DIVERSITY PATTERNS OF 16S rDNA OF BACTERIA AND *amoA* OF ARCHAEA AND BACTERIA FROM EASTERN HEMLOCK (*TSUGA CANADENSIS*) RHIZOSPHERE SOIL

By

Carter Thomas Dillow

A Thesis
Submitted to the
Faculty of the Graduate School
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of
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Committee:

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GLOSSARY

amoA – Ammonia Monooxygenase Subunit A

AOA – Ammonia Oxidizing Archaea

AOB – Ammonia Oxidizing Bacteria

GSMNP – Great Smoky Mountain National Park

HWA – Hemlock Woolly Adelgid

rDNA – Ribosomal Deoxyribonucleic Acid

T-RFLP – Terminal Restriction Fragment Length Polymorphism

PCA – Principal Components Analysis

RFU – Relative Fluorescent Unit

ABSTRACT

DIVERSITY PATTERNS OF 16S rDNA OF BACTERIA AND *amoA* OF ARCHAEA AND BACTERIA FROM EASTERN HEMLOCK (*TSUGA CANADENSIS*) RHIZOSPHERE SOIL

Carter T. Dillow, M.S.

Western Carolina University (December 2009)

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Traditionally microbial communities were assessed through culturing, which limits the populations detected. Molecular techniques are becoming more popular when assessing microbial communities. To illustrate gene variability between sites terminal restriction fragment length polymorphism (T-RFLP) was used in this study.

Bacterial 16S rDNA and archaeal and bacterial *amoA* diversity was examined from Albright Grove, Cataloochee, and Purchase Knob in Great Smoky Mountains National Park (GSMNP) using T-RFLP. Bacterial 16S rDNA T-RFLP electoferrogram peak data revealed that Cataloochee and Purchase Knob had a greater diversity compared to Albright Grove, with 60 different peaks and 17 unique peaks at each site, and 60 different peaks and 18 unique peaks, respectively, while Albright Grove had a total of 45 different peaks and 9 unique peaks. A comparison showed 28.6% of major peaks were shared between all three. Archaeal *amoA* T-RFLP electoferrogram peak data revealed that Cataloochee and Purchase Knob had a greater diversity compared to Albright Grove, with 105 different peaks and 30 unique peaks, and 103 different peaks and 22 unique peaks at each site, respectively, while Albright Grove had a total of 82 different peaks and 13 unique peaks at the site. No major peaks were shared between all three sites.

PCR products for bacterial *amoA* were only produced from Purchase Knob and generated a total of 10 peaks.

Overall diversity appeared to be higher in the disturbed sites of Cataloochee and Purchase Knob compared to the undisturbed site of Albright Grove. Since, the rhizosphere is a complex system a number of other variables could impact the structure of the microbial community, and must be taken into consideration when examining diversity. This study has provided insight into the spatial variability of the microbial community in the Eastern Hemlock rhizosphere, and further examination may help mediate the devastating loss of the hemlock due to an exotic adelgid species.

INTRODUCTION

History of Microbial Ecology

Since as early as Aristotle there has been interest in the diversity of organisms. At first everything was classified as a plant or animal. With advancement in observational skills, such as the development of the microscope, and the development of Carolus Linnaeus' classification system, organisms were divided by phenotypic characteristics into five kingdoms, monera, protista, fungi, plantae, and animalia (four eukaryotic groups and one prokaryotic group) (Starr & Taggart 2006). When developments in molecular biology allowed for DNA sequencing of an organism's genes, the old classification system of five kingdoms changed. Organisms can now be compared and classified through their genetics. Comparison of similar genes among organisms allows for a common yardstick in determining divergence (Avise 2004). Carl Woese utilized the new molecular techniques, specifically the sequencing of rRNA, and proposed a new tree of life consisting of three domains, *Bacteria, Archaea,* and *Eukarya* (Figure 1) (Madigan et al. 2009).

The new tree of life was far different from the five kingdom approach, which divided life into one prokaryotic and four eukaryotic groups, the new tree was divided into three groups or domains, two prokaryotic (*Bacteria* and *Archaea*) and one eukaryotic (*Eukarya*). According to the tree, *Bacteria* and *Archaea* are more ancestral groups to *Eukarya* and are located closer to the root of the tree (Figure 1). Originally *Archaea* were thought to be the most ancient, but an analysis of ribosomal genes along with other genes showed that *Archaea* are more closely related to *Eukarya* than *Bacteria* (Brown and

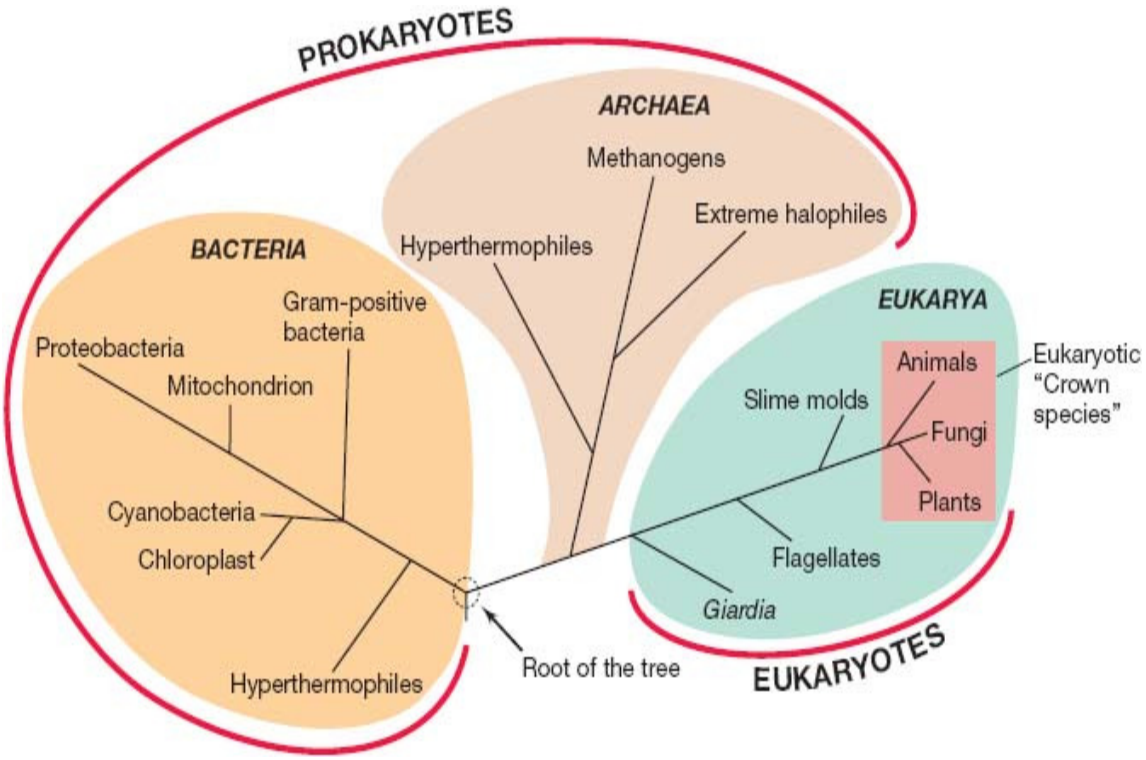

Figure 1. Universal phylogenetic tree built on 16S and 18S rDNA sequences. The scale Figure acquired from Madigan et al. 2009.

Doolittle 1997). Carl Woese's three domain approach is now considered the most inclusive tree of life (Figure 1) (Pace 1997).

Much debate has ensued over the best molecular marker to use when establishing evolutionary relationships (Avise 2004). A good molecular marker, such as 16S rRNA, must be common amongst all organisms, have similar functionality, be evolutionarily conserved, and have adequate length for comparison between organisms (Madigan et al. 2009, Woese 1987). In the 1970's, Carl Woese established the use of the 16S ribosomal RNA gene region as the best possible molecular marker for prokaryotic phylogenetics (Woese 1987). The 16S rRNA is an approximate 1,500 base pair subunit of the 30S ribosomal unit of prokaryotes (Brosius et al. 1978). Woese chose 16S rRNA because of its unique characteristics of high conservation with slight variability, small likelihood of lateral gene transfer, and presence in all prokaryotes (Delong and Pace 2001). The use of 16S rRNA as a molecular marker has been reaffirmed through a variety of other studies using markers such as 23S rRNA, ATPase subunits, elongation factors, and RNA polymerase genes (McArthur 2006). Current phylogenies using 16S rRNA genes have established 53 bacteria phyla (Figure 2) and two archaea phyla, *Crenarcheota* and *Euryarchaeota* (Figure 3) with three proposed phyla, *Thaumarchaeota*, *Nanoarchaeota*, and *Korarchaeota* (Not in Figure 3) (Brochier et al. 2005; Brochier-Armanet et al. 2008; Madigan et al. 2009; Pace 1997).

Microorganisms are present, abundant, and play important roles in all environments. A large portion of the microbial community occurs in soil, where in one gram of soil there can be 10^9 bacterial cells and in 10 grams of soil there can be 10^7 bacterial species (Gans et al. 2005). The dynamics of soil allow for a large surface area to

volume ratio leading to an immense number of microorganisms. Soil is heterogeneous and porous with a multitude of nutrients and microenvironments allowing for an infinite number of hypothetical niches (Garbeva et al. 2004). Soil microbes have been shown to be key components in maintaining soil function by forming plant root symbioses, toxin removal, and nutrient cycling of carbon, nitrogen, phosphorus, iron, and sulfur (Madigan et al. 2009; van Elsas and Trevors 1997). Bacteria, archaea, and fungi all play important roles in helping plants obtain essential compounds. One of the best examples of plant-microbe association is the nitrogen cycle, most importantly nitrogen fixation. Archaea and bacteria are also important in the nitrogen cycle through ammonia-oxidization and nitrite-oxidation (Leininger et al. 2006; Madigan et al. 2009). In recent studies the archaea ammonia-oxidizing gene has been shown to be abundant in soils, and has introduced the idea that archaea could play a more important role than previously thought (Adair and Schwartz 2008; Leininger et al. 2006).

Ammonia Oxidization

Functional gene markers, such as the ammonia oxidizing gene, play an important role in establishing phylogenetic relationships, and are important when examining genetic or metabolic diversity of a microbial environment. Both ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) contribute to the cycling of environmental nitrogen. The ammonia oxidizing gene in bacteria is composed of three subunits (A, B, C), which code for an enzyme, ammonia monooxygenase, that catalyses the conversion of ammonia to hydroxylamine (Treusch et al. 2005). The *amoA* subunit is an

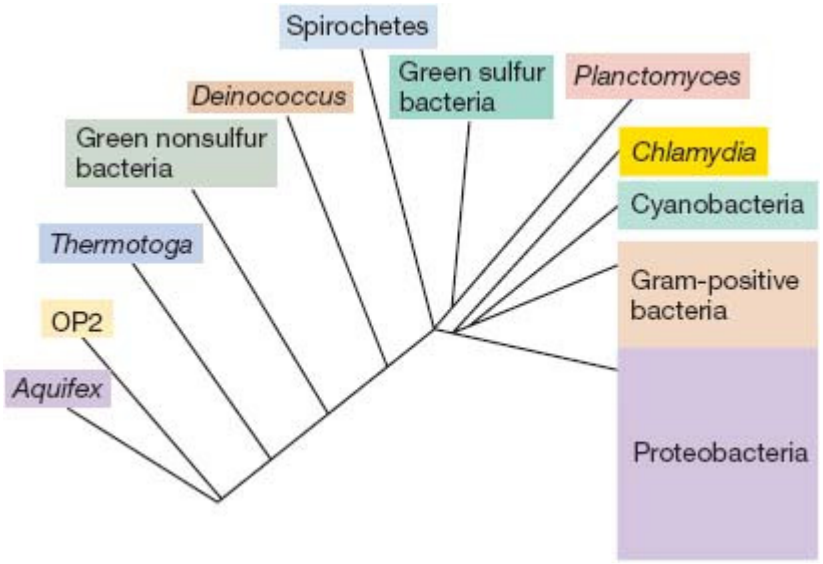

Figure 2. Phylogenetic tree of Bacteria based on 16S rDNA. The relative sizes of the colored boxes reflect the number of known genera and species in each of the groups. Figure was taken from Madigan et al. 2009.

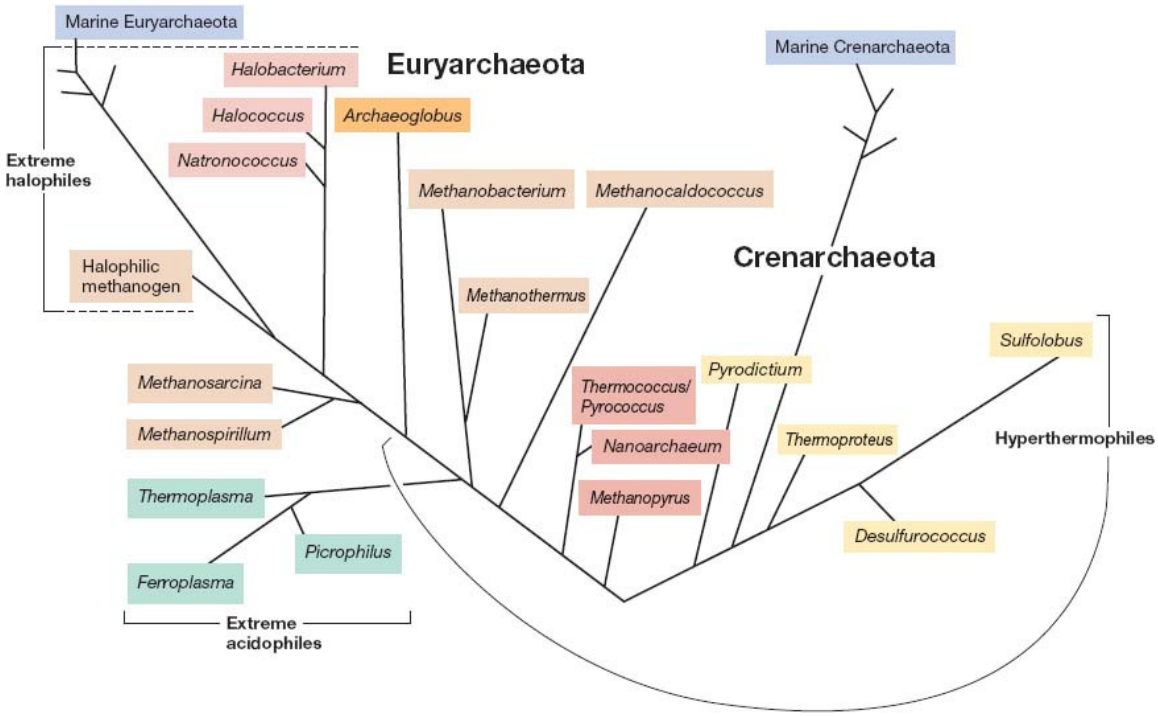

Figure 3. Phylogenetic tree of Archaea based on 16S rDNA showing the two accepted phyla (*Crenarchaeota* and *Euryarchaeota*) and excluding the three proposed phyla. Figure acquired from Madigan et al. 2009.

approximately 830 bp region, and because of the diversity in the *amoA* subunit it provides increased resolution in conjunction with 16S rRNA genes in studies examining genetic differences (McTavish et al. 1993; Rotthauwe et al. 1997; Treusch et al. 2005). The sequences of archaeal *amoA* and *amoB* genes show low but sufficient differences compared to bacterial genes to distinguish them from each other. An archaeal subunit C has recently been linked to *amoB* sequences meaning archaea are likely to have a full complement of genes that code for ammonia monooxygenase (Treusch et al. 2005).

Nitrogen compounds can enter the soil in many different forms such as urea from animal waste, decomposing organic material, atmospheric nitrogen, and fertilizer. Urea can be broken down into ammonia by bacterial enzymes called ureases, while symbiotic nitrogen fixing bacteria and archaea can fix atmospheric nitrogen into ammonia. Ammonia can be used by plants or converted by prokaryotes through the process of ammonia-oxidation into nitrite, and then nitrate, which plants can assimilate into amino acids (Madigan et al. 2009). Ammonia-oxidation is usually the rate limiting factor in nitrification and is centered around an electron transport system in the cytoplasmic membrane (Salyers and Whitt 2001). Ammonia is first converted to an intermediate hydroxylamine, which then diffuses across the cytoplasmic membrane, where it is converted into nitrous acid. The electrons released from the production of nitrous acid travel down an electron transport chain where the final electron acceptor is oxygen (Salyers and Whitt 2001). The nitrous acid when dissolved in solution produces hydrogen ions and nitrite. The nitrite can be further oxidized in a similar reaction as previously described resulting in nitrate (Salyers and Whitt 2001).

The diversity of soil communities can be impacted by the amount of available organics (Lynch and Whipps 1990; Wardle 1992). The level and chemical species of organics in soil can be altered by the types of plant life present thus changing the diversity of microbes present (Grayston et al. 1997). Many of the microbes present in the soil are associated with the rhizosphere, which is the area around the root system influenced by root exudates such as sugars, lipids, amino acids, hormones, and vitamins (Mukerji et al. 2006; Madigan et al. 2009). The microbes metabolize organics released from the plant roots, while providing the plant with simple compounds such as NH_3 (Mukerji et al. 2006). It has also been shown that microbes assist in controlling soilborne plant diseases and promoting plant growth (Doran et al. 1996).

Eastern Hemlock

Eastern Hemlock (*Tsuga canadensis*) has been a major component of eastern forest systems, but due to harvesting practices and hemlock woolly adelgid infestations its prominence has dropped (McClure et al. 2001). Hemlock stands provide food, cover, nesting and shelter for many species including grouse, turkey, deer, rabbit, and nearly 90 bird species (McClure et al. 2001). The Eastern Hemlock is a dominant tree species in Great Smoky Mountains National Park (GSMNP) where it thrives because of its ability to grow in the shade and in broad topographic gradients (GSMNP 2006). Over the past 25 years the hemlock woolly adelgid (HWA) has migrated into Western North Carolina attacking many hemlock stands and wiping out up to 80 percent of the trees in these areas (GSMNP 2006).

