VERRUCOMICROBIA: A MODEL PHYLUM TO STUDY THE EFFECTS OF DEFORESTATION ON MICROBIAL DIVERSITY IN THE AMAZON FOREST

by

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ABSTRACT

VERRUCOMICROBIA: A MODEL PHYLUM TO STUDY THE EFFECTS OF DEFORESTATION ON MICROBIAL DIVERSITY IN THE AMAZON FOREST

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The Amazon rainforest is known for having a very high diversity of plants and animals. However, it is one of the least understood ecosystems regarding microbial diversity. Microorganisms are important for the ecological balance of any ecosystem and play important role in various biogeochemical cycles. As the Amazon rainforest undergoes to rapid deforestation, loss of its biodiversity is expected. This research aims to determine the effects of deforestation on the soil microbial diversity of the Amazon forest. Toward this, we selected the phylum *Verrucomicrobia* as a model for observing changes in the microbial structure of rainforest soils. Samples were collected from a research site in the Eastern Amazon basin, Fazenda Nova Vida, State of Rondonia, Brazil. Three different treatments were considered during sampling: a primary forest, a 20 year old pasture, and a secondary forest that was developed after the pasture has been abandoned. Total soil DNA was extracted and used for amplification of the gene 16S rRNA through PCR with specific primers targeting *Verrucomicrobia*. PCR amplicons were cloned and transformation was carried out into *Escherichia coli*. After screening, positive clones were sequenced and analysis of the 16S rRNA gene was performed. A total of 750 sequences have been analyzed both at taxonomic and phylogenetic levels. Contrary to our predictions, alpha diversity was higher for pasture and secondary forest, indicating that land use did not decrease local species richness. However, deforestation changed the diversity and distribution of verrucomicrobial population.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Introduction

The Amazon rainforest is the largest tropical rainforest of the world and covers two fifth of the entire continent of South America. The area of the Amazon rainforest is enormous and stretches across nine countries with most of it lies in Brazil followed by Peru, Colombia, Venezuela, Ecuador, Guyana, Suriname, Bolivia, and French Guiana. In fact, about 60% of the total Amazon rainforest is found in 7 out of 27 states of Brazil. Geographically, the Amazon rainforest is located between the Tropic of Cancer and the Tropic of Capricorn and is approximately 2-4^{o} south of Equator. The sunlight strikes at about 90^{o} angle in this region, 12 hours a day and is responsible for the intense heat and humidity. The extreme heat causes the formation of a convection zone where the hot rising air loses its moisture through recurrent rainstorms. Heavy rainfall is quite common in the Amazon rainforest and it rains 130-250 days per year. The Average precipitation is 2,200 mm/year and the humidity remains constant at 80%. The average temperature in the rainforest is 26^{o}C and the temperature difference between day and night is more than that of the difference between the seasons.

Amazon, world's largest river in terms of area of drainage basin runs across the Amazon rainforest. Often described as the lifeline of Amazon rainforest, the River Amazon plays a vital role in supporting life in Amazon rainforest. Originating from the Peruvian Andes, the river is 6500 kilometers long and run across Northern South America. The annual average discharge is $6\times10^{12}m^3$ into the Atlantic Ocean (Richey, 1986). This is interesting because the Amazon basin receives a total influx of $14.1\times10^{12}m^3$ of water every year which means that about 50-60% of the precipitation goes back to the atmosphere (Salati, 1986). The recycling of water across the ecosystem is primarily achieved by the

process of evapotranspiration mediated by the enormous vegetation of the Amazon rainforest (Victoria *et al*., 1991). The rainforest adds water to the atmosphere in form of water vapor through transpiration leading to the formation of clouds which returns the rainwater back to the forest. The rainforest helps to maintain the hydrologic cycle across the basin as well as affects other biogeochemical cycles within the basin (Richey, 1989). The condensational energy produced due to the convection precipitation within the basin has been found to affect global climate patterns (Paegle, 1987). This is extremely important and relevant in the present times when Amazon rainforest is undergoing unprecedented deforestation and global warming has emerged as the biggest problem faced by mankind.

Intense heat, high temperature, and plentiful precipitation year round, and prevalent nutrient recycling has led the Amazon rainforest to support the most diverse plant and animal species on earth. Amazon rainforest is often described as the richest and most threatened reservoir of plant and animal life on earth. The rainforest is also home to 1,294 birds, 427 amphibians, 427 mammals, and 378 reptiles and about 3,000 fish species (Rylands *et al*., 2000). The Amazon basin has about 40, 000 plant species of which 12,500 are tree species and 3,248 tree species have a population size of more than 1 million individuals (Hubbell, 2008). The pioneer work of T.L. Erwin on insect diversity estimated 12,488 species of beetle per hectare of tropical forest canopy. He also estimated about 30 million species of insects to be present in this region. The number of endemic species supported by the Amazon rainforest is exceptional. Jaguar, tapir, giant otter, pink river dolphin, sloth, anacondas, boas, poison dart frog, piranha, electric eel, harpy eagle, toucan, manatee, to name just a few of the many marvels of nature in Amazon. Over the course of millions of years, the Amazon rainforest has evolved into a complex system where different species are intertwined and interdependent on each other and this enormous biodiversity helps this ecosystem to function properly. The immense biodiversity provides resilience and resistance against environmental change (Chapin III *et al*., 2000). Loss of biodiversity leads to changes in the ecologically crucial processes of nutrient recycling and pollination (Powell and Powell, 1987).

The Amazon rainforest not only sustains the enormous biodiversity but it also acts as the major terrestrial sink of carbon dioxide. Carbon dioxide is a major greenhouse gas and humans emit large amount of this gas in the atmosphere by burning biomass and fossil fuels. The Amazon rainforest recycles carbon dioxide into oxygen through photosynthesis and respiration and helps to maintain global carbon balance. Infact, about 15% of the global photosynthetic activity takes place in Amazon rainforest (Malhi *et al*., 1998). Various studies have suggested a carbon uptake of 0.85-6.0 t C ha^{-1} $year^{-1}$ by the Amazon rainforest (Ometto *et al*., 2004, Grace *et al*., 1995, Malhi *et al*., 1998). The Amazon rainforest stores huge quantities of carbon dioxide not only in its vegetation but also in the soil. The soil in Brazilian rainforest may contain up to 136Pg of carbon (Fearnside and Barbosa, 1997) while the vegetation in Brazil holds about 70Pg of carbon.

1.2 Destruction of Amazon rainforest and its causes

The Amazon rainforest is undergoing deforestation at an alarming rate. About 80% of the deforestation takes place in Brazil which contains more than 60% of the rainforest. Untill 1960's, human impact in this Brazilian rainforest was kept to minimum but with the construction of the Belém-Brasilía highway, the destruction of Amazon increased tremendously (Moran, 1993). The highway cut through the largely intact forest and exposed it to exploitation by cattle ranchers and landless farmers. Extensive tax incentives offered for the economic growth by the government between 1964 and1985 further accelerated the deforestation in Brazil. Cattle ranching, mining, logging of timber trees, and hydroelectric projects were undertaken at huge scale which profoundly affected the Amazon rainforest. Deforestation has already caused a loss of 6-11% of the original forest (Fearnside and Barbosa, 1998) and the total forest loss was estimated to be 837,000 km^2 in 2001 (Malhi, 2007). The average deforestation rate between 1988 and 2008 has been about 18,000 km^2 (Figure 1.1). However, the rate of deforestation escalated to new heights between 2002 and 2005 partly credited to a boost in the soybean and beef demand in the international market (Fearnside, 2005).

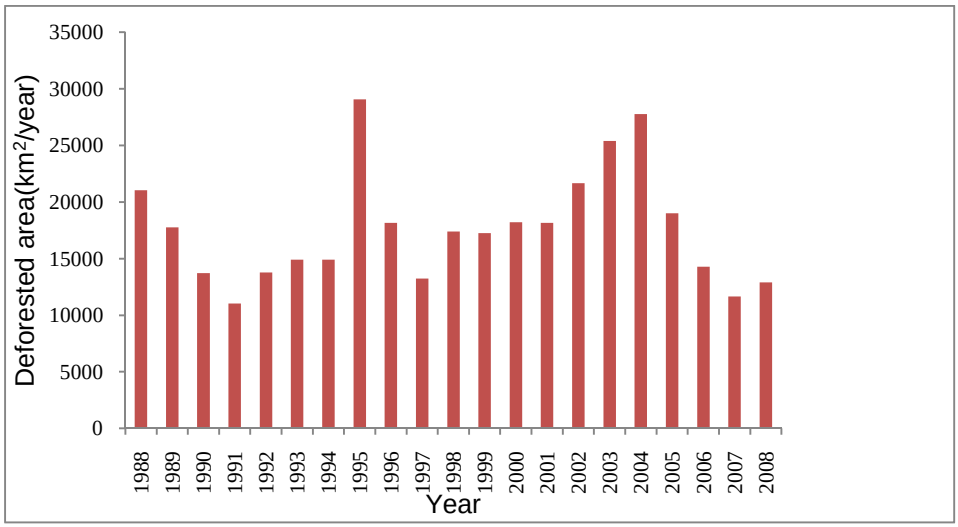


Figure 1.1: Annual rate of deforestation in the Amazon 1988 -2008, INPE (2010).

Cattle ranching for beef production has emerged as the most significant cause of clearing of Amazon rainforest. As mentioned previously, Brazilian government encouraged cattle ranching during 1965-1980 through favorable tax incentives (Moran, 1994). Thousands of square miles of Brazil's rainforest were cut down to make space for pastures. Pastures were mostly developed by the practice of slash and burn technique and then planting fast growing grasses. The extent of the growth of cattle ranching sector between the periods of 1965-1980 can be imagined by the facts that the number of cattle in this period increased from almost 0 to about 5 million and annual forest-pasture conversion rate was about 8,000-10,000 km^2 (Mahar, 1988). The forest-pasture conversion continues to drive deforestation in Amazon rainforest and the cattle number has surpassed 60 million. Today, cattle ranching has become a vital part of Brazil's economy. Brazil is presently the largest exporter of beef and the beef export generates revenue of three billion dollars a year for Brazil government. Cattle ranching not only catalyses the rate of deforestation but it also has a disastrous effect on the rainforest soil. In the absence of deep rooted trees on a pasture land, the rate of erosion is high which drains out the nutrients from the top layer of the soil making it incapable of supporting the larger fauna with extensive root system again. The fast

growing grasses which most of the times are not indigenous to Brazil suck up the remaining nutrients and further deteriorate the soil quality.

