EVALUATING THE SUITABILITY OF AFLP TECHNOLOGY FOR GENOTYPING STRAINS OF *SERRATIA MARCESCENS*

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CHAPTER I

INTRODUCTION

The terrorist attacks against the United States in 2001 brought about different security challenges not only for travel and the distribution of mail, but also for the protection of the food supply (Oberst, 2004). The citizens of the United States receive food from the safest food production system in the world under current safety and security programs (Oberst, 2004). However, current security programs are not designed to detect nor respond to intentional acts of bioterrorism that use biological agents to contaminate the food supply directly or indirectly through processing and distribution systems (Oberst, 2004). Thus, agriculture is considered by many to be an ideal target for bioterrorism, a new threat known as agroterrorism (Davis, 2001). Political scientist, Peter Chalk, defines agroterrorism as "the deliberate introduction of a disease agent, either against livestock or into the food chain for the purposes of undermining stability and/or generating fear" (Liebert, 2004). "Every link in the agricultural production chain is susceptible to attack with a biological weapon" (Monke, 2004). Therefore, it would be difficult to manage an agroterrorist attack because certain aspects of agriculture pose unique problems for protection. This point has been addressed by Monke (2004) who stated "production is geographically disbursed in unsecured locations, livestock is often

concentrated in confined locations, and then transported and co-mingled with other herds, pest and disease outbreaks can quickly halt economically important exports, and many veterinarians lack experience with foreign animal diseases that are resilient and endemic in foreign countries."

Pathogens that could be used in an agroterrorist attack include viruses, fungi, and bacteria (Monke, 2004). The consequences of a bioterrorist attack on the agricultural industry would include economic crises, loss of confidence in the government, and human casualties.

Economically, the effects of agroterrorism would be devastating for individuals, businesses, and governments not only in the U.S., but worldwide as well. Specifically in Oklahoma, there were 87,000 farms and ranches in 2002 that accounted for $4.1 billion of the agricultural economy (Chiappe and Nelson, 2003). In 2005, agricultural exports nationwide generated $62.4 billion according to the Economic Research Service of the USDA (Brooks and Jerado, 2007). Furthermore, U.S. agriculture depends twice as much on overseas marketing when compared to the general export economy. Targets of agroterrorism include, but are not limited to animals, plants, water, farm workers, producers, grain elevators, ships, food handlers, restaurants, and grocery stores.

Despite the fact that attacks against agriculture are not new and have been perpetrated by nation-states and substate organizations throughout history, agriculture and food production in the U.S usually receive less attention in counter-terrorism and homeland security efforts when compared to security for travel and the distribution of mail. However, agriculture has recently been gaining more attention within the expanding field of terrorism studies. To address the reality of agroterrorism, laboratory

and response systems are being evaluated, and modified to develop preparedness in this area. (Monke, 2004)

A bioterrorist attack on the agricultural sector may include some distinctive features that might dictate how the situation should be handled. For example, there are some agents which are not hazardous to the perpetrators thereby allowing easier production, storage, and dissemination (Wheelis, 2000). In addition many pathogens produce their deleterious effects long after the host has been infected, delaying the recognition by law enforcement that a crime has been committed. Finally, some agents can produce the maximum effect (crop destruction, economic losses) through just a few outbreaks (Wheelis, 2000).

In September of 2006, an outbreak of *E. coli* O157:H7 was linked to the consumption of fresh spinach (Brackett, 2006). Through a collaborative effort between the FDA and a State of California field investigation team, the same strain of *E. coli* O157:H7 that was involved in the illness outbreak was found in a stream, and in cattle, and wild pigs on a ranch that appeared to have been connected with the outbreak (Brackett, 2006). Additionally, the investigation team found evidence that the wild pigs had entered the spinach fields (Brackett, 2006). A total of 205 persons were infected with this strain of *E. coli* in 26 states in the U.S. (Tomlinson et. al, 2007). Three confirmed deaths were connected with the outbreak (CDC, 2006) and the spinach industry suffered major economic consequences that included a reduction in sales of fresh and processed spinach by at least 20 percent in Texas alone (Tomlinson et. al, 2007). According to California State Senator, George Runner, the economic impacts of the spinach recall due to the outbreak had the greatest effect on California's economy (Runner, 2007). Seventy-

five percent of all harvested acreage of lettuce and fresh spinach in the United States is located in California. He further stated that "the total acreage utilized in California for leafy greens is around 278,000 acres, which is valued at $1.3 billion annually." In 2005, fresh spinach sales totaled $157 million, which was only seven percent of the total sale of all leafy greens. According to the United Fresh Produce Association, the estimated losses from processors alone were between $50 and $100 million. This figure does not include losses from spinach growers or retailers, or from other fresh-cut products. The recall heavily impacted the sale of other fresh cut products as well. After September 2006, salad mix sales declined about 50%.

Since there is a high incidence of naturally occurring outbreaks, the intentional release of a pathogen at a single locale could be mistaken for a natural outbreak and not recognized as a criminal act (Wheelis, 2000). Additionally, a pathogen could be introduced into animal feed or fertilizers, creating a multiple site outbreak that spans a large area (Wheelis, 2000). Section 126 of the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 addresses the evaluation of new and emerging technologies relevant to bioterrorist attacks and other public health emergencies. Genomic technologies used in the development of new vaccines, pesticides, and diagnostic reagents may be useful also detecting or countering a bioterrorist attack (Wheelis, 2000).

Microbial forensics is an emerging field that encompasses "the detection of reliably measured molecular variations between related microbial strains and their use to infer origin, relationships, or transmission route of a particular isolate" (Cummings and Relman, 2002). Several programs exist for detection and surveillance of agricultural

crops pests and biological control agents. However, ideal preparedness would incorporate protocols and instrumentation currently present in crime laboratories that would allow strains of bacteria to be genotyped as a part of the investigation of suspected agroterrorism incidents.

Amplified fragment length polymorphism (AFLP) mapping is a molecular DNA analysis technique that involves restriction nuclease digestion and polymerase chain reaction to amplify thousands of genomic DNA fragments, some of which are polymorphic (Hoyle, 2006). The profile of DNA fragments produced using AFLP can be unique for an individual organism and therefore serve as specific identifiers in a manner similar to that of a fingerprint. AFLP mapping has advantages over other DNA typing techniques in that it requires a small amount of DNA, does not require information about the nucleotide sequence of the genome, and is more reproducible than other "generic" DNA typing techniques.

Serratia marcescens is a gram-negative, rod-shaped bacterium (Buchholz, 2004). It occupies several environmental niches including soil and water, as well as human, animal, insect, and plant hosts (Bruton et al., 2003). *S. marcescens* can be pathogenic and causes cucurbit yellow vine disease (CYVD) which causes heavy losses to watermelon, pumpkin, cantaloupe, and squash production in the United States (Bruton et al., 2003). *S. marcescens* can be transmitted by the squash bug, *Anasa tristis* (Bruton et al.., 2003), which is capable of infecting almost all cucurbits (Capinera 2003). It usually feeds on the foliage, and sometimes the fruit of cucurbits. While feeding the squash bug pierces the plant and sucks out the sap, while secreting highly toxic saliva into the plant (Hitchner and Kuhar, 2005). As a result, the foliage wilts and dies after being upon and

"the amount of damage occurring on a plant is directly proportional to the density of squash bugs" (Capinera 2003). Geographically, *S. marcescens* is widely distributed across the U.S.; it is found in midwestern states like Texas and Oklahoma and as far as north and east as Connecticut.

Annual grower income in Texas and Oklahoma from cucurbit production is over $100 million (Bruton et al., 1998). In the fields that are affected with CYVD the loss can range from 5% to 100%. Symptoms of infected plants usually include stunting, yellowing, and steady decline that starts 10 to 14 days pre harvest (Bruton et al., 2003).

S. marcescens is a good model for a bacterial strain diversity study because of the variety of ecological niches it can inhabit and the fact that it is found in many geographical locations. Thus, *S. marcescens* is a good model source of genomic DNA to evaluate the suitability of AFLP mapping as a molecular tool for discriminating different strains of bacteria. Moreover, if AFLP is effective for genotyping *S. marcescens* strains, then it can reasonably be assumed this technique could also be suitable for DNA typing of other plant pathogens as well. This study will consist of an evaluation of the suitability of AFLP for genotyping and discriminating strains of *S. marcescens*. In addition methods to communicate the characteristics of the AFLP profile were investigated through the development of a binary code.

We hypothesize that AFLP mapping technology is suitable for forensic investigation to characterize and attribute bacterial pathogens. *Serratia marcescens* was selected as our model pathogen.

Research questions

Can AFLP mapping technology be used with current instrumentation in crime laboratories?

Are the results that are obtained from AFLP technology reproducible?

Are the AFLP profiles discriminatory?

CHAPTER II

REVIEW OF LITERATURE

Preparing for an Agroterrorist Attack

The Public Health Security and Bioterrorism Preparedness and Response Act of 2002 contains provisions that address; expanding the Food and Drug Administration's authority over manufacturing and imported goods, tightening the control of biological agents and toxins, authorizing increased security at United States Department of Agriculture facilities, and imposes criminal penalties for using animals and select agents for terrorist attacks (FDA, 2002). The Homeland Security Presidential Directive 7 (HSPD-7) of 2003; Critical Infrastructure Identification, Prioritization, and Protection directed agencies to protect the national critical infrastructure by developing plans to deter, and mitigate a terrorist threat (Bush, 2003).

In 2004 the Defense of United States Agriculture and Food, Homeland Security Presidential Directive 9 (HSPD-9) was released. HSPD-9 provides directives for several agencies including the Department of Homeland Security (DHS), USDA, Health and Human Services (HHS), the Environmental Protection Agency (EPA) and the Central Intelligence Agency (CIA) to work together to prepare for , protect against, respond to, and recover from an agroterrorist attack (Monke, 2004).

HSPD-9 states that nationwide laboratory networks should be established to work in conjunction with State and Federal laboratory resources to develop diagnostic protocols and procedures that address food, veterinary health, plant health, and water quality (Bush, 2004). Item 23 in HSPD-9, regarding Research and Development, instructs administrators of the previously mentioned agencies to develop new technologies and countermeasures to respond to the intentional introduction or natural occurrence of catastrophic animal, plant, and zoonotic diseases (Bush, 2004). The purpose of Item 23 is to create methods for detection, prevention, agent classification, and assessment of dose response relationships among highly destructive agents that affect food and water (Bush, 2004).

Legislation exists to address an agroterrorist attack, and the National Research Council suggests an approach that includes deterrence and prevention, detection and response, and recovery and management (Monke, 2004). This study focuses on the second aspect of the Directive, detection and response. According to Monke, in order to effectively detect an agroterrorist attack, there must be a heightened sense of awareness and the ability to quickly determine the level of threat.

Several factors must be considered in preparation for an agroterrorist attack. These include the documentation of disease characteristics, sampling potential crime scenes, identifying the pathogen (s), selecting an appropriate response, identifying the suspected source of the pathogen, and either attributing or excluding that source to a particular pathogen (Nutter and Madden, 2005).

Disease management for many crops relies heavily on accurate identification and early detection (Le`vesque, 2001). Unfortunately, using morphological characteristics to

identify plant pathogens can be laborious and requires a background in taxonomy. On the other hand, molecular based technique can accurately and quickly provide results that can be used for disease management. According to Le`vesque there are two major types of technology that can be used for molecular detection of plant pathogens; antibody-based detection and DNA based detection . Some of the antibody based methods that have been used to identify viral and bacterial plant pathogens include ELISA (enzyme-linked immunosorbent assay) and indirect fluorescent antibody staining (Fletcher et al., 2006).

Numerous DNA typing methods exist that can be used to genotype pathogens. These methods can be traditional, specific, or generic (Ward et al., 2004, Fletcher et al., 2006, Toth et al., 1999, Najam et al., 2003). Within the categories mentioned above, typing methods can be immunologically or molecularly based. This study reviews five molecular based techniques that have been used to genotype plant pathogens. They include: Restriction length fragment polymorphism analysis (RFLP), random amplified polymorphic DNA, (RAPD), simple sequence repeats (SSR), suppressive subtractive hybridization (SSH), DNA-DNA Hybridization and amplified fragment length polymorphism (AFLP) (Waleron et al., 2002, Udupa et al., 1998, Mace et al., 2006, Huang et al., 2007, Zhang et al., 2003, Groenewald et al., 2005).

Immunological Techniques versus Molecular Detection Techniques

When immunological and molecular genetic techniques are compared in this context there are several advantages and disadvantages associated with each. In a comparison study performed by Ward et al. (2004), PCR-based technologies can be quickly developed, whereas immunological techniques are time-consuming and

developing and identifying antibodies with required specificity can be expensive. Sample preparation for PCR-based technologies usually requires minimal preparation and unprocessed DNA can be used (although purification may be necessary). On the other hand, sample preparation for antibody based techniques, can be minimal to complex depending on the plant material and the presence of other substances that might inhibit antibody-antigen interactions (Ward et al., 2004). PCR based techniques are usually complex, require training, measures must be taken to avoid contamination, and the assay can be performed in one day. Immunological techniques can be complex, can be also be performed in one day, and some assays provide results in as little as 15 minutes. With respect to portability, some antibody based assays can often be used in the field, whereas the majority of PCR based technologies must be performed in the laboratory. Immunological assays offer good specificity for viruses, however, cross reactivity between fungal species is problematic (Ward et al., 2004). PCR-based techniques offer excellent specificity which can be tailored to be genus, species or isolate specific. Immunological techniques generally are less sensitive than PCR-based technologies. Some formats of antibody-based techniques are directly quantitative, while PCR-based techniques may be tedious to quantitate unless real-time PCR is used. It is difficult to detect multiple pathogens using immunological techniques; however, detection using multiplex PCR is relatively easy. An immunological assay can generally only be used to detect living material and may require a specific morphological form of a pathogen. PCR-based techniques can usually be used detect to both living and non-living material (with the exception of reverse transcriptase PCR) in all morphological forms. RT-PCR can be used only on living organisms. Lastly, some immunological assays are relatively

inexpensive, while PCR-based techniques can be expensive. This is particularly true for real time PCR.

