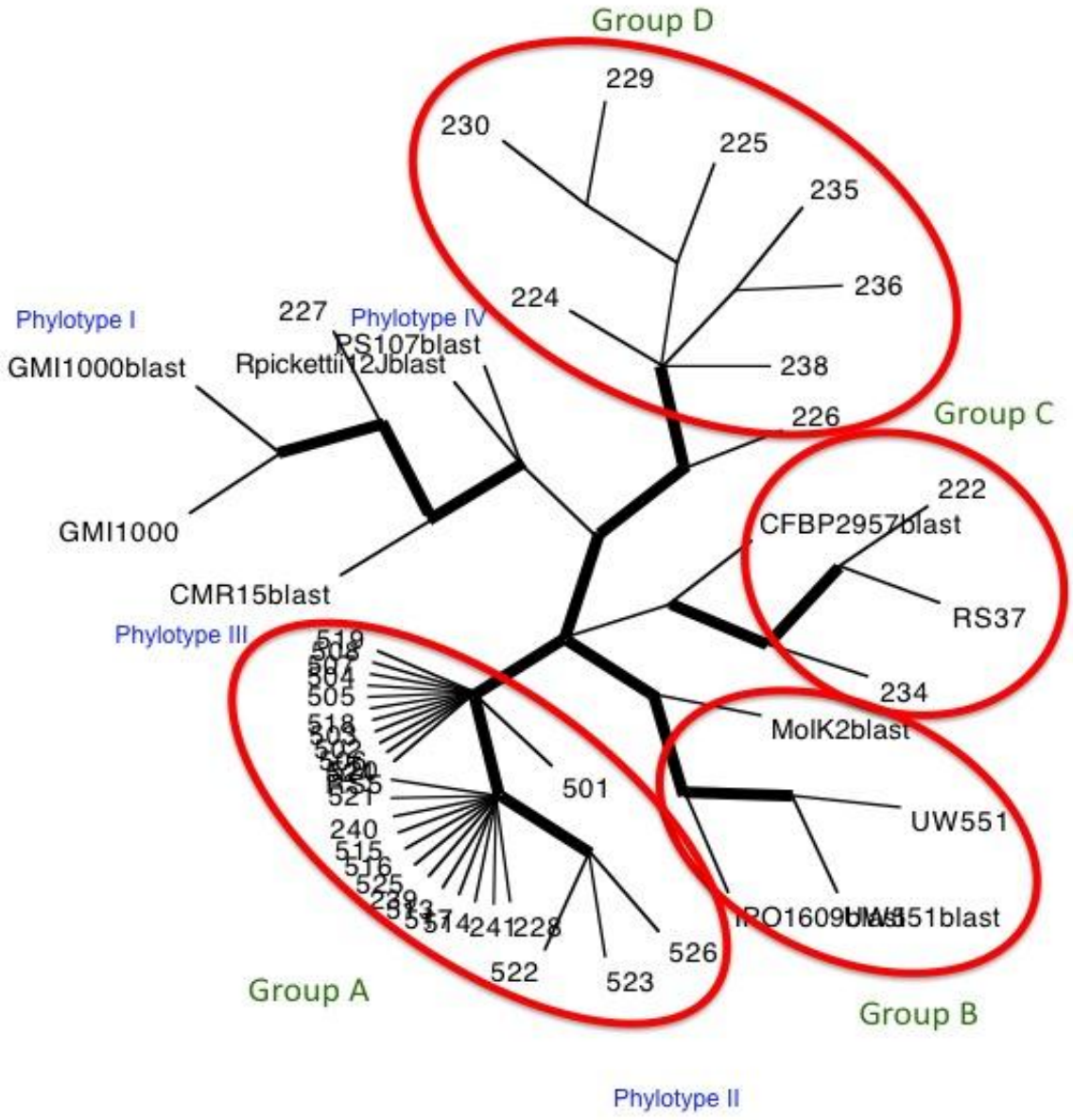


Figure 4.15 An unrooted majority rule consensus tree created by Bayesian analysis of all three MALs. Weighted line indicates a posterior probability values at 95 or greater and bootstrap value of 75% or greater for parsimony and likelihood. Groups A-D are part of phylotype II. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

# CHAPTER 5
# OVERALL SUMMARY AND DISCUSSION

*Ralstonia solanacearum,* the causal agent of bacterial wilt, can cause serious yield loss for many economically important crops. This study focused on investigating a method for controlling the disease, characterizing strains from the southeastern U.S., and determining if microsatellites could be used to differentiate infrasubspecies groups. The first objective was to determine the effectiveness of the application of thymol and acibenzolar-*S*-methyl (ASM) for decreasing bacterial wilt disease incidence on tomato. The second objective was to distinguish subspecies populations of *R. solanacearum* strains from the southeastern U.S. by applying classical and current characterization techniques. The third objective was to determine the effectiveness of using microsatellites, microsatellite-associated loci (MALs) and the combination of MALs for distinguishing subspecies groups within strains collected in the southeastern U.S.

