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Molecular methods for species –specific identification of Bacillus species

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Molecular methods for species –specific identification of *Bacillus* species

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Abstract

Bacillus. oleronius is a non-motile Gram-negative endospore forming bacterium, isolated for the first time in 1995 from the hindgut of termites. This bacterium has been reported to be the likely cause of demodecosis in humans and therefore it is considered to be a pathogen. The aim of this study was to design novel species-specific primers for *B. oleronius* and to prove that the primers designed by Szkaradkiewicz et al., 2012 are not specific to *B. oleronius* as claimed by the authors.

In order to design-species specific primers, the first objective was to obtain sequence information. Because the bacterium could be cultures easily, the hope was obtain sufficient genomic DNA for single molecule real time sequencing. Extraction the DNA of *B. oleronius* was challenging. Two extraction kits and three protocols were used to detect which would be the best purification methods for obtaining large amounts of high-quality genomic DNA. The DNA extracts obtained with three different DNA extraction protocols (Gentra Puregene Yeast/Bact kit Gram-positive protocol; Gentra Puregene Yeast/Bact kit Gram-negative protocol; DNeasy ® Blood & Tissue) were measured by spectrophotometry. DNA extract quantity and purity was varied between the different extraction kits and A260/A280 and A260/A230 ratios that used to assess the purity of DNA and nucleic acid respectively indicated presence of contamination during the extraction procedure. Based on the obtained results, all the purification methods used in this study failed to produce pure and high-quality genomic DNA for sequencing. The next objective was to design universal primers for four vary fast evolving genes, single copy genes (*rpoB, recA, gyrB* and *ytcP*) and the ITS region of *Bacillus* species for Sanger sequencing and to design species-specific 16S RNA primers. To do this, the genetically closest Bacillus species to B. oleronius were identified first through a phylogenetic analysis.

Through this study, it was possible to design two species-specific primer sets for *B. oleronius* and prove that the primers published by Szkaradkiewicz et al., 2012 are not specific. The new designed primer sets with the publisher's primers were tested using PCR for *B. oleronius* as positive control and other bacterial species *B. amyloliquefaciens*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, *B. sporothermodurans*, *B. aquimaris*, *B. carboniphalius* and *Amphibacillus. tropicus* as negative controls. The results showed that the new primer sets bound specifically to *B. oleronius* while the publisher's primers amplified non-specifically fragments of varying sizes of all *Bacillus* species. Based on the results, there is no evidence that *B. oleronius* was positively identified as being associated with demodecosis as claimed by Szkaradkiewicz et al., 2012.

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Abbreviations

16rRNA	16S ribosomal RNA
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
AFLP	Amplified Fragment Length Polymorphism
RFLP	Restriction Fragment Length Polymorphism
FAME	Fatty Acid Methyl Esters
TSA	Trypticase Soy Agar
Blast	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
recA	DNA recombination/repair protein
rpoB	RNA polymerase β subunit
gyrB	Grease B subunit
ITS	Intergenic transcribed spacer
pyrE	Orotate phosphoribosyltransferase
ytcP	Putative ABC transporter permease protein
SMRT	Single Molecule Real Time Sequencing
Tm	Melting temperature
Ta	Annealing temperature

Keywords: *Bacillus* species, 16S rRNA, *B. oleronius,* species-specific primers, PCR

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CHAPTER ONE

1. Introduction

1.1. The Genus *Bacillus*

When the *Bacillus* genus was described for the first time in 1872 by Ferdinand Cohn for bacteria associated with plants, no one expected that this genus would become a source of attention for scientific researchers and taxonomists far beyond plant biology (Cohn, 1872). The genus *Bacillus* is large, capable of growing in the presence of oxygen and is ubiquitous, being found in environments such as soil, water, and in some laboratories. It is characterized by rod shaped bacteria being part of the phylum of the Firmicutes, most of which belong to the low G+C Gram positive group (Økstad and Kolstø, 2011). The genus *Bacillus* includes large numbers of heterogeneous species and there could be more than 60 genetically diverse species (Ash et al., 1993). These species display phenotypic variations and are characterized by complex nutritional requirements, physiological and metabolic diversity and variation of the G+C content of their DNA (Xu and Cote, 2003).

Bacillus species are Gram positive bacteria producing single endospores and having the ability to grow under aerobic conditions. The spores are cylindrical, oval or rounded in shape and are resistant to various adverse conditions. *B. subtilis* is considered to be the type species of the *Bacillus* genus (Økstad and Kolstø, 2011). *Bacillus* species exhibit a wide range of phenotypes such as psychrophiles, thermophiles, acidophiles, halophiles, chemolithotrophs, alcalophiles and facultative anaerobes. The diversity of the ecological niches for *Bacillus* species is an indicator of the heterogeneity of the genus (Dropniewski, 1993).

Members of the *Bacillus* species play an important role in human life for several reasons, including the following: (1) Endospores produced by *Bacillus* species are resistant to adverse treatments, such as detergents (Økstad and Kolstø, 2011); (2) Some species of bacteria can contribute to the industrial sector through their use in the manufacture of products such as pesticides, enzymes, antibiotics and some detergents; (3) Some *Bacillus* species belong to the aetiology of various diseases in humans. These include *B. anthracis*, which is responsible for causing anthrax, and *B. cereus*, which is known to cause foodborne diseases (Priest, 1993).