Table 1 contains a summary of the advantages and disadvantages of both techniques (Ward et al., 2004).

Table 1. *Comparison of immunological and PCR techniques for the diagnosis and detection of plant pathogens*

Process	Immunological techniques	PCR techniques
Ease of development	Time-consuming and costly to develop and identify antibodies with the required specificity.	Rapid if sequence information (e.g. rDNA sequences or specific gene sequence) is available. Alternatively, techniques such as RAPDs can be used to quickly identify species-specific markers.
Sample preparation	Minimal to complex depending on plant material and presence of substances which may inhibit antibody-antigen interactions.	Minimal, crude DNA preparations can be used in many cases, however DNA purification may be required.
Procedure	Generally complex but some formats can be used by non-specialists. Can be performed in one day and some immunodiagnostic kits can give answers in 15 min.	Complex, requires expert staff and appropriate measures should be taken to avoid cross-contamination of samples. Can be performed in 1 day.
Portability	Assays can be adapted for use in the field.	With a few exceptions[a] assays can only be done in a laboratory.
Specificity	Often good for viruses, but cross reactivity between fungal species is a common problem.	Excellent, technique can be designed to be genus-, species-, or isolate-specific.
Sensitivity	Varies depending upon amount of antigen produced by the pathogen. Generally less sensitive than nucleic acid-based techniques.	Excellent, can detect a single copy of the target DNA sequence which corresponds to a single cell of the target organism.
Quantification	Some formats (e.g. ELISA) are directly quantitative.	Quantification is laborious in standard PCR. Simple and rapid quantification is possible using real-time PCR.
Detection of multiple pathogens	Difficult.	Relatively easy using multiplex PCR.
Detection of viable organism	Usually detects only viable material and may be specific for particular morphological forms/ stages of the life cycle.	Generally will detect both living and dead material[b] and all morphological forms.
Costs	Some formats e.g. ELISA are very cost effective for high-throughput applications.	Expensive, particularly real-time PCR, although costs are decreasing with increased adoption of the technologies.

[a] See Schaad *et al.* (2002)
[b] Except reverse transcriptase PCR

Ward et al., 2004

Restriction Fragment Length Polymorphism Mapping

In 1978 David Botstein developed restriction fragment length polymorphism (RFLP) mapping (Holland, 2005). The technique is based on the size variation of DNA fragment

banding patterns stemming from differences in the spatial arrangement of restriction enzyme recognition sites. The patterns can be seen when restriction digests of DNA are separated by electrophoresis (Lefers, 2004). The presence or absence of certain restriction sites will define the length of a fragment (ASIco, 2006). The presence or absence of particular restriction fragments has been used to identify certain species of organisms or even subpopulations of individuals within a species (ASIco, 2006). RFLP is the oldest DNA- based technique for revealing polymorphic loci and can provide the highest degree of discrimination per locus (ASIco, 2006, Rudin and Inman, 2002). Disadvantages of this technique include the facts that large amounts of DNA are required, it can be difficult to find polymorphisms associated with a restriction site, and analysis of the results can be complicated (ASIco, 2006).

Researchers in Poland have genotyped bacteria formerly classified in the genus *Erwinia* using PCR-RFLP. *Erwinia* species are gram-negative, non-spore-forming, facultatively anaerobic, bacilli and are epiphytic or saprophytic plant pathogens (Waleron et al., 2002). Using *Rec*A PCR-RFLP, 177 strains of pectinolytic *Erwinia* from 19 different species were distinguished.

Random Amplified Polymorphic DNA

In 1990 Welsh and McClelland (1990) and Williams et al. (1990) developed random amplified polymorphic DNA (RAPD) (Lui et al., 2005). RAPD is a simple technique that randomly amplifies anonymous loci using PCR (ASIco, 2001). RAPD uses 10 base pair arbitrary primers to direct amplification of segments of genomic DNA, some of which are polymorphic (Baillie et al., 2005). Changes in the RAPD pattern of amplification

products are caused by sequence differences in the genomic DNA template that occur in the priming region or change the length of the DNA amplicons between the priming sites. As a result, RAPD analysis can provide a simple and reliable way of assessing genetic variation.

RAPD does not require prior knowledge of the DNA sequence of the target organism (Fani, 1993) However, it relies on a large intact DNA template sequence, which may not be available if the DNA is degraded (Wong, 2004). Other disadvantages of RAPD include lower resolving power for the complex collection of amplicon products when compared to other specifically targeted DNA targets in the genome, difficulty in reproducing results, and the subjective nature of determining the precise characteristics of bands on a gel (Wong, 2004).

Even with these limitations, RAPD is an inexpensive technique useful for strain typing of bacteria (Schiliro et al., 2001 and Wang et al., 1993). RAPD and microsatellites markers have been employed to genotype the pathogen A*scochyta rabiei,* which causes accochyta blight disease of the chickpea (Udupa et al., 1998).

Simple Sequence Repeats

Simple sequence repeats (SSR), also known as microsatellite or short tandem repeats, are simple DNA sequences repeated numerous times at different loci in an organism's DNA (ASIco, 2001). These repeats are variable thereby allowing the polymorphic loci to be used as markers for identification (ASIco, 2001). Assessments of, SSRs is less ambiguous than RAPDs and AFLPs, and unlike RFLPs can be performed with small amounts of DNA. However, use of SSR technology requires prior knowledge

of genome sequence (Rudin and Inman, 2002). An additional limitation of SSR typing is that not all microbial species contain simple sequence repeats in their genomes. Microsatellites are especially useful for analyzing forensic evidence that may degraded and/or contain limited amounts of DNA (Tamaki and Jeffreys, 2005). SSR analysis is very sensitive when compared to other methods and can recover information at the level of a single cell (Tamaki and Jeffreys, 2005).

Researchers have successfully used SSRs patterns as tools to detect molecular genetic diversity in cultivated groundnut (peanut) germplasm (Mace et al., 2006).

Suppressive Subtractive Hybridization

Suppressive subtractive hybridization (SSH) developed by CLONTECH Laboratories in 1996 (Diatchenko et al., 1996), is used to identify DNA fragments present in one organism and absent in another organism (Zhang et al., 2005). "The substrate for SSH is melted double-stranded (ds) cDNA (the tester) containing specifically expressed sequences to be extracted (the target) and melted ds cDNA lacking the target sequence (the driver) that is used for comparison (Evrogen, 2007). This method works well for two organisms that are closely related (Zhang et al., 2005). SSH was used at Oklahoma State University-Stillwater (OSU-Stillwater), to identify two gene clusters present in strains of *S. marcescens* that cause CYVD (cucurbit yellow vine disease), that were not present in closely related strains of *S. marcescens* that are non-phytopathogenic. This technique is useful for identifying molecular markers; however, only two markers can typically be compared in one SSH and the results depend on the efficacy of the ligating adaptors (Huang et al., 2007).

DNA-DNA Hybridization

DNA-DNA hybridization was developed by Sibley and Alqhuist in the 1980s (Guerra and Speed, 1996). "DNA-DNA hybridization measures the degree of genetic similarity between complete genomes by measuring the amount of heat required to melt the hydrogen bonds between the base pairs that form links between the two strands of duplex DNA" (Sibley and Ahlquist, 1994). This technique can be used to compare two DNA strands of an individual or of different individuals. The conditions of the experiment allow a "hybrid" of the double stranded DNA to be formed from the single strands of DNA from two species. By melting the hybrid molecules in a thermal gradient using regulated conditions the melting temperature of the hybrid duplex can be calculated. The parameters of the experiment are set up such that only homologous sequences can form double-stranded DNA products. DNA-DNA hybridization and rep-PCR were used at OSU-Stillwater to compare 29 strains of *Serratia marcescens* that cause CYVD (Zhang et al., 2003). Their results revealed 100% similarity among these strains. This method was also useful in discriminating strains associated with CYVD and those strains of *S. marcescens* not associated with CYVD. DNA-DNA hybridization is efficacious because it provides greater discrimination when compared to 16S rDNA sequencing. However it is unpopular because pairwise cross hybridizations can be laborious, isotopes are required, and it is impossible to establish a central database (Cho and Tiedje, 2001).

Amplified Fragment Length Polymorphism

In the early 1990s Keygene N.V research company developed AFLP technology (Witterndorp, 2007), a technique that produces DNA fingerprints from restriction

fragments of genomic DNA through PCR amplification (Vos et al., 1995). AFLP analysis is similar to RFLP analysis, but is less laborious (Groenewald et al., 2005). DNA is first cut with two restriction enzymes, a rare cutter *EcoRI* and a frequent cutter *MseI* that generate restriction fragments for amplification. In general the restriction fragments produced have a rare cutter sequence at one end and a frequent cutter sequence at the other end. Oligonucleotide linkers are then ligated to the sticky ends of the restriction fragments that have target sites for PCR primers to direct fragment amplification. Amplification of a subset of the restriction fragments sequence occurs during a second PCR reaction that uses primers complimentary to the linker and restriction site sequences, but extended one to two nucleotides into the restriction fragment; this applies selectivity to the amplification strategy and reduces the complexity of the resulting profile (Vos et al., 1995).

The primers used in AFLP are usually 17 to 21 nucleotides long and anneal completely to their target sequences (Vos et al., 1995). Small variations in the amplification parameters such as thermal cyclers programming, template concentration, or PCR profile do not affect the AFLP therefore making it a reliable and robust technique (Witterndorp, 2007).

Figure 1. The selective principle of the AFLP technology. (Keygene)

AFLP technology has been used worldwide and is one of the most popular genetic fingerprinting technologies. In India, researchers used AFLP to asses the genetic variability in pearl millet downy mildew (*Sclerospora graminicola*) (Singru et al., 2002). Fourteen AFLP primer combinations produced 184 polymorphic bands of 19 fungal isolates. Selective amplification primers included five *EcoRI* primers with two selective nucleotides and seven *MseI* primers with three selective nucleotides.

In South Africa, AFLP technology was used to genotype Foc (*fusarium oxysporum f. sp cubense*) isolates that cause Fusarium wilt of bananas (Groenewald et al., 2005). In this study seven genotypic groups were identified using 5 primer pairs.

AFLP is advantageous when compared to other techniques because it rapidly and reproducibly generates hundreds of highly reproducible markers of any organism (Mueller and Wolfenbarger, 1999). AFLP is time and cost efficient, reproducible, and provides superior discrimination for minimal effort when compared to RAPDs, RFLPs,

and microsatellites. However, one disadvantage of AFLP is that "scoring of the presence or absence of an AFLP band of dizygotic organisms yields dominant markers, and accurate quantitation of band intensities and special software are needed to discriminate homozygotic and heterozygotic signals for codominant scoring" (Savelkoul et al., 1999). Table 2 contains comparative data for AFLP, RAPD, SSR, and RFLP.

Table 2: Comparison of techniques that generate genetic markers

Criterion [a]	AFLP	RAPD	SSR	RFLP
Quantity of information	High	High	High	Low
Replicability	High	Variable	High	High
Resolution of genetic differences	High	Moderate	High	High
Ease of use and development	Moderate [b]	Easy	Difficult	Difficult
Development time	Short	Short	Long	Long

The scoring scheme follows closely those in Hillis et al..(2) and Karp and Edwards (49).
b Analysis of amplified fragment length polymorphism (AFLP) markers is easy with the help of an automated
genotyper, or when using low-resolution agarose gel electrophoresis (24), but manual polyacrylamide electrophoresis requires a certain amount of experience.
(Mueller and Wolfenbarger, 1999).

CHAPTER III

METHODOLOGY

Twelve bacterial cultures of *S. marcescens* (ATCC-13880, ATCC-29844, db11, H01-A, P01-A, R02-A, 731-31, 90-166, W01-A, W01-C, Z01-A, and Z01-B) were obtained (kindly provided by Dr. Jacqueline Fletcher, Dept. Entomology & Plant Pathology, Oklahoma State University).

DNA Isolation

Using a sterile loop, a colony of each strain was inoculated into a tube containing 2 mL of Brain Heart Infusion Broth (Becton, Dickinson and Company Sparks, MD). The tubes were incubated with shaking overnight at 37^{o} C to obtain stationary-phase growth. After incubation, a 1 mL aliquot of each culture was centrifuged at 10,000 xg for three minutes at room temperature to obtain the cellular pellet. The supernatant was removed without disturbing the pellet.

DNA Extraction

The bacterial pellet was resuspended in 500 uL of extraction buffer that consisted of 0.02g of lysozyme dissolved 1mL of TNE (10mM Tris-Cl pH8.3, 0.2MNaCl, 1mM EDTA). The pellet was incubated at room temperature for ten minutes. A volume of

25uL of 20% sodium dodecyl sulfate (SDS) and 25 uL of 20 mg/mL of Protease K (in 10mM Tris-Cl, 20mM $CaCL_2$, 50% glycerol) were then added to each lysozyme-treated bacterial cell pellet. Samples were incubated for 1 hour at 65°C.