## Management of Bacterial Wilt in Tomatoes with Thymol and Acibenzolar-*S*-Methyl

In this study we showed that the application of thymol as a soil fumigant and foliar application of ASM was able to control bacterial wilt on moderately resistant tomato cultivars and to a lesser degree on susceptible tomatoes. This would provide another tool in the small arsenal of defense against the causal agent of bacterial wilt. Previously, the two products were applied separately in field conditions and both were able to decrease disease incidence and increase fruit yield (Ji et al., 2005; Pradhanang et al., 2005). We determined that the application of both products will not have a negative effect on tomato production. We were able to show that the combination of both products numerically increased fruit yield and decreased disease incidence for the susceptible cultivar. In both trials moderately resistant plants that received the

combination of both chemicals had statistically significant lower disease incidence and increased fruit yield, compared to the untreated control (UTC) and in one experiment compared to thymol alone.

Previous studies have shown that both products are effective at decreasing the incidence of different plant diseases (Ji et al., 2005; Pradhanang et al., 2005). Further research should be done to determine the effectiveness of the combination of both products against multiple plant pathogens. If these chemicals are successful in controlling a wide spectrum of diseases, they could be another alternative to methyl bromide. The next step would be to determine the minimum inhibitory concentration of thymol in field conditions to determine the most effective concentration that is economically beneficial to growers. Grafting is a fairly recent method that has shown promise for controlling the disease (Rivard and Louws, 2008). Further research is also needed to determine if grafting can be used as part of an integrated management strategy with thymol and ASM.

**Diversity Amongst *Ralstonia solanacearum* strains from the southeastern United States**

Currently one of the largest concerns pertaining to *R. solanacearum* is importation of exotic strains, especially sequevar 1 (race 3 biovar 2). In order to determine if exotic strains have been introduced into a country, we must first understand the characteristics of the native strains. The major incidence of bacterial wilt is restricted predominantly to the southeastern U.S. Strains were collected representing three different geographic regions, the Carolinas, Georgia, and Florida. The objective of the study was to determine the attributes of these strains, and then characterize an exotic strain.

Strains collected in this study were characterized based on pathogenicity tests, biovar, HR tests on tobacco, and phylotype using multiplex PCR. All the strains that were pathogenic were able to wilt tomato plants, and a few were able to wilt pepper. Most of the Carolina strains did not elicit a hypersensitive response (HR) on tobacco, while most of the Florida strains were HR positive. All identified strains native to the U.S. belonged to phylotype II.

We determined sequevar by phylogenetic analysis of *egl* gene. Strains were assigned to sequevar based on cladistic grouping with previously published sequences of strains (Fegan and Prior, 2005, Wicker et al., 2007 and Ji et al., 2008). Native U.S. strains grouped within sequevar 7. Strains that grouped within sequevars 4, 5/6 and an undefined sequevar were identified as exotic strains. The designation of an undefined sequevar was due to a lack of previously identified strains within this clade. Previously, it was reported that the undefined sequevar was part of sequevar 5 (Ji et al., 2008) or 4NPB (Hong et al, 2008). However, by including more taxa than the previous two studies used in their analyses, these strains did not group with sequevar 5 or 4NPB strains.

One strain that belonged to the undefined sequevar was further characterized. This strain, RS37, was isolated in North Florida from the waterways, tomato fields, and different plant species (Hong et al., 2008). The typical sequevar 7 strains were not detected in any of the samplings. It was determined that RS37 was able to produce a bacteriocin against RS5, a sequevar 7 strain, previously isolated in North Florida. We further characterized the strain by performing pathogenicity tests on cucurbits and *Musa* species. This strain was not pathogenic on the cucurbit cultivars tested. We were able

to show that inoculation of RS37 was deleterious to the growth of *Musa* plants. Plants inoculated with RS37 had less root mass and were shorter in height compared to the water control or race 1 inoculated plants. We also showed that symptoms can vary depending on *Musa* genotype.

