Endophytic prokaryotic diversity associated with sea grass beds of *Thalassia testudinum* from *Cabo Rojo, Lajas,* and *Vieques,* Puerto Rico

By

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Abstract

Sea grass beds are considered a vital component of marine ecosystems, which serve as a nutrient source for many marine organisms, while also providing a critical habitat for many endangered species. In *Puerto Rico* sea grass bed communities are beneficial to different marine ecosystems, though this habitat has been threatened by developmental projects, pollution and global warming which consequently are causing its decay. The scope of this research project was to determine the prokaryotic endophytic diversity of *T. testudinum* beds and to establish differences in community structure of four geographical areas in *Puerto Rico* that have been exposed to different anthropogenic impacts. The objectives were addressed by culture dependent and culture independent techniques. A total of 1,279 strains were recovered from surface sterilized leaves. Phylogenetic analysis revealed the prevalence of *gamma-Proteobacteria (Enterobacter (5%), Pseudomonas(1%) and Cobetia(4%), Actinobacteria (Cellulosimicrobium, Nesterenkonia and Micrococcus (1%))*, and the *Firmicutes (Bacillus(55%), Geobacillus(<1%), Staphylococcus(18%) and Halobacillus(11%)*. Furthermore the complexity and diversity patterns of the prokaryote community associated with total DNA extracted was evaluated based on Terminal Fragment Length Polymorphism (TRFLP) profiles and the analysis of clone libraries of PCR amplified 16S rRNA genes. TRFLP patterns revealed shared similarities in community structure as well as unique patterns for each sampling site, while the analysis of 16S rRNA clone libraries revealed the dominance of *alpha-Proteobacteria, gamma Proteobacteria, epsilon-Proteobacteria* and *Firmicutes*. *Staphylococcus* isolates predominated in Buyé Beach (39%) in contrast to the reduced community at the other sites (< 7%). In addition, *Bacillus* isolates were predominant in all sites, excluding Buyé which confirms the unfavorable influence of higher impact to this site, since members of *Bacillus* are recognized for establishing beneficial

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associations with terrestrial plants. Culture independent techniques revealed low diversity of TRF's and shifts of predominant OTU's for Buyé clone libraries. Statistical analysis confirmed that this site is significantly different from the others, suggesting that Buyé higher levels of impact are causing alterations to the basal endophytic communities. By knowing the assemblages of these endophytes is setting bases for the development of new strategies for restoration programs to address the decay of sea grasses.

Resumen

Las praderas de hierbas marinas son un componente vital del ecosistema marino, sirviendo como fuente de nutrientes para muchos organismos marinos, y también proveen un habitat crítico para especies en peligro de extinición. En Puerto Rico las praderas de hierbas marinas son beneficiosas para diferentes ecosistemas marinos, pero este habitat ha sido amenazado por el desarrollo de proyectos, la contaminación y calentamiento global, causando como consecuencia su decaimiento. El enfoque de este proyecto de investigación consistió en determinar la diversidad de endófitos procariotas asociados a las praderas de *Thalassia testudinum* y establecer la estructura de comunidades de cuatro areas geográficas de *Puerto Rico* que están expuestas a diferentes impactos antropogénicos. Los objetivos fueron realizados por técnicas dependientes de cultivo e independiente de cultivo. Se obtuvo un total de 1,279 cepas de hojas esterilizadas superficialmente. Análisis filogenéticos demostraron la prevalencia de *gamma-Proteobacteria (Enterobacter (5%), Pseudomonas(1%) y Cobetia(4%), Actinobacteria (Cellulosimicrobium, Nesterenkonia y Micrococcus (1%))*, y *Firmicutes (Bacillus(55%), Geobacillus(<1%), Staphylococcus(18%) and Halobacillus(11%)*. Tambien los patrones de diversidad y complejidad de la comunidad procariota asociada al DNA total extraído fue evaluado por perfiles de "Terminal Fragment Length Polymorphism" (TRFLP) y análisis de bibliotecas de clones del gen 16S rRNA amplificadas por PCR. Los patrones de TRFLP demostraron similaridades compartidas en la estructura de la comunidad, como también patrones únicos por cada area de muestreo, mientras que el análisis de las bibiliotecas de clones de 16S rRNA demostraron la dominancia de *alpha-Proteobacteria, gamma Proteobacteria, epsilon-Proteobacteria* y *Firmicutes*. Cepas de *Staphylococcus* fueron las predominantes en Buyé Beach (39%), en contraste con la reducida comunidad presente en las otras areas (< 7%). En adición, las

cepas de *Bacillus* predominaron en todas las áreas, excluyendo a Buyé confirmando la desfavorable influencia del impacto en este lugar, ya que los miembros de *Bacillus* se reconocen por establecer asociaciones beneficiosas con plantas terrestres. Las técnicas independientes de cultivo revelaron baja diversidad de TRF's y cambios en la predominancia de OTU'S para las librerias de clones en Buyé. El análisis estadístico confirma que este lugar es significativamente diferente a las demás areas, sugiriendo que los niveles elevados de impacto en Buyé están causando alteraciones a la comunidad endófita basal. El conocer la composición de estos endófitos está sentando las bases para desarrollar nuevas estrategias para programas de restauración enfocados en prevenir el decaimiento de las hierbas marinas.

Dedication

I want to dedicate all my work to the memory of my grandfather, Rigoberto Rodríguez. He has been a great inspiration throughout the development of my thesis project. Through this process I have comprehended why he was so passionate about science and teaching.

Also I want to dedicate my work to both my parents, Marisa and Luis Couto, whom always rejoice through the accomplishment of my goals. Both of you have always been there to support me and give me advice. Thank you, without you I would never grown to be what I am now.

Acknowledgements

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1. Introduction

Thalassia testudinum is a halotolerant marine angiosperm that has adapted to life at sea (Uku, 2005). Sea grass beds of *Thalassia testudinum* are considered a vital component of the marine ecosystems. They provide key ecological services, including organic carbon production and export, nutrient cycling, sediment stabilization, enhanced biodiversity and trophic transfers to adjacent habitats in tropical and temperate regions(Orth et al., 2006). In addition, they serve as a nutrient source for many marine organisms, including those of economic importance, while also providing a critical habitat for many endangered species such as the manatee (*Trichechus manatus*) and the hawksbill turtle (*Chelonia mydas*). This habitat has been threatened by development projects, pollution and global warming which consequently are causing its decay at scales of square meters to hundreds of square kilometers. Increased coastal development leading to harsher contamination is causing rapid large scale sea grass losses over relatively short temporal scales throughout the world (Orth et al., 2006). As a result, efforts for protection and conservation of *T. testudinum* are essential for the health of this marine ecosystem, thus requiring research focused in its preservation and proliferation.

Numerous studies have confirmed the relevance of symbiotic associations such as plant-microbe interactions for the survival of most terrestrial plants. Most researchers have focused their interest in documenting and studying endophytes associated with their hosts but little is known about those present in marine plants. Preliminary studies have confirmed the existence of fungal endophytes related to sea grasses (Maldonado et al., 2003; Wilson, 1995) but there are no reports about the bacterial or archaeal endophytic diversity and the beneficial role that these endophytes might provide. Endophytes might

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be implicated in promoting health, nitrogen fixation, phosphate solubilization and may also provide protection against phytopathogens. Hence, the study of endophytic bacteria or archaea in healthy mature tissues of sea grasses will help us understand their ecological role and importance in this environment.

In *Puerto Rico* there are several sea grass beds communities that are beneficial to different marine organisms and support other ecosystems, such as mangrove forests and coral reef communities. In this research we assessed the microbial endophytic diversity from sea grass beds located in the intertidal zone of four geographical areas of Puerto Rico which has been exposed to different anthropogenic impacts. These areas include Buyé Beach and *Los Morillos* in *Cabo Rojo, Cayo Enrique* in *Lajas* and Puerto *de la Libertad* in *Vieques* Island. Buyé Beach has been a recreational area for many years and has been exposed recently to construction projects, *Los Morillos* have been exploited mostly for salt production and recently experienced the development of two hotel complexes. *Cayo Enrique* in *La Parguera* has been exposed to commercial fishing and is constantly under recreational and developmental pressures. On the other hand, the western coast of Vieques is a conserved beach that has not received the same amount of impact but is the prospect location for the construction of a new seaport.In this research, it was hypothesized that if the endophytic microbial community is affected by the anthropogenic impact, then the diversity will change according to the level of impact. For studying the endophytic community present in *T. testudinum* there were two main objectives established: (1) determine the endophytic prokaryotic diversity present in *Thalassia testudinum* by culture dependent and culture independent techniques and (2)

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compare patterns of diversity among four geographical sites of Puerto Rico with different anthropogenic impact

      According to the level of anthropogenic impact at the sampling location the pattern in community structure was different. Moreover, shared species were recovered among the sampling areas, which might be putative endogenous microorganisms that are playing an important role in the health and establishment of *T. testudinum*. By constructing environmental libraries, it was possible to carry out the presumptive identification of uncultivable sea grass endophytes, which may lead to design better culturing and preservation techniques for microbial endophytes in *T. testudinum*. In addition, the endophytes isolated were cryopreserved awere used for constructing the Sea Grass Endophytic Culture Collection (SGECC) that serves as reference for future research. The endophytes were cryopreserved in an ultrafreezer located in the Biology building (Room B218 A) at UPRM.This work is an important contribution to the marine ecology of *T. testudinum* and is setting the bases for research applications which are relevant for improving the health and management efforts of this vital marine ecosystem. Eventually, it will be possible to know and determine the role of these endophytes, in terms of their interaction with the host and how this benefits the ecosystem.

2.Literature Review

2.1Endophytes

Taken literally the word endophyte means "in the plant". In Greek, the term endon means "within" and phyton means "plant" (Chanway, 1996). The term endophyte was defined by Wilson (1995) to describe fungi or bacteria which for all or part of their life cycle colonize the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues. Endophytic bacteria have been isolated from all plant compartments such as leaves roots, thalus and seeds (Posada and Vega, 2005) residing intercellularly as well as intracellularly within host tissues and therefore are able to form a more intimate relationships with the host plant than most other plant associated bacteria. Endophytes obtain nutrition from the host plant and protection from environmental stresses and microbial competition (Bai, 2003). In return, they confer enhanced fitness to the host (Tan and Zou, 2001). It has been estimated that the natural endophyte concentration in different crops range between 10^3 and 10^6 CFU/g fresh mass (Frommel et al., 1991, Hallman et al. 1997, McInroy and Kloepper, 1995, Reiter and Sessitsch, 2006).

The importance of bacterial endophytes in plants has been traditionally underestimated relative to their fungal counterparts (Stone et al., 2000). This, however, is far from correct as it is clear that healthy plant tissues are frequently colonized by endophytic, intracellular prokaryotes (Hollis, 1951; Kloepper et al., 1992). Although prokaryotes are usually stigmatized as pathogenic, many bacteria have neutral and beneficial effects on their host plant (Davison, 1988; Hoflich et al., 1994; Hallman et al., 1997; Lodewyckx et al., 2002; Kado, 1992).

Within the prokaryotic endophytic species reported, some are said to be strictly endophytes that confer enhanced benefits for the health of its host. Improvements in plant health and productivity are mediated by three different ecological mechanisms: antagonism of pests and pathogens, promotion of host nutrition and growth, and stimulation of plant host defenses (McSpadden, 2004). These organisms can promote plant growth by deterring insect and animal herbivory, and occupying an ecological niche similar to that of phytopathogens (Berg et al., 2005). Intensive work has shown that endophytes have the capacity to control insects and different microbial pathogens (Azevedo et al., 2000; Petrini et al**.,** 1989), as some exhibit strong anti-fungal activity (Brooks et al., 1994; Hinton and Bacon, 1995; Mukhopadhyay et al., 1997), antagonize harmful bacteria (Van Buren et al., 1993) and control plant parasitic nematodes (Hallman et al., 1997). Endophytes can also increase plant growth and development by a number of similar mechanisms **(**Lazarovits and Nowak**,** 1997). In some cases, endophytes can also accelerate seedling emergence and promote plant establishment under adverse conditions (Chanway, 1997) by cycling of nutrients like nitrogen fixation (Davison, 1988), phosphate solubilization activities (Verma et al., 2001; Wakelin et al., 2004), indole acetic acid production (Lee et al., 2004), phytohormone and enzyme production (Lambert and Joos, 1989), and iron sequestration from the soil by the production of siderophores (Kloepper et al., 1986; Costa and Loper, 1994; Ryan et al., 2008). In plant tissues bacterial endophytes may originate from seeds (Mundt and Hinkle, 1976; McInroy and Kloepper, 1995), vegetative material (Sturz, 1995), soil (McInroy and Kloepper, 1995) and the phylloplane (Raaijmakers et al., 1995) Entry of these bacteria into the plant can be through sites of emergence of lateral roots, wounds, as well as natural openings

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including stomata,lenticels and germinating radicles (Algam et al., 2005; Hallman et al., 1997)

Endophytic bacteria isolated from the internal plant tissues of healthy plants such as roots, stems, and leaves, comprise over 129 species representing over 80 genera, with *Pseudomonas, Bacillus, Enterobacter* and *Agrobacterium* being the most commonly isolated genera (Cho et al., 2002; Gardner et al., 1982; Hallman et al., 1997; Mahaffee and Kloepper, 1997; McInroy and Kloepper, 1995; Mundt and Hinkle, 1976; Sturz, 1995). Also, bacterial representatives from 82 genera have been found in a broad range of plants (Lodewyckx et al., 2002) including woody plants (Brooks et al., 1994) and arable crops (Fisher et al., 1992; McInroy and Kloepper, 1995). Commonly, several to hundreds of endophytic species can be isolated from a single plant, with at least one species showing host specificity (Tan and Zou, 2001). In agriculture, many efforts have focused in the study of microbial endophytes as potential biocontrol agents of typical crop diseases. Sugarcane, for example, has been cultivated continuously for 100 years in parts of Brazil without addition of nitrogen, which is supplied by the endophytic nitrogen fixing bacterium *Gluconacetobacter diazotrophicus* (Bodey et al., 1991). In this type of symbiosis, nitrogen fixation is the basis for a mutualistic association between plant and microbe (Chanway et al., 1996).

Many species of *Pantoea* produce a spectrum of inhibitory antibiotics which are effective for the control of fire blight disease caused by *Erwinia amylovora* in pear and apple trees (Beer et al., 1984, Johnson et al., 1993). *Pantoea agglomerans* strain YS19

has shown nitrogen-fixing activity *in vitro*, producing four different phytohormones, including indole acetic acid, therefore promoting plant growth (Feng et al., 2006).

Previous studies have documented the putative use of *Bacillus spp.* and actinomycetes as biological control agents (Filonow and Lockwood, 1985; Huang et al., 1993; McSpadden, 2004). *Bacillus spp.* and actinomycetes share several features that make them attractive candidates as biological control agents, including their abundance in soil and the production of various biologically active metabolites (Silo-Suh et al., 1994). Numerous strains of *Bacillus* and *Paenibacillus* can suppress pests and pathogens or promote plant growth (McSpadden- Gardener, 2004). A number of these strains already have been developed commercially as biological fungicides, insecticides, and nematicides or generic plant growth promoters, and their use in agriculture has recently been reviewed (Lacey et al., 2001, McSpadden-Gardener and Fravel, 2002, Paulitz and Belanger, 2001, Siddiqui and Mahmood, 1999). For example, *Bacillus cereus* and *Bacillus thuringiensis* have the capability of forming crystalline inclusion bodies within their spores that allow the disruption of the digestive tract of some nematodes and insects (McSpadden, 2004, Wei et al., 2003). *Bacillus spp.* can also contribute to abiotic stress adaptation in pepper (Sziderics et al., 2007) and serve as biocontrol agent against phytopatogenic fungi (Cho et al., 2007). Also, Toro et al., 1997, demonstrated that endophytic *Bacillus* species have the capability of solubilizing phosphorous and nitrogen which are limiting nutrient supplies needed for biomass development.

2.2 Sea grass beds

Sea grasses are higher plants comprising a group of marine angiosperms adapted to live in the sea. These adaptations allow them to grow completely submerged in the

marine environment. These plants have long thin straplike leaves and a monopoidal growth form in addition of possessing an anchoring system comprised of roots and rhizomes to withstand wave action. They attach to all types of substrates, particularly to those with soft consistency (Calumpong and Ménez, 1997).   In tropical coastal zones they extend from the intertidal zone to a depth of 20 m or more and often are located between mangrove communities and coral reefs (Uku, 2005).

Sea grass communities arehighly productive systems and globally important coastal habitats (Hemminga and Duarte, 2000) of ecological and biogeochemical significance (Adhitya et al., 2007;Duarte and Chiscano, 1999; Moncreiff et al., 1992) Its productivity is supported by a high demand for nutrients, including inorganic nitrogen (Adhitya et al., 2007 Further, submerged vegetation can affect sediment accretion rates (Fonseca and Fisher, 1986) and be a significant source of carbon in shallow systems (Papadimitriou et al., 2005). Sea grasses can either accumulate or export detritus (Cebrian and Duarte, 2001; Hemminga et al., 1991; Kenworthy et al., 1982), thus playing an important role in the carbon cycle in coastal environments (Adhitya et al., 2007). The high production and input of organic carbon make sea grass meadows sites of elevated microbial activity, as they serve as a substrate for bacteria and microalgae. This enhances animal abundance and promotes high heterotrophic activity such as macroalgae, invertebrates and vertebrates (Harlin, 1975; Borowitzka et al., 1990; Hemminga and Duarte, 2000; Middelburg et al., 2005).

Rapidly growing sea grass leaves and associated macroalgae serve as basis for a detrital food web, while the canopy structure formed by these leaves offers shelter and protection from predation for innumerable small organisms, such as invertebrates and

fishes, many of which are the juveniles of important commercial species (García et al., 1998; Zieman and Zieman, 1989). In addition, they contribute with significant amounts of primary production to the ecosystem and exert on profound effects on abiotic attributes of the aquatic environment such as sediment and water chemistry, sediment stability and water movement (Ailstock et al., 1991). These plants help stabilize their habitat by retarding erosion, in addition to promoting sedimentation of particles by retarding current flow and water velocity with their leaves.   Also, sea grasses have been shown to take up nutrients from the sediments, transporting them through the plant and releasing the nutrients into the water column through the leaves, thus acting as nutrient pumps (Zieman and Zieman, 1989).

2.3 Sea grass beds of Thalassia testudinum in Puerto Rico

Of the twelve recognized genera of sea grasses distributed throughout the temperate and tropical coastal zones of the world, four are found in the Caribbean and Puerto Rico. These four genera are represented by seven local species: turtle grass (*Thalassia testudinum)* the sea vines (*Halophila decipiens*, *H. baillonis* and *H. engelmannii)*, manatee grass (*Syringodium filiforme)*, the shoal grass (*Halodule wrightii)*, and the widgeon grass (*Ruppia maritima)*. Of these species, *Thalassia testudinum*, *Syringodium filiforme*, *Halophila decipiens*, and *Halodule wrightii* inhabit the insular shelf zones on both the Atlantic and Caribbean coasts of Puerto Rico as well as the nearby islands of *Vieques* and *Culebra.*

Sea grass beds are one of the most common coastal ecosystems in *Puerto Rico*, being *Thalassia testudinum* the most abundant in the Caribbean. They are widespread along the south and east coast of the island, but they can be found in areas lacking rough

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wave action at the north and west coast. The extensive sea grass beds that occur in southwestern Puerto Rico, in close proximity to some of the island's most pristine coral reefs and mangrove habitats, provide basic nutrients, primary productivity, and stable habitats serving as nursery and feeding grounds to a variety of organisms such as echinoderms, fish juveniles and endangered marine species as the West Indian manatee, *Trichechus manatus*, and the green sea turtle, *Chelonia mydas.* One significant characteristic of *Thalassia* beds is the velocity in which they store energy due to their photosynthetic activity (primary productivity), documented is one of the highest between benthic systems and similar in magnitude to that of terrestrial crop systems (García et al., 1998).

Large areas of *Thalassia* on the reef flat are periodically exposed to air, resulting in increased temperature and desiccation. In 1968 Glynn observed that *Thalassia* leaves do not survive when temperatures reach their upper tolerance levels of 35-40°C but that the rhizomes are apparently unaffected, due primarily to the thermal buffering effect of fine-grained sediments covering the underground portion of the plants. Tidal fluctuations accompanying strong spring tides can cause extreme temperature shifts that, coupled with desiccation, kill vast quantities of leaves, which then are shed by the plants. The process occurs sporadically throughout the year and seems to be an integral part of the ecology of the sea grasses, with no apparent negative long-term effect on the population as a whole (García et al., 1998).

In southern *Puerto Rico*, *La Parguera* has large sea beds mostly comprised of *Thalassia* and *Syringodium* which are widely distributed over the insular shelf and also in the back-reef zones of middle shelf reefs. The most extensive sea grass beds are found

within the 2m depth contour, fringing the red mangrove coastline. The distribution of *Thalassia* near the offshore reefs and mangrove islets is generally restricted to the lee (north) side of these formations and commonly occurs in association with coral species such as fire coral (*Millepora complanata*), staghorn coral (*Acropora cervicornis* and *A. prolifera*), and the finger coral (*Porites spp*). Sea grasses are absent from the exposed reef fronts. On the inundated central portion of the reef flats and in the shallow lagoon side of the reefs, *Thalassia* develops among the coral rubble and sand, providing a rich feeding ground for diurnal reef residents. Prominent grazers in the sea grass beds include the long-spined sea urchin (*Diadema antillarum*), the variegated sea urchin (*Lytechinus variegatus*), the West Indian sea egg (*Tripneustes ventricosus*), the queen conch (*Strombus gigas*), the green sea turtle (*Chelonia mydas*), and a sizable assemblage of herbivorous fishes (Cintrón et al., 1978).

Thalassia testudinum is characterized for having long strap-like leaves with an average size of 25 cm long and 10 mm wide and strong subterraneous rhizomes that extend generally from 3 to 15 cm below the sediment. Short shoots develop from the rhizomes at regular interval between 9 to13 nods and 3 to 7 leaf groups emerge from the shoot. The top part of the shoots and the basal part of the leaves are protected by a pod. Also, from the rhizomes emerge the roots, which are more reduced than those of terrestrial plants. In sea grasses salt and water intake also happen in the leaves and other structures (García et al., 1998).

Reproduction of sea grasses occurs vegetatively (rhizome propagation) and sexually (incomplete dioecious flowers). In Puerto Rico, male and female *Thalassia* flowers begin to appear in March, reaching peak abundance in April and May, after

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which time flower production begins to wane. The reproductive season normally ends in June. The percentage of shoots with reproductive structures (buds, flowers, or fruits) varies from 4% to 54% during May. Inter-annual variation is dependent on factors such as wave energy, sediment load, turbidity, and water temperature, all of which affect the success of the reproductive season (Vicente et al., 1980).

2.4 Prokaryotic diversity associated with sea grass beds

Previous studies concerning the epiphytic prokaryotic diversity of sea grass communities in the East African coastline have revealed the presence of *Cytophaga-Flavobacteria-Bacteroides* and potential nitrogen-fixing bacteria such as cyanobacteria associated with land sources (Uku, 2005). The nitrogen fixing potential of associated epiphytes has been documented by various authors (Goering and Parker., 1972; Capone et al., 1979; Capone, 1982; Moriarty and O'Donohue, 1993; McGlathery et al., 1998; Pereg et al., 2002) and they have shown that epiphytes on sea grass leaves were responsible for nitrogen fixation and thus were important contributors to the nitrogen budget of sea grass communities.

Some of the epiphytic taxa reported to be associated with sea grass beds communities include members of the gamma-*Proteobacteria* related to the genera *Pseudomonas*, *Vibrio*, *Marinomonas* and *Oceanospirillum*. Additionally, representatives of the alpha-*Proteobacteria* (*Hyphomonas, Roseobacter, Ruegeria,* and *Rhizobiaceae)* and affiliates from the orders *Verrucomicrobiales* and *Planctomycetales*, as well as members of the phylum *Holophaga/Acidobacterium* have been detected (Weidner et al., 2000).

2.5 Sampling Areas and Anthropogenic Impact

The endophytic community assessed in this study pertained to four beaches from *Puerto Rico*: Buyé Beach, *Los Morillos, Cayo Enrique*, and *Puerto de la Libertad*, which are exposed to different levels of anthropogenic impact (Figure 1). Buyé Beach in *Cabo Rojo* (N18º2'25" W67º12'22") is characterized by white sands and rocky shores, coral reefs, and sea grass beds. The beach has been impacted intensively by various anthropogenic activities that include thousands of bathers, small boats, marine motorcycles, and occasionally floating houses. Also, in the last twenty years, hotel complexes and housing structures were constructed in the marine-terrestrial zone. Although we selected an area secluded from bathers and boating activities, at both sampling events people in small boats anchor near the sampling site. The augmentation of urban development near the sea grass communities are constantly contributing to discharges, which are a threat for the health of such ecosystem. Also, sea grass communities from Buyé Beach are only feets away from the coast which makes them more susceptible to human impact.

Los Morillos also in *Cabo Rojo* (N17º56'5" W67º11'7") comprises 1,249 acres of diverse ecosystems (mangroves, hypersaline ponds, marine lagoons, dry forest, coral reefs, and sea grass beds). The area was identified as category 1 or of high priority for conservation, by the Fish and Wildlife Service, due to its unique and irreplaceable value at national and regional level. The ecological importance of this area rely on its role as a feeding ground for endangered and threatened species such as *Trichechus manatus manatus* and as an important habitat for the queen conch. The main anthropogenic impact in this area has been the commercial extraction of salt. Also, the marine ecosystems have been impacted

by the construction of hotels and restaurants in the area, as well as by bathers, local fishermen, and marine sport enthusiasts.

Cayo Enrique reef in *La Parguera, Lajas* (N17º57'12" W67º2'48") is located 1.6km offshore from an area severely impacted by aquatic motorcycles, cargo ships, private boats, and floating houses. Normally, during the summer, hundreds of boats are anchored to the roots of the mangrove trees, resting over the sea grass beds. Usually, bathers disrupt the area by trampling over the sea grasses. and the area historically has been designated a good fishing spot being impacted by oil spills occasionally.

The sea grass bed sampled at *Puerto de la Libertad* in Vieques (N18º6'7" W65º26'45") is located in the Northwest coast and comprises approximately 4, 225 acres. The sampling site is part of a tranquil, almost secluded beach at the left of the breakwater where a new port will be habilitated for the ferry and cargo ships. The area is visited by scuba divers, local fishermen and camping activity a few feet away the seashore. In the past, the US military used the current port facilities for ammunition disembarkation and for military practices. The area is currently one of the two components encompassed in the Western Vieques Conservation Area, as identified in the 1983 Memorandum of Understanding (MOU) between the Commomwealth of Puerto Rico and the Secretary of the Navy. The ecological importance of this area as a feeding ground for endangered and threatened species such as *C. mydas*, *T. manatus manatus*, and *Pelecanus occidentalis* was recognized in the 1983 MOU. This site is also an important habitat for the Queen Conch (*Strombus gigas*) and other species of commercial and recreational value (Maldonado-Ramírez and Montalvo-Rodríguez, 2008).

3. Hypothesis

If the endophytic microflora of *Thalassia testudinum* is affected by anthropogenic impact, then the diversity changes according to the level of impact received.

4. Objectives

For demonstrating the hypothesis the project was focused to address to main objectives:

- Determine the endophytic prokaryotic diversity present in *Thalassia testudinum* by culture dependent and culture independent techniques.

- Compare patterns of diversity among four geographical sites of *Puerto Rico* with different levels of anthropogenic impact.

5. General Materials and Methods

The project aimed to assess the endophytic diversity of *T. testudinum* and also, attempted to compare the diversity among four geographical locations of Puerto Rico. Figure 1.1 is a schematic representation that resumes the whole experimental design of this project.
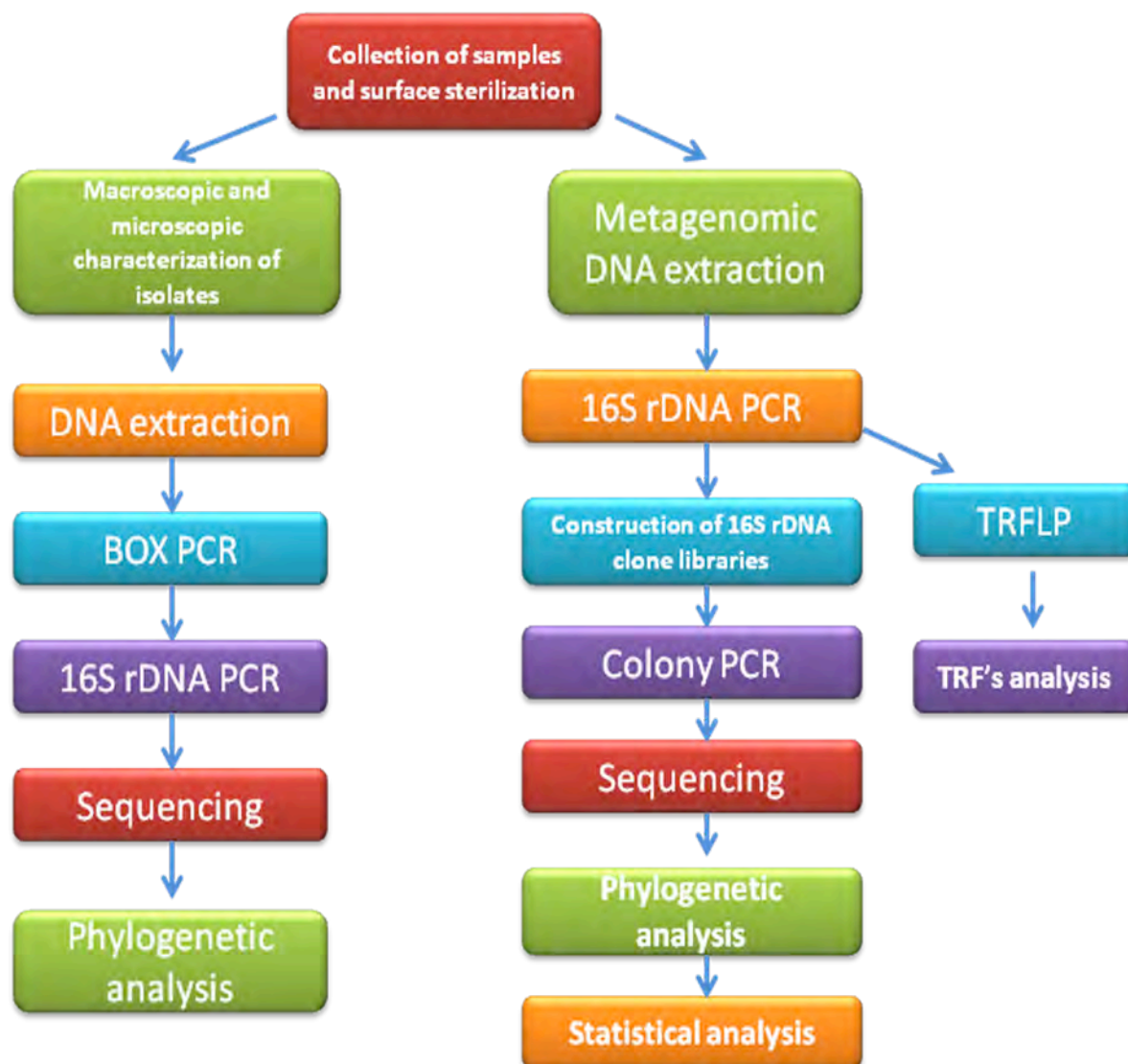
Figure 5.1 Schematic representation of materials and methods used to address the problem established for this study

6. Assesment of Endophytic Prokaryotes associated with *Thalassia testudinum* by Culture Dependent Techniques

Thalassia testudinum is a marine angiosperm that profoundly influences the physical, chemical and biological environments in marine coastal waters (Wright and Jones, 2006). Furthermore, the deterioration and decay of this vital plant could become a significant threat for the marine ecosystem. Previous research with terrestrial plants has provided evidence that plant-microbe interactions are essential for the health and establishment of the host plant. In the sea grass bed ecosystem the information related to the identity, role and distribution of the endophytic microflora inhabiting healthy tissue and the relationship with its host does not exist.

This project intended to assess the endophytic community of *T. testudinum* focusing in two main approaches. The first part of this project is aimed in the isolation and characterization of bacterial endophytes present in *T. testudinum*, which relies on basic microbiological techniques and molecular characterization of pure isolated strains. Strains were morphologically identified using colony characterization, Gram staining and Scanning electron microscopy, while the molecular characterization consisted in BOX-PCR fingerprinting and 16S rRNA gene phylogenetic analysis. BOX-PCR is oriented to the identification of the frequency and localization of an intergenic repetitive sequence which aids in profiling the molecular fingerprint of isolated strains. This technique is helpful for molecular classification of cultures recuperated with a resolution up to subspecies. After identifying the molecular profiles present in the endophytic community, representative fingerprints were chosen for further molecular and phylogenetic analysis of strains. The amplification of 16S rRNA gene allowed us to establish the identity and phylogenetic position of a recovered strain with a resolution up to genus.

Several endophytic strains were recovered which might be aiding in the proliferation of sea grasses. Within the isolates, it was possible to detect previously reported endophytes such as *Bacillus, Enterobacter* and *Pseudomonas* which.might suggests the acquisition of similar roles of these bacteria to that of their terrestrial counterparts. Also, several marine bacteria were isolated such as *Halobacillus, Exiguobacterium, Pseudoalteromonas* and *Microbulbifer* suggesting the possible adaptation of these organisms to establish symbiotic interactions with the marine plant *T. testudinum.*

The study and isolation of bacterial endophytes associated to *T. testudinum* is important for understanding the physiological advantages supplied by the endophyte. Pure strains could be later used for addressing other problems such as increasing availability of nutrients for the plant, isolation of novel secondary metabolites and identification of growth promoting bacteria that could aid in the restoration of this marine ecosystem. Also, comprehending the *Thalassia*-microbe interactions could be helpful for the implementation of new ecosystem management strategies. Cultures isolated were deposited in the SGECC for future reference and further experimental applications.

6.1 Materials and Methods

I. *Sampling areas:* During the extension of this research we collected 120 plants from sea grass beds of *Thalassia testudinum*. Samples were collected twice per site (fifteen samples at each event) from Buyé Beach and *Los Morillos* in *Cabo Rojo*, *Cayo Enrique* in *Lajas*, and *Puerto de la Libertad* in *Vieques* (Figure 6.1). First sampling events took place from August-December, while second sampling events took place from April to July.
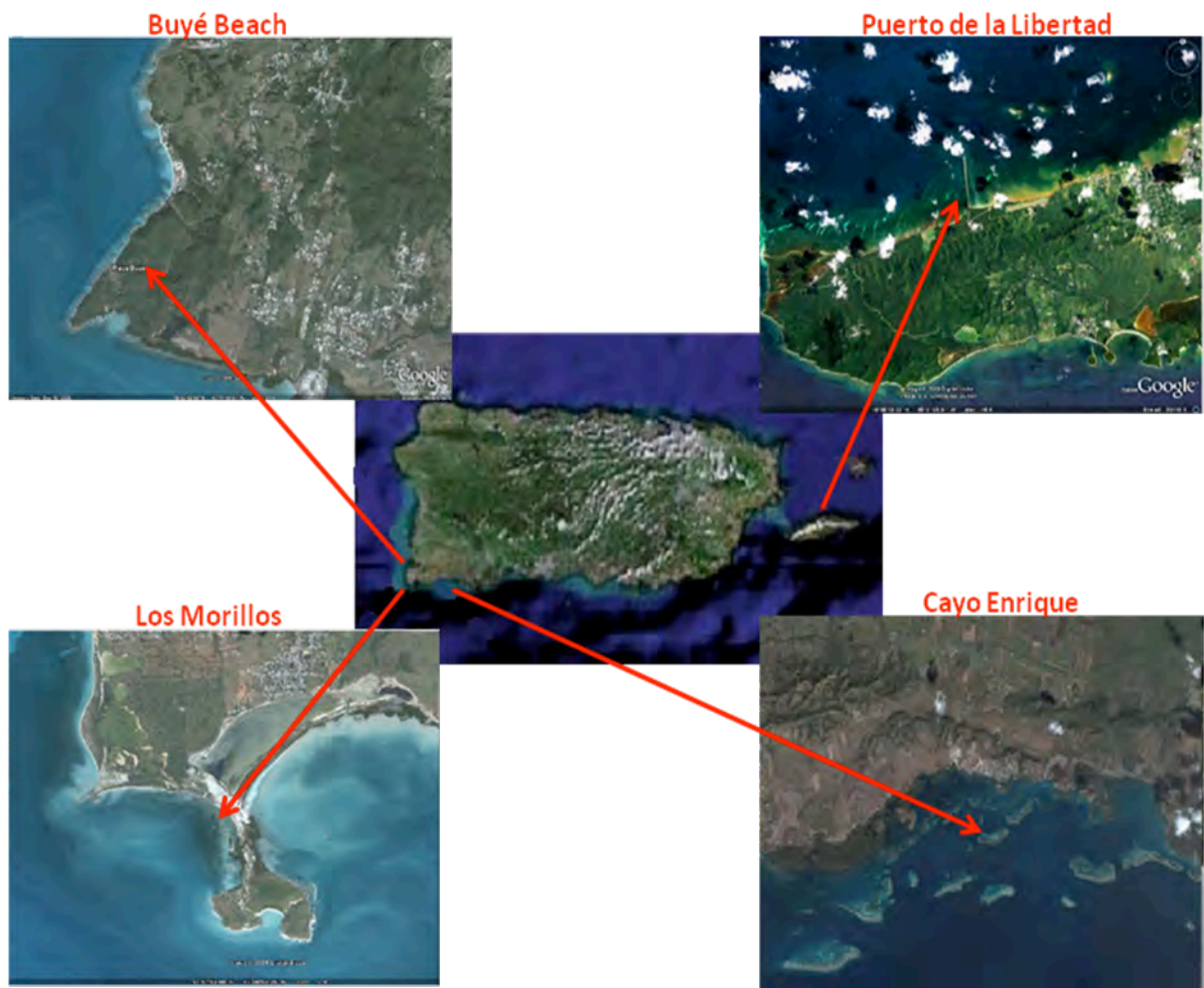
Figure 6.1 Location of sea grass beds of *Thalassia testudinum* evaluated in this research. Maps were obtained from Google Earth.

II. *Sample collection*: In this study, the transect area needed for sampling had to consist of a continous patch of *Thalassia testudinum*. The biggest sea grass bed area that could be covered when comparing all sites was a 10x30m transect. Three quadrants (10x30m) were established at each sampling location (Figure 6.2). A shovel was used to loosen the sediment and fifteen plants of *Thalassia testudinum* (five plants per quadrant) were collected randomly. Each plant was collecedt separately with seawater and sediment in sterile, labeled plastic bags. The bags were stored in a cooler for maintaining the temperature of the samples and were processed under aseptic conditions within three hours of collection. Salinity and temperature measures were taken at the sampling sites.
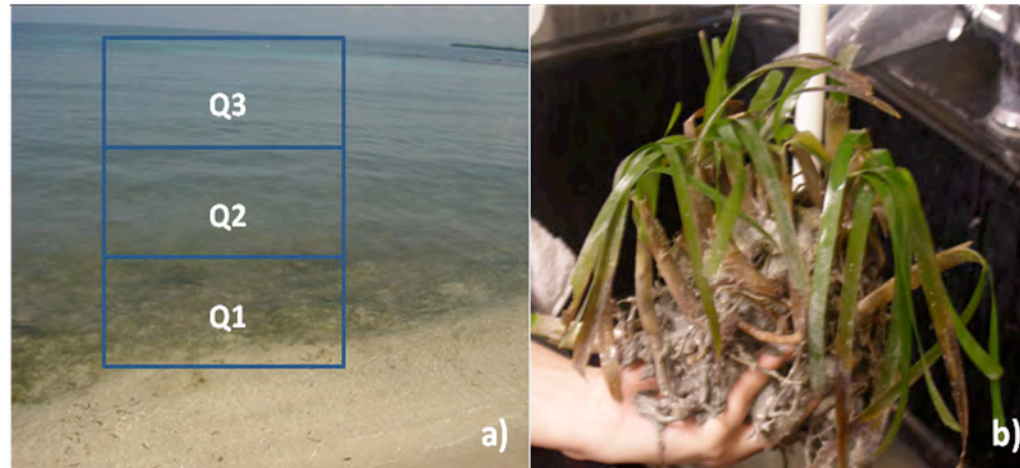
Figure 6.2 Sampling area at Buyé Beach with representation quadrant distribution strategy used for the collection of samples (a) and *T. testudinum* sample retrieved for processing (b).

III. *Sample processing and surface sterilization:* From each individual plant of *T. testudinum* we collected two healthy mature leaves with no evident sign of damage or disease. Leaves were surface sterilized individually using sodium hypochlorite (0.5%) as described by Maldonado et al. (2003) after

modification of the method used by Lodge et al., (1996) under aseptic conditions. Following surface sterilization, leaves were placed in sterile paper towels to remove excess water. Each leave was divided in apex, center and base. Aseptically, each fragment was transferred to a sterile Petri dish and segmented into nine sections of approximately $1cm^2$. Subgroups of three sections were placed equidistant to each other in Petri dish (100x15mm) containing Marine Agar (Difco) for the isolation of marine bacteria and possibly archaea (Figure 6.3). The plates were sealed with parafilm and incubated at 30ºC for four weeks. The plates were monitored daily and as soon as growth, emerging from the edge was observed, subcultures were made for further purification of isolates. Representative strains were transferred periodically and pure cultures were obtained using the four quadrant streak method.
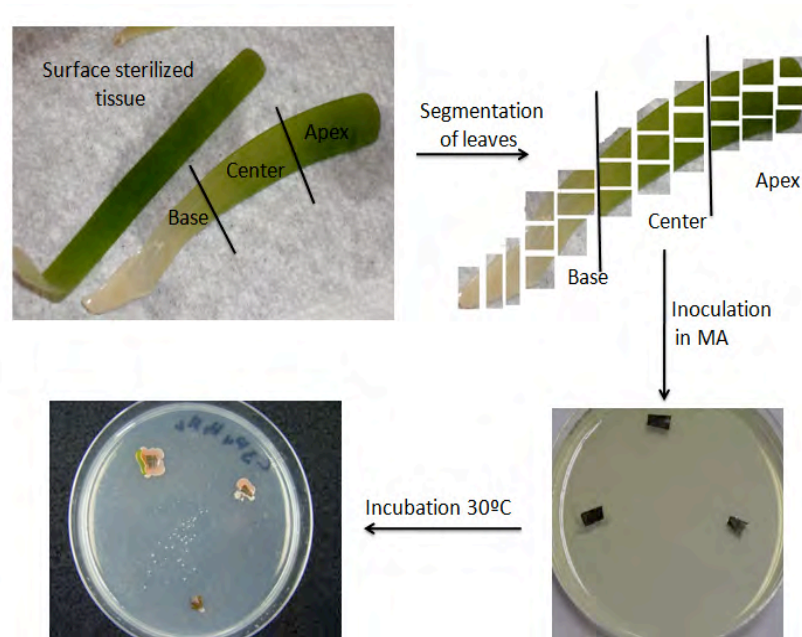
Figure 6.3 Schematic representation of processing and inoculation approaches for achieving surface sterilization of leaf segments from *T. testudinum* for isolation of endophytic prokaryotes.

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IV. *Morphological characterization:* Macroscopic characteristics of the colonies were documented using classical microbiological approaches. Gram staining was performed using smears fixed in acetic acid 5% (Dussault, 1955) in addition to spore and flagella stains.

V. **Preservation of isolates:** Pure isolates were cryopreserved at -80ºC and deposited in the Sea Grass Endophytic Culture Collection (SGECC). The bacterial strains were grown in 1.5 mL microtubes containing 930 µL of Marine Broth (Difco). When bacterial cultures reached logarithmic phase, 30µL of Dimethyl Sulfoxide (DMSO) was added. The culture was then flash frozen using -80ºC chiller units and stored in the SGECC ultra-freezer. All of the pure isolates were stored in an ultra freezer in room B218A at the Biology Building where the SGECC is located.

VI. *Genomic DNA isolation*: Strains were grown in MA at 30˚C. Genomic DNA was extracted according to a modified version of the protocol described by Benlloch et al., (2001). The isolated DNA was resuspended in 50 µl of 1X TE (Tris-EDTA, pH 8) and treated with RNAse (final concentration of 20 µg/µl) for 30 minutes at 37ºC. The quality of the DNA was verified on 0.8% agarose gels after staining with ethidium bromide (10µg/mL). All genomic DNA's were used as templates for subsequent BOX PCR amplification.

VII. *BOX-PCR*: In BOX PCR DNA fingerprinting, PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain specific DNA fingerprints which can be easily analyzed by pattern recognition (Dombek et al., 2000). Because of the specificity of this

technique we were able to discriminate and classify isolates into groups with a resolution up to serotype, reducing the amount of isolates used for further experiments. Isolated strains that have similar patterns were clustered in groups and one representative was submitted to further 16S rRNA phylogenetic analysis. Genomic DNA from each isolate was independently used as templates for subsequent BOX-PCR amplification as described previously (Rademaker et al., 1997; Versalovic et al., 1998; Dombek et al., 2000). PCR products were verified on a 2.5% horizontal low melting agarose gel containing ethidium bromide (10μg/mL).

VIII. ***PCR amplification and gel electrophoresis:*** The molecular characterization of isolates with unique genotypes was based on the analysis of conserved regions of the 16S rRNA gene, which were PCR-amplified using the bacterial primers 799F and 1492R. The reaction mixture consisted of ddH$_2$O, buffer 1X, MgCl$_2$ 2.5mM, dNTP's 250mM, 1pmol, of each primer, 25ng of template DNA and Taq Polymerase 0.026U/μl in a reaction volume of 30μL. PCR was performed with an initial denaturation for 4 minutes at 94°C followed by 30 cycles consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C and polymerization for 3 min at 72 °C (Hezayen et al., 2002). PCR products were resolved in 1% agarose gels after staining with ethidium bromide (10μg/mL). Prior to sequencing analysis, PCR products were purified using Wizard SV Gel and PCR cleanup System purification Kit (Promega Inc., Wisconsin, USA) according to manufacturer's protocol prior to sequencing analysis.

IX.   *Sequencing and phylogenetic analysis:* Selected PCR products of 16S rRNA were sent to the UPR Genomics and Sequencing Facility (Río Piedras, PR.) and to the UW-htseq Sequencing Facility (Washington University at St. Louis, MO) for sequencing analysis . DNA sequences were identified with respect to publicly available sequences in GenBank with the BLAST program (Altschul et al., 1990) to identify their closest relatives based on sequence similarities. A multiple-sequence alignment was made using the Clustal W program (Thompson et al., 1994) with DNA sequences of closely related organisms (as determined by BLAST analysis). The sequences similarity values were calculated by pairwise comparison of the sequences within the alignment. Distance analysis of sequence similarity was performed using the programs contained in the PHYLIP software package (3.5.1). Seqboot program was used to generate 500 bootstrapped data sets. Distance matrices were calculated with the DNADIST program. One hundred trees were inferred by using the Neighbor program. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. The Consense program was used to construct a consensus tree with the most frequent branching order. The final tree was drawn using TREEVIEW (Felsenstein, 1993; Page, 1996).

6.2 Results

6.2.1 Sample Collection and Physical Parameters

Salinity and temperature measures of the environment where sea grass beds of *T. testudinum* thrive is presented in Table 1.1. Temperature and salinity from each sampling site were within the same range.

Table 6.1 Summary of average temperature and salinity measurements of sea grass beds sampled.

Sampling Site	Temperature (ºC)	Salinity (w/v %)
Buyé Beach	25 +/-	3.4 +/-
Cayo Enrique	27+/-	3.2 +/-
Los Morillos	26+/-	3.5 +/-
Puerto de la Libertad	25+/-	3.2 +/-

6.2.2 Isolation of Endophytic Bacteria

All *T. testudinum* tissue samples were surface sterilized and segmented in 9 fragments per leaf area (apex, center and base), summing up to a total of 27 fragments per leaf processed (Figure 1.3). A total of 3,240 leaf fragments were processed from 60 individual plants. A total of 1,987 leaf fragments exhibited colonization of endophytic prokaryotes, which represents 61% of the samples. Prokaryotic colonies emerging from leaf fragments were visible in MA plates at 30°C after 24 hours of incubation (Figure 1.4). Representative colonies were selected and subcultivated to MA plates until pure cultures were recovered. A total of 1,274 isolates were obtained among all the sampling sites. The distribution of isolates per sampling site was: 522, 269, 288 and 199 isolatesfor Buyé Beach, *Cayo Enrique, Puerto de la Libertad* and *Los Morillos*, respectively.

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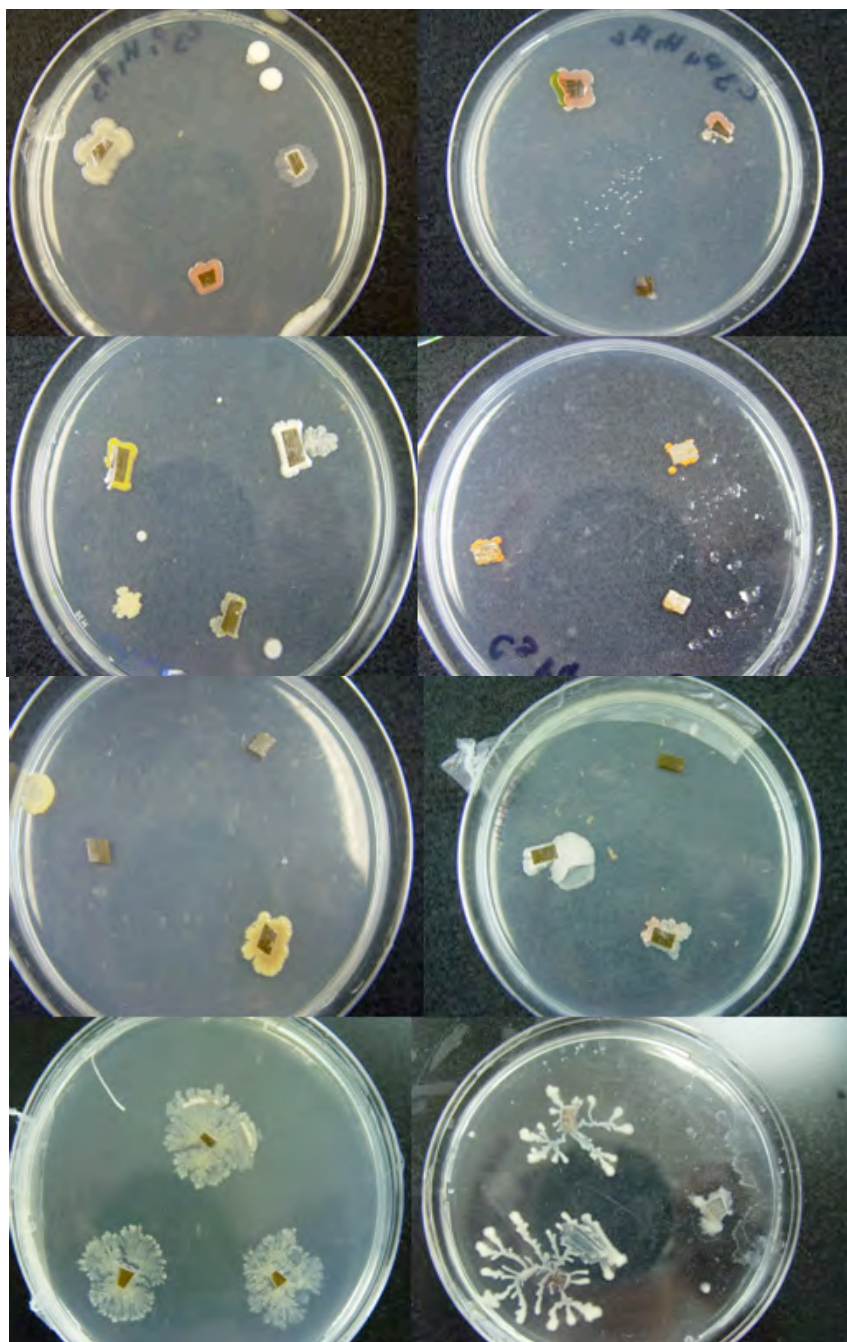
Figure 6.4 Representative endophytic colonies isolated emerging from surface sterilized *T. testudinum* leaves.

Figure 6.5 shows the distribution and relative frequency of isolated strains in percentage per sampling site. Also, Figure 1.6 displays the distribution of isolates retrieved from the different sections of the leaf, demonstrating an average homogenous allocation within the different sections.
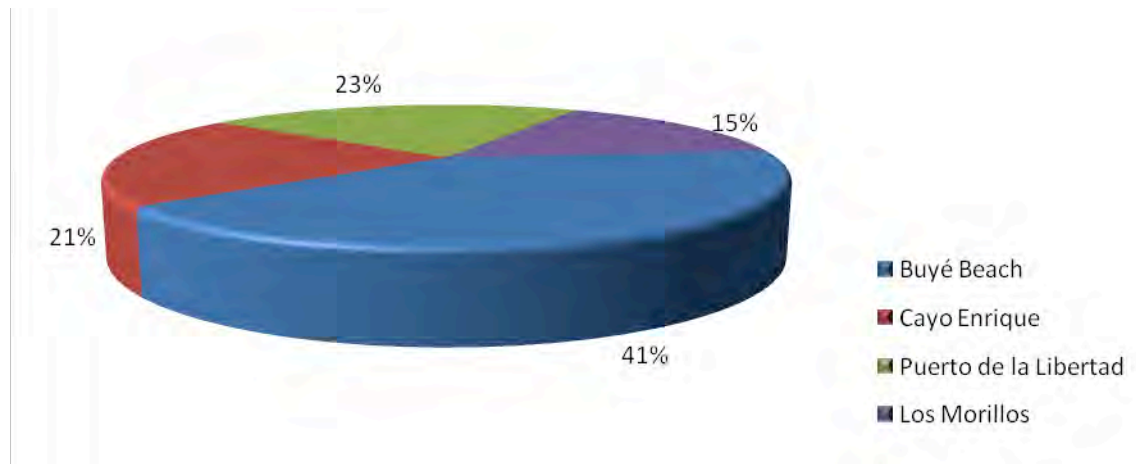
Figure 1.5 Pie chart representing the distribution of isolated prokaryotic endophytes per sampling site (N=1274).
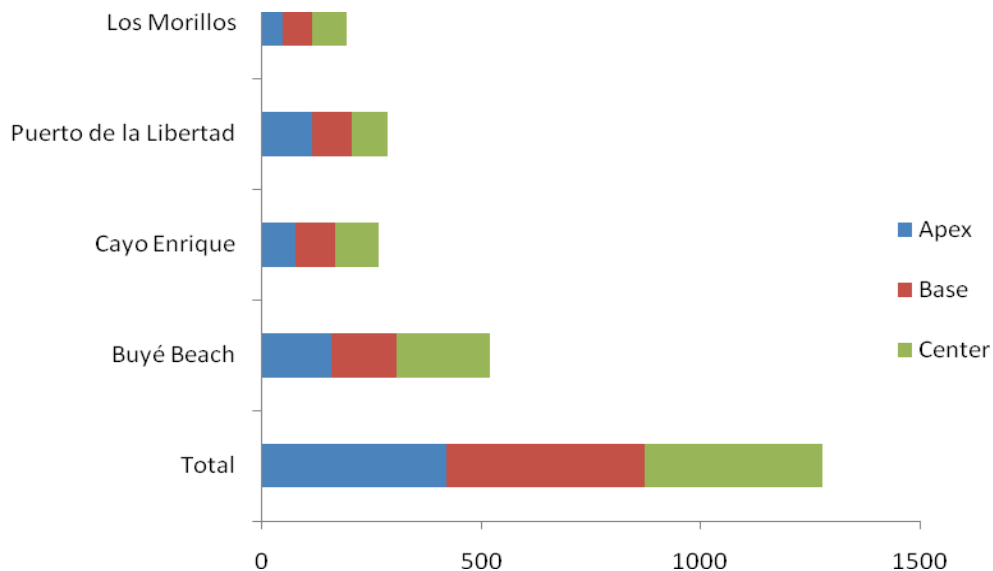
Figure 6.6 Bar graphic representing the distribution of isolated strains per leaf segment from different sampling sites. Almost equitative distribution can be observed in all segments.

6.2.3 Morphological characterization of isolates

Macroscopic characteristics were documented using the classical characterization of colony appearance (Appendix 1). Gram staining was performed using acetic acid fixation smears as described by Dussault, 1955. The microscopical morphology of representative strains was also examined by scanning electron microscopy (SEM). Electron microscopy procedures were performed with modifications as previously described (Díaz-Muñoz and Montalvo-Rodríguez, 2005). In addition SEM was performed to cross sections of *T. testudinum* leaves to confirm presence of endophytes within the tissue (Figure 1.7, Figure 1.8).