Organic extraction was performed on each extraction using an equal volume of phenol:chloroform/isoamyl alcohol (9:0.96:0.4). Samples were centrifuged at 10,000 xg for 3 minutes to separate the phases. The aqueous layer, that contained genetic material was removed and placed in a clean tube. Samples were then extracted with an equal volume of chloroform:isoamyl alcohol (24:1), centrifuged at 10,000 xg for 3 minutes and the aqueous layer was removed and placed in a clean tube.

Two volumes of 95% ethanol were then added to each sample to precipitate the DNA. The sample was vortexed and a sterile disposable inoculating loop was used to remove the fibrous clot of DNA to a clean tube containing 40 uL of TE^{-4} (10mM Tris-Cl, pH 8.0, 0.1mM EDTA). Table 3 lists each sample, its concentration, and the *Serratia* strain from which it was isolated.

DNA Quantitation

The quantity of DNA from each sample was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Two uL of each sample was placed in the UV light path of the spectrophotometer and the amount of DNA was estimated assuming 1.0 A_{260} equals 50 ug/ml of double stranded DNA.

Table 3: Isolates of *Serratia marcescens* used to compare genetic diversity

Isolate	**DNA ng/uL**	**Source of Strain**
ATCC-13880	1702.28	Pond water
ATCC-29844[1]	934.04	Spring water
db11	765.69	Drosophila melanogaster
H01-A	667.41	Human
P01-A	188.63	Pumpkin
R02-A	841.90	Rice endophyte
W01-A	103.17	Watermelon
W01-C	562.51	Watermelon
Z01-A	572.73	Zucchini
Z01-B	510.15	Zucchini
7-31-1	452.24	Unknown insect
90-166	1766.35	Cotton root endophyte

[1] ATCC-29844 is *Serratia fonticola*; all other strains are *Serratia marcescens*

DNA Digestion

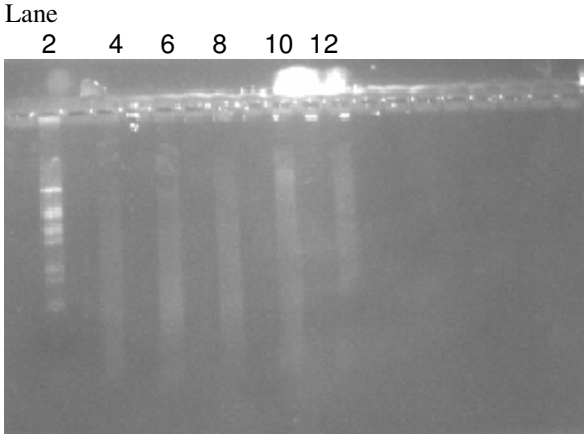
Samples of DNA were digested with *EcoRI* and *MseI* using a sequential digestion approach. The first digestion consisted of approximately 500 ng of DNA (this amount varied slightly per strain based on the concentration of DNA) 1 uL of *MseI* at 10,000 u/ml (New England BioLabs Inc., Ipswich, MA), 2 uL of NEB buffer (New England BioLabs Inc); and dH_2O was added to a the final volume of 20 uL. Samples were incubated in a 37^{o}C water bath for 1 hour then incubated at 65^{o}C heat block for five minutes to inactivate further enzyme activity, and finally placed on ice for five minutes.

The second digestion consisted of 20 uL from the first digest, 1uL of *EcoRI* at 20,000 U/ml (New England BioLabs Inc.), 5 uL of *EcoRI* buffer (New England BioLabs Inc.), and 24 uL of dH_2O to a final volume of 50 uL. Samples were again incubated for 1 hour at 37°C, and then at 65°C heat block for five minutes to inactivate the *EcoRI* and finally placed on ice for five minutes.

To assess the digestion, samples were electrophoresed on a 0.8% agarose gel, equilibrated and run in TAE buffer (10mM Tries-acetate pH 8.3 with 1mM EDTA). One uL of BstE II size standards (~ 250 ng) (New England BioLabs Inc.) and plus 19 uL of 1X loading buffer was added to one well. In the remaining wells, 20 uL of digest was mixed with 5 uL of 5x loading buffer dye (0.25% bromphenol blue, 0.25% xylene cyanol, in 5X TAE with 25% Ficol). After placing the gel into the gel box, it was completely immersed in 1X TAE Buffer. Electrophoresis was carried out at 65 volts for 90 minutes.

To visualize the restriction digest, the gel was stained with ethidium bromide (0.2% in dH2O, J.T. Baker, Philipsburg, NJ). Two hundred uL of ethidium bromide, the agarose gel and TAE buffer were placed in a Pyrex baking dish at room temperature for 10 minutes. The gel was destained for 5-10 minutes with tap water, and examined on a UV transilluminator to view and photograph the digest.

Figure 2: Photograph of DNA digestion



Lane 2 is BstE II size standard; Lanes 4-12 are strains H01-A, ATCC-29844, R02-A, 7-31-1, and Z01-A, respectively

DNA Ligation

EcoRI and *MseI* restriction fragments were ligated to oligonulceotide adaptors supplied with the AFLP Microbial typing kit (Applied Biosystems, Foster City, CA), that would subsequently serve as priming sites for PCR amplification using the following steps: an enzyme master mix was prepared using 1 uL of 10x T4 ligase buffer, 1 uL of 0.5M NaCl, 1 uL of 10u/ uL *MseI*, 2.5 uL of 20 U/ul *EcoRI*, and 2.5 units of T4 DNA ligase at 400 u/ul (all from New England BioLabs Inc. Ipswich, MA), and 3.5 uL of dH2O. The enzyme master mix was stored on ice until used. In a new tube, 1 uL of double digested DNA, 1 uL of 10X T4 ligase buffer, 1 uL, 0.5M NaCl, 0.5 uL of Bovine serum albumin (BSA, New England BioLabs Inc. Ipswich, MA) at 10mg/ml, 1 uL of *MseI* adaptor, 1 uL of *EcoRI* adaptor, 1 uL of enzyme master mix from above and 3.5 uL of dH20 were combined. The samples were incubated in a 37^{o}C water bath for 2 hours and then placed at room temperature overnight. A volume 189 uL of TE^{-4} was added to each ligation reaction, which was stored at 2-6^{o}C if not immediately used.

AFLP-I – Preselective Amplification

The AFLP procedure was performed according to the Applied Biosystems AFLP Microbial Fingerprinting protocol (Applied Biosystems). In a 200 uL PCR reaction tube the following components were mixed: 4.0 uL of the diluted restriction-ligation reaction from above, 0.5uL of AFLP *EcoRI* core solution, 0.5 uL of AFLP *MseI* core solution, and 15 uL of AFLP Amplification core mix (all from Applied Biosystems, Foster City, CA) for a total volume of 20 uL. The samples were placed in the thermocycler at ambient temperature then amplified using the PCR cycling parameters shown in Table 4.

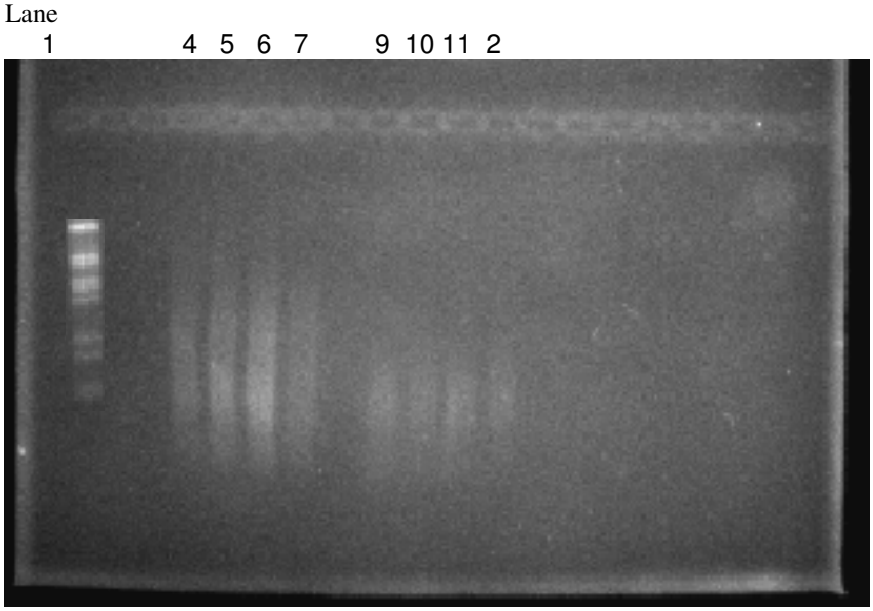
Table 4: Thermal cycler parameters for preselective amplification

HOLD	**CYCLE** **Each of 20 Cycles**			**HOLD**
72°C 2 min	94°C 20 sec.	56°C 30 sec.	72°C 2min.	4°C

Following the cycling program, 10 uL of the preselective product was combined with 190 uL of TE^{-4} and vortexed. The product was stored at 2-6°C if not immediately used.

The remaining 10 uL of the preselective product was combined with 10 uL 2X loading buffer and loaded onto a 0.8% agarose gel equilibrated in TAE buffer. Electrophoresis was at 60 volts for 90 minutes. The gel was stained with ethidium bromide and viewed as previously described. A hazy smear of amplification products seen in a gel track confirmed that the ligation step and the subsequent amplification of those modified restriction fragments were successful.

Figure 3: Photograph of DNA Digestion and Ligation



Lane 1 is *BstE II* size standard; Lanes 4-7 are digestion fragments for strains ATCC-29844, H01-A, R02-A, and 7-31-1, respectively. Lanes 9-12 are the ligation fragments for strains ATCC-29844, H01-A, R02-A, and 7-31-1, respectively.

AFLP-II – Selective amplification

The first PCR amplification step is non-selective in that all restriction fragments bearing adaptors on each end are suitable amplification targets. However, for large, complex genomes such as those in bacteria it is desirable to reduce the complexity of the AFLP profile to simplify analysis. Therefore, a second selective amplification reaction was included as a part of the AFLP typing process. Selectivity in the second PCR reaction results from the use of primers, identical to those used in the first pre-selective reaction, but also extending 1-2 nucleotides beyond the adaptor sequence into the restriction fragment. Thus, only those restriction fragments bearing the adaptor AND having a complimentary nucleotide sequence upstream from the adaptor will successfully bind the primer and be amplified. Depending upon the number of nucleotides extended

beyond the end of the adaptor, a 4-64 fold reduction in PCR products may be expected.

The following components were mixed in a PCR reaction tube: 1.5 uL of the diluted preselective amplification product, 0.5 uL *MseI* –A primer (2.5 pmoles), 0.5 uL dye labeled selective *EcoRI* primer (0.5 pmoles) labeled with one of three fluorescent dyes [NED (yellow), FAM (blue), or JOE (green)], and 7.5 uL of AFLP Core Amplification mix (all reagents from Applied Biosystems). The primer labeled with NED (yellow) has a one nucleotide extension of cytosine, that labeled with FAM (blue) has one nucleotide extension of adenosine and that labeled with JOE (green) has a one nucleotide extension of guanosine. Thus, three separate selective PCR reactions are prepared from each pre-selective PCR reaction. The samples were mixed and subjected to the cycling program shown in Table 5.