Based on the results of this study, it was determined that exotic strains have become established in the U.S., and they have different genotypic and phenotypic characteristics than the native strains. We recommend further characterization of RS37. Although we were able to show that RS37 produces a bacteriocin, the next step would be to determine if this strain is more competitive than RS5 and other sequevar 7 strains. Further characterization of the other exotic strains identified in this study should be performed to determine if other important economic crops could be potential hosts.

### Multiple Loci Variable Number Tandem Repeat Analysis Among *Ralstonia solanacearum* Strains from the southeastern United States

In this study we identified microsatellites with their flanking regions (MALs) that could be used for determining *R. solanacearum* infrasubspecies groups. These microsatellites were identified using GMI1000 and the draft sequences of other strains. Primers were designed for the amplification of these microsatellites and their flanking regions. Three SSRs were identified that were consistently amplified in a panel of *R. solanacearum* strains. Using the strains in our collection these three SSRs were amplified and sequenced. Phylogenetic analysis was performed on microsatellites alone, the MALs, and then the combination of the three MALs together. Trees produced by the analysis of combination of the three MALs were the more informative than the SSRs or the MALs alone trees. The trees produced by the combination of the MALs displayed 4 groups within phylotype II. These groups correspond to clades (sequevars)

found in the *egl* phylogenetic trees. This confirms that these loci were able to determine sequevar. The trees produced by the *egl* and these loci were able to differentiate between strains that were native to the U.S. and were exotic. Using the three methods for phylogenetic analysis, maximum parsimony, maximum likelihood and Bayesian, we had high confidence that groupings created in the trees were correct. Further research in combining microsatellites with phenotypic characteristics could give more support to infrasubspecies groups.

To date the quickest method for identifying sequevar 1 (race 3 biovar 2) strains is by real-time PCR; however, at times this method can be unreliable. As noted in this study some SSRs are unique to a specific population. It could be possible to identify SSRs or regions in the flanking regions of the SSRs that would be specific to sequevar 1 strains. This would provide another confirmation test when identifying specific groups of strains.

Microsatellites were useful in determining infrasubspecies groups within *R. solanacearum*. Analysis of these loci was able to determine with confidence the regional origin of the strains tested in our collection. This information could be vital for identifying the potential geographic origin when bacterial outbreaks occur. Further research should be done to determine if MALs could be located in other phytobacteria and used to identify subspecies groups. Hopefully by identifying these populations researchers will be able to distinguish epidemiological and ecological groupings, and thus be able to predict the characteristics of unknown strains. This information should be important for plant breeders, pathologists and quarantine officials.