Another agricultural practice which plays a major role in the destruction of Amazon rainforest is soybean plantation. Inexpensive land, favorable government policies and climate conditions have made Brazil a lucrative destination for soybean farming (Kirby *et al*., 2005). The area for soybean farming in Brazil has increased from 1.7 million hectares in 1994 to 21.0 million in 2003 (US Department of Agriculture). The tremendous growth of biofuel industry in the last decade has also increased the demand of soybean. Due to an increase in the prices of soybean worldwide, the Brazilian government has encouraged its plantation through heavy investments in transportation sectors including highways, waterways, and railways (Fearnside, 2005). Infrastructure development, especially highway construction, puts the rainforest in jeopardy by making it more vulnerable to logging of timber trees. Timber trees, especially mahogany, are in a great demand in the International market. The timber trees are not uniformly distributed in the rainforest but appear in patches. Hence, once the trees are cut, new logging roads are constructed to transport trees to the mills, which further cause the destruction of rainforest. About 3,000 km of roads across have been estimated to be constructed by loggers (Verissimo *et al*., 1994). After logging, the free land is converted into cattle pastures or soybean plantations. Hence, soybean farming initiates a vicious cycle of events leading to rainforest destruction at every step.

The cumulative effect of cattle ranching, soybean farming, timber logging, and infrastructure improvement has been detrimental to the Brazilian rainforest and unless the government takes stringent action to curb deforestation, the boundaries of the world's greatest natural resource will continue to diminish.

1.3 Effects of deforestation

The Amazon rainforest has an indispensable role in the Earth's ecosystem. The largest biological reservoir of plant and animal species controls temperature and precipitation as well as balances the flux of atmospheric gases. The enormous destruction of forest due to deforestation is expected to have a

considerable effect in the functioning of Earth's environment. The Amazon rainforest produces half of its rainfall through water recycling. Reducing the vegetation could decrease the amount of evapotranspiration and disrupt the hydrological cycle operating in this region (Shukla *et al*., 1990). Deforestation affects regional as well as global precipitation patterns as the water vapor from the basin is transported to south and south central Brazil, Paraguay, Uruguay, Argentina and southern Africa (Fearnside, 2005). These changes in the rainfall patterns increase the incidences of floods and droughts. In 2005, the southwestern region of Amazon rainforest faced one of the worst droughts of the decade when the river level was reduced to about 40 feet (http://news.mongabay.com/2005/0930-amazon_river.html). Droughts also increase the probability of forest fires which destroy rainforest and its inhabitants at very large scales. Loss of vegetation cover across Amazon also leads to increased soil erosion. In the absence of extensive root system of large trees, the nutrients present in the soil wash away very quickly leading to loss of soil fertility. This is extremely important for Amazon rainforest, as most of the nutrients are present in the topmost layer of the soil and soil erosion can be disastrous for future growth of plants.

Figure 1.2: Satellite pictures of the city of Ariquemes, the state of Rondonia, Brazil documents the extent of deforestation for the years 1975, 2001, and 2008.

Deforestation in Amazon rainforest impacts the global climate not only by disrupting hydrological cycle but also by destructing the largest carbon sink on Earth. As the vegetation cover of Amazon continues to diminish due to large scale deforestation, the carbon dioxide content in our atmosphere increase leading to enhanced greenhouse effects which in turn can increase the temperature of Earth by

as much as $4^{o}C$ (Malhi *et al*., 2008). The Amazon rainforest corresponding to 0.40% of Earth's total area is responsible for 5-20% of the global emissions (Ometto *et al*, 2005). Studies conducted over the past few decades indicate that climate change induced by anthropogenic factors has severe and possibly irreversible effects on the extent and composition of the rainforests (Malhi *et al.*, 2008).

The Amazon rainforest is regarded as a carbon sink with both living and non-living components of this biome storing huge quantities of carbon dioxide. Owing to deforestation, the Amazon rainforest has actually become a source of carbon dioxide. The deforestation in the Amazon is followed by burning the remaining biomass to clear the field for pastures. This practice of slash and burn releases large amounts of carbon dioxide into the atmosphere. Deforestation, in year 2003 alone, was found to produce about approximately 429×10^{6} tons of carbon dioxide equivalent carbon emissions (Fearnside, 2005). The decline in forest cover and the increasing rate of carbon dioxide emissions in the Amazon have continued to grow in last few decades and a number of models have already predicted the decrease in precipitation and increase in global temperature due to forest-pasture conversions (Nobre *et al*., 1991; Shukla and Nobre, 1990). Conservation of the Amazon forest has become extremely important to reduce the carbon dioxide emissions and stabilize the climate on Earth.

The destruction of Amazon rainforest along with disrupting the biggest carbon sink and hydrological cycle also endangers the million of plant animal species residing in the region. Most of the animal groups including mammals, primates, and birds have been found to decline or extinct due to fragmentation of Amazon rainforest (Lovejoy *et al*., 1986; Rylands and Keuroghlian, 1988; Bierregaard and Stouffer, 1997). The population of insects including beetles, ants, and butterflies is also negatively impacted by the process of deforestation (Klein, 1989; Brown and Hutchings, 1997; Carvalho and Vasconcelos, 1999). Moreover, construction of roads for deforestation makes the wildlife especially primates, birds and large carnivores vulnerable to hunting and poaching. Destruction of the Amazon rainforest has put the biodiversity in this region under tremendous pressure and the biodiversity is expected to diminish if the deforestation is not controlled.

1.4 Bacterial diversity in the Amazon rainforest

The Amazon rainforest has been a center of curiosity for ecologists due to its rich plant and animal diversity. About 10% of the world's known species live in the Amazon rainforest (Lewinsohn and Prado, 2005). Despite its vast plant and animal diversity and the long history of diversity studies, the Amazon rainforest is among the least understood ecosystems regarding bacterial diversity. There have been very few studies done to characterize the soil bacterial diversity present in the Amazon rainforest.

In one of the first studies to characterize bacterial diversity in Amazon rainforest, Manfio and Goodfellow (1995) used extraction methods to detect the presence of *Streptomyces* in the soil. Linhares (1995) used dispersion and centrifugation techniques and identified *Actinomycetes* in the Amazon rainforest soil. However, the methods used in both these studies were culture based methods which are the not best indicators of bacterial diversity. In fact, culture based methods have been found to isolate less than 1% of the total soil microbes (Amann *et al.,* 1995).

Bornemann and Triplett (1997) reported the first culture independent study of soil microbial biodiversity in the Amazon area. They sampled 98 bacterial 16S rRNA gene sequences from two different soil samples in the state of Para and concluded that all sequences were unique. Schloss and Handelsman used Distance Based OTU and Richness determination (DOTUR) to estimate the microbial diversity and concluded the presence of only two pairs of identical sequences indicating the enormous diversity in this region. Contradicting these results, Fierer and Jackson (2006) compared bacterial communities in 98 soil samples across North and South America based on the T-RFLP technique, and showed that a Peruvian Amazon soil had low bacterial diversity. Periera *et al*., (2006) identified several bacterial populations including *Proteobacteria, Bacteroidetes, Firmicutes, Acidobacteria* and *Actinobacteria* in the forest soil and concluded that the Amazon rainforest had high bacterial diversity. Kim *et al.,* (2007) reported that the bacterial communities were of moderate taxonomic richness in the pristine forest of Western Amazon and recognized *Acidobacterium* as the predominant phylogenetic

group to be present. *Proteobacteria, Actinobacteria, Planctomycetes* and *Verrucomicrobia* were the other groups identified in this region.

Owing the deforestation, there is an increased interest on characterizing the bacterial community structure in the Amazon rainforest. Although very few publication have addressed this question, they all agree that bacterial communities are affected by the ongoing deforestation and pasture development (Jesus *et al*., 2009; Cenciani *et al*., 2009)

We expect the Amazon rainforest to have a very high microbial diversity based on two observations. Firstly, the diversity of plants and animals is enormous in the Amazon forest (Kreft and Jetz, 2007) which could indicate diverse environmental conditions. Such differences in the environment can lead to higher microbial diversity. Secondly, the higher plant diversity means a variety of substrates and niches for microbes to grow and hence a higher microbial diversity.

1.5 *Verrucomicrobia*

Due to the enormously high diversity in the microbial world, a practical approach is to focus on one phylum. I will study the diversity within the phylum *Verrucomicrobia* and use this phylum as model to study the effect of deforestation on microbial diversity.

The bacterial phylum *Verrucomicrobia* has been divided into divided into seven monophyletic subdivisions based on the 16S rRNA gene studies (Schlesner *et al*., 2006). However, only three subdivisions namely *Verrucomicrobiaceae* (subdivision 1)*, Xiphinematobacteriaceae* (subdivision2), *and Opitutaceae*(subdivision 4) contain cultured isolates and are recognized in the Bergey's manual of Systematic Bacteriology (Garrity *et al.,* 2003). First recognized as a separate phylum about 20 years ago, it now consists of 12 genera including *Verrucomicrobium* (Schlesner, 1987), *Prostheobacteria* (Staley *et al*., 1976), *Akkermansia* (Derrien *et al*., 2004), *Xiphinematobacter* (Vandekerckhove *et al*., 2000), *Chthoniobacter* (Sangwan *et al*., 2004), *Opitutus* (Chin *et al*., 2001), *Rubritalea* (Scheuermayer *et al*., 2006), and *Victivallis* (Zoetendal *et al*., 2003). All the isolates of *Verrucomicrobia* are Gram-negative;

cells can be coccoid or rods shaped and divide by binary fission or unequal cell division (Schlesner, 2006). The oxygen requirement varies throughout this phylum; the members are strict aerobes, facultative anaerobes or strict anaerobes.