Table 5: Thermal cycler parameters for selective amplification

HOLD	**CYCLE**			**Number of Cycles**
94°C 2 min	94°C 20 sec.	66°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	65°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	64°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	63°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	62°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	61°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	60°C 30 sec.	72°C 2min.	1

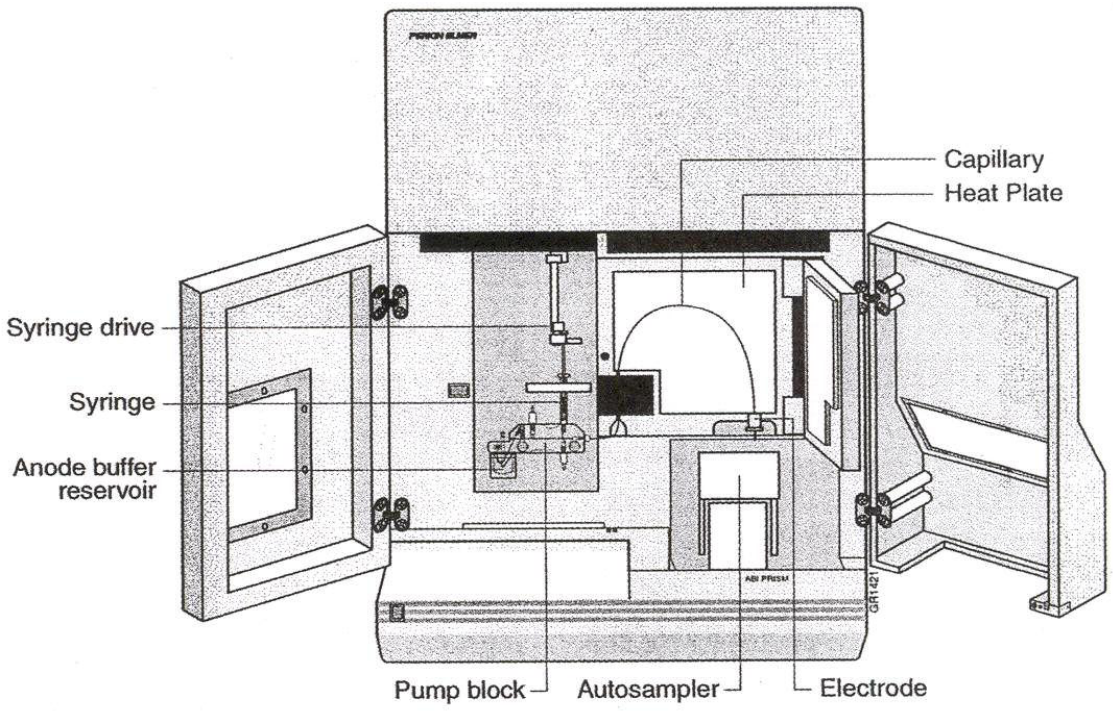
-	94°C 20 sec.	59°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	58°C 30 sec.	72°C 2min.	1
-	94°C 20 sec.	57°C 30 sec.	72°C 2min.	1
94°C 2 min	94°C 20 sec.	56°C 30 sec.	72°C 2min.	20
60°C 30 min		-		1
4°C forever		-		1

Capillary Electrophoresis

In a capillary electrophoresis (CE) system, such as the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), (Figure 4) the ends of the capillary are immersed in electrode buffers, which are connected to a high voltage power supply. The capillary is filled with a polymer buffer that acts as a molecular sieve. The sample is introduced into the capillary by replacing one of the buffer reservoirs with a sample. Electrophoresis is initiated and amplicons in the PCR reaction "electro-inject" into the capillary thereby loading the capillary for the run. Following injection, the capillary moves back to the buffer reservoir and electropheretic separation of the products occurs approximately over a 30 minute period (Butler, 2005). Capillary electrophoresis is advantageous over agarose gels for a variety of reasons including resolution, speed, and the ability to quantitate the amount of material in a sample (Butler, 2005). The AB1 310 Genetic Analyzer consists of a capillary, two vials of buffer, two electrodes are connected to a high-voltage power supply, a laser excitation source, a CCD camera, an autosampler that holds the sample tubes, and a computer that controls sample injection and stores data

from each run (Butler, 2005).

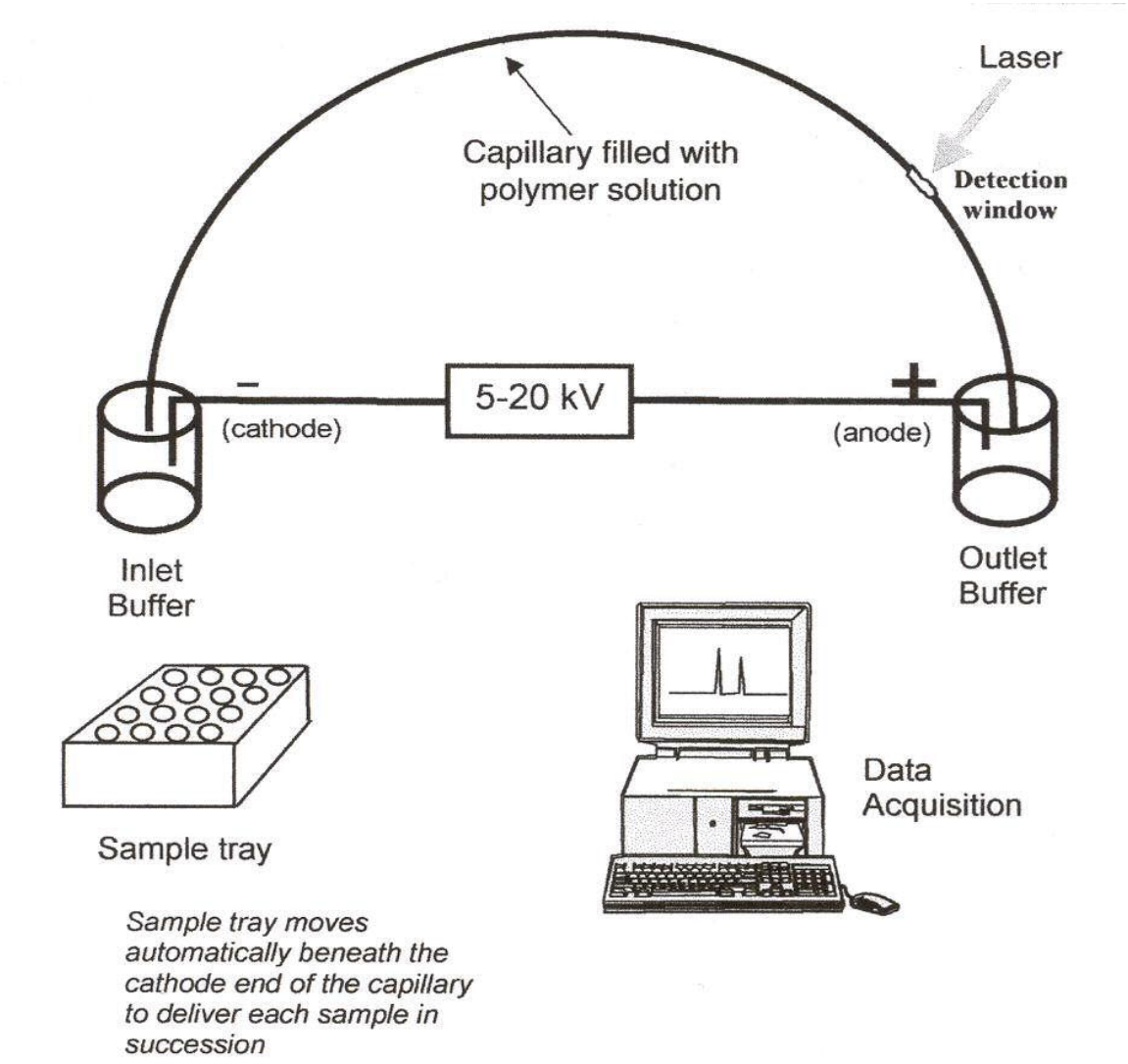
Figure 4: 310 Genetic Analyzer used for capillary electrophoresis (Budowle, 2000).



Once the selective amplification reaction was complete, samples to be analyzed were prepared as follows: In a tube 24.5 uL of Hi-Di Formamide, and 0.5 uL GeneScan-500 Liz size standards (both from Applied Biosystems), and 1.5 uL of selective amplified product were mixed together. The selective PCR products amplified with each selective primer (blue, green, and yellow) were pooled into a single sample tube. The tubes were placed in a 48-well sample tray in the ABI Prism 310 Genetic Analyzer (Applied Biosystems), each sample was electrophoresed for 28 minutes. The data from the CE are captured in the form of an electropherogram (Frazier et al., 2000). Electropherograms plot the migration of amplicons throughout the run that are detected as they pass a detection window in the capillary (Frazier et al., 2000). The detector response is based

on UV-visible absorbance or fluorescence and is usually concentration dependent (Frazier et al., 2000). The x axis of the electropherogram represents the fragment sizes in base pairs and the y axis shows the intensity of the fluorescence, (also called the relative fluorescence units) (RFU) (Jain et al., 2005).The process of capillary electrophoresis is depicted in Figure 5.

Figure 5: Capillary electrophoresis (Butler, 2005).

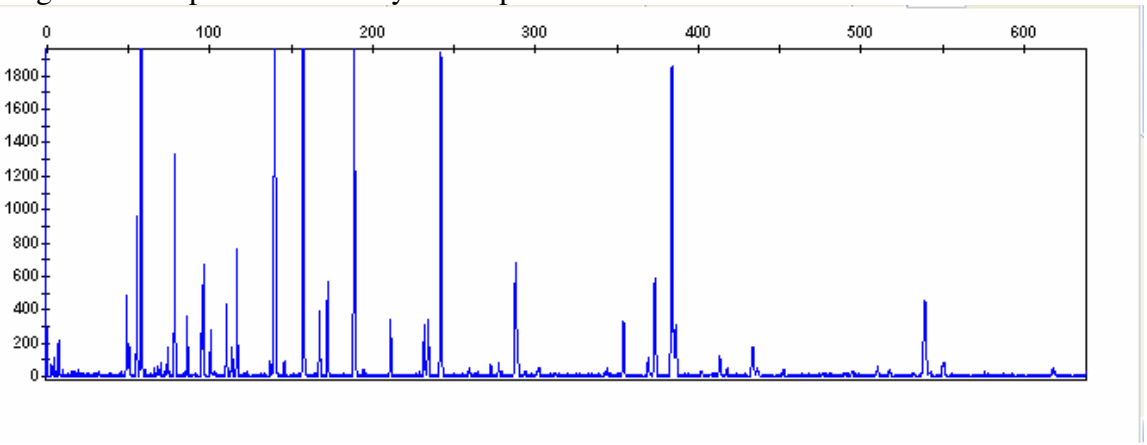


Data Analysis

The data contained in the electropherogram were analyzed using GeneMapper ID v3.2.1

software (Applied Biosystems). Figure 7 shows an electropherogram for *S. marcescens* train Z01-B for restriction fragments labeled with the FAM dye.

Figure 6: Electropherogram of Z01-B strain of *S. marcescens* The x-axis represents fragment base pair sizes. The y axis represents relative fluorescence units



Genemapper ID software calculates the base pair sizes and RFU peak heights. Using the program, the similarities and differences between samples can be compared. The size and peak data generated from the electropherogram were exported to Microsoft Excel. The data were studied and the AFLP profile was converted to a binary code using the following steps: amplicons of less than 100 bp were removed from the analysis as were as amplicons greater than 500 bp. The average rfu for the remaining fragments was calculated and set as a threshold. All amplified fragments containing rfu less than the threshold were eliminated from further analysis. The average rfu for the remaining fragments were then recalculated. Using this approach, only the most abundant restriction fragments in the original digest were included in the AFLP mapping process. This "filtering step" assisted with the reproducibility of the assay. Each individual fragment rfu was then divided by the recalculated average. Any value greater than 1.0 was designated "1" and values less 1.0 were designated "0." This simple coding scheme allowed the fluorescent intensity of each restriction fragment in each strain to be compared, resulting in a comparison of relatedness among strains. The binary code

generated from each strain was also used to determine the discriminatory power of the AFLP technique. Even though this method is useful in discriminating among strains of *S. marcescens* it does not incorporate the actual size of the amplicon and the rfu in to the code. In order to overcome this limitation, the entire size range of fragments included in the AFLP profile was divided into fixed 10 base pair size bins. If a restriction fragment included in the AFLP profile fell within a bin, a code designation of "1" was assigned; if a bin was empty a code of "0" was assigned. In order for this coding process to be effective we used the same start and stop points (restriction fragment size, of 100 bp and 350 bp respectively) for each strain.

CHAPTER IV

RESULTS

Our evaluation of the suitability of AFLP for genotyping strains of *S. marcescens* was, in part based on the feasibility of applying the technique with instrumentation currently present in crime laboratories. Other important considerations to be explored with AFLP mapping included reproducibility and the discriminatory capability of the assay. To investigate the utility of AFLP analysis as a forensic tool, selected strains of *Serratia macescens* were chosen as a model pathogen. Even though the strains are different they should be reasonably closely related genetically, which would allow us to assess the discriminatory power of AFLP analysis. *Serratia macescens* is an ideal pathogen because this bacterial species can be pathogenic to both humans and plants of agricultural importance.

AFLP technology was performed on twelve strains of *S. marcescens* to generate a "DNA fingerprint" for each strain. Twelve strains (ATCC-13880, ATCC-29844, H01-A, PO1-A, R02-A, W01-A, W01-C, Z01-A, Z01-B, 73-1-1, and 90-166) were evaluated. Successful profile generation was dependent on accurate DNA quantitation. In early attempts at producing DNA profiles the quantity of DNA was calculated using ethidium bromide stained yield gels that compared bacterial DNA with known concentrations of DNA from lambda phage. Although yield gels are acceptable for quantitating DNA for

some procedures, the technique provides only a "ballpark" estimate and is very subjective to the person comparing ethidium bromide fluorescence in an unknown with one of the lambda standards.

Early attempts to produce AFLP profiles also identified the amount of T4 DNA ligase needed to ligate adaptors to restriction fragments as a key step in the procedure. The protocol requires 100 weiss units of T4 DNA ligase, however the T4 DNA ligase purchased from New England Biolabs is in cohesive units. 100 weiss units are equal to 67 cohesive units. After increasing the amount of T4 DNA ligase ten fold for ligation reactions, reproducible AFLP profiles were produced.

Feasibility with Crime Laboratory Instrumentation

All of the instruments with the exception of the spectrophotometer are equipment that are commonly found in a DNA crime laboratory. While an accurate quantitation of DNA is crucial for reproducibly producing an AFLP profile, other techniques besides a spectrophotometer, like real time PCR or even a yield gel with careful analysis, can be used to accurately estimate the amount of DNA present. The two PCR steps used in our analysis were completed using a thermocycler and a genetic analyzer that are both normally present in a DNA crime laboratory.

