BIOGEOGRAPHY OF COPPER-RICH SOIL MICROBIAL COMMUNITIES ASSOCIATED WITH *GLEICHENELLA PECTINATA* AND *STICHERUS BIFIDUS* AT BOSQUE DEL PUEBLO (ADJUNTAS, PR) AND BOSQUE ESTATAL DE MARICAO (SABANA GRANDE, PR)

By

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ABSTRACT

The biogeography of microbial communities in tropical environment is poorly understood. Recent works in soil microbial ecology have focused on cataloging the diversity of soil bacteria, but few of them have documented how soil bacterial communities are affected by a wide range of biotic (fern species) and abiotic (season and study sites, including soil properties) factors.The objective of this work was to analyze biotic and abiotic factors that could affect the biogeography and composition of microbial communities associated with the rhizosphere of two common ferns, *Gleichenella pectinata* (GP) and *Sticherus bifidus* (SB) at a copper deposit in Bosque del Pueblo, Barrio Vegas Arriba in Adjuntas (BPA) and a reference site (Bosque Estatal de Maricao, Barrio Tabonuco in Sabana Grande, BEM) during the dry and the wet seasons. The biogeography of rhizosphere-associated microbial communities was evaluated using Terminal Restriction Fragment Length Polymorphisms (TRFLP) and clone libraries. Abiotic factors such as available phosphorus (P), exchangeable calcium (Ca), magnesium (Mg) and potassium (K), organic matter percentage (OM %), total copper (Cu) content and pH were also studied. Data were analyzed using a combination of statistical tools, such as multivariate analysis. Differences in soil microbial community composition were observed and associated with the presence of Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria, Nitrospira, Fibrobacteres and Bacteroidetes. Microbial communities associated with both ferns appeared to be more influence by season, than by fern specie or study site. At BPA, microbial communities from samples collected appeared to be influenced by Cu, P and K while those from BEM were apparently influenced by Ca, Mg, pH taken in H_2O and in $CaCl_2$. Microbial communities associated with GP site collected at BEM revealed that their biogeographical distribution during the dry season appeared to be more similar than during the wet season. In contrast, those

associated with the SB site seems to have a similar biogeographical distribution during both seasons. The biogeography of microbial communities associated with the GP collected at BPA appeared to be different regardless of the seasons. In contrast, surface communities associated with SB during both seasons and the subsurface communities during the wet season appeared to be similar, but communities at different depths during the dry season were not. This study indicates that different tropical forests within the same geographical region can have different microbial communities due to differences in soil properties, seasons, fern species and study sites.

RESUMEN

La biogeografía de comunidades microbianas en ambientes tropicales es pobremente entendida. Trabajos recientes en ecología microbiana del suelo se han enfocado en la clasificación de la diversidad de las bacterias del suelo, pero pocos de ellos han documentado cómo las comunidades bacterianas del suelo son afectadas por una amplia gama de factores bióticos (especie de helechos) y abióticos (época y lugares de muestreo, incluyendo propiedades del suelo). El objetivo de este trabajo fue analizar factores bióticos y abióticos que podían afectar la biogeografía y estructura de las comunidades microbianas asociadas a la rizósfera de dos helechos comunes, *Gleichenella pectinata* (GP) y *Sticherus bifidus* (SB), en un depósito de cobre en el Bosque del Pueblo, Bo. Vegas Arriba en Adjuntas (BPA) y en un lugar de referencia (Bosque Estatal de Maricao, Bo. Tabonuco en Sabana Grande, BEM) durante la época seca y la húmeda. La biogeografía de comunidades microbianas asociadas a la rizósfera fue evaluada utilizando polimorfismos de la longitud de los fragmentos terminales de restricción (TRFLP, por sus siglas en inglés) y una biblioteca de clones. Además, factores abióticos como fósforo disponible (P), calcio (Ca), magnesio (Mg) y potasio (K) intercambiable, porcentaje de materia orgánica (OM %), contenido de cobre (Cu) total y pH tomado en H_2O y en $CaCl_2$ fueron estudiados. Los datos fueron analizados usando una combinación de herramientas estadísticas, como análisis multivariados. Diferencias en la composicion de comunidades microbianas fueron observadas y asociadas a la presencia de Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria, Nitrospira, Fibrobacteres y Bacteroidetes. Comunidades microbianas asociada a ambos helechos aparentaron estar más influenciadas por la época, que por la especie del helecho o lugar de muestreo. En BPA, las comunidades microbianas de los suelos colectados aparentaron estar influenciadas por Cu, P and K mientras que aquellas del

BEM aparentaron estar influenciadas por Ca, Mg, pH tomado en H_2O y en $CaCl_2$. Comunidades microbianas asociadas al área de GP colectadas en BEM revelaron que su distribución biogeografíaca durante la época seca aparentó ser más similar que durante la época húmeda. En contraste, aquellas asociadas al área de SB parecieron tener una distribución biogeografía similar durante ambas épocas. La biogeografía de las comunidades microbianas asociadas a GP colectadas en BPA aparentó ser diferente sin importar la época. En contraste, comunidades de la superficie asociadas a SB durante ambas épocas y las comunidades profundas durante la época húmeda aparentaron ser similares, pero aquellas observadas a las distintas profundidades durante la época seca fueron menos similares. Los resultados de este estudio indican que, diferentes bosques dentro de la misma región geográfica pueden tener diferentes comunidades microbianas debido a las especies de helechos presentes, temporadas, lugares de muestreo y propiedades del suelo.

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INTRODUCTION

Since the eighteenth century, biologists have investigated the geographic distribution of plant and animal diversity to understand the ecosystem where they live. More recently, the biogeography (the study of biodiversity distribution over space and time) for microorganisms has been examined (Hughes *et al.,* 2006). Some researchers have argued that the smaller size of microbial prokaryotes and their high abundance make them less likely to be bounded by biogeographical barriers than microbial eukaryotes. According to Horner-Devine *et al.* (2004), understanding patterns of bacterial biodiversity is important because bacteria comprise the majority of biomass and perhaps the greatest species diversity, which are key for many environmental processes that maintain life on Earth.

However, the distribution of microbial diversity is poorly understood and for that reason the principle of "*everything is everywhere, but the environment selects*", is not defined. According to Fierer and Jackson (2006), microbial biogeography in terrestrial ecosystems is controlled primarily by soil variables which differ fundamentally from the biogeography of macroorganisms.

Recent work in soil microenvironments has focused on the diversity of soil bacteria and how these communities are affected by a wide range of biotic and abiotic factors (Fierer and Jackson, 2006). Hackl *et al.* (2004) argued that soil bacterial communities in forests with distinct vegetation and soil chemical properties appeared to be well differentiated based on 16S rRNA gene phylogeny. Above-ground plant communities have been assumed to drive below-ground microbial diversity, but very little is known about how plant species composition and diversity influence the community composition of microorganisms in soil (Kowalchuk *et al.,* 2002).

Walker *et al.* (2003) argued that plants can change the biochemical and physical properties of the soil by their exudates (organic and inorganic substances secreted by roots). Rhizodeposition (the total carbon transfer from plant roots to soil as exudates) can regulate the soil microbial community composition in the immediate vicinity of roots known as the rhizosphere (Singh *et al.*, 2004). These exudates provide carbon to soil microorganisms, which in turn provide nitrogen and phosphorus to the plant by mineralization and immobilization of organic matter (Walker *et al.*, 2003; Singh *et al.*, 2004). Bacteria respond in different ways to the presence of compounds released by plant roots, while different root exudates are expected to select exclusive rhizosphere bacterial communities (Garbeva *et al.* 2004). According to Garbeva *et al.* (2004), variation in physical, chemical, and biological properties of the rhizosphere, compared with those of the root-free bulk soil, are responsible for changes in microbial diversity and the increase in number and activity of microorganisms in the rhizosphere. Particle size distribution, pH, cation exchange capacity, and organic matter content in soil can affect microbial community composition either directly, by providing a specific habitat that selects specific microorganisms, or indirectly, by affecting plant root functioning and exudation in a soil-specific manner. In contrast, the rhizosphere microbial communities influence above-ground ecosystems by contributing to plant nutrition, soil composition and soil fertility. These communities can help plants deal with adverse biotic and abiotic factors such as pathogens and soil contamination (Kirk *et al*., 2004).

Plants have the ability to accumulate metals which are essential for their growth and development. These metals include: Mg, Fe, Mn, Zn, Cu, Mo and Ni. Certain plants also have the ability to accumulate heavy metals that do not have any known biological function, such as Cd, Cr, Pb, Ag, Se and Hg.

In general, plants have developed three basic strategies for growing on metalliferous soils (Baker and Walker, 1990). These plants are described as: (i) metal excluders: plants that effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil; however, they can still contain large amounts of metals in their roots, (ii) metal indicators: these plants accumulate metals in their above-ground tissues and the metal levels in their tissues generally reflect metal levels in the soil, and (iii) metal accumulators: these plant species (eg. hyperaccumulators) can concentrate metals in their above-ground tissues to levels far exceeding those present in the soil (Memon *et al.,* 2001). Those plants containing more than 0.1% of Ni, Co, Cu, Cr or Pb or 1% of Zn in its leaves on a dry weight basis are considerated as hyperaccumulators, irrespective of the metal concentration in the soil (Baker and Walker, 1990).

Metal accumulator plants distribute metals internally in many different ways. For example, they can localize selected metals mostly in roots and stems, or accumulate and store other metals in a nontoxic form for latter distribution and use. One mechanism for tolerance or accumulation in some plants involves the potential binding of toxic metals at cell walls of roots and leaves, away from sensitive sites within the cell or storing them in a vacuolar compartment (Memon *et al.,* 2001). Large concentrations of easily bioaccessible heavy metals can be aggregated in the rhizosphere and be able to affect the microbiota. Heavy metals affect microorganisms in many aspects such as: causing a shift in the composition of microbial populations, impoverishing their diversity, and affecting the activity of indigenous microorganisms (Kelly *et al.,* 2003; Konopka *et al.,* 1999; Lugauskas *et al.,* 2005; Ranjard *et al.,* 2006). The large concentration of easily bioaccessible heavy metals present in the rhizosphere

can provide a niche for heavy metal resistant bacteria. For that reason, bacteria in the rhizosphere have developed mechanisms to tolerate large concentration of heavy metals.

Plant metal uptake, which depends on many parameters such as soil properties, and plant species, also varies according to seasons (Langille and Maclean, 1976; Bidar *et al.*, 2009). According to Sims and Boswell (1978), soil properties such as: Cation Exchange Capacity (CEC), pH, organic matter and phosphorus can govern heavy metal uptake by plant. They argued that: (1) soils with low CEC such as sands have a much lower heavy metal binding power as compared to clay with a high CEC, (2) heavy metals are more available to plants below pH 6.5, (3) solubility of most heavy metals or trace elements increase as the acidity of soil increases, (4) organic matter, because it increase soil CEC, chelates soil heavy metals, and (5) phosphorus combines with metals ions to form soluble or insoluble complexes, for example, when orthophosphates are present, sorption of Cu in soils decrease.

Copper is an essential trace metal required for a number of metabolic enzymes such as oxidases, superoxide dismutases and lysyl oxidases (Rogers *et al.,* 1991; Lim and Cooksey, 1993; Rensing *et al.*, 2000; Voloudakis *et al.,* 2005). Various studies have demonstrated that copper can also catalyze harmful redox reactions resulting in oxidation of lipid membranes, damage to nucleic acids, and generation of free radicals from hydrogen peroxide (Rogers *et al.,* 1991; Munson *et al.*, 2000; Gracía *et al.*, 2002). Copper is ranked as the fifth most toxic substance to soil bacteria, fungi, and actinomycetes when compared with 16 other metals (Cha and Cooksey, 1991). Since copper ions are cytotoxic agents, microorganisms have developed several mechanisms to protect themselves and still ensure their nutritional requirements (Hasman and Aarestrup, 2002; Voloudakis *et al.*, 2005). These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and

reduction of the heavy metal ions to a less toxic state (Spain, 2003). For example; ***Pseudomonas syringae*** has a copper resistance mechanism via accumulation that is encoded by the ***cop*** operon on a plasmid (Cooksey, 1990; Lim and Cooksey, 1993). Periplasmic CopA and CopC, outer membrane (CopB), and inner membrane (CopD) proteins work together to compartmentalize copper away from sensitive cellular functions (Spain, 2003).

According to Idris ***et al.*** (2004) rhizosphere microorganisms play an important role by increasing the availability of heavy metals for plant uptake. Yang ***et al.*** (2007) argued that rhizobacteria have been shown to possess several traits that can alter heavy metal bioavailability through the release of chelating substances and acidification of the microenvironment. For example, Delorme ***et al***. (2001) found that the acidification of soil in the rhizosphere of the ***Thalaspi caerulescens*** facilitates the metal ion uptake by increasing the metal ion mobility around the roots. Representatives of the genera ***Pseudomonas*** and ***Bacillus*** are described as having plant growth-promoting rhizobacteria (PGPR) (Barea ***et al.***, 2005). These populations include a diverse group of free-living soil bacteria that can improve host plant growth and development in heavy metal contaminated soils by mitigating toxic effects of heavy metals on the plants (Belimov ***et al***., 2004).

Knowledge of the biogeographical distribution of microbial community associated with the rhizosphere of metal accumulator plants is limited. Duineveld ***et al.*** (2001) argued that only a small proportion (0.1 to 10%) of the total bacteria present in the rhizosphere can be cultured. Molecular genetics technologies have revealed that past culture-based studies greatly underestimated the microbial diversity and therefore the debate of whether microorganisms exhibit biogeography patterns still exists (Merrill ***et al.,*** 2005). The 16S rDNA approach combined with other molecular techniques have many important advantages including the ability

to: (i) rapidly evaluate gross similarities and differences within microbial communities, (ii) provide a rapid means of identifying bacterial isolates, (iii) detect and identify those bacteria that are not viable or cultivable, and (iv) identify the presence of individual uncultured bacterial species within a complex community. These advancements have been possible by analyzing total environmental DNA using techniques such as gene cloning and terminal restriction fragment length polymorphism (T-RFLP) of PCR-amplified 16S rRNA genes.

OBJECTIVES

1. To determine the natural copper-accumulating capacity of *Gleichenella pectinata* and *Sticherus bifidus.*
2. To determine the influence of biotic (fern species) and abiotic (season and study sites, including soil properties) on the biogeography and composition of the rhizosphere associated microbial community of *Gleichenella pectinata* and *Sticherus bifidus* in two tropical forests during the wet and the dry seasons.

LITERATURE REVIEW

Biogeography is defined as the study of the geographical distribution of biodiversity in both space and time (Horner-Devine *et al.,* 2004; Hughes *et al.,* 2006). Fierer and Jackson (2006) constructed a continental-scale description of soil bacterial communities and the environmental factors which influenced biodiversity. Soil samples from North and South America were analyzed by rDNA fingerprinting to compare the bacterial community composition and diversity quantitatively across sites. The results showed that bacterial diversity was higher in neutral soils and lower in the acidic ones, including the Peruvian Amazon. Bacterial diversity was unrelated to site temperature, latitude, geographic distance, and other variables that typically predict plant and animal diversity. It was concluded that microbial biogeography is controlled primarily by edaphic variables and differs fundamentally from the biogeography of macroorganisms.

Cho and Tiedje (2000) showed that genetic distance between fluorescent *Pseudomonas* species was related to geographic distances. Thirty-eight undisturbed soil samples from 10 sites of four continents including Australia, Africa, North and South America were collected. A total of 248 isolates were confirmed as *Pseudomonas* sp. by fluorescent pigmentation production and with specific 16S ribosomal DNA primers. The isolates were analyzed by molecular methods including: 16S rDNA restriction analysis (ARDRA), 16S-23S rDNA intergenic spacer-restriction fragment length polymorphism (ITS-RFLP) analysis, and repetitive extragenic palindromic PCR genomic fingerprinting. Their results showed very similar restriction patterns, while some ITS-RFLP types were exclusive to the site of origin, indicating weak endemicity at every geographic scale.

Genetic differences among purple non-sulfur bacteria were observed by Oda *et al.* (2003) along a 10 meter marsh transect. Thirty clones were isolated from each of five sampling

locations. A total of 150 clones were characterized by BOX-PCR fingerprinting. Cluster analysis yielded 26 distinct genotypes, and 106 clones constituted four major dominant genotypes. The main genotypes were represented by tentatively identified ***Rhodopseudomonas palustris*** based on phylogentic analyses of 16S rRNA gene sequences. Morisita-Horn similarity coefficients (CMH) used to compare the numbers of common genotypes found at pairs of sampling locations showed similarities between locations that were 1 cm apart, but there was almost no similarity between locations that were more than 9 m apart. It was concluded that natural populations of ***R. palustris*** are assemblages of genetically distinct ecotypes and the distribution of each ecotype is inconsistent.

Bowman and McCuaig (2003) used 16S rDNA clone library analysis to evaluate the prokaryotic diversity and community structural changes through a superficial sediment core obtained at Mertz Glacier Polynya (MGP) in Antarctic. The clone libraries were constructed from the core at 0.0 to 0.4 cm, 1.5 to 2.5 cm, and 20.0 to 21.0 cm (depth positions). Their results indicated that at the oxic sediment surface (depth, 0 to 0.4 cm) the microbial community appeared to be dominated by opportunistic species, resulting in lower species richness. At a depth of 1.5 to 2.5 cm, the species richness was higher, with a community composed by numerous γ- and δ-*Proteobacteria*. At a depth of 20.0 to 21.0 cm a decline in the species richness was observed, accompanied by a larger number of phylogenetically divergent phylotypes and a decline in the predominance of *Proteobacteria*. Oline (2006) compared the bacterial communities from serpentine soils and immediately adjacent non-serpentine soils. This study included a site-based replication of the serpentine to non-serpentine community comparison over a regional scale (100 km) at three different subalpine forest sites in northern California and southern Oregon by producing 16S rRNA clone libraries. The pattern of results

showed that serpentine communities tended to be more similar to each other than they were to non-serpentine communities. Some differences were observed in two non-serpentine communities suggesting that the geographical site may control community composition as well.

Molecular methods have revealed that culture-based studies missed most microbial diversity. These culture-independent methods have allowed recent studies to sample microbial diversity more deeply and broadly than ever before, being a continuous debate whether microorganisms exhibit any biogeography patterns (Merrill *et al.*, 2005). According to Torsvik *et al.* (1990), one problem with the culture-based methods is that they can only be used with bacteria which can be isolated and cultured. They argued that most (99.5 to 99.9%) of the soil bacteria observed in the fluorescence microscope cannot be isolated and cultured on laboratory media.

Borneman and Triplett (1997) reported the first description of microbial diversity in eastern Amazonian soils using a library construction. Among 100 small-subunit rRNA sequences obtained by PCR amplification with universal primers, 98 of them were bacterial and 2 were archaeal. Eighteen percent of bacterial sequences could not be classified in any known phylum**;** two sequences represent a exclusive branch between the vast majority of bacteria and the deeply branching, predominantly thermophilic bacteria. Five sequences formed a clade that may represent a novel group within the *Proteobacteria*.

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent technique developed for microbial diversity characterization (Pandey *et al.*, 2007) which also measures the spatial and temporal changes within bacterial communities (Mummey and Stahl, 2003; Ikeda *et al.*, 2004). The T-RFLP method involves a PCR in which one of the two primers used is fluorescently labeled at the 5' end. The PCR product is digested with restriction enzymes

and the fluorescently labeled terminal restriction fragments are identified by using an automated DNA sequencer. The resulting banding profile can be used to measure species richness and evenness as well as similarities among samples (Liu ***et al.,*** 1997; Kirk ***et al.,*** 2004). According to Egert and Friedrich (2003) since T-RFLP analysis is based on PCR amplification, all biases related to this technique apply and a number of important parameters related to PCR have been identified; it has been found that initial DNA template concentration, number of PCR cycles, annealing temperature, and the choice of ***Taq*** DNA polymerase from different manufacturers may affect the composition of T-RFLP profiles.

Ikeda ***et al.*** (2004), observed the temporal changes of soil microbial communities collected from a same region of an experimental field in the University of Tsukuba, Japan during August, October, and December 2003 by means of T-RFLP. A high degree of similarity in the fingerprinting patterns was obtained for summer and fall samples, and some exclusive bands were observed in the winter sample reflecting a seasonal difference in the soil microbial community. Mummey and Stahl (2003) evaluated temporal variability of bacterial TRF's derived from soil of an ecosystem having homogeneous grass cover to provide insights into seasonal shifts. Spatial analysis of T-RFLP profiles derived from ecosystems having homogeneous and heterogeneous plant cover was also determined to elucidate plant community influences. The homogeneous grassland (HG) was dominated by ***Bouteloua gracilis*** and the shrubland (SL) was dominated by ***Artemisia tridentata***. No trends toward dissimilarity were detected with temporal (180 days) or spatial (up to 100 m) distance in the HG system. T-RFLP profiles of the SL site exhibited pronounced small scale spatial variability (670 cm), although spatial analysis indicated weak spatial autocorrelation to distances greater than 36 cm. Average dissimilarity values differed greatly between the two sites (0.27 and 0.59 for HG and SL sites, respectively). These

results suggested that plant community composition strongly influences bacterial community composition in these semiarid ecosystems.

Lipson and Schmidt (2004) constructed 16S rDNA clone libraries from alpine soil collected at the Niwot Ridge Long Term Ecological Research area in the Front Range of the Colorado Rocky Mountains during winter, spring, and summer. Their results showed that: (1) the *Acidobacterium* phylum was most abundant in the spring; (2) the winter community had a higher proportion of *Actinobacteria* and members of the *Cytophaga/ Flexibacter/Bacteroides* (CFB) group; (3) the summer community had a higher proportion of the *Verrucomicrobium* phylum and the β-*Proteobacteria,* (5) α-*Proteobacteria* were equally abundant in all seasons, and (6) a number of sequences from currently uncultivated phyla were found, including two novel candidate phyla.While the cultures and clone libraries produced distinct groups of organisms, the two approaches gave consistent accounts of seasonal changes in microbial diversity.

The composition and diversity of microbial communities can be influenced by abiotic factors such as: temperature, water, heavy metals (Díaz-Raviña and Bååth, 1996) and biotic factors such as the above ground plant communities (Kowalchuk *et al.,* 2002). Abiotic factors such as heavy metals affect microorganisms in soil in many aspects, causing a shift in the composition of microbial populations, impoverishing their diversity, and affecting the activity of indigenous microorganisms (Lugauskas *et al.,* 2005; Ranjard *et al.,* 2006).

De la Iglesia *et al.* (2006) studied the effect of copper and other abiotic factors on the presence of bacteria in two abandoned tailing dumps in central Chile, using T-RFLP to compare the composition of the bacterial communities. Results showed that elevated available copper content in tailings had a strong effect over bacterial communities. In addition, they argued that

factors like pH and organic carbon content also play an important role in the composition of these communities. Zhou *et al.* (2002) tried to define some factors, including organic carbon, which drives microbial community composition in soil. Twenty-nine soil samples from four geographically distinct locations taken from the surface, vadose zone, and saturated subsurface were studied using a SSU 16S rRNA based clone library analysis. It was observed that surface soils from low carbon sites exhibited a uniform diversity pattern quite distinct from the pattern of saturated subsurface soils, which exhibited a more common competitive diversity pattern found in most biological communities. In contrast, microbial communities from high carbon sites displayed a uniform diversity pattern regardless of depth or water content of the sample, as shown by results based on the reciprocal of Simpson's (1/*D*) and the log series indexes, respectively.

Another factor that affects microbial diversity is the above-ground plant communities. Above-ground plant communities have been assumed to drive below ground microbial diversity, but very little is known about how plant species composition and diversity influence the community composition of microorganisms in the soil (Kowalchuk *et al.,* 2002). Walker *et al.* (2003) established that plants can change biochemical and physical properties of the soil using the organic and inorganic substances secreted by roots. Kowalchuk *et al.,* (2002) examined this relationship in fields exposed to different above-ground biodiversity and in fields experimentally designed to examine the influence of plant species on soil microbial communities. To assess the most dominant bacterial populations in the bulk and at the rhizosphere (root–soil interface zone) of these experimental fields, Denaturing Gradient Gel Electrophoresis (DGGE) profiling was used to determine microbial diversity and collector's curves of 16S rDNA types in clone libraries

were done. Comparisons between the influence of the two plants *Cynoglossum officinale* and *Cirsium vulgare* on the soil bacterial communities showed that detectable differences in microbial community composition were limited to the rhizosphere. The rhizosphere from experimental plots with lower plant diversity showed lower bacterial diversity than bulk soil. These results demonstrated that the level of coupling between above-ground plant communities and below-ground microbial communities is related to the tightness of the interactions involved. Finally, plant species composition and community composition appear to cause clear changes in microbial community composition and diversity observed in the rhizosphere.

Kuske *et al.* (2002) compared bacterial communities associated with the rhizospheres of the native *Stipa hymenoides* and *Hilaria jamesii*, the invading annual grass *Bromus tectorum*, and the interspaces colonized by cyanobacterial soil crusts at three depths (0 to 10, 10 to 20, and 20 to 30 cm). The compositions of total bacterial community and the prevalence of the pylum *Acidobacteria* in the soil crust interspaces were significantly different from those of the plant rhizospheres. The total bacterial community and the abundance of the *Acidobacteria* were affected by soil depth in both the interspaces and plant rhizospheres. T-RFLP profiles demonstrated that bacterial communities from the soil rhizospheres were very different from communities from the interspaces. Great differences in the soil bacterial communities from the uninvaded interspaces and *Bromus* invaded soils were observed which suggests that *Bromus* invasion alters the composition of the soil bacterial community.

Hackl *et al.* (2004) used T-RFLP and 16S rRNA sequence analysis to compare the diversity and composition of bacterial communities in soils from six natural forests at eastern Austria which differed in type of vegetation. Ten individual samples (0 to 10 cm depth) from

each forest stand were collected at intervals of 5 m along transects of 50 m in length. T-RFLP profiling and clone sequence analysis revealed that bacterial communities in soils under Austrian pine forests were distinct from those in soils under zonal oak-hornbeam and spruce-fir-beech forests, which were more similar in community composition. Clones derived from an Austrian pine forest soil were mostly affiliated to high G+C gram-positive bacteria (49%), followed by members of the α-*Proteobacteria* (20%) and the *Holophaga/Acidobacterium* (12%). Clones from oak-hornbeam and spruce-fir-beech forest soils were mainly related to the *Holophaga/Acidobacterium* (28 and 35%), followed by members of the *Verrucomicrobia* (24%) and the α-*Proteobacteria* (27%), respectively. Soil bacterial communities in forests with distinct vegetation and soil chemical properties appeared to be well differentiated based on 16S rRNA gene phylogeny.

Singh *et al.* (2004) established that one of the major difficulties that plant biologists and microbiologists face when studying the interactions between microorganisms and plants, is that many groups of microorganisms that inhabit this zone are not cultivable in the laboratory. For Idris *et al.* (2004), the rhizosphere microorganisms may play an important role, by increasing the availability of heavy metals for plant uptake. Soil microorganisms may improve the metal solubility and availability by reducing the soil pH or by producing chelators. In this study, the rhizosphere and endophytic bacterial populations of *Thlaspi goesingense* growing in a serpentine soil in eastern Austria were characterized. Microbial populations were studied by means of T-RFLP, and 16S rRNA genes cloning and sequencing. In the rhizosphere the *Holophaga/Acidobacterium* division accounted for 27% of the clones examined, whereas 22% classified as α-*Proteobacteria*. The remaining clones belonged to the high-GC gram-positives (16%), the *Cytophaga/Flexibacter/Bacteroides* division (10%), or the γ- or β-*Proteobacteria* (8

and 4%). From surface-sterilized shoot, the majority of clones affiliated with the α-, β-, γ-, and δ-*Proteobacteria* (20, 29, 12, and 1%) and bacteria belonging to the *Cytophaga/Flexibacter/Bacteroides* division accounted for 17%. The remaining clones belonged to the low-GC grampositives (12%), the *Holophaga/Acidobacterium* division (5%), and the high-GC gram-positive species (4%). These findings demonstrate that highly diverse microbial communities live in association with the Ni hyperaccumulator ***T. goesingense.***

METHODOLOGY

Study sites

The dominant presence of *Gleichenella pectinata* (Willdenow) Ching (1940) **(GP)** and *Sticherus bifidus* (Willdenow) Ching (1940) **(SB) (Appendix I)** were used to select two study sites at the Central Cordillera Mountains of Puerto Rico: the former mining zone at Bosque del Pueblo (Adjuntas, Barrio Vegas Arriba) (18°10'59''N, 66°40'35''W) **(BPA) (Figure 1)** and from a reference forest Bosque Estatal de Maricao (Sabana Grande, Barrio Tabonuco) (18°08'04''N, 66°57'17''W) **(BEM) (Figure 1).**

Bosque del Pueblo is the first community-managed forest. This site has 530 ha (Borges ***et al.***, 2006). It receives 31.5- 35.0 cm (Junta Rectora Consejo de Manejo Comunitario, 2002) of annual rainfall and has an elevation range of 500 to 600 m above sea level (U.S.G.S., topographic map, 7.5' series, Adjuntas quadrangle, 1960). This forest is located on the most important copper, gold and silver deposits of the island (Massol ***et al.***, 2006).

