Microbial Diversity of Halotolerant and Halophilic Endophytes from the Mangrove *Avicennia germinans* Located at the Solar Salterns of Cabo Rojo, Puerto Rico

By:

Jéysika Zayas Rivera

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Approved by:

_____	_____
Rafael Montalvo Rodríguez, PhD President, Graduate Committee	Date
_____	_____
Dimaris Acosta Mercado, PhD Member, Graduate Committee	Date
_____	_____
Carlos Santos Flores, PhD Member, Graduate Committee	Date
_____	_____
Carlos Rodríguez Minguela, PhD Member, Graduate Committee	Date
_____	_____
Kurt Allen Grove, PhD Representative of Graduate School	Date
_____	_____
Ana V. Vélez Díaz, MS Chair, Department of Biology	Date

Abstract

The relationship between plants and their environment has been described as a continuum, in which changes of a variable may have an effect on another. Ecological shifts in temperature, humidity, and soil chemistry can alter the microbiome associated with superficial or the internal plant structures. Halophytes are plants that require the abiotic stress of salinity for optimum growth and development. Previous studies have highlighted the differences in their unique microflora in contrast with mesophilic plants.

Avicennia germinans, also known as black mangrove, is an ecologically important halophyte found at the intertidal areas of tropical and subtropical regions. This study was targeted at evaluating the biodiversity of the prokaryotic halophilic or halotolerant endophytes in different strata in *Avicennia germinans*, as well as their community shifts based on salinity gradients using culture-independent techniques. These analyses can increase our understanding on the associated microbiota and metabolic processes that help the black mangrove thrive in such harsh conditions in their ecosystems, as there are no studies that assess the endophytic prokaryotic diversity in this plant species.

Two mangrove patches located at the Solar Saltern System of Cabo Rojo, Puerto Rico, denominated in this study as HS and Sal, were selected based on their variable water source (saline pond vs. hypersaline pond). Using classical culture techniques and phylogenetic analyses, one archaeal and eight different bacterial genera were recovered as endophytes of black mangroves. Diversity among sampling sites was determined using Shannon, Simpson and Jaccard diversity indexes. Based on the taxonomic analysis of three strains, this study proposes a bacterial and an archaeal new species and one new bacterial genus. Culture-independent analyses of prokaryotic

endophytes of black mangrove retrieved 16,285 OTUs total, of which 9,445 OTUs were obtained from HS samples and 6,840 OTUs from Sal samples. A total of five phyla were identified in both sampling sites (HS and Sal). On a genus level, 58 different genera were retrieved, of which only 39 are shared among both sampling sites. Shannon, Simpson and Jaccard indexes were calculated to determine the diversity, evenness and similarities between samples. Highly represented genera were consistent in both samples, raising the question of the ecological role these organisms play in the development and physiology of the plant.

Resumen

La relación entre las plantas y su medio ambiente ha sido descrita como un continuo en el que cambios en una variable pueden causar efectos en otra variable. Cambios ecológicos en temperatura, humedad y química del suelo pueden traer consigo alteraciones en el microbioma de las plantas, presentes en la superficie como en estructuras internas. Los halófitos son plantas que requieren altas concentraciones de sales en el suelo para tener un crecimiento y desarrollo óptimo; estudios previos han resaltado las diferencias del microbioma asociada a halófilos en contraste con plantas mesofílicas.

Avicennia germinans, también conocido como mangle negro, es un halófilo ecológicamente importante localizado en las zonas entre mareas de regiones tropicales y subtropicales. Este estudio fue destinado a evaluar la biodiversidad cultivable de endófitos procariotas halofílicos y halotolerantes a través de distintas estratas de la planta, así como los cambios en comunidades de endófitos de mangle basados en gradientes de salinidad utilizando técnincas independientes de cultivo. Estos análisis pueden incrementar nuestro entendimiento acerca de la presencia microbiana en estos ambientes, así como los procesos que puedan permitir que el mangle negro se establezca en estas condiciones severas debido a que, hasta el momento, no existen estudios que se enfoquen en la diversidad de endófitos en esta especie de planta.

Dos áreas de bosque de mangle fueron seleccionados en el sistema de Salinas de Cabo Rojo, Puerto Rijo, denominadas en este estudio como HS y Sal (laguna hipersalina y laguna salina, respectivamente). Utilizando técnicas dependientes de cultivo y análisis filogenéticos, ocho distintos géneros del domino Bacteria y uno del dominio Arquea fueron aislados como endófitos de *Avicennia germinans*. La diversidad en cada lugar de muestreo fue determinada utilizando los

índices de diversidad Shannon, Simpson, así como el índice de similitud de Jaccard. Basado en la identificación taxonómica de tres cepas aisladas, este estudio propone una nueva especie del dominio Bacteria y una del dominio Arquea, así como un género bacteriano novel. En cuanto a los análisis independientes de cultivo, 16,285 OTUs totales fueron adquiridos, de los cuales 9,445 pertenecían a las muestras HS y 6,840 a las muestras Sal. Un total de 5 filos fueron identificados en ambas muestras. Al nivel de género, 58 distintos géneros fueron adquiridos, de los cuales sólo 39 son compartidos entre ambos sitios de muestreo. Los índices de Shannon, Simpson y Jaccard, fueron calculados para determinar la diversidad y similitud entre las muestras. Los géneros altamente representados fueron similarmente dominantes en ambas muestras, aumentando la incertidumbre del rol ecológico que estos organismos tienen en el desarrollo y fisiología del mangle negro.

Dedication

Para mi gotita de agua. Te amo.

Acknowledgments

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Introduction

Mangroves are woody plants that possess a variety of morphological and physiological adaptations for life. They are usually found in the intertidal areas of tropical and sub-tropical regions, with a world-wide total coverage area of 137,760 km^2 in the 2000 [1]. These plants provide a suitable habitat for multiple species, including animals, plants, fungi, and prokaryotes, mainly due to the litter production that allow biogeochemical processes to occur [2–5]. Thus, mangrove forests are considered the major coastal ecosystem [2]. These forests are vital for the protection of extensive diversity of marine life including coral reefs, sea grass beds and associated organisms [6,7]. Furthermore, they contribute to the stabilization of coastlines, avoid shore erosion, and protect the shore from tidal waves [4,8–11]. However, a high incidence of mangrove mortalities have been reported in the past decades due to anthropogenic impact [12].

In Puerto Rico, the mangrove coverage includes 4 main species: *Rhizophora mangle, Laguncularia racemosa, Conocarpus erectus*, and *Avicennia germinans* [13]. The latter, also known as black mangrove, is a halophyte that grows in tropical and subtropical areas, predominating in hot, arid climates with nutrient deprived soils and high concentration of precipitated salts [10,12,14]. These conditions often come in hand with anoxia, periodical flooding, and heavy metal deposition [15,16]. To endure these conditions, black mangroves have developed morphological and physiological adaptations including pneumatophores – aerial roots to optimize oxygenation [14,17], dwarfism [13] and salt excretory glands [18]. Although these morphological traits have enabled the plant to thrive in hostile environments, it is desired to determine other factors that can promote their establishment and growth.

Studies regarding microbial-plant interactions have elucidated that several naturally occurring processes in plants can be improved or mediated by organisms associated to their host [19–21]. To understand these relationships, it is imperative to know the microbial organisms that colonize the plant, and the source of diversity – if its host dependent or environment dependent. Few studies have been conducted to understand the prokaryotic diversity associated to *Avicennia germinans* and its importance for plant functioning. These studies include the analysis of the microflora associated with the rhizosphere and surface of *Avicennia germinans* [6,11,22–24]. Thus, the analysis of endophytic microorganisms associated with the black mangrove could increase our understanding about the potential metabolic processes that help this plant thrive in such harsh conditions in their ecosystems. This information can be useful to further develop mangrove conservation strategies, as well as to improve crop production in salinized soils. The main goal of this study is to determine the prokaryotic biodiversity of halophilic or halotolerant endophytes within the black mangrove, focusing on the diversity in different areas of the plant and among different environments (saline and hypersaline), as a stepping stone towards the understanding of mangrove-microbe interactions.

Literature review

1. Halophytes

Globally, 950 million hectares of dry land and 250 Mha of agricultural land, are in contact with sea-water or affected by soil salinization [25,26]. This phenomenon, carried by the deposition of salts in the soil, generally Na^+ and Cl^- ion, can be caused naturally or anthropogenically. Naturally-occurring salinity stresses mostly happen as result of high evaporation rates, causing drought in the soil and an increase in salt ion deposition [27]. Known sites where the associated soil presents this trait include salt marshes, solar salterns, salt lakes, coastal shores, among others. Anthropogenically impacted soils can also increase soil salinization, especially after continuous irrigation with low-quality water. Reports by Munns and Tester show that currently 20% of the irrigated soils have been reported with an increase in salt deposition, with a yearly estimate increase of 0.5-1% [28].

Even though most plants, denominated as glycophytes, cannot withstand the osmotic stresses caused by increased salinity in soil, a specialized group of plants can. Halophytes are plants that, can grow in the presence of salt in the soil [29]. This definition has been analyzed and modified multiple times, to the extent where the creation of subcategories has occurred. Real halophytes require soil salinization to complete their life cycle, whereas natural halophytes has been attributed to plants that do not live in saline conditions but can tolerate it [30]. The range of salt tolerance varies from one species to another, however, “true” halophytes can resist over 200 mM NaCl [31,32].

This group encompasses a wide variety of plants, both terrestrial and marines. Highly studied terrestrial-halophytes include to *Suaeda maritima, Atriplex spongiosa, Puccinellia*

peisonis, Spartina townsendii, and *Arthrocnemum machrostachyum* [30]. On the other hand, mangroves and seagrasses are marine halophytes studied as well. [6,11,22–24,33]. Both these groups are subject of research to understand some salt tolerance strategies developed for survival.

1.1 Salt Tolerance Traits

Increased soil salinity can be detrimental for plant development in the absence of tolerance mechanisms. Ions precipitated in the soil, including Na^+ and Cl^-, can be thoroughly absorbed and transported from the roots to the rest of the plant. In this scenario, photosynthesis can become impaired, and cell toxicity due to the excess generation of reactive oxygen species (ROS) can end in the fatality of the plant[34]. Lacking the necessary machinery to thrive in higher salinity and decreased water potential, can cause the cellular homeostasis to be impaired, for which physiological/morphological modifications occur [35]. Halophytes have developed diverse salt tolerance mechanism to bear various abiotic stresses, being salinity the primordial stressor. These strategies include osmoregulation, ion exclusion, and ion compartmentalization.

Increased soil salinity has been associated with diminished water uptake through the roots causing plant dehydration. To avoid the inhibition of water uptake, halophytes developed a positive turgor adjustment with the accumulation of solutes in the cells [30,31]. Nevertheless, no glycophyte or halophyte can thrive with high concentrations Na^+ in the cytoplasm, for which halophytes developed specialized vacuoles to store these ions through an Na^+/H^+ antiporter [34,35]. The functionality of this antiporter is mediated by Salt Overly Sensitve (SOS) stress signaling, a pathway that, in the presence of salt, produces a series of proteins that activate the antiporter [35]. Halophytes have these proteins overexpressed for tolerance. In the case of halophytic succulents from the family Chenopodiaceae, two thirds of the solute concentration in

the cells was attributed to the solutes Na^+ and Cl^- in the specialized internal structures[30], a phenomena observed in dry soils to maintain turgor [28]. On the other hand, other plants that accumulate Na^+ internally require epidermal bladder cells to tolerate the salinity stress. In these cells, or glands, after salt ions are accumulated, an energy-expensive process of salt excretion to the environment occurs [36]. Halophytes exhibiting this trait are denominated as recretohlophytes, a term used to explain their salt exclusion modifications [37]. Even with this physiological change, the plant also suffers morphological changes to compensate for the low water potential. As a response to osmotic stress, halophytes might also reduce cell expansion causing young leaves to grow smaller, as well as roots [28].

Another technique for salinity tolerance is the accumulation of osmolytes. Osmolytes are compatible solutes that do not interfere with natural cell metabolism [35]. These include proline, glycine betaine, sugars and polyols [38]. In *Avicennia marina*, the solute accumulated varies according to the life stage of the plant [39]. In young plants, the Na^+ excess is being compensated by glycine betaine in the leaves, whereas in the stem a distribution of glycine betaine and the sugar stachyose equally compensate for the salt presence. As the organism matures, less glycine betaine is produced to compensate for the osmotic stress in the leaves, as well as in the stem. In the roots, a low content of osmolytes are produced [39]. In contrast, the mangrove *Aegiceras corniculatum* has a preference forwards mannitol as compatible solute, whereas pinitol is the predilected osmolyte of *Aegialitis annulata* [40].

2. Mangroves

Mangroves are woody plants that comprise around 200,000 km^2 of tropical and subtropical coastal vegetation (figure 1) [1,41–43]. They are divided in 9 orders, 20 families, 27 genera and

over 70 species [7]. These are characterized by growing in the sea-land interface, usually in anaerobic soils with variable salinity concentrations, high temperatures, and high tides [44,45]. Despite the fact that these characteristics may limit nutrient uptake, mangrove forests are considered enriched ecosystems based primarily on their productivity, diversity and uses [9]. Simultaneously, such forests contribute to the carbon cycle allowing carbon fixation into biomass. When extrapolated, due to their limited coverage (2% of the worlds surface), they can be easily compared to tropical forests [46]. Ecologically, they contribute to the stabilization of coastlines, storm and erosion protection, and production of organic matter; also, mangroves serve as habitat for other plants, animals and microorganisms, among others [9]

Figure 1: Worldwide distribution of mangrove forests (green lines). [1]

In spite of their ecological importance, tolerance to variable environmental conditions and their role in coastal stabilization, these forests have decreased world-wide since 1980 at a rate of 2.1% per year [45]. This population decline has been linked mostly to anthropogenic factors, such as increase in human population, industrialization, land use and aquaculture [7,12,45]. However, in Puerto Rico, the decline of mangrove forests occurred from the 1800s up to the 1972, when the legal protection of all mangroves in the island was approved [45]. Up to this date, 2000 hectares of mangrove forests have been recovered.

In Puerto Rico, mangrove forests have been reported to be found in 97 different locations throughout the island [45], composed of four different species: *Rhizophora mangle* (red mangrove), *Laguncularia racemosa* (white mangrove), *Avicennia germinans* (black mangrove) and a pseudo-mangrove *Conocarpus erectus* (buttonwood mangrove) [13]. Each species, excluding buttonwood mangroves, exhibit morphological adaptations based on soil oxygenation and salinity. Red mangroves present aerial roots, that allow the plant to situate in the interface between sea water and shore, as well as to inhabit a wide variety of marine life [47]. Black and white mangroves may exhibit pneumatophores, a root modification that is correlated to soil anoxia and heavy metal deposition [48]. Other adaptations include accelerated canopy development and mechanisms for nutrient retention [7].

2.1 Black mangrove, Avicennia germinans

Avicennia germinans, from the family *Avicenniaceae* and known as black mangrove, is classified as a recretohalophyte due to the presence of salt excretion glands for salt tolerance and its soil salinity requirements [49]. In the south of Puerto Rico, such trait allows *A. germinans* to become the colonizing species during secondary succession in *Rhizophora* and *Laguncularia*-dominated forests, avoiding mortality due to salinity increase in the soil [13].

Multiple prokaryotic organisms have been reported in association with the rhizosphere and leaf surface of *A. germinans*. Some include representatives from the genera *Staphylococcus, Pontibacillus*, *Kushneria* and *Halobacillus* [6,11,23,24]. However, few studies have been conducted on the prokaryotic diversity of the endosphere of the black mangrove and its relative importance. Studies regarding fungi-mangrove interactions have reported various representatives, including *Colletotrichum, Glomerella, Nodulisporium Phomopsis and Sordaria,* as endophytes of

the *Avicennia* individuals [50]. Even though most of these genera were were ubiquitous in the three mangrove genera under study (*Avicennia, Laguncularia* and *Rhizophora*), the genera *Hormonema, Microsphaeropsis* and *Phoema* were only found in *Laguncularia,* whereas *Phyllosticta and Sphaerosporium* were specific for *Rhizophora* [50].

3. *Endophytes*

Endophytes are eukaryotic or prokaryotic organisms that live within a plant at some point of their life, without causing harm to their host [51–53]. Multiple studies have identified that endophytes provide the plant with: insecticide, antifungal or antibiotic metabolites [52,53], in addition to hormones, and enzymes [17,54]. Furthermore, it has been elucidated that the endophytic production of the mentioned compounds provides the host with the ability to resist pathogens, tolerate adverse environmental conditions and thus, to expand their habitat. All of these may be mediated through: (a) phytohormone signaling [55], a process where certain bacteria regulate plant growth by producing hormones required by the plant; (b) nutrient acquisition [55–57],where specialized bacteria perform nutrient recycling within the plant, (c) protection against pathogens [51,52,58]; and (d) tolerance towards abiotic stress (i.e. drought, low temperatures) [55]. Since the high presence of diluted salts in the soil is considered an abiotic stress, a plant-microbe interaction that provides the plant with halotolerance is suggested.

3.1.1 Phytohormone and phytocompound production

Direct benefits microbes can provide to plants are germination and growth, performed by plant growth-promoting (PGP) organisms [59–61]. For instance, Navarro-Torre et al. [62,63] have isolated and analyzed the germination and growth-promotion that bacterial endophytes provide to

their host, *Arthrocnemum macrostachyum*. This study unveiled the increase in velocity and count of germinated seeds in presence of endophytic PGP bacteria of the genera *Bacillus* and *Gracibacillus* in the presence of NaCl [63]. Another halophyte analyzed for its PGP endophytes was *Limonium sinense*, by Qin et al. [64]. Aiming to determine the potential endophytes to provide stress tolerance during germination, the endophytes *Isoptericola sp.*, *Arthrobacter sp.*, *Streptomyces sp.*, and *Bacillus sp.* were tested. With an increase in salinity of the germination media, a decrease in germination was observed in the control. Similarly, a decrease in germination was observed in the inoculated plants. Nevertheless, a higher germination rate when compared to the control was observed, indicating that retardation of the effects of salinity stress is provoked with the inoculation of these endophytes. The best yield in germination was provided by *Bacillus sp.* [64].

PGP properties have been associated with the production or modification of compounds. Desale et al. [65] discovered some moderate halophiles are capable of providing the plants with phytohormones. In this study, the genera *Halobacillus* and *Halomonas* were analyzed for their production of indole-3-acetic acid (IAA), a phytohormone required for plant growth and development. These two genera were able to produce 95-168 µg/mL of this hormone in low concentrations of heavy metals. Other studies in the halophyte *Limonium sinense*, also determined the potential of endophyte-mediated IAA production [64]. In this study, out of the multiple endophytes isolated, some strains identified within the genera *Streptomyces, Klebsiella, Serratia, Pseudomonas,* and *Bacillus* were producers of IAA. In contrast with the studies by Desale et al. [65], lower IAA production was observed (ranging from 0.15-8.24µg/mL), where *Bacillus sp.* was the isolate with lower IAA production rate and *Streptomyces sp.* with the highest [64]. These results go in hand with studies in glycophytes, as observed in the studies by Phecharat and

Duangpaeng [66]. This study identified *Pseudomonas sp., Bacillus sp., Azotobacter sp.,* and *Enterobacter sp.* as endophytes of rice with the potential of IAA production. All strains produced IAA in values higher than 10µg/mL, however the highest value of IAA production was not higher than 15 µg/mL. In a similar manner, researchers reported multiple endophytic strains in *Solanum lycopersicum* (tomato), among which *Bacillus sp.* presents the highest IAA production (8.7µg/mL) [67]. In these scenarios, both glycophytes and halophytes have associated PGP microbiota but the phytohormone production by *Halobacillus spp.* and *Halomonas spp.* is substantial.

3.1.2 Nutrient cycling

The endosphere of halophytes can be an environment with nutrient limitations due to low transport of compounds in a usable redox state. Endophytes have been known to collaborate in biogeochemical processes for nutrient availability. Phosphate solubilization is a trait multiple endophyte provide the plant with. In this process, microorganisms transform inorganic phosphate compounds to soluble phosphate compounds [68]. In studies analyzing the PGP bacteria from rice, the genera *Flavobacterium* was denominated as the phosphate solubilizer with the highest potency and highest tolerance to salinity stress [69]. Other investigations focused on detecting the capability of phosphate solubilization include other bacterial genera such as *Bacillus, Halobacillus, Pseudomonas, Paenibacillus, Enterobacter* and *Proteus* [68,70–72].