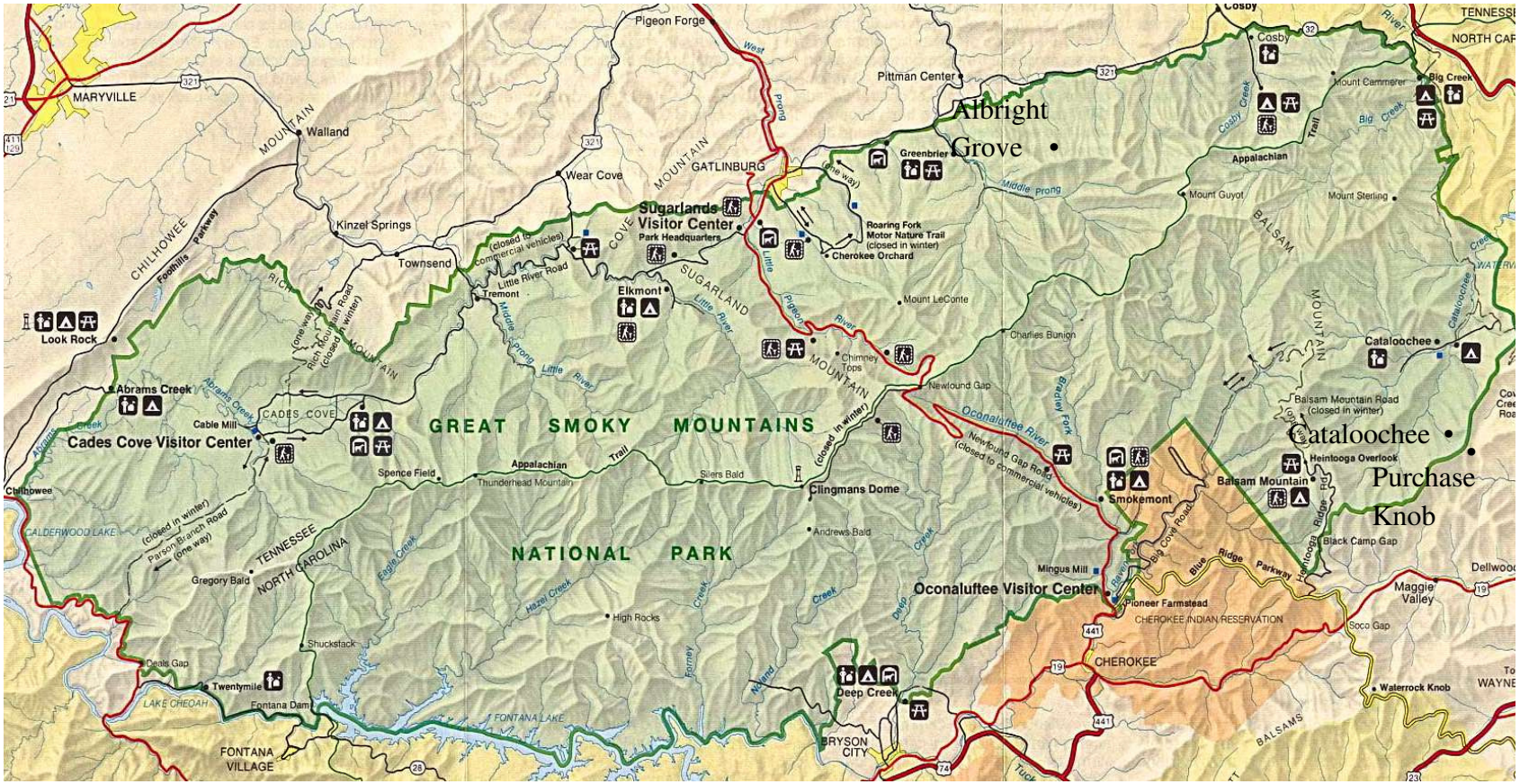

Figure 4. Map of Great Smoky Mountains National Park (GSMNP) showing the location of sampling sites Albright Grove, Cataloochee, and Purchase Knob. Albright Grove is an old growth forest located on the on the Tennessee side of the park. Cataloochee and Purchase Knob are disturbed sites from Chestnut blight and logging, respectively, in North Carolina.

The hemlock woolly adelgid is an aphid that was introduced from Asia, and was first observed in western North America in the 1920's and had moved east by the 1950's (McClure et al. 2001). In North America HWA can occur in four different tree species including Mountain Hemlock (*Tsuga mertensiana*), Western Hemlock (*Tsuga heterophylla*), Eastern Hemlock, and Carolina Hemlock (*Tsuga caroliniana*). Mountain and Western Hemlock species are resistant to HWA, while in Eastern and Carolina Hemlock HWA is a destructive pest. HWA has a stylet for feeding, which it inserts at the base of a needle before the junction with the stem (McClure et al. 2001). The stylet will penetrate and enter into the plant vascular tissue where it will find the parenchyma cells of the xylem rays, which are important in transferring and storing nutrients (McClure et al. 2001). When a tree is infected with the woolly adelgid the needles begin to fall off and damage is done to the buds limiting new growth (McClure et al. 2001). An infested hemlock will usually die within four years of infestation. GSMNP hemlocks are in danger of being eradicated if there is no intervention.

Study Sites

In GSMNP there are 800 acres of old-growth Eastern Hemlock along with 90,000 acres of younger forest (GSMNP 2006). Many of the older forest stands are present because they occurred in regions inaccessible to logging, while the younger forest stands have arisen after logging or the loss of the chestnut by the chestnut blight (GSMNP 2006 & Sharkey 2001). Three distinct Eastern Hemlock stands of interest in the GSMNP are

Table 1. Site description of three Great Smoky Mountains National Park biodiversity plots established for the All Taxa Biodiversity Inventory and examined in this study (Collins 2006).

ATBI plot	Albright Grove	Cataloochee	Purchase Knob
Forest Class	Montane Cove	Mesic Oak	Northern Hardwood
Watershed	Indian Camp Creek	Cataloochee Creek	Cove Creek
Geology	Thunderhead Sandstone	Thunderhead Sandstone	Biotite Augen Gneiss
Disturbance	Undisturbed	Chestnut Blight	Logged
Elevation(ft)	3,390	4,530	5,020
Soil pH	4.3	4.3	4.8
Phosporus (P) ppm	18.7	13.3	12.0
Potassium (K) ppm	93.3	81.7	85.7
Calcium (Ca) ppm	224.8	222.8	274.3
Magnesium (Mg) ppm	35.3	35.2	42.7
Organic Matter (%)	3.9	3.8	3.5

located at Albright Grove, Cataloochee, and Purchase Knob (Sharkey 2001; O'Connell et al. 2007). Albright Grove is an undisturbed old growth forest. It is categorized as a Montane Cove forest type with thunderhead sandstone geology occurring at 3,390 ft (Table 1). Cataloochee is a moderately disturbed site, which was shaped as result of the chestnut blight. It is as a Mesic Oak forest type with thunderhead sandstone geology occurring at 4,530 ft. Purchase Knob was logged and is a secondary growth forest. It is categorized as a Northern Hardwood forest type with biotite augen gneiss geology occurring at 5,020 ft.

Characterizing microbial communities of different Eastern Hemlock rhizospheres is important in understanding the impact, if any, of soil differences on microbial diversity. If the baseline microbial diversity can be understood in context of soil variation then one can examine how different stressors can alter the community. In a recent study it was shown that increased levels of nitrogen and carbon compounds occurred in soil surrounding moderate and heavily infested hemlocks (Cobb et al. 2006). The shift in nutrient availability could alter the presence of active and non-active microbes in the hemlock rhizosphere thus altering the compounds available to the tree. A change in the compounds available to the tree could inhibit a trees' ability to fight disease and grow (Doran et al. 1996). If we better understood these communities our knowledge could assist in the preservation and restoration of the Eastern Hemlock. Re-establishment of root health could be achieved by inoculating the roots with essential microbes or chemical compounds, which have been shown to stimulate and promote plant growth and health (Sunman et al. 2005; Safronova et al. 2004; Prithiviraj et al. 2002).

Methods in Microbial Ecology

Diversity in soil can be examined by a number of ways. Historically culturing and isolating bacteria were the only ways to evaluate microbial communities. Isolation of bacterial colonies is very informative in the characterization and classification of microorganisms through colony morphology, microscopy, and metabolic and biological processes. However, the reconstruction of the specific growth parameters required by most bacteria in the lab is not possible, thus many of the ecologically important bacteria are not represented using culturing methods (O'Connell et al. 2007).

Molecular cloning has also become a popular method of assessing microbial diversity directly from the environment. Molecular cloning incorporates isolated DNA from the environment into a plasmid. The plasmid is then transformed into *Escherichia coli*, which is allowed to grow into colonies. The colonies that have taken up the plasmid with the incorporated DNA are selected for DNA sequencing. Molecular cloning allows for the classification of DNA sequences without culturing microbes from environmental samples, and has allowed for discovery of previously undescribed bacteria and archaea (Janssen 2006; O'Connell et al. 2007; York 2008).

Terminal restriction fragment length polymorphism (T-RFLP) is a method that allows for comparison of community diversity. T-RFLP fingerprints a community by analyzing the polymorphism of a specific gene (Gruntzig et al. 2002). DNA is isolated from a sample and the gene of interest is amplified using PCR with a fluorescently labeled primer. The fluorescently labeled amplified DNA mixture is digested with a restriction enzyme, which results in DNA fragments of different sizes. The DNA

fragments are then separated by capillary electrophoresis, and a laser detects the labeled fragments and creates a profile based on fragment length (Boyle-Yarwood et al. 2008; Culman et al. 2008; Gruntzig et al. 2002; Li et al. 2007; Liu et al. 1997; Park et al. 2006).

A recent examination of three GSMNP sites mentioned previously showed a variation in diversity and richness in soil surrounding hemlock (O'Connell et al. 2007). Figure 4 shows the phylum-level diversity patterns represented at all three sites based on molecular cloning approaches.

An examination of T-RFLP profiles will give an insight into the level of diversity and richness of bacterial and archaeal genes within and between study sites. This will allow us to perhaps infer the impact of root exudates and soil type on the microbial diversity as well as to monitor hemlock rhizospheres as trees die. It is important to understand how these two variables impact the microbial life and tree health when developing a program for protection and reestablishment. The purpose of this study was to compare bacterial 16S rDNA, bacterial ammonia oxidation gene, and archaeal ammonia oxidation gene diversity at three different hemlock stands in GSMNP.

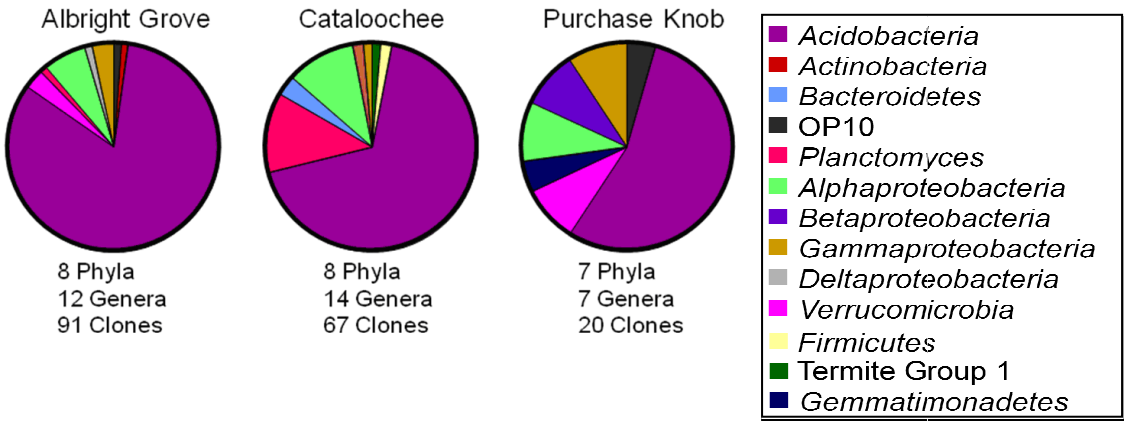

Figure 5. Phylum level diversity of Bacterial 16S rDNA clones from Albright Grove, Cataloochee, and Purchase Knob bulk soil as classified by RDP II. Data from a previous study (O'Connell et al., 2007).

Hypotheses

Hypothesis one. The T-RFLP community fingerprint of the bacterial 16S rDNA genes from the hemlock rhizospheres for the three study sites will show distinct communities for each site examined. Distinct communities have previously been shown for bacterial 16S rDNA gene diversity at the three GSMNP study sites (Figure 4) (O'Connell et al. 2007). Due to the geological and botanical differences (Table 1) between the three sites the available nutrients will select for metabolically distinct organisms.

Hypothesis two. The T-RFLP community fingerprint of the archaeal ammonia-oxidizing genes from hemlock rhizospheres for the three study sites will show distinct communities for each site examined. Since the three sites have shown distinct microbial communities the diversity of the ammonia-oxidizing gene should also be diverse. (Figure 4) (O'Connell et al. 2007). The geological and botanical differences (Table 1) between the three sites will lead to a diverse community.

Hypothesis three. The T-RFLP community fingerprint of the bacterial ammonia-oxidizing genes from hemlock rhizospheres for the three study sites will show distinct communities for each site examined. Since the three sites have shown distinct microbial communities the diversity of the ammonia-oxidizing gene should also be diverse. (Figure 4,) (O'Connell et al. 2007). The geological and botanical differences (Table 1) between the three sites will lead to a diverse community.

METHODS AND MATERIALS

Soil Sample Collection

Soil was collected from hemlock rhizospheres from three established study sites in GSMNP including Albright Grove, Cataloochee, and Purchase Knob in December of 2008 for molecular fingerprinting (T-RFLP). Aseptic techniques were used in collecting soil samples, including use of flame sterilized tools (shovel, garden trowel, and scissors). Collection was from three trees at each site that were not infested with HWA and were of similar size, 15 – 30 ft tall and at least 10 ft apart. A primary root was located and followed to a secondary root, where soil clumped to the secondary root was removed with flame sterilized scissors and placed in a 50 mL collection vial. The sample for each tree consisted of 8.0 – 20.0 g of soil from secondary roots below the soil surface. The samples were frozen on dry ice and transported to Western Carolina University where they were stored at -70^{o}C until analyzed (O'Connell et al. 2007).

DNA Extraction

Between 5 – 6 g of soil was added to 20 – 23 mL, (depending on weight of soil) of sterile deionized water in an Erlenmeyer flask with a magnetic stir bar. Samples were stirred at ~200 rpm for 5 minutes. A volume of 2 mL of each sample was pipeted into a microcentrifuge tube and centrifuged at 10,000 x g for 10 minutes. The supernatant was poured off and each tube was weighed. Centrifugation was repeated until all sample tubes were of similar weight (sample weight range 0.25 – 0.35 g). DNA was extracted

from the centrifuged hemlock rhizosphere soil samples using the MoBio® Power Soil DNA Extraction Kit utilizing the alternative lysis method (MoBio® Laboratories Inc. Solana Beach, CA) and a bead beater instead of a vortex for cell lysis. The Power Soil DNA Extraction Kit is designed to be used with difficult environmental samples eliminating PCR inhibitors such as high humic acid content.

PCR Amplification of Bacterial 16S rDNA

The full bacterial 16S rDNA gene (~1500 bp) of mixed root soil DNA extract was amplified by polymerase chain reaction (PCR) using forward primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) labeled with dye 6-FAM and reverse primer 1492R (5-GGY TAC CTT GTT ACG ACT T-3'), which corresponds to positions 8 to 27 and 1473 to 1492 of the open reading frame of *E. coli*, respectively (Braker et al. 2001; O'Connell et al. 2007). Triplicate PCR reactions were performed in a 50 µL reaction mix containing nuclease free water (Promega, Inc., Madison, WI), 1x Promega Master Mix, 0.5 mM $MgCl_2$, 0.25 pmol/µL primer, and one µL of Dilute DNA extract (10% strength of MoBio yield). Thermocycler (Eppendorf Corp., Westbury, NY) parameters used were initial denaturation at 94ºC for 3 minutes, followed by 30 cycles of denaturing at 94ºC for one minute, annealing at 55ºC for one minute, and extension at 72ºC for two minutes, followed by final extension for 10 minutes at 72ºC (O'Connell et al. 2007). PCR products were visualized on a 1% agarose gel run at 75 V for 45 minutes and compared to a Promega 100 bp ladder. PCR products were stored at 4ºC until further analysis.

PCR Amplification of Archaeal amoA *Genes*

A partial section of archaeal ammonia oxidation subunit A gene (~557 bp) of root soil DNA extract was amplified using forward primer *amo*111F (5'-TTY TAY ACH GAY TGG GCH TGG ACA TC-3', Y = C or T, H = A, C, or T) labeled with 6-FAM and reverse primer *amo*643R (5'-TCC CAC TTW GAC CAR GCG GCC ATC CA-3'; W = G or C) (Boyle-Yarwood et al. 2008; Treusch et al. 2005). Triplicate PCR reactions were performed in a 50 µL reaction mix containing nuclease free water (Promega), 1.x Promega Master Mix, 3.0 mM $MgCl_2$, 0.25 pmol/µL primer, and one µL of 10% DNA extract. Thermocycler parameters used were initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of denaturing at 94ºC for one minute, annealing at 55ºC for one minute, and extension at 72ºC for 1.5 minutes, followed by final extension for 7 minutes at 72ºC as modified from Boyle-Yarwood et al. (2008). PCR products were visualized on a 1% agarose gel run at 75 V for 45 minutes using Promega 100 bp ladder. Gel bands were cut from the gel using a sterile razor blade. Cut bands were placed in a 2 mL collection tube with 100 µL of Tris-EDTA (pH 8) and incubated at 37°C while shaking at 150 rpm overnight. One µL of this gel-excised DNA was subject to another round of PCR and visualized on 1% agarose gel as previously described. PCR products were stored at 4ºC until further analysis.

PCR Amplification of Bacterial amoA *Genes*

A partial section of the bacterial ammonia oxidation subunit A gene (~491 bp) of root soil DNA extract was amplified using forward primer amoA-1F (5'-GGG GGT TTC

TAC TGG TGG TGG T-3') labeled with dye 6-FAM and reverse primer amoA-2R (5'-CCC CTC KGS AAA GCC TTC TTC-3'; K = G or T), which corresponds to positions 332 to 349 and 802 to 822, for *Nitrosomonas europaea* respectively (Boyle-Yarwood et al. 2008; Mintie et al. 2003). Triplicate PCR reactions were performed as modified from Boyle-Yarwood et al. (2008) in a 50 µL reaction mix containing nuclease free water (Promega) 1x Promega Master Mix, 3.0 mM $MgCl_2$, 0.25 pmol/µL Primer, and 2 - 5 µL of 10% DNA extract. Thermocycler parameters the same as for archaeal *amoA*. PCR products were visualized same as for other samples. PCR products were stored at 4ºC until further analysis.

Terminal Restriction Fragment Polymorphism (T-RFLP) of Bacteria 16S rDNA PCR products

Triplicate PCR products from each sample were combined and purified using Montage PCR centrifugal filtration device (Promega, Inc., Madison, WI) following the manufacturer's protocol. Purified PCR products were visualized and quanitified on a 1% agarose gel run at 75 V for 45 minutes compared to a Lamda Hind III ladder. Twenty microliter T-RFLP digestion reactions were prepared with 13.8 µL of purified PCR product, 1X Buffer B, 0.1 µL BSA (Promega, Inc., WI), 10U of RsaI restriction enzyme, and 10U of MspI restriction enzyme (Boyle-Yarwood et al. 2008). Digestion reactions were incubated on a thermocycler with activation step of 3 hours at 37ºC and deactivation step of 15 minutes at 75ºC. Six and a half µL formamide (HiDi, Applied Biosystems, Forest City, CA), 2.5 µL of digested sample, and 1 µL of ROX 500 sizing standard were combined, denatured, and loaded into a microplate. Digested samples were analyzed on

an ABI 3130 Genetic Analyzer using GeneMapper 4.0 and run through a 36 cm capillary array for 45 minutes.