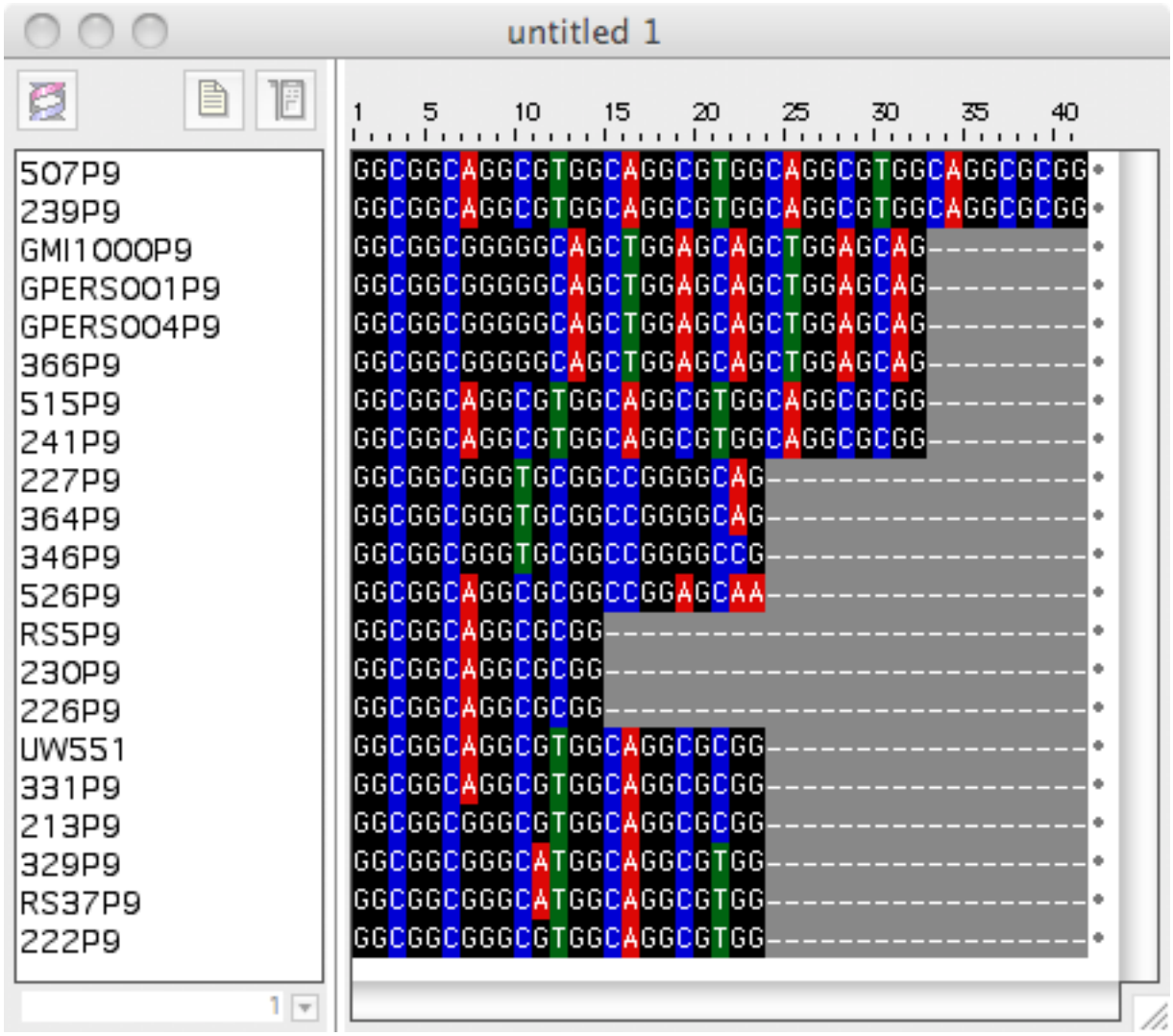


Figure A.1. An example of the diversity of SSR 9 of *R. solanacearum strains* isolated. Strains without P9 at the end of its name indicate strains obtained from NCBI-BLAST.