Reproducibility

AFLP technology is known for being highly reproducible and was found to be reproducible in this study as well. AFLP reactions were performed at least twice for each strain. In general, 87% of all AFLP fragments were reproducibly produced in replicate assays. For example AFLP typing was performed twice on strain db11, isolated from *Drosophila melanogaster* and data from each of the fluorescently-labeled primers (FAM,

JOE, and NED) showed nearly identical amplicon sizes and peak heights in AFLP profiles. Although the results were very reproducible, we occasionally encountered problems with "pull-up" peaks. According to Carrie Rowland, of Forensic Bioinformatics, "pull-up" peaks occur when the analysis software fails to discriminate between the different dye colors used while interpreting sample results (Rowland, 2006). "A signal from a locus labeled with blue dye, for example, might also mistakenly be interpreted as yellow or green signal, thereby creating false peaks at the yellow or green loci" (Rowland, 2006). "Pull-up" peaks can be identified through careful analysis of the size of amplicons across the color spectrum (Rowland, 2006). The "pull-up" peak height is usually 10-30% less than the "true peak."

Table 6 shows the components of the AFLP profile that were generated by strain db11 for the 1st and 2nd reactions after removing "pull-up" peaks and peaks that had a weak signal (i.e., fell below the threshold). For the blue primer, (FAM), the size differences among amplicons in the replicates were 0.25 base pairs or less. For the green primer, (JOE), the size differences among amplicons in the replicates were 0.97 base pairs or less. For the yellow primer, (NED), the size differences among amplicons in the replicates were 0.24 base pairs or less.

Table 6: 1st and 2nd assay results AFLP profiles blue, green, and yellow primers for db11

FAM		JOE		NED	
1st Size	2nd Size	1st Size	2nd Size	1st Size	2nd Size
38.95	38.82	48.93	49.16	69.96	69.94
46.46	46.52	59.44	59.49	76.5	76.69
57.73	57.81	67.7	67.93	86.26	86.39
96.03	95.78	74.25	74.37	88.19	88.39
110.22	110.34	98.82	99.05	98.6	98.84
116.66	116.67	106.05	105.94	101.84	102
119.22	119.3	132.02	132.12	112.21	112.4
139.61	139.36	140.59	140.57	125.29	125.27
151.72	151.47	163.47	163.32	132.69	132.45
178.69	178.93	171.97	172.07	198.33	198.47
188.5	188.71	184.91	184.94	283.75	283.6
246.42	245.87	210.62	210.14		
328.27	328.19	234.1	233.92		
373.07	372.87	253	252.98		
470.41	469.36	302.08	301.8		
		304.84	303.87		
		342.76	342.41		
		376.07	375.23		
		433.86	433.11		

AFLP was performed 3 times on strain Z01-A, a CYVD strain isolated from zucchini. For the three primers, the size differences among replicates were 0.52 base pairs or less for FAM, (blue), 0.74 base pairs or less for JOE (green) and 0.66 base pairs or less for NED (yellow). Results in Table 7 records the values obtained for peak sizes after removing "pull-up" amplicons and peaks with a weak signal.

Table 7: 1st, 2nd and 3rd assay results AFLP profiles blue, green, and yellow primers for Z01-A

FAM			JOE			NED		
1ST	2ND	3RD	1ST	2ND	3RD	1ST	2ND	3RD
55.13	54.96	55.02	49.15	48.92	48.91	99.15	98.49	98.58
57.91	57.65	57.75	74.36	74.25	74.24	110.25	110.07	110.05
78.42	78.23	78.27	86.11	85.98	86.02	112.23	112.08	112.08
95.09	94.93	94.97	98.93	98.81	98.8	114.65	114.53	114.55
96.05	95.79	95.96	100.4	100.31	100.31	125.24	125.16	125.08

110.25	110.07	110.05	172.15	172.15	172.17	135.17	135.11	135.05
116.65	116.56	116.5	210.9	210.88	210.93	166.67	166.68	166.76
139.86	139.62	139.76	233.9	233.87	234.02	188.14	188.07	188.16
157.34	157.27	157.36	272.09	272	272.42	220.89	220.95	221.03
188.48	188.53	188.51	353.96	353.8	354.01			
241.65	241.67	241.8	368.91	368.7	369.09			
287.67	287.79	287.65	413.19	412.91	413.21			
373.22	373.09	373.3	417.22	416.88	417.38			
383.66	383.35	383.85	433.35	433.04	433.35			
386.22	385.7	386.1	452.2	451.7	452.44			

Occasionally, AFLP products were observed in one replicate and not in the other. Such spurious discrepancies can most likely be attributed to incomplete digestion or ligation; this underscores the need to carefully monitor these steps in the AFLP typing process. Another aspect of the analysis process that contributed to the reproducibility in AFLP profiles is the use of a minimum fluorescent threshold for including fragments in the profiles. The method used here eliminates all but the strongest fluorescent signals captured by the genetic analyzer further contributing to reproducibility of the AFLP typing technique.

Genetic Relatedness

To evaluate the suitability of AFLP for genotyping strains of *S. marcescens* the genetic relatedness of 8 strains were evaluated. The eight strains of *Serratia* were: ATCC-29844 (spring water), dbll (insect), H01-A (human), R02-A (rice endophyte), W01-A (watermelon), Z01-A (zucchini), 7-31-1 (unknown insect), and 90-166 (cotton endophyte).

The amount of genetic relatedness was obtained by counting the number of AFLP fragments common amongst strains compared to the total number of fragments in the strains being compared. Table 8 summarizes the degree of genetic relatedness among the strains. Strain ATCC-29844 represents a different species of *Serratia* and therefore when compared to the other 7 strains exhibited 20% or less AFLP profile similarity was

seen. Since strain ATCC-29844 is species *fonticola* the decreased amount of genetic relatedness is to be expected. Strain db11, isolated from an insect showed 50% or less AFLP profile similarity to the other strains. Strain H01-A which was isolated from human exhibits 40% or less of genetic relatedness to the other strains while strain R02-A, a rice endophyte (an organism that lives within a plant without causing disease) had a genetic relatedness to several other strains as high as 68-70%. W01-A, a CYVD pathogen was isolated from watermelon and showed 100% genetic relatedness to Z01-A (and vice versa) another CYVD pathogen that was isolated from zucchini. Strain 7-31-1, that was isolated from an insect exhibited genetic relatedness of 43% or less when compared to the other seven strains. Lastly, strain 90-166 a cotton endophyte strain showed genetic relatedness as high as 68% when compared to the other strains.

Table 8: Genetic relatedness amongst eight strains

				Degree of Genetic Relatedness				
Strain:	**ATCC-29844**	**DB11**	**H01-A**	**R02-A**	**W01-A**	**Z01-A**	**7-31-1**	**90-166**
ATCC-29844	**1.00**	0.17	0.15	0.16	0.12	0.10	0.12	0.20
dB11	0.17	**1.00**	0.50	0.27	0.34	0.25	0.30	0.13
H01-A	0.15	0.50	**1.00**	0.32	0.31	0.27	0.40	0.20
R02-A	0.16	0.27	0.32	**1.00**	0.70	0.68	0.35	0.68
W01-A	0.12	0.34	0.31	0.70	**1.00**	**1.00**	0.37	0.55
Z01-A	0.10	0.25	0.27	0.68	**1.00**	**1.00**	0.43	0.47
7-31-1	0.12	0.30	0.40	0.35	0.37	0.43	**1.00**	0.19
90166	0.20	0.13	0.20	0.68	0.55	0.47	0.19	**1.00**

Legend: Genetic relatedness ≤ 15%
Genetic relatedness ≥ 50%

In general strain R02-A was more related to selected other strains when a comparison was made. Sixty eight percent genetic relatedness (observed twice) and 70% genetic relatedness was observed when strain R02-A was compared to Z01-A, 90-166, and W01-

A respectively. The close relatedness of R02-A confirms the finding of (Zhang et al., (2005) to the CYVD strains. In another study at OSU-Stillwater 16S rDNA and *gro*E sequence analysis revealed more than 97 % sequence similarity between strains W01-A and Z01-A, both of with are isolated from cucurbits with CYVD. (Rascoe et al., 2003). To assess diversity among *S. marcescens* populations within a single plant, Z01-A and Z01-B (different strains of *S. marcescens* isolated from the same plant) were compared. Table 9 contains data showing the genetic relatedness of the two different strains isolated from zucchini.

Table 9: Genetic relatedness of strains Z01-A and Z01-B

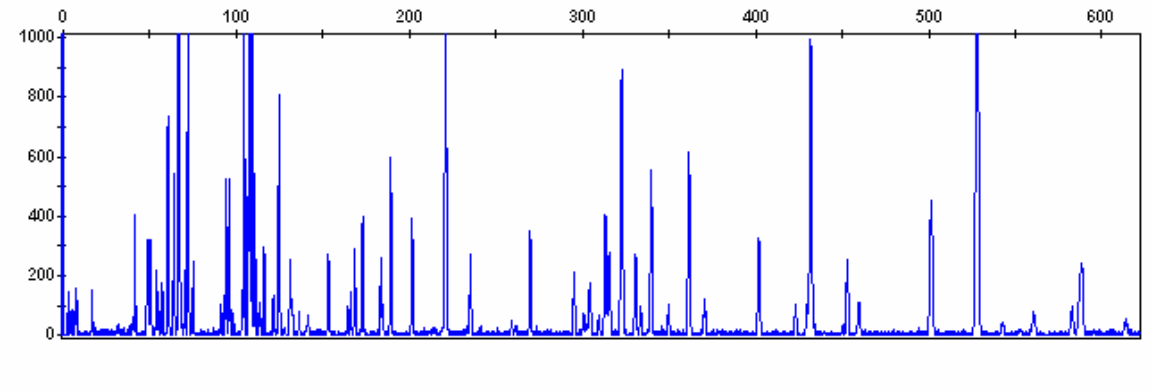
AFLP Fragment	Z01-A total fragments	Z01-B total fragments	# of identical fragments	Genetic Relatedness
FAM	13	17	8	47%
JOE	13	13	7	53%
NED	12	13	7	53%

Visual Comparison

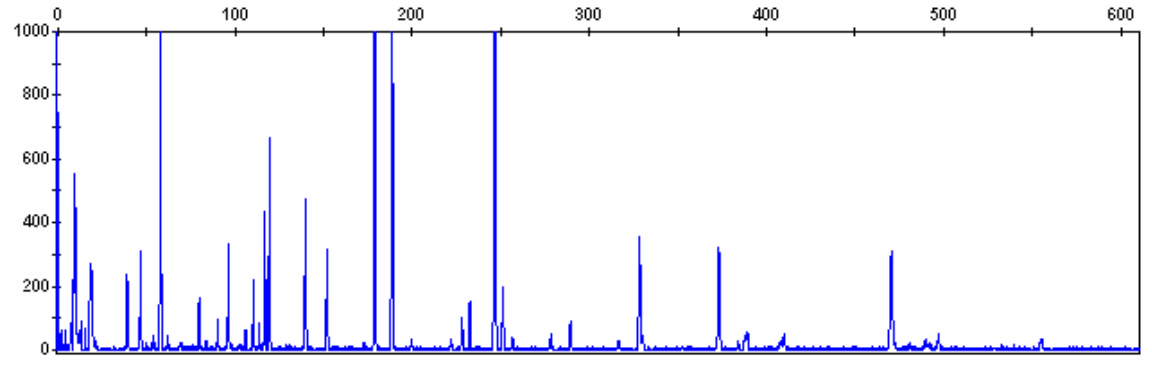
The electropherograms produced for each strain of *Serratia* were compared directly to evaluate the effectiveness of the AFLP technique. The AFLP profiles on the electropherogram captured enough variation to show the differences amongst strains. Figures 7-9 show the electropherograms of the AFLP products generated using the blue primer, FAM, which contains the selective nucleotide adenosine, the green primer, JOE, which has a nucleotide of guanosine and, the yellow primer, NED, which has a nucleotide of cytosine each for strains ATCC-29844, db11, and H01-A.. A visual comparison of the electropherogram shows that these strains can be clearly distinguished from each other.

