METAGENOMICS FOR THE IDENTIFICATION OF PLANT VIRUSES IN THE TALLGRASS PRAIRIE PRESERVE

By

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METAGENOMICS FOR THE IDENTIFICATION OF PLANT VIRUSES IN THE TALLGRASS PRAIRIE PRESERVE.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Viral genomics, the study of an organism's entire genome, has typically focused on uncontaminated, easily available samples such as viruses that replicate readily in culture or are easily propagated in plants. Such culturable viruses represent only a fraction of the total number of viruses currently existing in the environment as exemplified by the study of viruses in marine communities (Breitbart , 2002; Culley , 2006) and in the human gut (Breitbart , 2003). Many plant viruses go undetected as they fail to produce visible symptoms in their hosts or fail to replicate in a laboratory culture. Sequence-based methods circumvent these obstacles as the viral nucleic acid present in plants can be directly isolated and sequenced without the necessity to isolate and culture the viruses. This has led to the emergence of a new field referred to as viral metagenomics.

This thesis is focused on the development of a high-throughput metagenomic technique for the identification of unknown viruses present in plants growing in natural settings. The technique developed will help in increasing our knowledge about plant virus biodiversity and help in the estimation of the true number of virus species associated with plants.

HYPOTHESIS

The number of species of viruses associated with plants is much larger than the number of species currently recognized.

To test this hypothesis the following 4 objectives were set -

OBJECTIVES

1. Develop a high throughput metagenomic technique for the detection of both known and unknown plant viruses from plants growing in natural settings.
2. Apply the developed metagenomic technique to process plant specimens collected from the Tallgrass Prairie Preserve.
3. Analyze the sequences obtained to identify plant viruses.
4. Classify the contiguous nucleotide sequences (contigs) and identify sequences corresponding to other organisms.

1.1 *Viruses*

Viruses are obligate intracellular parasites that do not possess the molecular mechanism to replicate without the help of the host. Viruses are not considered free-living, as they cannot replicate outside a living cell. They are communicable agents found in all life forms, including humans, plants, animals, fungi and bacteria (Hull, 2002). Viruses consist of genetic material either DNA or RNA, which can be single stranded or double stranded. The genetic material is surrounded by a protective coat of protein called a capsid with or without a surface lipid envelope. The capsid can be made from a single type, or multiple types of coat proteins. A plant virus takes advantage of a break in the plant cell wall to

gain access to the plasma membrane, where the virus can enter the cell wall without interacting with specific receptors. Once the viral genome has entered the cell, the viral genes must be decoded and translated for the virus to replicate and spread. In the last stage of infection, new virus particles are formed. The viruses move between plant cells through the plasmodesmata. Viruses are rarely lethal to plants, but they can severely affect the host plants in quality, fecundity and longevity (Hamilton, 1981). A wide range of symptoms such as abnormal leaf color, abnormal vein patterns of leaves, mottling of leaves, abnormal leaf shape or/and spotting on leaves are recognized as expressions of viral diseases in plant. There are also irregularities in the colors of flowers, and the shapes, sizes and colors of fruits due to virus infection (Kristensen, 1966). Symptoms are often typical of a specific virus on a specific host plant and hence are often the chief criteria used to identify plant virus diseases (Zaitlin, 1987).

Most of the plant viruses that have caught immediate attention of people have caused noticeable disease symptoms in plants of economic value. Severe symptoms such as chlorotic spots, mosaic and general yellowing shown in wheat grown in the High Plains region of USA (Jensen, 1996.), led to the identification of *High plains virus* as the causative agent and its further characterization (She, 2004). The occurrence of leaf gall and leaf discoloration in sugarcanes grown in Australia and Fiji, led to the identification of *Fiji disease virus* (Rohozinski, 1981). Plant viruses that do not produce visible symptoms in plants are left undetected. For example the Cacao swollen shoot disease (Partiot, 1980) is a widespread disease initially found in West Africa. Despite the removal of infected trees, the disease continued to spread as the trees, appearing healthy, continued to harbor mild strains of the virus, which spread through insect vectors to

adjacent healthy trees. Such asymptomatic viruses that exist in plants can spread unnoticed and might cause a sudden serious outbreak if they find a susceptible host. Such outbreaks might be devastating if the host is a crop of economic value. For example a survey of cut flower roses of multiple varieties in ten grower's facilities (Curtis, 1986) revealed an overall 42% incidence rate of *Prunus necrotic ringspot virus*. Many of the infected plants did not show any symptoms. Lilies infected with *Lily symptomless virus* are close to symptomless in appearance (Asjes, 2000), but the cut flowers have reduced longevity, which decreased the economic value of the crop. Thus the reliance on healthy appearance in plants as virus-free can be misleading and might lead to severe disaster. There are only a few reports on discovering viruses associated with plants in natural environments regardless of the presence or absence of symptoms (Bodaghi , 2004; Fraile , 1997; Raybould , 1999).

The Plant Virus Biodiversity and Ecology (PVBE) project has been initiated to survey the biodiversity of viruses affecting vascular plants in The Nature Conservancy's 39000-acre Tallgrass Prairie Preserve in northeastern Oklahoma, home to over 700 plant species (Wren, 2006). The PVBE effort is not directed toward studying economically important or symptomatic plants, but to discover viruses affecting plants growing in a natural setting. As mentioned above, most of the currently identified viruses were recognized only after they caused a visible symptom in a plant host. The plants that raise concern due to viral infection are those that are of economic value. Thus, asymptomatic viruses and viruses infecting plants in non-managed ecosystems usually go undetected.

1.2 *Discovery of Viruses*

Viruses were first discovered in plants (Hull, 2002). The first virus to be discovered was the *Tobacco mosaic virus* (TMV), Adolf Mayer in 1883 described a disease of tobacco that could be transmitted to healthy plants by inoculation with extracts from diseased plants. Demitri Ivanowsky in 1893 showed that the extract could be filtered through filters that held back bacteria and called the extracts as filterable agents. For several decades viruses were called filterable agents and later the term virus (Latin for "poison") came into use. Martinus Beijerinck in 1897 called the agent a "contagium vivum fluidum" or a "contageous living liquid" and stated that the agent was not a bacterium, fungus or other culturable pathogen (Hull, 2002). Following the discovery of viruses in plants, viruses were soon discovered in animals and bacteria. F.A.J. Loeffler and P. Frosch in 1898 with Robert Koch in Germany discovered the first vertebrate virus, foot-and-mouth disease virus, with filters that held back bacteria. Frederick Twort discovered viruses that infect bacteria in 1915. Along with the increase in discovery of new viruses, developments were made to improve the study of viruses. In the 1940's the development of electron microscopy revealed that viruses have different shapes. Brakke in 1951 invented density gradient centrifugation to facilitate rapid purification of viruses. Sanger in 1977 developed the dideoxynuclotide sequencing technique (Sanger , 1977) and Maxam and Gilbert developed a chemical method for sequencing (Maxam, 1977). The first genomes sequenced were those of viruses. Fiers in 1978 sequenced the double-stranded DNA genome of SV40. Butler's Group at Cambridge in 1980 sequenced the TMV RNA genome.

Understanding the various properties of viruses, shed light on ways to prevent or cure viral diseases. Beachy in 1985 developed a general strategy for obtaining virus disease resistance in plants. He showed that transgenic plants expressing viral genes are protected against viruses related to the expressed viral genes. In 1978 smallpox eradication was a stunning success. Polio might soon be the second viral disease to be eradicated (Quadros, 2002). An effective worldwide strategy is being implemented for hepatitis B (Zuckerman , 2007) and measles (Orenstein , 2000). But as more effective treatment and prevention strategies are being developed and implemented for the currently known viruses, more potent and vicious viruses like the HIV, SARS virus, Ebola virus, Hanta virus etc. continues to emerge and cause serious outbreaks (Nichol , 2000). A good approach to prevent future epidemics by other novel viruses in the new century is to identify benign or asymptomatic viruses before they cause a serious disease. Knowledge about such unknown viruses existing in our environment will help us be better prepared for future outbreaks.

1.3 *Total Number of Viruses is underestimated.*

The International Union of Microbiological Societies (IUMS) charged the International Committee on Taxonomy of Viruses (ICTV) with the task of developing and maintaining a universal virus taxonomy for categorizing the multitude of known viruses by a single classification scheme that reflects their individual phylogenies. Based on the most recent ICTV report; there are 3 orders of viruses, 73 families, 9 subfamilies, 287 genera and 1938 virus species (Fauquet, 2005). Recent sequencing of samples from marine environments (Breitbart, 2002; Culley, 2006; Venter, 2004) suggests that the total

number of viral species reported by ICTV could be a gross underestimate. Thus it is not surprising to discover the International Nucleotide Sequence Database Collaboration, comprising the DNA databank of Japan, the European Molecular Biology Laboratory, and GenBank, which is located at the National Center for Biotechnology Information (NCBI) in the U.S. National Institute of Health has collected sequences belonging to 3142 species of viruses that are not present in the most recent ICTV list (Fauquet, 2005). Data on viruses that infect terrestrial organisms, including plants are small (Wren , 2006), compared to those reported from the marine environment . To-date 73 genera and 49 families of plant viruses are recognized by ICTV (Fauquet, 2005) .

1.4 *Studying Plant Viruses*

Plant viruses are accountable for the ever-increasing worldwide economic losses (Vlugt, 2006). The total worldwide estimated damage due to plant viruses is as high as US$ 6 x 10^{10} per year (Agrios, 1997). Therefore, it is essential to identify plant viruses and develop strategies to protect crop plants against these viruses. Plant viruses are easier to study as compared to their animal counterparts as they do not infect humans and hence are safe to work with. Also, plants are immobile hence easier to sample. The spread of viruses between plants is easier to study and there are fewer ethical concerns in studying plants than animals. Sampling and crushing plants for isolation of viruses will raise less concern compared to sampling and crushing of animals. Recent progress in the field of molecular biology and biotechnology have played a major role in the development of specific, rapid and sensitive assays for detection of plant viruses (Khan, 1998).

1.5 *Detection of Plant Viruses*

Both DNA and RNA viruses have been identified in terrestrial, marine and freshwater environments (Breitbart, 2005). Viruses have also been detected in the deepest depths of the ocean and far below the earths surface (Le Romancer, 2007). Most of the methods used for plant virus detection were developed to target the detection of a particular known virus from a given host plant with prior knowledge of which virus might be present in that plant. These methods are not suitable for detection of asymptomatic and novel viruses. These methods include serological techniques like immunofluorescence (IF) and Enzyme-Linked ImmunoSorbent Assay (ELISA) (Ball, 1974), molecular techniques such as, Polymerase Chain Reaction (PCR) based amplification, real-time or quantitative PCR, multiplex PCR, multiplex nested Reverse Transcriptase PCR (RT-PCR) (Lopez , 2003), DNA microarrays (Lee , 2003) etc. Though the panel of methods for rapid, sensitive and specific plant virus detection has improved over the years, these techniques have three significant disadvantages in testing for the presence of asymptomatic and novel viruses. The first is that the researcher must have a predetermined idea of what viruses may be present in the plant material to decide what would be the appropriate method for virus detection. This is not always possible, as many viruses present in wild ecosystems might not produce visible symptoms. The second is that most tests aim for the detection of single viruses. Plants are capable of harboring more than one virus, and thus to detect the presence of all the known viruses in a single plant specimen would require performing multiple assays. Finally viruses are genetically and antigenically diverse, and hence can escape detection by molecular and serological techniques. If a plant virus has novel

nucleotide sequences and antigens, it can go undetected if the detection method is based on primer specific PCR or antigen specific ELISA.

A related set of methods based on sequence-independent amplification, sub-cloning and sequencing of purified viral nucleic acids followed by computer based searches for sequence similarities to known viruses is increasingly being utilized to identify known and unknown viruses from environmental samples such as the identification of RNA viruses from coastal communities (Culley , 2006), identification of viruses in the human gut (Breitbart , 2003) etc. This procedure of non-specific amplification and sequencing of viral nucleic acid to identify viruses in environmental sample is known as viral metagenomics (Delwart, 2007). The key advantages of viral metagenomics are simplicity, rapidity, lack of bias towards a specific viral group, require no specific reagents and have the ability to detect novel viruses (Edwards, 2005).

1.6 *Metagenomics*

Metagenomics is defined as the sequence-based detection of collective microbial genomes contained in an environmental sample. Viral metagenomics was used in studies examining virus (phage) diversity in marine environments and in the human gut. The study of marine phages (Breitbart, 2002) involved metagenomic analysis of two uncultured marine viral communities. Overall about 2000 viral sequences were obtained and over 65% of the sequences were not significantly similar to previously reported sequences. This suggests that much of the diversity of viruses in the marine environment is yet to be characterized. In another study, the phage population of the human gut was investigated using the metagenomic approach (Breitbart, 2003). Virus particles were

isolated from human fecal samples from which viral nucleic acid was extracted. A library was constructed containing random fragments of the viral nucleic acid and about 500 clones were sequenced. Around 59% of the sequences did not show significant similarity to previously reported sequences. Approximately 1200 viral genotypes were found in the human gut, which is much greater than the number of bacterial genotypes found in the human gut. Another study of uncultivated coastal RNA virus communities uncovered a diverse group of marine picorna-like viruses, some of which were related to the viruses that infect higher plants and arthropods (Culley , 2006). A sequence-independent PCR amplification method was used to identify viral nucleic acids in the blood plasma of humans (Jones, 2005). Three novel DNA viruses were detected, a *parvovirus* and two viruses related to *TT virus*. Most of the viral metagenomic studies were focused on marine phages and viruses in the human gut. The viruses present in plants are yet to be explored. This study was undertaken to search for both known and unknown plant viruses present in plants obtained from the Tallgrass Prairie Preserve. The development of a successful plant-tissue virus metagenomic method requires a suitable procedure for breaking of plant tissue, enrichment of virus-like particles from plant tissue homogenates, isolation of viral nucleic acid, sequence-independent amplification, sub-cloning and sequencing of purified viral nucleic acids followed by computer-based searches for sequence similarities to known viruses.

1.7 *Development of a Metagenomic technique for the Identification of Plant Viruses*

The strategy used for a metagenomic based viral discovery is to increase the levels of the viral nucleic acids and reduce the background of eukaryotic, bacterial and archeal nucleic acids (Edwards, 2005). The experimental approach used must allow the enrichment of the viral nucleic acids prior to their sequence-independent amplification, cloning and sequencing followed by in-silico exploration for sequence similarities to known viruses.

1.7.1 *Homogenization of Plant Tissue*

The first step in the enrichment of viral nucleic acids is to break open the plant tissue to release the virus-like particles. The method used for homogenization of plant tissue must be easy to use, capable of handling large number of plant samples, must maintain virus particle stability and should prevent cross contamination. There are multiple ways to break open plant tissues. Rotor-stator homogenizers also called colloid mills or Willems homogenizers were used in studies such as extraction of wildtype *Cowpea mosaic virus* from plants (Nichols, 2002). The homogenizer draws the cellular material inside the apparatus by a rotor located within a static tube. The material is then centrifugally extruded through the holes on the tip of the tube. These homogenizers are bulky and not reliable to use for a large number of samples, as they are extremely prone to wear. Blade homogenizers or blenders have been used in studies such as purification of *Tobacco necrotic dwarf virus* and *Potato leafroll virus* from infected plant tissues (Takanami, 1979). The blender consists of a set of cutting blades made of stainless steel rotating at high speed inside a glass or stainless steel container. Blenders are not good for processing large numbers of samples as they are difficult to clean and can lead to cross-contamination. Liquid-nitrogen for breaking plant tissues was used in different studies

such as extraction of *Barley yellow dwarf virus* and *Sugarcane yellow leaf disease-associated virus* from plant tissue (Maia, 2000). The plant tissue is frozen in liquid nitrogen and then ground using a mortar and pestle. This technique is extremely time consuming and requires a large quantity of liquid nitrogen for processing a large number of plants. Also, thorough cleaning of the mortar and pestle is required after each extraction to prevent cross-contamination or the technique requires use of plenty of mortars and pestles. Grinding has been used in studies such as the extraction of *Tomato yellow leaf curl Thailand virus* from plant tissue (Leamkhang, 2005). In this technique, the plant material is pulverized in a mortar or tube with fine sand, alumina or glass powder. Cleaning of the tube or mortar after homogenization of each plant is time consuming and not suitable for processing large number of plant samples. Ultrasonic disintegrators have been used in studies such as extraction of *Red clover vein mosaic virus* from plant tissue (Varma , 1970). This technique makes use of sonic pressure waves to disrupt the plant cells. The chamber used for disrupting plant tissue is bulky and difficult to clean and hence can cause cross-contamination. The bead mill homogenizer has been used in studies involving extraction of *Citrus psorosis virus* (Barthe, 1998), *Citrus tristeza virus* (Nolan, 2005) etc from plant tissue. This homogenizer uses a procedure in which a large number of minute glass or ceramic beads are vigorously agitated by stirring or shaking. Disruption of plant cells occurs by crushing action of the glass beads as they collide with the cells. There are two types of bead mill homogenizer, the rotor-type bead mills that uses a rotor to agitate the beads for disruption of plant cells. The shaking-type bead mills, which uses shaking to agitate the beads for the disruption of plant cells. The advantages of using the shaking-type bead mills are that they make use of

2 ml disposable vials for each sample, hence there is absolutely no clean-up required after processing of each plant sample, which also reduces the chances of cross-contamination. The process is rapid and easy to use. BioSpec Products manufactures four high energy shaking machines: the Mini-BeadBeater series, MBB-1, MBB-8, MBB-16 and MBB-96. These are high-throughput machines capable of processing up to 48 microvials or using deep well microplates with up to 192 samples. Since the mini-beatbeaters are quick, easy to use, high-throughput machines capable of handling large number of plant samples, easy to clean and prevent cross contamination, they are the most suitable homogenizers for this study.

1.7.2 *Isolation of Virus-like Particles*

After the disruption of the plant tissue, the next step is the enrichment of virus-like particles (VLPs). The VLPs must be separated from other debris present in the plant homogenate. The team investigating marine phages (Breitbart, 2002) used ultra-centrifugation through sucrose and cesium chloride gradients to purify VLPs from other lower and higher density materials. Ultracentrifugation was also used on blood plasma for the purification of human DNA viruses (Simmonds, 1998). Viruses have different sizes (20 nm to 1200 nm) and sedimentation coefficients (80S to 200S) compared to most of the host cell components (Hull, 2002). Sedimentation coefficient is the ratio of the velocity of sedimentation of a molecule to the centrifugal force required to produce this sedimentation. The value is given in Svedberg units. The sedimentation coefficient of plant ribosomes is similar to that of most plant viruses. Hence, when plant viruses are pelleted by ultra-centrifugation, there is a high probability that the pellet might also

contain plant ribosomes. The sedimentation of the virus particle will depend on the mass, shape, partial specific volume of the virus as well as solvent density, rotor size and the rate of rotation.

The sedimentation (s) and diffusion coefficient (D) are related with the molar mass (M) by the Svedberg equation-

$$D = \frac{sRT}{M(1 - \bar{v}\rho)}$$

Where-

v	=	protein partial-specific volume
ρ	=	solution density
R	=	gas constant
T	=	absolute temperature

The higher the molar mass, the greater is the sedimentation coefficient. Thus, enrichment of plant viruses can be achieved by subjecting the plant homogenate to ultra-centrifugation through a sucrose cushion using an appropriate centrifugal force that would pellet the majority of plant viruses.

1.7.3 ***Isolation of Viral Nucleic Acid***

Important to a good preparation of viral nucleic acid is the removal of non-viral nucleic acid such as the host DNA and RNA. Gardner and Shepherd described a procedure for rapid isolation of *Cauliflower mosaic virus* (CaMV) DNA (Gardner, 1980). This protocol is based on the addition of DNase I to remove naked host DNA through endonuclease digestion. The host RNA is degraded by endogenous RNase released internally due to cell lysis. The viral coat protein protects the viral nucleic acid from enzymatic digestion

(Allander , 2001). Note that viral nucleic acids that are not protected by a protein coat are also subjected to nuclease digestion and hence this technique is not suitable for isolation of viral nucleic acids of viruses that lack protein coats. Chelating agents like ethylenediaminetetraacetic acid (EDTA) are used to inactivate DNase I by chelating the ions required for the enzyme activity. The capsid protected viral nucleic acids are extracted by degradation of the protein coat by using detergents like sodium dodecyl sulfate (SDS) and by protein digesting enzymes like proteinase-K. SDS is an anionic surfactant and works by disrupting non-covalent bonds in the proteins, thereby denaturing the protein molecules and making them lose their native conformation. Proteinase K or Endopeptidase K is a broad-spectrum serine protease, which remains active even in the presence of SDS and EDTA. The released viral nucleic acid can be purified using standard phenol extraction followed by ethanol precipitation. This procedure can be adopted for isolation of nucleic acids from most of the plant viruses that have a protein coat as this method is capable of extracting nucleic acid from CaMV virions which are known to be among the most obstinate for extraction of viral nucleic acid (Hull, 1978.). If the nucleotide sequence of the isolated viral nucleic acid is unknown then it must be subjected to sequence independent nucleic acid amplification.

1.7.4 Sequence Independent Nucleic Acid Amplification

There are several different ways to perform sequence independent nucleic acid amplifications. DeRisi's group used a random PCR amplification technique for labeling

targets with fluorescent dyes for microarray analysis (Wang, 2003). This method is based on the amplification of all nucleic acids present using PCR primers with a random nucleotide sequence at the 3' end and a defined sequence at the 5' end. For RNA viruses, reverse transcription is first performed with such a random primer at a low annealing temperature of 37 ^{o}C to permit randomly primed cDNA extensions. An additional single round of extension with the same primer is then performed following denaturation of the dsDNA or the cDNA/RNA hybrid, and primer annealing at low temperature followed by Sequenase DNA polymerase extension. Then, using a PCR primer complementary to the defined 5' sequence of the initial primer, 30-40 cycles of PCR are carried out at higher annealing temperature using Taq DNA polymerase. This method was also used for characterization of new human parvoviruses and identification of a large number of viruses from the human respiratory tract (Allander, 2005). The advantage of using this method is the possibility of amplifying both DNA and RNA templates. Since most of the plant viruses have RNA as the genetic material, this could be the most suitable method for sequence-independent amplification of viral nucleic acids.

Apart from the random PCR amplification technique, there are other sequence independent amplification techniques such as the amplification based on restriction digestion of target DNA followed by ligation of an adapter complementary to the overhanging bases followed by amplification of the sequence between the restriction sites by using a primer complementary to the adapter. Though this technique has been successfully used for the identification of a new *parvoviruses* from human plasma (Fryer, 2006), it will not be suitable for plant viruses because most plant viruses have RNA as their genetic material which cannot be subjected to restriction digestion. Other techniques

that have been used for sequence independent nucleic acid amplification include, arbitrarily primed PCR which is a technique recently used for cloning of new human *pneumovirus* (Hoogen, 2001); PhiX29 DNA polymerase-based amplification recently used in amplification of DNA *annellovirus* (Biagini, 2007) and rolling circle amplification which is a method that has been successfully used to amplify many circular DNA viral genomes like those of *Paillomaviruses* (Rector, 2005), *Geminiviruses* (Haible , 2006) and *Begomoviruses* (Inoue-Nagata, 2004).

Most of the above-mentioned techniques used for non-specific PCR amplification require the use of a DNA template. The most suitable technique should allow the amplification of both DNA and RNA obtained from environmental samples, hence the most appropriate method for this study is the technique devised by DiRisi's team –the random PCR amplification technique.

1.7.5 ***Cloning and sequencing of Amplified Viral Nucleic acid.***

A good cloning system (vector and host) is required for the isolation of amplified viral nucleic acid from the unknown heterogeneous gene pool. Different types of cloning systems are used depending upon the sizes of the amplified DNA fragments. Plasmid vectors that can accommodate small size inserts in the range of 0.5-10 kb are used in different metagenomic studies. The studies involving shotgun sequencing of entire marine communities of the Sargasso sea by J Craig Venter made use of plasmid vectors like a pBR322 derivative (Venter, 2004). The pSMART plasmid vector was used in the metagenomic analysis of uncultured marine viral communities (Breitbart, 2002) and uncultured viral communities in human feces (Breitbart, 2003). TOPO-TA vectors are

extremely ‘user-friendly’ with respect to rapidity and efficiency of cloning compared to other plasmid vectors that requires additional steps such as restriction digestion and ligation. TOPO-TA vectors were used in the metagenomic analysis of coastal RNA virus communities (Culley, 2006) and in the genome analysis of soil microbial communities (Rondon, 2000). The TA-cloning method takes advantage of the terminal transferase activity of Taq polymerase. This enzyme adds a single 3’-A overhang to each end of the PCR product, which makes it possible to clone it directly into the linear vector with single 5’-T overhangs. The Topoisomerase-I enzyme that is covalently bound to the 3’ phosphate of the vector performs the ligation. The TOPO-TA vector has a small multiple cloning site that positions the T7 and T3 priming sites only 33 bp away from the inserted PCR product and hence more of insert and less of vector is sequenced. Apart from the plasmid vectors, other cloning vectors such as cosmids or fosmids that can accommodate an insert size of 30- 45 kb were used in different metagenomic studies, for example in the characterization of metagenome-derived esterase genes from oil-contaminated soil (Elend, 2006). Bacteriophage vectors such as lambda phages that can carry an insert size of 7- 20 kb have been used in studies involving uncultured marine picoplankton communities (Schmidt, 1991). Bacterial artificial chromosomes (BAC) that can accommodate an insert size of 80-200 kb have been used in the study of the soil metagenome (Rondon, 2000). BAC vectors are increasingly being preferred over yeast artificial chromosome vectors (YAC) that can carry large size inserts of 0.2-1.5 Mb due to the tedious steps involved in construction of YAC libraries and low yields of YAC insert DNA. Such large size insert libraries are mostly used for screening of clones with activities or genes of interest. Thus, for sequence based identification of uncultured

microbial communities, the most predominant vectors that are used are the plasmid vectors and for high-throughput cloning of amplified nucleic acids from environmental samples, the most efficient plasmid vectors that are used are based on TOPO-TA cloning reactions.

In most of the studies involving the identification of uncultured microbial communities from environmental samples that were based on sequencing, the preferred method of sequencing was Sanger's method. Sanger's method, which is also referred to as dideoxy sequencing or chain termination, is based on the use of dideoxynucleotides (ddNTPs) in addition to the regular nucleotides (NTPs) that are found in DNA. Sanger's dideoxy method of DNA sequencing has been used in various studies such as the study of uncultured marine communities, soil metagenomics, and study of uncultured viral communities from human feces.

Another method for sequencing of uncultured microbial communities from environmental samples that is gaining in popularity is pyrosequencing. This method is based on detection of the activity of DNA polymerase with the help of a luminescent enzyme. Sequencing of a single-stranded DNA is performed based on the detection of pyrophosphate release when a solution of single dNTP (A, C, G or T) complements the unpaired base of the template. Pyrosequencing has been used in different studies such as the study of deep mine microbial ecology (Edwards, 2006) and sequencing of 27000 year old mammoth DNA (Gibbons, 2005). Though pyrosequencing generates large numbers of sequences at a low cost, it has an inherent disadvantage of generation of short sequence fragments, which limits the ability of most computational analyses used for similarity searches and sequence assembly. Also, another problem with pyrosequencing

is the difficulty in discrimination of homopolymeric runs in the sequence data. Thus the most appropriate sequencing method for obtaining uncultured plant virus sequences would be Sanger's dideoxy sequencing method.

1.7.6 *Sequence Data Analysis*

An important issue in metagenomics is avoiding errors in genome assembly i.e. to combine all the reads into contigs based on sequence similarities between the individual reads. The basic obstacle to assembly of genomes is the cost of achieving sufficient sequence coverage of the single microbe present in a community that might contain many different species. The biggest challenge in assembling genomes from metagenomic data is population heterogeneity due to genomic rearrangements and sequence polymorphisms. Most of the assembly algorithms such as Phrap (Gordon, 1998), CAP3 (Huang, 1999), and TIGR assembler (Kosack, 2004) are fairly robust to sequence polymorphisms and genomic rearrangements. In various metagenomic studies such as studies of uncultured microbial communities in soil, the Saragossa sea, the human gut most of the sequences obtained remain in small contigs or as unassembled reads. The simplest and the most common method of assigning these sequences into phylogenetic groups were based on the best BLAST hit. Each of the contigs obtained was analyzed either using nucleotide-nucleotide BLAST (BLASTn) or translated query vs. protein database (BLASTx). Based on all the previous studies involved in the identification of uncultured viruses from environmental samples, this research was undertaken to develop a metagenomic technique for the identification of uncultured viruses from the tissue of plants obtained from the unmanaged ecosystem of the Tallgrass Prairie Preserve.

CHAPTER II

METHODOLOGY

2.1 *Initial Preparation of Plants*

Plants were collected from different locations in the Tallgrass Prairie Preserve (TPP) in 2005, 2006 and 2007, by a team of botanists from the Department of Botany, Oklahoma State University. The collected plant samples were weighed and placed into zipper bags (7.5" x8" Ziploc bags). Small aliquots of the plant samples (100 mg) were placed in 1.5 ml self-standing screw cap microtubes (United Scientific Products). The microtubes and the zipper bags were labeled appropriately and stored at -80^{o}C.

Plants were also collected from Stillwater, Oklahoma near the Noble Research Center. These samples were labeled as non-TPP plants and were used for the initial standardization experiments.

2.2 *Preparation of Plant Homogenate*

The 100 mg aliquot of the plant material stored in the 1.5 ml self-standing screw cap microtube was used for preparation of a plant homogenate. The amount of plant material used for processing all the plants from the Tallgrass Prairie Preserve (TPP) was 100 mg. For the initial standardizing experiments 100, 200, 300, 400 and 500 mg of non-TPP

plants were used. Twelve to fourteen sterile beads were added to the plant material. Sterile glass beads (diameter, 2.5 mm) were used for the TPP samples. Sterile zirconium (2.5 mm), steel (2.0 mm) and glass beads of various diameters (2.5 mm, 3.0 mm and 1.0 mm) were used for the initial standardization experiments. The addition of sterile beads was followed by addition of 750 μl of 0.1 M sodium citrate pH 6.5 (citrate buffer) and 6.3 μl of 0.25M iodoacetamide. The tube was kept on ice for 10 min. All the TPP samples were treated at 4600 rpm for 3 min at room temperature in a Mini-Beadbeater (Biospec Products, Bartlesville, OK). In initial experiments, some of the non-TPP plants were frozen using liquid nitrogen prior to homogenization using the mini-bead beater. In other initial experiments, some of the non-TPP plants were treated in a Mini-Beadbeater 96 (Biospec Products, Bartlesville, OK) that can hold 45 microvials; the samples were subjected to 2100 rpm for 15 minutes. The plant homogenate obtained was subjected to 15 min of centrifugation (Micro-centrifuge Hermle Labnet Z233M) at 12000 g at room temperature; 137.5 μl of the supernatant was transferred to a sterile 1.5 ml microcentrifuge tube followed by the addition of 12.5 μl of 33.3% v/v of Triton X-100. This supernatant- Triton X-100 mixture was stored at 4 ^{o}C for a maximum of 4 days. In initial experiments, the mixture was stored at room temperature, -20 ^{o}C and -80 ^{o}C for 3-4 days.

2.3 ***Isolation of Virus-like Particles***

The mixture consisting of the supernatant and Triton X-100 was transferred to a clean ultracentrifuge tube (Beckman cellulose propionate 7 x 20 mm centrifuge tube, part number-342303) and was underlaid with 50 μl of 20% sucrose solution in citrate buffer.

This tube was subjected to ultra-centrifugation (Beckman Optima LE-80K ultracentrifuge) at 70000 g for 45 min at room temperature, in a Beckman Ti 42.2 rotor. The pellet was resuspended in 200 μl of 0.5X citrate buffer, transferred to a clean 0.5 ml microcentrifuge tube and centrifuged for 10 min at 8000 g at room temperature. The supernatant (150 μl) was transferred to another ultracentrifuge tube and underlaid with 50 μl of 0.5X citrate buffer and centrifuged at 150000 g for 65 min at room temperature. The pellet was resuspended in 250 μl of 0.1M Tris-HCl pH 7.5, 2.5mM $MgCl_2$ (viral resuspension buffer) and transferred into a clean microcentrifuge tube.

2.4 ***Nucleic acid Isolation from Virus-like Particles***

The sample was divided into a 50 μl aliquot and a 200 μl aliquot. Glycerol (15% final concentration) was added to the 50 μl aliquot and stored at -20^{o} C. This aliquot will be used later for electron microscopy studies. The 200 μl aliquot was used for the isolation of nucleic acid.

To this sample, 2 μl of 1 mg/ml (w/v) DNase I (Sigma) was added and the tube was kept at 37^{o} C for 10 min. Four microlitres of 0.5M EDTA (Fisher Biotech), 50 μl of 2.5 mg/ml Proteinase K (Invitrogen) and 12.5 μl of 20% sodium dodecyl sulfate (Fisher Scientific) were added to the sample and the mixture was incubated at 65^{o} C for 30 min. Six microlitres of 5M NaCl was added and the suspension was extracted with 300 μl of buffer-saturated phenol (MP Biomedicals, Inc.). The tube was mixed and centrifuged at 12000 g for 5 min. The upper aqueous phase was transferred to a clean microcentrifuge tube and extracted with 1ml of diethyl ether (Fisher Scientific). The tube was centrifuged at 12000 g for 1 min and the upper phase was discarded. Four microlitres of 5-mg/ml

linear polyacrylamide (Ambion) were added to the solution followed by addition of 95 % ethanol (AAPER alcohol and Chemical Co.) and incubated overnight at –20 ^{o} C to precipitate the nucleic acid and the co-precipitant linear polyacrylamide. The precipitate was pelleted by centrifugation at 12000 g for 15min. The pellet was washed with 75% ethanol and was vacuum dried in a Savant Speed Vac plus sc 110A.The pellet was dissolved in 30 µl of 10mM Tris-HCl pH 7.5, 1mM EDTA, 10mM NaCl (TEN buffer).

2.5 *Random PCR Amplification*

Nucleic acid was amplified by a procedure adapted from Dirisi's lab protocol (Wang, 2002).

Round A: First strand synthesis using Reverse transcriptase

The extracted viral nucleic acid (VNA) from most of the TPP samples was mixed with 1 µl of 40-pmol/µl primer CCTGGATTCGGATCCTCCNNNNNNNNNNN (primer A12) (Integrated DNA Technologies, Inc.) to a final volume of 10 µl in a 0.65ml RNase/DNase free tube (Life Sciences Products, Inc.) For the first ten of the TPP samples primer A (CCTGGATTCGGATCCTCCNNNNNN) was used and for some of the standardization experiments primer A2 (GATGAGGGAAGAGATGGAGNNNNNNNNNNN) was used instead of primer A12. The VNA-primer mixture was incubated at 65 ^{o} C for 5 min and placed at room temperature for 5 min. A 10 µl master mix comprised of 2 µl of 10X Stratascript buffer, 2 µl of reverse transcriptase (MMLV-RT, Stratascript, 50 U/µl), 0.4 µl 25mM dNTP (Fisher Scientific), 2.0µl 0.1M DTT and 3.6 µl of water (Ambion Nuclease free water) was added and the mixture was incubated for 42 ^{o} C for 30 min followed by 65 ^{o} C

for 5 min and was then kept at room temperature for 5 min. 1 μl of 50 U/μl of reverse transcriptase was added to this mixture and incubated at 42 ^{o}C for 30 min.

Second strand synthesis with Sequenase

The round A product was incubated at 94 ^{o}C for 2 min and held at 10 ^{o}C for 5 min. 10 μl of Sequenase mix comprising of 2 μl of 5X sequenase buffer, 0.3 μl of 13 units/μl Sequenase (USB) and 7.7μl of nuclease free water was added to the round A product and the mixture was subjected to a ramp from 10 ^{o}C to 37 ^{o}C over 8 min in a thermal cycler (PTC-100 MJ Research Inc.) followed by incubation at 37 ^{o}C for 8 min, 94 ^{o}C for 2 min and 10 ^{o}C for 5 min. 1.2 μl of 1:4 diluted Sequenase enzyme was added to the above mixture and the mixture was subjected to another round of ramp from 10 ^{o}C to 37 ^{o}C over 8 min in a thermal cycler. The tube was then incubated at 37 ^{o}C for 8 min followed by 94 ^{o}C for 8 min and was kept at 4 ^{o}C.

Round B

A master mix comprised of 4 μl of 50 mM $MgCl_2$, 10 μl of 10X PCR buffer, 1 μl of 5 U/μl Taq Polymerase (Invitrogen) 1 μl of 25 mM dNTP (Fisher Scientific), 1 μl of 100 pmol/μl CCTGAATTCGGATCCTCC (primer B) (Integrated DNA Technology, Inc.) and 77 μl of nuclease free water was added to 6 μl of Round A product to make a final volume of 100 μl. In initial experiments in which primer A2 was used for Round A, primer B2 (GATGAGGGAAGAGATGGAG) was used for the Round B amplification. The master mix was subjected to 30 cycles of 94 ^{o}C for 30 sec, 40 ^{o}C for 30 sec, 50 ^{o}C for 30 sec and 72 ^{o}C for 1 min followed by 5 min of extension at 72 ^{o}C in a thermal cycler. In the initial standardizing experiments different numbers of PCR cycles (20,25,30,35,40 and 45) were used.

2.6 *Electrophoresis*

An aliquot of 8 μl of the round B product was subjected to agarose gel electrophoresis on a 2% agarose gel (Fisher Scientific). The agarose gel was made with Tris-acetate-EDTA (TAE) electrophoresis buffer. The nucleic acid samples were mixed with 6x loading buffer and transferred to the wells of the gel using a pipette. The electrophoresis was run at approximately 4 V/cm in a high-resolution gel electrophoresis apparatus (International Biotechnologies Inc. Cat no. 54000). The gel was stained in ethidium bromide solution (0.5 μg/ml) for about 15-30 minutes. The stained gel was place on a transilluminator (UV light of wavelength 254 nm) and a photograph was taken.

2.7 *Ligation of the PCR product to a Cloning Vector*

The first 129 PCR positive TPP samples were ligated to a TOPO-TA cloning vector (Invitrogen) An aliquot of 2 μl of the round B PCR product was added to a 4 μl master mix comprised of 1 μl of 1.2 M NaCl, 0.06M $MgCl_2$ (salt solution), 1 μl of pCR 2.1-TOPO vector (Invitrogen) and 2 μl of sterile water. This ligation mixture was mixed gently and incubated for 30 min at room temperature.

The remaining samples were ligated to a StrataClone vector (Stratagene). An aliquot of 2 μl of the round B PCR product was added to a 4 μl master mix comprised of 3 μl of StrataClone cloning buffer and 1 μl of StrataClone vector mix. This ligation mixture was mixed gently by repeated pipetting and incubated for 5 minutes at room temperature.

All the cloning reactions were stored at -20 ^{o}C.

2.8 *Transformation*

In case of the PCR products that were ligated with the TOPO-TA vector, a 2 μl aliquot of the ligation mixture was added to a vial of one-shot TOP10 chemically competent cells (Invitrogen) and was incubated on ice for 30 min. These cells were subjected to heat shock by placing them at 42 ^{o} C for 30 sec and immediately transferring them to ice.

In case of the PCR products that were ligated to the StrataClone vector, a 2 μl aliquot of the ligation mixture was added to a tube of StrataClone SoloPack competent cells (Stratagene) and was incubated on ice for 20 minutes. These cells were subjected to heat shock by placing them at 42 ^{o} C for 45 sec and immediately transferring them to ice.

SOC medium (250 μl) was added to these cells and the mixture was incubated at 37 ^{o} C for 1 h. The transformation mixture was separated into a 200 μl and a 100 μl aliquot. The 200 μl aliquot was inoculated into 100 ml Luria Bertani (LB) broth (Fisher scientific) in a 250 ml flask with kanamycin (50 μg/ml) in the case of the TOPO-TA vector and ampicillin (50 μg/ml) in the case of the StrataClone vector. The flask was shaken in an incubator shaker (New Brunswick Scientific) at 37 ^{o} C for 16-18 h. 15% glycerol stocks of 1 ml of the bacterial growth were prepared by addition of 850 μl of bacterial growth and 150 μl of sterile glycerol in a clean 1.7 ml microcentrifuge tube (Life Sciences Products, Inc) and stored at –80 ^{o} C. The 100 μl aliquot was subjected to double dilution with sterile water and spread on LB agar (Fisher Scientific) plates containing the corresponding antibiotic and 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal) (40 mg/ml) and was incubated at 37 ^{o} C for 16-18 h. The presence of white colonies indicated success of transformation.

2.9 *Plasmid Preparation*

An aliquot of 5 ml of the bacterial growth was used for isolation of plasmids by the QIAprep Spin Miniprep Kit protocol (Qiagen). An aliquot of 8 μl of the plasmid preparation was subjected to agarose gel electrophoresis on a 0.8% agarose gel (Fisher Scientific) .The gel electrophoresis protocol mentioned in section 2.6 was followed.

2.91 *Restriction Digestion*

A 2 μl aliquot of the plasmid DNA (TOPO-TA vector) was subjected to restriction digestion using 2 μl of *Alw*N1; 2 μl of 10X buffer NE4 (New England Biolabs) and 14 μl of nuclease free water. The mixture was incubated at 37 ^{o}C for 2 h.

In case of the plasmid DNA containing the StrataClone vector, a 2 μl aliquot was subjected to restriction digestion using 2 μl of *Nsp*1; 2 μl of 10X buffer NE2 (New England Biolabs) and 14 μl of nuclease free water, the mixture was incubated at 37 ^{o}C for 2 h. An aliquot of 8 μl of the cut plasmid DNA was subjected to agarose gel electrophoresis on a 0.8% agarose gel (Fisher Scientific). The gel electrophoresis protocol mentioned in section 2.6 was followed.

2.92 *Sequencing*

The 15 % glycerol stock of bacterial growth that contained plasmids having large inserts (>500bp) was sent to the Advanced Center for Genome Technology (ACGT), University of Oklahoma at Norman for sequencing.

At ACGT Norman, the glycerol stock was subjected to a 10^{-4} serial dilution followed by plating on LB-Agar plates containing ampicillin. After incubation, plasmids were isolated from a random library of 384 colonies. The isolated plasmids were sequenced using a ABI 3730 capillary DNA sequencer and the chromatograms obtained were transferred to a Sun Workstation cluster where they were assembled into contiguous sequences (contigs) using Phred and Phrap.

2.93 ***Analysis of the Sequences***

At ACGT Norman, each of the contigs obtained were analyzed using nucleotide-nucleotide BLAST (BLASTn) and translated query vs. protein database (BLASTx). Some of the samples were also subjected to tBLASTx, which compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. Analysis in each case was against the Genbank non-redundant databases for nucleotides (nt) and proteins (nr), respectively, using an E-value cutoff of 10^{-3}.The top five-blastn and blastx results was used for contig classification.