1.2. Taxonomy of Bacillus species

1.2.1. Traditional Taxonomy

In 1973, *Bacillus* species were divided into three large groups based on the morphology of spores and sporangia. Group 1 are Gram positive rods that contain spores of different shapes, for example, central, terminal or cylindrical. This group includes medically significant species, such as *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. megaterium*. Group 2 are Gram variable and include *B. pumilus*, *B. subtilis*, *B. circulans*, *B. coagulans*, *B. licheniformis*, *B. alvei*, *B. brevis* and *B. macerans*. They produce central and terminal spores. The noticeable feature of group 3 is the presence of heterogeneous species of Gram-variable bacilli such as *B. sphaericus* (Dropniewski, 1993). Although the foregoing division is traditional, these classifications have proved to be important in reducing species to more manageable groups. However, this division may lead to incorrect classification of some species because of the difficulty of identification (Priest, 2011).

1.2.2 Modern Taxonomy

Scientific progress in taxonomy through the discovery of modern molecular and biochemical techniques has contributed to determining a new classification for *Bacillus* species. Modern techniques for determining of the C+G content of DNA have provided evidence that *Bacillus* species have greater genetic diversity than was previously realised (Priest, 2011). Determination of the components of the DNA is considered the ideal way to indicate the genetic diversity among the species in a genus. It is known that the variation between species is limited to between 10-12 mol% G+C. *Bacillus* species are an exception, however, the range of some *Bacillus* species being about 33 % (*B. anthracis*) or 65% (*B. schlegelii*) and that demonstrate that the diversity of *Bacillus* is controversial for a single genus (Ash et al., 1993). The genetic diversity provides one justification for dividing the genus into several taxa. In accordance with numerical classification, which depends on the phenotypic features, Priest et al. in 1988 divided *Bacillus* species into five or six large groups or clusters (Xu and Cote, 2003).

Group 1 encompasses *B. polymyxa*, which has been adopted as a reference organism and includes a number of species, such as *B. alvei*, *B. circulans* and *B. macerans*. The spores produced by these species are oval and swollen. These bacteria have a capacity for the fermentation of sugars, the utilization of amino acids and vitamins as a source of growth and the secretion of extracellular carbohydrases, such as amylases and B-glucanases, including cellulases, pectinases and pullulanases (Priest, 2011).

Group 2 encompasses *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus*, which produce oval spores that are not distended. Members of this group of bacteria are

strictly aerobic, except *B. subtilis*, which works on fermentation of sugars and grows easily in the presence of air and glucose. There are some species that depend on air to grow, such as *B. anthracis*, *B. cereus*, *B. licheniformis and B. thuringiensis.* In addition, these species secrete enzymes, such as amylases, B-glucanases and proteases that play a vital role in industry and commerce. A prominent species of group 3 is *B. brevis*, which is strictly aerobic and characterized by an inability to produce acids from sugars. Its spores are oval-shaped and swollen. This group includes species such as *B. badius* and *B. freudenreichii*, which are characterized by physiological heterogeneity (Priest, 2011).

The characteristic of homogeneity applies to group IV, which includes *B. sphaericus,* the psychrophiles *B. insolitus* and *B. psychrophilus*. They produce spherical endospores. The ability of these bacteria to change meso-diaminopimelic acid found in the cell wells to lysine or ornithine distinguishes them from other bacilli. All these bacteria are classified as strict aerobes, except for *B. sphaericus*, and they do not need sugars as a growth source replacement. The thermophilic bacilli have come to form a separate group (group V) according to the numerical classification system. Physiological and morphological heterogeneity of the species is the most prominent characteristic of this group, as well as different forms and patterns of metabolism, including obligate aerobics and microaerophilic types. (Ash et al., 1991). This group includes species such as *B. schlegelii*, *B. thermooleovorans* and *B. pallidus* (Priest, 2011).

The thermophilic bacilli are characterized by phylogenetic diversity, while acidophilic thermophiles have been assigned to the genus *Alicyclobacillus* (group VI) (Wisotzkey et al., 1992). In view of this finding, it is highly probable that Numerical classification has paved the way to showing that there is a relationship which combines bacilli and other groups.

1.3. Bacillus oleronius

As was mentioned earlier, some species of the genus *Bacillus* are causative agents for human diseases, such as *B. anthracis* which causes anthrax and *B. cereus* which causes foodborne illness. Several studies have demonstrated that one bacterium of the genus *Bacillus, B. oleronius*, plays a role in causing blepharitis which is an inflammation that infects the eyelid margins, causing severe itching, lack of vision and redness of the skin. *B. oleronius* is a non-motile Gram-negative endospore forming bacterium, isolated for the first time in 1995 from the hindgut of termites. Hitherto, this bacterium has been cultured from patients with blepharitis and from a parasite called the *Demodex* mite, found in the skin of patients with demodicosis. Pathological role for *B. oleronius* is still undetermined so far, however, recent studies have been indicated that *B*. *oleronius* is an opportunistic bacterium and plays a potential role in the

pathogenesis of some skin diseases such as acne, rosacea in humans and demodecosis (Szkaradkiewicz et al., 2011).

Demodicosis is a rare cutaneous disease that afflicts humans and animals and is caused by the *Demodex* mite, which belongs to the class Arachnida and the subclass Acarina (Jarmuda et al., 2012). There are about 100 species of *Demodex* mites, most of which are primarily found in mammals such as cattle, bats, pigs, sheep, cats, mice, and dogs. *Demodex canis* affects dogs and research into this species is on-going. The species that infect human beings of both sexes are *D. folliculorum* and *D. brevis,* which reside in particular parts of the body, especially the scalp, upper chest and face (Kojima et al., 2011). *D. folliculorum* is large and long approximately 0.3-0.4 mm and resides mainly in the hair follicles of adults in the form of cluster aggregates with a density of more than 5 mites/cm², while *D. brevis* is characterized by its small size, about 0.2-0.3mm, and slender legs, and is found in the sebaceous gland (Figure 1)(Jarmuda et al., 2012).