Archaea have been observed in metagenomes as part of endophytic communities, but yet have to be isolated from plant tissue. Nevertheless, studies conducted by Yadav et al. [73] selected previously described strains of haloarchaea to test their phosphate solubilization potential. Strains used are part of the following genera: *Halolamina, Halobacterium, Halococcus, Haloferax, Halosarcina, Haloterrigena, Haloarcula, Halobacterium, Halostagnicola., Natrinema,* and

Natrialba [73]. From these strains, the ones that solubilized the highest quantities of phosphate were *Natrinema sp.* and *Halococcus sp.* with 134.61 and 112.56 mg/L, respectively. This raises the question of the ecological role archaea in plants.

Nitrogen fixation is a role commonly associated with soil bacteria or rhizobacteria [74]. However, endophytic bacteria have also been reported to conduct this process. As reported by Li et al. [75], the capacity of nitrogen fixing will vary from one endophyte to the other. The endophytic isolates of elephantgrass (*Sphingomonas sp.*, *Bacillus sp.*, *Pantoea sp.*, and *Enterobacter sp.)* presented differences in nitrogen fixing potential. In this study, all strains were able to fix nitrogen, but *Sphingomonas sp.* and *Pantoea sp.* had higher fixing capacities. In contrast, all strains had a very low capacity of ammonia production [75].

3.1.3 Pathogen protection

Endophyte-mediated resistance to infections occurs with the production compounds that alleviate, repel or suppress infections. The first study that recorded pathogen protection by endophytes was focused on the Dutch elm disease in the plant *Ulmus glabara*, caused by *Ceratocystis ulmi* [76]. The infection was avoided thanks to the fungal endophyte *Phomopsis oblanga* by decreasing the pathogenesis of *C. ulmi* with the production of toxic compounds that repelled the vector [51]. This study was conducted by Joan Webber in 1981 [76], serving as stepping stone for the analysis of endophytes for their protection properties.

One group of compounds that can be regulated by the presence of endophytes in plants are the flavonoids. These have been suggested to work as microbial-infection suppressors. That is the case of the cedar-apple rust, caused by the fungal pathogen *Gymnosporangium yamadai*, where

this compound is key in the signal-transduction pathway to combat pathogenesis. Thus, flavonoid production by the plant limits the pathogenic infection [77].

Flavonoids tend to be produced and accumulated in the plant *Limonium sinense* due to abiotic stress. In the study performed by Qin et al. [64], salinity stresses and endophyte-mediated flavonoid production were assessed. The individual inoculum of the endophytes *Arthrobacter sp, Streptomyces sp., Isoptericola sp.*, and *Bacillus sp*. showed a dramatic increase of flavonoid production was observed compared to the control. Interestingly, all endophytes presented lower in flavonoid production in 200mM NaCl, but this value increased with the salinity increase [64]. This pattern indicates that in the presence of endophytes less pathogenic diseases may occur due to a rapid infection response.

The production of bioactive compounds by endophytes has also been recorded. The endophytic fungi *Pestalotiopsis jester*, isolated in Papua New Guinea, produces a compound denominated as jesterone. This secondary metabolite presents activity against oomycetes (now Stramenopiles), fungal-like protists [78]. The importance of this compound relies on the catastrophic damages on the agriculture caused by oomycetes with diseases including root rots, downy mildews, blights, and damping off [79]. Plant treatments for these infections in the absence of compounds specified for oomycetes require the mixture of fungicides, a practice that affects the natural microbiota, rather than eliminating the pathogen. Other isolated and identified bioactive compounds from endophytes include the antifungals: cryptocandin A and cryptocin, obtained from the fungi *Cryptosporiopsis* cf. *quercina*; and ambuic acid, produced by *Pestalotiopsis microspora* [78].

3.1.4 Abiotic stress tolerance

Studies previously discussed by Navarro-Torre et al. [63] have also assessed the tolerance towards saline stresses by the halophyte *Arthrocnemum macrostrachyum*. For this, plants inoculated with the PGP bacteria (genera: *Bacillus sp.* and *Gracibacillus sp.*) and uninoculated plants were exposed to two salinities, 510 mM NaCl– optimum for plant growth – and 1030 mM NaCl. No significant differences were observed in shoot to root dry mass, since the plants were growing on an optimum level. However, a decrease in plant growth was observed in 1030mM NaCl, but the effect was diminished in inoculated plants, suggesting a resistance towards the abiotic stress [63].

To test the effects of salt in *Limonium sinense*, four endophytes (*Arthrobacter sp, Streptomyces sp., Isoptericola sp.,* and *Bacillus sp.*) were selected as inoculum. Also, four variations in NaCl concentration (0 mM, 100 mM, 200 mM and 250 mM NaCl) were used as treatment. Changes in the morphology of the uninoculated plant were observed in salinity increases over 100 mM NaCl, since this is an optimum concentration for growth. In 200 and 250 mM NaCl, a decrease in root and leaf presence was evident. However, once again the endophytes buffered the effect on the salinity in the plant, suggesting that there is mitigation of the abiotic effects [64].

In 2016, Li et al. [75] decided to test the abiotic resistance mediated by endophytes from the glycophyte *Pennisetum purpureum* (elephant grass) to a Hybrid *Pennisetum*. Four strains from the genera *Sphingomonas, Bacillus, Pantoea,* and *Enterobacter* were isolated from the elephant grass, and co-inoculated into a Hybrid *Pennisetum*. After exposure to control and high salinity conditions, the plants with the inoculum showed increased growth in leaf and roots when compared to the uninoculated. Simultaneously, at 200 mM NaCl, the plants with the inoculum had better growth patterns than those inoculated at 0 mM NaCl. At 300 mM NaCl, the growth of inoculated plants decreased, indicating that salinity tolerance will plateau after a certain salinity

concentration. As a conclusion this study showed the viability of endophyte-mediated transferred resistance to abiotic stresses.

3.2 Systematics of endophytes

Systematic studies regarding the microbial composition of halophyte's endosphere remain scarce. However, metagenomic diversity analysis performed by Mora-Ruiz et al. [80] elucidated the endophytic composition of various halophytes of the subfamily *Salicornioideae*, originated from Chile and Spain. Representatives of the genera *Chromohalobacter, Salinicola, Halomonas, Kushneria, Marinococcus, Halobacillus, Staphylococcus* and *Brevibacterium* were observed as part of the microbiota of these plants [80]. A similar genera composition was reported from the halophyte *Arthrocnemum macrostachyum*, in the family Amaranthaceae. This plant presents physiological traits that allow growth in a wide range of salinities [62,81]. Additional genera were recorded in this study (*Rudaea, Psychrobacter, Pseudomonas, Marinobacter, Marinimonas, Vibrio, Enterobacter, Burkholderia, Alkalibacillus, Rhodovibrio* and *Streptococcus*) suggesting that certain communities are shared among various halophytes [81]. In contrast to previous research, Mora-Ruiz et al. [81] demonstrated the compartmentalization of microbiome of *Arthrocnemum macrostachyum*. This suggests that the relative abundance of each represented genus varies according to their location within the plant. The evolution of this plant-microbe site-specific interaction should be further explored since it may reveal biotechnological applications as well as conservation insights for the plant. In this study, it was also elucidated how the microflora associated with the internal tissues of the plant increased abiotic-stress tolerance towards salt, allowing the normal internal processes to occur [63].

Objectives

1. To assess the diversity of halotolerant and halophilic prokaryotes within *Avicennia germinans* by culture dependent techniques.
2. Determine if there are differences in community composition among tree strata, and between saline and hypersaline forests.
3. Provide a systematic metagenomic survey of black mangrove's prokaryotic endophytes in saline and hypersaline environments.

CHAPTER 1: DETERMINING THE BIODIVERSITY OF PROKARYOTIC ENDOPHYTES OF *AVICENNIA GERMINANS* USING CULTURE-DEPENDENT TECHNIQUES

Specific Aim

To isolate, categorize and quantify the culturable halotolerant/halophilic prokaryotes within *Avicennia germinans*, and to assess differences in culturable diversity in mangrove forests due to proximity to aquatic systems with variable salinity.

Methodology

Description of Sampling Sites

To assess the culturable variability in the diversity of halotolerant and halophilic prokaryotic endophytes of *Avicennia germinans* due to the proximity of the trees to saline and hypersaline ponds, the National Wildlife Refuge at Cabo Rojo (Figure 1) was selected as sampling location. Two mangrove forests were identified based on similar radiation, precipitation, and temperature parameters; nevertheless, the water systems proximal to the forests differed. The first sampling site (HS) was located near the crystallizer ponds (average NaCl content: 37% (w/v)) of the Solar Salterns of Cabo Rojo (coordinates: 17.9520001, -67.1958519), whereas the second sampling site (Sal) was located near a seawater-fed pond (average NaCl content: 3.5% w/v) (coordinates: 17.9538635, -67.2004355) (Figure 2).

Figure 2: Aerial view of sampling locations in National Wildlife Refuge at Cabo Rojo, Puerto Rico. The black dot is representative of the mangrove forest located near crystallizer ponds at the Solar Salterns (HS), whereas the red dot is indicative of the forest adjacent to the lagoon fed by seawater (Sal).

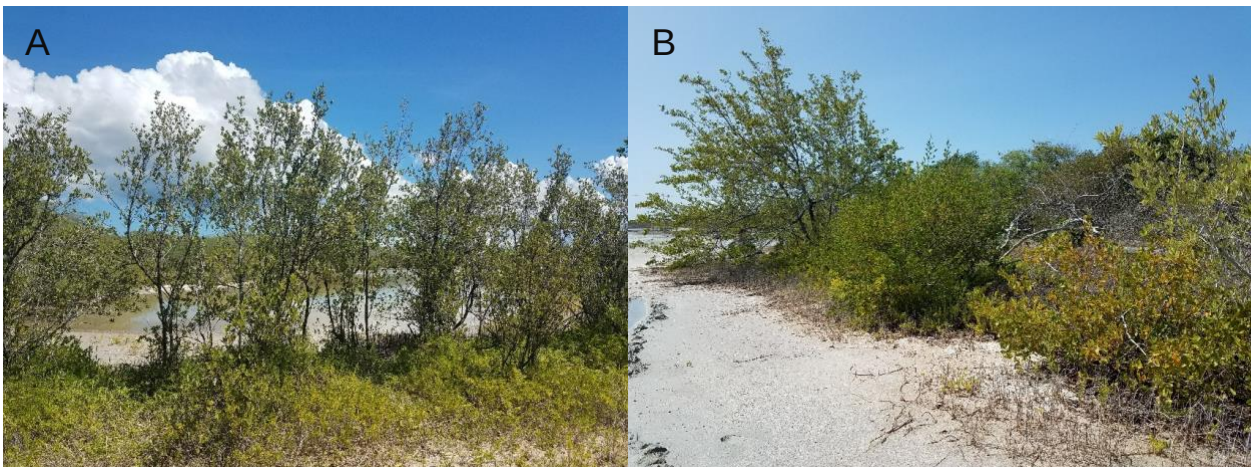

Figure 3: Black mangrove forest patches used near seawater-fed lagoon (Panel A) and crystallizer ponds at the Solar Saltern systems (Panel B), located at the National Wildlife Refuge at Cabo Rojo, Puerto Rico.

Samples Collection and Processing

Three trees were selected per sampling site for leaf collection. Sun/shade exposure, height (3.0-3.5 m) and average distance (1.5 m) between each tree were remained constant in both sampling regions. Each tree was measured and divided into 3 equivalent strata (bottom, center, and top), from which 9 leaves from the tip of the branch were selected. Samples collected from each section of the tree were stored in sterile Whirl-Pak® bags and transported to the laboratory, where surface sterilization was conducted as indicated by Couto [33]. Sample collection was conducted twice per sampling site (HS: May 2016 and September 2017; Sal: July 2016 and September 2017).

Culture, Isolation, and Purification

Sterile leaves were sectioned using a sterile hole puncher, and the petiole was cut longitudinally. The obtained leaf disks and petiole fragments were placed in three different solid Sehgal-Gibbons (SG) culture medium having three different NaCl concentrations (10%, 15% and 20% w/v), with three replicates per culture medium (Figure 3A). The inoculated Petri dishes were incubated at 30°C until growth was observed. The SG culture medium was prepared according to Sehgal (1960), using 20g $MgSO_4 \cdot 7H_2O$, 3g trisodium citrate, 2g KCl, 2.3 µg/L $FeCl_2$, 10g yeast extract, 7.5g casamino acids and 20g agar per liter[82]. The NaCl content was adjusted to the desired final salt percentages. The pH was adjusted to 7.1-7.3 using 1M NaOH.

During incubation, growth was observed around the leaf disks and petiole fragments (Figure 3B). Different morphotypes were selected and transferred to SG medium. Gram staining for each isolate was performed, and morphology was determined.

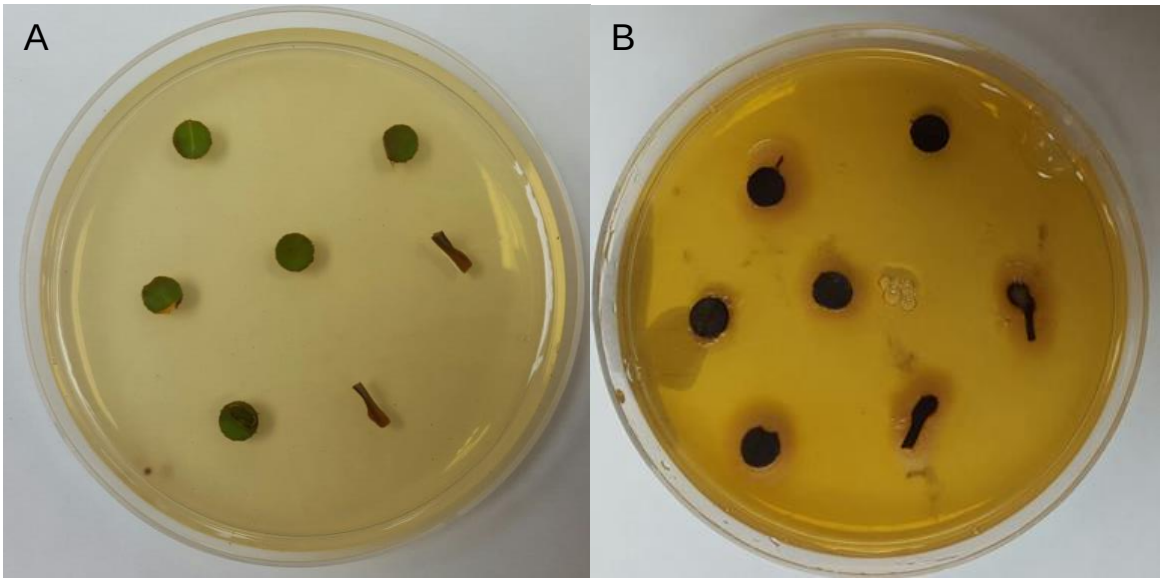

Figure 4: Sterile mangrove-leaf disks and petiole fragments placed in SG medium (**A**) prior incubation and (**B**) post-incubation at 30°C. Prokaryotic growth can be observed emerging from the edges of the disks, however, no growth is observed on top of the plant tissue.

Strain Cryopreservation

Pure cultures were transferred to 930µL of liquid medium, with the salinity percentage of the solid culture media the strain was originally isolated in. Then, 70µL of DMSO was added to prevent cell death due to freezing. The mixture was homogenized thoroughly and stored at -80°C until further use.

Genomic DNA Extraction Amplification of Molecular Marker: 16S rRNA gene

Extraction of genomic DNA was performed to each strain using physical and chemical methods. This was completed by lysing the cells with glass beads, lysis buffer (Tris-Acetate 40mM pH 8.0, Sodium Acetate 20mM pH 8.0, 1mM EDTA pH 8.0, SDS 1% w/v, sucrose 0.75M), lysozyme and potassium acetate. DNA was cleansed using phenol-chloroform, precipitated with isopropanol 100%, and ethanol 70%. Nucleic acids were resuspended using PCR-grade water and

treated with RNase. ThermoScientific® NanoDrop™ spectrophotometer was used to determine the DNA concentration and quality, as well as 0.8% (w/v) agarose gel.

PCR Product Purification and Sequencing

PCR amplifications of the molecular marker 16S rRNA gene were performed using specific primers for bacterial and archaeal 16S. The primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[83] were used to amplify bacterial 16S rRNA genes, whereas the primers 7F (5'-TTCCGGTTGATCCTGCCGGA-3')[84] and 927R (5'-CCCGCCAATTCCTTTAAGTTT-3')[85] were employed to target archaeal 16S rRNA genes. In the lack of amplification, the primers 519F (5'-CCGTCAATTCMTTRAGTTT-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') [86] were preferred. The PCR reactions were performed in a My Cycler™ thermocycler (BioRad) via the following conditions: 95°C – 5 min, (95°C – 1 min, 52°C – 1 min, 72°C – 3 min) x 25 cycles, 72 °C – 10min, 4°C. After 16S rRNA gene amplification, PCR products were purified by precipitation using isopropanol 100% and ethanol 70%, and resuspended with PCR-grade water. Amplicon concentration and quality were measured using NanoDrop™ spectrophotometer from ThermoScientific® and 1% (w/v) agarose gel. All amplicons were shipped for sequencing to Macrogen USA facilities.

Data Analysis

Phylogenetic analysis

The retrieved sequenced data was edited and concatenated in BioEdit[87] and uploaded to EZ-Taxon [88], where FASTA sequences of 16S rRNA gene from phylogenetically-relative strains were retrieved. Pairwise and multiple sequence alignments of each amplicon and closely related species were performed using ClustalW, and a final sequence edition was conducted in Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software [89]. Phylogenetic distances were generated with the neighbor-joining tree method [90] using p-distance models parameters. An outgroup was selected according to the analyzed genus.

Statistical Analysis

To determine presence and absence of different taxa among tree strata and between sampling sites a Jaccard index analyses were performed using a preferred richness index from culture-dependent procedures. Statistical analyses were conducted using the Paleontological Statistics Software (PAST). Venn Diagrams were created using R software and the *VennDiagram* package.

Results

The 16S rRNA analysis revealed the composition of halophilic and halotolerant endophytes in sampling site HS and Sal, as well as in each stratum. Table 1 shows the number of isolated strains based successfully identified as archaea and bacteria per sampling site and strata. In both HS and Sal sampling sites, a similar overall number of isolated strains were 87 and 80, respectively. Seventeen percent of the isolates from Sal samples belonged to the *Archaea* domain, whereas a smaller percentage of this domain (7.5%) was found in the HS samples.

Table 1: Comparison of quantity of sequenced strain per tree stratum in HS and Sal sampling sites.

Sampling Site	**Sequenced Strains Per Strata**						**Total**
	Superior		Center		Inferior		
	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	
HS	38	1	17	2	19	3	**80**
Sal	36	6	17	3	19	6	**87**

Sequencing and bioinformatics methods revealed that the isolated strains were closely related to the following: *Palleronia spp.* (Figure 5), *Kushneria spp.* (Figure 6), *Marinococcus spp.* (Figure 7), *Halobacillus spp.* (Figure 8), *Salinisphaera spp.* (Figure 9), *Bacillus spp.* (Figure 10), *Staphylococcus spp.* (Figure 11), *Pontibacillus spp.* (Figure 12) and *Halococcus spp.* (Figure 13). Of all 9 represented genera, only *Halococcus spp.* belongs to the *Archaea* domain. Phylogenetic trees were generated using the best curated sequences, and not every strain recovered from the genus is represented.