Located between the municipalities of San Germán, Sabana Grande and Maricao, the Bosque Estatal de Maricao has an extension of 4,150 ha. The elevation ranges from 600 to 700 m above sea level (U.S.G.S., topographic map, 7.5' series, Maricao quadrangle, 1960). Precipitation ranges from 5.7 cm in January to 37.5 cm in October (Delannoy, 1997). Eighty-five percent of Bosque Estatal de Maricao soils are derived from serpentines, a rock of volcanic origin.

The USDA Soil Survey of Ponce Area of Southern Puerto Rico (1979) and USDA Soil Survey of San Germán Area of Southwestern Puerto Rico (2008) were used to determine the soil series **(Appendix II)** for each forest. The soil series found at the Bosque del Pueblo site is ***Adjuntas*** **(Figure 2)**. The ***Adjuntas*** series is derived from volcanic rock high in quartz

(**Appendix XVIII**) and is strongly acidic (Soil Survey Staff, 1999). It belongs to the order **Oxisols** (**Ox**, F. *oxide,* oxide; **sols**, L. *solum*, soil), suborder **Udox** (**Ud**, L. *udus,* humid, udic soil moisture regime), great group **Hapludox** (**Hapl**, Gr. *haplous*, simple, minimum horizon development), subgroup **Inceptic Hapludox**, and family **very-fine, parasesquic, isohyperthermic**. According to the Soil Survey Staff (1999) soils of the order **Oxisols**: "(1) have low fertility, (2) have a very low cation exchange capacity (CEC), (3) have phosphorus as the most restricted nutrient, (4) have organic matter much higher than is indicated by the soil color, which may result from red staining of the associated iron oxides and (5) have a loamy or clayey texture (sandy loam or finer)."

Based on the USDA–NRCS National Cooperative Soil Survey (http://www2.ftw.nrcs.usda.gov/osd/dat/E/EL_CACIQUE.html) our sampling point in the Bosque Estatal de Maricao belongs to the ***El Cacique*** soil series **(Figure 3)**. This series was formed with material weathered from serpentine bedrock (**Appendix XVIII**). ***El Cacique*** belongs to the order **Mollisols** (**Oll**, L. *mollis*, soft**; sols**, L. *solum*, soil), suborder **Udolls** (**Ud**, L. *udus,* humid, udic soil moisture regime), great group **Argiudolls** (**Argi**, L. *argilla*, white clay, presence of argillic horizon), subgroup **Typic Argiudolls**, and family **clayey, mixed, and magnesic, isohyperthermic.** Representatives from the order **Mollisols** have the following characteristics: "(1) a combination of a very dark brown to black surface horizon (mollic epipedon, results from the long-term addition of organic materials derived from plant roots), (2) a moderate or high cation exchange capacity (CEC), (3) have a high calcium supply, (3) an organic rich surface horizon, (4) are among the most productive soils in the world, and (5) a slow accumulation of humified organic matter (Soil Survey Staff, 1999)."

Fern Taxa

Two dominant species of ferns were used in this study, comb forked fern, *Gleichenella pectinata,* and Mexican umbrella fern, *Sticherus bifidus. Gleichenella pectinata* and *Sticherus bifidus* are classified in the *Gleicheniaceae* fern family, easily recognized by its pseudodichotomous branching pattern with periodically or permanently dormant laminar buds at the fork of a pair of axes (Gonzales, 2003). This family contains five genera: *Diplopterygium* (Diels) Nakai with about 25 species, only one of them widespread in tropical America; *Dicranopteris* Bernh. with 4 of 12 species in the Neotropics; *Gleichenella* Ching with one Neotropical species; *Gleichenia* J. E. Smith with about ten species confined to the Old World; and *Sticherus* C. Presl with a pantropical distribution of about 90 species of which 54 are found in the Neotropics (Gonzales, 2003). The family *Gleicheniaceae* is a conspicuous element of disturbed habitats, eg., roadsides, landslides and sometimes agricultural areas, especially in humid habitats, but a few species also regularly occur in lowland forest and inside closed forests (Gonzales, 2003).

Soil physical, chemical and microbiological study: sampling strategy

To evaluate microbial community diversity, a soil sample of approximately 1 kg was collected at the surface (0 to 5 cm depth) in an area of 1 ft^2. Those samples were collected in sites with (**Figure 5**, labels 1-2 and label 3) or without ferns (**Figure 5**, label 3) at the two tropical forests during the wet (July 2006) and the dry (January 2007) seasons (**Appendix XVII**). In addition, samples collected during the wet season were used to determine the degree of variability of soil physical and chemical parameters within sites. A soil sample of approximately 100 g each was collected from fern rhizospheres to analyze the microbial community composition and determine total copper content.To evaluate the biogeography of the microbial

community in a separate fern monoculture of *Gleichenella pectinata* or *Sticherus bifidus* at the two tropical forests in both seasons, soil samples were collected using the sampling strategy of Oda *et al.* (2003) with some modifications. The ferns from each monoculture were carefully removed and soil samples of approximately 3 g were collected from different depths (0, 5, 10, 15, 20, and 25 cm) **(label A in Figure 6)** and surface distances (0, 10, 100, and 1,000 cm) **(label B in Figure 6)**. In addition, during November 2007 approximately 200 g at each depth (0, 5, 10, 15, 20, and 25 cm) were collected to determine the degree of variability of some soil physical and chemical parameters (available phosphorus, exchangeable potassium, calcium, and magnesium, organic matter percentage and pH) within sites and depths. Physical and chemical analyses were performed at the UPRM Agronomy and Soils Department with the collaboration of Emmanuel Feliciano Justiniano.

Physical and chemical analysis of soil samples

Interactions of numerous physical, chemical, and biological properties in soils control the availability of nutrients. Soil nutrients are either bound to the soil or remain dissolved in the soil solution (water which surrounds soil particles). Available phosphorus, exchangeable potassium, calcium, and magnesium were determined from both forests. In addition, percentage organic carbon and organic matter, and pH taken in H_2O and in $CaCl_2$ were determined. Each of the analysis was performed at the UPRM Agronomy and Soils Department with collaboration of Emmanuel Feliciano Justiniano.

Available Phosphorus

To obtain the available phosphorus a protocol based on the Bray-1 Method according to Frank *et al.* (1998) with some modifications was used. Approximately 1 g of gridded air-dried soil (n=3 per soil pool) was placed into a 50 mL centrifuge tube, 10 mL of extracting solution

(0.025 N HCl in 0.03 N ammonium fluoride, NH_4F pH 2.6 ± 0.5) was added. The solution was mixed and centrifuged (2,500 rpm) for 5 min. After centrifuging, the extraction was filtered through a Whatman 42 filter paper. Acid molybdate, and ascorbic acid, $C_6H_8O_6$ stock solutions were prepared. The acid molybdate was prepared as follow: (1) 60 g ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ were dissolved in 200 mL of distilled water, (2) 1.455 g antimony potassium tartrate, $K(SbO)C_4H_4O_6$ were dissolved in the $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution and (3) 700 mL of sulfuric acid, H_2SO_4 were added to dilute to a final volume of 1, 000 mL. As well, 132 g of $C_6H_8O_6$ were dissolved in distilled water and diluted to a final volume of 1,000 mL. Both solutions were store in the dark under refrigeration. Two milliliters of soil extracts were collected in a 10 mL bottle and mixed with 8 mL of working solution (preparation: 25 mL of acid molybdate stock solution were added to about 800 mL distilled water and then 10 mL $C_6H_8O_6$ stock solution were gathered to brought to a final volume of 1, 000 mL). After 10 min, the optical density (OD) was measured using a Thermo Spectronic Genesys 20 Spectrophotometer at 882 nm. A standard curve (0, 0.2, 0.5, 1, 2, 3, 4, 5 and 6 ppm) was prepared from several working standards prepared as follows:

mL of 50 ppm of stock solution [1]	**Final volume** [2]	**Concentration of working standard (ppm)**
1	250	0.2
1	100	0.5
2	100	1.0
4	100	2.0
6	100	3.0
8	100	4.0
10	100	5.0
12	100	6.0

[1]Potassium dihydrogen phosphate, KH_2PO_4 (preparation: (1) 0.2197 g of KH_2PO_4 [dried in a desiccator] was dissolved in 25 mL of distilled water and diluted to a final volume of 1,000 mL with extraction solution, 0.025 N HCl in 0.03 N NH_4F pH 2.6 ± 0.05. This stock was placed under refrigeration.

[2]extracting solution, 0.025 N HCl in 0.03 N NH_4F pH 2.6 ± 0.05

The standard curve was prepared by aliquoting 2 mL of the working standards and 8 mL of working solution to measure the intensity in the same manner as the soil extracts. The intensity was plot against concentration of working standards to determine concentration of soil extracts from intensity and standards curve. Then the ppm concentration in filtrate obtained was converted to concentration in the soil using the following equation:

Equation 2:

ppm P in soil = ppm P filtrate x DF
Where;
ppm P filtrate = calculated using; y =mx + b
DF= dilution factor

Exchangeable Potassium (K^+), Calcium (Ca^{2+}) and Magnesium (Mg^{2+})

To obtain the exchangeable potassium, calcium and magnesium a protocol based on the "Unbuffered Salt Extraction Method" according to Sumner and Miller (1996), with some modification, was used. Approximately 5 g of soil (n=3 per soil pool) was added to a 50 mL centrifuge tube and 30 mL of 0.2 M ammonium chloride (NH_4Cl) was added. The solution was centrifuged (2,500 rpm) for 5 min. After centrifuging, the extraction was filtered through Whatman 42 filter paper into a 250 mL volumetric flask, and 30 mL of 0.2 M NH_4Cl was added, the soil was homogenized using a vortex, mixed and centrifuged (2,500 rpm) for 5 min. Supernatants were decanted through filter paper into a 250 mL volumetric flask. This process was repeated three additional times and the volume brought to 250 mL with 0.2M NH_4Cl to pool the solution prior to the analysis. Exchangeable calcium and magnesium in soils was determined in the resultant digests using a Perkin Elmer Air Acetylene Analyst 100 Atomic Absorption Spectrometer. Exchangeable potassium was determined using a Perkin Elmer Air Acetylene Analyst 300 Atomic Absorption Spectrometer at the USDA Tropical Agriculture Research

Station (TARS) with collaboration of Delvis Pérez and Ulises Chardón. The following parameters were used:

K^+ = **Wavelength:** 324.8 nm, **Lamp current:** 25 mA, **Slit:** 0.7 nm, **Standards used:** 2.0, 4.0, 6.0, 8.0, and 10.0 ppm

Ca^{2+} = **Wavelength:** 422.7 nm, **Lamp current:** 25 mA, **Slit:** 0.7 nm, **Standards used:** 1.0, 2.0, 3.0, and 4.0 ppm

Mg^{2+} = **Wavelength:** 285.2 nm, **Lamp current:** 25 mA, **Slit:** 0.7 nm, **Standards used**: 0.3, 0.4, 0.5, 0.6, and 1.0 ppm

The standard curve was determined using the standards with observed correlation coefficients not less than 0.99. A linear-intercept type equation was calculated. A test of interference was performed once every ten samples using one standard. The fuel used was C_2H_2: 3.0 L.min^{-1} and the oxidant air was: 10.0 L.min^{-1}. The exchangeable K^+, Ca^{2+} and Mg^{2+} were determined using the following conversion:

$$\frac{\text{mg cation}}{\text{L soln.}} \times \frac{\text{1 L soln.}}{\text{1000 mL soln.}} \times \frac{\text{250 mL}}{\text{dry wt. of sample (g)}} \times \frac{\text{1000 g of soil}}{\text{1 kg of soil}} \times \frac{\text{1 mmol cation}}{\text{cation molecular wt. (mg)}} \times \frac{\text{1 mol cation}}{\text{1000 mmol cation}} \times \frac{\text{cmol}_{(c)}\text{ cation}}{\text{1 mol cation}}$$

Where;

mg cation/ L soln. = reading given by the AA-espectrophotometer
dry wt. of the spl. (g) = gridded air-dried soil samples weight
cation mol. wt. (mg) = for K^+ is 39.10, for Ca^{2+} is 40.08 and for Mg^{2+} is 24.30
cmol $_{(c)}$ cation = for K^+ is 100, for Ca^{2+} is 200 and for Mg^{2+} is 200

Percentage of organic carbon (% OC) and organic matter (% OM)

To obtain the percentage of organic carbon we used a modified protocol based on the Walkley-Black method according to Nelson and Sommers (1996). According to Schumacher (2002) this method of extraction involves the rapid dichromate oxidation of organic matter. In this procedure, potassium dichromate ($K_2Cr_2O_2$) and concentrated sulfuric acid (H_2SO_4) are

added to a soil sample. Prior to adding water to halt the reaction, the solution is swirled and allowed. As a result of the incomplete oxidation and in the absence of a site-specific correction factor, a correction factor of 1.33 is applied to the results to adjust the organic C recovery. Upon completion of the sample extraction phase, the quantity of organic carbon present in the soil or sediment can be determined through a manual titrimetric quantitation where an indicator solution is added to the digestate. One of the most common indicators used is ortho-phenanthroline ferrous complex (Ferroin). The excess $Cr_2O_7^{2-}$ is titrated with ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] or ferrous sulfate ($FeSO_4$) until color change occurs in the sample. Color change associated with this indicator is green to reddish brown for the ortho-phenanthroline ferrous complex. Approximately 0.5 g of gridded air-dried soil (n=3 per soil pool) was transferred to a 500 mL wide-mouth Erlenmeyer flask. Ten milliliters of 1 N potassium dichromate ($K_2Cr_2O_7$) and 20 mL of sulfuric acid (H_2SO_4) were added. After 30 min, 200 mL of distilled water were added to filter the solution through a Whatman 42 filter paper. After filtering the solution we added 5 drops of 0.025 M *o*-phenanthroine-ferrous indicator and titrated the solution with 0.5 N ammonium ferrous sulfate, $Fe(NH_4)_2(SO_4)_3$. Similarly, but without soil, a blank control was prepared. After the end point determination we calculated the percentage of organic carbon and then the percentage of organic matter using a correction factor of 1.33 to adjust the organic C recovery determined with the following equations

Equation 3:

$$\%\ OC = \frac{[(mL\ K_2Cr_2O_7)(N\ K_2Cr_2O_7) - (mL\ Fe(NH_4)_2(SO_4)_3)(N\ Fe(NH_4)_2(SO_4)_3)]}{\text{dry weight of the sample (g)}} \frac{[0.003\ gC]}{1\ meq\ C} \times 100\%$$

Where;

dry weight of the sample (g) = gridded air-dried soil sample weight
mL $K_2Cr_2O_7$ = 10 mL
N $K_2Cr_2O_7$ = 1N
mL $Fe(NH_4)_2(SO_4)_3$ = mL used for sample tiltrated
(N $Fe(NH_4)_2(SO_4)_3$)] = calculated using; $x = \frac{0.5\ N}{20\ mL}$ x mL blank tiltrated

Equation 4:

% OM= (% OC) (1/0.77) (1/0.58)

Where;

% OC= Organic Carbon Percentage
(1/0.77)= corresponds to the amount of organic carbon that is oxidized by potassium dichromate, because this method is not 100 % effective and therefore must be corrected by that value (77 %).
(1/0.58)= corresponds to the estimated amount of organic carbon in organic matter. This means that for every 100 units of organic matter, we will have 58 units of organic carbon.

pH H_2O and pH $CaCl_2$

The pH in H_2O was measured with approximately 5 g of sieved, air-dried soil (n=3 per soil pool) in a 10 mL bottle. Five milliliters of distilled water were added, the solution was mixed for 5 min and the pH in H_2O was read with an Orion 310 PerpHecT pH/Temperature Meter. Similarly, pH in $CaCl_2$ was determined using a 0.01 M $CaCl_2$ solution.

Copper analysis in soil and plant tissue samples

To determine the total copper content in soils, samples of approximately 1.5 g of soil (n= 5 samples per soil pool) were oven dried (105 °C for 18 to 20 hrs), incinerated (600 °C for 24 hrs) and copper extracted in porcelain dishes, as specified in the protocol "Analysis of Soil and Sediments: Total Cations Protocol" (Atomic Absorption Spectrometry, pp. 136-137, Perkin Elmer Instruments LLC). In general, samples were digested in a hot mixture consisting of 2 mL of concentrated nitric acid (HNO_3) and 6 mL of concentrated hydrochloride acid (HCl) to solubilize the metals. The digests were filtered using a Whatman 40 filter paper into 100 mL volumetric flasks and brought to volume with 10% HCl. To standardize the procedure, spike samples were prepared with 0.5 mL of 100 ppm copper stock in 100 mL volumetric flasks and brought to volume with sample digest. Standard stocks of 0.05, 0.10, 1.0, 2.0, and 5.0 ppm from a 1,000ppm copper stock solution were prepared in a volumetric flask and brought to volume

with10% HCl. Standards and soil samples were stored in plastic bottles at room temperature until analysis.

To provide evidence of natural copper-accumulating capacity of *Gleichenella pectinata* and *Sticherus bifidus*, total copper level in fronds, rhizome and roots were determined. At the two tropical forests, and in both seasons approximately 60 g of fronds, rhizome and roots from randomly selected specimens of each fern (n= 20 ferns for *S. bifidus* and n=30 ferns for *G. pectinata*) were collected from a 1 ft^2 area. Species were identified by botanists at Biology Department Herbarium at the UPR Mayagüez Campus **(Figures 4a to 4c)**. The samples were rinsed with distilled water and stored in large plastic bags prior to analysis.

The fern tissue samples were processed using the protocol based on Díaz and Massol (2003) with some modifications. Approximately 3.0 g of frond, rhizome or root sample (n= 3 for roots; n= 5 for rhizomes and fronds) were oven dried (70 °C for 18 to 20 hrs), incinerated (585 °C for 24 hrs), and copper extracted. After the incineration, the samples were treated to solubilize the metals by digestion with a hot mixture of 5 mL 20% HNO_3 and 5 mL 10% HCl. Digests were filtered through a Whatman 40 filter paper into 50 mL volumetric flasks and brought to volume with 10% HCl. Spiked samples used for standardization were prepared with 0.5 mL of 100 ppm copper stock in 50 mL volumetric flasks and brought to volume with sample digest. Spikes and soil samples were stored in plastic bottles at room temperature until analysis.

Total copper content in soil samples and plant tissue was carried out on the resultant digests using a Perkin Elmer Air Acetylene Analyst 100 Atomic Absorption Spectrometer (AA-Spectrometer) at the Tropical Environmental Microbial Ecology Laboratory (TEMEL, UPRM Biology Department). The following parameters were used: Wavelength: 324.8 nm, Lamp

current: 25 mA, Slit: 0.7 nm, Fuel C_2H_2: 3.0 $L.min^{-1}$, Oxidant air: 10.0 $L.min^{-1}$. A linear-intercept type equation was calculated. The standard curve was determined using five standards (0.05, 0.10, 1.0, 2.0, and 5.0 ppm) with observed correlation coefficients not less than 0.99. Total copper was determined after using the following equation:

Equation 1:

$$\text{Cu concentration (mg/kg)} = \frac{\text{reading of F (mg/L) x total volume of the sample x R}}{\text{dry weight of the sample (g)}}$$

Where,

reading of F (mg/mL) = reading of Cu in the sample
dry weight of the sample (g) = (weight of dried sample) – (weight of porcelain dish)
R= reciprocal of the dilution

Statistical analysis

Ryan-Joiner (R-J) normality test

To determine if the soil physical and chemical parameters and total copper content in tissue and soil sample data came from a non-normal distribution, a Ryan-Joiner normality test using MiniTab 15 was performed. Salafranca ***et al.*** (2005) argued that the Ryan-Joiner normality test is based on the technique of Shapiro-Wilks, and therefore is extremely useful for small sample sizes ($n < 30$). The normality tests evaluate the null hypothesis (H_0) that the data follow a normal distribution. If the p-value for the test is less than the chosen α-level, then the null hypothesis must be rejected and it is concluded that the data do not follow a normal distribution. According to Osborne (2002), many statistical procedures assume that the variables are normally distributed. A significant violation of the assumption of normality can seriously increase the chances of the researcher of committing either a Type I (overestimation) or Type II (underestimation) error. Thus, one reason that researchers utilize data transformations is to

improve the normality of variables. For our study, the non-normal distributed data were transformed using square roots.

General Analysis of Variance (ANOVA)

To determine significant differences (less than the selected α-level threshold, typically 0.05) between physical and chemical parameters including total copper content in tissue and soil samples, a General Analysis of Variance was performed using MiniTab 15.

Microbial community analysis: Terminal Restriction Fragment Length Polymorphism (T-RFLP)

To evaluate the composition and complexity of the microbial community associated with the rhizosphere of the fern species; the T-RFLP method was employed. The total community DNA was extracted from soil samples of approximately 0.25 g obtained from rhizosphere material, and in soil areas with or without ferns during the two seasons in both tropical forests using the Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories Inc.). After extraction 100 µg of total community DNA was used as a template for the 16S rDNA amplification using the universal primers: 27F (5'-AGAGTTTGATCMTGGCTC-3') labeled with IR700 at the 5' terminal and 1392R (5'-ACGGGCGGTGTGTACA-3'). The PCR reactions were set up with the least possible exposure to light.

Each PCR reaction consisted of 100 ng of template DNA, 5 µL of 10X buffer, 6.0 µL of 25 mM $MgCl_2$, 1.0 µL of dNTP's mix [2.5 mM each], 1.0 µL of each primer [50 pmol/ µL] and 1.0 µL [5 U/µL] of ***Taq*** DNA polymerase (Promega®), adjusted with ddH_2O to a final reaction volume of 50 µL. Amplification was performed using a Perkin Elmer Gene Amp PCR System 2400. The cycling parameters were: denaturation at 95 °C for 5 min, followed by 37 cycles of melting at 94 °C for 1 min, annealing at 52 °C for 1 min 30 s, extension at 72 °C for 2 min and a

final extension at 72 ºC for 7 min. The resulting amplicons were resolved in a 1.0 % agarose gel (1X TAE) using 1 µg of Lambda Hind III DNA molecular marker to estimate their concentrations prior to restriction enzyme digestions.

A total of 200 ng of each labeled 16S rDNA product was used for restriction digestions with the following enzymes: ***Rsa***I [10 U/µL], ***Hae***III [10U/µL] and ***Msp***I [10 U/µL] (Promega®). Each digestion reaction consisted of 2.0 µL of 10 X reaction buffer, 0.5 µL of restriction enzyme and adjusted with ddH_2O to final volume of 20 µL. The digestions were incubated at 37 ºC in a water bath for 4 hrs followed by 10 min at 65 °C to inactivate the enzymes. Digestions were run on a LI-COR 4300 DNA Analysis System (Biology Department, UPR Mayagüez). Aliquots of each digestion were prepared by mixing with an equal amount of IR2 stop solution (LI-COR Biosciences) in a 1:1 proportion. A 5.5 % acrylamide gel (acrylamide gel matrix KB^{Plus}-LICOR) of 0.25 mm of thickness was prepared with 150 µl of 10 % ammonium persulfate and 20 mL of 15 % TEMED with a polymerization time of 90 min. The samples were denatured at 94 ºC for 3 min and kept at 4 ºC until loading the gel. A pre-run step for 20 min was performed using TBE 1X buffer (KB^{Plus}-LICOR) with the following parameters: voltage 1,500 (V), current 40 (mA), and power 40 (W). After the pre-run, one microliter of each denatured sample was loaded onto the gel, including a molecular sizing standard of 50-700 bp (KB^{Plus}-LICOR) in the first, middle and last lanes. The samples were run for 3 hrs 30 min with the same pre-run parameters. The T-RFLP fingerprints were collected in a TIF image and analyzed using the Gel Pro Analyzer 4.5 software. For this analysis the T-RF with a molecular weight between 50-700 bp were used. The fluorescence intensity signal observed in the electropherogram was used to determine the relative abundance of each T-RF after standardization by dividing the height of each peak by the sum of the height of all peaks of the same sample. After obtaining the data of T-RF detected and their

relative abundances, noise signals were removed after the creation of a ***PEAKS script*** described previously using the Common Lisp Implementation for ANSI (CLISP) software, version 2.38 *CLisp* (Caro-Quintero, 2008).

Text box 1:

> **PEAKS script-** created to detect the minimal values that can separate fragments peaks from noise based in a modification of statistical criteria previously proposed by Abdo ***et al.*** (2006). According to Caro-Quintero (2008) the peak identification relies on the difference of signal intensity of background noise and labeled DNA fragments. Noise values cluster around the signal median of T-RFLP scan points, while real peaks are distant from median values. Therefore, real peaks can be separated from background using a dispersal measure. Detection of real peaks is based on exclusion of values larger than the median plus three standard deviations ($\mu+3\sigma$), this calculation are done recursively until no larger values are removed from data set. To the end, the script saves the last calculated value as the threshold to consider real peaks.

After separated fragments peaks from noise, a ***BINNING script*** was created to group similar length fragments (peaks) from different samples as the same OTU. A final matrix of T-RF was constructed to identify with which possible phylogenetic groups the extracted fragments of the T-RFLP profiles could be associated. The molecular weights of all T-RF fragments were compared with the results of the PAT+ (http://mica.ibest.uidaho.edu/pat.php). Those were selected as possible phylogenetic groups.

Text box 2:

> **BINNING script-** created to group similar length fragments (peaks) from different samples as the same OTU. According to Caro-Quintero (2008) the script explores all possible binning groups and organizes individual sets of data

> in a unified matrix, allowing easy modification and posterior analysis. BINNING uses different criteria to detect possible binned groups, (1) fragment size, (2) congruence and (3) number of peaks within a possible OTU. The fragment size criteria established the maximum binning size of an OTU depending on fragment size as suggested by Brown *et al.* (2005) for Automatic rRNA Intergenic Spacer Analysis (ARISA). Windows of 2, 3, 5, or 10 bp are used for fragments of 200-400, 400- 700, 700-1000 and <1000 bp respectively. Congruence criteria discard binned groups that cluster two or more peaks of the same profile into the same OTU, script subdivide the grouping on 2 o more new OTUs. Finally the script counts the number of peaks that belong to each possible OTU and favor larger binned groups. Scripts output is a two space delimited text file, matrix merged file, and binning possible groups files. Final decision of binning groups is done using these two files in a Microsoft Office Excel 2003 spread sheet, where fragment lengths that are binned together are represented by their average size to create a final matrix.

The final matrix was also used to perform several multivariate statistical analyses (using PAlaeontological STatistics 1.86) such as: Non-metric multidimensional scaling (NMDS), Analysis of similarity (ANOSIM), Canonical Correspondence Analysis (CCA), Similarity Percentage (SIMPER), and Principal Components Analysis (PCA).

Text box 3:

> **NMDS** was used to determine the degree of similarity between T-RFLP biogeography samples. According to Reiss *et al.* (2009), the distance between samples on the multidimensional scaling plot corresponds to the degree of similarity between the samples. Previous to the analysis, the coefficients of similarity of Bray-Curtis (Bray and Curtis, 1957) were calculated.

ANOSIM was used to determine if the differences between the observed T-RFLP biogeography samples in the non-metric multidimensional scaling graphs were statistically significant. This analysis reports R- statistics and the corresponding p-value (that indicates if the R value is statistically significant). An R-statistic close to 1 indicates that there are significant differences between the two groups of samples compared, whereas an R-statistic close to 0 indicates that there are no significant differences between the two groups of samples (Reiss *et al*., 2009). Greater the R-statistic value, greater the dissimilarity between groups. The same distance matrix used in non-metric multidimensional scaling calculated with the coefficient of similarity of Bray-Curtis was used.

SIMPER was used to identify taxa primarily responsible for differences between T-RFLP biogeography samples. According to Clarke and Gorley (2001) the species will be listed in decreasing order of their importance in contributing to the average dissimilarity between two sample groups. This method is performed with the raw data, because it implicitly calculates a distance matrix with the Bray-Curtis similarity coefficient.

CCA was used to determine the relationships between microbial community and the physical and chemical parameters. According to Ter Braak (1995) in canonical correspondence analysis the communities are represented by points and environmental variables represented by arrows (vectors). Each vector has a length and a particular direction. The length of the vector corresponds to the significance that the environmental variables have in relation to the community. The position of the samples with respect to the vectors indicates how the changes in this community are related to the parameter. All the values used in canonical correspondence analysis (relative abundance of T-RF and the values of the different environmental parameters) were transformed by their square root.

PCA was used to visualize the relationships among soil microbial communities.

Cloning analysis of 16S rDNA

The dominant phylogenetic groups present in the rhizosphere microbial community of *Sticherus bifidus* (the ferns that accumulated more copper in their tissue) in the two seasons in both tropical forests were identified by sequencing analysis of clone libraries constructed with 16S rDNA genes that were PCR-amplified (approximately 1.4 Kb) from rhizosphere total community DNA. Total DNA extraction and PCR reaction were carried out as described in the TRFLP section, except that the primes used were unlabelled.