Recent studies have proved that the presence of *Demodex* mites out a high density in the dermis makes the subject a prime candidate for many skin diseases, such as pityriasis folliculorum, demodicosis gravis, papulopustular rosacea, rosacea-like demodicosis and blepharitis (Hsu et al., 2008). A study conducted by Jackson (2008) indicated that there is a correlation between chronic blepharitis, (the symptoms of which are described above), and *Demodex* mites, particularly *D. folliculorum* (Jackson, 2008). It is noteworthy that in healthy people *Demodex* does not cause any symptoms or signs which would enable the occurrence of the disease to be predicted, although this is not the case with people suffering from immune deficiency (Szkaradkiewicz et al., 2011).

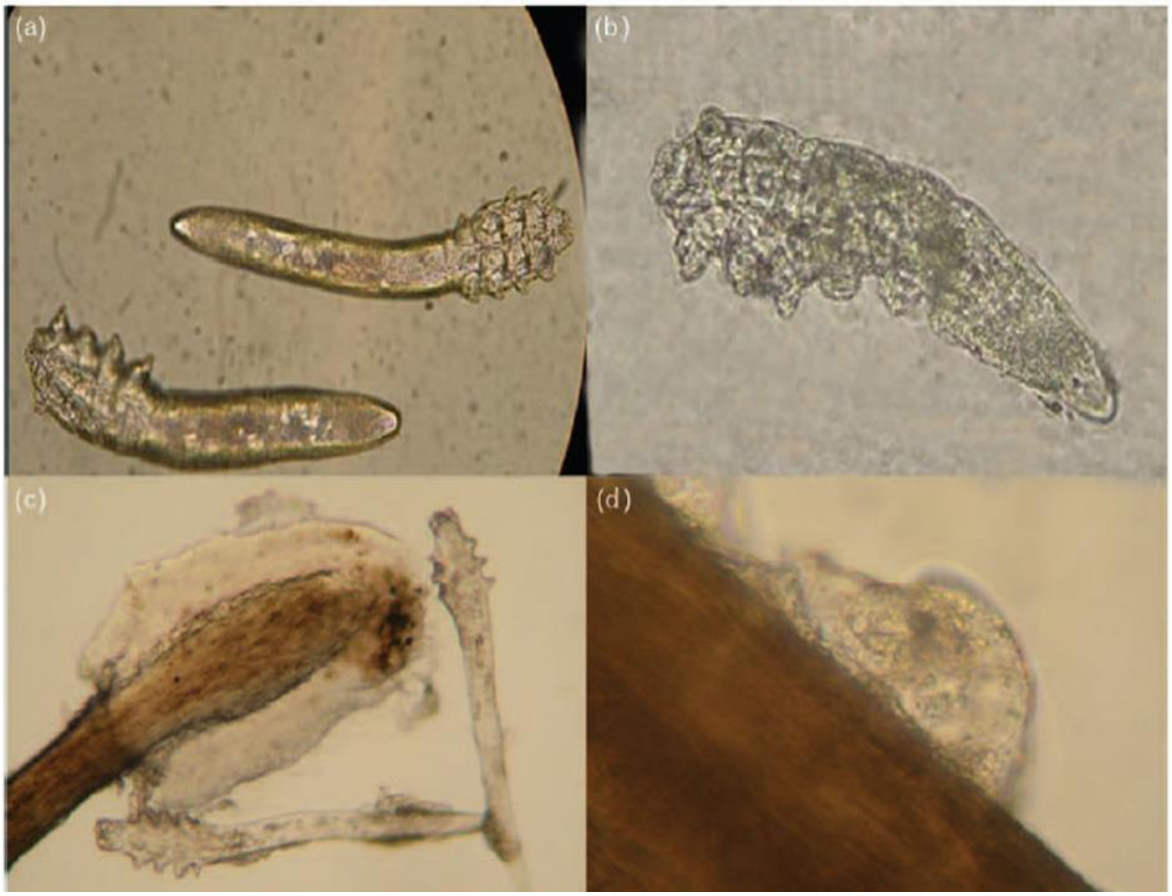


Figure 1: Photograph of *Demodex* mite under microscope represents (a) *D*. *folliculorum*, (b) *D*. *brevis*, (c) the larva of *Demodex* and (d) egg of *Demodex* (Liu et al., 2010).

A study conducted by Phillips (2012) in La Sal del Rey Lake, in South Texas (USA), which is characterized by severe salinity demonstrated that one of the most micro-organisms that had been detected there was belongs to the genus *Bacillus*. Five isolates (A2D, A3, A9, A12, and A16) indicated a great similarity 95.8% between these isolates and *B. oleronius* (Table 1) (Phillips et al., 2012).

Table 1: Identification of isolated organisms from La Sal de Rey Lake (Phillips et al., 2012).

Isolate No.	Closest Species	Identity (%)	Confidence Level
A1	*Bacillus fastidiosis*	95.3	Genus
A2Y	*Bacillus flexus*	87.4	No match
A2D	*Bacillus oleronius*	96.1	Genus
A3	*Bacillus oleronius*	95.8	Genus
A4	*Pseudomonas fulva*	98	Genus
A7	*Bacillus megaterium*	99.9	Species
A8	*Exiguobacterium acetylicum*	92.1	No match
A9	*Bacillus oleronius*	95.3	Genus
A10	*Pseudomonas fulva*	98	Genus
A11R	*Halomonas aquamarina*	99.2	Species
A12	*Bacillus oleronius*	95.3	Genus
A13W	*Bacillus thuringiensis*	99.9	Species
A13Y	*Bacillus horikoshii*	99.1	Species
A14	*Exiguobacterium acetylicum*	92.1	No match
A15	*Bacillus firmus*	97.8	Genus
A16	*Bacillus oleronius*	95.3	Genus
A17	*Exiguobacterium*	92.1	No match

Another study indicated the presence of *B. oleronius* in raw milk and feed concentrate (which is a large repository for micro-organisms) (Heyndrickx et al., 2012). Vaerewijck et al., (2001) reported that the contamination of animal feed with micro-organisms can be due to either contamination the soil leading, to contamination of crops used as food for animals, or the feed may shelter microorganisms and bacteria originating from agricultural crops, such as citrus and coconut, and the bacteria may end up in raw milk through what is fed to the animals, or through contamination of the udder by faeces containing the bacterium (Vaerewijck et al., 2001). Another suggestion is that there may be contamination of feed during processing or storage, especially when appropriate environmental conditions exist for the dispersal of spores; or alternatively there may be contamination of equipment and devices used in factories and thus the transmission of bacteria to animal feed.