The contigs were classified into plant, animal, bacterial, fungal, virus, retrotransposon or uncertain, based on the top five blastn and blastx results. To assign a category to a contig the following rules were used-

a. If there was "No hits found" for both blastx and blastn, the contig was assigned as "uncertain"
b. If there was "No hits found" in one search, but not the other the genome identified in the other was used for assigning a category to the contig.

c. If there were hits in both searches and they were from the same genome the choice was obvious.

d. If there was a disagreement between the blastn and blastx results, the result with a lower E-value was used for assigning a category to the contig.

2.94 ***Reverse transcriptase PCR (RT-PCR) of Maize necrotic streak virus.***

For initial standardizing experiments 100 mg of maize plant infected with *Maize necrotic streak virus* (MNeSV) was used for isolation of the viral nucleic acid (VNA) as mentioned above. The isolated VNA was subjected to RT-PCR using the forward primer MNeSV1756c (TCCACCATCAGGTACATACC) and the reverse primer MNe1107 (CTGGCAAACCTGAAAAGG). The extracted VNA was mixed with 1 µl of 100 ng MNeSV1756c and 1µl of 100 ng MNe1107 followed by addition of 5 µl of 10X RT-PCR buffer, 1 µl of reverse transcriptase (Stratagene) and 1 µl of 40mM dNTPs. The total reaction volume was made 50 µl by addition of RNase free water. This mixture was incubated at 42 ^{o}C for 15 min, 95 ^{o}C for 1 min, followed by 40 cycles of 95 ^{o}C for 30 sec, 42 ^{o}C for 30 sec, 68 ^{o}C for 2 min followed by 10 min of extension at 68^{o}C in a thermal cycler. An aliquot of 8 µl of the cut plasmid DNA was subjected to agarose gel electrophoresis on a 2.0 % agarose gel (Fisher Scientific). The protocol mentioned in section 2.6 was followed.

2.95 Surface Sterilization of Plant specimens.

The plant specimens were washed in running water to remove soil particles. The washed plant tissues were immersed in 70% (v/v) ethanol for 5 minutes and transferred to a solution of hypochlorite solution (0.9%) for 20 minutes. The plant tissues were washed in sterile water for 3 times to wash away the sterilization agents.

CHAPTER III

RESULTS

3.1 Optimization of Plant Homogenization Conditions

3.1.1 *Determination of Optimum Amount of Plant Tissue.*

The collected non-Tallgrass Prairie Preserve (non-TPP) samples were classified into grass, hard leaves, tree leaves, soft leaves and fern leaves. Multiple aliquots of 100, 200, 300, 400 and 500 mg of each of these samples were placed in 1.5 ml self-standing screwcap microtubes. These tubes were stored at -80^{o} C for 3-4 days.

About 12-15 sterile glass beads (2.5 mm diameter) were added to each of these tubes.

After addition of citrate buffer and iodoacetamide, all the samples were treated at 4600 rpm for 3 min in a Mini- Beadbeater. The beaten samples were scored based on visual observation for the presence of non-homogenized plant tissue.

Non-homogenized tissue was not observed in the 100 mg aliquot of all the different types of plant leaves. Though the soft leaves and fern leaves of 200 mg aliquots were homogenized, the hard leaves, grass and tree leaves samples contained non-homogenized plant tissue. The 300, 400 and 500 mg plant aliquots showed the presence of non-homogenized plant tissue in all the samples.

Thus 100 mg of plant tissue was determined to be the maximum amount of plant tissue that can be efficiently homogenized under the above-mentioned conditions.

3.1.2 *Determination of the Optimum Bead Material.*

The optimum diameter of glass beads was determined to be 2.5 mm among 1.0, 2.5 and 3.0 mm glass beads, from previous studies by Fenqui Zhang in our laboratory. To determine the optimum material of beads that would efficiently homogenize plant tissues, the 100 and 200 mg aliquots of all the non-TPP samples were subjected to homogenization in the mini-beater using about 12-15 2.5 mm glass beads, 2.0 mm zirconium beads and 2.3 mm chrome steel beads. The beaten samples were scored based on visual observation for presence of non-homogenized plant tissue.

There were no non-homogenized plant tissues observed in any of the 100 mg tubes. The soft leaves and fern leaves of the 200 mg samples were completely homogenized by all the three kinds of bead material but the plant homogenate obtained using glass beads appeared less turbid as compared to the homogenate obtained using the zirconium and steel beads. Thus glass bead material was determined to be the optimum bead material that can be used for efficient plant homogenization.

3.1.3 *Pretreatment of Plant Tissue.*

To determine the optimum homogenization conditions, the non-TPP plant tissues were subjected to different pretreatment conditions such as freezing at -80^{o} C or freezing by using liquid nitrogen. Aliquots of the non-TPP samples (100 mg) were stored at -80^{o} C

and 4^{o} C. After addition of citrate buffer and iodoacetamide, all the samples were treated at 4600 rpm for 3 min in a Mini- Beadbeater. Some of the samples that were stored at -80^{o} C were immersed in liquid nitrogen for about 10 seconds before homogenization.

There were no visible differences between the homogenate obtained from plant samples stored at -80^{o} C and the plant samples treated with liquid nitrogen. Non-homogenized plant tissue was observed in plant tissues that were stored at 4 ^{o} C and were not pretreated with liquid nitrogen before homogenization. Freezing the plant tissues at -80^{o} C is less expensive as compared to freezing using liquid nitrogen and hence was determined to be the most cost-effective way for preparing plant tissue for homogenization.

3.1.4 *Type of Bead-Beater*

A small aliquot (100 mg) of the non-TPP plant tissues was subjected to homogenization using both the Mini- Beadbeater (4600 rpm for 3 min) and the Mini-Beadbeater-96 (2100 rpm for 15 min). About 12-15 glass beads were added to each of the samples followed by addition of citrate buffer and iodoacetamide. The results were recorded based on visual observation for presence of non-homogenized plant tissue.

A large portion of the plant tissues was not homogenized by the Mini-Beadbeater-96. The Mini- Beadbeater produced a better homogenate as compared to the homogenate produced by the Mini- Beadbeater-96. Thus the Mini- Beadbeater was determined to be more efficient than the Mini- Beadbeater-96 for processing of plant samples.

3.2 Storage of Plant samples.

3.2.1 *Storage of Plant Homogenate.*

Maize plants harboring the *Maize necrotic streak virus* (MNeSV) were homogenized and aliquots of the supernatants obtained were stored at room temperature (S-RT), 4 ^{o} C (S4), -20 ^{o} C (S20), and -80 ^{o} C (S80) for 4 days. The stability of the virus in the stored supernatant was tested by performing a RT-PCR using MNeSV specific primers. The RT-PCR products were subjected to agarose gel electrophoresis (figure 1).

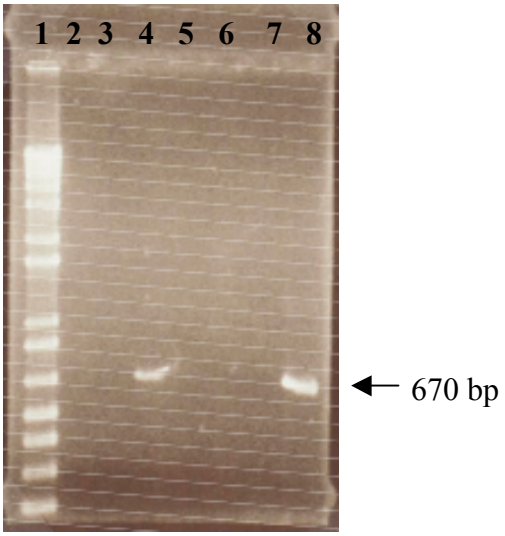


Figure 1: Storage of plant homogenate. Lane1- 1 Kb+ ladder, Lane2-Empty, Lane3- S-RT, Lane4- S4, Lane5- S20, Lane6- Negative control (uninfected maize plant), Lane7- S80, Lane8- Positive control (MNeSV RNA)

Only the supernatant stored at 4 ^{o} C showed a positive PCR band. This suggests that viruses are not stable in supernatants stored at room temperature, -20 ^{o} C, and -80 ^{o} C. Thus during processing of the TPP samples the supernatants were stored at 4 ^{o}C for a maximum of 4 days.

3.2.2 *Storage of Plant tissue at -80^{o} C*

As mentioned above, an aliquot of 100 mg of the plant tissues were stored at -80^{o} C to increase the efficiency of homogenization and to prevent plants from getting spoilt.

To test the stability of viruses in the plant tissue stored at -80^{o} C, 100 mg of maize plant tissue infected with MNeSV was stored at -80^{o} C for 14 days and the presence of the virus was detected by RT-PCR using virus specific primers. Uninfected maize plant tissue was used as a negative control. MNeSV RNA was used as a positive control. The RT-PCR products were subjected to agarose electrophoresis (figure 2).

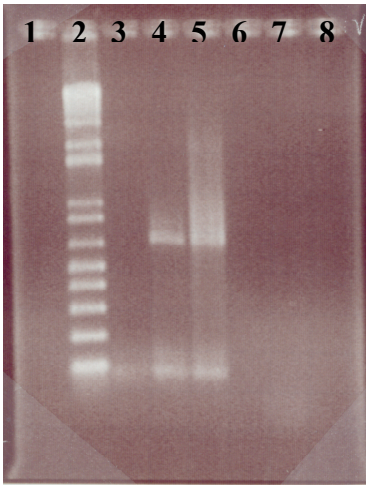


Figure 2: Storage of plant tissue. Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- uninfected maize plant tissue, Lane4- maize plant tissue infected with MNeSV, Lane5- positive control, Lane6, 7, 8- Empty.

The maize plant tissue infected with MNeSV was PCR positive; this suggests that plant tissues can be stored at -80^{o} C without destabilizing plant viruses. All the TPP-samples were weighed into 100 mg aliquots, placed in screwcap microtubes and stored in -80 ^{o}C until further use.

3.3 Optimization of Random PCR conditions.

3.3.1 *Random PCR of Maize necrotic streak virus.*

Both the uninfected maize plant (U) and the maize plant infected with MNeSV (M) were homogenized and the supernatants obtained were stored at room temperature (U-RT, M-RT), 4 ^{o} C (U4, M4), -20 ^{o} C (U20, M20), and -80 ^{o} C (U80, M80) for 4 days. A separate set of infected and non-infected maize plant tissues was homogenized and the supernatants obtained were processed directly (U-direct, M-direct) without storing them. All the samples were subjected to the VLP-VNA protocol for the isolation of viral nucleic acid. The isolated viral nucleic acid was subjected to random PCR using degenerate primers. The PCR products were subjected to agarose gel electrophoresis (Figure 3). Two negative controls were included in this experiment: first, the PCR negative control, which was nuclease free water used in the random PCR reaction without any template second, a nuclease-free water-negative control was subjected to the VLP-VNA protocol, this control was processed along with the plant samples.

Both the MNeSV infected and uninfected plant samples were PCR positive. The typical pattern of overlapping bands obtained when a nucleic acid is subjected to random PCR is designated as a smear. The presence of a smear indicates the presence of a nucleic acid, which served as a template for the amplification reaction. Since the employed protocol was based on enrichment of VLPs and isolation of VNA, the presence of a smear was supposed to indicate the possible presence of viruses.

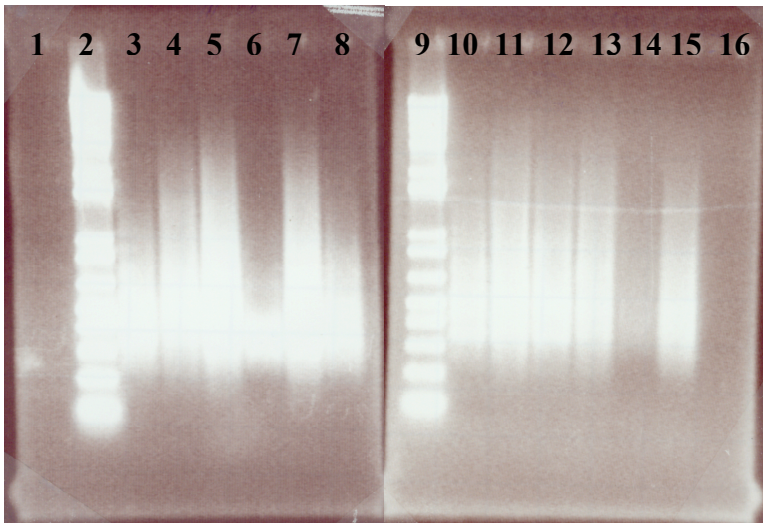


Figure 3: Random PCR of MNeSV and uninfected maize plant. Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- M-direct, Lane4- U-direct, Lane5- M-RT, Lane6- U-RT, Lane7- M4, Lane8- U4, Lane9- 1 Kb+ Marker, Lane10- M20, Lane11-U20, Lane12- M80, Lane13-U80, Lane14- PCR negative control, Lane15- Nuclease free water- (negative control), Lane16- Empty.

The occurrence of a smear in the negative controls and in the uninfected plant samples were hypothesized to be due to one of the following 3 conditions-

1. The presence of microbes on the surface of the plant tissue, which provides the nucleic acid template for the amplification reaction.
2. The random primers are self-annealing and producing amplicons.
3. The reagents used in the protocol are contaminated and are the source of the template for the random PCR amplification.

3.3.2 *Surface Sterilization of Plant Tissue*

To test for microbes on the plant tissue surface being the source of template for the random PCR reaction, two aliquots of the uninfected maize plant were used; one of them was subjected to surface sterilization as mentioned in Materials and Methods. A small

aliquot of the surface sterilized leaf was inoculated into 100 ml of LB broth for checking the sterility of the leaf (Table 1).

Table1: Observation for growth of surface sterilized and non-surface sterilized plants.

Leaf	Growth
Surface Sterilized	No
Non-Surface Sterilized	Yes
None	No

Both the surface sterilized and non-surface sterilized maize plant tissue was subjected to the VLP-VNA protocol. The isolated VNA was subjected to random PCR using degenerate primers and the PCR products were subjected to agarose gel electrophoresis (Figure 4).

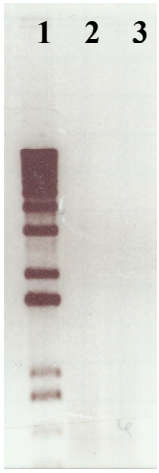


Figure 4: Surface sterilization of plant tissue. Lane1- 1 Kb+ Marker, Lane2- Surface sterilized plant tissue, Lane3- Non-surface sterilized plant tissue.

If microbes on the surface were the true source of template for the random PCR reaction, then only the non-surface sterilized sample would have been PCR positive, but a smear was observed in both surface-sterilized and non-surface sterilized uninfected maize plant

tissue. Thus microbes on the surface are not the reason for observing smears in the uninfected plant tissue samples.

3.3.3 *Sequencing of Negative control and MNeSV PCR products.*

A straightforward way to show that the self-annealing of the random primers produces amplicons in the negative control would be to clone and sequence the amplicons obtained in the negative control. If the obtained sequences consist of repetitive primer sequences , then it can be concluded that the true reason of obtaining amplicons in the negative control is self-annealing of the random primers.

The maize plant tissue infected with the MNeSV was subjected to the VLP-VNA protocol along with a nuclease-free water negative control. The obtained nucleic acid was subjected to random PCR amplification and the PCR products were subjected to agarose gel electrophoresis (Figure 5).

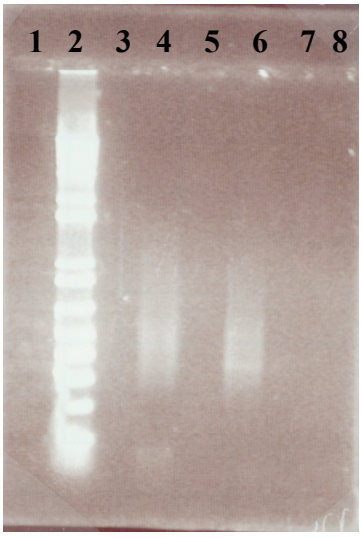


Figure 5: Sequencing of Negative control and MNeSV PCR products. Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- Empty, Lane4- Nuclease free water negative control, Lane5- Empty, Lane6- maize plant infected with MNeSV, Lane7, 8- Empty.

The PCR products obtained were ligated to a TOPO-TA vector and transformed into *E.coli* one-shot-TOP10 chemically competent cells. The transformation mixture was plated on a LB agar medium containing ampicillin. Colonies obtained from the transformation mixture of both the MNeSV containing plant samples and the negative control were picked and plasmids were extracted (Figure 6).

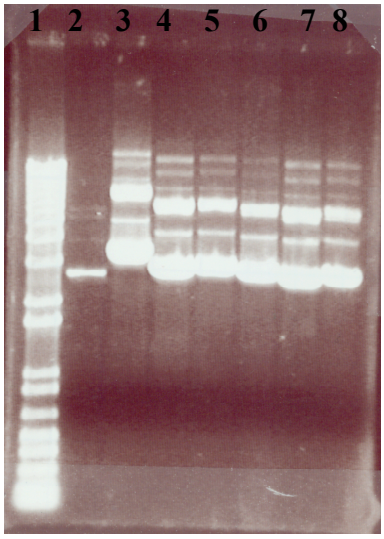


Figure 6: Plasmid preparation of MNeSV infected plants and negative control. Lane1- 1 Kb+ Marker, Lane2, 3,4,5 – plasmid prepared from colonies obtained from negative control, Lane6, 7, 8- plasmid prepared from colonies obtained from plant samples infected with MNeSV.

The plasmids obtained were subjected to restriction digestion using *Alw*N1 to determine the size of the insert. The restriction digestion reaction was subjected to agarose gel electrophoresis (figure 7).

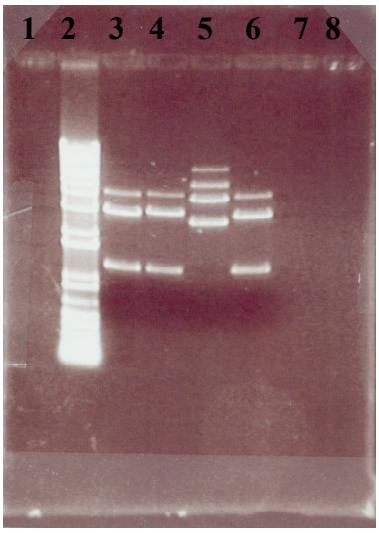


Figure 7: Restriction digestion of plasmids. Lane1- Empty, Lane2- 1Kb+ Marker, Lane3, 4- Restriction digestion of plasmids prepared from colonies obtained from negative control, Lane5, 6- Restriction digestion of plasmids prepared from colonies obtained from MNeSV infected plant tissue, Lane7, 8- Empty.

Based on the size of the insert, three plasmid preparations from the negative control and three plasmid preparations from the MNeSV infected plant tissue were sequenced.

The sequences obtained were identified by comparing with the sequence database using BLASTn (Table 2).

Table 2: BLASTn analysis of sequences obtained from MNeSV infected plant and nulclease-free water negative control.

	SEQUENCE	TOP HIT ON BLASTN
MNeSV infected plant	Colony 1	TVCV
	Colony 2	MNeSV
	Colony 3	MNeSV
Negative control	Colony 1	TVCV
	Colony 2	Mus musculus chromosome
	Colony 3	TVCV

TVCV = *Turnip vein clearing virus*

The following conclusions were drawn based on the obtained results.

1. The VLP-VNA technique can be used for identification of plant viruses from plant tissues. Two out of three colonies showed MNeSV-like sequences as the top hit in BLASTN analysis.
2. The sequences obtained in the negative control did not contain repetitive primer sequences; this suggests that the smear obtained in the negative control is not due to the self-annealing of the random primers.
3. The top-hit in the BLASTn analysis of the negative control was the TVCV, a virus that has been previously studied in our laboratory. This result suggests that the probable cause of a smear in the negative control was contamination of the reagents used in the protocol.

3.3.4 *Contamination in the Reagents.*

To determine the source of contamination, fresh PCR reagents were purchased and random PCR was performed using primer A and B on *Potato virus X* (PVX) RNA along with a nuclease free water negative control. The PCR products were subjected to agarose gel electrophoresis (Figure 8).

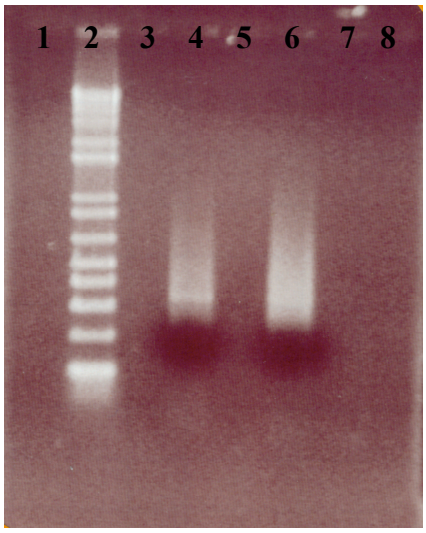


Figure 8: Contamination in the reagents. Lane1- Empty, Lane2- 1 Kb+ Ladder, Lane4- Nuclease free water –negative control, Lane6- PVX RNA, Lane3, 5, 7, 8- Empty.

Both the PVX RNA and the nuclease free water were PCR-positive. The contamination might be due to any one or more of multiple sources such as dust particles, contaminated pipettes due to lack of filters in pipette tips, improper sterilization of microfuge tubes or contamination of primers.

To solve the problem of contamination the following actions were undertaken:

1. A UV-hood was set up and all the regents were added strictly inside the hood. The tubes were never opened outside the hood.
2. Fresh reagents were prepared, sterilized and distributed into small aliquots; a single aliquot once opened was never used again.

3. New pipettes and filtered pipette tips were purchased.
4. DNase and RNase free microfuge tubes were purchased.
5. A new set of primers was purchased.

By following all the above-mentioned strategies, the problem of contamination was almost eradicated. Each run included negative controls to monitor for contamination.

3.3.5 *Optimization of the Number of PCR Cycles.*

Even after taking the necessary precautions, a faint smear was still observed in many of the negative controls after the PCR reactions. This was hypothesized to be due to the large number of PCR cycles (40 cycles). To find the optimum number of PCR cycles that would show presence of smears in the positive control and absence of smear in the negative control, the following experiment was conducted.

PVX RNA was used as the positive control (P) and nuclease free water was used as the negative control (N) and the random PCR was performed using primer A and primer B.

The PCR reaction was run under the following conditions with 5 sets of positive and negative controls (Table 3). One set of tubes was removed after 20 (P20, N20), 25 (P25, N25), 30 (P30, N30), 35 (P35, N35) and 40 (P40, N40) cycles.

Table3: Optimization of number of PCR cycles.

STEP	TEMPERATURE	TIME
1	94 ^{o}C	30 S
2	40 ^{o}C	30 S
3	50 ^{o}C	30 S

4	72 ^{o} C	60 S
5	GOTO Step 1- 20 more times	
6	21 ^{o} C	5 MIN
7	94 ^{o} C	30 S
8	40 ^{o} C	30 S
9	50 ^{o} C	30 S
10	72 ^{o} C	60 S
11	GOTO Step 7- 5 more times	
12	21 ^{o} C	5 MIN
13	94 ^{o} C	30 S
14	40 ^{o} C	30 S
15	50 ^{o} C	30 S
16	72 ^{o} C	60 S
17	GOTO Step 13- 5 more times	
18	21 ^{o} C	5 MIN
19	94 ^{o} C	30 S
20	40 ^{o} C	30 S
21	50 ^{o} C	30 S
22	72 ^{o} C	60 S
23	GOTO Step 19- 5 more times	
24	21 ^{o} C	5 MIN
25	94 ^{o} C	30 S
26	40 ^{o} C	30 S
27	50 ^{o} C	30 S
28	72 ^{o} C	60 S
29	GOTO Step 25- 5 more times	
30	4 ^{o} C	STORE

The PCR reaction was subjected to agarose electrophoresis (Figure 9).

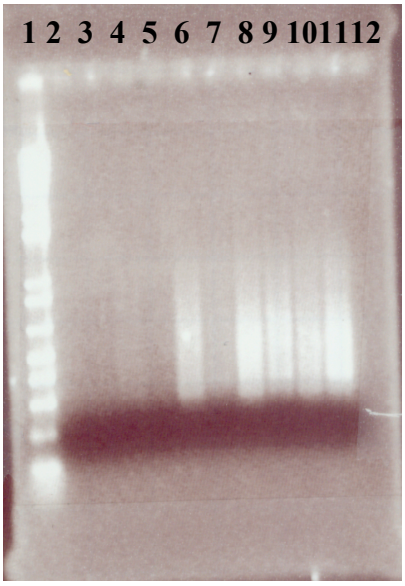


Figure 9: Optimization of number of PCR cycles. Lane1- 1 Kb+ Marker, Lane2- P20, Lane3- N20, Lane4- P25, Lane5- N25, Lane6- P30, Lane7- N30, Lane8- P35, Lane9- N35, Lane10- P40, Lane11- N40, Lane12- Empty.

No smear was observed after 20 and 25 cycles. After 30 cycles, a smear was observed in the positive control but not in the negative control. After 35 and 40 cycles, smears were observed in both the negative and positive control. Thus, 30 cycles was determined to be the optimum number of PCR cycles.

3.4 Selection of primers

3.4.1 *Primer A vs. Primer A12*

Increasing the length of the random primer was reported to increase the size and yield of the resulting cDNA (Stangegaard, 2006). Primer A (hexamer) was compared to Primer A12 (dodecamer) to determine the primer that is capable of producing fewer smaller-size

PCR products. Small-size PCR products have less sequence information and hence should be avoided in the VNA library.

VNA was isolated from the TGP plant 05TGP00289 and divided into two aliquots. Random amplification was performed in these aliquots using primer A and primer B in one aliquot and primer A12 and primer B in the other. The PCR products were subjected to agarose gel electrophoresis (Figure 10).

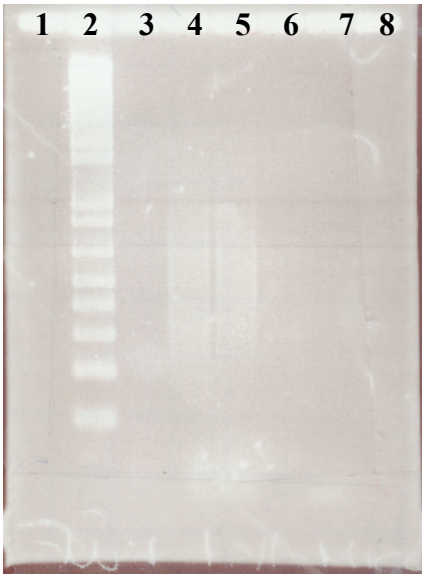


Figure 10: Primer A vs. Primer A12 Lane1- Empty, Lane2- 1 Kb+ marker, Lane4- Primer A used for amplification Lane5- Primer A12 used for amplification, Lane3, 6, 7, 8- Empty.

Primer A produced smaller size PCR products starting from 100 bp, while primer A12 gave PCR products starting from 300 bp. Thus Primer A12 was used instead of primer A for processing of the TGP samples.

3.4.2 *Primer A12, Primer B vs. Primer A2, Primer B2*

The 5' end sequence of primer A12 is self-complementary (ability to form 2^{o} structures such as hairpins); this might lower the yield of PCR products. Primer A2 and primer B2 were designed to be non self-complementary.

VNA isolated from a TGP sample that gave positive PCR product with primer A12 and primer B (05TGP00040) and a sample that did not produce amplicons with primer A12 and primer B (05TGP00028) was used for testing primer A2 and primer B2. The PCR products were subjected to agarose gel electrophoresis (Figure 11).

Figure 11: Primer A12, Primer B vs. Primer A2, Primer B2 Lane1- Negative sample (05TGP00028) using primer A12 and primer B, Lane2- Positive sample (05TGP00040) using primer A12 and primer B, Lane3-05TGP00028 + 05TGP00040 using primer A12 and primer B, Lane4- positive sample (05TGP00040) using primer A2 and primer B2, Lane5- nuclease free water (negative control) using primer A12 and primer B, Lane6- nuclease free water (negative control) using primer A2 and primer B2.

The nuclease free water negative control that was amplified using primer A2 and primer B2 was PCR-positive and produced a smear that was similar to the smear obtained with the positive sample. This could have been due to contamination of the primers. But this puzzle was left unsolved as the primer A12 and primer B was capable of producing a good yield of PCR products and did not produce amplicons in the negative control.

3.5 Processing of TGP samples.

A total of 1187 plants were collected from the Tallgrass Prairie Preserve over a period of three years. A total of 687 plants were subjected to the VLP-VNA method, of which 404 plants gave smears after random PCR. Figure 12 shows the agarose gel electrophoresis profiles of the first 5 plants that were processed. The profiles of the other plants are in the appendix.

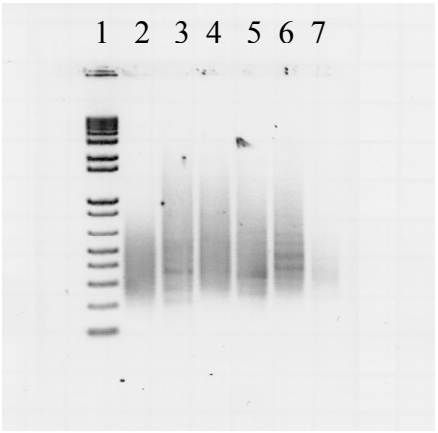


Figure 12: Random PCR amplification of TGP samples. Lane 1- 1Kb+ Marker, Lane 2- VNA_05TGP00295_001, Lane 3- VNA_05TGP00248_002, Lane 4-VNA_05TGP00289_003, Lane 5- VNA_05TGP00162_004, Lane 6- VNA_05TGP00296_005, Lane 7- Negative control.

3. 6 Smear Results.

Of the 1187 plant samples that were collected from the Tallgrass Prairie Preserve, 687 plants were subjected to the VLP-VNA method followed by random PCR amplification. About 122 plants are in process, i.e. the VNA have been isolated from these plants but they are yet to be subjected for random PCR amplification. About 378 plants are yet to be processed as represented in the pie chart (figure 13).

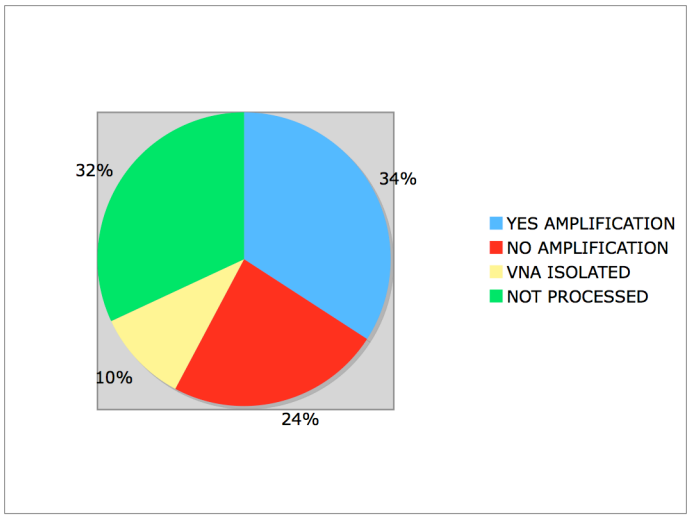


Figure 13: Pie chart representing processed samples, in-process samples and the yet to be processed samples.

The result of amplification of the VNA obtained from the first 5 plant samples is summarized in table 4. The complete amplification results of all the processed plant samples can be found in the appendix.

Table 4: **Smear results for the first 5 TGP samples.**

Virion ID	Plant ID	Plant Species	AMPLIFICATION
VNA_05TGP00295_001	05TGP00295	*Ambrosia psilostachya*	YES AMPLIFICATION
VNA_05TGP00248_002	05TGP00248	*Asclepias viridis*	YES AMPLIFICATION
VNA_05TGP00289_003	05TGP00289	*Asplenium rhizophyllum*	YES AMPLIFICATION
VNA_05TGP00162_004	05TGP00162	*Dichanthelium oligosanthes*	YES AMPLIFICATION
VNA_05TGP00296_005	05TGP00296	*Panicum virgatum*	YES AMPLIFICATION

A smear graph was constructed based on the number of positive and negative PCR results obtained for each plant species. The graph shown in figure 14 represents only those plant species for which more than 5 specimens were collected.

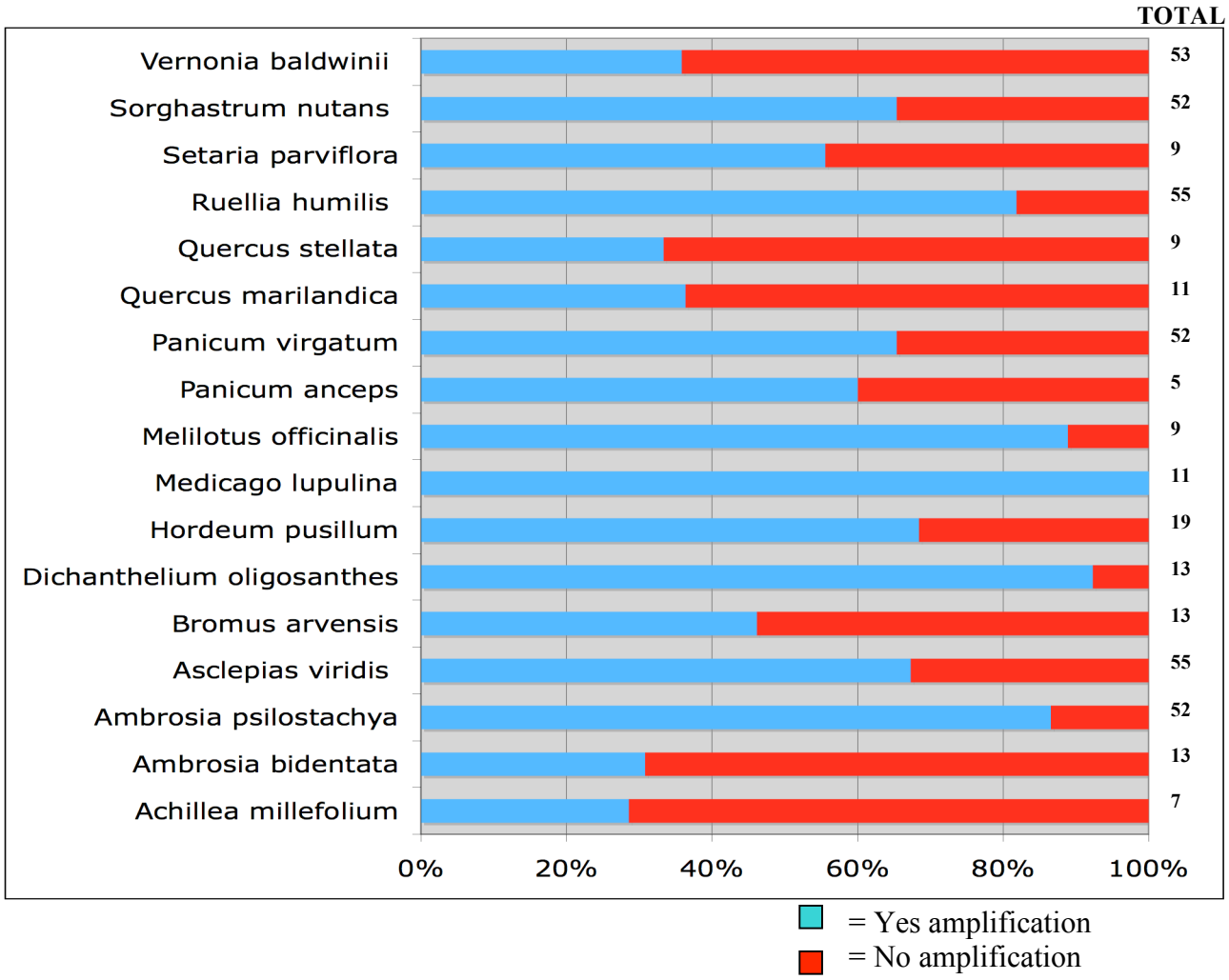


Figure 14: Bar graph representing percentage of positive and negative PCR results obtained for specific plant species.

As shown (figure 14), the distribution of smears among plant species does not follow any particular pattern, for example more than 60% of *Asclepias viridis* and more than 80% of *Ambrosia psilostachya* plants were PCR positive while only less than 40% of *Vernonia*

baldwinii were PCR positive. There was at least one plant specimen that produced amplification among all the plant species that were processed.

3.7 Sending Samples for Sequencing

Only those TGP samples that were PCR positive were further processed and they were sent to ACGT, Norman for sequencing. The PCR positive samples were ligated to a plasmid vector and were transformed into chemically competent cells. The protocols suggested by Invitrogen and Stratagene were tested and confirmed by the presence of blue and white colonies on the LB agar plates containing the appropriate antibiotic. Plasmids extracted from the transformed cells were subjected to agarose gel electrophoresis (figure 15 and 16).

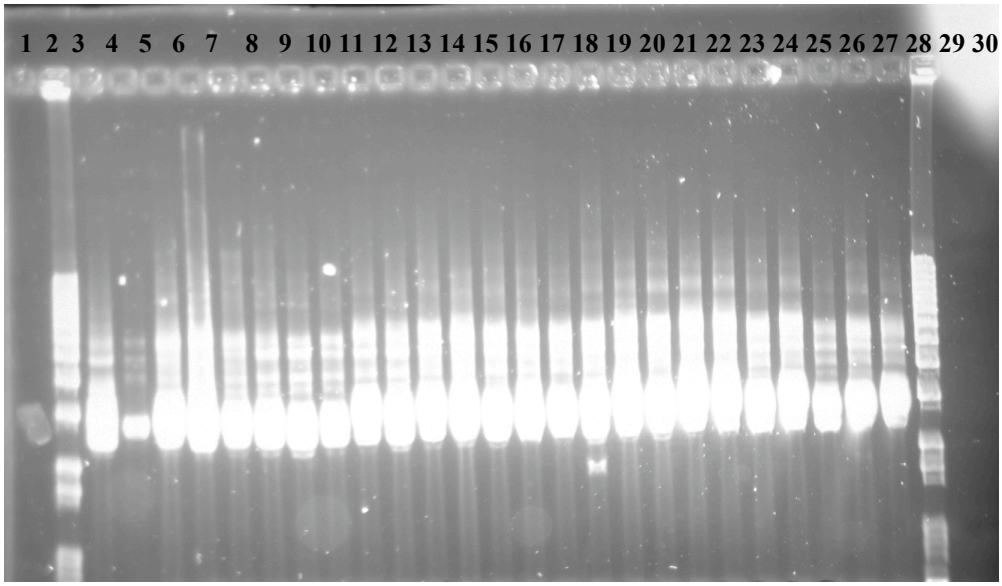


Figure 15: Plasmid preparation (TOPO-TA vector). Lane1-Empty, Lane2- 1 Kb+ Marker, Lane3 to 27- TGP samples, Lane29,30-Empty.

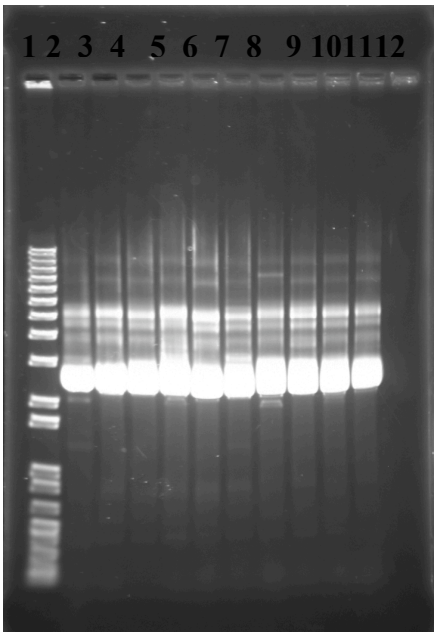


Figure 16: Plasmid preparation (Stratagene vectors) Lane1- 1 Kb+ marker, Lane2 to 11- TGP samples, Lane12- Empty

The plasmids were subjected to restriction digestion to check the size of inserts. The TOPO TA plasmids were cut using *Alw*N1 while the Stratagene plasmids were cut using *Nsp*1. The *Alw*N1 enzyme cuts the plasmid into two segments, a 2326 bp segment and a 1574 bp + insert segment. The *Nsp*1 enzyme cuts the Stratagene vector into two segments, a 1246 bp segment and a 2221 bp + insert segment. The restriction digestion reactions were subjected to agarose gel electrophoresis (figure 17 and 18).

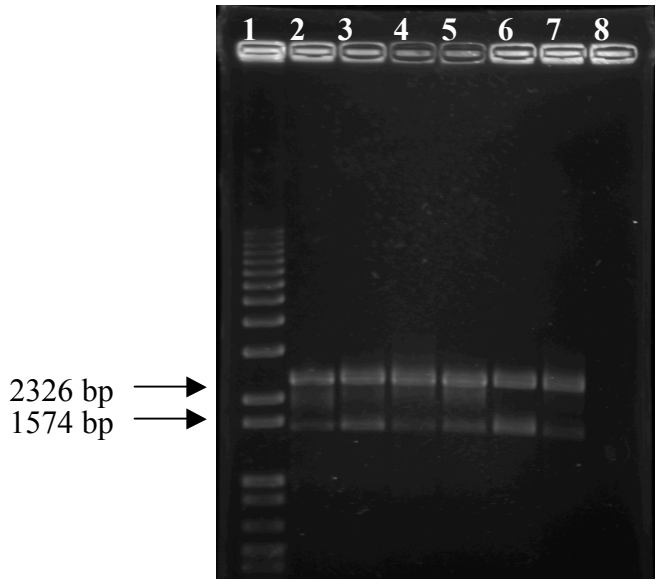


Figure 17: Restriction digestion (TOPO TA vector) using *Alw*NI enzyme.
Lane1- 1 Kb+ marker, Lane2 to 7- TGP samples, Lane8- Empty.