Verrucomicrobia is a universally distributed phylum and has been known to be present in a variety of habitats. This phylum is mostly represented by a large collection of 16S rRNA genes with a very few cultured isolates. About 5% of all the 16S rRNA genes recognized till now belong to phylum *Verrucomicrobia* (Sangwan *et al,* 2004). First observed in freshwater (Henrici and Johnson, 1935), this phylum has already been discovered in termite gut, human intestines, and sea cucumbers as well as in very extreme environments including hot springs (60^{o}C), fumaroles (70^{o}C, pH 0.8-2.0), and Antarctica (Islam *et al.,* 2008; Pol *et al*., 2007; Derrien *et al.,* 2004; Stevenson *et al.,* 2004; Pearce *et al.,* 2003; Sakai *et al*., 2003; Borneman and Triplett, 1987). *Verrucomicrobia* is also one of the most important members of the soil bacterial community. Members of this phylum have been found to make up 1-10% of the bacterial 16S rRNA in soils (Buckley and Schmidt, 2003). As a numerically abundant phylogenetic group, *Verrucomicrobia* was estimated to range from 10^{6} to 10^{8} cells per gram of dry weight soil (Felske and Akkermans, 1998). The abundance of this phylum in the soil indicates a significant role in the various global chemical cycles. In spite of being one of the most abundant bacterial groups present in soil, not much in known about their ecological importance or factors that affect their distribution and diversity in soil.

Lack of enough cultured isolates has further restricted our understanding of this phylum. There are more than 9,000 verrucomicrobial 16S rRNA gene sequences currently available in the Ribosomal Database Project (http://rdp.cme.msu.edu); interestingly 99.9% of these sequences are from uncultured organisms. The few cultured species of this phylum have revealed them to have characteristics atypical of other bacteria. Three members of this phylum, *Prostheobacter dejongeii*, *Prosthecobacter vanneervenii* and *Prosthecobacter debontii* have been shown to posses the eukaryotic tubulin homologs BtubA and BtubB (Jenkins *et al*. 2002). Tubulin is a eukaryotic protein involved in the formation of microtubules for

cell division. In prokaryotes, the process of cell division is achieved by FtsZ and this protein is universally present in Domain Bacteria. The presence of tubulin homologue in place of FtsZ protein in the three species of *Verrucomicrobia* represents a link between prokaryotes and eukaryotes and can provide insight into the process of evolution.

Most of the *Verrucomicrobia* isolates have been found to utilize carbohydrates for their energy needs. However, three very recent studies have shown members of this phylum to be involved in methanotrophy. These three bacteria were isolated from three geographically distant locations; a fumarole in Southern Italy, a steaming soil in New Zealand, and an acidic hot spring in Russia (Dunfield *et al*., 2007; Pol *et al.*, 2007; Islam *et al.*, 2008) and all were found to use methane (CH_4) as their carbon source. This finding has made *Verrucomicrobia* the only group that contains aerobic methanotrophs outside of the *Proteobacteria.* Another reason which makes this discovery even more significant is that all three members have been isolated from extreme environmental conditions of pH 1-3.5 and temperatures of 50^oC-70^oC. None of the known methanotrophs can grow at temperatures more than 60^oC and pH below 4. This discovery has proved the methantrophs to be more genetically and ecologically diverse than previously thought and indicated role of phylum *Verrucomicrobia* in the metabolic pathways of C_1 utilization.

Another interesting aspect of *Verrucomicrobia* is their probable role in cellulose degradation. Preliminary analysis of the genome of the *Verrucomicrobium spinosum* has indicated its genomic capability to degrade cellulose. Presently, five strains of *Verrucomicrobia* namely TAV1-TAV5 (Termite Associated *Verrucomicrobium*) are being tested for cellulose degradation (Jantiya Isanapong, personal communication). The five strains have been isolated from the hindgut of wood consuming termite *Reticulitermis flavipes*. Degradation of cellulose to fermentable sugar is a major hindrance in the field of biofuel production. Further research to understand the principles governing the ecology and diversity of *Verrucomicrobia* can open new avenues in the field of biofuel production.

Another reason to choose phylum *Verrucomicrobia* in this study is that it is already known to be present in the Amazon soil (Borneman and Triplett, 1997). In a study, *Verrucomicrobia* was one of the bacterial groups found in the pristine forests soils of Western Amazon (Kim *et al*., 2006).

Based on the current lack of knowledge about the ecological and functional role of *Verrucomicrobia*, its abundance in Amazon rainforest soil, ability to utilize methane, and probable role in cellulose degradation it would be interesting to study the distribution and diversity of *Verrucomicrobi*a in Amazon rainforest soil.

1.6 16S rRNA gene analysis

I will assess the *Verrucomicrobia* diversity in different soil samples from the Amazon based on the natural sequence variation of the 16S rRNA gene. First developed by Woese in 1980's, this method revolutionized the field of microbial ecology and helped to define the three domains of life. The 16S rRNA gene sequence analysis is being extensively used to study the microbial diversity and phylogeny (Barns *et al*., 1996; Dunbar *et al*., 2000; Hill *et al*., 2000; Hugenholtz *et al*., 1996; Weisburg *et al*., 1991). In this analysis, the 16S rRNA gene is recovered directly from the natural environments and the phylogenetic diversity of the microorganisms present is determined. The 16S rRNA sequence is about 1,500 bp long and consists of conserved and variable regions. The variable region acts a signature sequence and helps to differentiate among bacterial species.

The 16S rRNA analysis along with other culture independent methods has helped to define the 80 phylum level divisions within *Eubacteria* domain (Pace*,* 1997). Most of these divisions are poorly represented by cultured organisms. Culture based methods have been found to isolate less than 1% of the total soil microbes (Amann *et al.,* 1995). The 16S rRNA method has obviated the need to culture microorganisms to study microbial diversity and has emerged as "gold standard" in microbial ecology studies. Genebank, a database of genetic sequence contains more than 100 million deposited sequences of which, about 280,000 are 16S rRNA sequences.

In the Ribosomal Database Project, *Verrucomicrobia* is one division which is defined by a large collection of 16S rRNA gene sequences, approximately 8,400 obtained from a wide range of environments. This makes the 16S rRNA analysis even more appealing in this study as we already have a repository of 16S rRNA sequences for comparison. The diversity of the total soil community using 16S rRNA analysis was found to be at least 200 times higher than the diversity of bacterial isolates from the same soil (Torsvik, 1998). Additionally, it is not necessary to sequence the whole 1500bp long 16S rRNA gene for identification as most of the variability in the 16S rRNA gene lies in the first 500bp (Kattar *et al.,* 2001).

Thus, the 16S rRNA analysis provides a more convenient and rapid alternative to culture based method to study bacterial diversity.

HYPOTHESIS

The conversion of forest to pasture will decrease the diversity of the phylum Verrucomicrobia in the Amazon.

CHAPTER 2

VERRUCOMICROBIA: A MODEL PHYLUM TO STUDY THE EFFECTS OF DEFORESTATION ON MICRBIAL DIVERSITY IN THE AMAZON RAINFOREST

2.1 Introduction

The Amazon rainforest is the largest equatorial forest of the world with an area over 6.9 millions square kilometers. It maintains the largest fresh water river of the world and regulates temperature and precipitation patterns (Malhi *et al*., 2008). The extensive evaporation and condensation processes taking place in this region help to generate rainfall across South America. It also controls the flux of atmospheric gases and lowers the Earth's average temperature. The Amazon rainforest is also known to be one of the most biodiverse regions on Earth (Mittermeier, 1988). To date, at least 40,000 vascular plant, 5,500 vertebrate, and 100,000 invertebrate species have been scientifically classified (Da Silva *et al.,* 2005; Lewinsohn and Prado, 2005), making this region the largest repository of animal and plant species on the planet. Considering the abundance of these life forms and the favorable living conditions prevalent throughout the year, it is tenable to suggest the prevalence of a wide variety of microorganisms in this region. However, the Amazon soil microbial diversity is especially poorly characterized, with few published studies presenting starkly conflicting views.

It is especially imperative to study the microbial diversity in the Amazon rainforest as this ecosystem has been under threat due to rapid large scale deforestation. Approximately 648.5 x 10^3 square kilometers of Amazon rainforest have already been cleared (Fearnside, 2003), to make space for pastures and agricultural farms (Fearnside and Barbosa, 1998). Though the Amazon rainforest lies in nine nations, most of the deforestation occurs in Brazil. As deforestation has decreased the biodiversity of plant and animal species in the Amazon substantially, it is expected to reduce the diversity of

microorganisms as well. Deforestation is expected to alter the structure of the bacterial communities in the Amazon. Understanding the consequences of this extensive deforestation requires insight into the diversity of rainforest microbes and their response to it.

This research is aimed to determine the effects of such destruction on microbial diversity. We used the 16S rRNA gene analysis to study the diversity within the bacterial phylum *Verrucomicrobia*. This is a universally distributed phylum of *Bacteria* and is a numerically abundant member of soil bacterial community (Buckley and Schmidt, 2003). It comprises up to10% of the total active bacteria in soil. It has been found in extremely diverse environments including termite gut, human intestines, and sea cucumbers as well as in very extreme environments including hot springs (60^{o}C), fumaroles (70^{o}C, pH 0.8-2.0), and Antarctica (Islam *et al.,* 2008; Pol *et al*., 2007; Derrien *et al.,* 2004; Stevenson *et al.,* 2004; Pearce *et al.,* 2003; Sakai *et al*., 2003; Borneman and Triplett, 1987). Despite their widespread distribution in the environment, their biological role is not very well understood mostly due to the very few cultured isolates available. Three members of this phylum have been shown to posses the eukaryotic tubulin homologs BtubA and BtubB (Jenkins *et al*. 2002). Recently, certain *Verrucomicrobia* are found to be involved in methanotrophy. All the methane utilizing bacteria discovered till now belonged to *Proteobacteria*. However, this methane utilizing ability of *Verrucomicrobia* has widened their metabolic diversity. *Verrucomicrobia* are also found to be involved in cellulose degradation (Jantiya Isanapong, personal communication) and nitrogen fixation (Khadem, 2010). The lack of information about the ecology of *Verrucomicrobia,* their abundance in soil, and role in nitrogen fixation, methanotrophy, and cellulose degradation makes them an attractive choice of phylum in this study. We studied the change in the diversity of *Verrucomicrobia* in three soil treatments, primary forest, a 20 year old pasture, and secondary forest or fallow in order to understand the effects of deforestation on the bacterial diversity in Amazon rainforest.