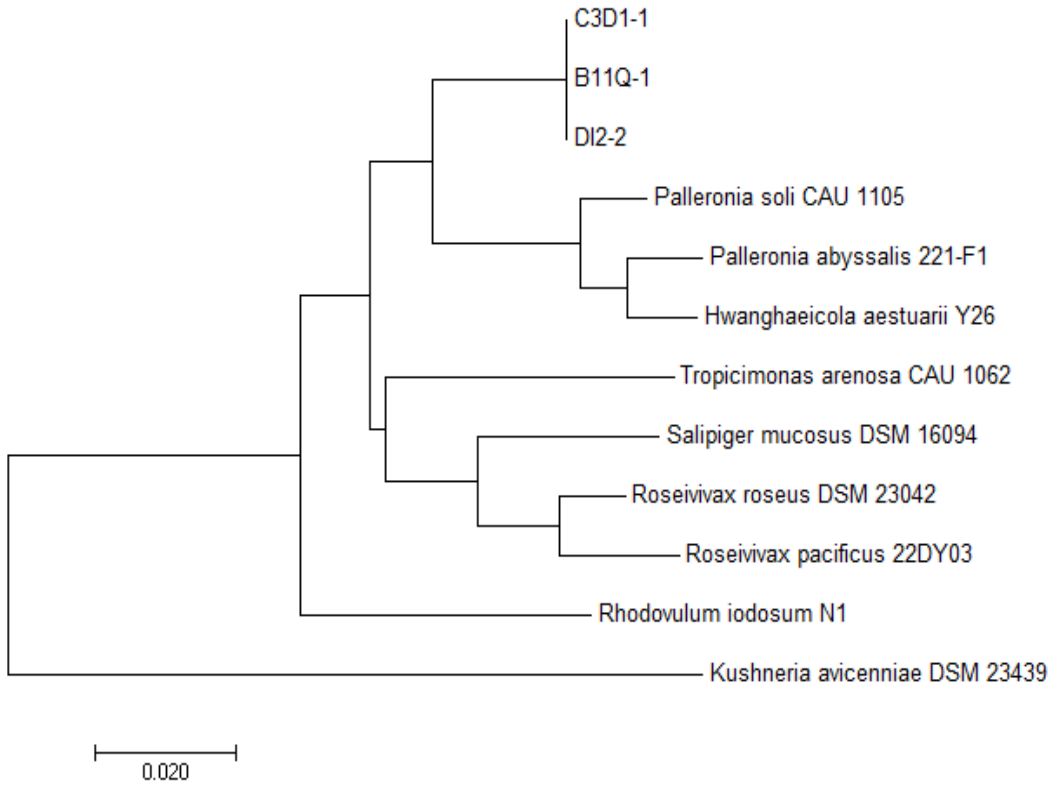

Figure 5: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates C3D1-1, B11Q-1, DI2-2 from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 2 substitutions per 10 nucleotides. *Kushneria avicenniae* DSM 23439 was used as an outgroup.

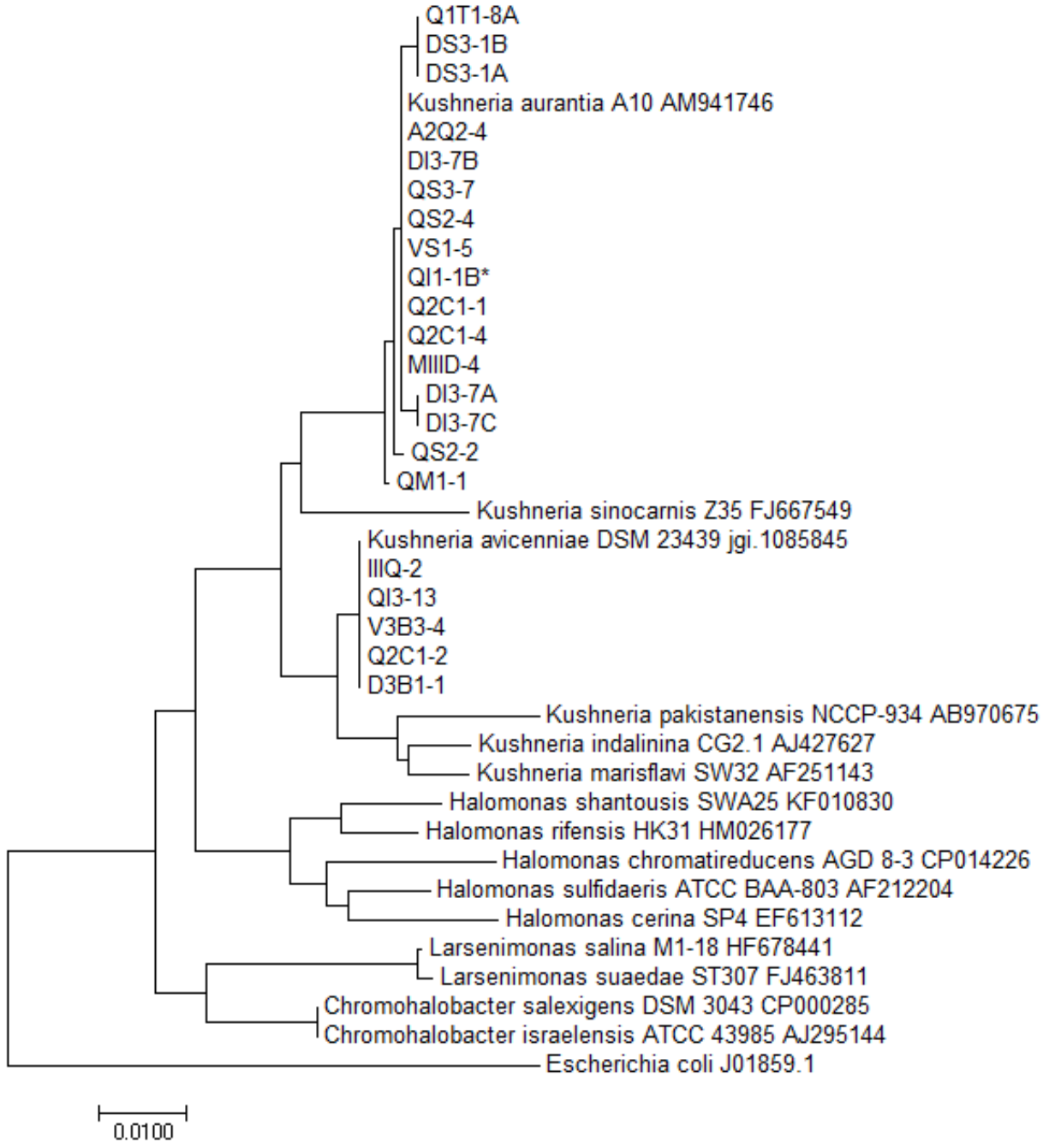

Figure 6: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (Q1T1-8A, DS3-1B, DS3-A, A2Q2-4, DI3-7B, QS3-7, QS2-4, VS1-5, QI1-1B*, Q2C1-1, Q2C1-4, MIIID4, DI3-7A, DI3-7C, QS2-2, QM1-1, IIIQ-1, QI3-13, V3B3-4, Q2C1-2, D2B1-1) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 1 substitution per 10 nucleotides. *Escherichia coli* J01859.1 was used as an outgroup.

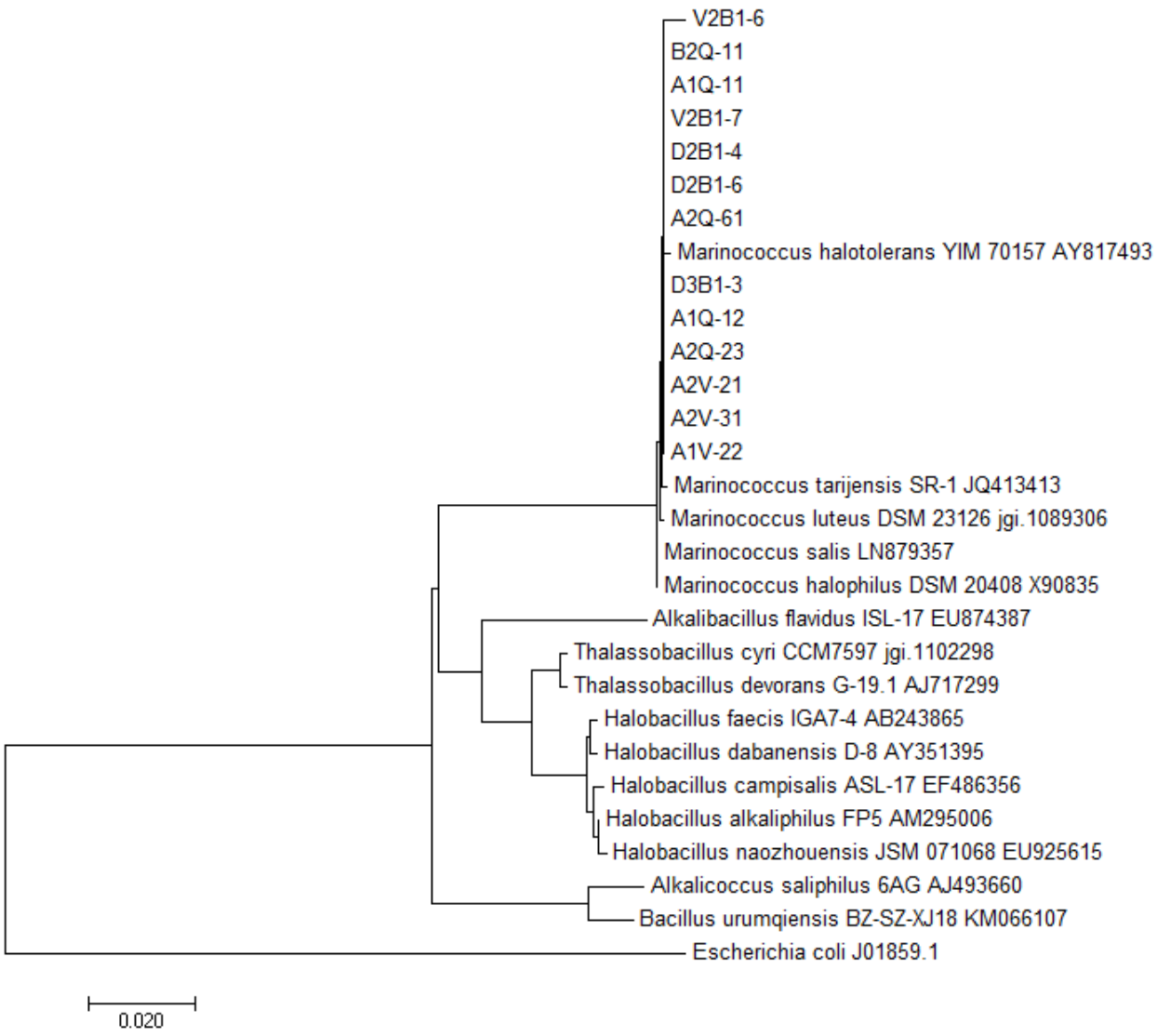

Figure 7: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (V2B1-6, B2Q-11, A1Q-11, V2B1-7, B2B1-4, A2Q-61, D3B1-3, A1Q-12, A2Q-23, A2V-21, A2V-31, A1V-22) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 2 substitutions per 10 nucleotides. *Escherichia coli* J01859.1 was used as an outgroup.

Figure 8: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (B1Q-11, Q1T1-6) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 2 substitutions per 10 nucleotides. *Escherichia coli* J01859.1 was used as an outgroup.

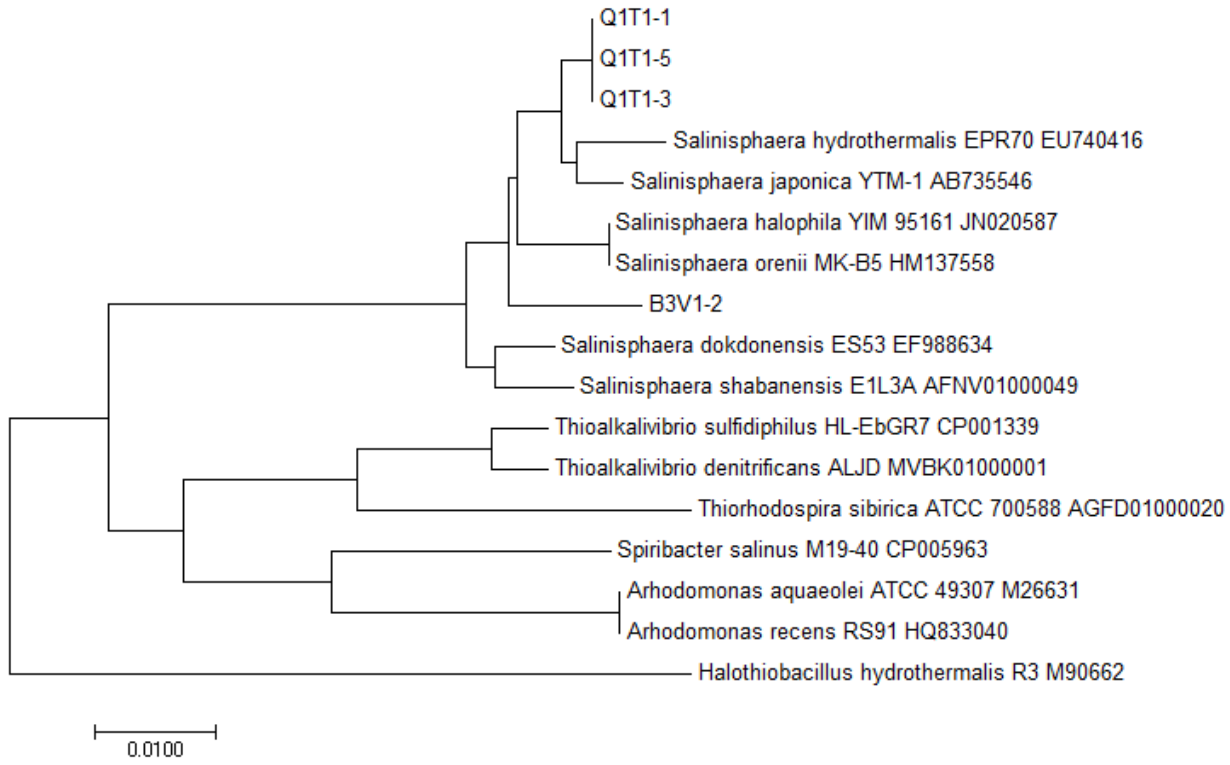

Figure 9: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (Q1T1-1, Q1T1-3, Q1T1-5, B3V1-2) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 1 substitution per 10 nucleotides. *Halothiobacillus hydrothermalis* R2 M90662 was used as an outgroup.

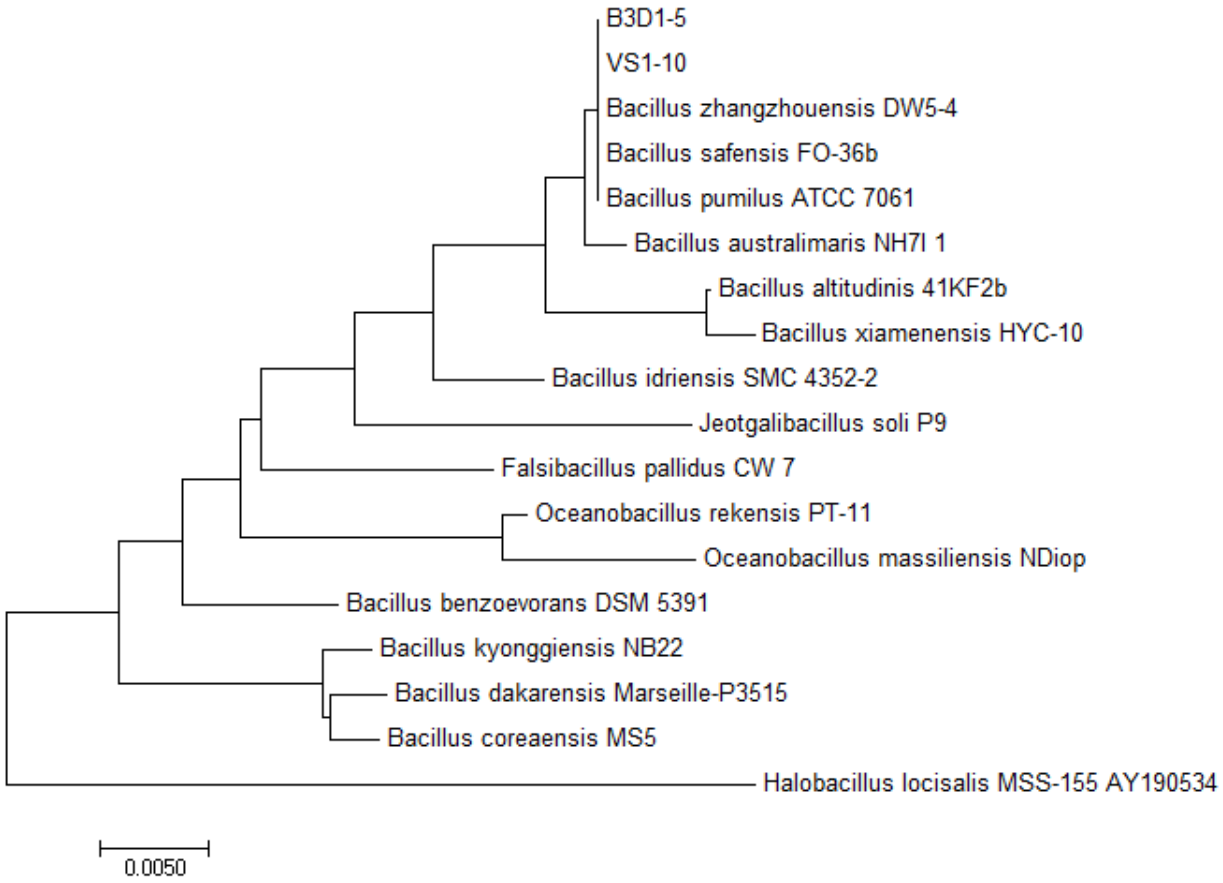

Figure 10: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (B3D1-5, VS1-10) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 5 substitutions per 100 nucleotides. *Halobacillus locisalis* MSS-155 was used as an outgroup.

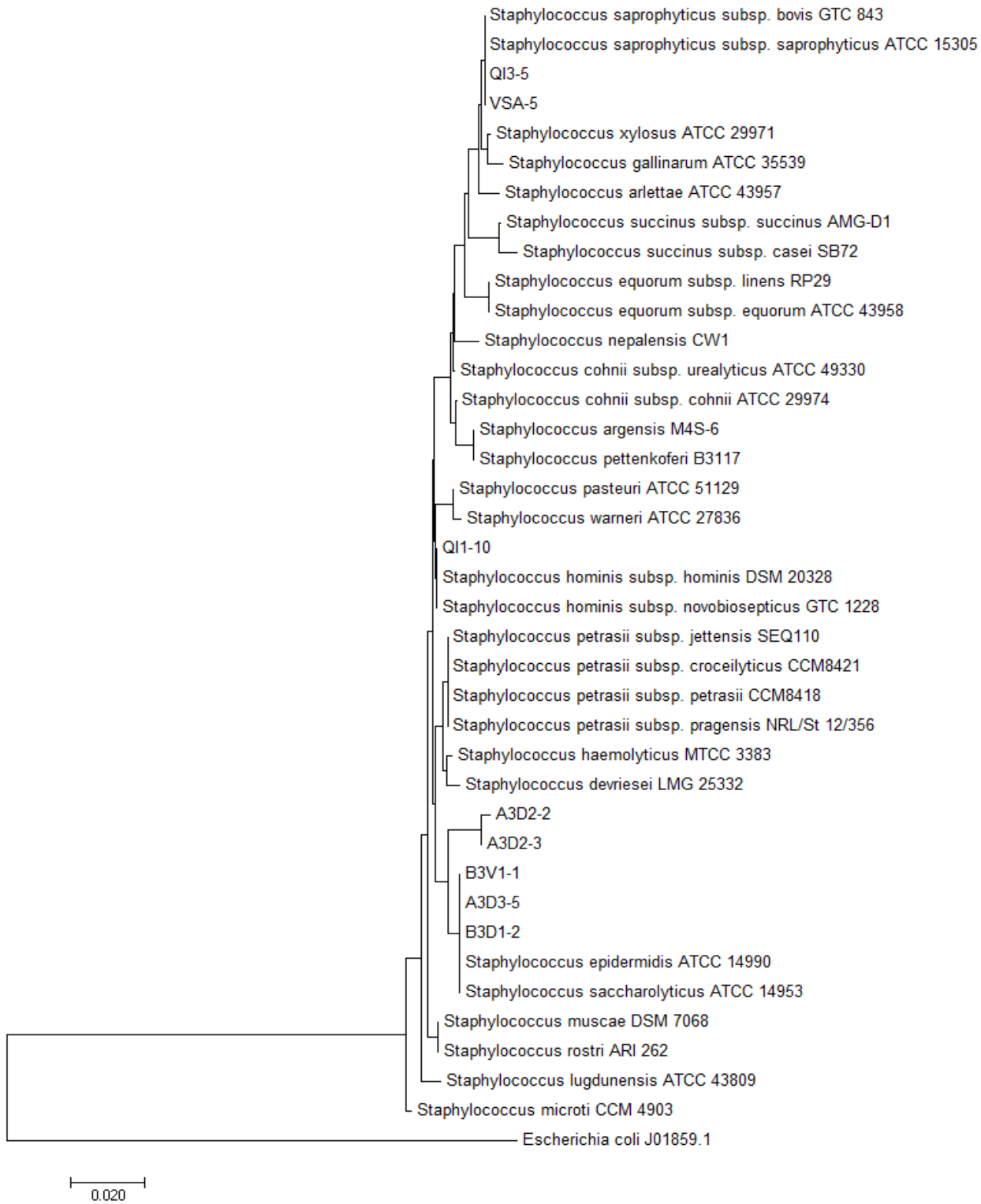

Figure 11: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected bacterial isolates (QI3-5, VSA-5, QI1-10, A3D2-2, A3D2-3, B3V1-1, A3D3-5, B3D1-2) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 2 substitutions per 10 nucleotides. *Escherichia coli* J01859.1 was used as an outgroup.