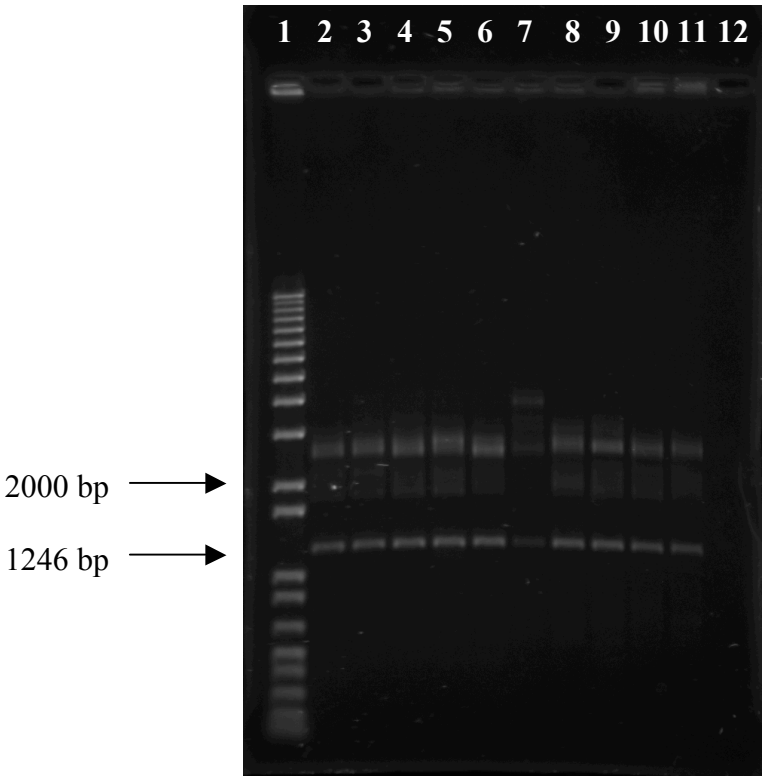


Figure 18: Restriction digestion (Stratagene vector) using *Nsp*1 enzyme. Lane1- 1 Kb+ marker, Lane2 to 11- TGP samples, Lane12- Empty.

Glycerol stocks of samples that have >500 bp insert sizes were sent to ACGT, Norman , for sequencing.

3.8 Contig Classification:

Sequences were obtained for 68 plant samples (Table 5). The sequences were assembled into contigs and the contigs were classified based on the protocol mentioned in the Materials and Methods. Table 6 represents the classification of the contigs obtained from an *Asclepias viridis* plant (05TGP00248).

Table 5: List of plants for which sequences were obtained.

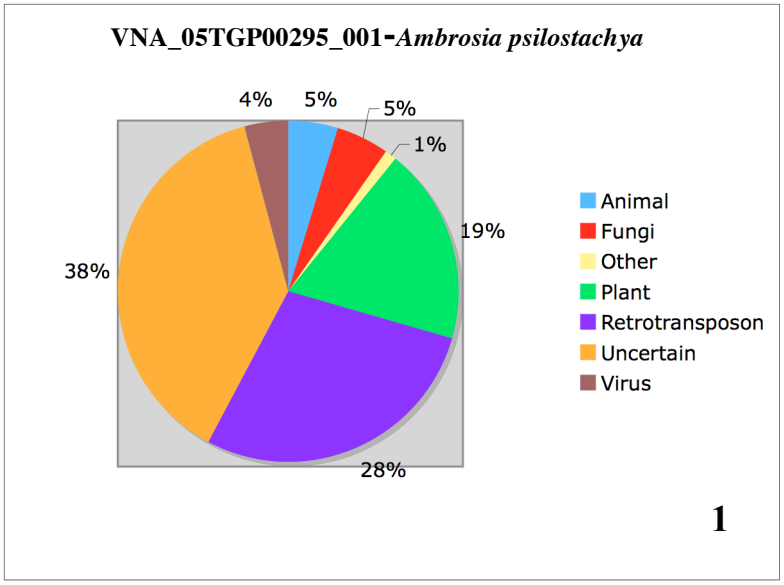
Plant ID	Plant Species
05TGP00295	Ambrosia psilostachya
05TGP00248	Asclepias viridis
05TGP00289	Asplenium rhizophyllum
05TGP00162	Dichanthelium oligosanthes
05TGP00296	Panicum virgatum
05TGP00307	Asclepias viridis
05TGP00312	Ambrosia psilostachya
05TGP00316	Panicum virgatum
05TGP00321	Ambrosia psilostachya
05TGP00323	Asclepias viridis
05TGP00332	Sorghastrum nutans
05TGP00335	Ruellia humilis
05TGP00337	Asclepias viridis
05TGP00339	Panicum virgatum
05TGP00342	Coreopsis tinctoria
05TGP00351	Asclepias viridis
05TGP00192	Sedum nuttallianum
05TGP00248	Asclepias viridis
05TGP00294	Asclepias viridis
05TGP00379	Ambrosia psilostachya
05TGP00403	Asclepias viridis
05TGP00029	Apocynum cannabinum
05TGP00031	Symphoricarpos orbiculatus
05TGP00050	Echinacea pallida
05TGP00096	Baptisia australis

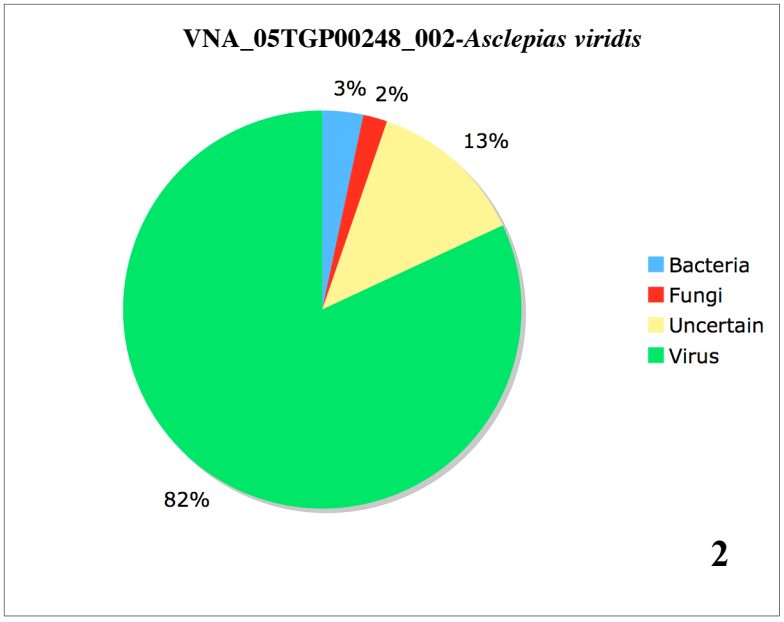
05TGP00112	Carex vulpinoidea
05TGP00182	Festuca subverticillata
05TGP00193	Xanthisma texanum
05TGP00040	Vitis sp
05TGP00044	Lepidium virginicum
05TGP00060	Phalaris caroliniana
05TGP00065	Tradescantia ohiensis
05TGP00079	Ludwigia palustris
05TGP00088	Allium canadensis
05TGP00094	Erigeron tenuis
05TGP00100	Ambrosia psilostachya
05TGP00106	Xanthium strumarium
05TGP00117	Maclura pomifera
05TGP00165	unk sp
05TGP00374	Typha domingensis
05TGP00037	Prunus serotina
05TGP00041	Arisaema dracontium
05TGP00042	Rubus flagellaris
05TGP00048	Amorpha canescens
05TGP00052	Artemisia ludoviciana
05TGP00053	Psoralidium tenuiflorum
05TGP00061	Callirhoe alcaeoides
05TGP00062	Desmanthus illinoensis
05TGP00066	Amorpha fruticosa
05TGP00453	Diodia teres
05TGP00464	Asclepias viridis
05TGP00576	Asclepias verticillata
06TGP01051	Asclepias viridis
05TGP00251	Panicum virgatum
05TGP00265	Panicum virgatum
05TGP00304	Panicum virgatum
05TGP00333	Panicum virgatum
05TGP00378	Panicum virgatum
05TGP00386	Panicum virgatum
05TGP00415	Panicum virgatum
05TGP00425	Panicum virgatum
05TGP00444	Panicum virgatum
05TGP00555	Panicum obtusum
05TGP00619	Panicum rigidulum
O5TGP00244	Sorghastrum nutans
05TGP00264	Sorghastrum nutans
05TGP00298	Sorghastrum nutans
05TGP00315	Sorghastrum nutans
05TGP00325	Sorghastrum nutans
05TGP00256	Chara globularis

Table 6: Contig classification for sample VNA_05TGP00248_002 (*Asclepias viridis*).

Contig#	No. of Reads	Category	Comment
1	1	Virus	Tymovirus
2	1	Virus	Tymovirus
3	2	Virus	Tymovirus/Pseudovirus
4	3	Virus	Tymovirus
5	4	Virus	Tymovirus
6	4	Virus	Tymovirus
7	5	Virus	Tymovirus
8	5	Uncertain	
9	6	Virus	Tymovirus
10	6	Virus	Tymovirus
11	7	Virus	Tymovirus
12	7	Virus	Tymovirus
13	7	Virus	Tymovirus
14	8	Virus	Tymovirus
15	9	Virus	Tymovirus
16	9	Virus	Tymovirus
17	10	Virus	Tymovirus
18	11	Virus	Tymovirus
19	11	Uncertain	
20	11	Fungi	Gibberella
21	12	Virus	Tymovirus
22	12	Virus	Tymovirus
23	12	Virus	Tymovirus
24	13	Virus	Tymovirus
25	13	Virus	Tymovirus
26	14	Virus	Tymovirus
27	14	Uncertain	
28	14	Virus	Tymovirus
29	15	Virus	Tymovirus
30	15	Virus	Tymovirus
31	17	Virus	Tymovirus
32	19	Uncertain	
33	19	Bacteria	Burkholderia
34	19	Virus	Tymovirus
35	21	Virus	Tymovirus
36	21	Virus	Tymovirus
37	22	Virus	Tymovirus
38	25	Uncertain	
39	26	Virus	Tymovirus
40	27	Virus	Tymovirus
41	28	Virus	Tymovirus
42	30	Virus	Tymovirus
43	37	Virus	Tymovirus

The percentage of reads corresponding to each group was represented as a pie chart. Figure 17 represents the read pie chart of the first 5 plant samples. The pie charts of the rest of the samples are given in the appendix.





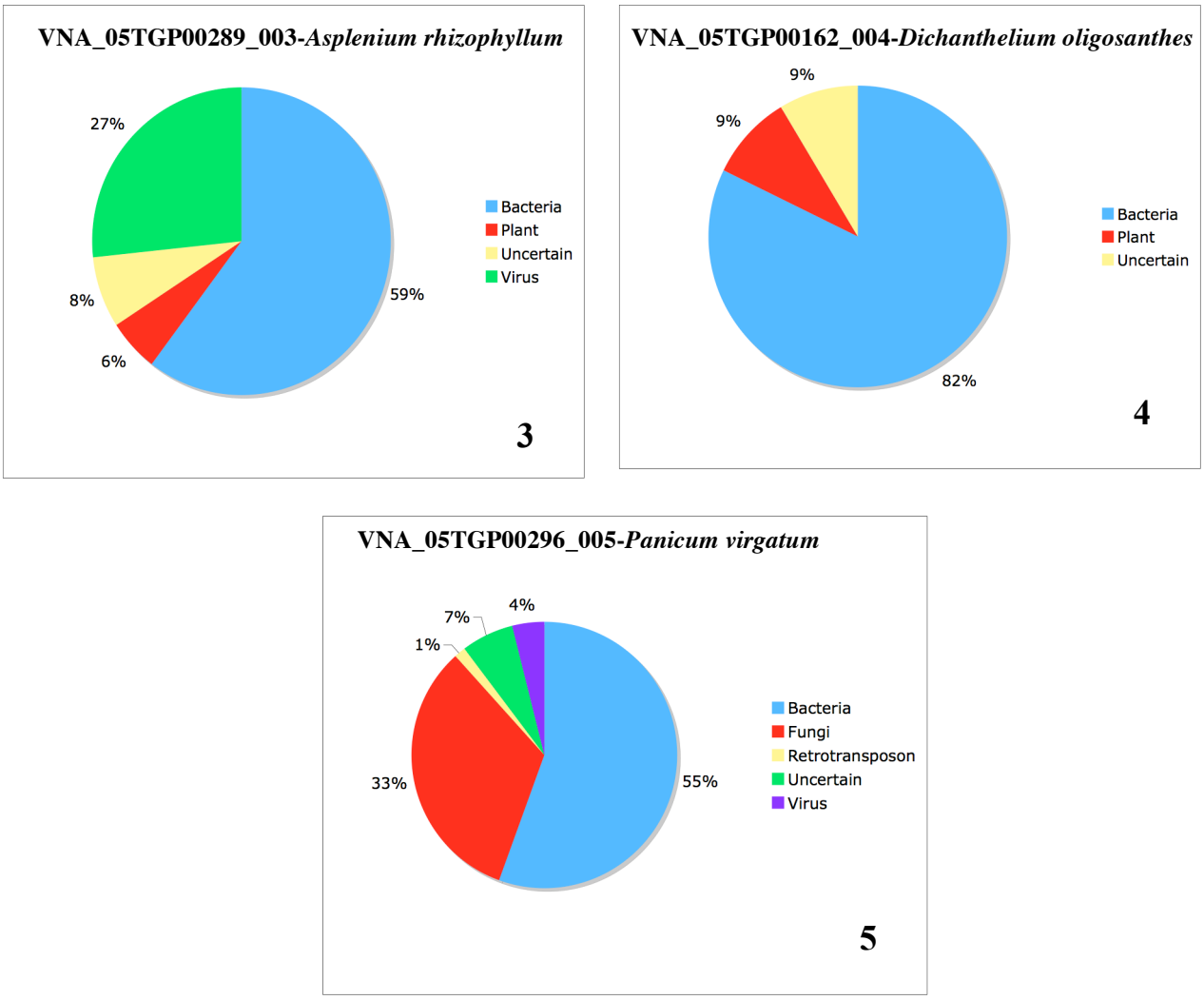


Figure19: Pie charts representing the classification of reads of the first 5 plant samples.

All the reads obtained from the 68 plant samples were pooled together and the percentage of reads representing each group was calculated (Figure 20)

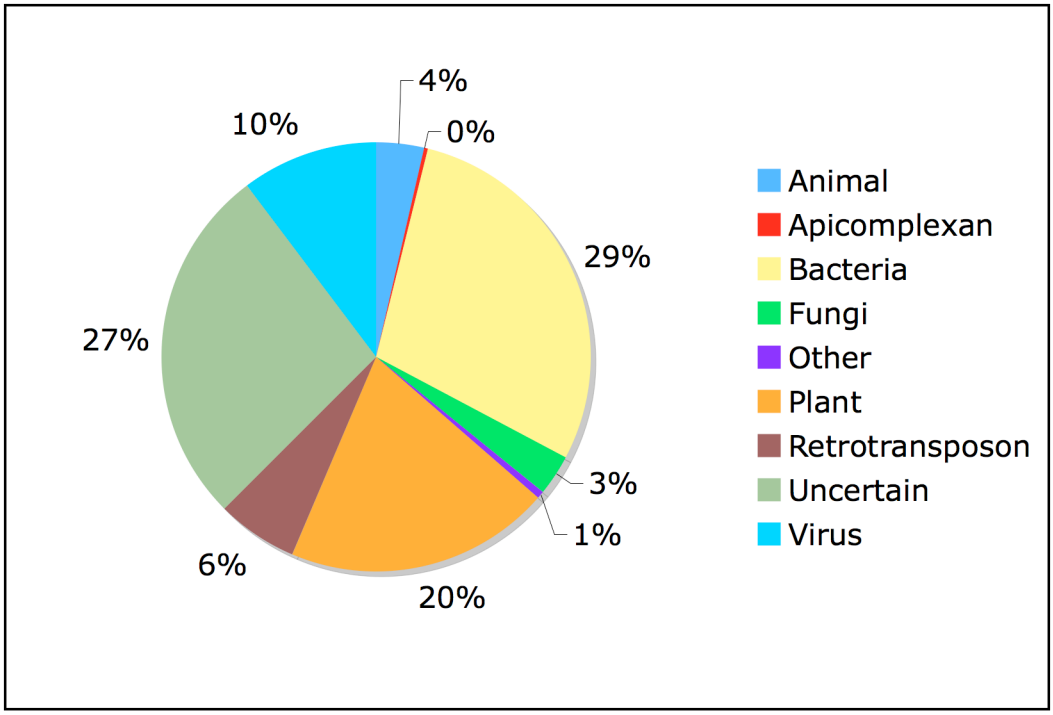


Figure 20: Pie chart representing all the reads obtained from 68 plant samples pooled together.

3.9 Virus Sequences Identified.

Sequences obtained for the 68 samples yielded evidences of at least 9 different viruses (table 6), which include plant viruses such as positive-sense single-stranded RNA viruses (related to *Tymovirus, Flexiviridae, Comovirus, Closterovirus*), double-stranded DNA virus (related to *Badnavirus*) and double-stranded RNA virus (related to *Endornavirus*), a mycovirus (related to *Chrysovirus*), animal viruses (related to MMLV, *Ascovirus)* and a bacteriophage (Lambda-like phage).

Most of the putative viral sequences identified, although assigned to known families and, often genera of viruses, represent novel viruses that have not been previously described by the sequences of their nucleic acid. Viruses belonging to the same genus were found in multiple specimens of the same plant species for example the *Tymovirus* was found in 5 different *Asclepias viridis* plants. Viruses belonging to the same genus was found in different plant species for example beside *Asclepias viridis* the *Tymovirus* was also found in *Panicum virgatum*, *Desmanthus illinoensis* and *Amorpha fruticosa*.

Four plant specimens yielded sequences similar to more than one virus species. A single *Asclepias viridis* plant (05TGP00307) yielded sequences similar to both the *Tymovirus* and the *Comovirus.* A single *Ambrosia psilostachya* plant (05TGP00312) yielded sequences related to a *Badnavirus, Potexvirus* and an unclassified virus from the Closteroviridae family and another *Ambrosia psilostachya* plant (05TGP00321) yielded sequences that were related to unclassified virus from the Flexiviridae family and a *Badnavirus.* A tBLASTx search of the general protein database suggested evidence of the presence of an *Endornavirus* and *Closterovirus* in an *Ambrosia psilostachya* plant (05TGP00295).

The plants were collected from the Tallgrass Prairie Preserve without regard to any visible symptoms of disease. The presence of evidence of viruses does not correlate with diseased appearance of plants. All the plants from which viruses were identified in this study were asymptomatic.

Further evidence for the presence of viruses was obtained from electron microscopy (EM) (figure 21). EM results were obtained for VLPs obtained from only 5 plant samples. The sequences obtained from a *Panicum virgatum* plant (05TGP00339) did not

indicate presence of virus-like sequences in the BLASTn and BLASTx results. But the observation of helically constructed filamentous particles in the EM field led to further analysis of the sequences using tBLASTx which yielded evidence for the presence of a member of the *Closteroviridae* family. The VLPs isolated from all the 687 plant samples were stored in 20% glycerol and have been submitted for EM studies at the University of Tulsa, Oklahoma. We are awaiting the results.

Table 7: Virus sequences identified.

Plant ID	*Plant Species*	*Virus*
05TGP00289	*Asplenium rhizophyllum*	*Chrysovirus*
05TGP00339	*Panicum virgatum*	*Closterovirus*
05TGP00298	*Sorghastrum nutans*	*Emiliana huxleyi virus*
05TGP00052	*Artemisia ludoviciana*	*Emiliana huxleyi virus, Ascovirus*
05TGP00295	*Ambrosia psilostachya*	*Endornavirus, Closterovirus* [a]
05TGP00321	*Ambrosia psilostachya*	*Flexiviridae family, Badnavirus*
05TGP00061	*Callirhoe alcaeoides*	*Murine leukemia virus* [c]
05TGP00312	*Ambrosia psilostachya*	*Potexvirus, Badnavirus,Closteroviridae*[b]
05TGP00248	*Asclepias viridis*	*Tymovirus*
05TGP00337	*Asclepias viridis*	*Tymovirus*
05TGP00351	*Asclepias viridis*	*Tymovirus*
05TGP00294	*Asclepias viridis*	*Tymovirus*
05TGP00062	*Desmanthus illinoensis*	*Tymovirus*
05TGP00066	*Amorpha fruticosa*	*Tymovirus*
05TGP00307	*Asclepias viridis*	*Tymovirus, Comovirus*
05TGP00100	*Ambrosia psilostachya*	*VWB phage, Moloney murine leukemia virus* [c]

a - tBLASTx required for identification of sequences.
b - tBLASTx required for identification of sequences; electron micrograph spurred search.
c - Might be contaminants from the reverse transcriptase enzyme.

Two plant specimens, *Ambrosia psilostachya* (05TGP00100) and *Callirhoe alcaeoides* (05TGP00061) yielded *Moloney murine leukemia virus* (M-MLV) like sequences. The

reverse transcriptase that was used in the random PCR amplification reaction was purified from M-MLV. The presence of the M-MLV sequences could be due to contamination of the reverse transcriptase enzyme with M-MLV nucleic acid.

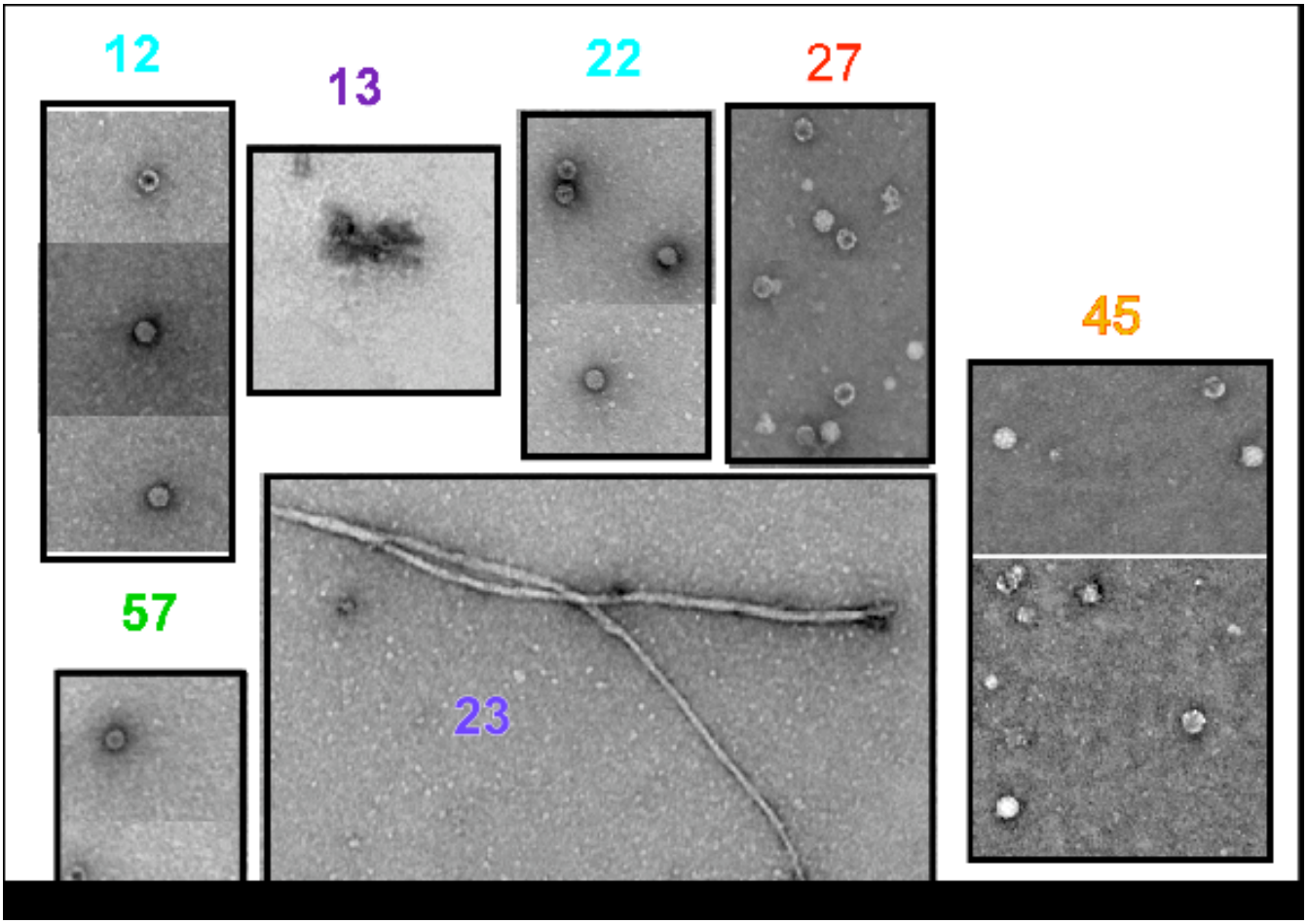


Figure 21: Electron micrograph of VLPs extracted from plant samples. (12) VNA_05TGP00307_012 - *Tymovirus, Comovirus*, (13) VNA_05TGP00312_013-*Flexivirus, Badnavirus,* (22) VNA_05TGP00337_022- *Tymovirus*, (23) VNA_05TGP00339_023- *Closterovirus,* (27) VNA_05TGP00351_027- *Tymovirus,* (45) VNA_05TGP00248_045- *Tymovirus,* (57) VNA_05TGP00294_057- *Tymovirus*

3.91 Other Sequences.

The majority of the reads (29%) consisted of putative bacterial sequences (Figure 20). BLASTn and BLASTx results of the 68 plant specimens showed that most of the plants contained putative bacterial sequences. Most of the bacterial sequences obtained were similar to ribosomal sequences belonging to the enterobacteriace family that includes *Escherichia, Vibrio, Salmonella* and *Shigella*.

About 3% of the reads consisted of fungal-like sequences (figure 20). Of the 68 plants analyzed, 50% had putative fungal sequences. Sequences similar to sequences of fungi such as *Pycnoporus, Phaesospharia, Ustilago, Gibberella, Aspergillus* and *Alternaria* were obtained.

About 6% of the total contigs consisted of putative retrotransposon sequences (figure 20). Sequences that were classified under retrotransposons were similar to sequences of retroviruses, gag-pol polyprotein, retrotransposon protein, reverse transcriptase etc. Most About 50% of the 68 plants analyzed contained retrotransposon-like sequences. Most of the retrotransposon-like sequences were obtained from *Ambrosia, Panicum* and *Sorghastrum* plant specimens.

About 20% of the total reads consisted of putative plant sequences (figure 20). Almost all of the 68 plant samples analyzed, consisted of sequences similar to plant sequences. Most of the putative plant sequences were similar to either 18S and 23S ribosomal RNA, or chloroplast and mitochondrial sequences of plants. About 4% of the total reads were putative animal sequences (figure 20) most of which were similar to human DNA sequences possibly due to investigators contamination. About 27% of the total reads were

classified as uncertain (figure 20) i.e. they could not be assigned with any certainty to a source.

3.92 Negative PCR Amplicons.

As mentioned above only the VNA isolated from plants that were PCR positive were subjected to cloning and sequencing. To test whether VNA that produces no detectable smear after random PCR can yield any sequence information, six *Ambrosia psilostachya* plants (table 8) that were not PCR-positive were chosen.

The VNA isolated from these samples were subjected to random PCR and gel electrophoresis of the PCR reaction confirmed that that the samples did not produce smears. The PCR product was ligated to the Stratagene vector, followed by transformation and plating on a LB Agar media containing ampicillin. Sample number 05TGP00387 did not yield any white colonies. Five white colonies were picked for the remaining samples. Plasmids were extracted and from all the five clones and subjected to restriction digestion using *Nsp*I to determine the plasmid having the largest insert. This plasmid was submitted for sequencing. The sequences obtained were subjected to BLASTn analysis.

Table 8: BLASTn analysis of the sequences obtained from PCR-negative samples.

Plant ID	Top HIT in BLASTn
05TGP00303	*Escherchia coli*
05TGP00361	Bad sequence
05TGP00387	Blue colonies only
05TGP00426	Cloning vector
05TGP00408	Uncultured bacterium
05TGP00417	*Azoarcus*

Though the samples did not produce any detectable smears after random PCR, they were capable of generating sequences similar to *Escherchia coli* and *Azoarcus.* Hence the negative samples cannot be completely ignored and should be further processed.

CHAPTER IV

DISCUSSION

The central hypothesis of the plant virus biodiversity and ecology (PVBE) project is that the distribution and evolutionary patterns of viruses are determined by complex environmental interactions among many factors including distribution of hosts, vectors, other viruses and climatic variation. To test this hypothesis, the project was divided into two different phases, phase I is the surveying of plant virus biodiversity and phase II is to make use of the uncovered viral biodiversity for studying the ecology of viruses, which includes: studying distance decay, which measures how the similarity between two plots in species composition varies with distance between the plots; effect of burning on virus species abundance; and patterns of virus distribution. This study was completely focused on phase I of the PVBE project.

This study was undertaken to develop a method for detection of both known and unknown plant viruses from plants growing in natural settings. A metagenomic technique has been successfully developed. The experiments with plants infected with *Maize necrotic streak virus* (Figure 1), demonstrated that this technique is capable of obtaining virus-like sequences only from plants that are infected with viruses, as there were no virus-like sequences obtained from the uninfected maize plants. These sequences are referred to as putative virus sequences, as the source of these sequences can be confirmed

only after the viruses are isolated and characterized from these plants. The problem of contamination leading to smears in the negative control was one of the biggest preliminary hurdles in this study. This problem has been almost eradicated and smears are rarely observed in the negative controls. The developed technique is capable of processing 96 plants in one week. The rate-limiting step of the process has always been the sequencing step as the samples were sent to ACGT, Norman, for sequencing.

The developed metagenomic technique also referred to as the VLP-VNA technique was applied to about 687 plants collected from the Tallgrass Prairie Preserve; sequences were received for 68 plants. BLASTn, BLASTx and tBLASTx analysis of the sequences led to the identification of at least 9 different viruses. As the data suggests, the technique was capable of identifying sequences similar to plant viruses, mycoviruses, animal viruses and even bacteriophages. The VLP-VNA method does not establish that the plants mentioned in Table 6 were infected with viruses. That conclusion awaits isolation and further characterization of these viruses.

This technique can identify putative sequences of viruses having different genetic material like positive-sense single-stranded RNA (*Tymovirus, Potexvirus*), double-stranded DNA (*Badnavirus*) and double-stranded RNA (*Endornavirus*). As observed in the results this technique was capable of identifying virus-like sequences from a wide range of plant species that includes dicots (*Ambrosia, Asclepias*), monocots (*Panicum*), fern (*Asplenium*), legumes (*Desmanthus*). This suggests that this technique is capable of obtaining viral sequences irrespective of the genetic material of the virus or the type of plant species. The data also suggest that plants from natural environments are frequently infected with multiple viruses (Table 7). Of the 68 plants analyzed, 16 plants contained

virus-like sequences and six of the plants containing virus-like sequences, had sequences similar to more than one virus genus.

Apart from putative virus sequences, the developed technique also yielded sequences highly similar to bacterial and fungal sequences. These sequences are referred to as putative as the bacterial or fungal source can be confirmed only after the bacteria or the fungi are isolated and characterized. Most of the putative bacterial and fungal sequences were similar to ribosomal sequences. As mentioned above, the enrichment of the VLPs was based on filtration of the plant homogenate by ultracentrifugation through a sucrose cushion. The VLPs were separated from the other components of the plant homogenate based on their density. As the density of both 70S and 80S ribosomes are comparable to the density of most of the viruses these ribosomes might have accumulated along with the VLPs in the pellet, giving rise to ribosomal sequences. The putative bacterial sequences obtained were similar to the sequences of potent human pathogens such as *Vibrio, Yersinia, Salmonella, and Escherichia*. The putative fungal sequences obtained were similar to known fungal sequences. Some of the sequences were similar to the sequences of potentially pathogenic fungi such as *Pycnoporus, Phaesospharia, and Aspergillus* etc. Presence of these sequences should not be mistaken to the presence of bacteria or fungi in these plants. But these sequences do serve as an important evidence for the possible presence of novel bacteria or fungi associated with these plants. This demonstrates that the developed technique is not only useful in identifying viral pathogens, but can also be used for identifying bacterial and fungal pathogens associated with plants growing in natural settings.

The majority of reads were classified under uncertain (about 27%) i.e. they could not be assigned with any certainty to a source. Although these sequences might be plant-derived mRNA sequences from genes that are not highly conserved in the genomes of sequenced higher plants, they were not observed in all the processed samples especially the plant specimens that produced a high percentage of virus-like sequences and hence there exists a possibility that the uncertain sequences represent viruses whose sequences are unrelated to those of known viruses. A basic problem is how to detect the presence of novel viral sequences when the sequences obtained are extremely divergent from the sequences currently in Genbank. A major fraction of virus sequences obtained from environmental samples show no significant nucleotide and amino acid similarities to any of the sequences currently available in Genbank (Edwards, 2005). The origin of these sequences could be of great interest as they could represent novel and highly distinct viruses. Search algorithms like tBLASTx, which compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database, can help in classifying some of the contigs previously classified as uncertain. A tBLASTx search of the uncertain hits to the EST database revealed that some of the unassigned sequence determinations could be assigned, mostly to plant-derived sequences (Melcher, unpublished).

A tBLASTx score of <0.001 to a known viral sequence has previously been used to classify a sequence as belonging to a virus (Breitbart, 2003; Culley, 2006; Cann, 2005). Some researchers have also used a more stringent cutoff of $E<10^{-5}$ (Allander, 2005) . To solve the fundamental problem of classification of extremely divergent and novel viral sequences (possibly the uncertain hits that show up in BLASTn and BLASTx), several

approaches can be instigated. Firstly, the use of a substitution matrix, which is basically used to quantify protein sequence similarities, derived from a viral rather than prokaryotic and eukaryotic protein alignments (Delwart, 2007). This might help in detection of viruses that are showing even a very low-level of similarity to current viruses. Secondly, it is possible to computationally generate theoretical ancestral sequences of the various virus groups. Though these sequences are ambiguous, they can be used for sequence similarity searches (Stenger , 2002). This might help to identify the highly divergent sequences, as the genetic distance of the novel viruses to their common ancestor might be shorter when compared to the distances of the present species. Finally, it is possible to search for conserved protein motifs (Koonin, 1993) and specific RNA folds (Simmonds, 2004) that might help in the classification of the novel virus sequences.

Out of the 687 plants that were processed using the VLP-VNA method and subjected to PCR amplification, 59% of the plants processed were PCR positive (smear observed in the agarose gel) and 41% of the plants were PCR negative (no smear detected). Only the VNA isolated from plants that were PCR positive were subjected to cloning and sequencing. The absence of amplicons does not necessarily mean that the plant samples were devoid of viruses. There are two possible ways by which viruses present in the plant sample can escape amplification and hence not get detected. Either, the viruses are present in extremely low titer and hence fail to give visible smears in the agarose gel or the VNA preparation might contain contaminants that might interfere and inhibit the PCR reaction. The first dilemma can be solved by subjecting the VNA to further rounds of PCR to detect the presence of low titer viruses in the plant sample. The second problem can be addressed by dilution of the VNA sample before subjecting it to PCR

amplification. Dilution of sample might result in the dilution of the PCR inhibitor preventing it from interfering with the PCR reaction. The presence of amplicons does not necessarily mean that the plant samples have viruses, as mentioned before there is a possibility of obtaining sequences other than virus-like sequences using this VLP-VNA method. The VNA isolated from plants that did not produce positive amplicons are stored at -20 ^{O}C and will be subjected later to cloning and sequencing.

All the presumed virus sequences obtained via the VLP-VNA approach have not been previously described. This observation supports the argument that the actual number of virus species present in the world is much greater than the number currently recognized (Wren , 2006). Thus this study can contribute to the increase in knowledge about the biodiversity of plant viruses. Identification and characterization of plant viruses present in natural settings will help in answering a number of ecology-based questions in phase II of this project, like the pattern of distribution of viruses, effect of burning on virus population and effect of bison or cattle grazing on the distribution of viruses. The study of viruses infecting wild plants will help in better understanding of interaction between viruses and their hosts

The identified novel viruses will provide information for fundamental research on mechanism of virus replication, virus assembly, movement of virus particle and might provide new tools for biotechnology like novel vectors for knock-down studies.

The novel viruses identified now might later become future crop pathogens. Knowing what viruses are out there and more importantly knowing how to identify them will better prepare us against possible outbreaks. This study also helps us in determining the background noise in forensics i.e. whether a plant disease is the result of a release of an

infectious agent or whether the agent was already lurking in the environment.

The research work reported identifies a method that is capable of rapidly expanding the universe of plant-associated viruses. Out of the 1187 plants that were collected from the Tallgrass Prairie Preserve, 687 plants have been subjected to the VLP-VNA method. The processing of the rest of the plants is in progress. Sequences were obtained for 68 plants; we are still awaiting sequence results for the rest of the plant samples. The obtained virus-like sequences need to be further analyzed to confirm the preliminary identification of the viruses. Also, the sequences need to be studied for detection of possible overlaps among the obtained viral sequences. The gaps present in the putative viral sequences can be filled by conducting a primer walking experiment to get the complete sequence of the viruses. Once the entire viral sequence is obtained, the viral sequence can be subjected to phylogenetic analysis to estimate the relationship between the different viral sequences. For example, the *Tymovirus* was found in 7 different plant specimens, the relationship between these *Tymovirus* could be studied by construction of a phylogenetic tree. Also, the NCBI reference sequences available for different viruses can be used for studying the relationship between the identified viruses and the currently known viruses. This can be accomplished by subjecting the NCBI reference sequences and the obtained sequences to multiple sequence alignment followed by phylogenetic analysis. The presence of viruses in the plant specimens can be confirmed only after the virus has been isolated and characterized. The viruses present in the plant specimens could be propagated into indicator plants, isolated and further characterized. Similarly the bacteria and fungi present in the plant specimens can be enriched, isolated and further characterized.

The VLPs isolated from all the 687 plant samples are stored in 20% glycerol and have been submitted for EM studies at the University of Tulsa, Tulsa, Oklahoma. Based on the information obtained from the EM studies regarding the shape, size and any additional surface features of the VLPs, the identification and classification of the viruses can be further confirmed.

Apart from the VLP-VNA technique, a double-stranded RNA (ds RNA) isolation technique is being pursued at the Noble foundation, Ardmore, Oklahoma. Samples of all collected plants have been processed for ds RNA isolation and analyzed by gel electrophoresis. The isolated ds RNA were amplified and subjected to pyrosequencing using the 454-sequencing instrument at ACGT, Norman, Oklahoma. Though this technique was able to obtain putative viral sequences, it has an inherent inability to identify DNA viruses. Another approach currently being developed in our laboratory is the microarray approach for detection of viruses. Oligonucleotides have been designed for each genus (or subgenus) of viruses known to be associated with plants or fungi. Microarray analysis has the potential of recognizing viruses regardless of the nature of their genomes or their packaging status, but only if they are related currently recognized viruses whose sequences are available. The VLP-VNA method continues to be the best method for processing plant samples for obtaining virus-like sequences as this method can recognize viruses regardless of their genomes, is capable of identifying novel viruses and yields a enriched population of VLPs that can be used for EM studies.

As mentioned above, all the putative viral sequences identified, although they could be assigned to known families and often, genera of viruses, represents viruses that have not been previously described by the sequences of their nucleic acids. This observation supports the hypothesis of this study that the number of species of viruses associated with plants is much larger than the number of species currently recognized.

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APPENDIX I

COMPLETE PROTOCOL

VIRUS-LIKE PARTICLE PREPARATION

REAGENTS:

1. Citrate buffer (0.1M Sodium Citrate pH 6.5)
2. 0.25M Iodoacetamide
3. 20% Sucrose solution in Citrate buffer (w/v) (20 g of Sucrose q.s. 100 ml with Citrate buffer)
4. 0.5X Citrate buffer
5. 20% Sucrose solution in 0.5X Citrate buffer (w/v) (20 g of Sucrose q.s. 100 ml with 0.5 X Citrate buffer)
6. 33.3% Triton X-100
7. Viral resuspension buffer (0.1M Tris-HCl pH 7.5 , 2.5mM MgCl2)

MATERIALS:

1. Mini Bead beater (Biospec Products)
2. Sterile 2.5 mm glass beads.
3. Bead-beater tubes (United Scientific Products 1.5 ml Self-standing screw cap microtube)
4. Micro-centrifuge (Hermle Labnet Z233M)
5. Ambion RNase free 1.5 ml microfuge tubes
6. Beckman Ti 42.2 rotor
7. Beckman cellulose propionate centrifuge tubes
8. Beckman ultracentrifuge

PROCEDURE:

Preparation of Plant Homogenate:

1. Weigh out approximately 100 mg of plant sample into a Bead beater tube.
2. Add 12-14 glass beads.
3. Add 750 microL of citrate buffer and 6.3 microL of 0.25 M Iodoacetamide. Addition of polyvinyl pyrolidone is recommended for samples rich in tannins.
4. Keep on ice for 10 min.
5. Homogenize the plant sample using the Bead beater at 4600 rpm for 3 min.

6. Freeze the remaining plant material at -80 C
7. Centrifuge for 15 min at 12000 g.
8. Transfer 137.5 microL of the supernatant to a clean 1.5 ml microcentrifuge tube.
9. Add 12.5 microL of Triton X -100.

If necessary the samples can be stored overnight at 4 C.
Do not freeze.

****

ISOLATION OF VIRION-LIKE PARTICLES:

1. Transfer 150 microL of the mixture to a Beckman ultracentrifuge tube and underlay with 50 microL of 20 % sucrose solution in citrate buffer.
2. Centrifuge at 70,000g for 45 min at 21 C in the Beckman Ti 42.2 rotor.
3. Discard the supernatant slowly by pipetting and resuspend the pellet in 200 microL of 0.5 X Citrate buffer.
4. Transfer the sample to a clean microcentrifuge tube and centrifuge at 8000 g for 10 min.
5. Transfer 150 microL of the supernatant to a Beckman ultracentrifuge tube and underlay with 50 microL of 20 % Sucrose solution in 0.5 X Citrate buffer.
6. Centrifuge at 150,000 g for 65 min at 21 C in the Beckman Ti 42.2 rotor.
7. Discard the supernatant slowly by pipetting and resuspend the pellet in 250 microL of viral resuspension buffer.
8. Transfer 50 microL of the mixture to a clean microcentrifuge tube. Stretch some parafilm around the cap of the tube and store at -20 C and record location in the database. This tube might be used for analysis using electron microscopy.
9. The remaining 200 microL is used for isolation of viral nucleic acid.

If necessary, the 200 microL sample resuspended in VRB (step 9) can be stored overnight at -20 C.

Note:

The samples must be handled with extreme caution to avoid contamination. Working inside a UV-hood is advisable. RNase free pipette tips and microfuge tubes must be used.

NUCLEIC ACID ISOLATION FROM VIRUS-LIKE PARTICLES:

REAGENTS:
1. DNase I (Sigma)(1 mg/ml) (W/V) 1mg of DNase I dissolved in 1 ml of water.