2.2 Material and Methods

A combination of molecular and bioinformatic analyses were used to address the *Verrucomicrobia* diversity in soils under three different vegetation treatments.

2.2.1 Site description and soil collection

The soil samples for this study were collected from the research site, Fazenda Nova Vida situated in the western Amazon Basin state of Rondonia, Brazil. This region has undergone rapid deforestation due to agriculture and logging and it provides an ideal model to study the response of microbial diversity to deforestation and agricultural conversion. Fazenda Nova Vida is about 22,000 ha in area and the climate is mostly tropical. It has two seasons: the dry season and the wet season. The annual precipitation reaches 2200 mm and the annual temperature averages 25.5^{o}C (Bastos and Diniz, 1982). In this region, the primary forest is being constantly cut to make space for pastures. The pasture is developed by burning the vegetation left after the logging of the hard timber trees. The land is planted with the fast growing grasses *Brachiaria brizantha* and *Panicum maximum* in order to accelerate the development of these pastures for commercial benefits. The pasture is developed without using any chemical fertilizer or agricultural machinery. Once the soil becomes unproductive, the land is abandoned. Later, secondary forest (fallow) develops on the abandoned land which is not as diverse as the original forest (Pires and Prance, 1986).

Soil samples were collected for the following treatments: a primary forest, a 20 year old pasture developed by cutting down the primary forest in 1987, and fallow which was used as pasture till 1994 (Cenciani *et al*., 2009). The soil samples were collected during the wet season (February, 2004) from a depth of 10 cm. Each soil treatment (forest, pasture, and fallow) had five replicates. The soil samples were sieved and stored on ice and transported to the laboratory where they have been stored at -80℃ until use.

2.2.2 Extraction of DNA and PCR amplification

Total genomic DNA from soil was extracted using the PowerSoil DNA MoBio DNA Extraction kit (Mobio Laboratories Inc., Carlsbad, CA). The 16S rRNA gene was amplified using the verrucomicrobial specific forward primer (VER_37F: 5'—TGG CGG CGT GGW TAA GA—3') and verrucomicrobial specific reverse primer (VER_673R: 5'—TGC TAC ACC GWG AAT TC—3'). As per the Invitrogen AccuPrime *Taq* DNA polymerase system protocol, a 25 µL PCR reaction was prepared with 10X AccuPrime PCR buffer II, 0.2µM of each primer, ~10-50ng DNA template, and 0.25 U of the AccuPrime *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The 10X AccuPrime PCR buffer contains thermostable AccuPrime protein, 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 20mM $MgCl_2$, 10% glycerol, 10% glycerol deoxyribonucleotide triphosphates (2 mM dGTP, 2 Mm dATP, 2 mM dTTP, 2 mM dCTP) concentrations sufficient to allow amplification during PCR. The PCR reaction mix was run in the C1000 Thermo Cycler (BIO RAD, Hercules, CA) at the following conditions: (1) initial denaturation at 95℃ for 5 minutes; (2) followed by 30 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 50 ℃ for 30 seconds and extension at 68 ℃ for 60 seconds and a final extension at 68℃ for 10 minutes. The PCR product was run through a 1% agarose gel in 1X Tris acetate-EDTA buffer (Sambrook and Russel, 2001) and was stained with 0.5 µg/ml ethidium bromide solution (Promega Corporation, Madison, WI). The gel was observed under UV light for the presence of bands confirming the amplification of the DNA fragment.

2.2.3 Preparation of clone libraries

A clone library (50 clones per library) was constructed for each replicate of the three soil samples (forest, pasture, and fallow). The amplified PCR product was cloned into a plasmid vector (pCR 2.1-TOPO) using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The chemically competent One Shot TOP10 *Escherichia coli* cells were transformed as described in the TOPO TA cloning kit protocol. The transformed cells were screened using the X-gal blue-white colony selection method (Sambrook and Russel, 2001). White colonies were transferred to Luria Bertani plate containing kanamycin (50µg/ml) and was grown overnight at 37℃. In order to preserve the cells, 50 colonies were transferred to culture

tubes containing 15% glycerol LB broth with 50µg/ml kanamycin, grown overnight at 37℃ in orbital shaker at the speed of 250 rpm and stored at -80℃.

2.2.4 Sequencing of DNA inserts

Whole colony PCR targeting the cloned 16S rRNA gene was performed with the primers M13F (5'—GTT GTA AAA CGA CGG CCA GTG—3') and M13R (5'—CAC ACA GGA AAC AGC TAT G—3') using the PCR conditions mentioned above in section 3.2 except for the DNA template which was replaced by a single bacterial colony. The amplified DNA was treated with the ExoSAP-IT (USB Corporation, Cleveland, OH) according to manufacturer's instructions. Briefly, ExoSAP was diluted 1:10 in DNA grade water. Next, 1 µL of diluted ExoSAP was added to 10 µL of PCR product and was incubated for 20 minutes at 37℃, followed by 20 minutes at 80℃. Samples were collected at the bottom of the tubes by centrifugation and immediately chilled on ice. The sequencing reaction consisted of the DNA template (5-10 ng of ExoSAP treated PCR product) was mixed with 6.4 pmol of M13F primer in a final volume of 10µL. To 5µL of the template/primer mix, 1.6 µL 2.5X Big dye reaction buffer, 1.5 µL Big dye v3.1 (Big dye Terminator v3.1 cycle sequencing kit, ABI, Foster city, CA) and 1µL of DNase free water was added so that the final volume is 10µL. The plate was briefly centrifuged. The PCR reaction was run under following conditions: (1) 96℃ for 3 minutes; (2) 25 cycles of 96℃ for 10 seconds, 50℃ for 15 seconds, 60℃ for 4 minutes; and (3) stored at 4℃. DNA Sequencing was performed using the Applied Biosystem (ABI) 3130xl genetic analyzer (Foster city, CA). The standard ABI3130xl precipitation protocol was followed for sequencing (Bougon, 2009).

2.2.5 Data analysis

The 16S rRNA gene sequences were analyzed using Sequencher 4.2 (Genes code Corporation, Ann Arbor, MI). A BLAST search was conducted to confirm the presence of *Verrucomicrobia* 16S rRNA sequence match at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned with Infernal (Nawrocki & Eddy, 2007). The sequences were clustered using the Ribosomal Database Project's Pyrosequencing pipeline.

Rarefaction and Jaccard-Sorensen indices were calculated. Jaccard-Sorensen distance dendrogram and heat maps were also generated using Ribosomal Database Project (RDP) to compare diversity between each replicate of all the soil treatments. We calculated the mean Jaccard-Sorensen similarity for each treatment as well as pairwise comparison of the forest, pasture, and fallow treatments. EstimateS 7.5 (Colwell, 2004) was used to determine non parametric richness estimators including Chao, Shannon, Simpson, and ICE (Incidence based Coverage Estimator) indices. A cut-off value for sequence similarity of 99% was used to define operation taxonomic unit (OTU) according to Stackebrandt and Ebers (2006). Statistical analysis (one way ANOVA) was performed using the freely available software package R (http://www.r-project.org). Phlyogenetic trees analysis was done by aligning the 750 sequences using MUSCLE (Edgar, 2004) and performing a neighbor joining algorithm using MEGA version 4.0 (Tamura *et al*., 2007). Circular trees were constructed and displayed using Interactive Tree of Life (http://itol.embl.de)

2.3 Results

The data analysis was performed using the sequence similarity value of 99% for the definition of an Operational Taxonomic Unit (OTU). Since we are studying diversity within one phylogenetic group, the OTU definition of 99% similarity was more apt as well as sensitive to the differences in diversity.

2.3.1 Rarefaction curve

A rarefaction curve was generated using the RDP to compare species richness among different samples (Figure 2.1). Complete linkage clustering was performed using RDP to produce a cluster file for 250 sequences from each treatment of primary forest, pasture and fallow. This cluster was used as an input file for generating rarefaction curve. A rarefaction curve is a plot of number of OTU as a function of number of clones sampled.

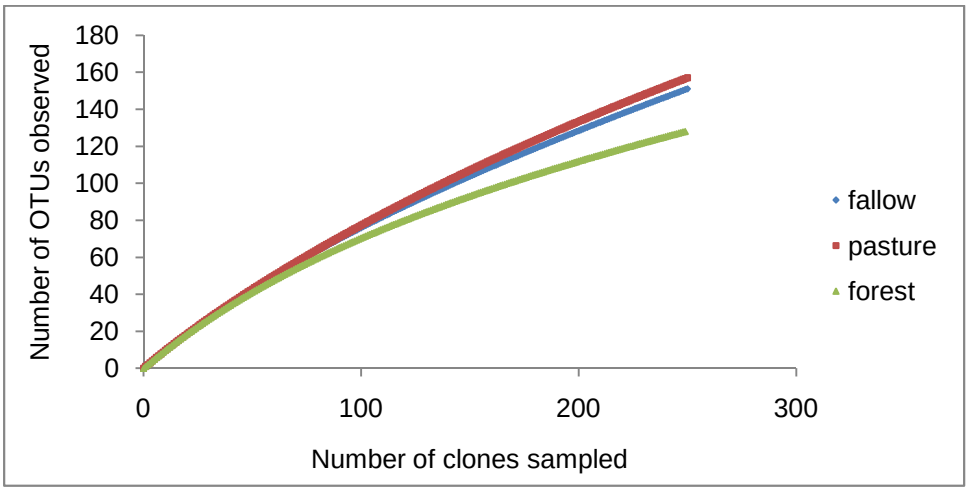
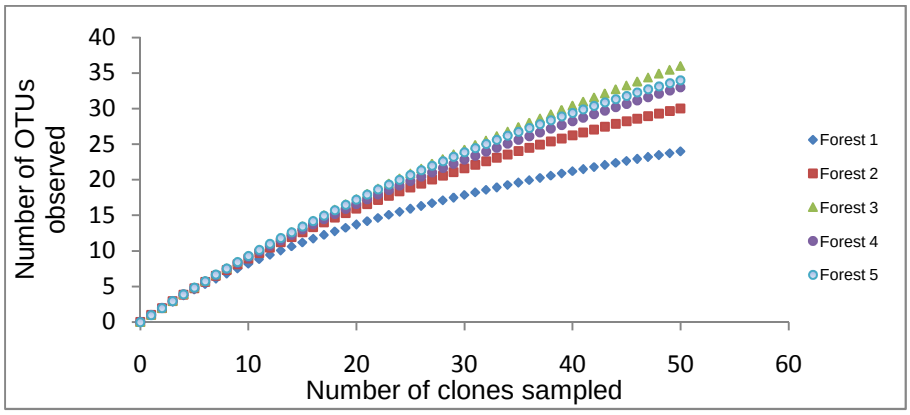