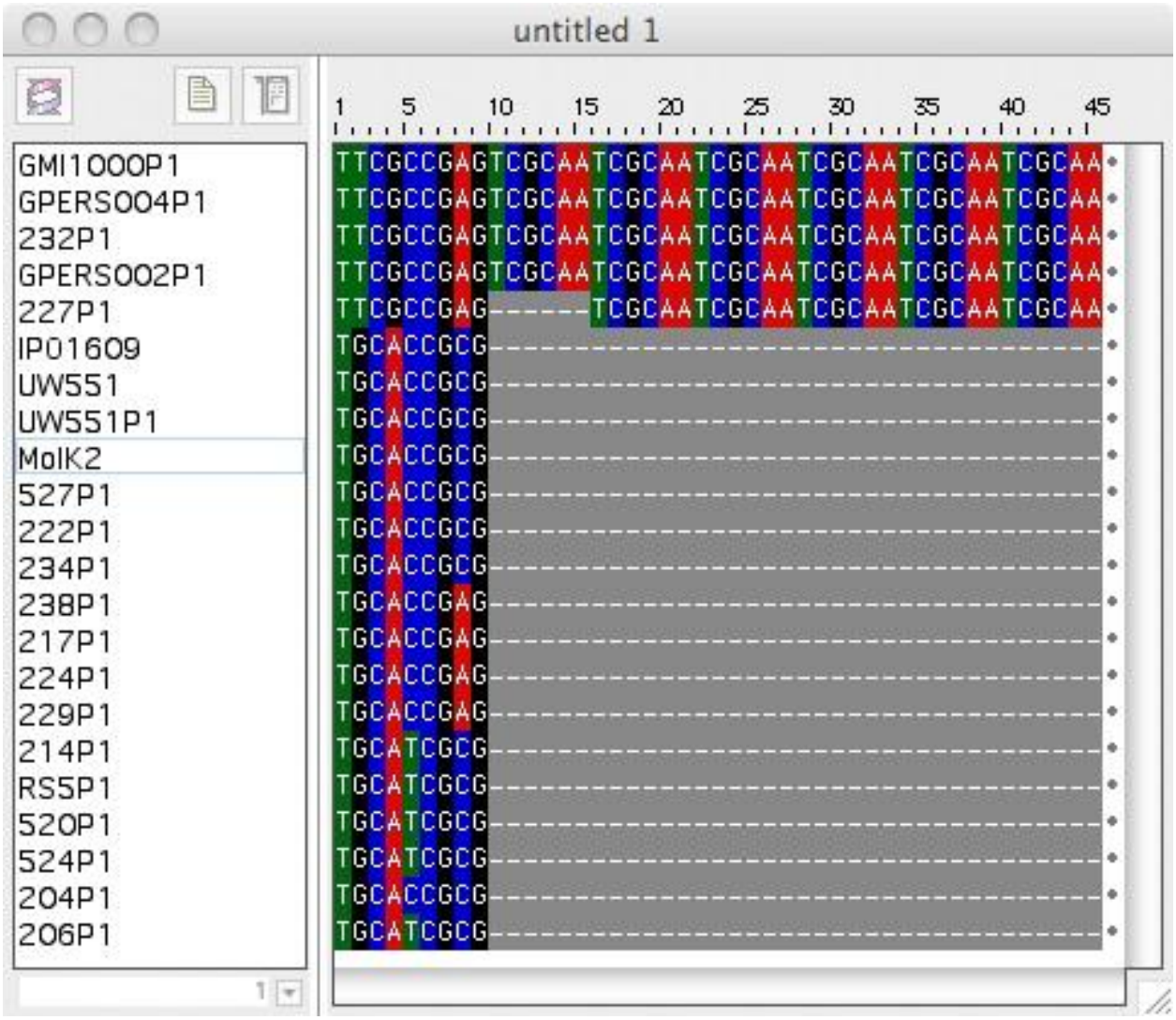


Figure A.2. An example of the diversity of SSR 1 of *R. solanacearum strains* isolated. Strains without P1 at the end of its name indicate strains obtained from NCBI-BLAST.