2. 0.5M EDTA
3. Proteinase K (Invitrogen) (2.5 mg/ml) 2.5 mg of Proteinase K dissolved in 1 ml water.
4. 20% SDS
5. 5M NaCl
6. Phenol (buffer saturated) pH 6.6 (MP Biomedicals)
7. Diethyl ether
8. 95% Ethanol
9. 75% Ethanol
10. Linear acrylamide (Ambion)
11. TEN buffer (10mM Tris-HCl pH 7.5,1mM EDTA, 10mM NaCl)

MATERIALS:

1. Micro-centrifuge (Hermle Labnet Z233M)
2. Ambion RNase free 1.5 ml microfuge tubes

PROCEDURE:

1. Add 2 microL of DNase I solution to the virion-like particles resuspended in 200 microL of VRB and incubate at 37 C for 10 min.
2. Add 4 microL of EDTA, 50 microL of Proteinase K and 12.5 microL SDS. Mix and incubate at 65 C for 30 min.
3. Extract with 6 microL of NaCl and 300 microL of buffer saturated phenol.
4. Mix and centrifuge for 5 min at 12,000 g.
5. Transfer the aqueous (upper) phase to a clean microfuge tube.
6. Extract with 1 ml of ether. Mix and spin for 1 min at 12,000 g. Remove and discard ether (upper) phase.
7. Add 4 microL linear acrylamide and 0.75 ml of 95% ethanol and mix.
8. Keep at –20 C overnight to precipitate the nucleic acid.
9. Centrifuge at 12,000 g for 15 min and decant the supernatant.
10. Wash the pellet with 1 ml of 75% ethanol.
11. Repeat step 9
12. Air dry in the hood.
13. Dissolve the pellet in 30 microL of TEN buffer.
14. Store the samples at -20 C and record the location in the database.

ROUND A/B RANDOM AMPLIFICATION:

REAGENTS:

Nucleic acid sample

Round A
Reverse transcriptase (Stratascript)
10X Reverse transcription buffer

25 mM dNTP mix
0.1 M DTT
40 pmol/µl primer A12 (CCTGAATTCGGATCCTCCNNNNNNNNNNN)
Sequenase (13unit/microL) USB cat#70775
5X Sequenase buffer
Sequenase dilution buffer

Round B
10X PCR buffer (500 mM KCl, 100 mM Tris pH 8.3)
50mM MgCl2
25mM dNTP mix
5 unit/microL Taq polymerase**
100 pmol/microL Primer B (CCTGAATTCGGATCCTCC)

Thermal cycler

PROCEDURE:

Round A
1st strand synthesis with RT
1. Mix nucleic acid sample with 1 microL primer A12 to a final volume of 10 microL in a 500microL tube.
2. Incubate at 65˚C for 5 min. Then keep at RT for 5 min
3. Make master mix (2X enzyme mix) by mixing following reagents:
   2.0 microL 10X RT buffer
   0.4 microL 25 mM dNTP mix
   3.6 microL H2O
   2.0 microL 0.1 M DTT
   2.0 microL Reverse Transcriptase (Stratascript)
4. Add 10 microL of master mix to nucleic acid-primer mixture.
5. Incubate for 42˚C 30 min>65˚C 5 min>RT 5 min>
6. Add 1.0 microL Reverse Transcriptase
7. Incubate 42˚C 30 min

2nd strand synthesis with Sequenase
1. Incubate Round A product at 94˚C for 2 min. Then cool and hold at 10˚C for 5min
2. Prepare Sequenase mix

5 X Sequenase Buffer	2.0 microL
water	7.7 microL
Sequenase	0.3 microL

3. Add 10 microL sequenase mix.
4. Use a thermal cycler to ramp from 10˚C to 37˚C over 8 min.
5. Then 37˚C 8 min>94˚C 2 min>10˚C 5 min
6. Add 1.2 µl of diluted (1:4) sequenase.
7. Ramp from 10˚C to 37˚C over 8 min.

8. 37˚C 8 min>94˚C 8 min, then 4˚C hold

Round B
1. Mix following reagents:
   6 µl Round A template
   4 µl 50 mM MgCl2
   10µl 10X PCR buffer
   1 µl 25 mM dNTP
   1 µl Primer B
   1 µl Taq Polymerase (hot start)
   77 µl H2O
2. (94˚C 30 sec>40˚C 30 sec> 50˚C 30 sec>72˚C 1 min)*30>72˚C 5 min> 4˚C

Run an aliquot 8 microL plus 2 microL 5x stop reagent on agarose gel electrophoresis. A smear indicates success in amplification.

APPENDIX II

RANDOM PCR OF TALLGRASS PRAIRIE SAMPLES**:**

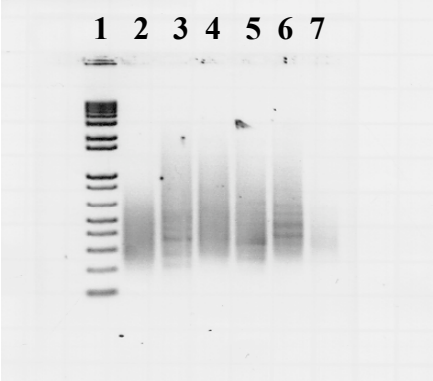

Figure 1: Sample 1-5 Lane 1- 1Kb+ Marker, Lane 2- VNA_05TGP00295_001, Lane 3- VNA_05TGP00248_002, Lane 4- VNA_05TGP00289_003, Lane 5- VNA_05TGP00162_004,Lane 6- VNA_05TGP00296_005, Lane7- Negative control.

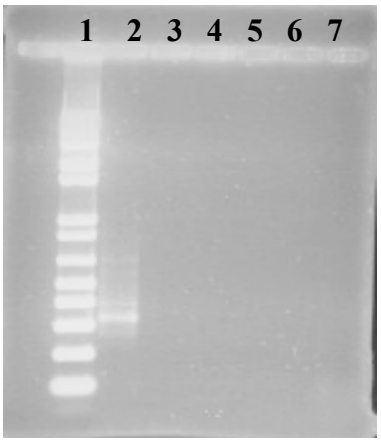

Figure 2: Sample 6-10 Lane 1- 1 Kb+ Marker, Lane 2- VNA_05TGP00305_006, Lane 3- VNA_05TGP00262_007, Lane 4- VNA_05TGP00283_008, Lane 5- VNA_05TGP00212_009 Lane 6- VNA_05TGP00328_010, Lane 7- Negative control

Figure 3: Sample 11-62. Lane1-1Kb+ Marker, Lane2- Negative control, Lane3-VNA_05TGP00303_011, Lane 4- VNA_05TGP00307_012, Lane5- VNA_05TGP00312_013, Lane 6-VNA_05TGP00313_014, Lane7- VNA_05TGP00316_015, Lane8- VNA_05TGP00318_016, Lane9-VNA_05TGP00321_017, Lane 10- VNA_05TGP00323_018, Lane11-VNA_05TGP00326_019, Lane12- VNA_05TGP00332_020, Lane13-Negative control, Lane14-VNA_05TGP00335_021, Lane15- VNA_05TGP00337_022, Lane16-VNA_05TGP00339_023, Lane 17-VNA_05TGP00342_024, Lane18- VNA_05TGP00343_025, Lane19-VNA_05TGP00344_026, Lane20- VNA_05TGP00351_027, Lane21- VNA_05TGP00352_028, Lane22-VNA_05TGP00353_029, Lane23- VNA_05TGP00360_030, Lane24- VNA_05TGP00361_031, Lane25-VNA_05TGP00362_032, Lane26- VNA_05TGP00364_033, Lane27- VNA_05TGP00365_034, Lane28-VNA_05TGP00192_035, Lane29-VNA_05TGP00199_036, Lane30- 1 Kb+ Marker, Lane31- 1 Kb+ Marker,Lane32-VNA_05TGP00201_037, Lane33- VNA_05TGP00211_038, Lane34-VNA_05TGP00214_039, Lane35- VNA_05TGP00219_040, Lane36- VNA_05TGP00220_041, Lane37-VNA_05TGP00237_042, Lane38- VNA_05TGP00238_043, Lane39- VNA_05TGP00241_044, Lane40-VNA_05TGP00248_045, Lane41- VNA_05TGP00255_046,Lane42- VNA_05TGP00256_047, Lane43-VNA_05TGP00258_048,Lane44- VNA_05TGP00269_049, Lane45- VNA_05TGP00276_050, Lane46-VNA_05TGP00279_051, Lane47- VNA_05TGP00282_052, Lane48- VNA_05TGP00285_053, Lane 49-VNA_05TGP00287_054,Lane50- VNA_05TGP00288_055, Lane51- VNA_05TGP00290_056, Lane52-VNA_05TGP00294_057,Lane53- VNA_05TGP00297_058, Lane54- VNA_05TGP00379_059, Lane55-VNA_05TGP00383_060,Lane56- VNA_05TGP00401_061, Lane57- VNA_05TGP00402_062, Lane58-PCR Negative control, Lane59-PCR Positive control, Lane60- 1Kb+ Marker.

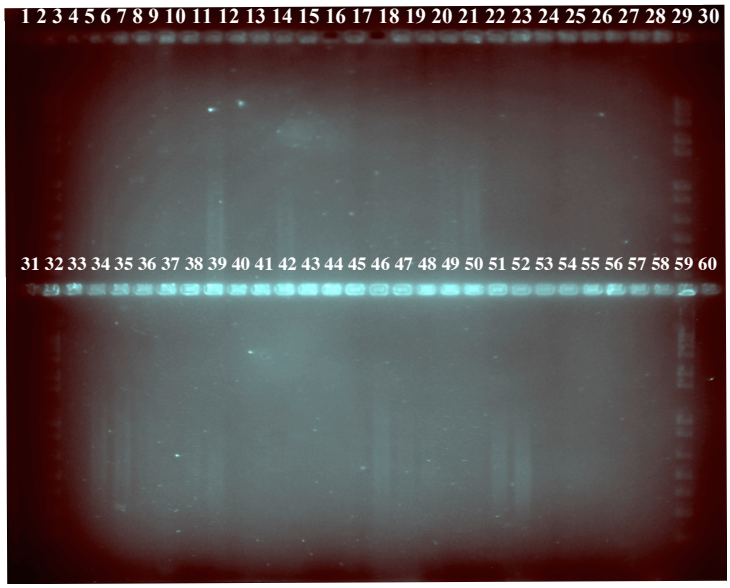

Figure 4: Sample 63-100 Lane1- Empty, Lane2- 1Kb+ Marker, Lane3- Empty, Lane4- VNA_05TGP00403_063, Lane5-VNA_05TGP00406_064, Lane6- VNA_05TGP00407_065, Lane7- VNA_05TGP00408_066, Lane8- VNA_05TGP00413_067, Lane9- VNA_05TGP00417_068, Lane10- VNA_05TGP00418_069, lane11-VNA_05TGP00419_070, Lane12- VNA_05TGP00420_071, Lane13- VNA_05TGP00422_072, Lane14-VNA_05TGP00426_073, Lane15- VNA_05TGP00427_074, Lane16- VNA_05TGP00428_075, Lane17-VNA_05TGP00429_076, Lane18- VNA_05TGP00430_077, Lane19- VNA_05TGP00431_078, Lane20-VNA_05TGP00432_079, Lane21- VNA_05TGP00434_080, Lane22- VNA_05TGP00435_081, Lane23-VNA_05TGP00437_082, Lane24- Empty, Lane25- Empty, Lane26- Empty, Lane27- Empty, Lane28-Empty, Lane29- 1Kb+ Marker, Lane30- Empty, Lane31- Empty, Lane32- 1Kb+ Marker, Lane33- empty,Lane34- VNA_05TGP00029_083, Lane35- VNA_05TGP00031_084, Lane36- VNA_05TGP00032_085,Lane37- VNA_05TGP00049_086, Lane38- VNA_05TGP00050_087, Lane39- VNA_05TGP00057_088,Lane40- VNA_05TGP00058_089, Lane41- VNA_05TGP00070_090, Lane42- Negative control,Lane43- VNA_05TGP00075_091, Lane44- VNA_05TGP00080_092, Lane45- VNA_05TGP00089_093,Lane46- VNA_05TGP00096_094, Lane47- VNA_05TGP00111_095, Lane48-VNA_05TGP00112_096,Lane49-VNA_05TGP00114_097,Lane50- VNA_05TGP00116_098, Lane51- VNA_05TGP00139_099,Lane52- VNA_05TGP00167_100, Lane53- Negative control, Lane54 to 58- Empty,
Lane59- 1 Kb+ Marker, Lane60- Empty.

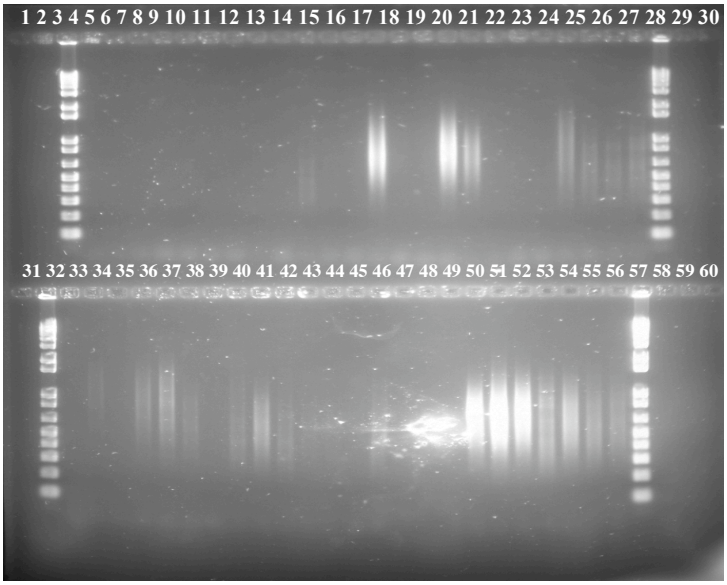

Figure 5: Sample 6-223. Lane1, 2- Empty, Lane3- 1Kb+ Marker, Lane4- Negative control, Lane5- VNA_05TGP00305_006, Lane6- VNA_05TGP00199_036, Lane7- VNA_05TGP00383_060, Lane8- VNA_05TGP00402_062, Lane9- VNA_05TGP00408_066, Lane10-VNA_05TGP00417_068, Lane11- VNA_05TGP00420_071, Lane12- VNA_05TGP00431_078, Lane13- VNA_05TGP00432_079, Lane14- VNA_05TGP00032_085, Lane15- Negative control, Lane16- VNA_05TGP00057_088, Lane17- VNA_05TGP00089_093, Lane18- VNA_05TGP00111_095, Lane19- VNA_05TGP00139_099, Lane20- VNA_05TGP00167_100, Lane21- VNA_05TGP00076_193, Lane22- VNA_05TGP00136_194, Lane23- VNA_05TGP00173_195, Lane24- VNA_05TGP00221_196, Lane25- VNA_05TGP00243_197, Lane26- VNA_05TGP00260_198, Lane27- VNA_05TGP00331_199, Lane28- 1Kb+ marker,
Lane29 to 31- Empty, Lane32- 1Kb+ marker, Lane33- VNA_05TGP00336_200, Lane34- VNA_05TGP00416_201, Lane35- VNA_05TGP00424_202, Lane36- VNA_05TGP00443_203, Lane37- VNA_05TGP00453_204, Lane38- VNA_05TGP00464_205, Lane39- VNA_05TGP00489_206, Lane40- VNA_05TGP00565_207, Lane41- VNA_05TGP00576_208, Lane42- VNA_06TGP01051_209, Lane43- VNA_06TGP01079_210, Lane44- VNA_06TGP01092_211, Lane45- VNA_06TGP01109_212, Lane46- VNA_06TGP01122_213, Lane47- VNA_06TGP01123_214, Lane48- VNA_06TGP01126_215, Lane49- VNA_05TGP00226_216, Lane50- VNA_05TGP00251_217, Lane51- VNA_05TGP00265_218, Lane52- VNA_05TGP00304_219, Lane53- VNA_05TGP00333_220, Lane54- VNA_05TGP00378_221, Lane55- VNA_05TGP00386_222, Lane56- VNA_05TGP00415_223, Lane57- 1 Kb+ Marker,
Lane 58 to 60- Empty.

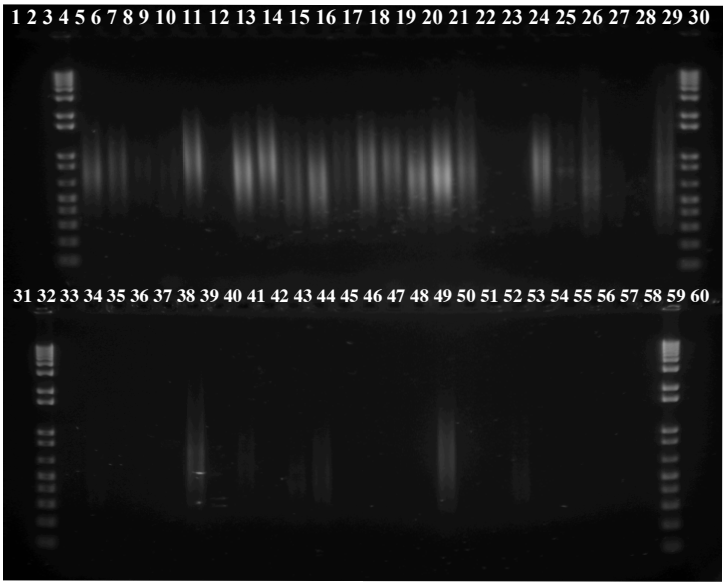

Figure 6: Sample 223-269. Lane1,2- Empty, Lane3- VNA_05TGP00425_224, Lane4- VNA_05TGP00444_225, Lane5- VNA_05TGP00555_226, Lane6- VNA_05TGP00619_227, Lane7- VNA_05TGP00244_228, Lane8- VNA_05TGP00252_229, Lane9- VNA_05TGP00264_230, Lane10- VNA_05TGP00298_231, Lane11- VNA_05TGP00315_232, Lane12- VNA_05TGP00325_233, Lane13- VNA_05TGP00341_234, Lane14- VNA_05TGP00393_235, Lane15- VNA_05TGP00410_236, Lane16- VNA_05TGP00447_237, Lane17- VNA_06TGP01085_238, Lane18- VNA_06TGP01137_239, Lane19- VNA_06TGP01141_240, Lane20- VNA_06TGP01150_241, Lane21- VNA_06TGP01156_242, Lane22- VNA_06TGP01158_243, Lane23- VNA_06TGP01158_243, Lane24- VNA_06TGP01168_244, Lane25- VNA_06TGP01178_245, Lane26- VNA_06TGP01189_246, Lane27- VNA_06TGP01198_247, Lane28- 1 Kb+ Marker, Lane29, 30,31- Empty, Lane32- 1 Kb+ Marker, Lane33- VNA_06TGP01212_248, Lane34- VNA_06TGP01223_249, Lane35- VNA_06TGP01224_250, Lane36- VNA_06TGP01234_251, Lane37- VNA_06TGP01243_252, Lane38- VNA_06TGP01258_253, Lane39- VNA_06TGP01261_254, Lane40- VNA_06TGP01271_255, Lane41- VNA_06TGP01277_256, Lane42- VNA_06TGP01094_257, Lane43- VNA_06TGP01105_258, Lane44- VNA_06TGP01121_259, Lane45- Negative control, Lane46- VNA_06TGP01128_260, Lane47- VNA_06TGP01139_261, Lane48- VNA_06TGP01144_262, Lane49- VNA_06TGP01151_263, Lane50- VNA_06TGP01162_264, Lane51- VNA_06TGP01167_265, Lane52- VNA_06TGP01171_266, Lane53- VNA_06TGP01180_267, Lane54- VNA_06TGP01191_268, Lane55- VNA_06TGP01201_269, Lane56- Negative control, Lane57- 1 Kb+ Marker, Lane58, 59,60- Empty.

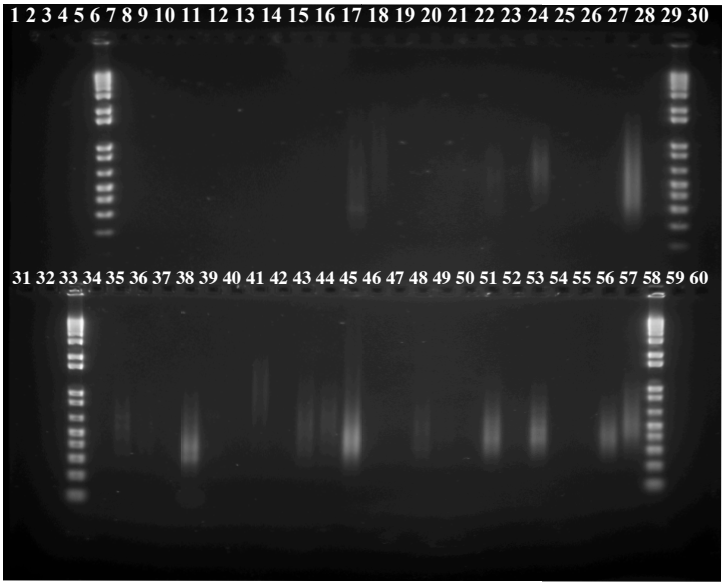

Figure 7: Sample 101-146 Lane1, 2,3- Empty, Lane4- 1Kb+ Marker, Lane5- Negative control, Lane6- VNA_05TGP00239_101, Lane7- VNA_05TGP00345_102, Lane8- VNA_05TGP00359_103, Lane9- VNA_05TGP00363_104, Lane10- VNA_05TGP00366_105, Lane11- VNA_05TGP00195_106, Lane12- Negative control, Lane13- VNA_05TGP00370_107, Lane14- VNA_05TGP00371_108, Lane15- VNA_05TGP00372_109, Lane16- VNA_05TGP00376_110, Lane17- VNA_05TGP00377_111, Lane18- VNA_05TGP00380_112, Lane19- VNA_05TGP00381_113, Lane20- VNA_05TGP00382_114, Lane21- VNA_05TGP00385_115, Lane22- VNA_05TGP00387_116, Lane23- VNA_05TGP00389_117, Lane24- VNA_05TGP00390_118, Lane25- VNA_05TGP00391_119, Lane26- VNA_05TGP00392_120, Lane27- VNA_05TGP00394_121, Lane28- VNA_05TGP00396_122, Lane29- 1 Kb+ Marker, Lane30, 31, 32- Empty, Lane33- 1Kb+ Marker, Lane34- VNA_05TGP00435_123, Lane35- VNA_05TGP00034_124, Lane36- VNA_05TGP00059_125, Lane37- VNA_05TGP00064_126, Lane38- VNA_05TGP00067_127, Lane39- VNA_05TGP00068_128, Lane40- VNA_05TGP00072_129, Lane41- VNA_05TGP00073_130, Lane42- VNA_05TGP00085_131, Lane43- VNA_05TGP00087_132, Lane44- VNA_05TGP00093_133, Lane45- VNA_05TGP00095_134, Lane46- VNA_05TGP00113_135, Lane47- VNA_05TGP00118_136, Lane48- VNA_05TGP00122_137, Lane49- VNA_05TGP00135_138, Lane50- VNA_05TGP00137_139, Lane51- VNA_05TGP00149_140, Lane52- VNA_05TGP00151_141, Lane53- VNA_05TGP00158_142, Lane54- VNA_05TGP00170_143, Lane55- VNA_05TGP00175_144, Lane56- VNA_05TGP00182_145, Lane57- VNA_05TGP00193_146, Lane58- 1Kb+ Marker, Lane59, 60- Empty.

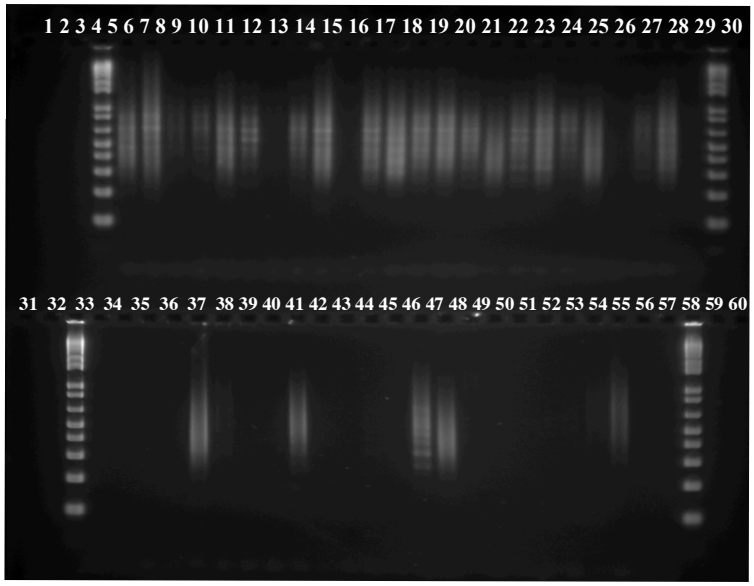

Figure 8: Sample 147-192. Lane1, 2, 3- Empty, Lane4- 1Kb+ Marker, Lane5- VNA_05TGP00030_147,Lane6- VNA_05TGP00040_148, Lane7- VNA_05TGP00044_149, Lane8- VNA_05TGP00051_150, Lane9- VNA_05TGP00055_151, Lane10- VNA_05TGP00060_152, Lane11- VNA_05TGP00063_153, Lane12- VNA_05TGP00065_154, Lane13- VNA_05TGP00069_155, Lane14- VNA_05TGP00072_156, Lane15- VNA_05TGP00077_157, Lane16- VNA_05TGP00079_158, Lane17- VNA_05TGP00082_159, Lane18- VNA_05TGP00086_160, Lane19- VNA_05TGP00088_161, Lane20- VNA_05TGP00094_162, Lane21- VNA_05TGP00100_163, Lane22- VNA_05TGP00106_164, Lane23- VNA_05TGP00117_165, Lane24- VNA_05TGP00119_166, Lane25- VNA_05TGP00157_167, Lane26- VNA_05TGP00165_168, Lane27- VNA_05TGP00374_169, Lane28- VNA_05TGP00026_170, Lane29- 1Kb+ Marker, Lane30, 31,32- Empty, Lane33- 1Kb+ Marker, Lane34- VNA_05TGP00027_171, Lane35- VNA_05TGP00028_172, Lane36- VNA_05TGP00033_173, Lane37- VNA_05TGP00035_174, Lane38- VNA_05TGP00036_175, Lane39- VNA_05TGP00037_176, Lane40- VNA_05TGP00038_177, Lane41- VNA_05TGP00041_178, Lane42- VNA_05TGP00042_179, Lane43- VNA_05TGP00043_180, Lane44- VNA_05TGP00045_181, Lane45- VNA_05TGP00046_182, Lane46- VNA_05TGP00047_183, Lane47- VNA_05TGP00048_184, Lane48- VNA_05TGP00052_185, Lane49- VNA_05TGP00053_186, Lane50- Negative control, Lane51- VNA_05TGP00054_187, Lane52- VNA_05TGP00056_188, Lane53- VNA_05TGP00061_189, Lane54- VNA_05TGP00062_190, Lane55- VNA_05TGP00066_191, Lane56- VNA_05TGP00071_192, Lane57- Negative control, Lane58- 1 Kb+ Marker, Lane59, 60 – Empty.

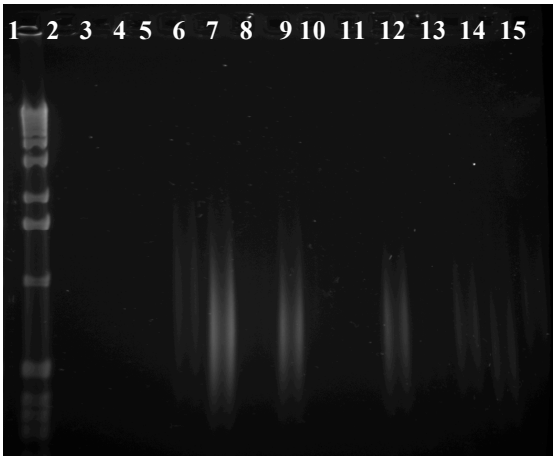

Figure 9: Sample 270-314 (1A). Lane1- 1 Kb+ Marker, Lane2- Empty, Lane3- Negative control, Lane4- VNA_06TGP01217_270, Lane5- VNA_06TGP01228_271, Lane6- VNA_06TGP01237_272, Lane7- VNA_06TGP01247_273, Lane8- VNA_06TGP01253_274, Lane9- VNA_06TGP01263_275, Lane10- Negative control, Lane11-VNA_06TGP01273_276, Lane12- VNA_06TGP01080_277, Lane13- VNA_06TGP01095_278, Lane14-VNA_06TGP01108_279, Lane15- VNA_06TGP01118_280.

Figure 10: Sample 270-314 (1B) Lane1- VNA_06TGP01129_281, Lane2- VNA_06TGP01138_282, Lane3- VNA_06TGP01143_283A, Lane4- VNA_06TGP01143_283B, Lane5- VNA_06TGP01154_284, Lane6-VNA_06TGP01163_285, Lane7- VNA_06TGP01173_286, Lane8- VNA_06TGP01179_287, Lane9-VNA_06TGP01194_288, Lane10- VNA_06TGP01202_289, Lane11- VNA_06TGP01214_290, Lane12, 13, 14- Empty, Lane15- 1 Kb+ Marker.

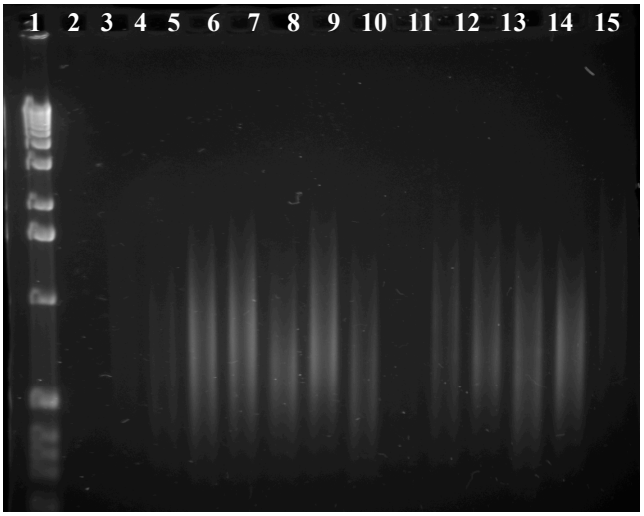

Figure 11: **Sample 270-314 (2A)** Lane1- 1 Kb+ Marker, Lane2- Empty, Lane3- VNA_05TGP00340_298, Lane4- VNA_05TGP00330_297, Lane5- VNA_05TGP00261_296, Lane6- VNA_05TGP00249_295, Lane7-VNA_05TGP00240_294, Lane8- VNA_05TGP00005_293, Lane9- VNA_06TGP01239_292, Lane10-VNA_06TGP01227_291, Lane11- VNA_05TGP00350_299, Lane12- VNA_05TGP00397_300, Lane13-VNA_05TGP00445_301, Lane14- VNA_05TGP00461_302, lane15- VNA_06TGP01082_303.

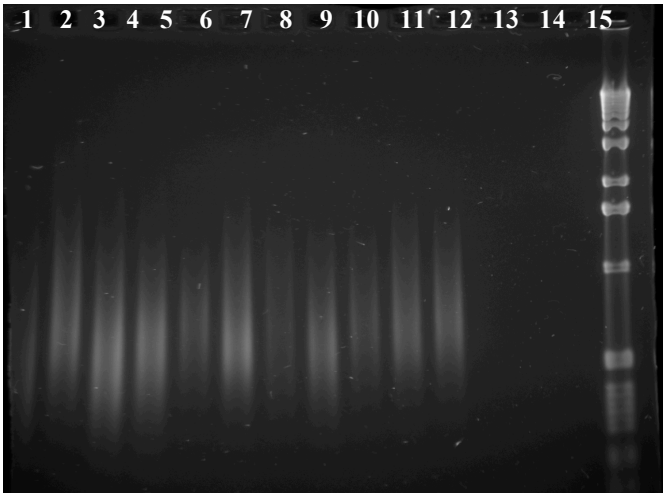

Figure 12: Sample 270-314 (2A) Lane1- VNA_06TGP01097_304, Lane2- VNA_06TGP01107_305, Lane3-VNA_06TGP01119_306,Lane4- VNA_06TGP01125_307, Lane5- VNA_06TGP01135_308,Lane6-VNA_06TGP01145_309, Lane7- VNA_06TGP01155_310, Lane8- VNA_06TGP01159_311, Lane9-VNA_06TGP01169_312, Lane10- VNA_06TGP01176_313, Lane11- VNA_06TGP01192_314, Lane12, 13, 14- Empty, Lane15- 1Kb+ Marker.

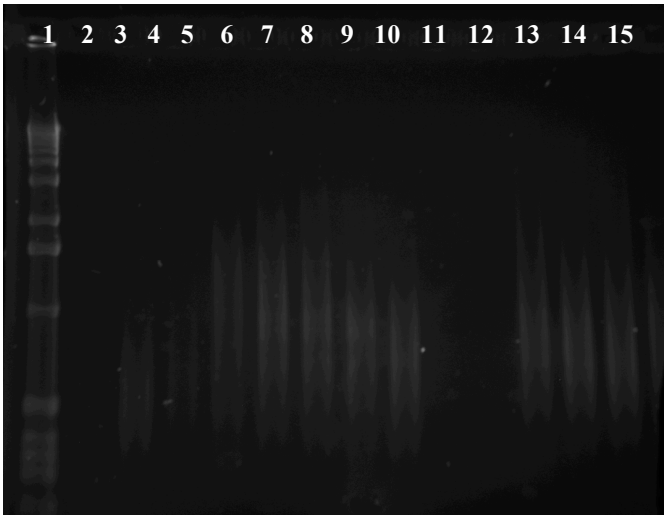

Figure 13: Sample 315-360 (1A) Lane1- 1Kb+ Marker, Lane2- Empty, Lane3- VNA_06TGP01200_315, Lane4- VNA_05TGP00107_316, Lane5- VNA_05TGP00250_317, Lane6- VNA_05TGP00263_318, Lane7-VNA_05TGP00299_319, Lane8- VNA_05TGP00306_320, Lane9- VNA_05TGP00314_321, Lane10-VNA_05TGP00324_322, Lane11- VNA_05TGP00338_323, Lane12- VNA_05TGP00354_324, Lane13-VNA_05TGP00388_325, Lane14- VNA_05TGP00395_326, Lane15- VNA_05TGP00409_327.

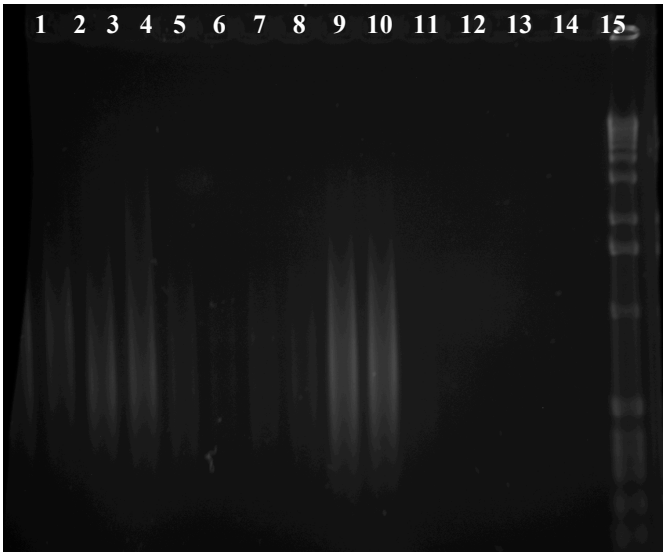

Figure 14: Sample 315-360 (1B) Lane1- VNA_05TGP00433_328,Lane2- VNA_05TGP00446_329, Lane3-VNA_05TGP00462_330, Lane4- VNA_06TGP01084_331, Lane5- VNA_06TGP01093_332, Lane6- VNA_06TGP01213_333, Lane7- VNA_06TGP01222_334, Lane8- VNA_06TGP01226_335, Lane9- VNA_06TGP01236_336, Lane10- VNA_06TGP01244_337, Lane11- VNA_06TGP01255_338, Lane12, 13, 14- Empty, Lane15- 1Kb+ Marker

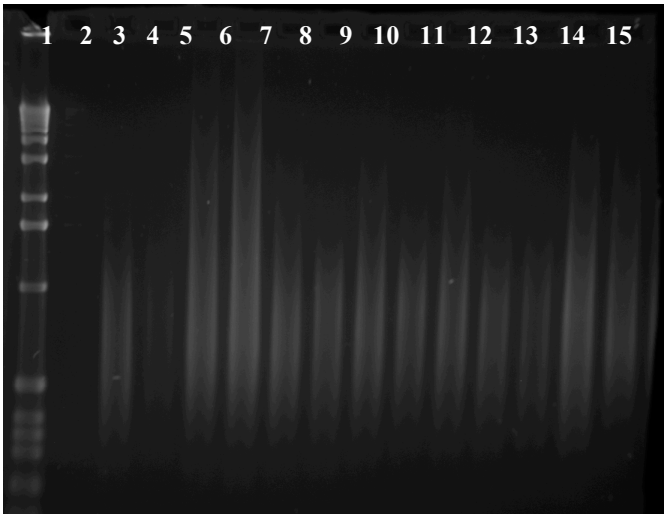

Figure 15: Sample 315-360 (2A) Lane1- 1Kb+ Marker, Lane2- Empty, Lane3- VNA_06TGP01106_339, Lane4- VNA_06TGP01117_340, Lane5- VNA_06TGP01131_341, Lane6- VNA_06TGP01136_342, Lane7- VNA_06TGP01142_343, Lane8- VNA_06TGP01153_344, Lane9- VNA_06TGP01160_345, Lane10-VNA_06TGP01170_346, Lane11- VNA_06TGP01187_347, Lane12- VNA_06TGP01195_348, Lane13- VNA_06TGP01199_349, Lane14- VNA_06TGP01215_350, Lane15- VNA_06TGP01225_351.

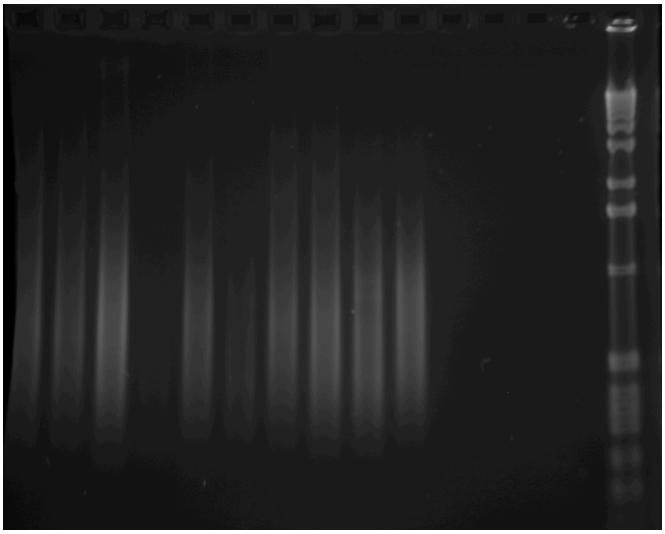

Figure 16: Sample 315-360 (2B) Lane1-VNA_06TGP01235_352, Lane2- VNA_06TGP01245_353, Lane3- VNA_06TGP01251_354, Lane4- Negative control, Lane5-VNA_06TGP01262_355, Lane6-VNA_06TGP01272_356, Lane7-VNA_06TGP01276_357, Lane8-VNA_05TGP00123_358,Lane9-VNA_05TGP00181_359,Lane10-VNA_05TGP00244_360, Lane11- Negative control, Lane12, 13, 14-Empty, Lane15- 1 Kb+ Marker

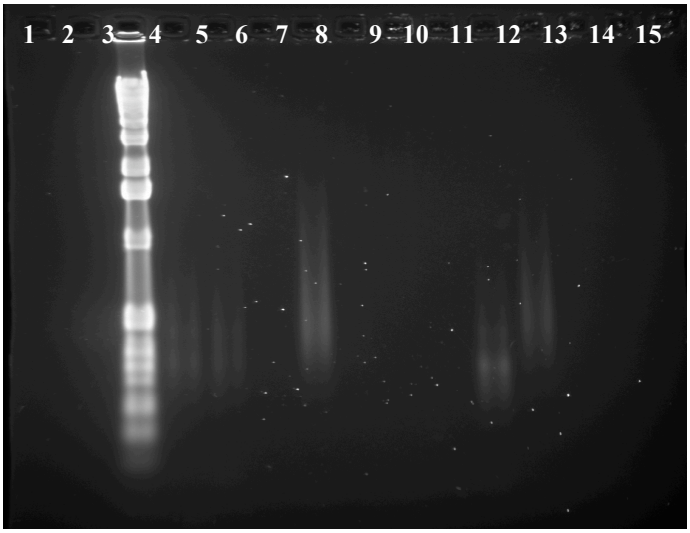

Figure 17: Sample 361-406 (1A). Lane1, 2- Empty, Lane3- 1 Kb+ Marker, Lane4- Negative control, Lane5- VNA_06TGP01246_361, Lane6- VNA_05TGP00242_362, Lane7- VNA_05TGP00247_363, Lane8-VNA_05TGP00242_364, Lane9- VNA_05TGP00322_365, Lane10- VNA_05TGP00334_366, Lane11-Negative control, Lane12- VNA_05TGP00355_367, Lane13- VNA_05TGP00411_368, Lane14-VNA_05TGP00448_369, Lane15- VNA_05TGP00465_370.

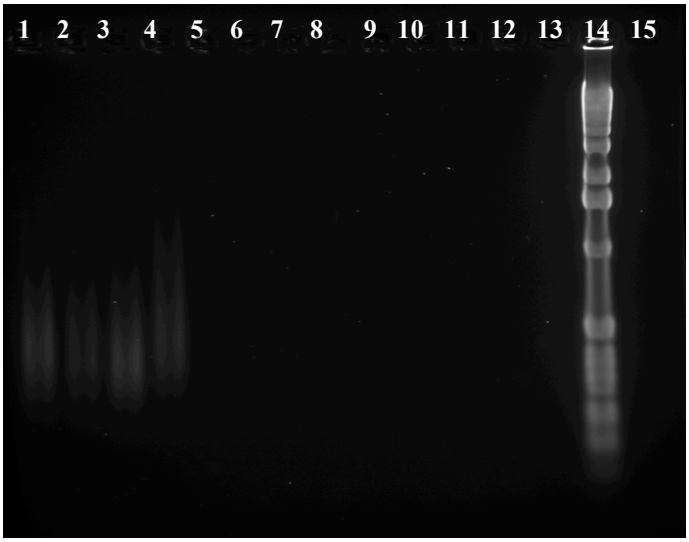

Figure18: Sample 361-406 (1B)
Lane1- VNA_06TGP01254_371, Lane2- VNA_06TGP01264_372, Lane3- VNA_06TGP01274_373, Lane4- VNA_06TGP01081_374, Lane5- VNA_06TGP01096_375,Lane6- VNA_06TGP01110_376, Lane7- VNA_06TGP01120_377, Lane8- VNA_06TGP01127_378, Lane9- VNA_06TGP01140_379, Lane10- VNA_06TGP01146_380, Lane11- VNA_06TGP01152_381, Lane12- VNA_06TGP01164_382, Lane13- Empty, Lane14- 1 Kb+ Marker, Lane15- Empty.