The colonies' morphotypes isolated from Buyé Beach and *Puerto de la Libertad* exhibited more diversity when compared to those of *Los Morillos* and *Cayo Enrique*. Interestingly, the sampling sites had a common predominant rhizoid colony which exhibited very aggressive growth. Microscopically, rod-shaped cells were the most abundant morphology representing 76% of the isolates, followed by cocci-shaped cells which represented 24 % among all surveys (Figure 1.9). Gram staining revealed that 88% of the rod shaped cells were Gram positive while the remaining 12% were Gram negative rod-shaped cells. All cocci-shaped cells were Gram positive. A detailed distribution of the microscopic morphologies found in the isolated strains per sampling site is shown in Figure 1.10. Some of the representative strains are shown in Figures 1.11- Figure 1.22.
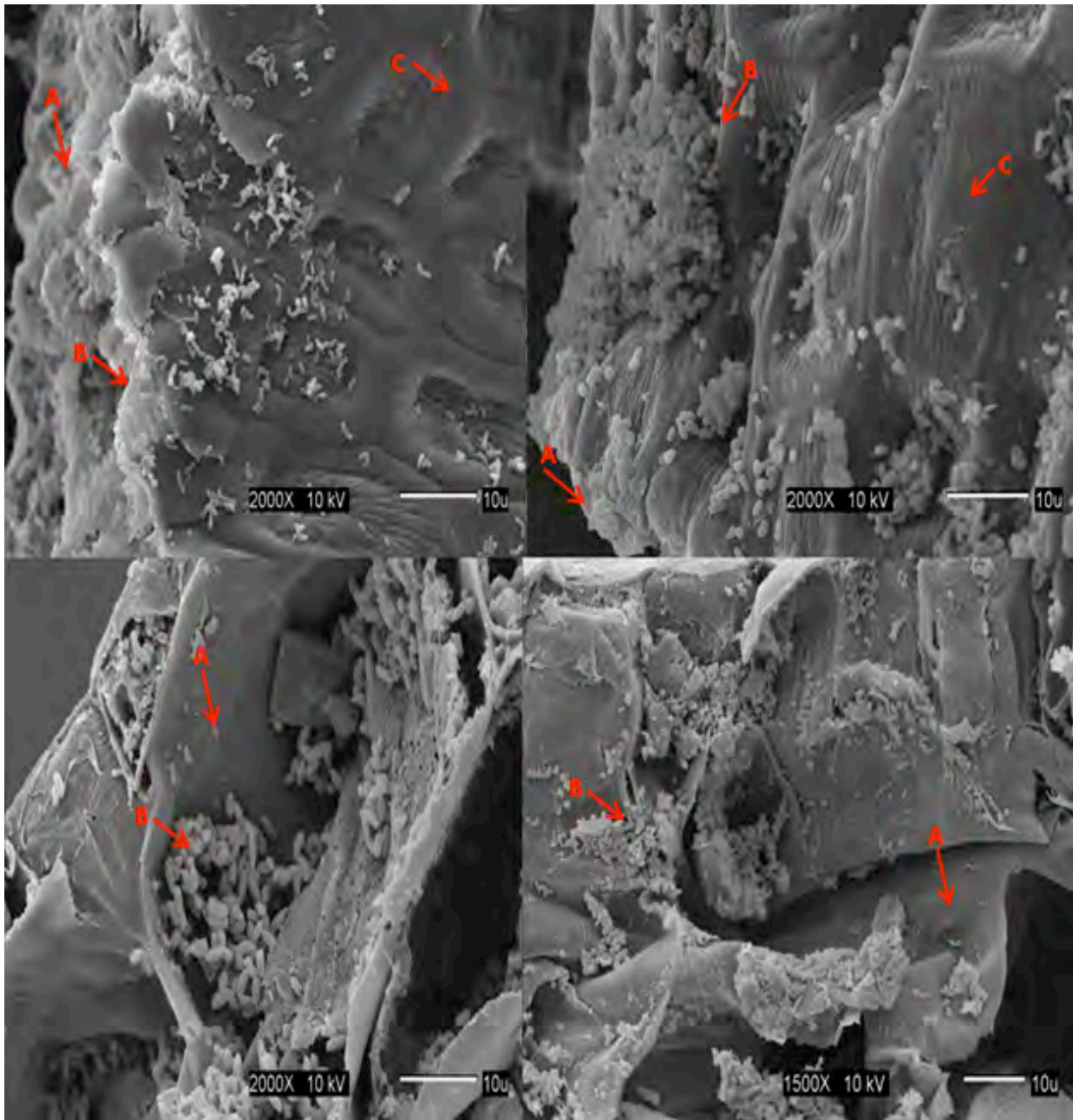
Figure 6.7 Scanning Electron Microscopy cross section of *T. testudinum* leaf showing the presence of endophytic bacteria within the parenchyma (a), *T. testudinum* leaf parenchyma with endophytic bacteria emerging from the leaf (b) and *T. testudinum* leaf surface (c).
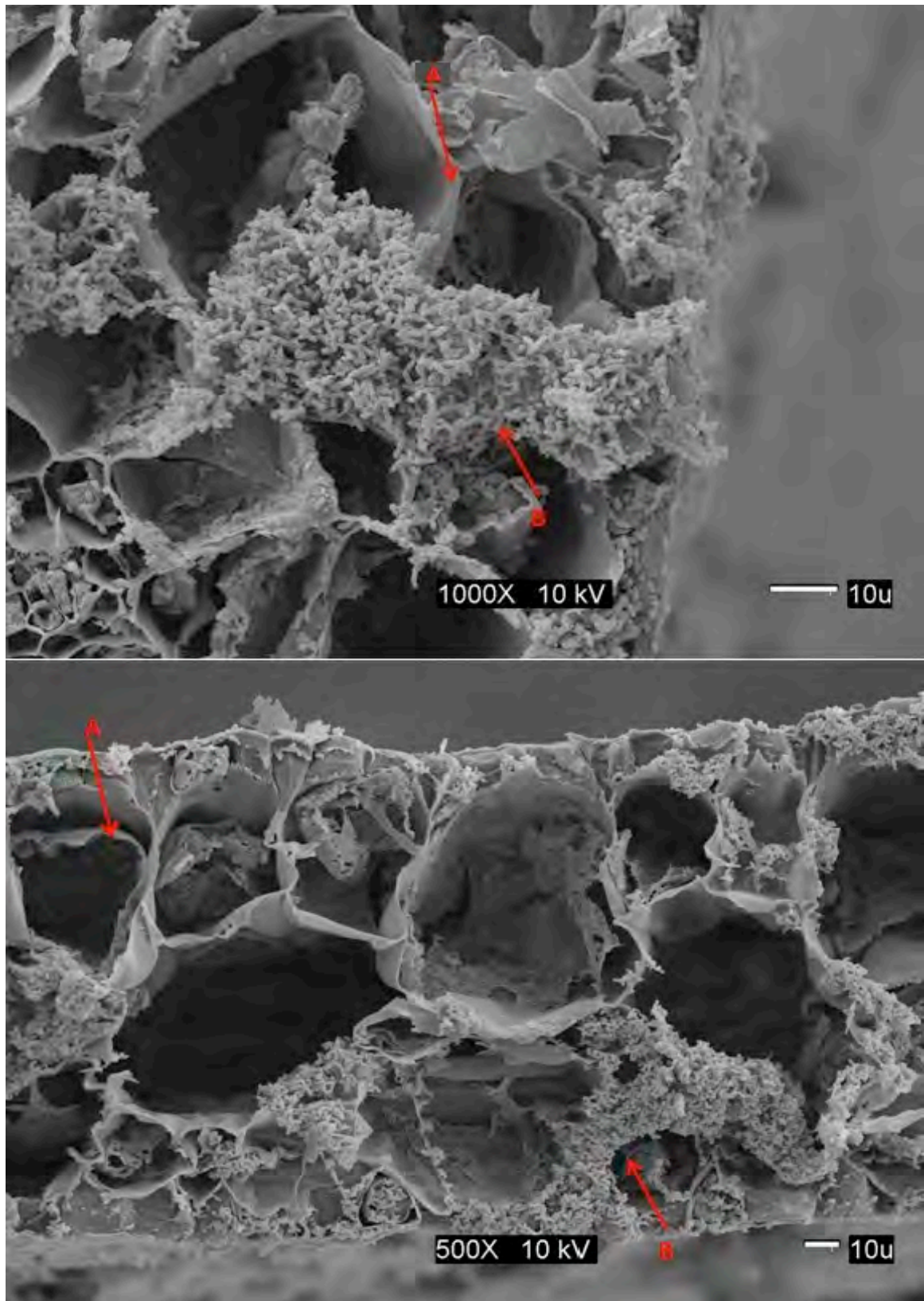
Figure 6.8 Scanning Electron Microscopy cross section of *T. testudinum* leaf parenchyma (a*)* and *T. testudinum*parenchyma harboring endophytic bacteria (b).
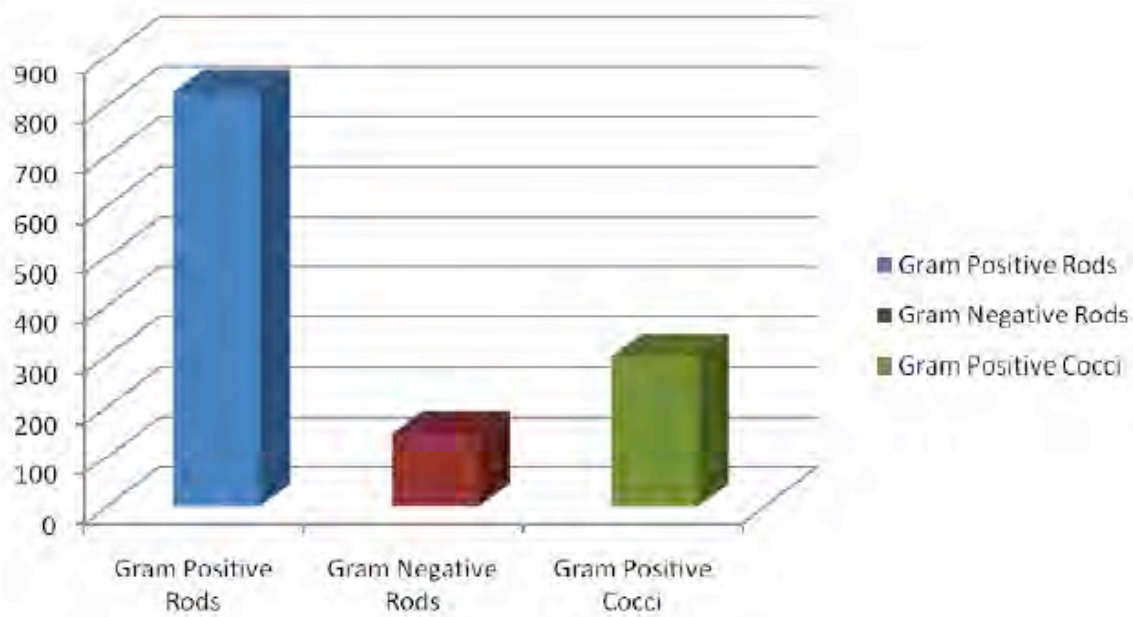
Figure 6.9 Bar chart representing the distribution of total isolated strains from all sampling sites based on microscopical characteristics. The y-axis denotes number of isolates.
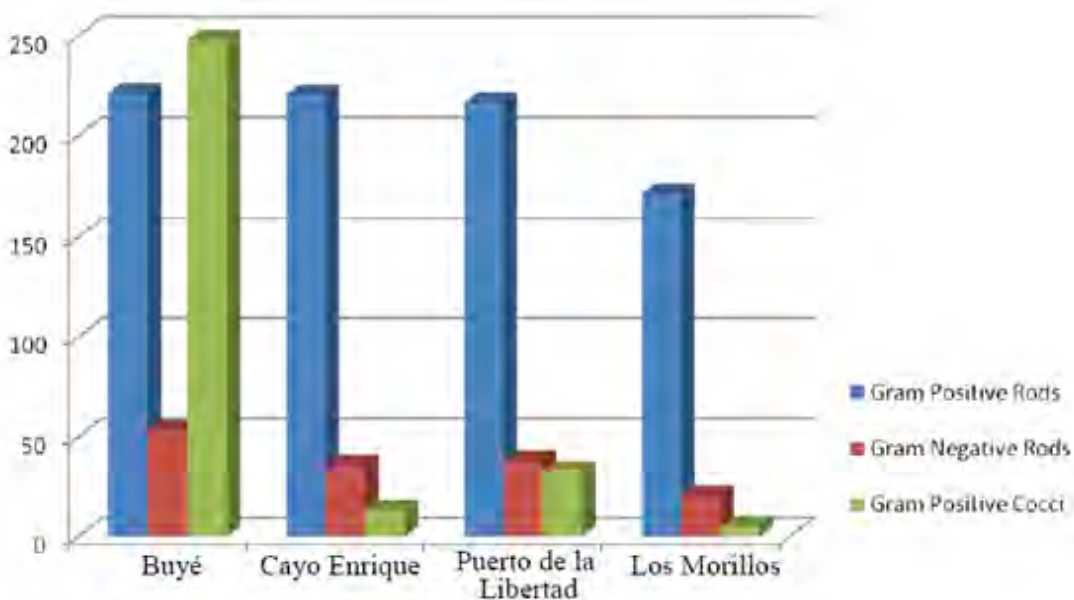
Figure 6.10 Bar chart representing the distribution of isolated strains among sampling sites based on Gram staining and morphology. The y-axis denotes number of isolates.

31
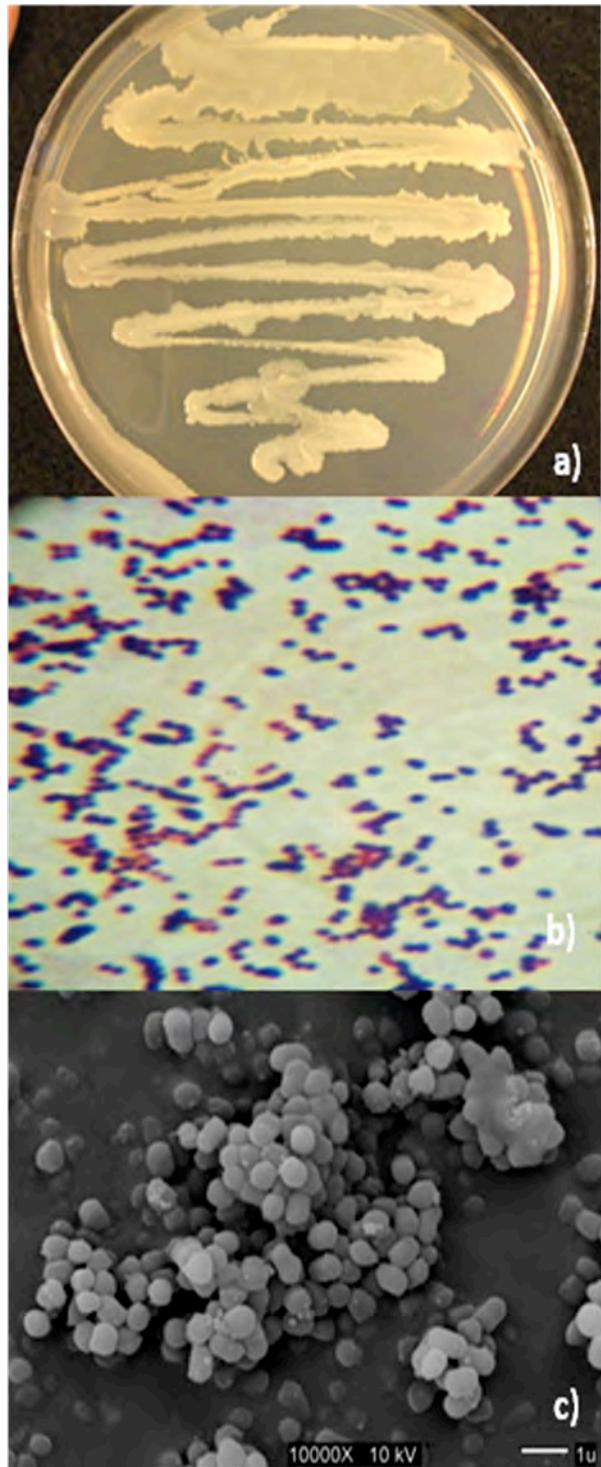
Figure 6.11 Macroscopical and microscopical characteristics of strain L20 on MA (a), Gram positive stain on light microscopy (b), and SEM of cocci shaped cells (c).
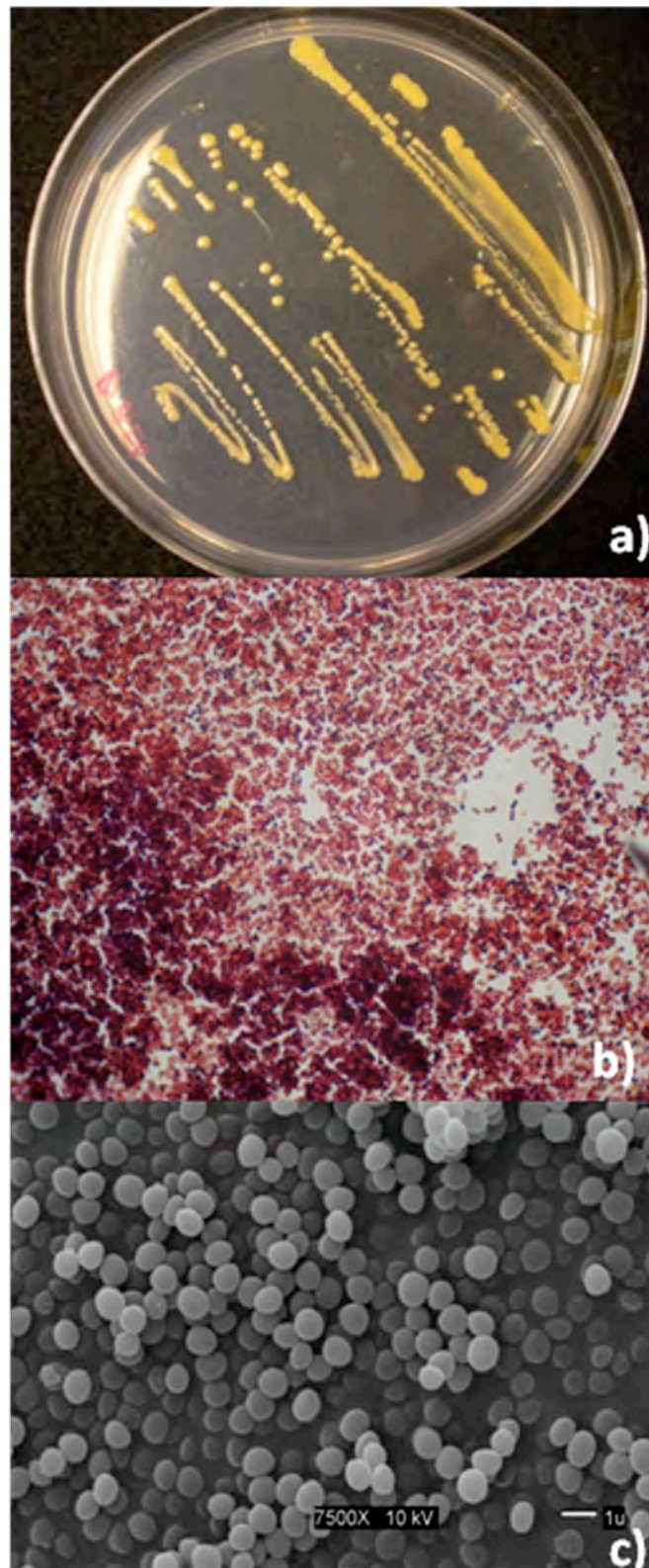
Figure 6.12 Macroscopical and microscopical characteristics of strain B 390 on MA (a), Gram positive stain on light microscopy (b), and SEM of cocci shaped cells (c).
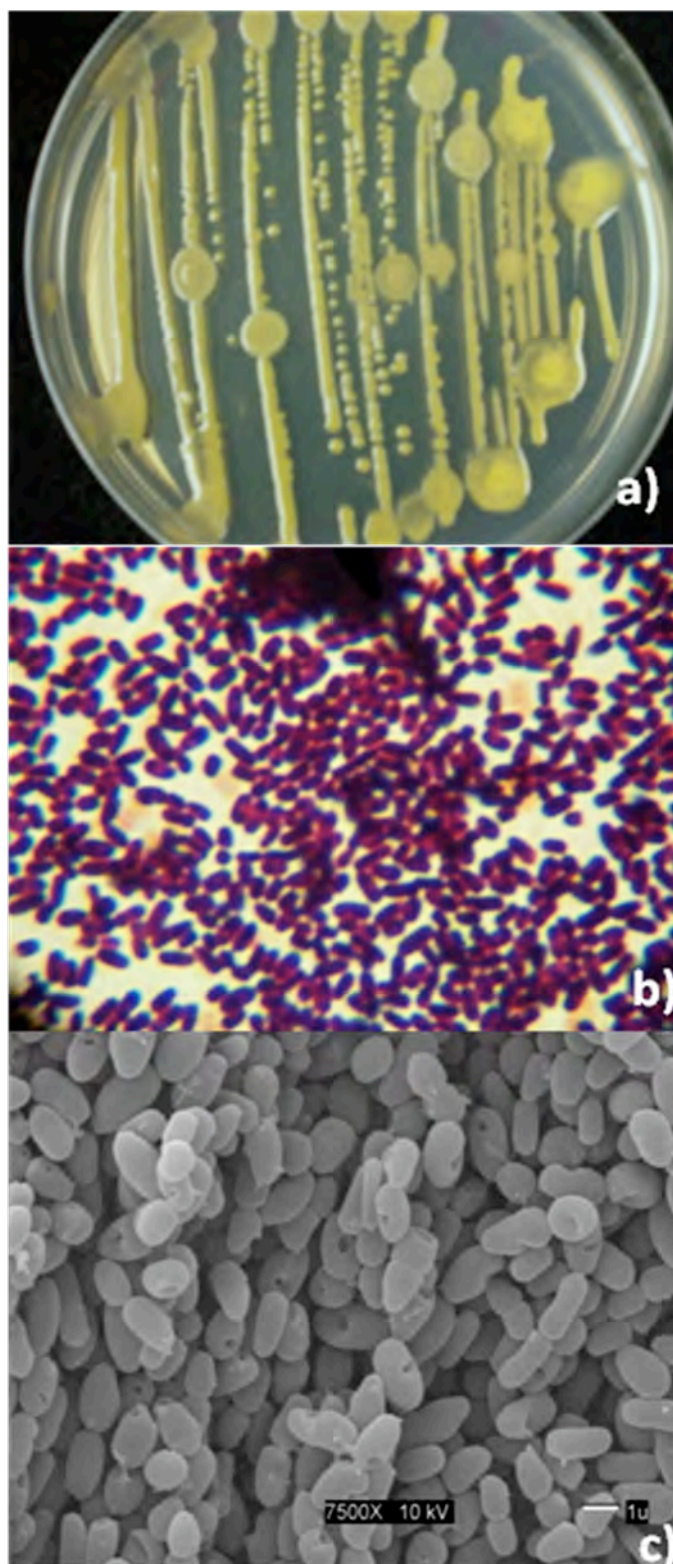
Figure 6.13 Macroscopical and microscopical characteristics of strain B419 on MA (a), Gram positive stain on light microscopy (b), and SEM of rod shaped cells (c).
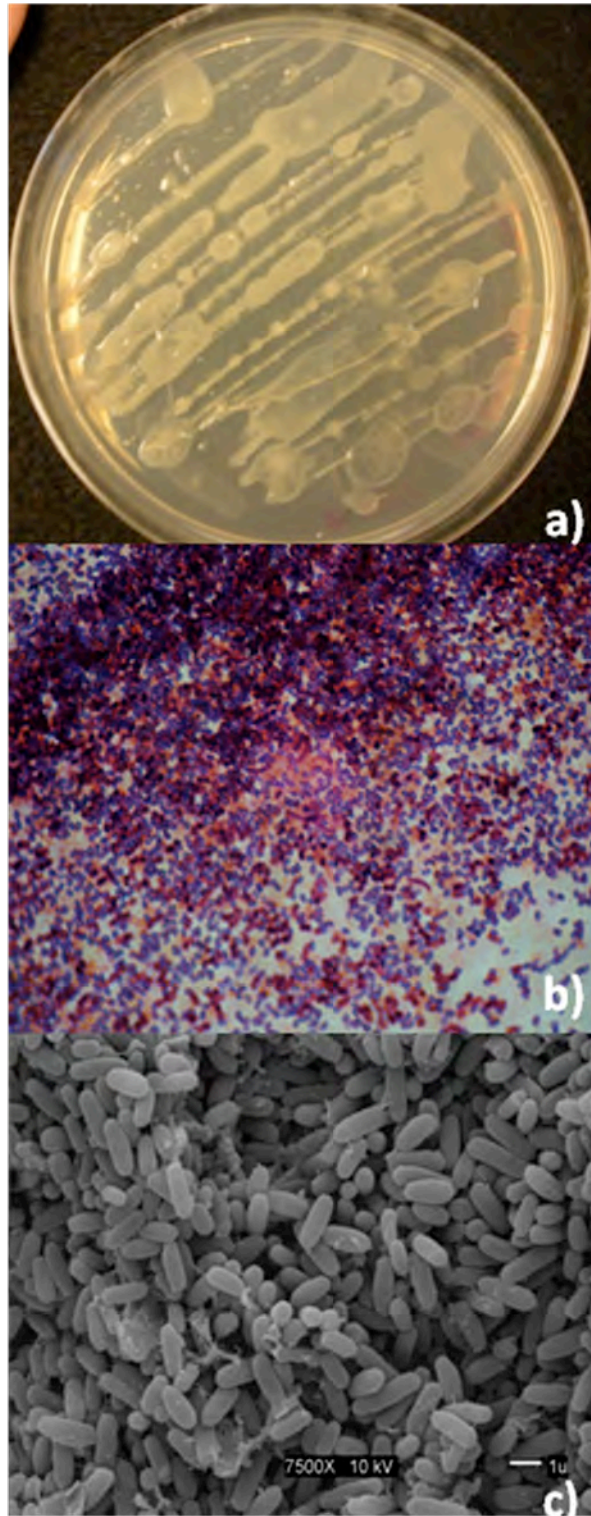
Figure 6.14 Macroscopical and microscopical characteristics of strain B 326 on MA (a), Gram positive stain on light microscopy (b), and SEM of rod shaped cells (c).
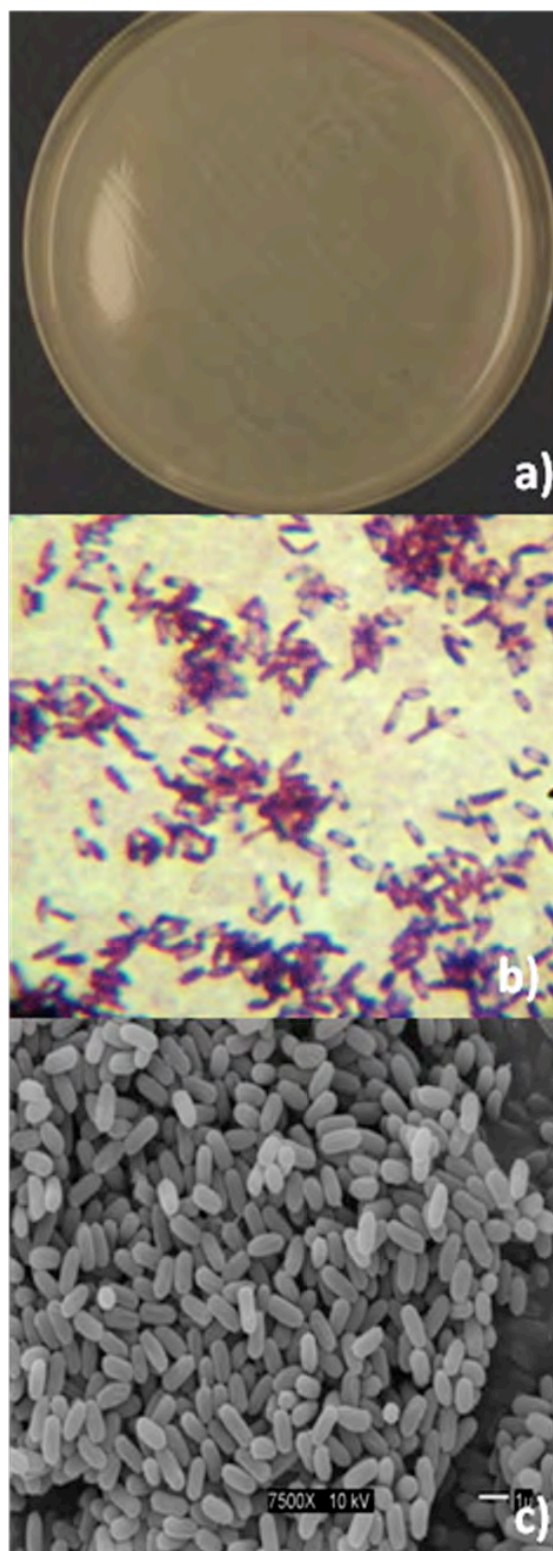
Figure 6.15 Macroscopical and microscopical characteristics of strain VQ 81 on MA (a), Gram positive stain on light microscopy (b), and SEM of rod shaped cells (c).
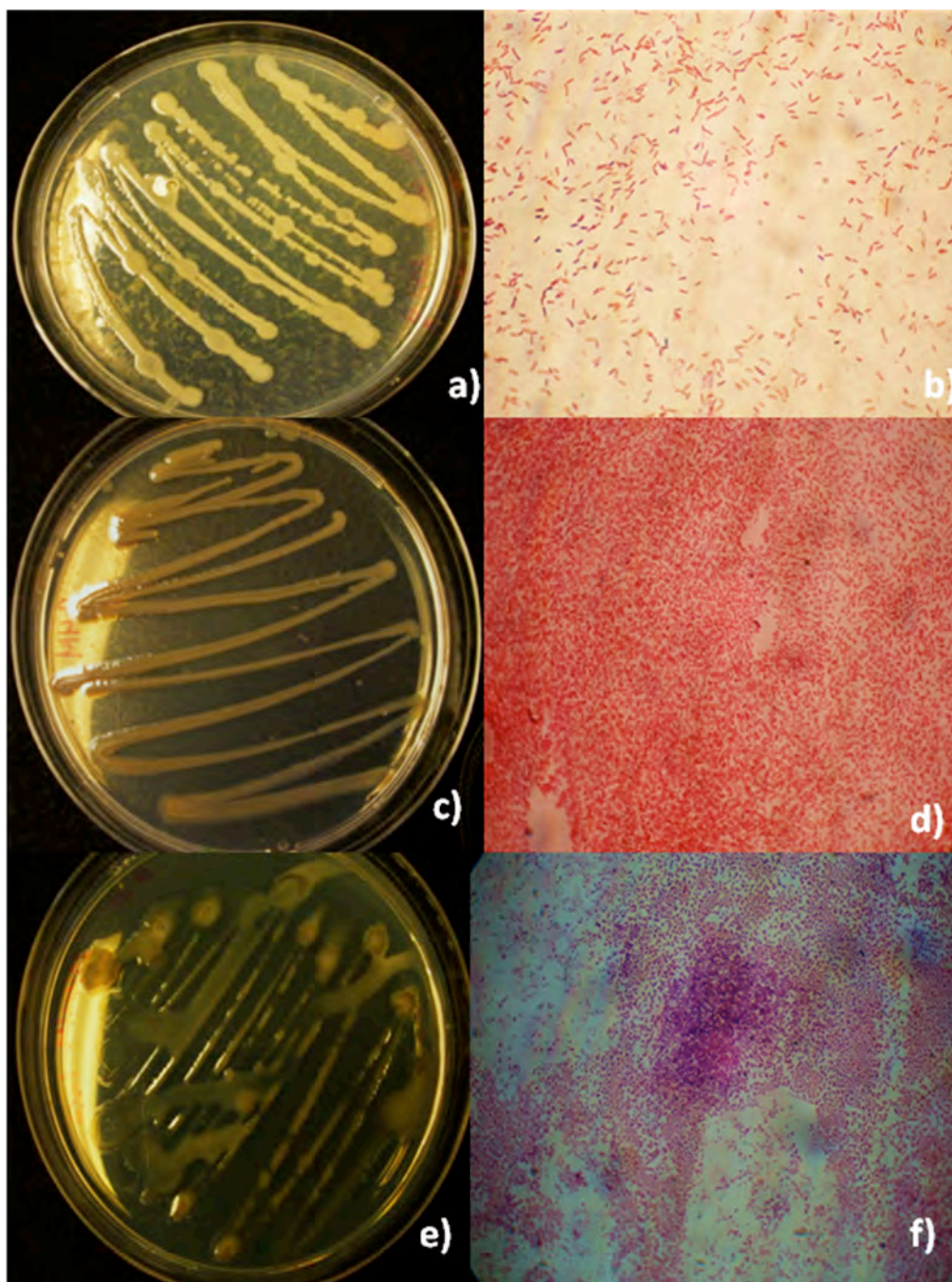
Figure 6.16 Macroscopical and microscopical characteristics of Strains B 208 on MA (a), Gram negative stain on light microscopy (b), Strain B 414 on MA (c), Gram negative stain on light microscopy (d), Strain B 117 on MA (e) and Gram positive strain on light microscopy (f).
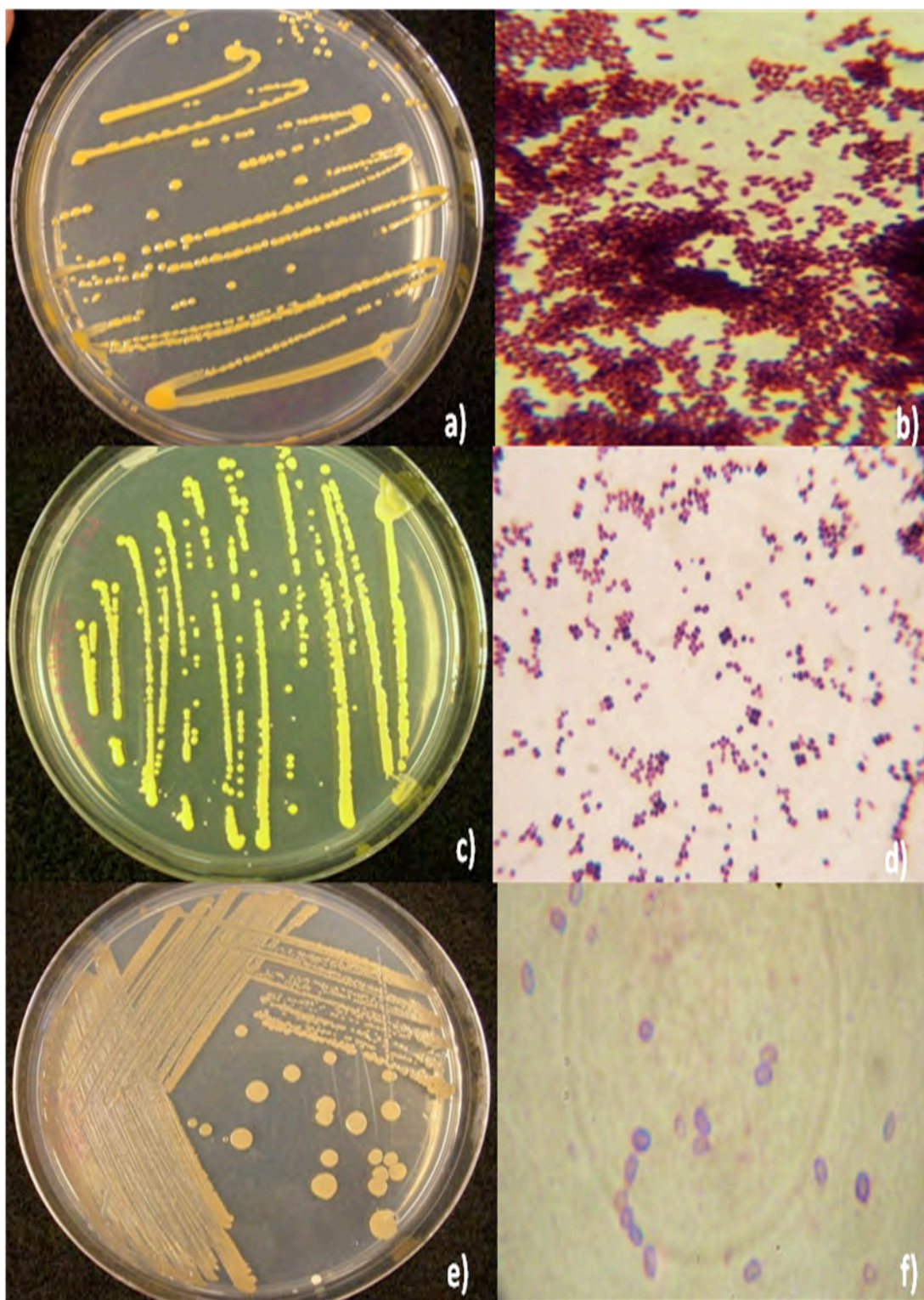
Figure 6.17 Macroscopical and microscopical characteristics of Strain VQ 37 on MA (a), Gram positive stain on light microscopy (b), Strain L19 on MA (c), Gram positive stain on light microscopy (d), Strain B 287 on MA (e), and Gram positive strain on light microscopy (f).
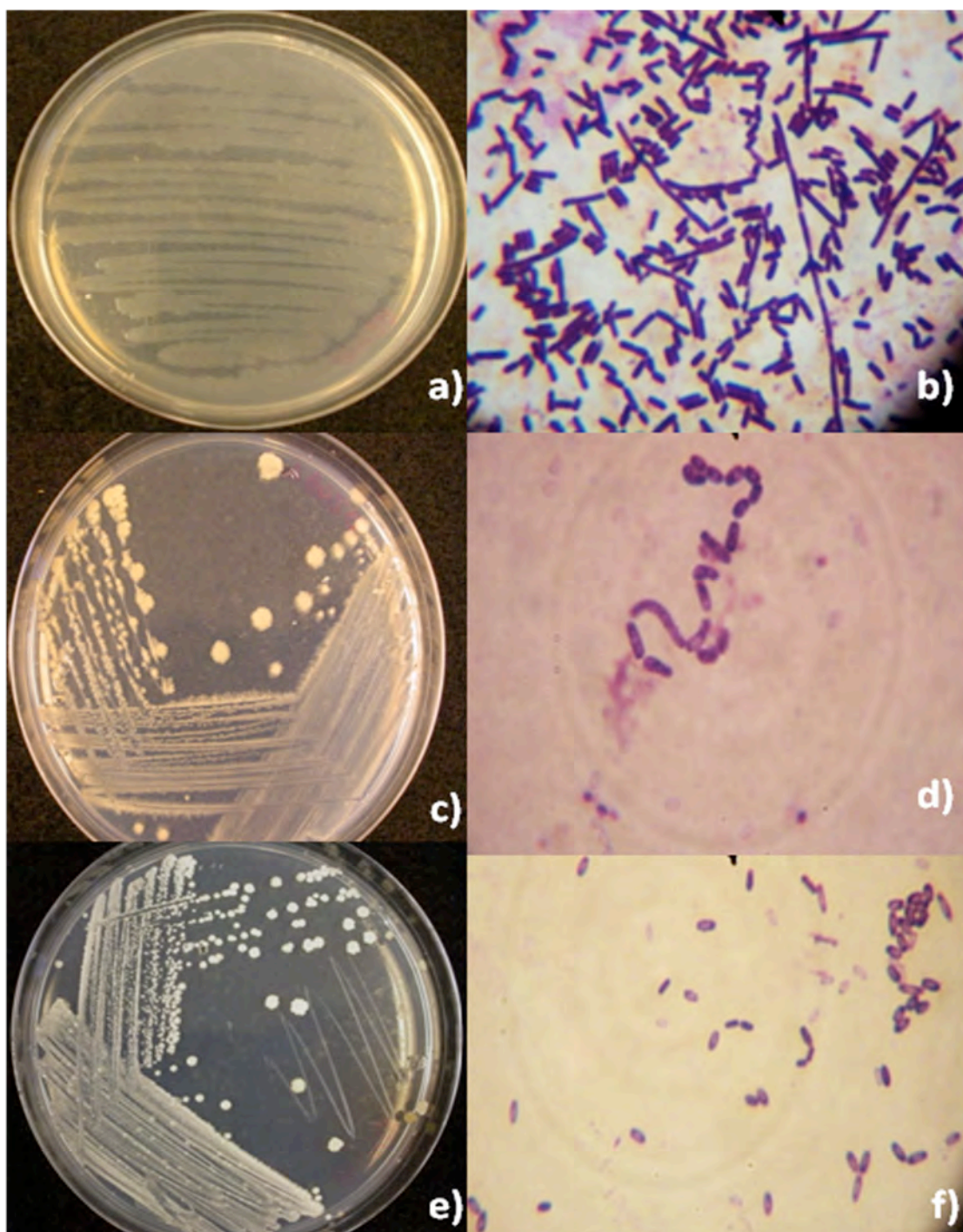
Figure 6.18 Macroscopical and microscopical characteristics of Strain B 420 on MA (a), Gram positive stain on light microscopy (b), strain B 268A on MA(c), Gram positive stain on light microscopy (d), Strain B 146 on MA (e), and Gram positive strain on light microscopy (f).
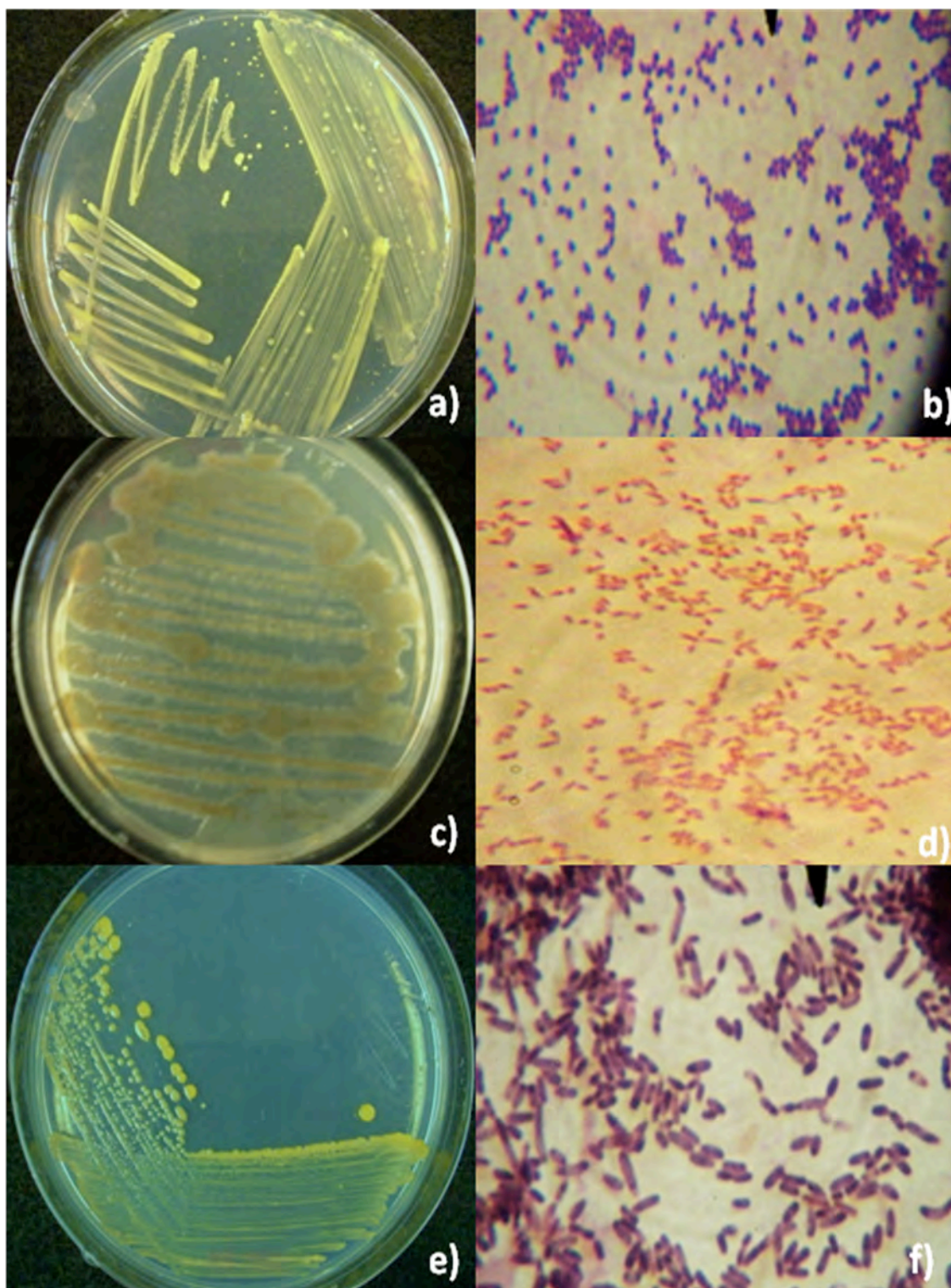
Figure 6.19 Macroscopical and microscopical characteristics of Strain VQ 57 on MA (a), Gram positive stain on light microscopy (b), strain B 110 on MA(c), Gram negative stain on light microscopy (d), Strain VQ 229 on MA (e), and Gram positive strain on light microscopy (f).
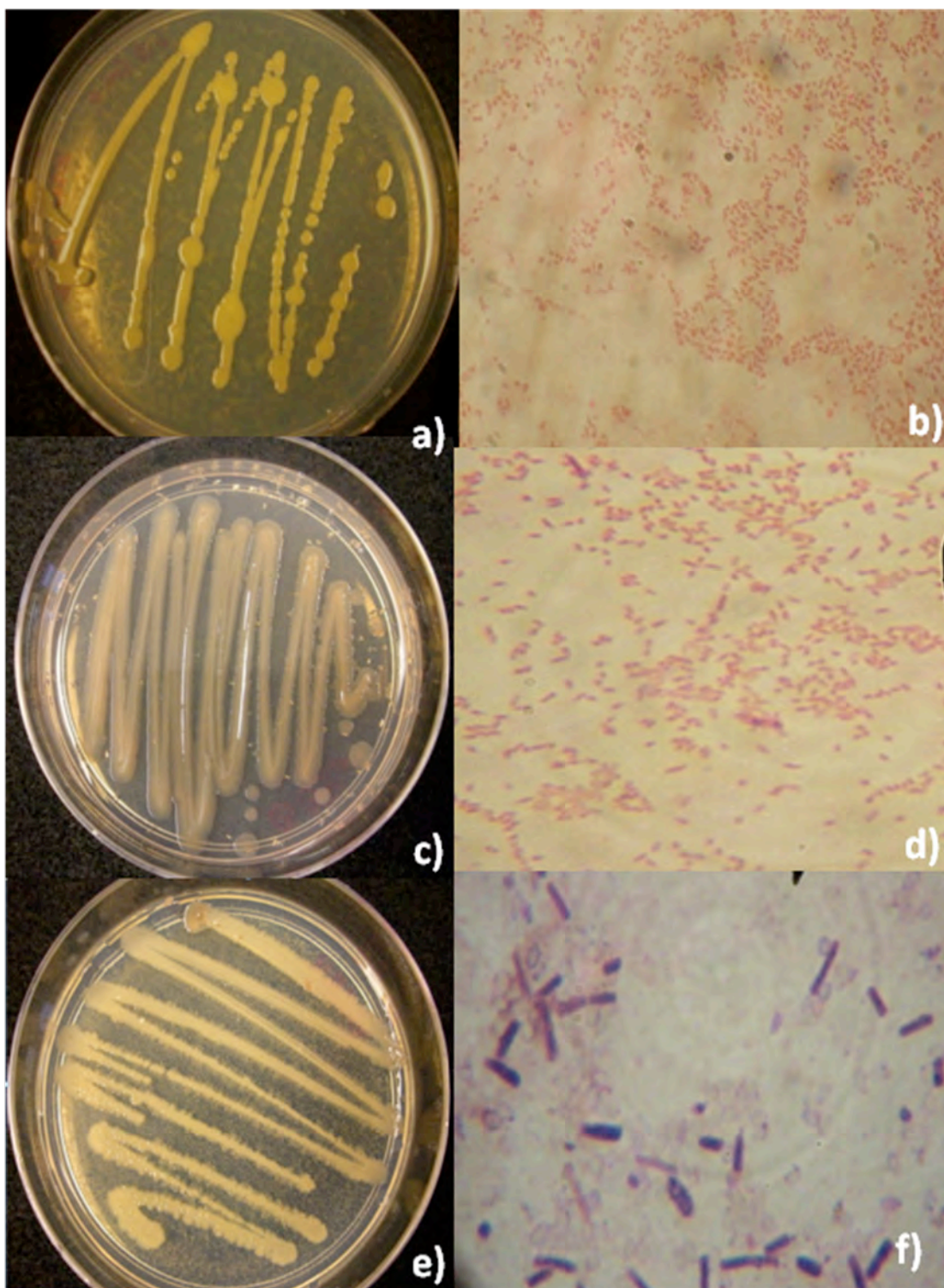
Figure 6.20 Macroscopical and microscopical characteristics of Strain VQ65B on MA (a), Gram negative stain on light microscopy (b), Strain B 356 on MA(c), Gram negative stain on light microscopy (d), Strain B 294 on MA (e), and Gram positive strain on light microscopy (f).
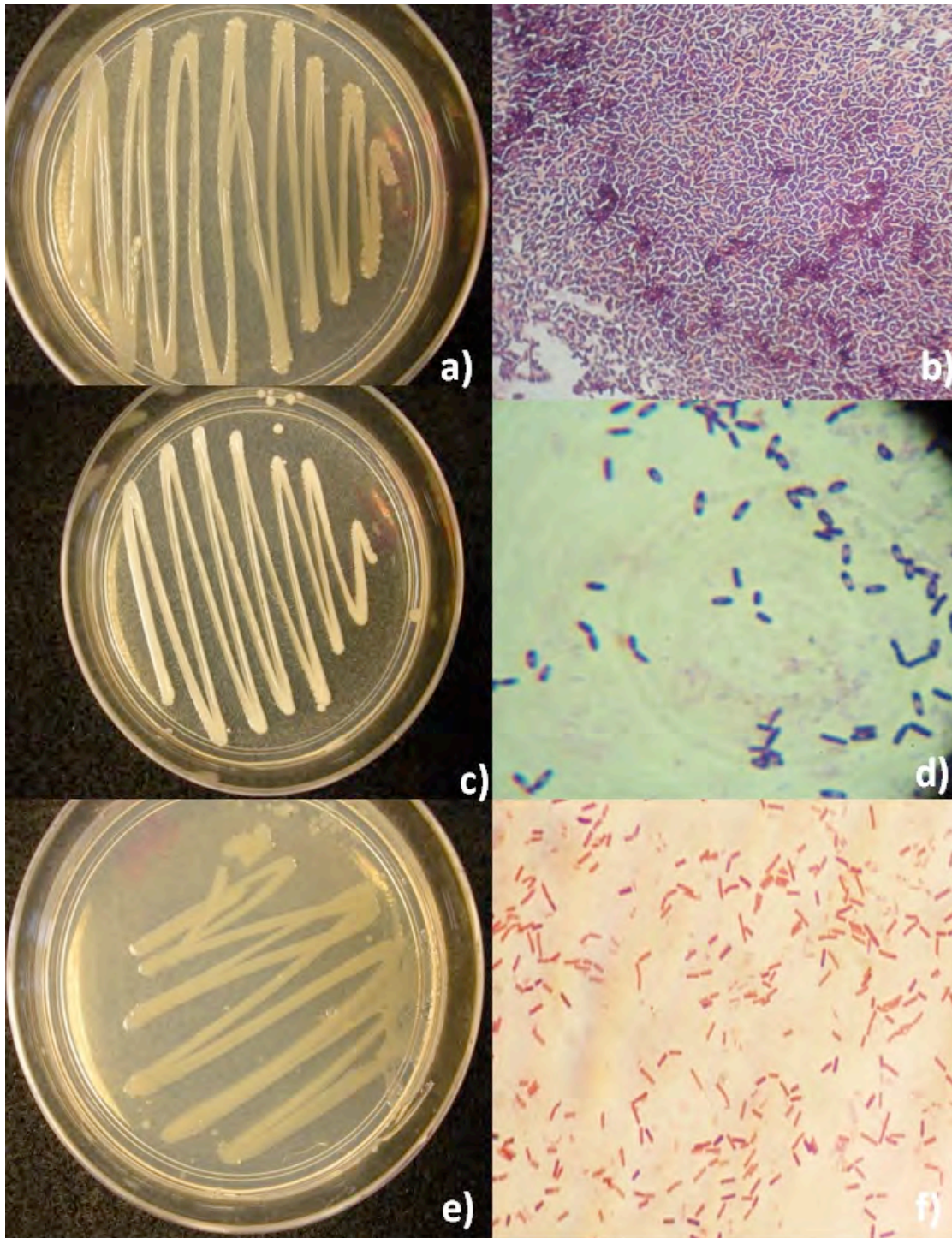
Figure 6.21 Macroscopical and microscopical characteristics of Strain B 399 on MA (a), Gram positive stain on light microscopy (b), strain B 52 on MA(c), Gram positive stain on light microscopy (d), Strain VQ 90 on MA (e), and Gram positive strain on light microscopy (f).
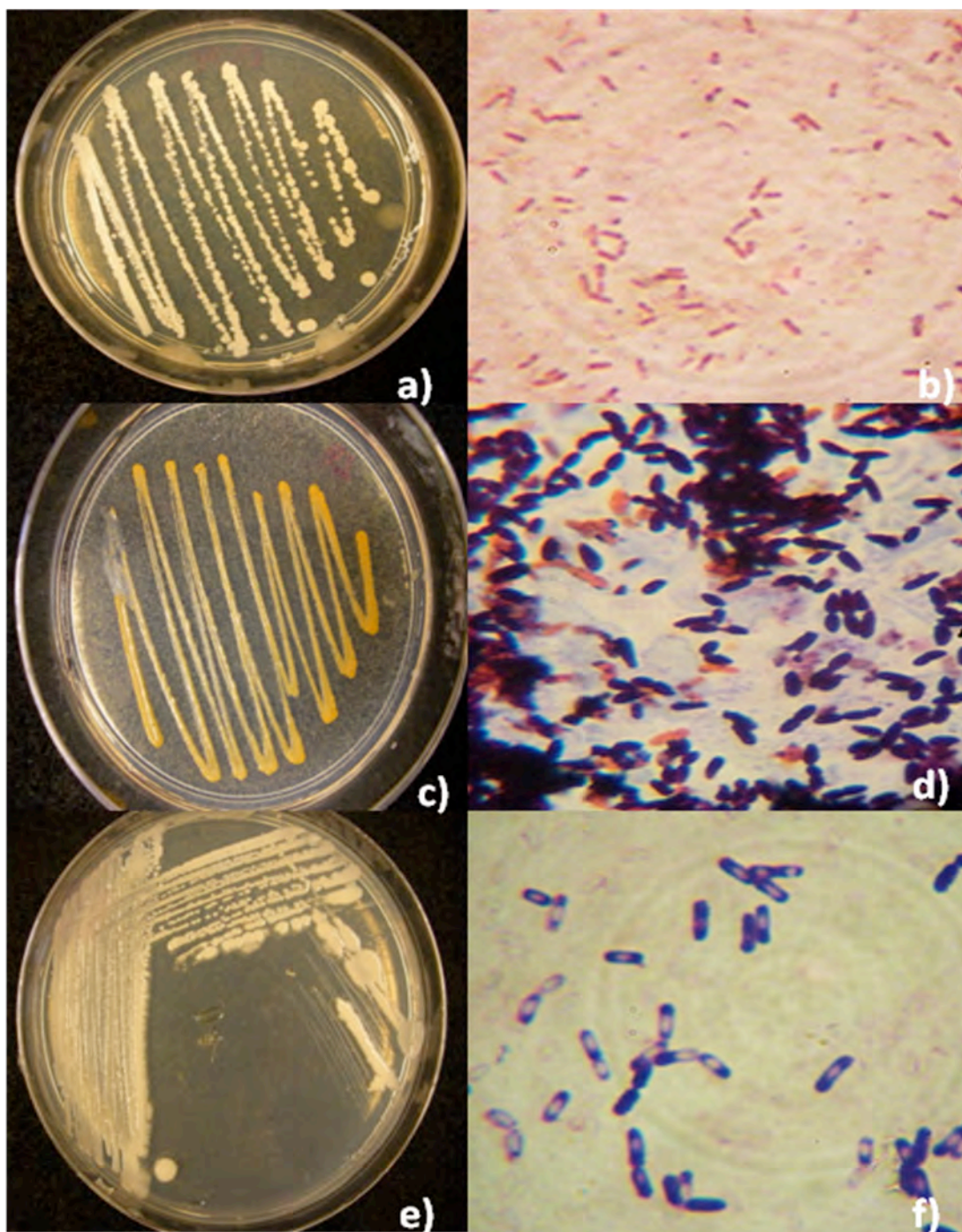
Figure 6.22 Macroscopical and microscopical characteristics of strains Strain B 34 on MA (a), Gram negative stain on light microscopy (b), Strain L8 on MA(c), Gram positive stain on light microscopy (d), Strain B 63 on MA (e), and Gram positive strain on light microscopy (f).

6.2.4 Molecular analysis of pure cultures

After macroscopical and miscroscopical characterization, strains were further characterized using molecular tools. The amplification of the 16S rRNA region was used for obtaining the phylogenetic affiliation of the strains after BOX-PCR analysis. Each isolate was submitted to a phenol/chloroform DNA extraction, Figures 1.23 to 1.25 demonstrate the quality of genomic DNA obtained from representative strains.
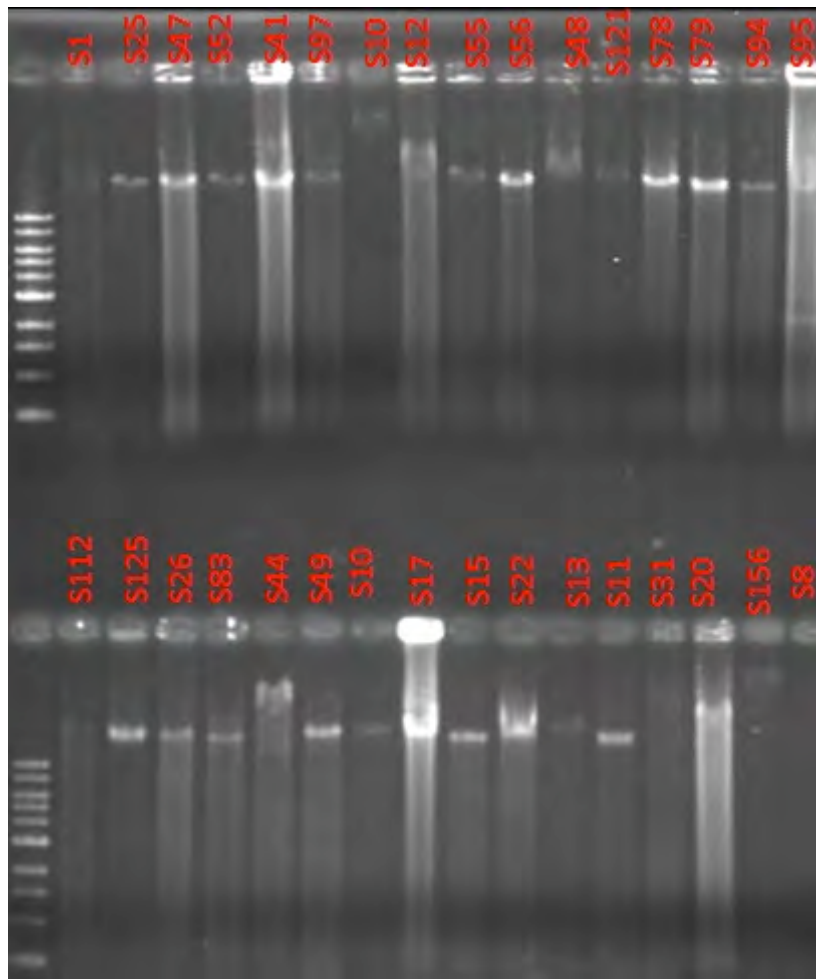
Figure 6.23 Genomic DNA extractions of endophytic strains isolated from *T. testudinum* from *Los Morillos s*howing high concentration yields of extraction protocol.
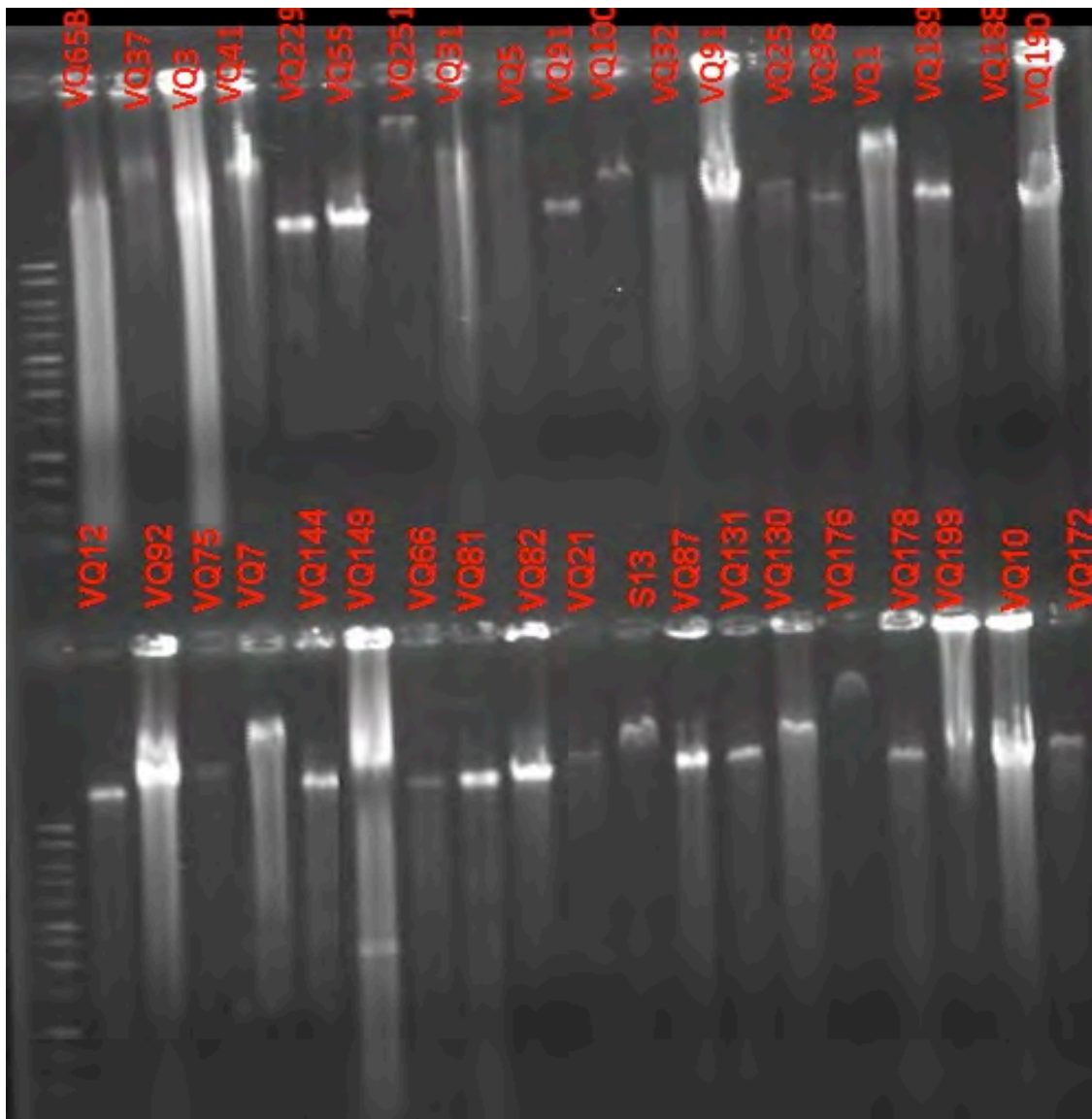
Figure 6.24 Genomic DNA extractions of endophytic strains isolated from *T. testudinum* of *Puerto de la Libertad, Vieques s*howing high concentration yields of extraction protocol.
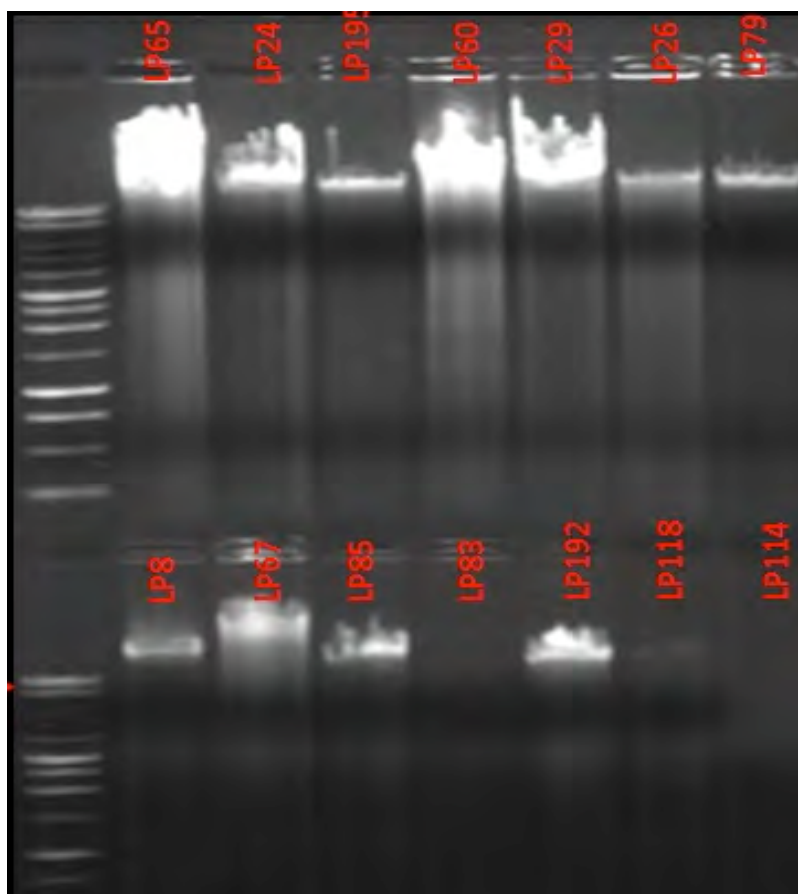
Figure 6.25 Genomic DNA extractions of endophytic strains isolated from *T. testudinum* of *Cayo Enrique, Lajas s*howing high concentration yields of extraction protocol.