Figure 7: Electropherograms for strains ATCC-29844, db11, and H01-A. The x-axis represents fragment base pair sizes. The y axis represents relative fluorescence units

ATCC-29844



db11



H01-A

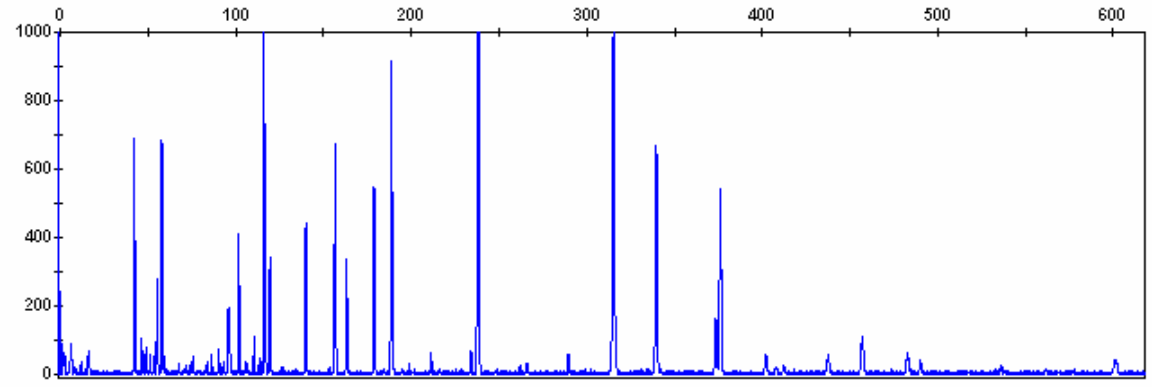
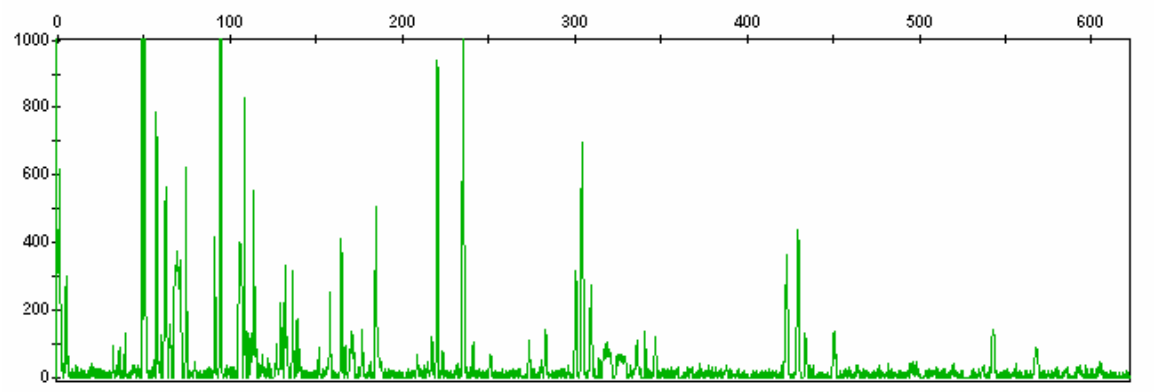
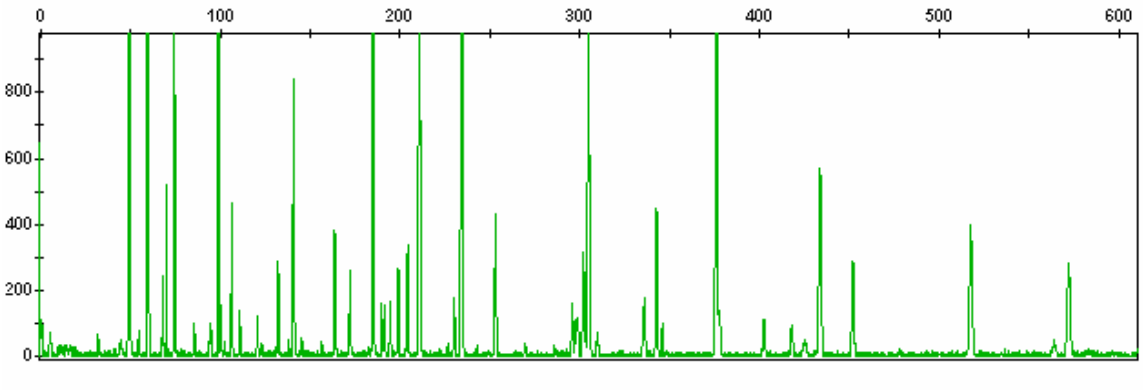


Figure 8: Electropherograms for strains ATCC-29844, db11, and H01-A for the green primer JOE. The x-axis represents fragment base pair sizes. The y axis represents relative fluorescence units.

ATCC-29844



db11



H01-A

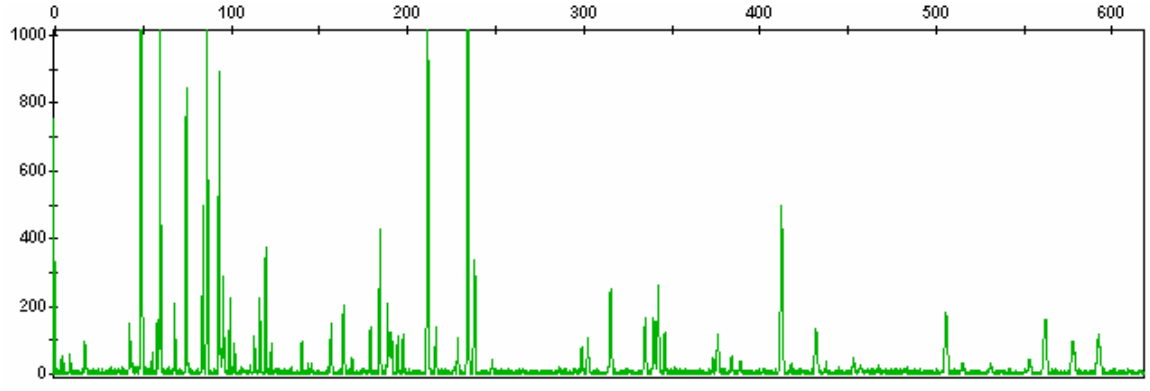
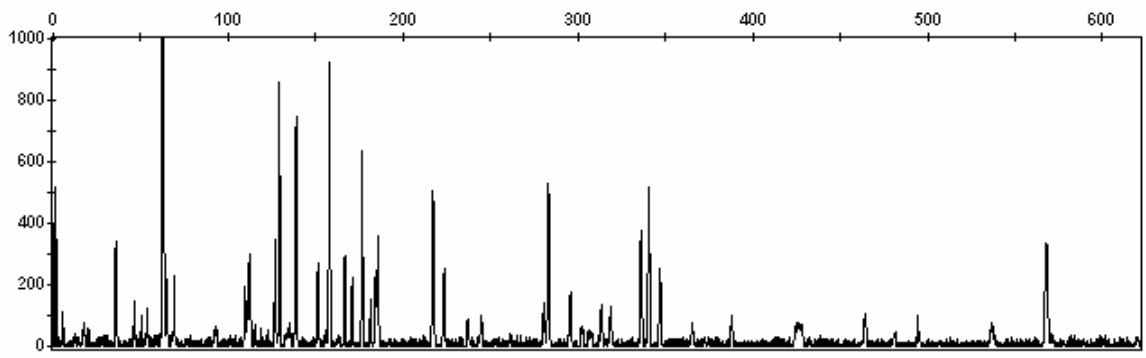
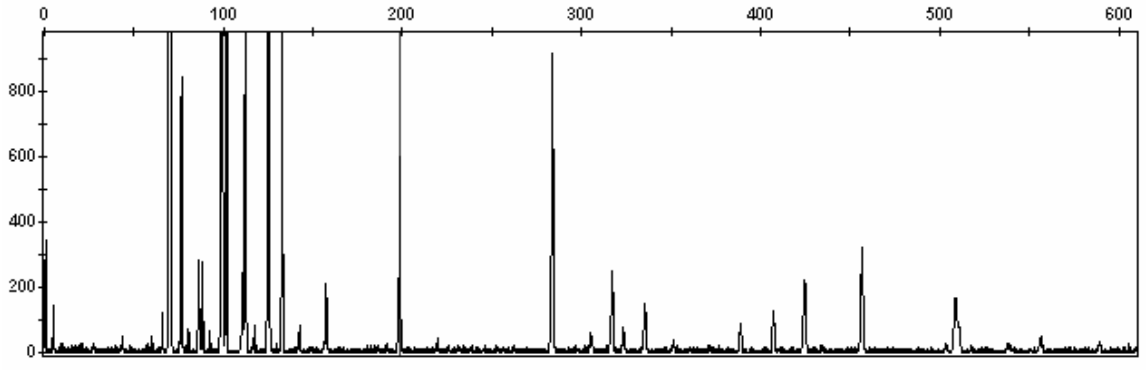


Figure 9: Electropherograms for strains ATCC-29844, db11, and H01-A for the yellow primer NED. The x-axis represents fragment base pair sizes. The y axis represents relative fluorescence units

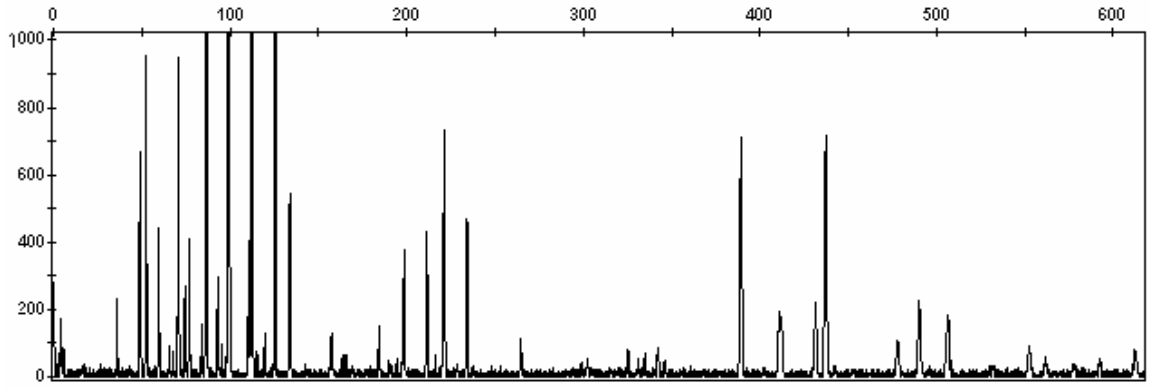
ATCC-29844



db11



H01-A



Discriminatory Power

The discriminatory power of AFLP typing can be assessed in several ways: one way is through a comparison of the binary codes developed for each strain. Each strain produces a unique code which allows them to be distinguished from one another. Table 10 shows all 12 strains of *Serratia* and the binary code used to describe their respective AFLP profiles.

Table 10: 12 strains of *Serratia* with binary code

ATCC-13880	ATCC-29844	db11	H01-A	P01-A	R02-A	W01-A	W01-C	Z01-A	Z01-B	73-1-1	90-166
0	1	0	0	1	1	1	1	1	0	0	0
0	1	0	0	0	0	0	0	0	1	1	0
1	1	1	1	0	1	1	0	1	0	0	0
0	0	0	0	0	0	1	0	0	1	1	1
1	0	1	0	0	0	0	1	0	0	1	1
1	0	1	1	1	0	0	0	0	1	1	0
0	0	0	1	1	1	0	0	1	0	0	0
1	0	1	1	1	0	1	0	0	0	0	1
1	1	1	1	1	1	1	0	0	0		1
0	1	1	1	0	0	0	1	0	1		0
1	1	0	0	1	0	1	0	1	0		
0	0	0	0	0	0	1	1	1	0		
0	1	0	0	0	0	0	0	0	0		
	1	0	0	0	0	0	0	0	1		
	1	0	0	0	0	0	0	0	1		
	1	0	0	0	0	0	0	0	1		
	0	0	0	0	0	0	0	0	0		
	0	0	0	0	0	0	0	0	0		
	0	0	0	0	0	0	0	0	0		

One limitation of the binary code developed without the consideration of specific size of each AFLP fragment is the possibility different amplicons from different strains may exhibit the same code. To compensate for this limitation, the entire size range of fragments included in the analysis of AFLP profiles were divided into fixed, 10 base pair sizes bins. If a restriction fragment included in the AFLP profile fell within a bin and reached the threshold for fluorescent intensity, a code designation of "1" was assigned: if a bin was empty or contained an amplicon below threshold, a code of "0" was assigned. As long as the starting point and stopping points for analysis of electropherograms was

uniform, a comparison of binary code among *Serratia* strains were much more meaningful. The AFLP profiles displayed as binary codes for the different strains of *Serratia* strains developed using this methodology is shown in the appendix 1.

CHAPTER V

DISCUSSION

The goals of this study were to evaluate the suitability of AFLP profiling for genotyping disparate strains of *S. marcescens* and to investigate the utility of a system of nomenclature using a binary code to capture and communicate DNA profiles of different strains. In the event of agroterrorist attacks in different areas, it is imperative to have a strategic plan for laboratories to use nationwide to communicate whether or not they may be working with the same isolate so it can be attributed to a source. Twelve strains of *S. marcescens* from different ecological niches were evaluated. The reproducibility, genetic relatedness among strains, and the overall discriminatory power of the technique were also examined. The AFLP profiles were reproducible as long as the amount of input genomic DNA was carefully controlled and restriction digestion and ligation reactions went to completion. Assessment of these steps in the AFLP protocol was performed using the subjective technique of agarose gel electrophoresis and ethidium bromide staining. While a lack of restriction digestion would be clearly revealed using agarose gels, partial digestion affecting the final AFLP profile would escape detection early in the protocol and might not be revealed even in the final result. Therefore, a more effective assessment method for digestion is needed. Possible modifications include incorporation into the digest of cloned linear DNA fragments with only a single *MseI* or *EcoRI* site in

their sequence that would escape amplification in later steps of the process. Digestion of such indicator fragments would document complete digestion of genomic DNA by allowing the visualization of an expected banding pattern as the indicator rather than a hazy smear of genomic restriction fragments. Such method was developed by Allen et al. (1989) for use as a digestion indicator for human genomic DNA being subjected to restriction digestion as part of the RFLP mapping process.

The second critical step affecting reproducibility is the ligation step. The efficiency of ligation depends both on the completeness of digestion with restriction enzyme and the T4 DNA ligase activity. The former is currently assessed using agarose gel electrophoresis of products from the non-selective PCR amplification step. If the hazy smear of amplicons is not visualized, a problem with ligation must be assumed because with failed ligation, primer binding sites for the first PCR reaction will not exist. It is possible that the cloned "indicator" DNA used to assess restriction digestion could also serve a role as an indicator of ligation as it recreates detectable amounts of intact DNA, visible on the agarose gel used to resolve amplicons from the non-selective PCR reaction.