Terminal Restriction Fragment Polymorphism (T-RFLP) of Archaea amoA *Gene PCR products*

Triplicate PCR products were combined and purified using Montage PCR centrifugal filtration devices (Millipore Corp., Bedford, MA). Purified PCR products were visualized and quantified on a 1% agarose gel run at 75 V for 45 minutes using a Lamda Hind III ladder. Twenty microliters of T-RFLP digestion reactions were prepared with 11.8 µL of purified PCR product, 1.2X Buffer B, 0.1 µg/µL BSA (Promega, Inc., WI), 10U of RsaI restriction enzyme, 10U of MspI restriction enzyme, and 10U of CfoI restriction enzyme (Boyle-Yarwood et al. 2008). Digestion of reactions, and electrophoresis were as above.

Terminal Restriction Fragment Polymorphism (T-RFLP) of Bacteria amoA *Gene PCR products*

Triplicate PCR products were combined and purified using Montage PCR centrifugal filtration devices (Millipore Corp., Bedford, MA). Purified PCR products were visualized and quanitified on a 1% agarose gel run at 75 V for 45 minutes with a Lamda Hind III ladder. Twenty microliter T-RFLP digestion reactions were prepared with 11.8 µL of purified PCR product, 1X Buffer C, 0.1 µg/µL BSA (Promega, Inc., WI), 10U of TaqI restriction enzyme, 10U of AluI restriction enzyme, and 10U of CfoI

restriction enzyme (Boyle-Yarwood et al. 2008). Digestion reactions were incubated on a thermocycler with two steps, 3 hours at 37ºC and 3 hours at 65ºC. Reactions were electrophoresis as described above.

Data Analysis

For each gene (16S rDNA, *amoB*, *amoA*) sample electroferrograms were screened for distinguishable peaks greater than 15 relative frequency units (RFU). The fragment length of each of these peaks was entered into a spreadsheet for each replicate sample and the peak height assigned to it. These data were plotted as histograms for easier comparison. Principal components analysis (PCA, Systat, SPSS, Chicago) was performed on samples comparing nucleotide fragment length, peak height, and presence or absence of each peak (i.e. species). Differences between communities from each site were screened for *amoA* and bacterial 16S rDNA. The *amoB* gene study yielded results for only Purchase Knob.

RESULTS

Amplification for the bacterial 16S rDNA gene was achieved from all nine samples. Amplification of archaeal *amoA* gene was achieved from all nine samples as well, while amplification of bacterial *amoA* was only achieved from three samples, which came from Purchase Knob. DNA yield after PCR cleanup for bacterial 16S rDNA resulted in 7 ng/µL for Albright Grove replicates 1, 2, and 3, Cataloochee replicates 1 and 2, and Purchase Knob replicates 1 and 2 (Figure 6b). Catalochee 3 and Purchase Knob 3 had a yield of 2 ng/µL and 5 ng/µL respectively (Figure 6b).

The first round of *amoA* PCR contained non-specific amplification (Figure 7a), while the second round of PCR saw a decrease in non-specific amplification (Figure 7b). DNA yield after PCR cleanup for archaeal *amoA* resulted in 14 ng/µL for Albright Grove replicates 1 and 2, Cataloochee replicates 1 and 3, Purchase Knob replicates 1, 2, and 3 (Figure 7c). Albright Grove 3 and Catalooche 2 had a yield of 20 ng/µL and 7 ng/µL, respectively (Figure 7c). Purchase Knob replicates 1, 2, and 3 each yielded 2 ng/µL after DNA cleanup for bacterial *amoA* (Data not shown).

Bacterial 16S rDNA T-RFLP profiles are located in Figure 8, 9, and 10. Table 2 shows a summary of 16S rDNA T-RFLP electoferrogram peak data. Albright Grove had a total of 45 different peaks, an average of 28.7±8.7 peaks per sample, and 9 peaks unique to the site. Cataloochee had a total of 60 different peaks, an average of 29.3±17.2 peaks per sample, and 17 peaks unique to the site. Purchase Knob had a total of 60 different peaks, an average of 31±2.6 peaks, and 18 peaks unique to the site. Figure 11 shows a principal components analysis plot which groups Albright Grove profiles group

together more closely than Cataloochee and Purchase Knob. A comparison of major peaks in bacterial 16S rDNA T-RFLP profiles showed 14 major peaks across all sites with 28.6% of major peaks shared between all three sites and 57.1% of peaks occurring in at least two sites (Table 3).

AOA T-RFLP profiles are located in Figures 12, 13, and 14 and AOB T-RFLP profiles are located in Figure 16. Table 4 is a summary of AOA and AOB T-RFLP electroferrogram peak data. Albright Grove had a total of 82 different peaks for AOA, with an average of 47.7±9.3 peaks per sample, and 13 unique peaks (Table 4). Cataloochee had a total of 105 different peaks for AOA, an average of 54.3±1.2 peaks per site, and 30 unique peaks (Table 4). Purchase Knob had a total of 103 different peaks for AOA, 10 for AOB, an average of 50.7±11.7 peaks per site for AOA, 7±1 peaks per site for AOB, and 22 unique peaks for AOA, and 10 unique peaks for AOB (Table 4). Figure 15 shows a principal components analysis plot grouping Albright Grove and Purchase Knob *amoA* profiles together and with less variation than Cataloochee samples. A comparison of major peaks in AOA T-RFLP profiles showed 33 major peaks across all sites and none of the major peaks were shared between all three sites while 18.2% of peaks occurred in at least two sites (Table 5).

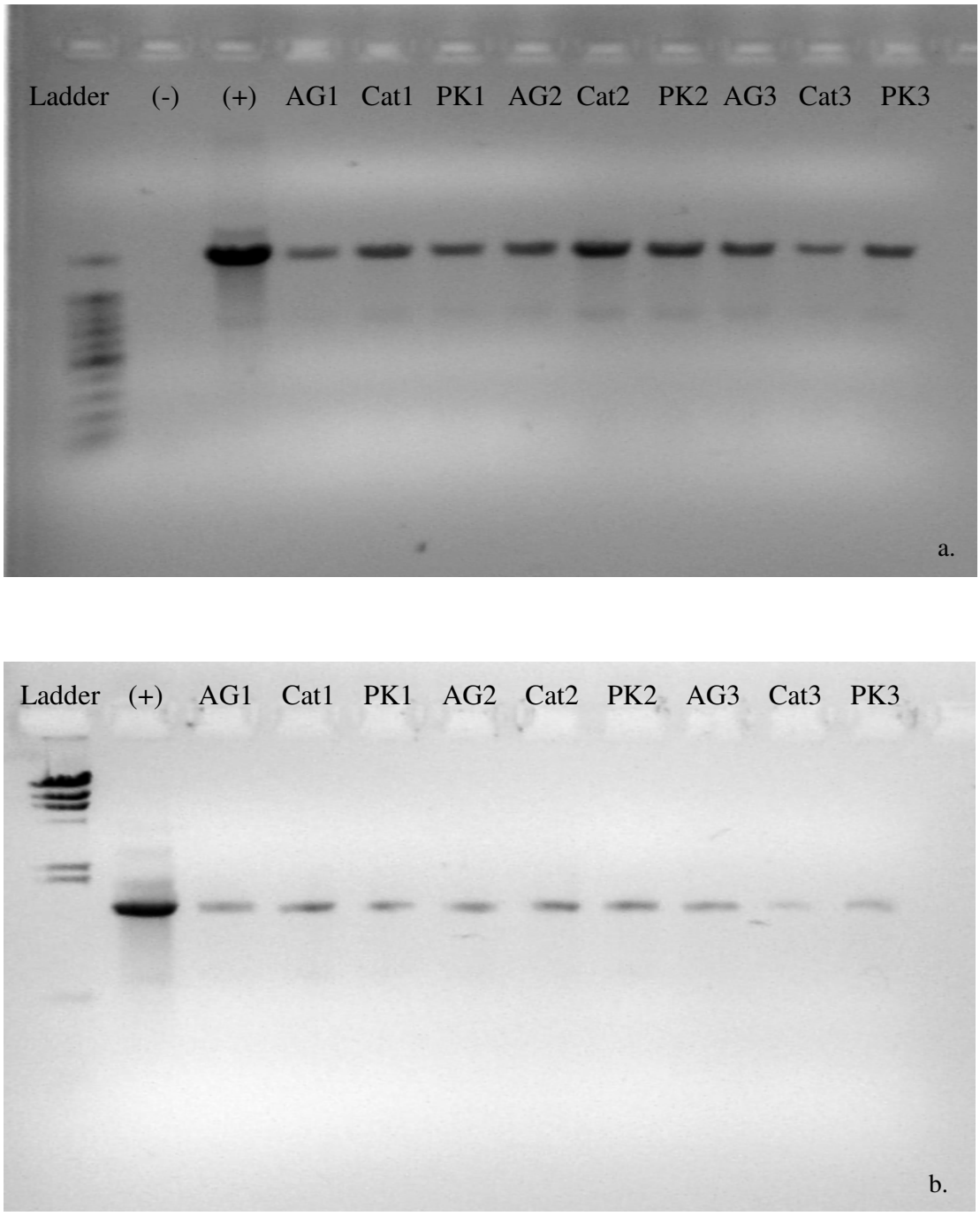

Figure 6. (a.) Agarose gel image of bacterial 16S rDNA PCR product and (b.) PCR cleanup quantification gel ((-) = Negative control, (+) = Positive control, AG = Albright Grove, Cat = Cataloochee, PK = Purchase Knob).

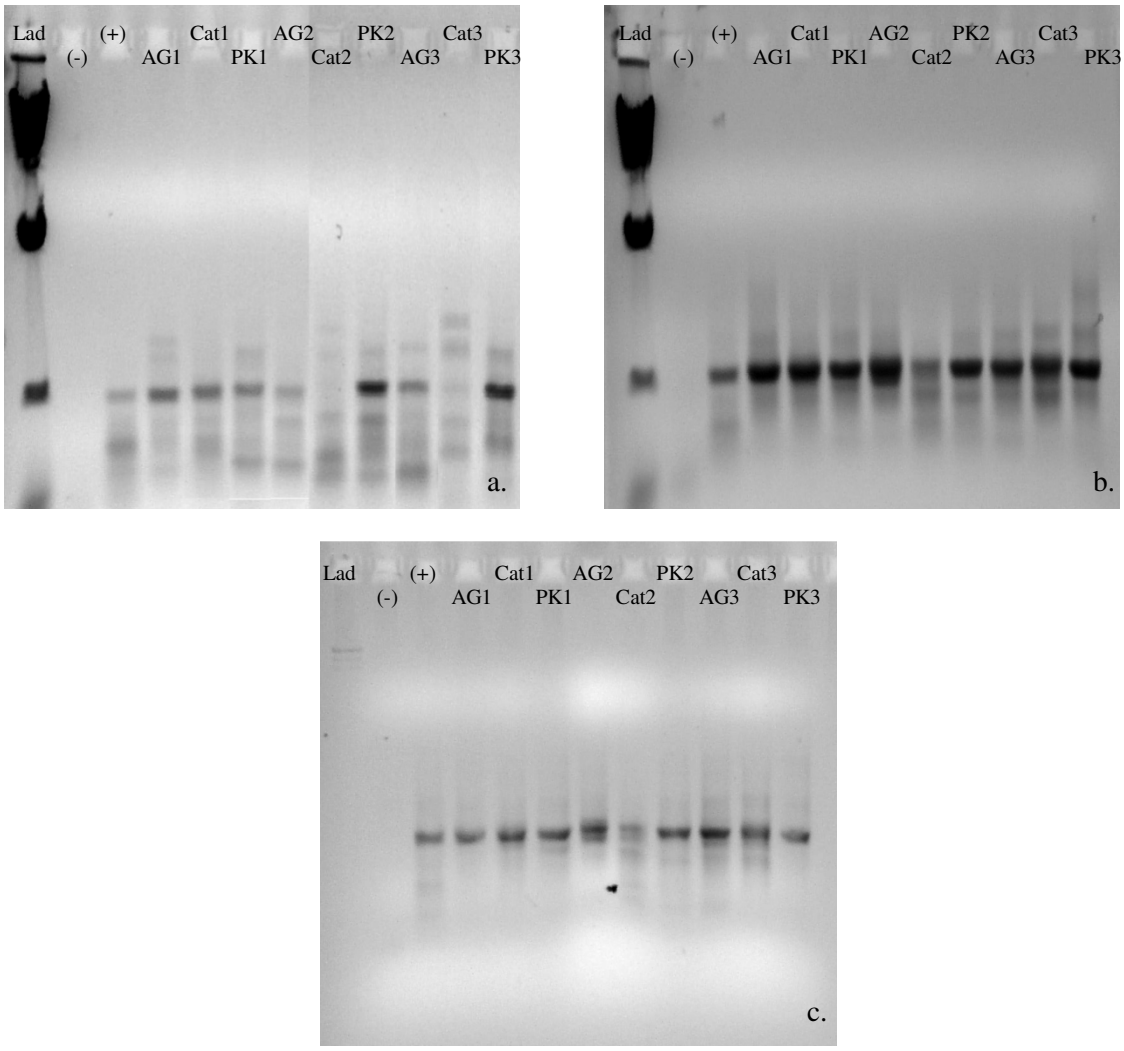

Figure 7. (a.) Results of first round of archaea *amoA* PCR. (b.) Second round of PCR of gel extracted bands from first PCR. (c.) Cleaned PCR product used for digestion (Lad = PCR Ladder, (-) = Negative control, (+) = Positive control, AG = Albright Grove, Cat = Cataloochee, PK = Purchase Knob).

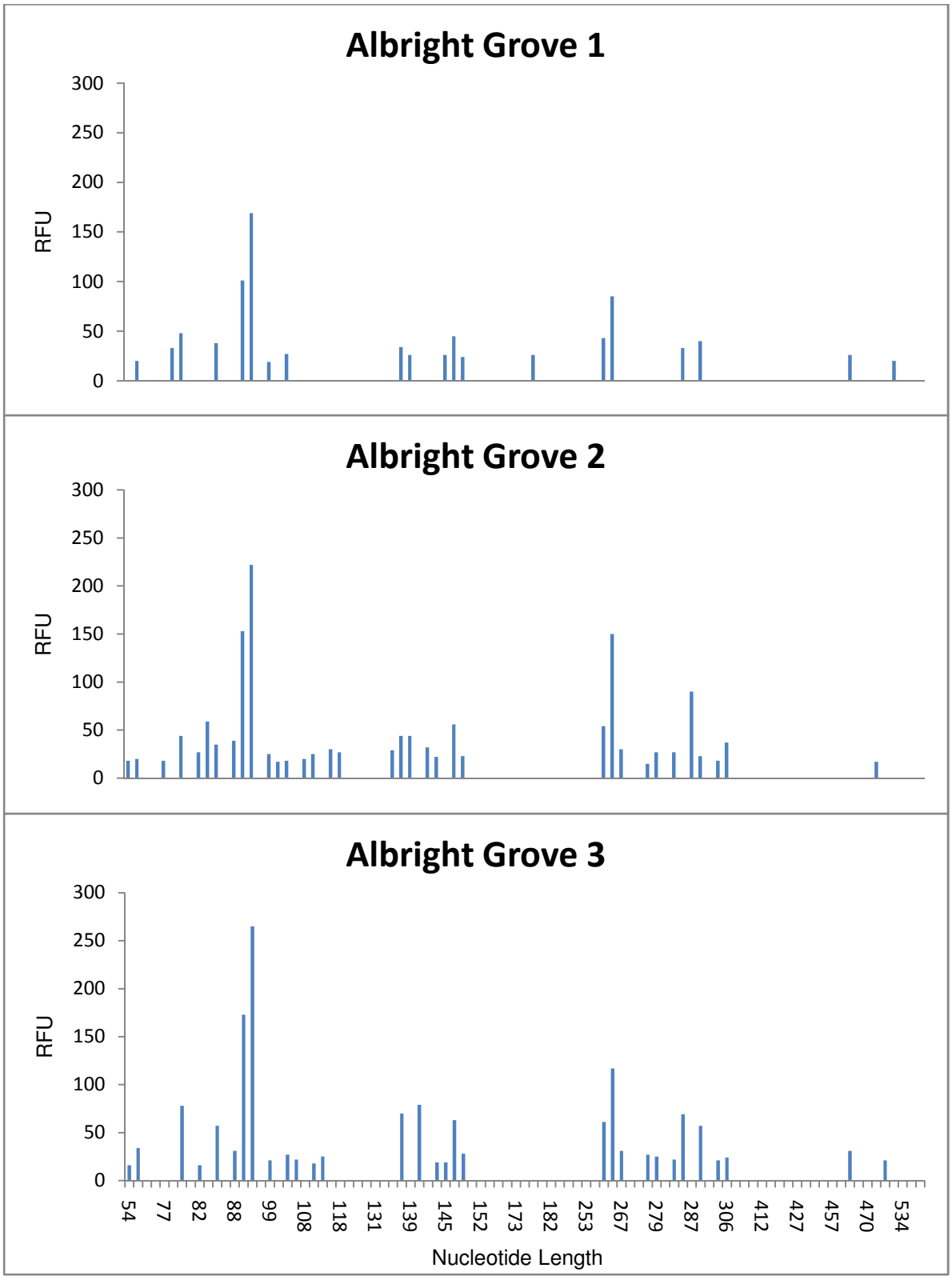

Figure 8. Histograms of T-RFLP peaks of bacterial 16S rDNA PCR products digested by RsaI and MspI from Albright Grove. (RFU = relative fluorescent unit).