Due to the numerous diversity of isolates, a preliminary screening was performed using the BOX PCR technique. In this fingerprinting method, PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain specific DNA fragments which can be easily analyzed by pattern recognition (Dombek et al., 2000). Due to the high resolution of this technique, the amplicon patterns allowed discrimination and classification of isolates into groups. Therefore, one strain from each group was selected and used for subsequent molecular and phylogenetic analysis. Because this approach has not been employed extensively on endophytic microorganisms, several optimizations were performed before obtaining optimal and

reproducible fingerprint patterns. Genomic DNA was quantified using the Nanodrop ND-1000 Spectrophotometer© and concentration was adjusted so that 50 ng of template DNA were added to the BOX-PCR amplification. Fingerprints were verified in a 2.5 % low melting agarose gel and further analyzed for group classification (Figure 1.26 – Figure 1.31).

Genotypic BOX-PCR fingerprints corroborated the high diversity observed on colony morphotypes. It is important to point out the contrast of diversity observed within the the sampling sites. Buyé Beach and *Puerto de la Libertad* demonstrated high diversity in fingerprint patterns, while *Cayo Enrique* and *Los Morillos* were more homogenous having low diversity of fingerprints. Interestingly, the most frequent pattern in *Cayo Enrique* is the same for *Los Morillos*.
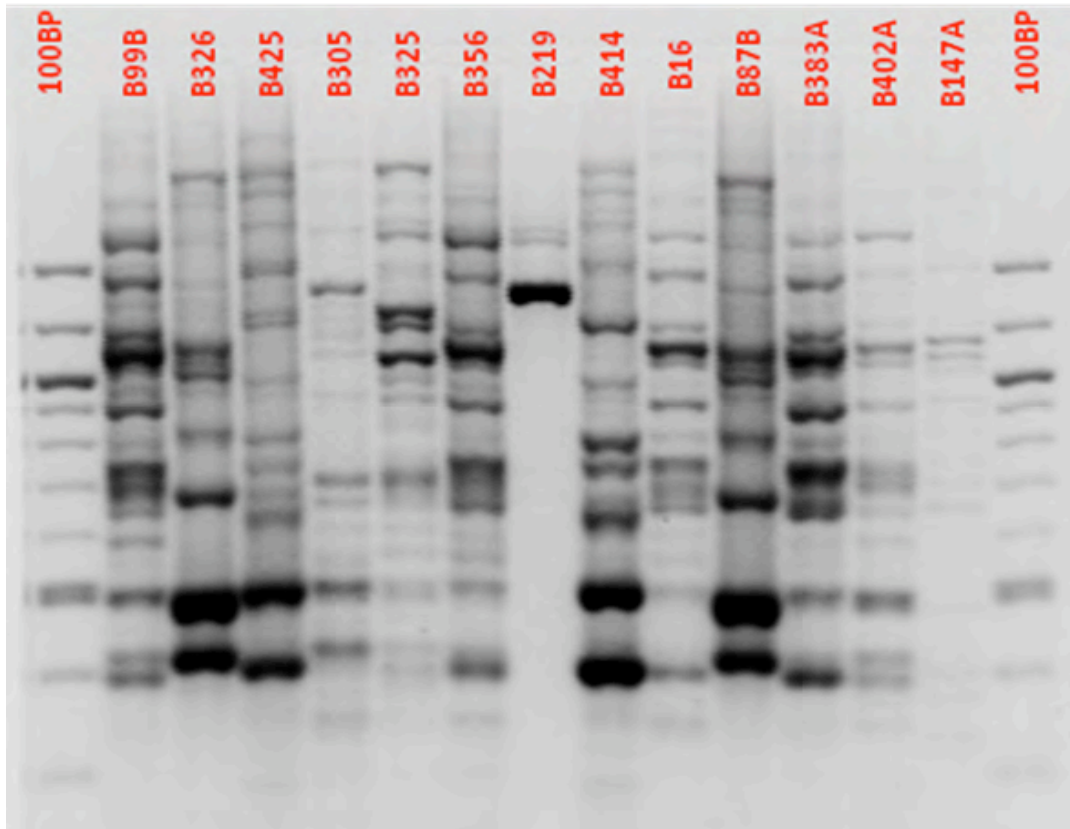
Figure 6.26 BOX-PCR fingerprints obtained from representative cultures recovered from Buyé Beach, *Cabo Rojo* demonstrating high diversity of patterns
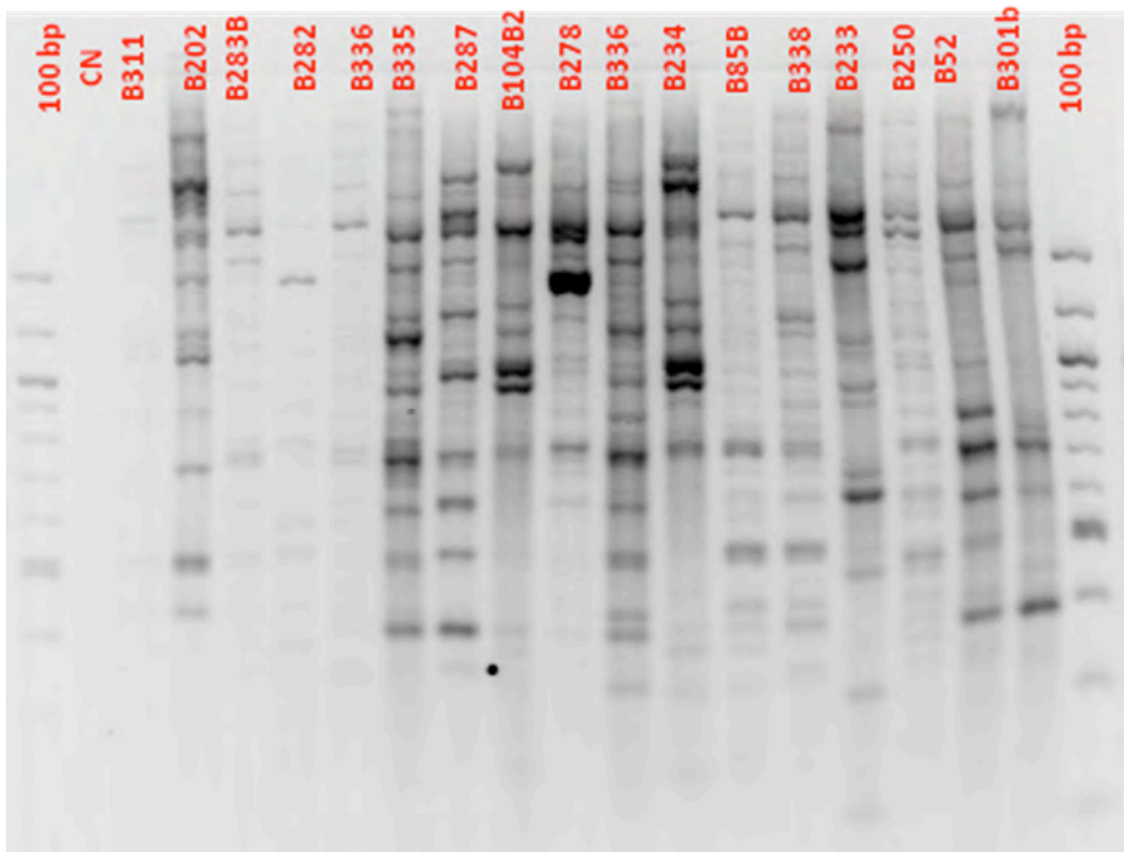
Figure 6.27 BOX-PCR fingerprints obtained from representative cultures recovered from *Buyé Beach, Cabo Rojo* demonstrating high diversity of patterns
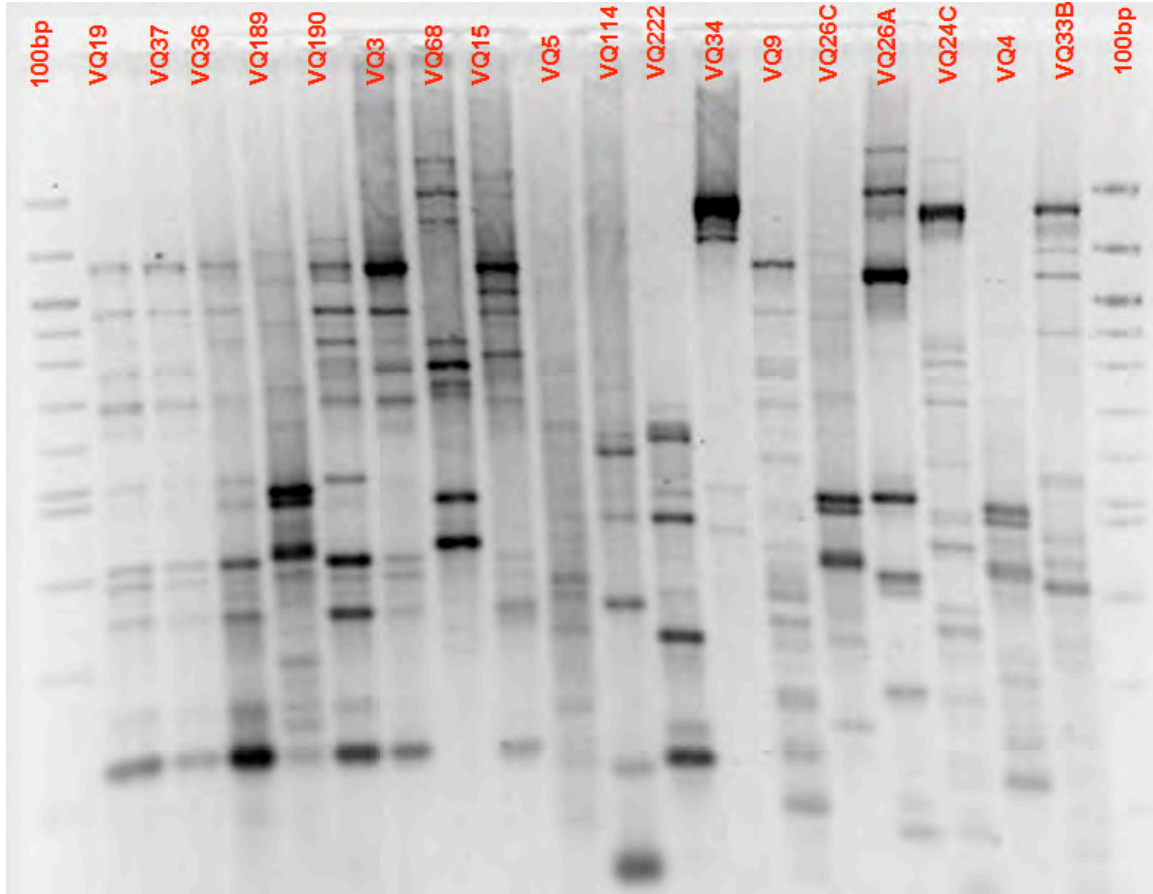
Figure 6.28 BOX-PCR fingerprints obtained from representative cultures recovered from *Puerto de la Libertad, Vieques* demonstrating high diversity of patterns
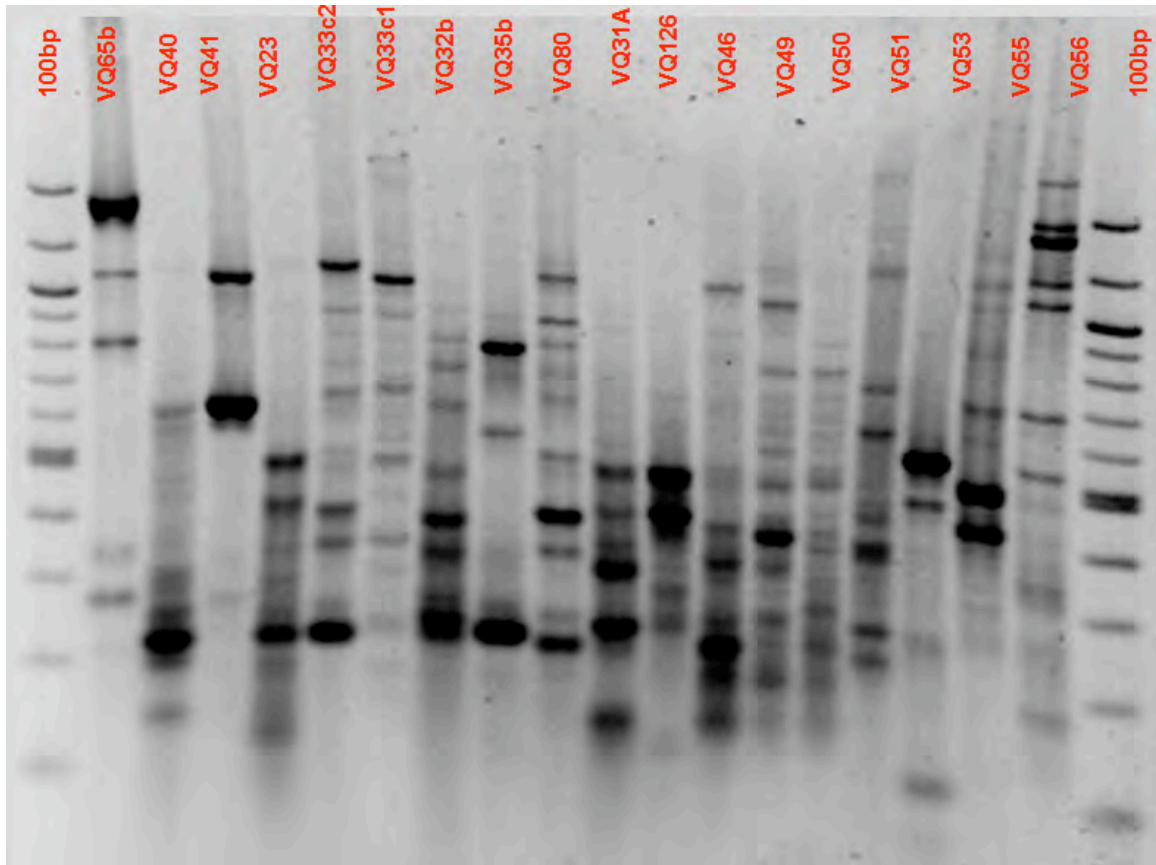
Figure 6.29 BOX-PCR fingerprints obtained from representative cultures recovered from *Puerto de la Libertad, Vieques* demonstrating high diversity of patterns
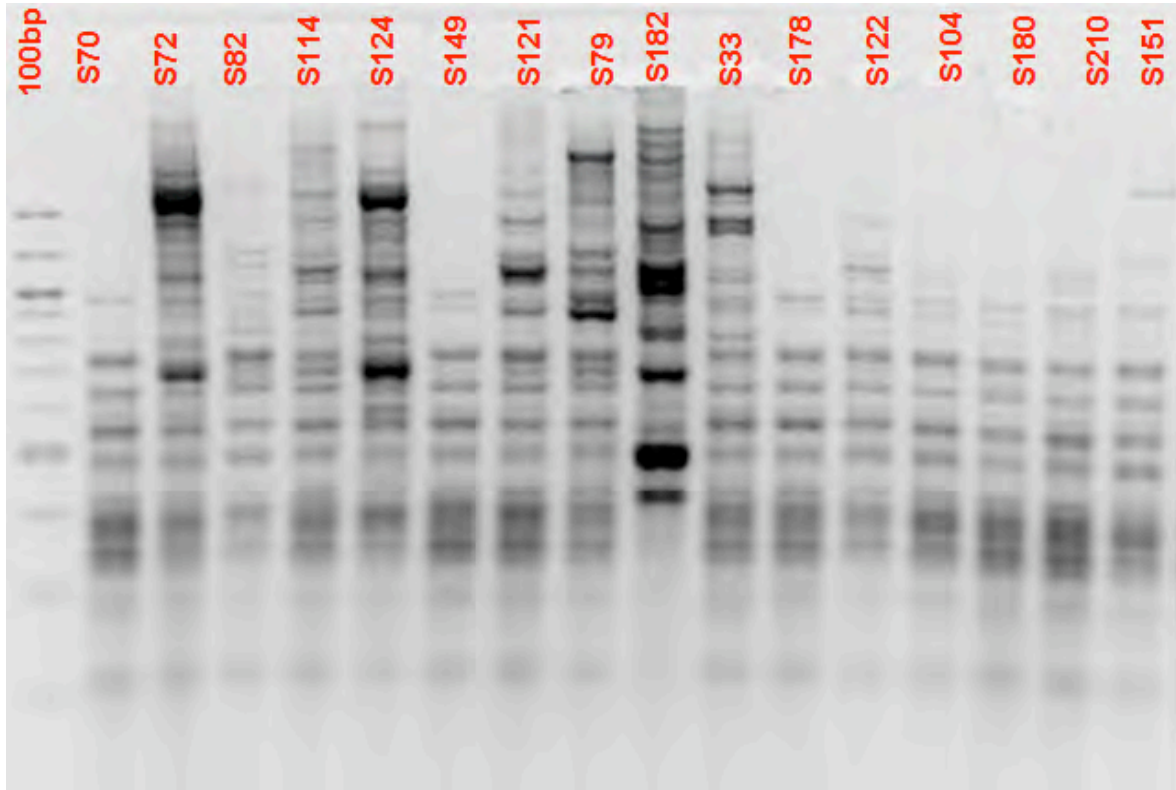
Figure 6.30 BOX-PCR fingerprints obtained from representative cultures recovered from *Los Morillos, Cabo Rojo* showing low diversity of patterns.
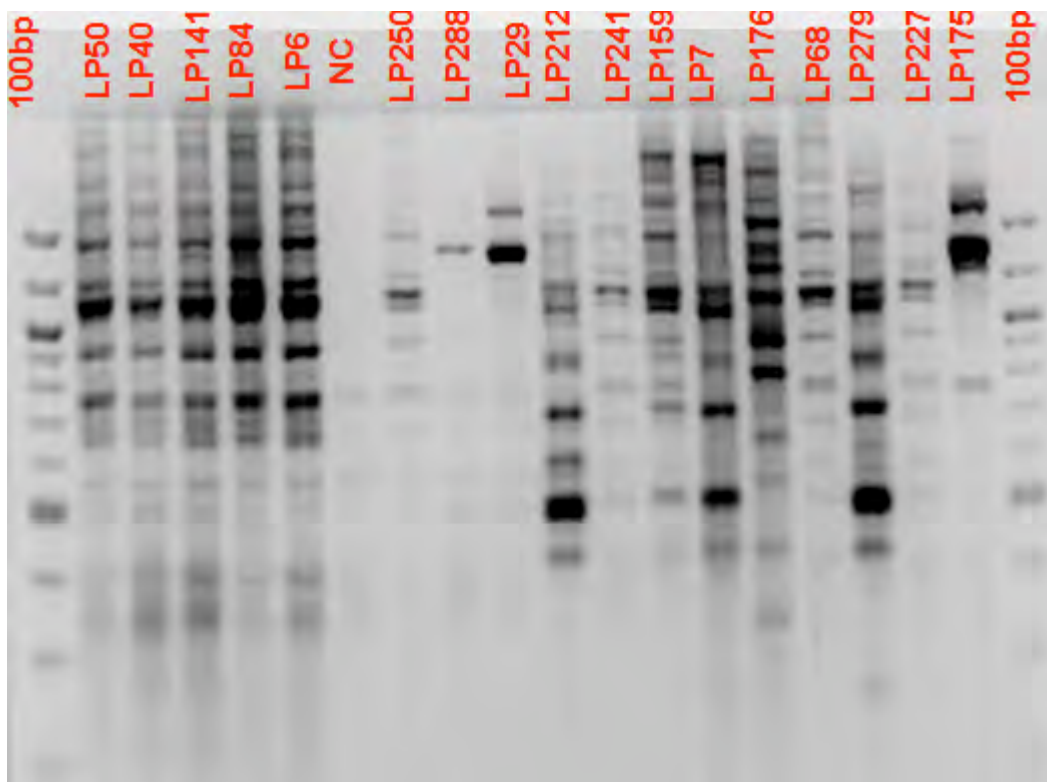
Figure 6.31 BOX-PCR fingerprints obtained from representative cultures recovered from *Cayo Enrique, Lajas* showing low diversity of patterns.
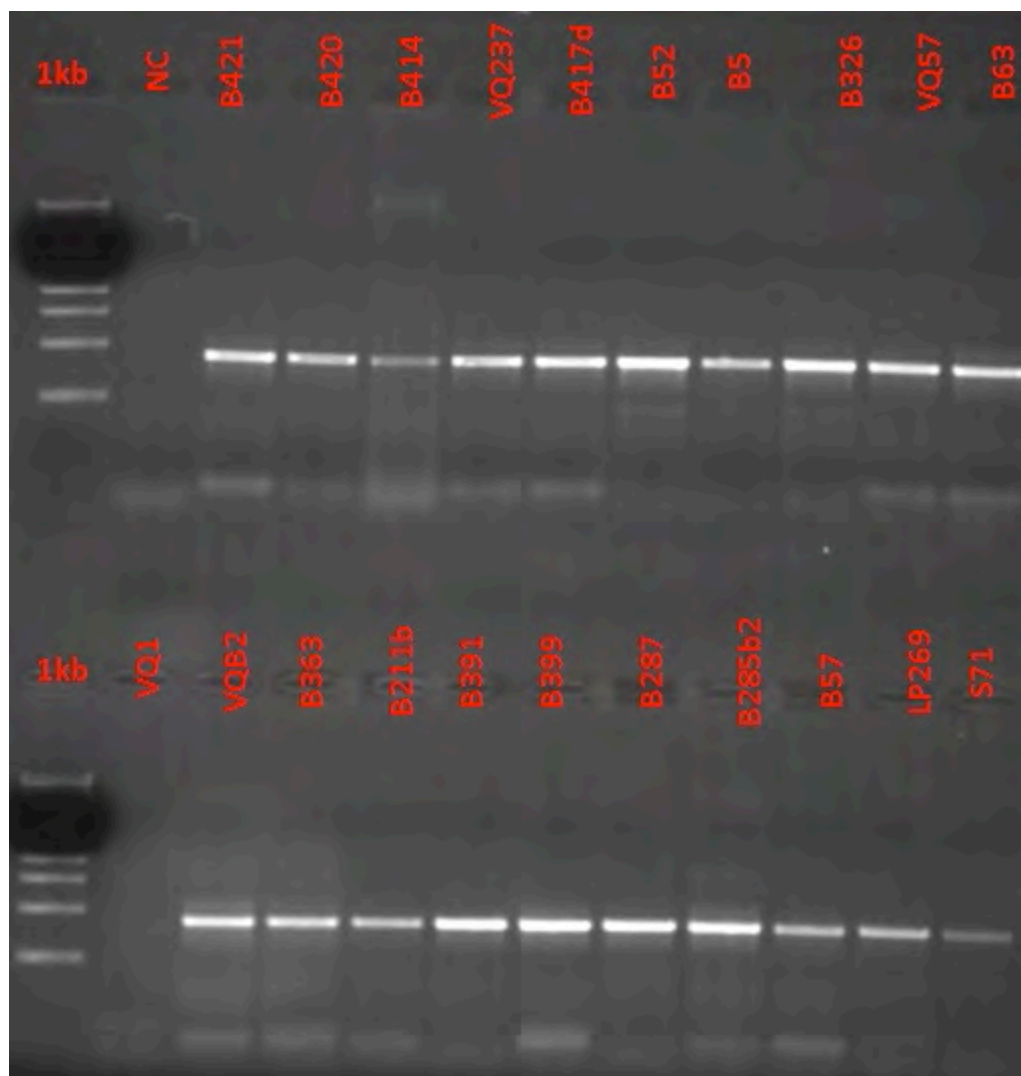
Figure 6.32 16S rRNA amplification using universal primers for representative strains isolated from *T. testudinum.Molecular marker corresponds to 1kb ladder DNA marker(New England, Biolabs, MA, USA)*

For the analysis of BOX-PCR fingerprints, the molecular weights of DNA fragments were estimated using the migration rates in the agarose gel as described by Bloom et al. (1996). A total of 107 different patterns were observed in with the BOX-PCR reactions performed.   Strains representing different BOX-PCR patterns were selected for further *in silico* analysis, and then a 16S rRNA PCR reaction was performed to a representative of each group (Figure 1.32). PCR amplicons with an approximate size

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of 873bp were purified and sent to UW-htseq Sequencing Facility. Sequences were verified for quality and the phylogenetic relationships were determined by constructing a neighbor joining tree. Although 107 fingerprints were observed, some of the strains that exhibited different BOX PCR patterns matched with the same 16S rRNA from a type strain, so only one isolate was selected for the construction of phylogenetic trees.

The phylogenetic analysis revealed that the majority of the strains recoveredbelonged to the *Firmicutes* group. The predominant genus within the *Firmicutes* was *Bacillus ,* which are ubiquitous and versatile microorganisms that have been isolated from diverse environments (Figures 1.45 and 1.46). Different strains within the *Bacillus* genus were the most frequently recovered bacteria in this study. Strain B63 was clustered with *Bacillus thuringiensis, B. anthracis* and *B. cereus.* Strain B399 formed a branch with *B. gibsonii* and *B. plakortidis*, while B218 was closely related with *B. firmus*. Strain LP 89 formed a cluster with *B. megaterium* and *B. flexus* and strain VQ 251 is phylogenetically affiliated to *B. endophyticus*. Strains VQ190, VQ33B, VQ7, VQ37B, VQ182, VQ81b and VQ209 formed a cluster with *B. pumilus, B. aerius* and *B. altitudinis* as closest relatives. Strain B421 formed a branch in association with *B. algicola*, while B 426 formed a branch with *B. safensis* and *B. pumilus*. Strain VQ 237 deeply branched within *B. pocheonensis, B. isabelliae* and *B. acidicola.*

Other genera found within the *Firmicutes* were *Exiguobacterium, Staphylococcus, Geobacillus and Halobacillus.* The second most represented genera within the *Firmicutes* were *Halobacillus spp.* (Figure 1.44). Strains B108, VQ419 and VQ 81 are closely related to *Halobacillus kuroshimensis*, strain VQ150 is related to *H. profundi* and strain B430b is associated with *H. mangrovi*. The third most frequent group corresponded t*o*

Staphylococcus sp. (Figure 1.43). Strains B363 is phylogenetically close to *Staphylococcus succinus* and *S. saprophyticus subsp. bovis* and strain 211B deeply branched within this cluster. Strain B326b formed a cluster with *S. xylosus* and *S. saprophyticus subsp. saprophyticus*. Strain VQ 71 is associated with *Exiguobacterium aestuarii* and *E. profundum* (Figure 1.35). Finally, strain L8 was related to *Geobacillus vulcani* and *G. thermoleoverans* (Figure 1.47).

Another abundant group recuperated in this study were members from the *Actinobacteria* related to *Micrococcus, Nesterenkonia* and *Rhodococcus.* Strain L25 belonged to the *Rhodococcus* genus and forms a branch with *Rhodococcus gordoniae* (Figure 1.37). Strain L20 was related to *Nesterenkonia* species being *Nesterenkonia aethiopica* the closest relative (Figure 1.33). Strain L19 was affiliated to *Micrococcus luteus* (Figure 1.36). Finally, strain VQ 57 was associated with *Cellulosimicrobium*, forming a branch with *Cellulosimicrobium funkei* and *Cellulosimicrobium cellulans* (Figure 1.38).

Various isolates pertained to the *gamma-Proteobacteria* group, which were associated with *Pseudomonas, Pseudoalteromonas, Enterobacter, Chromohalobacter, Microbulbifer* and *Cobetia*. This group was one of the most diverse but was less frequent within the isolated strains. Strain B5 was closely related to the marine strain *Cobetia marina* (Figure 1.32), while strain S25 was affiliated to *Chromohalobacter salarius* (Figure 1.49). *Pseudomonas* strains B268, B414, B417d, B87b2, LP254 and B425 formed a cluster with *Pseudomonas aeruginosa* (Figure 1.40). Strain B158b formed a deep branch where its closest relatives were *Pseudomonas lini* and *Pseudomonas cedrina* (Figure 1.41). Strain VQ65 was a close relative of *Microbulbifer celer* (Figure 1.48),

while strain S1 was related to *Pseudoalteromonas rubra* (Figure 1.50). The most represented genus was *Enterobacter* where strains B385, B326, B56, VQ95A, B222 and B208 formed a cluster with strains *Enterobacter carcenogenus, Enterobacter hormaechei, Enterobacter ludwigii* and *Enterobacter absuriae* (Figure 1.42).

Finally, the least represented group were members from the *alpha-Proteobacteria* which were recovered in small frequencies. The *alpha-Proteobacteria* was represented by the genus *Pseudovibrio.* Strains related to B57 formed a cluster with *Pseudovibrio denitrificans* in the phylogenetic analysis (Figure 1.39).

A frequency distribution was constructed after BOX PCR fingerprints and phylogenetic analysis. The distribution of the strains isolated from all sampling sites is observed in Figure 1.51. The most frequent strains recovered at all sites pertained to *Bacillus, Hallobacillus* and *Staphylococcus.*Most of the genera were recovered at all sampling sites, but there were two genera, *Pseudoalteromonas* and *Chromohalobacter* that were only isolated from *Los Morillos.*
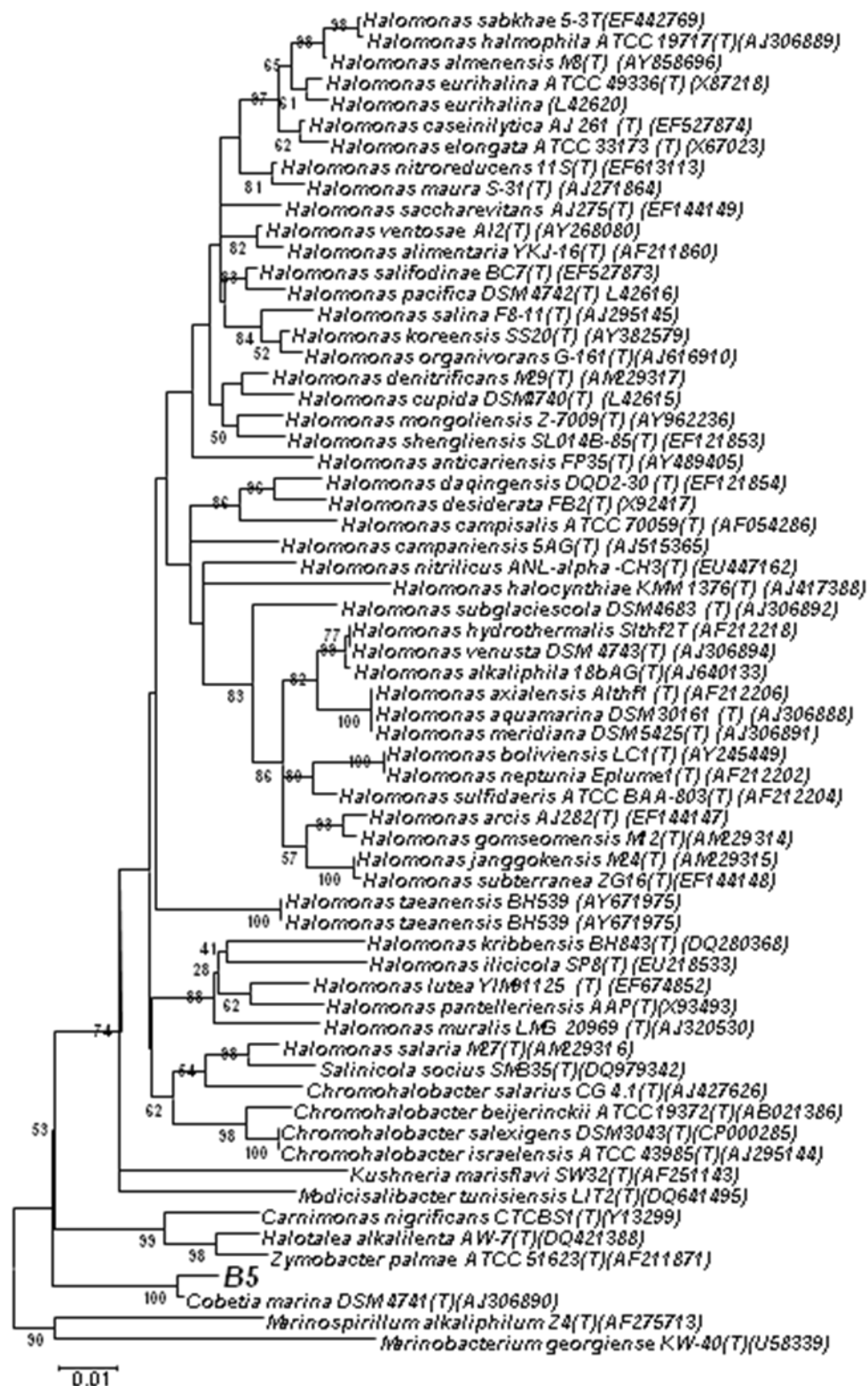
Figure 6.33 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain B5 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

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Figure 6.34 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain L20 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Kytococcus sedentarius* (X87755) served as outgroup.
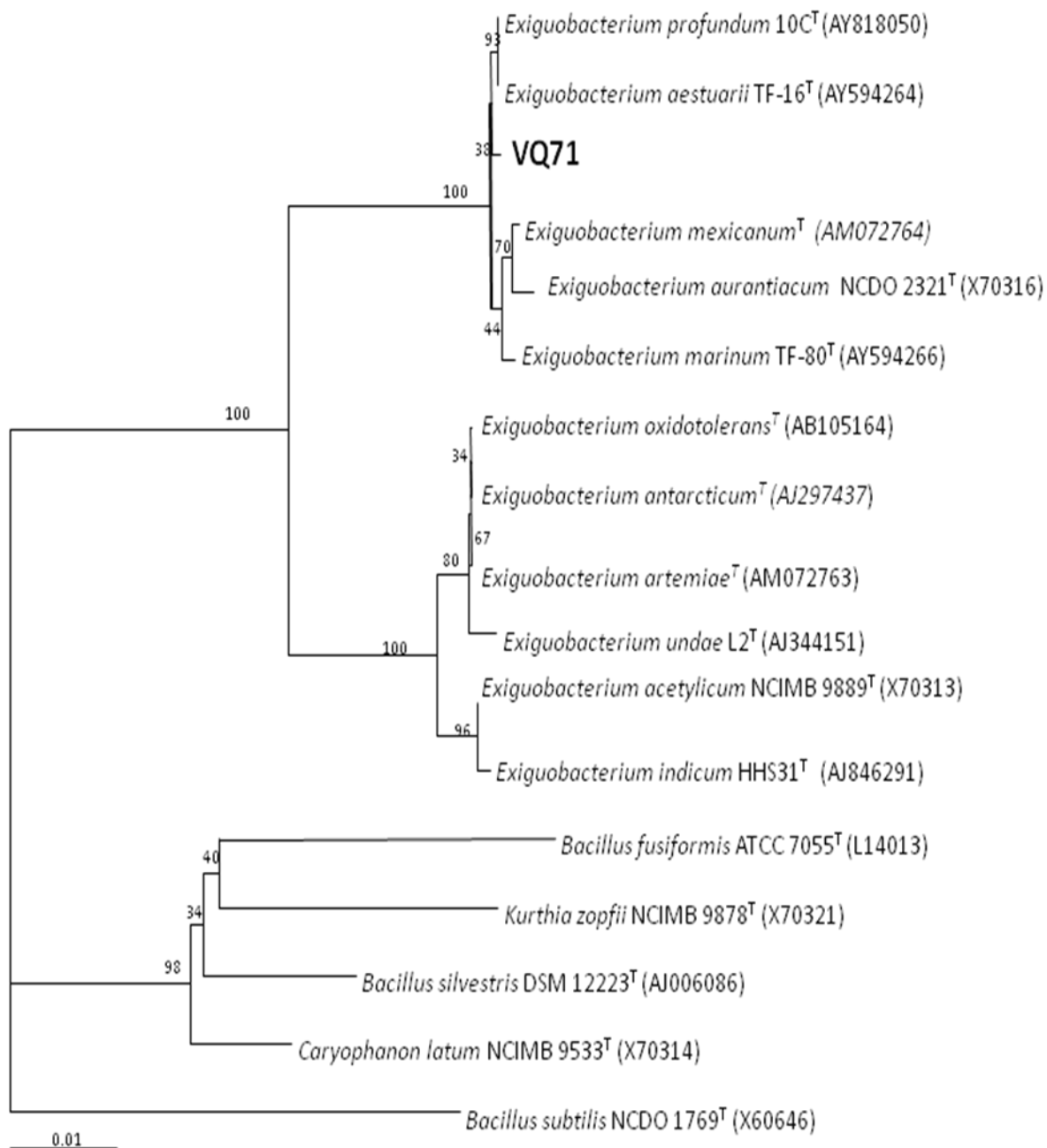
Figure 6.35 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain VQ71 isolated from *T. testudinum.* Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown. *Bacillus subtilis* (X60646) served as outgroup.
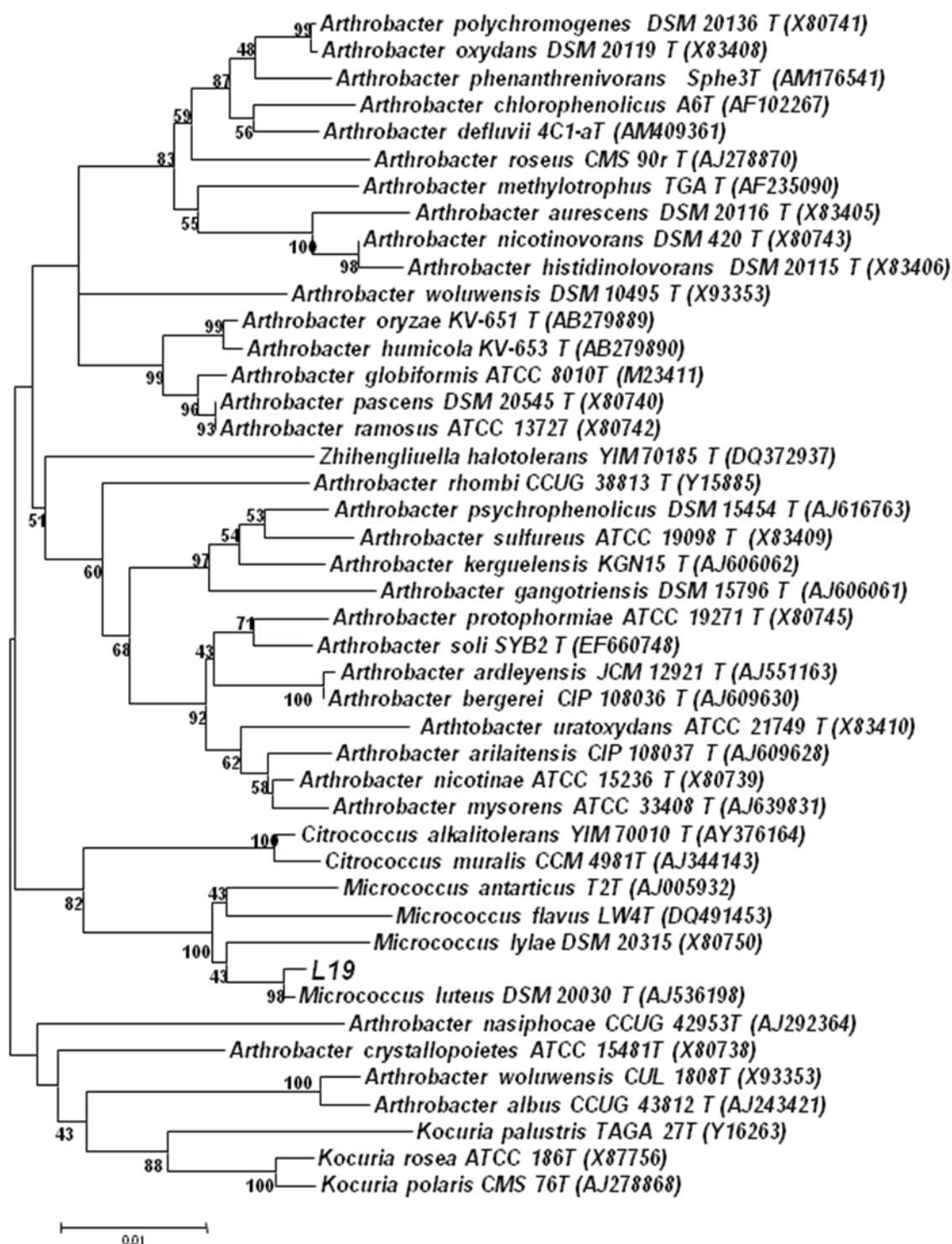
Figure 6.36 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain L19 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

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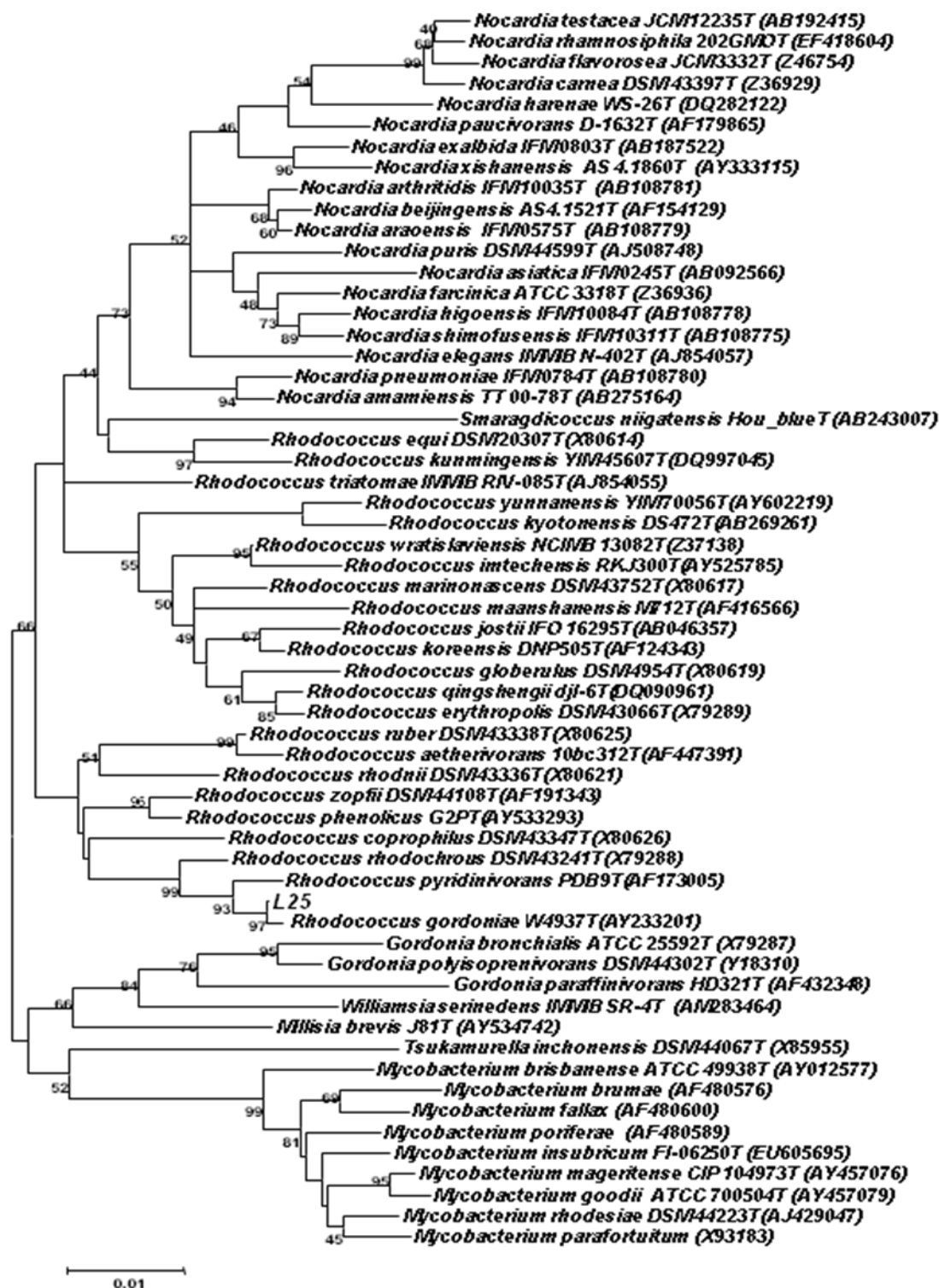
Figure 6.37 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain L25 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
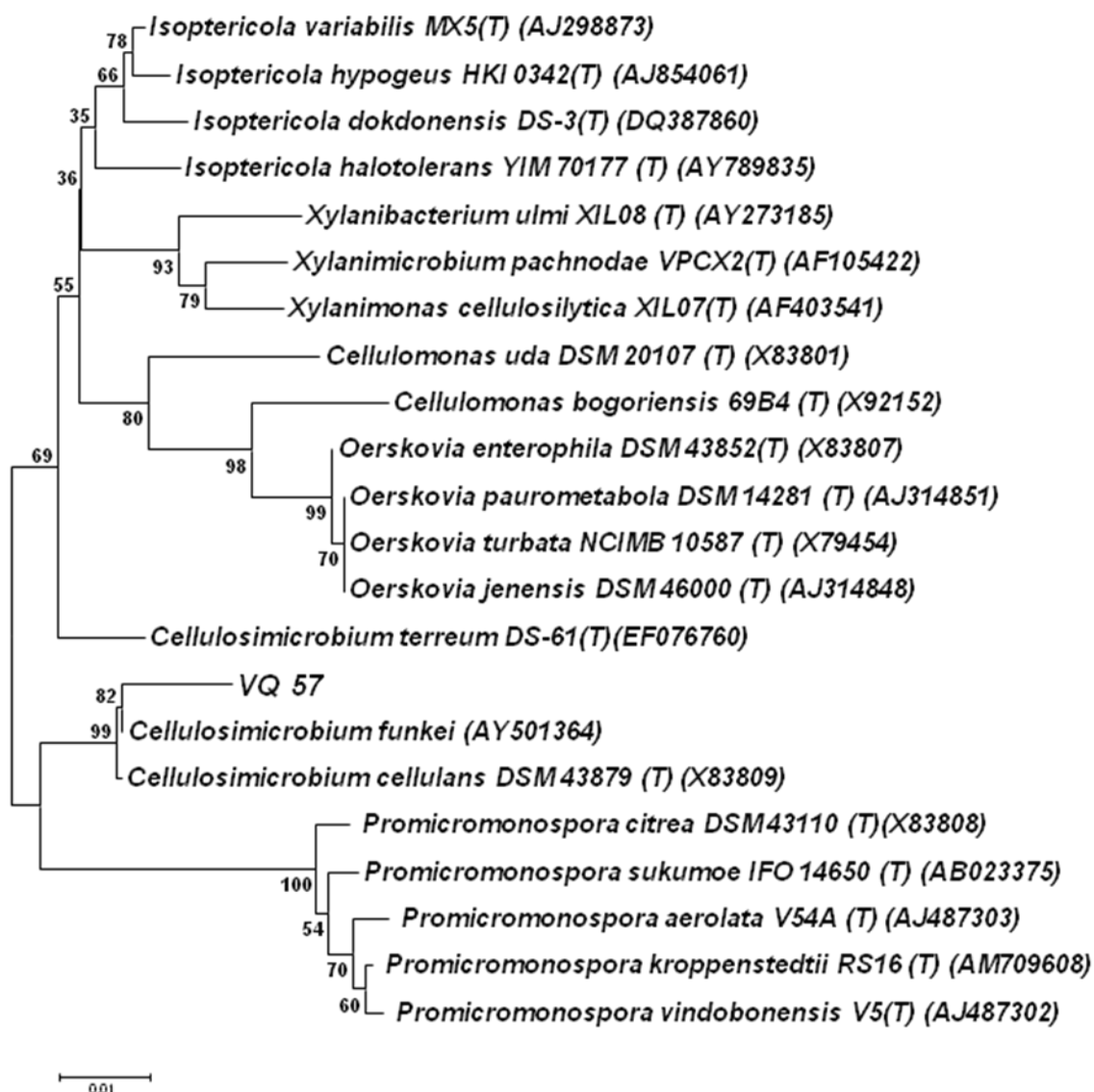
Figure 6.38 Neighbor-joining distance tree using the 16S rRNA sequences genes showing the phylogenetic relationship of strain VQ57 isolated from *T. testudinum* tissue. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown
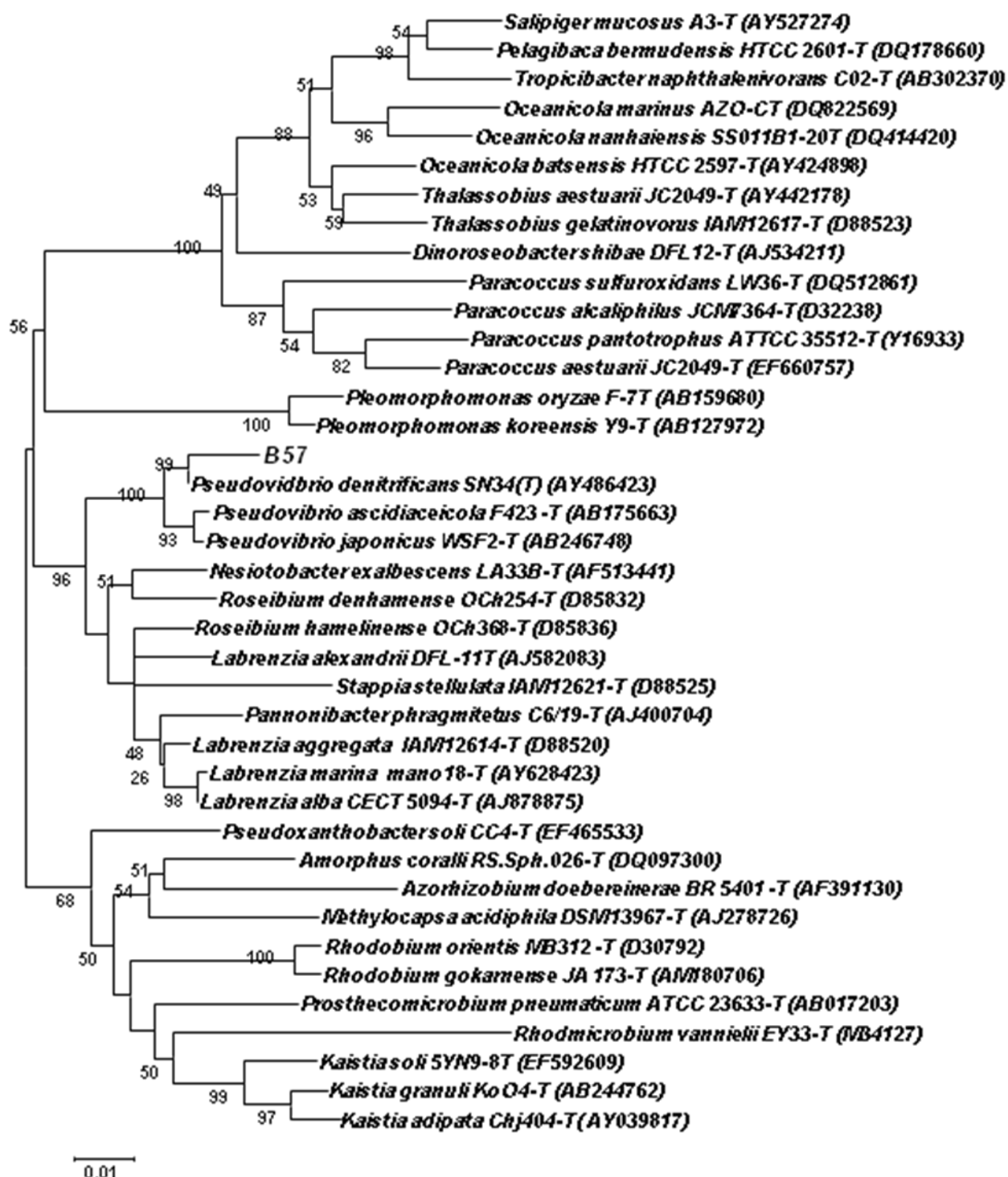
Figure 6.39 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain B57 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown
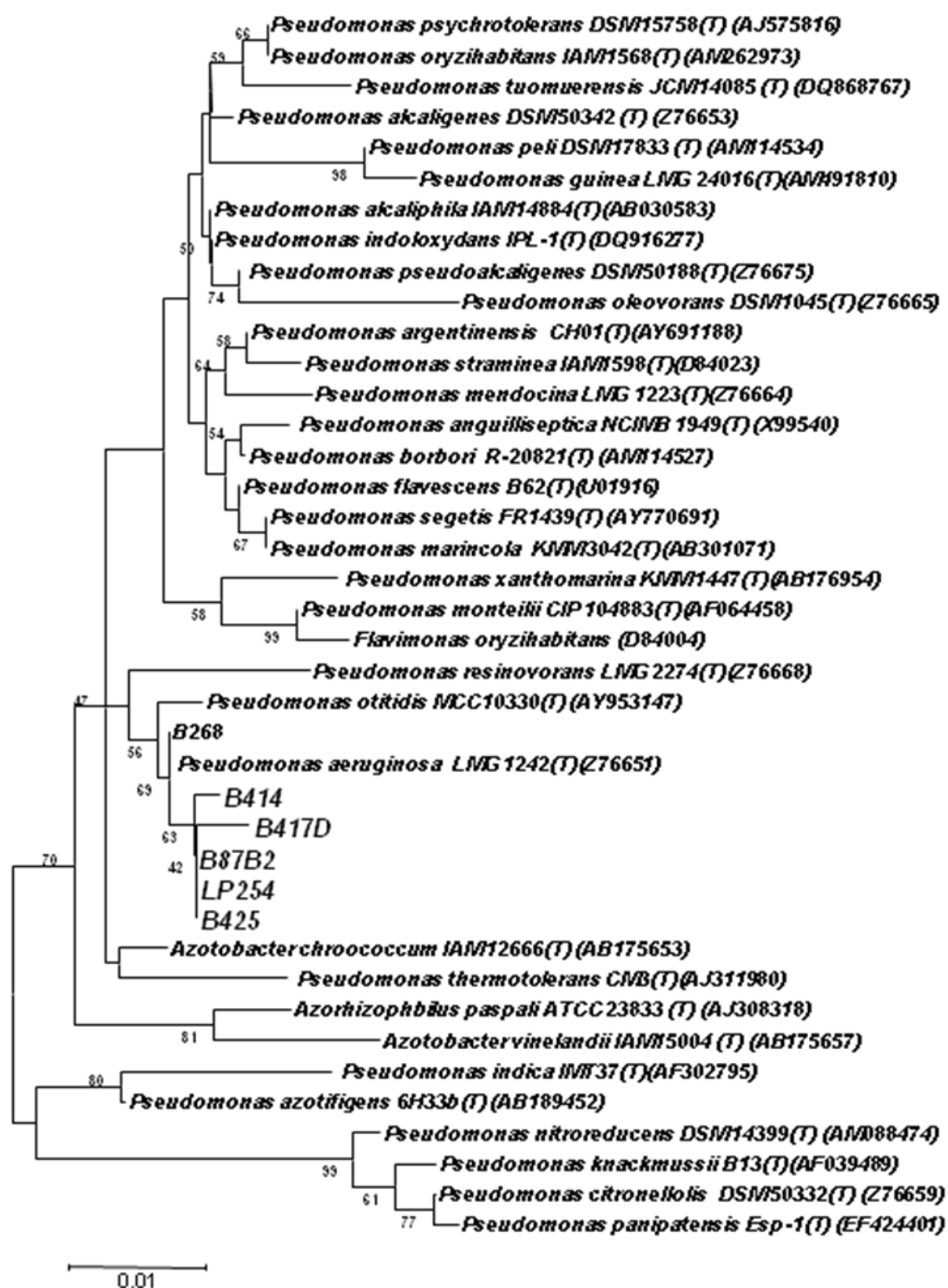
Figure 6.40 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strains B414, B268, B417D, B87B2, LP254, B425 isolated from *T. testudinum.*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
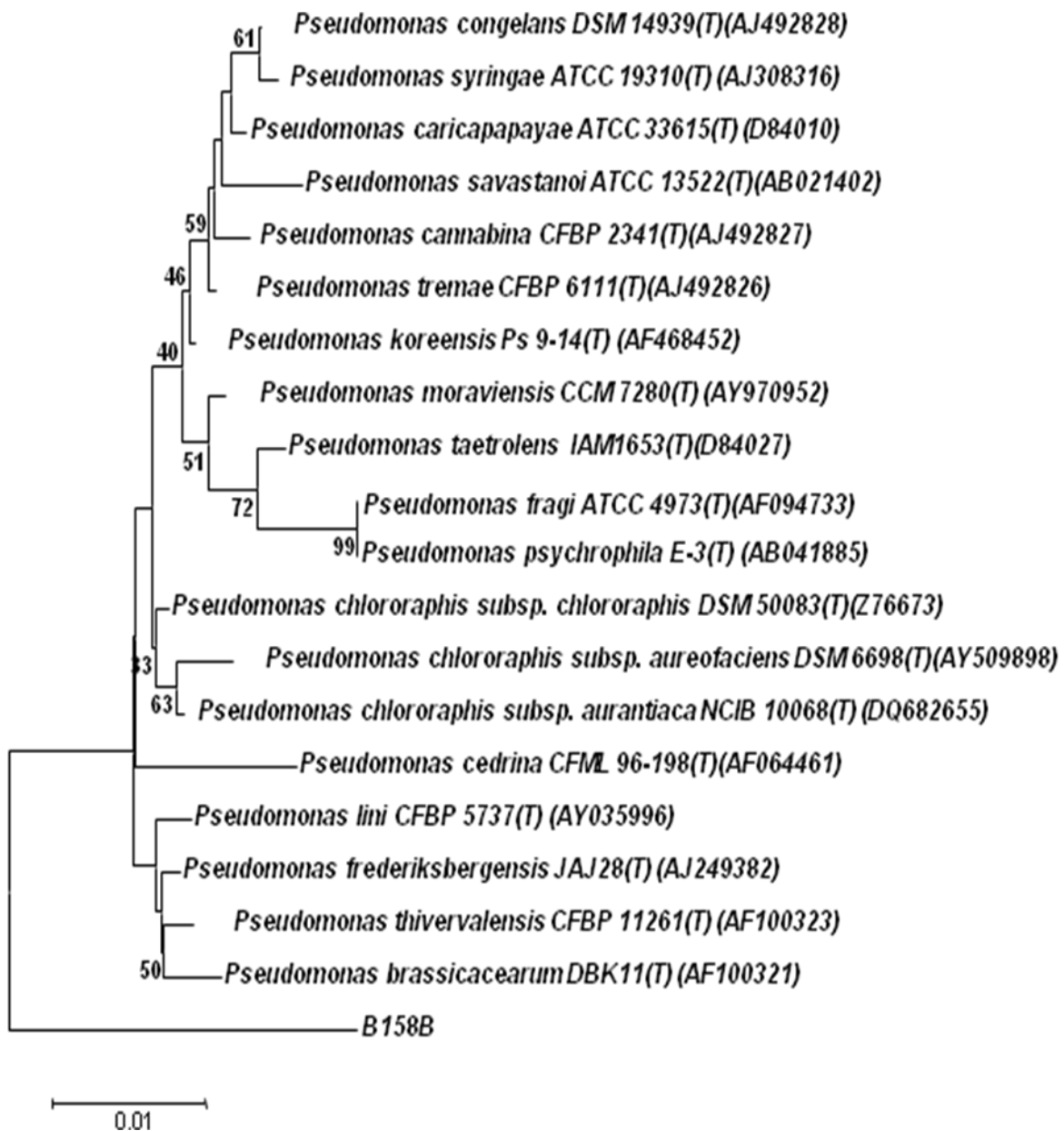
Figure 6.41 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain B158B isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
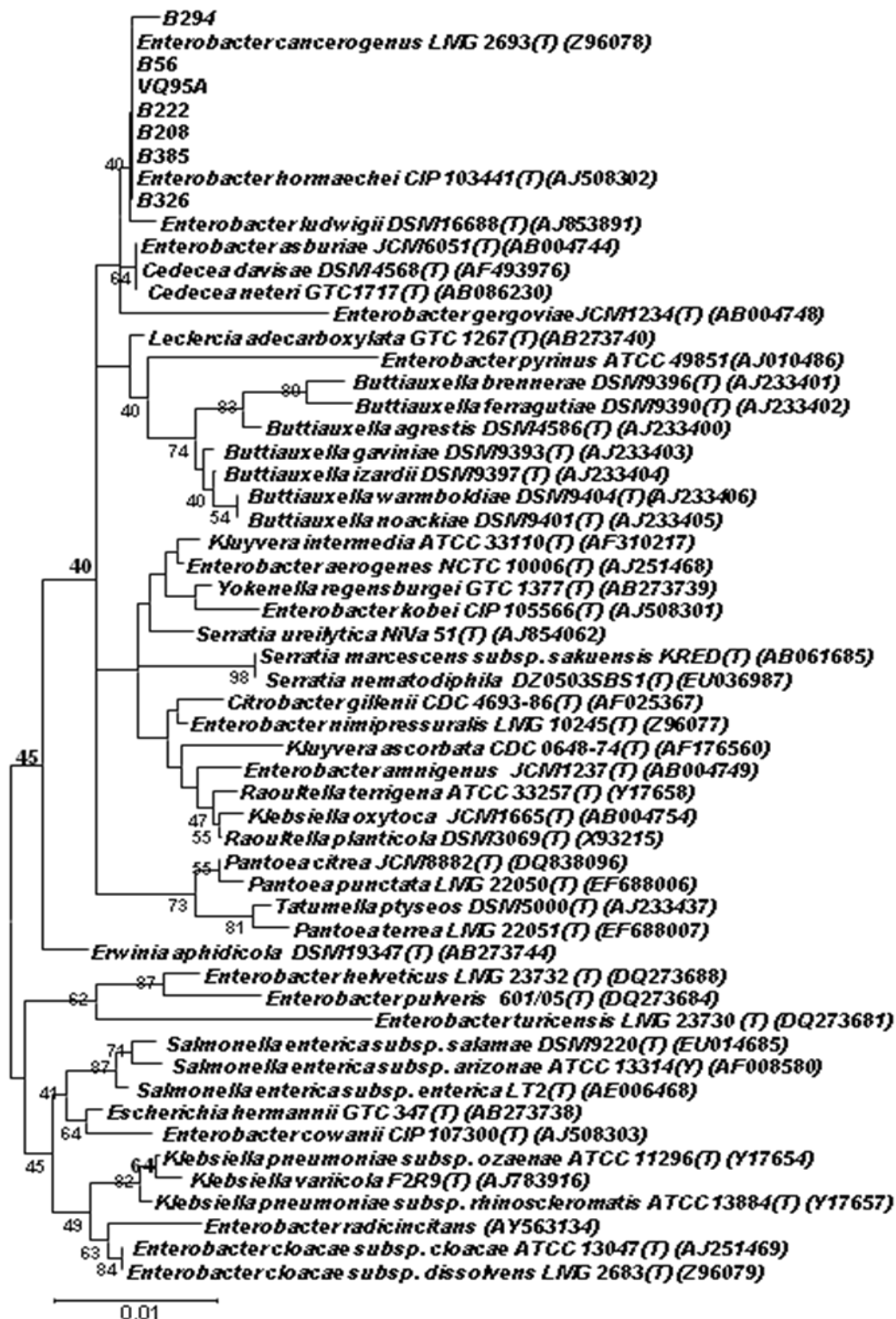
Figure 6.42 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strains B294, B56, VQ95A, B222, B208, B385 and B326 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
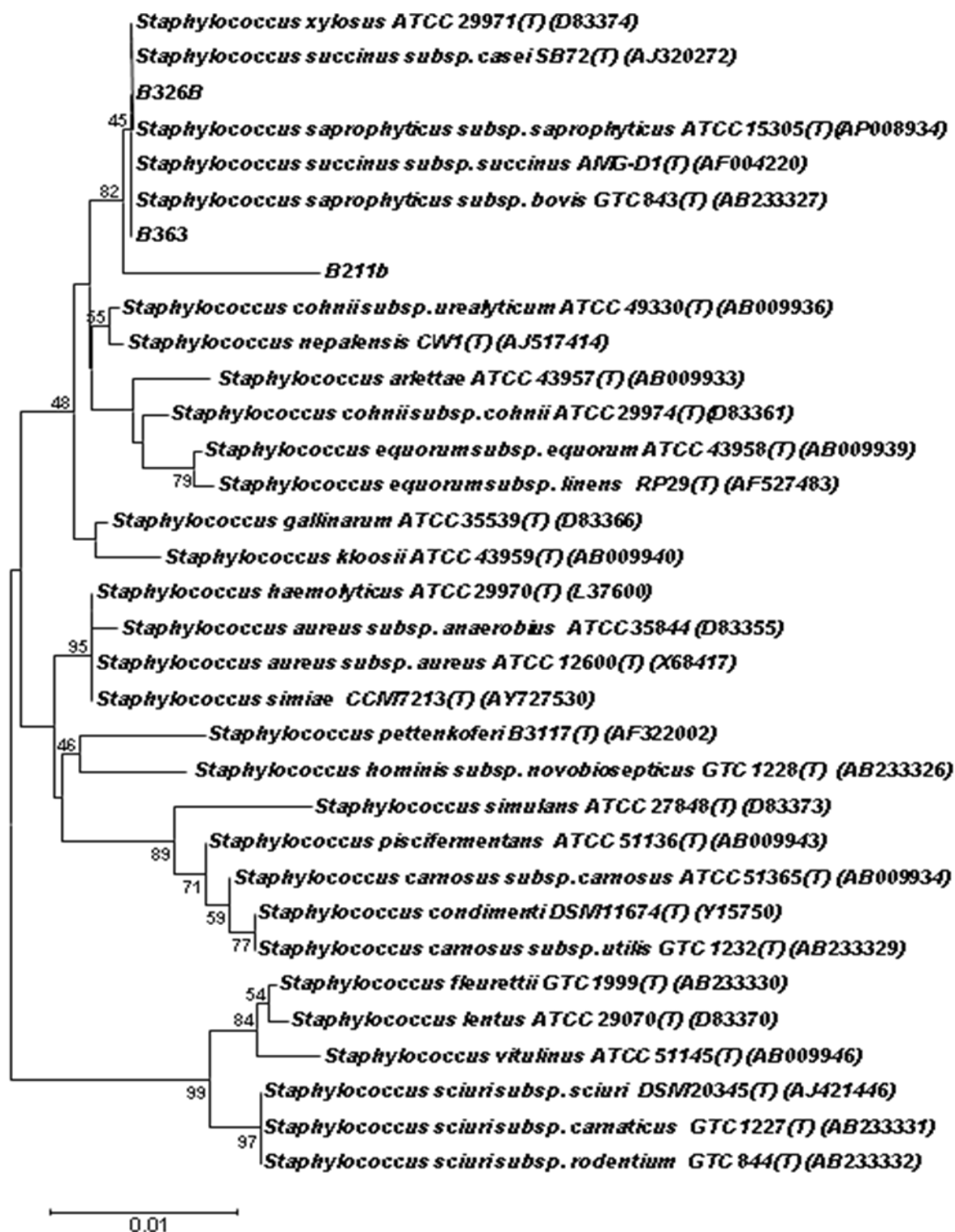
Figure 6.43 Neighbor-joining distance tree using the 16S rRNA sequences genes showing the phylogenetic relationship of strains B326B, B363 and B211b isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