Using AFLP typing method, strains of *S. marcescens* could be discriminated from each other by visually comparing profiles and by converting the profile into a binary code. We have therefore developed a system of nomenclature that can be shared among laboratories to communicate whether or not two strains might be identical. Although the current study did not incorporate a statistically significant number of strains to generate clear confidence levels, our hypothesis, to be tested in future research is that, if strains share the same binary code, they can be considered "positive" for a presumptive test of

the identity. Further side by side comparisons of the questioned strains would be needed to confirm the AFLP profiles identical characteristics.

Applications for AFLP include the ability to genotype plants, fungi, animals, nematodes and bacteria DNA. Using AFLP in conjunction with the binary code provides a novel approach to efficiently and effectively compare and possibly attribute pathogens to a source.

Traditional methods of pathogen detection and identification may include visual assessment of the pathogenic symptoms in a host accompanied by identification in the laboratory by growth of the pathogen on selective media and/or microscopy by to make a diagnosis (Ward et al., 2004). While these methods are useful and inexpensive, they are time-consuming and usually require extensive training and microbiological expertise. More generic typing methods are needed that can be quickly and easily taught to a somewhat unskilled staff. A generic DNA-based test may overcome some the drawbacks of conventional methods that can include long waiting periods for samples that are cultured, difficulty in distinguishing closely related organisms based on morphology alone, and a lack of sensitivity.

Several methods can be used to attribute pathogens to a source. Specific typing methods include BIOLOG (Hayward, California), ERIC (enterobacterial repetitive intergenic consensus), and ELISA (enzyme linked immunosorbent assay), phage typing and 16S rRNA sequencing. The BIOLOG assay provides a physiological profile that can be used to identify pathogens; however the identification of microbes is limited by the number and types of strains that are included in databases (Fletcher et al., 2006). Rep-PCR ERIC analysis uses specific primers to locate repetitive sequences present in a

genome (Toth et al., 1999). However for many bacterial species, repetitive sequences do not exist or are not terribly polymorphic. ELISA can be used to detect antibody binding to a specific pathogen using an enzyme-mediated color change reaction. The degree of color change can be used to quantify the pathogen present, however it may be difficult to detect multiple pathogens and sensitivity of the assay is dependent upon the amount of antigen that the pathogen produces (Ward et al., 2004). Phage typing can assist in the identification of bacteria by their susceptibility or resistance to various bacteriophages; however, it does not discriminate between all strains of bacteria (Najam et al., 2003). When the genomes of bacteria and fungi are sequenced in areas such as 16S rRNA and 23S rRNA, information can be obtained to assist in the identification of the pathogen (Ward et al., 2004, Fletcher et al., 2006). These regions are highly variable and are known as the internal transcribed spacer regions (Ward et al., 2004). While all of the techniques mentioned above are useful, a generic typing method that can be applied to a variety of pathogens is ideal.

Generic typing methods include RAPD and AFLP. Because no prior knowledge of the genomic sequence is required, RAPD could be used to type numerous pathogens. "RAPD uses non-specific primers which bind randomly to regions over the entire genome" (Toth et al.,1999). In a comparison study of phenotypic and molecular techniques to determine the diversity in *Erwinia carotovora,* RAPD analysis proved to be more discriminatory than ERIC analysis because it is more sensitive and can detect not only large sequences but small changes as well (Toth et al., 1999). In another comparison study of DNA fingerprinting techniques for tetraploid potato *(Solanum tubersum L)* germplasm, out of 39 potato cultivars, RAPD primer analysis yielded 38

different genotypes; only two of the cultivars were indistinguishable (McGregor et al., 1999).

AFLP analysis is another method that does not require any prior knowledge of about genome sequence expanding its utility for pathogen characterization. In the same comparison study for tetraploid potato germplasm, AFLP analysis also successfully distinguished 39 cultivars (Toth et al., 1999) Additionally, with two AFLP primer combinations 244 polymorphic fragments were produced (Toth et al., 1999). Twelve genotypes of *Phytophthora ramorum* have been identified using AFLP typing (Jones, 2003). Eighty two percent of the isolates were of one genotype (Jones, 2003). The previously mentioned studies underscore the utility of AFLP profiling as an effective tool for the attribution of plant pathogens in the event of an agroterrorist attack.

Culture methods such as biological inoculation and microscopic observation can be used to identify pathogens as well (Chang, 2003). Biological inoculation, a more traditional method, involves the deliberate infection of a susceptible plant host. The usefulness of biological inoculation is dependent upon a good system of different hosts which can be inoculated and observed for differential symptom expression. Drawbacks of this method are that it requires a greenhouse or growth chamber, skilled labor is needed to maintain the different hosts and it may take several days or even weeks for infection to become apparent. Microscopic observation is ideal for small sample sizes. However, microscopic observation requires sophisticated facilities and experienced staff and is not terribly discriminatory.

An ideal typing method to be used for microbial forensic application is generic, discriminatory, fast, portable, and works with existing instruments. The AFLP method

with its binary code assignment meets these requirements. In order to confirm general applicability, other species will need to be evaluated. Once an ideal technique has been identified, there are still several other aspects of plant pathogen forensics that must be considered. One of the first aspects to be considered is whether or not a crime has occurred. Since natural plant disease outbreaks are common a thorough investigation of the immediate area, weather conditions, and other geographic locations must be conducted. If it is determined to be a criminal act, what should be sampled, and how samples are collected and preserved are the next questions that need to be addressed. As seen in this study, one plant can be infected with two different strains of *Serratia*, and one strain can infect more than one plant, so it is imperative to collect a sample that is representative of an entire field in order to effectively investigate the outbreak. Extension agents or other individuals who are involved in sample collection must be educated about crime scene investigation and sample preservation until the arrival of law enforcement. Additionally, accurate characterization of pathogens using AFLP typing methods is dependant on all investigators using the same methods to produce and interpret profiles thereby allowing binary codes translated from AFLP profiles to be compared and making the information portable.

Other applications of this technique include studies of the host pathogen and vector-pathogen relationships. The possibility that one vector could transmit more than one strain to the host should be considered. Also, if particular elements of the AFLP profile contain unique fragments that are specific to a species of a pathogen, the technique is even more useful because a database of theses conserved fragments could be profiles from different species and could be diagnostic for a particular species of

pathogen. In summary, an ideal laboratory test to be used in the investigation of agroterrorism acts should be reliable, discriminatory, and portable to enable information sharing among law enforcement agencies. The AFLP mapping process developed and evaluated in this study seems to fulfill the requirements of an ideal test, at least with strains of the model pathogen *Serratia marcescens*. Future studies applying the technique to pathogens from other species will be needed to confirm the general utility of the assay.

REFERENCES

Allen, R. W., & Pearson, A. (1989). Characteristics of a DNA probe (pa3'HVR) when used for paternity testing. *Tranfusion, 29*(6), 477.

ASIco (Aquatic Stock Impovement Company). (2001). *PCR (A method for multiplying DNA).* Retrieved July 25, 2007, from http://www.asicoaquaticmarkers.com/PCRMethods.htm

Baillie, M.; Higgins, K.; Kerestes, R.; McGrew, D.; Richards, B.L.; Richards, J.; Salb, A.. (2005). *Use of random amplified polymorphic DNA (RAPD) to study genetic diversity within a population of crayfish orconectes rusticus from stony brook, mount holyoke college, south hadley, massachusetts.* Retrieved October 26, 2007, from www.mtholyoke.edu/~damcgrew/crays.html - 40k

Brackett, R. E. (2006). *FDA's role in tracking and resolving the recent E.coli spinach outbreak.* Retrieved on October 22, 2007, from http://www.fda.gov/ola/2006/foodsafety1115.html

Brooks, N., & Jerardo, A. (2007). *U.S. agricultural exports, year-to-date and current months*
Retrieved October 22, 2007, from http://www.ers.usda.gov/Data/FATUS/#monthly

Bruton, B. D., Fletcher, J. P., S.D., Shaw, M., & Sittertz-Bhatkar, H. (1998). Association of a phloem-limited bacterium with yellow vine disease in cucurbits. *Plant Disease, 82*(5), 512.

Bruton, B. D., Mitchell, F. L., Pair, J., Wayadande, A., Melcher, U., Brady, J., et al. (2003). *Serratia marcescens*, a phloem-colonizing, squash bug-transmitted bacterium: Causal agent of cucurbit yellow vine disease. *Plant Disease, 87*(8), 937.

Buchholz, K. (2004). *Serratia marcescens* Retrieved August 2, 2007, from http://web.umr.edu/~microbio/BIO221_2004/S_marcescens.htm

Budowle, B., Smith, J., Moretti, T., & DiZinno, T. (2000). *DNA typing protocols: Molecular biology and forensic analysis.* Natick, MA: Eaton Publishing.

Bush, G. W. (2003). *December 17, 2003 homeland security presidential Directive/HSPD-7.* Retrieved September 24, 2007, from http://www.whitehouse.gov/news/releases/2003/12/20031217-5.html

Bush, G. W. (2004). *January 30, 2004 homeland security presidential Directive/HSPD-9.* Retrieved September 24, 2004, from http://www.whitehouse.gov/news/releases/2004/02/print/20040203-2.html

Butler, J. (2005). *Forensic DNA typing: Biology, technology and genetics of STR marker books* (2nd ed.)Elsevier Science & Technology Books.

Capinera, J. (2003). *Featured creatures: Squash bug-anasa tristis* . Retrieved October 6, 2007, from http://creatures.ifas.ufl.edu/veg/leaf/squash_bug.htm

CDC. (2006). *Update on multi-state outbreak of E. coli O157:H7 infections fresh spinach, October 6, 2006.* Retrieved October 22, 2007, from http://www.cdc.gov/foodborne/ecolispinach/100606.htm

Chang, C. A. (2003). *Advanced technology for producing healthy seeds of vegetative material*. Taiwan ROC: Food & Fertilizer Technology Center.

Chiappe, J., & Nelson, S. (2003). *Economic impact of oklahoma agriculture enhancement & diversification program and the cooperatives & farms that it supports.* Retrieved September 24, 2007 from www.swosu.edu/cebd/docs/oklahoma_aed_program.pdf database.

Cho, J. C., & Tiedje, J. M. (2001). Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Applied and Environmental Microbiology, 67*(8), 3677-3682.

Cummings, C. A., & Relman, D. A. (2002). Genomics and microbiology. microbial forensics-"cross-examining pathogens". *Science (New York, N.Y.), 296*(5575), 1976-1979.

Davis, R. G. (2001). *Agricultural bioterrorism.* Retrieved March 4, 2007, from http://www.actionbioscience.org/newfrontiers/davis.html

Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., et al. (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the United States of America, 93*(12), 6025-6030.

Evrogen. *Suppression subtractive hybridization (SSH).* Retrieved October 6, 2007, from www.evrogen.com/t6.shtml

Fani, R., Damiani, G., Di Serio, C., Gallori, E., Grifoni, A., & Bazzicalupo, M. (1993). Use of random amplified polymorphic DNA (RAPD) for generating specific DNA probes for microorganisms. *Molecular ecology, 2*(4), 243-250.

FDA. (2002). *The bioterrorism act of 2002.* Retrieved July 31, 2007, from http://www.fda.gov/oc/bioterrorism/bioact.html

Fletcher, J., Bender, C., Budowle, B., Cobb, W. T., Gold, S. E., Ishimaru, C. A., et al. (2006). Plant pathogen forensics: Capabilities, needs, and recommendations. *Microbiology and molecular biology reviews : MMBR, 70*(2), 450-471.

Frazier, R. A., Ames, J. M., & Nursten, H. E. (2000). Background theory and principles of capillary electrophoresis. *Capillary electrophoresis for food analysis* (pp. 1). Burlington House London: RSC Publishing.

Groenewald, S., Van Den Berg, N., Marasas, W. F., & Viljoen, A. (2006). The application of high-throughput AFLP's in assessing genetic diversity in fusarium oxysporum f. sp. cubense. *Mycological Research, 110*(Pt 3), 297-305.

Guerra, R., & Speed, T. Statistical issues arising in the analysis of DNA-DNA hybridization data. *Systematic Biology, 45*(4), October 26, 2007 from links.jstor.org/sici?sici=1063-5157(199612)45%3A4%3C586%3ASIAITA%3E2.0.CO%3B2-M

Hitchner, E., & Kuhar, T. P. (2005). *Squash bug. Virginian cooperative extension, 444-031.* Retrieved October 31, 2007, from http://www.ext.vt.edu/pubs/entomology/444-031/444-031.html.

Holland. (2005). *Short history of DNA technology.* Retrieved September 1, 2007, from www8.utsouthwestern.edu/vgn/images/portal/cit_56417/40/39/178088histbiotech.pdf -

Hoyle, B. (2005). *Amplified fragment length polymorphism.* Retrieved November 5, 2007, from http://www.bookrags.com/research/amplified-fragment-length-polymorph-wog/

Huang, X., Li, Y., Niu, Q., & Zhang, K. (2007). Suppression subtractive hybridization (SSH) and its modifications in microbiological research. *Applied Microbiology and Biotechnology, 76*(4), 753-760.

Jain, S. K., Persaud, D., Perl, T. M., Pass, M. A., Murphy, K. M., Pisciotta, J. M., et al. (2005). Nosocomial malaria and saline flush. *Emerging infectious diseases, 11*(7), 1097-1099.