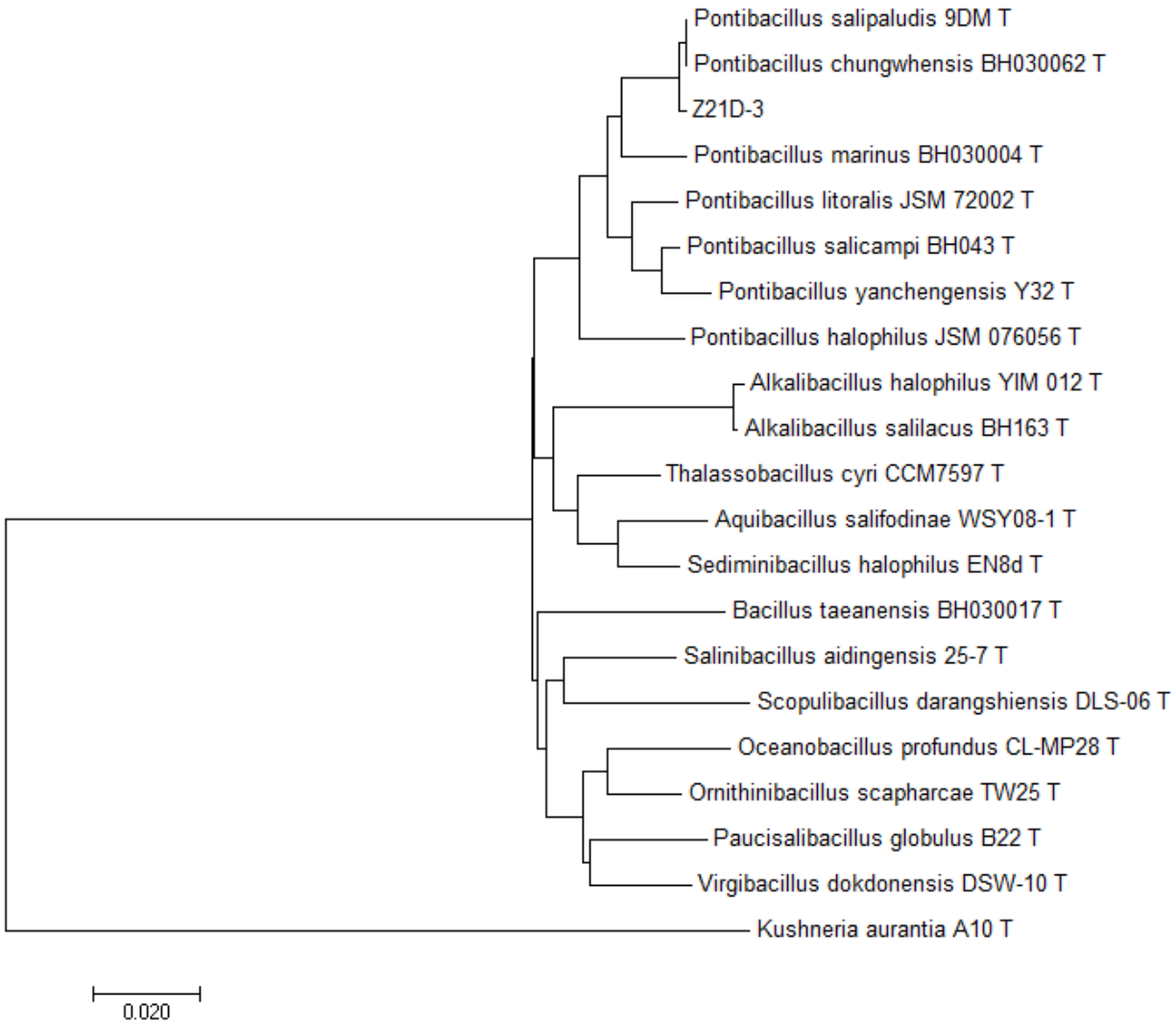

Figure 12: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of bacterial isolate Z21D-3 from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 2 substitutions per 10 nucleotides. *Kushneria aurantia* A10 T was used as an outgroup.

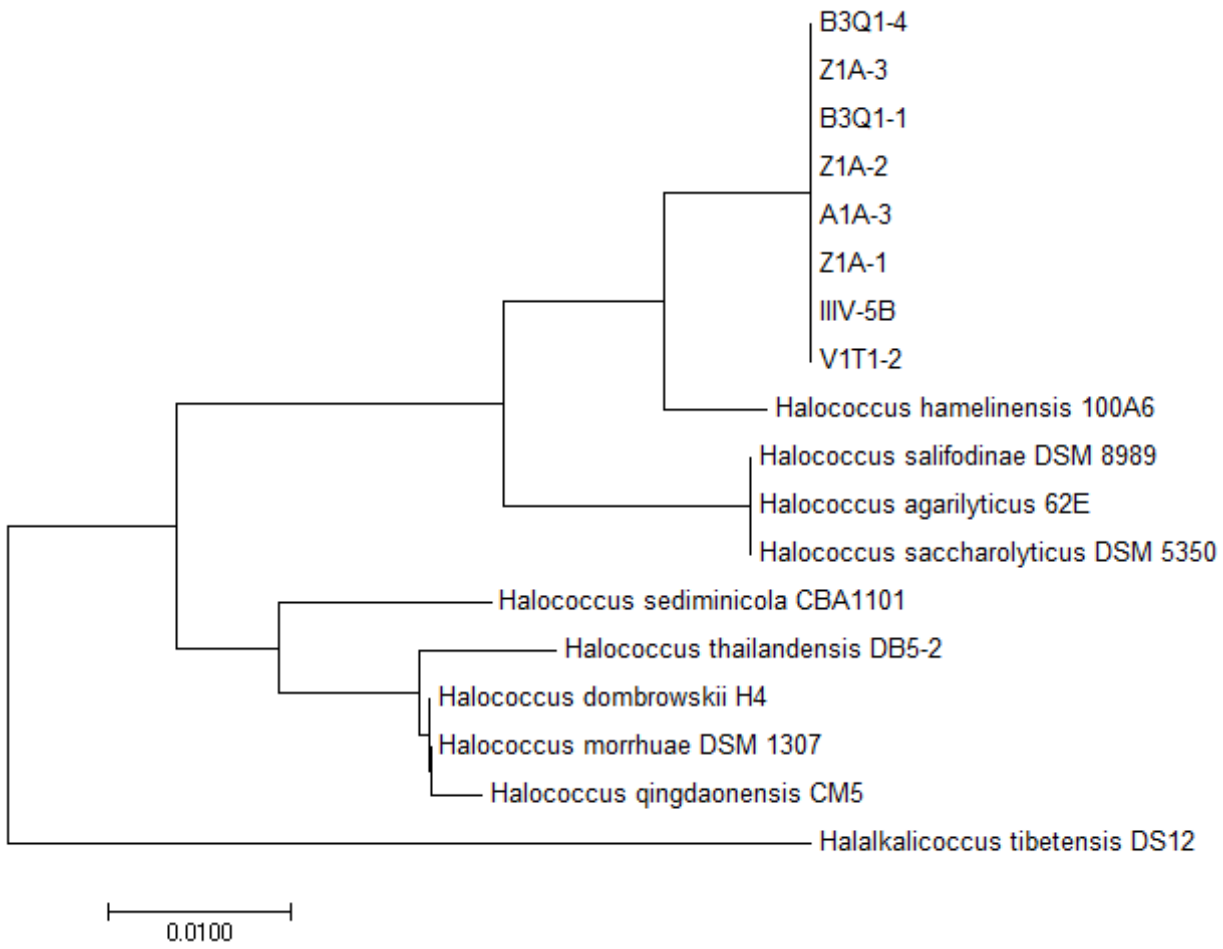

Figure 13: Phylogenetic analysis using the neighbor-joining method of partial 16S rRNA gene sequences of selected archaeal isolates (B3Q1-4, ZIA-3, B3Q1-1, Z1A-2, A1A-3, Z1A-1, IIIV-5B, V1T1-2) from *Avicennia germinans* leaf tissue and closely related strains. Bar represents 1 substitution per 10 nucleotides. *Halalkalicoccus tibetensis* DS12 was used as an outgroup.

Table 2: Number of strains and their closely related genera per strata in sampling sites HS and Sal.

Genera	HS			Total	Sal			Total
	Superior	Center	Inferior		Superior	Center	Inferior	
Staphylococcus	0	0	0	**0**	4	2	2	**8**
Bacillus	0	0	0	**0**	1	1	0	**2**
Halobacillus	5	1	0	**6**	2	0	0	**2**
Pontibacillus	0	0	1	**1**	0	0	0	**0**
Marinococcus	8	1	9	**18**	4	0	0	**4**
Kushneria	14	14	8	**36**	21	9	13	**43**
Salinisphaera	10	1	1	**12**	4	4	2	**10**
Palleronia	1	0	0	**1**	0	1	2	**3**
Halococcus	1	2	3	**6**	6	3	6	**15**

Table 2 shows the genera presence per sampling site, as well as per strata. The HS sample had representatives of the genera *Halobacillus, Pontibacillus, Marinococcus, Kushneria, Salinisphaera, Palleronia* and *Halococcus.* Sal isolates also were categorized in the genera *Halobacillus, Marinococcus, Kushneria, Salinisphaera, Palleronia* and *Halococcus;* however, no *Pontibacillus* representative was isolated from the Sal samples. The presence of *Staphylococcus spp.* and *Bacillus spp.* was also recorded in Sal sampling site (Figure 14). In both regions, *Kushneria spp.* showed the highest frequency of isolation, with 36 strains in HS (45%) and 43 isolates in Sal (49%). Considering only the different isolates per sampling site, the calculated Jaccard index between the HS and Sal sampling sites had a shared frequency of ~0.67.

Table 3: Jaccard index analysis of culture-dependent samples, comparing the genera presence in both HS and Sal sampling sites.

	SAL
HS	0.66666667

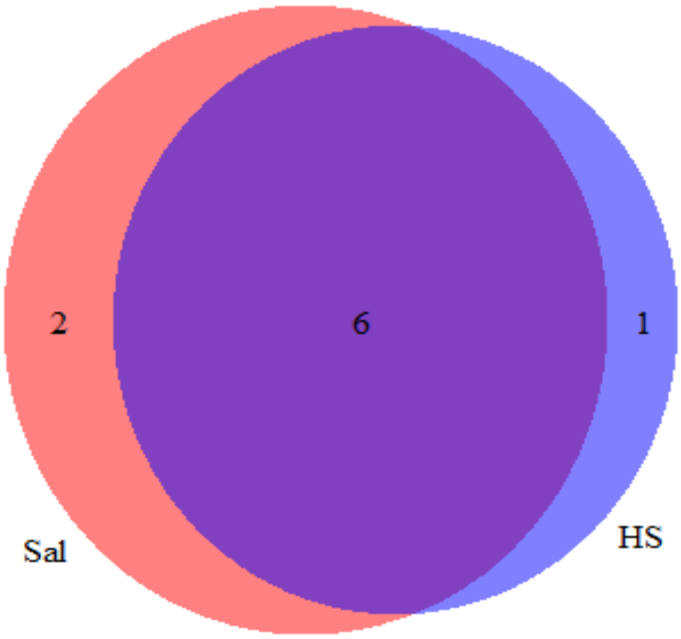

Figure 14: Venn diagram of total isolated and shared genera between samples from HS and Sal collection sites.

Stratum variances were also tested focusing on isolated genera. In HS samples, as represented in Figure 15, a total of 6 genera (*Halobacillus*, *Marinococcus*, *Kushneria.*, *Salinisphaera.*, *Palleronia* and *Halococcus.)* were isolated from the superior section of the tree. Similar to the superior stratum, *Halobacillus spp.*, *Marinococcus spp.*, *Kushneria spp.*, *Salinisphaera spp.*, *Palleronia spp.* and *Halococcus spp.* were isolated from the central area; however, the genera *Palleronia spp.*was not recovered from this region. The inferior region of the tree also shared the genera *Marinococcus*, *Kushneria*, *Salinisphaera* and *Halococcus.* Furthermore, the genera *Pontibacillus* was also isolated from the lower stratum. Table 4 includes the Jaccard indexes of the comparisons among strata in the HS samples. The superior and center stratum of the mangroves presented frequency of shared genera of ~0.83, while the superior and

inferior regions showed an index of ~0.57. Furthermore, the Jaccard index calculated for the center and inferior regions was ~0.67.

Table 4: Jaccard index of similarity of culture-dependent samples recovered from different strata in the HS sampling site.

	SUPERIOR	CENTER	INFERIOR
SUPERIOR	1	0.83	0.57
CENTER	0.83	1	0.67
INFERIOR	0.57	0.67	1

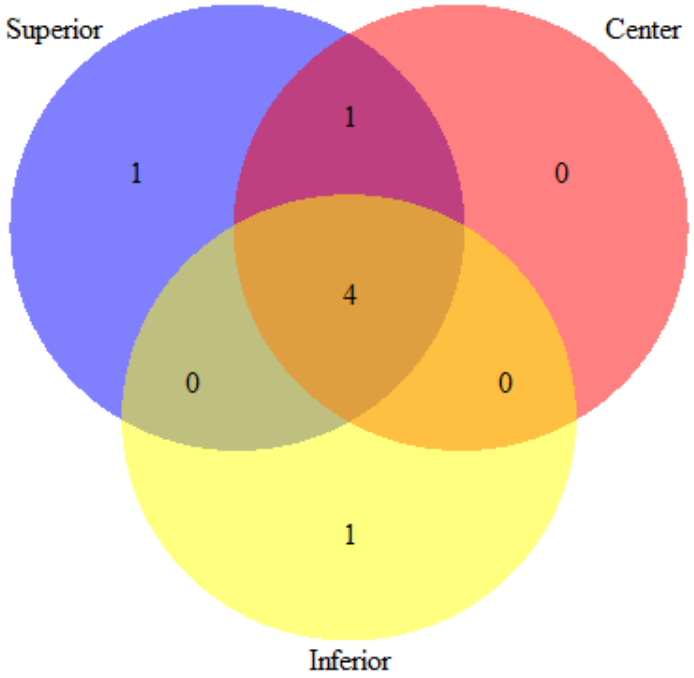

Figure 15: Venn diagram of isolated and shared genera within each stratum in HS sampling site.

In Sal samples, the highest number of isolates (42) were recovered from the superior stratum of the tree (Table 1). These included *Staphylococcus spp., Bacillus spp., Halobacillus spp., Marinococcus spp., Kushneria spp., Salinisphaera spp.,* and *Halococcus spp.* (Table 2). On the central region of the tree, 20 strains were identified and related to *Staphylococcus spp., Bacillus sp., Kushneria spp., Salinisphaera spp., Palleronia spp.,* and *Halococcus spp.*. Finally, on the

lower strata, the genera were similar to those of the central region of the tree, excluding *Bacillus spp.* Even though the superior region presented 2 unique genera, the remaining taxa identified were shared between strata (Figure 16). The calculated Jaccard indexes based on taxon presence per stratum shown in Table 6 indicate a frequency of ~0.63 of shared genera in superior and center stratum, 0.5 between the superior and inferior regions and ~0.83 between the center and inferior areas of the mangroves.

Table 5: Jaccard index of similarity of culture-dependent samples recovered from different strata in the Sal sampling site.

	SUPERIOR	**CENTER**	**INFERIOR**
SUPERIOR	1	0.63	0.5
CENTER	0.63	1	0.83
INFERIOR	0.5	0.83	1

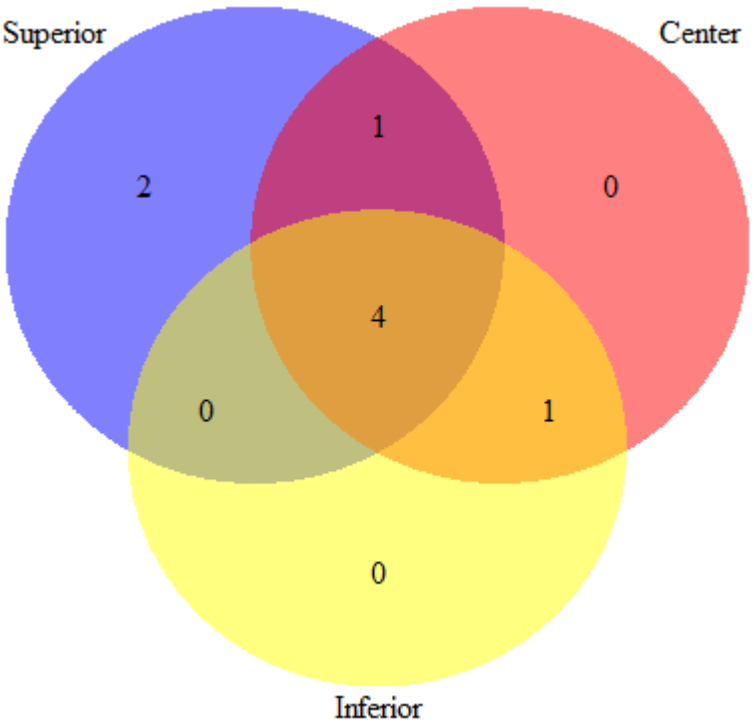

Figure 16: Venn diagram of isolated and shared genera within each stratum in Sal sampling site.

Discussion

Plants are part of the soil-plant-atmosphere continuum, which creates an intrinsic relationship between each component for an ultimate balance [91]. In the context of this continuum, plant associated microbiota will shift as an effect of an environmental or plant related disturbances. This concept fits the microbial composition of black mangroves because it is expected to have differences in culturable microbiota based on the changes in the soil. In this case, soil changes come as a result of the water source to which each forest is near. For the HS sampling site, these water sources are the crystallizer ponds of the Solar Salterns (Figure 2). On the other hand, the water source for the Sal sampling site is a naturally occurring pond fed by seawater (Figure 2).

From these sampling sites a total of 167 strains were isolated, purified and identified by phylogenetic analysis (Table 1). A similar number of isolates per sampling site was collected and identified (HS: 80; Sal: 87), and a higher percentage of those isolates belonged to the *Bacteria* domain (HS: 92.5%; Sal: 83.7%). Within each sampling site, the region from which a higher number of strains were isolated was the superior strata. Similar number of strains were collected from both center and inferior strata in both sampling sites. Other studies using culture-dependent techniques to assess the culturable diversity of endophytes have only been able to isolate bacterial strains [63,80,81]. However, the strain count values are not to be taken as significative since culture-dependent procedures are not able to represent all the present community and can be biased towards the organisms that grow optimally in the medium used [92]. Nevertheless, it culturable diversity is important in the study of the metabolic potential of endophytic organisms.