58 │ *B108*
57 │ *Halobacillus kuroshimensis IS-Hb7(T) (AB195680)*
└ *B419*
VQ81
B350
Halobacillus profundi IS-Hb4(T)(AB189298)
B430b
80 *Halobacillus mangrovi M510(T) (DQ888316)*
Halobacillus halophilus NCIMB 9251(T) (X62174)
Halobacillus litoralis SL-4(T) (X94558)
68 *Halobacillus salinus HSL-3(T) (AF500003)*
Halobacillus alkaliphilus FP5(T) (AM295006)
Halobacillus locisalis MSS-155(T) (AY190534)
40 *Halobacillus campisalis ASL-17(T) (EF486356)*
47 *Halobacillus seohaensis ISL-50(T) (EF612763)*
94 *Halobacillus faecis IGA7-4(T) (AB243865)*
Halobacillus yeomjeoni MSS-402(T) (AY881246)
Halobacillus dabanensis D-8(T) (AY351395)
Halobacillus karajensis DSM14948(T) (AJ486874)
40 *Halobacillus trueperi DSM10404(T) (AJ310149)*
45 *Halobacillus aidingensis AD-6(T) (AY351389)*
Salimicrobium album DSM20748(T) (X90834)
99 *Salimicrobium luteum BY-5(T) (DQ227305)*
97 *Salimicrobium halophilum VDSM4771(T) (AJ243920)*
Thalassobacillus devorans G-19.1(T) (AJ717299)
99 *Pontibacillus marinus BH030004(T) (AY603977)*
Pontibacillus chungwhensis BH030062(T) (AY553296)
60 *Aquisalibacillus elongatus SH4s (T) (AM311047)*
40 *Piscibacillus salipiscarius RBU1-1(T) (AB194046)*
75 *Tenuibacillus multivorans 28-1(T) (AY319933)*
98 *Filobacillus milosensis DSM13259(T) (AJ238042)*
Alkalibacillus filiformis 4AG(T) (AJ493661)
99 *Alkalibacillus silvisoli BM2(T) (AB264528)*
45 *Bacillus foraminis CV53(T) (AJ717382)*
93 *Amphibacillus tropicus Z-7792(T) (AF418602)*
55 *Natronobacillus azotifigens 24KS-1(T) (EU143681)*
48 *Gracilibacillus halotolerans NN(T) (AF036922)*
Gracilibacillus lacisalsi BH312(T) (DQ664540)
63 *Gracilibacillus quinghaiensis YIM-C229(T) (EU135723)*
81 94 *Gracilibacillus boraciitolerans T-16X(T) (AB197126)*
51
0.01

 Figure 6.44 Neighbor-joining distance tree using the 16S rRNA sequences of *Halobacillus* strains VQ81, B108, B419, B350 and B430B isolated from *T. testudinum* tissue. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

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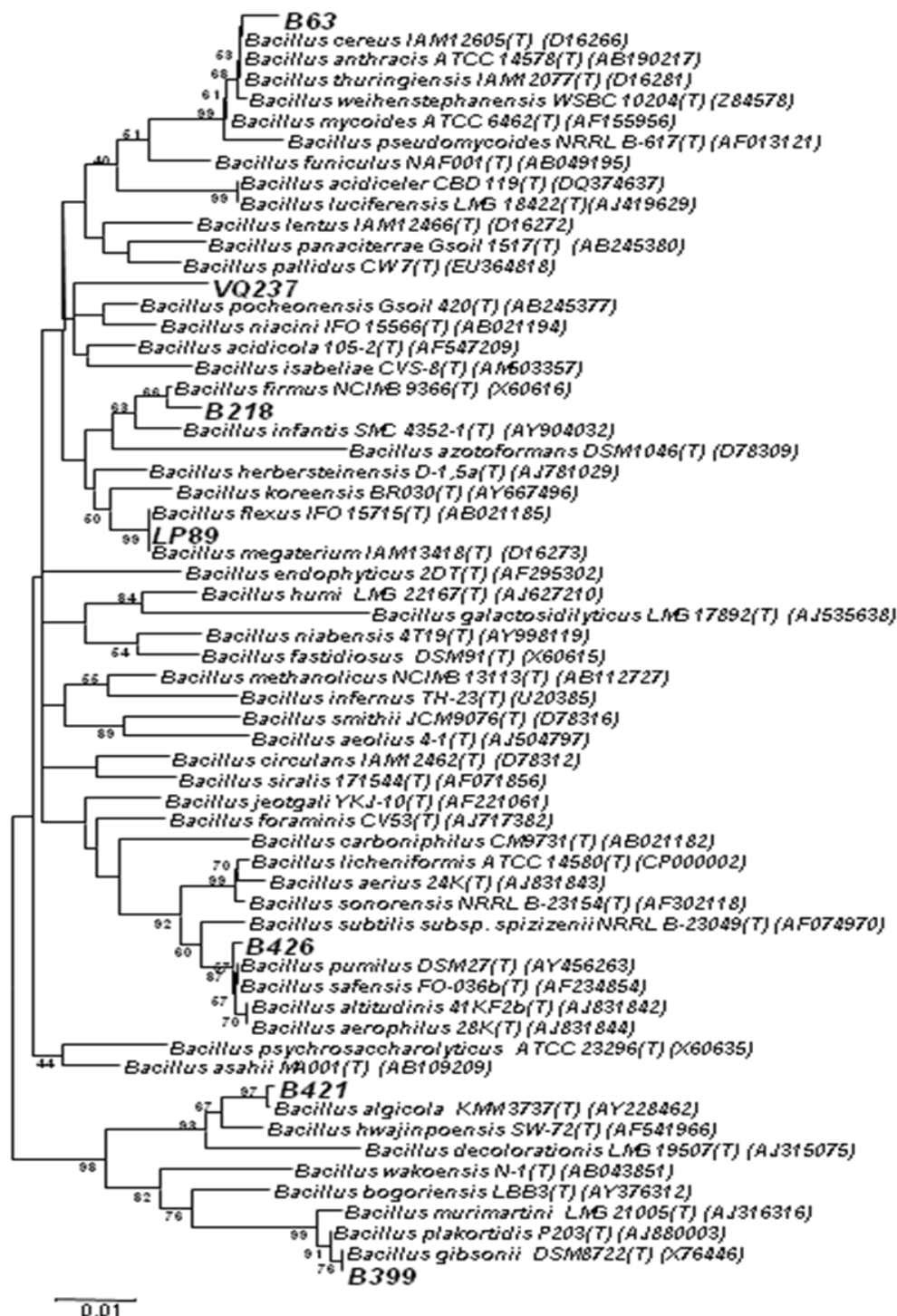
Figure 6.45 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strains B399, B421, B426, LP89, B218, VQ237 and B63 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
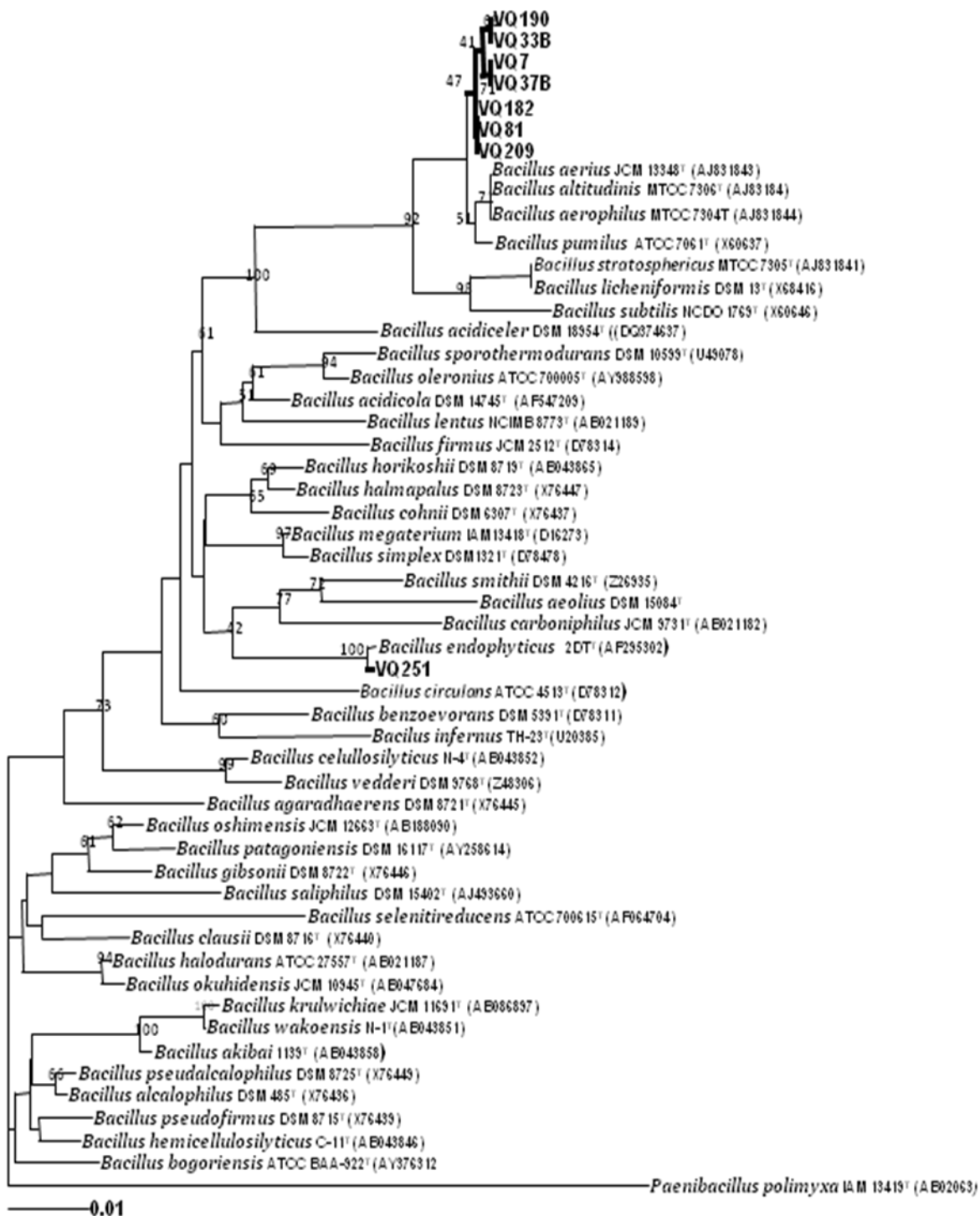
Figure 6.46 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strains VQ251, VQ190, VQ33B, VQ7, VQ37B, VQ182, VQ91 and VQ109 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
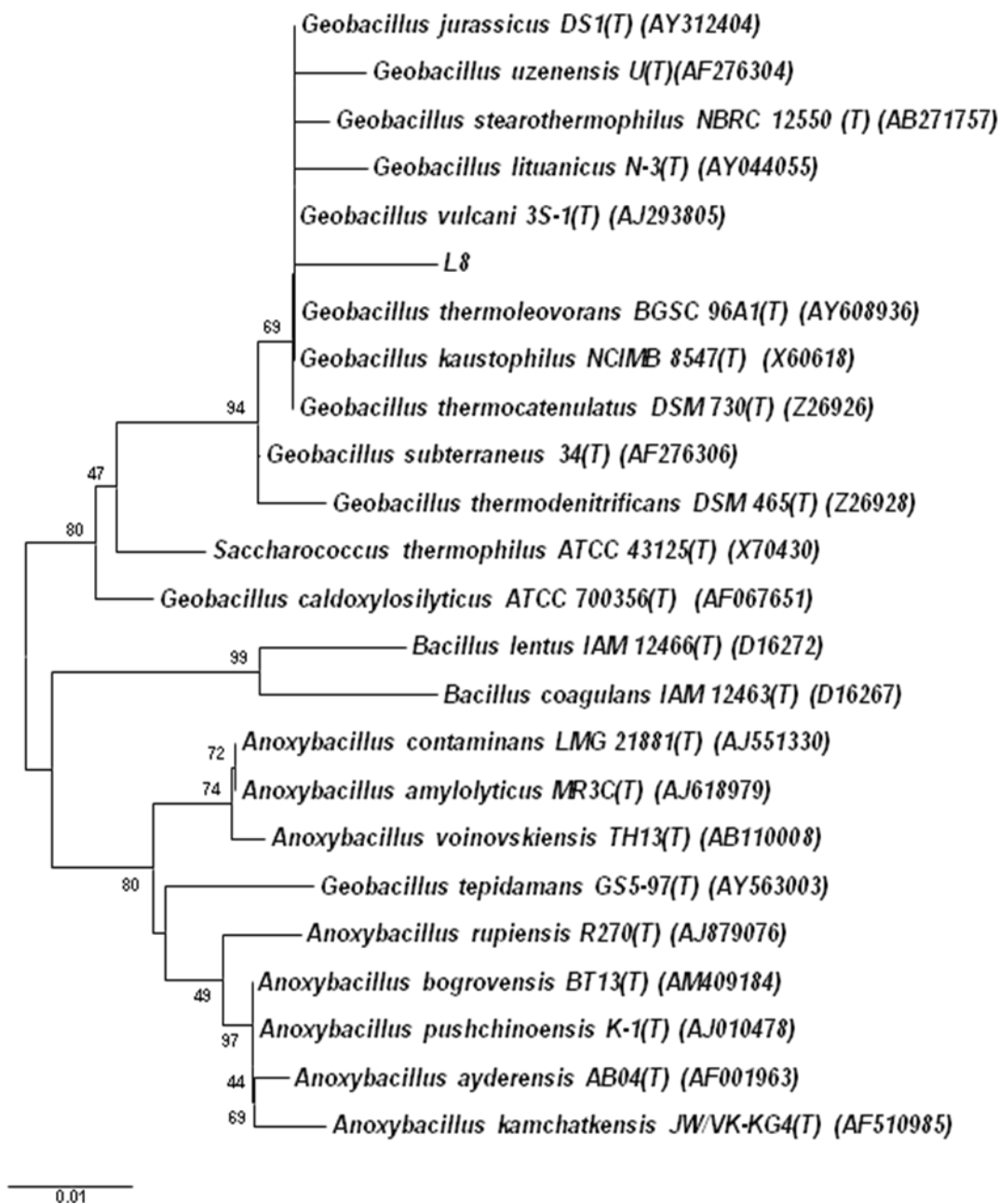
Figure 6.47 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain L8 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
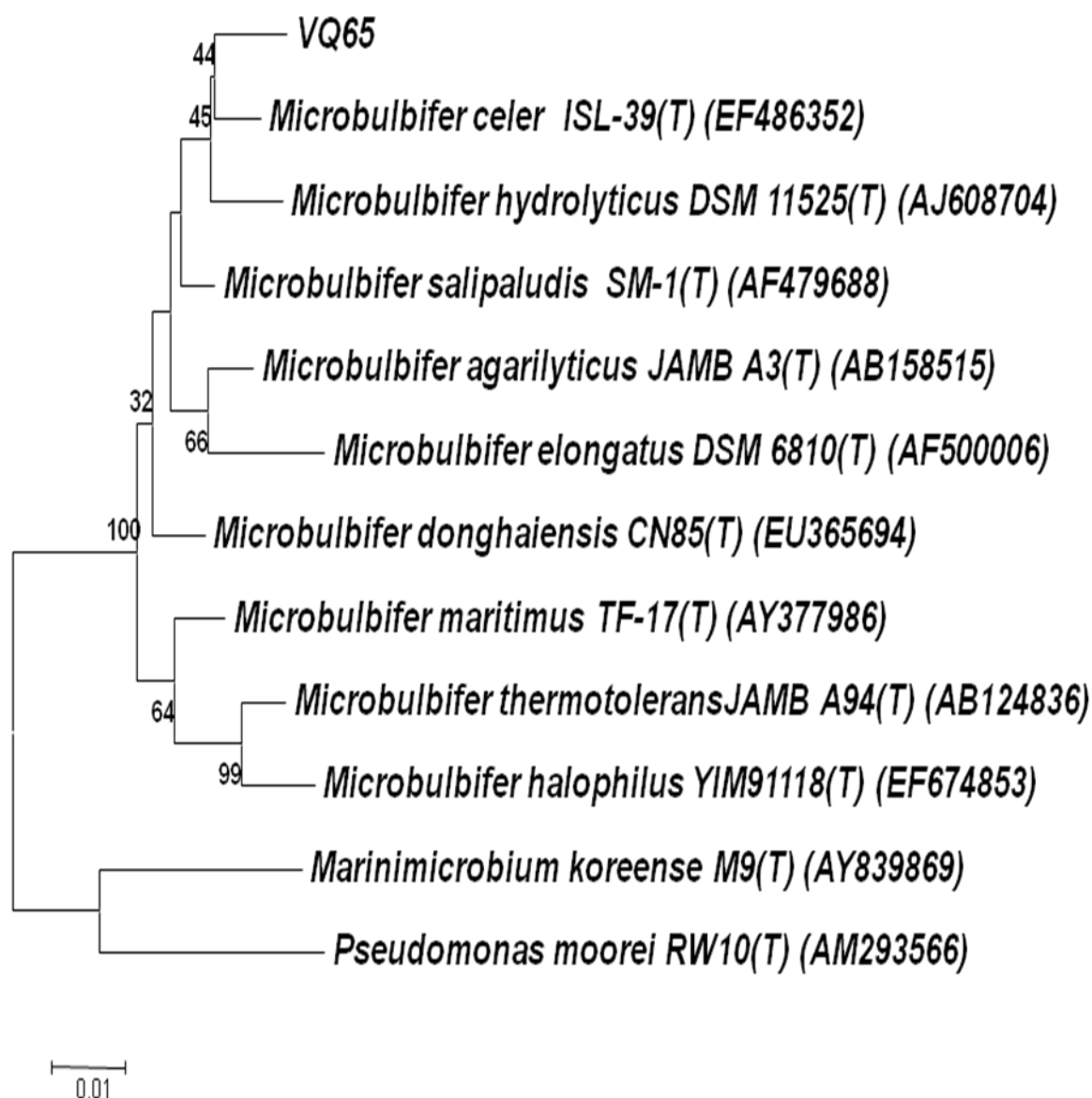
Figure 6.48 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain VQ65 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.
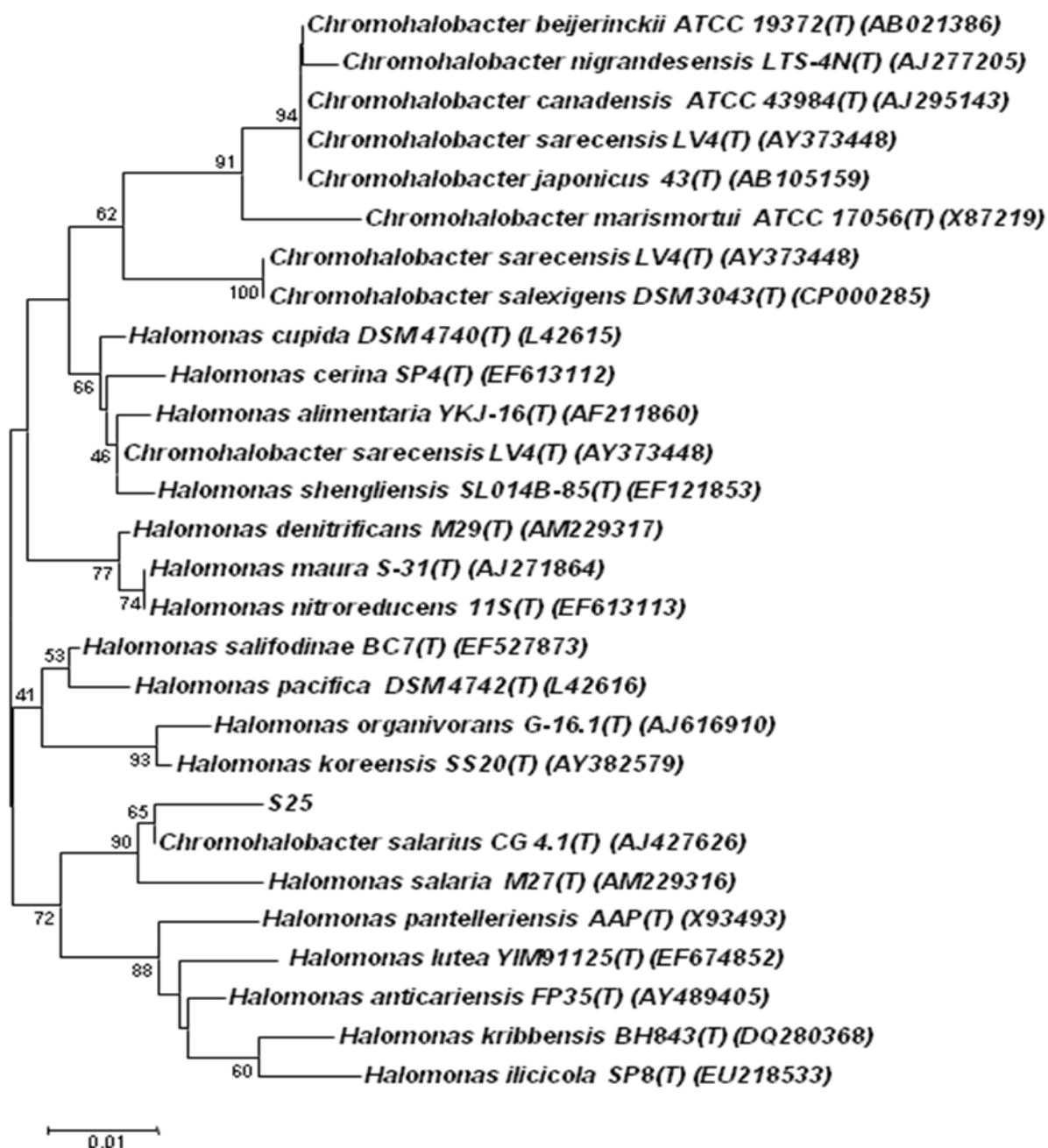
Figure 6.49 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain S25 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

74
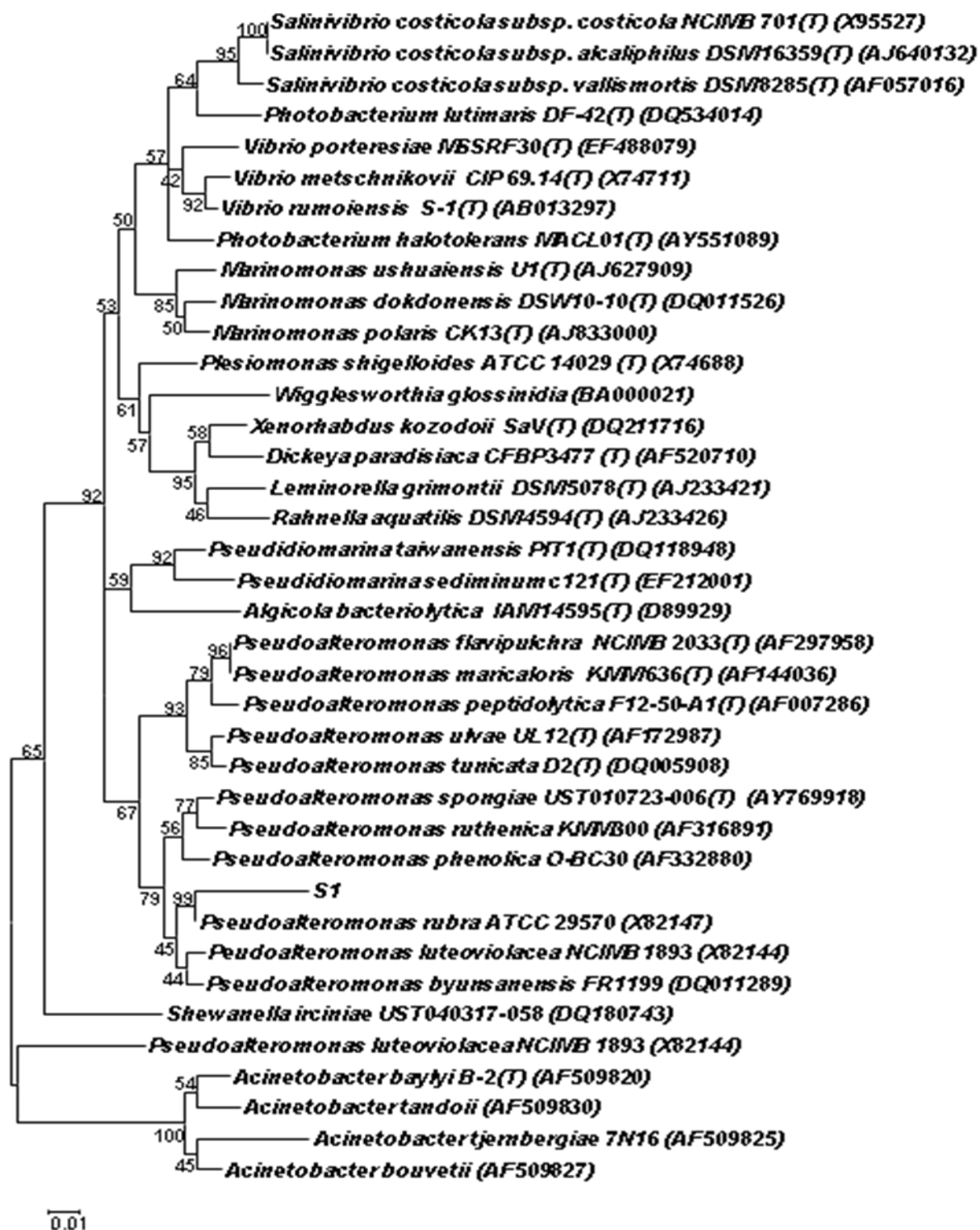
Figure 6.50 Neighbor-joining distance tree using the 16S rRNA genes showing the phylogenetic relationship of strain S1 isolated from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Bootstrap values higher than 40% are shown.

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Legend (Figure 6.51):
- Staphylococcus
- Nesterenkonia
- Micrococcus
- Bacillus
- Halobacillus
- Enterobacter
- Cellulosimicrobium
- Vibrio
- Pseudovibrio
- Pseudoalteromonas
- Cobetia
- Microbulbifer
- Rhodococcus
- Geobacillus
- Pseudomonas
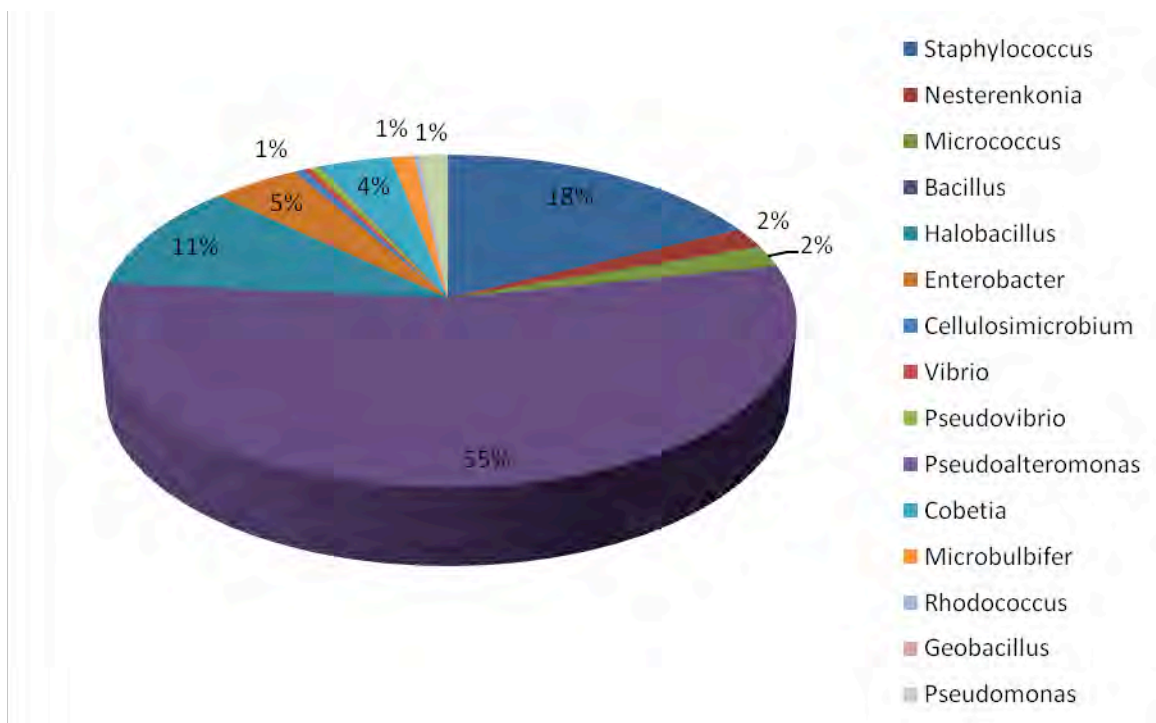
Figure 6.51 Pie chart representing the **g**enus affiliation and frequency distribution of endophytic bacteria isolated from surface sterilized tissue of *T. testudinum* from all sampling sites**.**

Legend (Figure 6.52):
- Staphylococcus
- Nesterenkonia
- Micrococcus
- Bacillus
- Halobacillus
- Enterobacter
- Cellulosimicrobium
- Vibrio
- Pseudovibrio
- Pseudoalteromonas
- Cobetia
- Microbulbifer
- Rhodococcus
- Geobacillus
- Pseudomonas
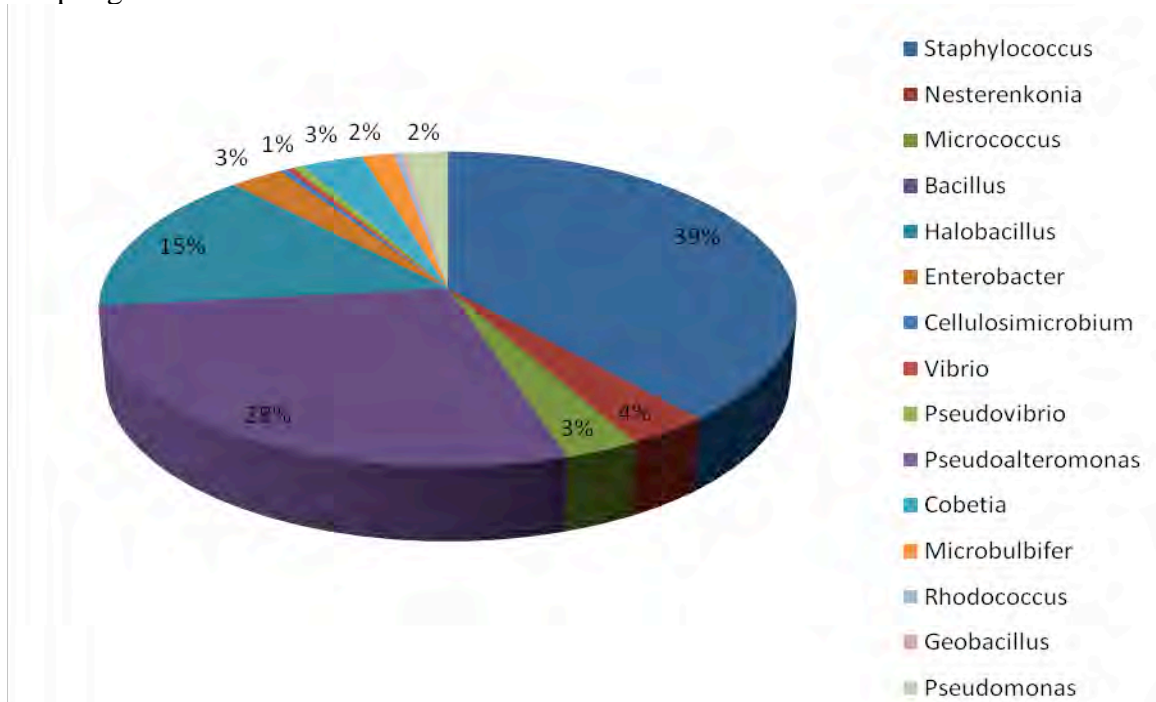
Figure 6.52 Pie chart representing genus affiliation and frequency distribution of endophytic bacteria isolated from *T. testudinum* tissues at Buyé Beach.
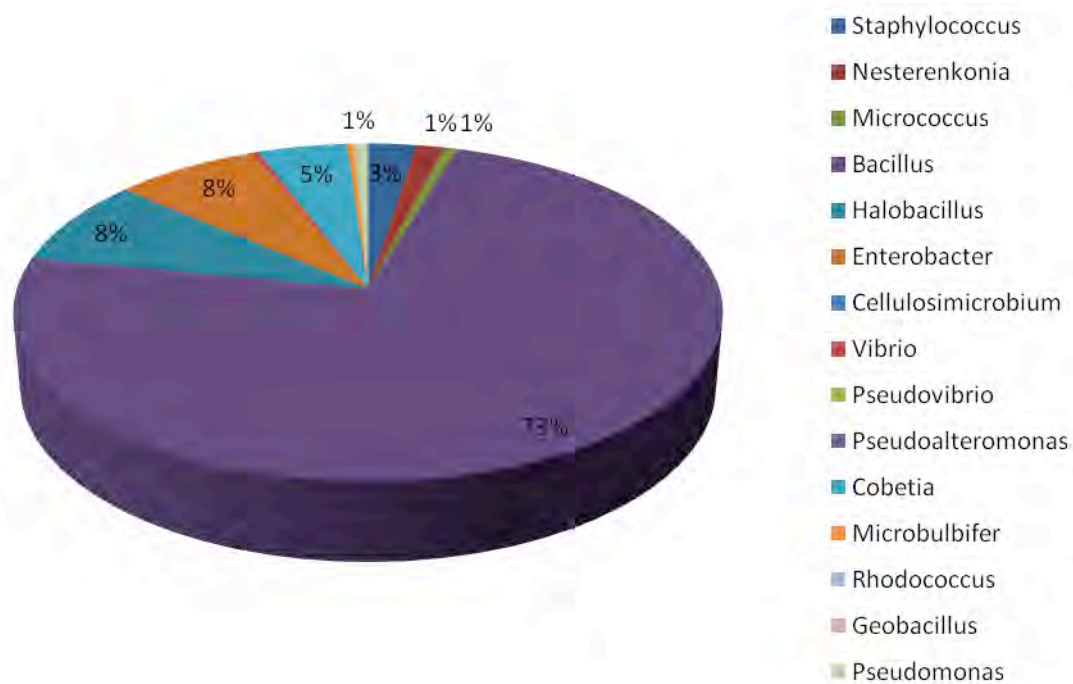
Figure 6.53 Pie chart representing genus affiliation and frequency distribution of endophytic bacteria isolated from *T. testudinum* tissues at *Cayo Enrique*.
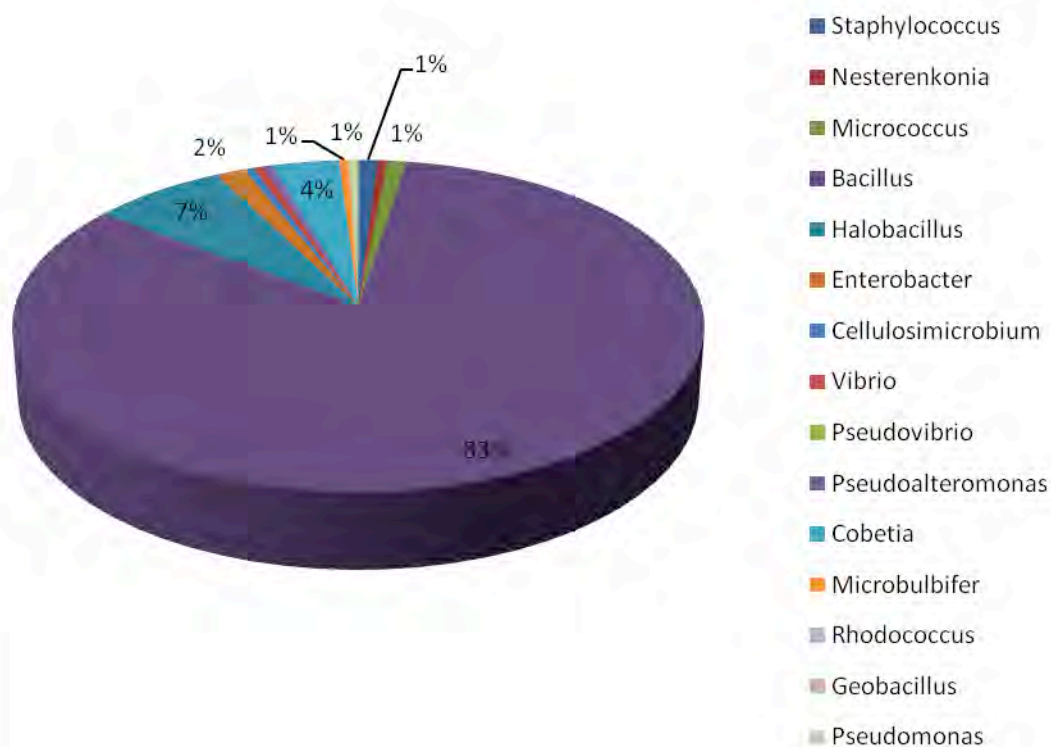
Figure 6.54 Pie chart representing genus affiliation and frequency distribution of endophytic bacteria isolated from *T. testudinum* tissues at *Los Morillos*.
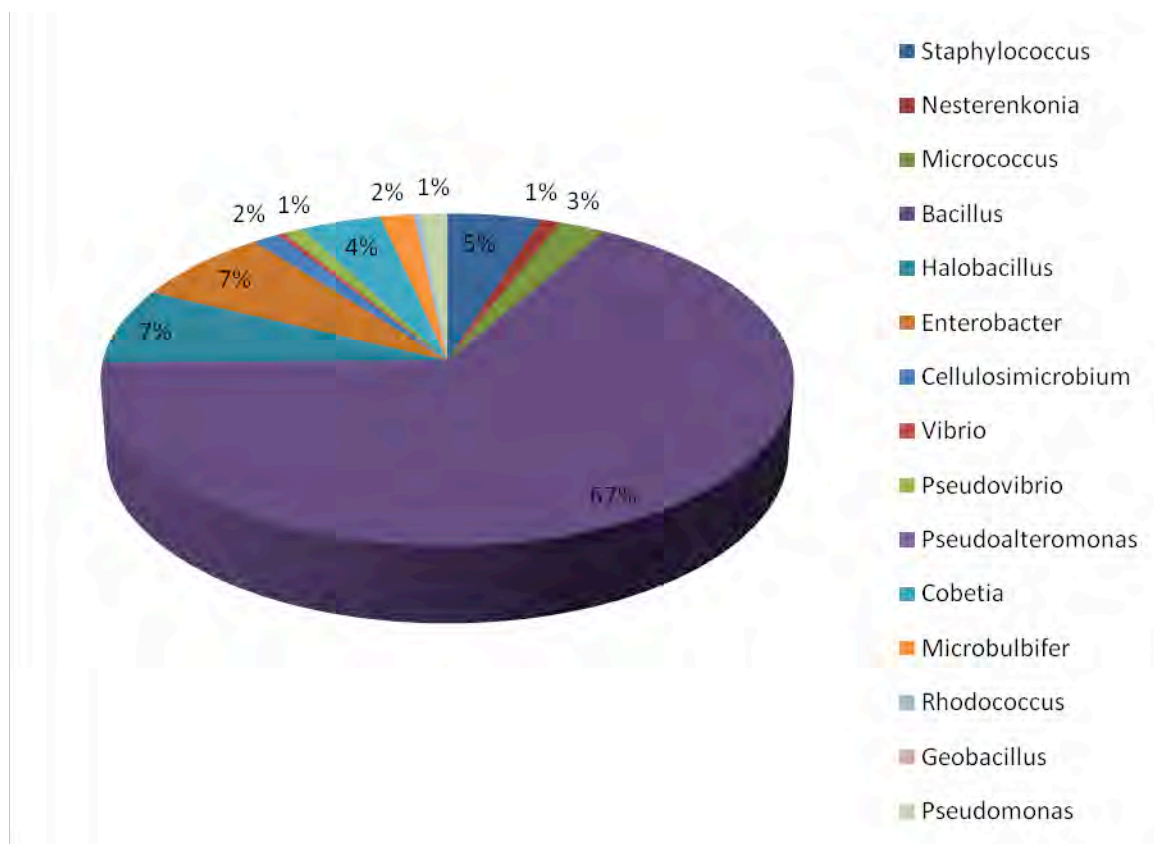
Figure 6.55 Pie chart representing genus affiliation and frequency distribution of endophytic bacteria isolated from *T. testudinum* tissues at *Puerto de la Libertad.*

6.3 Discussion

6.3.1   Culture dependent diversity of bacterial endophytes associated with healthy mature leaves of *T. testudinum*

The scope of the culture dependent techniques of this project was focused on the isolation and characterization of endophytic strains associated with surface sterilized healthy foliar tissue of the sea grass *Thalassia testudinum*. This study represents the first attempt to assess the prokaryotic endophytic community of *this plant*. Since *T. testudinum* thrives in marine habitats, on site salinity and temperature measures were taken for mimicking the environment where these microorganisms live in isolation experiments. The salinity in the Marine Agar medium was 3.5% NaCl, which concords with the average salinity levels of the sampling sites. Leaf samples were surface sterilized with sodium hypochlorite and sterilization effectivity was verified by impression of the leaf on the medium. Controls were incubated under the same conditions of the leaves used for isolation. There was no growth of bacterial colonies on control plates after two weeks of incubation.

Surface sterilized tissues used for isolation were cut in nine fragments per leaf and three fragment sets, representative of three sections of the leaves were used to inoculate plates wit MA medium.. The samples were incubated at room temperature and after 24-48 hours growth was observed in most of the media. Bacterial colonies were subcultivated until achieving pure cultures and 1,274 strains were recovered during the extension of this project. After macroscopical and microscopic characterization, molecular characterization was made for identification of phylogenetic affiliation. A bead beating based DNA extraction protocol was optimized several times because most of the

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strains were Gram positive and very fastidious for extraction. The use of glass beads ensured better breakage of the Gram positive bacterial cell wall and higher concentrations of DNA (up to 3ug/ul) were obtained.

After extracting good quality DNA, molecular fingerprinting of the isolates was performed. BOX-PCR is a technique that has proved to give high resolution and differentiation of bacterial subspecies can be achieved with this technique (Meintanis et al., 2008; Rademaker et al., 2005; von der Weid et al., 2000). After the generation of all the fingerprints, a total of 107 groups were identified. One representative for each group was selected for further phylogenetic analysis. The sequences were submitted to databases searchesin GenBank, the Ribosomal Database project, and EZ-taxon (Chun et al., 2007; http://147.47.212.35:8080/) for characterization and detection of related phylotypes.. An important fact was that some strains that exhibited dissimilar BOX-PCR patterns matched with the same type strain based on the 16S rRNA However, 16S rRNA gene sequences sometimes show limited variation for members of closely related taxa (Fox et al.,1992) due to the conserved nature of this gene (Ki et al., 2009). Evenmore, it is relevant to point out that the BOX-PCR is a technique which gives resolution up to subspecies, while the 16S rRNA only has resolution until genus. Though for *in silico* analysis, only one sequence was used for construction of phylogenetic trees.

Phylogenetic analysis showed that the Gram-positive bacteria belonging to the phyla *Firmicutes* and *Actinobacteria* were among the endophytic strains present in *T. testudinum*. The *Firmicutes* was represented by the genera *Bacillus, Halobacillus, Geobacillus, Staphylococcus,* and *Exiguobacterium*.

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The vast majority of the strains (55%) were affiliated to various *Bacillus* species. The genus *Bacillus* is distributed widely across many terrestrial and aquatic habitats (Ivanova et al., 1999; Siefert et al., 2000), including marine sediments (Miranda et al., 2008). Within the isolated *Bacillus* some were associated phylogenetically with *B. cereus, B. licheniformis, B. firmus, B. pumilus, B. mycoides*, and *B. lentus* which have been detected in marine environments (Ivanova et al., 1999). Also, representatives from this genus have been recovered associated with the rhizosphere and internal tissues of many terrestrial plants such as cotton, potato, sweet pepper, maize, coffee, papaya, rice, soybean and oak (Bai et al., 2002; Garberva et al., 2001; Jiménez-Salgado et al., 2003; McInroy and Kloepper, 1995; Rasche et al., 2006; Thomas et al., 2004; Vega et al., 2005).

Strains VQ190, VQ33B, VQ7, VQ37B, VQ182, VQ81b, B426 and VQ209 formed a cluster with *B. pumilus, B. safensis, B. aerius* and *B. altitudinis* as closest relatives. *B. safensis* is a spore forming bacterium highly resistant to gamma and UV radiation, which was first isolated from a spacecraft-assembly facility of the Jet Propulsion Laboratory, Pasadena, CA (Satomi et al., 2006), thus its role in the tissues of *T. testudinum* is unclear. *B. pumilus* is a bacterium that protects cotton roots against *Fusarium oxysporum f. sp. vasinfectum* (Chen et al., 1995). Also, it has been found that it has a strong growth-promoting activity by the secretion of high amounts of physiologically active gibberellins (Gutiérrez-Mañero et al., 2008). *B. pumilus* has been documented as a biocontrol agent in agriculturally important crops, such as tomato reducing significantly whitefly crawlers, nymphs, and pupae which threatened the plant (Kloepper et al., 2004).

Strain VQ 251 was closely related to the endophytic bacterium *B. endophyticus* which was isolated from inner tissue of healthy cotton plants (Reva et al., 2002). Strain B63 was clustered with *B. thuringiensis, B. anthracis* and *B. cereus. B. thuringiensis* is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle (Schnepf et al., 1998*). B. thuringiensis* has been characterized as an insect and nematode pathogen, being one of the major biopesticides used. *B. thuringiensis* has been used for the control of certain insect species among the orders *Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, and Mallophaga* and against nematodes, mites, and protozoa (Beegle and Yamamoto, 1992; Feitelson et al., 1992; Feitelson, 1993; Schnepf et al., 1998).

Strain B399 formed a branch with *B. gibsonii* and *B. plakortidis*. *B. plakortidis* is an alkalitolerant marine bacterium found associated with the sponge *Plakortis simplex* (Borchert et al., 2007). *B. gibsonii* is a spore forming alkalitolerant that have been isolated from soil (Nielsen et al., 1995). B218 was closely related with *B. firmus*, while strain LP 89 formed a cluster with *B. megaterium* and *B. flexus.* It has been previously observed that *B. megaterium* and *B. firmus* are applied as seed treatment to colonize the endorhizosphere and increase the plant biomass (Chanway et al. 2000).

Strain B421 formed a branch in association with *B. algicola*, while Strain VQ 237 deeply branched within *B. pocheonensis, B. isabelliae* and *B. acidicola*. *B. algicola* was isolated in enrichment cultures during degradation of the thallus of the brown alga *Fucus evanescens*. This bacterium is chemoorganotrophic, tolerant to 3% NaCl, alkalitolerant, and alginolytic (Ivanova et al., 2004). *B. pocheonensis* is a halotolerant and

nitrate reducer bacteria that was first isolated from a soil sample of a ginseng field in Pocheon Province, South Korea (Ten et al., 2007).

Moreover, some studies have demonstrated the vital role of *Bacillus* strains for stimulating plant defense mechanisms and providing enhanced health and fitness of its host plant. *Bacillus spp.* can also contribute to abiotic stress adaptation by production of indole acetic aceid in pepper (Sziderics et al., 2007) and serve as biocontrol agent against phytopatogenic fungi (Cho et al., 2007). One of the most important studies was conducted by Toro and collaborators (1997) where they showed that endophytic *Bacillus* species can solubilize phosphorous and nitrogen for the plant to use as biomass being an extremely important asset to nutrient supply. Probably, *Bacillus* species have an important role in the health and establishment of *T. testudinum*.

Other members of the *Firmicutes* frequently isolated in this study are associated with *Halobacillus* species. Strains of this genus are mostly halophilic and have been isolated from various habitats such as salt marshes (Claus et al., 1983), hypersaline soils (Amoozegar et al., 2003), the Yellow Sea in Korea (Yoon et al., 2004) the salt lakes of East Sea in Korea (Yoon et al., 2003), and marine solar salterns in Korea (Yoon et al., 2005). Strains B108, VQ419 and VQ 81 are closely related to *Halobacillus kuroshimensis*, strain VQ150 was related to *Hallobacillus profundi* and strain B430b is associated with *Halobacillus mangrovi*. *Halobacillus kuroshimensis* and *Halobacillus profundi* were isolated from a deep-sea carbonate rock at a methane cold seep in Kuroshima Knoll, Japan (Hual et al., 2007). *Halobacillus mangrovi* was found associated with the leaf surface of the black mangrove *Avicennia germinans* (Soto-Ramírez et al.,

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2008). The common abundance of *Halobacillus* within marine habitats such as mangroves might explain the presence of this bacterium associated with *T. testudinum*.

Staphylococcus was another abundant group especially at Buyé Beach. Members of the genus *Staphylococcus* are ubiquitous and diverse, and have been isolated from soil, plants, animals and humans. *Staphylococcus spp.* has been reported as endophytes in carrot *(Daucus carota L. var. sativu),* sweet corn, sweet pepper and cotton (McInroy and Kloepper, 1995; Rasche et al., 2006; Surette et al., 2003).

Strain VQ71 was also classified within the *Firmicutes* group closely associated with *Exiguobacterium aestuarii* and *Exiguobacterium marinus,* which were isolated from a tidal flat of the Yellow Sea in Korea (Kim et al., 2005). Representatives of this genus are common in marine habitats (Crapart et al., 2007; Kim et al., 2005) Also, Mahaffee and Kloepper (1997) reported the presence of *Exiguobacterium* in the rhizophere of cucmber plants (*Cucumis sativa L.*), whereas *Exiguobacterium acetylicum* have been found associated with rice seeds from the Phillipines (Cottyn et al., 2001). The fact that *Exiguobacterium* have been reported associated with plants and since *Exiguobacterium* species closely related to VQ 71 are marine bacteria might indicate that these species have adapted to inhabit within *T.testudinum* tissues.

One interesting finding was the strain L8 belonging to *Geobacillus* isolated from Buyé Beach. There are no reports about the presence of this genus *Geobacillus* as an endophyte or even to thrive at mesophilic conditions. Known members of *Geobacillus* are characterized as thermophiles with an optimal growth above 50ºC, also including *Geobacillus vulcani* which is a marine representative of the genus. Strain L8 grows

optimally at 30ºC. The phylogenetical position of this strain together with some physiological properties suggests that strain L8 might be a novel species for this genus.

The *Actinobacteria* isolated was represented by *Nesterenkonia, Micrococcus, Cellulosimicrobium,* and *Rhodococcus*. *Actinobacteria* have been well characterized due to their ability of producing secondary metabolites which are of economic and medical importance, such as antibiotics (Berdy, 1989; Kieser et al., 2000). Some *Actinobacteria* are also known to form more intimate associations with plants and colonize their internal tissues, as endophytic or plant-pathogenic species (Coombs and Franco, 2003).

Strain L19 was related to *Micrococcus luteus*, which has been previously reported in association to lettuce promoting root growth by secretion of indole acetic acid (Barazani and Friedman, 1999). Strain L20 was associated with *Nesterenkonia aethiopica*, an alkaliphilic, moderate halophile isolated from an Ethiopian soda lake (Delgado et al., 2006). Most of the strains of *Nesternekonia* have been isolated from hypersaline environments and has been found in soda lake wetlands associated with reed beds of *Phragmites australis* (Ruznyak et al., 2007), but there is no report of its association to marine plants. Strain VQ57 was closely related to *C. funkei* and *C. cellulans*. Interestingly, this two species only have been reported in clinical samples (Brown et al., 2006; Tucker et al., 2008) and associated with duck faeces (Murphy et al., 2005), thus the role of this organism within *T. testudinum* tissue is still unclear.