The amplified products were purified as described in the manufacturer's instructions for the Wizard® SV Gel and PCR Clean-up system (Promega®) and stored at -20 °C until cloning. The 16S rDNA gene product was cloned following the manufacturer's protocol for pGEM® T-Vector System (Promega® cat. No. A3600). One hundred microliters of each transformed live cell suspension in Luria Bertani (LB) was scattered on agar containing X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), IPTG (isopropyl-β-D-thio-galactopyranoside) and 50 μg/mL of ampicillin. These culture plates were incubated in the dark at 37 ºC for 18-20 hrs. Seventy-five clones of each library were randomly selected for sequencing. A volume of 6 mL of Luria Broth with 50 mg/mL of ampicillin enrichment was prepared to grow all the clones and they were incubated at 37°C in a rotary shaker at 120 rpm overnight. Plasmid extraction was prepared for each clone using the Wizard® Plus SV Minipreps DNA purification system (Promega® No. A1330). The presence of plasmids was verified and quantified by agarose gel electrophoresis, then concentrated using a Savant 120 DNA SpeedVac® system (Thermo Scientific®). Plasmid samples were placed in 96 well PCR plates and sent for single-strand sequencing to the High-Throughput Genomics Unit at the University of Washington State

acoording to the facility instructions. For preliminary identification, the sequences of each clone were analyzed with the data base of Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Detection of chimeric sequences among libaries was performedwith the Chimera-Check program available through the Ribosomal Dabase Project (RDP, http://rdp.cme.msu.edu/) whereas the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Bellerophon program was used to verify the absence of chimeric sequences in multiple alignments.

Clone libraries were labeled as: (1) **JBPS**- "Julio Bosque del Pueblo *S. bifidus*" for samples collected during the wet season at BPA and (2) **EBPS**- "Enero Bosque del Pueblo *S. bifidus*" for samples collected during the dry season at BPA, and (3) **JBMS**- "Julio Bosque Estatal de Maricao *S. bifidus*" for samples collected during the wet season at BEM and (4) **EBMS**- "Enero Bosque Estatal de Maricao *S. bifidus*" for samples collected during the dry season at BEM.

A virtual restriction digest or *In Silico* analysis using Hae III, Rsa I and Msp I restriction enzymes was performed using the program Restriction Mapper version 3 (http://www.restrictionmapper.org/).

RESULTS

Phytoaccumulation of copper by fern species

The natural copper accumulating capacity of GP and SB was determined **(Table 1)**. According to ANOSIM copper accumulation in: (1) fern roots change by fern species, by study site and by season (p-value: 0.00), (2) in rhizomes by fern species (p-value: 0.00) and (3) in fern fronds by study sites (p-value: 0.00). The results indicate that up to 7 times higher copper content was detected in roots than rhizomes and 5 times higher than fronds. When there was differences between plants species, SB tissue samples generally showed higher copper concentration than GB tissue**.**

Physical and chemical parameters of soil samples

Soil samples associated with GP and SB were examined for copper content, available phosphorus, exchangeable potassium, calcium and magnesium, organic matter and carbon percentage and pH taken in H_2O and $CaCl_2$. According to ANOVA available phosphorus, exchangeable potassium, calcium, magnesium, pH change by study site (p-value: 0.00). In addition, exchangeable potassium and calcium change by sampling area (p-value: 0.00). Soils collected at BPA presented a higher total copper content during both seasons with concentrations as much as 3 times higher than samples collected at BEM (**Table 2)**. According to ANOVA total copper content on soil change: (1) by study site and sampling area (p-value: 0.00) and (2) by season (p-value: 0.01).

Results related to available phosphorus, exchangeable calcium, magnesium and potassium, organic matter and carbon percentage and pH taken in H_2O and $CaCl_2$ **(Table 3)** indicates that: (1) available phosphorus was higher in soil samples at BPA, with concentrations 2 or 3 times higher than BEM, (2) exchangeable calcium and magnesium were higher in BEM,

with 2.23 cmol $_{(c)}$ Ca^{2+}/kg soil and 1.34 cmol $_{(c)}$ Mg^{2+}/kg soil, (3) exchangeable potassium was higher in SB site at BPA, with concentration 2 times higher than SB site at BEM. Whereas for the GP site the concentration of potassium at both locations was similar.Organic matter and carbon percentage were similar at both sites but in BPA the organic matter at the GP site was higher than the SB site, the opposite happened in the BEM. Collected soils appeared to be less acidt at BEM than those from BPA.

To determine if physical-chemical parameters can affect the vertical distribution of bacterial populations soil samples collected during November 2007 at different depths (5, 10, 15, 20, and 25 cm) at the GP and SB site in BPA **(Table 4)** and BEM **(Table 5)** were analyzed. According to ANOVA (1) OM%, OC% (p-value: 0.02), total Cu content, exchangeable K, Ca and Mg (p-value: 0.00) change by depth, (2) pH taken in H_2O, total Cu content (p-value: 0.02), exchangeable Ca and Mg (p-value 0.00) change by fern species, OM%, OC% (p-value 0.04) and (3) total Cu content, pH taken in $CaCl_2$ and in H_2O, exchangeable K, Ca and Mg (p-value: 0.00) change by study site.

Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

Fern influenced microbial communities and reference locations were examined by the culture independent 16S rDNA T-RFLP during the wet **(Appendix III)** and the dry **(Appendix IV)** seasons at BPA and BEM. The T-RF's profiles of 16S rRNA genes were in the range of 50 to 700 bp representing a wide range of bacterial populations within the soil microbial communities during both seasons **(Appendix V)**. A total of 5 and 9 exclusive phylotypes were observed in T-RF's profiles from soil microbial communities during the wet season when Hae III and Msp I were used. In contrast, during the dry season 7 and 1 exclusive phylotypes were

detected. During both seasons 6 exclusive phylotypes were observed when Rsa I was used **(Table 6).**

To evaluate the biogeography of microbial communities at both forests during the wet **(Appendices VI and VII)** and the dry **(Appendices VIII and IX)** season, culture independent 16S rDNA T-RFLP were performed **(Appendix X)**. T-RF's profiles from soil microbial communities collected at different depth and surface on GP site during both seasons revealed a total of 2 exclusive phylotypes when Hae III was used. In addition, when Msp I was used 4 exclusive phylotypes were observed during the wet season and 1 during the dry season. On SB site, only 1 exclusive phylotype was found during the dry season when Hae III was used **(Table 7)**.

Cloning analysis of 16S rDNA

To access soil metagenomic DNA from the rhizosphere of SB and its dominant phylogenetic groups, four environmental libraries were constructed using rhizosphere samples at BPA and at BEM during both seasons **(Appendices XI-XIV).**

After sequencing the recombinant plasmid, all the sequences smaller than 600 bp, sequences of poor quality or considered as chimeras (a gene sequence derived from more than one DNA template) and those occupied by parts of the cloning vector were discarded. The good sequences were divided in two groups, according to the sequenced gene direction, 5' → 3' or 3' → 5' **(Appendix XV).** Fragments within a library that had a one base length difference were considered in the T-RFLP as the same terminal fragment. Fragments greater than 700 bp or less than 50 bp could not be located in the T-RFLP, because they were outside the measurement range provided by the molecular weight markers. The dominant phylogenetic groups in SB rhizosphere at BPA and BEM during both seasons were: α-proteobacteria, γ-proteobacteria, δ-

proteobacteria β-proteobacteria, Acidobacteria, Planctomyces, Verrucomicrobia, Firmicutes and Bacteroidetes **(Figures 8-11)**.

To determine if the clones were also detected through the T-RFLP, an *in silico* analysis **(Appendices XVI)** were performed. The *in silico* analysis revealed that the 100% of JBPS clones were found in the soil and biogeography study TRFLP when Hae III and Msp I were used and the 67% in the soil study TRFLP when Rsa I was used. In contrast, the 86% of JBMS clones were found with Hae III, 71% with Msp I and 27% with Rsa I. The metagenomic library EBPS revealed that 100% of clones were found in the soil and biogeography study TRFLP when Hae III and Msp I were used and the 25% in the soil study TRFLP when Rsa I was used. In contrast, for EBMS the 87% of clones were found with Hae III, 100 % with Msp I and 50% with Rsa I.

Statistical analysis

Principal Components Analysis (PCA)

To determine the degrees of similarity and differences between the microbial communities PCA was performed. According to the analysis can observe the following: (1) samples collected during the wet season are more similar than during the dry season, (2) microbial communities associated with GP and with SB are different and (3) rhizosphere and fern site microbial communities for each fern are similar **(Figure 12)**. In the biogeography study at SB site **(Figure 14)** one finds that: (1) microbial communities at BPA (with the exception of sample 9), during both seasons are similar and (2) at BEM the microbial communities during both seasons are different, but if we analyze them separately (the dry and the wet season) can conclude that they are similar. The biogeography study at GP **(Figure 13)** revealed that: (1) microbial communities during the wet season at both sites are different, but if we analyze them individually (BPA and BEM), can observe a similar pattern in samples 1 to 6

and 7 to 9 and (2) microbial communities during the dry season (with the exception of samples: 1 from both seasons, 5 from BEM and 2 and 8 from BPA) are different.

Analysis of Similarity (ANOSIM) and Similarity Percentage (SIMPER)

To determine if there are significant differences between microbial communities compared and determine which taxa were primarily responsible for differences among compared samples we performed ANOSIM and SIMPER method, respectively. Significant differences between microbial communities compared were found **(Table 8)**. According to the results, microbial communities from GP at BPA during both seasons were the comparative samples that differed more (R=0.8351). According to SIMPER, those differences can be related with the following taxa: Firmicutes, α-proteobacteria, γ-proteobacteria, Tenericutes, Actinobacteria, Fusobacteria, Thermotogae and Spirochaetes **(Table 9 a and b).** In contrast, the comparative samples which differed less were SB at BPA during both seasons (R=0.3741).

Canonical Correspondence Analysis (CCA)

To determine which physico-chemical parameters affect the soil microbial communities we made CCA**.** The results revealed that **(Figure 28)** some samples are similar affected by certain soil nutrient:(1) sample 2 from BPA during both seasons are similar affected by exchangeable potassium and (2) sample 1 from BEM during both season are similar affected by exchangeable Ca and Mg. In the biogeography study at SB site **(Figure 29)** we observed that: microbial communities during the dry season (with the exception of samples 8 and 9) are more affected by OM %, OC %, exchangeable K and available P than during the wet season. At GP site **(Figure 30)** we found that some samples are similar affected by certain soil physical and chemical parameter: (1) samples 5 and 6 at BPA during both seasons are similar affected by total

Cu content and (2) sample 4 at BEM during both seasons are similar affected by OC % and OM%.

Non-Metric Multidimensional Scaling (NMDS)

To examine the biogeographical pattern of microbial communities a NMDS was performed. At GP site **(Figure 15)** samples 4 and 9 during both seasons and study sites are more distantly from sample 1 than the other samples**.** The biogeography study at SB site **(Figure 16)** revealed that: (1) samples collected at BEM during both seasons are similar; (2) samples collected at BPA during the dry season are different and during the wet season are similar.

DISCUSSION

Copper accumulating capacity of *Gleichenella pectinata* and *Sticherus bifidus*

Studying heavy metal accumulation by plants has important implications in the field of phytorremedation, because they can be used to restore disturbed environments or function as ***bio-indicators*** of contamination. For example, in a research done by Al-Farraj and Al-Wabel (2007) they evaluated concentration of heavy metals in plant species to identify the best adapted for the uptake of a specific metal, to use them as a bio-indicator. The enhancement of plants to accumulate more heavy metals by adding nutrients that promote growth or the modification of soil properties is sometimes necessary in phytorremedation. Hence, biotic and abiotic factors that may influence the accumulation have to be studied. For example, in a research done by Tongbin ***et al.*** (2002), they examined the effect of phosphorus on arsenic accumulation in the hyperaccumulator fern ***Pteris vittata*** L to increase the heavy metal uptake. They found that phytoremediation using that fern can be elevated by the phosphorus addition at high rates. Research like this is important, because if some area like a forest is contaminated with heavy metal we can increase the heavy metal uptake by plants that live there.

G. pectinata and *S. bifidus* are not copper hyperaccumulator plants **(Table 1)**, because (1) they do not transport the accumulated copper from roots to fronds and (2) they do not accumulated more than 1,000 mg kg^{-1} dry weight (DW) of copper in their tissues (Baker and Brooks, 1989). Significant differences suggest that fern species, study site and seasons influence the accumulation of copper by ferns. According to Kabata-Pendias and Pendias (1984) the type of plant root system and the response of plants to elements in relation to seasonal cycles control the heavy metal uptake. In addition, according to Ghosh and Singh (2005) metal solubility in

soils is predominantly controlled by pH, amount of metals, cation exchange capacity and organic carbon content.

Effect of biotic and abiotic factors over microbial communities associated with ferns

According to ANOSIM **(Table 8)**, significant differences were obtained in a greater part of samples compared. These significant differences suggest that microbial communities associated are different among the samples that were compared. Flores-Mireles *et al.* (2007) used a combination of TRFLP, ANOSIM and SIMPER to analyze the compositions and compositions of N_2 fixers (*nifH*) and denitrifiers (*nirS* and *nirK)* associated with mangrove roots growing at three different kinds of soils. Using the SIMPER **(Tables 7 a and b)** we conclude that those differences can be associated with the contribution of taxa related with following phylum: Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria, Nitrospira, Fibrobacteres and Bacteroidetes. According to Fierer *et al.*, 2007, the six soil dominant bacterial phyla are: Acidobacteria, Bacteroidetes, Firmicutes, Actinobacteria, α-proteobacteria, and the β-proteobacteria. According to the results some OUT was related with: **Firmicutes**: *Thiothrix eikelboom*, **Actinobacteria**: *Kitasatospora kifunensis*, **Proteobacteria**: *Oligotropha carboxidovorans, Bacillus caryophylli, Bacillus subtilis, Bradyrhizobium elkanii, and Rhodomicrobium vannielli, Pseudomonas sp.* and *Burkholderia sp.* and **Bacteroidetes**: *Flavobacterium sp.*

To determine which factors contribute to differences in microbial communities we evaluated the effect of fern species, season, and soil properties. In a study done by Marschner *et al.* (2004) they concluded that the composition of soil microbial communities are affected by physical and chemical characteristics of the soil and environmental factors such as climate and vegetation, the latter being the one with the strongest impact. The Principal Components

Analysis (PCA) **(Figure 12)** suggests that microbial communities are more influenced by seasons, than by fern species or study sites. According to CCA **(Figure 17)**, microbial communities from BPA collected samples seem to be more influenced by total copper, available phosphorus and exchangeable potassium and those from BEM collected samples which seemed to be more influenced by exchangeable calcium and magnesium, pH. Values for these parameters (total copper, available phosphorus and exchangeable potassium) were higher at BPA collected samples **(Tables 2 and 3)** relative to BEM collected samples which in contrast showed a more alkaline pH as well as higher concentrations of exchangeable calcium and magnesium **(Table 3)**.

According to NMDS microbial communities associated with the GP site (**Figure 15)** at BEM collected samples revealed that their biogeographical distribution at the different sampling points collected during the dry season was more similar than during the wet season. In contrast, surface communities at BPA collected samples during both seasons and depth communities during the wet season were similar, but those observed at different depths during the dry season were different. At the SB site **(Figure 16)**, the biogeography of microbial communities at BEM collected samples during both seasons were similar. In contrast, soil surface communities at BPA collected samples during both seasons and from different depth during wet season were similar, but those observed at different depths during dry season were different.To determine which factors can affect the biogeography of microbial communities, the effect of soil properties, study sites and season were evaluated. According to CCA **(Figure 19)**, we conclude that total copper, available phosphorus and exchangeable potassium seem to influence the biogeography of microbial communities associated with SB at BPA collected samples during both seasons. In contrast, exchangeable calcium and, magnesium, pH taken in H_2O and in $CaCl_2$, the percentage

of organic carbon and matter seem to influence the biogeography of microbial communities at BEM collected samples. Canonical Correspondence Analysis (CCA) also showed that total copper, available phosphorus, exchangeable potassium as well as the percentage of organic carbon and matter seems to influence the biogeography of microbial communities associated with GP at BPA collected samples during both seasons **(Figure 18)**. In contrast, exchangeable calcium and magnesium, pH taken in H_2O and in $CaCl_2$ seem to influence the biogeography of microbial communities at BEM collected samples. According to PCA **(Figure 13)**, sampling sites and seasons seem to influence the biogeography of microbial communities associated with GP during both seasons and study sites. Principal Components Analysis (PCA) **(Figure 14)** also showed that the biogeography of microbial communities associated with SB at BPA collected samples during both seasons was apparently influenced by sampling site. In contrast, sampling sites and seasons seem to dictate microbial community composition associated with SB during both seasons at BEM collected samples.

Phylogenetic groups associated with *Sticherus bifidus* rhizosphere

Results **(Appendices XI to XIV)** revealed that some metagenomic clones were related to heavy metal resistant bacteria like *Bradyrhizobium sp.* (Tong and Sadowsky, 1994), *Pseudomonas sp.*, *Flavobacterium sp.* and *Bacillus sp.* (Rajbanshi, 2008). Some examples are: *Bradyrhizobium japonicum* (clone JBPS 15) and *Bradyrhizobium elkanii* (clone EBMS 44).

According to our results **(Figures 8 to 11)**, the microbial communities associated with the Acidobacteria phyla were observed at both sites during both seasons. In contrast, those associated with the phyla Planctomyce were detected at both sites only during the wet season. These results suggest that temporal and climate-related factors have a strong selective role on the presence of phyla. Based on the results from ANOSIM (R=0.5936) and SIMPER analyses it was

observed that during the dry season microbial communities at BEM collected samples appeared to be more diverse than those at BPAcollected samples.This can be true for the higher concentration of copper at BPA soils and the low leaching of nutrients that is normally observed during the dry season.

Additionally, ANOSIM revealed that during the dry season the microbial communities at BEM collected samples seemed to be more diverse with respect to that of the wet season (R=0.7277). These results provide further evidence in support of the notion that season can be a possible factor affecting the diversity. In studies done by Marschner ***et al.*** (2002) they concluded that during the dry season, a large portion of the microbial biomass of rhizosphere dies resulting in a change in community composition. These observation can be also related with the precence or abscence of Planctomyces phyla detected at both sites only during the wet season. According to CCA **(Figure 17)** soil pH as well as calcium and magnesium availability seem to be other important factors determining the diversity of microbial communities at SB rhizosphere.

After making a study of this kind, we can observe that two ferns in different forests within a same country have different microbial communities due to soil properties, season, and study sites and fern species. This makes us think about the existing and yet unknown microbial diversity present in tropical environments.

CONCLUSIONS

- ✓ Niether GP and SB were copper hyperaccumulator plants.
- ✓ Copper accumulation in fern roots was influenced by fern species, study site and season. Copper accumulation in rhizomes was influenced by fern species.
- ✓ In general, differences in microbial community composition are associated with the presence of Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria, Nitrospira, Fibrobacteres and Bacteroidetes.
- ✓ Microbial communities seem to be more influence by seasons, than by ferns species or study sites.
- ✓ Microbial communities at BPA seem to be influenced by copper, available phosphorus and exchangeable potassium while those from BEM were apparently influenced by exchangeable calcium and magnesium, and pH taken in H_2O and in $CaCl_2$.
- ✓ Microbial communities associated with the GP site at BEM revealed that their biogeography (depth and surface) distribution during the dry season was more similar than during the wet season.
- ✓ The biogeography of microbial communities associated with GP at BPA was different regardless of the season.
- ✓ Under SB cover at BEM, microbial communities were similar during both seasons.
- ✓ Surface communities at BPA were similar during both seasons. Subsurface communities during the wet season were also similar. Communities observed at different depths during the dry season were not similar.
- ✓ Sampling sites and seasons influenced the biogeography of microbial communities associated with GP during both seasons and at both study sites.

- ✓ The biogeography of microbial communities associated with SB at BPA during both seasons was influenced by sampling site.
- ✓ Sampling sites and seasons influenced microbial communities associated with SB during both seasons at BEM.
- ✓ Microbial communities associated with the Acidobacteria phyla were observed at both sites during both seasons. Planctomyce were observed at both sites, but only during the wet season.
- ✓ During the dry season, microbial communities at BEM were more diverse during the dry season than those at BPA. Microbial communities at BEM were more diverseduring the dry than during the wet season.
- ✓ Fithteen clones were related to heavy metal resistant bacteria (*Bradyrhizobium sp. Pseudomonas sp., Flavobacterium sp.* and *Bacillus sp.*)
- ✓ Soil pH and calcium and magnesium availability were factors that determined the diversity of microbial communities in the SB rhizosphere.

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TABLES

Table 1. Copper content (mg/kg) in *G. pectinata* and *S. bifidus* tissue collected from Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet seasons.

Study site species	Fern	Fronds		Rhizomes		Roots	
		Wet season	Dry season	Wet season	Dry season	Wet season	Dry season
Bosque del Pueblo	***G. pectinata***	11.7 (3.83)	15.6 (2.73)	6.8 (2.11)	9.4 (7.51)	49.2 (10.52)	25.8 (3.64)
	S. bifidus	14.8 (2.03)	9.8 (2.40)	11.3 (1.61)	10.7 (1.72)	63.1 (21.69)	52.0 (9.58)
Bosque Estatal de Maricao	***G. pectinata***	11.9 (4.84)	3.0 (0.38)	6.2 (3.25)	6.4 (2.29)	29.8 (1.82)	14.7 (2.70)
	S. bifidus	9.3 (2.35)	8.3 (1.61)	10.7 (2.43)	14.7 (2.70)	48.4 (10.94)	19.3 (2.06)

average (standard deviation); n=3 for roots and n=5 for rhizomes and fronds

Table 2. Total copper content (mg/kg) in soil samples collected from Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet seasons.

average (standard deviation); n=5

Study site	Sampling area	Total copper content (ppm)	
		Wet season	Dry season
Bosque del Pueblo	*G. pectinata* site	297.1 (52.45)	211.0 (29.02)
	S. bifidus site	270.5 (27.71)	182.9 (12.94)
	Without ferns	136.9 (7.71)	158.4 (13.05)
Bosque Estatal de Maricao	*G. pectinata* site	84.2 (1.93)	91.5 (1.61)
	S. bifidus site	90.0 (3.44)	76.4 (1.02)
	Without ferns	84.9 (3.82)	78.2 (1.22)

Table 3. Physical and chemical parameters in soil samples collected from Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR).

Parameter	Bosque del Pueblo			Bosque Estatal de Maricao		
	***G. pectinata* site**	***S. bifidus* site**	**Without ferns**	***G. pectinata* site**	***S. bifidus* site**	**Without ferns**
Available phosphorus[a]	3.70 (0.200)	2.20 (0.700)	2.20 (0.046)	1.23 (0.252)	1.37 (0.379)	ND
Exchangeable potassium[b]	0.17 (0.014)	0.12 (0.010)	0.08 (0.01)	0.13 (0.011)	0.06 (0.009)	0.07 (0.003)
Exchangeable calcium[b]	0.94 (0.018)	0.55 (0.016)	1.09 (0.019)	1.58 (0.062)	1.17 (0.307)	2.23 (0.027)
Exchangeable magnesium[b]	0.73 (0.124)	0.31 (0.009)	0.71 (0.568)	1.04 (0.514)	1.24 (0.172)	1.34 (0.335)
Organic Carbon Percentage	1.97 (0.112)	1.17 (0.055)	1.52 (0.070)	1.33 (0.036)	1.66 (0.216)	2.03 (0.035)
Organic Matter percentage	4.42 (0.255)	2.61 (0.127)	3.40 (0.155)	2.98 (0.082)	3.72 (0.485)	4.55 (0.075)
pHH_2O	4.19 (0.111)	4.64 (0.140)	4.81 (0.149)	5.27 (0.159)	4.99 (0.158)	5.03 (0.157)
pH$CaCl_2$	2.90 (0.057)	3.34 (0.064)	3.38 (0.076)	4.15 (0.44)	3.81 (0.038)	4.20 (0.172)

average (standard deviation); n= 3 for the other physical and chemical parameters; [a]ppm; [b]cmol $_{(c)}$ K^+/kg soil, cmol $_{(c)}$ Ca^{2+}/kg soil or cmol $_{(c)}$ Mg^{2+}/kg soil; ND= Not detected with the method used

Table 4. Physical and chemical parameters at various depths at *G. pectinata* and *S. bifidus* sites in Bosque del Pueblo (Vegas Arriba, Adjuntas PR).

	Bosque del Pueblo									
	***G. pectinata* site**					***S. bifidus* site**				
	Depth (cm)									
Parameter	**5**	**10**	**15**	**20**	**25**	**5**	**10**	**15**	**20**	**25**
Total copper	267.5 (22.00)	341.0 (28.54)	205.9 (13.44)	258.4 (24.54)	216.6 (3.10)	245.9 (45.16)	204.1 (1.73)	167.8 (20.19)	179.3 (28.57)	204.6 (15.16)
Available phosphorus[a]	1.91 (0.237)	1.54 (0.165)	0.93 (0.046)	0.67 (0.046)	0.38 (0.182)	0.65 (0.000)	0.44 (0.046)	0.49 (0.079)	0.75 (0.456)	3.17 (1.617)
Exchangeable potasium[b]	0.14 (0.003)	0.11 (0.004)	0.07 (0.009)	0.04 (0.007)	0.03 (0.006)	0.07 (0.012)	0.05 (0.007)	0.03 (0.011)	0.03 (0.010)	0.02 (0.005)
Exchangeable calcium[b]	0.42 (0.010)	0.29 (0.054)	0.30 (0.094)	0.12 (0.017)	0.14 (0.047)	0.31 (0.009)	0.21 (0.016)	0.16 (0.028)	0.14 (0.016)	0.12 (0.006)
Exchangeable magnesium[b]	0.38 (0.004)	0.25 (0.005)	0.20 (0.001)	0.11 (0.000)	0.10 (0.002)	0.14 (0.004)	0.11 (0.003)	0.09 (0.002)	0.08 (0.016)	0.09 (0.004)
Percentage organic carbon	1.65 (0.108)	1.18 (0.136)	0.87 (0.021)	0.23 (0.087)	ND	0.43 (0.135)	0.37 (0.017)	ND	ND	ND
Percentage organic matter	3.69 (0.237)	2.65 (0.304)	1.99 (0.047)	0.51 (0.196)	ND	0.96 (0.301)	0.83 (0.035)	ND	ND	ND
pH H_2O	3.97 (0.238)	3.99 (0.211)	4.15 (0.127)	4.64 (0.061)	4.88 (0.180)	4.93 (0.072)	5.20 (0.146)	4.91 (0.529)	4.53 (0.092)	4.91 (0.042)
pH $CaCl_2$	2.96 (0.120)	3.02 (0.010)	3.22 (0.072)	3.33 (0.055)	3.42 (0.064)	3.56 (0.044)	3.64 (0.021)	3.58 (0.017)	3.74 (0.020)	3.84 (0.025)

average (standard deviation); n=5 for total copper content and n=3 for the other physical and chemical parameters; [a]ppm; [b]cmol $_{(c)}$ K^+/kg soil, cmol $_{(c)}$ Ca^{2+}/kg soil or cmol $_{(c)}$ Mg^{2+}/kg soil; ND= Not detected with the method used

Table 5. Physical and chemical parameters at various depths at *G. pectinata* and *S. bifidus* sites in Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR)

	Bosque Estatal de Maricao									
	***G. pectinata* site**					***S. bifidus* site**				
	Depth (cm)									
Parameter	**5**	**10**	**15**	**20**	**25**	**5**	**10**	**15**	**20**	**25**
Total copper	75.4 (9.55)	77.5 (4.63)	70.5 (1.82)	93.2 (9.45)	103.7 (2.71)	115.9 (4.05)	99.2 (4.47)	7 8.1 (10.57)	89.4 (8.76)	81.2 (5.1428)
Available phosphorus[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Exchangeable potasium[b]	0.10 (0.005)	0.02 (0.004)	0.01 (0.002)	0.01 (0.002)	ND	0.06 (0.004)	0.05 (0.005)	0.05 (0.002)	0.05 (0.005)	0.02 (0.002)
Exchangeable calcium[b]	1.21 (0.038)	0.44 (0.065)	0.31 (0.012)	0.21 (0.024)	0.31 (0.441)	2.22 (0.061)	1.81 (0.214)	1.82 (0.066)	1.30 (0.035)	0.54 (0.022)
Exchangeable magnesium[b]	1.02 (0.042)	0.26 (0.006)	0.21 (0.008)	0.15 (0.003)	0.08 (0.003)	2.58 (0.112)	1.72 (0.098)	1.49 (0.085)	1.35 (0.026)	0.63 (0.013)
Percentage organic carbon	1.46 (0.295)	0.54 (0.080)	0.48 (0.035)	0.35 (0.017)	0.22 (0.017)	1.45 (0.055)	1.52 (0.050)	1.72 (0.238)	1.62 (0.099)	0.90 (0.023)
Percentage organic matter	3.26 (0.660)	1.21 (0.174)	1.07 (0.075)	0.79 (0.040)	0.49 (0.040)	3.25 (0.127)	3.41 (0.116)	3.86 (0.533)	3.63 (0.223)	2.02 (0.052)
pH H_2O	4.98 (0.090)	5.22 (0.074)	5.12 (0.021)	5.20 (0.081)	4.95 (0.110)	5.17 (0.133)	5.22 (0.178)	4.97 (0.253)	4.73 (0.123)	4.73 (0.199)
pH $CaCl_2$	4.37 (0.075)	4.83 (0.042)	4.90 (0.025)	5.05 (0.130)	5.22 (0.095)	4.38 (0.482)	3.76 (0.197)	4.06 (0.226)	4.04 (0.020)	4.09 (0.021)

average (standard deviation); n=5 for total copper content and n=3 for the other physical and chemical parameters; [a]ppm; [b]cmol $_{(c)}$ K^+/kg soil, cmol $_{(c)}$ Ca^{2+}/kg soil or cmol $_{(c)}$ Mg^{2+}/kg soil; ND= Not detected with the method used

Table 6. Exclusive phylotypes detected in *G. pectinata* and *S. bifidus* site collected at Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet season.