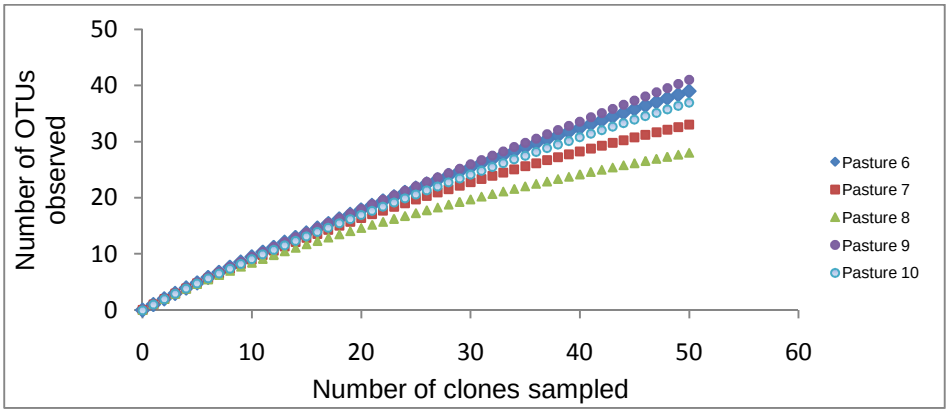
Figure 2.1: Rarefaction analysis of verrucomicrobial community from the Amazon rainforest. The 16S rRNA sequences recovered from forest, pasture, and fallow soil samples were used to construct rarefaction curve with an OTU definition of 99% similarity.

Results from the rarefaction curve demonstrated that the number of OTUs present in 250 sequences of pasture was higher than the number of OTU's in 250 sequences of fallow treatment. Also, the number of OTUs present in pasture and fallow were higher in comparison to forest. The number of OTUs observed in the 250 clones of pasture was 157, followed by fallow with 151 unique OTUs followed by the primary forest for which 128 OTUs were observed. Rarefaction curves of the fallow and pasture overlapped signifying the extent of verrucomicrobial diversity in the two treatments to be similar. The rarefaction curve along with estimating the species richness also provides information about how well a community was sampled. The forest, pasture, and fallow curves were shown not to reach an asymptote even at 250 clones signifying the enormous diversity. In order to understand the diversity patterns within a soil treatment, rarefaction curves for all the replicates from each of the soil treatment were also generated (Figure 2.2 a, b, c).

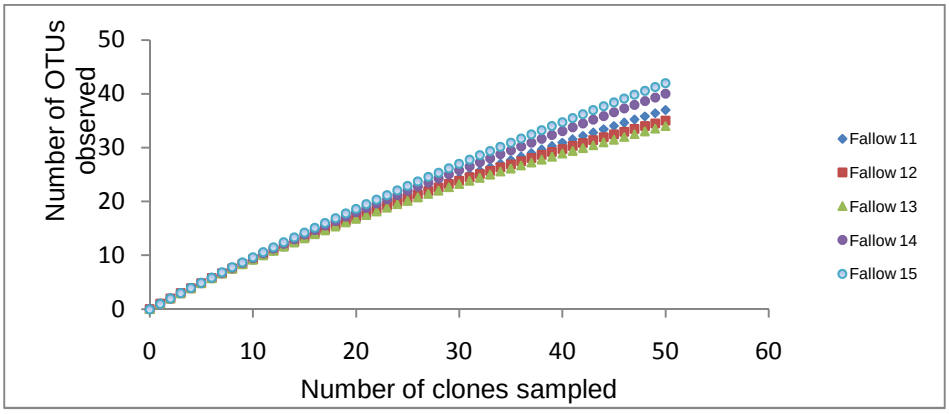
Though the rarefaction curve suggested that the *Verrucomicrobia* community to be more diverse in fallow and pasture than forest, statistical significance of these results could not be established. Hence, diversity indices were calculated.



(a)



(b)



(c)

Figure 2.2: Rarefaction analysis of verrucomicrobial community in each replicate of forest (a), pasture (b), and fallow (c) soil samples from the Amazon rainforest. OTUs are defined at 99% similarity.

2.3.2 Diversity indices

Non-parametric estimators were used to assess the diversity of *Verrucomicrobia* in forest, pasture, and fallow soil samples. The richness estimators, Chao and Incidence based coverage estimators (ICE) were calculated using Ribosomal database project and EstimateS respectively (Table 2.1). Since the richness estimators take into account only the species richness and not the evenness, diversity indices were also calculated. Two diversity indices, Shannon and Simpson indices were calculated using RDP and EstimateS respectively. All the indexes were represented as bar graphs (Figure 2.3) to visualize the pattern of verrucomicrobial diversity in the forest, pasture, and fallow soil samples.

Table 2.1: Comparison of the verrucomicrobial diversity including estimated diversity (Shannon and Simpson diversity indices) and estimated OTUs richness (Chao 1 and ICE) based on 16S rRNA analysis from the primary forest, pasture, and fallow soil samples of the Amazon

Soil treatments (*n*)	Richness estimators		Diversity indices	
	Chao	ICE	Shannon	Simpson
Forest 1 (50)	37	24	2.93	21
Forest 2 (50)	42	30	3.25	38
Forest 3 (50)	94	36	3.46	61
Forest 4 (50)	75	33	3.35	47
Forest 5 (50)	53	34	3.43	64
Forest (250)	261	128	4.60	112
Pasture 6 (50)	138	39	3.56	76
Pasture 7 (50)	56	33	3.32	37
Pasture 8 (50)	47	28	3.06	23
Pasture 9 (50)	374	41	3.59	76
Pasture 10 (50)	109	37	3.43	42
Pasture (250)	364	157	4.79	115
Fallow 11(50)	87	37	3.44	44
Fallow 12(50)	77	35	3.43	58
Fallow 13(50)	109	34	3.38	51
Fallow 14(50)	180	40	3.58	81
Fallow 15(50)	127	42	3.67	136
Fallow (250)	445	151	4.78	142

[n]Number of 16S rRNA sequences

According to Chao and Simpson index, the highest verrucomicrobial diversity was present in fallow followed by pasture and forest. However, ICE and Shannon index gave slightly higher diversity of

Verrucomicrobia in pasture when compared to fallow. The diversity of *Verrucomicrobia* remained the lowest in primary forest for all the indices.

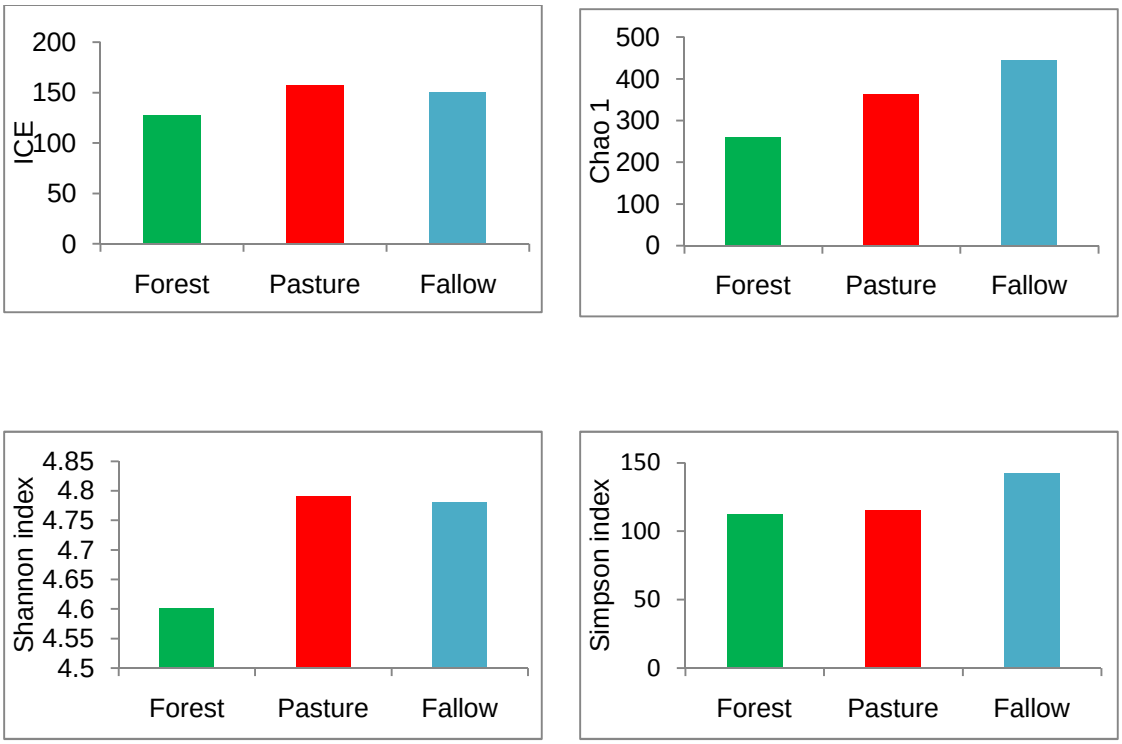


Figure 2.3: Chao, ICE, Shannon, and Simpson diversity indices for *Verrucomicrobia* 16S rRNA sequences with OTUs defined at 99% similarity.

One way Analysis of variance of the diversity index values was performed to ascertain the statistically significant differences of the verrucomicrobial community between the forest, pasture, and fallow treatments. The p values were tabulated (Table 2.2). The diversity of *Verrucomicrobia* in forest was found to be significantly lower than fallow for Chao index and moderately significant for Shannon index. However, significant p values were not obtained for rest of the differences.