Another theory is that this bacterium comes from contaminated bedding materials, such as straw or wood shavings, used in animal living quarters, leading to the adhesion of the bacterium to the teat and its consequent transmission into the milk ducts, causing mastitis and thus contamination of raw milk. It is worth mentioning that used bedding has a greater proportion of *B. oleronius* than unused bedding (Vissers and Driehuis, 2008).

There is to date no scientific evidence indicating the presence of *B. oleronius* naturally in the udders of healthy cows, but it is possible that this bacterium is transmitted to raw milk through the dust of straw and feedstuff, or through contamination of the teat by faeces due to the spores spreading through the air and onto the udders of cattle, leading to the contamination of raw milk. On this basis it is possible to assume that *B. oleronius* may be present naturally in the gut of healthy cows, or this bacterium may exist in the gut of some vectors such as insects or flies and therefore, these flies are transmitted through the air, leading to contamination of raw milk.

It is possible to describe the process of the contamination of raw milk with bacteria as follows:

Soil or water → Feed → Gastrointestinal duct → Dung ↺

Udder → Milk devices → Raw Milk

A study recently conducted in the Cochin estuary in India aimed to isolate and study microorganisms in this region, which has been contaminated by heavy metals. One of these organisms that were detected was *B. oleronius* particularly in Vypin and Munambam of the estuary (Table 2) (Jose et al., 2011).

Table 2: Variety of bacteria of water and residue in the Cochin estuary (Jose et al., 2011).

Bacterial species	Water			Sediment		
	Vypin	Munambam	Eloor	Vypin	Munambam	Eloor
Proteobacteria						
Acinetobacter baumannii	–	+	–	+	+	–
Chromobacterium violaceum	+	–	+	–	–	–
Citrobacter koseri	–	–	–	+	–	–
Comamonas acidovorans	–	–	+	+	–	+
Comamonas testosteroni	–	+	+	+	–	+
Kluyvera ascorbata	+	–	–	+	+	–
Listonella anguillarum	+	–	+	+	+	+
Proteus mirabilis	–	–	–	–	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Pseudomonas huttiensis	–	+	+	+	+	+
Serratia marcescens	–	–	+	+	–	+
Shewanella putefaciens	–	–	+	+	–	+
Vibrio cholerae	–	–	+	–	+	+
Vibrio furnissii	+	–	+	–	+	+
Xanthomonas arboricola-pruni	–	–	+	+	–	+
Yersinia pseudotuberculosis	–	–	+	+	–	+
Firmicutes						
Bacillus cereus	+	–	–	+	–	–
Bacillus filicolonicus	+	+	+	+	–	–
Bacillus marinus	+	+	–	–	+	–
Bacillus mycoides	+	+	–	+	+	–
Bacillus oleronius	+	+	–	–	+	–
Bacillus pumilus	+	+	+	+	+	+
Bacillus sphaericus	+	+	–	+	–	–
Bacillus sp	+	–	–	–	+	–
Exiguobacterium acetylicum	–	–	–	+	–	–
Paenibacillus larvae pulvifaciens	+	–	–	+	–	–
Staphylococcus gallinarum	+	–	+	–	–	+
Actinobacteria						
Cellulomonas turbata	+	–	+	+	+	–
Bacteriodetes						
Myroides odoratus	–	+	–	+	+	–
Shannon H'	2.788	2.458	2.774	3.087	2.692	2.66

The latest study, conducted by Edward in 2012, demonstrated that *B. oleronius* can be isolated in petroleum contaminated soil, which is viewed as one of the most appropriate environments for its detection, due to the capacity of this bacterium to resist organic solvents and heavy metals. In the same study, this bacterium displayed effective resistance to several heavy metals, such as copper chloride, lead acetate, potassium dichromate, nickel chloride, cobaltous chloride, while the least resistance was detected in cadmium chloride and silver nitrate. According to the results above, *B. oleronius* has showed variation in its resistance to different types of heavy metals (Edward et al., 2012).

The results of this study demonstrated biochemical characteristics of the strains of *B. oleronius*. It has been found that this bacterium Gram negative, milky or creamy colour on the agar plate and it is negative for both methyl red and Voges Proskauer test in addition to, the difficulty of converting glucose to ethanol or butanediol and glucose to acetoin. It has been noticed that *B. oleronius* is positive for production catalase enzyme while it is negative and unable for growth in MacConkey agar (Table 3) (Edward et al., 2012).

Table 3: Morphological and Biological features of *B. oleronius* strains (Edward et al., 2012).

Biochemical test	Observation
Grams staining	Gram negative
Methyl Red	Negative
Voges proskauer	Negative
Citrate utilization	Positive
Catalase	Positive
MacConkey agar	Negative
Pigmentation	Negative
Shape	Rods

The origin of the name *B. oleronius* can be traced to the island of L'ile d'Oleron in France where the bacterium was first found in the hindgut of a termite. The most diagnostic feature of *B. oleronius* is the average size of the rods that are found in the form either of pairs or singles. The spores are ellipsoidal and located in the centre or near the end of the swollen sporangia. The endospores are characterized as having a weak resistance to high temperatures (Heyndrickx et al., 2012). The relationship between *B. oleronius* and the *Demodex* mite is symbiotic. The bacterium lives inside the parasite and because of the absence of an anus in *Demodex*, *B. oleronius* plays a key role in facilitating its process of digestion (Lacey, 2007).