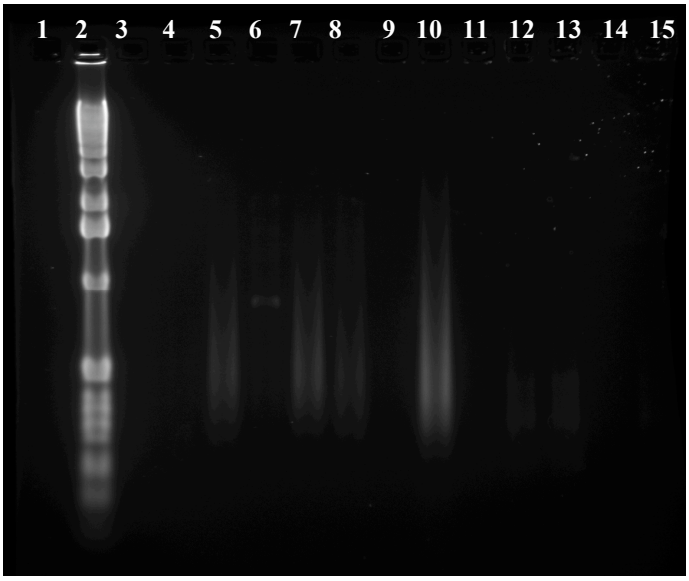

Figure 19: Sample 361-406 (2A) Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- VNA_06TGP01172_383, Lane5- VNA_06TGP01186_384, Lane6- VNA_05TGP00125_385, Lane7- VNA_05TGP00186_386, Lane8-VNA_05TGP00190_387, Lane9- VNA_05TGP00308_388, Lane10- VNA_05TGP00542_389, Lane11-VNA_06TGP01012_390, Lane12- VNA_06TGP01040_391, Lane13- VNA_06TGP01048_392, Lane14-VNA_06TGP01054_393, Lane15- VNA_06TGP01056_394.

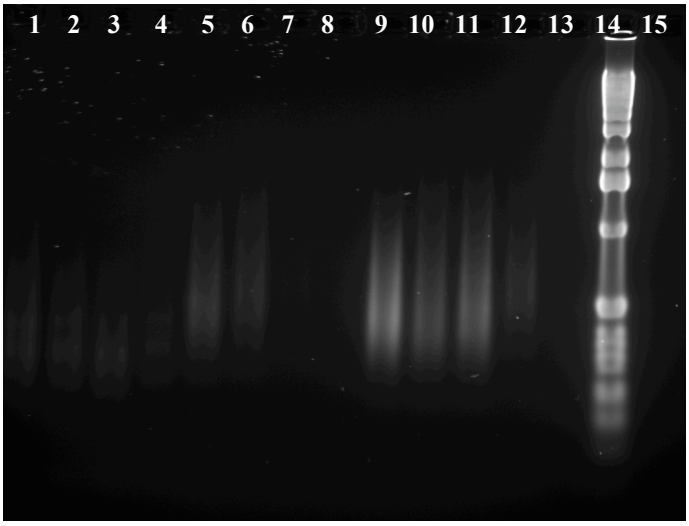

Figure 20: Sample 361-406 (2B) Lane1- VNA_06TGP01060_395, Lane2- VNA_06TGP01067_396, Lane3-VNA_06TGP01069_397, Lane4- VNA_06TGP01072_398, Lane5- VNA_06TGP01190_399, Lane6-VNA_06TGP01203_400, Lane7- VNA_06TGP01216_401, Lane8- VNA_06TGP01229_402, Lane9-VNA_06TGP01238_403, Lane10- VNA_06TGP01248_404, Lane11- VNA_06TGP01252_405, Lane12-VNA_06TGP01266_406, Lane13- Empty, Lane14- 1 Kb+ Marker, Lane15- Empty.

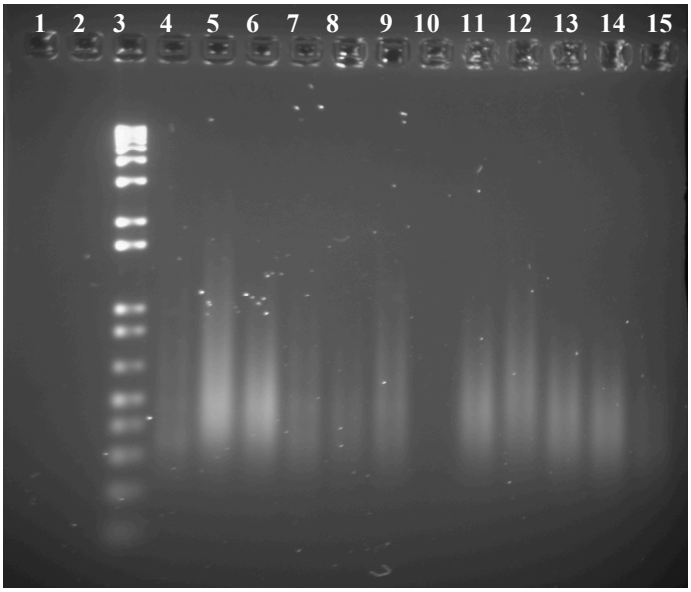

Figure 21: Sample 407- 452 (1A)
Lane1, 2- Empty, Lane3- 1 Kb+ Marker, Lane4- VNA_06TGP01275_407, Lane5- VNA_05TGP00110_408, Lane6- VNA_05TGP00273_409, Lane7- VNA_05TGP00478_410, Lane8- VNA_05TGP00488_411, Lane9- VNA_05TGP00527_412, Lane10- VNA_05TGP00528_413, Lane11- VNA_05TGP00529_414, Lane12- VNA_05TGP00572_415, Lane13- VNA_05TGP00598_416, lane14- VNA_06TGP01005_417, Lane15- VNA_06TGP01007_418.

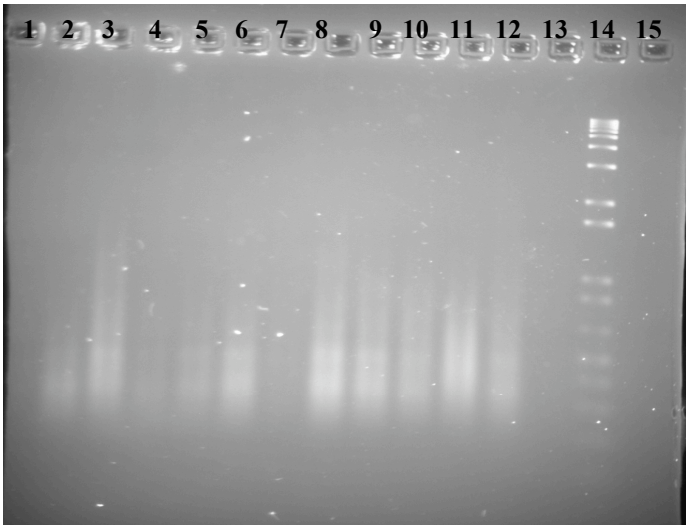

Figure 22: Sample 407- 452 (1B)
Lane1- VNA_06TGP01019_419, Lane2- VNA_06TGP01025_420, Lane3- VNA_06TGP01039_421, Lane4- VNA_06TGP01046_422, Lane5- VNA_06TGP01047_423, Lane6- VNA_06TGP01055_424, Lane7- VNA_06TGP01066_425, Lane8- VNA_06TGP01074_426, Lane9- VNA_06TGP01083_427, Lane10- VNA_06TGP01116_428, Lane11- VNA_06TGP01130_429, Lane12- VNA_06TGP01161_430, Lane13- Empty, Lane14- 1 Kb+ Marker, Lane15- Empty.

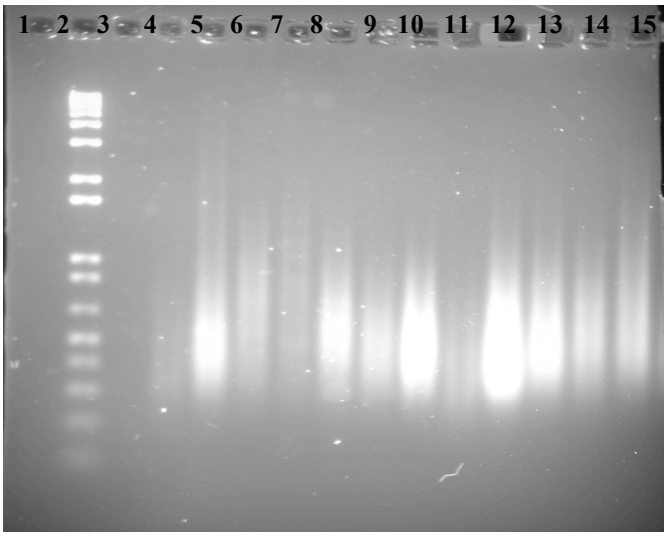

Figure 23: Sample 407- 452 (2A)
Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- Empty, Lane4- VNA_05TGP00196_431,Lane5- VNA_06TGP01003_432, Lane6- VNA_06TGP01016_433, Lane7- VNA_06TGP01018_434, Lane8- VNA_06TGP01061_435, Lane9- VNA_06TGP01062_436, Lane10- VNA_06TGP01063_437, Lane11- VNA_06TGP01068_438, Lane12- VNA_06TGP01070_439, Lane13- VNA_06TGP01073_440, Lane14-VNA_06TGP01076_441, Lane15- VNA_06TGP01132_442.

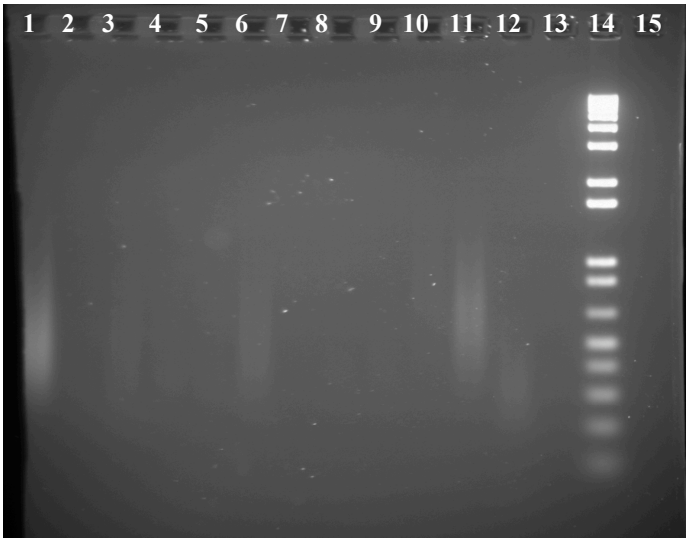

Figure 24: Sample 407- 452 (2B)
Lane1- VNA_06TGP01133_443, Lane2- VNA_06TGP01177_444, Lane3- VNA_06TGP01188_445, Lane4- VNA_06TGP01193_446, Lane5- Negative control, Lane6- VNA_06TGP01197_447, Lane7- VNA_06TGP01221_448, Lane8- VNA_06TGP01230_449, Lane9- VNA_06TGP01240_450, Lane10- VNA_06TGP01256_451, Lane11- VNA_06TGP01257_452, Lane12- Negative control, Lane13- Empty, Lane14- 1 Kb+ Marker, Lane15- Empty.

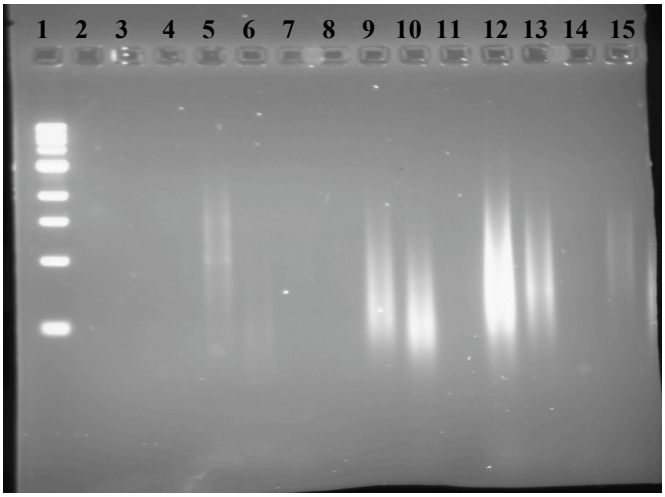

Figure 25: Sample 453-498 (1A)
Lane1- 1 Kb+ Marker, Lane2, 3- Empty, Lane4- Negative control, Lane5- VNA_06TGP01269_453, Lane6-VNA_05TGP00121_454, Lane7- VNA_05TGP00126_455, Lane8- VNA_05TGP00218_456, Lane9-VNA_05TGP00229_457, Lane10- VNA_05TGP00280_458, Lane11- Negative control, Lane12- VNA_05TGP00286_459, Lane13- VNA_05TGP00348_460, Lane14- VNA_05TGP00475_461, Lane15- VNA_05TGP00507_462.

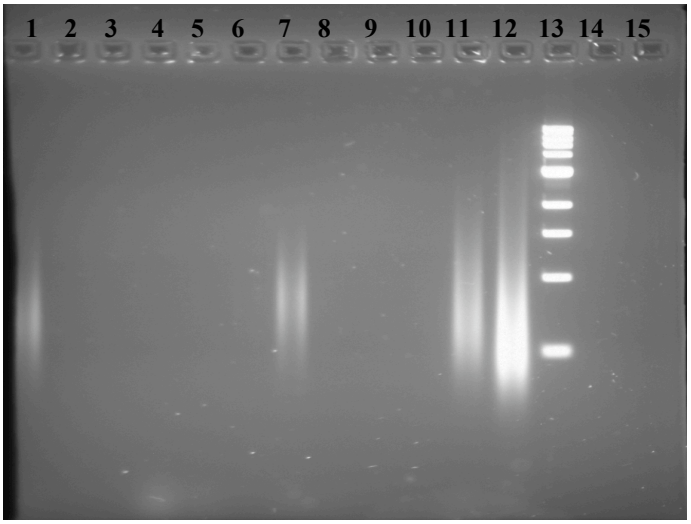

Figure 26: Sample 453-498 (1B)
Lane1- VNA_05TGP00540_463, Lane2- VNA_05TGP00557_464, Lane3- VNA_05TGP00602_465, Lane4- VNA_06TGP01006_466, Lane5- VNA_06TGP01009_467, Lane6- VNA_06TGP01075_468, Lane7- VNA_06TGP01089_469, Lane8- VNA_06TGP01090_470, Lane9- VNA_06TGP01111_471, Lane10- VNA_06TGP01147_472, Lane11- VNA_06TGP01148_473, Lane12- VNA_06TGP01231_474, Lane13- 1 kb+ Marker, Lane14,15- Empty.

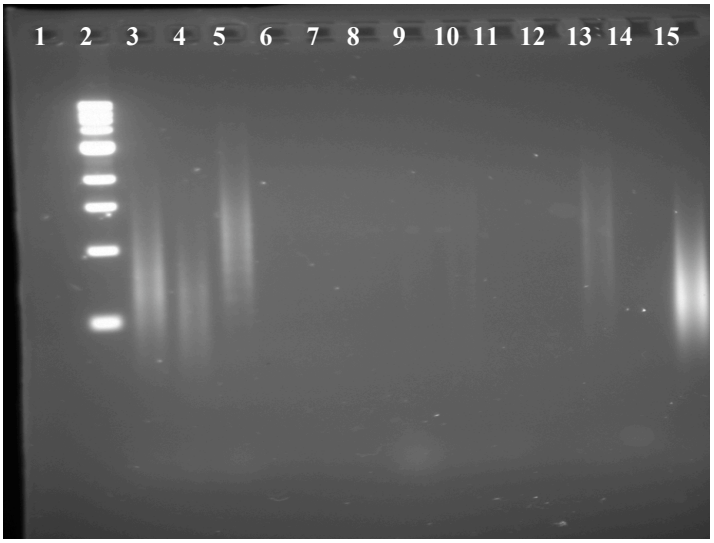

Figure 27: Sample 453-498 (2A)
Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- VNA_06TGP01267_475, Lane4- VNA_06TGP01268_476, Lane5- VNA_05TGP00105_477, Lane6- VNA_05TGP00115_478,Lane7- VNA_05TGP00194_479, Lane8- VNA_05TGP00202_480, Lane9- VNA_05TGP00203_481, Lane10- VNA_05TGP00204_482, Lane11- VNA_05TGP00227_483, Lane12- VNA_05TGP00274_484, Lane13- VNA_05TGP00414_485, Lane14- VNA_05TGP00472_486, Lane15- VNA_05TGP00481_487

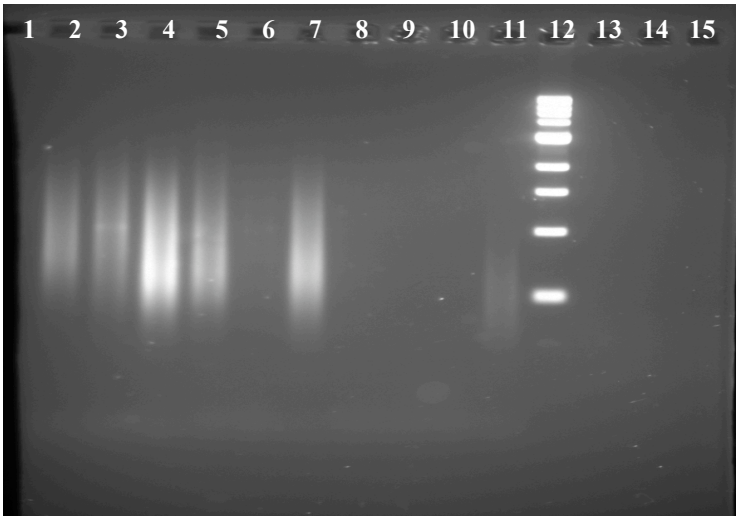

Figure 28: Sample 453-498 (2B)
Lane1- VNA_05TGP00486_488, Lane2- VNA_05TGP00499_489, Lane3- VNA_05TGP00543_490, Lane4- VNA_05TGP00585_491, Lane5- VNA_05TGP00591_492, Lane6-

VNA_06TGP01033_493, Lane7- VNA_06TGP01088_494, Lane8- VNA_06TGP01112_495, Lane9- VNA_06TGP01209_496, Lane10- VNA_06TGP01241_497, Lane11- VNA_06TGP01259_498, Lane12- 1 Kb+ Marker, Lane13, 14, 15- Empty.

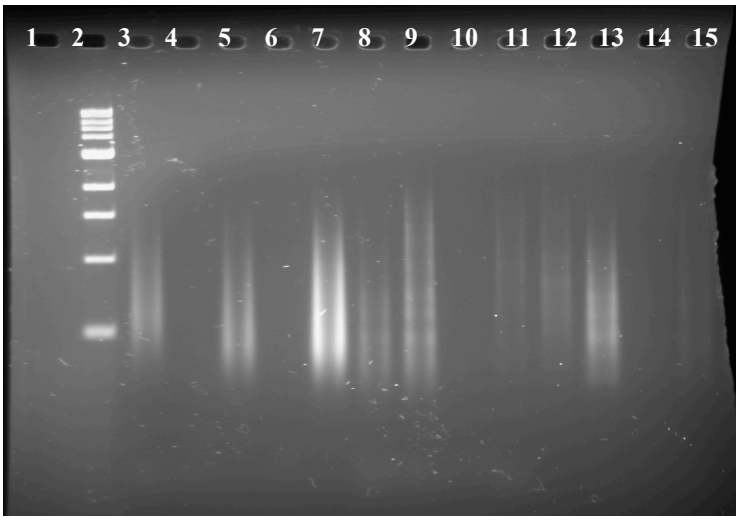

Figure 29: Sample 499-544 (1A)
Lane1- Empty, Lane2- 1Kb+ Marker, Lane3- VNA_06TGP01270_499, Lane4- VNA_06TGP01124_500, Lane5- VNA_05TGP00098_501, Lane6- VNA_05TGP00161_502, Lane7- VNA_05TGP00091_503, Lane8- VNA_06TGP01043_504, Lane9- VNA_05TGP00081_505, Lane10- VNA_05TGP00536_506, Lane11- VNA_05TGP00487_507, Lane12- VNA_05TGP00480_508, Lane13- VNA_06TGP01166_509, Lane14- VNA_05TGP00207_510, Lane15- VNA_06TGP01242_511.

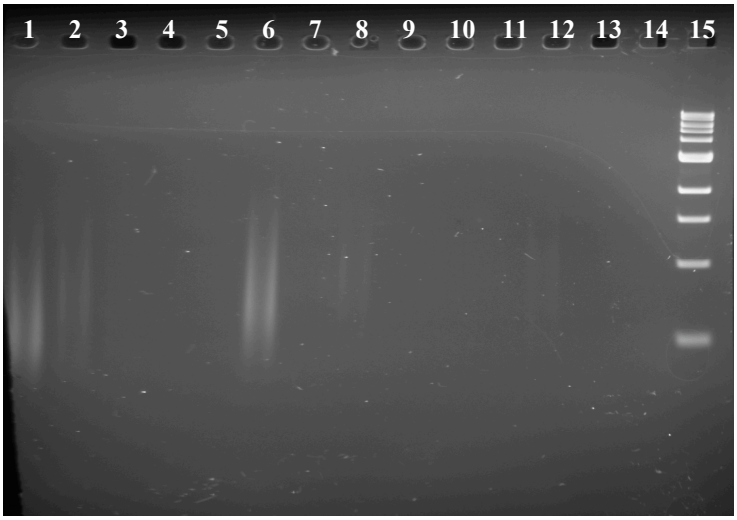

Figure 30: Sample 499-544 (1B)
Lane1- VNA_05TGP00228_512, Lane2- VNA_06TGP01091_513, Lane3- VNA_05TGP00458_514, Lane4- VNA_06TGP01174_515, Lane5- VNA_06TGP01031_516, Lane6- VNA_05TGP00099_517, Lane7- VNA_06TGP01022_518, Lane8- VNA_05TGP00492_519, Lane9- VNA_05TGP00268_520, Lane10- VNA_06TGP01206_521, Lane11- VNA_05TGP00246_522, Lane12- VNA_05TGP00259_523, Lane28,29- Empty, Lane30- 1 Kb+ Marker.

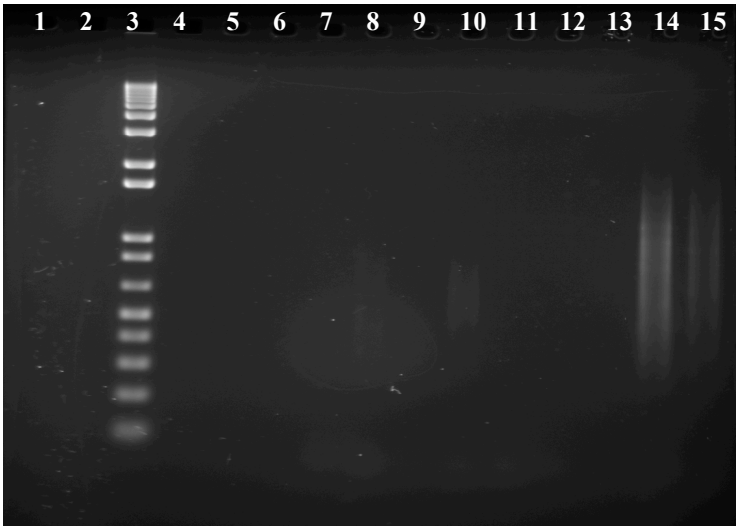

Figure 31: Sample 499-544 (2A)
Lane1, 2- Empty, Lane3- 1Kb+ Marker, Lane4, 5- Empty, Lane6- VNA_05TGP00509_524, Lane7- VNA_05TGP00423_525, Lane8- VNA_06TGP01044_526, Lane9- VNA_06TGP01030_527, Lane10- VNA_05TGP00469_528, Lane11- VNA_05TGP00571_529, Lane12- VNA_06TGP01059_530, Lane13- VNA_06TGP01014_531, Lane14- VNA_06TGP01037_532, Lane15- VNA_06TGP01050_533.

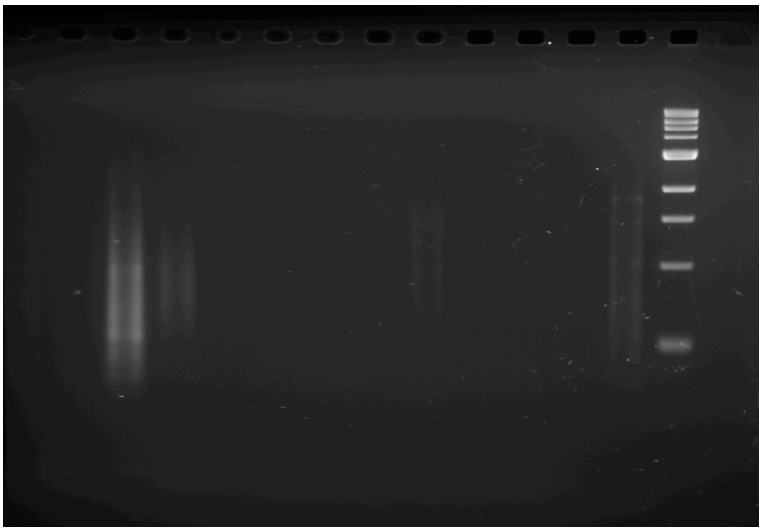

Figure 32: Sample 499-544 (2B) Lane1- VNA_06TGP01052_534, Lane2- VNA_06TGP01057_535, Lane3-VNA_06TGP01064_536, Lane4- VNA_06TGP01078_537, Lane5- VNA_06TGP01182_538, Lane6-Negative control, Lane7- VNA_06TGP01219_539, Lane8- VNA_05TGP00083_540, Lane9- VNA_06TGP01013_541, Lane10- VNA_06TGP01038_542, Lane11- VNA_06TGP01049_543, Lane12- VNA_06TGP01065_544, Lane13- Negative control, Lane14- 1 kb+ Marker, Lane15- Empty.

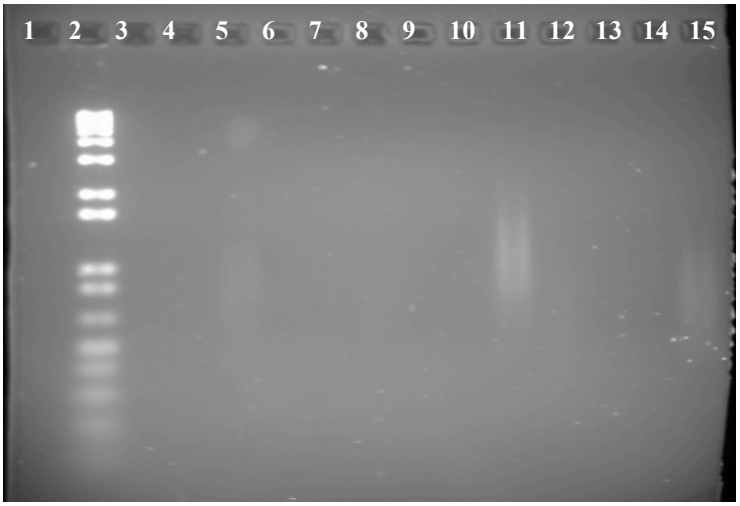

Figure 33: Sample 545-590 (1A) Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- Negative control, Lane4- VNA_06TGP01077_545, Lane5- VNA_06TGP01181_546, Lane6- VNA_06TGP01120_547, Lane7- VNA_05TGP00153_548, Lane8- VNA_06TGP01100_549, Lane9- VNA_06TGP01175_550, Lane10- Negative control, Lane11- VNA_05TGP00156_551, Lane12- VNA_05TGP00271_552, Lane13- VNA_05TGP00185_553, Lane14- VNA_05TGP00103_554, Lane15- VNA_06TGP01053_555.

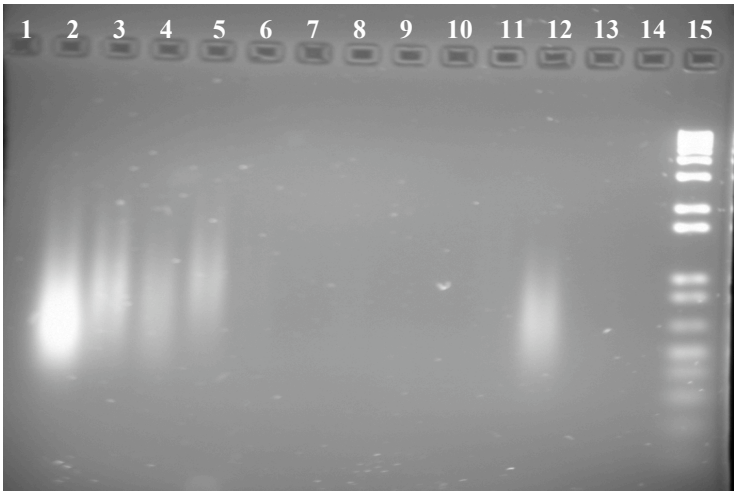

Figure 34: Sample 545-590 (1B) Lane1- VNA_05TGP00442_556, Lane2- VNA_05TGP00578_557, Lane3-VNA_06TGP01001_558,Lane4- VNA_06TGP01029_559, Lane5- VNA_05TGP00311_560,Lane6-VNA_05TGP00520_561, Lane7- VNA_05TGP00502_562, Lane8- VNA_05TGP00206_563, Lane9-VNA_06TGP01113_564, Lane10- VNA_05TGP00622_565, Lane11- VNA_05TGP00583_566, lane12-VNA_05TGP00168_567, Lane13, 14- Empty, Lane15- 1 Kb+ Marker.

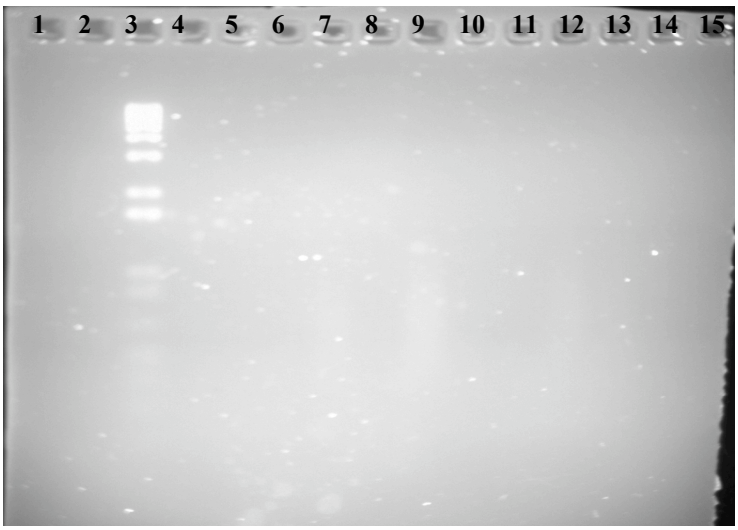

Figure 35: Sample 545-590 (2A) Lane1, 2- Empty, Lane3- 1Kb+ Marker, Lane4, 5-Empty, Lane6-VNA_06TGP01087 _568, Lane7- VNA_05TGP00146_569, Lane8- VNA_05TGP00510_570, Lane9-VNA_05TGP00131_571, Lane10- VNA_05TGP00152_572, Lane11- VNA_05TGP00568_573, Lane12-VNA_07TGP00001_574,Lane13- VNA_07TGP00002_575, Lane14- VNA_07TGP00003_576, Lane15-VNA_07TGP00004_577.

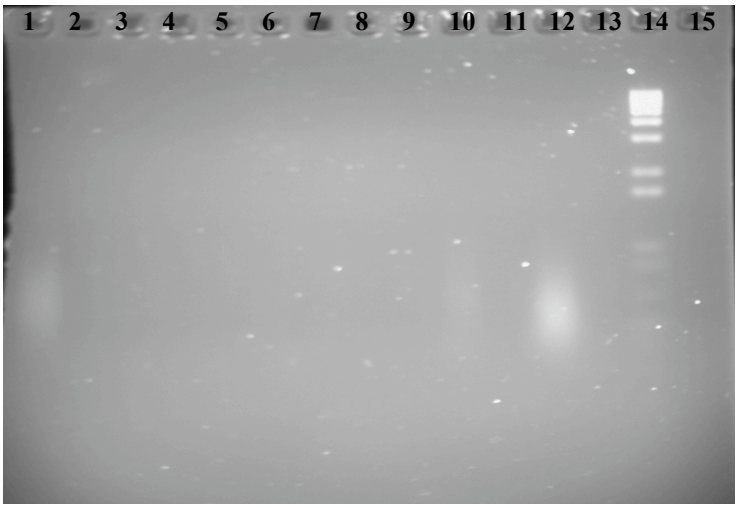

Figure 36: Sample 545-590 (2B) Lane1- VNA_07TGP00005_578, Lane2- VNA_07TGP00006_579, Lane3- VNA_07TGP00007_580, Lane4- VNA_07TGP00008_581, Lane5- VNA_07TGP00009_582, Lane6-VNA_07TGP00010_583, Lane7- VNA_07TGP00011_584, Lane8- VNA_07TGP00012_585, Lane9-VNA_07TGP00013_586, Lane10- VNA_07TGP00014_587, Lane11- VNA_07TGP00015_588, Lane12-VNA_07TGP00016_589, Lane13- VNA_07TGP00017_590, Lane14-1 Kb+ Marker, Lane15- Empty.

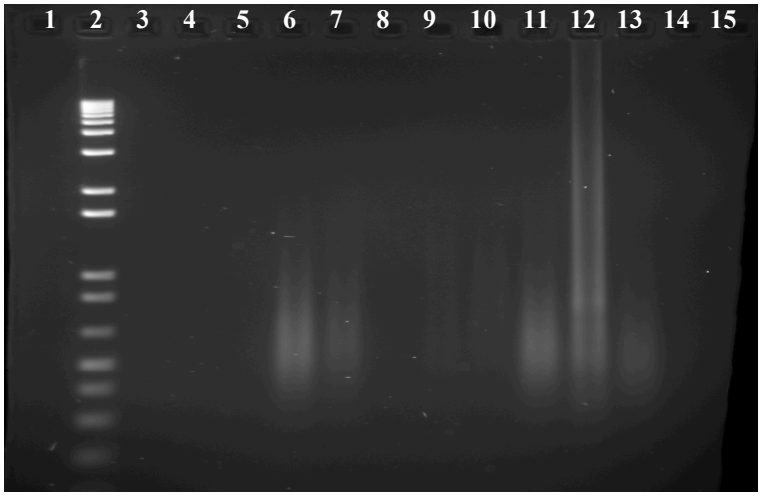

Figure 37: Sample 591-634 (1A) Lane1- Empty, Lane2- 1 Kb+ Marker, Lane3- VNA_07TGP00018_591, Lane4- VNA_07TGP00019_592, Lane5- VNA_07TGP00020_593, Lane6- VNA_07TGP00021_594, Lane7-VNA_07TGP00022_595, Lane8- VNA_07TGP00023_596, Lane9- VNA_07TGP00024_597, Lane10-VNA_07TGP00025_598, Lane11- VNA_07TGP00026_599, Lane12- VNA_07TGP00027_600, Lane13-VNA_07TGP00028_601, Lane14- VNA_07TGP00029_602, Lane15- VNA_07TGP00030_603.

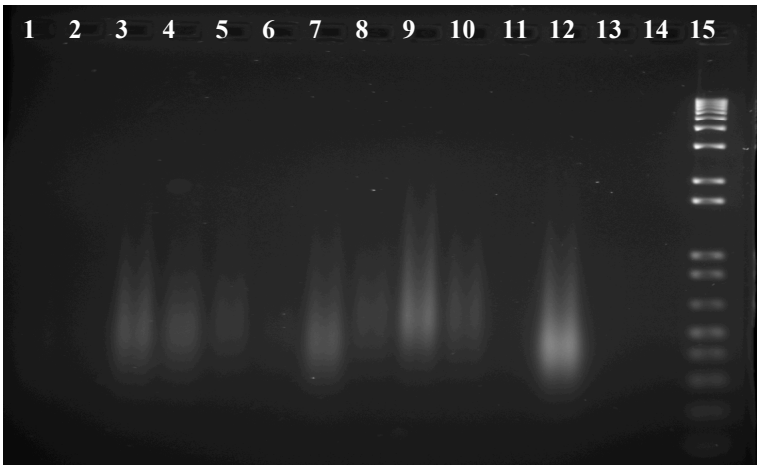

Figure38: Sample 591-634 (1B) Lane1- VNA_07TGP00031_604, Lane2- VNA_07TGP00032_605, Lane3- VNA_07TGP00033_606, Lane4- VNA_07TGP00034_607, Lane5- VNA_07TGP00035_608, Lane6-VNA_07TGP00036_609, Lane7- VNA_07TGP00037_610, Lane8- VNA_07TGP00038_611, Lane9-VNA_07TGP00039_612,Lane10-VNA_07TGP00040_613,Lane11 VNA_07TGP00041_614,Lane12- VNA_07TGP00042_615, Lane13, 14- Empty, Lane15- 1 Kb+ Marker.

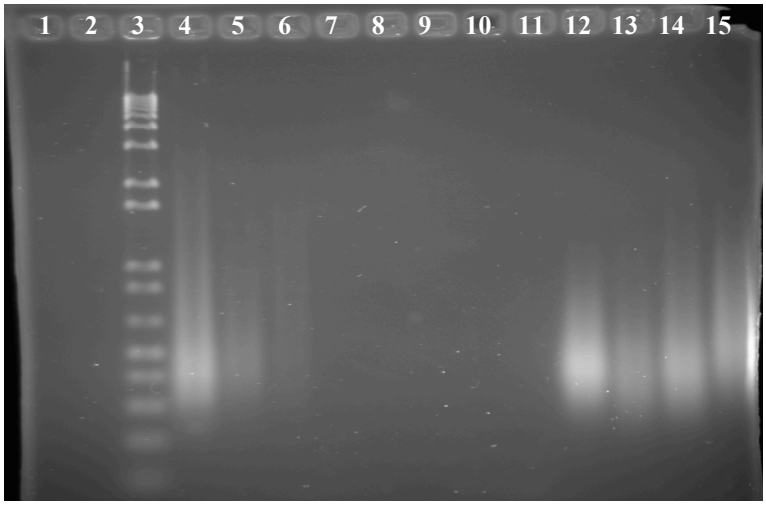

Figure 39: Sample 591-634 (2A) Lane1, 2- Empty, Lane3- 1 Kb+ Marker, Lane4- VNA_07TGP00043_616, Lane5- VNA_07TGP00044_617, Lane6- VNA_07TGP00045_618, Lane7- VNA_07TGP00046_619,Lane8,9-Empty,Lane10-VNA_07TGP00047_620,Lane11-

VNA_07TGP00048_621, Lane12- VNA_07TGP00049_622, Lane13- VNA_07TGP00050_623, Lane14-VNA_07TGP00051_624, Lane15-VNA_07TGP00052_625.

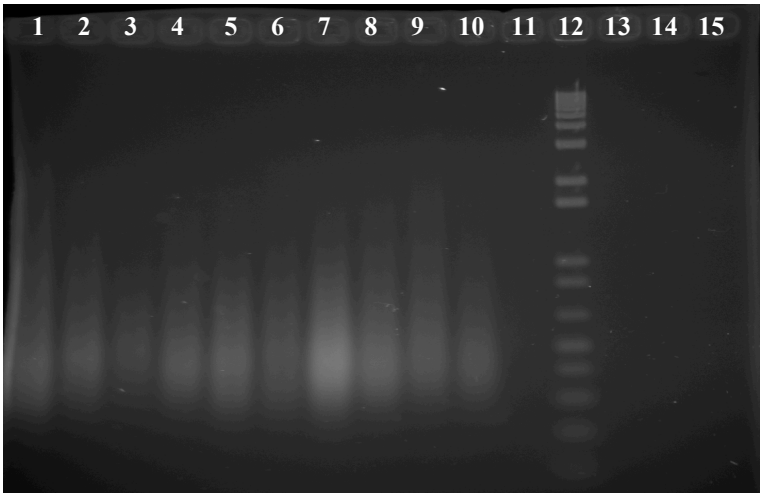

Figure 40: Sample 591-634 (2B) Lane1- VNA_07TGP00053_626, Lane2- VNA_07TGP00054_627, Lane3- VNA_07TGP00055_628, Lane4- VNA_07TGP00056_629, Lane5- VNA_07TGP00057_630, Lane6- Negative control, Lane7- VNA_07TGP00058_631, Lane8- VNA_07TGP00059_632, Lane9-VNA_07TGP00060_633, Lane10- VNA_07TGP00061_634, Lane11- Negative control, Lane12-1Kb+ Marker, Lane13, 14, 15- Empty.

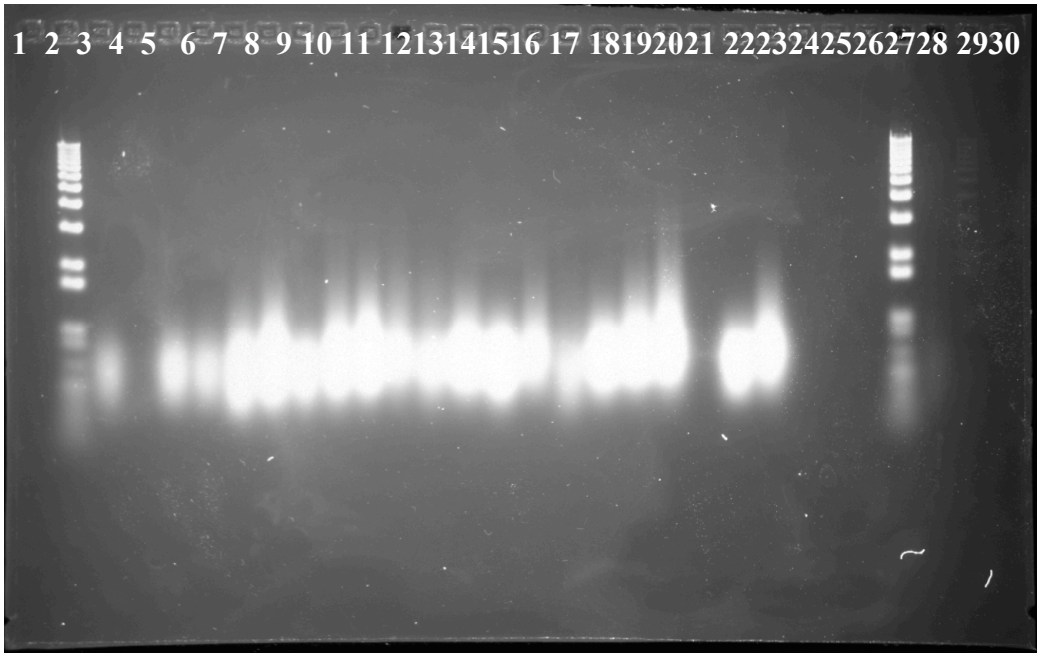

Figure 41: Sample 635-658 Lane1- empty, Lane2-1 Kb+ Marker, Lane3- VNA_07TGP00062_635,Lane4-VNA_07TGP00063_636, Lane5- VNA_07TGP00064_637, Lane6- VNA_07TGP00065_638, Lane7-VNA_07TGP00066_639, Lane8- VNA_07TGP00067_640, Lane9- VNA_07TGP00068_641, Lane10-VNA_07TGP00069_642, Lane11- VNA_07TGP00070_643, Lane12- VNA_07TGP00071_644, Lane13-VNA_07TGP00072_645-, Lane14- VNA_07TGP00073_646, Lane15- VNA_07TGP00074_647, Lane16-VNA_07TGP00075_648, Lane17- VNA_07TGP00076_649, Lane18- VNA_07TGP00077_650, Lane19-VNA_07TGP00078_651, Lane20- VNA_07TGP00079_652, Lane21- VNA_07TGP00080_653, Lane22-VNA_07TGP00081_654, Lane23- VNA_07TGP00082_655, Lane24- VNA_07TGP00083_656, Lane25-VNA_07TGP00084_657, Lane26- VNA_07TGP00085_658, Lane27- 1 Kb+ Marker, Lane28- Negative control, Lane29,30- Empty.