Samples	Phylotypes (bp)					
	Hae III		Msp I		Rsa I	
	Wet	Dry	Wet	Dry	Wet	Dry
BPAGR	172 and 390	-	267, 327 and 590	-	-	-
BPAGS	-	117, 433, 438, 459 and 462	-	-	-	-
BPASR	-	-	-	-	-	-
BPASS	-	-	286	280	-	256
BPAWF	-	181	232 and 297	-	-	-
BEMGR	-	-	-	-	164, 213, 311 and 559	139 and 231
BEMGS	NRS	-	NRS	-	NRS	-
BEMSR	-	-	-	-	-	-
BEMSS	273, 341 and 423	-	252, 254 and 576	-	163 and 629	-
BEMWF	-	596	-	-	-	249, 525 and 659

NRS= the restriction enzyme not recognize any restriction site; - = exclusive phylotypes not detected

Table 7. Exclusive phylotypes from samples detected at different depths in *G. pectinata* and *S. bifidus* site collected at Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet season.

Samples		Phylotypes (bp)			
		Hae III		Msp I	
		Wet	Dry	Wet	Dry
GP 0HP	BPA	NRS	-	NRS	-
	BEM	-	-	-	-
GP 10HP	BPA	-	-	-	-
	BEM	-	-	-	-
GP 100HP	BPA	-	-	425	-
	BEM	-	-	-	-
GP 1000HP	BPA	-	-	329, 378 and 410	-
	BEM	-	-	-	190
GP 5VP	BPA	-	-	-	-
	BEM	-	-	-	-
GP 10VP	BPA	-	-	-	-
	BEM	-	-	-	-
GP 15VP	BPA	-	-	-	-
	BEM	-	590 and 684	-	
GP 20VP	BPA	-	-	-	-
	BEM	-	-	-	-
GP 25VP	BPA	-	-	-	-
	BEM	458 and 497	-	-	-

NRS= the restriction enzyme not recognize any restriction site; - = exclusive phylotypes not detected

Table 7. (continuation)

SB 0HP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 10HP	BPA	-	-	-	-
	BEM	-	449	-	-
SB 100HP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 1000HP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 5VP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 10VP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 15VP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 20VP	BPA	-	-	-	-
	BEM	-	-	-	-
SB 25VP	BPA	-	-	-	-
	BEM	-	-	-	-

- = exclusive phylotypes not detected

Table 8. Analysis of Similarity (ANOSIM) for soil samples collected at *G.pectinata* and *S. bifidus* site at Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet season.

Samples compared	R	p-value
GPBPA and SBBPA wet season	0.7092	0.00
GPBPA and SBBPA dry season	0.5521	0.00
GPBEM and SBBEM wet season	0.6166	0.00
SBBPA and SBBEM wet season	0.6108	0.00
SBBPA and SBBEM dry season	0.5936	0.00
GPBPA and GPBEM dry season	0.5610	0.00
GPBEM and SBBEM dry season	0.6879	0.00
GPBPA and GPBEM wet season	0.4661	0.00
SBBPA both seasons	0.3741	0.00
GPBPA both seasons	0.8351	0.00
GPBEM both seasons	0.6509	0.00
SBBEM both seasons	0.7277	0.00

If R is close to 1 (R›0.5) indicates that there are significant differences between two groups of samples compared, whereas an R close to 0 (R‹0.5) indicates that there are no significant differences. P-value indicates if the R is statistically significant.

Table 9. a. Similarity Percentage (SIMPER) for soil samples collected in *G. pectinata* and *S. bifidus* site at Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet season.

Comparison	Taxon[1]	Contribution[2]	Fragment size (bp)	Phylum
SB BPA Dry season vs. SB BEM Dry season	65M	4.436	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
	239H	2.788	237	Actinobacteria
			238	Firmicutes
			239	δ-Proteobacteria, Firmicutes
			240	Firmicutes
GP BPA Wet season vs. GP BEM Wet season	271M	3.889	268	γ-Proteobacteria, Fusobacteria
			269	-
			272	Thermotogae
			273	-
	245H	3.709	242	Firmicutes
			243	α-Proteobacteria, Firmicutes
			244	γ- Proteobacteria, Firmicutes, Tenericutes
			245	Fusobacteria, Actinobacteria, Firmicutes
			246	Actinobacteria, Firmicutes
			247	Thermotogae, γ-Proteobacteria, Actinobacteria, Firmicutes
GP BPA Wet season vs. GP BPA Dry season	245H	4.347	242	Firmicutes
			243	α-Proteobacteria, Firmicutes
			244	γ-Proteobacteria, Tenericutes, Firmicutes
			245	Fusobacteria, Actinobacteria, Firmicutes
			246	Actinobacteria, Firmicutes
			247	Thermotogae, γ-Proteobacteria, Actinobacteria, Firmicutes
	271M	3.772	268	Spirochaetes, Fusobacteria
			269	-
			272	Thermotogae
			273	-

[1] *Number:* Average of the taxon range and *Letter:* Restriction enzymes - **H:** HaeIII (restriction site: GG^CC), **M:** MspI (restriction site: C^CGG) and **R:** RsaI (restriction site: GT^AC); [2] 10% of overall averages dissimilarity; - Not found in the data base

Table 9 a. (continuation)

GP BEM Wet season vs. GP BEM Dry season	197H	2.208	196	α-Proteobacteria, Actinobacteria, Tenericutes
			197	α-Proteobacteria, δ-Proteobacteria, Firmicutes, Tenericutes
			198	β-Proteobacteria, Firmicutes
	216H	2.059	214	Deinococcus-Thermus, Firmicutes
			215	δ-Proteobacteria, β-Proteobacteria, Firmicutes
			216	Actinobacteria, Firmicutes
			217	β-Proteobacteria, δ-Proteobacteria, γ-Proteobacteria, Deinococcus-Thermus, Firmicutes
			218	Actinobacteria, Firmicutes
	443M	1.617	442	α-Proteobacteria
			443	Cyanobacteria, α-Proteobacteria
			444	γ-Proteobacteria
	160M	1.444	157	Actinobacteria, Firmicutes, Tenericutes,
			158	Firmicutes, Actinobacteria
			159	Actinobacteria, Tenericutes, Firmicutes, Synergistetes
			160	δ-Proteobacteria, Actinobacteria, Firmicutes
			161	Actinobacteria, Firmicutes, δ-Proteobacteria, Fusobacteria
			162	Nitrospira, δ-Proteobacteria, Actinobacteria
GP BPA Dry season vs. GP BEM Dry season	65H	2.451	64	α-Proteobacteria, Actinobacteria
			65	-
			66	Actinobacteria
	239H	2.45	237	Actinobacteria, Firmicutes
			238	Firmicutes
			239	δ-Proteobacteria, Firmicutes
			240	Firmicutes
	65M	2.047	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
SB BPA Wet season vs. SB BEM Wet season	239H	3.453	237	Actinobacteria, Firmicutes
			238	Firmicutes
			239	δ- Proteobacteria, Firmicutes
			240	Firmicutes
			264	Deinococcus-Thermus
			265	Deinococcus-Thermus, Thermotogae, ε-Proteobacteria
	266M	3.044	266	-
			267	Fibrobacteres

[1] *Number:* Average of the taxon range and *Letter:* Restriction enzymes - **H:** HaeIII (restriction site: GG^CC), **M:** MspI (restriction site: C^CGG) and **R:** RsaI (restriction site: GT^AC); [2] 10% of overall averages dissimilarity; - Not found in the data base

Table 9 a. (continuation)

SB BPA Wet season vs. SB BPA Dry season	239H	3.551	237	Actinobacteria, Firmicutes
			238	Firmicutes
			239	δ-Proteobacteria, Firmicutes
			240	Firmicutes
	65M	2.652	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
SB BEM Wet season vs. SB BEM Dry season	65M	2.41	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
	216H	2.327	214	Deinococcus-Thermus, Firmicutes
			215	δ-Proteobacteria, β-Proteobacteria, Firmicutes
			216	Actinobacteria, Firmicutes
			217	β-Proteobacteria, δ-Proteobacteria,γ-Proteobacteria, Deinococcus-Thermus, Firmicutes, Actinobacteria
			218	Actinobacteria, Firmicutes
	141M	2.292	140	Bacteroidetes, Firmicutes, Actinobacteria
			141	β-Proteobacteria, γ-Proteobacteria, Actinobacteria, Firmicutes
			142	Bacteroidetes, γ-Proteobacteria, β-Proteobacteria, Firmicutes

[1] *Number:* Average of the taxon range and *Letter:* Restriction enzymes - **H:** HaeIII (restriction site: GG^CC), **M:** MspI (restriction site: C^CGG) and **R:** RsaI (restriction site: GT^AC); [2] 10% of overall averages dissimilarity; - Not found in the data base

Table 9. b. Similarity Percentage (SIMPER) for soil samples collected at *G. pectinata* and *S. bifidus* site at Bosque del Pueblo (Vegas Arriba, Adjuntas PR) and Bosque Estatal de Maricao (Tabonuco, Sabana Grande PR) during the dry and the wet season.

Comparison	Taxon[1]	Contribution[2]	Fragment size (bp)	Phylum
GB BPA Wet season vs. SB BPA Wet season	245H	4.686	242	Firmicutes
			243	α-Proteobacteria, Firmicutes
			244	γ-Proteobacteria, Tenericutes, Firmicutes
			245	Fusobacteria, Actinobacteria, Firmicutes
			246	Actinobacteria, Firmicutes
			247	Thermotogae, γ-Proteobacteria, Actinobacteria, Firmicutes
	271M	4.057	268	Spirochaetes, Fusobacteria
			269	-
			272	Thermotogae
			273	-
GP BPA Dry season vs. SB BPA Dry season	65M	4.268	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
	65H	3.189	64	α-Proteobacteria, Actinobacteria
			65	-
			66	Actinobacteria
GB BEM Wet season vs. SB BEM Wet season	197H	2.912	196	α-Proteobacteria, Actinobacteria, Tenericutes
			197	α-Proteobacteria, δ-Proteobacteria, Firmicutes, Tenericutes
			198	β-Proteobacteria, Firmicutes
	195H	2.59	194	α-Proteobacteria, Actinobacteria
			195	α-Proteobacteria, γ-Proteobacteria, Actinobacteria

[1] *Number:* Average of the taxon range and *Letter:* Restriction enzymes - **H:** HaeIII (restriction site: GG^CC), **M:** MspI (restriction site: C^CGG) and **R:** RsaI (restriction site: GT^AC); [2] 10% of overall averages dissimilarity;- Not found in the data base

Table 9 b. (continuation)

GP BEM Dry season vs. SB BEM Dry season	65M	2.074	63	-
			64	Firmicutes
			65	Firmicutes
			66	Firmicutes
	245H	1.815	242	Firmicutes
			243	α-Proteobacteria, Firmicutes
			244	Firmicutes, γ-Proteobacteria, Tenericutes
			245	Fusobacteria Firmicutes, Actinobacteria,
			246	Actinobacteria, Firmicutes,
			247	Thermotogae,γ-Proteobacteria, Actinobacteria, Firmicute
	216H	1.722	214	Deinococcus-Thermus, Firmicutes
			215	δ-Proteobacteria, β-Proteobacteria, Firmicutes
			216	Actinobacteria, Firmicutes
			217	β-Proteobacteria, γ-Proteobacteria, δ-proteobacteria, Deinococcus-Thermus, Firmicutes
			218	Actinobacteria, Firmicutes

[1] *Number:* Average of the taxon range and *Letter:* Restriction enzymes - **H:** HaeIII (restriction site: GG^CC), **M:** MspI (restriction site: C^CGG) and **R:** RsaI (restriction site: GT^AC); [2] 10% of overall averages dissimilarity;- Not found in the data base

FIGURES

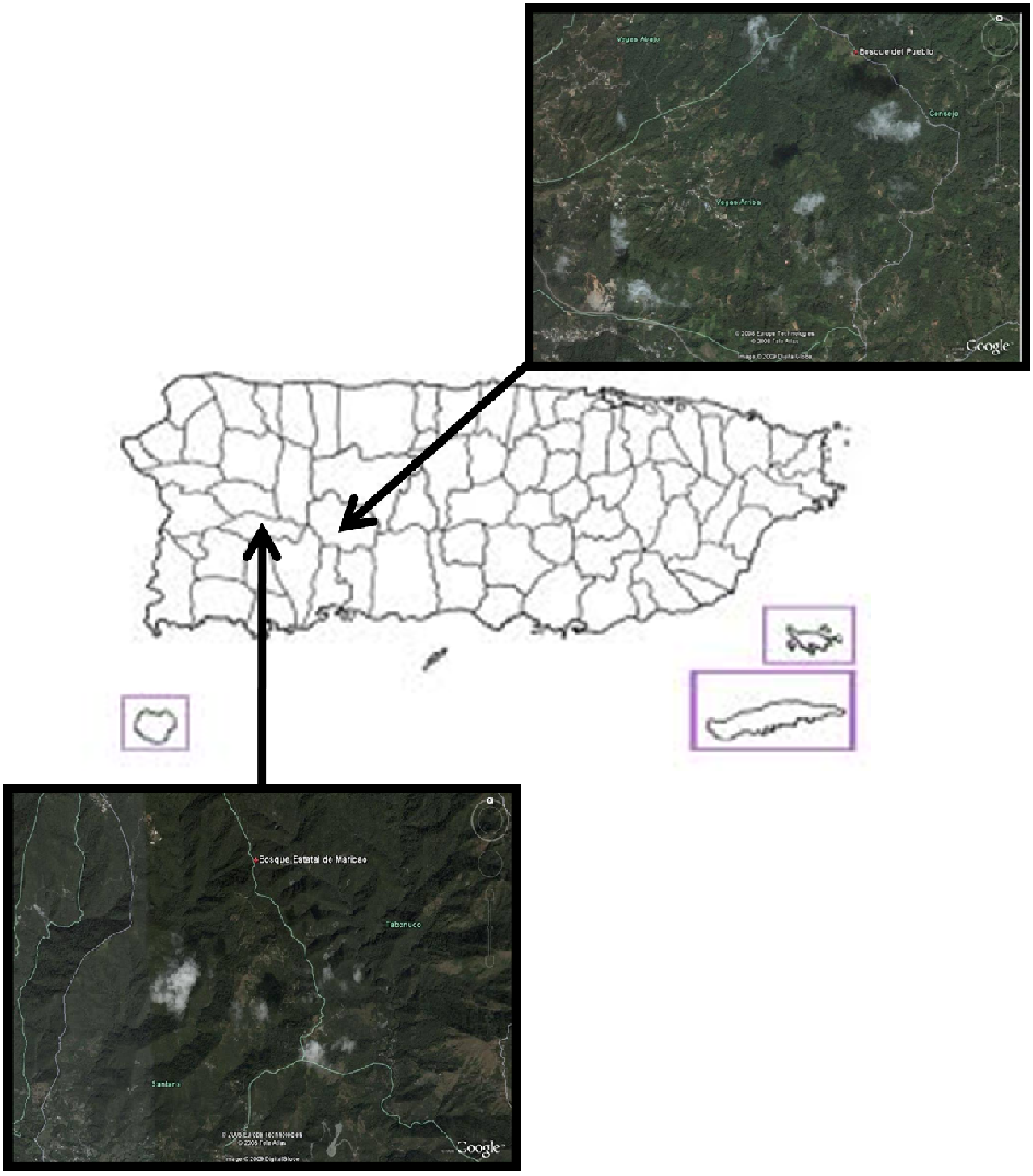


Figure 1. Bosque del Pueblo in Barrio Vegas Arriba in Adjuntas and Bosque Estatal de Maricao in BarrioTabonuco in Sabana Grande (Image taken from http://earth.google.com/).

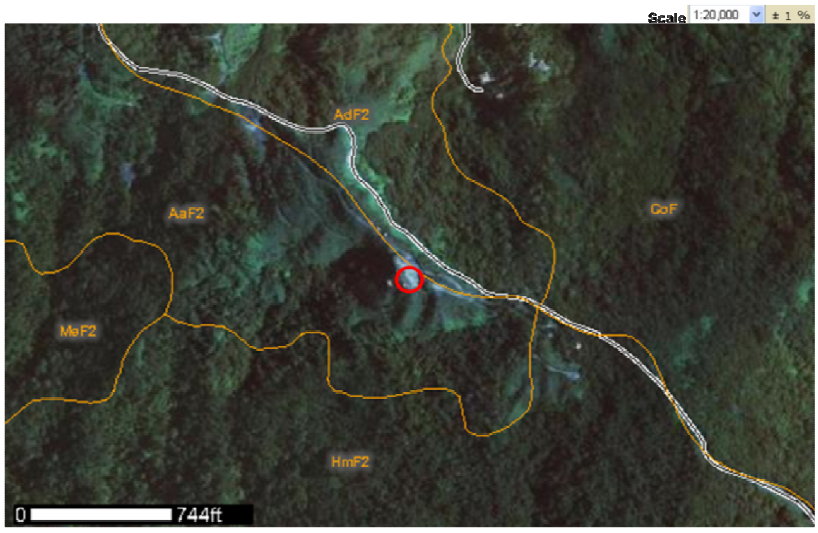


Figure 2. Location of the sampling area in Bosque del Pueblo, Barrio Vegas Arriba in Adjuntas. The sampling site is located within the *Adjuntas Soil Series* (AaF2) (USDA Soil survey of Ponce area of southern Puerto Rico, 1979) (Image from Web Soil Survey 2.0).

Figure 3. Location of the sampling area in Bosque Estatal de Maricao, Barrio Tabonuco in Sabana Grande. The sampling site is located within the *El Cacique Soil Series* (EcG) (USDA Soil survey of San Germán area of southwestern Puerto Rico, 2008) (Image from Web Soil Survey 2.0).

Figure 4 a. Herbarium specimen of *Sticherus bifidus* aerial section (fronds) and subaerial section (rhizomes and roots) from Bosque Estatal de Maricao, Barrio Tabonuco in Sabana Grande road 120 km 9.2 (18°08’04’’N, 66°57’17’’W) (Picture taken by Doralis Villanueva)

Figure 4 b. Herbarium specimen of *Gleichenella pectinata* aerial section (fronds) and subaerial section (rhizomes and roots) from Bosque Estatal de Maricao, Barrio Tabonuco in Sabana Grande road 120 km 9.2 (18°08’04’’N, 66°57’17’’W) (Picture taken by Doralis Villanueva)

Figure 4 c. Herbarium specimen of *Sticherus bifidus* and *Gleichenella pectinata* aerial section (fronds) and subaerial section (rhizomes and roots) from Bosque del Pueblo, Barrio Vegas Arriba in Adjuntas road 143 int. 521 18°10’59’’N, 66°40’35’’W) (Picture taken by Doralis Villanueva)

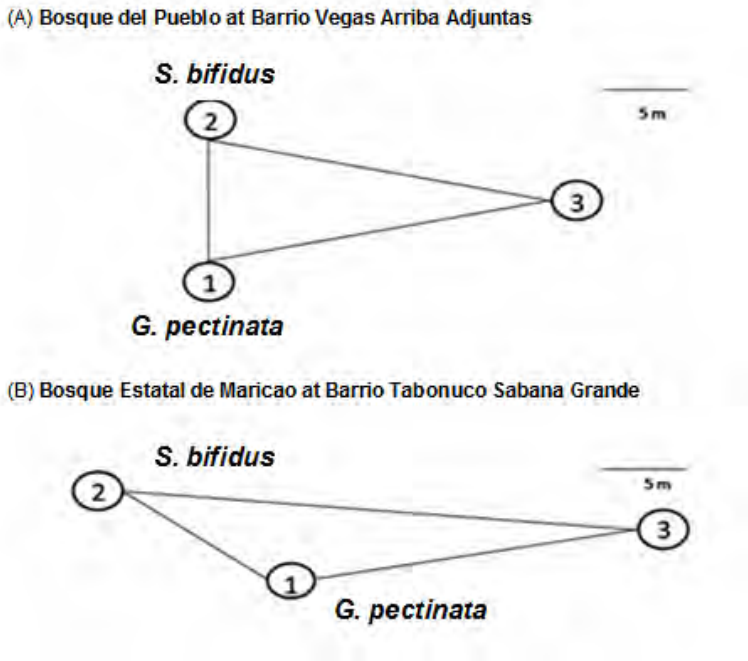


Figure 5. Sampling diagram. Soil samples were collected at (1) *Gleichenella pectinata* site, (2) *Sticherus bifidus* site and (3) without ferns (Diagram by Dubiezel Medina Bonilla).

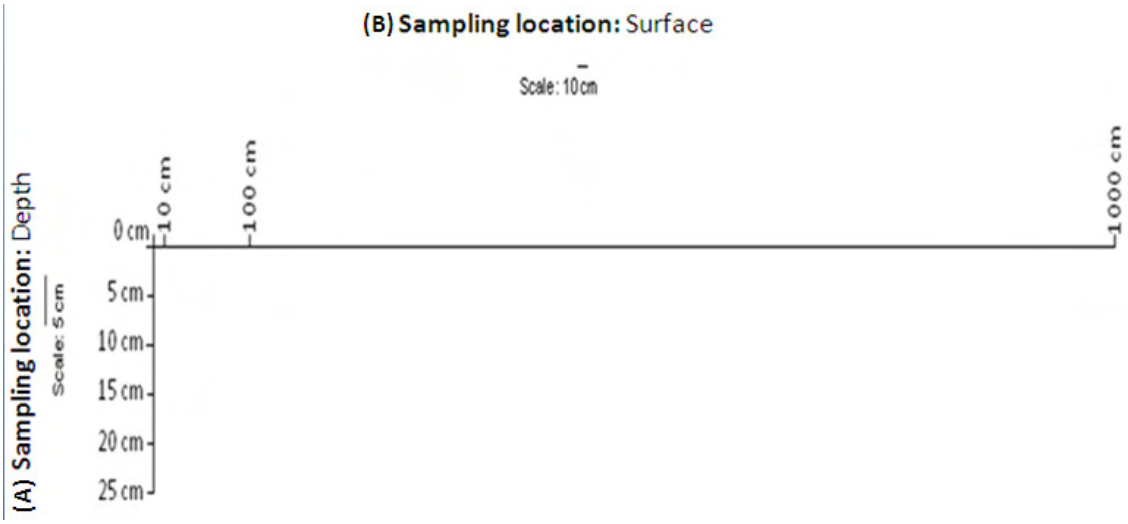


Figure 6. Sampling diagram. Soil samples were collected at two transects, (A) depth (vertically) and (B) surface (horizontally), in Bosque del Pueblo, Barrio Vegas Arriba Adjuntas and Bosque Estatal de Maricao, Barrio Tabonuco Sabana Grande for *Gleichenella pectinata* and *Sticherus bifidus* sites during the dry and the wet seasons (Diagram by Dubiezel Medina Bonilla)

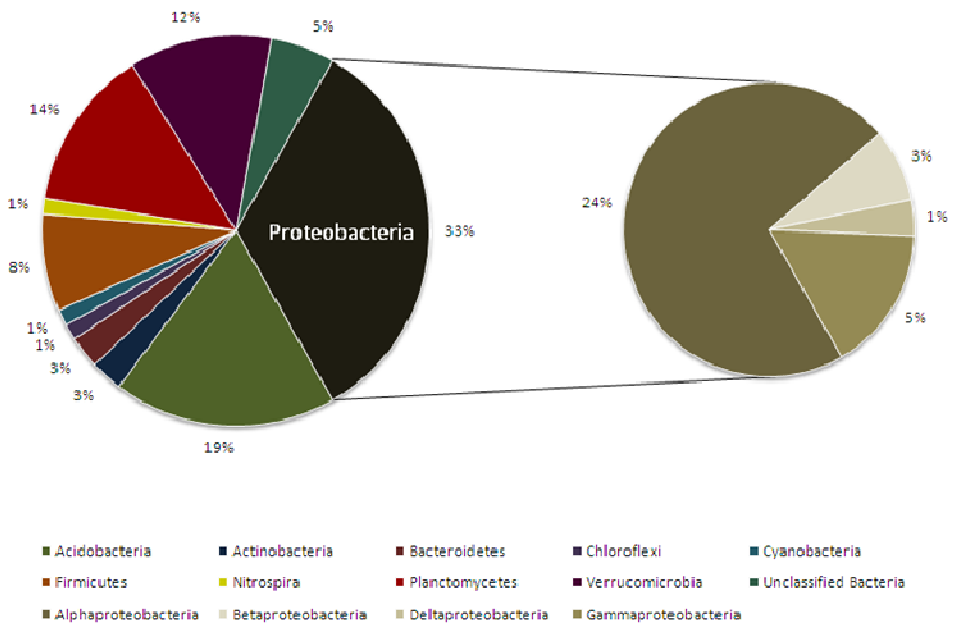


Figure 7. Dominant phylogenetic groups in *S. bifidus* rhizosphere at Bosque Estatal de Maricao in Tabonuco Sabana Grande during the dry season.

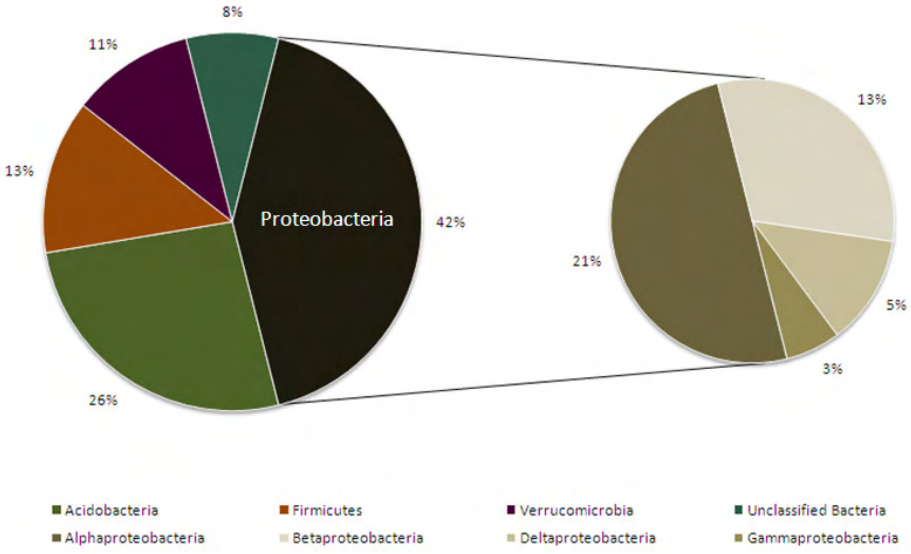


Figure 8. Dominant phylogenetic groups in *S. bifidus* rhizosphere at Bosque Estatal de Maricao in Tabonuco Sabana Grande during the wet season.

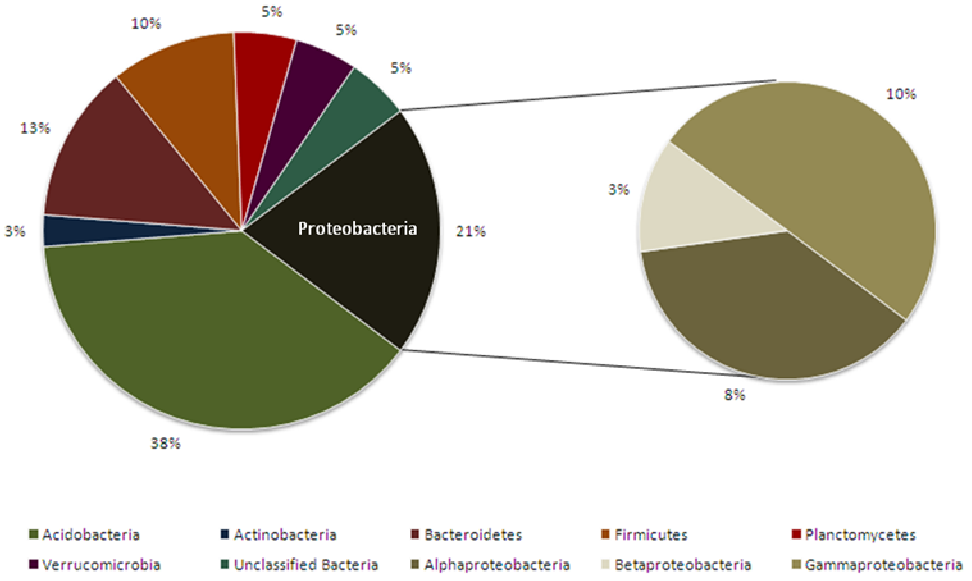


Figure 9. Dominant phylogenetic groups in *S. bifidus* rhizosphere at Bosque del Pueblo in Vegas Arriba Adjuntas during the dry season.