1.3.1. The role of *B. oleronius* in rosacea

Rosacea is a complex and chronic skin disease that infects humans, especially in the region of the face and eyes (Figure 2). The aetiology of rosacea is currently unknown, but several studies in the past 10 years have shown that this disease has a strong correlation with *B. oleronius*. It has been noted that the antibiotics used for the treatment or control of rosacea can increase the chances of killing *B. oleronius*. It is worth mentioning that the antigens released by *B. oleronius* have a potential role in the pathogenesis of rosacea. Large numbers of serological studies have been carried out in order to discover whether there is a correlation between this bacterium and rosacea. A study conducted by Lacey showed that the bacteria produces two types of stimulatory antigens (62 and 83 kDa) responsible for a stimulating skin infections and the induction of rosacea. Serum samples were taken from patients with rosacea and controls and the results indicated that the presence of *B. oleronius* in the sera of patients with rosacea was significantly greater, (around 72%), compared to the controls (29%) (Lacey et al., 2007). Bacterial antigens play an important role and are effective in stimulating neutrophils and also in stimulating the inflammation, especially in the pilosebaceous units (O'Reilly et al., 2012).

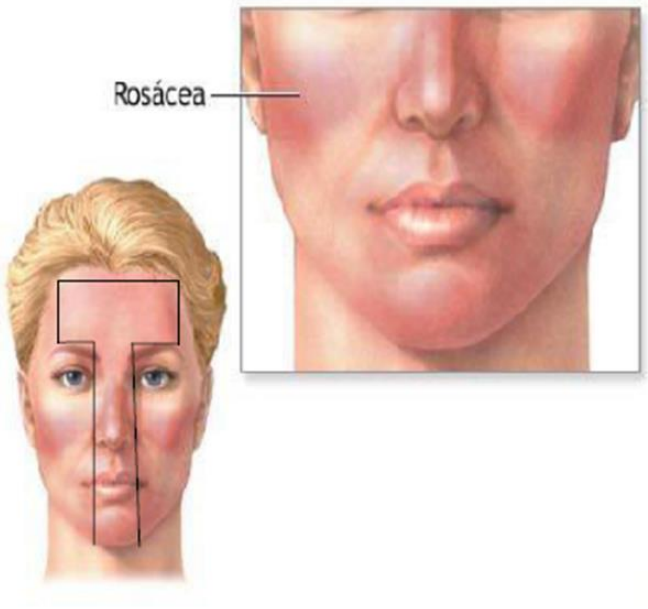


Figure 2: Diagram showing the most commonly regions in the face where rosacea symptoms appear.

[Taken from the website: www.getacnehomeremedies.info/search/overview-on-rosacea-acne-treatment-causes-and-prevention]

O'Reilly and colleagues (2012) proved that antibiotics such as metronidazole, tetracyclines and erythromycin, used to control rosacea, kill the bacteria inside the *Demodex* mite and thus the bacterium is unable to produce antigens, leading to a clearance of the symptoms. After killing the bacterium, the metabolism of the mite will be adversely affected, since the bacterium lives inside the parasite. But when the use of the antibiotics is halted, the parasite is able to return again, thus increasing the number of bacteria and the incidence of infection (O'Reilly et al., 2012). In the same study it has been provided that *B. oleronius* very sensitive to some antibiotics such as tetracycline, doxycycline and minocycline.

Two genes of *B. oleronius* have so far been sequenced: the 16S rRNA gene and the glutamate-5-semialdehyde dehydrogenase-like gene (NCBI). As mentioned later, the 16S rRNA approach fails in the identification of particular species belonging to related taxa, especially when there is a similarity between these species (Fox et al., 1992), whereas the second gene has not been tested so far. *B. oleronius* has a close relationship with two neighbouring species, *B. sporothermodurans* and *B. licheniformis* genotypically more than phenotypically (Figure 3).