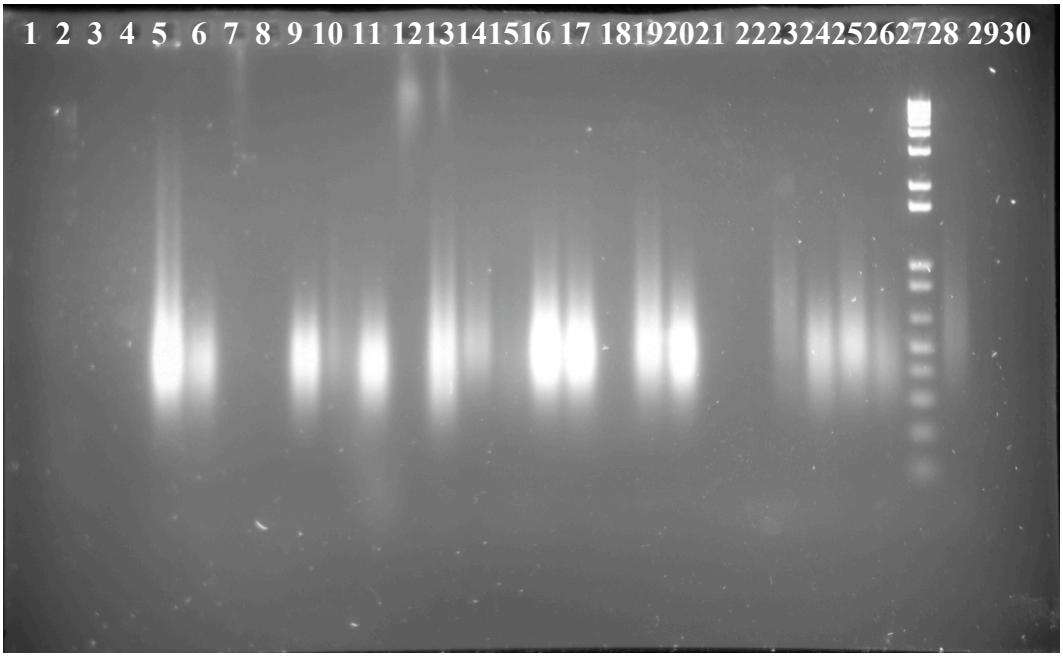

Figure 42: Sample 659-680. Lane1- Empty, Lane2- 1 Kb+ Ladder, Lane3- VNA_07TGP00084_657, Lane4- VNA_07TGP00085_658, Lane5- VNA_07TGP00086_659, Lane6- VNA_07TGP00087_660, Lane7- VNA_07TGP00088_661, Lane8- VNA_07TGP00089_662, Lane9- VNA_07TGP00090_663, Lane10- VNA_07TGP00091_664, Lane11- VNA_07TGP00092_665, Lane12- VNA_07TGP00093_666, Lane13- VNA_07TGP00094_667, Lane14- VNA_07TGP00095_668, Lane15- VNA_07TGP00096_669, Lane16- VNA_07TGP00097_670, Lane17- VNA_07TGP00098_671, Lane18- VNA_07TGP00099_672, Lane19- VNA_07TGP00100_673, Lane20- VNA_07TGP00101_674, Lane21- VNA_07TGP00102_675, Lane22- VNA_07TGP00103_676, Lane23- VNA_07TGP00104_677, Lane24- VNA_07TGP00105_678, Lane25- VNA_07TGP00106_679, Lane26- VNA_07TGP00107_680, Lane27- 1 Kb+ Marker, Lane28- Negative control, Lane29.30- Empty.

APPENDIX III

SMEAR RESULTS

Virion ID	Plant ID	Plant Species	AMPLIFICATION
VNA_05TGP00295_001	05TGP00295	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00248_002	05TGP00248	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00289_003	05TGP00289	Asplenium rhizophyllum	YES AMPLIFICATION
VNA_05TGP00162_004	05TGP00162	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_05TGP00296_005	05TGP00296	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00305_006	05TGP00305	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00262_007	05TGP00262	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00283_008	05TGP00283	Aesculus glabra	NO AMPLIFICATION
VNA_05TGP00212_009	05TGP00212	Boehmeria cylindrica	NO AMPLIFICATION
VNA_05TGP00328_010	05TGP00328	Bothriochloa saccharoides	NO AMPLIFICATION
VNA_05TGP00303_011	05TGP00303	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00307_012	05TGP00307	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00312_013	05TGP00312	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00313_014	05TGP00313	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00316_015	05TGP00316	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00318_016	05TGP00318	Helianthus mollis	NO AMPLIFICATION
VNA_05TGP00321_017	05TGP00321	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00323_018	05TGP00323	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00326_019	05TGP00326	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00332_020	05TGP00332	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00335_021	05TGP00335	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00337_022	05TGP00337	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00339_023	05TGP00339	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00342_024	05TGP00342	Coreopsis tinctoria	YES AMPLIFICATION
VNA_05TGP00343_025	05TGP00343	Pyrrhopappus carolinianus	NO AMPLIFICATION
VNA_05TGP00344_026	05TGP00344	Chrysopsis pilosa	NO AMPLIFICATION
VNA_05TGP00351_027	05TGP00351	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00352_028	05TGP00352	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00353_029	05TGP00353	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00360_030	05TGP00360	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00361_031	05TGP00361	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00362_032	05TGP00362	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00364_033	05TGP00364	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00365_034	05TGP00365	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00192_035	05TGP00192	Sedum nuttallianum	YES AMPLIFICATION

VNA_05TGP00199_036	05TGP00199	Chaetopappa asteroides	NO AMPLIFICATION
VNA_05TGP00201_037	05TGP00201	Tragopogon dubius	NO AMPLIFICATION
VNA_05TGP00211_038	05TGP00211	Ranunculus sceleratus	NO AMPLIFICATION
VNA_05TGP00214_039	05TGP00214	Ellisia nyctelea	NO AMPLIFICATION
VNA_05TGP00219_040	05TGP00219	Triodanis perfoliata	NO AMPLIFICATION
VNA_05TGP00220_041	05TGP00220	Schedonorus pratensis	NO AMPLIFICATION
VNA_05TGP00237_042	05TGP00237	Brickellia eupatorioides	NO AMPLIFICATION
VNA_05TGP00238_043	05TGP00238	Eupatorium altissimum	NO AMPLIFICATION
VNA_05TGP00241_044	05TGP00241	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00248_045	05TGP00248	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00255_046	05TGP00255	Ceratophyllum demersum	NO AMPLIFICATION
VNA_05TGP00256_047	05TGP00256	Chara globularis	YES AMPLIFICATION
VNA_05TGP00258_048	05TGP00258	Potamogeton nodosus	NO AMPLIFICATION
VNA_05TGP00269_049	05TGP00269	Oligoneuron rigidum	NO AMPLIFICATION
VNA_05TGP00276_050	05TGP00276	Sorghum halepense	NO AMPLIFICATION
VNA_05TGP00279_051	05TGP00279	Ampelopsis cordata	NO AMPLIFICATION
VNA_05TGP00282_052	05TGP00282	Ptelea trifoliata	NO AMPLIFICATION
VNA_05TGP00285_053	05TGP00285	Asimina triloba	NO AMPLIFICATION
VNA_05TGP00287_054	05TGP00287	Dryopteris marginalis	NO AMPLIFICATION
VNA_05TGP00288_055	05TGP00288	Rubus occidentalis	NO AMPLIFICATION
VNA_05TGP00290_056	05TGP00290	Asplenium trichomanes	NO AMPLIFICATION
VNA_05TGP00294_057	05TGP00294	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00297_058	05TGP00297	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00379_059	05TGP00379	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00383_060	05TGP00383	Rumex crispus	NO AMPLIFICATION
VNA_05TGP00401_061	05TGP00401	Juncus marginatus	NO AMPLIFICATION
VNA_05TGP00402_062	05TGP00402	Lespedeza violacea	NO AMPLIFICATION
VNA_05TGP00403_063	05TGP00403	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00406_064	05TGP00406	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00407_065	05TGP00407	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00408_066	05TGP00408	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00413_067	05TGP00413	Helianthus maximiliani	NO AMPLIFICATION
VNA_05TGP00417_068	05TGP00417	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00418_069	05TGP00418	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00419_070	05TGP00419	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00420_071	05TGP00420	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00422_072	05TGP00422	Lespedeza capitata	NO AMPLIFICATION
VNA_05TGP00426_073	05TGP00426	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00427_074	05TGP00427	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00428_075	05TGP00428	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00429_076	05TGP00429	Euphorbia corollata	NO AMPLIFICATION
VNA_05TGP00430_077	05TGP00430	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00431_078	05TGP00431	Panicum virgatum	NO AMPLIFICATION
VNA_05TGP00432_079	05TGP00432	Ambrosia psilostachya	YES AMPLIFICATION

VNA_05TGP00434_080	05TGP00434	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00435_081	05TGP00435	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00437_082	05TGP00437	Spiranthes vernalis	NO AMPLIFICATION
VNA_05TGP00029_083	05TGP00029	Apocynum cannabinum	YES AMPLIFICATION
VNA_05TGP00031_084	05TGP00031	Symphoricarpos orbiculatus	YES AMPLIFICATION
VNA_05TGP00032_085	05TGP00032	Plantago virginica	NO AMPLIFICATION
VNA_05TGP00049_086	05TGP00049	Arnoglossum plantagineum	NO AMPLIFICATION
VNA_05TGP00050_087	05TGP00050	Echinacea pallida	YES AMPLIFICATION
VNA_05TGP00057_088	05TGP00057	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00058_089	05TGP00058	Baptisia australis	NO AMPLIFICATION
VNA_05TGP00070_090	05TGP00070	Agrimonia parviflora	NO AMPLIFICATION
VNA_05TGP00075_091	05TGP00075	Carex microdonta	NO AMPLIFICATION
VNA_05TGP00080_092	05TGP00080	Eryngium yuccifolium	NO AMPLIFICATION
VNA_05TGP00089_093	05TGP00089	Chasmanthium latifolium	NO AMPLIFICATION
VNA_05TGP00096_094	05TGP00096	Baptisia australis	YES AMPLIFICATION
VNA_05TGP00111_095	05TGP00111	Bouteloua dactyloides	NO AMPLIFICATION
VNA_05TGP00112_096	05TGP00112	Carex vulpinoidea	YES AMPLIFICATION
VNA_05TGP00114_097	05TGP00114	Bromus arvensis	NO AMPLIFICATION
VNA_05TGP00116_098	05TGP00116	Aegilops cylindrica	NO AMPLIFICATION
VNA_05TGP00139_099	05TGP00139	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00167_100	05TGP00167	Silphium integrifolium	YES AMPLIFICATION
VNA_05TGP00239_101	05TGP00239	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00345_102	05TGP00345	Hieracium longipilum	NO AMPLIFICATION
VNA_05TGP00359_103	05TGP00359	Arenaria serpyllifolia	NO AMPLIFICATION
VNA_05TGP00363_104	05TGP00363	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00366_105	05TGP00366	Setaria parviflora	NO AMPLIFICATION
VNA_05TGP00195_106	05TGP00195	Juncus brachycarpus	NO AMPLIFICATION
VNA_05TGP00370_107	05TGP00370	Scleria ciliata	NO AMPLIFICATION
VNA_05TGP00371_108	05TGP00371	Polygala incarnata	NO AMPLIFICATION
VNA_05TGP00372_109	05TGP00372	Spermolepis inermis	YES AMPLIFICATION
VNA_05TGP00376_110	05TGP00376	Medicago lupulina	YES AMPLIFICATION
VNA_05TGP00377_111	05TGP00377	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00380_112	05TGP00380	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00381_113	05TGP00381	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00382_114	05TGP00382	Calylophus serrulatus	NO AMPLIFICATION
VNA_05TGP00385_115	05TGP00385	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00387_116	05TGP00387	Ambrosia psilostachya	NO AMPLIFICATION
VNA_05TGP00389_117	05TGP00389	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00390_118	05TGP00390	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00391_119	05TGP00391	Cyperus acuminatus	YES AMPLIFICATION
VNA_05TGP00392_120	05TGP00392	Pascopyrum smithii	NO AMPLIFICATION
VNA_05TGP00394_121	05TGP00394	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00396_122	05TGP00396	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00435_123	05TGP00435	Vernonia baldwinii	NO AMPLIFICATION

VNA_05TGP00034_124	05TGP00034	Verbena urticifolia	YES AMPLIFICATION
VNA_05TGP00059_125	05TGP00059	Verbascum thapsus	YES AMPLIFICATION
VNA_05TGP00064_126	05TGP00064	Hordeum pusillum	NO AMPLIFICATION
VNA_05TGP00067_127	05TGP00067	Quercus velutina	YES AMPLIFICATION
VNA_05TGP00068_128	05TGP00068	Cornus drummondii	NO AMPLIFICATION
VNA_05TGP00072_129	05TGP00072	Symphyotrichum oolentangiense	NO AMPLIFICATION
VNA_05TGP00073_130	05TGP00073	Hypoxis hirsuta	YES AMPLIFICATION
VNA_05TGP00085_131	05TGP00085	Viola bicolor	NO AMPLIFICATION
VNA_05TGP00087_132	05TGP00087	Antennaria parlinii ssp. fallax	YES AMPLIFICATION
VNA_05TGP00093_133	05TGP00093	Pyrrhopappus grandiflorus	YES AMPLIFICATION
VNA_05TGP00095_134	05TGP00095	Melilotus officinalis	YES AMPLIFICATION
VNA_05TGP00113_135	05TGP00113	Hymenopappus scabiosaeus	NO AMPLIFICATION
VNA_05TGP00118_136	05TGP00118	Physalis pumila	NO AMPLIFICATION
VNA_05TGP00122_137	05TGP00122	Gleditsia triacanthos	YES AMPLIFICATION
VNA_05TGP00135_138	05TGP00135	Polytaenia nuttallii	YES AMPLIFICATION
VNA_05TGP00137_139	05TGP00137	Sanicula canadensis	NO AMPLIFICATION
VNA_05TGP00149_140	05TGP00149	Lespedeza virginica	YES AMPLIFICATION
VNA_05TGP00151_141	05TGP00151	Vernonia arkansana	NO AMPLIFICATION
VNA_05TGP00158_142	05TGP00158	Parietaria pensylvanica	YES AMPLIFICATION
VNA_05TGP00170_143	05TGP00170	Juglans nigra	NO AMPLIFICATION
VNA_05TGP00175_144	05TGP00175	Verbesina virginica	NO AMPLIFICATION
VNA_05TGP00182_145	05TGP00182	Festuca subverticillata	YES AMPLIFICATION
VNA_05TGP00193_146	05TGP00193	Xanthisma texanum	YES AMPLIFICATION
VNA_05TGP00030_147	05TGP00030	Galium circaezans	YES AMPLIFICATION
VNA_05TGP00040_148	05TGP00040	Vitis sp	YES AMPLIFICATION
VNA_05TGP00044_149	05TGP00044	Lepidium virginicum	YES AMPLIFICATION
VNA_05TGP00051_150	05TGP00051	Chaerophyllum tainturieri	YES AMPLIFICATION
VNA_05TGP00055_151	05TGP00055	Carex gravida	YES AMPLIFICATION
VNA_05TGP00060_152	05TGP00060	Phalaris caroliniana	YES AMPLIFICATION
VNA_05TGP00063_153	05TGP00063	Delphinium carolinianum	YES AMPLIFICATION
VNA_05TGP00065_154	05TGP00065	Tradescantia ohiensis	YES AMPLIFICATION
VNA_05TGP00069_155	05TGP00069	Chaerophyllum tainturieri	YES AMPLIFICATION
VNA_05TGP00072_156	05TGP00072	Symphyotrichum oolentangiense	NO AMPLIFICATION
VNA_05TGP00077_157	05TGP00077	Scirpus pendulus	YES AMPLIFICATION
VNA_05TGP00079_158	05TGP00079	Ludwigia palustris	YES AMPLIFICATION
VNA_05TGP00082_159	05TGP00082	Krigia cespitosa	YES AMPLIFICATION
VNA_05TGP00086_160	05TGP00086	Claytonia virginica	YES AMPLIFICATION
VNA_05TGP00088_161	05TGP00088	Allium canadensis	YES AMPLIFICATION
VNA_05TGP00094_162	05TGP00094	Erigeron tenuis	YES AMPLIFICATION
VNA_05TGP00100_163	05TGP00100	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00106_164	05TGP00106	Xanthium strumarium	YES AMPLIFICATION
VNA_05TGP00117_165	05TGP00117	Maclura pomifera	YES AMPLIFICATION
VNA_05TGP00119_166	05TGP00119	Thelesperma filifolium	YES AMPLIFICATION
VNA_05TGP00157_167	05TGP00157	Carex corrugata	NO AMPLIFICATION

VNA_05TGP00165_168	05TGP00165	unk sp	YES AMPLIFICATION
VNA_05TGP00374_169	05TGP00374	Typha domingensis	YES AMPLIFICATION
VNA_05TGP00026_170	05TGP00026	Poa pratensis	NO AMPLIFICATION
VNA_05TGP00027_171	05TGP00027	Trifolium campestre	NO AMPLIFICATION
VNA_05TGP00028_172	05TGP00028	Oenothera laciniata	NO AMPLIFICATION
VNA_05TGP00033_173	05TGP00033	Phytolacca americana	NO AMPLIFICATION
VNA_05TGP00035_174	05TGP00035	Quercus macrocarpa	NO AMPLIFICATION
VNA_05TGP00036_175	05TGP00036	Menispermum canadense	YES AMPLIFICATION
VNA_05TGP00037_176	05TGP00037	Prunus serotina	YES AMPLIFICATION
VNA_05TGP00038_177	05TGP00038	Sisyrinchium campestre	NO AMPLIFICATION
VNA_05TGP00041_178	05TGP00041	Arisaema dracontium	NO AMPLIFICATION
VNA_05TGP00042_179	05TGP00042	Rubus flagellaris	YES AMPLIFICATION
VNA_05TGP00043_180	05TGP00043	Calylophus serrulatus	NO AMPLIFICATION
VNA_05TGP00045_181	05TGP00045	Oenothera speciosa	NO AMPLIFICATION
VNA_05TGP00046_182	05TGP00046	Cirsium undulatum	NO AMPLIFICATION
VNA_05TGP00047_183	05TGP00047	Mimosa nuttallii	NO AMPLIFICATION
VNA_05TGP00048_184	05TGP00048	Amorpha canescens	YES AMPLIFICATION
VNA_05TGP00052_185	05TGP00052	Artemisia ludoviciana	YES AMPLIFICATION
VNA_05TGP00053_186	05TGP00053	Psoralidium tenuiflorum	YES AMPLIFICATION
VNA_05TGP00054_187	05TGP00054	Carex microdonta	NO AMPLIFICATION
VNA_05TGP00056_188	05TGP00056	Senecio plattensis	NO AMPLIFICATION
VNA_05TGP00061_189	05TGP00061	Callirhoe alcaeoides	YES AMPLIFICATION
VNA_05TGP00062_190	05TGP00062	Desmanthus illinoensis	YES AMPLIFICATION
VNA_05TGP00066_191	05TGP00066	Amorpha fruticosa	YES AMPLIFICATION
VNA_05TGP00071_192	05TGP00071	Rhus copallinum	NO AMPLIFICATION
VNA_05TGP00076_193	05TGP00076	Cephalanthus occidentalis	NO AMPLIFICATION
VNA_05TGP00136_194	05TGP00136	Galium circaezans	NO AMPLIFICATION
VNA_05TGP00173_195	05TGP00173	Asclepias sullivantii	NO AMPLIFICATION
VNA_05TGP00221_196	05TGP00221	Asclepias tuberosa	YES AMPLIFICATION
VNA_05TGP00243_197	05TGP00243	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00260_198	05TGP00260	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00331_199	05TGP00331	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00336_200	05TGP00336	Sabatia campestris	NO AMPLIFICATION
VNA_05TGP00416_201	05TGP00416	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00424_202	05TGP00424	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00443_203	05TGP00443	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00453_204	05TGP00453	Diodia teres	YES AMPLIFICATION
VNA_05TGP00464_205	05TGP00464	Asclepias viridis	YES AMPLIFICATION
VNA_05TGP00489_206	05TGP00489	Asclepias viridiflora	NO AMPLIFICATION
VNA_05TGP00565_207	05TGP00565	Galium triflorum	YES AMPLIFICATION
VNA_05TGP00576_208	05TGP00576	Asclepias verticillata	YES AMPLIFICATION
VNA_06TGP01051_209	06TGP01051	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01079_210	06TGP01079	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01092_211	06TGP01092	Asclepias viridis	YES AMPLIFICATION

VNA_06TGP01109_212	06TGP01109	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01122_213	06TGP01122	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01123_214	06TGP01123	Apocynum cannabinum	NO AMPLIFICATION
VNA_06TGP01126_215	06TGP01126	Asclepias viridis	NO AMPLIFICATION
VNA_05TGP00226_216	05TGP00226	Panicum anceps	NO AMPLIFICATION
VNA_05TGP00251_217	05TGP00251	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00265_218	05TGP00265	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00304_219	05TGP00304	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00333_220	05TGP00333	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00378_221	05TGP00378	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00386_222	05TGP00386	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00415_223	05TGP00415	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00425_224	05TGP00425	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00444_225	05TGP00444	Panicum virgatum	YES AMPLIFICATION
VNA_05TGP00555_226	05TGP00555	Panicum obtusum	YES AMPLIFICATION
VNA_05TGP00619_227	05TGP00619	Panicum rigidulum	YES AMPLIFICATION
VNA_05TGP00244_228	O5TGP00244	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00252_229	05TGP00252	Sorghastrum nutans	NO AMPLIFICATION
VNA_05TGP00264_230	05TGP00264	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00298_231	05TGP00298	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00315_232	05TGP00315	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00325_233	05TGP00325	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00341_234	05TGP00341	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00393_235	05TGP00393	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00410_236	05TGP00410	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00447_237	05TGP00447	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01085_238	06TGP01085	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01137_239	06TGP01137	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01141_240	06TGP01141	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01150_241	06TGP01150	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01156_242	06TGP01156	Asclepias tuberosa	YES AMPLIFICATION
VNA_06TGP01158_243	06TGP01158	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01168_244	06TGP01168	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01178_245	06TGP01178	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01189_246	06TGP01189	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01198_247	06TGP01198	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01212_248	06TGP01212	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01223_249	06TGP01223	Cephalanthus occidentalis	YES AMPLIFICATION
VNA_06TGP01224_250	06TGP01224	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01234_251	06TGP01234	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01243_252	06TGP01243	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01258_253	06TGP01258	Asclepias viridis	YES AMPLIFICATION
VNA_06TGP01261_254	06TGP01261	Asclepias viridis	NO AMPLIFICATION
VNA_06TGP01271_255	06TGP01271	Asclepias viridis	YES AMPLIFICATION

VNA_06TGP01277_256	06TGP01277	Cynanchum laeve	NO AMPLIFICATION
VNA_06TGP01094_257	06TGP01094	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01105_258	06TGP01105	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01121_259	06TGP01121	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01128_260	06TGP01128	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01139_261	06TGP01139	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01144_262	06TGP01144	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01151_263	06TGP01151	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01162_264	06TGP01162	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01167_265	06TGP01167	Panicum anceps	YES AMPLIFICATION
VNA_06TGP01171_266	06TGP01171	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01180_267	06TGP01180	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01191_268	06TGP01191	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01201_269	06TGP01201	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01217_270	06TGP01217	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01228_271	06TGP01228	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01237_272	06TGP01237	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01247_273	06TGP01247	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01253_274	06TGP01253	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01263_275	06TGP01263	Panicum virgatum	NO AMPLIFICATION
VNA_06TGP01273_276	06TGP01273	Panicum virgatum	YES AMPLIFICATION
VNA_06TGP01080_277	06TGP01080	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01095_278	06TGP01095	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01108_279	06TGP01108	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01118_280	06TGP01118	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01129_281	06TGP01129	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01138_282	06TGP01138	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01143_283A,B	06TGP01143	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01154_284	06TGP01154	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01163_285	06TGP01163	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01173_286	06TGP01173	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01179_287	06TGP01179	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01194_288	06TGP01194	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01202_289	06TGP01202	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01214_290	06TGP01214	Sorghastrum nutans	NO AMPLIFICATION
VNA_06TGP01227_291	06TGP01227	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01239_292	06TGP01239	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00005_293	05TGP00005	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00240_294	05TGP00240	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00249_295	05TGP00249	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00261_296	05TGP00261	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00330_297	05TGP00330	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00340_298	05TGP00340	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00350_299	05TGP00350	Ambrosia psilostachya	YES AMPLIFICATION

VNA_05TGP00397_300	05TGP00397	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00445_301	05TGP00445	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00461_302	05TGP00461	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01082_303	06TGP01082	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01097_304	06TGP01097	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01107_305	06TGP01107	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01119_ 306	06TGP01119	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01125_307	06TGP01125	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01135_308	06TGP01135	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01145_309	06TGP01145	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01155_310	06TGP01155	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01159_311	06TGP01159	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01169_312	06TGP01169	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01176_313	06TGP01176	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01192_314	06TGP01192	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01200_315	06TGP01200	Ambrosia psilostachya	YES AMPLIFICATION
VNA_05TGP00107_316	05TGP00107	Justicia americana	YES AMPLIFICATION
VNA_05TGP00250_317	05TGP00250	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00263_318	05TGP00263	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00299_319	05TGP00299	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00306_320	05TGP00306	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00314_321	05TGP00314	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00324_322	05TGP00324	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00338_323	05TGP00338	Ruellia humilis	NO AMPLIFICATION
VNA_05TGP00354_324	05TGP00354	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00388_325	05TGP00388	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00395_326	05TGP00395	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00409_327	05TGP00409	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00433_328	05TGP00433	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00446_329	05TGP00446	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00462_330	05TGP00462	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01084_331	06TGP01084	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01093_332	06TGP01093	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01213_333	06TGP01213	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01222_334	06TGP01222	Justicia americana	YES AMPLIFICATION
VNA_06TGP01226_335	06TGP01226	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01236_336	06TGP01236	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01244_337	06TGP01244	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01255_338	06TGP01255	Ambrosia psilostachya	YES AMPLIFICATION
VNA_06TGP01106_339	06TGP01106	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01117_340	06TGP01117	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01131_341	06TGP01131	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01136_342	06TGP01136	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01142_343	06TGP01142	Ruellia humilis	YES AMPLIFICATION

VNA_06TGP01153_344	06TGP01153	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01160_345	06TGP01160	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01170_346	06TGP01170	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01187_347	06TGP01187	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01195_348	06TGP01195	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01199_349	06TGP01199	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01215_350	06TGP01215	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01225_351	06TGP01225	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01235_352	06TGP01235	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01245_353	06TGP01245	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01251_354	06TGP01251	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01262_355	06TGP01262	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01272_356	06TGP01272	Ruellia humilis	YES AMPLIFICATION
VNA_06TGP01276_357	06TGP01276	Ruellia humilis	YES AMPLIFICATION
VNA_05TGP00123_358	05TGP00123	Ruellia strepens	YES AMPLIFICATION
VNA_05TGP00181_359	05TGP00181	Ruellia strepens	YES AMPLIFICATION
VNA_05TGP00244_360	05TGP00244	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01246_361	06TGP01246	Sorghastrum nutans	YES AMPLIFICATION
VNA_05TGP00242_362	05TGP00242	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00247_363	05TGP00247	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00242_364	05TGP00302	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00322_365	05TGP00322	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00334_366	05TGP00334	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00355_367	05TGP00355	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00411_368	05TGP00411	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00448_369	05TGP00448	Vernonia baldwinii	NO AMPLIFICATION
VNA_05TGP00465_370	05TGP00465	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01254_371	06TGP01254	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01264_372	06TGP01264	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01274_373	06TGP01274	Sorghastrum nutans	YES AMPLIFICATION
VNA_06TGP01081_374	06TGP01081	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01096_375	06TGP01096	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01110_376	06TGP01110	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01120_377	06TGP01120	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01127_378	06TGP01127	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01140_379	06TGP01140	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01146_380	06TGP01146	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01152_381	06TGP01152	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01164_382	06TGP01164	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01172_383	06TGP01172	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01186_384	06TGP01186	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00125_385	05TGP00125	Asplenium platyneuron	YES AMPLIFICATION
VNA_05TGP00186_386	05TGP00186	Ambrosia trifida	YES AMPLIFICATION
VNA_05TGP00190_387	05TGP00190	Ambrosia bidentata	YES AMPLIFICATION

VNA_05TGP00308_388	05TGP00308	Ambrosia artemisiifolia	NO AMPLIFICATION
VNA_05TGP00542_389	05TGP00542	Ambrosia bidentata	YES AMPLIFICATION
VNA_06TGP01012_390	06TGP01012	Bromus arvensis	NO AMPLIFICATION
VNA_06TGP01040_391	06TGP01040	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01048_392	06TGP01048	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01054_393	06TGP01054	Bromus arvensis	NO AMPLIFICATION
VNA_06TGP01056_394	06TGP01056	Bromus arvensis	NO AMPLIFICATION
VNA_06TGP01060_395	06TGP01060	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01067_396	06TGP01067	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01069_397	06TGP01069	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01072_398	06TGP01072	Bromus arvensis	YES AMPLIFICATION
VNA_06TGP01190_399	06TGP01190	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01203_400	06TGP01203	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01216_401	06TGP01216	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01229_402	06TGP01229	Vernonia baldwinii	NO AMPLIFICATION
VNA_06TGP01238_403	06TGP01238	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01248_404	06TGP01248	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01252_405	06TGP01252	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01266_406	06TGP01266	Vernonia baldwinii	YES AMPLIFICATION
VNA_06TGP01275_407	06TGP01275	Vernonia baldwinii	YES AMPLIFICATION
VNA_05TGP00110_408	05TGP00110	Hedeoma hispida	YES AMPLIFICATION
VNA_05TGP00273_409	05TGP00273	Dichanthelium linearifolium	YES AMPLIFICATION
VNA_05TGP00478_410	05TGP00478	Dichanthelium clandestinum	YES AMPLIFICATION
VNA_05TGP00488_411	05TGP00488	Dicliptera brachiata	YES AMPLIFICATION
VNA_05TGP00527_412	05TGP00527	Equisetum hyemale	YES AMPLIFICATION
VNA_05TGP00528_413	05TGP00528	Dichanthelium sphaerocarpon	NO AMPLIFICATION
VNA_05TGP00529_414	05TGP00529	Dichanthelium acuminatum	YES AMPLIFICATION
VNA_05TGP00572_415	05TGP00572	Dichanthelium malacophyllum	YES AMPLIFICATION
VNA_05TGP00598_416	05TGP00598	Dicliptera brachiata	YES AMPLIFICATION
VNA_06TGP01005_417	06TGP01005	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01007_418	06TGP01007	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01019_419	06TGP01019	Hordeum pusillum	NO AMPLIFICATION
VNA_06TGP01025_420	06TGP01025	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01039_421	06TGP01039	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01046_422	06TGP01046	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01047_423	06TGP01047	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01055_424	06TGP01055	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01066_425	06TGP01066	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01074_426	06TGP01074	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01083_427	06TGP01083	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01116_428	06TGP01116	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_06TGP01130_429	06TGP01130	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01161_430	06TGP01161	Hordeum pusillum	YES AMPLIFICATION
VNA_05TGP00196_431	05TGP00196	Iva annua	YES AMPLIFICATION

VNA_06TGP01003_432	06TGP01003	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01016_433	06TGP01016	Leucobryum albidum	YES AMPLIFICATION
VNA_06TGP01018_434	06TGP01018	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01061_435	06TGP01061	Melilotus officinalis	YES AMPLIFICATION
VNA_06TGP01062_436	06TGP01062	Melilotus officinalis	YES AMPLIFICATION
VNA_06TGP01063_437	06TGP01063	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01068_438	06TGP01068	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01070_439	06TGP01070	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01073_440	06TGP01073	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01076_441	06TGP01076	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01132_442	06TGP01132	Melilotus officinalis	YES AMPLIFICATION
VNA_06TGP01133_443	06TGP01133	Melilotus officinalis	YES AMPLIFICATION
VNA_06TGP01177_444	06TGP01177	Hordeum pusillum	NO AMPLIFICATION
VNA_06TGP01188_445	06TGP01188	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01193_446	06TGP01193	Hordeum pusillum	YES AMPLIFICATION
VNA_06TGP01197_447	06TGP01197	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01221_448	06TGP01221	Hordeum pusillum	NO AMPLIFICATION
VNA_06TGP01230_449	06TGP01230	Melilotus officinalis	NO AMPLIFICATION
VNA_06TGP01240_450	06TGP01240	Hordeum pusillum	NO AMPLIFICATION
VNA_06TGP01256_451	06TGP01256	Hordeum pusillum	NO AMPLIFICATION
VNA_06TGP01257_452	06TGP01257	Medicago lupulina	YES AMPLIFICATION
VNA_06TGP01269_453	06TGP01269	Iva annua	YES AMPLIFICATION
VNA_05TGP00121_454	05TGP00121	Pellaea atropurpurea	YES AMPLIFICATION
VNA_05TGP00126_455	05TGP00126	Bromus pubescens	NO AMPLIFICATION
VNA_05TGP00218_456	05TGP00218	Bromus arvensis	NO AMPLIFICATION
VNA_05TGP00229_457	05TGP00229	Arctium minus	YES AMPLIFICATION
VNA_05TGP00280_458	05TGP00280	Allium vineale	YES AMPLIFICATION
VNA_05TGP00286_459	05TGP00286	Pleopeltis polypodioides	YES AMPLIFICATION
VNA_05TGP00348_460	05TGP00348	Aristolochia tomentosa	YES AMPLIFICATION
VNA_05TGP00475_461	05TGP00475	Bouteloua curtipendula	NO AMPLIFICATION
VNA_05TGP00507_462	05TGP00507	Bothriochloa ischaemum	YES AMPLIFICATION
VNA_05TGP00540_463	05TGP00540	Selaginella rupestris	YES AMPLIFICATION
VNA_05TGP00557_464	05TGP00557	Antennaria neglecta	NO AMPLIFICATION
VNA_05TGP00602_465	05TGP00602	Agastache nepetoides	NO AMPLIFICATION
VNA_06TGP01006_466	06TGP01006	Bromus arvensis	NO AMPLIFICATION
VNA_06TGP01009_467	06TGP01009	Allium canadense	NO AMPLIFICATION
VNA_06TGP01075_468	06TGP01075	Bromus arvensis	NO AMPLIFICATION
VNA_06TGP01089_469	06TGP01089	Xanthium strumarium	YES AMPLIFICATION
VNA_06TGP01090_470	06TGP01090	Bothriochloa laguroides ssp. torreyana	NO AMPLIFICATION
VNA_06TGP01111_471	06TGP01111	Amorpha canescens	NO AMPLIFICATION
VNA_06TGP01147_472	06TGP01147	Antennaria neglecta	NO AMPLIFICATION
VNA_06TGP01148_473	06TGP01148	Brickellia eupatorioides	YES AMPLIFICATION
VNA_06TGP01231_474	06TGP01231	Melilotus officinalis	YES AMPLIFICATION

VNA_06TGP01267_475	06TGP01267	Melilotus officinalis	YES AMPLIFICATION
VNA_06TGP01268_476	06TGP01268	Melilotus officinalis	YES AMPLIFICATION
VNA_05TGP00105_477	05TGP00105	Cercis canadensis	YES AMPLIFICATION
VNA_05TGP00115_478	05TGP00115	Evolvulus nuttallianus	NO AMPLIFICATION
VNA_05TGP00194_479	05TGP00194	Daucus pusillus	NO AMPLIFICATION
VNA_05TGP00202_480	05TGP00202	Elymus virginicus	NO AMPLIFICATION
VNA_05TGP00203_481	05TGP00203	Elymus canadensis	NO AMPLIFICATION
VNA_05TGP00204_482	05TGP00204	Dianthus armeria	NO AMPLIFICATION
VNA_05TGP00227_483	05TGP00227	Diospyros virginiana	NO AMPLIFICATION
VNA_05TGP00274_484	05TGP00274	Kummerowia striata	NO AMPLIFICATION
VNA_05TGP00414_485	05TGP00414	Lespedeza cuneata	YES AMPLIFICATION
VNA_05TGP00472_486	05TGP00472	Digitaria cognata	NO AMPLIFICATION
VNA_05TGP00481_487	05TGP00481	Diarrhena obovata	YES AMPLIFICATION
VNA_05TGP00486_488	05TGP00486	Elymus villosus	NO AMPLIFICATION
VNA_05TGP00499_489	05TGP00499	Cucurbita foetidissima	YES AMPLIFICATION
VNA_05TGP00543_490	05TGP00543	Kummerowia stipulacea	YES AMPLIFICATION
VNA_05TGP00585_491	05TGP00585	Datura stramonium	YES AMPLIFICATION
VNA_05TGP00591_492	05TGP00591	Eragrostis spectabilis	YES AMPLIFICATION
VNA_06TGP01033_493	06TGP01033	Chasmanthium latifolium	NO AMPLIFICATION
VNA_06TGP01088_494	06TGP01088	Cirsium undulatum	YES AMPLIFICATION
VNA_06TGP01112_495	06TGP01112	Lespedeza cuneata	NO AMPLIFICATION
VNA_06TGP01209_496	06TGP01209	Ipomoea pandurata	NO AMPLIFICATION
VNA_06TGP01241_497	06TGP01241	Diospyros virginiana	NO AMPLIFICATION
VNA_06TGP01259_498	06TGP01259	Elymus virginicus	YES AMPLIFICATION
VNA_06TGP01270_499	06TGP01270	Elymus canadensis	YES AMPLIFICATION
VNA_06TGP01124_500	06TGP01124	Monarda fistulosa	NO AMPLIFICATION
VNA_05TGP00098_501	05TGP00098	Oenothera laciniata	YES AMPLIFICATION
VNA_05TGP00161_502	05TGP00161	Oenothera linifolia	NO AMPLIFICATION
VNA_05TGP00091_503	05TGP00091	Oenothera speciosa	YES AMPLIFICATION
VNA_06TGP01043_504	06TGP01043	Oenothera triloba	YES AMPLIFICATION
VNA_05TGP00081_505	05TGP00081	Fimbristylis puberula var. puberula	YES AMPLIFICATION
VNA_05TGP00536_506	05TGP00536	Perilla fructescens	NO AMPLIFICATION
VNA_05TGP00487_507	05TGP00487	Oxalis sricta	YES AMPLIFICATION
VNA_05TGP00480_508	05TGP00480	Phryma leptostachya	YES AMPLIFICATION
VNA_06TGP01166_509	06TGP01166	Paspalum setaceum	YES AMPLIFICATION
VNA_05TGP00207_510	05TGP00207	Penstemon tubiflorus	NO AMPLIFICATION
VNA_06TGP01242_511	06TGP01242	Lespedeza cuneata	YES AMPLIFICATION
VNA_05TGP00228_512	05TGP00228	Lespedeza procumbens	YES AMPLIFICATION
VNA_06TGP01091_513	06TGP01091	Lespedeza procumbens	YES AMPLIFICATION
VNA_05TGP00458_514	05TGP00458	Lespedeza repens	NO AMPLIFICATION
VNA_06TGP01174_515	06TGP01174	lespedeza virginica	NO AMPLIFICATION
VNA_06TGP01031_516	06TGP01031	Leucanthemum vulgare	NO AMPLIFICATION
VNA_05TGP00099_517	05TGP00099	Lolium perenne ssp.	YES AMPLIFICATION