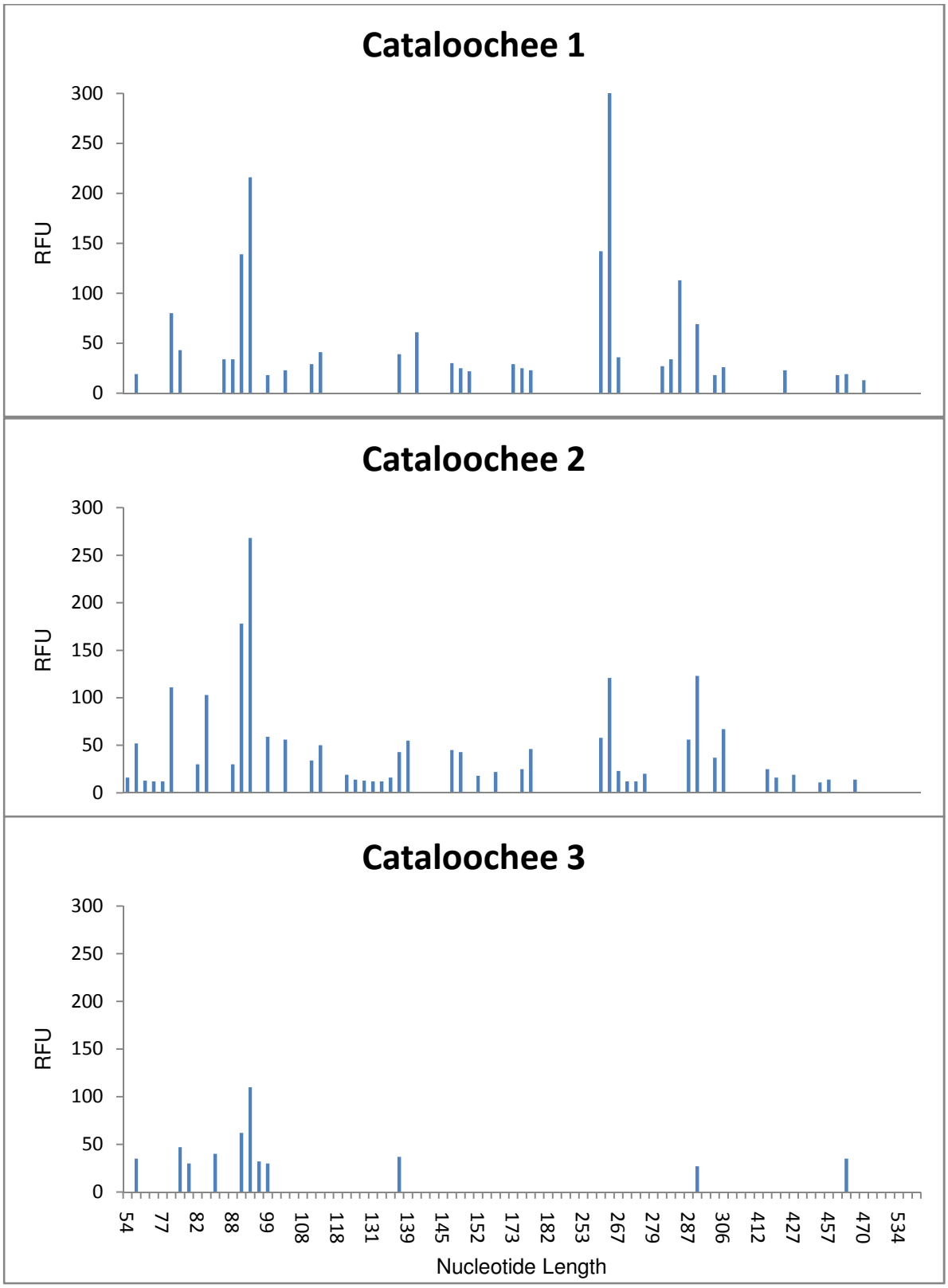

Figure 9. Histograms of T-RFLP peaks of bacterial 16S rDNA PCR products digested by RsaI and MspI from Cataloochee. (RFU = relative fluorescent unit).

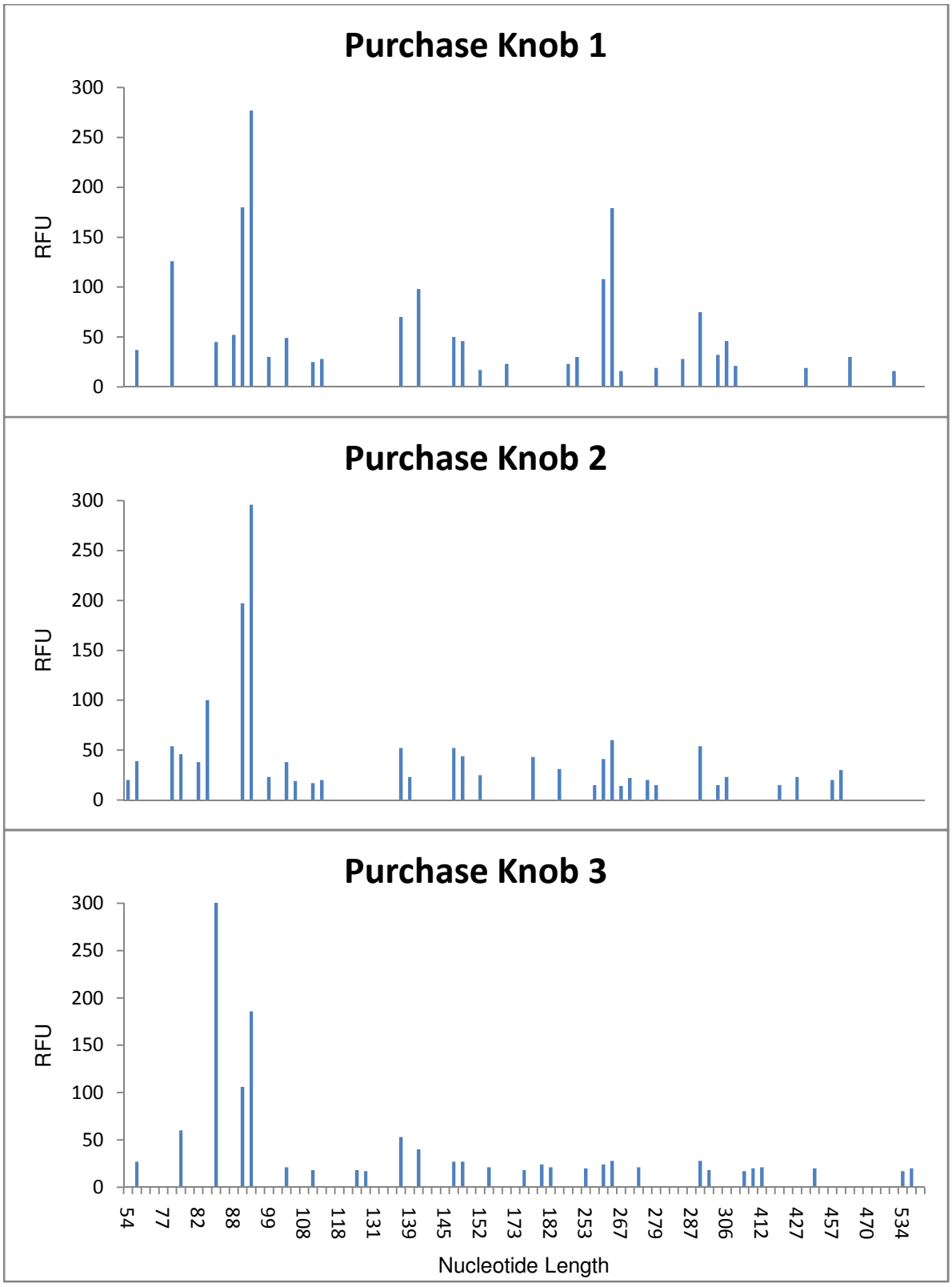

Figure 10. Histograms of T-RFLP peaks of bacterial 16S rDNA PCR products digested by RsaI and MspI from Purchase Knob. (RFU = relative fluorescent unit).

Table 2. Summary of results of T-RFLP electroferrograms for bacterial 16S rDNA.

Site	Total Peaks	Mean Peaks	Unique Peaks
Albright Grove	45	28.7 ± 8.7	9
Cataloochee	60	29.3 ± 17.2	17
Purchase Knob	60	31 ± 2.6	18

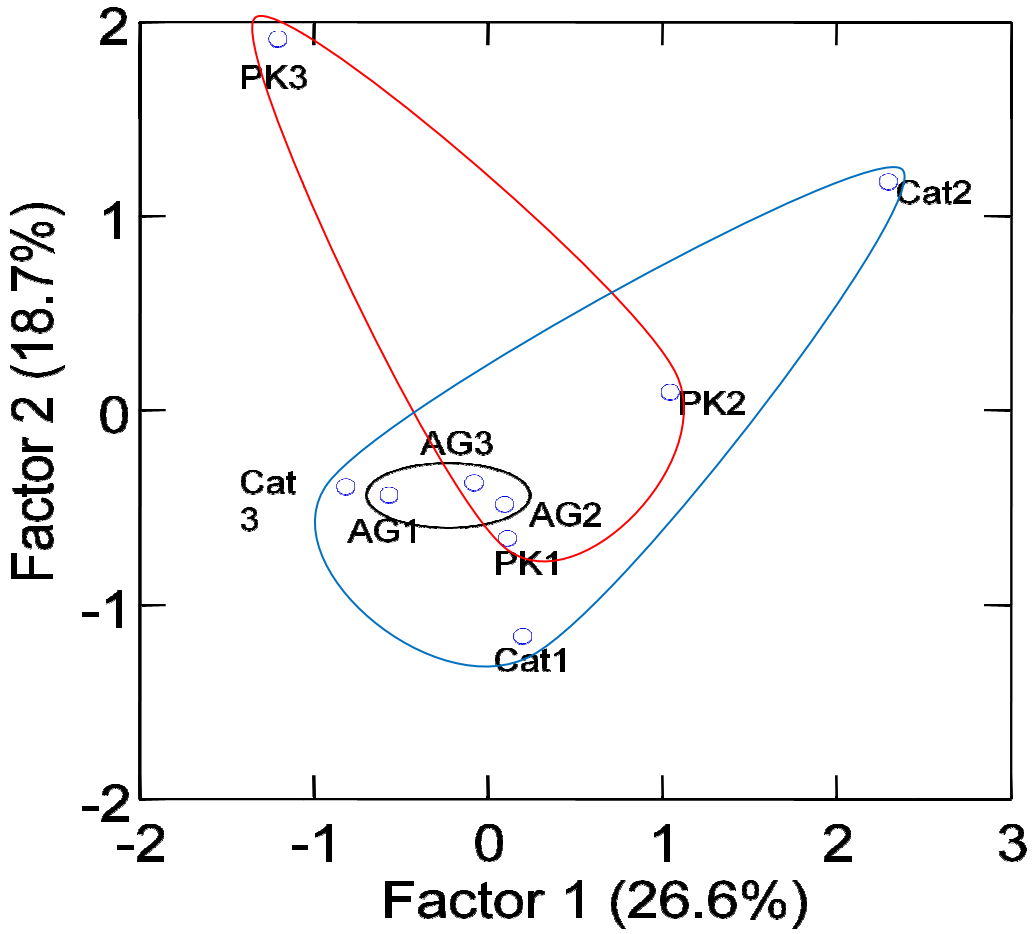

Figure 11. Principal components analysis from T-RFLP profiles of bacterial 16S rDNA communities from the hemlock rhizosphere.

Table 3. Comparison of the five dominant peaks from each 16S rDNA T-RFLP profile; 57.1% of the dominant peaks occurred in at least two sites and 28.6% of the dominant peaks were shared between all three sites (nucleotide length is indicated in the first column, +++ = occurred in all samples, ++ = occurred in two samples, + = occurred in one sample, blank = did not occur).

	Albright Grove	Cataloochee	Purchase Knob
90	+++	+++	+++
93	+++	+++	+++
265	+++	++	++
80	++	+	+
79		+	++
84	+		+
85		+	+
138		+	+
140	+		
148	+		
263			+
286		+	
287	+		
288		+	

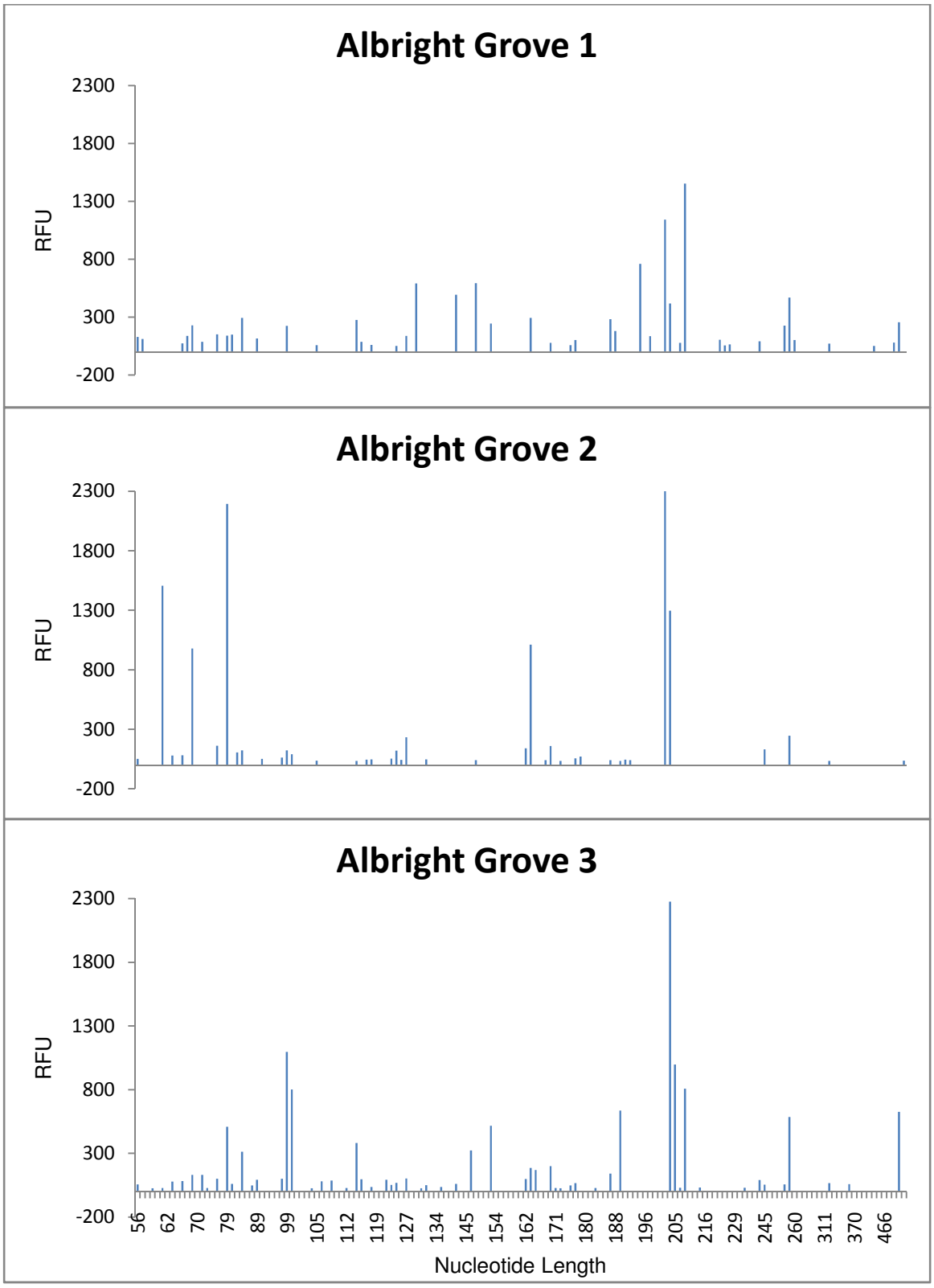

Figure 12. Histograms of T-RFLP peaks of ammonia oxidizing archaea PCR products digested by RsaI, MspI, and CfoI from Albright Grove. (RFU = relative fluorescent unit).

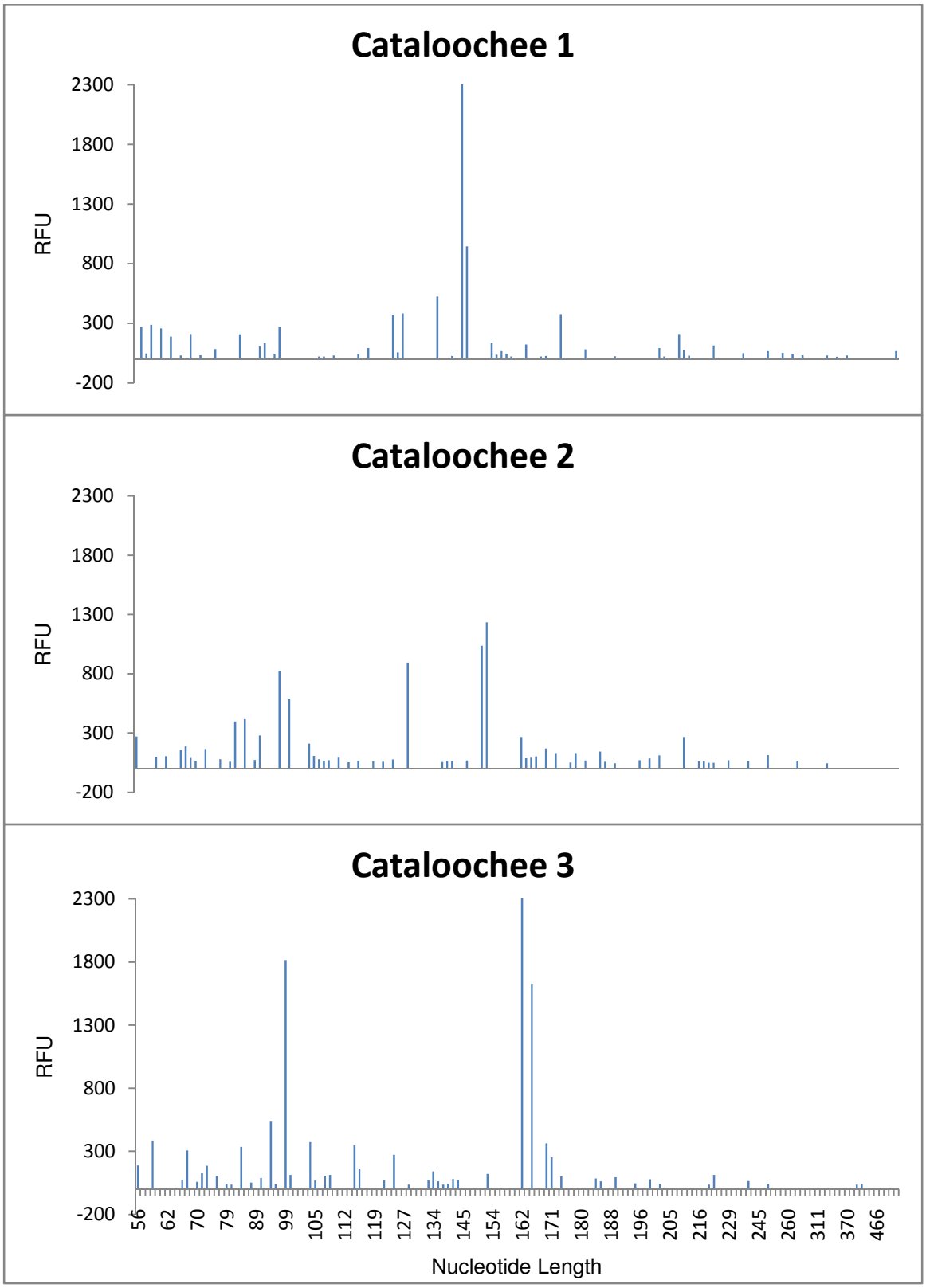

Figure 13. Histograms of T-RFLP peaks of ammonia oxidizing archaea PCR products digested by RsaI, MspI, and CfoI from Cataloochee. (RFU = relative fluorescent unit).