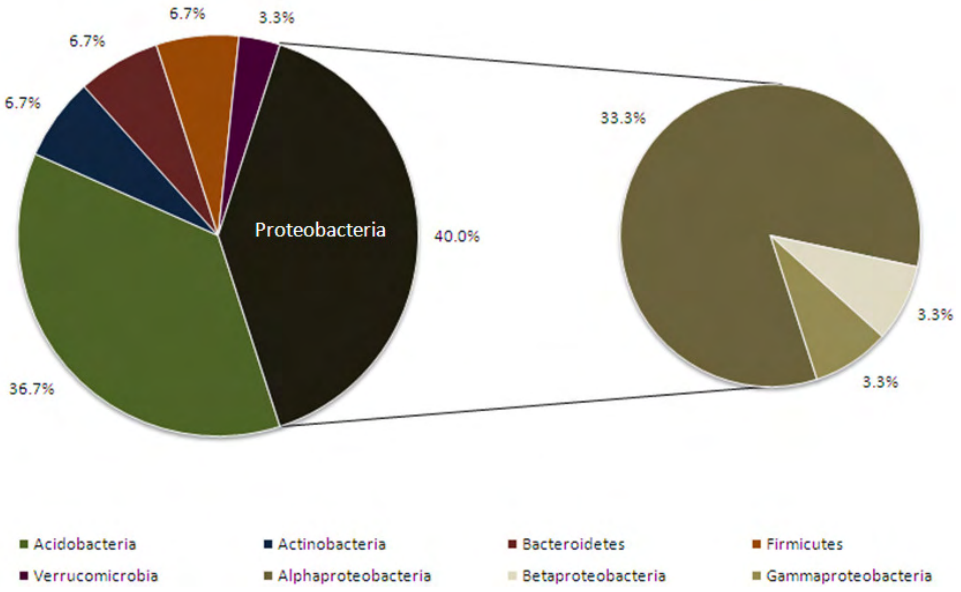


Figure 10. Dominant phylogenetic groups in *S. bifidus* rhizosphere at Bosque del Pueblo in Vegas Arriba Adjuntas during the wet season.

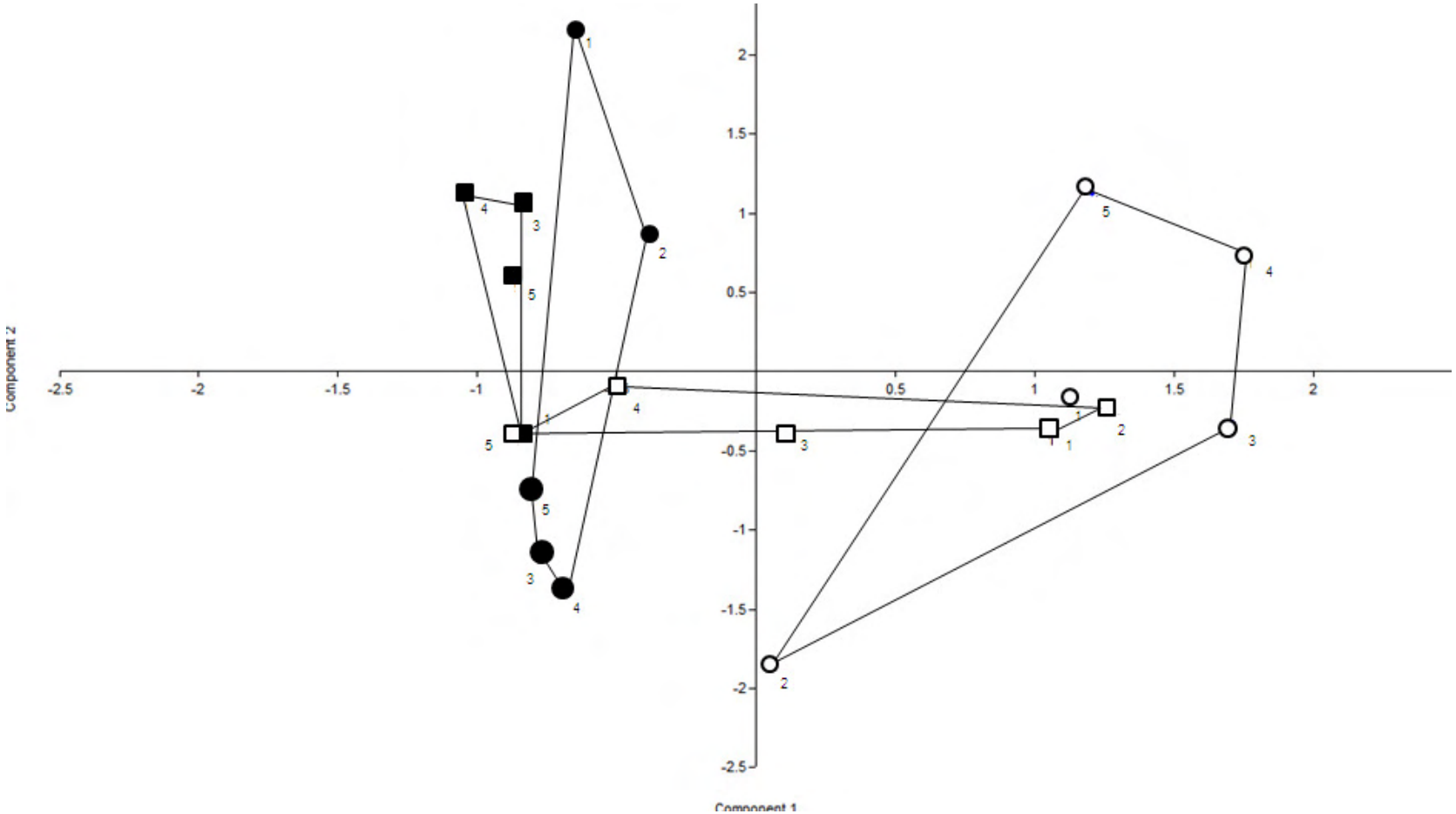


Figure 11. Principal Components Analysis (PCA) for soil study (1=GR, 2=GS, 3=SR, 4=SS, and 5=WF) at BPA (circles) during the wet (filled) and the dry season (empty) and BEM (squares) during the wet (filled) and the dry (empty) season.

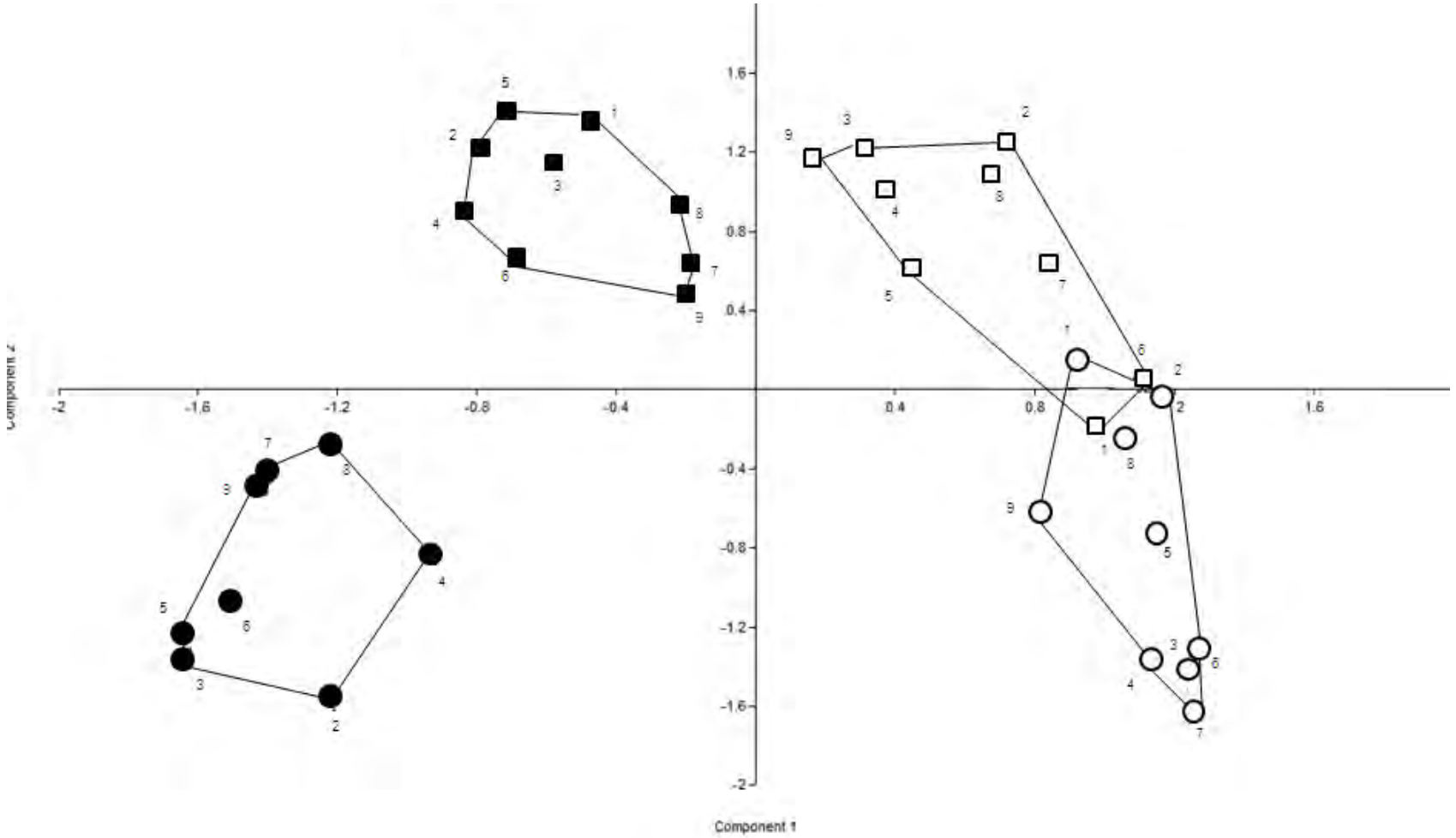


Figure 12. Principal Components Analysis (PCA) for biogeography study (1 to 4 soil samples collected horizontally and 5 to 9 soil samples collected vertically) at *G. pectinata* site at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry (empty) season.

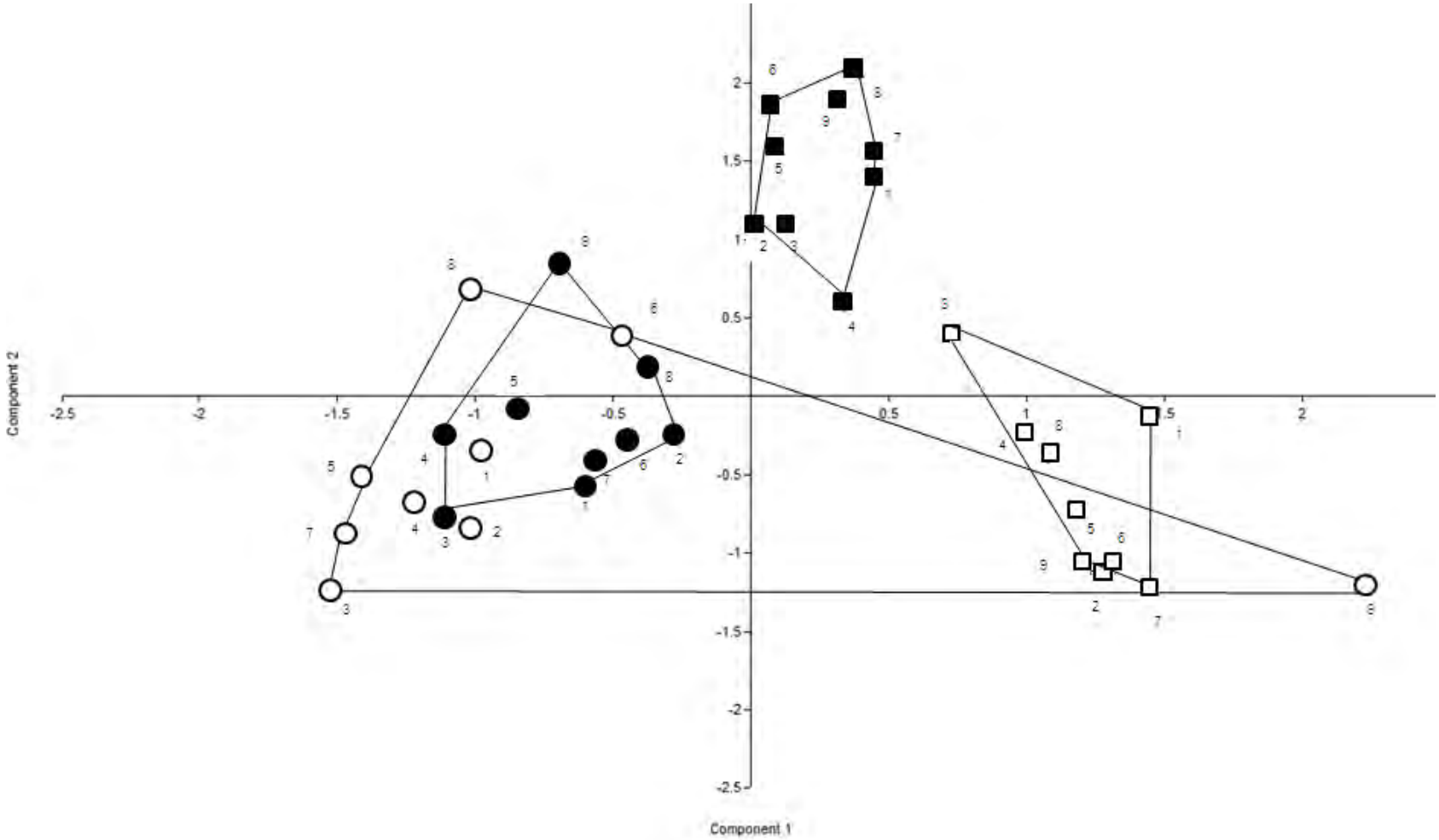


Figure 13. Principal Components Analysis (PCA) for biogeography study (1 to 4 soil samples collected horizontally and 5 to 9 soil samples collected vertically) at *S. bifidus* site at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry (empty) season.

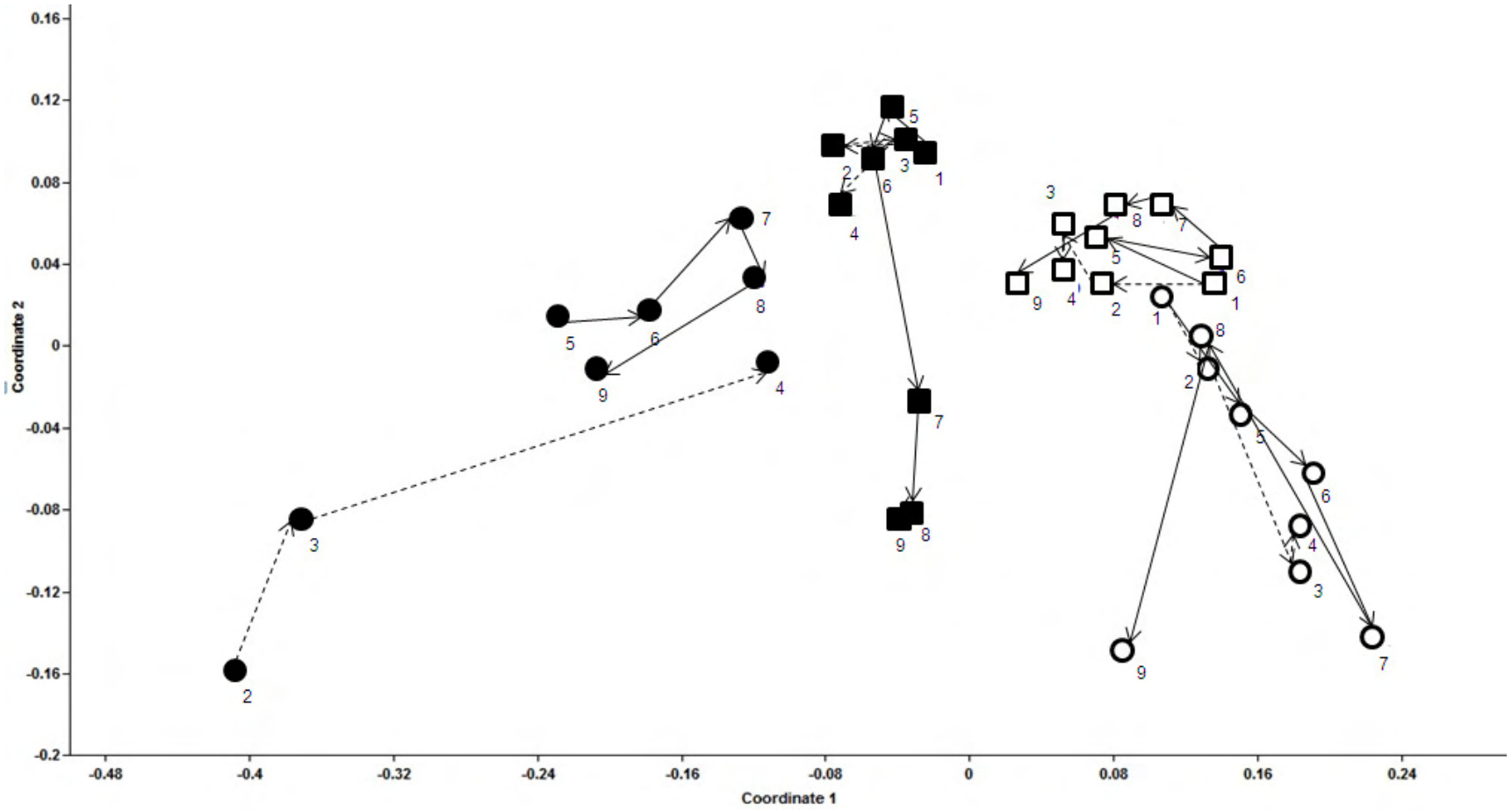


Figure 14. Non-Metric Multidimensional Scaling (NMDS) for biogeography study (1 to 4 soil samples collected horizontally, ***dashed lines*** and 5 to 9 soil samples collected vertically, ***solid lines***) study at *G. pectinata* site at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry (empty) season.

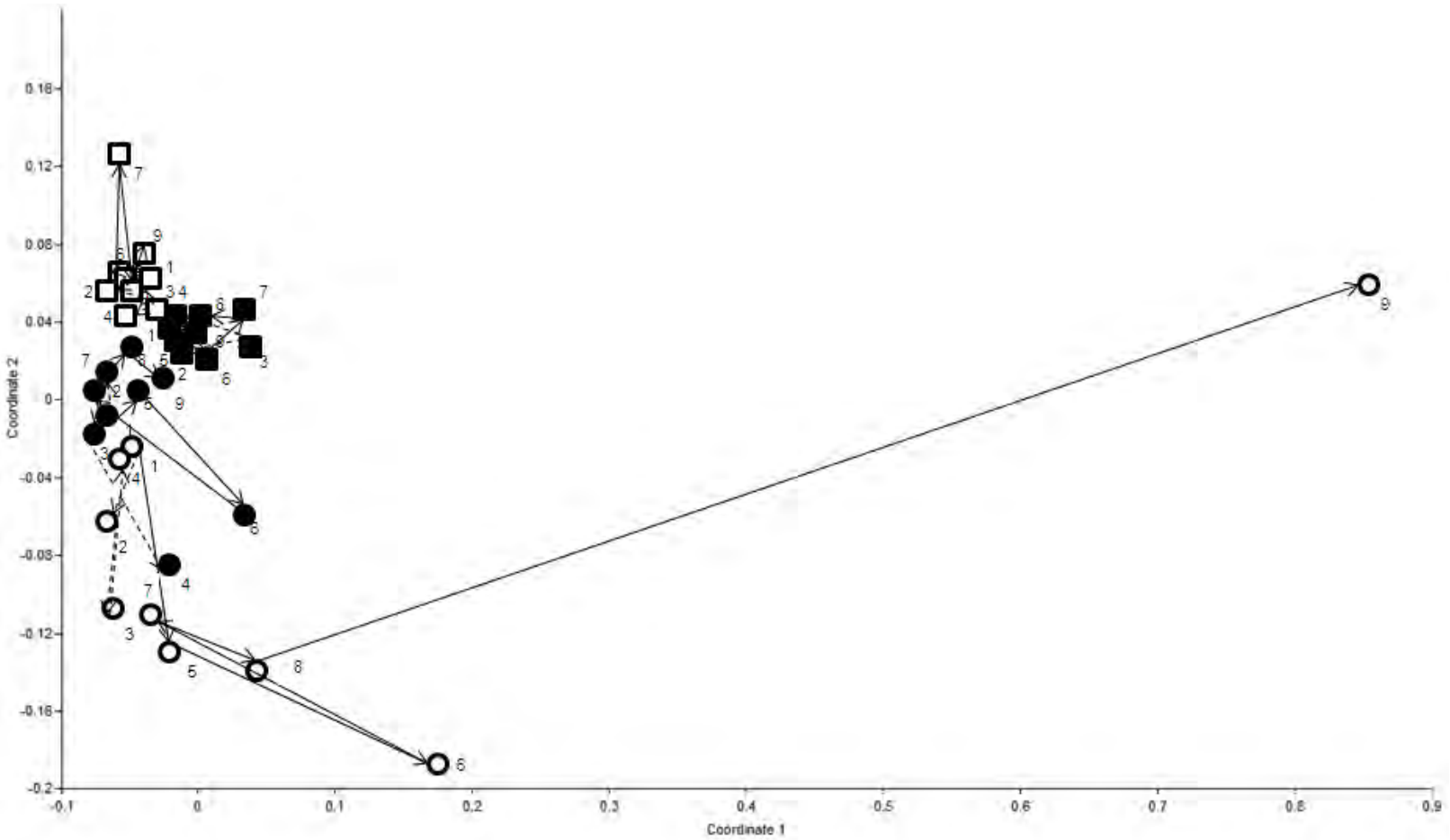


Figure 15. Non-Metric Multidimensional Scaling (NMDS) for biogeography study (1 to 4 soil samples collected horizontally, ***dashed lines*** and 5 to 9 soil samples collected vertically, ***solid lines***) study at ***S. bifidus*** site at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao during the wet (squares) and the dry (empty) season.

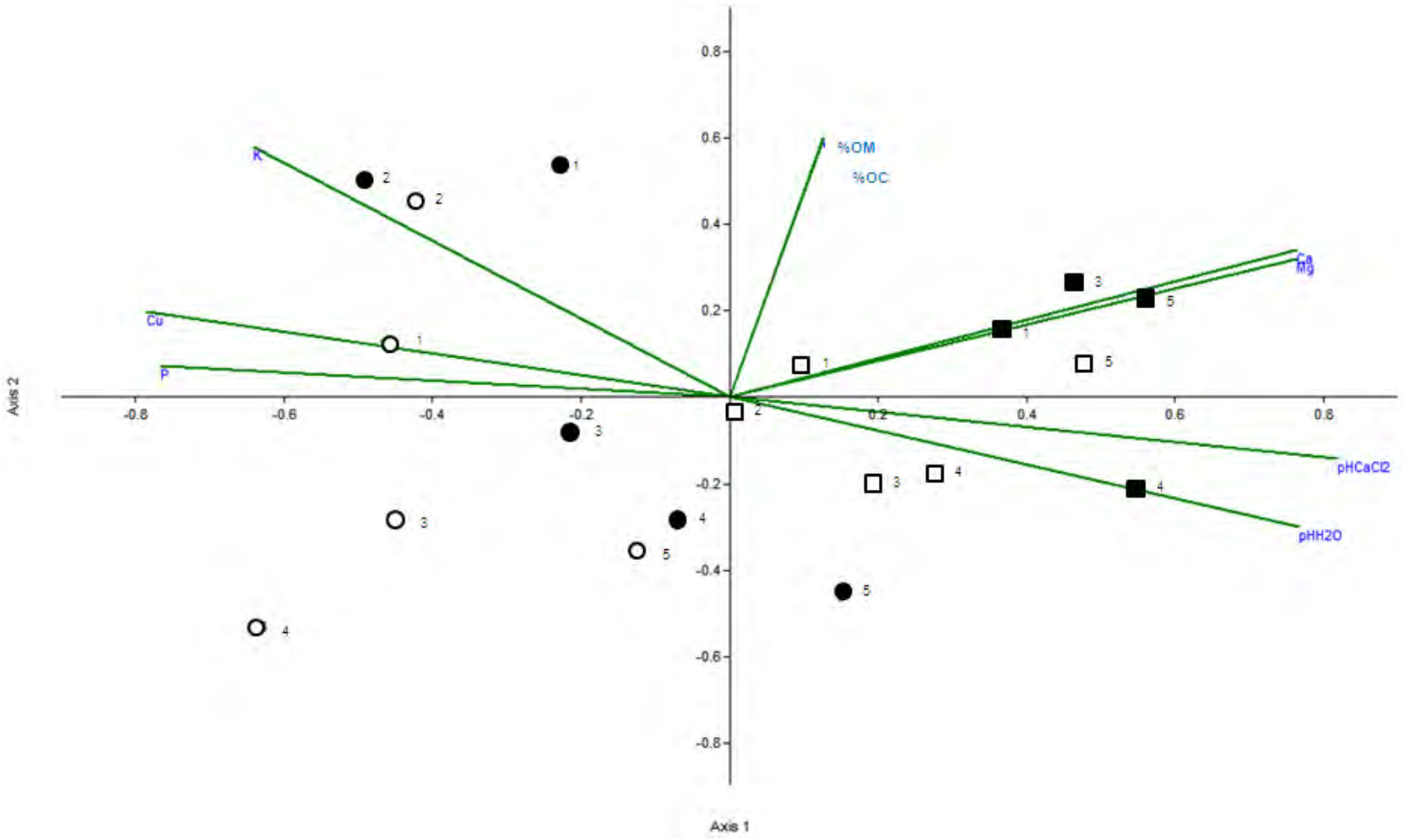


Figure 16. Canonical Correspondence Analysis (CCA) soil study (1=GR, 2=GS, 3=SR, 4=SS 5=WF) at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry season (empty).

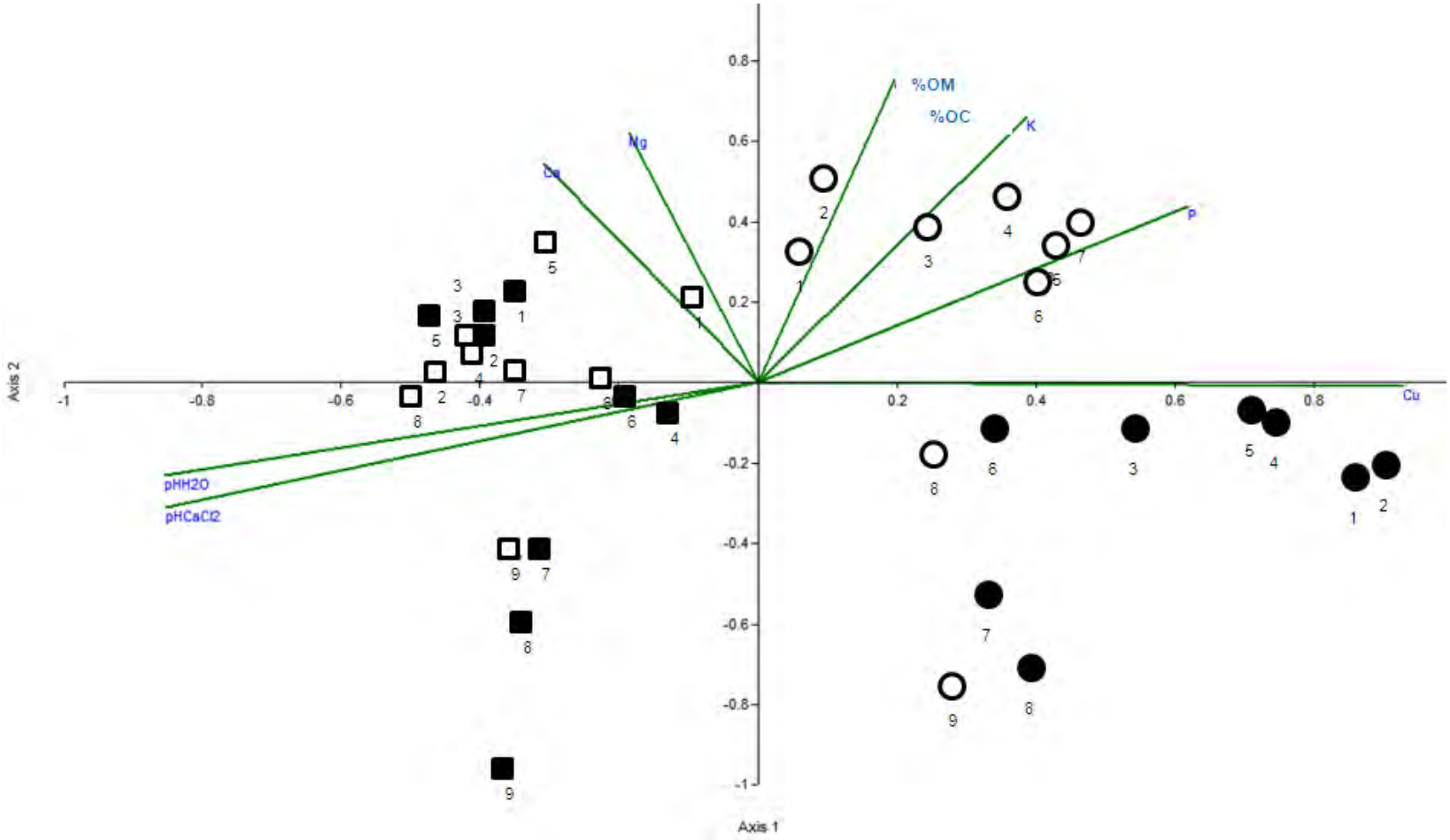


Figure 17. Canonical Correspondence Analysis (CCA) for biogeography study (1 to 4 soil samples collected horizontally and 5 to 9 soil samples collected vertically) at *G. pectinata* site at Bosque del Pueblo (circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry season (empty).