Figure 5 shows strains C3D1-1, B11Q-1, DI2-2 and their closest relatives in the genus *Palleronia*. This genus was first isolated from saline soil in 2005 [93] and currently consists of two species: *Palleronia abyssalis* [94] and *Palleronia soli* [95]. Even though *Palleronia spp.* seem to be the closest relatives, the error bar presents a difference of ~0.04, which translates to an ~96% homology. The accepted cut-off value for genus identification is >97% homology. Recent studies have argued for the use of 16S rRNA for classification up to the species level, using a cutoff of 98.56% homology[96]. The results acquired from the latter require other steps for corroboration, including whole genome sequencing and average nucleotide identity or DNA-DNA hybridization [96–98]. These values indicate that the isolated strains are sufficiently variable genetically to their closest relatives, thus we propose these strains might belong to a new genus. With this in mind, these strains have to be studied further for proper classification.

Figure 6 shows the phylogeny of the strains Q1T1-8A, DS3-1B, DS3-A, A2Q2-4, DI3-7B, QS3-7, QS2-4, VS1-5, QI1-1B*, Q2C1-1, Q2C1-4, MIIID4, DI3-7A, DI3-7C, QS2-2, QM1-1, IIIQ-1, QI3-13, V3B3-4, Q2C1-2, and D2B1-1 that are clustered in the genus *Kushneria*. These strains form two clusters, one with *Kushneria aurantia* and another with *Kushneria avicenniae.* Virtually, no difference between these strains in each cluster with its closest relative can be seen, suggesting that these are the same species. This genus, initially part of the *Halomonas* genera, consists of 7 species, including *K. aurantia, K. avicenniae, K. indalinina, K. marisflavi, K. pakistanensis,* and *K. sinocarnis.* Interestingly, these two species *K. aurantia* and *K. avicenniae* were discovered as epiphytic isolates from *Avicennia germinans* in the Solar Salterns of Cabo Rojo [23,24]. This raises the question: are the *Kushneria spp.* present in the black mangrove transported to the surface of the leaf? Other plant diversity studies focusing on the halophytes *Arthrocnemum*

macrostachyum and orther members of the *Salicornioidae* have identified this organism as a regular member of the plant microbiome [80,81].

Phylogenetic analysis of V2B1-6, B2Q-11, A1Q-11, V2B1-7, B2B1-4, A2Q-61, D3B1-3, A1Q-12, A2Q-23, A2V-21, A2V-31, and A1V-22 clusters these strains within the genus *Marinococcus* (Figure 7). Similar to Figure 6, in Figure 7 no differences between the strains and close relatives are shown, suggesting that none of the isolates is a new species within the genus. This genus consists of 5 different species: *Marinococcus halotolerans, M. tarijensis, M. luteus, M. salis*, and *M. halophilus.* This genus was first proposed in the 1984, where two species were described (*M. albus* and *M. halophilus*) [99]. Currently the genus is emended, as *M. albus* was relocated to another genus. Usual isolation sites for these organisms include salt marshes, salt mines and salt lakes [100–103]. Similar studies, regarding the microbial composition of halophytes, have also identified *Marinococcus spp.* as part of their microbiota [22,80,81].

Strains B1Q-11 and Q1T1-6, clustered within the genus *Halobacillus*, show differences in percentage of identity in the 16S rRNA gene sequence due to their placement on the phylogenetic tree (Figure 8). The genus *Halobacillus* is composed by 21 different species, one of which was also first reported as an epiphyte of the black mangrove in Cabo Rojo [11], and was also detected in the rhizosphere of *Avicennia germinans* [22]. Studies have reported the plant growth-promoting (PGP) capabilities of this genus [65], suggesting that these organisms partake in the salinity tolerance and growth of black mangrove.

Figure 9 shows the phylogenetic distribution of strains Q1T1-1, Q1T1-3, Q1T1-5 and B3V1-2. These cluster correctly within the genus *Salinisphaera;*. however, differences can be observed. The strain B3V1-2 presents a 0.015 divergence with respect to its closest relative, suggesting this could be a new species within the genus. Longer sequences for 16S rRNA gene

indicate that Q1T1-3 and Q1T1-5 have lower homology with their closest relative, but 100% homology between them. This can result in the discovery of another species of *Salinisphaera.* This genus contains 8 identified species, including *Salinisphaera hydrothermalis, S. japonica, S. halophila, S. orenii, S. dokdonensis,* and *S. shabanensis.* All species have been isolated from saline environments, including saline water-systems, saline sediment and the surface of fishes [104–109]. Studies have associated this organism to halophytes as well [80].

The strains B3D1-5 and VS1-10 were classified within the genus *Bacillus* (Figure 10). A small representation of the closely related species was selected, since *Bacillus* contains over 200 species. This is one of the highly reported organisms in association with plants and has been reported numerously as a plant-growth promoting bacteria (PGP) [67,75,110,111]. The closest relative to the isolated strains is *Bacillus zhangzhouensis*, a species isolated in the 2016 from shrimp-aquaculture waters [112]. Low to no evolutionary distances were observed from the closely related strains to the isolates B3D1-5 and VS1-10.

Figure 11 shows the strains QI3-5, VSA-5, QI1-10, A3D2-2, A3D2-3, B3V1-1, A3D3-5 and B3D1-2 clustered within the genus *Staphylococcu*s. The strains are distributed across the tree, indicating differences between strains, but no new species were detected. *Staphylococcus* is a genus that, similarly to *Bacillus spp.*, includes a wide variety of species. This genera has also been found in endophytic relationships with plants, like *Corchorus olitorus* [113]. In this plant, *Staphylococcus* expresses an ACC deaminase for the production of IAA. Furthermore, this genus was also identified as a part of the surface microbiome of black mangroves in past studies [6], suggesting that it might play an important role in the production of phytohormones for plant development.

Pontibacillus was the genus in which the strain Z21D-3 was clustered, but few differences between the strain and its closest relative were observed (Figure 12). This genus contains 7 species, including *Pontibacillus salipaludis, P. chungwhensis, P. marinus, P. litoralis, P. salicampi, P. yanchengensis* and *P. halophilus.* Studies regarding both the ecto-microbiome and rhizo-microbiome of black mangroves have detected this genus as a frequent member of the microbiota [6,22]. Even though it has been described as part of the microbiome of mangroves, in this study it was not easily isolated, showing the concept of culture bias clearly.

Figure 13 shows the phylogeny of strains B3Q1-4, ZIA-3, B3Q1-1, Z1A-2, A1A-3, Z1A-1, IIIV-5B and V1T1-2, and its closest relatives. These strains cluster within the genus *Halococcus*, a genus composed by *Halococcus hamelinensis, H. salifonidae, H. agarilyticus, H. saccharolyticus, H. sediminicola, H. dombrowskii, H. morrhuae* and *H. qingdaonensis.* This genus has been shown to have PGP properties, with exceptional performance in phosphate solubilization [73]. This suggests that this archaeon may have a role within the plant to maintain homeostasis and thrive in hostile environments. Significative differences in sequence homology were observed in the 16S rRNA gene of all of the isolates, suggesting a new species within this genus.

Based on the strain distribution in Table 2, a trend of isolation can be observed. The genera with the highest number of isolates include *Kushneria , Salinisphaera* and *Marinococcus* With the results in Table 2, the Jaccard's similarity index was calculated. This index formulates a proportion indicating the shared strains per location [114]. Table 3 shows that based on total identified genera, the similarity between HS and Sal locations was ~0.67. Of the nine overall-identified genera, six are shared between sampling sites, as shown in Figure 14. Possible explanations for these phenomena are: 1) a bias towards *Kushneria, Salinisphaera* and *Marinococcus* because of the

culture media; 2) actual higher abundances of these strains in the plant; 3) the presence and absence of species are based on the salinity stresses in the sample.

Using the Jaccard Index with the stratification data revealed that in the HS samples, the superior and central strata share 0.83 of their genera, the superior and inferior strata share 0.57, and the central and inferior share 0.67 (Table 4). This means that, of the 9 total identified genera, 4 are present in every stratum (*Marinococcus, Kushneria, Salinisphaera* and *Halococcus*), one is shared only between the superior and central strata (*Halobacillus*), and each of the superior and inferior include one unique genus (*Palleronia* and *Pontibacillus*, respectively) (Figure 15). Table 5 highlights the Jaccard indexes for shared genus among strata in Sal sampling sites, in which the plants have a different water source. The superior and center presented a proportion of 0.63 shared genera, whereas the superior and inferior shared only half of the genera. Inferior and central strata presented a proportion of 0.833, presenting the highest Jaccard index value. The visualization in Figure 16 demonstrates how, once again, 4 of the 9 identified genera are present in all strata (*Staphylococcus, Kushneria, Palleronia* and *Halococcus*). The superior and central strata, as well as the central and inferior strata share one genus (*Bacillus* and *Palleronia*, respectively), and the superior strata has more unique genera represented (*Halobacillus* and *Marinococcus*). These results suggest that the culturable biodiversity will similar in adjacent regions, whereas distant strata will differ.

The analysis of diversity in terms of plant compartmentalization provides us with insights regarding the ecological and functional roles of the microbial isolates. We could argue that an actual compartmentalization is occurring at low scales; for instance, in Sal samples the genera *Halobacillus spp*. was only isolated from the superior region of the mangrove, but in HS samples, the same genera was acquired from the superior and central strata. Nevertheless, the genera

Salinisphaera spp., Kushneria spp. and *Halococcus spp.* were ubiquitous in all stratum of mangroves in both sampling sites. There is a low basis of comparison because few articles have used culture dependent techniques to explain prokaryotic stratification in the plant. Mora et al. used compartmentalization to determine the plant microbiome in the rhizosphere, and internal structures, including the roots. Using metagenomics, the changes in abundance and maintenance of prokaryotic communities across the roots, stems and soil were observed in this study.

CHAPTER 2: DRAFT GENOME SEQUENCING OF STRAIN Q1T1-3, A NOVEL SPECIES OF THE GENUS *SALINISPHAERA*

Specific Aim

To conduct a small subunit rRNA gene-based identification and genomic description of Q1T1-3, a novel species within the *Salinisphaera* genus.

Methodology

Genomic DNA extraction

Cryopreserved Q1T1-3 strain was cultured in Sehgal-Gibbons medium containing NaCl 15% (w/v), prepared as previously described, and incubated at 30°C. After sufficient growth was observed, genomic DNA extraction was performed using the Promega™ Wizard™ Genomic DNA Purification Kit. DNA concentration and quality were determined using NanoDrop™ Spectrophotometer from ThermoScientific® and 0.8% (w/v) agarose gel.

Draft Genome Sequencing

Aiming to obtain a draft genome sequence of Q1T1-3, 100 µL of diluted DNA of the sample (100 ng/µL) was shipped for sequencing to MicrobesNG facilities (http://www.microbesng.uk) at Birmingham, UK. Illumina HiSeq platform, with a 250bp paired end protocol, was used to perform the genomic sequencing. At the facility, the adapters were trimmed using Trimmomatic 0.30 [115], quality was verified and contigs were annotated with Prokka 1.11[116].

Data analysis

Subsystem category distribution

Following sequencing, the retrieved genome was uploaded for Rapid Annotation using Subsystem Technology server (RAST) [117–119]. A subsystem-based classification of coding sequences was obtained from the server where putative function of coding sequences within the genome were inferred.

Phylogenetic divergence

To determine phylogenetic distance from other *Salinisphaera spp.*, the whole 16S rRNA gene was obtained from the RAST server and uploaded to EZ-Taxon [88], where taxonomically-close relatives were observed. FASTA sequences were downloaded, aligned using ClustalW, and edited in Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software [89]. Neighbor-joining tree method [90] and p-distance model were used to determine phylogenetic distances between Q1T1-3, closely related and distantly related (outgroup) strains.

Average Nucleotide Identity

Average Nucleotide Identity (ANI) of Q1T1-3 and its *Salinisphaera hydrothermalis* was performed to observe genetic difference between genomes. Using the Enveomics tool collection [120], both genomes were uploaded to the Average Nucleotide Identity calculator tool in FASTA format, and ANI was calculated.

Genomic Description of New Species

With the isolation and characterization of strain Q1T1-3 in the genus *Salinisphaera*, to further study this putative new species, a draft genome sequencing was performed at the MicrobesNG facilities (http://www.microbesng.uk). Genome sequencing of the strain can be retrieved using the accession number **PRJNA490533**. The obtained sequencing data was uploaded to the RAST server, and the complete 16S rRNA gene was downloaded and compared against multiple type strains available in the EZ-Taxon databases. This search retrieved a 97.40% homology of Q1T1-3 with *Salinisphaera hydrothermalis*^{T} EPR70 (Figure 17), a value lower than the cutoff value do differentiate species [97].

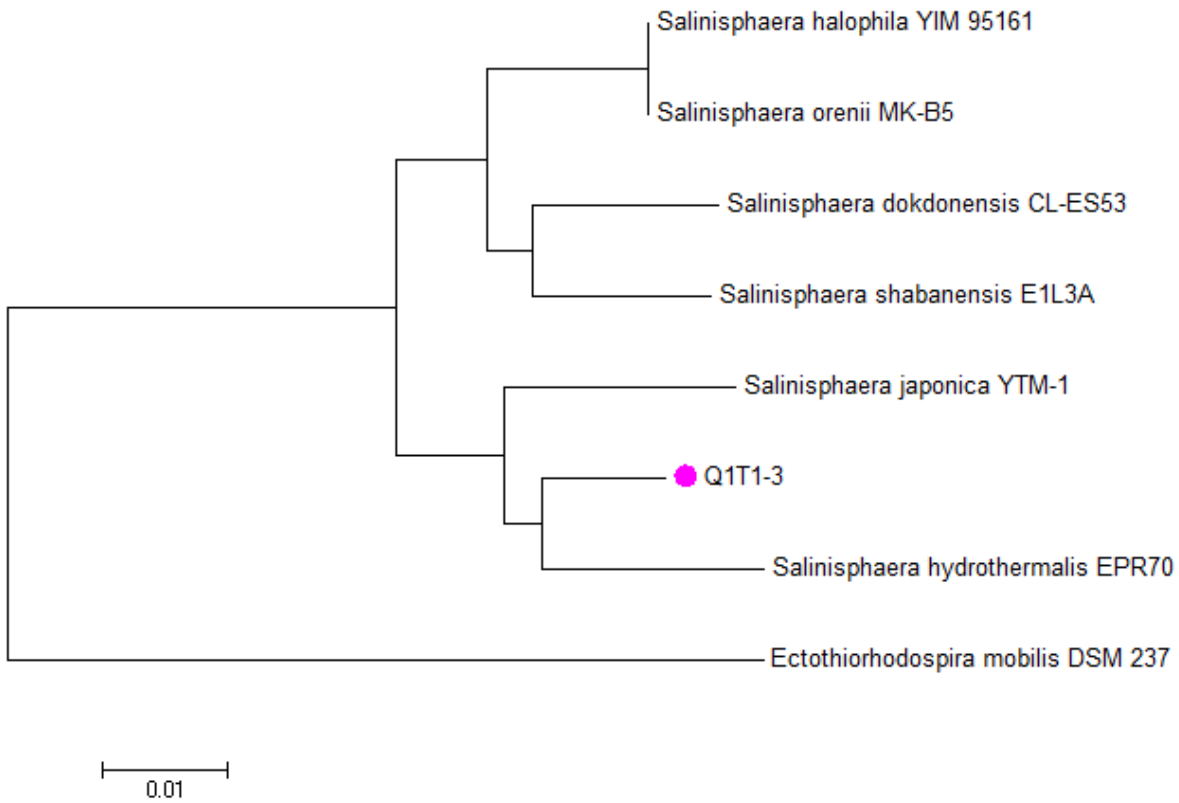

Figure 17: Evolutionary inference using Neighbor-Joining method based on full 16S rDNA gene sequence of strain Q1T1-3. Bar represents 1 substitution per every 10 nucleotides. *Ectothiorhodospira mobilis*^{T} DSM237 was used as an outgroup.

Table 6: Genomic features in the draft genome of Q1T1-3 strain isolated from HS sampling sites in Cabo Rojo, Puerto Rico.

Characteristic	
Assembly size (bp)	3,875,893
G+C content (%)	64.2
Number of contigs	64
Coding sequences	3539
Ribosomal RNA genes	2
Transfer RNA genes	50
CRISPR repeats	2

Based on the RAST annotation capabilities, the recovered draft genome had a size of 3.9 Mb size with a G+C content of 64.2% (Table 6). Within this genome, 2 CRISPR repeats, 2 rRNA genes and 50 tRNA genes were identified, as well as 3,539 coding sequences (cds). Using RAST functional classification, these putative genes were clustered in subsystems, as shown in Figure 18. A high number of cds from this genome are classified in the following subsystems: Carbohydrates (312 cds), Amino Acids and Derivatives (300 cds), Cofactors, Vitamins, Prosthetic Groups and Pigments (272 cds), Protein Metabolism (261 cds), Fatty Acids, Lipids and Isoprenoids (172 cds), RNA metabolism (141 cds), Stress Response (123 cds), DNA Metabolism (119 cds), Respiration (113 cds), and Cell Wall and Capsule (105 cds). Underrepresented subsystems include: Virulence, Disease and Defense (79 cds), Potassium Metabolism (20 cds), Photosynthesis (6 cds), Miscellaneous (49 cds), Phages, Prophages, Transposable Elements, Plasmids (1 cds), Membrane Transport (86 cds), Iron Acquisition and Metabolism (18 cds), Nucleosides and Nucleotides (87 cds), Cell Division and Cell Cycle (34 cds), Motility and Chemotaxis (60 cds), Regulation and Cell Signaling (42 cds), Secondary Metabolism (4 cds), Nitrogen Metabolism (20 cds), Metabolism of Aromatic Compounds (30 cds), Sulfur Metabolism (24 cds), and Phosphorus Metabolism (38 cds).

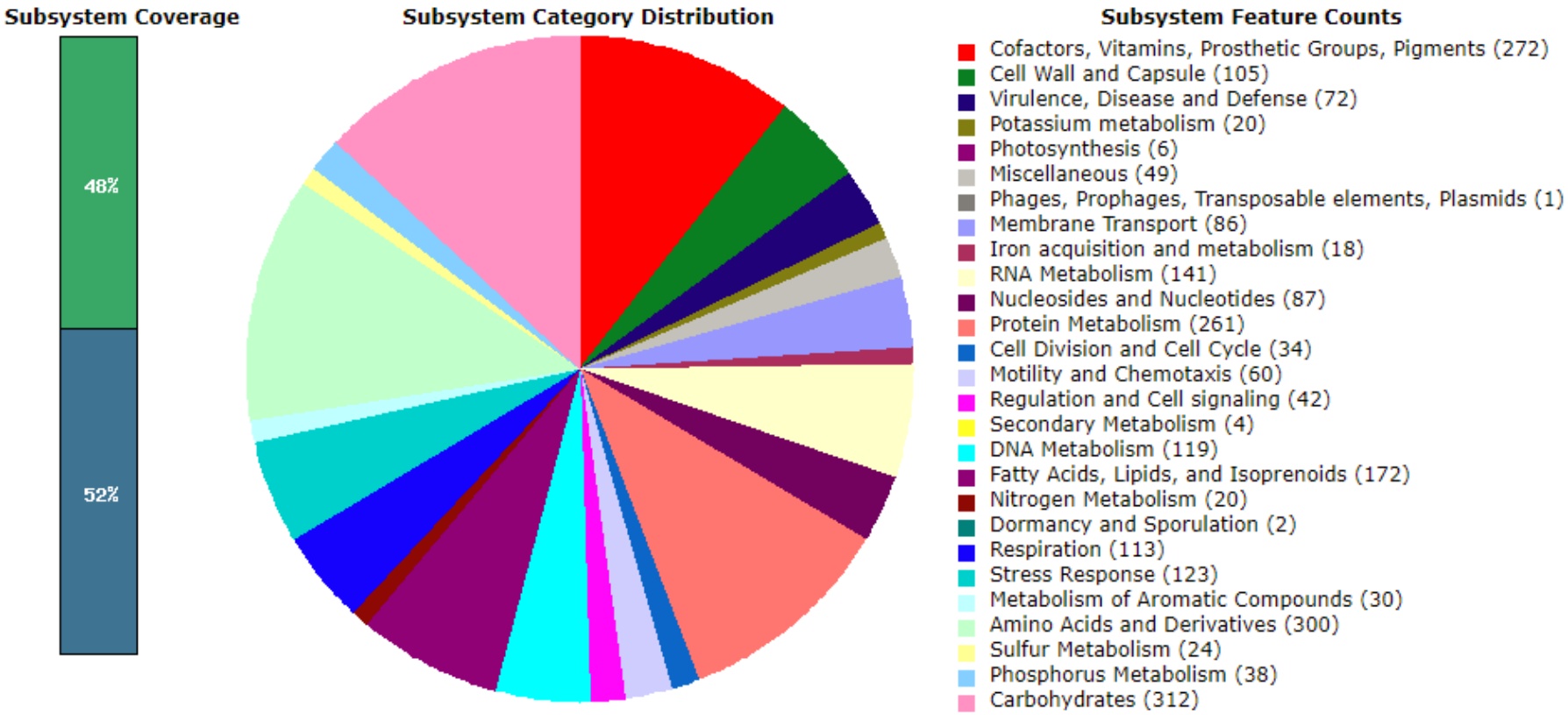

Figure 18: Subsystem category distribution of strain Q1T1-3 (*Salinisphaera endophytica*) retrieved from RAST server. Numbers in parentheses indicate associated proteins within each subsystem.