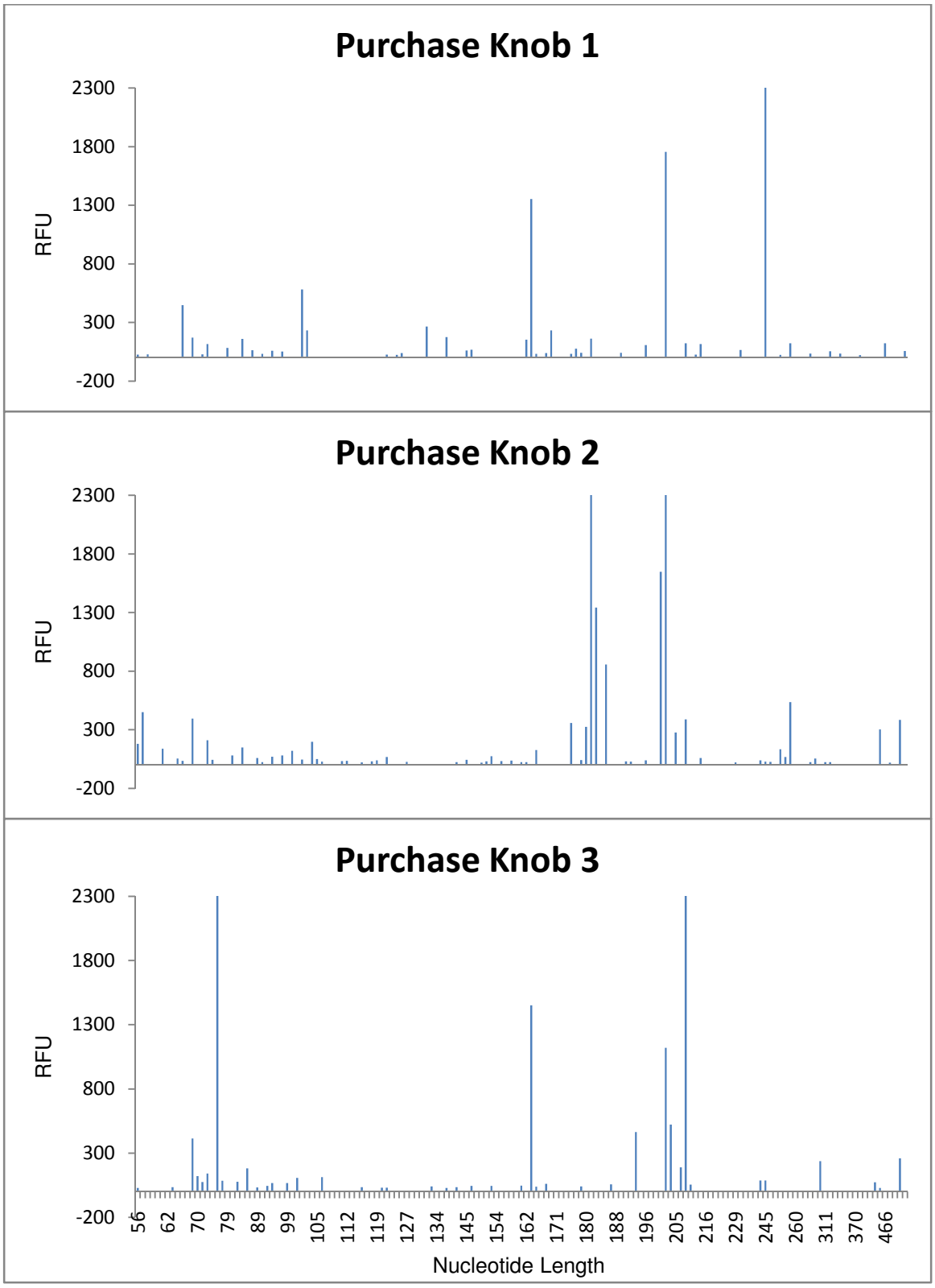

Figure 14. Histograms of T-RFLP peaks of ammonia oxidizing archaea PCR products digested by RsaI, MspI, and CfoI from Purchase Knob. (RFU = relative fluorescent unit).

Table 4. Summary of results of T-RFLP electroferrograms for ammonia oxidizing archaea and ammonia oxidizing bacteria (NA = no amplification of gene via PCR).

Site (AOA/AOB)	Total Peaks (AOA/AOB)	Mean Peaks (AOA/AOB)	Unique Peaks (AOA/AOB)
Albright Grove	82 / NA	47.7±9.3 / NA	13 / NA
Cataloochee	105 / NA	54.3±1.2 / NA	30 / NA
Purchase Knob	103 / 10	50.7±11.7 / 7±1	22 / 10

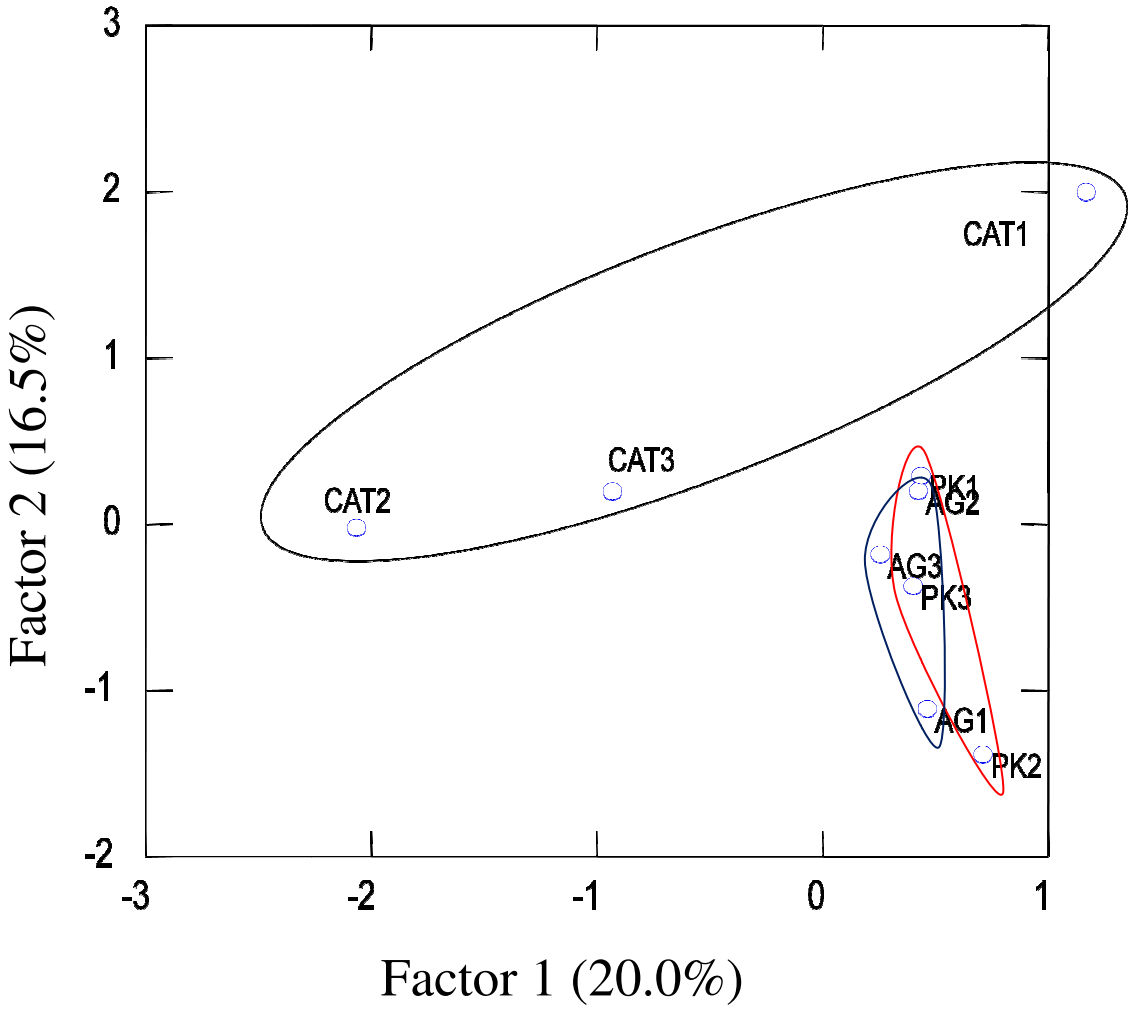

Figure 15. PCA results from T-RFLP profiles of the ammonia oxidizing archaea community from the hemlock rhizosphere.

Table 5. Comparison of the five dominant peaks from each ammonia oxidizing archaea T-RFLP profile; 18.2% of the dominant peaks occurred in at least two sites and none were shared between all three sites (+++ = occurred in all samples, ++ = occurred in two samples, + = occurred in one sample, blank = did not occur).

	Albright Grove	Cataloochee	Purchase Knob
203	++		+++
207	++		+++
204	++		+
164	+		++
99	+	+	
100	+	+	
61	+		
79	+		
129	+		
148	+		
195	+		
205	+		
59		+	
94		+	
97		+	
127		+	
128		+	
136		+	
145		+	
147		+	
150		+	
152		+	
162		+	
165		+	
174		+	
65			+
75			+
102			+
181			+
182			+
186			+
202			+
245			+

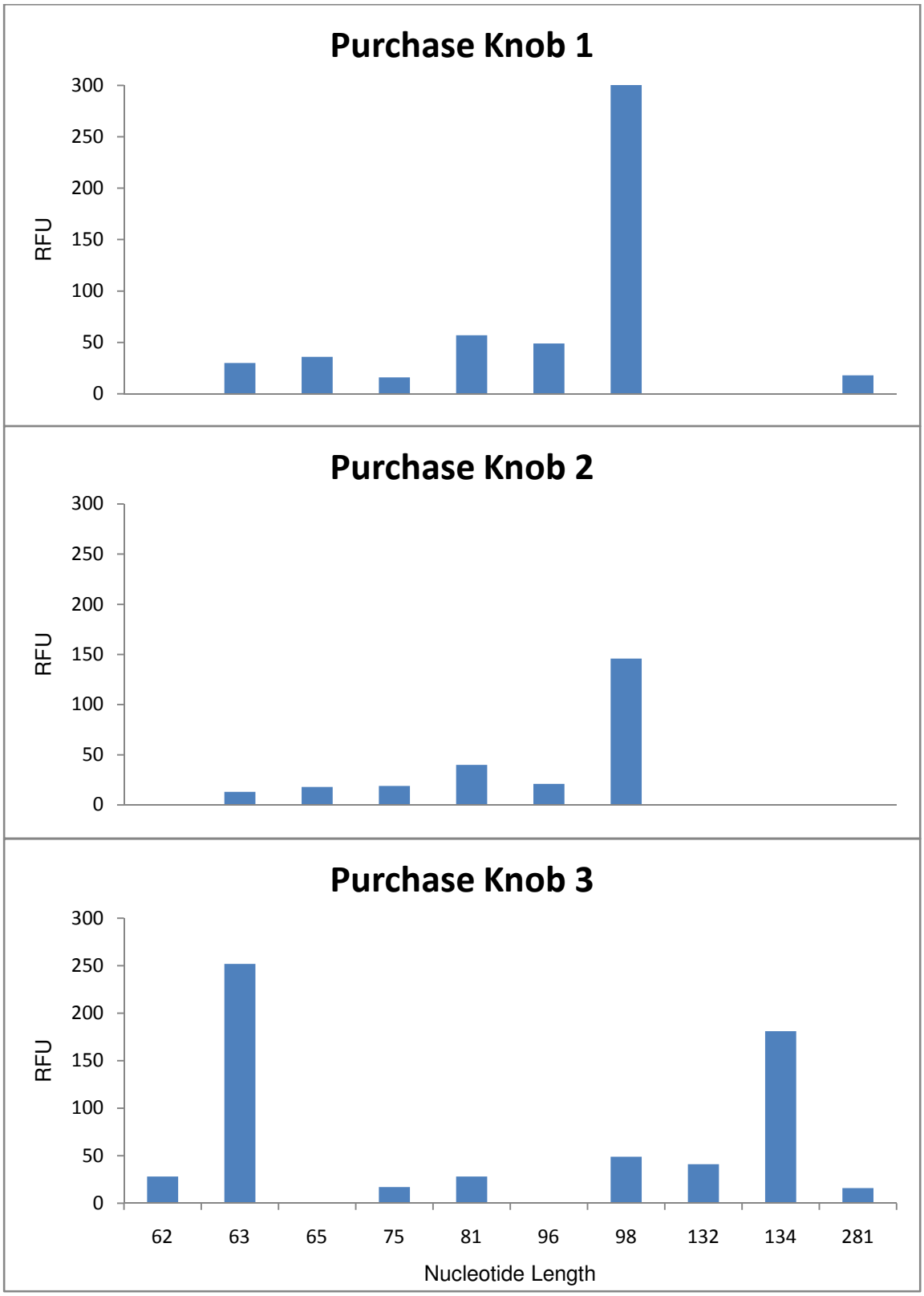

Figure 16. Histograms of T-RFLP peaks of ammonia oxidizing bacteria PCR products digested by TaqI, AluI, and CfoI from Purchase Knob.

DISCUSSION

Archaea and bacteria are ubiquitous and abundant almost everywhere. Microbial diversity has been examined through metagenomic, 16S rDNA, and culturing studies and have shown that microbial communities are highly diverse locally and globally (Fierer et al. 2007; Janssen et al. 2002, 2006). A very low percentage of the total microbial diversity has been phenotypically described. Soil contains a large portion of Earth's biomass and diversity in the form of microorganisms (Whitman et al. 1998). The dynamics of soil; high surface area to volume ratio, heterogeneity, porosity, multitude and variation of nutrients such as carbon, nitrogen, phosphorus, and sulfur provide unlimited niches along with incalculable diversity of microorganisms. Soil variants can have a drastic impact on what microbes can grow, especially the soil in close contact with plant roots (Marschner et al. 2001; Oburger et al. 2009; Bomberg & Timonen, 2009; Berg & Smalla, 2008).

Bacterial 16S rDNA

In this study, an examination of the bacterial 16S rDNA diversity associated with Eastern Hemlock rhizospheres was conducted in three different forests sites (Albright Grove, Cataloochee, Purchase Knob) in GSMNP (Hypothesis One). Hypothesis one predicted that the bacterial 16S rDNA diversity would vary between sites. Ongoing work has been done on the bulk soil from these sites and has shown that the diversity is higher at the Cataloochee and Purchase Knob sites, which have both experienced disturbances,

compared to Albright Grove, which is an undisturbed site (O'Connell et al. 2007; Collins 2006). Since each T-RFLP peak represents an individual species, the number of peaks present and their height in an electroferrogram is an indication of the number of species present and their abundance, respectively. In this study the results indicated similar trends as previously described. The difference in the total number of peaks per site was statistically insignificant suggesting that the total number of species between sites is similar or variation is great ($p = 0.93$ using ANOVA). Although the total number of peaks present was not different the general trend of the 16S rDNA T-RFLP data suggest that Cataloochee and Purchase Knob have a greater number of species present compared to Albright Grove. This is seen in the total number of unique peaks per site with Cataloochee and Purchase Knob having higher numbers compared to Albright Grove, suggesting that they have a greater level of diversity (Table 2). A higher level of diversity is not surprising because of previous results from these sites, but is surprising because these sites are disturbed. Disturbed sites are often associated with decreased diversity in oligotrophic soil microbes because of stress to delicate environments which alter and decrease the number of microhabitats (Bruce et al. 1995; Buckley & Schmidt 2001). This is in contrast to my results the disturbed sites appear to have an increase in diversity. The results of the bacterial 16S rDNA PCA indicate that Albright Grove is less variable than Cataloochee and Purchase Knob. The relative distance of the Albright Grove points on the PCA plot indicates the similarity and low variance amongst those samples. The relative spacing of the Cataloochee and Purchase Knob points on the PCA plot indicate higher variability between these communities and greater variance compared to Albright Grove (Figure 11).

Ammonia Oxidizing Archaea

Microorganisms play an important role in the nitrogen cycle and are the rate limiting factor in the ammonia oxidation step of the cycle. Historically bacteria have been thought to be the key contributors in ammonia oxidation. In recent years, analyses of environmental *amoA* genes have shown that archaea are a major component of the *amoA* gene pool in soil (Leininger et al. 2006). Hypothesis two examined the AOA diversity patterns of the three sites and it was postulated that they would differ based on differences between sites such as soil chemistry, site history, and other environmental factors.

Previous studies along with results of this study have shown bacterial diversity patterns are variable between sites (Collins 2006). It is possible that ammonia oxidizing archaea (AOA) patterns would follow similar trends as 16S rDNA. A previous study examined soil close to Eastern Hemlocks from the same study sites in this study. Using DGGE this study examined the diversity patterns of archaea 16S rDNA and it showed Albright Grove was distinct from Cataloochee and Purchase Knob, with little variability between the two disturbed sites (Drummond 2006). Amplification of AOA occurred for all nine samples. This is the first time PCR amplification of archaeal *amoA* gene from the Eastern Hemlock rhizospheres has been documented, and could mean that archaea play an important role in the nitrogen cycle in hemlocks. AOA diversity patterns were also variable between sites. The diversity patterns of AOA show a similar trend found in the T-RFLP profiles of bacterial 16S rDNA. Cataloochee and Purchase Knob, although not statistically significant, appear to have a greater abundance of species and a greater diversity than Albright Grove ($p = 0.68$ with ANOVA). This is particularly interesting

because Cataloochee and Purchase Knob are disturbed forests. PCA of AOA T-RFLP peaks showed that Albright Grove and Purchase Knob are similar and communities overlap, while Cataloochee was more variable and different from the other sites (Figure 15).

Ammonia Oxidizing Bacteria

Hypothesis three examined the ammonia oxidizing bacteria (AOB) diversity patterns of the three sites and it was suggested that AOB would reflect on differences previously stated. AOB was only amplified from three of the nine samples, and all were from one site. The diversity patterns of AOB could not be established because amplification was only achievable from Purchase Knob, but the T-RFLP peak patterns from these samples may provide some insight into the relative abundance and diversity of AOB. The AOB T-RFLP profiles from Purchase Knob showed little variability in peak patterns (Figure 16).

A recent study has shown AOA occurs in greater abundance than AOB in a wide range of soil types (Adair & Schwartz 2008; Leininger et al. 2006). A variety of protocols were used in this study to achieve optimum amplification of AOA and AOB genes (Tables 6 & 7). AOB was only amplified from three samples, but PCR inhibition can be ruled out. If PCR inhibitors were present amplification of 16S rDNA and AOA would have also been affected. The presence of AOA PCR products and the lack of AOB amplification suggests that AOA template existed at a higher concentration. If the concentration levels of AOA are higher this may indicate that AOA have a greater

importance than AOB in hemlock root systems. The implications of this could provide insight into the importance of AOA in hemlock roots.