Table 2.2: Univariate tests for significance using ANOVA analysis for Chao 1, Shannon, and Simpson index

Soil treatments	p value		
	Chao 1	Shannon	Simpson
Forest-Pasture	0.60	1.0	0.70
Pasture-Fallow	0.18	0.17	1.30
Fallow-Forest	0.02*	0.08	0.17

* The difference is statistically significant

We also calculated the mean of the diversity indices for the five replicates of each soil treatment and a bar graph was plotted (Appendix 1). The results remain unchanged and the same pattern of verrucomicrobial diversity was observed. Forest remained the least diverse for the phylum *Verrucomicrobia*.

2.3.3 Similarity analysis

A comprehensive comparison of the verrucomicrobial community present in forest, pasture, and fallow was performed by calculating the Jaccard coefficient for community similarity. This index is based on the presence or absence of different *Verrucomicrobia* in the three soil treatments. Jaccard similarity was calculated for each pairwise comparison of the forest, pasture, and fallow and a heatmap was generated to allow visualization of the pairwise comparison. The verrucomicrobial community in forest was very distinct from pasture. However, the verrucomicrobial community in the forest was more similar to fallow than it was to pasture (Figure 2.4).

Figure 2.4: Comparison of verrucomicrobial communities in forest, pasture, and fallow soil samples. Values for the Jaccard coefficient values are presented in a heatmap as a color coded distance matrix, with the most similar communities represented by red and the most dissimilar communities represented by white color.

In order to better understand the verrucomicrobial community structure, a pairwise comparison of all the replicates from each treatment was conducted and a heatmap was produced for each soil treatment (Figure 2.5). The heatmap showed that *Verrucomicrobia* communities were more different to each other in forest than pasture. We further analyzed the beta diversity at phylogenetic level, the results of which are discussed in section 2.4.

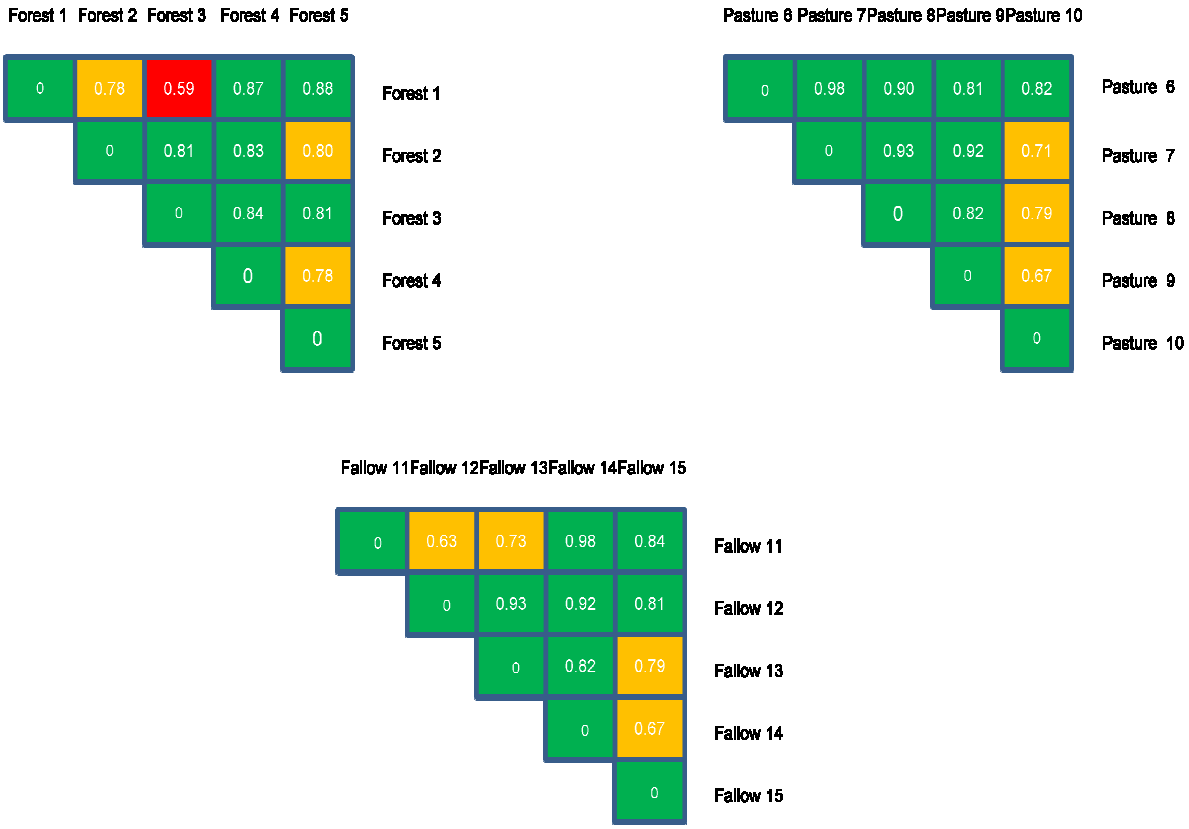


Figure 2.5: A pairwise comparison of verrucomicrobial community forest, pasture, and fallow soil treatment. More similar replicates are represented in green and less similar in yellow color.

A multiple sample analysis was performed using Mothur (www.**mothur**.org) to evaluate the differences in shared OTUs between the communities and the results were represented as a Venn diagram (Figure 2.6). The number of OTUs unique to forest was found to be 70; the number of unique OTUs for pasture was 113 while for fallow it was 99. The forest shared 25 OTUs with fallow and 15 OTUs with pasture while pasture shared 28 OTUs with fallow. The results showed that diversity of *Verrucomicrobia* was highest in pasture followed by fallow and forest. Twenty three OTUs were found to be common to all the three treatments.

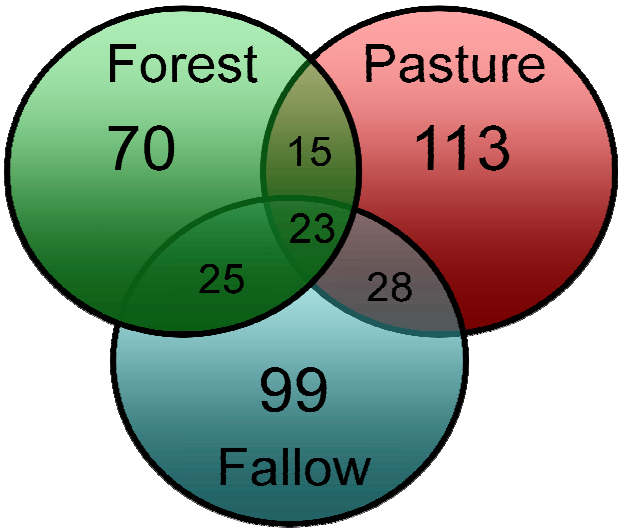
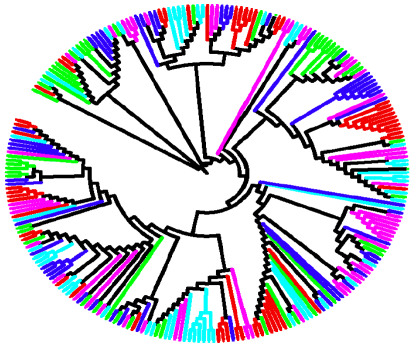


Figure 2.6: Venn diagram depicting the shared and unshared OTUs among forest, pasture, and fallow soil samples from the Amazon. The OTUs are defined at 99% similarity.

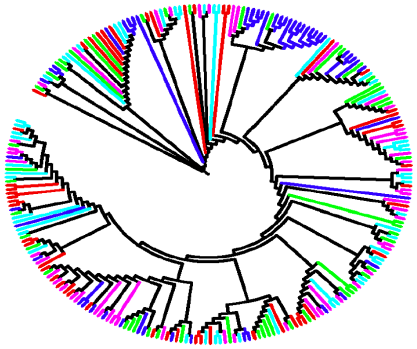
2.3.4 Phylogenetic analysis

We analyzed our data at phylogenetic level to better understand and further corroborate the distribution of *Verrucomicrobia* in the three soil treatments. Phylogenetic trees of the 250 sequences from each soil treatment were constructed using PhyML (http://atgc.lirmm.fr/phyml/) and displayed using ITOL (http://itol.embl.de/index.shtml). The verrucomicrobial diversity found in Amazon is illustrated by three phylogenetic tree analyses of 250 16S rRNA sequences from each soil treatment (Figure 2.7) and a tree of all the 750 sequences from all the three soil treatments (Figure 2.8).

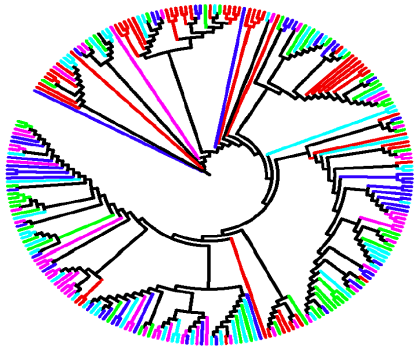
The 16S rRNA sequences from forest, as shown using an arc A-B, were found to be clustering together on one side of the phylogenetic tree indicating the presence of some *Verrucomicrobia* to be specific to forest soil samples. The 16S rRNA from pasture also clustered towards one side of the tree. However, the pattern was not as obvious as the forest treatment. The fallow 16S rRNA sequences were uniformly distributed throughout the phylogenetic tree (Figure 10).



Forest



Pasture



Fallow

Figure 2.7: Maximum likelihood tree calculated for 250 *Verrucomicrobia* 16S rRNA sequences from each soil treatment. Each replicate is designated in a different color; (Forest 1=red, forest 2=green, forest 3=blue, forest 4=dark blue, forest 5=pink), (pasture 6=green, pasture7=dark blue, pasture8=blue, pasture9= 32, pasture 10=red), (Fallow 11= dark blue, fallow 12=blue, fallow 13=pink, fallow 14=red, fallow 15= green).