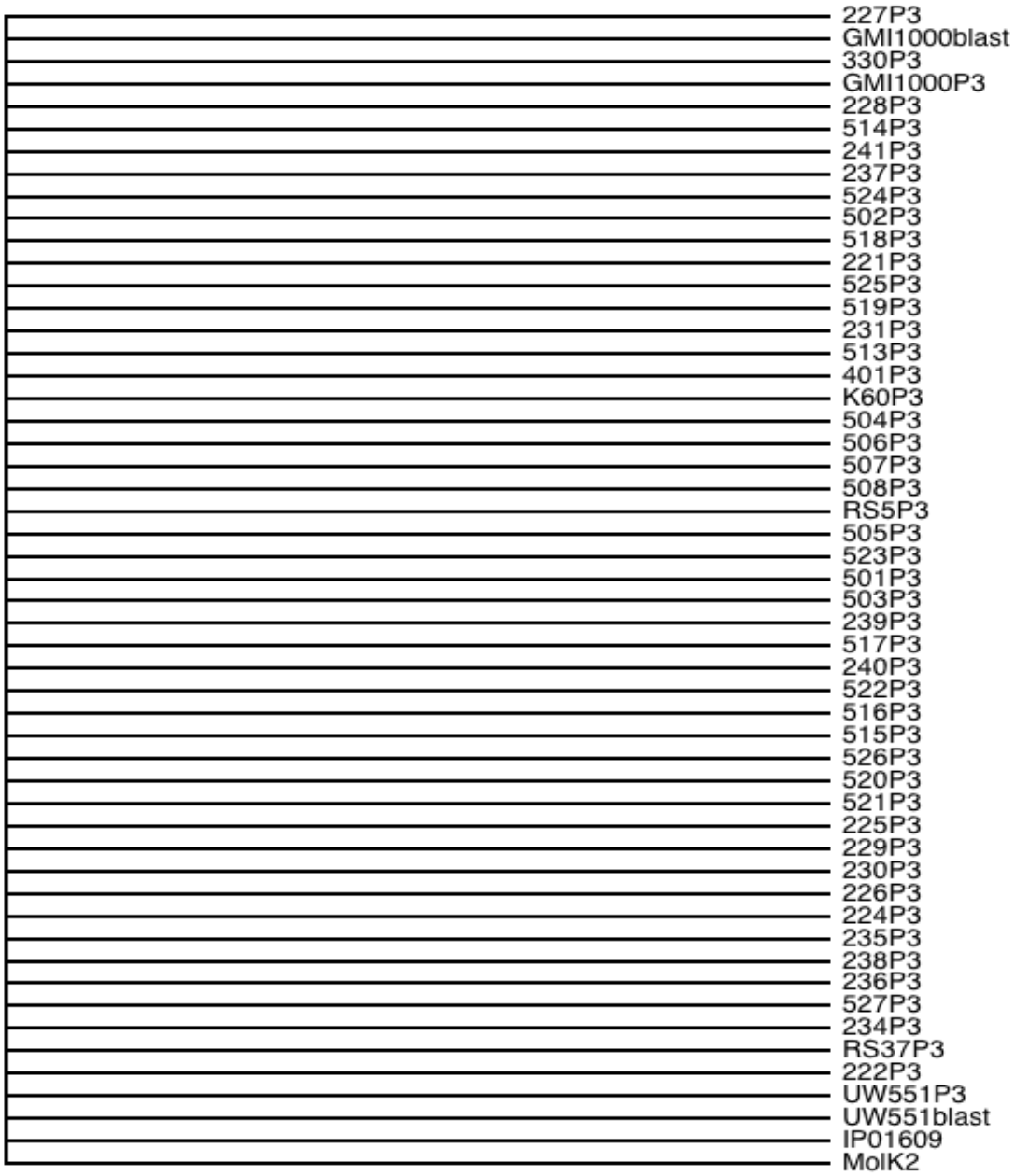


Figure A.3. A majority rule consensus rooted tree created by Bayesian analysis of SSR 3. Numbers indicated the confidences of correct position of clades based on creating the consensus tree. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

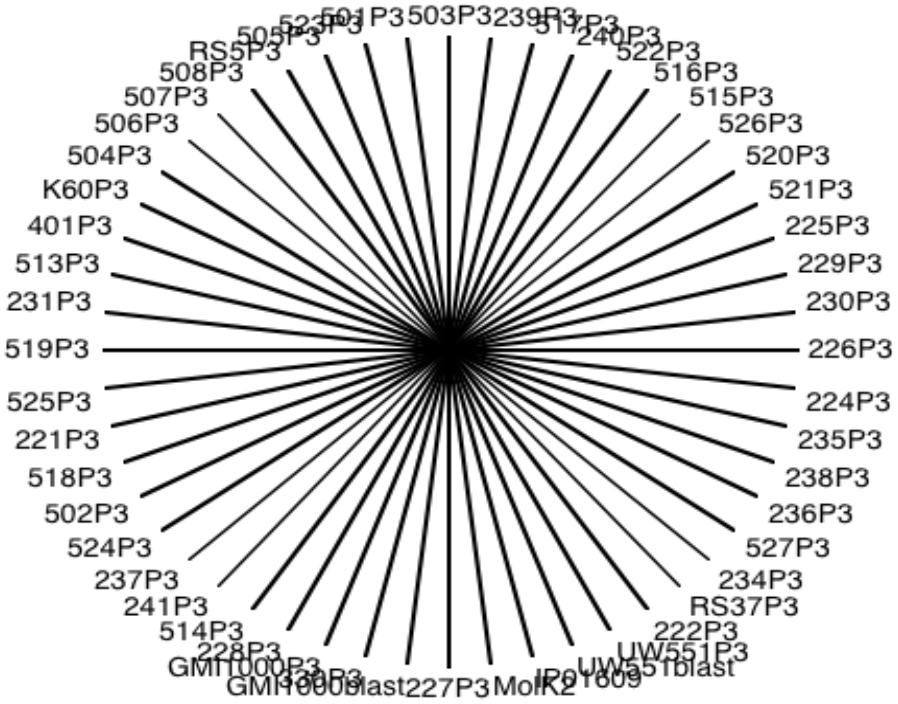


Figure A.4. A majority rule consensus unrooted tree created by Bayesian analysis of SSR 3. Numbers indicated the confidences of correct position of clades based on creating the consensus tree. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