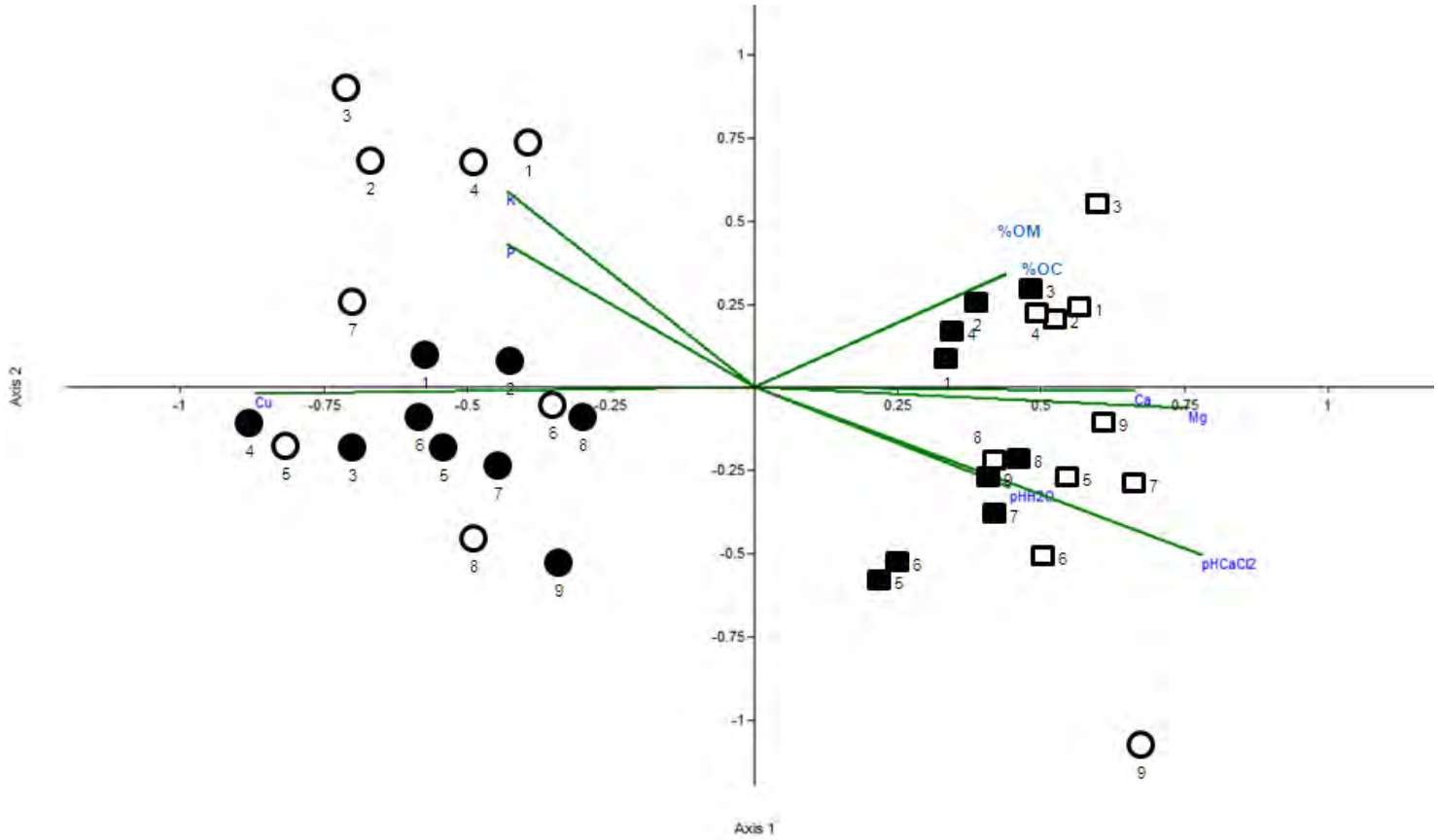


Figure 18. Canonical Correspondence Analysis (CCA) for biogeography study (1 to 4 soil samples collected horizontally and 5 to 9 soil samples collected vertically) at *S.bifidus* site at Bosque del Pueblo(circles) during the wet (filled) and the dry season (empty) and Bosque Estatal de Maricao (squares) during the wet (filled) and the dry season (empty).

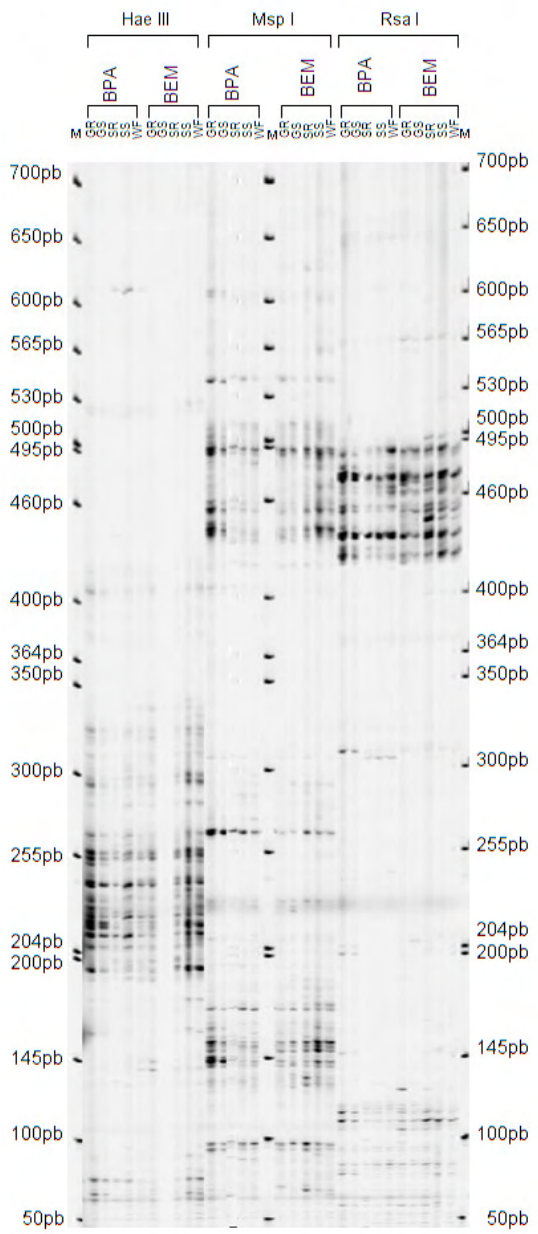
APPENDICES

Appendix I. Characteristics, distribution and habitat of *Gleichenella pectinata* Ching (1940) and *Sticherus bifidus* Ching (1940) according to Proctor (1989).

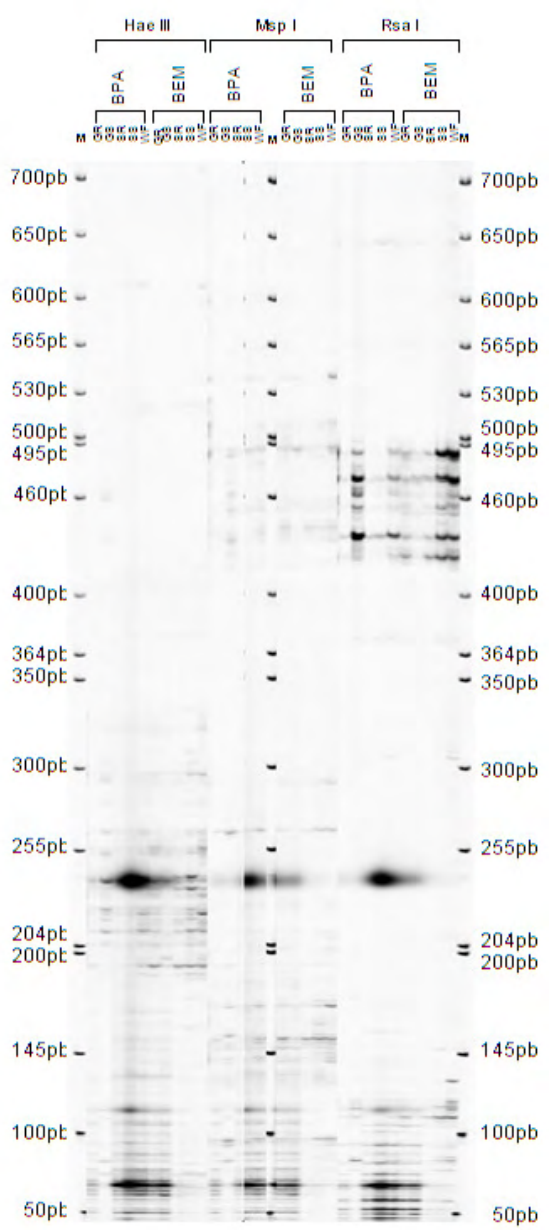
Fern name	*Gleichenella pectinata* (Willdenow)	*Sticherus bifidus* (Willdenow)
Synonym and Basionym	**Syn.** *Dicranopteris pectinata* (Willdenow) Underwood (1907) **Bas.** *Mertensia pectinata* Willdenow (1804)	**Syn.** *Dicranopteris bifida* (Willdenow) Maxon (1909), *Gleichenia bifida* (Willdenow) Sprengel (1827) **Bas.** *Mertensia bifida* Willdenow (1804)
Key characteristics	Frond forks without accessory pinnae; tissue sometimes sparsely stellate-pubescent beneath.	Tissue concealed on abaxial side by dense woolly tomentum, not glaucous. Scales of costae beneath numerous, larger, ovate-deltate and basally attached, pale brownish, and woolly-ciliate; rhizome scaly (especially toward apex), reddish-brown.
Rhizome	3-5 mm thick, light brown, scabrous, clothed with numerous deciduous, castaneous, articulate hairs	3-4 mm thick, reddish-brown, bearing narrow, attenuate, short-ciliate scales 1.5-2 mm long especially near apex
Primarly frond	axis 3-6 mm thick; primary lateral branches consisting of several opposite pairs, stipulate in the forks, repeatedly and unequally forking, producing a naked flexuous secondary axis, the shorter branches once- or twice- forked; primary internodes 2-16 cm long or more, the others shorter	axis 1.5-4 mm thick, bearing a few pale brown, ciliate, deciduous scales, similar scales occurring abundantly on all the main vascular parts of the blade beneath; primary lateral branches consisting of two to several pairs, each branch 1- to 2- forked a secondary axis seldom developed
Pinnae	variable, 10-25 cm long, 1-6 cm broad, very glaucous, glabrous beneath or sometimes sparsely stellate-pubescent; segment usually notched at the apex; veins 3- to 5-forked	25-50 cm long, 3-7cm broad, often arcuate; segments numerous, linear from a dilatate base, densely woolly beneath with light rusty-brown tomentum; veins once-forked at base
Distribution in Puerto Rico	Viny terrestrial in Northern Limestone Hills (Lares), Río Abajo Forest Reserve, Sierra de Luquillo, Caguas Valley (Las Piedras), Sierra de Cayey, Central Cordillera, Western Cordillera.	Viny terrestrial in Northern Limestone Hills (Lares), Río Abajo Forest Reserve, Sierra de Luquillo, Eastern Coast (Cuchilla de Panduras), Caguas Valley (Caguas), Sierra de Cayey, Central Cordillera, Western Cordillera, Cerro de las Mesas.
Habitat	Exposed banks, open hillsides, clearing, and moist borders of thickets in acidic soils at low to high elevations (sea-level-1300 m), common. This species often forms extensive patches growing so densely as to exclude all other vegetation. It often invades areas that have been burned, and is characteristic of open road cuts in areas of high rainfall	Moist thickets, clearings, banks, and open hillsides in acidic soils at middle to high elevations (400-1300m), common, often abundant, forming dense tangles. This species and *G. pectinata* frequently grow in the same localities

Appendix II. Soil taxonomy consists of six categories: *order, suborder, great group, subgroup, family,* and *series* (Beinroth *et al.*, 2003)

categories	description
order	category groups soils on the basis of the results of major soil-forming process and has twelve orders: *Alfisols, Andisols, Entisols, Gelisols, Histosols, Inceptisols, Mollisols, Oxisols, Spodosols, Ultisols,* and *Vertisols,* of these, nine have been established in the Puerto Rico
suborder	factors that control the major processes are considered, mainly the soil temperature and soil moisture regimes
great group	reflect extreme expression of pedologic or maturity of a soil profile process such as high degree of weathering or cementation of soil layers
subgroup	subdivide the great groups in one of three kinds of subgroups: (i) *typic*- soils representing the central concept of the great group, (ii) *integrades*- soils that have some properties of another order, suborder or great groups and (iii) *extragrades*- soils that have properties that are not representative of the great group but are not transitional to any other known kind of soil
family	subgroups are differentiated on the basis of physical, chemical, mineralogical and climatic properties that affect the growth of plants
series	generally is derived from a town or landmark in or near the area where the soil was first recognized; areas with similar soils are grouped and labeled as series because their have similar origins, chemical, and physical properties



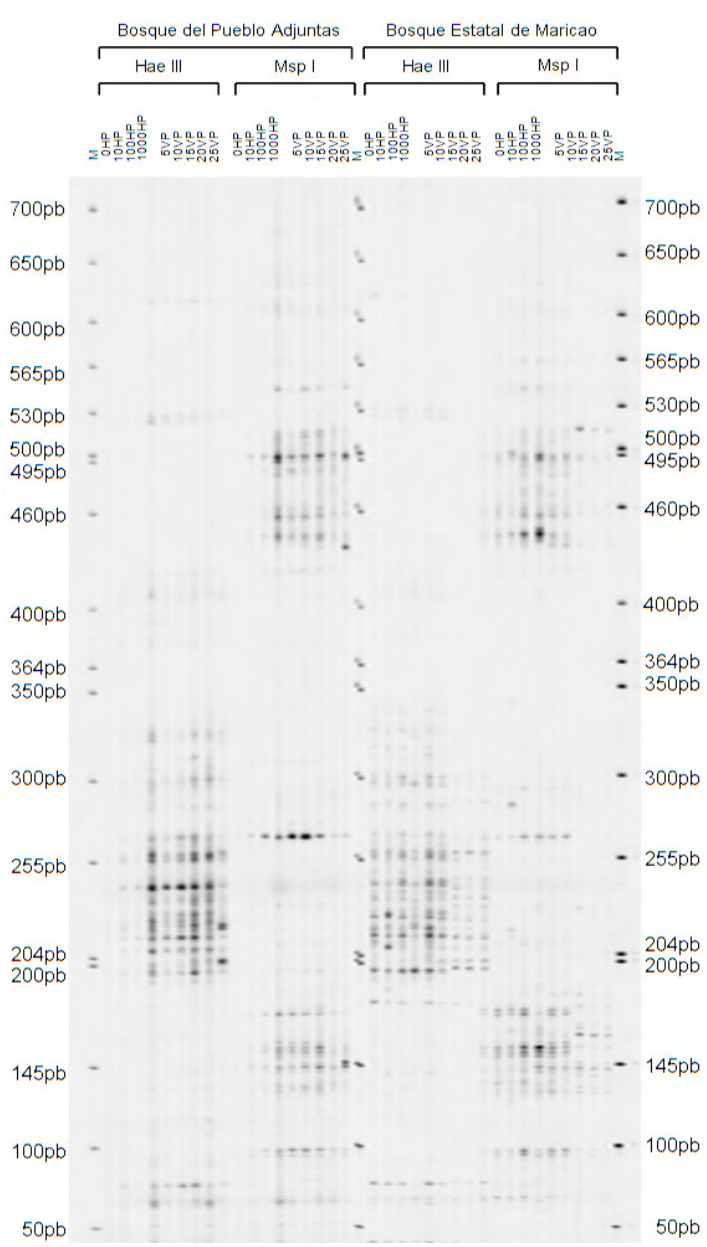
Appendix III. TRFPL from samples collected during the wet season. BPA= Bosque del Pueblo Barrio Vegas Arriba, Adjuntas PR; BEM= Bosque Estatal de Maricao Barrio Tabonuco, Sabana Grande PR; GR= *G. pectinata* rhizosphere; SR= *S. bifidus* rhizosphere; GS= *G. pectinata* site; SS= *S. bifidus* site; WF= without ferns.



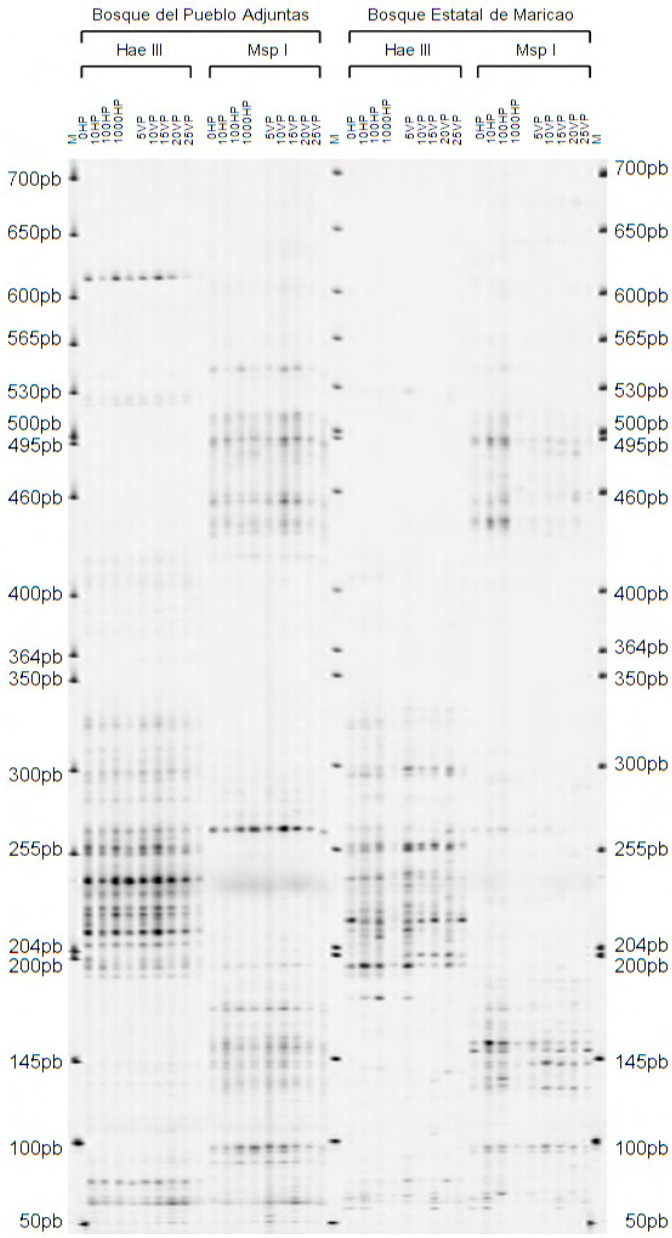
Appendix IV. TRFPL from samples collected during the dry season. BPA= Bosque del Pueblo Barrio Vegas Arriba, Adjuntas PR; BEM= Bosque Estatal de Maricao Barrio Tabonuco, Sabana Grande PR; GR= *G. pectinata* rhizosphere; SR= *S. bifidus* rhizosphere; GS= *G. pectinata* site; SS= *S. bifidus* site; WF= without ferns

Appendix V. Phylotypes from samples GR, GS, SR, SS and WF collected at Bosque del Pueblo and Bosque Estatal de Maricao during both seasons obtained when restriction enzymes Hae III, Msp I and Rsa I were used.

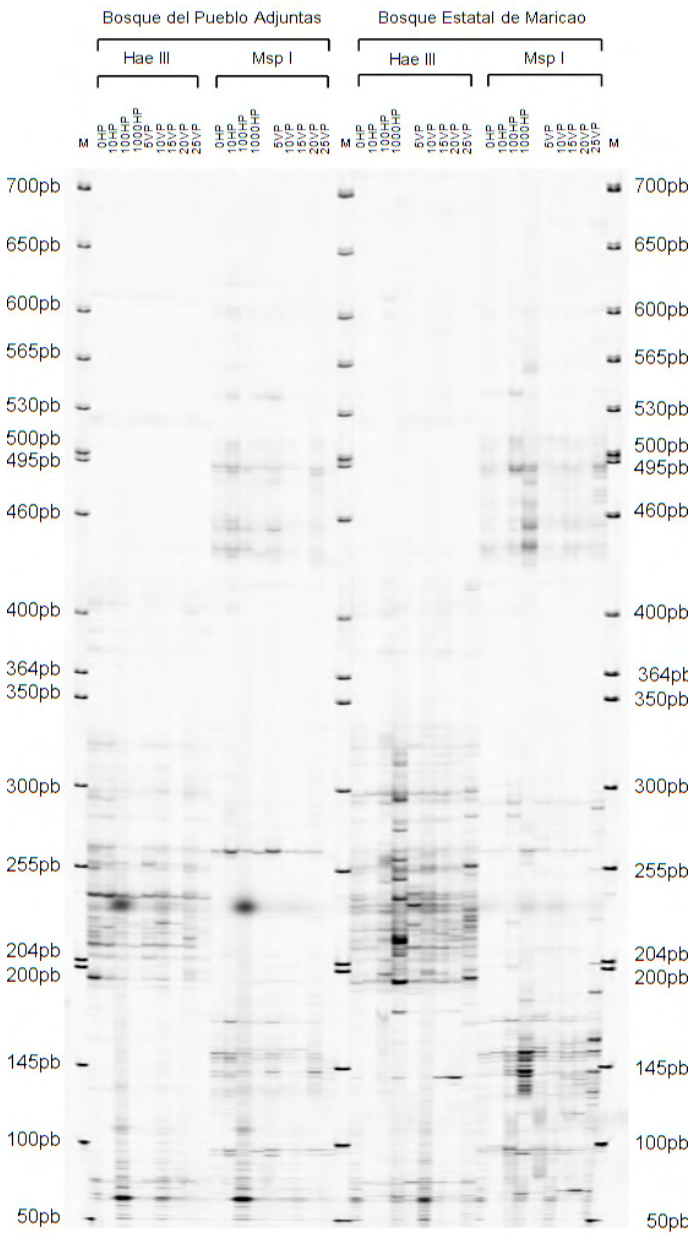
Sample name		Season	Restriction enzyme (# of phylotypes)		
			HaeIII	**MspI**	**RsaI**
BPA	GR	Wet	39	42	23
		Dry	17	23	19
	GS	Wet	19	26	17
		Dry	16	23	23
	SR	Wet	15	29	16
		Dry	26	14	16
	SS	Wet	21	23	23
		Dry	33	36	18
	WF	Wet	13	30	20
		Dry	34	34	27
BEM	GR	Wet	23	30	18
		Dry	35	33	28
	GS	Wet	0	32	23
		Dry	35	26	25
	SR	Wet	15	40	24
		Dry	20	17	13
	SS	Wet	40	35	15
		Dry	24	23	22
	WF	Wet	37	15	13
		Dry	22	22	22



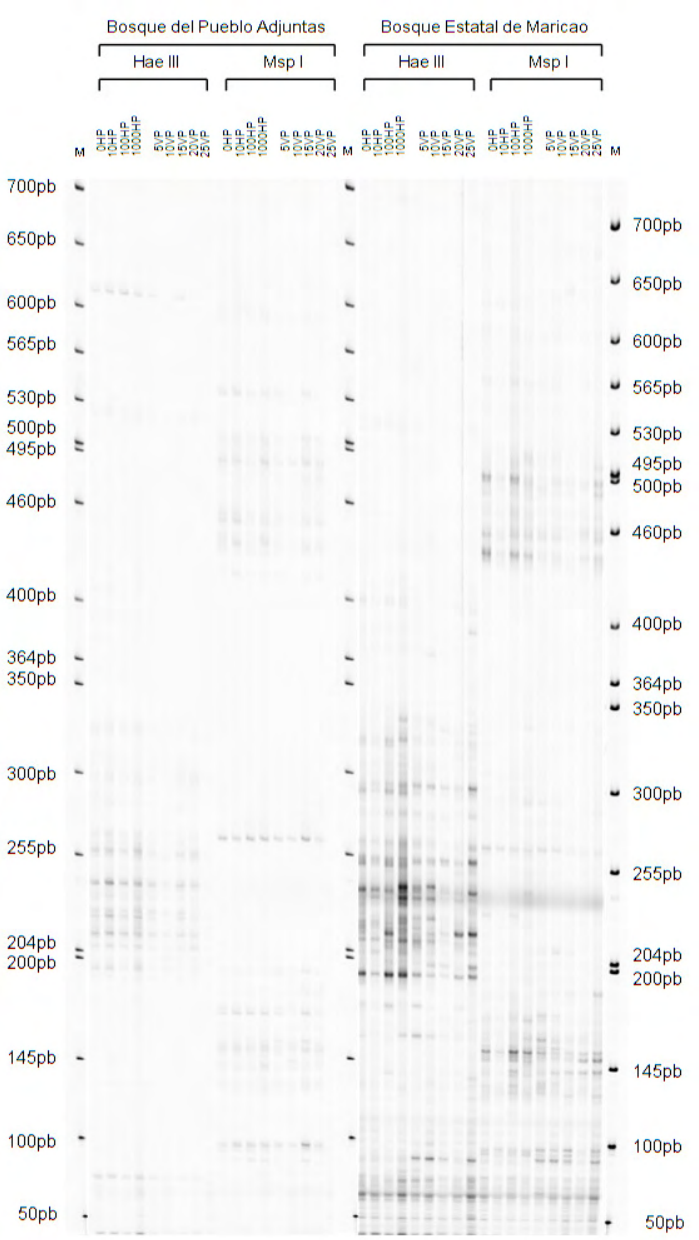
Appendix VI. TRFLP from samples collected at *G. pectinata* site during the wet season 0HP= Horizontal Profile at 0 cm; 1HP= Horizontal Profile at 1 cm; 10HP= Horizontal Profile at 10 cm; 100HP= Horizontal Profile at 100 cm, 1000HP= Horizontal Profile at 1000 cm; 5VP= Vertical Profile at 5 cm; 10VP= Vertical Profile at 10 cm; 15VP= Vertical Profile at 15 cm; 20VP= Vertical Profile at 20 cm; 25VP= Vertical Profile at 25 cm



Appendix VII. TRFLP from samples collected at *S. bifidus* site during the wet season. 0HP= Horizontal Profile at 0 cm; 1HP= Horizontal Profile at 1 cm; 10HP= Horizontal Profile at 10 cm; 100HP= Horizontal Profile at 100 cm, 1000HP= Horizontal Profile at 1000 cm; 5VP= Vertical Profile at 5 cm; 10VP= Vertical Profile at 10 cm; 15VP= Vertical Profile at 15 cm; 20VP= Vertical Profile at 20 cm; 25VP= Vertical Profile at 25 cm



Appendix VIII. TRFLP from samples collected at *G. pectinata* site during the dry season. 0HP= Horizontal Profile at 0 cm; 1HP= Horizontal Profile at 1 cm; 10HP= Horizontal Profile at 10 cm; 100HP= Horizontal Profile at 100 cm, 1000HP= Horizontal Profile at 1000 cm; 5VP= Vertical Profile at 5 cm; 10VP= Vertical Profile at 10 cm; 15VP= Vertical Profile at 15 cm; 20VP= Vertical Profile at 20 cm; 25VP= Vertical Profile at 25 cm



Appendix IX. TRFLP from samples collected at *S. bifidus* site during the dry season.0HP= Horizontal Profile at 0 cm; 1HP= Horizontal Profile at 1 cm; 10HP= Horizontal Profile at 10 cm; 100HP= Horizontal Profile at 100 cm, 1000HP= Horizontal Profile at 1000 cm; 5VP= Vertical Profile at 5 cm; 10VP= Vertical Profile at 10 cm; 15VP= Vertical Profile at 15 cm; 20VP= Vertical Profile at 20 cm; 25VP= Vertical Profile at 25 cm

Appendix X. Phylotypes from samples collected at different depths at GP and SB site at Bosque del Pueblo and Bosque Estatal de Maricao during both seasons obtained when restriction enzymes Hae III and Msp I were used.