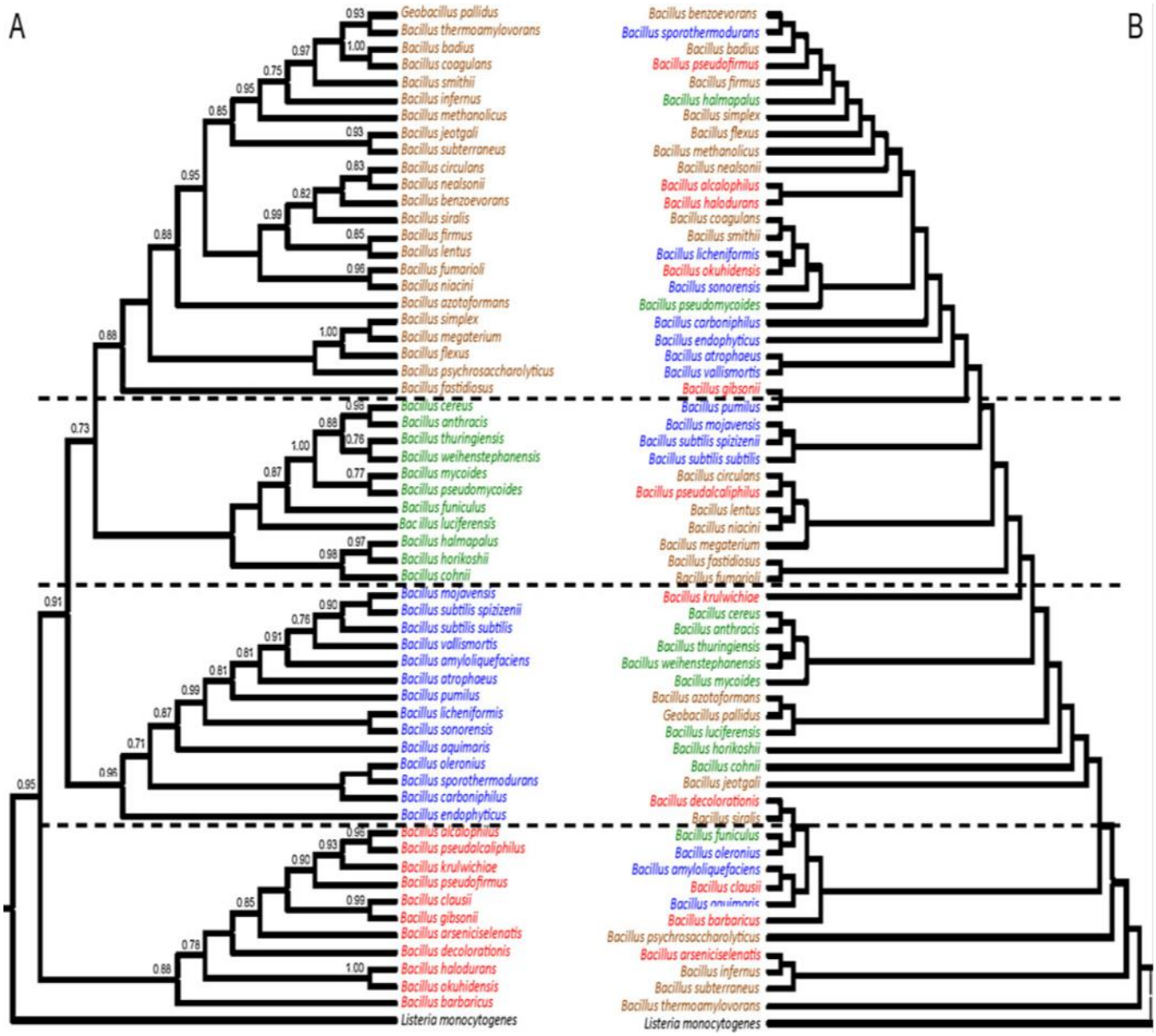


Figure 3. Phylogenetic relationships of 59 *Bacillus* species based on the 16S rRNA sequence data. *B. oleronius* is boxed in blue. *B. oleronius* has a close relationship with *B. licheniformis*. (A) Represents the analysis of the location of 16S rDNA; (B) represent rearranging the branches in A in order to reduce the number of evolutionary changes in 11 phenotypes related to: minimum and maximum growth temperature, growth in the presence of air, the ability to produce acids from glucose, arabinose and mannitol, the presence of flagella, the shape of spores and the swell of sporangium (Maughan and Auwera, 2011).

1.4. Molecular methods for the identification of *Bacillus* species

Molecular biology techniques have contributed to the progress of a large number of DNA markers capable of determining and recognizing genetic polymorphism. PCR (Polymerase chain reaction) is an important invention that has been widely used in molecular biological laboratories because of its proven ability to amplify large numbers of DNA in a short period of time, and also because it is easy to use and gives accurate results. All these features have popularized the PCR technique. Several molecular techniques have been developed in order to find a fast, accurate and cheap method for the identification of various species and strains of bacteria such as those of the *Bacillus* genus, one of these being the RAPD technique.

1.4.1. Randomly Amplified Polymorphic DNA (RAPD) technique

In the latter part of the twentieth century, Randomly Amplified Polymorphic DNA, known as the RAPD technique, has emerged as a technique for the identification and development of DNA markers associated with PCR. The RAPD technique requires the presence of a short, single primer without the need for a prior background of DNA sequence. The technique has several advantages for researchers, including its superior ability to acquire a significant number of genetic markers requiring the presence of small amounts of DNA (around 10 ng per reaction), without the need to provide the required conditions in the molecular characterization, such as DNA probes, cloning and DNA sequencing (Bardakci, 2001). RAPD technique can be used in genetic diversity studies using a random primer for several species of bacteria, particularly the genus *Bacillus*, which includes heterogeneous and diverse species (Kumar and Gurusubramanian, 2001).

The study of Stephan (1996), which included the identification of 25 strains of *B. cereus* using the RAPD technique, showed the efficiency and ability of this technique in the classification of *B. cereus* strains. Another study using the RAPD method was conducted on *B. sphaericus*, a virulent pathogen, to determine the genetic relationships that combine 31 pathogenic and 14 non-pathogenic strains of *B. sphaericus*. The results of the RAPD technique demonstrated that the *B. sphaericus* species is heterogeneous, and the technique was successful in separating the pathogenic strains from others (Woodburn et al., 1995).

The RAPD method became widely employed after it was shown that it could be used in several applications, such as polymorphism, population and evolutionary genetics. Despite the applications mentioned above, however, the technique is not without its drawbacks, for example, the dominance of RAPD markers and the difficulty of interpreting the results of RAPD due to a mismatching of PCR products, such as primers and templates (Kumar and Gurusubramanian, 2001).

1.4.2. Amplified fragment length polymorphism (AFLP)

Researchers in molecular biology have used specific molecular techniques for the identification of living organisms, and one of these techniques is Amplified Fragment Length Polymorphism (AFLP). It is a genetic marker technique and is preferred to other techniques for several reasons: the analysis is fast and easy, no prior knowledge of the organism is required, costs are low, the results are accurate and there is significant coverage of the genome (Vos et al., 1995). The technique relies on restriction of fragments such as *Eco*RI and *Mse*I with adapter-homologous primers (+0/+0 primers) (Keim et al., 1997).