Although AOB diversity patterns could not be established, a comparison of peak patterns still can be done. The average number of peaks present in Purchase Knob AOB T-RFLP profiles (7±1) was far less than the average number of peaks in the AOA T-RFLP profiles (50.7±11.7) (Table 3; Figure 14 & 16). The diversity patterns of Purchase Knob samples indicate that AOB are less abundant and less diverse than AOA in Purchase Knob samples, and it can probably be inferred the other sites follow the same pattern if AOB are present at all. A recent study has shown that AOA and AOB community structure is related to the pH level. That study showed a shift in AOA to AOB dominance as the pH increased (Nicol et al. 2008). The three study sites have acidic soils, which could partially explain the abundance and diversity differences of AOA and AOB. It has been shown that the concentration of ammonia substrate available may have an impact on the structure of AOA and AOB communities. Recently it was shown that AOA had a higher affinity for ammonium than comparable AOB species (Martens-Habbena et al. 2009). If the concentration of ammonium is higher than ammonia there would be an increased level of competition for available nitrogen, which may favor AOA. It is likely that ammonium is at a higher concentration because it occurs more readily at a low pH. It is possible that competition is high between AOA present, because of the diversity and differences of major species within and across the sites.

Sample Volume (µL)	[Mg2+]	[MM]*	[Primer]	Initial Denaturation (min@ 94°C)	Cycles	Denatureation (min@ 94°C)	Annealing (min@55°C)	Extension (min@72°C)	Final Extension (min@72°C)
1, 2.5, 5	0.5	1.0X	0.25	5	30	0.5	0.5	0.75	7
1.0	1.0	1.0X	0.25	5	30	0.5	0.5	0.75	7
1.0	2.0	1.0X	0.25	5	30	0.5	0.5	0.75	7
1.0	2.0	1.0X	0.25	5	30	1	1	1.5	7
1, 2.5	2.0	1.0X	0.25	5	35	1	1	1.5	7
2.5, 5	3.0	1.0X	0.25	5	35	1	1	1.5	7
2.5	3.0	1.1X	0.25	5	35	1	1	1.5	7
2.5	**3.0**	**1.2X**	**0.25**	**5**	**35**	**1**	**1**	**1.5**	7

Table 6. PCR method development for AOA including parameters which were changed one at a time.
(total volume = 50 µL, * = Promega Master Mix)

Sample Volume (µL)	[Mg2+]	Adjuvant	[MM]*	[Primer]	Cycles	Denaturation (min@94 °C)	Annealing	Extension (min@72°C)
1, 2.5, 5	1.5	NA	1X	0.25	30	1	1min @ 60°C	1.5
1, 2.5, 5	2.5	NA	1X	0.25	30	1	1min @ 60°C & 55°C	1.5
1	2.5	IgePal 0.05%	1X	0.25	30	1	1min @ 55°C	1.5
1 Conc., PCR products	2.5	NA	1X	0.25	30	1	1min @ 55°C	1.5
1 Conc., PCR products	2.0	NA	1X	0.25	30	1	1min @ 55°C	1.5
1 Conc., PCR products	2.0	NA	1X	0.25	35	1	1min @ 55°C	1.5
1 Conc., PCR products, Filtered Genomic DNA, & PCR product	**3.0**	**NA**	**1.2X**	**0.25**	**35**	**1**	**1min @ 55°C**	**1.5**
1 Conc.	3.0	BSA 0.05%	1.2X	0.25	35	1	1min @ 55°C	1.5

Table 7. PCR method development for AOB including parameters change one at a time. Initial denaturation and final extension were constant across all reactions (5 min @ 94°C and 7 min @ 72°C, respectively, Conc. = Concentrated DNA extract, PCR product are from previous reactions, Filter Genomic DNA & PCR products were cleaned with Montage PCR centrifugal filtration device).

The presence of archaea in soil is commonly associated with the mycorrhizosphere of plants (Bomberg & Timonen 2007). Plants with mycorrhizal roots contain higher diversity of archaea than uncolonized roots or bulk soil (Simon et al. 2005). The AOA diversity may be a result of mycorrhizal symbionts associated with Eastern Hemlock roots (Bomberg & Timonen 2009). Mycorrhizal fungi have been shown to have some control over the exudates released from roots in to the soil minimizing the direct effect of the tree on soil composition (Bomberg & Timonen 2007). The mycorrhizal root relationship is established early in development of plants roots and depending on the species can lead to spatial variability within and between sites (Goddard et al. 2001). Most plant roots contain a mycorrhizae and in a natural system plant roots are often entangled enabling interaction from neighboring mycorrhizosphere (Bomberg & Timonen 2007). Thus a microbial community associated with the roots of one plant could shift depending on surrounding flora. The three study sites in this study have distinct flora, which may have added to the variation seen in this study (Jenkins 2007).

Molecular Techniques

When using molecular techniques, one must always address biases associated with them. Bias can occur in any step of the molecular analysis beginning with contamination during collection, cell lysis and DNA extraction, PCR amplification, and analysis of PCR products (von Wintzingerode et al. 1997).

Aseptic techniques were used during soil collection and throughout the molecular process, which should have minimized contamination. The beginning step of cell lysis can lead to a large portion of PCR bias, because some cells are resistant or protected by soil structures to lysis (Frostegård et al. 1999). Homogenization of soil and use of the MoBio alternate lysis method during DNA extraction should have exposed a greater proportion of the microbial community to PCR amplification. Harsh conditions during cell lysis and DNA extraction can cause fragmented nucleic acids leading to PCR artifacts like chimeras, mutations, non-specific alleles, and heteroduplexes, which can lead to overestimation of diversity (Qui et al 2001). Chimeras can occur in PCR when there is fragmented DNA and extension begins on one strand, but is interrupted and continues on another strand (Pääbo et al. 1990). Chimeras, mutations, and heteroduplexes were not addressed in this study and have to be considered when examining the amount of diversity seen. Non-specific amplification was an issue with AOA and was addressed, but could not be completely eliminated (Figure 7). Non-specific amplicons were decreased and probably played a minimal role in the diversity patterns seen, because of their concentration level compared to the target gene.

Universal primers provide the greatest spectrum of amplification, but are a source of bias associated with PCR. Universal primers are not capable of amplifying all genes of interest in a mixture of environmental templates (Baker et al. 2003). In mixtures of high or low concentration primers will associate more often with the most common DNA strands, which will then dominate the reaction (Baker et al. 2003). This will create a disproportionate representation of the microbial community, but at least reflect the most abundant templates

T-RFLP is an important microbial ecology tool that can be utilized to monitor diversity and structure and measure populations in a statistical manner (Leuders & Friedrich 2003). T-RFLP has been shown to be a repeatable and reliable quantitative technique capable of establishing community variability (Leuders & Friedrich 2003, Li et al. 2007). In this study, consistency of T-RFLP profiles was established by examination of the similarity between runs. Electroferrograms for multiple runs showed little variation and provided confidence in the diversity patterns being seen. If there were any skewed T-RFLP patterns it was most likely due to PCR artifacts previously mentioned (Leuders & Friedrich 2003). Principal components analysis (PCA) is a common tool used to analyze T-RFLP profiles. PCA is a robust tool that has been shown to be a capable analysis method for T-RFLP (Culman et al. 2008). In this study PCA allowed for analysis of large amounts of data and provided an illustration of the diversity being seen. The distribution patterns seen through PCA, and the consistency of these patterns with previous work justifies its use as the analysis method in this study (Culman et al. 2008). In this study PCA picked up 45.3% of the total variance for bacteria 16S rDNA data, which is accounting for nearly half of all the variance seen (Figure 11). The PCA for AOA only picked up 36.5% of the total variance, which illustrates that there was a lot of variability between the data (Figure 16).

CONCLUSION AND POSSIBLE FUTURE WORK

The root systems of Eastern Hemlock are highly complex and there are probably a large number of factors that could impact the variability of the microbial community of these systems including: dominant flora of sites, pH, mycorrhizae, site history, geology, and other environmental factors. This study has shown that there is spatial variation in the soil microbial community of the Eastern Hemlock rhizosphere. This suggests that the environment has an influence on the hemlock microbe selection and there is a large pool of microbial diversity.

Bacterial 16S rDNA sequences were diverse and variable between sites with Albright Grove being less diverse than Cataloochee and Purchase Knob. Archaeal *amoA* genes are present and highly diverse and probably play an important role in the nitrogen cycle in the roots of Eastern Hemlocks. Bacterial *amoA* genes proved to be hard to amplify and when they were amplified showed less diversity and abundance than AOA.

Possible future studies could examine a number of other variables that could impact the diversity of the Eastern Hemlock microbial community. Since plants impact the soil diversity an examination of how various tree species impact the Eastern Hemlock root microbial diversity could be conducted. Hemlocks could be grown in conjunction with other tree species and diversity patterns could be examined. Also mycorrhizae have shown to impact diversity. An examination of diversity of mycorrhizae associated with hemlock roots could be done along with bacterial and archaeal studies. Also, an examination of ammonia oxidation potential by examining the concentration of ammonia in the soil could be performed. Examination of mRNA transcripts of *amoA* genes would

allow for examination of active genes. Culturing AOA and AOB from hemlock rhizosphere would allow for identification of organisms. This would allow further insight into the diversity and abundance patterns of AOA and AOB.

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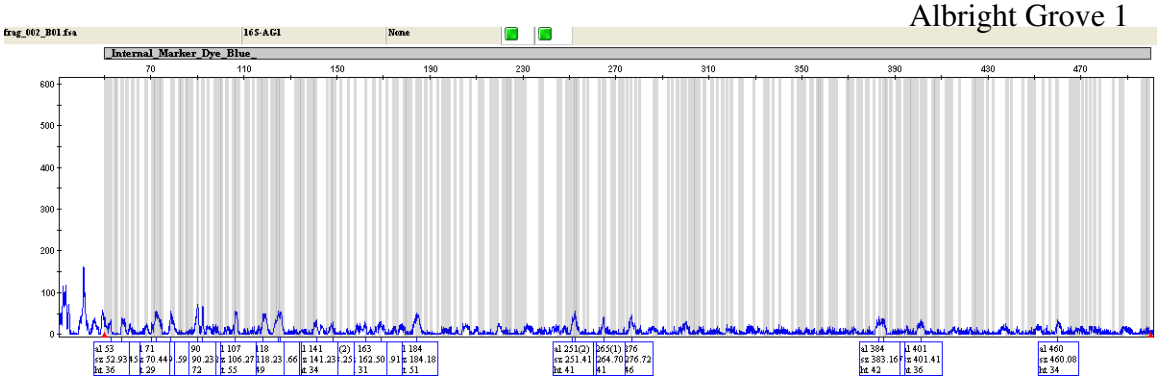
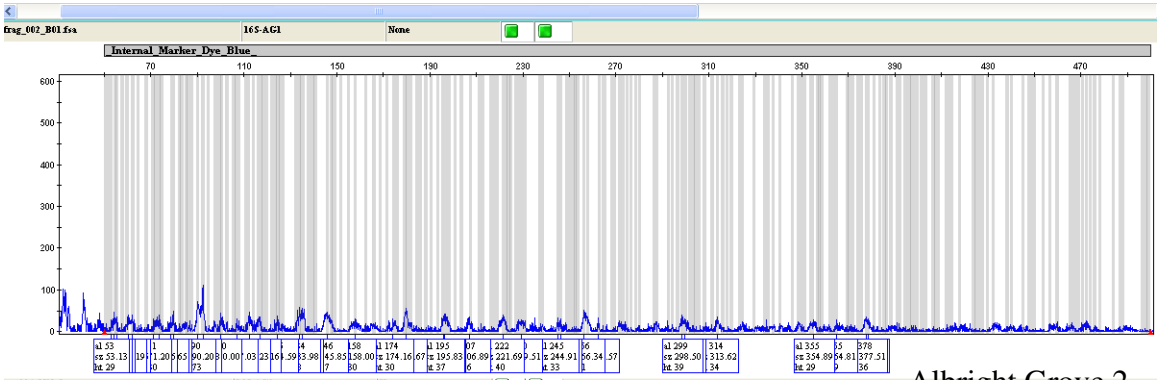
APPENDIX A

Bacterial 16S rDNA T-RFLP Electroferrograms

Albright Grove 1





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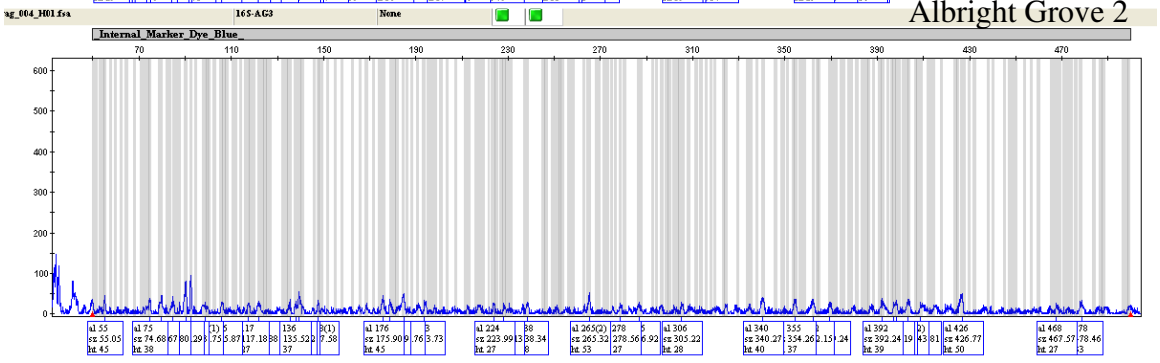

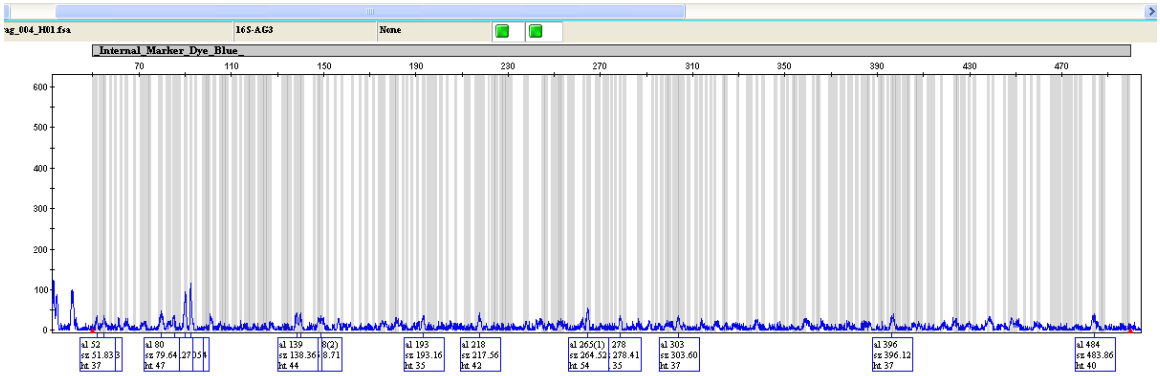

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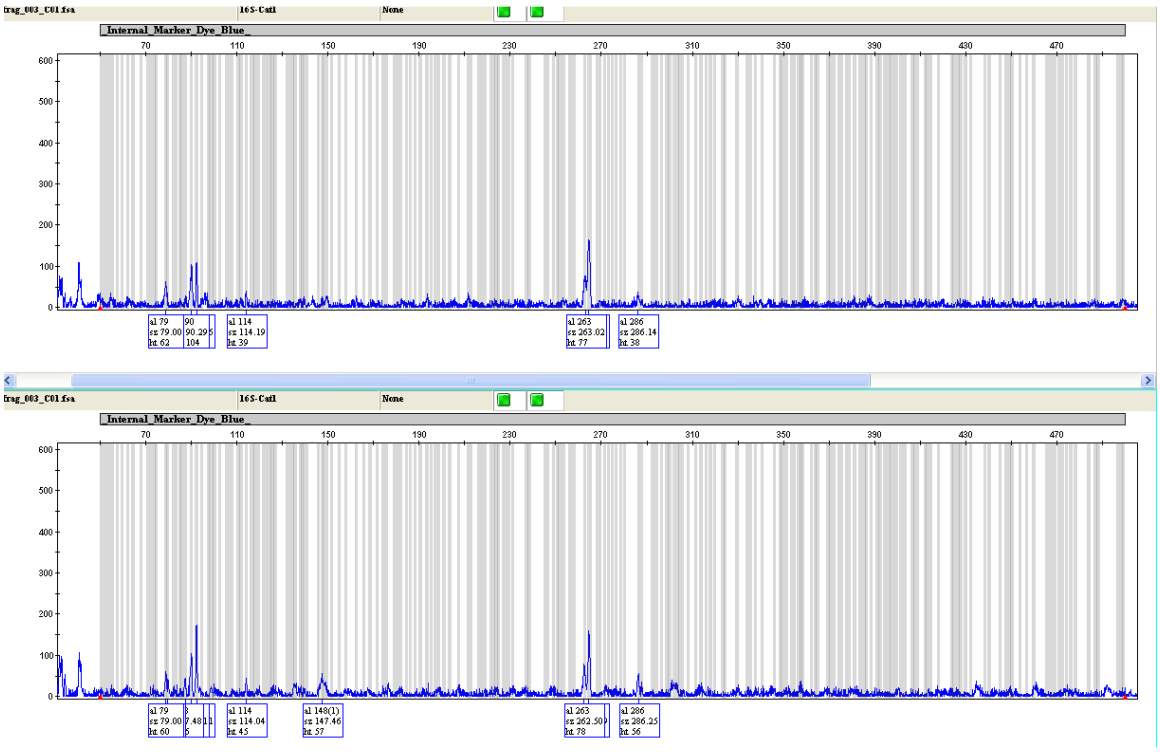

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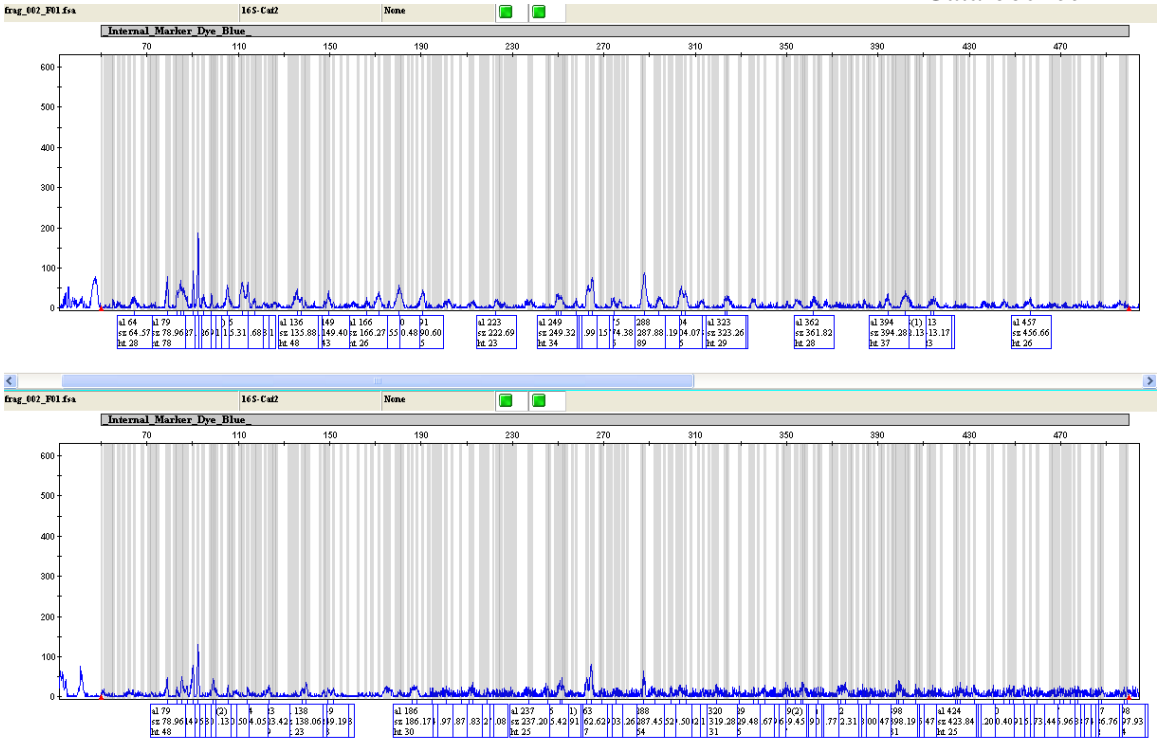