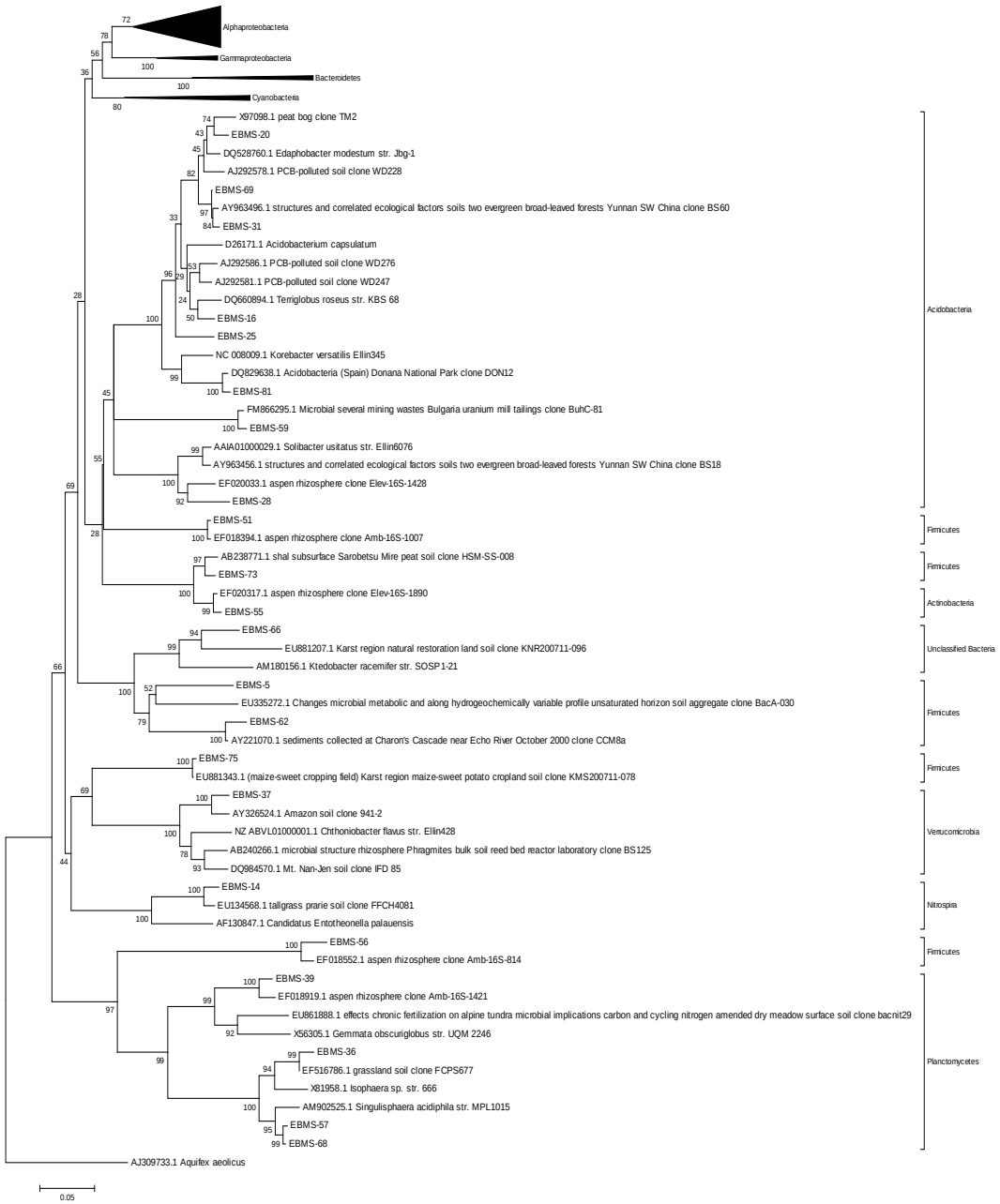
Sample name			Season	Restriction enzyme (# of phylotypes)	
				HaeIII	MspI
BPA	GP	0HP	wet	0	0
			dry	38	28
		10HP	wet	13	9
			dry	28	48
		100HP	wet	6	15
			dry	46	40
		1000HP	wet	36	51
			dry	19	28
		5VP	wet	18	23
			dry	21	30
		10VP	wet	25	25
			dry	38	21
		15VP	wet	32	31
			dry	25	15
		20VP	wet	31	27
			dry	26	29
		25VP	wet	17	21
			dry	15	11
	SB	0HP	wet	27	19
			dry	13	10
		10HP	wet	24	29
			dry	15	16
		100HP	wet	27	22
			dry	10	8
		1000HP	wet	22	24
			dry	18	12
		5VP	wet	31	32
			dry	9	8
		10VP	wet	35	31
			dry	6	5
		15VP	wet	32	35
			dry	7	16
		20VP	wet	25	21
			dry	6	6
		25VP	wet	16	13
			dry	1	1

Appendix X. (continuation)

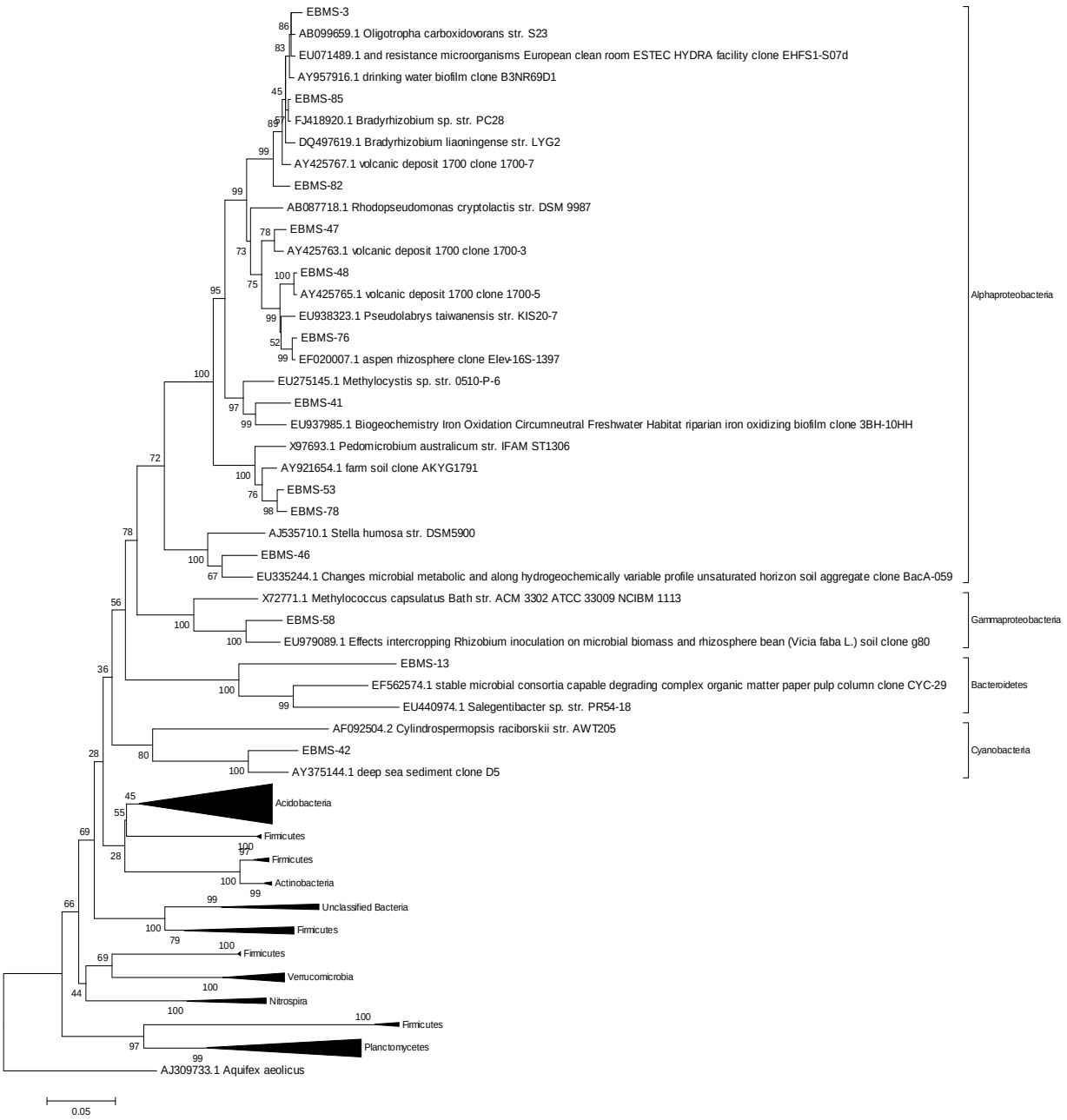
BEM	GP	0HP	wet	27	25
			dry	42	37
		10HP	wet	25	24
			dry	19	19
		100HP	wet	26	31
			dry	37	50
		1000HP	wet	21	31
			dry	52	53
		5VP	wet	30	31
			dry	32	39
		10VP	wet	24	28
			dry	53	45
		15VP	wet	19	19
			dry	38	37
		20VP	wet	17	11
			dry	32	34
		25VP	wet	29	12
			dry	42	56
	SB	0HP	wet	17	13
			dry	32	29
		10HP	wet	27	29
			dry	31	18
		100HP	wet	27	23
			dry	28	28
		1000HP	wet	10	5
			dry	34	23
		5VP	wet	24	7
			dry	27	24
		10VP	wet	17	9
			dry	31	30
		15VP	wet	22	13
			dry	22	21
		20VP	wet	19	17
			dry	25	21
		25VP	wet	10	10
			dry	33	29

Description of appendices XI-XIV: Phylogenetic analysis for metagenomic libraries Julio Bosque del Pueblo *S. bifidus* (JBPS), Julio Bosque Estatal de Maricao *S. bifidus* (JBMS), Enero Bosque del Pueblo *S. bifidus* (EBPS) and Enero Bosque Estatal de Maricao *S. bifidus* (EBMS).

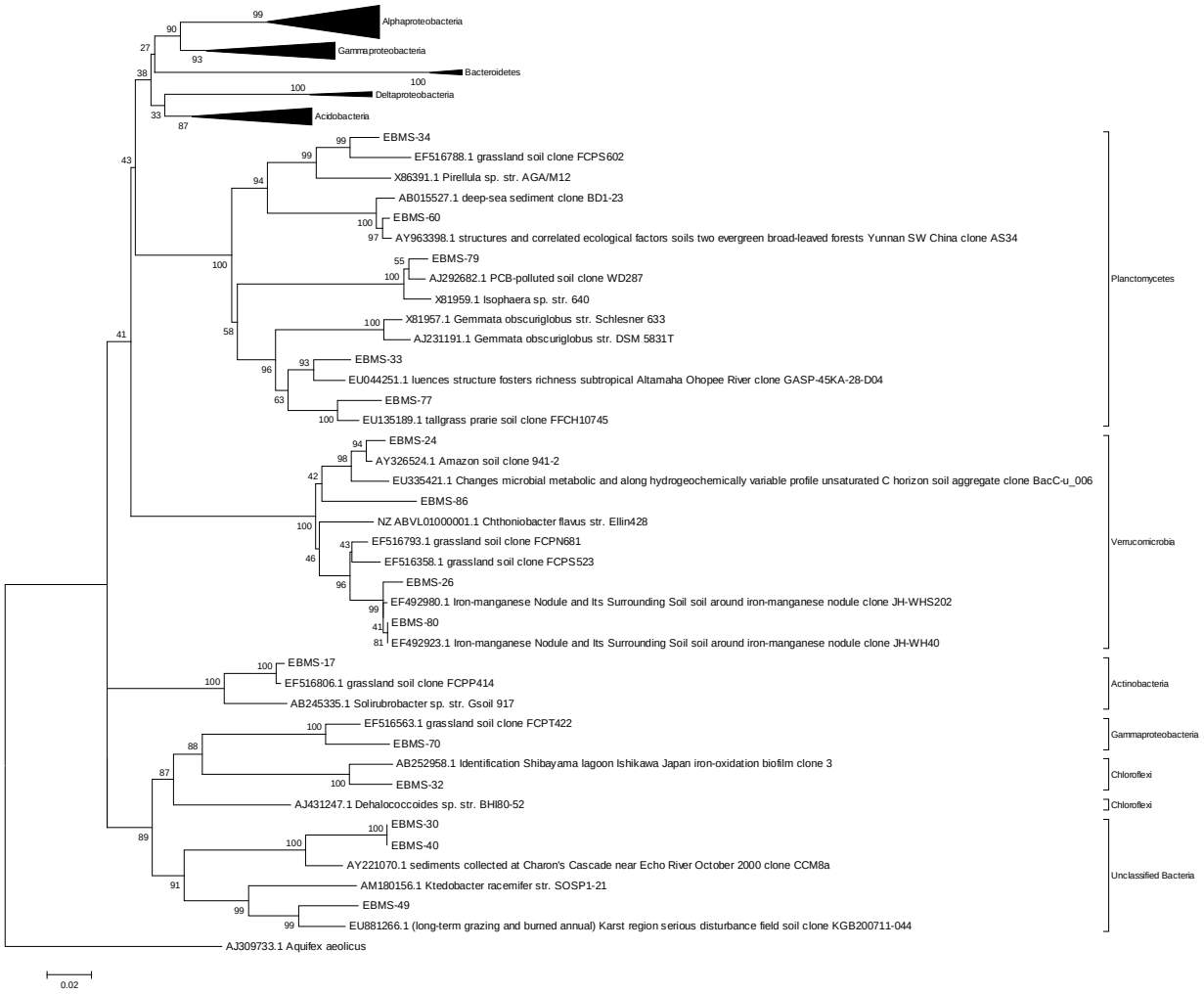
The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4.



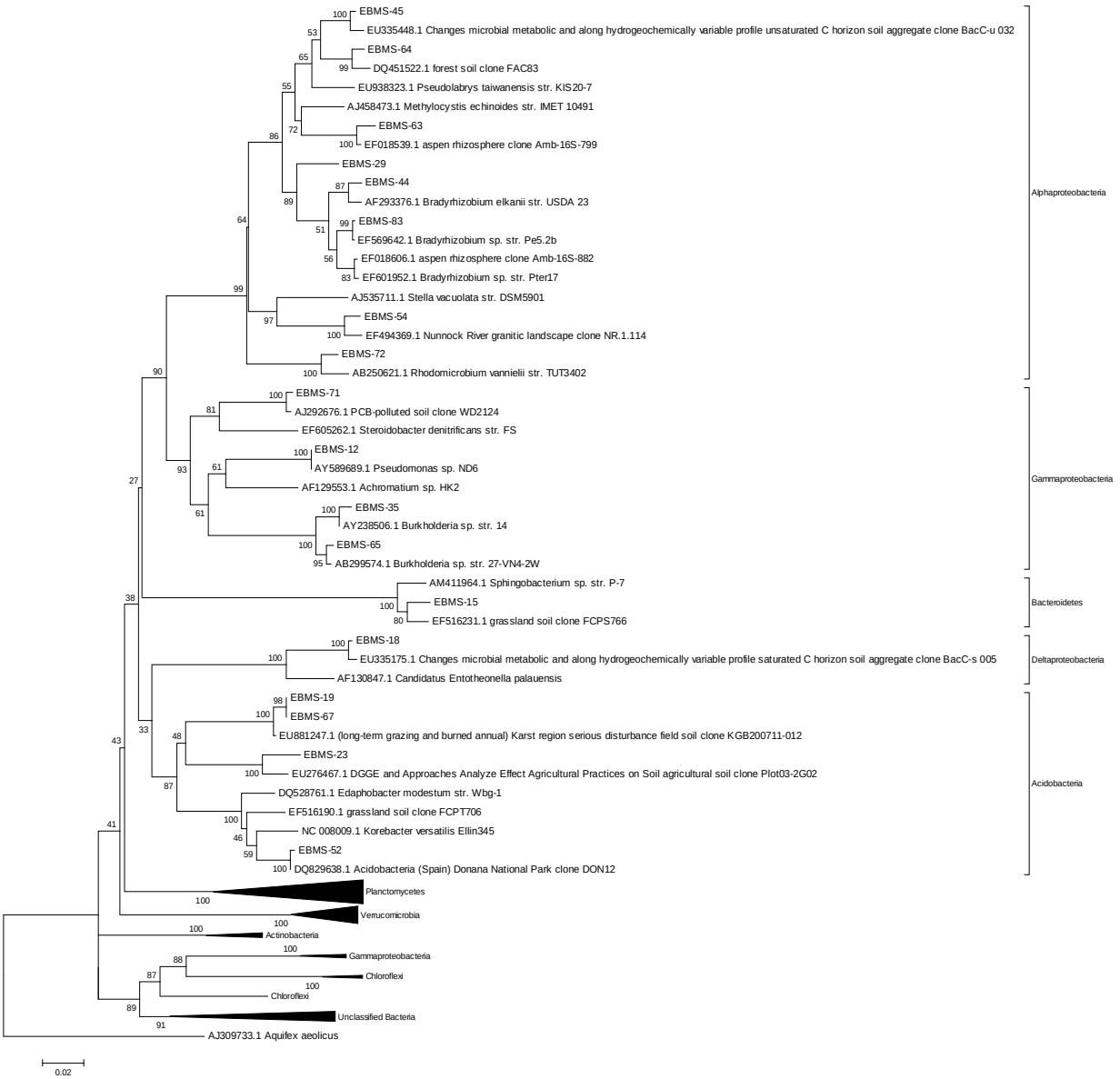
Appendix XI a. Phylogenetic tree for EBMS metagenomic library (27F). Bases: 560



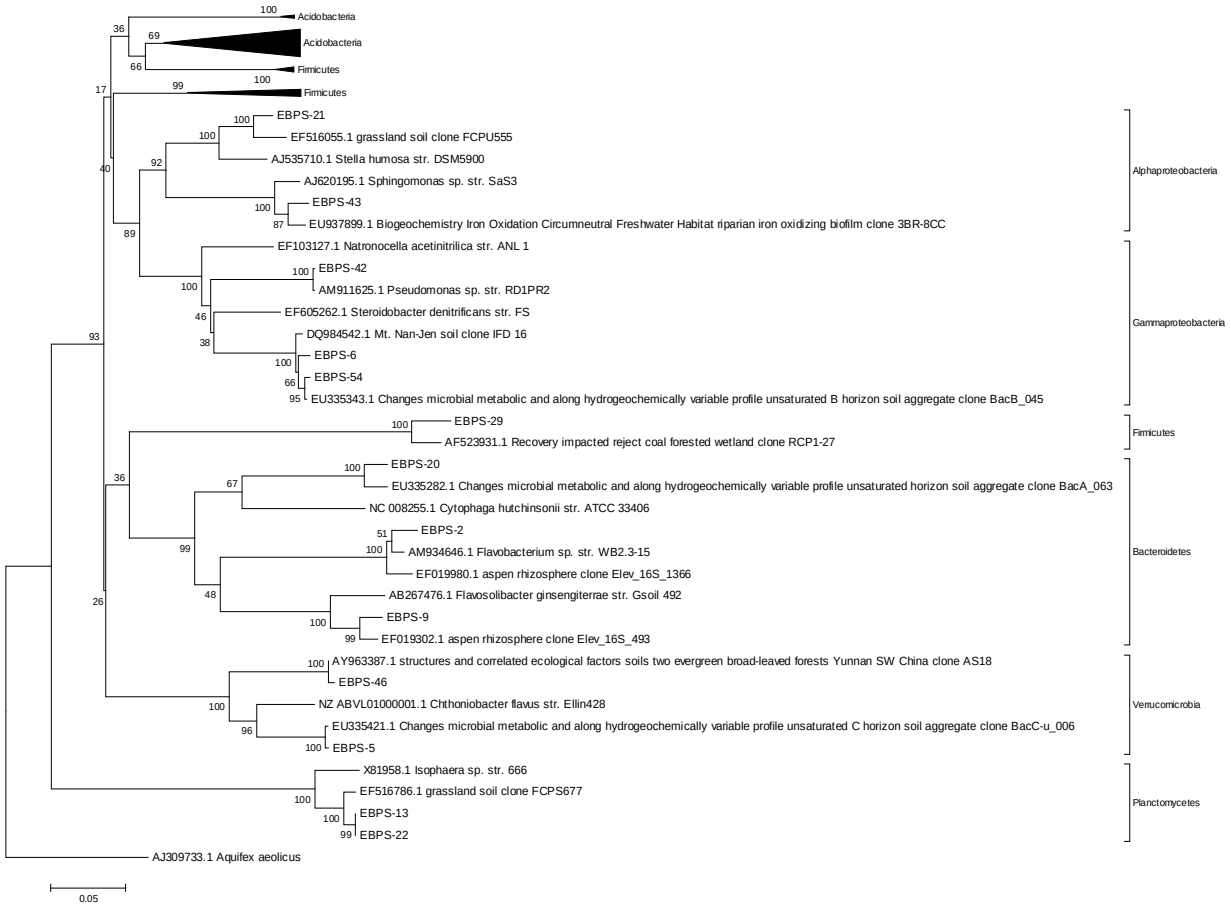
Appendix XI b. Phylogenetic tree for EBMS metagenomic library (27F). Bases: 560



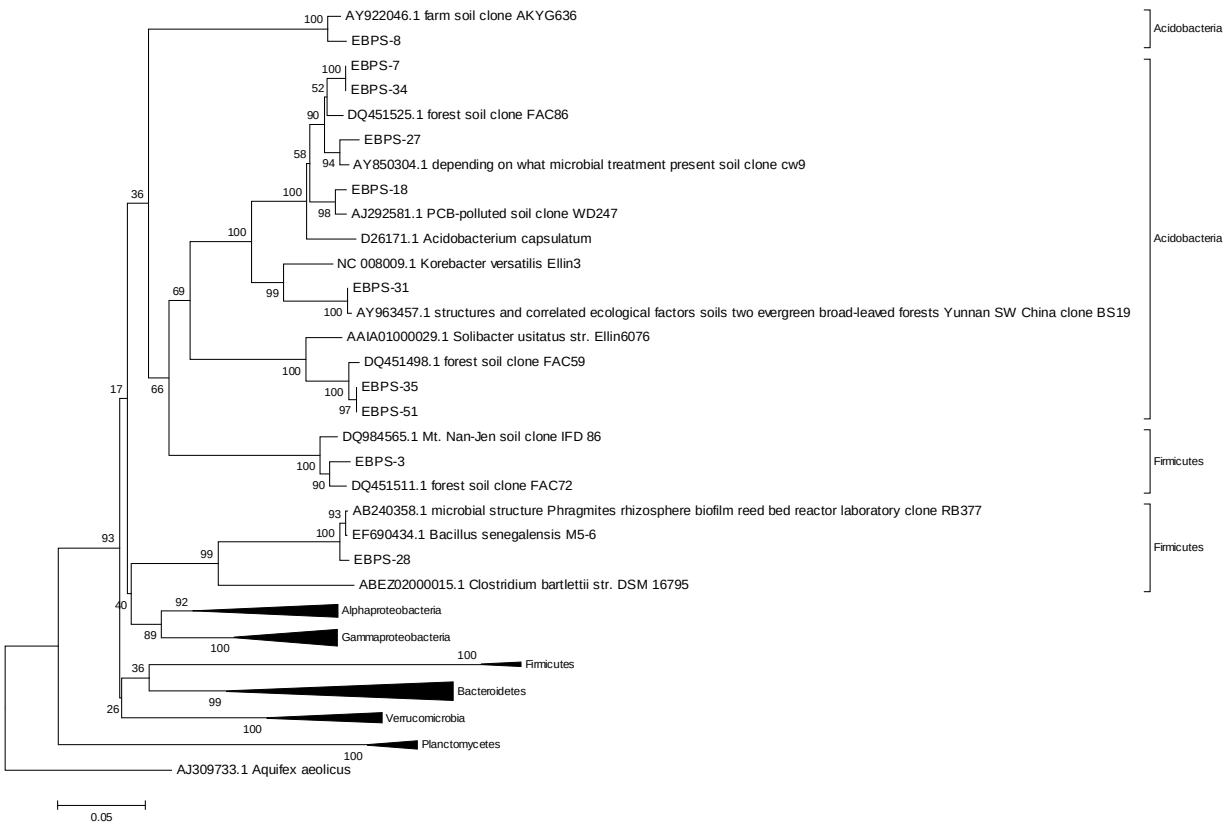
Appendix XI c. Phylogenetic tree for EBMS metagenomic library (1392R). Bases: 564



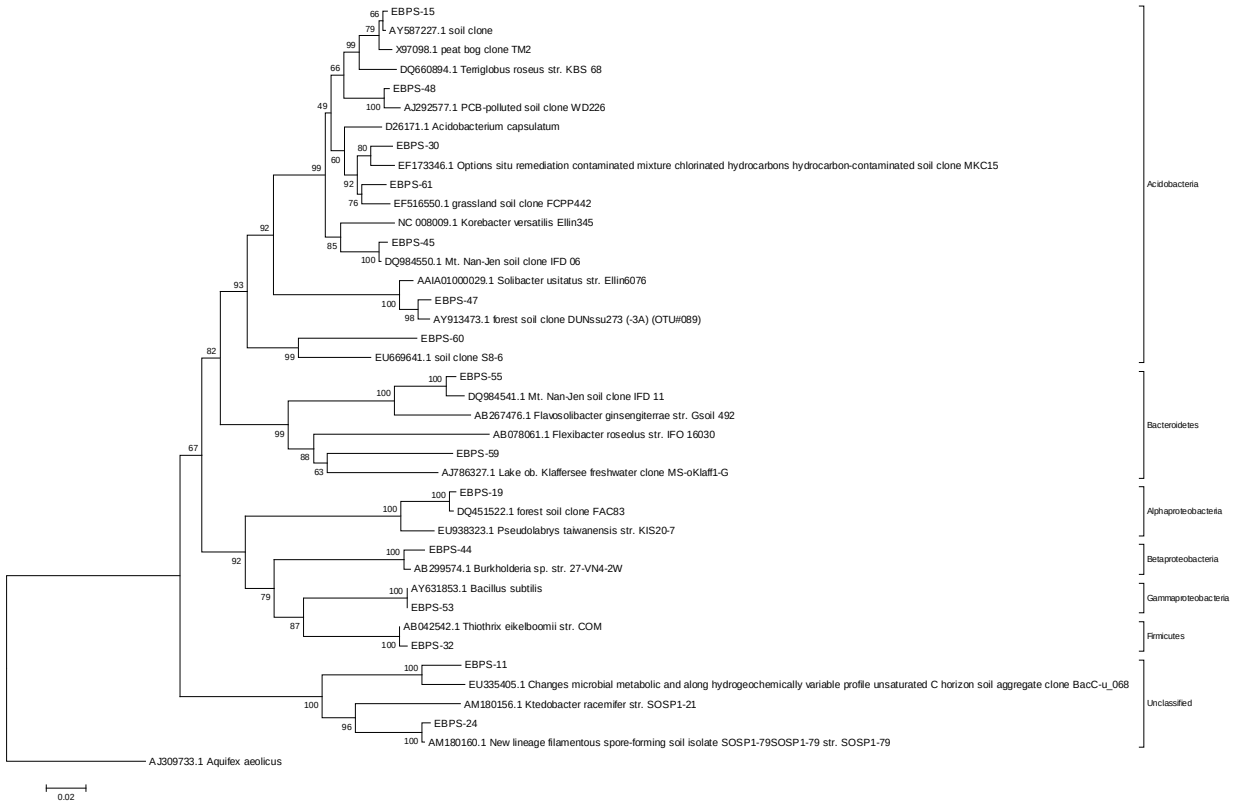
Appendix XI d. Phylogenetic tree for EBMS metagenomic library (1392R). Bases: 564



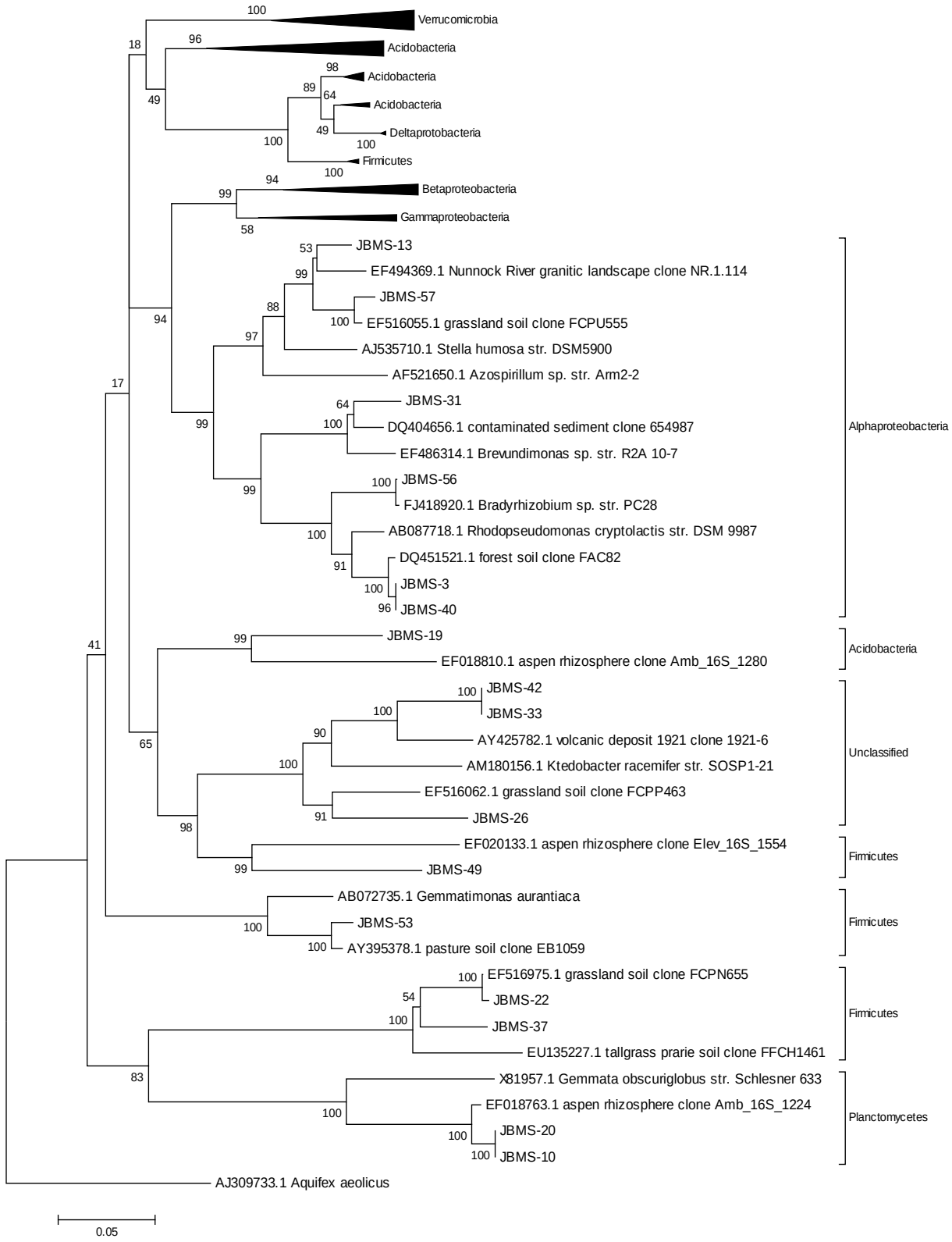
Appendix XII a. Phylogenetic tree for EBPS metagenomic library (27F). Bases: 572