All the Gram-negative bacteria characterized belonged to the phylum *Proteobacteria*, subphylum *gamma* and *alpha*. The representative genera were *Microbulbifer, Pseudomonas, Pseudoalteromonas, Chromohalobacter*, *Enterobacter* and *Pseudovibrio.* Some of these genera have been reported in the past as normal members of

the endophytic communities in terrestrial plants and as marine microorganisms. However, some of the isolated genera have never been reported as inhabitants of plant tissue.

Alpha-Proteobacteria was only represented by *Pseudovibrio*. All species reported so far are from marine environments isolated from sea water and sea squirts. Strain B57 was closely related to the denitrifying marine bacterium, *Pseudovibrio denitrificans*. Denitrification is a dissimilatory process in which oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limiting and consists of four reaction steps in which nitrate is reduced to dinitrogen gas (Zumft, 1997). The occurrence of denitrifying bacteria implies loss of fixed nitrogen and nitrogen is frequently a limiting nutrient for plants. Thus the role of *Pseudovibrio* in *Thalassia testudinum* has to be further explored.

Strains B268, B414, B417d, B87b2, LP254 and B425 were associated with *Pseudomonas* species, while strains B385, B326, B56, VQ95A, B222 and B208 were close to *Enterobacter* species. *Pseudomonas* and *Enterobacter* species has been extensively researched for their strong relationship with plants like production of phytohormones, aiding in nutrient intake, solubilization of phosphate, production of siderophoresand fixing nitrogen (Garbeva et al., 2001; Hinton and Bacon, 1995; Kuklinsky-Sobral et al., 2004). *Pseudomonas aeruginosa* was demonstrated to promote seedling emergence, root length, shoot length, dry weight and pod yield of groundnut in the field (Kishore et al., 2005) and also has broad-spectrum antifungal activity.

Strain VQ 65 was associated with *Microbulbifer,* which is a genus that has been associated with sea sediments and to mucus and tissues of *Oculina patagonica* (Koren and Rosenberg, 2008) but there are no records of the presence of this genus on sea

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grasses. Strain S25 was related to the moderately halophilic bacterium, *Chromohalobacter salarius,* which was recently characterized and isolated from from a solar saltern in Cabo de Gata at southern Spain (Aguilera et al., 2007).

Pseudoalteromonas is another Gram-negative rod detected once as an endophyte of *T. testudinum* from *Los Morillos*. Species of this genus have been shown to produce an array of low and high molecular weight compounds with antimicrobial, anti-fouling, algicidal and various pharmaceutically-relevant activities. Compounds formed include toxic proteins, polyanionic exopolymers, substituted phenolic and pyrolle-containing alkaloids, cyclic peptides and a range of bromine substituted compounds. Ecologically, *Pseudoalteromonas* appears significant and to date has been shown to influence biofilm formation in various marine econiches,. Furthermore, it is also involved in predator-like interactions within the microbial loop, influence settlement, germination and metamorphosis of various invertebrate and algal species, and may also be adopted by marine flora and fauna as defensive agents (Bowman, 2007).

Phylogenetic analysis revealed the presence of *Bacillus, Pseudomonas, Enterobacter, Rhodococcus* and *Micrococcus* which might be vital for the health of *Thalassia testudinum*, assuming roles similar to those previously reported in terrestrial plants. Also, recuperation of marine genera such as *Microbulbifer, Exiguobacterium, Nesterenkonia*, *Halobacillus, Pseudovibrio,* and *Vibrio* suggests that these bacteria have adapted to live inside the tissues of *Thalassia testudinum*, but their role is still unclear. This study about endophytic prokaryotic diversity associated with internal tissues of *T. testudinum* is the first approach to survey the community present in such environment. The data obtained for this study provide useful information for understanding the role of endophytes in the

health, establishment and proliferation of *T. testudinum*. Also, the identification of the endophytic flora will lead to isolate and characterize novel secondary metabolites that are important for the promotion of the plant's health.

6.3.2 **Ecology of culturable endophytes associated with healthy mature tissues of *T. testudinum***

In this study we attempted to compare the bacterial endophytes present in healthy mature tissues of *T. testudinum* from four geographical areas in *Puerto Rico* anthropogenically impacted in various ways. Buyé Beach have been under developmental and recreational pressures, *Los Morillos* has been exploited for salt, *Cayo Enrique* is nearby *La Parguera* which is impacted by boating and fishing activity while *Puerto de la Libertad* has received minimal anthropogenic, but is the place where the new port for the ferry will be constructed.

For each sampling site, there were differences observed in the number of isolates recuperated. Buyé Beach had the majority of isolates (522) while *Los Morillos* was the least numerous (126). *Puerto de la Libertad* and *Cayo Enrique* were very similar in the number of isolates recovered (~270-290). When looking at the distribution of recovered genera there are various differences in diversity depending on the sampling site.

One important fact is that *Bacillus* species were recovered from all sampling sites at the highest frequency of isolation. *Bacillus* represented 55% of the recovered strains, suggesting that these bacteria are part of the basal endophytic community of *T. testudinum*. *Bacillus* represented 67% of the strains from *Puerto de la Libertad*, 73% of the strains from *Cayo Enrique*, 83% from those of *Los Morillos* and only 28% at Buyé Beach.

Staphylococcus species were the second most frequently recovered, representing 18% of the endophytic strains. *Staphylococcus* was the predominant genus in Buyé Beach representing 39% of the isolated strains. On the other hand, endophytic *Staphylococcus* was significantly reduced in *Cayo Enrique, Puerto de la Libertad* and *Los Morillos* representing only 3%, 5% and 1%, respectively. The reduction of this bacterial community might be correlated with the type of anthropogenic impact received at the different beaches, but other environmental parameters have to be evaluated to better understand the predominance of *Staphylococcus* at Buyé Beach. *Halobacillus* was the third most predominant genus representing *11%* of all the isolated strains. The distribution of this bacterium was almost homogenous within all sampling sites representing 8% of *Cayo Enrique*, 7% of *Puerto de la Libertad*, 15% of Buyé Beach and 7% of *Los Morillos* endophytic community.

Other genera were recovered in smaller frequencies such as *Enterobacter, Pseudomonas, Cobetia, Nesterenkonia, Micrococcus,* and *Microbulbifer* representing 5%, 1%, 4%, 2%, 2% and 1% respectively. Significant differences were observed in the *Enterobacter* and *Microbulbifer* communities when comparing all sites. The *Enterobacter* community was significantly different in Buyé Beach when compared to the other three beaches. *Microbulbifer* was higher at *Cayo Enrique*, while the distribution was reduced homogenously in *Puerto de la Libertad, Los Morillos* and Buyé Beach.

Cellulosimicrobium, Pseudovibrio, Geobacillus, Rhodococcus, Vibrio, and *Pseudoalteromonas* were the least recovered strains. Vibrio was recovered at all sampling sites but only 1-2 strains were isolated. *Cellulosimicrobium* strains were not found at *Cayo Enrique*, while *Pseudovibrio* was only recovered at Buyé Beach and *Puerto de la*

Libertad. *Pseudoalteromonas* and *Chromohalobacter* were only isolated from *Los Morillos*, whereas *Geobacillus* and *Rhodococcus* strains were only found at Buyé Beach.

The endophytic community recuperated from the different beaches suggests the presence of basal endophytic communities from *T. testudinum* such as *Bacillus, Halobacillus, Staphylococcus, Microbulbifer, Nesterenkonia, Cobetia, and Enterobacter*. Buyé Beach reveals differences in the composition and distribution of the endophytic community when compared to the other three sampling sites. Buyé Beach community composition was dominated by the *Staphylococcus, a genus mostly associated to human,* which was a reduced community in the other sites. Probably the anthropogenic impact in Buyé Beach could be affecting the endophytes associated with *T. testudinum,* thus causing shifts in the community when compared to the other sites. Also, it would be interesting to look for effects among the overall endophytic community.

7. Assesment of the Endophytic Microbial Diversity by Culture-Independent Techniques

The second part of this project consisted on the identification of endophytic prokaryotes associated with *T. testudinum* by culture independent techniques. Due to the restrictions in microbial cultivation, many bacteria are difficult to isolate from their natural niche. In general, comprehensive description of microbial communities requires the use of techniques that sidestep cultivation because typically only a small fraction (<1%) of naturally occurring microorganisms is cultivable by standard techniques (Hugenholtz et al., 1998). For addressing the cultivation limitations, many culture independent techniques have been developed for trying to detect the microbial community thriving in a wide range of environments.

In this project two techniques were employed for the assemblage of endophytic community structure and distribution. One approach for the identification and assemblage of the endophytic community structure was to construct 16S rRNA gene metagenomic clone libraries. Through this approach it was possible to amplify the 16S rRNA genes obtained directly from environmental DNA through PCR based techniques, cloning and sequencing of such environmental genes. The second approach used was Terminal Restriction Fragment Length Polymorphism which intended to establish community profiles allowing detecting differences and similarities among the structure of microbial communities from different samples.

The profiles generated with these two techniques aided in understanding better the assemblage of endophytic population, which can't be detected by cultivation. Moreover, it was possible to establish differences in community profiles among different sites of

Puerto Rico. Endophytic community was significantly different in Buyé Beach, when compared to the other three beaches (*Los Morillos, Cayo Enrique* and *Puerto de la Libertad*). These results suggests that Buyé Beach endophytic microflora is exposed to higher anthropogenic impact, which is pertinent since Buyé Beach sea grass beds are increasingly threatened by many developmental pressures. By understanding the endophytes associated with *T. testudinum,* it will be possible to develop restoration strategies for the conservation of this essential marine ecosystem,

7.1 Materials and Methods

I. *Leaf Sterilization and leaf's processing*: Fifteen leaf samples per survey were collected, surface sterilized and processed using the same procedures described for the culture dependent techniques.

II. *Total metagenomic DNA extraction:* Metagenomic DNA was extracted using a modified protocol as described by Keb-Llanes et al, (2002). DNA was extracted from the plant tissue using an extraction buffer consisting of 10% SDS, 2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl, 10 mM ß-mercaptoethanol, 0.75 M sucrose and 5 mg/mL lysozyme. Proteins and salts were precipitated with cold 5 M potassium acetate, followed by isoamyl: chloroform extraction and isopropanol precipitation. The recovered DNA was resuspended in 50 µl of 1X TE (Tris-EDTA, pH 8) and treated with RNAse (final concentration of 20 µg/µl) for 30 minutes at 37ºC. Its quality was verified on 0.8% agarose gels after staining with ethidium bromide (10µg/mL).

III. *Terminal Restriction Fragment Length Polymorphism (TRFLP):* The TRFLP technique was used to establish differences and similarities among the structure of microbial communities between samples. This culture independent technique creates community profiles and patterns for each sample by fingerprinting that can be compared for further analysis. The 16S rRNA gene was amplified from total DNA extracted from surface sterilized leaves using the labeled primer pair 27 F/ FAM and 1392R. TRFLP fingerprinting was applied using three different restriction enzymes: Alu I, Hae III/HhA I and Msp I. Samples were processed using the LI-COR Biosciences NEN® DNA Analyzer Model 4300 (LICOR Inc.).

Gel Pro Analyzer Software® was used to evaluate accurately the level of differences of fingerprints between samples. Data obtained was statistically analyzed using Paleontological Statistics Program (PAST).

IV. *Amplification of the 16S rRNAgenes by PCR:* PCR reactions were performed as described by Chelius and Triplett (2001) using the set of primers 799F and 1492R which would amplify most bacterial sequences while excluding chloroplast DNA. PCR products were verified in 1% agarose gels after staining with ethidium bromide (10μg/mL). PCR products were purified by using Wizard SV Gel and PCR Cleanup System Purification Kit (Promega Inc.) according to manufacturer's protocol previous to cloning experiments.

V. *Cloning of PCR products and generation of environmental 16SrRNA clone libraries:* The products were ligated into the pGEM®-T cloning vector, and transformed into *E. coli* JM 109 high efficiency competent cells as described by the manufacturer (Promega Inc., Wisconsin, USA). Transformants were recovered in Luria-Bertani (LB) agar plates supplemented with ampicillin (100μg/ml).

VI. *Screening and Purification*: The Transformants were analyzed by Colony PCR (Güssow and Clackson, 1989) to confirm the presence of the the cloned fragments. The primers used for the PCR amplifications were T7 promoter and SP6 promoter (Promega ®) which flank the plasmid vector multiple cloning site. The reaction mixture consisted of ddH$_2$O, Promega buffer ® 1X, MgCl$_2$ 2.5mM, dNTP's 250mM, T7 promoter (5' TAA TAC GAC TCA CTA TAGGG 3') 1uM, SP6 promoter (5' ATT TAG GTG ACA CTA TAG AA 3') 1uM, Go*Taq* polymerase (Promega®) 0.026U/μl and 3uL of lysate from the clones as template for the reaction. PCR products were resolved by electrophoresis in agarose (0.8%) and visualized after

staining with ethidium bromide (10ug/mL). Amplicons of expected size, 755 bp, were purified using the MinElute PCR purification kit according to the manufacturer instructions (USA QIAGEN Inc.) and were sent to UPR Genomics and Sequencing Facility (Río Piedras, PR.) or UW-htseq Sequencing Facility for sequencing analysis. Sequences were characterized and phylogenetic trees were constructed as described above.

VII. *Statistical analysis of clones libraries*: To estimate the richness, evenness, and diversity of the eight clone libraries, both qualitative species-based measures, such as Chao 1 (Chao, 1984; Chao et al., 1993), ACE (Chao and Lee, 1992; Chazdon et al., 1998) and Jackknife (Burnham and Overton, 1979), and a quantitative species-based measure, Shannon index (Shannon and Weaver, 1949), have been calculated by using DOTUR (Schloss and Handelsman, 2005). In addition, rarefaction curves were plotted to estimate the expected species richness for each library and to study the effects of sampling on richness estimates (Brewer and Williamson, 1994).

The Chao1 is a richness estimator which uses the numbers of singletons and doubletons to estimate the number of missing species because missing species information is mostly concentrated on those low frequency counts. The ACE (Abundance-based Coverage Estimator) is a non-parametric estimator proposed by Chao and Lee (1992). In this estimator the observed species are separated as rare and abundant groups; only the rare group is used to estimate the number of missing species. The estimated coefficient of variation (CV) is used to characterize the degree of heterogeneity among species discovery probabilities. The Jacknife estimator is a used to estimate the bias and standard error when a

random sample of observations is used. Jacknife explores the statistic variability between subsamples by recomputing the estimator leaving out one observation at a time from the sample set, this makes it ideal for use in complex sample schemes and multistage samping (Chao, 2009).

These calculations of estimators were accomplished by first using ClustalW to align the sequences of each library with exclusions of gaps and corrections of multiple substitutions. Each of the resultant sequence sets were used as the input for the DNADIST program of PHYLIP (Felsentein, 1993) and distance matrixes were generated using the Jukes-Cantor distance correction model. Next, rarefaction curves and the richness estimators of Chao1, ACE, Jacknife and Shannon's index were obtained from DOTUR. The clones were clustered into OTUs at the level of sequence similarity corresponding to the distance between species of above 97% and the confidence intervals were calculated based on the 5% significant level.

Finally, the phylogenetic diversities among the three libraries were compared using the S-LIBSHUFF program (Schloss et al., 2004). This program determines wheter two libraries were drawn from the same population. In addition, it uses the coverage formula of Good (Good 1953) to determine homologous and heterologous coverage curves (C_x and C_{xy} respectively), and generated P values to test if two collections of sequences were sampled from the same population. The test statistics used to obtain P values was the Cramer-von Mises statistics (Pettitt, 1982), which also determined whether the coverage curves C_x and C_{xy} are significantly different at any evolutionary distance level. The distance matrix for

all of the sequences in three libraries were generated from M as described above and fed into S-LIBSHUFF to obtain coverage curves. The libraries were considered significantly different if the P value is less than 0.05.

VIII. *Construction of Phylogenetic Trees*: Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). A multiple-sequence alignment was made by using the Clustal W program with 16S rRNA gene sequences of close related organisms (as determined BLAST analysis). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Randomizing the input order was used to minimize any bias introduced by the order of sequence addition. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Replicate trees were made to generate 500 bootstrap data sets and the most frequent branching order was used for the final phylogenetic tree.

7.2 Results

7.2.1 Metagenomic DNA extraction from *T. testudinum* leaves

Since the research was focused in the study of microbial endophytes, surface sterilization was performed to eliminate epiphytic organisms. To validate the effectiveness of surface sterilization, concentrated genomic DNA (400ng/uL) was treated with sodium hypochlorite (0.5%) and DNA Away® surface decontaminant. After the assay, an agarose gel revealed no DNA in the sample treated with sodium hypochlorite (0.5%), but DNA treated with DNA Away® was partially degraded (Fig 2.1).
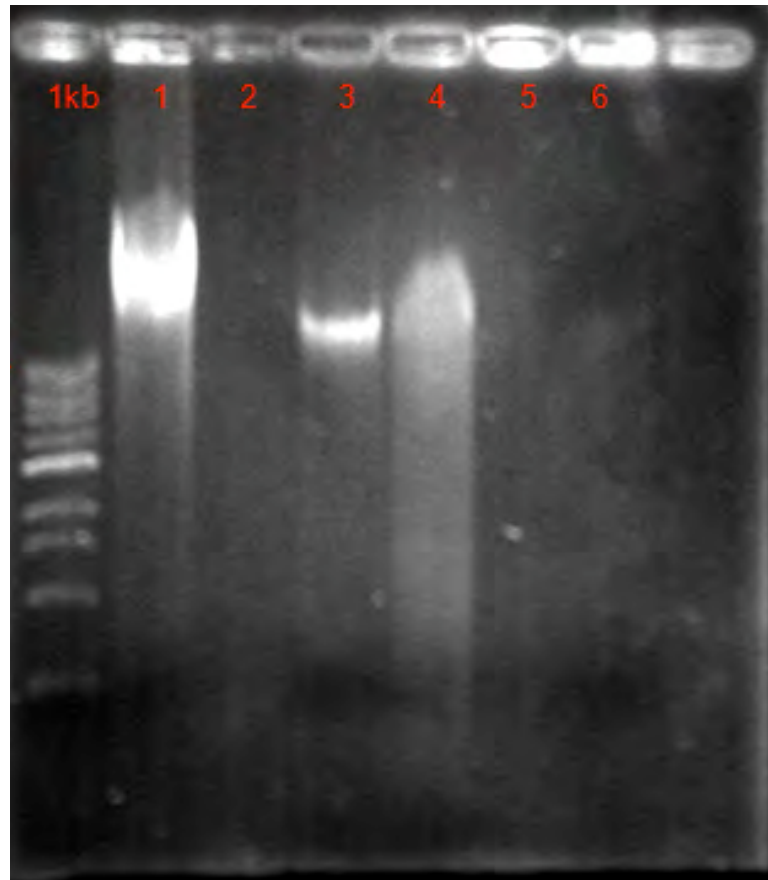
Figure .7.1 Validation of surface sterilization method with sodium hypochlorite solution. Genomic DNA of strain B 415 (1), B 415 treated with sodium hypochlorite solution (2), B 415 treated with DNA Away Solution (3), Genomic DNA of strain S 210 (4), S 210 treated with sodium hypochlorite solution (5) and S 210 treated with DNA Away solution (6).

After performing several DNA extractions from *Thalassia testudinum* leaves, good quality environmental DNA was obtained using a modified version of the protocol used by Keb-Llanes et al, (2002). DNA extractions were performed to fifteen leaves from each survey and named as Buyé (B, BB), *La Parguera (L, LB), Las Salinas (S, SB) and Vieques* (V, VB) which corresponded to the samples from the first and second survey accordingly (Figure 2.2).
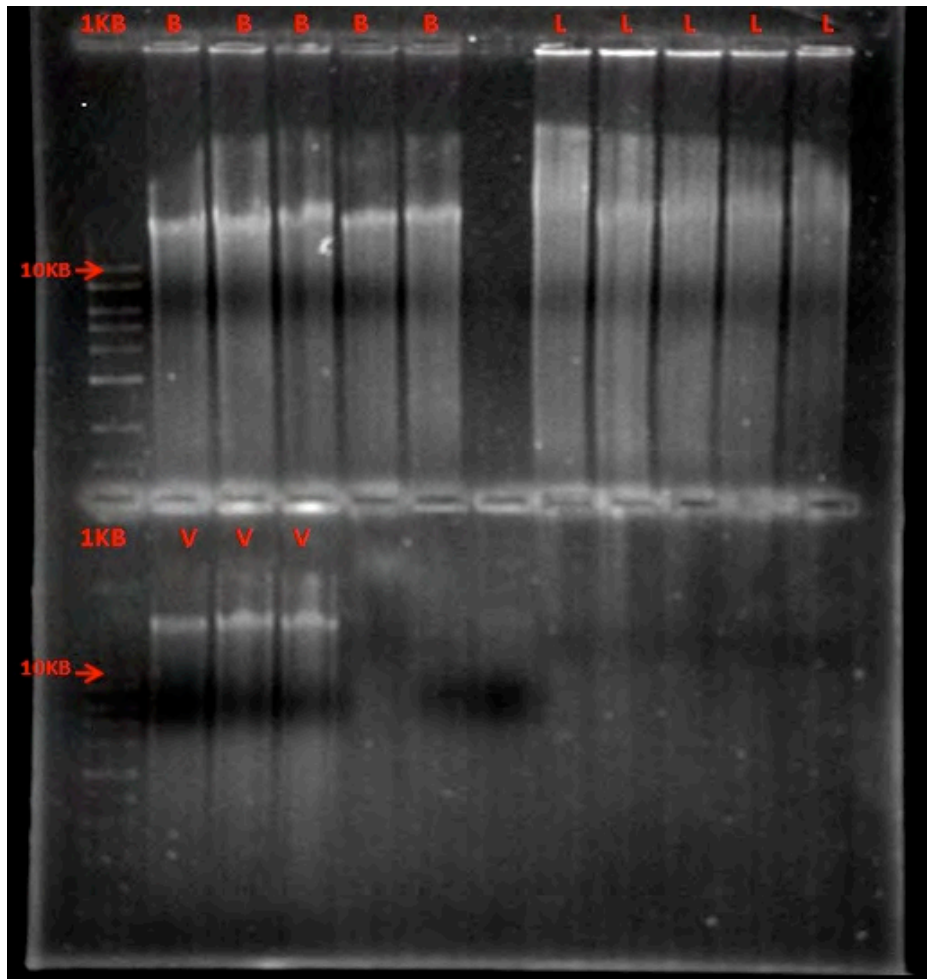
Figure 7.2 Metagenomic DNA from the leaves of *T. testudinum.* Samples: Buyé first survey (B), *Cayo Enrique, Lajas* first survey (L) and *Puerto de la Libertad*, Vieques first survey (V).

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7.2.2 Terminal Restriction Fragment Length Polymorphism Community Analysis

Small subunit 16S rRNA genes were from total community DNA was amplified using labeled primer pair Bacterial 27F/FAM and Universal 1392R for T-RFLP analysis (Figure 2.3). This technique is able to establish differences and similarities among the structure of microbial communities between samples. T-RFLP fingerprinting was applied using three different restriction enzymes. PCR amplicons were digested separately with Alu I, Msp I and a double digestion with HhA I/Hae III (Figure 2.3- 2.10) and the community T-RFLP patterns were analyzed in gel based DNA sequencer from LI-COR Biosciences NEN® DNA Analyzer Model 4300 (LICOR Inc.).
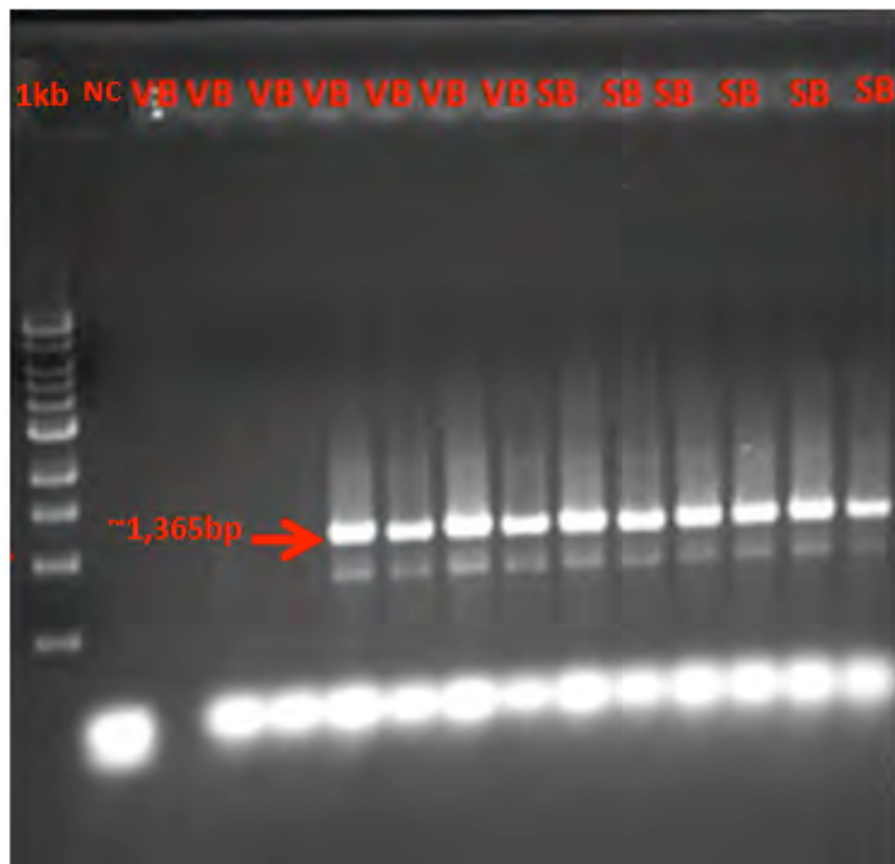
Figure 7.3 Amplification of the 16S rRNA genes from *T. testudinum* DNA using the labeled 27F/FAM and 1392R primers. Samples from *Puerto de la Libertad*, Vieques (VB) and *Los Morillos* (SB).
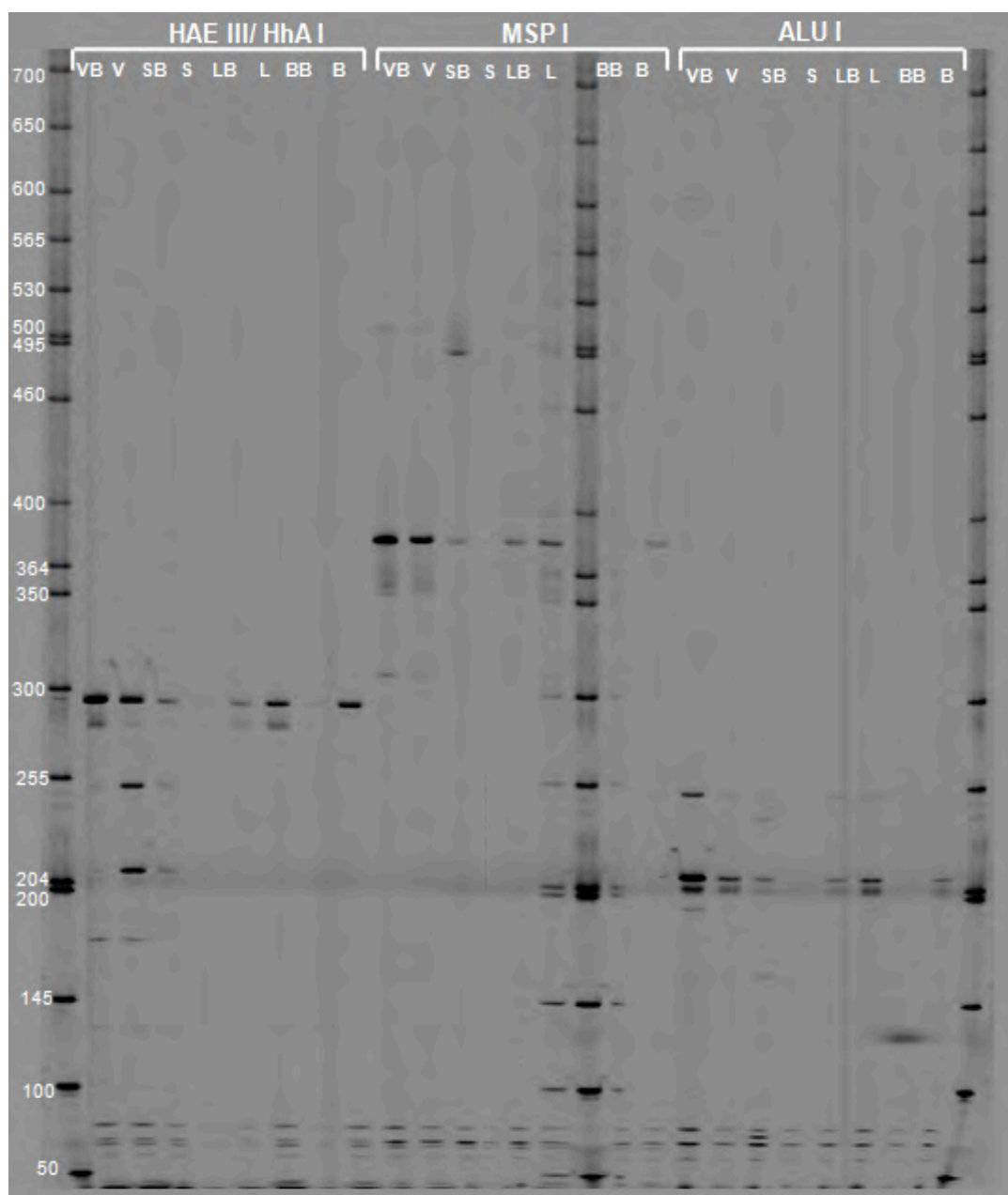
Figure 7.4 T-RFLP community profiles obtained by double digestion with HAE III/HhA I and single digestions with ALU I and MSP I observed in a gel sequencer**.** B= Buyé Beach first survey, BB= Buyé Beach second survey, L= *Cayo Enrique* first survey, LB= *Cayo Enrique* second survey, S= *Los Morillos* first survey, SB= *Los Morillos* second survey, V= *Puerto de la Libertad* first survey and VB= *Puerto de la Libertad* second survey.
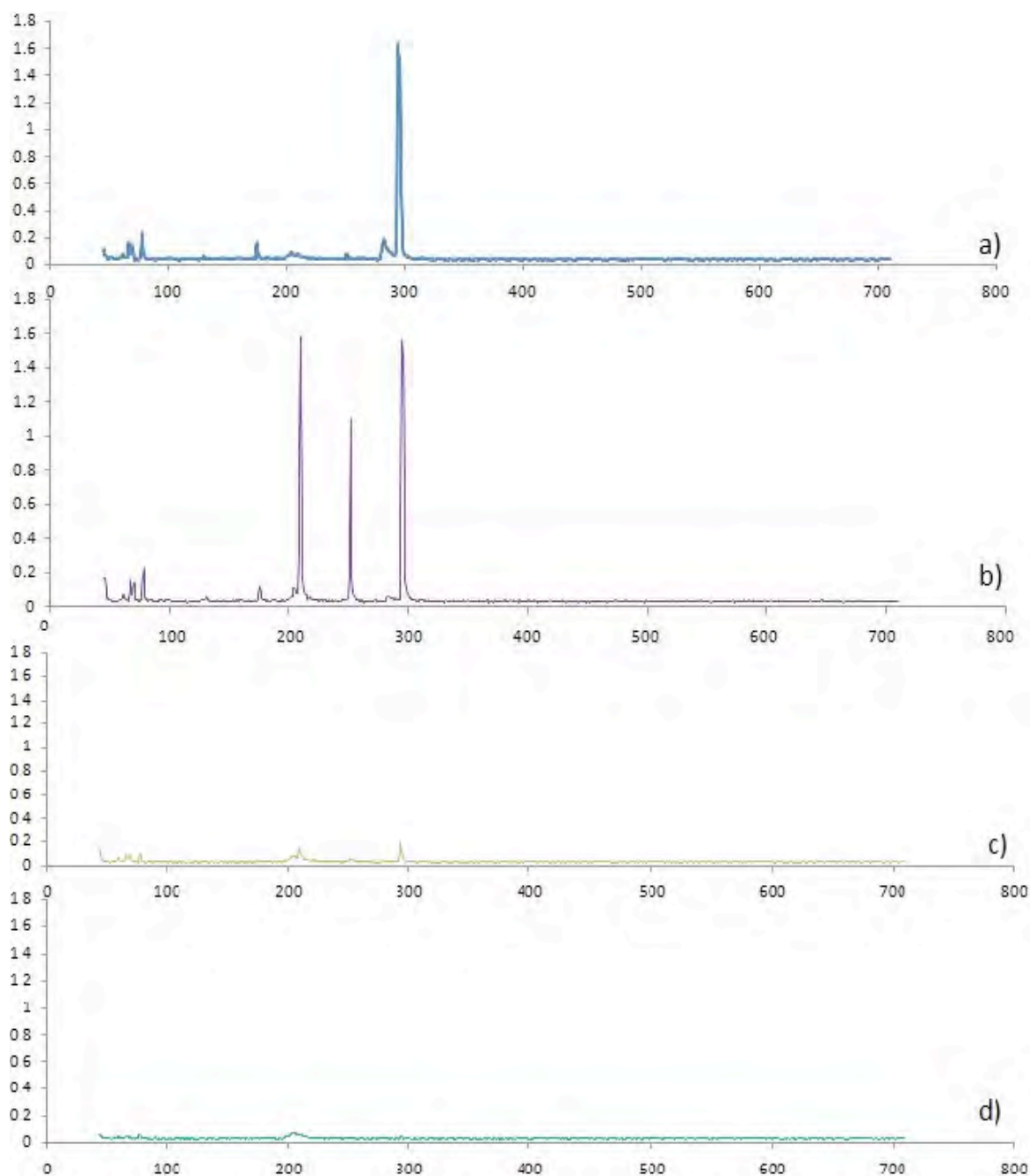
Figure 7.5 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by double digestion with Hae III/HhA I. Samples: *Puerto de la libertad*, Vieques second survey (a), *Puerto de la Libertad*, Vieques first survey (b), *Los Morillos* second survey (c) and *Los Morillos* first survey (d).
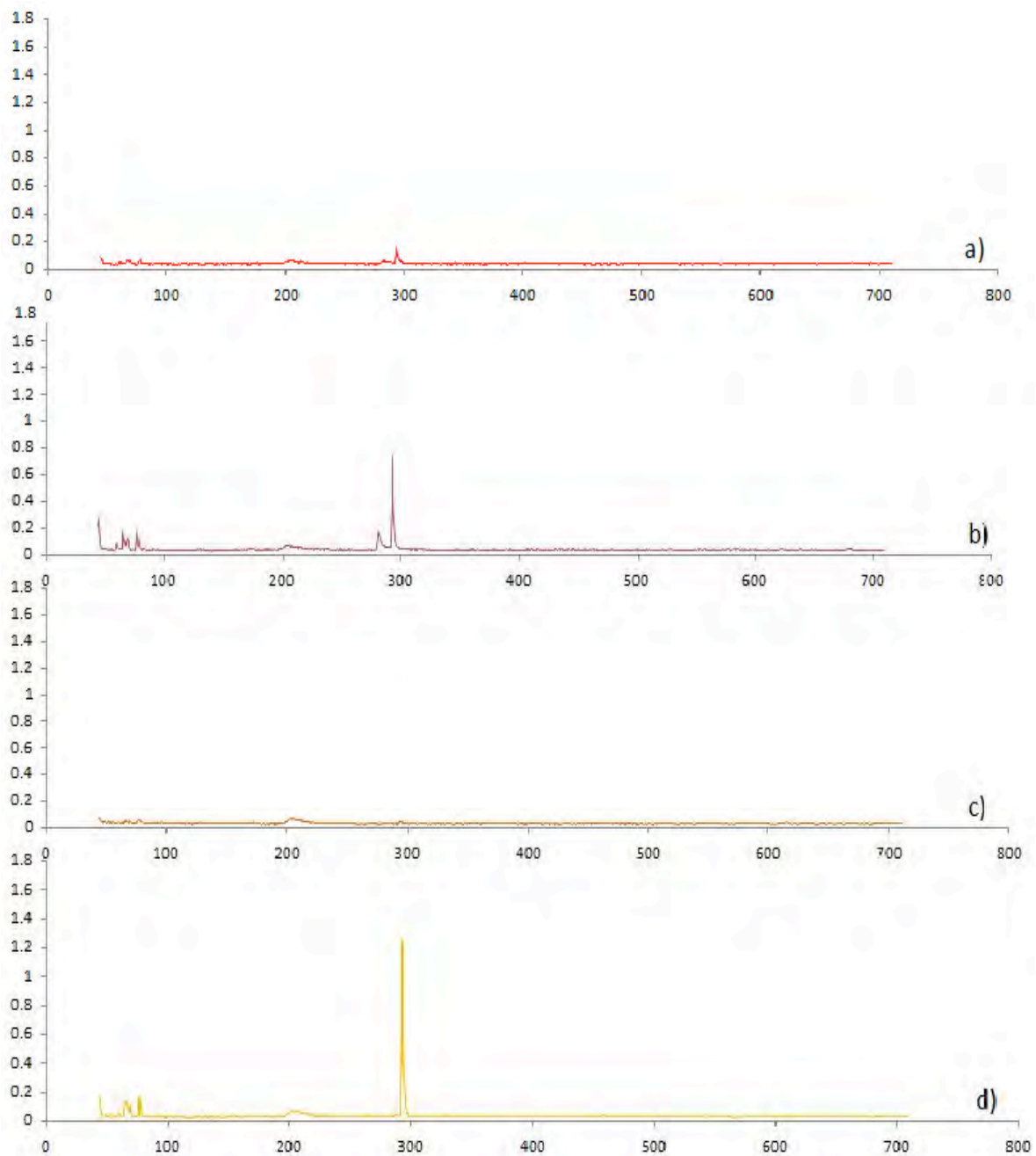
Figure 7.6 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by double digestion with Hae III/HhA I. Samples: *Cayo Enrique* second survey (a*), Cayo Enrique* first survey (b), Buyé Beach second survey (c) and Buyé Beach, first survey (d).
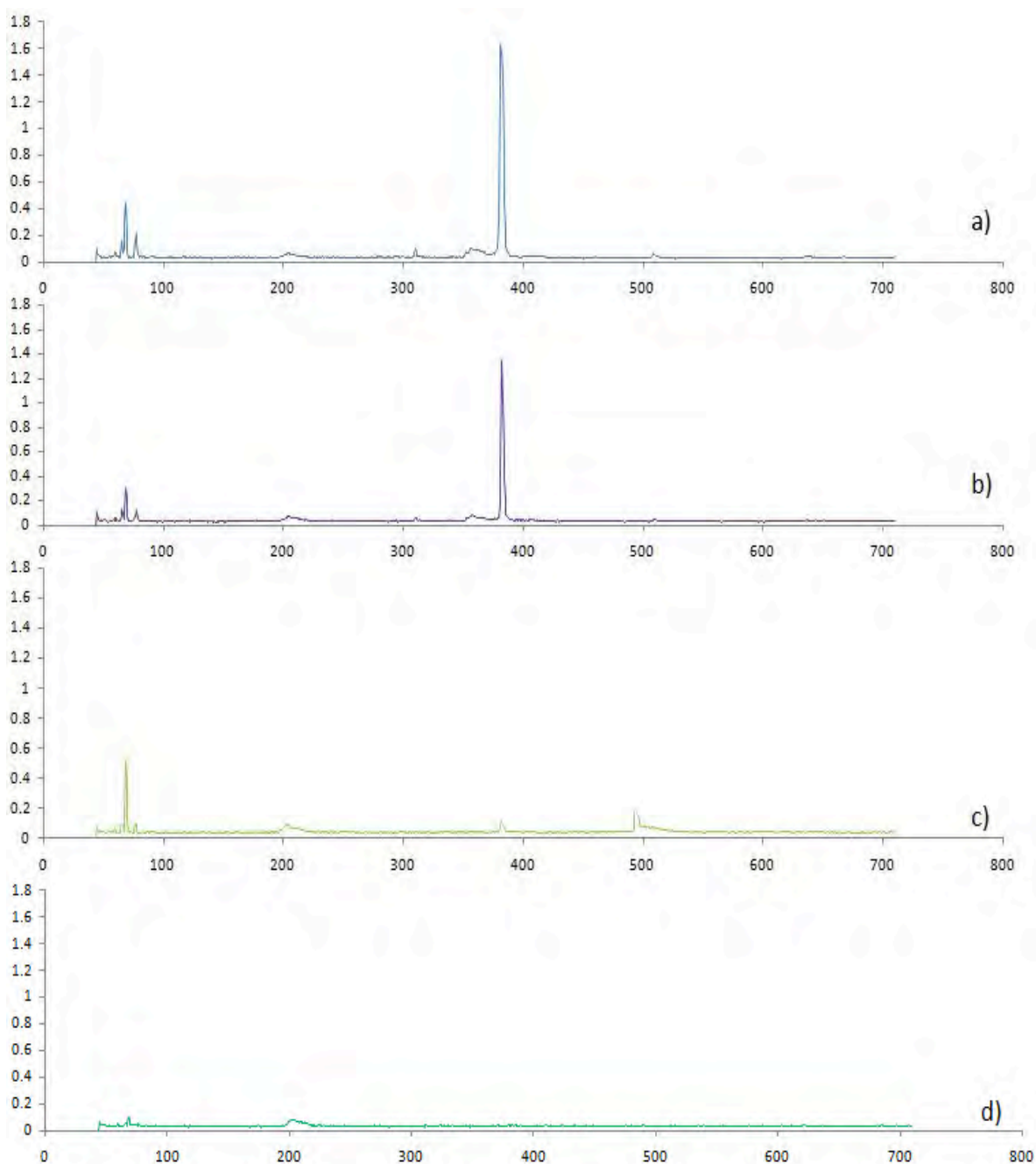
Figure 7.7 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by digestion with Msp I. Samples: *Puerto de la libertad*, Vieques second survey (a), *Puerto de la Libertad*, Vieques first survey (b), *Los Morillos* second survey (c) and *Los Morillos* first survey (d).

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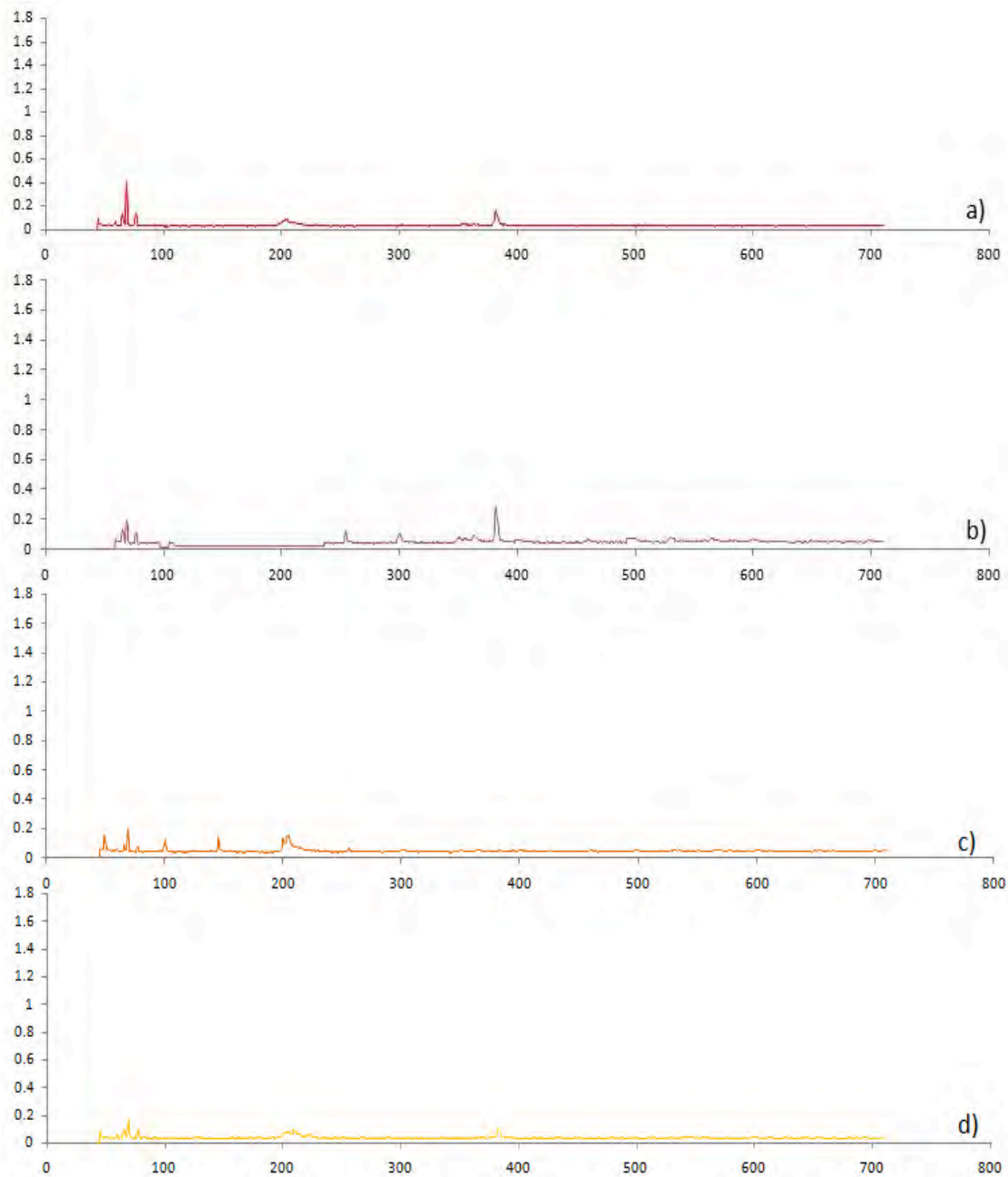
Figure 7.8 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by digestion with Msp I. Samples*: Cayo Enrique* second survey (a), *Cayo Enrique* first survey (b), Buyé Beach second survey (c) and Buyé Beach, first survey (d).
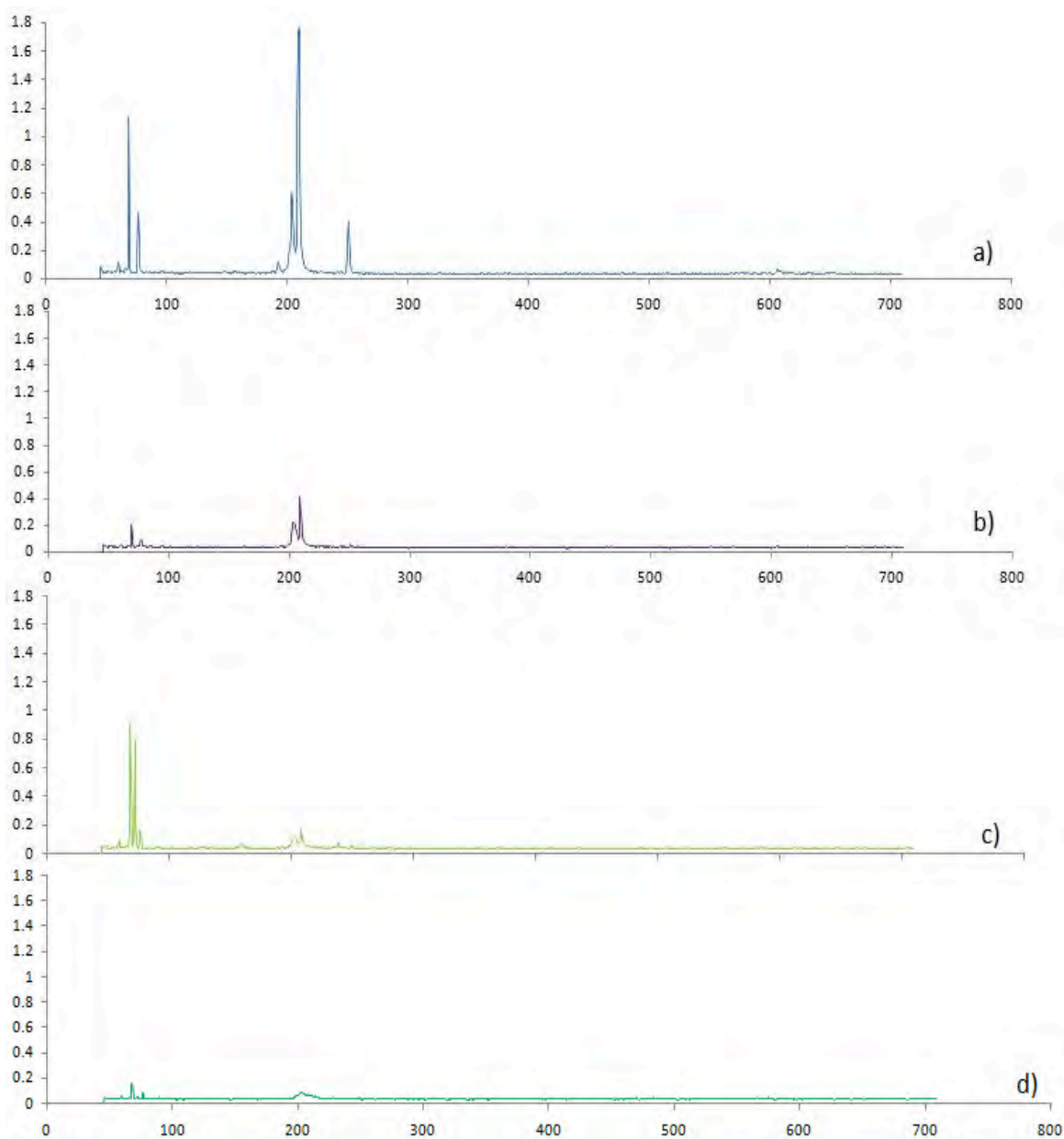
Figure 7.9 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by digestion with Alu I. Samples: *Puerto de la libertad*, Vieques second survey (a), *Puerto de la Libertad*, Vieques first survey (b*), Los Morillos* second survey (c) and *Los Morillos* first survey (d).

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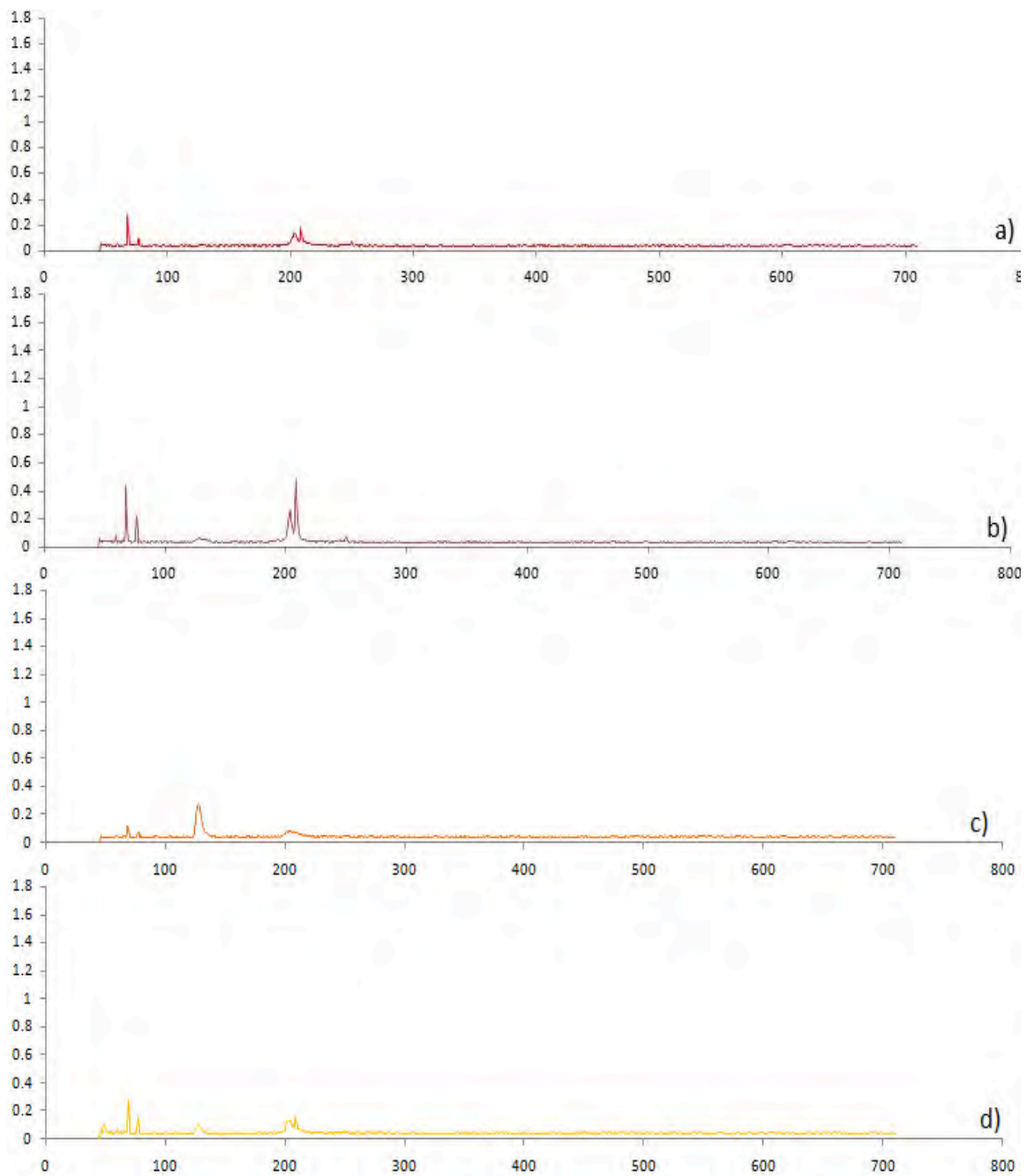
Figure 7.10 Electropherograms of T-RFLP patterns from the endophyte bacterial community associated with *T. testudinum* generated by digestion with Alu I. Samples*: Cayo Enrique* second survey (a), *Cayo Enrique* first survey (b), Buyé Beach second survey (c) and Buyé Beach, first survey (d).

Among the restriction endonucleases used for the T-RFLP community patterns, Alu I exhibited better resolution of T-RF's, followed by Msp I. The majority of the T-RF's generated with Alu I were small fragments with less than 300 bp (Figure 2.8 and Figure 2.9). All samples shared a T-RF of ~75bp and ~200bp. *Puerto de la Libertad* first survey reveals larger numbers of T-RF's in addition to higher peaks. The other six samples were homogenous with the number and position of peaks. The majority of Msp I digestions generated fragments of less than 500 bp (Figure 2.6 and Figure 2.7). This enzyme demonstrated a consistent peak at ~ 375 bp, excluding Buyé Beach second survey and *Los Morillos* first survey. The most diverse sample was *Puerto de la Libertad* second survey, while the least diverse was *Los Morillos* first survey. Finally, the double digestion with Hae III/HhA I showed lesser resolution of peaks (Figure 2.4 and Figure 2.5). The majority of T-RF's had a length of less than 300 bp and a shared 300 bp peak is consistent within all samples.

The T-RFLP patterns generated with each enzyme revealed shared and unique patterns within the different samples. *Puerto de la Libertad*, *Vieques* demonstrates consistent predominance in the quantity of T-RF's generated when compared to the other samplings, therefore being the most diverse sample. Interestingly, depending on the sampling event, differences were observed in the T-RF's pattern. On the other hand, *Los Morillos* samples consistently exhibited smaller T-RF's when compared to other samples. Shared peaks within all samples suggest the presence of endogenous endophytes associated with *T. testudinum* tissue. Also, the patterns seen from all digestions revealed a low diversity of endophytic bacteria associated with *T. testudinum*.

In addition, correspondence analysis of the T-RFLP patterns (Figure 2.11) was performed using the Paleontological Statistics Program (PAST). This analysis revealed that samples from *Puerto de la Libertad* and *Cayo Enrique* were clustered, indicating similarities within the T-RF patterns generated. *Los Morillos*, (second survey) was distant from the first survey suggesting a possible shift in bacterial diversity within seasons. In contrast, Buyé Beach sampling events exhibit a small difference in pattern within seasons, but have shared similarities with *Los Morillos* first survey.
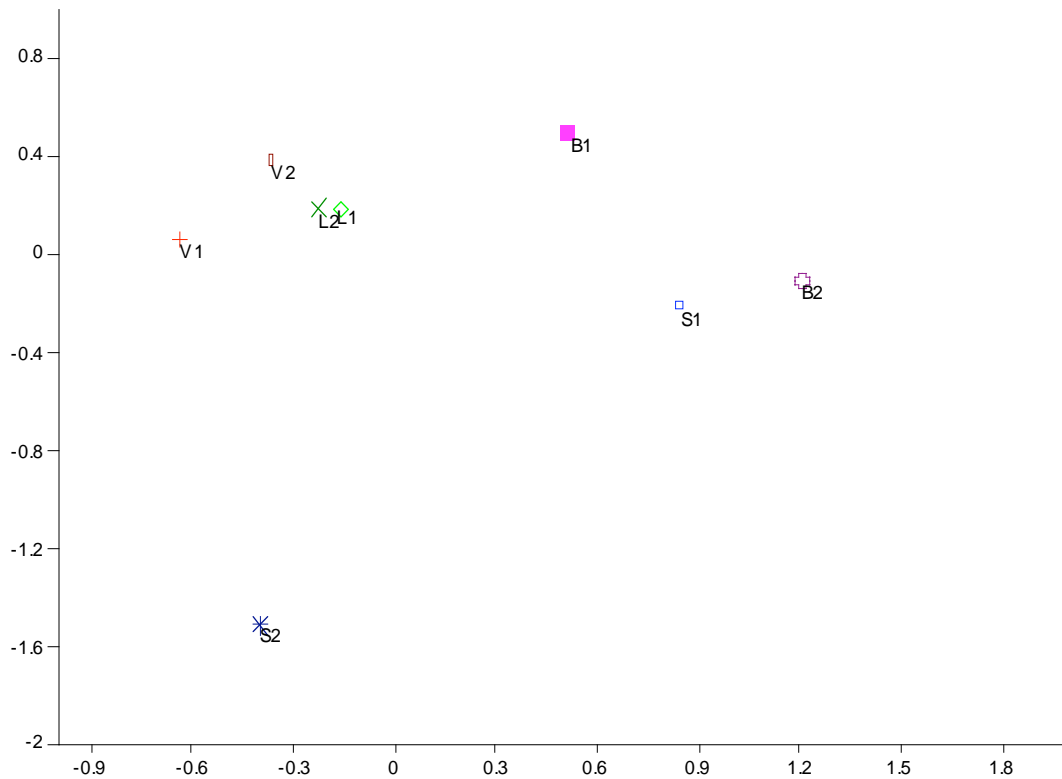
Figure 7.11 Correspondence analysis of T-RFLP profiles from 16S rRNA of endophytic community from *T. testudinum*. B1= Buyé Beach first survey, B2= Buyé Beach second survey, L1= *Cayo Enrique* first survey, L2= *Cayo Enrique* second survey, S1= *Los Morillos* first survey, S2= *Los Morillos* second survey, V1= *Puerto de la Libertad* first survey and V2= *Puerto de la Libertad* second survey.