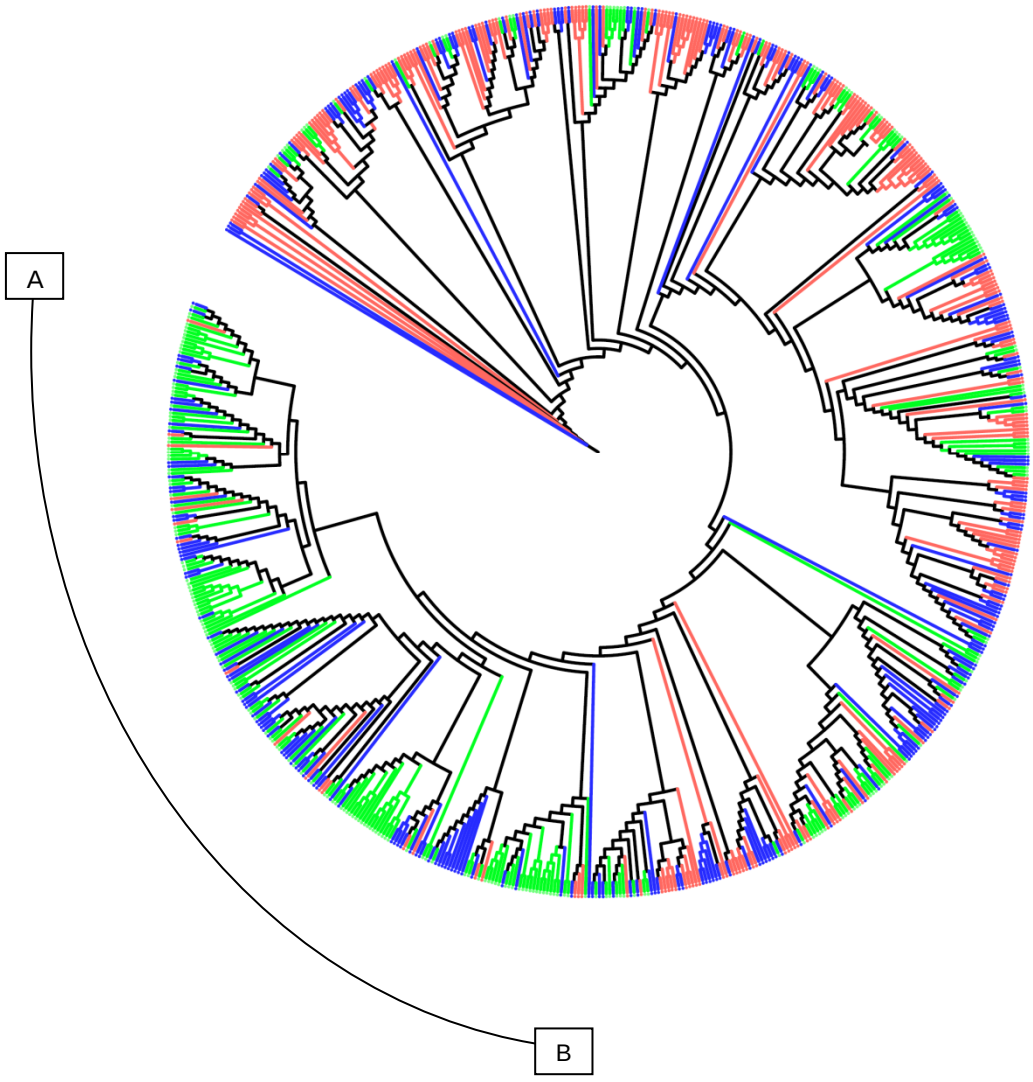


Figure 2.8: A maximum likelihood phylogenetic tree of 750 *Verrucomicrobia* 16S rRNA gene sequences from forest (green), pasture (red), and fallow (blue) soil treatments from the Amazon. The distribution of primary forest 16S rRNA is shown *via* an arc A-B.

We further calculated the distance matrix between the three soil treatments using UniFrac (Lozupone *et al*., 2007). The UniFrac metric measures the difference between two environments in terms of the branch length that is unique to one environment or the other. The verrucomicrobial community in forest was found to be most different from pasture. The difference between fallow-pasture was also found

to be more than the difference between fallow-forest (Figure 2.9). The results were found to be in complete agreement with the results from taxonomic analysis (Figure 2.4).

Figure 2.9: A color coded heatmap based on the distance matrix calculated using the unique branch length of a phylogenetic tree. Brighter color represents high similarity and the brightness decreases as the similarity decreases.

Next, to study the beta diversity at phylogenetic level, we calculated the distance matrix of all the replicates from each treatment using UniFrac (Table 2.3, 2.4, 2.5). The replicates of forest were found to be more different from each other than replicates of the pasture. The replicates of fallow were found to be more like forest in terms of similarity.

Table 2.3: Distance matrix of replicates of forest

SOIL TREATMENT	FOREST 1	FOREST 2	FOREST 3	FOREST 4	FOREST 5
FOREST 1	0	0.694444	0.715278	0.768421	0.719723
FOREST 2			0.650000	0.735915	0.682927
FOREST 3				0.748227	0.676157
FOREST 4					0.721014
FOREST 5					

Table 2.4: Distance matrix of replicates of pasture

SOIL TREATMENT	PASTURE 6	PASTURE 7	PASTURE 8	PASTURE 9	PASTURE 10
PASTURE 6		0.687285		0.644828	0.619863
PASTURE 7			0.750865	0.750000	0.733108
PASTURE 8				0.703833	0.673611
PASTURE 9					0.668966
PASTURE 10					

Table 2.5: Distance matrix of replicates of fallow

SOIL TREATMENT	FALLOW 11	FALLOW 12	FALLOW 13	FALLOW 14	FALLOW 15
FALLOW 11		0.621528	0.667774	0.808511	0.66899
FALLOW 12			0.660131	0.789474	0.660959
FALLOW 13				0.775439	0.614035
FALLOW 14					0.768657
FALLOW 15					

Our results have shown that the community structure of *Verrucomicrobia* is different among the three soil treatments. We have also demonstrated that the verrucomicrobial communities differ more among forest replicates when compared to pasture and fallow replicates using heatmaps generated through RDP (table 2.3.1, 2.3.2, 2.3.3). We further substantiated these results at phylogenetic level by performing a parsimony test using Mothur (http://www.mothur.org/). The parsimony test calculates the probability of two communities to have similar structure by chance. All the replicates of each treatment were compared to each other as well as with the replicates of the other treatment, generating a total 105 different combinations (Table 2.4). The probability of *Verrucomicrobia* communities being different (shown by the significance values highlighted in green) was found to be higher among forest replicates when compared to pasture replicates. Eight out of ten combinations among forest replicates were found to

differ. Pasture had only 2 combinations of replicates which were significantly different. Interestingly, fallow had 6 combinations for which the probability of being different was significant. These results indicated that verrucomicrobial communities in pasture were more similar to each other than in forest and fallow.

Table 2.6: Parsimony probability test

	FOREST					PASTURE					FALLOW				
	FOR 1	FOR 2	FOR 3	FOR 4	FOR 5	PAS 6	PAS 7	PAS 8	PAS 9	PAS10	FAL11	FAL12	FAL13	FAL14	FAL15
FOR 1															
FOR 2	<0.001														
FOR 3	<0.001	0.033													
FOR 4	<0.011	0.028	<0.001												
FOR 5	<0.001	0.006	0.009	0.012											
PAS 6	0.001	0.003	<0.001	<0.001	0.012										
PAS 7	<0.001	0.003	<0.001	<0.001	<0.001	0.006									
PAS 8	<0.001	<0.001	<0.001	<0.001	<0.001	0.329	0.002								
PAS 9	<0.001	0.015	<0.001	<0.001	<0.001	0.643	0.104	0.204							
PAS10	<0.001	<0.001	<0.001	<0.001	<0.001	0.12	0.016	0.108	0.116						
FAL11	<0.001	0.014	<0.001	0.019	<0.001	0.01	<0.001	<0.001	0.044	0.003					
FAL12	<0.001	0.062	<0.001	<0.001	0.019	0.033	<0.001	<0.001	0.061	<0.001	<0.001				
FAL13	<0.001	0.244	0.009	0.035	<0.001	0.026	0.003	<0.001	0.042	0.006	0.324	0.069			
FAL14	<0.001	<0.001	<0.001	<0.001	0.035	0.125	<0.001	0.127	0.061	<0.048	<0.001	<0.001	<0.001		
FAL15	<0.001	0.078	<0.001	<0.001	<0.010	0.04	0.004	<0.001	0.084	0.01	0.042	0.008	0.079	<0.001	

Significance p< 0.01667

2.4 Discussion

The Amazon rainforest has extremely favorable conditions for the growth of a wide variety of organisms ranging from huge trees and animals to minute microorganisms. Though the diversity of macroorganisms has been studied to some extent, the diversity of the microbial world in the Amazon remains largely uncharacterized. Moreover, the large scale deforestation taking place is expected to further alter the distribution and diversity of microorganisms in the Amazon. This study aimed to

understand the effects of deforestation on bacterial diversity using *Verrucomicrobia* as a model phylum. Most of the 16S rRNA studies done till now have targeted all the bacteria and studied less than 350 sequences. Here, we provided a robust analysis by studying diversity within only one phylogenetic group to decrease complexity and studying a total of 750 sequences; 250 from each soil treatment of primary forest, a 20 year old pasture, and secondary forest or fallow. A larger number of 16S rRNA sequences ensured a better and ecologically relevant estimate of *Verrucomicrobia* diversity in Amazon.

We used a combination of taxonomic and phylogenetic tools to analyze our data. Based on the richness estimators and diversity indices, the primary forest was found to have the lowest diversity of *Verrucomicrobia*. We used Chao 1 and rarefaction curves to estimate the richness. We also used the Shannon and Simpson indices to measure the diversity based on richness as well as evenness. The Chao 1 index measures richness based on the number of observed species represented by a single individual (singleton) and the number of species represented by 2 individuals (doubleton). Chao1 emphasizes the rare species and therefore tend to overestimate the species richness. Therefore, we utilized Simpson and Shannon indices to measure diversity, which are not or to a lesser extent biased towards rare species. All the richness estimators and diversity indices were calculated based on the 99% 16S rRNA sequences similarity OTU definition. Since we studied the diversity within one phylum, a 99% OTU similarity provided a better sensitivity to species level diversity differences (Klepac-Ceraj *et al*., 2004). Interestingly, all the measures of diversity calculated showed *Verrucomicrobia* to be least diverse in primary forest soil samples.