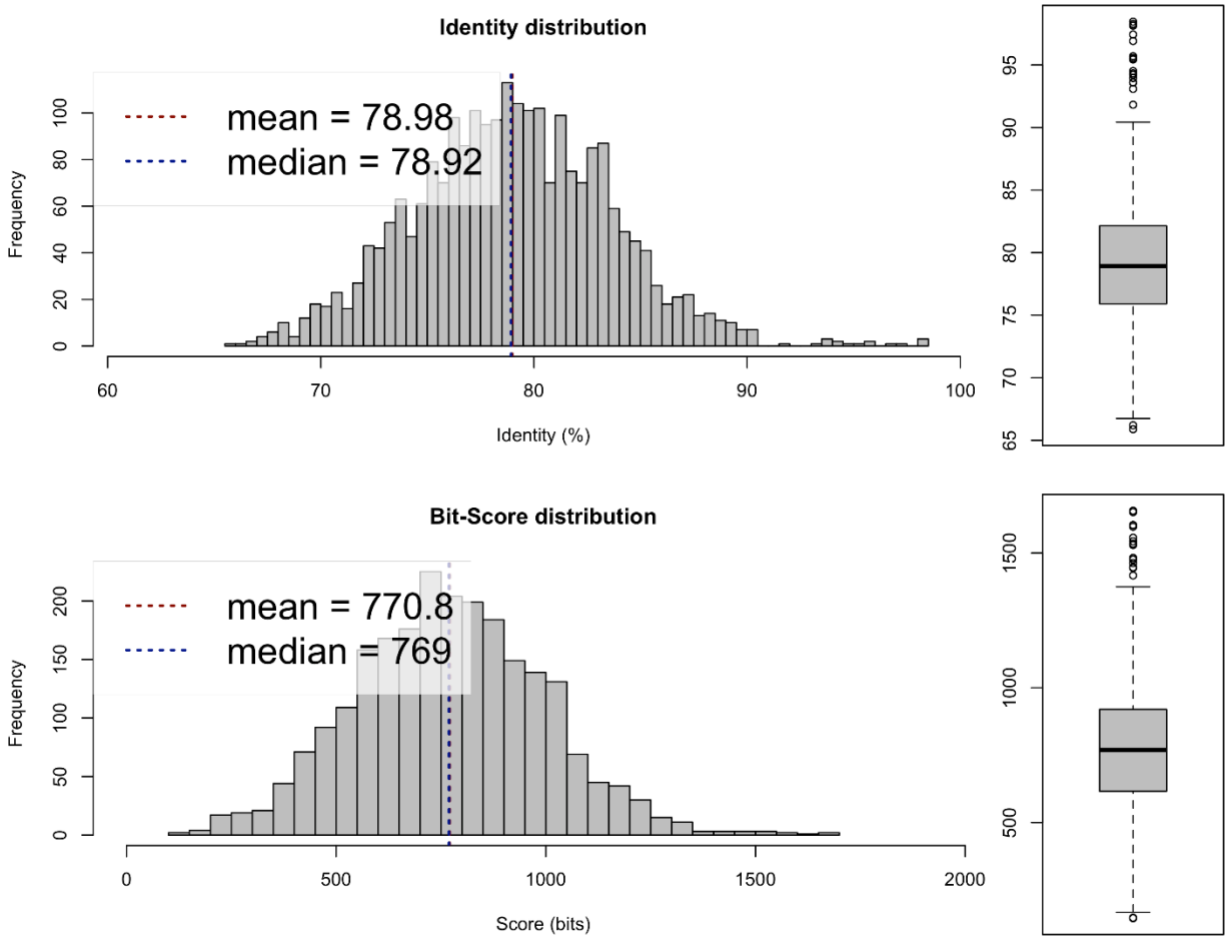

Figure 19: Identity distribution histogram of average nucleotide identity (ANI) of Q1T1-3 and *Salinisphaera hydrothermalis* ^{T} EPR70. The mean identity distribution between both genomes, obtained using the ANI calculator in the Enveomics toolset [120], retrieved a 78% nucleotide identity between genomes.

The ANI-calculator from the Enveomics tool collection [120] was used to determine the average nucleotide identity (ANI) of the strain Q1T1-3 and the closest related strain, *Salinisphaera hydrothermalis*. Therefore, based on the phylogenetic tree in Figure 19, the complete genome of *Salinisphaera hydrothermalis*^{T} EPR70 was used as basis of comparison. A 78.98% mean identity distribution is observed after comparing the nucleotide composition of both strains. The cutoff

value established for ANI to be used as a substitute of DNA-DNA hybridization is 95-96% [96], indicating that Q1T1-3 is, in fact, a new species.

This study proposes the etymology *Salinisphaera endophytica* for the new *Salinisphaera* species. *Salinisphaera endophytica* (en.do.phy'ti.ca. Gr. pref. endo- within; Gr. n. phyton plant; L. fem. suff. -ica adjectival suffix used with the sense of belonging to; N.L. fem. adj. endophytica within plant, endophytic).

CHAPTER 3: DRAFT GENOME SEQUENCING OF STRAIN IIIV-5B, A NOVEL SPECIES OF THE GENUS *HALOCOCCUS*

Specific Aim

To classify the strain IIIV-5B as novel species within the *Halococcus* genus using a genome-based approach.

Methodology

Genomic DNA extraction

Cryopreserved IIIV-5B strain was cultured in Sehgal-Gibbons media containing NaCl 15% (w/v), prepared as described in Chapter 1, and incubated at 30°C. After growth was observed, genomic DNA extraction was performed using the Promega™ Wizard™ Genomic DNA Purification Kit. DNA concentration and quality were determined using NanoDrop™ Spectrophotometer from ThermoScientific® and 0.8% (w/v) agarose gel.

Draft Genome Sequencing

Aiming to obtain a draft genome sequence of IIIV-5B, 100 µL of diluted DNA of the sample (100 ng/µL) was shipped for sequencing to MicrobesNG facilities (http://www.microbesng.uk) at Birmingham, UK. Illumina HiSeq platform, a 250bp paired end protocol was used to perform the genomic sequencing. At the facility, the adapters were trimmed

using Trimmomatic 0.30 [115], quality was verified and contigs were annotated with Prokka 1.11[116].

Data analysis

Subsystem category distribution

The sequenced genome was uploaded in the Rapid Annotation using Subsystem Technology server (RAST) [117–119]. A subsystem-based classification of coding sequences was obtained from the server where possible function of coding sequences within the genome was inferred.

Phylogenetic Tree Construction

To determine phylogenetic distance from other *Halococcus spp.*, the whole 16S rRNA gene was obtained from the RAST server and uploaded to EZ-Taxon [88], where taxonomically-close relatives were observed. FASTA sequences were downloaded, aligned using ClustalW, and edited in Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software [89]. Neighbor-joining tree method [90] was used to determine phylogenetic distances between IIIV-5B, closely related and distantly related (outgroup) strains.

Average Nucleotide Identity

Average Nucleotide Identity (ANI) of IIIV-5B and *Halococcus hamelinensis* was performed to observe genetic difference between genomes. Using the Enveomics tool collection [120], both genomes were uploaded to the Average Nucleotide Identity calculator tool in FASTA format, and ANI was calculated.

Genomic Description of New Species

Whole genome sequencing of strain IIIV-5B was performed in order to further investigate if this isolate is a new species of the genus *Halococcus*. After genomic DNA extraction, sequencing, and genome annotation, the complete 16S rRNA gene was obtained to classify the strain phylogenetically. A sequence of ~1400 bp was used to create a phylogenetic tree (Figure 20). This figure clusters the strain IIIV-5B (in red) in the genus *Halococcus*. A 98.06% of homology with the species *Halococcus hamelinensis*^{T} 100A6 was observed after comparison with the EZ-Taxon database [88]. This homology percentage was the homology percentage lower than the cutoff value for species differences [97], suggesting a new species.

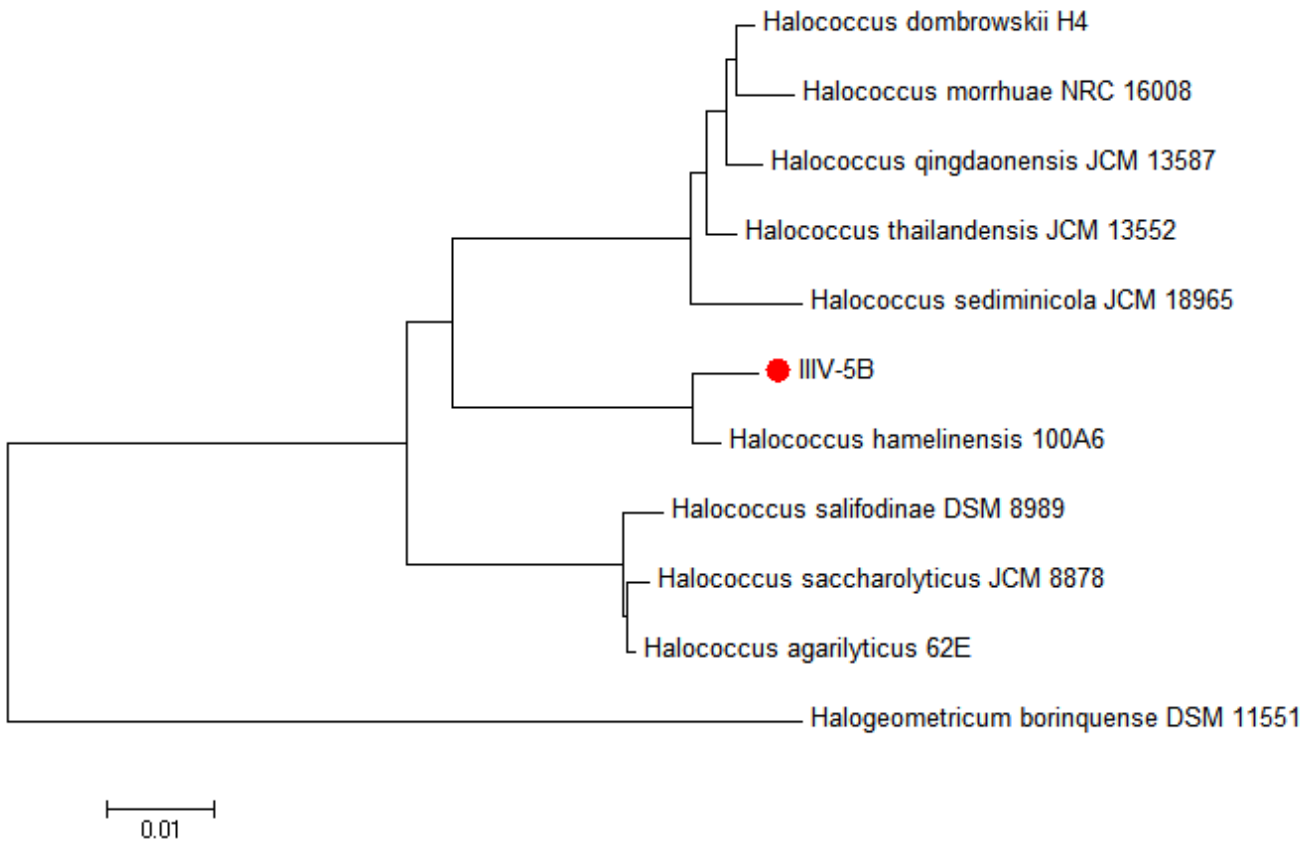

Figure 20: Evolutionary inference using Neighbor-Joining method based on full 16S rRNA gene sequence of IIIV-5B isolated from HS sampling sites in Cabo Rojo, Puerto Rico.

Table 7: Genomic features observed in the draft genome of IIIV-5B strain.

Characteristic	
Assembly size (bp)	3,877,752
G+C content (%)	63.9
Number of contigs	93
Coding sequences	3,812
Ribosomal RNA genes	3
Transfer RNA genes	46
CRISPR repeats	2

Within the genomic features of the strain IIIV-5B (table 7) a genome size of approximately 3.88 Mb and a G+C content of 63.9% are shown. This draft genome included 2 CRISPR repeats and around 3,812 coding sequences (cds), from which 3 are suggested to encode rRNAs and 46 encode tRNAs. Cds were grouped in the subsystems shown in Figure 21, retrieved from RAST server. Most coding sequences were classified in the subsystems related to: Amino Acids and Derivatives (267 cds), Carbohydrates (258 cds), Protein Metabolism (222 cds), Cofactors, Vitamins, Prosthetic Groups and Pigments (160 cds), Fatty Acids, Lipids and Isoprenoids (130), Membrane Transport (117), and RNA metabolism (106). Other subsystems with less number of cds include: Cell Wall and Capsule (46 cds), Virulence, Disease and Defense (19 cds), Potassium Metabolism (35 cds), Miscellaneous (17 cds), Iron Acquisition and Metabolism (7 cds), Nucleosides and Nucleotides (80 cds), Cell Division and Cell Cycle (6 cds), Motility and Chemotaxis (4 cds), Regulation and Cell Signaling (3 cds), Secondary Metabolism (6 cds), DNA Metabolism (79 cds), Nitrogen Metabolism (10 cds), Respiration (96 cds), Stress Response (79 cds), Metabolism of Aromatic Compounds (10 cds), Sulfur Metabolism (23 cds), and Phosphorus Metabolism (32 cds).

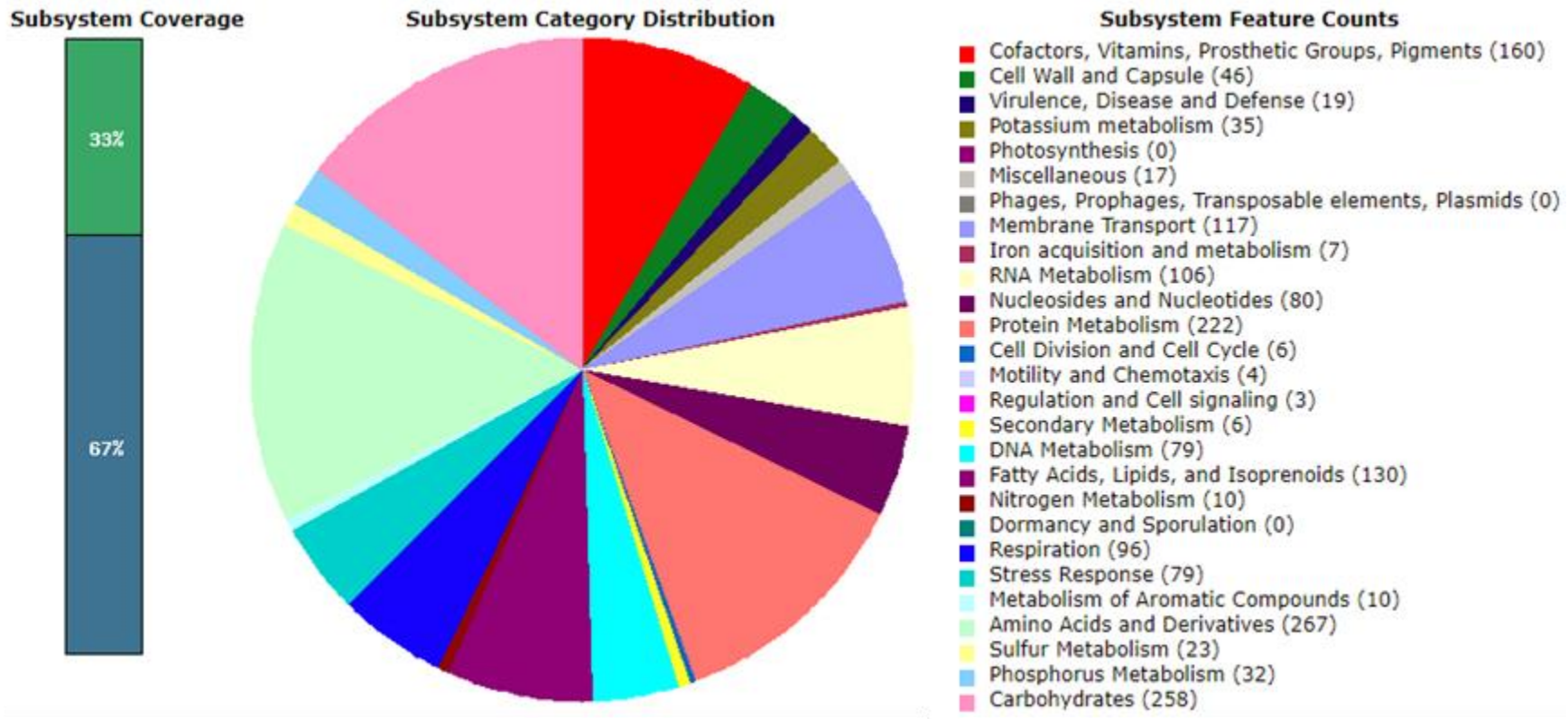

Figure 21: Subsystem category distribution of strain IIIV-5B (*Halococcus salsifolii*) retrieved from RAST server. Numbers in parentheses indicate the associated coding sequences within each subsystem.