7.2.3 Amplification of bacterial 16S rRNA from environmental DNA

Metagenomic DNA was used as template for PCR amplification of the 16S rRNA region. Initially, prokaryotic universal primers were used for PCR reactions, a doublet was observed in the amplifications products (Figure 2.12). The product of desired size (873bp) was excised from the agarose gel and purified. After sequencing, most of the PCR products obtained were from chloroplast 16S rRNA origin. To eliminate amplification of chloroplast 16S rRNA, bacterial endophyte primers 799F and 1492R (Chelius and Tripplett, 2001) were used for 16S rRNA PCR reactions yielding an amplicon of approximately 735 bp.
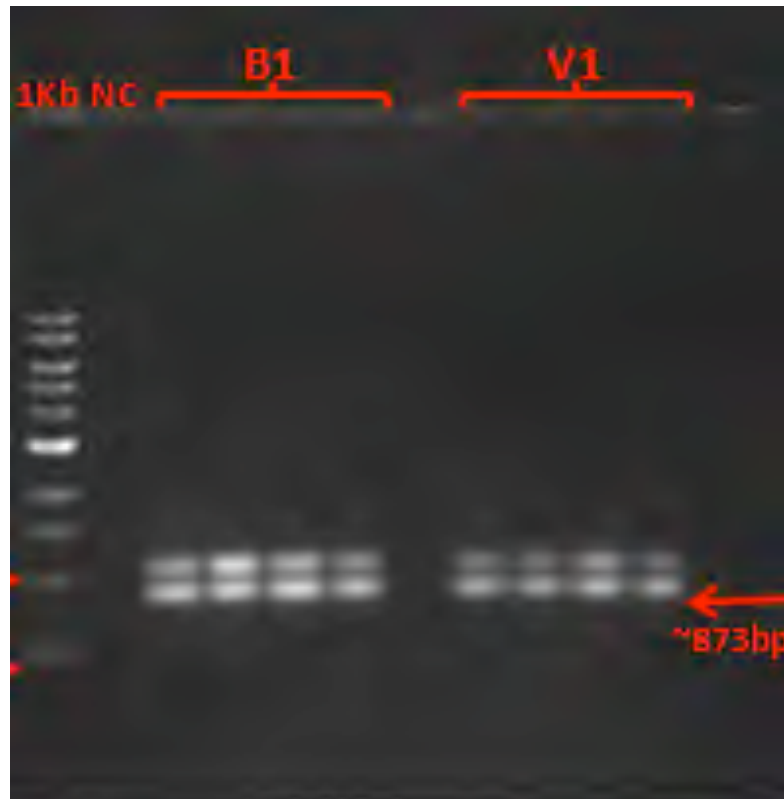
Figure 7.12 Four independent 16S rRNA PCR reactions (873bp) from *T. testudinum* leaves using universal primers. Samples: Buyé Beach first survey (B) and *Puerto de la Libertad,* Vieques first survey (V).

Amplicons exhibited a doublet were the heavier band corresponded to mitocondrial 16S rRNA and our product of interest was the lighter band of ~735bp (Figure 2.13). The amplicon of interest was excised from the 1% agarose gel and purified using the Wizard SV PCR Product Purification Kit as described by the manufacturer (Promega ®)
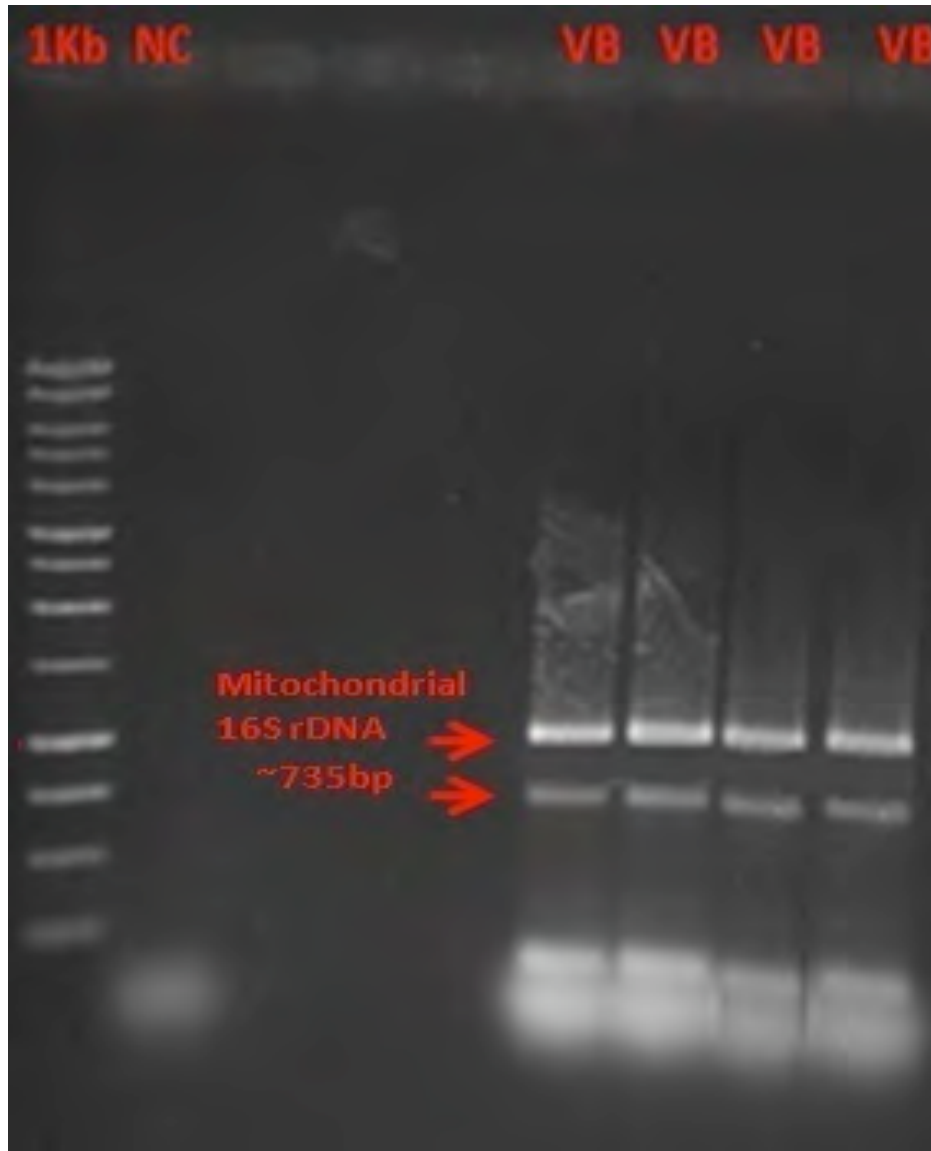
Figure 7.13 Four independent 16S rRNA PCR reactions (693bp) from *T. testudinum* leaves using bacterial primers. Samples: *Puerto de la Libertad*, Vieques (VB) first survey.

7.2.4 Construction of PCR amplified 16S rRNA genes clone libraries

Genomic 16S rRNA clone libraries were constructed with the purified PCR products described above. A total of 1,677 clones were obtained from all clone libraries. The distribution of clones obtained is showed in Table 2.1.

Table 7.1 Distribution of clones obtained per clone library

Genomic clone library	Sampling Site	# of clones
B	Buyé Beach	102
BB	Buyé Beach	135
L	*Cayo Enrique*	328
LB	*Cayo Enrique*	201
S	*Los Morillos*	76
SB	*Los Morillos*	231
V	*Puerto de la Libertad*	316
VB	*Puerto de la Libertad*	288

Colony PCR products revealed that 75% of the clones contained insert of the expected size (Figure 2.14). The amplification product was approximately 755 bp. For our study, 100 colony PCR products were chosen from each clone library for sequencing. Colony PCR products were purified using Wizard SV PCR Product Purification Kit as described by the manufacturer (Promega ®). Samples were sequenced at UW-htseq facility according to their specifications.

Sequences were screened for quality and vector contamination using VecScreen (BLAST). To check for possible chimeric sequences all clones were analyzed using the Chimera Check program from the RDP database (Cole et al., 2003) version 2.7 (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU). This analysis confirmed that no chimeric sequences were present. Among all clones sequenced, 104 OTU'S (<97% similarity) were chosen for further characterization. Sequences having a 97% similarity were considered the same OTU's. The closest relatives of representative OTU'S were

identified by searching in the GenBank database. Selected OTU'S closest relatives are listed in Table 2.2.
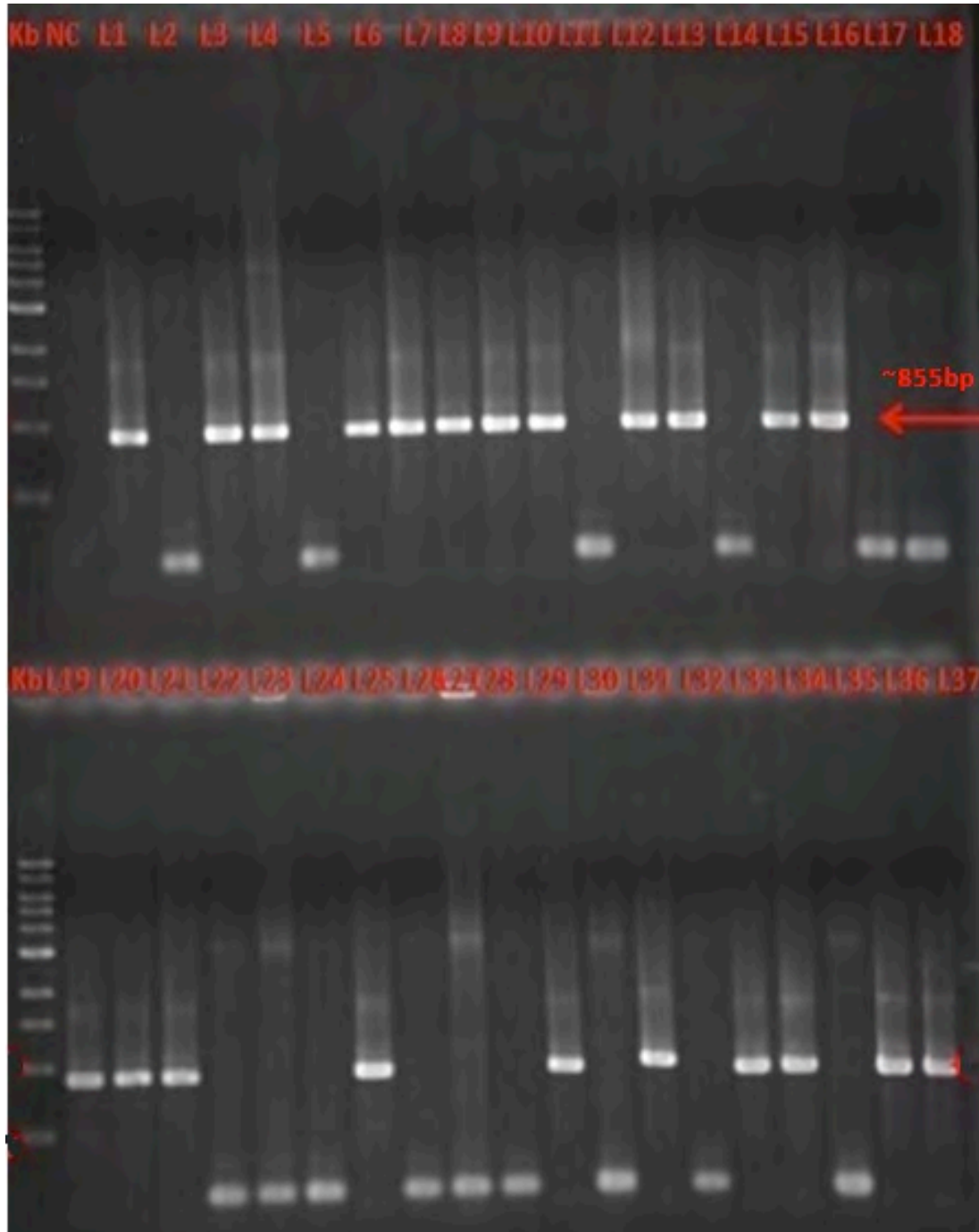
Figure 7.14 Colony PCR amplicons from L, *Cayo Enrique*, clone library using SP6 and T7 promoter primers. Bands show a PCR product of approximately 855 bp.

Table 7.2 16SrRNA sequences identified from *T. testudinum* tissue clone libraries

Sequence Representative of OTU's	Number of clones	Microorganism or clone with highest 16SrRNA sequence similarity		
		Taxon	Acession No.	% Identity
VB1	39	Alpha-proteobacterium	AY562567	95
SB22	24	*Enterobacter hormaechei subsp. steigerwaltii*	AJ853890	99
LB 48	7	Uncultured bacterium clone	FJ425593	98
V66	5	*Bacillus thuringensis*	FJ614243	96
VB26	10	*Bacillus sp.*	DQ448800	99
B47	8	*Bacillus sp.*	FJ461466	95
V6	20	Uncultured bacterium clone	DQ071482	99
LB70	12	Uncultured bacterium clone	FJ202916	97
LB42	8	*Hirschia baltica*	AJ421782	95
LB91	4	Uncultured bacterium clone	EU917585	96
B18	21	*Enterobacter sp.*	EU545406	96
LB106	5	Uncultured alpha proteobacterium	DQ644016	96
LB107	4	Uncultured *Rhodobactereaceae* clone	FJ403109	95
VB102	4	*Porphyromonas clone*	AF385561	99
SB57	14	*Microbulbifer cystodytense*	AJ620879	84
L208	4	*Bacillus sp.*	FJ461466	97
L214	6	*Bacillus cereus*	FJ455077	99
S21	3	Uncultured bacterium clone	FJ479547	88
L207	3	*Bacillus sp.*	FJ461466	86
V41	9	*Bacillus sp.*	FJ529033	95
V150	6	*Bacillus sp.*	FJ461466	99
LB83	2	Uncultured bacterium clone	FJ203462	98
LB29	2	Uncultured bacterium clone	EU917915	94
VB23	2	Uncultured bacterium clone	FM874666	99
LB31	2	Uncultured bacterium clone	EU799764	98
VB117	*4*	*Geobacillus sp.*	DQ642093	97
Lb95	*4*	*Erythrobacter citreus*	FJ607439	99
L127	*3*	*Uncultured Leptothrix clone*	FJ388370	99
VB88	*3*	*Uncultured Myxococcaceae bacterium clone*	FJ516764	94
L212	*6*	*Pelagiobacter variabilis*	FM180508	99
L176	*2*	*Microbulbifer sp.*	AB243106	94
Vb93	*5*	Uncultured bacterium clone	FJ479556	98
V122	*2*	Uncultured beta proteobacterium	AB294945	97
VB24	*2*	Uncultured *Acinetobacter* clone	EU491216	96
VB109	*7*	Uncultured low G+C Gram positive clone	AF348712	94
Lb98	*4*	Uncultured bacterium clone	FM875547	93
VB94	*2*	Uncultured *Firmicutes*	AM706669	96

7.2.5 Phylogenetic Analysis of Clones

Phylogenetic and molecular evolutionary analyses among a total of 37 OTU's was conducted using MEGA version 4 (Tamura, et al., 2007). A multiple-sequence alignment was made by using the Clustal W program with 16S rRNA gene sequences of close related organisms (as determined BLAST analysis). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Randomizing the input order was used to minimize any bias introduced by the order of sequence addition. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Replicate trees were made to generate 500 bootstrap data sets and the most frequent branching order was used for the final phylogenetic tree.

A total of 37 OTU's were obtained from all 16S rRNA clone libraries. Phylogenetic analysis revealed the presence of shared and unique clones among all sampling sites. After verifying the phylogenetic affiliation of OTU's, their distribution among major bacterial groups was analyzed (Figure 2.15).
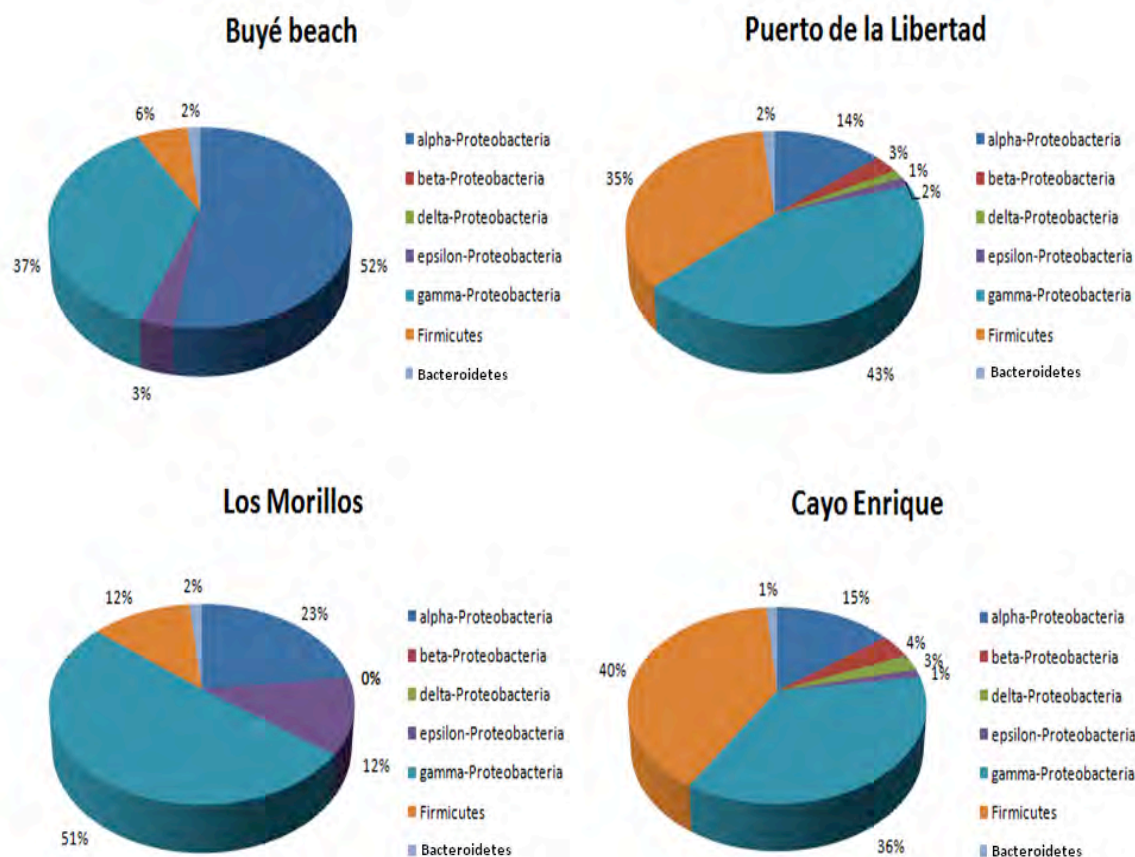
Figure 7.15 Pie charts representing the distribution OTU's among bacterial groups as observed per sampling sites.

 Cayo Enrique and *Puerto de la Libertad* clone libraries exhibited similar patterns in community structure when analyzing the affiliation of clones to major bacterial groups. At these two sampling sites clones affiliated to *Firmicutes* and *gamma-Proteobacteria* were predominant. Also, a reduced population of *Bacteroides* and *delta-Proteobacteria* was observed only at these two sites. In contrast, *Firmicutes* at Buyé Beach and *Los Morillos* is substantially reduced. At Buyé Beach, *alpha-Proteobacteria* were predominant, while *gamma-Proteobacteria* where most abundant in Los Morillos. Phylogenetic analysis performed by neighbor joining algorithm revealed that from the

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clones analyzed, 37 different operational taxonomic units were found among all sampling

sites.

The *alpha-Proteobacteria* group was found in all sampling sites and 68 clones

were obtained which were clustered in 8 different representative OTU's (Table 2.16).

OTU LB42 (representative of clones B69, LB42, S55, S60, S5, S100, SS52 and VB112)h

is closely related to *Hirschia baltica* (Figure2.16), a budding hyphal bacteria isolated

from the Baltic sea (Schlessner et al, 1990). OTU LB91 (representative of clones LB41,

S45, BB126 and VB76) formed a cluster with uncultured *alpha-Protebacteria* clone

(Figure 2.16) found associated with *Ulva australis*, a marine macroalgae. OTU VB1

(representative of clones B2, BB2, B13, B68, B22, B50, B96, B41, B62, B75, B90, B54,

B10, B72, B71, B101, BB127, B102, B57, B56,B66, B88, B34, B43, B94, B87, VB1,

L2, L400, S2, S51, S17, S22, S13, S3, and SB3) was closely related to an uncultured

Stappia clone (Figure 2.16), associated with the coral *Erythropodium caribaeorum.* OTU

LB107 (representative of clones B31, LB107, S42 and VB 86) was closely related to an

uncultured *alpha-Proteobacterium* clone WN-HWB-17 (Figure 2.16), isolated from

alkaline hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah et. al, 2007). Clones

LB29 and V44 are clustered in the same OTU, which was closely related to an uncultured

bacterium clone Cyano2E05 (Figure2.16) found in natural stromatolites, a type of

microbialite, collected from Exuma Sound, Bahamas (Haveman and Foster, 2008). LB

106 (representative of of clones B9, SB55, V138, V110 and LB106) was related to an

uncultured bacterial clone BBD HS216b-07 (Figure 2.16) isolated from corals affected

with black band disease. Clones LB83 and V51 were the same OTU, phylogenetically

related to uncultured bacterium clone SHFH515 (Figure 2.16), isolated from the

Caribbean coral *Montastraea faveolata.* Clones LB31 and VB84 were the same OTU, which was closely related to an uncultured bacterium clone 1C227412 (Figure 2.17).

The *beta-Proteobacteria* group was represented in low frequencies from *Puerto de la Libertad* and *Cayo Enrique* sampling sites. Five clones were recuperated which were clustered into 2 OTU. Clones V122 and LB79 were the same OTU which was closely related to an uncultured *beta-Proteobacterium* clone pItb-vmat-37 (Figure 2.18) found at a shallow submarine hydrothermal system occurring within a coral reef near Taketomi Island, Japan (Hirayama et al., 2007). Clones L127, L300, and V89 were classified as the same OTU. OTU L127 was related to uncultured *Leptothrix* clone DA185 (Figure 2.19), found associated with a citrus plant.

Also, the *delta-Proteobacteria* was only found in *Cayo Enrique* and *Puerto de la Libertad* sampling sites. Three clones were found which were represented in only one OTU sequence. Clones VB88, LB23, and LB73. OTU VB88 was phylogenetically affiliated to an uncultured *Myxococcaceae* bacterium clone TDNP Bbc97-142-1-36 (Figure 2.21) which was isolated from a semiarid wetland at Central Spain.

The *epsilon-Proteobacteria* group was observed from all sampling sites. Twelve clones were found clustered in only one OTU. OTU LB 70 (representative of clones B85, B48, LB70, S9, S31, S28, S30, S39, S10, S44, S54, and VB17) was closely related to an uncultured bacterium clone SGUS955 (Figure 2.20), found associated with the Caribbean coral *Montastrea faveolata*.

The *gamma-Proteobacteria* was consistently a predominant group at all sampling sites with 111 clones clustered into 12 OTU's. SB 22 (representative of clones SB26, SB43, SB9, SB41, SB35, SB15, SB59, SB22, SB11, SB3, SB21, S102, L162, L182, B8,

V21, VB99, V11, V22, V130, V13, V118, V157, and V48) was closely related to *Enterobacter hormaechei subsp. steigerwaltii* (Figure 2.24). OTU LB48 (representative of clones B17, B6, LB48, S53, V111, VB11 and V148) was closely related to an uncultured bacterium clone B78-102 (Figure 2.23), which was isolated from sediment of the Pacific. OTU V6 (representative of clones BB116, LB12, SB24, SB19, SB17, SB47, SB5, SB53, SB16, SB25, SB29, SB30, SB6, SB2, SB1, SB13 ,S26, S33, S37, and V6) was affiliated to an uncultured bacterium clone TuCc20 (Figure 2.25), isolated from the wood grouse *Tetrao urogallus*. B18 (representative of clones B18, B7, B84, B73, B37, B19, B51, B5, B42, B83, B67, B35, B26, B33, B45, B76, B14, B59, LB44, S24, and VB118) was associated with *Enterobacter sp*. XW122 (Figure 2.24), a strain isolated from sediment of a poultry-waste polluted river at Southwestern Nigeria. OTU L212 (representative of clones B89, L223, L212, L205, L129, L161, L192, L0, L156, L1, L3, LB6, SB57, and V119) formed a cluster with *Microbulbifer cystodytense* (Figure 2.22) which is a strain isolated from a colonial tunicate, *Cystodytes dellechiajei*. OTU S21 (representative of clones L21, S21, and V63 are represented in OTU S21) was phylogentically associated to an uncultured bacterium clone p8n02ok, isolated from an undisturbed tall grass prairie. OTU L212 (representative of clones L212, L225, L210, L204, L186, L126, V141, and VB75) was related to *Pelagiobacter variabilis* (Figure 2.22), a strain found associated with the marine sponge *Tedania anhelans*. Clones L176 and VB95 pertained to the same OTU which was affiliated to *Microbulbifer sp*. A4B-17 (Figure 2.22), a strain isolated from an ascidian in the coastal waters of Palau (Peng et al, 2006). OTU VB93 (representative of clones LB33, VB15, VB92, VB125, and VB93) was closely related to an uncultured bacterium clone U00096/1-7682 found at a tall grass

prairie. Clones LB5 and VB 24 were clustered as an OTU which is affiliated to an uncultured bacterium clone P9X2b7H04, which were found at seafloor lavas from the Loi'hi Seamount Pisces Peak X2 (Santelli et al., 2008). OTU LB98 (representative of clones LB98, VB 81, VB89, and VB 97) was related to an uncultured bacterium clone SD01A04 isolated from the skin surface.

The *Bacteroidetes* group was represented by 4 clones, one from each sampling site. All clones, BB1, LB67, S32, and VB102 pertained to the same OTU. This *Bacteroidetes* OTU was affiliated to *Porphyromonas* sp. oral clone BS077 (Figure 2.28), isolated from oral epithelial cells from humans.

The *Firmicutes* group was found widely distributed at all sampling sites, being predominant in *Puerto de la Libertad* and *Cayo Enrique*. A total of 65 clones were grouped into 12 OTU's. OTU V66 (representative of clones B3, LB50, S54, V66, and V85) was phylogenetically associated with *Bacillus thuringiensis* isolate LDC-391 (Figure 2.26). OTU VB 26 (representative of clones VB26, B82, B78, LB52, LB64, LB74, LB100, LB86, LB47, and S23) was closely related to *Bacillus sp.* CNJ937 PL04 (Figure 2.26), which was isolated from marine sediments (Gontang et. al, 2007). OTU B47 (representative of clones B47, L183, L198, L194, L200, L189, S36, and V108) was phylogenetically related to *Bacillus sp.* SCSSS10 (Figure 2.26) found at marine sediment from South China Sea. OTU L208 (representative of clones L208, L172, SB50 and V91) was affiliated to *Bacillus sp*. Tianshan512-1, isolated from a glacier in Tianshan Moutain from China. OTU L214 (representative of clones L214, L173, L174, L195, S0, and V153) was related to *Bacillus cereus* strain JHCFS2 (Figure 2.26). OTU V41 (representative of clones V41, L213, S25, V49, V51, V80, VB113, V155, V78, and V30)

was closely related to *Bacillus sp.* S-JS-3, isolated from soil of a contaminated oilfield. OTU V150 (representative of clones LB10, SB56, VB9, V142, V150, and V152) was related to *Bacillus pumilus* strain BSH-4 (Figure 2.26). OTU VB117 (representative of clones LB2, V25, V109, and VB117) was related to *Geobacillus sp.* O83 (Figure 2.27) isolated from a Santorini volcano habitat (Meintanis et. al, 2008). OTU LB45 (representative of clones LB45, LB60, LB40, LB103, LB62, LB66, and VB109) was closely related to an uncultured low G+C Gram-positive bacterium clone MPD-16, which thrives in a selenium-contaminated hypersaline evaporation pond (de Souza et al., 2001).
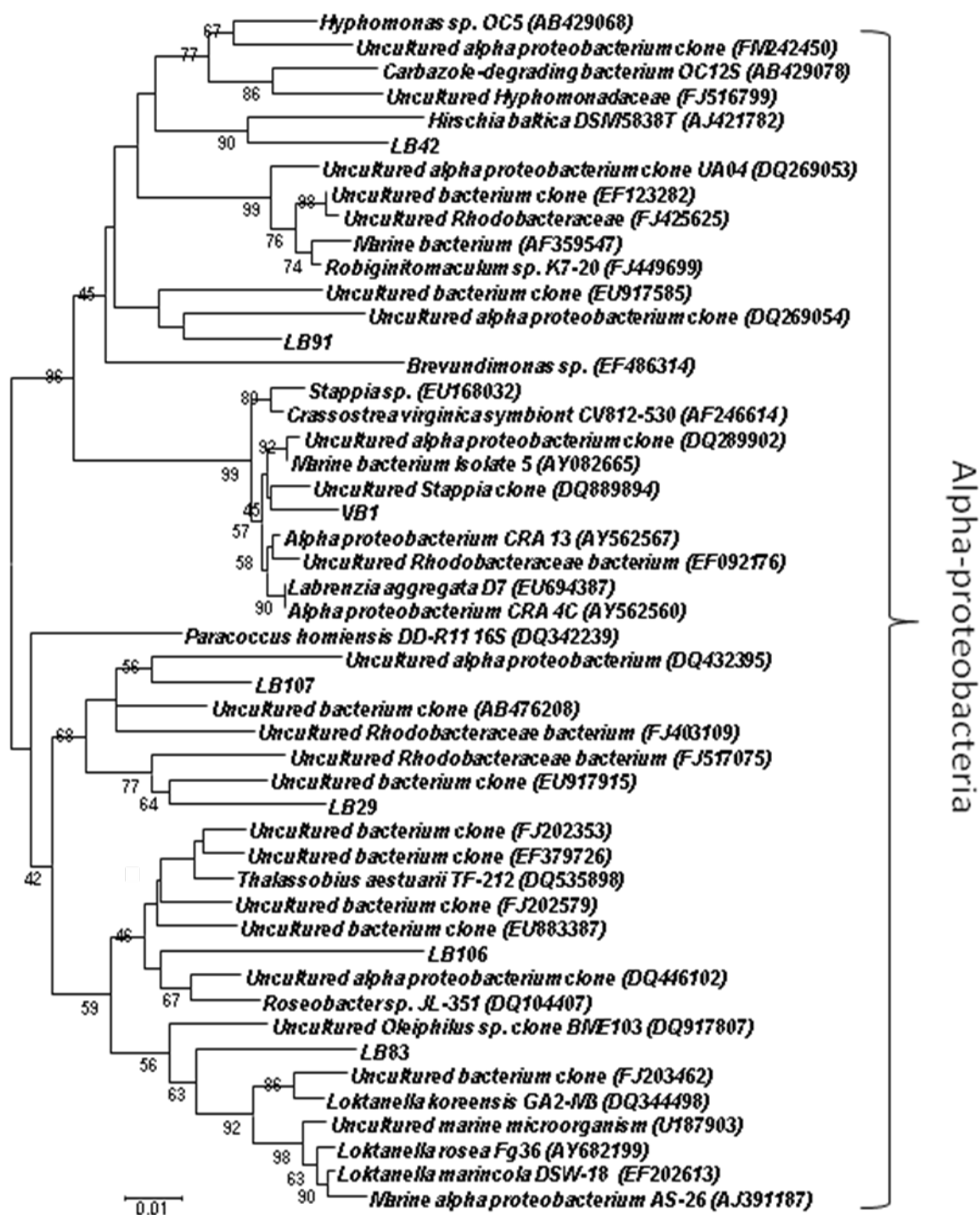
Figure 7.16 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *alpha-proteobacteria* OTU LB83, LB106, LB29, LB107, VB1, LB91, and LB42 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follow the designation of each taxon.
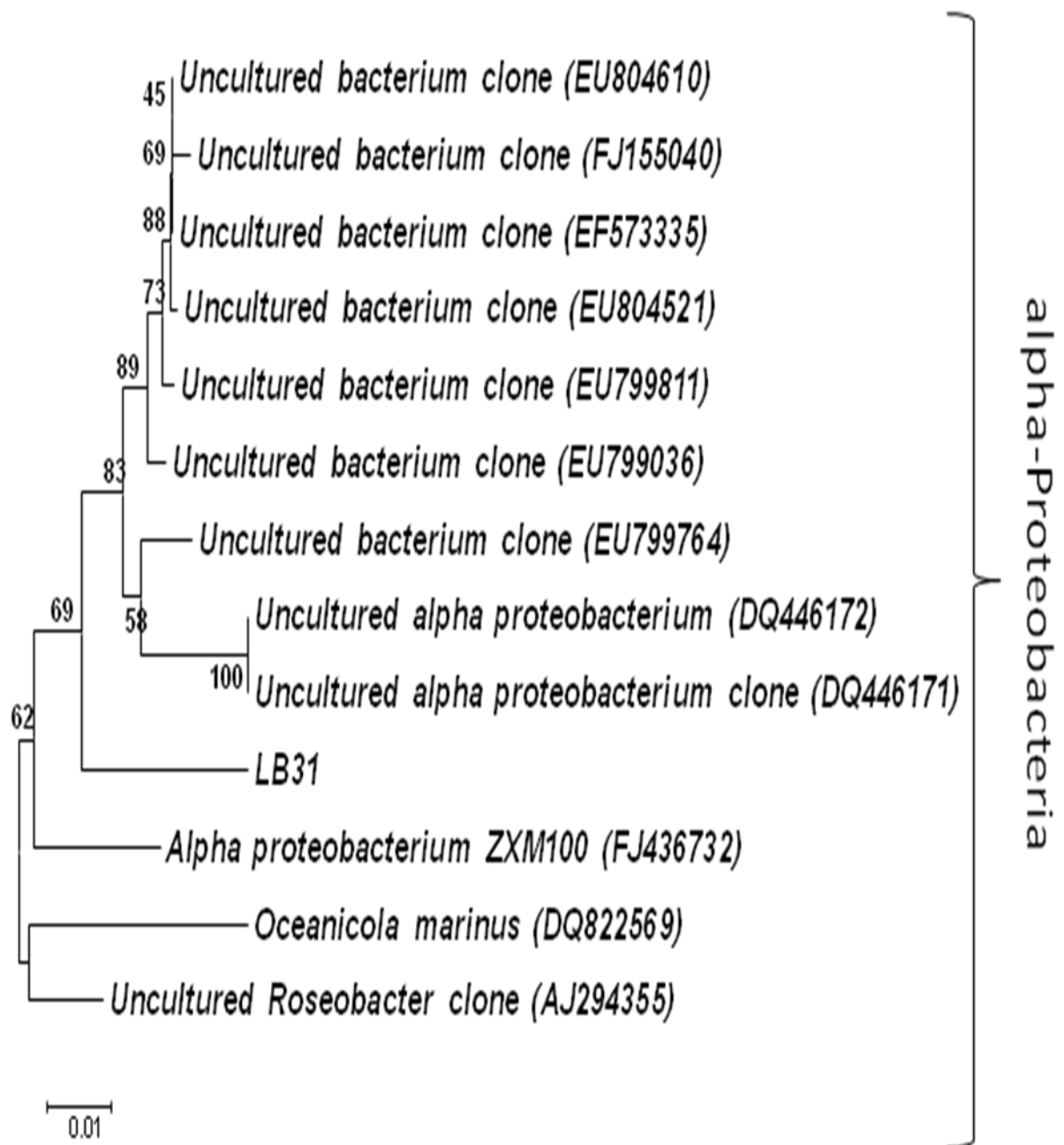
Figure 7.17 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *alpha-Proteobacteria* OTU LB31from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follow the designation of each taxon.
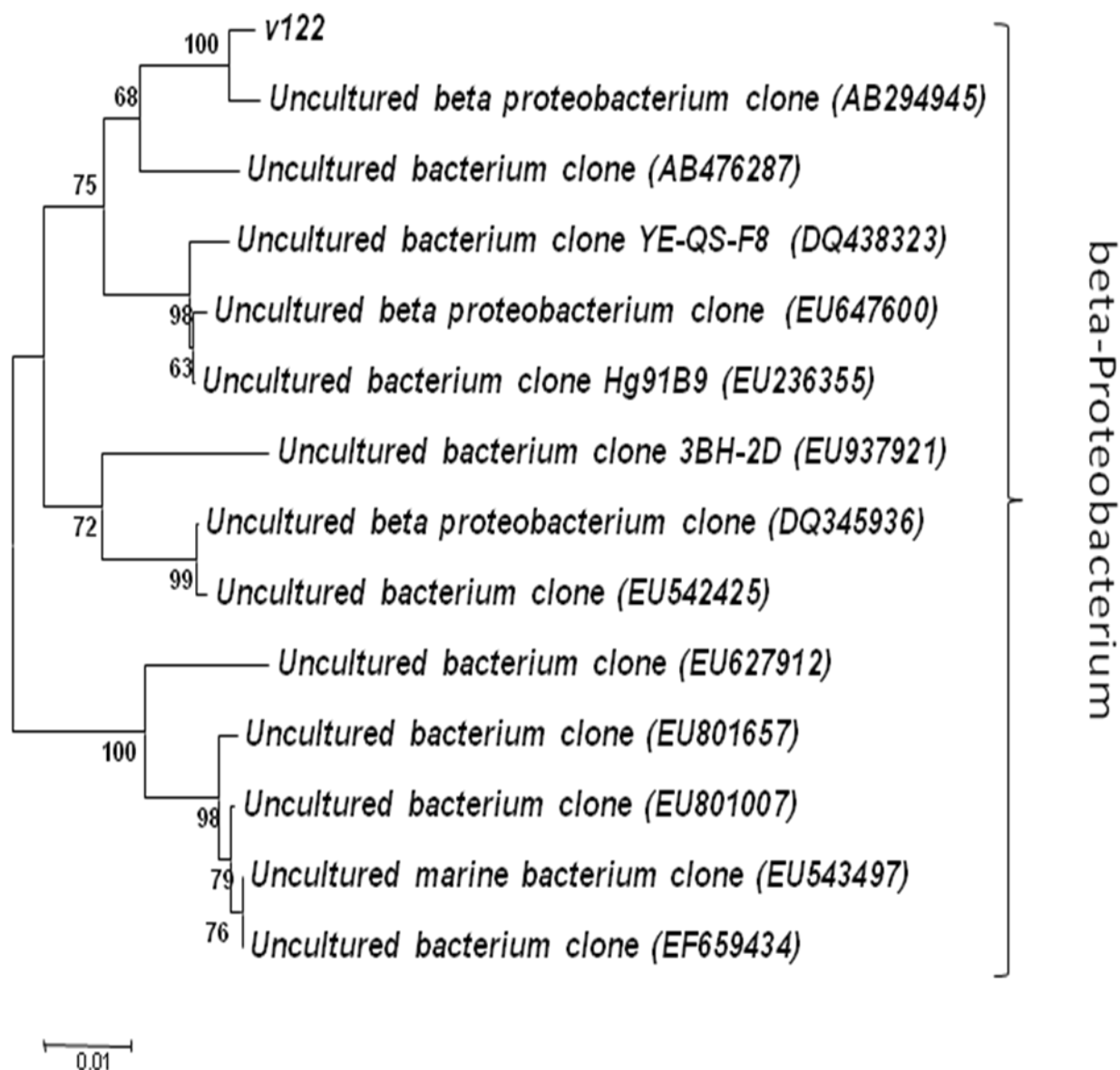
Figure 7.18 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *beta-Proteobacteria* OTU V122 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follow the designation of each taxon.
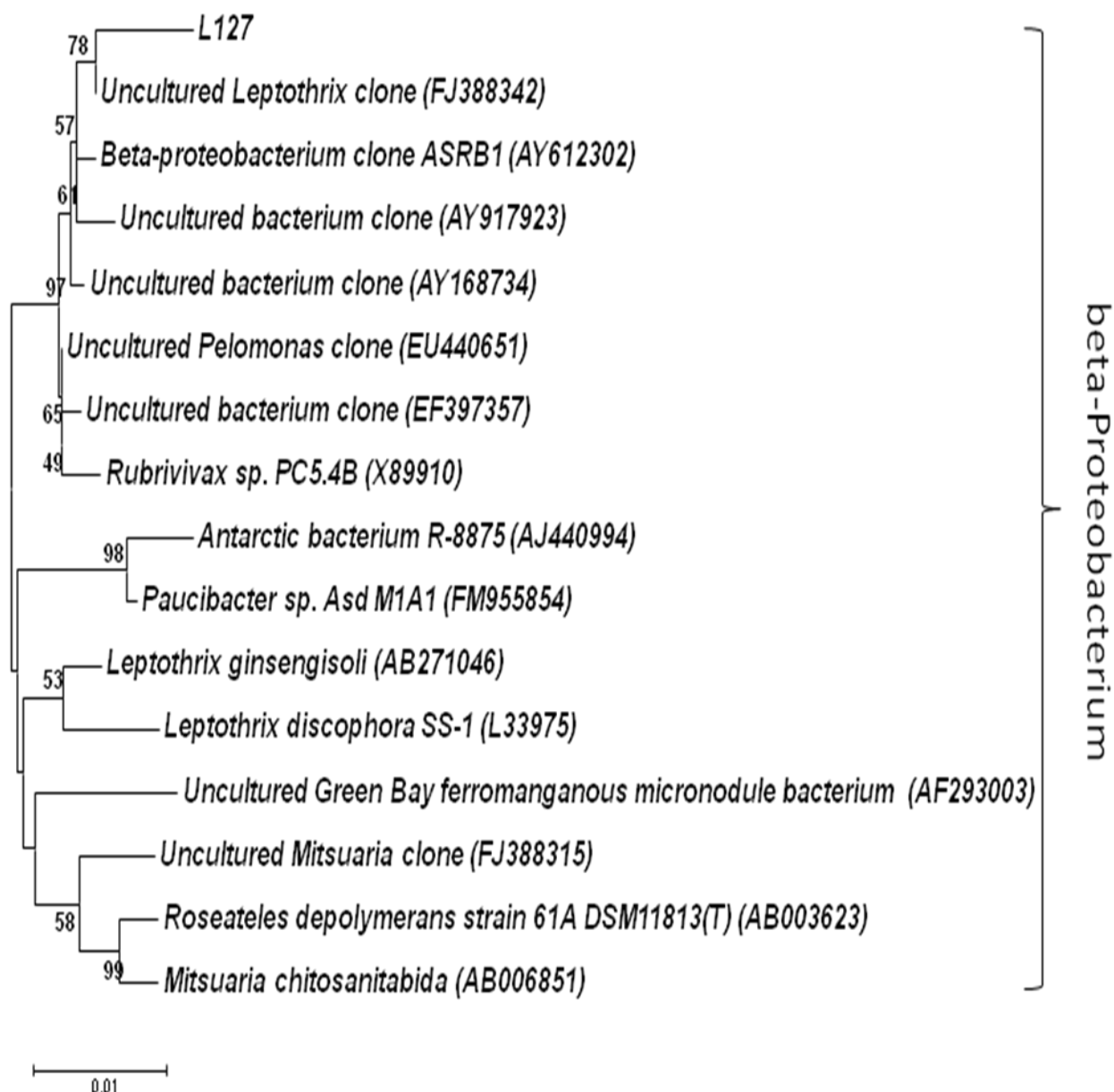
Figure 7.19 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *beta-Proteobacteria* OTU L127 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
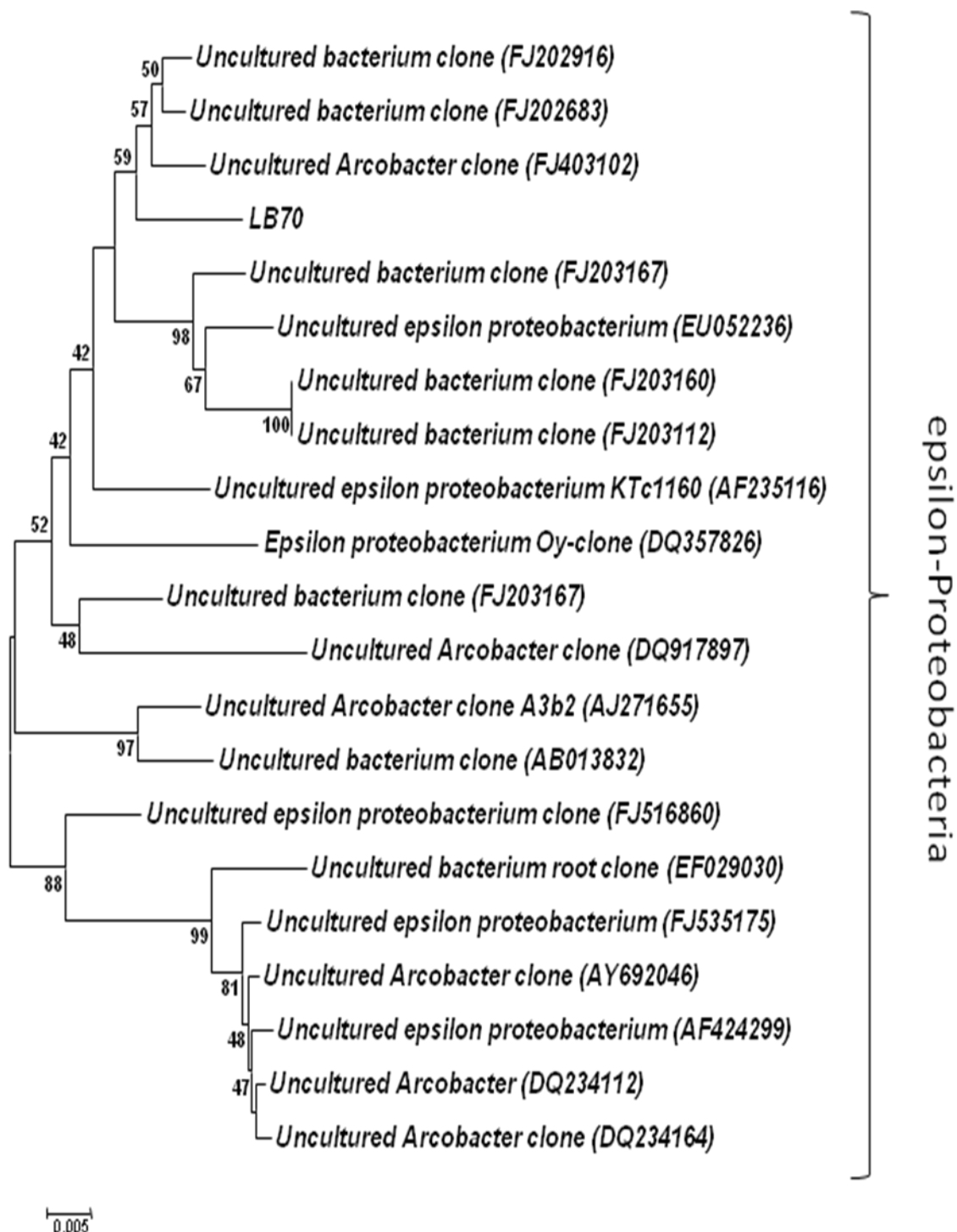
Figure 7.20 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *epsilon-Proteobacteria* OTU LB70 from *T. testudinum.* Bar represents 5 substitutions per 1000 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
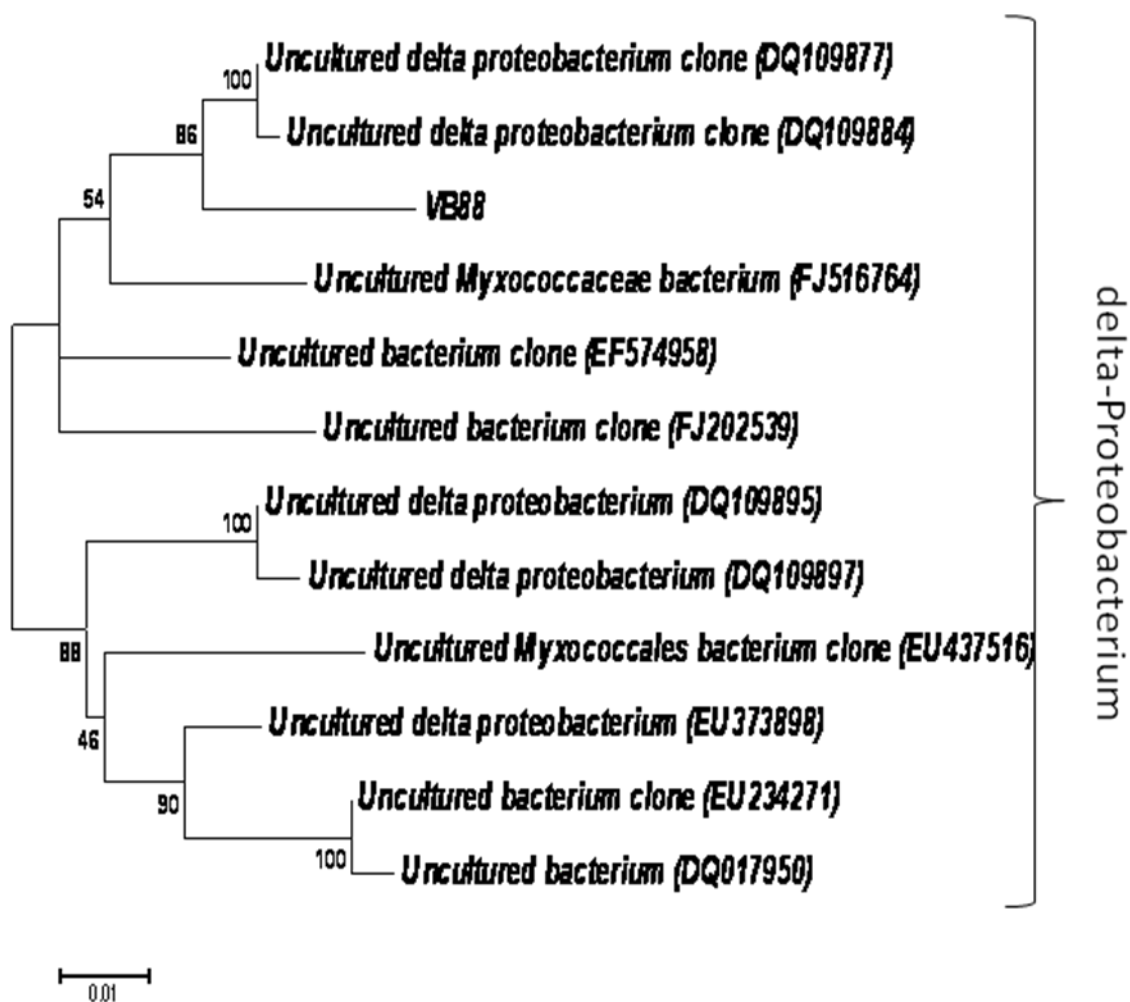
Figure 7.21 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *delta-Proteobacteria* OTU VB88 from *T. testudinum* . Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
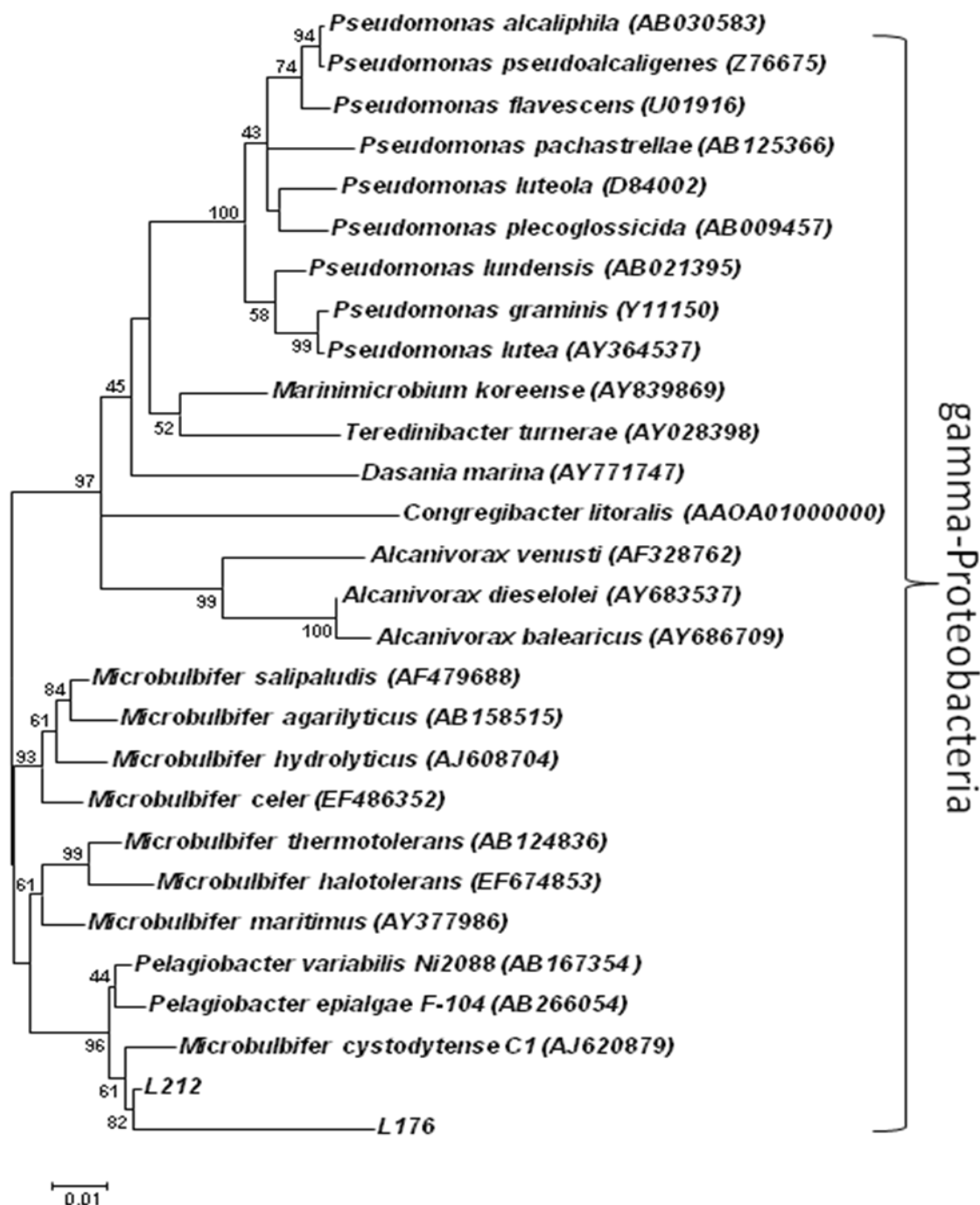
Figure 7.22 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *gamma-Proteobacteria* OTU L212 and L176 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
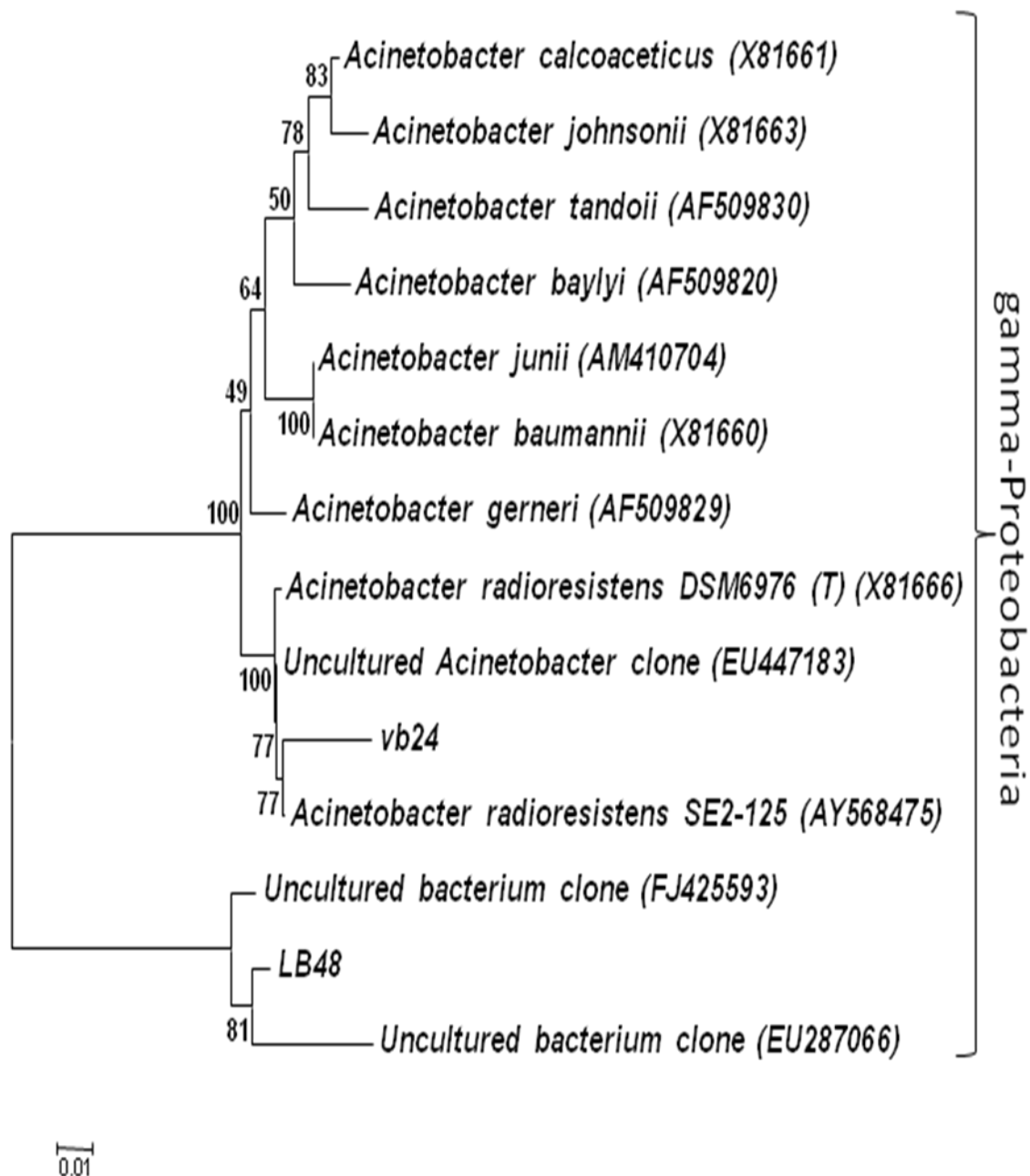
Figure 7.23 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *gamma-Proteobacteria* OTU VB24 and LB48 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
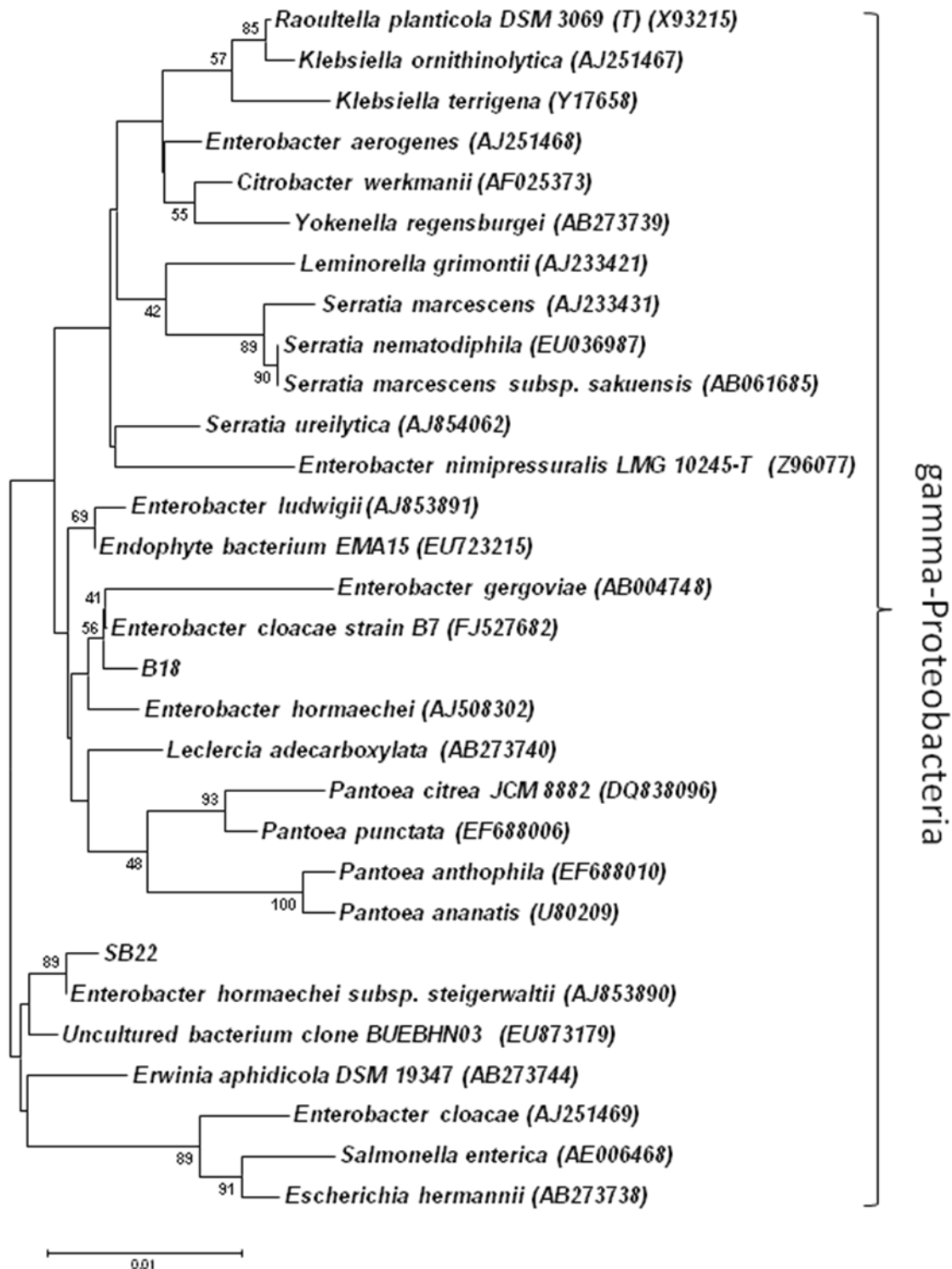
Figure 7.24 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *gamma-Proteobacteria* OTU SB22 and B18 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
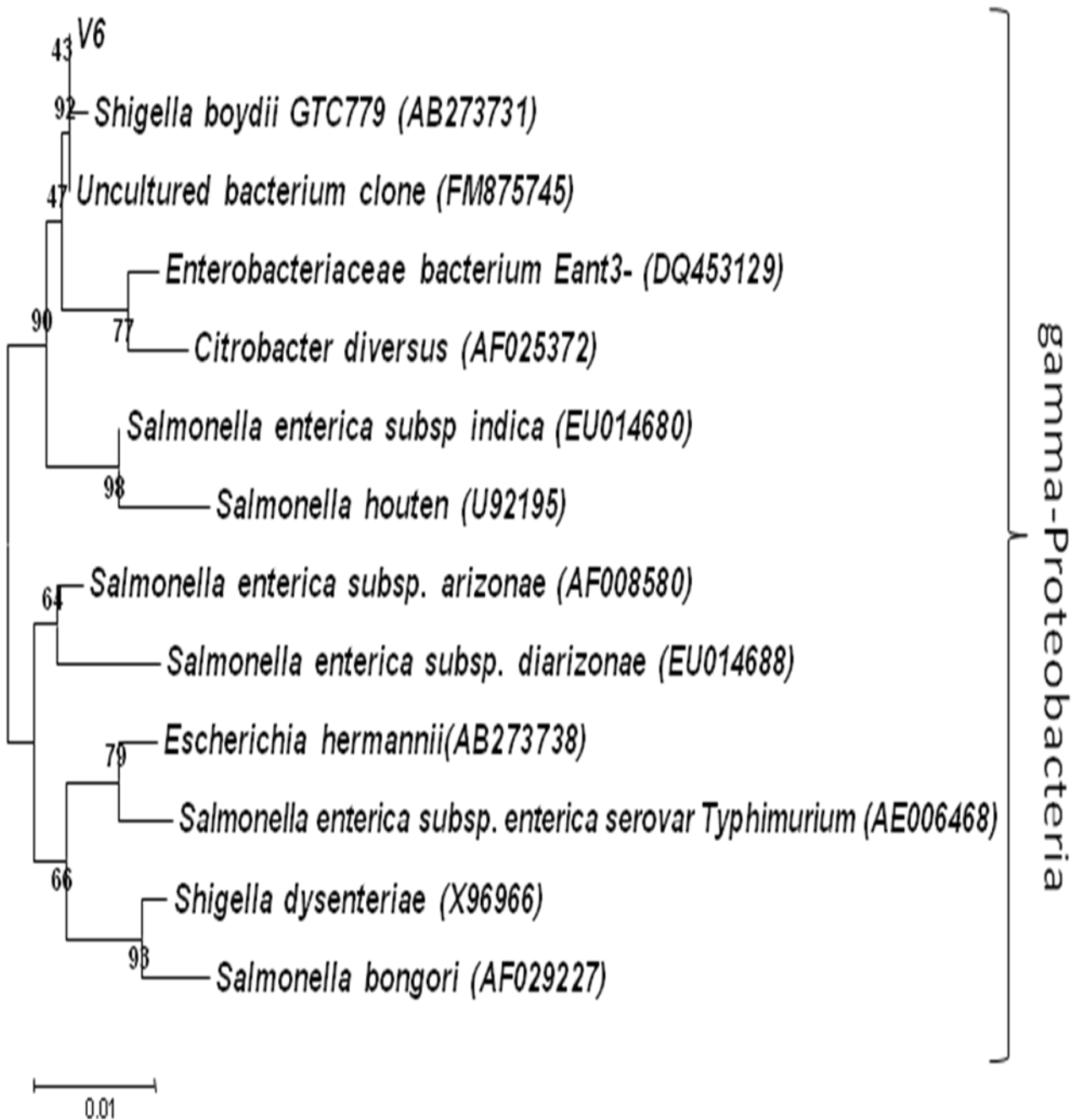
Figure 7.25 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *Shigella* OTU V6 from *T. testudinum*. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.