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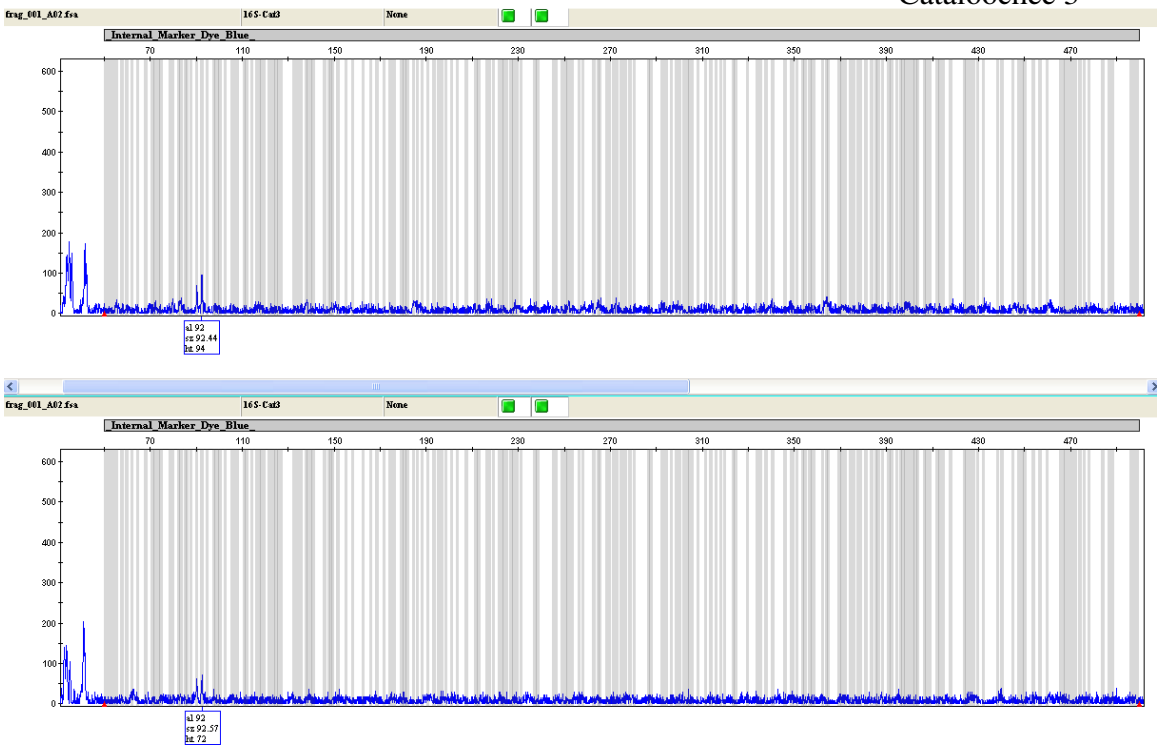

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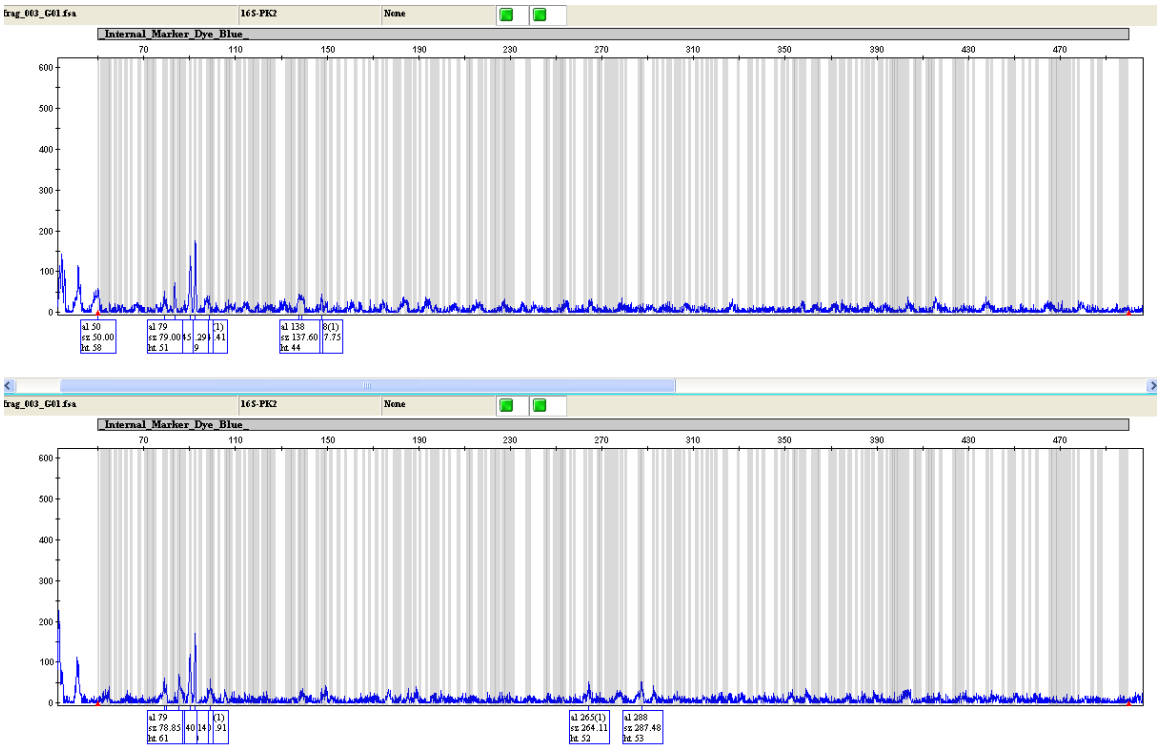

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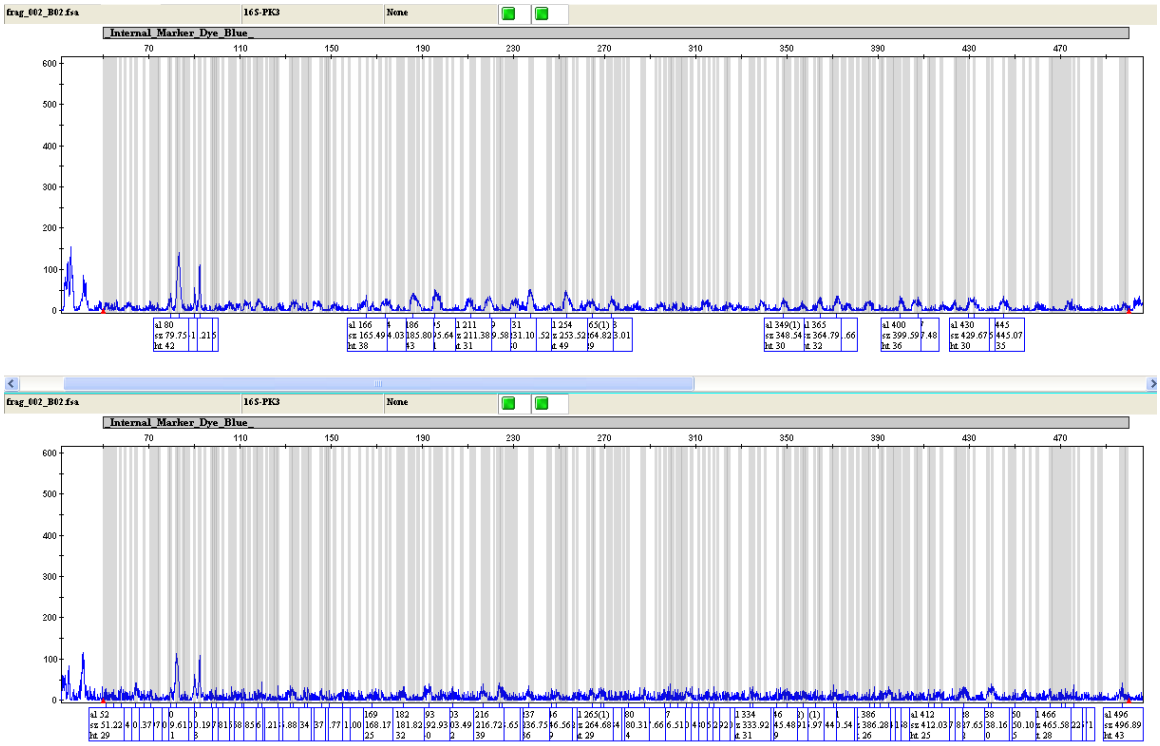

APPENDIX B

Archaeal *amoA* T-RFLP Electroferrograms

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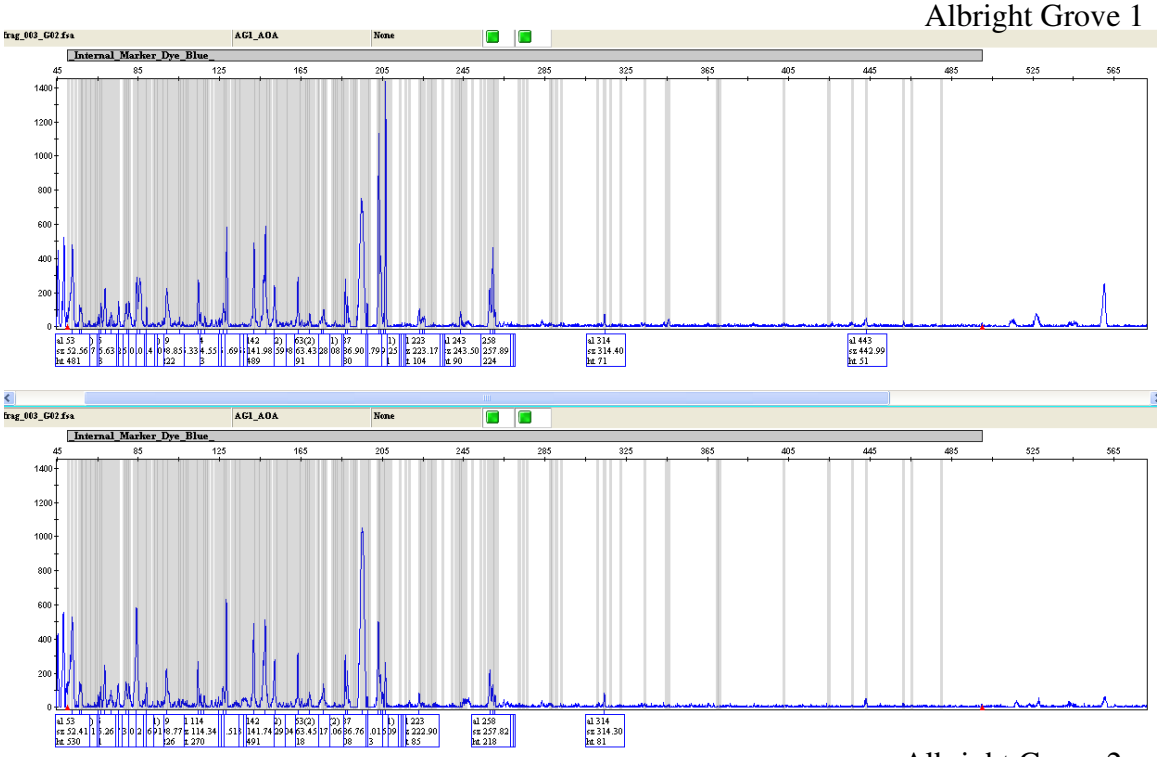

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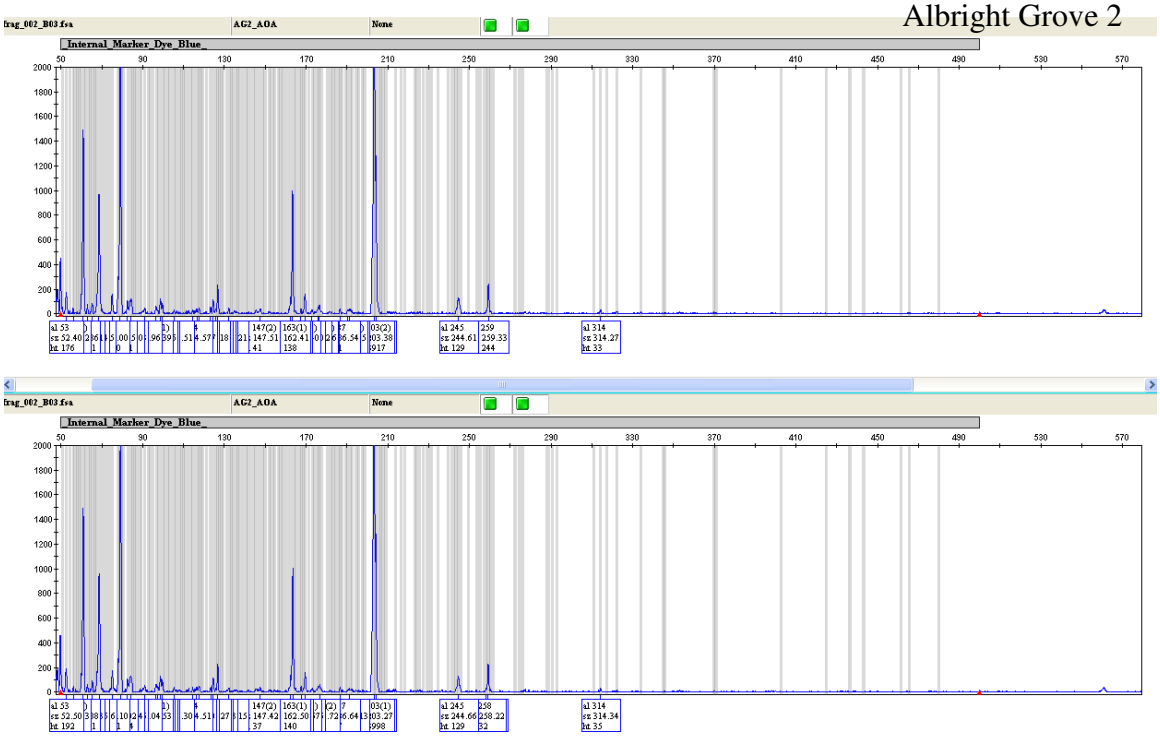

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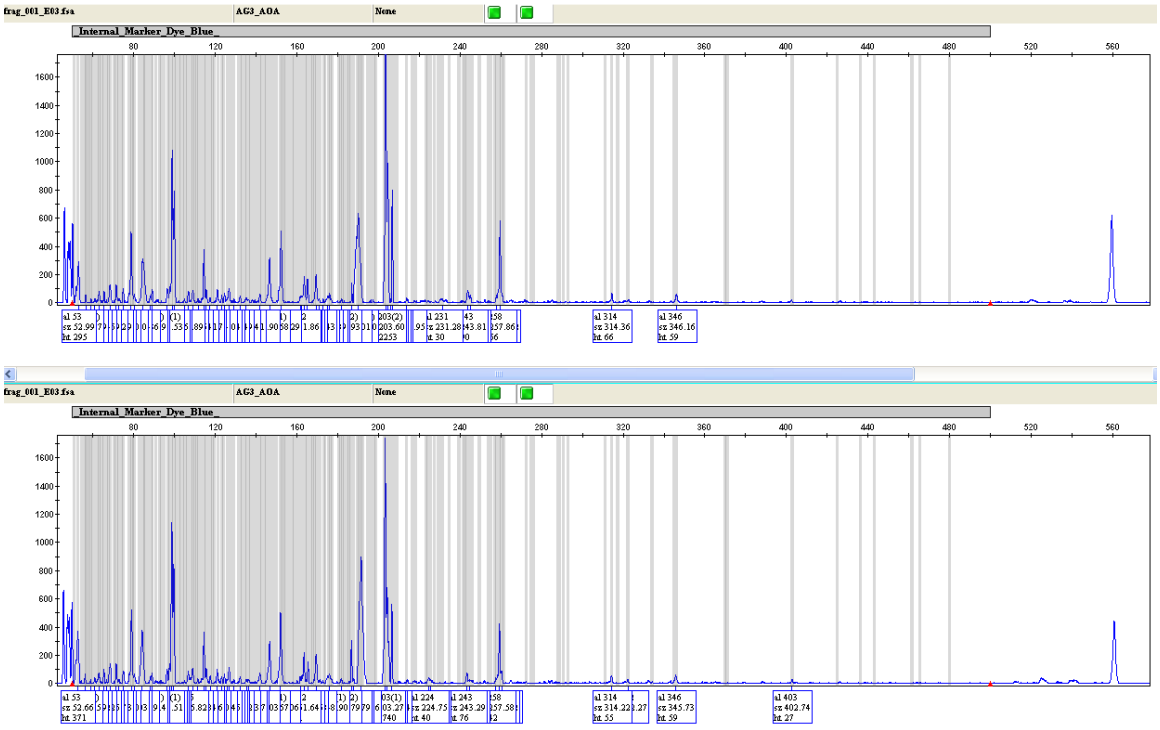