		Multiflorum	
		Lolium perenne ssp.	
VNA_06TGP01022_518	06TGP01022	Multiflorum	NO AMPLIFICATION
VNA_05TGP00492_519	05TGP00492	Marrubium vulgare	YES AMPLIFICATION
VNA_05TGP00268_520	05TGP00268	Monarda citriodora	NO AMPLIFICATION
VNA_06TGP01206_521	06TGP01206	Monarda citriodora	NO AMPLIFICATION
VNA_05TGP00246_522	05TGP00246	Monarda fistulosa	NO AMPLIFICATION
VNA_05TGP00259_523	05TGP00259	Phyla lanceolata	YES AMPLIFICATION
VNA_05TGP00509_524	05TGP00509	Physalis longifolia	NO AMPLIFICATION
VNA_05TGP00423_525	05TGP00423	Physalis pumila	NO AMPLIFICATION
VNA_06TGP01044_526	06TGP01044	Physalis virginiana	YES AMPLIFICATION
VNA_06TGP01030_527	06TGP01030	Phytolacca americana	NO AMPLIFICATION
VNA_05TGP00469_528	05TGP00469	Polygala sanguinea	YES AMPLIFICATION
VNA_05TGP00571_529	05TGP00571	Prunella vulgaris	NO AMPLIFICATION
VNA_06TGP01059_530	06TGP01059	Quercus macrocarpa	NO AMPLIFICATION
VNA_06TGP01014_531	06TGP01014	Quercus marilandica	NO AMPLIFICATION
VNA_06TGP01037_532	06TGP01037	Quercus marilandica	YES AMPLIFICATION
VNA_06TGP01050_533	06TGP01050	Quercus marilandica	YES AMPLIFICATION
VNA_06TGP01052_534	06TGP01052	Quercus marilandica	NO AMPLIFICATION
VNA_06TGP01057_535	06TGP01057	Quercus marilandica	NO AMPLIFICATION
VNA_06TGP01064_536	06TGP01064	Quercus marilandica	YES AMPLIFICATION
VNA_06TGP01078_537	06TGP01078	Quercus marilandica	YES AMPLIFICATION
VNA_06TGP01182_538	06TGP01182	Quercus marilandica	NO AMPLIFICATION
VNA_06TGP01219_539	06TGP01219	Quercus marilandica	NO AMPLIFICATION
VNA_05TGP00083_540	05TGP00083	Quercus stellata	NO AMPLIFICATION
VNA_06TGP01013_541	06TGP01013	Quercus stellata	YES AMPLIFICATION
VNA_06TGP01038_542	06TGP01038	Quercus stellata	NO AMPLIFICATION
VNA_06TGP01049_543	06TGP01049	Quercus stellata	NO AMPLIFICATION
VNA_06TGP01065_544	06TGP01065	Quercus stellata	NO AMPLIFICATION
VNA_06TGP01077_545	06TGP01077	Quercus stellata	NO AMPLIFICATION
VNA_06TGP01181_546	06TGP01181	Quercus stellata	YES AMPLIFICATION
VNA_06TGP01120_547	06TGP01120	Quercus stellata	NO AMPLIFICATION
VNA_05TGP00153_548	05TGP00153	Salvia azurea var. grandiflora	NO AMPLIFICATION
VNA_06TGP01100_549	06TGP01100	Salvia azurea var. grandiflora	NO AMPLIFICATION
VNA_06TGP01175_550	06TGP01175	Salvia azurea var. grandiflora	NO AMPLIFICATION
VNA_05TGP00156_551	05TGP00156	Scutellaria parvula	YES AMPLIFICATION
VNA_05TGP00271_552	05TGP00271	Silene antirrhina	NO AMPLIFICATION
VNA_05TGP00185_553	05TGP00185	Silene stellata	NO AMPLIFICATION
VNA_05TGP00103_554	05TGP00103	Solanum carolinense	NO AMPLIFICATION
VNA_06TGP01053_555	06TGP01053	Solanum carolinense	YES AMPLIFICATION
VNA_05TGP00442_556	05TGP00442	Solanum dimidiatum	NO AMPLIFICATION
VNA_05TGP00578_557	05TGP00578	Solanum elaeagnifolium	YES AMPLIFICATION
VNA_06TGP01001_558	06TGP01001	Solanum elaeagnifolium	YES AMPLIFICATION
VNA_06TGP01029_559	06TGP01029	Solanum physalifolium	YES AMPLIFICATION

VNA_05TGP00311_560	05TGP00311	Solanum rostratum	YES AMPLIFICATION
VNA_05TGP00520_561	05TGP00520	Solanum sarrachoides	NO AMPLIFICATION
VNA_05TGP00502_562	05TGP00502	Spartina pectinata	NO AMPLIFICATION
VNA_05TGP00206_563	05TGP00206	Sphenopholis obtusata	NO AMPLIFICATION
VNA_06TGP01113_564	06TGP01113	Sphenopholis obtusata	NO AMPLIFICATION
VNA_05TGP00622_565	05TGP00622	Sporobolus compositus	NO AMPLIFICATION
VNA_05TGP00583_566	05TGP00583	Sporobolus pyramidatus	NO AMPLIFICATION
VNA_05TGP00168_567	05TGP00168	Tripsacum dactyloides	YES AMPLIFICATION
VNA_06TGP01087 _568	06TGP01087	Tripsacum dactyloides	NO AMPLIFICATION
VNA_05TGP00146_569	05TGP00146	Valerianella radiata	YES AMPLIFICATION
VNA_05TGP00510_570	05TGP00510	Verbena bracteata	NO AMPLIFICATION
VNA_05TGP00131_571	05TGP00131	Verbena simplex	YES AMPLIFICATION
VNA_05TGP00152_572	05TGP00152	Verbena stricta	NO AMPLIFICATION
VNA_05TGP00568_573	05TGP00568	Verbena stricta	NO AMPLIFICATION
VNA_07TGP00001_574	07TGP00001	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00002_575	07TGP00002	Ruellia humilis	NO AMPLIFICATION
VNA_07TGP00003_576	07TGP00003	Asclepias viridis	NO AMPLIFICATION
VNA_07TGP00004_577	07TGP00004	Vernonia baldwinii	NO AMPLIFICATION
VNA_07TGP00005_578	07TGP00005	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00006_579	07TGP00006	Sorghastrum nutans	NO AMPLIFICATION
VNA_07TGP00007_580	07TGP00007	Dichanthelium oligosanthes	NO AMPLIFICATION
VNA_07TGP00008_581	07TGP00008	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00009_582	07TGP00009	Setaria parviflora	NO AMPLIFICATION
VNA_07TGP00010_583	07TGP00010	Vernonia baldwinii	NO AMPLIFICATION
VNA_07TGP00011_584	07TGP00011	Panicum virgatum	NO AMPLIFICATION
VNA_07TGP00012_585	07TGP00012	Ambrosia psilostachya	NO AMPLIFICATION
VNA_07TGP00013_586	07TGP00013	Ruellia humilis	NO AMPLIFICATION
VNA_07TGP00014_587	07TGP00014	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00015_588	07TGP00015	Sorghastrum nutans	NO AMPLIFICATION
VNA_07TGP00016_589	07TGP00016	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00017_590	07TGP00017	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00018_591	07TGP00018	Setaria parviflora	NO AMPLIFICATION
VNA_07TGP00019_592	07TGP00019	Panicum anceps	NO AMPLIFICATION
VNA_07TGP00020_593	07TGP00020	Achillea millefolium	NO AMPLIFICATION
VNA_07TGP00021_594	07TGP00021	Perideridia americana	YES AMPLIFICATION
VNA_07TGP00022_595	07TGP00022	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00023_596	07TGP00023	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00024_597	07TGP00024	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00025_598	07TGP00025	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00026_599	07TGP00026	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00027_600	07TGP00027	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00028_601	07TGP00028	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00029_602	07TGP00029	Vernonia baldwinii	NO AMPLIFICATION
VNA_07TGP00030_603	07TGP00030	Achillea millefolium	NO AMPLIFICATION

VNA_07TGP00031_604	07TGP00031	Setaria parviflora	NO AMPLIFICATION
VNA_07TGP00032_605	07TGP00032	Apocynum cannabinum	NO AMPLIFICATION
VNA_07TGP00033_606	07TGP00033	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00034_607	07TGP00034	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00035_608	07TGP00035	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00036_609	07TGP00036	Vernonia baldwinii	YES AMPLIFICATION
VNA_07TGP00037_610	07TGP00037	Ambrosia artemisiifolia	YES AMPLIFICATION
VNA_07TGP00038_611	07TGP00038	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00039_612	07TGP00039	Dichathelim oligosanthes	YES AMPLIFICATION
VNA_07TGP00040_613	07TGP00040	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00041_614	07TGP00041	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00042_615	07TGP00042	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00043_616	07TGP00043	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00044_617	07TGP00044	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00045_618	07TGP00045	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00046_619	07TGP00046	Vernonia baldwinii	NO AMPLIFICATION
VNA_07TGP00047_620	07TGP00047	Achillea millefolium	NO AMPLIFICATION
VNA_07TGP00048_621	07TGP00048	Paspalum setaceum	NO AMPLIFICATION
VNA_07TGP00049_622	07TGP00049	Setaria parviflora	YES AMPLIFICATION
VNA_07TGP00050_623	07TGP00050	Panicum anceps	YES AMPLIFICATION
VNA_07TGP00051_624	07TGP00051	Tripsacum dactyloides	YES AMPLIFICATION
VNA_07TGP00052_625	07TGP00052	Asclepias tuberosa	YES AMPLIFICATION
VNA_07TGP00053_626	07TGP00053	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00054_627	07TGP00054	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00055_628	07TGP00055	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00056_629	07TGP00056	Marsilea vestita	YES AMPLIFICATION
VNA_07TGP00057_630	07TGP00057	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00058_631	07TGP00058	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00059_632	07TGP00059	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00060_633	07TGP00060	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00061_634	07TGP00061	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00062_635	07TGP00062	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00063_636	07TGP00063	Veronia baldwinii	NO AMPLIFICATION
VNA_07TGP00064_637	07TGP00064	Dichanthelium sphaerocarpon	YES AMPLIFICATION
VNA_07TGP00065_638	07TGP00065	Diodia teres	YES AMPLIFICATION
VNA_07TGP00066_639	07TGP00066	Paspalum setaceum	YES AMPLIFICATION
VNA_07TGP00067_640	07TGP00067	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00068_641	07TGP00068	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00069_642	07TGP00069	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00070_643	07TGP00070	Ambrosia bidentata	YES AMPLIFICATION
VNA_07TGP00071_644	07TGP00071	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00072_645	07TGP00072	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00073_646	07TGP00073	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00074_647	07TGP00074	Veronia balwinii	YES AMPLIFICATION

VNA_07TGP00075_648	07TGP00075	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00076_649	07TGP00076	Panicum anceps	YES AMPLIFICATION
VNA_07TGP00077_650	07TGP00077	Dichanthelium acuminatum	YES AMPLIFICATION
VNA_07TGP00078_651	07TGP00078	Achillea millefolium	YES AMPLIFICATION
VNA_07TGP00079_652	07TGP00079	Veronia balwinii	YES AMPLIFICATION
VNA_07TGP00080_653	07TGP00080	Achillea millefolium	YES AMPLIFICATION
VNA_07TGP00081_654	07TGP00081	Ambrosia artemisiifolia	YES AMPLIFICATION
VNA_07TGP00082_655	07TGP00082	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00083_656	07TGP00083	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00084_657	07TGP00084	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00085_658	07TGP00085	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00086_659	07TGP00086	Ambrosia bidentata	YES AMPLIFICATION
VNA_07TGP00087_660	07TGP00087	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00088_661	07TGP00088	Panicum virgatum	NO AMPLIFICATION
VNA_07TGP00089_662	07TGP00089	Veronia baldwinii	NO AMPLIFICATION
VNA_07TGP00090_663	07TGP00090	Setaria parviflora	YES AMPLIFICATION
VNA_07TGP00091_664	07TGP00091	Paspalum setaceum	YES AMPLIFICATION
VNA_07TGP00092_665	07TGP00092	Dichanthelium sphaerocarpon	YES AMPLIFICATION
VNA_07TGP00093_666	07TGP00093	Helianthus mollis	NO AMPLIFICATION
VNA_07TGP00094_667	07TGP00094	Asclepias viridis	YES AMPLIFICATION
VNA_07TGP00095_668	07TGP00095	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00096_669	07TGP00096	Ambrosia psilostachya	YES AMPLIFICATION
VNA_07TGP00097_670	07TGP00097	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00098_671	07TGP00098	Dichanthelium oligosanthes	YES AMPLIFICATION
VNA_07TGP00099_672	07TGP00099	Ambrosia bidentata	NO AMPLIFICATION
VNA_07TGP00100_673	07TGP00100	Ruellia humilis	YES AMPLIFICATION
VNA_07TGP00101_674	07TGP00101	Setaria parviflora	YES AMPLIFICATION
VNA_07TGP00102_675	07TGP00102	Achillea millefolium	NO AMPLIFICATION
VNA_07TGP00103_676	07TGP00103	Vernonia baldwinii	NO AMPLIFICATION
VNA_07TGP00104_677	07TGP00104	Lotus unifoliolatus	YES AMPLIFICATION
VNA_07TGP00105_678	07TGP00105	Sorghastrum nutans	YES AMPLIFICATION
VNA_07TGP00106_679	07TGP00106	Panicum virgatum	YES AMPLIFICATION
VNA_07TGP00107_680	07TGP00107	Vernonia baldwinii	YES AMPLIFICATION
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VNA_07TGP00109_682	07TGP00109	Ambrosia psilostachya	VNA ISOLATED
VNA_07TGP00110_683	07TGP00110	Asclepias viridis	VNA ISOLATED
VNA_07TGP00111_684	07TGP00111	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00112_685	07TGP00112	Euthamia gymnospermoides	VNA ISOLATED
VNA_07TGP00113_686	07TGP00113	Setaria parviflora	VNA ISOLATED
VNA_07TGP00114_687	07TGP00114	Achillea millefolium	VNA ISOLATED
VNA_07TGP00115_688	07TGP00115	Dichanthelium acuminatum	VNA ISOLATED
VNA_07TGP00116_689	07TGP00116	Ambrosia bidentata	VNA ISOLATED
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VNA_07TGP00118_691	07TGP00118	Panicum virgatum	VNA ISOLATED

VNA_07TGP00119_692	07TGP00119	Asclepias viridis	VNA ISOLATED
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VNA_07TGP00121_694	07TGP00121	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00122_695	07TGP00122	Sorghastrum nutans	VNA ISOLATED
VNA_07TGP00123_696	07TGP00123	Vernonia baldwinii	VNA ISOLATED
VNA_07TGP00124_697	07TGP00124	Ruellia humilis	VNA ISOLATED
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VNA_07TGP00126_699	07TGP00126	Setaria parviflora	VNA ISOLATED
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VNA_07TGP00135_708	07TGP00135	Asclepias tuberosa	VNA ISOLATED
VNA_07TGP00136_709	07TGP00136	Ambrosia trifida	VNA ISOLATED
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VNA_07TGP00141_714	07TGP00141	Asplenium platyneuron	VNA ISOLATED
VNA_07TGP00142_715	07TGP00142	melica nitens	VNA ISOLATED
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VNA_07TGP00146_719	07TGP00146	Galium circaezans	VNA ISOLATED
VNA_07TGP00147_720	07TGP00147	Asclepias sullivantii	VNA ISOLATED
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VNA_07TGP00149_722	07TGP00149	Ruellia humilis	VNA ISOLATED
VNA_07TGP00150_723	07TGP00150	Ambrosia bidentata	VNA ISOLATED
VNA_07TGP00151_724	07TGP00151	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00152_725	07TGP00152	Ambrosia psilostachya	VNA ISOLATED
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VNA_07TGP00155_728	07TGP00155	Panicum virgatum	VNA ISOLATED
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VNA_07TGP00157_730	07TGP00157	Panicum anceps	VNA ISOLATED
VNA_07TGP00158_731	07TGP00158	Apocynum cannabinum	VNA ISOLATED
VNA_07TGP00159_732	07TGP00159	Achillea millefolium	VNA ISOLATED
VNA_07TGP00160_733	07TGP00160	Vernonia baldwinii	VNA ISOLATED
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VNA_07TGP00162_735	07TGP00162	Ambrosia psilostachya	VNA ISOLATED

VNA_07TGP00163_736	07TGP00163	Sorghastrum nutans	VNA ISOLATED
VNA_07TGP00164_737	07TGP00164	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00165_738	07TGP00165	Asclepias viridis	VNA ISOLATED
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VNA_07TGP00168_741	07TGP00168	Ophioglossum engelmannii	VNA ISOLATED
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VNA_07TGP00191_764	07TGP00191	Panicum virgatum	VNA ISOLATED
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VNA_07TGP00195_768	07TGP00195	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00196_769	07TGP00196	Ambrosia bidentata	VNA ISOLATED
VNA_07TGP00197_770	07TGP00197	Asclepias sullivantii	VNA ISOLATED
VNA_07TGP00198_771	07TGP00198	Setaria parviflora	VNA ISOLATED
VNA_07TGP00199_772	07TGP00199	Digitaria cognata	VNA ISOLATED
VNA_07TGP00200_773	07TGP00200	Diodia teres	VNA ISOLATED
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VNA_07TGP00202_775	07TGP00202	Panicum virgatum	VNA ISOLATED
VNA_07TGP00203_776	07TGP00203	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00204_777	07TGP00204	Sorghastrum nutans	VNA ISOLATED
VNA_07TGP00205_778	07TGP00205	Ambrosia psilostachya	VNA ISOLATED
VNA_07TGP00206_779	07TGP00206	Ruellia humilis	VNA ISOLATED

VNA_07TGP00207_780	07TGP00207	Asclepias viridis	VNA ISOLATED
VNA_07TGP00208_781	07TGP00208	Acalypha gracilens	VNA ISOLATED
VNA_07TGP00209_782	07TGP00209	Ambrosia bidentata	VNA ISOLATED
VNA_07TGP00210_783	07TGP00210	Panicum virgatum	VNA ISOLATED
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VNA_07TGP00212_785	07TGP00212	Sorghastrum nutans	VNA ISOLATED
VNA_07TGP00213_786	07TGP00213	Dichanthelium oligosanthes	VNA ISOLATED
VNA_07TGP00214_787	07TGP00214	Ambrosia psilostachya	VNA ISOLATED
VNA_07TGP00215_788	07TGP00215	Asclepias viridis	VNA ISOLATED
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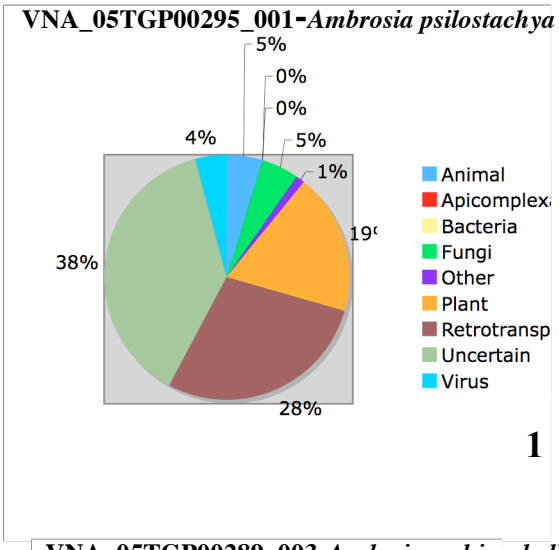
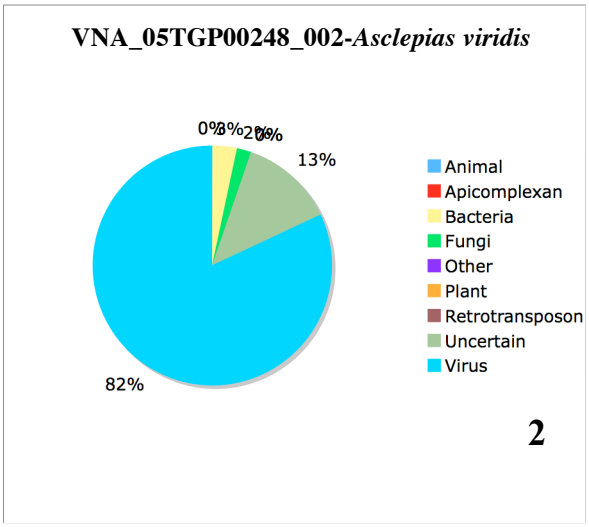
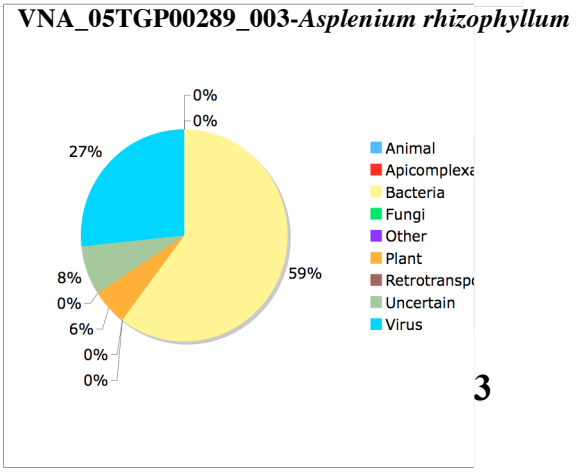
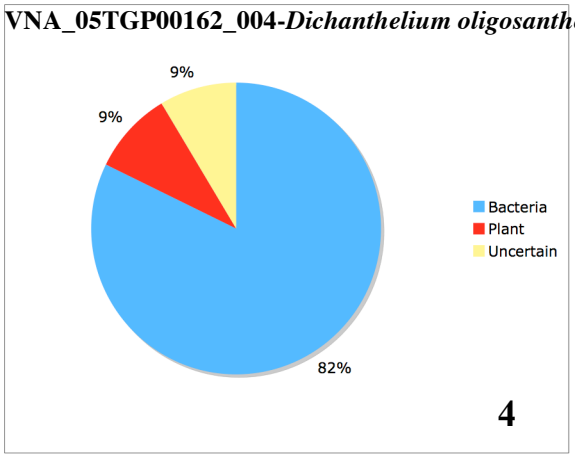
VNA_07TGP00217_790	07TGP00217	Glycyrrhiza lepidota	VNA ISOLATED
VNA_07TGP00218_791	07TGP00218	Panicum virgatum	VNA ISOLATED
VNA_07TGP00219_792	07TGP00219	Veronia baldwinii	VNA ISOLATED
VNA_07TGP00220_793	07TGP00220	Dichanthelium Oligosanthes	VNA ISOLATED
VNA_07TGP00221_794	07TGP00221	Asclepias viridis	VNA ISOLATED
VNA_07TGP00222_795	07TGP00222	Sorghastrum nutans	VNA ISOLATED
VNA_07TGP00223_796	07TGP00223	Ambrosia psilostachya	VNA ISOLATED
VNA_07TGP00224_797	07TGP00224	Ruellia humilis	VNA ISOLATED
VNA_07TGP00225_798	07TGP00225	Apocynum cannabinum	VNA ISOLATED
VNA_07TGP00226_799	07TGP00226	Asclepias sullivantii	VNA ISOLATED
VNA_07TGP00227_800	07TGP00227	Smilax bona-nox	VNA ISOLATED
VNA_07TGP00228_801	07TGP00228	Woodsia obtusa	VNA ISOLATED
VNA_07TGP00229_802	07TGP00229	Juniperus virginiana	VNA ISOLATED
VNA_07TGP00230_803	07TGP00230	Asclepias verticillata	VNA ISOLATED
VNA_07TGP00231_804	07TGP00231	Sabatia campestris	VNA ISOLATED
VNA_07TGP00232_805	07TGP00232	çephalanthus occidentalis	VNA ISOLATED
VNA_07TGP00233_806	07TGP00233	Dichanthelium linearifolium	VNA ISOLATED
VNA_07TGP00234_807	07TGP00234	Ambrosia psilostachya	VNA ISOLATED
VNA_07TGP00235_808	07TGP00235	Asclepias viridis	VNA ISOLATED
VNA_07TGP00236_809	07TGP00236	Diodia teres	VNA ISOLATED

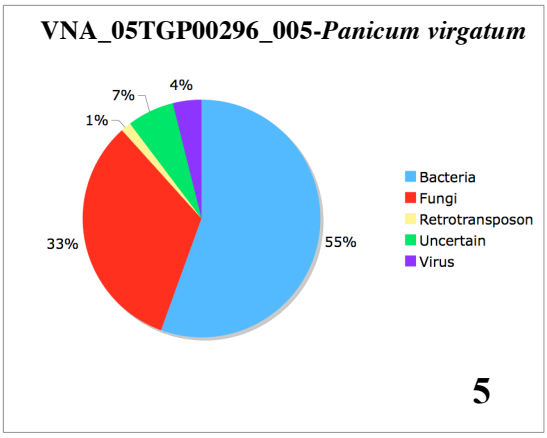
APPENDIX I
CLASSIFICATION OF READS

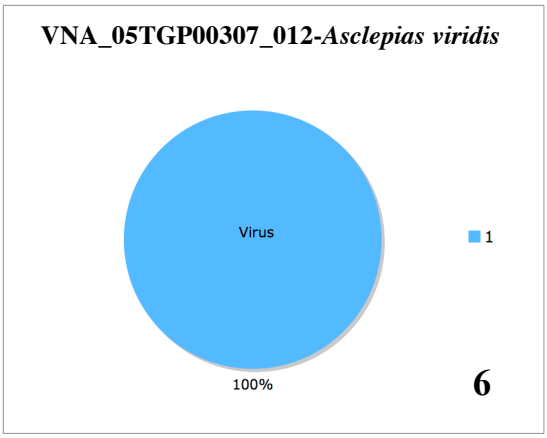




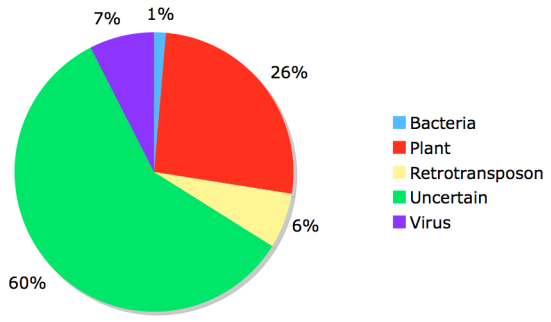






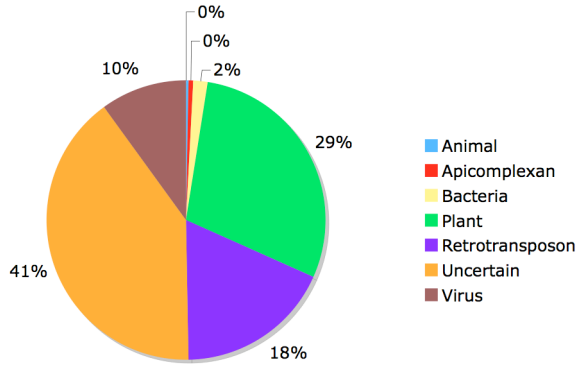


VNA_05TGP00312_013-*Ambrosia psilostachya*



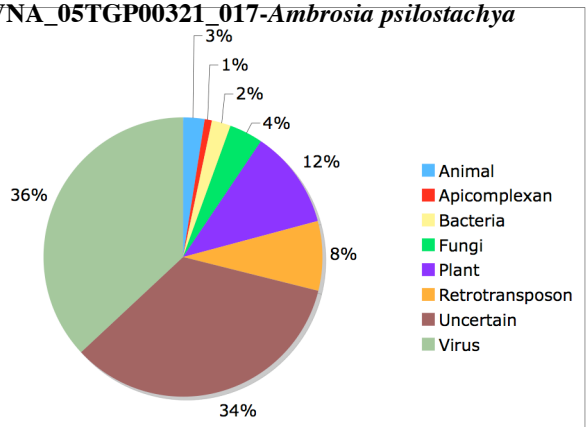
7

VNA_05TGP00316_015-*Panicum virgatum*



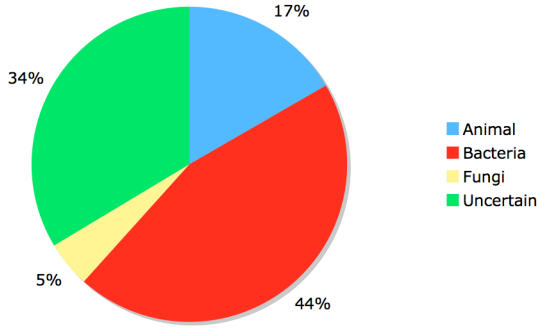
8

VNA_05TGP00321_017-*Ambrosia psilostachya*



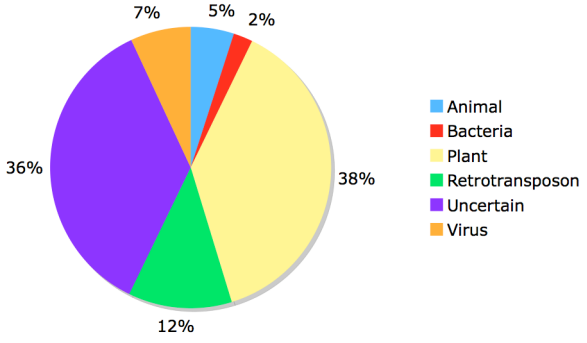
9

VNA_05TGP00323_018-*Asclepias viridis*



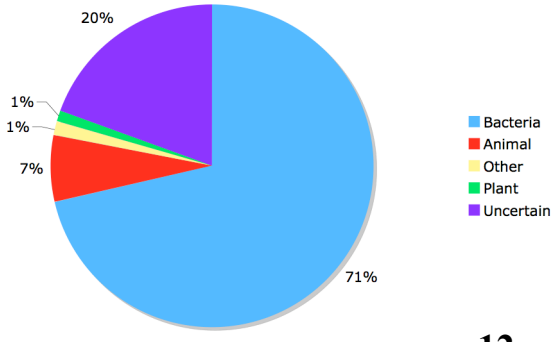
10

VNA_05TGP00332_020-*Sorghastrum nutans*



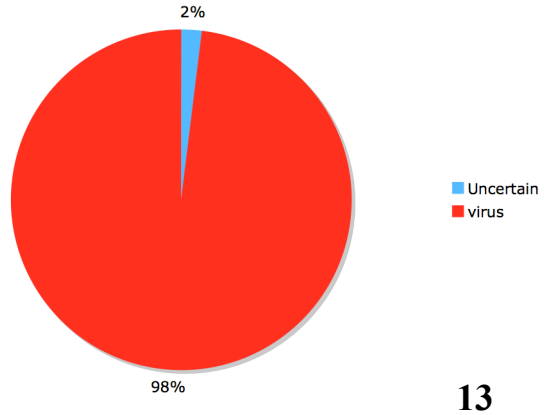
11

VNA_05TGP00335_021-*Ruellia humilis*



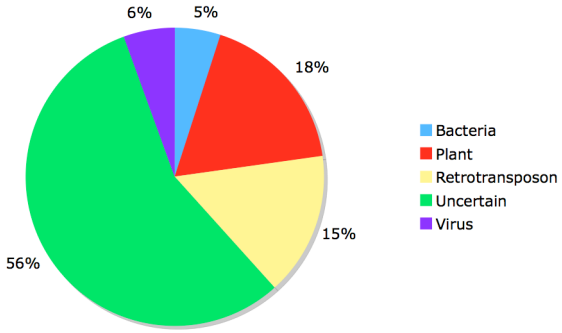
12

VNA_05TGP00337_022-*Asclepias viridis*



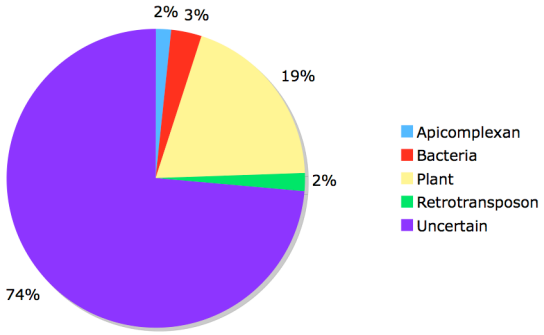
13

VNA_05TGP00339_023-*Panicum virgatum*



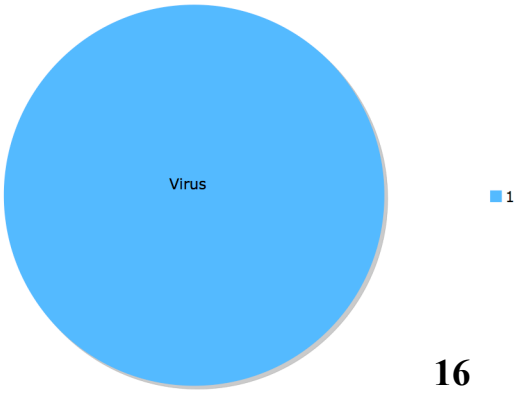
14

VNA_05TGP00342_024-*Coreopsis tinctoria*



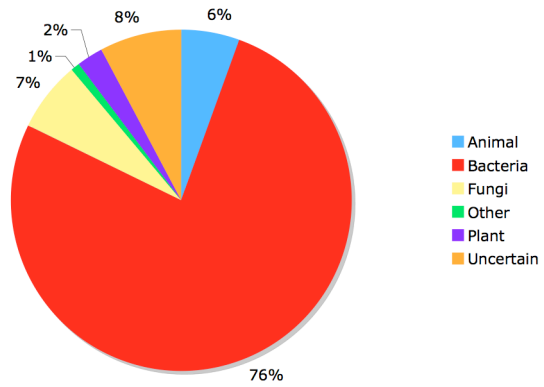
15

VNA_05TGP00351_027-*Asclepias viridis*



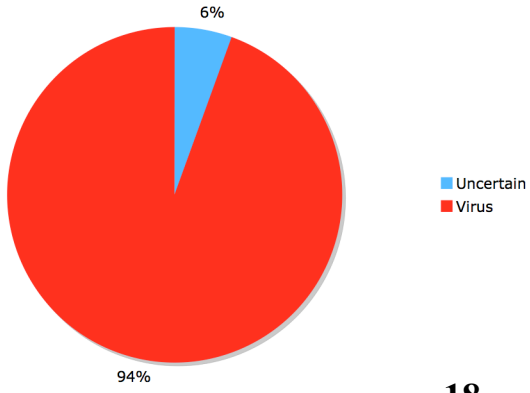
16

VNA_05TGP00192_035-*Sedum nuttallianum*

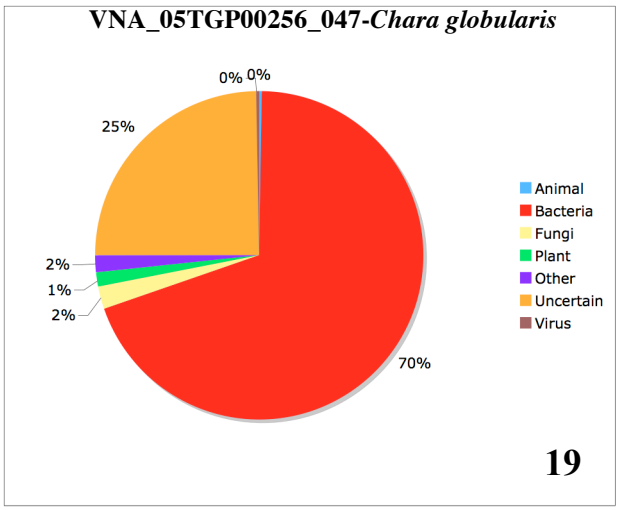


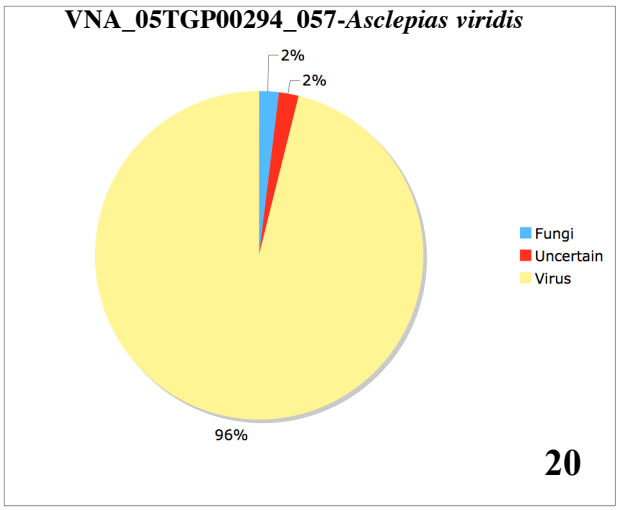
17

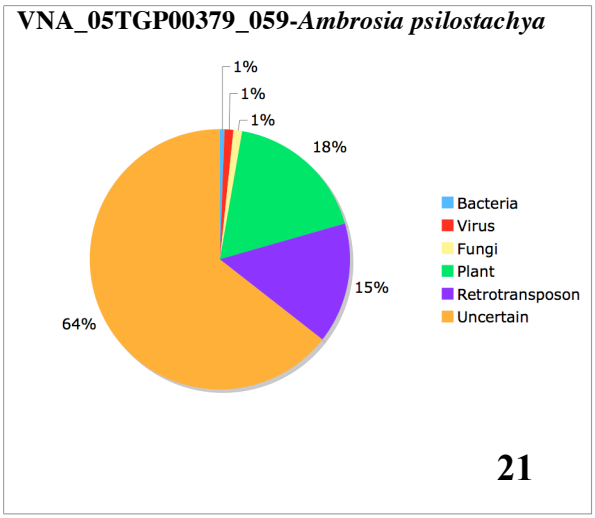
VNA_05TGP00248_045-*Asclepias viridis*

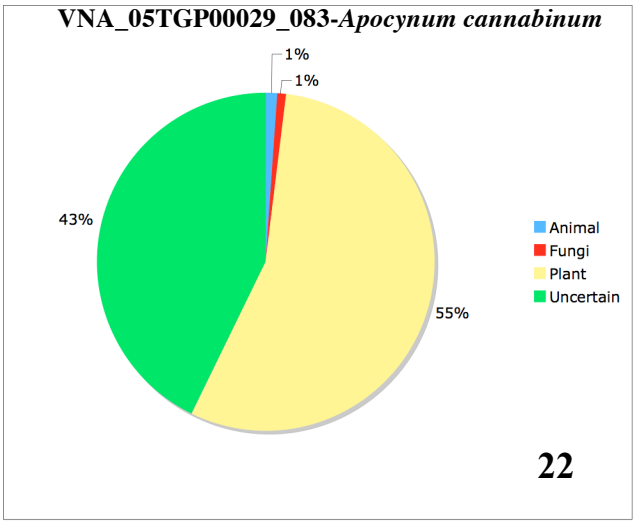


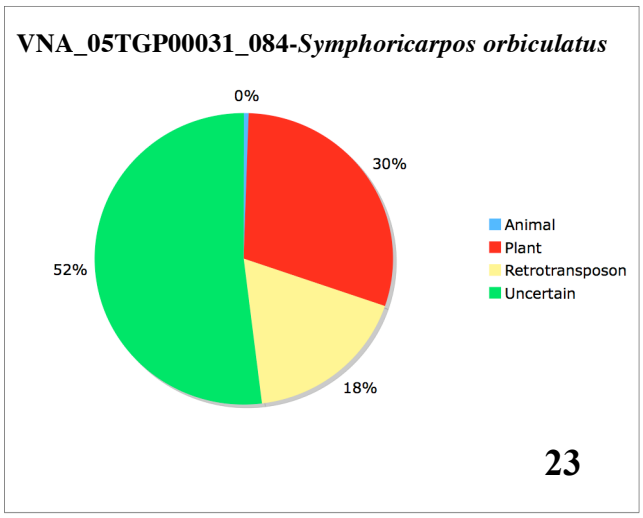
18

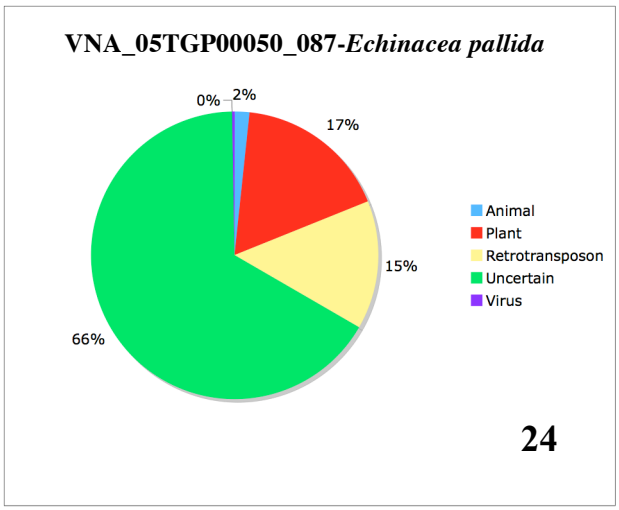

VNA_05TGP00256_047-Chara globularis
0%
0%
25%
2%
1%
2%
70%
Animal
Bacteria
Fungi
Plant
Other
Uncertain
Virus
19


VNA_05TGP00294_057-Asclepias viridis
2%
2%
96%
Fungi
Uncertain
Virus
20

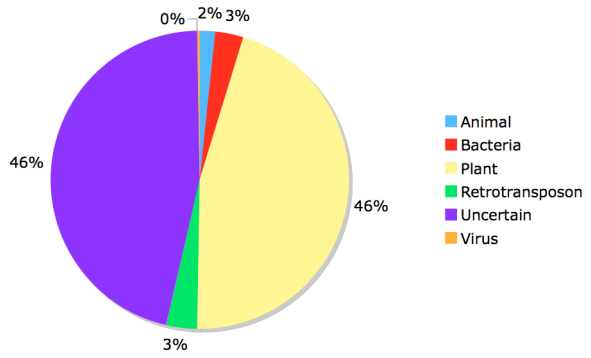

VNA_05TGP00379_059-Ambrosia psilostachya
1%
1%
1%
18%
15%
64%
Bacteria
Virus
Fungi
Plant
Retrotransposon
Uncertain
21


VNA_05TGP00029_083-Apocynum cannabinum
1%
1%
43%
55%
Animal
Fungi
Plant
Uncertain
22


VNA_05TGP00031_084-Symphoricarpos orbiculatus
0%
30%
52%
18%
Animal
Plant
Retrotransposon
Uncertain
23

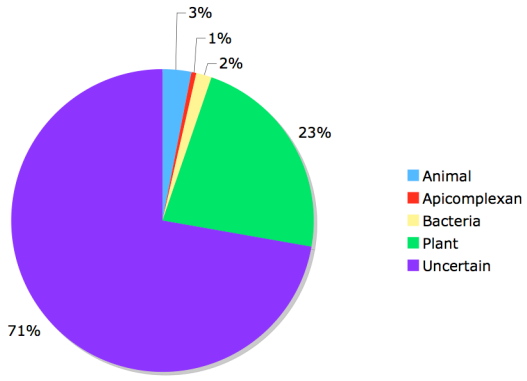

VNA_05TGP00050_087-Echinacea pallida
0%
2%
17%
15%
66%
Animal
Plant
Retrotransposon
Uncertain
Virus
24