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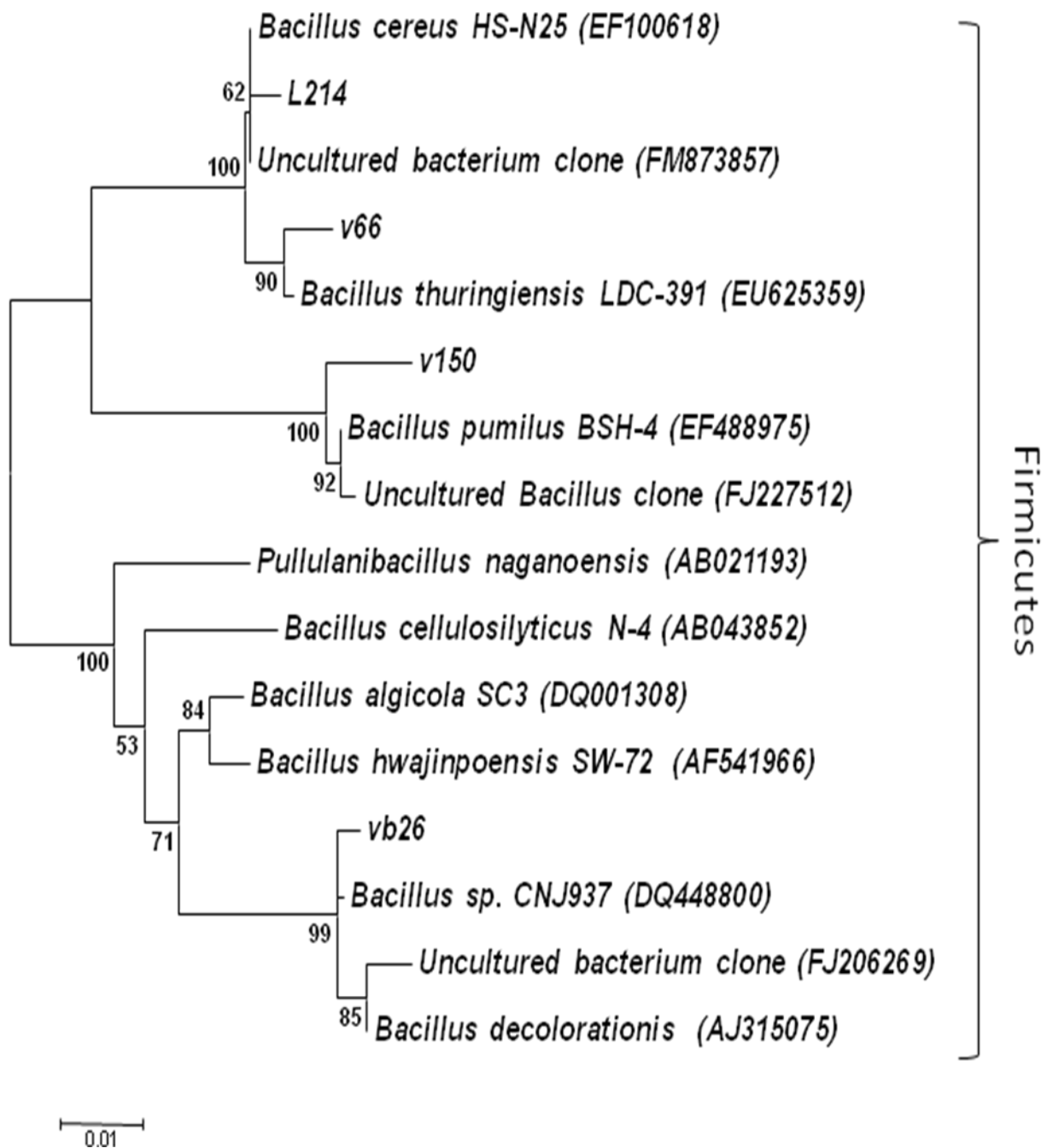
Figure 7.26 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *Firmicutes* OTU L214, V66, V150 andVB26 from *T. testudinum.* Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
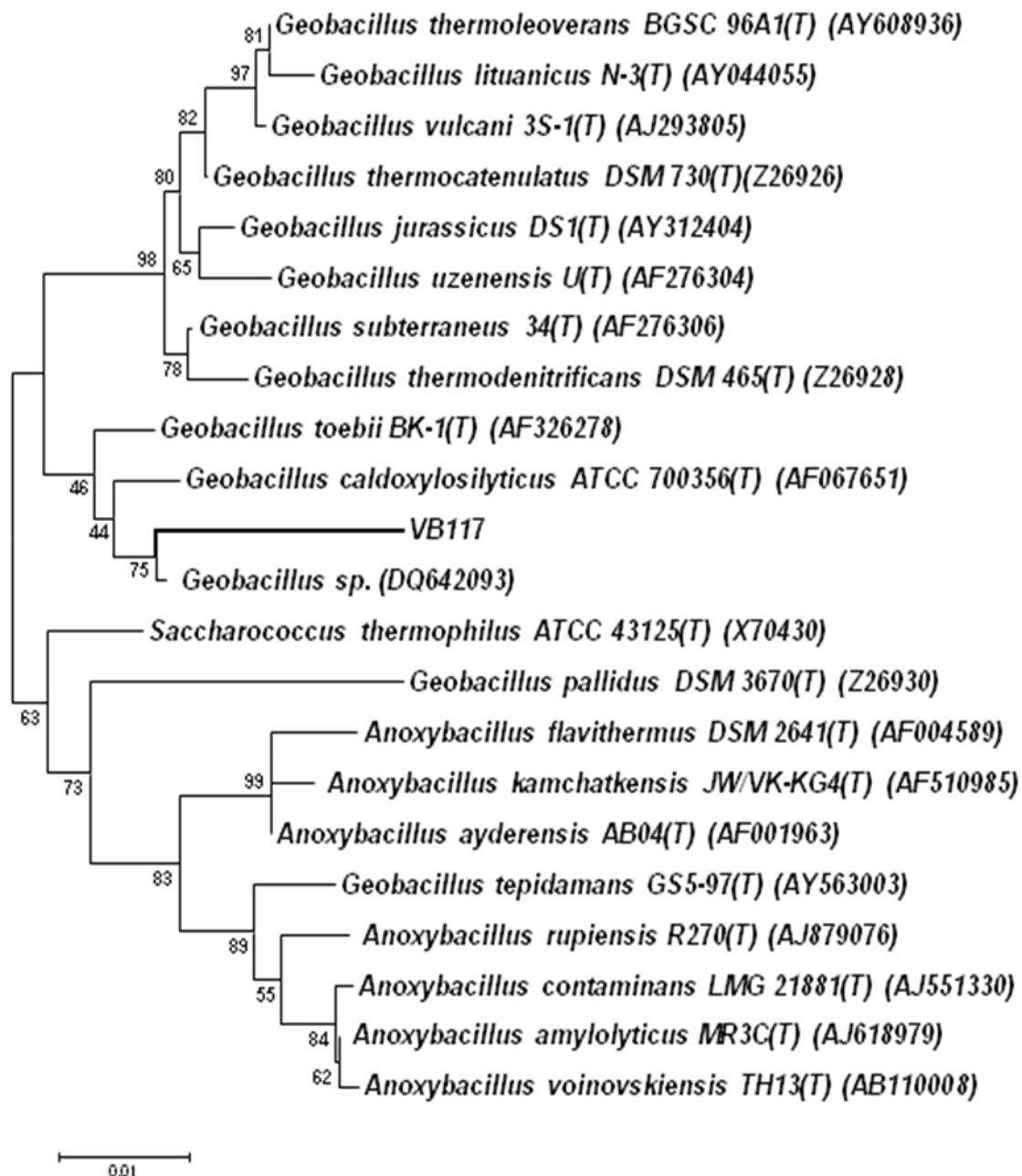
Figure 7.27 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *Geobacillus* OTU VB70 from *T. testudinum* . Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.
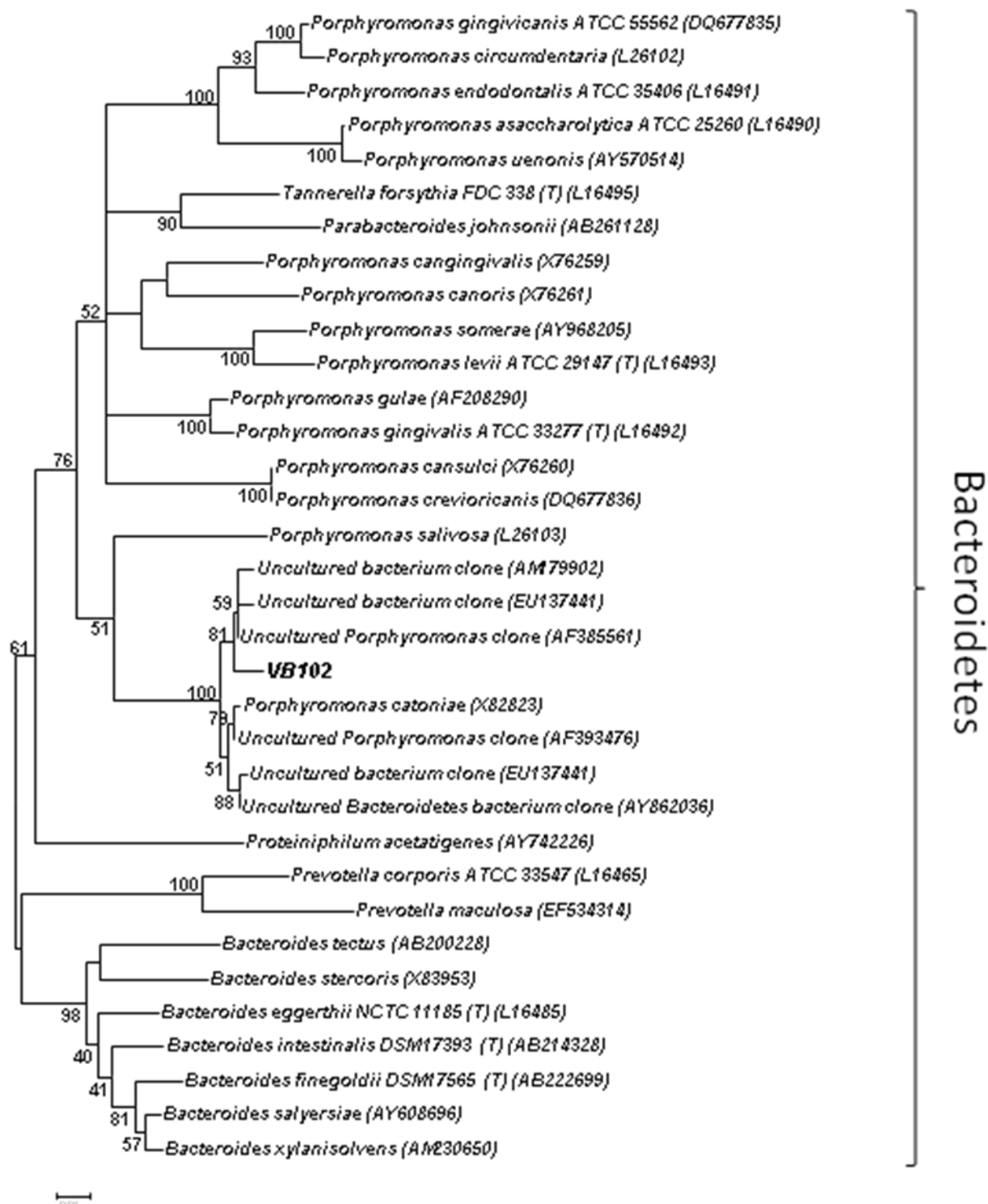
Figure 7.28 Neighbor-joining distance tree of partial 16SrRNA gene of representative phylotypes detected in *Bacteroidetes* OTU VB102 from *T. testudinum* tissues. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values higher than 40% shown. Gen Bank accession number follows the designation of each taxon.

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7.2.6 Statistical analysis

Statistical indices calculated from the environmental clone libraries of 16S rRNA gene, for *Puerto de la Libertad, Los Morillos*, Buyé Beach and *Cayo Enrique* are shown in Table 2.3. The statistical indexes were calculated using the DOTUR, which assists in assessing the completeness of a sequencing effort and reliability of richness estimates. Moreover, diversiy indices and richness estimators used are valuable for comparing the relative complexity of two or more communities and for estimating the completeness of sampling events of a community. The non-parametric richness estimators used in this study, such as ACE, Chao1, and Jackknife, enabled us to use observed frequencies of each OTU to estimate the richness of organisms in a community without having to sample each organism (Schloss and Handelsman, 2005).

The unique distance for defining an OTU from the eight libraries was 0.03 or 97% of sequence similarity (Dunbar, 2002; Singleton et al., 2001; Stout and Nusslein, 2005) between sampled clones. Richness was the number of phylotypes observed for each 16S rRNA gene clone library. Each phylotype consisted of either unique clone or a group of clones that has sequence similarities of over 97%.

Based on Chao1 index, there are no significant differences for the richness estimates among *Cayo Enrique, Los Morillos*, and *Puerto de la Libertad*. Though there are significant differences for richness estimates between Buyé Beach and the two beaches of *Los Morillos* and *Puerto de la Libertad*. In addition, based on Jackknife index, there are no significant differences for the richness estimates among *Cayo Enrique*, *Los Morillos,* and *Puerto de la Libertad*; but there are significant differences between Buyé Beach and the other sampling sites: *Cayo Enrique, Los Morillos* and *Puerto de la*

Libertad. For calculating the evenness of the samples, the Shannon index was used (Table 2.3). All four beaches have the Shannon's index >1suggesting that the individuals of each sampling site are almost equally distributed among species. However, Buyé beach has the smallest Shannon index, and it is considered significantly different from the other sites.

Rarefraction curves were done using the program DOTUR (http://www.plantpath.wisc.edu/fac/joh/dotur.html). This method is used for comparing observed richness between environments that have been unequally sampled (Hughes and Bohannan, 2004). The rarefraction curves generated (Figure 2.29) show that there are no significant differences of abundance and observed richness between *Puerto de la Libertad* and *Cayo Enrique*; but there are significant differences between Buyé Beach and the other three beaches of *Cayo Enrique, Los Morillos* and *Puerto de la Libertad*. There are also significant differences between *Los Morillos* and the two beaches of *Cayo Enrique* and *Puerto de la Libertad* based on the observed richness and abundance; thus the abundance and observed richness for Buyé Beach are the smallest ones.

In the Libshuff analysis all of the P-values are less than 0.05, so there are significant differences among sampling events based on Libshuff analysis. Overall, there are significant differences of observed richness, estimated richness, evenness, and abundance between the Buyé Beach and the other three sites. Also, there are significant differences of abundance and evenness between *Los Morillos* and the beaches of *Cayo Enrique* and *Puerto de la Libertad*. The observed richness increases in an order of Buyé Beach, *Los Morillos, Cayo Enrique*, and *Puerto de la Libertad*. There is no significant

difference of observed richness, estimated richness, evenness, and abundance between *Cayo Enrique* and *Puerto de la Libertad*.

To measure how well the samples represent the larger environment, the Good Coverage Index was calculated (Table 2.4) using the ASLO program (www.aslo.org/methods/free/2004/0114a.html). The coverages were 84% for Buyé Beach, 70% for Cayo Enrique, 75% for Los Morillos and 58% for Puerto de la Libertad. Coverage Index confirms that the samples used significantly represent the unculturable endophytic community present in *T. testudinum*.

Table 7.3 Statistical Indexes for 16S rRNA clone libraries from tissues of *T. testudinum*

Sampling site	Chao1	ACE	Jackknife	Shannon	Sequences
Buyé Beach	26.25	36.7	25.38	1.7	63
Buyé Beach	17.6522	20.6348	16.2905	1.3692	
Cayo Enrique	86.43	98.7996	87.23	3.32	86
Cayo Enrique	56.9123	64.6457	61.8273	3.1084	
Los Morillos	141	99.64	133.91	2.39	65
Los Morillos	67.2324	41.63	75.775	2.10399	
Puerto de la Libertad	132.5	132.22	134.83	3.33	67
Puerto de la Libertad	70.4541	76.3443	86.9536	3.09172	

* For each sampling site: the first row corresponds to statistical analysis with 99% confidence interval and the second row corresponds to 95 % confidence interval.

Table 2.4 Coverage indexes for genomic clone libraries

Sampling site	Coverage (%)
Buyé Beach	84
Cayo Enrique	70
Los Morillos	75
Puerto de la Libertad	58

Table 7.5 P-value table from S-Libshuff for testing community structure similarity based on Cramer-von Mises statistics

	Buyé Beach	Cayo Enrique	Los Morillos	Puerto de la Libertad
Buyé Beach		<0.0001	<0.0001	<0.0001
Cayo Enrique	<0.0001		<0.0001	0.028
Los Morillos	<0.0001	<0.0001		<0.0001
Puerto de la Libertad	<0.0001	0.028	<0.0001	
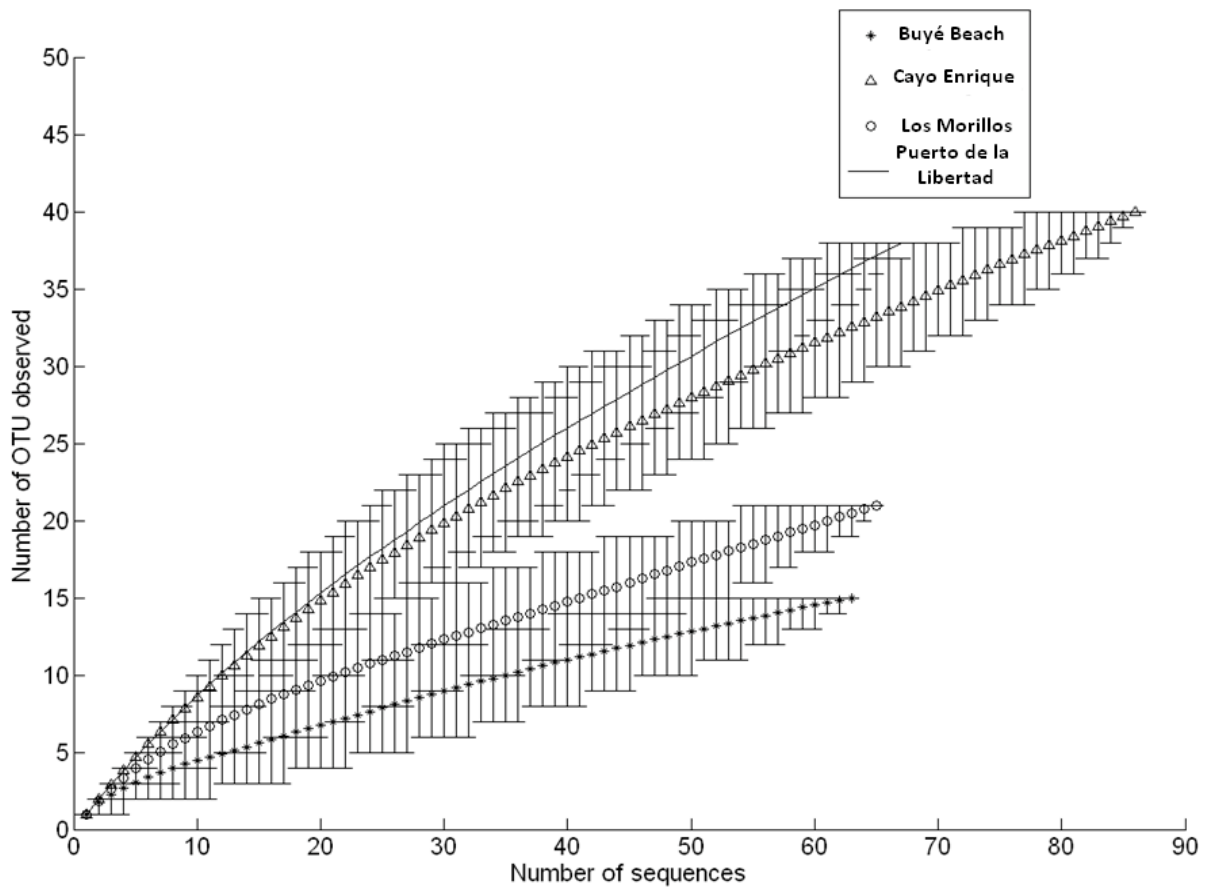
Figure 7.29 Rarefraction curves for the eight clone libraries sampled from *T.testudinum* tissues. OTU's were determined by $\geq$ 97% similarity. Bars represent the Standard deviation of samples.

7.3 Discussion

7.3.1 Culture independent diversity of bacterial endophytes associated with *T. testudinum*

Even though studies concerning microbial ecology and diversity have employed extensive use of culture independent techniques for assessing microbial communities, most of the work performed on endophytic diversity are based on characterization of isolates obtained from internal tissues (Adhikari et al. 2001; Elvira-Recuenco and van Vuurde 2000; Reiter and Sessitsch, 2006; Zinniel et al. 2002;). Because of insufficient knowledge about bacterial growth requirements, culture media for bacterial isolation is biased and many microorganisms enter a viable but not culturable state, thus not reflecting the actual community structure and also limiting recuperation of organisms (Reiter and Sessitsch, 2006; Tholozan et al. 1999). Consequently, the culture independent techniques employed in this study pretend to assess more broadly the concealed prokaryotic community associated with internal healthy mature tissues of *T. testudinum* based on 16S rRNA clone libraries and T-RFLP's. One difficulty that could have possibly affected the outcome of this study was contamination with epiphytic DNA present in the leaf. The approach used for verification of the efficiency of surface sterilization confirmed that potential interference of epiphytic DNA was avoided eliminated with treatment of sodium hypochlorite, favoring the amplification of DNA from endophytic sources.

The modified DNA extraction protocol of Keb-Llanes et al., (2002) allowed the isolation of DNA from *T. testudinum* and that of endophytic microorganisms. Other extraction methods were previously used but failed to give high yields of high molecular

weight DNA for PCR amplification. Also, PCR reactions had to be optimized several for efficient amplification. The first clone library of 16S rRNA gene was constructed using universal primers, Univ519F and Univ 1392R, then the right sized amplicon was purified and ligated to a pGEM TA vector (Promega). Transformants with plasmids containing the appropriate insert were sent to be sequenced. Sequencing results confirmed the ineffectiveness of the "universal primers" for amplification of endophytic 16S rRNA genes as most of the sequenced inserts corresponded to 16S rRNA sequences of chloroplast origin. However this result confirmed the effectiveness of the DNA extraction protocol and confirms that "universal primers" were not specific for bacterial and archaeal 16S rRNA genes.

In previous studies on endophytic diversity, Chelius and Tripplett (2001) described a set of bacterial primers, 799F and 1492, which discriminate against 16S rRNA from plant origin. The primer 799F was designed for amplifying most bacterial sequences to the exclusion of chloroplast DNA and gives a mitochondrial product that is approximately 1.5 times the size of the bacterial product. PCR amplification gave a doublet with a mitochondrial product of approximately 1,090 bp and a bacterial product approximately 735 bp which were able to be separated through electrophoresis and excised from an agarose gel for further experiments.

For studying the endophytic communities associated with healthy mature leaves of *T. testudinum,* two clone libraries per sampling site were constructed summing up to a eight clone libraries. A total of 1,678 clones were obtained from all libraries and 100 clones with the right insert per library were sequenced. Clones were selected randomly to avoid any bias in our analysis. Most of the OTU's were related to organisms or clones

associated with marine sediments, sea water, marine sponges, corals and terrestrial plants. Phylogenetic analysis revealed that the majority of the clones were affiliated to the *gamma-Proteobacteria, Firmicutes* and *alpha-proteobacteria.* Previous studies performed have documented predominance of *gamma-proteobacteria, Firmicutes, delta-Proteobacteria, alpha-Proteobacteria* and *epsilon-Proteobacteria* as endophytes of *Crocus albiflorus, Oryza sativa, Thlaspi goesingense* and citrus plants (Araújo et al., 2002; Idris et al., 2004; Reiter and Sessitsch,2006; Sun et al., 2007).

The majority of the clones were *gamma-Proteobacteria,* mostly clustered within OTU SB22 and B18, which are within the *Enterobacter sp.* Species pertaining to this genus have been previously described as endophytes of sweet potato, corn, Kentucky blue grass, cotton, spinach and potato. Some endophytic *Enterobacter* species are capable of producing phytohormones such as indole acetic acid, other auxins, cytokinins and gibberellins, which are important to plant development and are capable of changing root morphology for better nutrient intake. Also, species of *Enterobacter cloacae* possess siderophores such as aerobactin and enterochelin that apparently are involved in stimulation of plant growth (Asis and Adachi, 2003; Garbeva et al., 2001; Haahtela et al., 1990; Hinton and Bacon, 1995; Quadt-Hallmann and Kloepper , 1996; Tsuda et al., 2001). Also, many *Enterobacter* have been extensively studied for the capability of nitrogen fixation activity and solubilization of mineral phosphate (Kuklinsky-Sobral et al., 2004).

The remaining OTU's were classified within the *gamma Proteobacteria* and were phylogenetically similar to species of *Microbulbifer* and *Pelagiobacter*. OTU SB57 consisted of 14 clones closely associated with *Microbulbifer cystodydense*, which is

associated with the tunicate *Cystodytes dellechiajei*. Also, members of this genus have been isolated from sea sediments, living in calcareous marine sponge *Leuconia nivea* and associated with mucus and tissues of *Oculina patagonica* (Koren and Rosenberg, 2008; Tang et al., 2008; Quévrain et al.,2009).

The *alpha-Proteobacteria* subclass was the second most abundant group in this study. A total of 68 clones were affiliated to this group. OTU VB1 was the most abundant clone within all libraries. Its closest relative is an *alpha-Proteobacteria* clone isolated from the marine sponge *Chondrosia reniformis*. OTU LB91 formed a cluster with an Uncultured *alpha-Protebacteria* clone which was found associated with *Ulva australis*, a marine macroalgae. Interestingly, these *alpha-Proteobacteria* clones closest relative is *Labrenzia aggregata,* previously known as *Stappia aggregata*. Species pertaining to this genus are mostly isolated from marine environments such as the water column, sediments, phytoplankton, macroalgae, sponges as *Chodrosia reiniformis,* corals as *Erythropodium caribaeorum* and salt marshes (Boettcher et al., 2000; Buchan et al., 2001; Groben et al., 2000; King, 2003; Kurahashi and Yokota, 2002; Pichon et al., 2005; Pujalte et al., 2005, and Sfanos et al., 2005). Bacteria from this genus possess interesting physiological characteristics such as rhizobactin like siderophores, dioxygense genes, sodium channel-blocking agents, genes for the large subunit of RuBisCO and oxidize carbon monoxide. In addition, they participate in biogeochemical cycles such as CO oxidation and denitrification (Buchan et al., 2001; Groben et al., 2000; Kim et al., 2006; King, 2003; Martínez et al., 2003; Weber and King, 2007). OTU LB 48 is closely related to *Hirschia baltica,* thi*s* bacterium is related to *Hyphomonas* and *Caulobacter* and has

been found mostly thriving in marine sediments, marine water column, picoplankton (Schlessner et al., 1990; Uphoff et al., 2006).

OTU LB107 was closely related to an uncultured *alpha-Proteobacterium* clone WN-HWB-17, which was isolated from alkaline hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah et al., 2007). OTU LB29 was affiliated to an Uncultured bacterium clone Cyano2E05 found in natural stromatolites, a type of microbialite collected from Exuma Sound, Bahamas (Haveman and Foster, 2008). OTU LB106 was phylogenetically affiliated to an Uncultured bacterial clone BBD HS216b-07 isolated from corals affected with black band disease. OTU V51 was phylogenetically related to an Uncultured bacterium clone SHFH515 isolated from the Caribbean coral *Montastraea faveolata.* Clearly most of the *alpha-Proteobacteria* recuperated in this study are related to organisms found thriving in marine environments. Most of the clones are related to marine invertebrates such as sponges and corals, which are found nearby sea grass beds. These results suggest that these microorganisms are present within this marine ecosystem and some may have specialized to colonize and reside within *T. testudinum* tissues.

The closest relative of OTU LB95 was *Erythrobacter citreus*. This genus belongs to the family *Sphingomonadaceae,* where with the exception of *Sphingomonas alaskensis* (Vancanneyt et al., 2001), species that have been isolated from marine environments, to date, have only been found to belong to the genus *Erythrobacter* (Denner et al., 2002; Shiba and Simidu, 1982; Yurkov et al., 1994). *Erythrobacter citreus* was in fact, isolated from the water column where the benthos is characterized by an extensive sea grass system that extends down to 40m depth within the Bay of Calvi. *Erythrobacter spp.* are obligately aerobic bacteria containing bacteriochlorophyll *a* and are known as aerobic

anoxygenic phototrophs (Denner et al., 2002; Yurkov and Beatty, 1998). Also, one remarkable characteristic of this genus is their intense red/orange pigmentation due to their extremely complex carotenoid composition (Takaichi et al., 1988, 1990, 1991). *Eryhtobacter citreus* is a Mn (II) oxidizing bacterium, which may be implicated in the availabitity of Mn^{+2} at sea grasses. Mn^{+2} is the state used by plants for various processes such as   assimilation of carbon dioxide in photosynthesis, aids in the synthesis of chlorophyll and nitrate assimilation and functions in the formation of riboflavin, ascorbic acid, and carotene. However, higher levels of Mn^{+2} are toxic and detrimental, thus the balance of Mn^{+2} is essential for the proper survival of plants (Nealson et al., 1988, Yachandra et al., 1996).

There were only two OTU's associated with *beta-Proteobacteria*. OTU V122 was closely related to an uncultured *beta-Proteobacterium* clone isolated from a shallow submarine hydrothermal system in a tropical coral reef location (Hirayama et al., 2007). OTU L127 was similar to an uncultured *Leptothrix* clone which is an organism found associated with citrus leaf midribs that were infected by the greening disease pathogen or Huanglongbing pathogen (HLB) (Sagaram et al., 2009). Sheathed iron bacteria of the genus *Leptothrix* are found commonly in aquatic and terrestrial habitats containing aerobic-anaerobic interface zones in which *Fe* and *Mn* are cycled between their oxidized (insoluble) and reduced (soluble) forms (Emerson and Ghiorse, 1992; Ghiorse and Ehrlich, 1992). *Leptothrix* bacteria, in general, are known to be capable of oxidizing both iron(II) and manganese(II) although they are not known to receive energy out of the process (Nelson et al., 1999). The metal-oxidizing ability is a prime example of a biological mechanism for control of trace metals associated with ferromanganese

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minerals (Ghiorse, 1984), which is important in biogeochemical cycling of the metals and may have applications in metal recovery processes (Ghiorse and Ehrlich, 1992).

OTU VB88 was closely related to an uncultured *Myxococcaceae* clone which belongs to the delta-Proteobacteria. OTU VB102 is associated with a *Porphyromonas* clone. Both bacteria have the capability of forming biofilms in their environment, but *Porphyromonas* is mainly associated with human oral cavity (Cook et al., 2006). These bacterial species can physically interact with surfaces forming small aggregates and complex multicellular assembies. Some researchers suggest that the success of plant-microbe interactions is significantly influenced by the formation of bacterial biofilms. The success of the biofilms formation is dependent of the physical attachment mechanisms of the bacteria within plant tissues, such as presence of adhesions, secretion of exopolysaccharides and surface proteins with initial contact often mediated by active motility (Danhorn and Fuqua, 2007).

Firmicutes affiliated clones were the third most abundant group in the clone libraries. The majority of the clones within the *Firmicutes* like phylotypes were associated with *Bacillus spp.* Among the numerous *Bacillus* species, *B. badius B. subtilis, B. cereus, B. licheniformis, B. firmus, B. pumilus, B. mycoides*, and *B. lentus* have been detected in marine environments (Ivanova et al., 1999). Furthermore, *Bacillus* species are among the most common bacteria found to colonize plants endophytically (Lilley et al., 1996; Mahaffee and Kloepper, 1997) and it is likely that their endophytic ability could play a role in the biocontrol of vascular plant pathogens (Algam et al., 2005). Nevertheless, according to previous studies by Ivanova and colleagues (1992, 1999) the strains of *B. subtilis, B. horti* and *B. pumilus* are the most abundant among those

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associated with marine sponges, ascidians, soft corals, and are present in seawater as well. All of the strains studied were able to utilize a wide range of organic compounds, were halotolerant and alkalitolerant, which reflects their great metabolic flexibility.

OTU V66 closest relative was a strain of *Bacillus thuringiensis.* OTU L214 is phylogenetically affiliated to *Bacillus cereus* which is commonly found as a saprophyte. *B. cereus* and *B. thuringiensis* has been extensively studied as a potential biocontrol agent with the capability of forming crystalline inclusion bodies within their spores. *B. thuringiensis* inclusion bodies are known as the *Bt toxin* which allows the disruption of the digestive tract of some nematodes and insects, eventually causing their death (McSpadden, 2004). Hence, it has been used for many years as a commercial insecticide. Also, it is used for creating transgenic plants with insect resistance genes such as *Bt* corn and *Bt* cotton (Peferoen, 1997). *B. cereus* has been studied as a biocontrol agent for fungal phytopathogens in important agricultural crops. Research by Silo-Suh et al. (1994) demonstrated the capability of *B. cereus* to secrete antibiotics such as zwittermicin A which suppresses the growth of *Phytophtora medicaginsis*, associated with thedamping off disease in alfalfa. Milner et al., (1996) described the capacity of *B. cereus* to exude kanosamine, which is a highly inhibitory of plant pathogenic oomycetes and moderately inhibitory to certain fungi and bacterial strains.

OTU V150 was closely related to *Bacillus pumilus*. *B. pumilus* is a common bacterium in agricultural soils (Slepecky and Hemphill, 1992; Garbeva et al., 2003) and marine ecosystems (Siefert et al., 2000). This strain has been documented as endophyte of cotton, maize (McInroy and Kloepper, 1995), grape (Thomas, 2004), citrus (Araújo et al., 2002) and epiphytes in plants like rice (Cao et al., 2001). *B. pumilus* has been

documented to promote growth in *Pinus sp.* (Probanza et al., 2001). Also, it induces systemic disease protection like curcubit wilt, a disease in cucumber (Zehnder et al., 2001), late blight in tomato (Yan et al., 2003) and *Cercospora* leaf spot in sugar beets (Bargabus et al., 2002).

Interestingly, OTU VB117 was closely related to *Geobacillus sp.* There are no reports about the presence of the genus *Geobacillus* as an endophyte. In fact, members of this genus are characterized for being extremophiles having optimal growth above 50ºC. Thermophilic bacilli, including *Geobacillus*, are widely distributed and have been successfully isolated from all continents where geothermal areas occur (Sharp et al., 1992). Geobacilli have been also isolated from shallow marine hot springs and from deep sea hydrothermal vents, with Maugeri et al. (2002) recently describing the isolation of three novel halotolerant and thermophilic *Geobacillus* strains from three separate shallow marine vents off the Eolian Islands, Italy, which include the species *Geobacillus vulcani*.

The diversity of endophytic bacteria associated with *T. testudinum* determined by culture independent techniques have demonstrated various interesting facts about this microbial community. Bacterial species like *Bacillus spp.* are probably assuming a role of enhancing the fitness of the plant and controlling phytopathogens by various mechanisms described before. Other bacteria, such as *Leptothrix* and *Erythrobacter* are more involved in biogeochemical processes which aid in the availability of certain metals needed in small concentrations for the plant's physiological pathways. *Gamma*-proteobacteria, such as *Enterobacter spp.* are probably involved in stimulation of plant growth by secretion of phytohormones. Some of the bacterial groups reported in this study, such as *alpha-*

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Proteobacteria and *epsilon- Proteobacteria* have been previously reported as marine organisms but their relationships with tissues of *T. testudinum* are still not clear.

7.3.2 Ecology of bacterial endophytes associated with *T. testudinum* recuperated by culture independent techniques from four geographical areas of Puerto Rico.

The goal ofthis study was to compare the bacterial endophytes present in healthy mature tissues of *T. testudinum* from four geographical areas of Puerto Rico, anthropogenically impacted in various ways. Buyé Beach have been under developmental and recreational pressures, *Los Morillos* has been exploited for salt, *Cayo Enrique* is nearby *La Parguera* which is impacted by boats and fishermen and *Puerto de la Libertad* has not received the same amount of impact, but is the place where the new port for the ferry will be constructed.

Each site was surveyed twice, thus eight 16S rRNA clone libraries were constructed for studying the bacterial community associated with *T. testudinum*. After statistical and phylogenetic analysis, 37 OTU were identified for all sampling sites. *Cayo Enrique* and *Puerto de la Libertad* shared 37 OTU's, being the most diverse beaches, *Los Morillos* had a total of 21 OTU's and Buyé Beach was the least diverse site with only 15 OTU's.

Phylotypes related to the *gamma-Proteobacteria* were the most frequently detected. This group predominated at *Puerto de la Libertad* and *Los Morillos* at a relative frequency of 43% and 51%, respectively. In *Cayo Enrique* and Buyé Beach, the *gamma-Proteobacteria* were the second dominant group with 36% and 37% accordingly. This results are rather significant since many members of this group has been previously reported as plant associated bacteria such as *Pseudomonas, Enterobacter,* and *Klebsiella* which provide enhance fitness to its host plant. Another important fact is that this group

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comprises bacteria that are fecal contaminants, which is a major problem at recreational beaches.

The second most predominant group within the constructed clone library was the *alpha-Proteobacteria*. This group was more frequent at Buyé Beach (52%), followed by *Los Morillos* (23%) and had a very similar distribution at *Cayo Enrique* and *Puerto de la Libertad* (15% and 14% respectively).

The third most dominant group was the *Firmicutes*, which was mostly comprised by *Bacillus* species. This group was the most frequent at *Cayo Enrique* with 40% and at Puerto de la Libertad was the second most frequent group representing 35% of the population. Interestingly, this population was significantly reduced at *Los Morillos* and Buyé Beach representing 12% and 6% respectively. Members of *Firmicutes,* especially those pertaining to *Bacillus,* have been documented as plant associated bacteria with the capability of producing secondary metabolites, antibiotics, phytohormones and toxins that are of great importance to the health, establishment and proliferation of the host plant.(Bargabus et al., 2002; McInroy and Kloepper, 1995; McSpadden-Gardener, 2004).

Clones that belonged to the beta-*Proteobacteria, delta-Proteobacteria, epsilon-Proteobacteria* and *Bacteroidetes* were recovered in small frequencies when compared to the other groups. The *epsilon-Proteobacteria* was more frequent at *Los Morillos* and Buyé Beach representing 12% and 3% of the community; while at *Puerto de la Libertad* and *Cayo Enrique* the *epsilon-Proteobacteria* was poorly represented at 2% and 1%, respectively. The *beta-Proteobacteria* was almost equally distributed at all sampling sites representing 2-4% of the endophytic bacterial community of *T. testudinum*. The *delta-Proteobacteria* was obtained in small frequencies only at *Puerto de la Libertad* and

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Cayo Enrique. This community was not observed either at Buyé Beach nor *Los Morillos*. These *delta-Proteobacteria*-like phylotype OTU was closely related to an uncultured *Myxococcus*. The *beta-Proteobacteria* were found at all sampling sites representing a small fraction of the endophytic bacterial community of *T. testudinum*. This group was a bit more frequent at *Puerto de la Libertad* and *Cayo Enrique* representing 3% and 4%, respectively and represented 2% of the population at Buyé Beach and *Los Morillos*. Terminal Restriction Analysis was performed on samples obtained from all sampling sites. Metagenomic DNA's were used as templates for PCR reactions that amplified the almost complete 16S rRNA gene from prokaryotic microorganisms. PCR amplicons were used for enzymatic digestions and the resulting Terminal Restriction Fragments (TRF's) were loaded to a polyacrylamide sequencing gel using a LICOR sequencer. In the TRF's patterns obtained from all the sampling sites it is demonstrated that there are bands shared on all the samples even when several restriction enzymes are used. This shared TRF's probably representing the endogenous endophytic prokaryotic community of *T. testudinum*. There are specific TRF's which represent unique members of this community that are present in some samples and are not shared by other sea grass beds sites. However, when comparing the microbial community structure at all sampling sites reveals that *Cayo Enrique* and *Puerto de la Libertad* posses very similar community structure. This was also confirmed by the statistical analyses of species richness and abundance, which yielded similar results for both locations. *Los Morillos* was not significantly different to the former two beaches but it has a slightly dissimilar community composition. Buyé Beach was the least diverse site, with low richness and abundance indexes. These results suggest that the anthropogenic impact at Buyé Beach is

higher, thus affecting the microbial diversity associated with the sea grass beds of *T. testudinum*.

Historically, Buyé Beach is the most directly impacted site when compared to the other areas. Sea grass beds present at Buyé Beach are only a few feets away from the coast, while the sea grasses at *Los Morillos, Puerto de la Libertad* and *Cayo Enrique* are more distant from the coastal area. Therefore bathers that use Buyé Beach as a recreational area are in constant contact with the sea grasses. Also, nearshore activities and construction of houses and hotels are contributing to constant discharges to the ecosystem. The input of contaminants might be affecting the microbial communities present by augmenting levels of fecal coliforms, increasing concentration of ammonia present in urine, and causing lesions in *T. testudinum* when trampling over the plants. In contrast, the other three sites *(Los Morillos*, *Puerto de la Libertad* and *Cayo Enrique)* seem to be less impacted due to the longer distance of the sea grass communities from the areas where human activities take place.

One important detail is that overall, sea grasses collected from *Puerto de la Libertad* and *Cayo Enrique* were bigger and healthier than those collected from Buyé Beach. Therefore, bacterial communities exposed to more direct human impact may lead to predominance of a few species while causing attenuation on the diversity. On the other hand, less impacted beaches may be more prolific and favorable environments for the establishment of more bacterial species. Probably, having a higher bacterial variety increases the metabolic activity and as a result the plant has more availability of nutrients for its growth. It is readily observed that there are shared communities within the sea grass beds of Puerto Rico, suggesting a basal endophytic flora of *Thalassia testudinum.*

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However, the physical parameters and chemical composition within *T. testudinum* beds is variable depending on the amount and type of impact to which the plant is exposed to, which may be leading to shifts of predominant species within the basal endophytic flora.

8. Conclusions

- This study is the first attempt to assess the endophytic bacterial community associated with healthy mature leaves of *T. testudinum*.

- The isolated microorganisms in this study belonged to the phylums *Actinobacteria, Firmicutes, gamma-Proteobacteria,* and *alpha-Proteobacteria* which are groups with representatives associated with marine and plant-related environments microorganisms. These genera include the *Bacillus, Halobacillus, Staphylococcus, Micrococcus, Enterobacter, Pseudomonas, Cobetia* and *Nesterenkonia.*

- The genus *Bacillus* showed the highest frequency of isolation in culture dependent techniques and also was frequently recovered in 16S rRNA clone libraries.

- Isolates related to *Exiguobacterium, Nesterenkonia, Microbulbifer, Cobetia, Cellulosimicrobium, Pseudovibrio,* and *Geobacillus* were recovered and reported herein as endophytes for the first time.

- Culture independent techniques revealed OTU's associated with the phylums *Proteobacteria (alpha, beta, delta, epsilon and gamma)*, *Firmicutes* and *Bacteroidetes* as the dominant groups.

- Statistical analysis (Chao, Shannon, ACE, and Jacknife) revealed that Buyé Beach was the least diverse sampling site when compared to the other three sites. Also, differences in isolated strains were observed, thus suggesting the anthropogenic impact received might cause shifts in community structure.

- This research is the first attempt to elucidate the relationship between the anthropogenic activities and the composition of the resident endophytic microbial community.

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9. Recommendations

- More research is needed to understand and describe the role of prokaryotic endophytesassociated with *T. testudinum* tissues.

- Studying the bacterial localization within *T. testudinum* tissue might help us understand better the interactions occurring among the endophytic microbiota.

- To study *Bacillus* species isolated and the secondary metabolites that might be promoting health in *T. testudinum.*

- Construction of 16S rRNA libraries with archaeal specific primers for understanding the diversity and possible function of archea associated with *T. testudinum.*

- Research on the interactions within endophytic bacteria and fungi isolated from *T. testudinum* and the potential beneficial effects for the plant.

- Further characterization is required of putative novel species

- Study the secondary metabolites produced by *T. testudinum* endophytes such as phytohormones and antibiotics that might be helping to the health of the plant.

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Appendix 1 Macroscopic and microscopic characteristics of isolated strains associated to *T. testudinum*

Strain	Sampling site	Leaf's part	Color	Morphology	Gram staining	Cell morphology	Arrangement
B 1	Buyé	Apex	White	Rhizoid	-	Rods	Single bacilli
B 2	Buyé	Apex	White	Irregular	+	Cocci	Staphylococci
B 3	Buyé	Base	White	Rhizoid	+	Rods	Single Bacilli
B 4	Buyé	Center	White	Rhizoid	+	Rods	Single Bacilli
B 5	Buyé	Center	White	Rhizoid	-	Rods	Single bacilli
B 6	Buyé	Center	White	Circular	+	Long rods	Streptobacilli
B 7	Buyé	Center	White	Circular	+	Rods	Streptobacilli
B 8	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 9	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 10	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 11	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 12	Buyé	Base	White	Circular	+	Long Rods	Streptobacilli

Appendix 1 Continuation

B 13	Buyé	Base	White	Irregular	+	Cocci	Staphylococci
B 14	Buyé	Apex	White	Irregular	+`	Short rods	Single bacilli
B 15	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 16	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 17	Buyé	Center	White	Rhizoid	-	Rods	Single bacilli
B 18	Buyé	Center	White	Irregular	+	Cocci	Staphylococci
B 19	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 20	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 21	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 22	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 23	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 24B	Buyé	Center	Cream orange	Irregular	+	Short rods	Single bacilli

Appendix 1 Continuation

B 25	Buyé	Center	Orange	Circular	-	Rods	Single bacilli
B 26	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 27	Buyé	Apex	Transparent White	Rhizoid	+	Rods	Single bacilli
B 28	Buyé	Center	Transparent White	Rhizoid	+	Rods	Single bacilli
B 29	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 30	Buyé	Apex	Transparent White	Irregular	+	Rods	Single bacilli
B 31	Buyé	Base	Cream orange	Irregular	+	Cocci	Staphylococci
B 32	Buyé	Base	White	Rhizoid	+	Bacilli	Single bacilli
B 33	Buyé	Center	White	Irregular	+	Bacilli	Streptobacilli
B 35	Buyé	Base	Cream orange	Irregular	+	Cocci	Staphylococci
B 36	Buyé	Apex	Cream orange	Irregular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 37	Buyé	Base	Cream orange	Irregular	+	Cocci	Staphylococci
B 38	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 39	Buyé	Base	White	Circular	+	Rods	Single bacilli
B 40	Buyé	Center	Orange	Circular	+	Rods	Diplobacilli
B 41	Buyé	Center	Yellow	Circular	+	Cocci	Stapylococci
B 42	Buyé	Center	White	Circular	+	Cocci	Stapylococci
B 43	Buyé	Apex	Cream	Circular	+	Cocci	Stapylococci
B 44	Buyé	Base	Orange	Circular	-	Short rods	Single bacilli
B 45	Buyé	Base	Orange	Circular	-	Short rods	Single bacilli
B 46	Buyé	Center	White	Irregular	-	Rods	Single bacilli
B 46A	Buyé	Center	Cream	Circular	+	Cocci	Stapylococci
B 47	Buyé	Base	White	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 48	Buyé	Base	Cream	Circular	+	Cocci	Single cocci
B 49	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 50B	Buyé	Center	Bright yellow	Circular	+	Cocci	Staphylococci
B 51B	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 51	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 52	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 54	Buyé	Apex	Bright yellow	Circular	+	Cocci	Staphylococci
B 55	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 56	Buyé	Apex	White	Irregular	-	Rods	Single bacilli
B 56B	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 57	Buyé	Apex	Transparent brown	Circular	-	Rods	Single bacilli
B 58	Buyé	Center	White	Irregular	+	Cocci	Staphylococci

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B 59 A	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 59 B	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 59B1	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 59B2	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 60 B	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 60 B1	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 60 B2	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 60 B3	Buyé	Center	Bright yellow	Circular	+	Cocci	Staphylococci
B 61	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B62	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B62 A	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 62 B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci

B 63	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 64	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 65	Buyé	Center	Cream orange	Irregular	+	Cocci	Staphylococci
B 66	Buyé	Apex	Peach	Circular	+	Rods	Streptobacilli
B 67	Buyé	Center	Cream orange	Irregular	+	Cocci	Single cocci
B 67A	Buyé	Base	Cream	Irregular	+	Rods	Streptobacilli
B 67B	Buyé	Center	Cream orange	Irregular	+	Rods	Diplobacilli
B 68	Buyé	Apex	Cream	Circular	+	Cocci	Stapjhylococci
B 69	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 70	Buyé	Center	Cream	Circular	-	Short rods	Single bacilli
B 70 A1	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 70 A2	Buyé	Center	Yellow	Circular	+	Cocci	Staphylococci

B 71	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 71B	Buyé	Center	Cream	Irregular	+	Long rods	Streptobacilli
B 71B3	Buyé	Center	Yellow	Circular	+	Cocci	Staphylococci
B 72	Buyé	Base	Brown	Irregular	-	Rods	Streptobacilli
B 74	Buyé	Base	Cream white	Circular	+	Cocci	Staphylococci
B 74B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 75	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B76	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B77	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 78	Buyé	Base	Transparent white	Irregular	+	Rods	Single bacilli
B 79	Buyé	Base	Yellow	Circular	+	Cocci	Staphylococci
B80	Buyé	Apex	White	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 81	Buyé	Base	Cream	Circular	+	Rods	Streptobacilli
B 82	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 83	Buyé	Base	Transparent white	Irregular	+	Rods	Single bacilli
B 83B	Buyé	Base	Cream	Rhizoid	+	Rods	Single bacilli
B 84	Buyé	Apex	Bright orange	Circular	+	Rods	Streptobacilli
B 85	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 85B	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 86	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 87	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 87B	Buyé	Base	Cream	Irregular	-	Rods	Single bacilli
B 88	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 89	Buyé	Apex	White	Irregular	+	Rods	Single bacilli

Appendix 1 Continuation

B 90	Buyé	Apex	White	Irregular	+	Cocci	Staphylococci
B 90B	Buyé	Apex	White	Circular	-	Short rods	Diplobacilli
B 91	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 92	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 92B1	Buyé	Apex	Transparent white	Circular	+	Cocci	Staphylococci
B 93	Buyé	Apex	White	Rhizoid	-	Rods	Single bacilli
B 94	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B95	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 96	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 98	Buyé	Center	Transparent white	Circular	+	Cocci	Staphylococci
B 99B	Buyé	Apex	Transparent	Rhizoid	-	Rods	Single bacilli
B 100		Center		Circular			

	Buyé		Orange		+	Rods	Streptobacilli

B 100B	Buyé	Center	White	Circular	+	Rods	Streptobacilli
B 102	Buyé	Base	White	Circular	+	Rods	Streptobacilli
B 103B	Buyé	Apex	White	Rhizoid	-	Rods	Single bacilli
B 104	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 104B1	Buyé	Base	Cream	Irregular	+	Cocci	Staphylococci
B 104B2	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 107B	Buyé	Center	Transparent White	Rhizoid	+	Rods	Streptobacilli
B 106	Buyé	Base	Cream	Circular	-	Short rods	Single bacilli
B 106B	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 107A	Buyé	Center	Transparent White	Irregular	+	Cocci	Single cocci

B 107B	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 108	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 109	Buyé	Base	White	Circular	+	Cocci	Single cocci
B 110	Buyé	Base	Creamy white	Circular	+	Cocci	Staphylococci
B 111	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B112	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 113	Buyé	Center	Cream	Rhizoid	+	Rods	Streptobacilli
B 114	Buyé	Base	Creamy white	Circular	+	Cocci	Streptococci
B 115	Buyé	Base	Transparent white	Irregular	+	Rods	Single Rods
B 116	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B117	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 118	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci

B 120	Buyé	Apex	Dark Pink	Circular	-	Short rods	Streptobacilli
B 121	Buyé	Apex	Cream	Irregular	+	Rods	Streptobacilli

Appendix 1 Continuation

B121	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 122	Buyé	Apex	Cream	Rhizoid	+	Rods	Streptobacilli
B 123	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 124	Buyé	Center	Cream	Rhizoid	+	Rods	Streptobacilli
B 125	Buyé	Center	Cream	Rhizoid	+	Rods	Streptobacilli
B 126	Buyé	Center	Cream	Irregular	+	Rods	Streptobacilli
B 127	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli
B 128	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli
B 129	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli
B 130	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli

| B 130B | Buyé | Apex | Cream | Circular | + | Cocci | Staphylococci |
| B 131 | Buyé | Base | Cream | Rhizoid | + | Rods | Streptobacilli |

Appendix 1 Continuation

B 132	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 133	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 134	Buyé	Base	Cream	Rhizoid	+	Rods	Single bacilli
B 135	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 136	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 137	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 138	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 139	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 140	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 141	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 142	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli
B 143	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli

B 144A	Buyé	Base	White	Irregular	-	Rods	Streptobacilli
B 144B	Buyé	Base	Transparent white	Circular	+	Cocci	Diplococci
B 145B	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 146	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 147A	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 147B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B148	Buyé	Apex	White	Circular	+	Rods	Single bacilli
B 150	Buyé	Apex	White	Circular	+	Rods	Single bacilli
B152	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 153	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 153B	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
B 154	Buyé	Apex	White	Irregular	+	Rods	Single bacilli

B155	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 156	Buyé	Base	Orange	Irregular	+	Rods	Streptobacilli
B 157	Buyé	Base	Cream	Irregular	+	Rods	Streptobacilli
B 158A	Buyé	Base	Orange	Irregular	-	Rods	Single bacilli
B 158B	Buyé	Base	Cream	Circular	+	Rods	Streptobacilli
B 159A	Buyé	Base	Orange	Irregular	-	Rods	Single bacilli
B 160	Buyé	Base	Cream (AI)	Circular	+	Cocci	Staphylococci
B 161	Buyé	Apex	Cream	Irregular	+	Rods	Streptobacilli
B162	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 163	Buyé	Apex	White	Circular	+	Rods	Streptobacilli
B 164	Buyé	Apex	White	Irregular	+	Rods	Streptobacilli
B 164B	Buyé	Apex	Transparent	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 165	Buyé	Apex	Transparent white	Irregular	+	Rods	Single bacilli
B 166	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 166B	Buyé	Base	White	Circular	+	Rods	Streptobacilli
B 166C	Buyé	Base	Transparent cream	Irregular	+	Rods	Streptobacilli
B 166C2	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 167A	Buyé	Base	Orange	Circular	-	Short rods	Diplobacilli
B 167B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 168A	Buyé	Base	Creamy gray	Irregular	+	Cocci	Staphylococci
B 169A	Buyé	Center	Transparent white	Irregular	+	Rods	Streptobacilli
B 169B	Buyé	Center	Cream	Circular	+	Rods	Streptobacilli
B 171	Buyé	Center	Cream	Circular	+	Rods	Streptobacilli
B 171B	Buyé	Center	Transparent white	Irregular	-	Rods	Single bacilli

Appendix 1 Continuation

B 172	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 173	Buyé	Center	Cream	Circular	+	Rods	Single bacilli
B 174	Buyé	Center	White	Circular	+	Rods	Single bacilli
B 175	Buyé	Center	Cream	Circular	-	Short rods	Diplobacilli
B 177	Buyé	Center	White	Circular	+	Rods	Single bacilli
B 177 b1	Buyé	Center	Transparent white	Irregular	-	Rods	Single bacilli
B 178	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 179	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 179 B	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 180	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 180 B	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 181	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 182	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 182 B	Buyé	Center	Orange	Irregular	+	Rods	Streptobacilli
B 182 B2	Buyé	Center	White	Irregular	+	Rods	Streptobacilli
B 183	Buyé	Center	Cream	Circular	+	Rods	Single bacilli
B 184 A	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 184 B	Buyé	Center	Orange	Irregular	+	Rods	Streptobacilli
B 185	Buyé	Center	Orange	Irregular	+	Rods	Streptobacilli
B 186	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 187	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 188	Buyé	Center	White	CIrcular	+	Cocci	Staphylococci
B 189	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 190	Buyé	Base	White	Irregular	+	Rods	Single rods