Jones, D. R. (2003). *Forest research: Phytophora ramorum* No. 03-9947). York, UK:

Lefers, M. (2004). *Restriction fragment length polymorphism.* Retrieved September 16, 2007, from http://www.biochem.northwestern.edu/holmgren/Glossary/Definitions/Def-R/RFLP_mapping.html

Le'vesque, C. A. (2001). Molecular methods for detection of plant pathogens- what is the future? *Can. J. Plant Pathol., 24*, 333.

Liebert, M. (2004). Agroterrorism in the US: Key security challenges agroterrorism. *Biosecurity & Bioterrorism, 2*(2), 97-105.

Lui, W., Li, P. J., Qi, X. M., Zhou, Q. X., Zheng, T. H., & Sun, Y., Y.S. (2005). DNA changes in barley (*hordeum vulgare)* seedlings induced by cadmium pollution using RAPD analysis. *Chemosphere, 61*, 158.

Mace, E. S., Yuejin, W., Boshou, L., Upadhyaya, H., & Chandra, S. Crouch, J.H. (2007). Simple sequence repeats (SSR)-based diversity analysis of ground (arachis hypogaea L.) germplasm resistant to bacterial wilt. *Plant Genetic Resources: Characterization and Utilization, 5*(1), 27-36.

McGregor, C. E., Lambert, C. A., Greyling, M. M., Louw, J. H., & Warnich, L. (2000). A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP, and SSR) in tetraploid potato (*(solanum tuberosum* L.) germplasm. *Euphytica, 113*, 135.

Monke, J. (2004). *Agroterrorism: Threats and preparedness* (CRS Report for Congress No. RL32521)Library of Congress. Retrieved September 24, 2007 from www.fas.org/irp/crs/RL32521.pdf

Mueller, U. G., & Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Trends in ecology & evolution (Personal edition), 14*(10), 389-394.

Najam, F. Z., KONSTANTINOU, K., & ZERVOS, M. (2003). Role of molecular biology and nucleic acid technology in the study of human infection and epidemology.*Pathology & Laboratory Medicine, 127*(9), 1098. cat.inist.fr/?aModele=afficheN&cpsidt=15248485

Nutter, F., & Madden, L. V. (2005). Plant diseases as a possible consequence of biological attack. *Biodefense: Principles and pathogens* [Plant Diseases as a Possible Consequence of Biological Attack] ()Horizon Press.

Oberst, R. (2004). *Agricultural biosecurity taking on increase importance.* Retrieved July 27, 2007, from http://www.eurekalert.org/features/doe/2004-06/dnl-abt061

Rascoe, J., Berg, M., Melcher, U., Mitchell, F. L., Bruton, B. D., & Fletcher, J. (2003). Identification, phylogenetic analysis, and biological characterization of *serratia marcescens* strains causing cucurbit yellow vine disease. *Bacteriology, 93*(10), 1233.

Rowland, C. (2006). *Characteristics and tendencies of pull-up peaks (carrie rowland, forensic bioinformatics).* Retrieved October 26, 2007, from www.bioforensics.com/conference06/Pullup/index.html

Rudin, N., & Inman, K. (2001). *An introduction to forensic DNA analysis* (2nd ed.)CRC Press.

Runner, G. (2007). *Week in review april 2007: E.coli in california.* Retrieved October 31, 2007, from http://republican.sen.ca.gov/opeds/17/oped3827.asp

Savelkoul, P. H., Aarts, H. J., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., et al. (1999). Amplified-fragment length polymorphism analysis: The state of an art. *Journal of clinical microbiology, 37*(10), 3083-3091.

Schiliro, E., Predieri, S., & Bertaccini, A. (2001). Use of random amplified polymorphic DNA analysis to detect genetic variation in *pyrus* species. *Plant Molecular Biology Reporter, 19*, 271a. Retrieved November 2, 2007 from pubs.nrc-cnrc.gc.ca/ispmb/ispmb19/r01-035.pdf

Sibley, C. G., & Ahlquist, J. E. (1994). On the phylogeny and classification of living birds. *Journal of Avian Biology, 25*(2), 87. Retrieved October 7, 2007 from JSTOR database. links.jstor.org/sici?sici=0908-8857(199406)25%3A2%3C87%3AOTPACO%3E2.0.CO%3B2-6

Singru, R., Sivaramakrishnan, S., Thakur, R. P., Gupta, V. S., & Ranjekar, P. K. (2003). Detection of genetic variability in pearl millet downy mildew (sclerospora graminicola) by AFLP. *Biochemical genetics, 41*(11-12), 361-374.

Tamaki, K., & Jeffreys, A. J. (2005). Human tandem repeat sequences in forensic DNA typing. *Legal medicine (Tokyo, Japan), 7*(4), 244-250.

Tomlinson, M. N., Adcock, F. J., Palma, M., Pena, J. G. & Ribera, L. (2007). *An initial assessment of the economic impacts of E. coli on the Texas spinach industry.* Retrieved October 22, 2007, from cnas.tamu.edu/Spinach%20E%20Coli%20Impacts%20Final

Toth, I. K., Bertheau, Y., Hyman, L. J., Laplaze, L., Lopez, M. M., McNicol, J., et al. (1999). Evaluation of phenotypic and molecular typing techniques for determining diversity in erwinia carotovora subspp. atroseptica. *Journal of applied microbiology, 87*(5), 770-781.

Udupa, S. M., Weigand, F., Saxna, M. C., & Kahl, G. (1998). Genotyping with RAPD and microsatellite markers resolves pathotype diversity in the ascochyta blight pathogen of chickpea. *Theor Appl Genet, 97*, 299-307.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., et al. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic acids research, 23*(21), 4407-4414.

Waleron, M., Waleron, K., Podhajska, A. J., & Lojkowska, E. (2002). Genotyping of bacteria belonging to the former erwinia genus by PCR-RFLP analysis of a recA gene fragment. *Microbiology (Reading, England), 148*(Pt 2), 583-595.

Wang, G., Whittam, T. S., Berg, C. M., & Berg, D. E. (1993). RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic acids research, 21*(25), 5930-5933.

Ward, E., Foster, S. J., Fraaije, B. A., & McCartney, H. A. (2004). Plant pathogen diagnostics: Immunological and nucleic acid-based approaches. *Annals of Applied Biology, 145*, 1-16. Retrieved October 8, 2007 from http://www.blackwell-synergy.com.ezproxy.chs.okstate.edu/doi/abs/10.1111/j.1744-7348.2004.tb00354.x database.

Wheelis, M. (2005). *Agricultural biowarfare and bioterrorism: What are the special features of an attack on an agricultural sector?* Retrieved March 4, 2007, from http://www.fas.org/bwc/agr/main.htm

Wittendorp, J. (2007). *AFLP® - genetic fingerprinting.* Retrieved September 3, 2007, from http://www.keygene.com/techs-apps/index.htm

Wong, Y. (2004). *Random amplified polymorphic DNA.* Retrieved October 21, 2007, from http://www.ntu.edu.sg/home2004/WONG0172/RAPD.html

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., & Fletcher, J. (2005). Genomic comparison of plant pathogenic and nonpathogenic serratia marcescens strains by suppressive subtractive hybridization. *Applied and Environmental Microbiology, 71*(12), 7716-7723.

APPENDIX

BIN	Z01-B	Z01-A	W01-A	W01C	R02A	P01	DB11	ATCC	90166	731-1	H01A	ATCC298
100	0	0	0	0	0	0	0	0	0	0	0	1
110	0	0	0	0	0	0	0	0	0	0	1	1
120	0	0	0	0	0	0	0	0	0	0	0	0
130	1	1	1	1	0	0	0	0	0	0	0	0
140	0	0	0	0	0	0	0	0	0	0	0	0
150	1	1	0	0	1	1	0	1	1	1	1	0
160	0	0	0	0	0	0	0	0	0	0	0	0
170	0	0	0	0	0	0	1	1	0	0	1	0
180	1	1	1	1	1	1	1	1	1	1	0	0
190	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0
210	0	0	0	0	0	0	0	0	0	0	0	0
220	0	0	0	0	0	0	0	0	0	0	0	1
230	0	0	0	0	0	0	0	1	1	0	0	0
240	0	0	0	0	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0	0	0	0	0
260	0	0	0	0	0	0	0	0	0	0	0	0
270	0	0	0	0	0	0	0	0	0	0	0	0
280	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0
310	0	0	0	0	0	0	0	1	0	0	1	0
320	0	0	0	0	0	0	0	0	0	0	0	1
330	0	0	0	0	0	0	0	0	0	0	0	0
340	0	0	0	0	0	0	0	0	0	0	1	1
350	0	0	0	0	0	0	0	0	0	0	0	0

100	0	1	1	1	1	1	0	0	0	1	0	1
110	0	0	0	0	0	0	0	0	0	0	0	1
120	0	0	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0	0	0
140	0	0	0	0	0	0	1	0	0	1	0	0
150	0	0	0	0	0	0	0	0	1	0	0	0
160	0	0	0	0	0	0	0	0	0	0	0	0
170	1	1	1	1	1	1	0	0	1	0	0	0

180	0	0	0	0	0	0	1	1	0	0	1	0
190	0	0	0	0	0	0	0	0	0	0	0	0
200	1	1	0	0	0	0	0	0	0	0	0	0
210	0	0	1	1	1	1	1	1	1	0	1	0
220	0	0	0	0	0	0	0	0	0	0	0	1
230	1	1	1	1	1	1	1	1	1	1	1	1
240	0	0	0	0	0	0	0	0	0	0	0	0
	Z01-B	Z01-A	W01-A	W01C	R02A	P01	DB11	ATCC	90166	731-1	H01A	ATCC298
250	0	0	0	0	0	0	0	0	0	0	0	0
260	0	0	0	0	0	0	0	0	0	0	0	0
270	0	0	0	0	0	0	0	0	0	0	0	0
280	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	1	0	0	0	0	1
310	0	0	0	0	0	0	0	0	0	0	0	0
320	0	0	0	0	0	0	0	0	0	0	0	0
330	0	0	0	0	0	0	0	0	0	0	0	0
340	0	0	0	0	0	0	0	0	0	0	0	0
350	0	1	1	1	0	0	0	0	0	0	0	0

100	0	0	0	0	0	0	1	0	0	0	0	0
110	0	1	1	1	1	1	1	1	1	1	1	0
120	1	1	1	1	1	1	1	1	1	1	1	1
130	0	1	1	1	1	1	1	0	1	1	1	1
140	0	0	0	0	0	0	0	0	0	0	0	1
150	0	0	0	0	0	0	0	0	0	0	0	1
160	0	1	1	1	1	1	0	0	1	0	0	1
170	0	0	0	0	1	0	0	0	0	0	0	1
180	1	1	1	1	1	1	0	0	1	0	0	1
190	0	0	0	0	1	0	0	1	1	1	1	1
200	1	0	0	0	0	0	0	0	1	0	0	0
210	0	0	0	0	0	0	0	0	0	0	1	1
220	1	1	1	1	1	1	0	0	1	0	1	1
230	1	0	0	0	1	0	0	0	1	1	1	0
240	1	0	0	0	1	0	0	0	0	0	1	0
250	0	0	0	0	0	0	0	0	0	0	0	0
260	0	0	0	0	0	0	0	0	0	0	0	0
270	0	0	0	0	0	0	0	0	0	0	0	0
280	0	0	0	0	0	0	1	0	0	0	0	1
300	0	0	0	0	0	0	0	0	0	0	0	0
310	0	0	0	0	0	0	0	0	0	0	0	0
320	0	0	0	0	0	0	0	0	0	0	0	0
330	0	0	0	0	0	0	0	0	0	0	0	1
340	0	0	0	0	0	0	0	0	0	0	0	1
350	0	0	0	0	0	0	0	0	0	0	0	0

VITA

Charlene Nichelle Beauman

Candidate for the Degree of

Master of Science

Thesis: EVALUATING THE SUITABILITY OF AFLP TECHNOLOGY FOR GENOTYPING STRAINS OF *SERRATIA MARCESCENS*

Major Field: Forensic Sciences

Biographical:

Personal Data:

Education: Received Bachelor of Science degree from North Carolina Central University in 1999. Completed the requirements for the Master of Science in Forensic Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2007.

Experience:

Professional Memberships:

Name: Charlene N. Beauman Date of Degree: December, 2007

Institution: Oklahoma State University-CHS Location: Tulsa, Oklahoma

Title of Study: EVALUATING THE SUITABILITY OF AFLP TECHNOLOGY FOR GENOTYPING STRAINS OF *SERRATIA MARCESCENS*

Pages in Study: 59 Candidate for the Degree of Master of Science

Major Field: Forensic Sciences

Scope and Method of Study: The purpose of this study was to evaluate the suitability of AFLP (amplified fragment length polymorphism) for genotyping strains of *Serratia marcescens* as a model plant pathogen. Twelve strains of *Serratia* from various ecological niches were obtained from Oklahoma State University-Stillwater. DNA was extracted using organic extraction and quantitated using spectrophotometry. Strains were genotyped using the AFLP Microbial Fingerprinting kit from Applied Biosystems. Capillary electrophoresis and GeneMapper ID software was used to analyze the data.

Findings and Conclusions: AFLP profiles exhibited 87% reproducibility through replicate assays. Visual comparisons of electropherograms showed that all strains of *Serratia* tested can be distinguished from one another. The discriminatory power of the AFLP profiles was enhanced with the application of a binary code. The binary code allows laboratories nationwide to communicate whether are not the same strain maybe present, which is critical in attributing the pathogen to its source.

ADVISER'S APPROVAL: Robert W. Allen