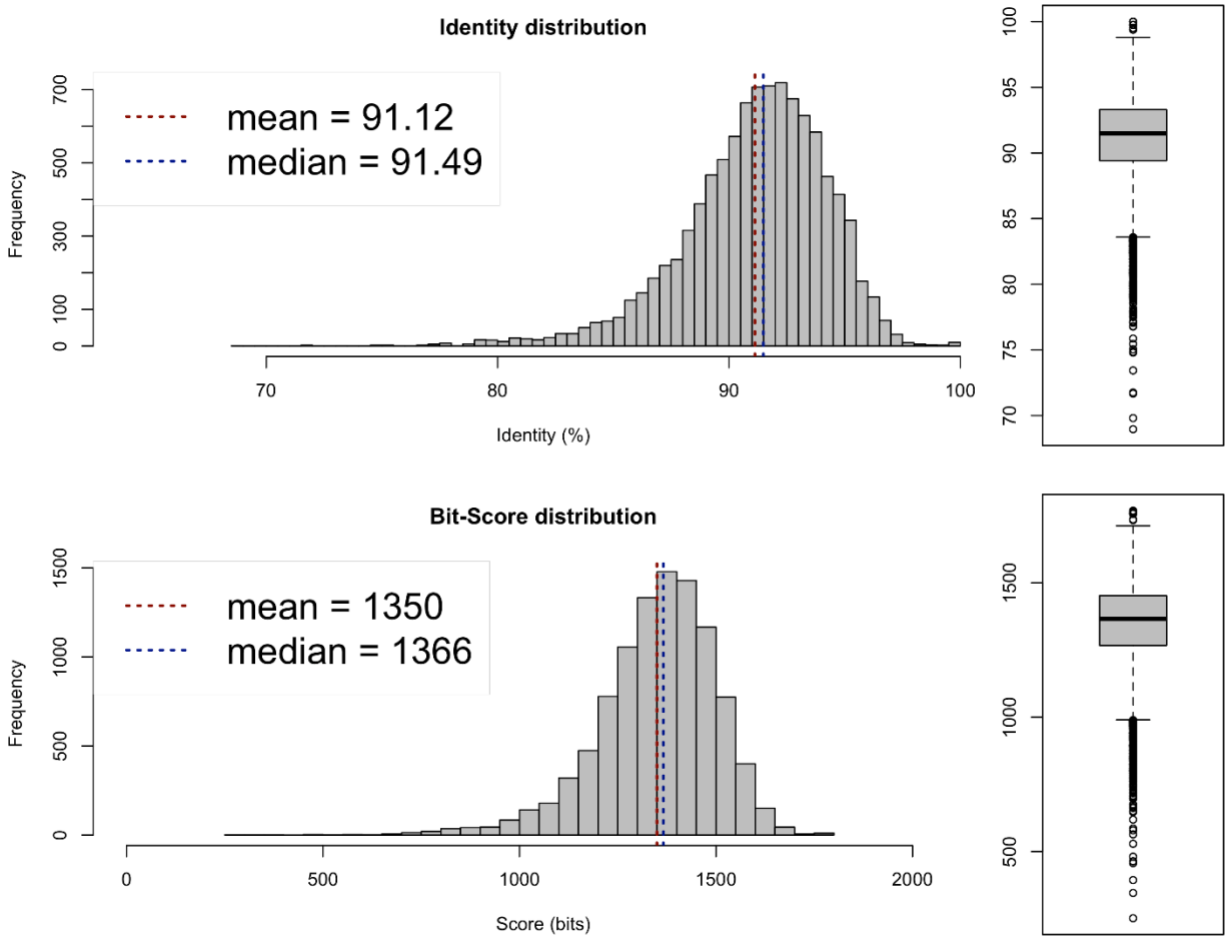

Figure 22: Identity distribution histogram of average nucleotide identity (ANI) of IIIV-5B and *Halococcus hamelinensis* [T] 100A6. The mean identity distribution between both genomes, obtained using the ANI calculator in the Enveomics toolset [120], retrieved a 91.12% nucleotide identity between genomes.

Average nucleotide identity (ANI) analysis retrieved from the ANI-calculator, using the Enveomics tool collection [120] is shown in Figure 22. A 91.12% mean identity distribution is observed after comparing nucleotide composition from the genomes of both strain IIIV-5B and *Halococcus hamelinensis* [T] 100A6. The cutoff value established for ANI to be used as a substitute of DNA-DNA hybridization is 95-96% [96]. This comparison states that IIIV-5B is a new species.

This study proposes the etymology *Halococcus salsifolii* for the new *Halococcus* species. *Halococcus salsifolii* (sal.si.fo'li.i. L. adj. salsus salted; L. neut. n. folium leaf; N.L. gen. n. salsifolii of a salted leaf).

CHAPTER 4: PHYLOGENETIC ANALYSIS OF STRAIN B11Q-1, AND THE PROPOSAL OF *SALINIROSEUM* AS NOVEL GENUS

Specific Aim

Small subunit rRNA gene-based identification of B11Q-1, a novel genus closely related to the genera *Palleronia* and *Hwanghaeicola*.

Methods

Genomic DNA Extraction and 16S rRNA Gene Amplification

Cryopreserved B11Q-1 strain was cultured in Sehgal-Gibbons media containing NaCl 15% (w/v), prepared as previously described, and incubated at 30°C. After sufficient growth was observed, genomic DNA extraction was performed using the mechanic, chemical and physical procedures, as indicated in Chapter 1. DNA concentration and quality were determined using NanoDrop™ Spectrophotometer from ThermoScientific® and 0.8% (w/v) agarose gel.

PCR of the molecular marker 16S rRNA gene were conducted using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [83], using the specifications indicated in Chapter 1. The amplification products were precipitated and purified using isopropanol and ethanol and subsequently resuspended with PCR-grade water. DNA concentration and quality were determined using NanoDrop™ Spectrophotometer from ThermoScientific® and 1% (w/v) agarose gel. Prior 16S rRNA gene amplification, purified samples were diluted and prepared for sequencing, as indicated by Macrogen USA sequencing facilities.

Phylogenetic Tree Construction

The obtained sequence (~1200bp) was uploaded to EZ-Taxon [88], where FASTA sequences of taxonomically-close relatives were retrieved. These sequences were aligned using Clustal W, and edited in Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software [89]. Neighbor-joining tree method [90] was used to determine phylogenetic distances between B11Q-1 and selected sequences.

Genomic Description of New Genus

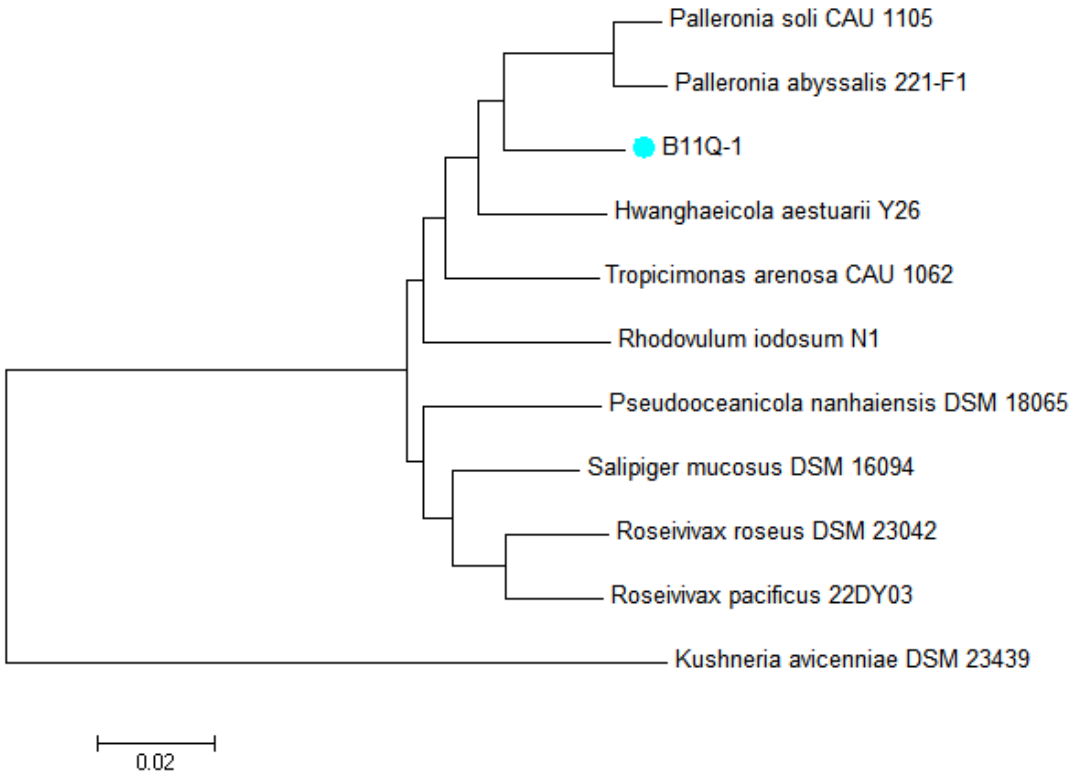

Figure 23: Phylogenetic analysis using the neighbor-joining method using partial 16S rDNA sequences of strain B11Q-1. Bar represents 2 substitutions per 10 nucleotides. *Kushneria avicenniae*^{T} DSM 23439 was used as outgroup.

Generated PCR amplicons of strain B11Q-1 were used for sequencing purposes. The results of partial 16S rRNA gene (~1200bp) sequencing was compared with available data of type strains in the server EZ-Taxon [88]. Full 16S rRNA gene was sequenced and used for the phylogenetic analysis of strain B11Q-1.

This analysis located the strain B11Q-1 evolutionarily related to the genera *Palleronia* and *Hwanghaeicola*, but low homology values (<97%) were recorded. These and other closely related type strains used to construct a phylogenetic tree in MEGA 6 software [89], also using partial 16S rRNA gene sequences and employing the neighbor-joining method [90]. The recovered cladogram

in Figure 23 locates B11Q-1 as closely related to the *Hwanghaeicola* and *Palleronia* genus, with 2 nucleotide substitutions per every 10 nucleotides. Research based on species description indicate that 97% homology is a cutoff value, representative of a new genus.

With this in mind, we propose the etymology of *Saliniroseum* as the genus, being *Saliniroseum caribbense* the type species. *Saliniroseum* (Sa.li.ni.ro'se.um. N.L. masc. adj. salinus saline; L. neut. adj. roseum rose-coloured; N.L. neut. n. Saliniroseum a rose-coloured saline organism.) *Saliniroseum caribbense* (ca.rib.be.en'se. N.L. neut. adj. caribbense of the Caribbean).

CHAPTER 5: ASSESSING THE TOTAL BIODIVERSITY OF PROKARYOTIC ENDOPHYTES WITHIN *AVICENNIA GERMINANS* USING CULTURE-INDEPENDENT TECHNIQUES

Specific Aim

Analysis of endophytic community composition of black mangroves at different locations (HS and Sal sampling sites) employing 16S rDNA-based metagenomics, and statistical comparisons of the biodiversity to test whether there are community shifts based on salt deposition in the soil.

Methodology

Sample Collection and Processing

To observe the metagenomic diversity and abundance of prokaryotic endophytes associated to *Avicennia germinans,* 27 leaves were collected from all the trees previously selected on each sampling site, as described in Chapter 1. All the leaves were pooled to create a compound sample per sampling region. As indicated in Chapter 1, the plant tissue was sterilized according to Couto (2009) using sodium hypochlorite 0.5% and prepared for total DNA extraction.

Total DNA Extraction and Purification

The pooled leaf samples corresponding to each mangrove forest were frozen using liquid nitrogen and thoroughly macerated until a fine leaf powder was obtained. From the macerated powder, 30 g of each pooled sample were treated with the PowerSoil® DNA Isolation Kit (Mo Bio), as indicated by the manufacturer's protocol. DNA quality and concentrations were determined using the ThermoScientific® NanoDrop™ spectrophotometer and a 0.8% (w/v) agarose gel, and both extraction products were shipped to Molecular Research DNA (MR DNA) (http://www.mrdnalab.com) laboratories for sequencing using Illumina MiSeq platform following the bTEFAP® methods established by Chiodini et al. [121]. The amplification of the 16S rRNA gene was performed using the primers 799F (5′-AACMGGATTAGATACCCKG-3') [122] and 1193R (5'- ACGTCATCCCCACCTTCC-3') [123] for bacterial 16S, and the primers UniArc8F (5'-YCYGKTTGATCCYGSCRG-3') [124] and a custom 515R for archaeal 16S. These primers were selected since they are prokaryote- limited and also to decrease plastid sequencing. All data quality verification and editing were performed by the bioinformatics department in the sequencing facility using QIIME bioinformatics pipeline [125].

Data Analysis

The retrieved data were modified to remove the sequences from virus and eukaryotes that were amplified using the bacterial and archaeal specific primers. The resulting sequences were utilized to create rarefaction curves and to determine the coverage of the samples. Similarly, relative abundance graphs and Venn diagrams were generated using the *vegan, fossil, reshape, plyr, ggplot2* and *VennDiagram* packages within the R statistical computing software. Statistical analyses to determine similarity, diversity, and richness were created in PAST [126] using the Shannon, Simpson and Jaccard indexes.

Results

The quantification and comparison of total community composition of endophytic prokaryotes within mangroves was completed with the leaf collection of three selected trees, as described in Chapter 1, and the creation of a composite sample per site. A total DNA extraction of the leaves was conducted, and the samples were sent for 16S rRNA gene sequencing, using specific primers for archaea and bacteria. The recovered data showed that from a total of 16,285 operational taxonomic units (OTUs), 9,445 corresponded to the HS samples and 6,840 were obtained from the Sal samples. These data, classified by genera, were used to create a rarefaction curve per sample, shown in Figure 24. Two curves representing species richness as function of the sample size can be observed. In both Sal and HS samples, a plateau is observed when 58 different species were identified but varied in the sample size necessary to target the full diversity. Sal samples reached a plateau with a smaller sample, whereas a larger amount of plant tissue was required to reach a plateau in HS samples.

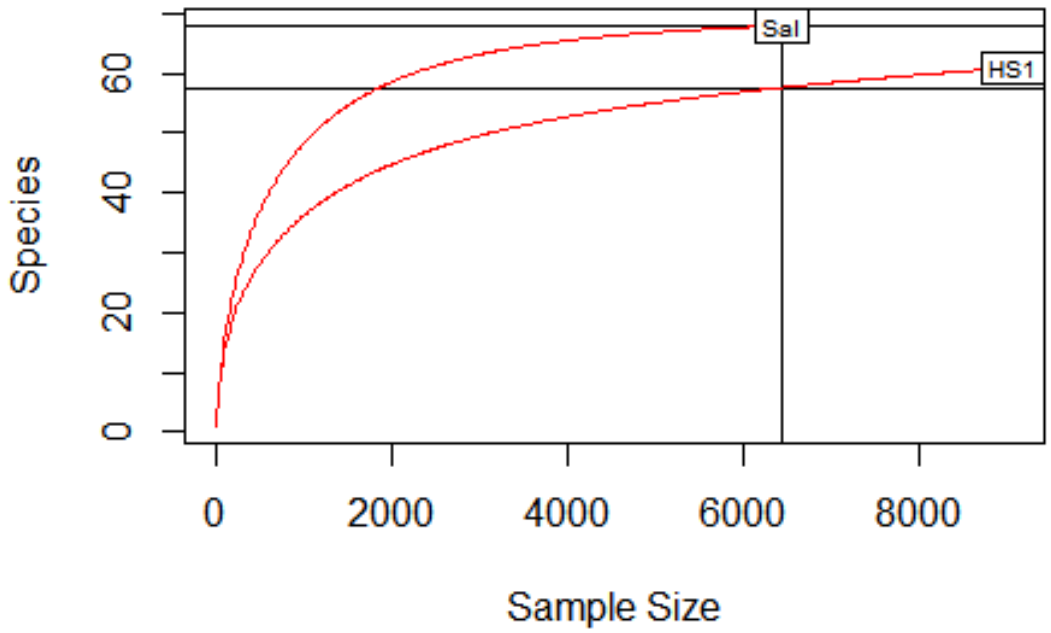

Figure 24: Rarefaction curve of OTUs sequenced per sampling site in the Solar salterns of Cabo Rojo Puerto Rico. The curve represents the accumulation of prokaryote species as a function of

OTUs found . Both samples present a plateau indicating that the sample size was sufficient to represent all the prokaryotic diversity of the sites.

Figure 25 presents a graphic visualization of the existing phyla per samples. Specific relative abundance of the identified phyla are shown in Table 8. The phyla *Euryarchaeota, Firmicutes, Proteobacteria, Bacteroidetes* and *Actinobacteria* were present in both sites but *Proteobacteria* was the predominant phylum, with a relative abundance of 0.78 and 0.84 in HS and Sal samples, respectively. However, different underrepresented genera were observed in each sampling site; for instance, *Actinobacteria* were less frequent on HS samples while the *Firmicutes* were less frequent on Sal samples. *Euryarchaeota* and *Bacteroidetes* phyla were found equally represented on both sample sites, with relative abundances of 0.07 and 0.11 on HS, and 0.06 and 0.10 on Sal, respectively.

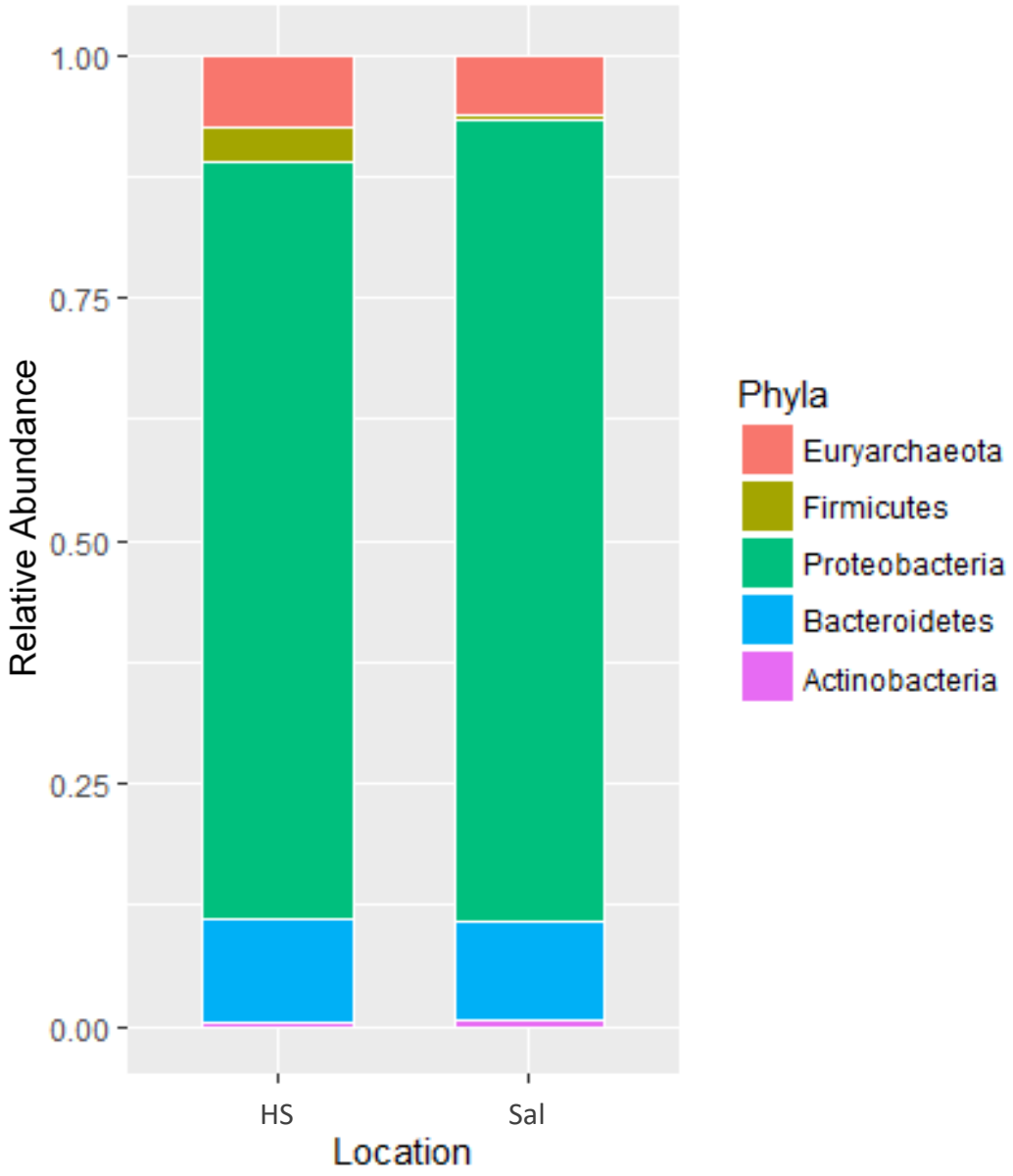

Figure 25: Visualization of relative abundance of represented phyla per sampling site. Bacterial representatives are seen represented in 4 different phyla, where *Proteobacteria* dominates in both samples (0.77 in HS and 0.82 in Sal).

Table 8: Relative abundance of prokaryote phyla in sampling sites HS and Sal. Phyla with highest abundance are shown in green whereas the phyla with lowest representation is shown in orange.

Phylum	Sampling Site	
	HS	Sal
Euryarchaeota	0.074885	0.062203
Firmicutes	0.035863	0.005232
Proteobacteria	0.779623	0.824735
Bacteroidetes	0.106572	0.102456
Actinobacteria	0.003057	0.005377

Statistical analyses were performed to determine differences in phyla abundance and shared/unique phyla per sampling site. Table 9 includes the values obtained for each calculated index (Shannon, Simpson and Jaccard indexes), used to estimate diversity and evenness. Shannon indexes for HS and Sal sites were 0.76 and 0.62, respectively. A similar result was observed using the Simpson index, where the index values obtained for HS and Sal were 0.63 and 0.69, respectively. The Jaccard index calculated based on phyla abundance was 1, restating that both samples were composed of the same phyla.