B 191	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 192	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 193	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 194	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 195	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 196	Buyé	Apex	White(ai)	Circular	+	Cocci	Staphylococci
B 197	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 198	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 199	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 200	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 202	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 203	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli

Appendix 1 Continuation

B 204	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 205	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 206	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 207	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 208	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 209	Buyé	Base	Creamy orange	Irregular	+	Rods	Single bacilli
B 209 a1	Buyé	Base	Cream	Irregular	+	Rods	Single bacilli
B 209 b	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 209 c	Buyé	Base	Transparent	Circular	+	Cocci	Staphylococci
B 210 b	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 211	Buyé	Apex	Orange	Irregular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 211 B	Buyé	Center	Transparent white	Irregular	+	Cocci	Staphylococci
B 211 C	Buyé	Apex	Transparent cream	Circular	+	Curved rods	Single bacilli
B 212	Buyé	Base	Transparent white	Rhizoid	+	Cocci	Staphylococci
B 213	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 214	Buyé	Center	White	Rhizoid	+	Cocci	Staphylococci
B 214 b	Buyé	Center	White	Irregular	+	Cocci	Staphylococci
B 215	Buyé	Center	White	Rhizoid	+	Cocci	Staphylococci
B 216	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 217	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 218	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 219	Buyé	Center	Transparent white	Rhizoid	+	Cocci	Single cocci
B 220	Buyé	Center	Cream	Circular	-	Rods	Streptobacilli

Appendix 1 Continuation

B 221	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 222	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 223	Buyé	Base	White	Circular	+	Rods	Single bacilli
B 224	Buyé	Base	White	Irregular	-	Short rods	Single bacilli
B 225	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 226 a	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 226 b	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 227	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 228	Buyé	Base	Creamy orange	Irregular	+	Rods	Diplobacilli
B 228 B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 229	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 229 B	Buyé	Base	White	Irregular	+	Rods	Streptobacilli

B 230	Buyé	Base	White	Irregular	+	Cocci	Staphylococci
B 231	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 232	Buyé	Base	Transparent	Rhizoid	+	Rods	Streptobacilli
B 233	Buyé	Center	Creamy white	Circular	+	Cocci	Staphylococci
B 234	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli
B 235	Buyé	Center	White	Rhizoid	-	Rods	Streptobacilli
B 236	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 236 b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 237 a	Buyé	Apex	White	Irregular	+	Rods	Streptobacilli
b 237 B	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 238	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 238 b	Buyé	Apex	White	Irregular	+	Rods	Streptobacilli

Appendix 1 Continuation

B 239	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 240	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 241	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 242	Buyé	Apex	White	Irregular	+	Rods	Streptobacilli
B 243	Buyé	Apex	White	Circular	+	Short rods	Single bacilli
B 244	Buyé	Center	White	Irregular	+	Rods	Streptobacilli
B 245	Buyé	Center	White	Rhizoid	+	Rods	Streptobacilli
B 246	Buyé	Center	White	Rhizoid	-	Rods	Streptobacilli
B 247	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 247 a	Buyé	Base	White	Rhizoid	+	Rods	Streptobacilli
B 247 B	Buyé	Base	White	Rhizoid	+	Rods	Streptobacilli
B 248	Buyé	Base	White	Rhizoid	+	Rods	Streptobacilli

Appendix 1 Continuation

B 249	Buyé	Base	Transparent white	Irregular	+	Rods	Single bacilli
B 250	Buyé	Base	Transparent white	Irregular	+	Rods	Single bacilli
B 251	Buyé	Center	Transparent cream	Irregular	+	Rods	Single bacilli
B 252	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 253	Buyé	Center	Transparent white	Irregular	+	Rods	Single bacilli
B 254	Buyé	Center	Cream	Irregular	+	Cocci	Staphylococci
B 255 A	Buyé	Center	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 255 b	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 256	Buyé	Apex	Transparent white	Irregular	-	Rods	Streptobacilli
B 257	Buyé	Apex	Creamy white	Circular	+	Cocci	Staphylococci
B 259	Buyé	Base	White	Irregular	+	Rods	Streptobacilli
B 260 b	Buyé	Base	Orange	Circular	+	Short rods	Diplobacilli

Appendix 1 Continuation

B 260 b1	Buyé	Base	White	Rhizoid	+	Short rods	Single bacilli
B 261	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 262	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 263 a	Buyé	Center	White	Irregular	+	Rods	Streptobacilli
B 263 b	Buyé	Apex	White	Rhizoid	+	Cocci	Single cocci
B 264a	Buyé	Base	White	Circular	+	Rods	Diplobacilli
B 264 b	Buyé	Base	White	Circular	+	Cocci	Single cocci
B 265	Buyé	Base	Cream	Circular	+	Cocci	Single cocci
B 266	Buyé	Base	White	Circular	+	Rods	Streptobacilli
B 267	Buyé	Base	White	Circular	+	Cocci	Single cocci
B 267 b	Buyé	Base	White	Rhizoid	+	Rods	Streptobacilli
B 268	Buyé	Center	Bright yellow	Circular	+	Cocci	Staphylococci

B 268 B	Buyé	Center	Cream	Circular	+	Cocci	Single cocci
B 269	Buyé	Center	Transparent cream	Irregular	+	Rods	Single bacilli
B 271	Buyé	Apex	Orange	Circular	+	Curved rods	Single bacilli
B 271 B	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 272	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 273	Buyé	Apex	Cream	Irregular	-	Short curved rods	Single bacilli
B 274	Buyé	Apex	Cream	Irregular	-	Short curved rods	Single bacilli
B 275	Buyé	Apex	White	Circular	+	Rods	Single bacilli
B 275 B	Buyé	Apex	Transparent	Circular	+	Rods	Single bacilli
B 276	Buyé	Apex	Orange	Circular	+	Short rods	Single bacilli
B277	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B277 b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 277 c	Buyé	Apex	Yellow	Circular	+	Cocci	Staphylococci
B277 d	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 278	Buyé	Base	Yellow	Irregular	+	Cocci	Staphylococci
B 278 b	Buyé	Base	Cream	Irregular	+	Cocci	Staphylococci
B278 C	Buyé	Base	Bright yellow	Circular	+	Short rods	Single bacilli
B 279	Buyé	Base	Cream	Rhizoid	+	Cocci	Staphylococci
B 280	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 280 B	Buyé	Base	Cream	Irregular	+	Rods	Single bacilli
B 281	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 282	Buyé	Base	Transparent white	Circular	+	Cocci	Single cocci
B 283	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 283b	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 284	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 285 a	Buyé	Center	Yellow	Circular	+	Cocci	Staphylococci
B285 b	Buyé	Center	Light orange	Rhizoid	-	Short rods	Single bacilli
B 286	Buyé	Base	Cream	Circular	-	Long rods	Streptobacillil
B 287	Buyé	Apex	Cream	Circular	+	Rods	Single bacilli
B287b	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B288	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 288 b1	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
B 288b2	Buyé	Apex	Transparent yellow	Circular	+	Short rods	Single bacilli
B289a	Buyé	Base	Cream	Irregular	+	Rods	Single bacilli
B289b	Buyé	Base	Cream	Rhizoid	+	Rods	Single bacilli

Appendix 1 Continuation

B290	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 291	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 292	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 292 b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 293	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 293b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 294	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 295	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 296	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 297	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 298	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 299	Buyé	Base	White	Circular	+	Cocci	Staphylococci

Appendix 1 Continuation

B 300	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 301 a	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 301 b	Buyé	Base	Yellow	Circular	+	Cocci	Staphylococci
B 302	Buyé	Base	Creamy orange	Irregular	+	Rods	Single bacilli
B 303	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 304	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 305	Buyé	Apex	Transparent white	Irregular	+	Cocci	Staphylococci
B 306	Buyé	Apex	Cream	Circular	+	Rods	Single bacilli
B 307	Buyé	Apex	White	Irregular	+	Cocci	Staphylococci
B 308	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 309	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 310	Buyé	Center	Creamy orange	Irregular	-	Rods	Single bacilli

Appendix 1 Continuation

B 311	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 312	Buyé	Center	Transparent white	Rhizoid	-	Rods	Single bacilli
B 312 b	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 313	Buyé	Center	White	Irregular	+	Rods	Single bacilli
B 314 A	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 314 B	Buyé	Center	Creamy orange	Irregular	+	Rods	Single bacilli
B 315	Buyé	Center	Creamy orange	Irregular	+	Rods	Single bacilli
B 316	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 317	Buyé	Center	White	Rhizoid	-	Rods	Single bacilli
B 318	Buyé	Center	Cream	Rhizoid	+	Cocci	Staphylococci
B 319	Buyé	Base	Transparent cream	Rhizoid	-	Rods	Single bacilli
B 320	Buyé	Base	White	Rhizoid	-	Rods	Single bacilli

Appendix 1 Continuation

B 321 a	Buyé	Base	White	Rhizoid	+	Rods	Single rods
B 321 B	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 322	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 323	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 324	Buyé	Base	Creamy orange	Irregular	+	Cocci	Staphylococci
B 325	Buyé	Base	Cream	Circular	+	Long rods	Streptobacilli
B 326	Buyé	Base	Cream	Rhizoid	+	Rods	Single bacilli
B 326 B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 327	Buyé	Base	Yellow	Circular	+	Cocci	Staphylococci
B 328	Buyé	Center	Creamy orange	Irregular	+	Cocci	Staphylococci
B 329	Buyé	Center	Mustard yellow	Irregular	-	Rods	Single bacilli
B 329 b	Buyé	Center	Cream	Circular	+	Rods	Single bacilli

Appendix 1 Continuation

B 330	Buyé	Base	Transparent	Circular	+	Cocci	Staphylococci
B 331	Buyé	Base	Cream	Circular	-	Rods	Single bacilli
B 331 b	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 332	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 333	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 334	Buyé	Center	Light orange	Circular	+	Rods	Streptobacilli
B 334 b	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 335	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 336	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 337	Buyé	Base	White	Circular	+	Cocci	Staphylococci
b 338	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 339	Buyé	Base	Creamy orange	Irregular	+	Rods	Single rods

Appendix 1 Continuation

B 341	Buyé	Base	Transparent cream	Circular	+	Rods	Single bacilli
B 342	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 343	Buyé	Base	Transparent white	Circular	+	Cocci	Staphylococci
B 344	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 345	Buyé	Base	Cream	Circular	-	Rods	Single bacilli
B 346	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 347	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 348	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 348 b	Buyé	Apex	Cream	Rhizoid	+	Rods	Single bacilli
B 349	Buyé	Apex	Transparent cream	Circular	+	Rods	Single bacilli
B 350	Buyé	Apex	Yellow	Circular	+	Cocci	Staphylococci
B 351 A	Buyé	Base	Transparent cream	Circular	+	Rods	Single bacilli

Appendix 1 Continuation

B 351 b	Buyé	Base	Transparent	Circular	+	Short rods	Single bacilli
B 352	Buyé	Apex	White	Irregular	+	Rods	Streptobacilli
B 353	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 354	Buyé	Apex	Cream	Irregular	+	Rods	Streptobacilli
B 355 A	Buyé	Apex	Cream	Circular	+	Short rods	Single bacilli
B 355 B	Buyé	Apex	White	Rhizoid	+	Rods	Streptobacilli
B 356	Buyé	Apex	Transparent white	Rhizoid	+	Rods	Streptobacilli
B 357	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 358	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 359	Buyé	Center Apex	Transparent	Rhizoid	-	Rods	Single bacilli
B 360	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli
B 360 b	Buyé	Apex	White	Rhizoid	+	Rods	Single bacilli

B 361	Buyé	Center	Cream	Irregular	+	Cocci	Staphylococci
B 363	Buyé	Center	Cream	Circular	+	Cocci	Single cocci
B 364	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 367	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 367b	Buyé	Base	White	Rhizoid	-	Rods	Single bacilli
B 368	Buyé	Apex	Cream	Irregular	+	Cocci	Staphylococci
B 369	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 369 b	Buyé	Center	Yellow	Circular	+	Cocci	Staphylococci
B 371	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 372	Buyé	Center	White	Rhizoid	+	Rods	Single bacilli
B 374	Buyé	Base	White	Rhizoid	+	Rods	Single bacilli

B 375 A	Buyé	Base	Cream	Rhizoid	+	Rods	Streptobacilli
B 375 B	Buyé	Base	Cream	Circular	+	Cocci	Diplococci
B 376	Buyé	Base	White	Circular	+	Cocci	Staphylococci
B 377 A1	Buyé	Base	Transparent	Irregular	+	Cocci	Staphylococci
B 377 A 2	Buyé	Base	Transparent	Irregular	+	Cocci	Staphylococci
B 377 A3	Buyé	Base	Cream	Circular	-	Short rods	Single bacilli
B 377 B	Buyé	Base	Brown	Circular	-	Rods	Single bacilli
B 377 C	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 377 D	Buyé	Base	Cream	Irregular	+	Rods	Streptobacilli
B 377 E	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 378 a	Buyé	Center	White	Circular	+	Rods	Single bacilli
B 378 b	Buyé	Center	Creamy orange	Irregular	-	Rods	Single bacilli

B 379	Buyé	Apex	Creamy orange	Irregular	+	Rods	Diplobacilli
B 379A	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 379 b	Buyé	Apex	White	Irregular	+	Rods	Single bacilli
B 380 a	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 380 b	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 381	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 382 A	Buyé	Center	Cream	Irregular	+	Rods	Single bacilli
B 382 b	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
b 382 C	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 383 A	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 384	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 385	Buyé	Base	Cream	Irregular	+	Rods	Single bacilli

Appendix 1 Continuation

B 385 B	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 385 C1	Buyé	Base	Transparent Cream	Circular	+	Cocci	Staphylococci
B 385 C2	Buyé	Base	Cream	Irregular	+	Rods	Single bacilli
B 386	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 388	Buyé	Base	White	Irregular	+	Rods	Single bacilli
B 389	Buyé	Base		Circular	+	Cocci	Staphylococci
B 390	Buyé	Apex	Creamy orange	Irregular	+	Rods	Single bacilli
B 391	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 392	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 394 b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 395	Buyé	Center	Transparent white	Rhizoid	+	Rods	Single bacilli

Appendix 1 Continuation

B 396	Buyé	Center	Cream	Irregular	+	Rods	Single bacilli
B 397	Buyé	Base	Yellow cream	Circular	+	Cocci	Staphylococci
B 399	Buyé	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
B 400 A	Buyé	Base	Creamy white	Circular	+	Short rods	Single bacilli
B 401	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 402 B	Buyé	Base	Creamy white	Circular	-	Short rods	Single bacilli
B 403	Buyé	Apex	Cream	Irregular	+	Rods	Streptobacilli
B 404	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 407	Buyé	Base	Cream	Irregular	-	Short rods	Single bacilli
B 408	Buyé	Apex	Cream	Irregular	+	Rods	Single bacilli
B 409	Buyé	Apex	White	Irregular	+	Rods	Single bacilli

Appendix 1 Continuation

B 409 B	Buyé	Apex	Transparent white	Irregular	+	Rods	Single bacilli
B 410	Buyé	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
B 411	Buyé	Center	White	Circular	+	Rods	Single bacilli
B 412 A	Buyé	Center	White	Circular	+	Cocci	Staphylococci
B 412 B	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 413	Buyé	Base	Yellow	Irregular	+	Cocci	Staphylococci
B 414	Buyé	Base	Brown	Circular	-	Short rods	Single bacilli
B 415	Buyé	Base	Brown	Circular	-	Short rods	Single bacilli
B 417 A	Buyé	Base	Cream	Circular	+	Cocci	Staphylococci
B 417 D	Buyé	Base	Cream	Irregular	-	Rods	Single bacilli
B 418	Buyé	Base	Brown	Rhizoid	-	Rods	Single bacilli

B 419	Buyé	Apex	Yellow orange	Circular	+	Rods	Single bacilli
B 420	Buyé	Apex	Cream	Circular	-	Rods	Single bacilli
B 421	Buyé	Apex	Yellow orange	Circular	+	Rods	Single bacilli
B 422 A	Buyé	Base	White	Circular	-	Rods	Single bacilli
B 422 B	Buyé	Base	White	Circular	-	Rods	Streptobacilli
B 424	Buyé	Center	Cream	Circular	+	Cocci	Staphylococci
B 425	Buyé	Apex	Brown	Irregular	-	Rods	Single bacilli
B 426	Buyé	Center	Cream	Circular	-	Short Rods	Single bacilli
B 426 B	Buyé	Center	White	Circular	-	Rods	Single bacilli
B 427 A	Buyé	Center	White	Rhizoid	-	Short rods	Single bacilli
B 428	Buyé	Center	White	Circular	+	Rods	Single bacilli

Appendix 1 Continuation

B 429	Buyé	Center	Cream	Rhizoid	+	Rods	Single bacilli
B 430 a	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 430 b	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
B 433 A	Buyé	Apex	White	Circular	+	Cocci	Staphylococci
B 433 B	Buyé	Apex	Cream	Circular	+	Cocci	Staphylococci
LP 1	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 1A	La Parguera	Base	Purple	Irregular	-	Rods	Streptobacilli
LP 2	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 3	La Parguera	Base	White	Rhizoid	-	Rods	Single bacilli
LP 4	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 5	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 6	La Parguera	Base	White	Irregular	+	Rods	Single bacilli

LP 7	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 8	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 9	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 10	La Parguera	Base	White	Rhizoid	-	Rods	Single bacilli
LP 11	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 13	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 14	La Parguera	Base	White	Irregular	-	Rods	Single bacilli
LP 15	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 16	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 17	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 18	La Parguera	Base	White	Irregular	+	Rods	Single bacilli

Appendix 1 Continuation

LP 19	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 20	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 21	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 22	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 23	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 24	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 25	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 26	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 27	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 28	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 29	La Parguera	Apex	Creamy orange	Irregular	+	Rods	Single bacilli
LP 30 A	La Parguera	Base	White	Irregular	-	Rods	Single bacilli

Appendix 1 Continuation

LP 31	La Parguera	Base	Cream	Irregular	-	Rods	Single bacilli
LP 32	La Parguera	Base	Cream	Irregular	-	Rods	Single bacilli
LP 33	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 34	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 35	La Parguera	Base	White	Irregular	-	Rods	Single bacilli
LP 36	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 37	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 38	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 39	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 40	La Parguera	Apex	Yellow	Circular	+	Cocci	Staphylococci
LP 41	La Parguera	Apex	Cream	Circular	+	Cocci	Staphylococci
LP 42	La Parguera	Center	Yellow	Circular	+	Cocci	Single cocci

Appendix 1 Continuation

LP 43	La Parguera	Base	Transparent white	Irregular	+	Rods	Single bacilli
LP 44	La Parguera	Base	Transparent white	Irregular	+	Rods	Single bacilli
LP 45	La Parguera	Center	Transparent cream	Irregular	+	Long rods	Single bacilli
LP 45 B	La Parguera	Center	Creamy orange	Irregular	-	Rods	Single bacilli
LP 46	La Parguera	Center	Cream	Irregular	+	Rods	Single bacilli
LP 47	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 49	La Parguera	Base	Cream	Circular	-	Rods	Single bacilli
LP 50	La Parguera	Base	White	Irregular	-	Rods	Single bacilli
LP 51	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 52	La Parguera	Apex	Cream	Circular	-	Short rods	Single bacilli
LP 53	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 53 A	La Parguera	Apex	Cream	Circular	-	Rods	Single bacilli

Appendix 1 Continuation

LP 54	La Parguera	Center	Cream	Irregular	+	Rods	Single bacilli
LP 55	La Parguera	Center	Cream	Irregular	+	Rods	Single bacilli
LP 56	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 57	La Parguera	Base	White	Circular	+	Cocci	Single cocci
LP 58	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 59	La Parguera	Center	White	Irregular	+	Cocci	Staphylococci
LP 60	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 61	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 62	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 63	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 64	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 65	La Parguera	Base	White	Irregular	+	Rods	Streptobacilli

Appendix 1 Continuation

LP 66	La Parguera	Apex	Creamy orange	Circular	+	Rods	Streptobacilli
LP 67	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 68	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 69	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 70	La Parguera	Apex	White	Irregular	+	Cocci	Single cocci
LP 71	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 72	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 73	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 74	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 75	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 76	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 77	La Parguera	Apex	White	Circular	+	Cocci	Staphylococci

LP 78	La Parguera	Apex	White	Irregular	+	Long rods	Streptobacilli
LP 79	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 80	La Parguera	Base	White	Irregular	+	Cocci	Single cocci
LP 81	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 82	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 83	La Parguera	Apex	White	Rhizoid	+	Rods	Streptobacilli
LP 84	La Parguera	Apex	Cream	Circular	-	Rods	Single bacilli
LP 85	La Parguera	Apex	Cream	Rhizoid	+	Rods	Single bacilli
LP 86	La Parguera	Apex	Cream	Circular	+	Rods	Streptobacilli
LP 87	La Parguera	Center	Cream	Rhizoid	+	Rods	Single bacilli
LP 88	La Parguera	Center	White	Irregular	+	Rods	Diplobacilli
LP 89	La Parguera	Apex	Creamy orange	Irregular	+	Rods	Diplobacilli

Appendix 1 Continuation

LP 90	La Parguera	Apex	Creamy orange	Irregular	+	Rods	Diplobacilli
LP 92	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 93	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 94	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 95	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 96	La Parguera	Base	Transparent white	Rhizoid	+	Cocci	Staphylococci
LP 97	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 98	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 99	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 100	La Parguera	Center	Transparent cream	Rhizoid	+	Rods	Single bacilli
LP 101	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 102	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli

Appendix 1 Continuation

LP 103	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 104	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 105	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 106	La Parguera	Apex	Transparent white	Rhizoid	-	Rods	Single bacilli
LP 107	La Parguera	Center	Creamy orange	Circular	+	Rods	Single bacilli
LP 108	La Parguera	Center	Creamy orange	Irregular	+	Rods	Single bacilli
LP 109	La Parguera	Base	Cream	Circular	-	Rods	Single bacilli
LP 110	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 111	La Parguera	Base	White	Rhizoid	-	Rods	Single bacilli
LP 113	La Parguera	Apex	Creamy yellow	Rhizoid	-	Rods	Single bacilli
LP 115	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 116	La Parguera	Apice	Transparent white	Rhizoid	+	Rods	Single bacilli

Appendix 1 Continuation

LP 117	La Parguera	Base	Creamy white	Irregular	+	Rods	Single bacilli
LP 118	La Parguera	Base	Cream	Rhizoid	+	Rods	Single bacilli
LP 119	La Parguera	Center	Creamy white	Rhizoid	+	Rods	Single bacilli
LP 120	La Parguera	Center	Cream	Irregular	-	Rods	Single bacilli
LP 121	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 122	La Parguera	Apex	White	Irregular	+	Rods	Single bacilli
LP 123	La Parguera	Apex	Creamy white	Circular	+	Rods	Single bacilli
LP 124	La Parguera	Apex	Cream	Irregular	-	Rods	Diplobacilli
LP 125	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 126	La Parguera	Center	White	Circular	+	Rods	Single bacilli
LP 128	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 129	La Parguera	Base	Cream	Irregular	-	Rods	Single bacilli

LP 130	La Parguera	Center	White	Irregular	+	Rods	Single bacilli
LP 131	La Parguera	Center	White	Circular	+	Rods	Single bacilli
LP 132	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 133	La Parguera	Base	Cream	Irregular	-	Rods	Single bacilli
LP 134	La Parguera	Base	White	Irregular	+	Rods	Single bacilli
LP 135	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 136	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 137	La Parguera	Apex	White	Circular	-	Rods	Single bacilli
LP 138	La Parguera	Apex	Transparent white	Rhizoid	-	Rods	Single bacilli
LP 139	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 140	La Parguera	Center	White	Circular	-	Rods	Single bacilli
LP 141	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli

Appendix 1 Continuation

LP 142	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 143	La Parguera	Apex	Creamy orange	Circular	+	Rods	Streptobacilli
LP 144	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 145	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 146	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 147	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 148	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 149	La Parguera	Apex	White	Rhizoid	+	Rods	Single bacilli
LP 150	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 151	La Parguera	Base	White	Rhizoid	+	Rods	Single bacilli
LP 152	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 153	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli

LP 154	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 155	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 156	La Parguera	Center	White	Rhizoid	+	Rods	Single bacilli
LP 157	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 158	La Parguera	Base	Cream	Irregular	-	Rods	Single bacilli
LP 159	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Streptobacilli
LP 160	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 161	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 162	La Parguera	Base	Transparent white	Rhizoid	-	Rods	Single bacilli
LP 163	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 164	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 167	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli

LP 168	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 169	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 170	La Parguera	Center	Transparent white	Rhizoid	-	Rods	Single bacilli
LP 171	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 172	La Parguera	Center	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 173	La Parguera	Apex	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 174	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 175	La Parguera	Base	Peach	Rhizoid	+	Cocci	Streptococci
LP 176	La Parguera	Base	Transparent white	Rhizoid	+	Rods	Single bacilli
LP 178	La Parguera	Apex	Cream	Irregular	-	Rods	Single bacilli
LP 179	La Parguera	Apex	White	Rhizoid	+	Rods	Streptobacilli
LP 180	La Parguera	Apex	Cream	Irregular	-	Rods	Streptobacilli

Appendix 1 Continuation

LP 181	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 182	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 183	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 184	La Parguera	Base	Rhizoid	White	-	Rods	Single bacilli
LP 185	La Parguera	Apex	Irregular	White Transparent cream	+	Rods	Streptobacilli
LP 186	La Parguera	Center	Rhizoid	White	+	Rods	Streptobacilli
LP 187	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 188	La Parguera	Center	Rhizoid	White Transparent cream	+	Rods	Single bacilli
LP 189	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 190	La Parguera	Apex	Irregular	White	+	Rods	Single bacilli
LP 191	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 192	La Parguera	Center	Rhizoid	White	-	Rods	Single bacilli

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LP 193	La Parguera	Center	Rhizoid	Creamy orange	+	Rods	Diplobacilli
LP 194	La Parguera	Apex	Irregular	Cream	-	Rods	Single bacilli
LP 195	La Parguera	Base	Circular	White	+	Cocci	Staphylococci
LP 196	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Streptobacilli
LP 197	La Parguera	Center	Rhizoid	White	+	Curved rods	Single bacilli
LP 198	La Parguera	Apex	Irregular	Cream	+	Cocci	Staphylococci
LP 199	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 200	La Parguera	Apex	Rhizoid	White	+	Long rods	Streptobacilli
LP 201	La Parguera	Base	Rhizoid	White	+	Cocci	Single cocci
LP 202	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 203	La Parguera	Base	Irregular	Creamy orange	+	Rods	Diplobacilli
LP 204	La Parguera	Center	Rhizoid	White	+	Short rods	Single bacilli

LP 205	La Parguera	Apex	Rhizoid	Cream	+	Short rods	Single bacilli
LP 206	La Parguera	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 207	La Parguera	Center	Rhizoid	Cream	+	Rods	Single bacilli
LP 208	La Parguera	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 209	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 210	La Parguera	Apex	Rhizoid	Cream	+	Short rods	Single bacilli
LP 211	La Parguera	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 212	La Parguera	Center	Rhizoid	Cream	+	Rods	Single bacilli
LP 213	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 214	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 215	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 216	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Diplobacilli

Appendix 1 Continuation

LP 217	La Parguera	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 218	La Parguera	Base	Circular	Cream	+	Rods	Single bacilli
LP 219	La Parguera	Base	Circular	White	+	Rods	Single bacilli
LP 220	La Parguera	Apex	Rhizoid	Creamy white	+	Rods	Single bacilli
LP 221	La Parguera	Apex	Rhizoid	Transparent	-	Rods	Single bacilli
LP 222	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 223	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 224	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 225	La Parguera	Apex	Circular	Creamy white	+	Short rods	Single bacilli
LP 226	La Parguera	Center	Irregular	White	+	Rods	Single bacilli
LP 227	La Parguera	Center	Circular	White	+	Rods	Single bacilli
LP 228	La Parguera	Center	Circular	Transparent white	+	Rods	Single bacilli

LP 229	La Parguera	Apex	Circular	White	Rods	+	Single bacilli
LP 230	La Parguera	Base	Rhizoid	Cream	Rods	-	Diplobacilli
LP 231	La Parguera	Apex	Circular	White	Rods	+	Single bacilli
LP 232	La Parguera	Apex	Rhizoid	White	Rods	+	Diplobacilli
LP 233	La Parguera	Base	Irregular	White	Rods	+	Single bacilli
LP 234	La Parguera	Base	Irregular	White	Rods	+	Single bacilli
LP 235	La Parguera	Center	Rhizoid	White	Rods	+	Single bacilli
LP 236	La Parguera	Apex	Rhizoid	White	Rods	+	Single bacilli
LP 237	La Parguera	Apex	Rhizoid	White	Rods	+	Single bacilli
LP 238	La Parguera	Base	Rhizoid	White	Rods	+	Single bacilli
LP 239	La Parguera	Base	Circular	White	Rods	+	Single bacilli
LP 240	La Parguera	Center	Rhizoid	Cream	Rods	+	Single bacilli

Appendix 1 Continuation

LP 241	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 242	La Parguera	Base	Irregular	White	+	Rods	Single bacilli
LP 243	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 244	La Parguera	Apex	Rhizoid	White	+	Rods	Single bacilli
LP 245	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 246	La Parguera	Center	Circular	White	+	Long rods	Single bacilli
LP 247	La Parguera	Base	Rhizoid	Cream	-	Rods	Single bacilli
LP 248	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 249	La Parguera	Base	Circular	White	+	Rods	Single bacilli
LP 250	La Parguera	Base	Rhizoid	White	+	Rods	Single bacilli
LP 251	La Parguera	Center	Circular	White	+	Rods	Single bacilli
LP 252	La Parguera	Base	Circular	White	+	Rods	Single bacilli

LP 253	La Parguera	Apex	Rhizoid	Transparent	+	Rods	Streptobacilli
LP 254	La Parguera	Center	Irregular	Green	+	Rods	Single bacilli
LP 255A	La Parguera	Center	Rhizoid	White	+	Rods	Single bacilli
LP 255b	La Parguera	Center	Rhizoid	Cream	+	Rods	Single bacilli
LP 257	La Parguera	Base	Circular	White	+	Rods	Single bacilli
LP 256	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 258	La Parguera	Center	Circular	Creamy yellow	+	Rods	Single bacilli
LP 259	La Parguera	Base	Circular	Creamy yellow	-	Rods	Single bacilli
LP 260	La Parguera	Apex	Irregular	Orange	+	Rods	Single bacilli
LP 261	La Parguera	Center	Circular	Creamy yellow	+	Rods	Single bacilli
LP 271	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP272	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli

LP 273	La Parguera	Base	Circular	White	+	Rods	Single bacilli
LP 274	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 275	La Parguera	Center	Circular	Creamy yellow	+	Rods	Single bacilli
LP 276	La Parguera	Base	Circular	Creamy yellow	-	Rods	Single bacilli
LP 277	La Parguera	Apex	Irregular	Orange	+	Rods	Single bacilli
LP 278	La Parguera	Center	Circular	Creamy yellow	+	Rods	Single bacilli
LP 279	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 282	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
LP 283	La Parguera	Apex	Irregular	Orange	+	Rods	Single bacilli
LP284	La Parguera	Center	Circular	Creamy yellow	+	Rods	Single bacilli
LP285	La Parguera	Center	Rhizoid	Transparent white	+	Rods	Diplobacilli
VQ 2	Vieques	Apex	Circular	Cream	+	Rods	Streptobacilli

VQ 3	Vieques	Center	Rhizoid	White	+	Rods	Streptobacilli
VQ 3 A	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 4	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 5	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 6	Vieques	Center	Circular	White	+	Rods	Single bacilli
VQ 6 A	Vieques	Center	Irregular	Transparent cream	+	Rods	Single bacilli
VQ 7	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 8	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 8A	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 10	Vieques	Center	Circular	Creamy brown	+	Rods	Single bacilli
VQ 11	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 12	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli

VQ 14	Vieques	Base	Irregular	White	+	Cocci	Staphylococci
VQ 15	Vieques	Base	Circular	Transparent white	+	Cocci	Staphylococci
VQ 15 B	Vieques	Base	Irregular	Cream	+	Rods	Single bacilli
VQ 16	Vieques	Center	Circular	Creamy orange	+	Short rods	Diplobacilli
VQ 17	Vieques	Center	Circular	Yellow	+	Rods	Single bacilli
VQ 18	Vieques	Base	Rhizoid	White	-	Rods	Single bacilli
VQ 19	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 20	Vieques	Base	Circular	White	+	Cocci	Staphylococci
VQ21	Vieques	Base	Circular	Cream	+	Rods	Single bacilli
VQ 22	Vieques	Base	Circular	Pink	+	Rods	Streptobacilli
VQ 23	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 23 A	Vieques	Apex	Irregular	Transparent cream	+	Rods	Single bacilli

Appendix 1 Continuation

VQ 23 B	Vieques	Apex	Circular	Cream	-	Rods	Single bacilli
VQ 24	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 25	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 25 B	Vieques	Base	Irregular	Cream	+	Rods	Single bacilli
VQ 25 C	Vieques	Base	Irregular	Pink	+	Rods	Streptobacilli
VQ 26 A	Vieques	Apex	Circular	Creamy pink	+	Rods	Single bacilli
VQ 26 B	Vieques	Apex	Irregular	Cream	+	Rods	Streptobacilli
VQ 26 C	Vieques	Apex	Rhizoid	White	-	Rods	Single bacilli
VQ 27	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 28	Vieques	Apex	Circular	Transparent cream	+	Rods	Single bacilli
VQ 29	Vieques	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 30	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli

Appendix 1 Continuation

VQ 31	Vieques	Apex	Circular	Light orange	+	Rods	Single bacilli
VQ 31 A	Vieques	Apex	Irregular	Creamy orange	+	Short rods	Single bacilli
VQ 31 B	Vieques	Apex	Circular	Cream	-	Rods	Single bacilli
VQ 32 B	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 33A	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 33 C	Vieques	Center	Rhizoid	Creamy orange	+	Cocobacilli	Single
VQ 33 C1	Vieques	Center	Rhizoid	Cream	+	Rods	Streptobacilli
VQ 33 C2	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 34	Vieques	Apex	Irregular	White	+	Rods	Streptobacilli
VQ 35	Vieques	Center	Irregular	Cream	-	Rods	Single bacilli
VQ 35 A	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 36	Vieques	Apex	Rhizoid	White	+	Rods	Streptobacilli

VQ 37 A	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 37 B	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 37 C	Vieques	Apex	Irregular	Creamy green	+	Rods	Single bacilli
VQ 38	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 38B	Vieques	Center	Circular	Transparent white	+	Curved rods	Single bacilli
VQ 39	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 40	Vieques	Apex	Circular	Cream	+	Cocci	Single cocci
VQ 40 B	Vieques	Apex	Circular	Cream	+	Rods	Single bacilli
VQ 41	Vieques	Center	Irregular	Cream	+	Rods	Streptobacilli
VQ 45	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 46	Vieques	Base	Irregular	White	-	Rods	Single bacilli
VQ 47	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 48	Vieques	Center	Irregular	White	+	Rods	Single bacilli

Appendix 1 Continuation

VQ 49	Vieques	Center	Irregular	Cream	+	Rods	Single bacilli
VQ 50	Vieques	Center	Irregular	Cream	-	Rods	Single bacilli
VQ 50 A	Vieques	Center	Irregular	Beige	+	Cocci	Staphylococci
VQ 51	Vieques	Center	Irregular	Beige	+	Cocci	Staphylococci
VQ 52	Vieques	Base	Rhizoid	White	+	Cocci	Diplococci
VQ 53	Vieques	Base	Irregular	Pink	+	Rods	Streptobacilli
VQ 54	Vieques	Base	Irregular	Pink	+	Rods	Streptobacilli
VQ 55	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQ 56	Vieques	Apex	Irregular	White	-	Rods	Single bacilli
VQ 57	Vieques	Base	Circular	Yellow	+	Rods	Single bacilli
VQ 58	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 59	Vieques	Center	Circular	Cream	+	Rods	Single bacilli

Appendix 1 Continuation

VQ 61	Vieques	Center	Irregular	White	-	Rods	Single bacilli
VQ 62	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 64	Vieques	Base	Irregular	Light brown	+	Rods	Single bacilli
VQ 65	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQ 65 B	Vieques	Apex	Irregular	Orange	-	Rods	Single bacilli
VQ 67	Vieques	Center	Irregular	Cream	+	Rods	Single bacilli
Vq 68	Vieques	Base	Irregular	Orange	+	Rods	Single bacilli
VQ 69	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 70	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 71	Vieques	Center	Irregular	Transparent cream	+	Rods	Single bacilli
VQ 72	Vieques	Apex	Circular	White	+	Coccibacilli	Single
VQ 73	Vieques	Apex	Irregular	Cream	-	Rods	Single bacilli

Appendix 1 Continuation

VQ 74	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 75	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQ 76 A	Vieques	Base	Irregular	Cream	-	Rods	Single bacilli
VQ 76 B	Vieques	Base	Irregular	Cream	-	Rods	Single bacilli
VQ 77	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQ 78	Vieques	Base	Irregular	Cream	+	Rods	Single bacilli
VQ 79	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 80	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 81	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 82	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 83	Vieques	Center	Circular	White	+	Rods	Single bacilli
VQ 85	Vieques	Apex	Rhizoid	Cream	-	Rods	Single bacilli
VQ 86	Vieques	Apex	Irregular	White	-	Rods	Single bacilli

VQ 87	Vieques	Base	Circular	Cream	+	Cocci	Staphylococci
VQ 87 A	Vieques	Apex	Circular	Cream	+	Rods	Streptobacilli
VQ 88	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 89	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 90	Vieques	Base	Circular	Transparent white	+	Long rods	Streptobacilli
VQ 90 A	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 91	Vieques	Apex	Circular	White	+	Cocci	Staphylococci
VQ 92	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 93	Vieques	Center	Circular	White	-	Rods	Single bacilli
VQ 94	Vieques	Center	Circular	White	+	Cocci	Staphylococci

Appendix 1 Continuation

VQ 95	Vieques	Base	Irregular	Transparent white	+	Rods	Single bacilli
VQ 95 A	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 97	Vieques	Apex	Irregular	Cream	+	Rods	Single bacilli
VQ 98	Vieques	Apex	Irregular	Cream	-	Rods	Streptobacilli
VQ 99	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 100	Vieques	Apex	Circular	Cream	+	Rods	Single bacilli
VQ 100 B	Vieques	Apex	Irregular	Transparent cream	+	Rods	Single bacilli
VQ 101	Vieques	Apex	Irregular	Transparent cream	+	Cocci-bacilli	Single bacilli
VQ 101	Vieques	Apex	Irregular	Cream	+	Cocci	Single bacilli
VQ 102	Vieques	Base	Irregular	Creamy orange	+	Rods	Single bacilli
VQ 102	Vieques	Base	Circular	Light pink	+	Rods	Streptobacilli
VQ 103	Vieques	Apex	Irregular	Light brown	+	Rods	Streptobacilli

VQ 104	Vieques	Apex	Circular	Orange	+	Rods	Single bacilli
VQ 105	Vieques	Apex	Circular	Transparent cream	+	Cocci-bacilli	SIngle
VQ 105	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 105 B	Vieques	Apex	Circular	Cream	+	Rods	Single bacilli
VQ 106	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 106	Vieques	Center	Irregular	Transparent	-	Rods	Single bacilli
VQ 107	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 108	Vieques	Base	Irregular	Yellow	+	Cocci	Staphylococci
VQ 109	Vieques	Base	Irregular	Creamy yellow	+	Rods	Single bacilli
VQ 109 B	Vieques	Base	Irregular	Transparent cream	+	Rods	Single bacilli
VQ 110	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 111	Vieques	Center	Irregular	White	+	Rods	Single bacilli

Appendix 1 Continuation

VQ 112	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 113	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 114	Vieques	Base	Circular	Transparent yellow	+	Rods	Single bacilli
VQ 115	Vieques	Base	Rhizoid	Transparent white	+	Long rods	Single bacilli
VQ 116	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 117	Vieques	Base	Irregular	Creamy orange	+	Rods	Single bacilli
VQ 118	Vieques	Base	Rhizoid	Cream	+	Rods	Streptobacilli
VQ 119	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 120	Vieques	Apex	Irregular	Creamy yellow	+	Rods	Single bacilli
VQ 121	Vieques	Apex	Circular	Creamy yellow	+	Rods	Streptobacilli
VQ 121 B	Vieques	Apex	Irregular	White	+	Rods	Streptobacilli
VQ 122	Vieques	Apex	Irregular	White	+	Rods	Streptobacilli

Appendix 1 Continuation

VQ 123	Vieques	Apex	Circular	Yellow	+	Cocci	Staphylococci
VQ 124	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 125	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 126	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 127	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 128	Vieques	Apex	Rhizoid	White	-	Rods	Single bacilli
VQ 129	Vieques	Base	Circular	White	+	Rods	Diplobacilli
VQ 130	Vieques	Base	Circular	Orange	-	Rods	Streptobacilli
VQ 131	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 133	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 134	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 135	Vieques	Center	Circular	Yellow	+	Cocci	Staphylococci

VQ 137	Vieques	Center	Circular	Orange	+	Rods	Streptobacilli
VQ 138	Vieques	Apex	Rhizoid	Transparent white	+	Short rods	Diplobacilli
VQ140	Vieques	Base	Irregular	White	+	Rods	Single bacilli
VQ 140 B	Vieques	Base	Circular	Orange red	+	Cocci	Staphylococci
VQ 141	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 142	Vieques	Center	Circular	Pink	+	Rods	Streptobacilli
VQ 143	Vieques	Center	Circular	Pink	+	Rods	Streptobacilli
VQ 143 B	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 144	Vieques	Center	Circular	Cream	+	Rods	Single bacilli
VQ 145	Vieques	Center	Irregular	Transparent white	+	Rods	Single bacilli
VQ 146	Vieques	Center	Circular	Yellow	+	Cocci	Staphylococci
VQ 148	Vieques	Center	Circular	White	+	Rods	Single bacilli

| VQ 149 | Vieques | Apex | Circular | Cream | + | Cocci | Diplococci |

Appendix 1 Continuation

VQ 150	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 152	Vieques	Apex	Rhizoid	Creamy white	+	Rods	Single bacilli
VQ 151	Vieques	Apex	Irregular	Creamy white	+	Rods	Single bacilli
VQ 154	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 155	Vieques	Apex	Irregular	Transparent white	-	Rods	Single bacilli
VQ 156	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 157	Vieques	Base	Circular	Cream	-	Rods	Single bacilli
VQ 161	Vieques	Base	Circular	Creamy orange	+	Rods	Diplobacilli
VQ 162	Vieques	Center	Irregular	Cream	+	Rods	Streptobacilli
VQ 164	Vieques	Center	Circular	Yellow	+	Cocci	Staphylococci
VQ 165	Vieques	Apex	Irregular	White	-	Rods	Single bacilli
VQ 166	Vieques	Apex	Irregular	Cream	-	Rods	Streptobacilli

Appendix 1 Continuation

VQ 167b	Vieques	Center	Irregular	Cream	+	Rods	Streptobacilli
VQ 168	Vieques	Center	Irregular	Creamy yellow	-	Short rods	Streptobacilli
VQ170	Vieques	Center	Circular	Pink	+	Rods	Streptobacilli
VQ 171	Vieques	Apex	Irregular	Cream	-	Rods	Single bacilli
VQ 172	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 173	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 174	Vieques	Apex	Circular	White	+	Rods	Single bacilli
VQ 175	Vieques	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 176	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 177	Vieques	Center	Circular	White	-	Rods	Single bacilli
VQ 178	Vieques	Center	Irregular	Creamy yellow	-	Rods	Streptobacilli
VQ 179	Vieques	Apex	Rhizoid	White	+	Rods	Streptobacilli

| VQ 180 | Vieques | Center | Circular | Cream | + | Rods | Streptobacilli |

Continuation Table

VQ 181	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 182	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 183	Vieques	Apex	Circular	Creamy orange	+	Rods	Single bacilli
VQ 184	Vieques	Apex	Circular	Creamy orange	+	Rods	Single bacilli
VQ 185	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 186	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 187	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 188	Vieques	Apex	Circular	Creamy orange	+	Rods	Single bacilli
VQ 189	Vieques	Base	Circular	Cream	+	Rods	Streptobacilli
VQ 190	Vieques	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 191	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 192	Vieques	Apex	Circular	Cream	+	Rods	Streptobacilli

Continuation Table

VQ 193	Vieques	Base	Circular	Cream	+	Rods	Single bacilli
VQ 194	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 195	Vieques	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 196	Vieques	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 197	Vieques	Base	Irregular	Cream	-	Rods	Single bacilli
VQ 198	Vieques	Base	Rhizoid	Transparent white	+	Short rods	Streptobacilli
VQ 199	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 200	Vieques	Apex	Circular	White	+	Cocci	Staphylococci
VQ 201	Vieques	Apex	Irregular	Grey	+	Rods	Single bacilli
VQ 202	Vieques	Center	Irregular	Cream	+	Cocci	Staphylococci
VQ 203	Vieques	Base	Irregular	Orange	+	Cocci	Staphylococci
VQ 204	Vieques	Base	Rhizoid	Transparent white	-	Rods	Streptobacilli

Continuation Table

VQ 205	Vieques	Base	Circular	Transparent	+	Rods	Single bacilli
VQ 206	Vieques	Base	Irregular	Transparent	+	Rods	Single bacilli
VQ 207	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 208	Vieques	Base	Circular	Mustard yellow	+	Curved rods	Single bacilli
VQ 209	Vieques	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
VQ 210	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 211	Vieques	Apex	Irregular	Transparent cream	-	Rods	Single bacilli
VQ 212	Vieques	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
VQ 213	Vieques	Center	Irregular	Cream	+	Rods	Single bacilli
VQ 214	Vieques	Apex	Irregular	Transparent white	-	Rods	Single bacilli
VQ 215	Vieques	Apex	Irregular	Transparent white	-	Rods	Single bacilli
VQ 216	Vieques	Base	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

VQ 217	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli
VQ 218	Vieques	Apex	Irregular	Cream	+	Rods	Single bacilli
VQ 219	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 220	Vieques	Apex	Rhizoid	Transparent white	-	Rods	Single bacilli
VQ 221	Vieques	Center	Circular	Creamy yellow	+	Rods	Single bacilli
VQ 222	Vieques	Apex	Rhizoid	Transparent	+	Short rods	Single bacilli
VQ 223	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 224	Vieques	Base	Circular	Cream	+	Rods	Single bacilli
VQ 225	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 226	Vieques	Base	Circular	White	+	Rods	Single bacilli
VQ 227	Vieques	Center	Irregular	Cream	+	Rods	Single bacilli
VQ 228	Vieques	Apex	Circular	White	+	Rods	Single bacilli

Continuation Table

VQ 229	Vieques	Apex	Circular	Yellow orange	+	Rods	Single bacilli
VQ 230	Vieques	Apex	Circular	Cream	+	Rods	Single bacilli
VQ 231	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 232	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQ 233	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQ 234	Vieques	Apex	Circular	White	+	Rods	Single bacilli
VQ 235	Vieques	Base	Rhizoid	Trasnparent	-	Rods	Single bacilli
VQ 236	Vieques	Base	Rhizoid	Transparent white	-	Rods	Single bacilli
VQ 237	Vieques	Center	Circular	White	+	Rods	Single bacilli
VQ 238	Vieques	Center	Circular	White	+	Rods	Single bacilli
VQ 239	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQ 240	Vieques	Apex	Rhizoid	Transparent	+	Rods	Single bacilli

Continuation Table

VQ 241	Vieques	Base	Irregular	Transparent	+	Rods	Diplobacilli
VQ 242	Vieques	Apex	Irregular	Transparent	+	Rods	Diplobacilli
VQ 243	Vieques	Apex	Irregular	Transparent	+	Rods	Diplobacilli
VQ 244	Vieques	Apex	Circular	Creamy brown	-	Rods	Single bacilli
VQ 245	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQ 246	Vieques	Center	Irregular	Orange brown	+	Rods	Single bacilli
VQ 247	Vieques	Apex	Circular	Orange	+	Rods	Single bacilli
VQ248	Vieques	Base	Irregular	Pink	+	Cocci	Staphylococci
VQ249	Vieques	Base	Irregular	Creamy yellow	+	Rods	Streptobacilli
VQ250	Vieques	Base	Circular	White	+	Long rods	Single bacilli
VQ251	Vieques	Center	Circular	Cream	+	Cocci	Staphylococci
VQ252	Vieques	Apex	Irregular	Cream	+	Rods	Streptobacilli

Continuation table

VQ253	Vieques	Apex	Irregular	Cream	+	Rods	Single bacilli
VQ254	Vieques	Apex	Circular	White	+	Rods	Single bacilli
VQ256	Vieques	Apex	Circular	Orange	+	Cocci	Staphylococci
VQ257	Vieques	Base	Circular	Light pink	+	Rods	Single bacilli
VQS 1	Vieques	Center	Irregular	White	+	Rods	Single bacilli
VQS 2	Vieques	Apex	Circular	Light orange	+	Rods	Single bacilli
VQS 3	Vieques	Apex	Circular	Light brown	-	Rods	Single bacilli
VQS 4	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli

| VQS 5 | Vieques | Base | Irregular | Transparent cream | + | Rods | Single bacilli |

Continuation Table

VQS 6	Vieques	Base	Rhizoid	White	+	Rods	Single bacilli
VQS 7	Vieques	Apex	Rhizoid	Transparent	+	Rods	Single bacilli
VQS 8	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQS 9	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQS 10	Vieques	Apex	Rhizoid	Transparent	+	Rods	Single bacilli
VQS 11	Vieques	Apex	Irregular	White	+	Rods	Single bacilli
VQS 12	Vieques	Apex	Rhizoid	White	-	Rods	Single bacilli
VQS 13	Vieques	Apex	Rhizoid	White	+	Rods	Single bacilli
VQS 14	Vieques	Apex	Irregular	Cream	-	Rods	Streptobacilli
VQS 15	Vieques	Apex	Rhizoid	White	+	Rods	Streptobacilli
VQS 16	Vieques	Apex	Circular	White	+	Rods	Single bacilli
VQS 17	Vieques	Center	Rhizoid	White	+	Rods	Single bacilli

Continuation Table

S1	Solar Salterns	Apex	Circular	Red	-	Rods	Single bacilli
S 3	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 4	Solar Salterns	Apex	Rhizoid	White	+	Long filamentous rods	Streptobacilli
S 5	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 6	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S7	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Diplobacilli
S 8	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 9	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 10	Solar Salterns	Apex	Irregular	White	+	Rods	Single bacilli
S 11	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S12	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 13	Solar Salterns	Center	Rhizoid	Transparent white	-	Rods	Single bacilli

Continuation Table

S 14	Solar Salterns	Center	Circular	Cream	+	Rods	Single bacilli
S 15	Solar Salterns	Center	Rhizoid	Transparent cream	+	Rods	Single bacilli
S 16	Solar Salterns	Apex	Rhizoid	Transparent cream	-	Rods	Single bacilli
S 17	Solar Salterns	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
S 18	Solar Salterns	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
S19	Solar Salterns	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
S 20	Solar Salterns	Apex	Rhizoid	Transparent cream	-	Rods	Single bacilli
S 21	Solar Salterns	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
S 22	Solar Salterns	Apex	Rhizoid	Transparent cream	-	Short rods	Single bacilli
S 23	Solar Salterns	Base	Circular	White	+	Rods	Single bacilli
S 24	Solar Salterns	Base	Rhizoid	Transparent cream	-	Rods	Single bacilli
S 25	Solar Salterns	Base	Circular	Light transparent orange	-	Short rods	Single bacilli

Continuation Table

S 26	Solar Salterns	Base	Irregular	White	+	Rods	Streptobacilli
S 27	Solar Salterns	Base	Rhizoid	Transparent white	-	Rods	Streptobacilli
S 29	Solar Salterns	Base	Rhizoid	Transparent white	-	Rods	Streptobacilli
S 31	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 32	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 33	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 34	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 35	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 36	Solar Salterns	Center	Irregular	White	-	Rods	Streptobacilli
S 37	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 38	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 39	Solar Salterns	Base	Circular	Transparent cream	+	Rods	Streptobacilli

Continuation Table

S 40	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 41	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 42	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Streptobacilli
S 43	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 44	Solar Salterns	Base	Rhizoid	Transparent white	-	Rods	Single bacilli
S 45	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S46	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 47	Solar Salterns	Apex	Circular	Transparent cream	-	Rods	Single bacilli
S 48	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 49	Solar Salterns	Base	Rhizoid	Transparent white	-	Rods	Single bacilli
S51	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 53	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

				Transparent			
S 54	Solar Salterns	Apex	Rhizoid	white	+	Rods	Single bacilli
S 55	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 56	Solar Salterns	Center	Circular	Transparent cream	+	Rods	Single bacilli
S 57	Solar Salterns	Center	Irregular	Transparent white	+	Rods	Single bacilli
S 58	Solar Salterns	Center	Irregular	Transparent white	+	Rods	Single bacilli
S 59	Solar Salterns	Base	Irregular	Transparent white	+	Rods	Single bacilli
S 60	Solar Salterns	Base	Circular	Transparent cream	+	Rods	Single bacilli
S 62	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 63	Solar Salterns	Base	Irregular	Transparent white	+	Rods	Single bacilli
S 64	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 65	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 66	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

				Transparent			
S 68	Solar Salterns	Base	Rhizoid	white	+	Rods	Single bacilli
S 69	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 70	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S71	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 72	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 73	Solar Salterns	Center	Irregular	Cream	+	Rods	Single bacilli
S 74	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S75	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 76	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 77	Solar Salterns	Apex	Circular	White	+	Rods	Single bacilli
S 78	Solar Salterns	Center	Irregular	Transparent	-	Rods	Single bacilli
S 79	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

S 80	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 81	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 82	Solar Salterns	Center	Irregular	Cream	+	Rods	Single bacilli
S83	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 84	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 85	Solar Salterns	Center	Circular	White	+	Rods	Diplobacilli
S 86	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 87	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 88	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 89	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 90	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 91	Solar Salterns	Base	Irregular	White	+	Rods	Single bacilli

Continuation Table

S 92	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S93	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 94	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 95	Solar Salterns	Center	Rhizoid	Transparent white	-	Rods	Single bacilli
S 96	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 97	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 98	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli
S 99	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 100	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 101	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 102	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 103	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

S 104	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 105	Solar Salterns	Center	Rhizoid	Transparent white	-	Rods	Single bacilli
S 106	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 107	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 108	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 109	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 110	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 111	Solar Salterns	Center	Irregular	Cream	+	Cocci	Staphylococci
S 112	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 113	Solar Salterns	Apex	Irregular	Cream	+	Rods	Single bacilli
S 114	Solar Salterns	Base	Irregular	Cream	+	Rods	Single bacilli
S 115	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

S 116	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 117	Solar Salterns	Base	Rhizoid	Cream	+	Rods	Single bacilli
S 118	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 119	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 120	Solar Salterns	Base	Rhizoid	White	+	Short rods	Single bacilli
S 121	Solar Salterns	Center	Circular	Cream	+	Short rods	Single bacilli
S 122	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 123	Solar Salterns	Base	Rhizoid	Cream	+	Rods	Single bacilli
S 124	Solar Salterns	Base	Rhizoid	Cream	+	Rods	Single bacilli
S 125	Solar Salterns	Apex	Rhizoid	White	+	Cocci	Streptococci
S 126	Solar Salterns	Center	Irregular	White	+	Rods	Single bacilli
S 127	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli

Continuation Table

S 128	Solar Salterns	Base	Rhizoid	White	+	Long rods	Single bacilli
S 129	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 130	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 131	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 132	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 133	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 134	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S 135	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 137	Solar Salterns	Base	Irregular	Transparent	+	Rods	Single bacilli
S 138	Solar Salterns	Base	Irregular	White	+	Rods	Single bacilli
S 139	Solar Salterns	Center	Rhizoid	White	-	Rods	Single bacilli
S 140	Solar Salterns	Center	Irregular	Cream	-	Rods	Single bacilli

Continuation Table

S 141	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli
S 142	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 143	Solar Salterns	Center	Circular	Cream	+	Rods	Single bacilli
S 144	Solar Salterns	Base	Rhizoid	Transparent	+	Rods	Single bacilli
S 145	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 146	Solar Salterns	Apex	Circular	White	+	Rods	Single bacilli
S 147	Solar Salterns	Center	Irregular	Cream	+	Rods	Single bacilli
S 148	Solar Salterns	Base	Irregular	Cream	+	Rods	Diplobacilli
S 149	Solar Salterns	Base	Rhizoid	Transparent	+	Rods	Single bacilli
S 150	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli
S 151	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli
S 152	Solar Salterns	Center	Irregular	White	-	Rods	Single bacilli

Continuation Table

S 153	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 154	Solar Salterns	Apex	Circular	Creamy white	+	Rods	Single bacilli
S 155	Solar Salterns	Center	Irregular	Transparent white	+	Rods	Single bacilli
S 156	Solar Salterns	Center	Circular	White	+	Rods	Single bacilli
S 157	Solar Salterns	Center	Rhizoid	White	-	Rods	Single bacilli
S 158	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S 159	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli
S 160	Solar Salterns	Apex	Circular	Cream	-	Rods	Single bacilli
S 161	Solar Salterns	Center	Rhizoid	Cream	+	Rods	Single bacilli
S 162	Solar Salterns	Center	Circular	White	+	Rods	Single bacilli
S 163	Solar Salterns	Apex	Rhizoid	Creamy white	+	Rods	Single bacilli
S 164	Solar Salterns	Apex	Rhizoid	Transparent white	+	Rods	Single bacilli

Continuation Table

S 165	Solar Salterns	Apex	Rhizoid	Creamy white	+	Rods	Single bacilli
S 166	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 167	Solar Salterns	Base	Rhizoid	White	+	Rods	Single bacilli
S 168	Solar Salterns	Center	Circular	Transparent yellow	+	Rods	Single bacilli
S 169	Solar Salterns	Center	Circular	Creamy white	+	Rods	Single bacilli
S 170	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli
S 171	Solar Salterns	Base	Rhizoid	Transparent white	+	Long rods	Single bacilli
S 172	Solar Salterns	Center	Rhizoid	Creamy white	+	Rods	Single bacilli
S 173	Solar Salterns	Center	Irregular	Creamy white	+	Rods	Single bacilli
S 174	Solar Salterns	Apex	Rhizoid	Creamy white	+	Rods	Single bacilli
S 175	Solar Salterns	Center	Rhizoid	Cream	+	Rods	Single bacilli
S 176	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli

Continuation Table

S 177	Solar Salterns	Center	Circular	Creamy white	+	Rods	Single bacilli
S 178	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S 179	Solar Salterns	Center	Rhizoid	Transparent cream	+	Rods	Single bacilli
S 180	Solar Salterns	Center	Circular	Transparent	+	Rods	Diplobacilli
S181	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S182	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S183	Solar Salterns	Base	Circular	Transparent cream	+	Rods	Single bacilli
S184	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S185	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S186	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli
S187	Solar Salterns	Base	Rhizoid	Creamy white	+	Rods	Single bacilli
S188	Solar Salterns	Apex	Irregular	Creamy white	+	Rods	Single bacilli

Continuation Table

S189	Solar Salterns	Apex	Rhizoid	Cream	+	Rods	Single bacilli
S190	Solar Salterns	Apex	Rhizoid	White	+	Rods	Single bacilli
S191	Solar Salterns	Center	Irregular	Cream	+	Cocci	Single cocci
S192	Solar Salterns	Center	Rhizoid	Transparent	+	Rods	Single bacilli
S193	Solar Salterns	Center	Circular	Yellow	+	Cocci	Staphylococci
S194	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S195	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S196	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S197	Solar Salterns	Base	Rhizoid	Transparent cream	+	Rods	Single bacilli
S198	Solar Salterns	Apex	Rhizoid	Transparent cream	+	Rods	Single bacilli
S199	Solar Salterns	Center	Rhizoid	White	+	Rods	Single bacilli

Continuation Table

S201	Solar Salterns	Apex	Circular	Yellow	+	Cocci	Single cocci
S 202	Solar Salterns	Apex	Irregular	Cream	+	Rods	Diplobacilli
S203	Solar Salterns	Center	Rhizoid	Transparent white	+	Rods	Single bacilli
S207	Solar Salterns	Center	Irregular	White	+	Rods	Streptobacilli
S208	Solar Salterns	Base	Irregular	White	+	Rods	Streptobacilli
S209	Solar Salterns	Base	Rhizoid	Transparent white	+	Rods	Single bacilli