AFLP analysis has been used to provide information about *B. anthracis* and its close relatives. The results using this analysis illustrated a few differences among bacterial isolates, while providing a large amount of information about *B. anthracis* that could not be obtained using other techniques of that time (Jackson et al., 1999).

1.4.3. Restriction Fragment Length Polymorphism (RFLP)

RFLP was one of the most efficient molecular methods based on genetic patterns, and has achieved great success as a tool of molecular classification for studying and assessing the genetic diversity of chromosomes and the relationships that combine bacterial species and subspecies with each other. Using DNA-DNA hybridization during RFLP analysis has given strong evidence of the ability of this technique to provide dependable results compared to other methods that rely on phenotypic data (Joung and Cote, 2001).

Joung and Cote (2001) have reported the use of the RFLP technique in phylogenetic analysis and in the determination of different *B. thuringiensis* serotypes, using the 16S rRNA gene. The results showed that 80 different serotypes had been detected. Determination of novel strains of *B. thuringiensis* can be achieved using 16S rRNA gene restriction pattern analysis. The use of the 16S rRNA gene in this technique provides an opportunity for grouping *B. thuringiensis* strains, in addition to allocating a place for them on the phylogenetic tree.

1.4.4. Fatty Acid Methyl Esters (FAME) analysis

Bacillus oleronius shows a close relationship with two species, *B. sporothermodurans* and *B. licheniformis*. Fatty acid methyl esters (FAME) have been used as a method for identification of the *B. oleronius* strains (Heyndrickx et al., 2010). FAME analysis is one of the most prominent and important methods, apart from PCR, for the classification and characterization of different types of bacteria, such as *Bacillus*. The genotype of organisms can be used to determine several types of fatty acids produced within a cell, and in addition can be used in the differentiation and discrimination of different species and strains. The fatty acid extraction process is still accessible because of the commercial systems that facilitated its spread after the basic role of fatty acid in the identification of several types of bacteria (Ehrhardt et al.,

2010). Simplicity, speed, and reliability are the most important features of this analysis (Yong et al., 2010).

B. sporothermodurans and *B. oleronius* have displayed genetic heterogeneity and FAME analysis was used to provide amended descriptions to discriminate between these species, due to the difficulty of discriminating the two species according to their biochemical and morphological properties. A study conducted to distinguish between *B. sporothermodurans* and *B. oleronius* showed that the strains of *B. oleronius* are non-motile, Gram-negative, rod bacilli, the spores are able to resist high heat, and the diameter of colonies are around 1-2 mm. When placed on the TSA (Trypticase Soy Agar) media, the colonies are milky or beige, glossy, its edges are very light and they do not need vitamin B12 for growth. *B. oleronius* grows in temperatures around 30-50°C, pH 5.7 and 6.8 % NaCl. The G+C content was 35.2 mol% and the fatty acids were iso-C15: 0, Anteiso-C15: 0 and anteiso-C17: 0 (Heyndrickx et al., 2012).

In contrast, the amended characterizations of *B. sporothermodurans* have shown that the cells are motile, Gram positive rods, needing vitamin B12 for growth. The colonies are cream, full, and spherical, around 2 mm in diameter, and spores have the ability to resist high temperatures. *B. sporothermodurans* grows in temperatures around 20-55°C, pH 5 and 5 % NaCl. The G+C content was 36.7 mol% and the fatty acids were iso-C15: 0, anteiso-C15: 0 and anteiso-C17: 0 (Heyndrickx et al., 2012). In view of these findings it is highly probable that *B. oleronius* can be included in group II of *Bacillus* species.

1.5. The Use of genes and probes for the identification of *Bacillus* species

In the past, *Bacillus* species have been classified into several clusters, depending on their phenotypic features, but a new method for studying *Bacillus* taxonomy and phylogeny has come to light, one that depends on the small subunit ribosomal 16S rRNA gene.

1.5.1. 16S rRNA gene

This gene is considered to be the most successful method so far discovered for use in the analysis of phylogenetic relationships. The main advantages of 16S rRNA are (i) It is permanently present in all living organisms; (ii) it is accurate when there is a change in the nucleotide sequences; (iii) the large size of 16S rRNA has contributed to it being useful in many informatics applications. Experts were able to arrange and reclassify *Bacillus* species on the basis of 16S rRNA (Økstad and Kolstø, 2011; Woese, 1987). Many medical laboratories have employed 16S DNA in scientific research as a fundamental criterion for the assessment of phylogenetic diversity in various bacterial populations and in the *Bacillus* genus in particular (Lane et al., 1985). The ability of 16S rRNA gene to provide many PCR primers easily has

contributed to the targeting of a large number of bacterial communities (Vos et al., 2012). The success of the use of the 16S rRNA gene in the classification of a number of bacterial genera has made it the gold standard, particularly with regard to the *Bacillus* genus (Ki et al., 2009).

However, the 16S rRNA gene is not without possible disadvantages. Sometimes the 16S rRNA approach fails in the identification of a particular species belonging to related taxa, especially when there is a similarity between these species' rRNA genes or in their morphological and biochemical properties (Fox et al., 1992). The reason for the difficulty of making the distinctions by using the 16S rRNA gene is due to the high level of conservation of the primary structure of the 16S rRNA gene. This case applies to some *Bacillus* species, which are characterized by containing closely related species, and there are 222 recognized species (Ki et al., 2009). For example, the use of 16S rRNA to distinguish *B. subtilis* from the closely related *B. licheniformis* or *B. amyloliquefaciens* is a major challenge due to insufficient evidence for the existence of any difference in their 16S rRNA sequences. Despite the fact that the genus of *Bacillus* is large and diverse, some species that belong to genus are characterized by a high degree of similarity between each other. Hence, distinguishing the *Bacillus* species remains a matter of some importance. Due to the above facts, a large number of genes are used today as substitutes for 16S rRNA. These include *recA*, *rpoB*, *gyrB*, genes (Kwon et al., 2009) and *dnaJ* gene (Shah et al., 2007).