Table 9: Diversity indexes, evenness and similarities of prokaryote communities using metagenomic data from HS and Sal sampling sites. Shannon and Simpson indexes characterize both the richness and evenness of the individuals represented in the sample whereas the Jaccard index presents a proportion of the shared phyla in the sample.

Sampling Site	Shannon Index (H)	Evenness (H/Hmax)	Simpson Index (D)	Jaccard Index
HS	0.76383	0.4293	0.62607	1
Sal	0.62068	0.372	0.69461	

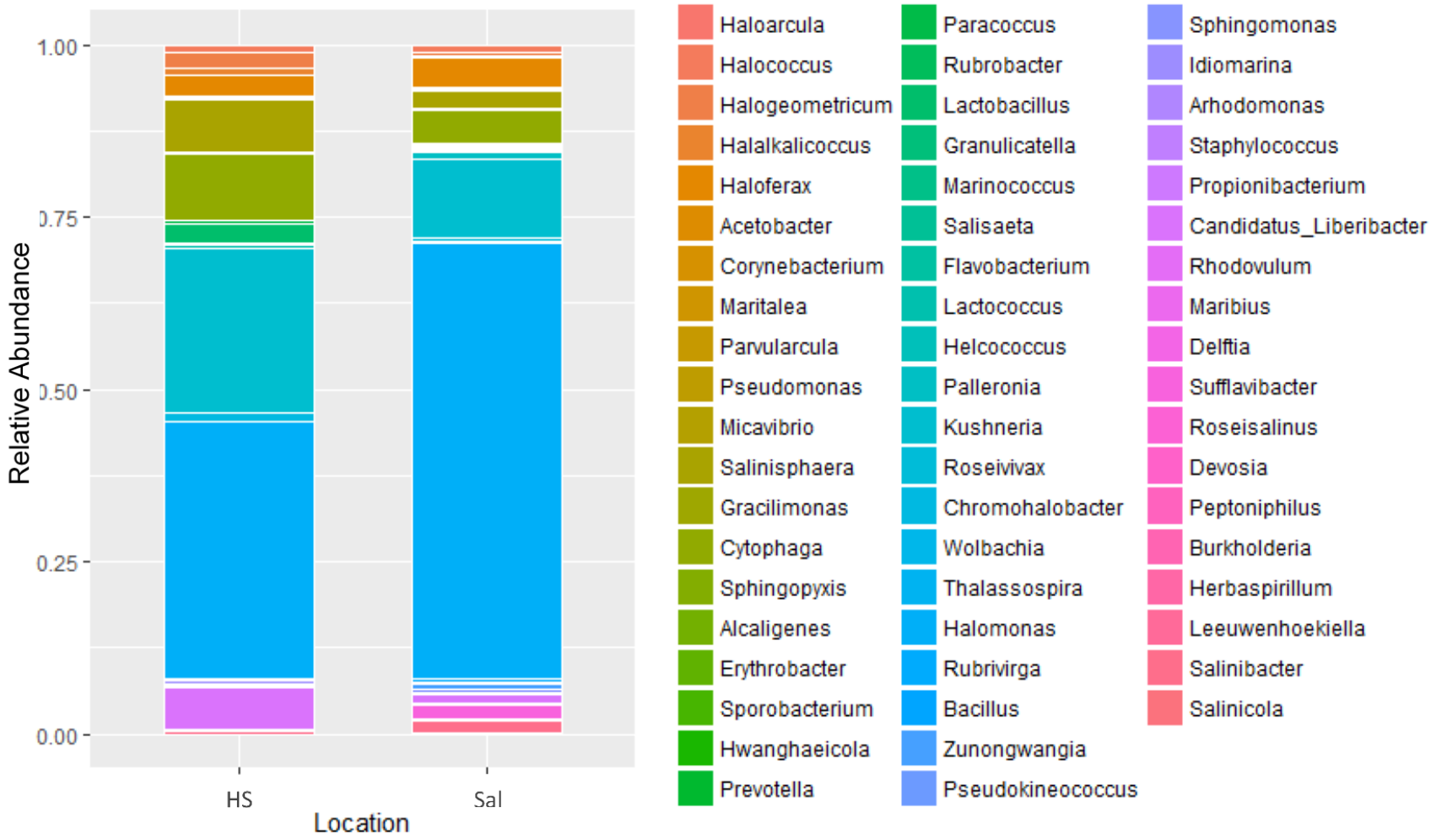

Figure 26: Visualization of relative abundance of the genera retrieved from the metagenomic analysis of the sampling sites HS and Sal. A high representation of the genera *Halomonas* and *Kushneria* is observed in both samples.

Table 10: Metagenomic- based relative abundance of genera present in sampling sites HS and Sal. The genus with highest abundance is shown in green whereas the genera with lowest representation are shown in orange.

Genus	Sampling Site	
	HS	Sal
Haloarcula	0.000611	0.000872
Halococcus	0.011207	0.009882
Halogeometricum	0.021192	0.004505
Halalkalicoccus	0.011513	0.002907
Haloferax	0.030362	0.044034
Acetobacter	0.001732	0.000145
Corynebacterium	0.000917	0.000581
Maritalea	0	0.000436
Parvularcula	0.000102	0.001017
Pseudomonas	0.001732	0.001599
Micavibrio	0	0.001017
Salinisphaera	0.076516	0.025868
Gracilimonas	0.003158	0.003052
Cytophaga	0.094855	0.046941
Sphingopyxis	0	0.000872
Alcaligenes	0.000917	0
Erythrobacter	0.000204	0.003052
Sporobacterium	0	0.000436
Hwanghaeicola	0.000509	0.001308
Prevotella	0.000713	0
Paracoccus	0.002853	0.000145
Rubrobacter	0	0.002471
Lactobacillus	0.028222	0.002616
Granulicatella	0.000408	0.000145
Marinococcus	0.001019	0.000436
Salisaeta	0.000509	0.001017
Flavobacterium	0.000306	0
Lactococcus	0.00489	0.000581
Helcococcus	0	0.000436
Palleronia	0.002038	0.010609
Kushneria	0.237799	0.114518
Roseivivax	0.000306	0.003633
Chromohalobacter	0.012837	0.000581
Wolbachia	0	0.001163
Thalassospira	0	0.001017
Halomonas	0.372899	0.632612
Rubrivirga	0.000917	0.005232

Table 10 Continued

Genus	Sampling site	
	HS	Sal
Bacillus	0.000306	0.000581
Zunongwangia	0.000306	0.007848
Pseudokineococcus	0.000102	0.001744
Sphingomonas	0.001223	0.003052
Idiomarina	0.004585	0
Arhodomonas	0.001426	0.002907
Staphylococcus	0.000306	0
Propionibacterium	0.002038	0.000581
Candidatus_Liberibacter	0.060621	0.011626
Rhodovulum	0	0.002035
Maribius	0.000102	0.002035
Delftia	0.000204	0.000436
Sufflavibacter	0.000611	0.01991
Roseisalinus	0	0.000872
Devosia	0	0.001163
Peptoniphilus	0.000713	0
Burkholderia	0.000408	0
Herbaspirillum	0.000408	0
Leeuwenhoekiella	0.000102	0.001017
Salinibacter	0.005094	0.017439
Salinicola	0.000204	0.001017

Sequenced data was also used to estimate a genus-based relative abundance. As shown in Figure 26, a notable predominance of the genus *Haloarcula* is observed in both samples (blue), but the relative abundance of this genus is strongly shown in HS (0.37) versus Sal (0.63). A similar trend is observed with the genus *Kushneria* (teal), where a high presence is shown but relative abundance shifts can also be detected. Table 10 include the specific relative abundance of all identified genera per sample. Dominant genera in both samples include *Halomonas* (HS: 0.37; Sal: 0.63), *Kushneria* (HS: 0.24; Sal: 0.11), *Cytophaga* (HS: 0.09; Sal: 0.05) and *Salinisphaera* (HS: 0.08; Sal: 0.03) (Table 10).

A Venn diagram in Figure 27 was generated using the R software and the *Venndiagram* package to visualize better the number of shared and unique genera detected in both samples. Out of the 58 genera identified in the metagenomes, 39 genera were shared in both samples (Jaccard index 0.67, Table 11). The HS sample had 8 unique genera when compared to the Sal sample. These include: *Alcaligenes*, *Prevotella*, *Flavobacterium*, *Idiomarina*, *Staphylococcus*, *Peptoniphilus*, *Burkholderia*, and *Herbaspirillum* (Table 10). In contrast, Sal sample included 11 exclusive genera, including *Maritalea, Micavibrio, Sphingopyxis, Sporobacterium, Rubrobacter, Helcococcus, Wolbachia, Thalassospira, Rhodovulum, Roseisalinus* and *Devosia* (Table 10).

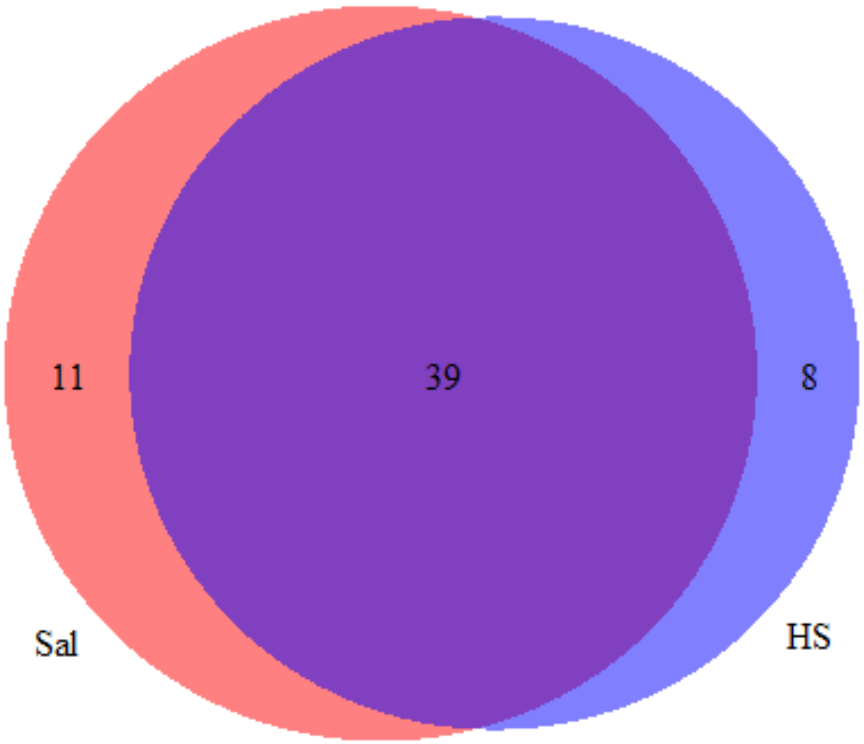

Figure 27: Venn diagram of genera count presenting each sampling site and shared among sites. Sal sampling site dominates with a higher amount (50) of identified OTUs, whilst HS site showed the presence of 47 genera, of which 8 are not shared with HS.

The Shannon and Simpson indexes were calculated to determine which sample represented higher genera diversity and evenness. The Shannon index for HS was higher than the index for Sal samples (2.01 versus 1.61 respectively). However, the Simpson diversity index revealed that Sal samples are less diverse (0.42) than HS (0.22).

Table 11: Statistical analyses for genera diversity and evenness of metagenomic studies in HS and Sal sampling sites. Shannon and Simpson indexes characterize both the richness and evenness of the individuals represented in the sample whereas the Jaccard index presents a proportion of the shared genera in the sample.

Sampling Site	Shannon Index (H)	Eveness (H/Hmax)	Simpson Index (D)	Jaccard Index
HS	2.0071	0.1583	0.21683	0.6724
Sal	1.6092	0.09998	0.41938	

Discussion

Metagenomics is a relatively recent branch of science that aims to assess the genetic information of all the organisms present in a given environment avoiding the isolation by culture [92,127]. Through this approach we identified all prokaryotic communities associated to the endosphere of *Avicennia germinans*. Based on the rarefaction curves, there were a total of 16,825 operational taxonomic units (HS: 9445 OTUs; Sal: 6840 OTUs) represented by 58 taxa, as indicated by the species count in Figure 24. This species accumulative curve revealed differences between the sampling sites. Furthermore, these curves suggest that the mangroves at the Sal sampling site provide the microbiome a more stringent habitat because of the fast identification of all the biodiversity.

The recovered 16,825 OTUs were distributed in 5 different phyla: *Euryarchaeota, Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria* (Figure 25). Table 8 shows the relative abundances of each phyla, where high values were shown for the phylum *Proteobacteria* in both samples. This goes in accordance with previous recorded data on endophytes of both glycophytes [128] and halophytes [129], as the Proteobacteria includes 3 subgroups highly recovered in environmental samples (*Alpha-*, *Beta-*, and *Gamma-proteobacteria*) [130]. As reviewed in literature, an average of 18%, 10% and 25% of the endophytic biodiversity is associated to the *Alpha-*, *Beta-*, and *Gamma-proteobacteria*, respectively [128]. The tendency of high proteobacterial strains in this study, as both HS and Sal samples present a similar relative abundance of this taxon. The second most abundant phyla, with relatively equal representation between sampling sites, were the *Bacteroidetes* and *Euryarchaeota*. The latter taxa presents a higher presence in this study than other halophyte-focused investigations [129]. On the other hand, the phyla *Actinobacteria* and Firmicutes were underrepresented in both samples. This is not

consistent with previous studies in both glycophytes and halophytes, as the Firmicutes is the second highest recovered phyla in endophytic studies (due to the high association of the *Bacillus* taxon to plants) followed by *Actinobacteria* [80,123,128–131]. The relative abundances between sampling sites is not proper metrics of diversity since the abundance and richness of the sample are not taken into consideration. Therefore, the Shannon and Simpson indexes for diversity were used, as well as the Jaccard index of similarity (Table 9) Shannon indexes for HS and Sal samples resulted in ~0.76 and ~0.62, whereas the Simpson indexes were ~0.62 and ~0.69, respectively. These values demonstrate how the species abundances are more even in the HS samples than in Sal samples. The Jaccard index of similarity for these samples is 1, as this index creates a proportion of shared taxa per sample. In this case, all taxa were present in both samples.

To analyze the prokaryotic diversity at a genus level, the relative abundances of the obtained OTUs were determined (Table 10) and a visualization was generated. Figure 10 includes the 58 genera present in both samples. Higher abundances per genera are observed in HS versus Sal samples, with the exception of the genera *Halomonas*, shown in sky-blue. This phenomenon correlates with other studies of the establishment and propagation of this genus in environments with lower salinity, as high salt concentrations tend to reduce growth rates and increase mortality in *Halomonas* [132] despite the fact that some species are tolerant up to 30% NaCl (w/v) [133]. Nevertheless, *Halomonas* appears to be the most abundant genus in both samples. The abundance of this genera contributes to the higher incidence of the phyla *Proteobacteria* in both samples, as shown in Figure 25. Previous studies have assessed the potency of *Halomonas sp*. to act as a PGP bacteria by producing the phytohormone IAA [65], thus acting as a mutualist symbiont.

The second most abundant genera reported was *Kushneria*, contributing also to the high abundance of the *Proteobacteria*. Multiple studies have associated this bacterial genus to plants,

as they have been found as epiphytes [6,23,24], endophytes [80], and part of the rhizosphere [22,80]. Other studies have also described several species of this genus as a PGP bacteria with phosphate solubilization properties [134].

Figure 26 and Table 10 also show less abundant genera that includes *Salinisphaera, Cytophaga sp., Lactobacillus sp., Chromohalobacter sp.* and Candidatus *Liberibacter*. *Salinisphaera sp.* has been associated with plants, but no PGP trait has yet been described, whereas *Chytophaga sp.* and *Chromohalobacter sp.* are bacteria that have been widely described as normal microbiota of the rhizosphere [81,135]. The latter has also been identified as a putative ammonia oxidizer, a trait that may attribute this genus a role in biogeochemical cycling [136]. An interesting finding was the presence of *Lactobacillus sp.* as part of the microbiome of *Avicennia germinans*. The plant-colonization capabilities of the members of *Lactobacillus* have been studied since late 1960s. Studies carried by Mundt and Hammer [137] aimed to determine the potential proliferation of this genera in plants, yet they were unsuccessful. To further question the high incidence of lactobacilli in both samples, studies of lactic-acid bacteria inhibition have been conducted using mangrove-derived extracts due to their high content of antibiotic compounds [138]. Data also shows presence of *Candidatus* Liberibacter, the genus of three phytopathogen that is without a free-living stage, this genus has evolved to infect plants but use insects as vectors [139]. As a phloem-specific pathogen, *Candidatus* Liberibacter has yet to be isolated, but symptomatology and molecular characterization are widely known. It can be argued that high abundances of this genus in black mangroves, especially in HS sampling site, might be the cause of the unexplained plant mortalities encountered while sampling. Nevertheless, no studies present the salinity stress as an inductor or as a preferred environment for pathogenesis.

Finally, other interesting groups observed as endophytes were the haloarchaea represented in the genera *Haloarcula, Halococcus, Halogeometricum, Halalkalicoccus* and *Haloferax* (Table 10). These genera are often associated to salt lakes, salt marshes, solar salterns, saline soil and other saline environments. However, the genus *Halococcus* has been associated with plants, but mostly as epiphyte or part of the root environment [6,80,140]. Other archaeal representatives have not been associated with plant systems, which represents a novel report.

The Venn Diagram was (Figure 27) shows that, of the 58 total genera identified in the metagenomes, 39 were shared between sampling sites. Sal samples included 11 unique genera, whereas HS only included 8. The high concentrations of salt ions the within black mangrove has been suggested as a factor that may reduce biodiversity [63]. However, HS samples showed a higher abundance of certain genera (indicated by the high Shannon index), whereas the Sal samples were richer and more even in genera (higher Simpson index) [141]. The HS sampling site provides a more restrictive environment, allowing specific organisms to colonize the plant and maintain evenness. On the other hand, the lower salinities associated with the Sal sampling site has more room for competition, which can cause a natural dominance of a few microorganisms. The Jaccard index revealed a community similarity of ~0.67 between HS and Sal (Table 11). The higher abundances but low richness in HS samples are expected since specialized groups may proliferate under stress conditions, while in the relatively mesophilic conditions provided by Sal sampling sites, more genera can establish in the plant, but with less abundance.

Conclusion

- Various representatives of the microbial diversity within mangroves can be isolated using culture media with high content of salt. Being able to isolate species of *Kushneria, Halobacillus, Pontibacillus, Bacillus, Staphylococcus, Salinisphaera, Palleronia*, and *Halococcus* using SG media with 10, 15 and 20% NaCl (w/v) suggests halophily for these strains.
- The strains IIIV-5B and Q1T1-3 were recommended as new species and the etymologies *Halococcus salsifolii* and *Salinisphaera caribbense* were proposed, respectively. Furthermore, the strain B11Q-1 was described as a new genus under the etymology *Saliniroseum caribbense.* There is still a high diversity of microorganisms to discover from halophytes with an immense promise for biotechnological and agricultural applications.
- HS samples presented higher evenness (fewer dominant species) when compared to sale, thus showing higher diversity using both Shannon and Simpson indexes. Metagenomic studies remind us that the microbiome encompasses an unimaginable diversity of prokaryotes, and that plants from the same species will present microbiome variability.
- Highly represented taxa in both samples, although with differences in relative abundances, included the genera *Halomonas* and *Kushneria*; the latter being the highest culture-isolated organism. Jaccard index for shared genera per sampling site indicated that around 67% of the identified taxa was present in both HS and Sal suggesting that even when a big part of the microbial communities is conserved among mangrove forests, the salinity conferred by the aquatic system feeding the vegetation has a role in shaping the microbiome.

Recommendations

- Select mangroves creating a gradient of distance from the water-source out to have measurements of distance and its effect on salt deposition.

- Analyze the chemical profiles from the soil and plant.

- Perform metagenomic analyses per stratum.

- Select multiple locations around the island of Puerto Rico to compare chemical profiles of the soil and their effect on the diversity.

- Study the microbiota transmissible by seeds.

- Observe the mechanisms by which *Halomonas sp.* and *Kushneria sp*. evade the plant's immune responses and their colonization capabilities.

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