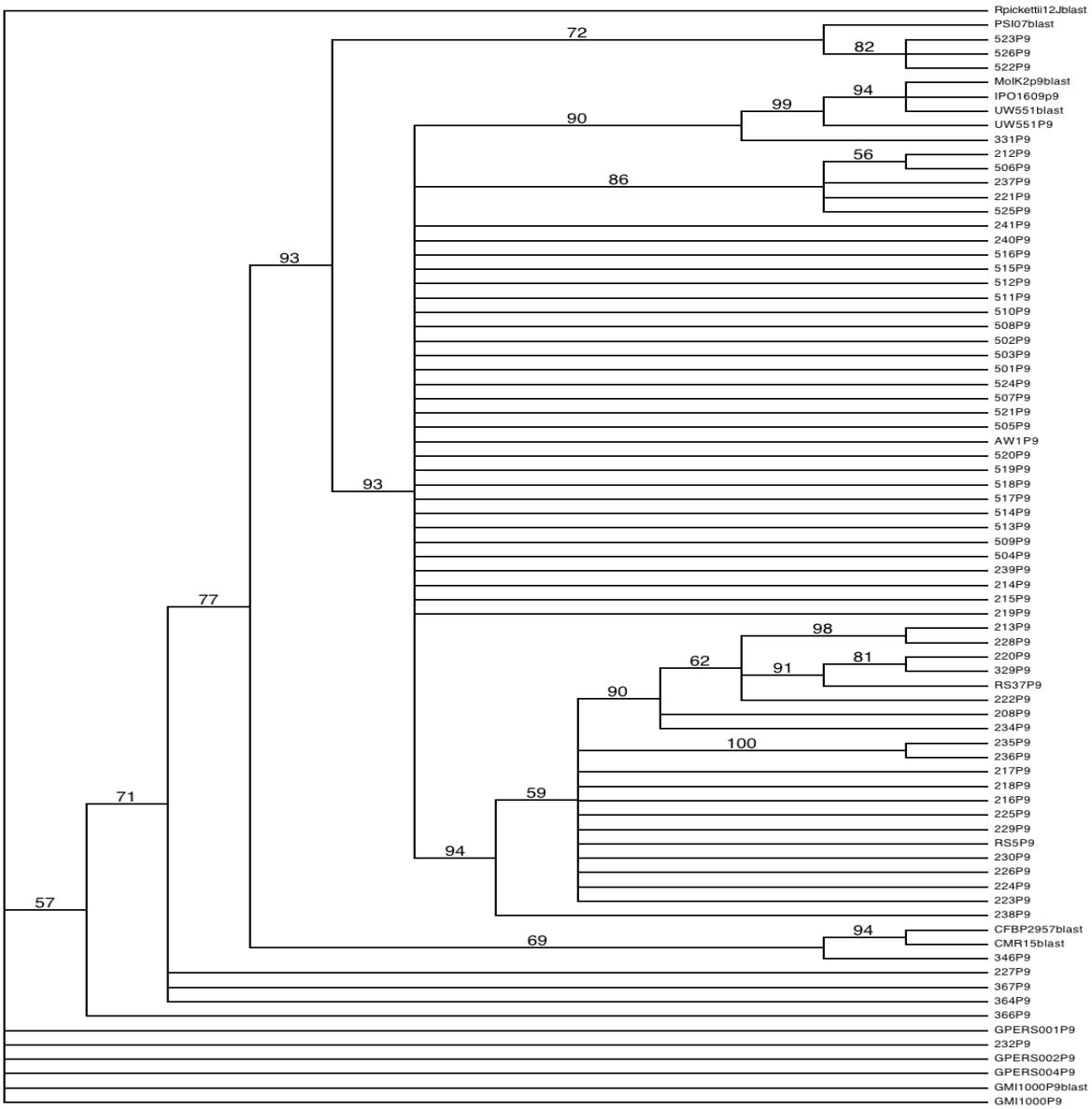


Figure A.5. A majority rule consensus rooted tree created by Bayesian analysis of MAL 9. Numbers indicated the confidences of correct position of clades based on creating the consensus tree. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