VNA_05TGP00096_094-*Baptisia australis*



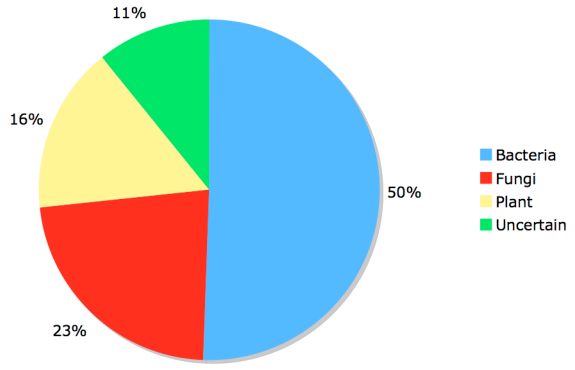
25

VNA_05TGP00112_096-*Carex vulpinoidea*



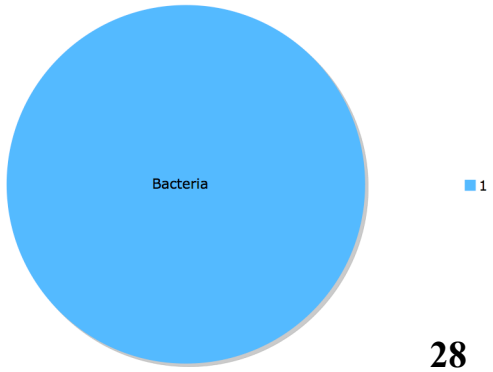
26

VNA_05TGP00453_204-*Diodia teres*



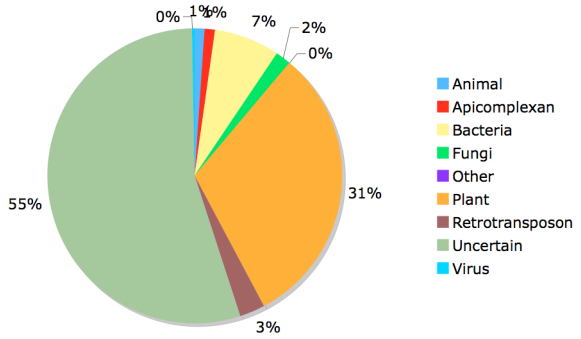
27

VNA_05TGP00464_205-Asclepias viridis



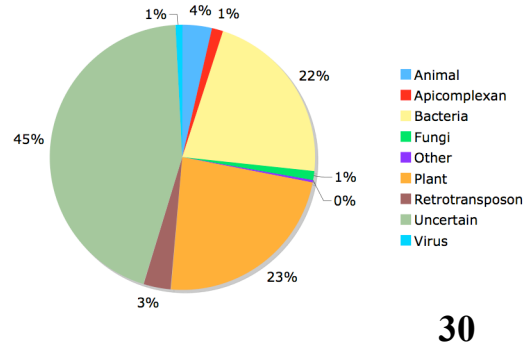
28

VNA_05TGP00576_208-*Asclepias verticillata*

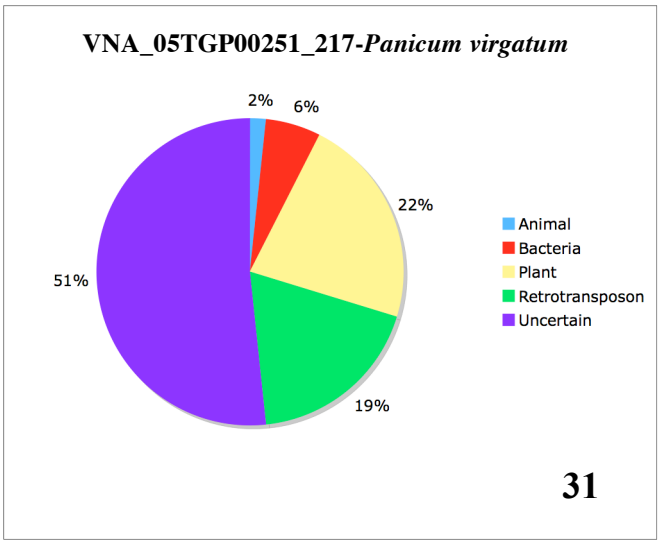


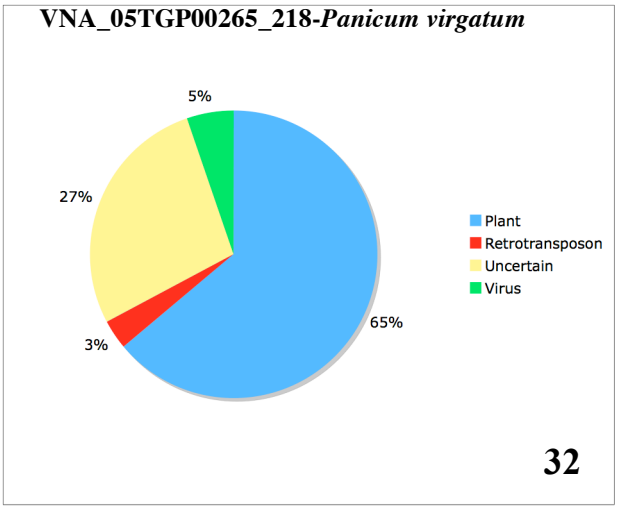
29

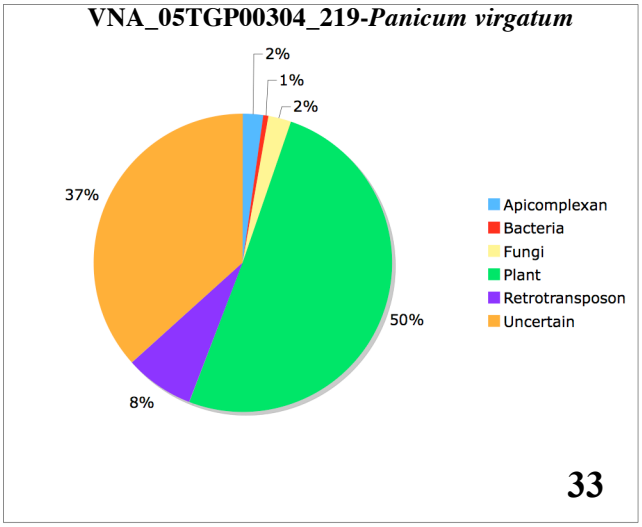
VNA_06TGP01051_209-*Asclepias viridis*

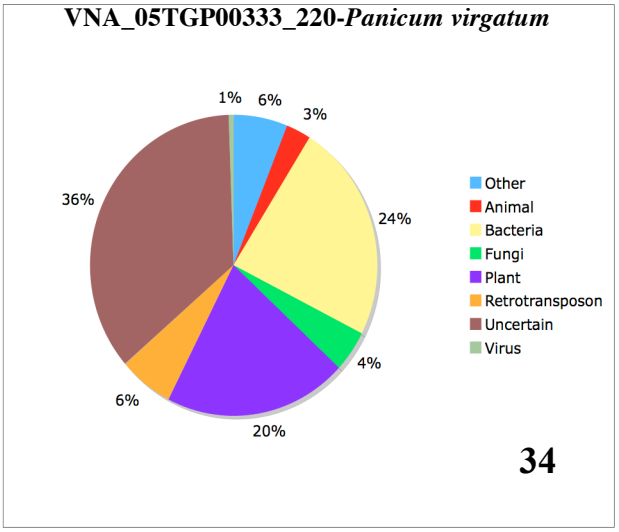


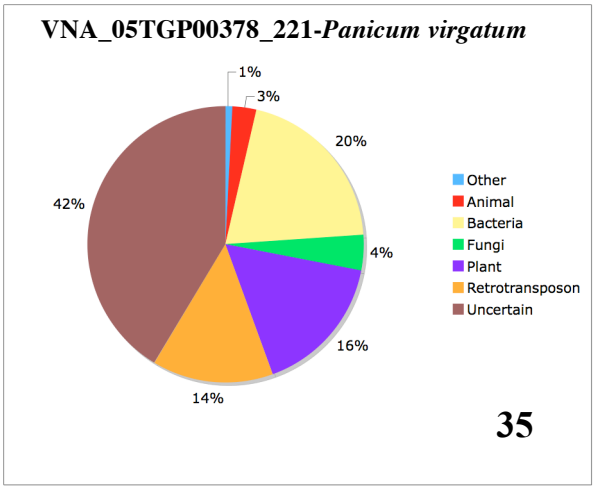
30

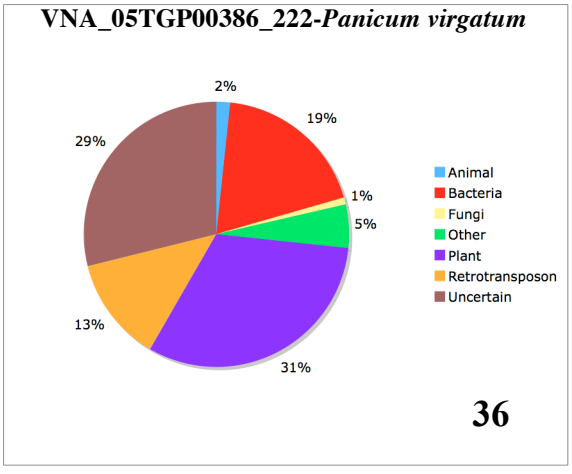

VNA_05TGP00251_217-*Panicum virgatum*
2%
6%
22%
51%
19%
Animal
Bacteria
Plant
Retrotransposon
Uncertain
31


VNA_05TGP00265_218-*Panicum virgatum*
5%
27%
3%
65%
Plant
Retrotransposon
Uncertain
Virus
32

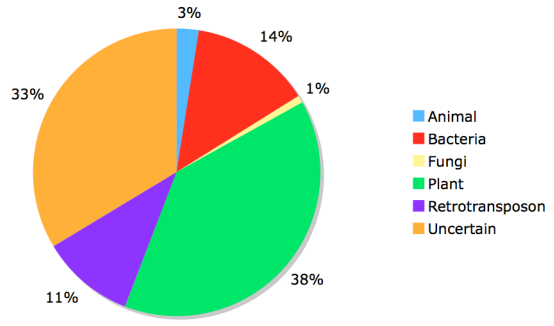

VNA_05TGP00304_219-*Panicum virgatum*
2%
1%
2%
37%
50%
8%
Apicomplexan
Bacteria
Fungi
Plant
Retrotransposon
Uncertain
33


VNA_05TGP00333_220-*Panicum virgatum*
1%
6%
3%
36%
24%
4%
6%
20%
Other
Animal
Bacteria
Fungi
Plant
Retrotransposon
Uncertain
Virus
34


VNA_05TGP00378_221-*Panicum virgatum*
1%
3%
20%
42%
4%
16%
14%
Other
Animal
Bacteria
Fungi
Plant
Retrotransposon
Uncertain
35

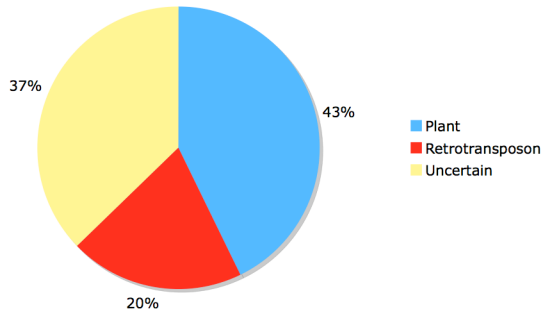

VNA_05TGP00386_222-*Panicum virgatum*
2%
19%
29%
1%
5%
13%
31%
Animal
Bacteria
Fungi
Other
Plant
Retrotransposon
Uncertain
36

VNA_05TGP00415_223-*Panicum virgatum*



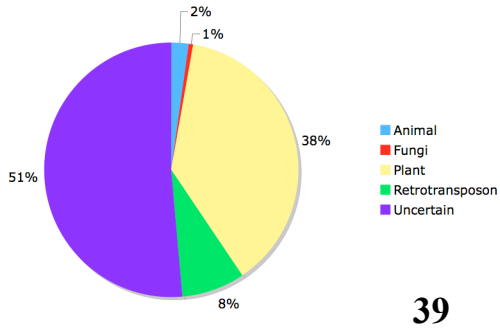
37

VNA_05TGP00425_224-*Panicum virgatum*



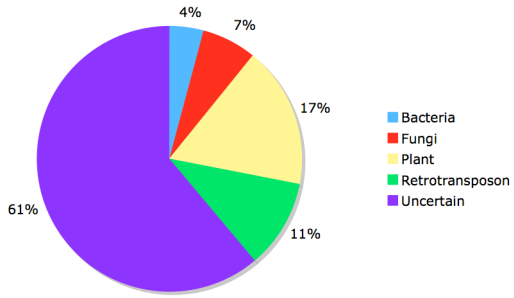
38

VNA_05TGP00444_225-*Panicum virgatum*



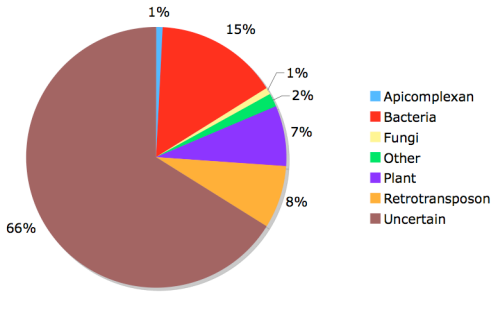
39

VNA_05TGP00555_226-*Panicum obtusum*



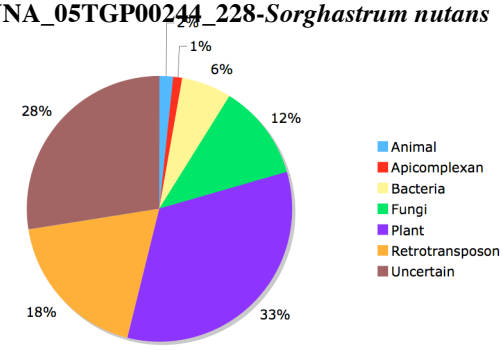
40

VNA_05TGP00619_227-*Panicum rigidulum*

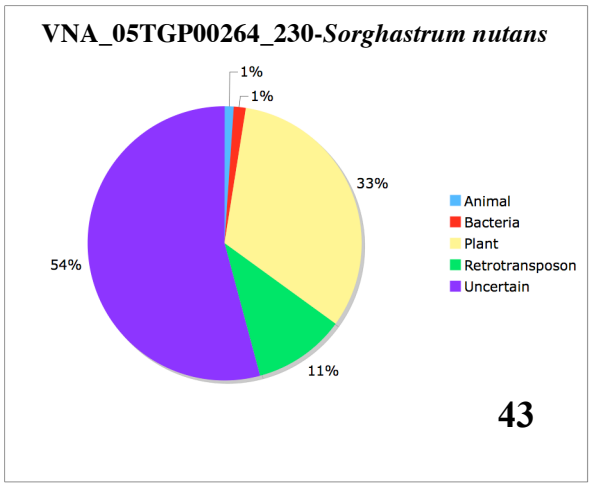


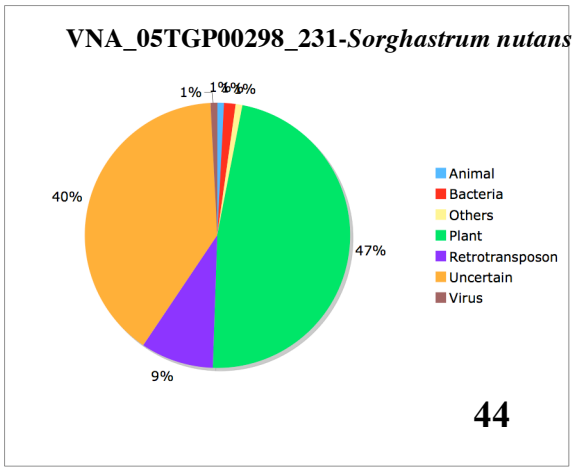
41

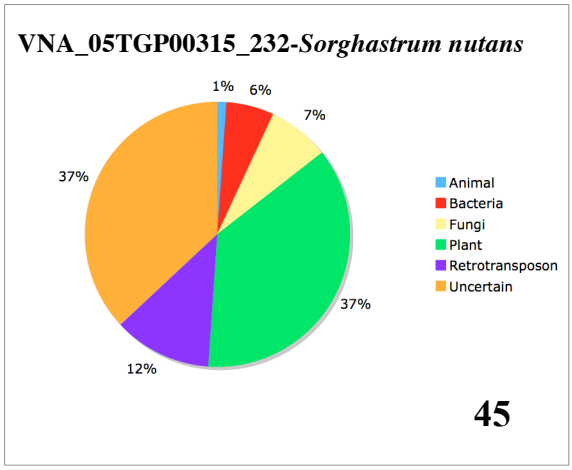
VNA_05TGP00244_228-*Sorghastrum nutans*

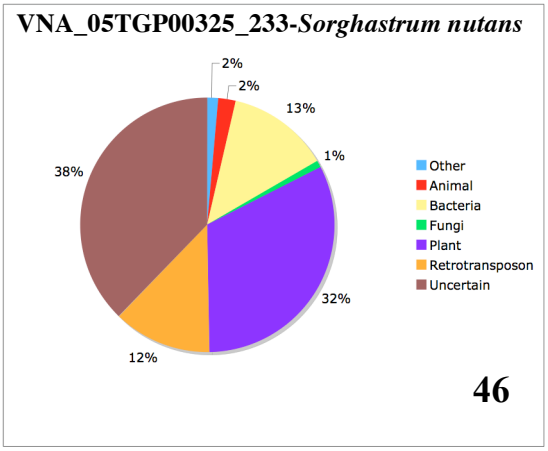


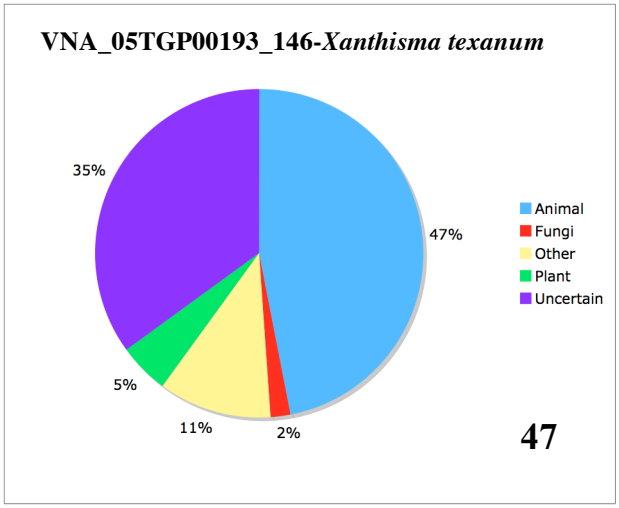
42

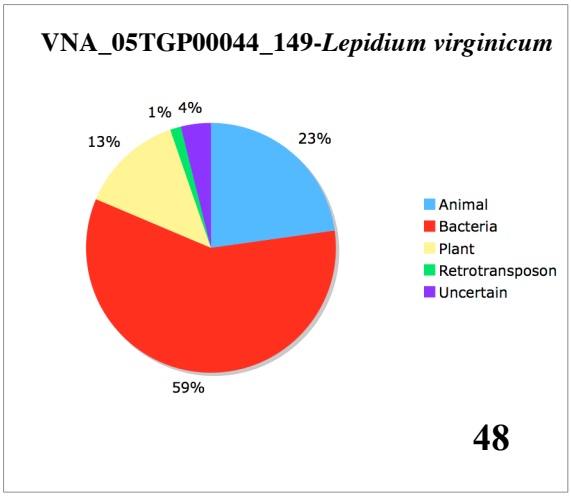

VNA_05TGP00264_230-Sorghastrum nutans
1%
1%
33%
54%
11%
Animal
Bacteria
Plant
Retrotransposon
Uncertain
43

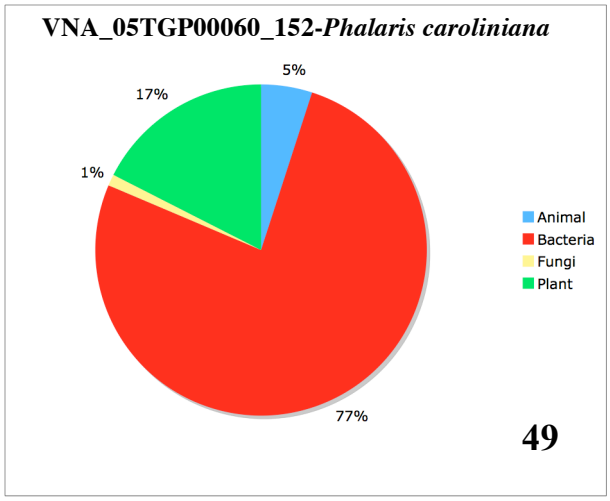

VNA_05TGP00298_231-Sorghastrum nutans
1%
40%
47%
9%
Animal
Bacteria
Others
Plant
Retrotransposon
Uncertain
Virus
44

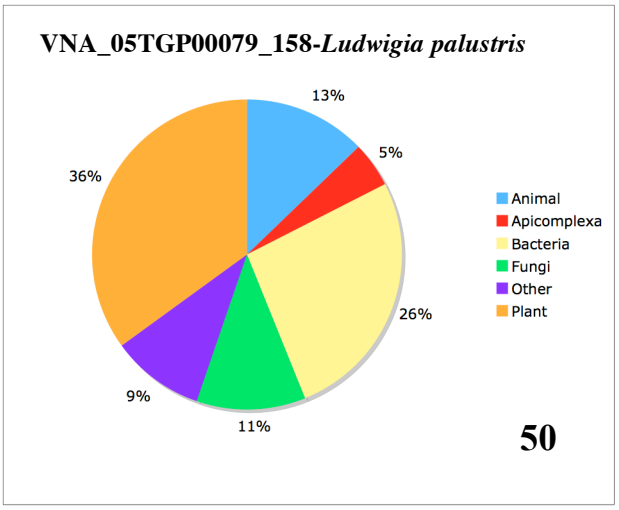

VNA_05TGP00315_232-Sorghastrum nutans
1%
6%
7%
37%
37%
12%
Animal
Bacteria
Fungi
Plant
Retrotransposon
Uncertain
45

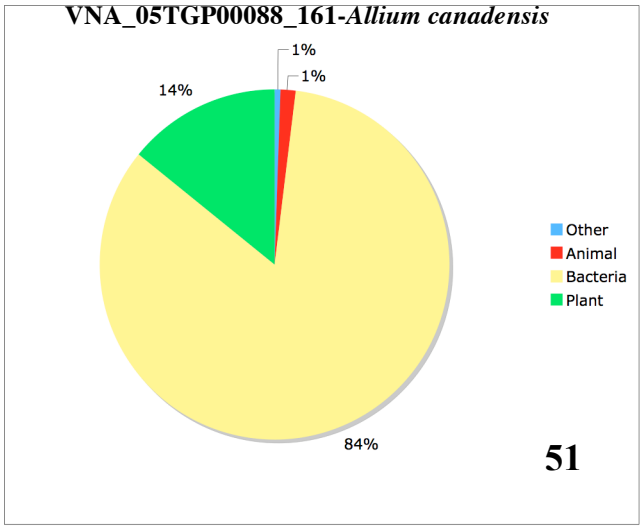

VNA_05TGP00325_233-Sorghastrum nutans
2%
2%
13%
1%
38%
32%
12%
Other
Animal
Bacteria
Fungi
Plant
Retrotransposon
Uncertain
46

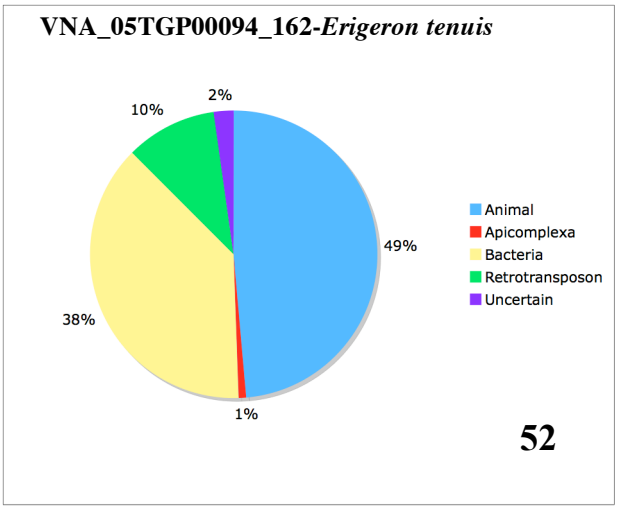

VNA_05TGP00193_146-Xanthisma texanum
35%
47%
5%
11%
2%
Animal
Fungi
Other
Plant
Uncertain
47

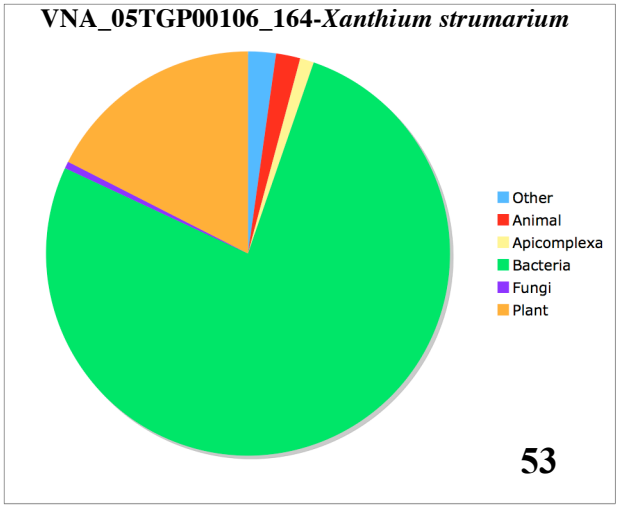

VNA_05TGP00044_149-Lepidium virginicum
1%
4%
13%
23%
59%
Animal
Bacteria
Plant
Retrotransposon
Uncertain
48

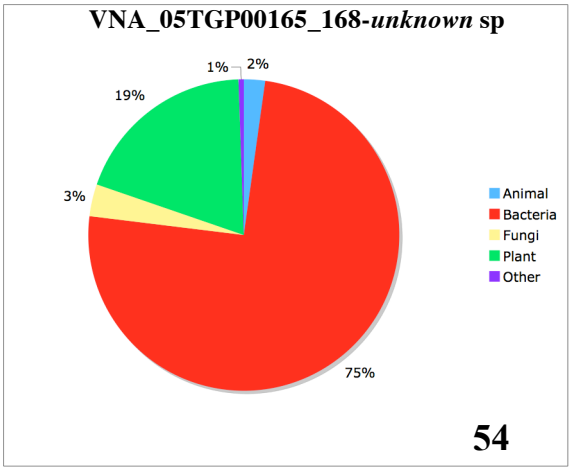

VNA_05TGP00060_152-Phalaris caroliniana
5%
17%
1%
77%
Animal
Bacteria
Fungi
Plant
49

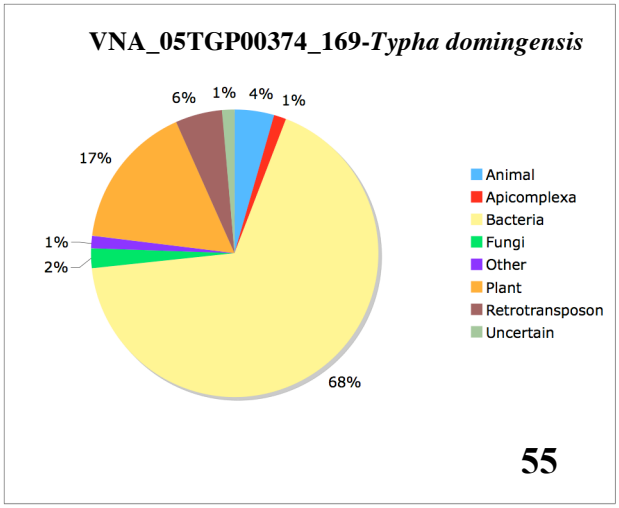

VNA_05TGP00079_158-Ludwigia palustris
13%
5%
36%
26%
9%
11%
Animal
Apicomplexa
Bacteria
Fungi
Other
Plant
50

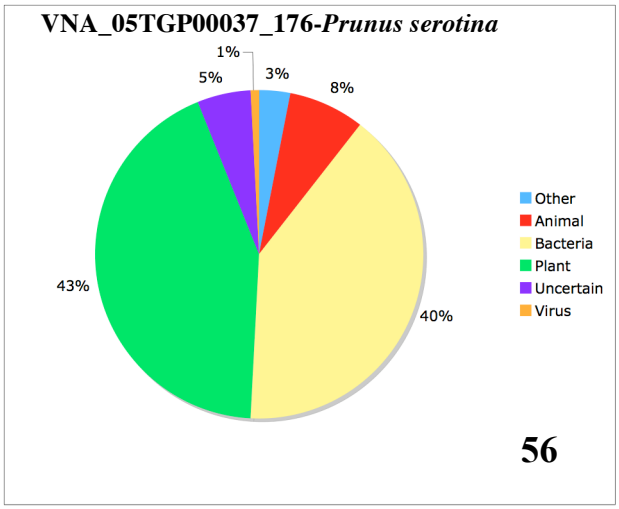

VNA_05TGP00088_161-Allium canadensis
1%
1%
14%
84%
Other
Animal
Bacteria
Plant
51

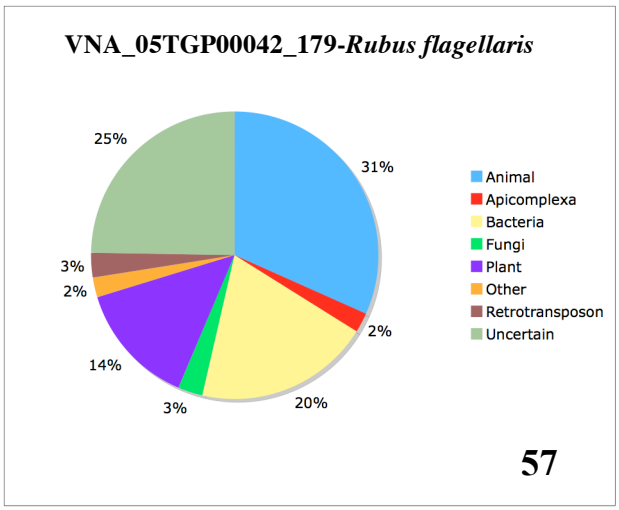

VNA_05TGP00094_162-Erigeron tenuis
2%
10%
49%
38%
1%
Animal
Apicomplexa
Bacteria
Retrotransposon
Uncertain
52

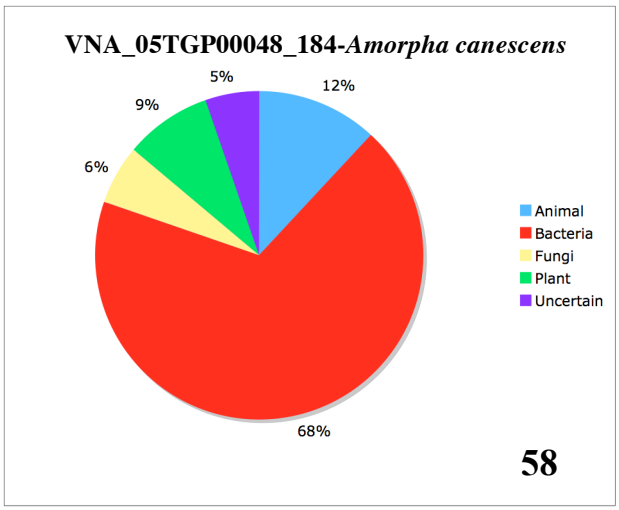

VNA_05TGP00106_164-Xanthium strumarium
Other
Animal
Apicomplexa
Bacteria
Fungi
Plant
53

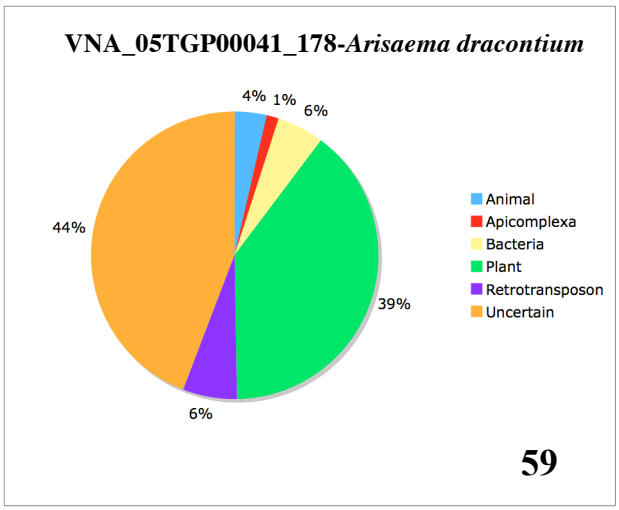

VNA_05TGP00165_168-unknown sp
1%
2%
19%
3%
75%
Animal
Bacteria
Fungi
Plant
Other
54

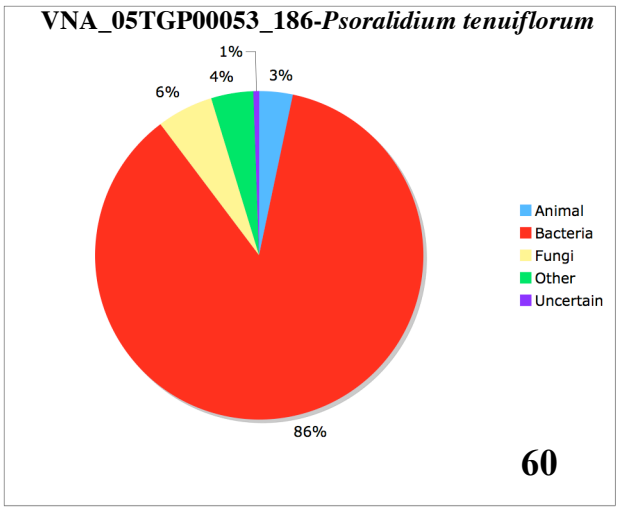

VNA_05TGP00374_169-Typha domingensis
6%
1%
4%
1%
17%
1%
2%
68%
Animal
Apicomplexa
Bacteria
Fungi
Other
Plant
Retrotransposon
Uncertain
55


VNA_05TGP00037_176-Prunus serotina
1%
5%
3%
8%
43%
40%
Other
Animal
Bacteria
Plant
Uncertain
Virus
56

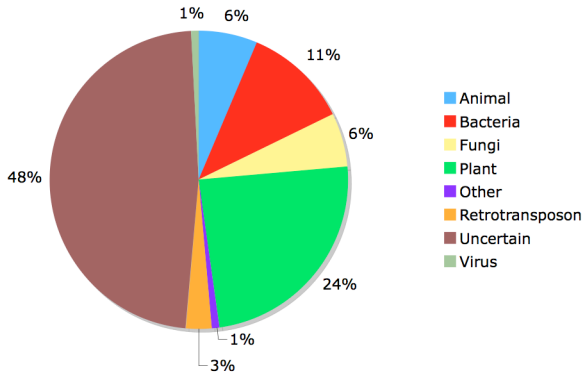

VNA_05TGP00042_179-Rubus flagellaris
25%
31%
3%
2%
2%
14%
3%
20%
Animal
Apicomplexa
Bacteria
Fungi
Plant
Other
Retrotransposon
Uncertain
57


VNA_05TGP00048_184-Amorpha canescens
5%
12%
9%
6%
68%
Animal
Bacteria
Fungi
Plant
Uncertain
58


VNA_05TGP00041_178-Arisaema dracontium
4%
1%
6%
44%
39%
6%
Animal
Apicomplexa
Bacteria
Plant
Retrotransposon
Uncertain
59

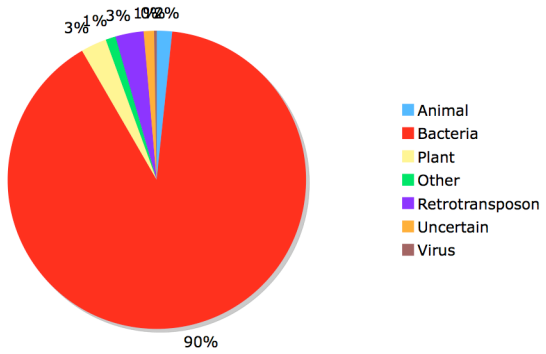

VNA_05TGP00053_186-Psoralidium tenuiflorum
1%
4%
3%
6%
86%
Animal
Bacteria
Fungi
Other
Uncertain
60

VNA_05TGP00182_145-*Festuca subverticillata*



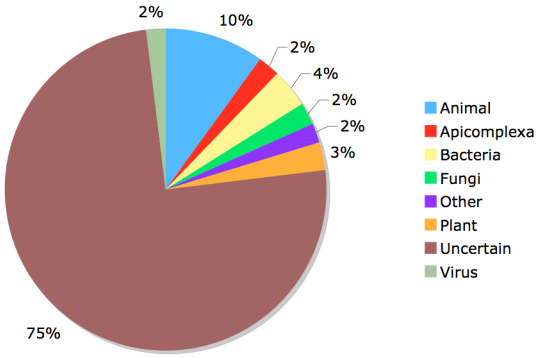
61

VNA_05TGP00117_165-*Maclura pomifera*



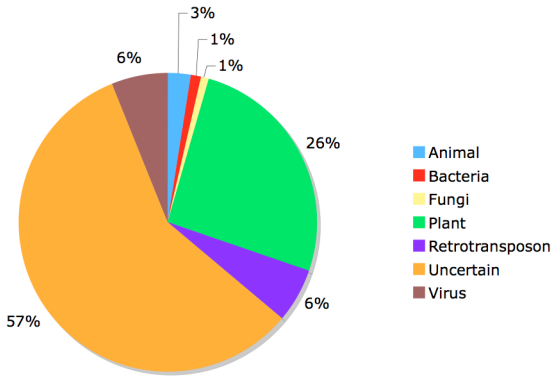
62

VNA_05TGP00052_185-*Artemisia ludoviciana*



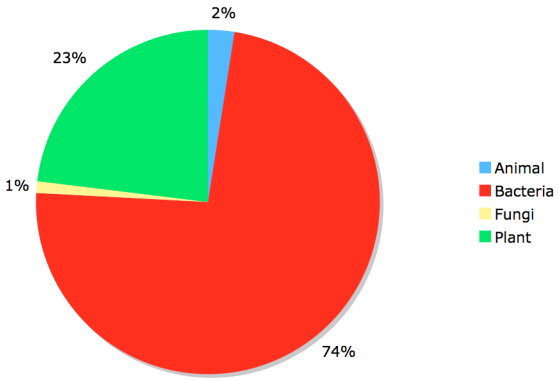
63

VNA_05TGP00062_190-*Desmanthus illinoensis*



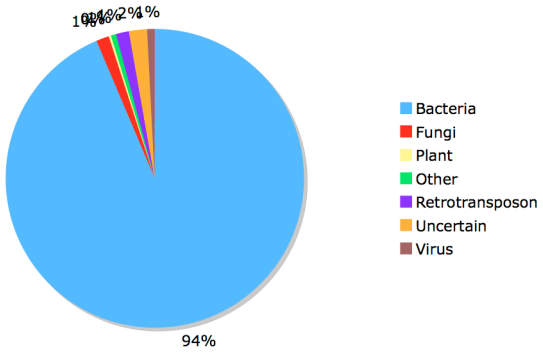
64

VNA_05TGP00040_148-*Vitis sp*

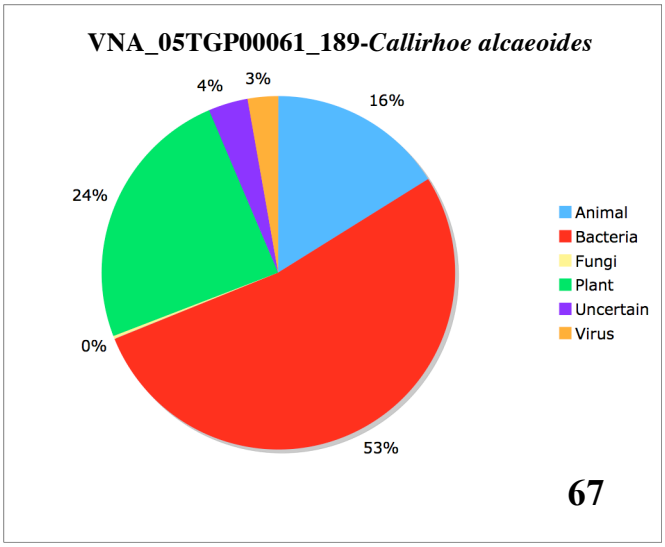


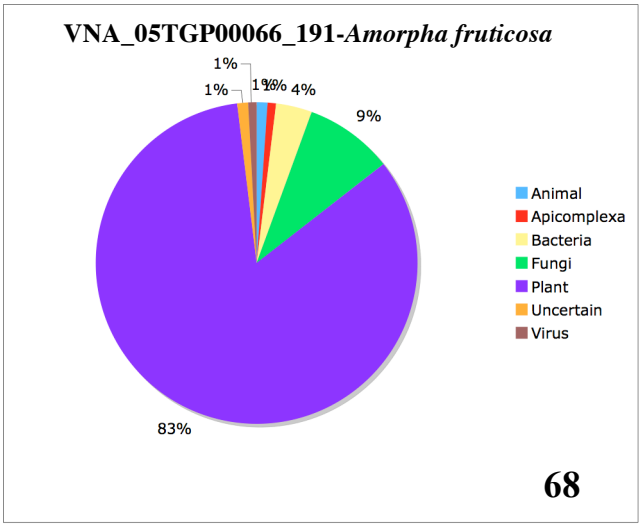
65

VNA_05TGP00100_163-*Ambrosia psilostachya*



66


VNA_05TGP00061_189-Callirhoe alcaeoides
4%
3%
16%
24%
0%
53%
Animal
Bacteria
Fungi
Plant
Uncertain
Virus
67


VNA_05TGP00066_191-Amorpha fruticosa
1%
1%
4%
9%
83%
Animal
Apicomplexa
Bacteria
Fungi
Plant
Uncertain
Virus
68

VITA

Vijay Muthukumar

Candidate for the Degree of

Master of Science

Thesis: METAGENOMICS FOR THE IDENTIFICATION OF PLANT VIRUSES IN THE TALLGRASS PRAIRIE PRESERVE.

Major Field: Biochemistry and Molecular biology.

Biographical:

Personal Data: Born in Chennai, India, June 18,1981, the son of Sakuntala Muthukumar and T.J. Muthukumar.

Education:

Bachelor of Science in Microbiology
May 2001 Pune University, Pune, India

Master of Science in Microbiology
May 2003 Pune University, Pune, India

Master of Science in Biochemistry and Molecular biology
*Expected May 2008***,** Oklahoma State University, Stillwater, Oklahoma

Professional Memberships:

American Phytopathological Society	*May 2007- June 2008*
Oklahoma Academy of Sciences	*December 2005- December 2006*
Advancing Science Serving Society	*November 2007- November 2008*
Biochemistry and Molecular Biology Graduate Student Association (Vice President)	*2006-2007*

Name: Vijay Muthukumar Date of Degree: May 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: METAGENOMICS FOR THE IDENTIFICATION OF PLANT VIRUSES IN THE TALLGRASS PRAIRIE PRESERVE.

Pages in Study: 147 Candidate for the Degree of Master of Science

Major Field: Biochemistry and Molecular biology.

Scope and Method of Study:

The number of virus species identified by the ICTV is likely to be far less than the actual number of viruses. This study was undertaken to develop a metagenomic technique to discover viruses from plants. Aliquots of plant material were subjected to homogenization and differential centrifugation to isolate virus-like particles. The pellet obtained was treated successively with DNase-I to remove contaminating plant DNA and proteinase-K, SDS to digest the DNase-I and capsid proteins. The viral nucleic acid was amplified by random PCR, cloned and sequenced. The sequences obtained were assembled and characterized by comparison with known viral sequences.

Findings and Conclusion:

Sequences were obtained from 68-plant specimens. Analysis of the sequences suggested the presence of at least 9 different viruses. Sixteen plants contained virus-like sequences and six double infections were found. Plant specimens yielding virus sequences showed no obvious symptoms of infection. Evidence of bacteria and fungi was also found in several samples.

ADVISER'S APPROVAL: Dr. Ulrich Melcher