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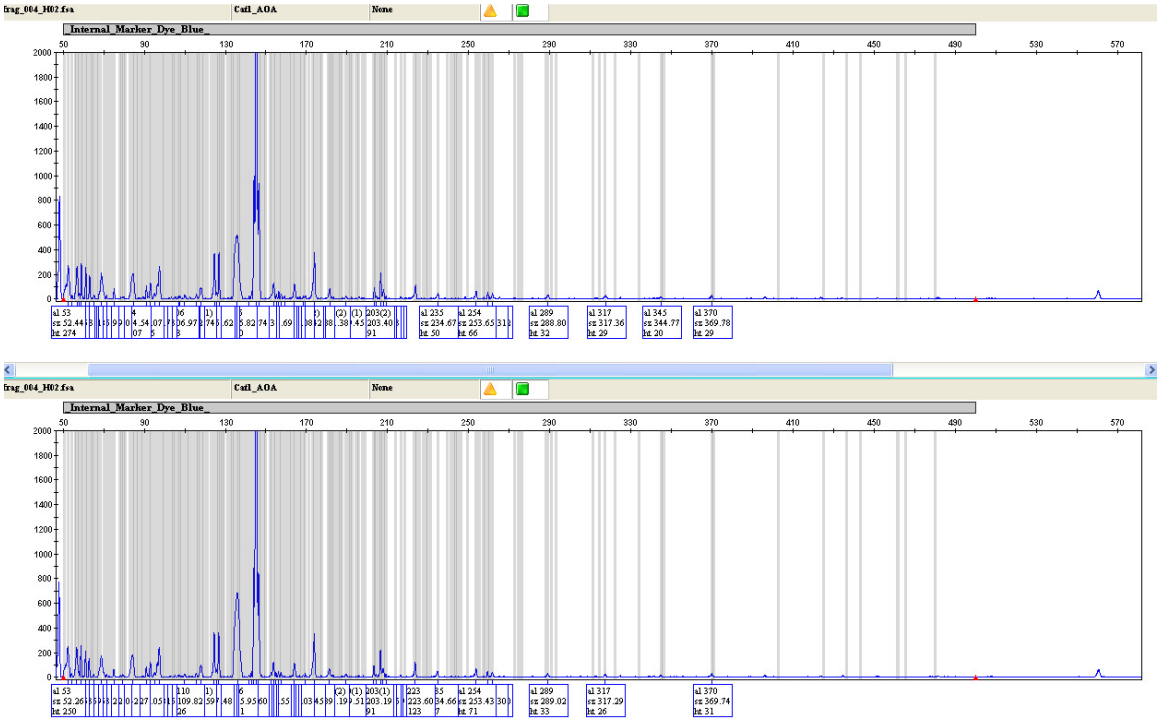

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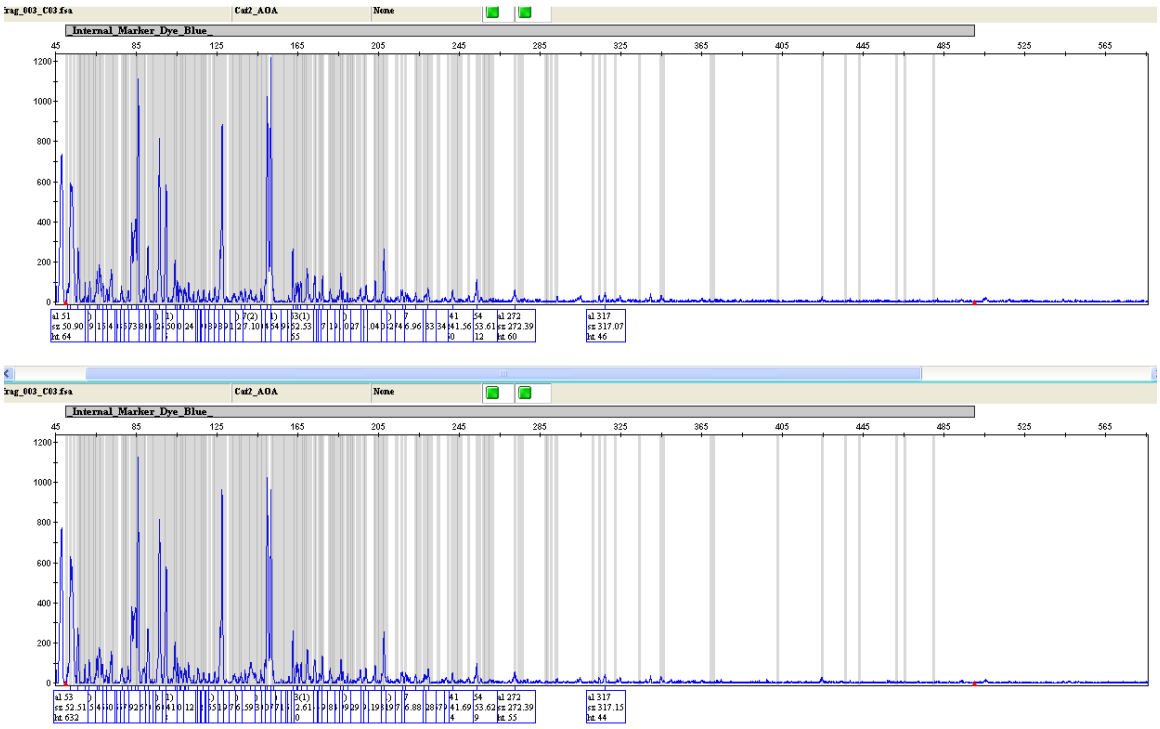

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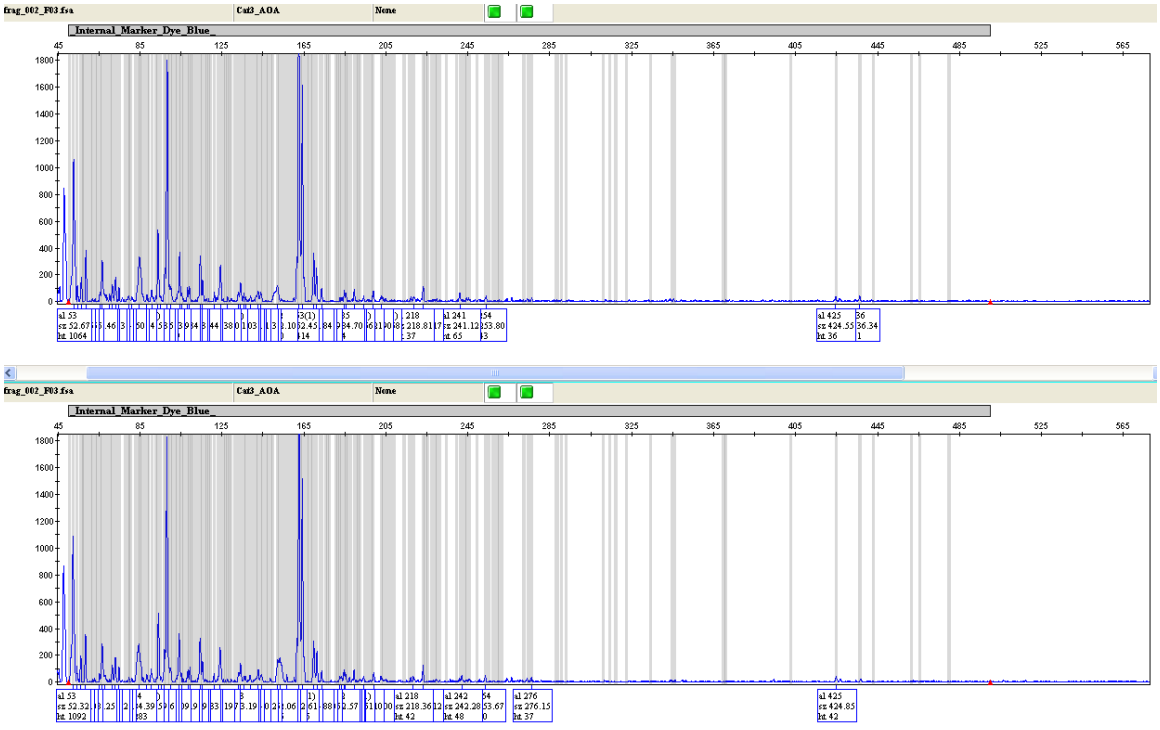

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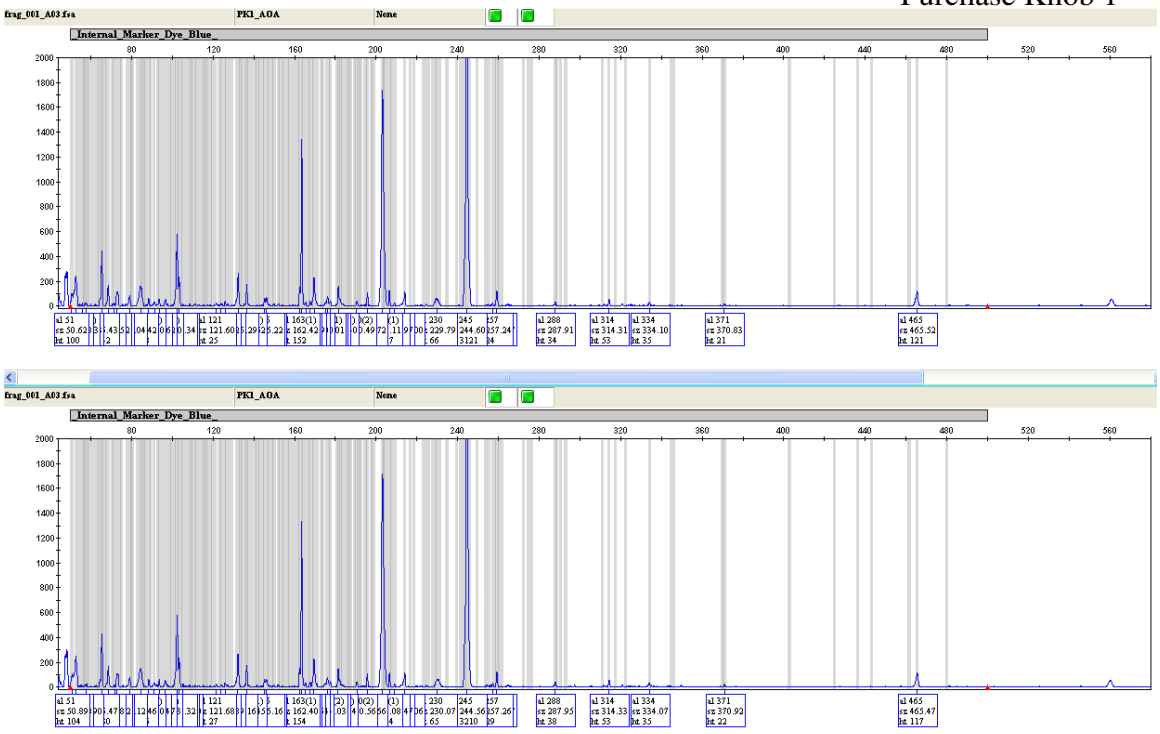

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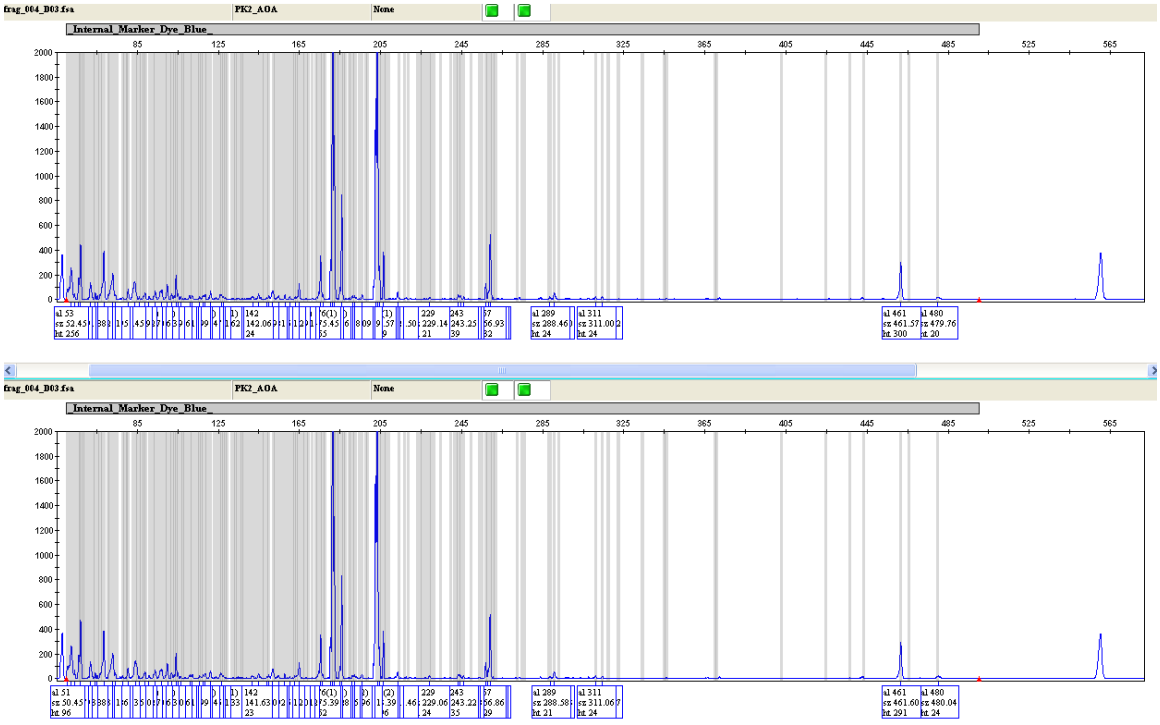

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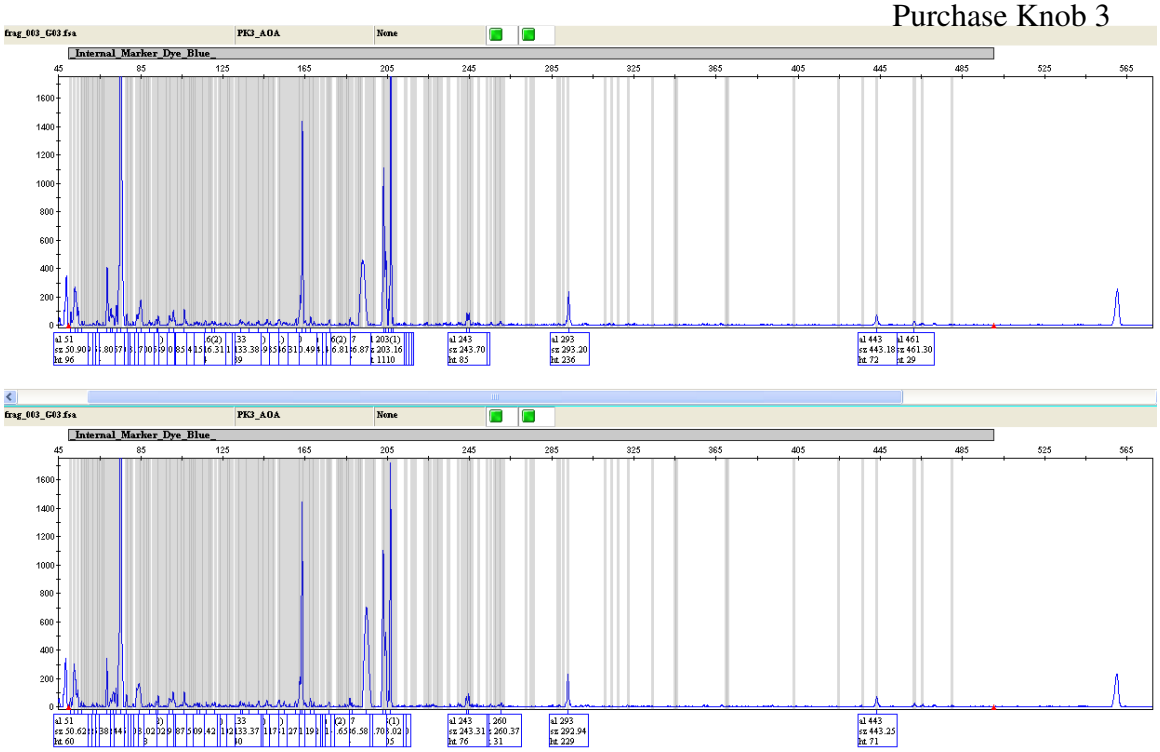

APPENDIX C

Bacterial *amoA* T-RFLP Electroferrograms

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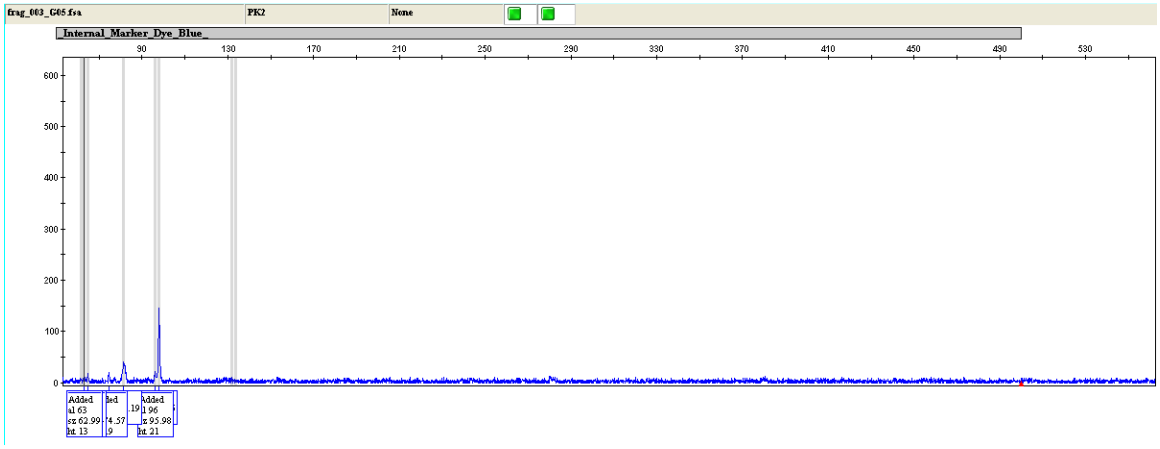

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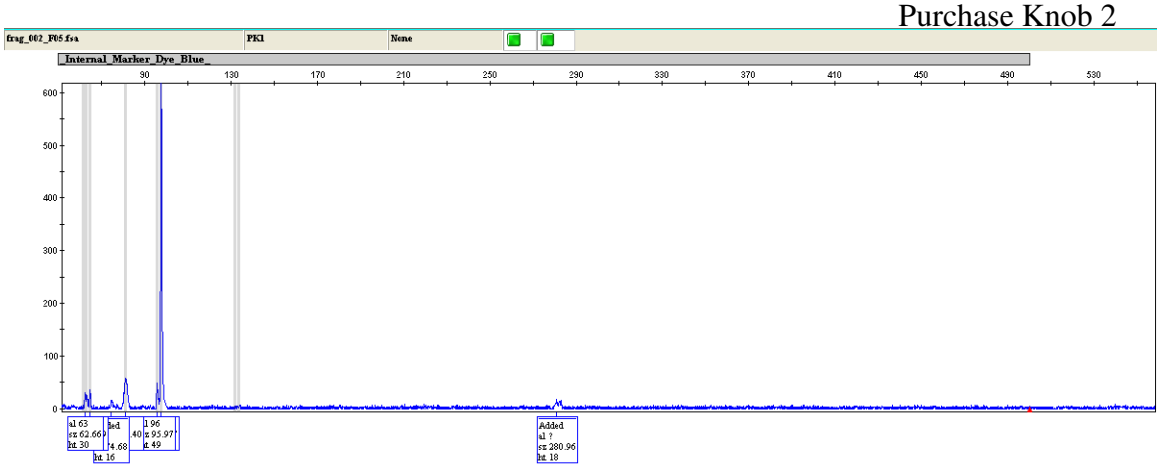

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