Though our results were in agreement with a previous study by Jesus *et al.* in 2009, we expected the forest soil samples to have the highest *Verrucomicrobial* diversity as stated in our hypothesis. A possible explanation was that deforestation has been found to lower the diversity of plants and animal species and we expected the bacterial communities to be affected in the same way. The diversity of plants and animal species decreases substantially as the widespread deforestation causes destruction of their habitat and leads to food shortage. However, for the bacterial community especially the soil bacterial

community, habitat is not destroyed but altered due to deforestation. The soil characteristics are changed considerably when a pristine forest is converted into pasture. Soil pH, Ca^{2+}/Mg^{2+} ratio as well carbon and nitrogen contents have been found to change after the conversion of forest to pasture (Neil *et al*., 2006, Faoro *et al*., 2010, Cenciani *et al*., 2009).These changes are expected to affect the diversity and distribution of bacterial community though it might not necessarily decrease the diversity. The forest-pasture conversion changes the soil chemistry and therefore probably provides an altered environment leading to the growth of new and different members of *Verrucomicrobia*. The new ecological niches that have been opened up during the conversion of forest to pasture are perhaps also found in the secondary forest and could be the reason for higher or equally high verrucomicrobial diversity in soil.

The increase in diversity of *Verrucomicorbia* can also be linked to the growth or reduction of other phylogenetic groups due to forest-pasture conversion. Probably, *Verrucomicrobia* is better adapted to the environmental conditions present in pasture than forest and is therefore displacing other phylogenetic groups and becoming more diverse. Some phylogenetic groups have been found to flourish better in one soil than the other. *Acidobacteria* prefer to grow in pristine forest while *Proteobacteria* population dominates in disturbed soil (Nusslein and Tiedje, 1999). Cenciani *et al* reported an increase in the nitrogen content when the primary forest was converted to pasture. The nitrogen fixing capability of *Verrucomicrobia* (Khadem *et al*., 2010) along with other nitrogen fixers probably helps it to sustain the changed soil condition of pasture as well as causing the increase in the amount of nitrogen in pasture soil.

Fallow or secondary vegetation that develops after the infertile pasture has been abandoned for many years might further change the soil ecology giving rise to new species of *Verrucomicrobia* accounting for the high Chao 1 and Simpson values.

Though the *Verrucomicrobial* community was clearly least diverse in the primary forest (Table 2.2), its level of difference in pasture and fallow was not very coherent. Two of the diversity measures, rarefaction and the Shannon index indicated a higher *Verrucomicrobia*l diversity in the pasture in

comparison to fallow while Chao1 and Simpson indices were higher for the fallow. According to the rarefaction curve, the number of OTUs found in pasture was 157 while fallow had 151 OTUs in the same number of observed clones. This indicates a similar level of diversity in pasture and fallow soil samples. In spite of similar levels of diversity in fallow and pasture, the *Verrucomicrobial* community in forest and fallow were more similar to each other versus forest and pasture as shown by the heatmap based on the Jaccard distance (Figure 2.4). A similar result was generated when we compared the distance matrix of the three soil treatments based on phylogeny (Figure 2.9).

In order to better understand the community structure within the three soil treatments, we compared the similarity of each replicate with the others within each treatment. At taxonomic level, the replicates of all the three soil treatments were found to be different from each other (Figure 2.5). However, at phylogenetic level, the replicates of forest were found to be more different from each other when compared to pasture based on the distance matrix (Table 2.3.1). The replicates of pasture were more similar to each other (Table 2.3.2b) while fallow replicates were again dissimilar (Table 2.3.3). A parsimony test which calculates the probability of two communities to be similar also gave the same results (Table 2.4). Each replicate of forest was more different to each other than the replicates of pasture. A primary forest is a pristine and undisturbed land and therefore responsible for the uneven distribution of *Verrucomicrobia*. On the other hand, pasture is a land where the soil and organic matter is mixed and same grass is planted all over, hence causing a uniform level of distribution and diversity of *Verrucomicrobia*.

Another interesting observation was that the verrucomicrobial community structure similar to primary forest seemed to getting restored with the growth of fallow. Cenciani *et al*., (2009), demonstrated that the soil chemical properties including C/N ratio of fallow are becoming more like primary forest. The verrucomicrobial diversity seems to follow the same trend in our study. To visualize the community structure, we constructed a phylogenetic tree of all 750 16S rRNA sequences using neighbor joining algorithm (Figure 10). Many 16S rRNA sequences from forest were found to cluster towards one side of

the tree indicating that some members of this phylum were found solely in forest. This observation in particular, was very interesting because it signifies that though the primary forest was least diverse in *Verrucomicrobia,* but the species that are found in primary forest are not found in pasture or fallow. However, fallow might be able to restore these species as it is becomes more like primary forest. The 16S rRNA sequences belonging to fallow seemed to be distributed more uniformly signifying that many members that are found in forest and pasture are also found in fallow.

Clearly, deforestation and pasture development are affecting the diversity of *Verrucomicrobia* in the Amazon. Moreover, the verrucomicrobial community, in terms of diversity, might be returning back to the structure it was present in forest.

Future Perspectives

The application of culture independent methods to study bacterial diversity has helped us to understand the ecology of world's most mysterious ecosystems. In this study, we have tried to characterize the bacterial diversity of the Amazon and its response to deforestation using *Verrucomicrobia* as a model phylum. It is imperative that diversity of the microbial world in Amazon is further analyzed to better understand the functioning of this complex ecosystem. It would be interesting to study the long term effects of deforestation on bacterial diversity. The soil samples used in this study were collected from a 20 year old pasture and a fallow which started developing after 1994 when the pasture was abandoned. We indicated that the *Verrrucomicrobial* community is returning to a form similar to the one found in primary forest. Analyzing the diversity in an older secondary forest or fallow will help to provide a clearer picture about the structure of bacterial diversity returning back to its original shape. In our study, we found that the diversity of *Verrucomicrobia* increased when primary forest was converted into fallow. However, we need an insight into the change in diversity of other bacterial groups and how they respond to the large scale deforestation in Amazon. An appealing observation would be to see if they respond in a similar manner as *Verrucomicrobia*. Major players of Amazon's soil bacterial community including*, Proteobacteria, Actinobacteria, and Planctomycetes* can be chosen for further studies. As

stated previously, the nitrogen fixers may have role in changing the nitrogen content of pasture; studying the diversity of major nitrogen fixers like *Acidobacteria* and *Burkholderia* in Amazon can be useful in understanding this observation.

The soil samples in this study were collected from a 20 year old pasture. A pasture in Brazil is developed without the application of any chemical fertilizers, pesticides, and mechanical machinery. However, agricultural land especially, soybean farms are developed using heavy machinery and phosphorous fertilization. The forest area in Brazil is continuously being converted into intensified cropping systems for soybean production. In fact, soybean farms are even displacing the pastures to pursue new opportunities for biofuel production. Extensive uses of chemical fertilizers have been found to affect the soil quality and bacterial diversity in the agricultural soil of Northern China (Ge *et al*., 2008). Studying the effects of soybean cultivation on bacterial diversity in the Amazon can help us to further understand the consequences of human disturbances on this ecosystem.

APPENDIX A

DIVERSITY INDICES BAR GRAPH

The mean of the diversity indices, Shannon and Simpson as well as richness estimators, Chao 1 and ACE for the 5 replicates of each soil treatment was calculated and represented as a bar graph.

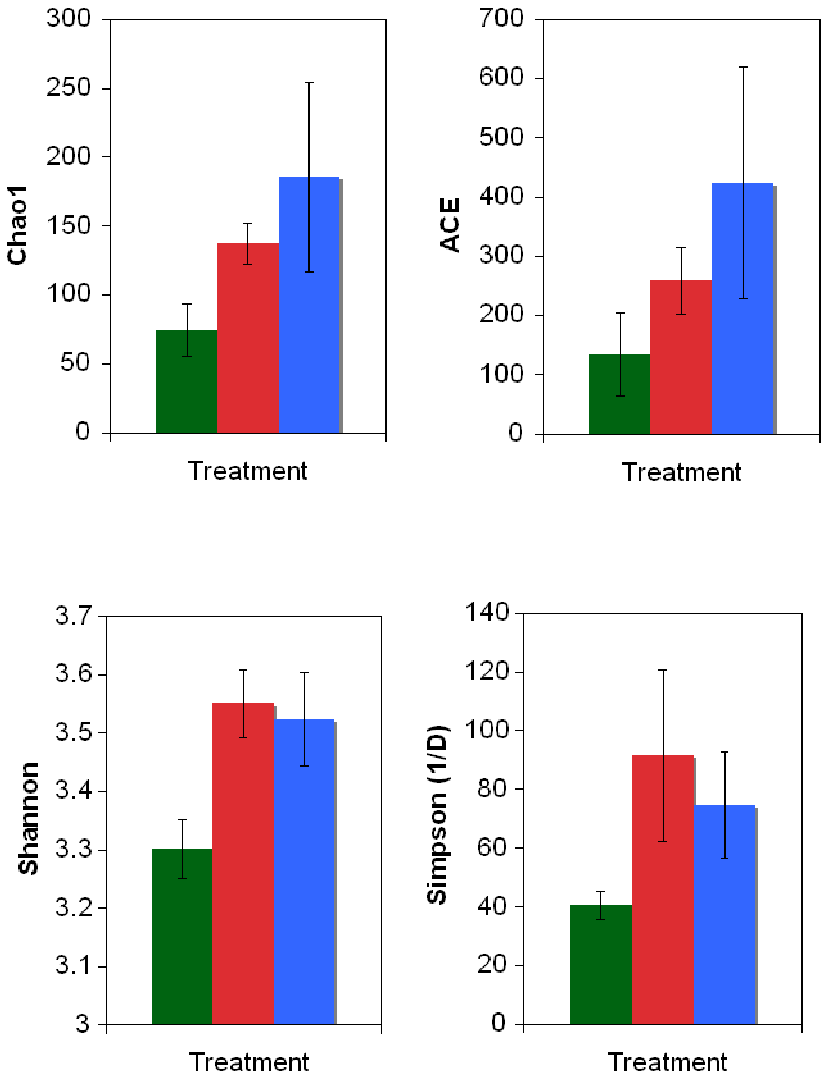


Figure 1: Mean of the diversity indices for each replicate from forest, pasture, and fallow.

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BIOGRAPHICAL INFORMATION

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