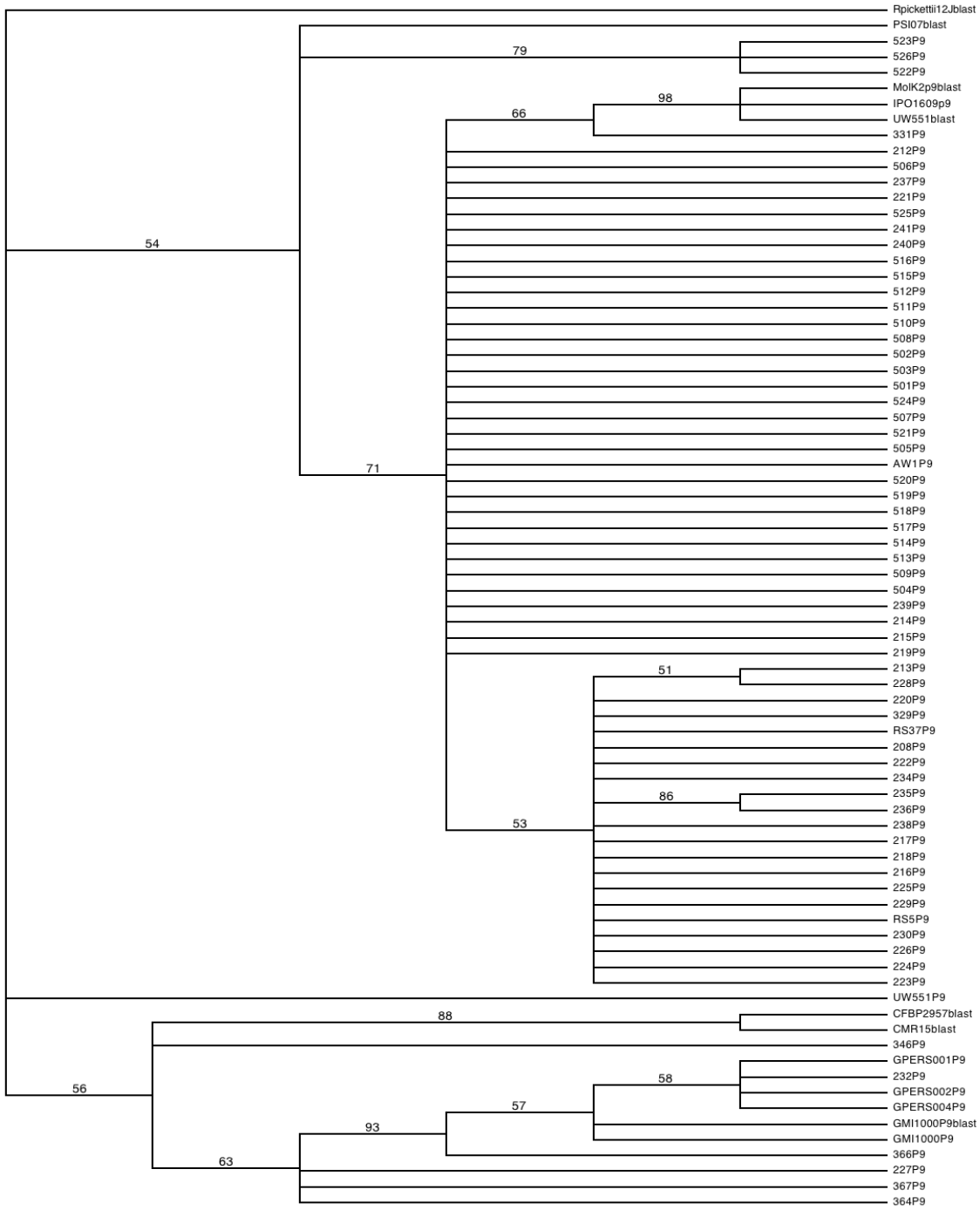


Figure A.6. A majority rule consensus rooted tree created by parsimony analysis of MAL 9. Numbers indicated the confidences of correct position of clades based on creating the consensus tree. The word “blast” after strain’s name indicates the sequence was acquired from GenBank.

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# BIOGRAPHICAL SKETCH

Jason Hong was born in Logan, Utah in 1978, but grew up in Wooster, Ohio. For the last two years of high school he was dual enrolled at the local community college and graduated with an Associate of Science the same year he graduated from high school. In 2003, he graduated from The Ohio State University with a Bachelor of Science degree in microbiology. During the summers of 2002 and 2003 he had internships with Dr. Jackson at AgriPhi (now Omnilytics) and was exposed to plant pathology, bacteriophage and research. In 2005 he graduated with his Master of Science from the University of Florida, College of Agricultural and Life Sciences, Department of Plant Pathology. His master's project focused on the survival of *R. solanacearum* strains in irrigation ponds, and determining methods for decreasing the bacterial population in the water. In 2005, he continued his research on *R. solanacearum* and was admitted into a Ph.D. program at the Plant Pathology Department at University of Florida from which he received his Ph.D. 2010. His research project focused on controlling bacterial wilt in the field and determining the biodiversity of *R. solanacearum* strains in the southeastern U.S., while looking at simple sequence repeats and microsatellites to aid in determining diversity.