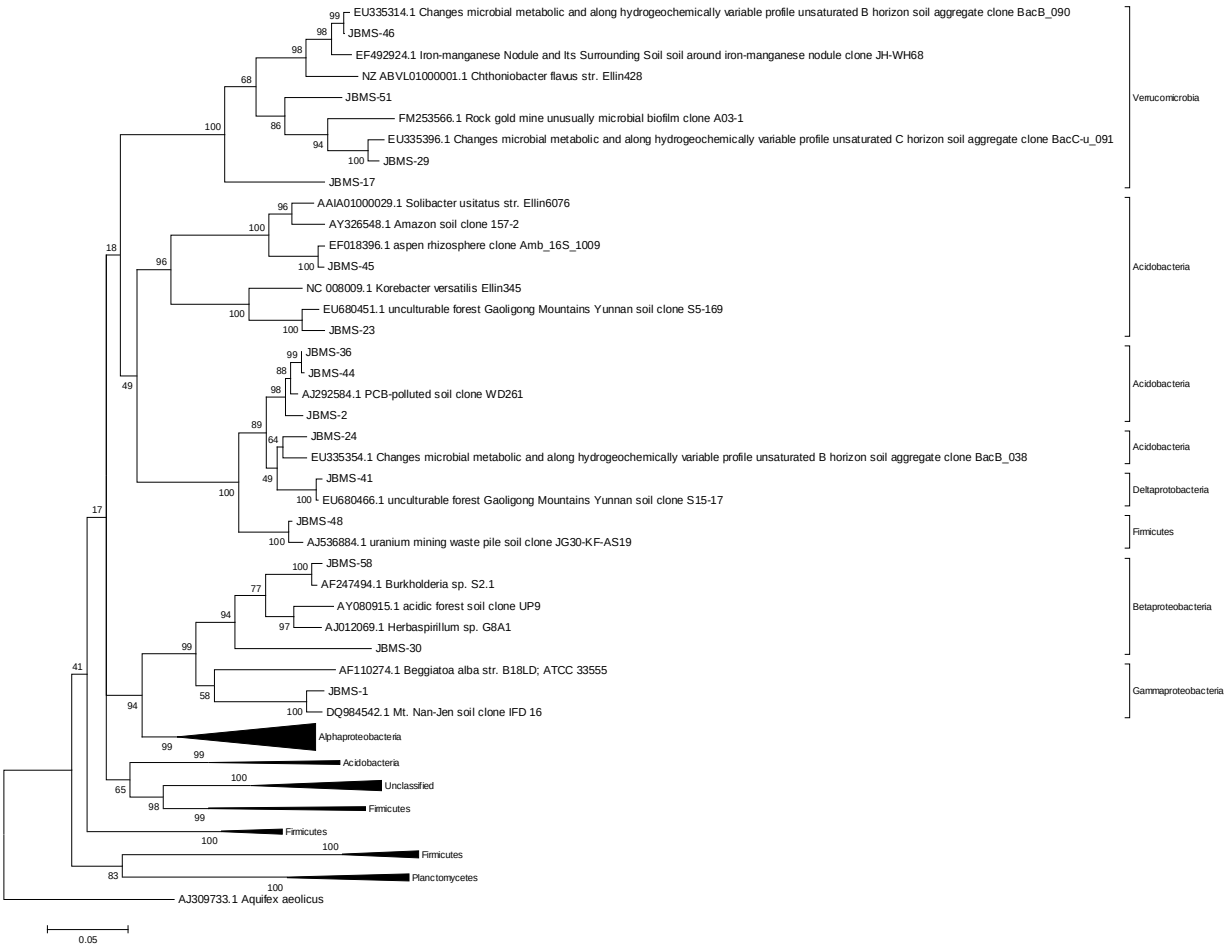
Appendix XII b. Phylogenetic tree for EBPS metagenomic library (27F). Bases: 572



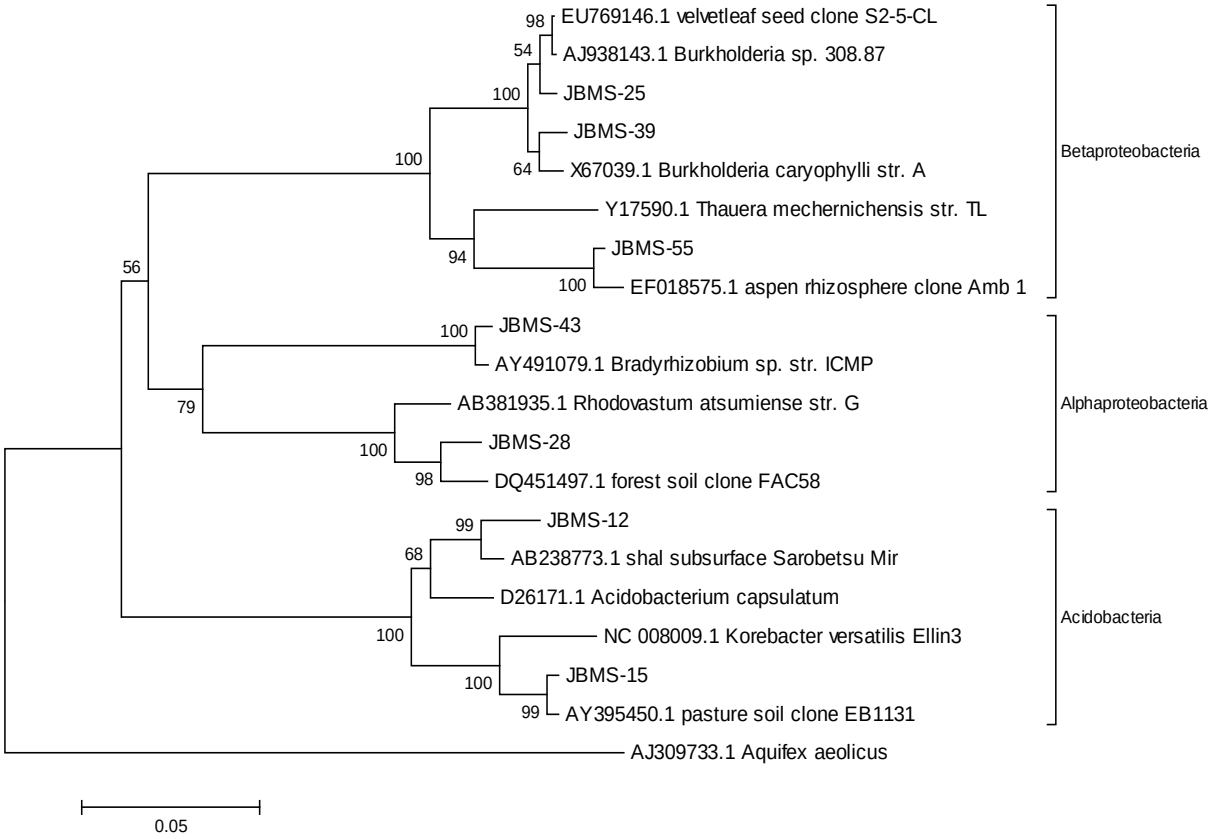
Appendix XII c. Phylogenetic tree for EBPS metagenomic library (1392R). Bases: 575



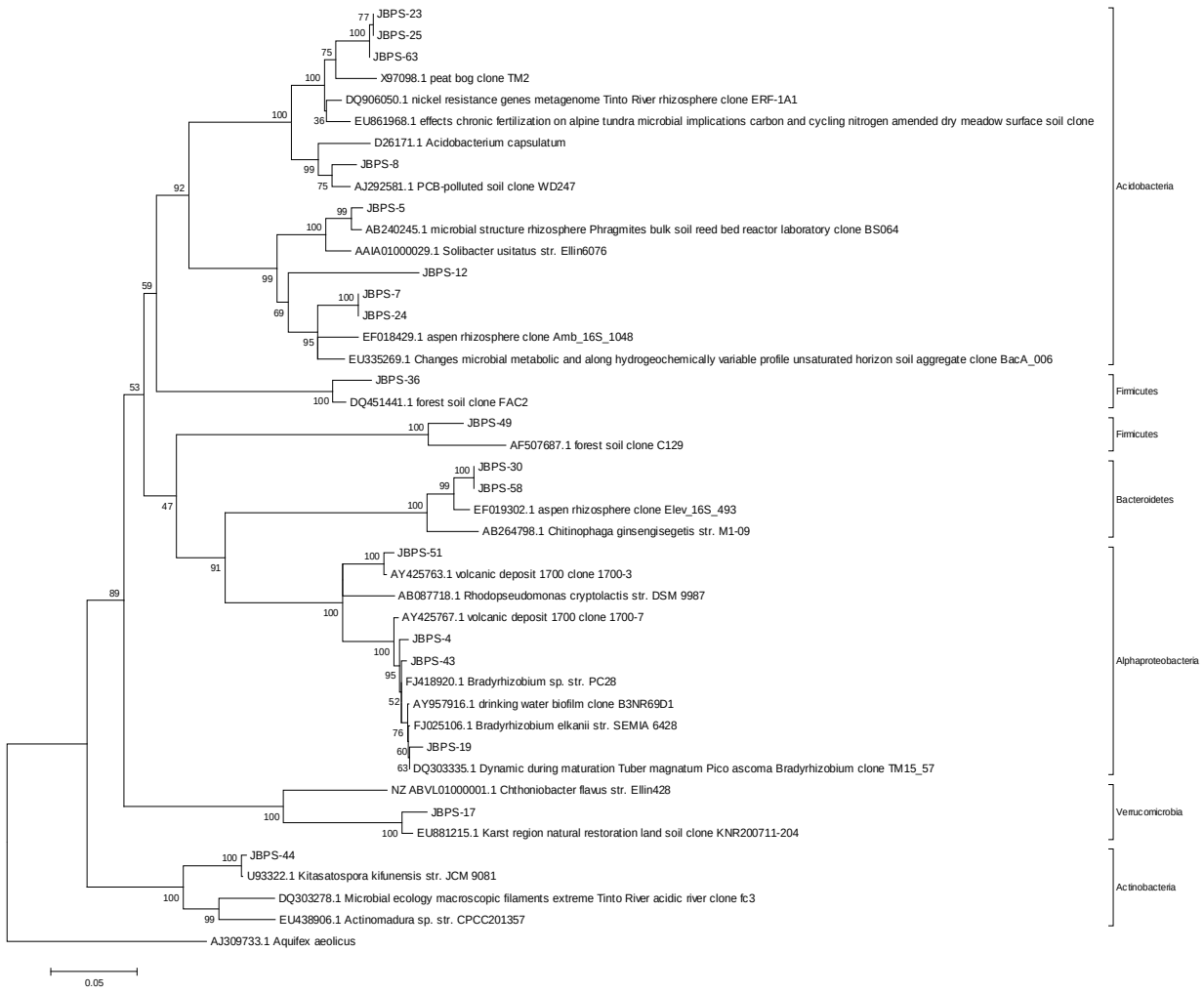
Appendix XIII a. Phylogenetic tree for JBMS metagenomic library (27F). Bases: 551



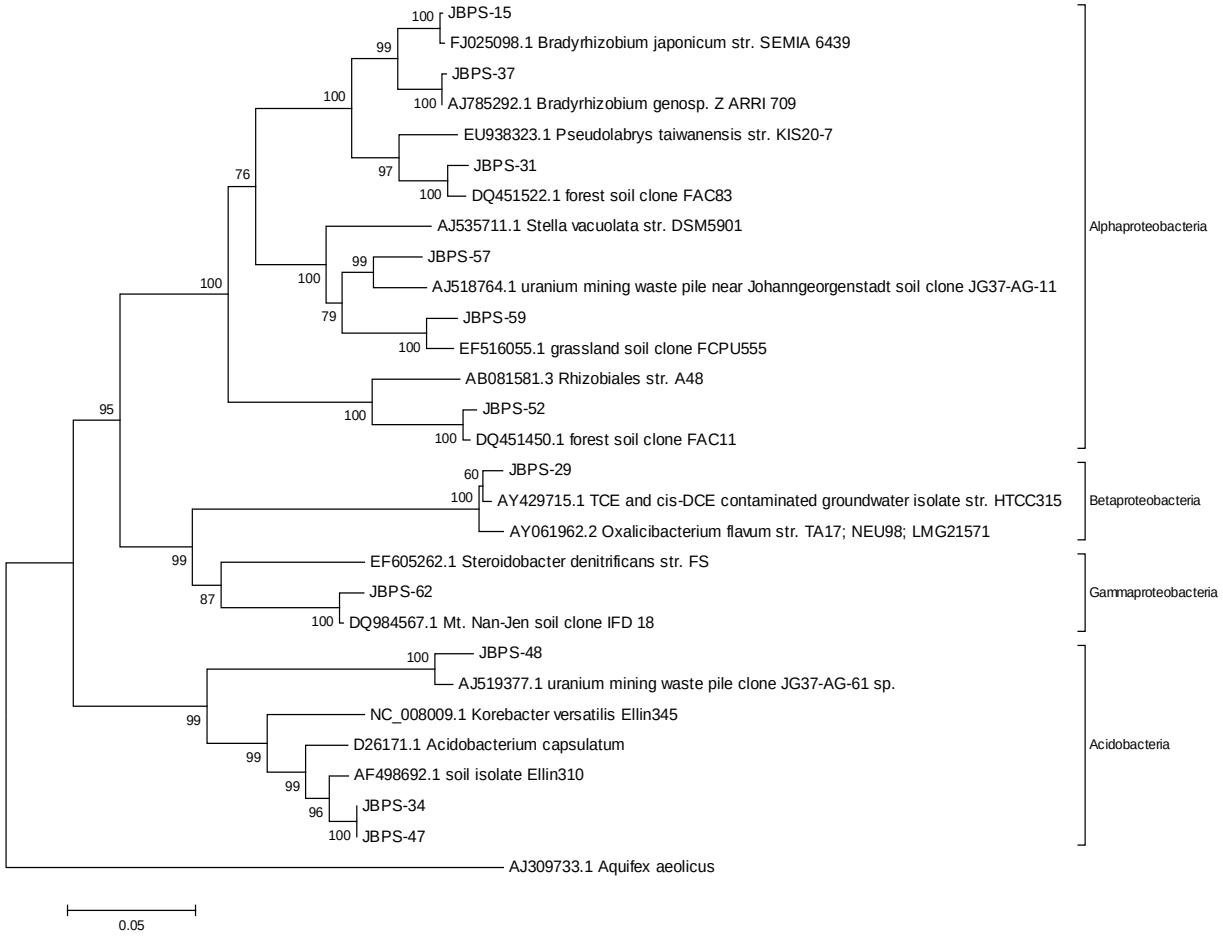
Appendix XIII b. Phylogenetic tree for JBMS metagenomic library (27F). Bases: 551



Appendix XIII c. Phylogenetic tree for JBMS metagenomic library (1392R). Bases: 629



Appendix XIV a. Phylogenetic tree for JBPS metagenomic library (27F). Bases: 580



Appendix XIV b. Phylogenetic tree for JBPS metagenomic library (1392R). Bases: 733

Appendix XV. Sequences for metagenomic libraries JBPS, JBMS, EBPS and EBMS according to the sequenced gene direction (5'→3' or 3' →5').

Library name	Sequences	
	5'→3'	**3' →5'**
EBMS	35	33
EBPS	23	15
JBMS	31	7
JBPS	18	11

Appendix XVI. *In Silico* analysis using restriction enzymes Hae III, Msp I and Rsa I for metagenomic libraries JBPS, JBMS, EBPS and EBMS.

Clone name	Hae III	Msp I	Rsa I
JBPS			
7	**191**	**146**	**90**
12	NRS	**281**	NRS
17	NRS	**68**	NRS
24	**191**	**146**	**90**
30	**258**	**91**	NRS
36	NRS	NRS	470
37	NRS	**292**	NRS
44	NRS	**159**	NRS
58	**258**	NRS	NRS
JBMS			
1	NRS	699	NRS
2	**530**	NRS	NRS
10	NRS	699	NRS
13	NRS	**456**	NRS
18	NRS	NRS	432
19	NRS	**276**	448
22	**249**	NRS	NRS
24	**261**	**178**	470
27	NRS	NRS	**486**
29	NRS	**622**	NRS
30	NRS	**487**	470
31	NRS	NRS	420
33	NRS	NRS	431
37	**292**	NRS	NRS
42	NRS	NRS	431
48	NRS	NRS	**475**
49	**246**	NRS	**442**
51	415	NRS	NRS
53	NRS	NRS	541
57	**175**	NRS	NRS

Red bold = phylotype was found in the TRFLP from biogeographical study; **Blue bold**=phylotypes was found in the TRFLP from soil study; **Green bold**= phylotypes was found in the TRFLP from both studies; NRS= restriction enzyme not recognize any restriction site; [1]the enzyme Rsa I was used only in soil study

Appendix XVI. (continuation)

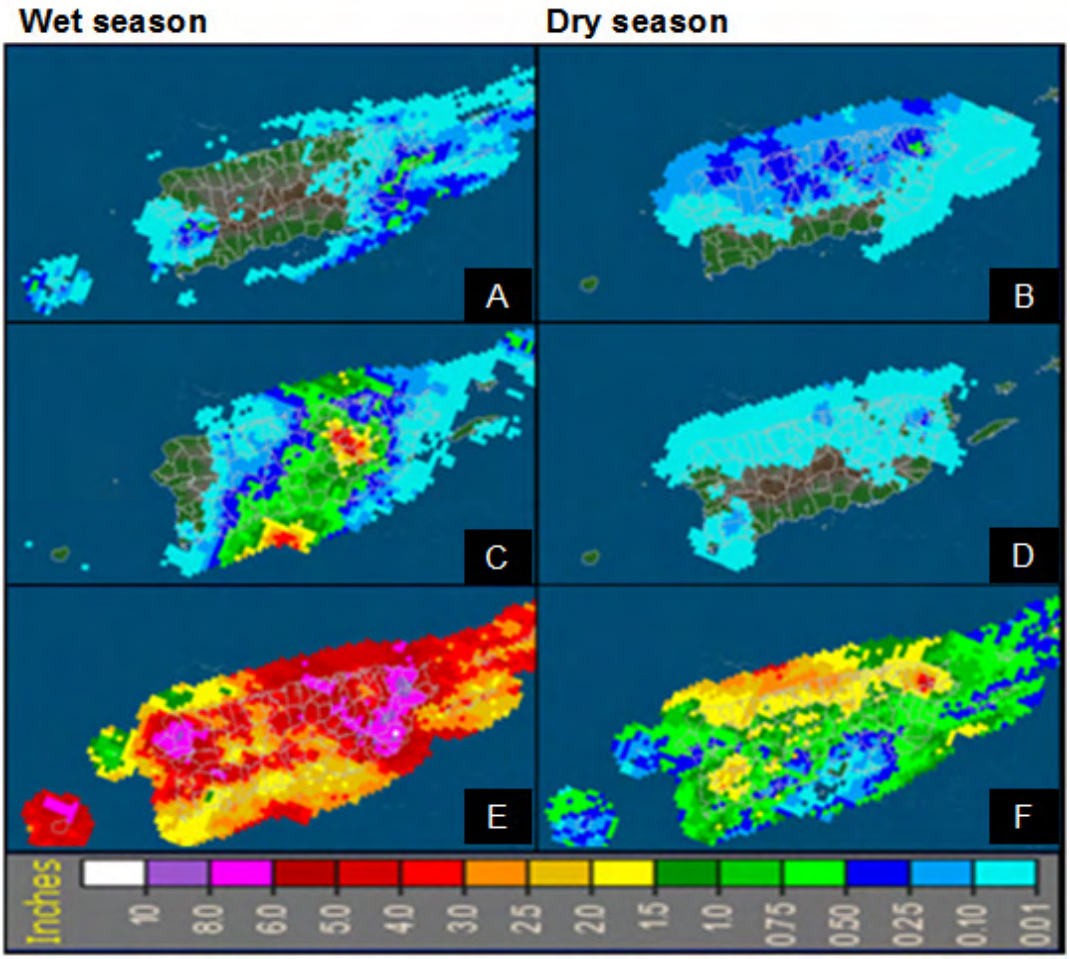
EBPS			
1	**202**	**174**	467
2	**614**	**85**	**306**
3	**286**	**174**	467
5	**227**	**158**	NRS
6	**255**	**492**	NRS
7	**214**	NRS	NRS
8	**298**	NRS	456
9	**258**	**91**	NRS
13	**63**	**170**	NRS
18	**268**	**275**	NRS
20	NRS	NRS	643
21	**211**	**173**	NRS
22	**63**	**170**	NRS
27	**214**	**265**	NRS
28	**231**	NRS	NRS
29	NRS	**493**	**306**
31	**241**	NRS	NRS
34	**214**	NRS	NRS
35	**214**	**150**	NRS
42	NRS	**490**	644
43	**76**	**161**	432
46	**123**	**169**	NRS
51	**214**	**150**	NRS
54	**255**	**492**	NRS

Red bold = phylotype was found in the TRFLP from biogeographical study; **Blue bold**=phylotypes was found in the TRFLP from soil study; **Green bold**= phylotypes was found in the TRFLP from both studies; NRS= restriction enzyme not recognize any restriction site; [1]the enzyme Rsa I was used only in soil study

Appendix XVI. (continuation)

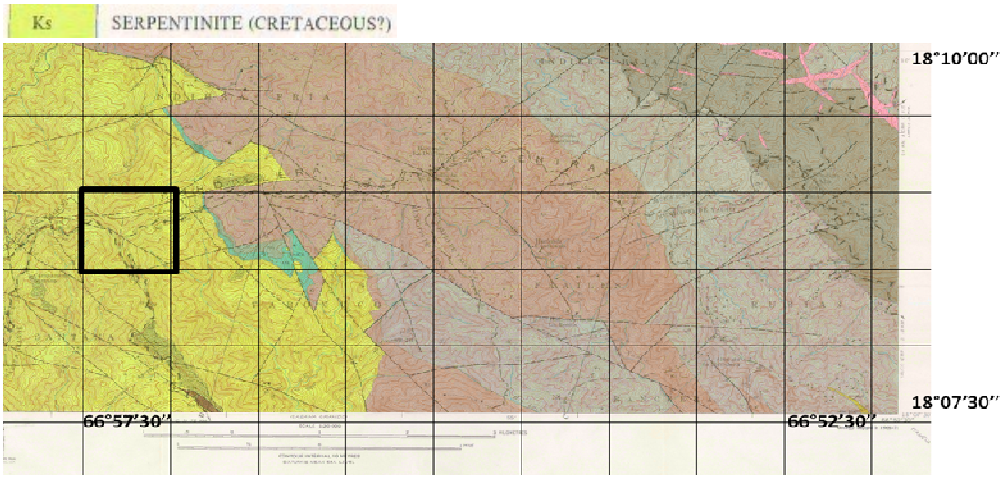
EBMS			
3	**193**	NRS	NRS
5	NRS	**502**	**106**
13	NRS	**538**	NRS
14	**188**	**507**	NRS
16	NRS	**264**	434
22	488	NRS	235
25	**214**	NRS	NRS
31	**214**	NRS	NRS
36	NRS	**176**	NRS
37	NRS	**457**	NRS
39	**264**	**83**	NRS
42	**244**	NRS	**442**
46	**82**	NRS	NRS
55	NRS	**435**	**466**
56	**284**	NRS	NRS
57	NRS	**166**	NRS
58	NRS	NRS	649
59	**299**	NRS	**491**
62	**237**	NRS	433
66	NRS	**499**	430
68	NRS	**167**	NRS
69	**214**	NRS	NRS
73	303	**489**	**472**
75	**245**	NRS	NRS
87	**197**	**81**	NRS

Red bold = phylotype was found in the TRFLP from biogeographical study; **Blue bold**=phylotypes was found in the TRFLP from soil study; **Green bold**= phylotypes was found in the TRFLP from both studies; NRS= restriction enzyme not recognize any restriction site; [1]the enzyme Rsa I was used only in soil study

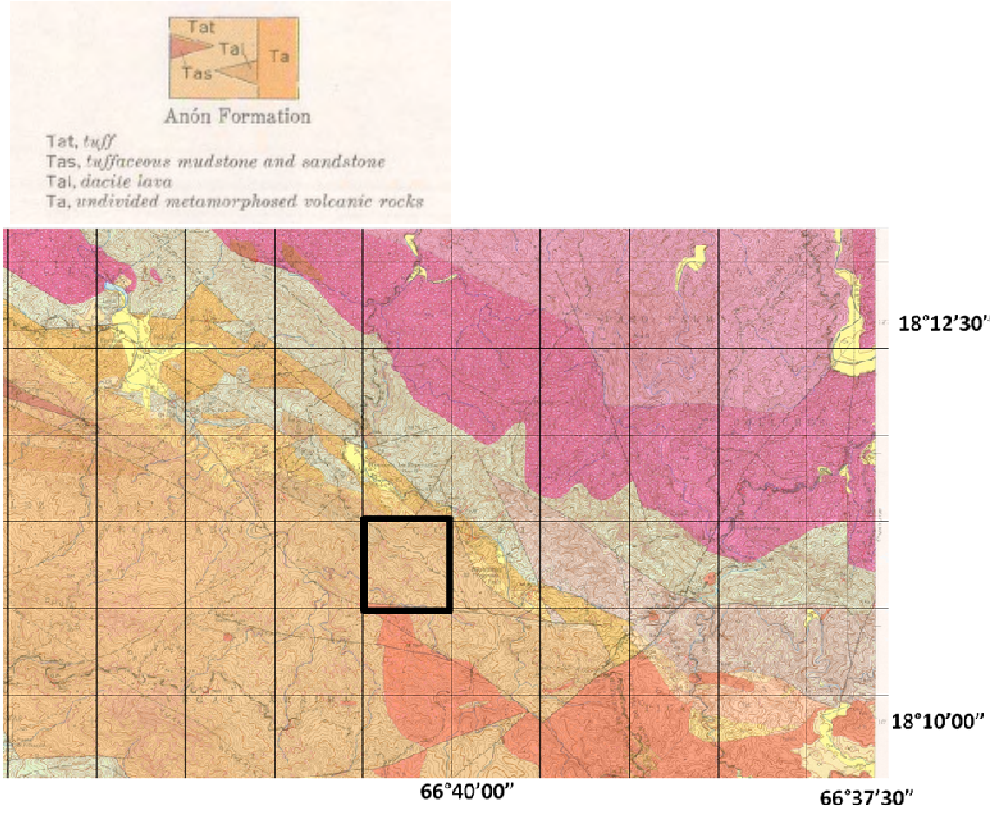


Appendix XVII. Precipitation: at Bosque del Pueblo Barrio Vegas Arriba in Adjuntas on July 7, 2006 (A) and on January 8, 2007 (B), at Bosque Estatal de Maricao Barrio Tabonuco in Sabana Grande on July 11, 2006 (C) and on January 7, 2007 (D). Precipitation during July 2006 (E), and during January 2007 (F) (Images taken from http://water.weather.gov/).

A



B



Appendix XVIII. Sampling area origin: (1) at Bosque Estatal de Maricao (Tabonuco, Sabana Grande) 18°08'04" N and 66°57'17" W (Images taken from USGS. Geologic map of the Maricao quadrangle, Western Puerto Rico. 1975) and (2) at Bosque del Pueblo (Vegas Arriba, Adjuntas) 18°10'59" N and 66°40'35" W (Images taken from USGS. Geologic map of the Adjuntas quadrangle, Puerto Rico. 1968)