1.5.2. The *recA* gene

This gene encodes part of the DNA recombination and repair system. It has been suggested as a potential biomarker with reference to the phylogeny of bacteria and it has been used to distinguish various bacterial species belonging to bacterial genera. For example, the *recA* sequence has been used to distinguish between the *Lactobacillus casei* and the *L. plantarum* groups (Felis et al., 2001 and Torriani et al., 2001). Rodríguez and colleagues (2006) have succeeded in differentiating some *Lactobacillus* species that contain heterogeneous strains by using the recA gene sequence. In addition, the *recA* gene has been employed to distinguish some *Bacillus* species that have a high similarity of rRNA genes (Kwon et al., 2009).

1.5.3. The *rpoB* gene

This gene encodes as the β subunit of DNA polymerase. It has been recommended as a molecular substitute of 16S rRNA and is a highly conserved housekeeping gene (Case et al., 2007). More compellingly, the *rpoB* gene has many advantages over the 16S rRNA gene, for example, the *rpoB* gene is characterized by homogeneity within the cell, due to the fact that it consists of a single copy gene; also its genetic sequences are quite long, approximately 3.5 kb in the genus of *Bacillus*, and the *rpoB* gene is typically present in public databases (Ki et al., 2009). The data of the *rpoB* gene is characterized by easy and fast interpretation, based

on the fact that it is a protein-encoding gene (Vos et al., 2012). The identification and classification of numerous types of bacterial genera, such as the *Streptomyces*, *Pseudomonas*, *Staphylococcus*, *Vibrio*, and *Mycobacterium* and *Bacillus* can be undertaken by using the *rpoB* sequences (Marianelli et al*.,* 2006).

To date, the sequencing of ten *Bacillus* species with the complete *rpoB* gene has been conducted using many of the *Bacillus* genome projects in the world. Likewise, determination of some *Bacillus* species by using partial *rpoB* gene sequences has been carried out for the purpose of discovering the *Bacillus* species that cause bacterial diseases, such as *B. cereus* and *B. anthracis* (Qi et al., 2001; Ko *et al*., 2003). Case and his colleagues (2007) have proposed a comparison between *rpoB* and 16S rRNA genes to select a substitute biomarker for microbial ecology. Their study has proved that the *rpoB* gene is a suitable alternative, particularly as it has a single copy gene, thus ensuring its accuracy in diversity by reducing errors resulting from heterogeneity.

1.5.4. The *gyrB* gene

More recently, researchers have relied mainly on protein encoding genes as a tool for determining bacterial phylogenetic relationships (Chelo et al., 2007). *GyrB* and *gyrA* are two subunits of bacterial DNA gyrase. The *gyrase B* gene (*gyrB* gene) encodes the subunit B protein of DNA gyrase. It can be considered as DNA topoisomerase II, which has a vital role in DNA replication and in cellular metabolism. The *gyrB* gene is widespread among the bacterial species (Watt and Hickson, 1994; Huang, 1996). Inferred rates of genetic evolution are sometimes calculated on the basis of *gyrB* gene sequences, rather than 16S rRNA sequences, because of the high speed and accuracy of the *gyrB* gene compared to rRNA genes (Yamamoto and Harayama, 1996). Several phylogenetic studies have been conducted to demonstrate the crucial role played by this gene in bacterial genera, such as *Salmonella*, *Mycobacterium*, *Escherichia* (Fukushima et al., 2002) and the *B. anthracis–cereus–thuringiensis* group (La Duc et al., 2004).

The results of the above studies demonstrate that *gyrB* is a useful biomarker for the construction of bacterial phylogenetic relationships and for studying the classification of some bacterial species, including those of the *Bacillus* genus (Wang et al., 2007). Another study, conducted by Huang and his colleagues (2012), has indicated that the *gyrB* gene could be used for the identification and differentiation of various species of *Bacillus*, such as *B. subtilis*, which has 13 related species.

1.6. DNA sequencing

The discovery of the double helix structure of DNA in 1953 by James Watson & Francis Crick gave way to the beginning of a new science that eventually originated the field of DNA sequencing. Despite being a great discovery, scientists did not pay a great attention to the importance of the DNA sequence. In fact, it was only 15 years after Watson & Crick's discovery that the first method for determining DNA sequences was develop by Wu and Kaiser, who were able to partially sequence DNA fragments of lambda bacteriophage. After that, scientist's interest in studying DNA sequences and life technologies increased with the first sequencing procedure described by Sanger and knickers in 1975 (Fei, 2014).

Sanger sequencing procedure, which is also called "plus and minus" sequencing, used two sequences of ΦX174 bacteriophage. In the Sanger method, DNA polymerase I was considered the basic material for extension of the primer oligonucleotide and copy DNA template using the four Deoxynucleotide Triphosphates (dNTPs) with one of these nucleotides marked with radioactive phosphate-32. The DNA molecules with different lengths were mixed together with the primer oligonucleotides. When the DNA purification was finished, the additional triphosphates were removed by dividing the DNA mixture into eight equal amounts for preparing the DNA polymerase I reactions to the next round. The process of DNA sequencing was completed using a specific sequence method that involved using either three of the four dNTPs (minus) or using only one of the four dNTPs (plus). Through separation of the eight reactions, it would be possible to determine the DNA sequence using gel electrophoresis. The most important advantages of this method was speed and simplicity; however, the disadvantage of this method was using only single stranded DNA to determine the DNA sequence (Figure 4) (Fei, 2014).

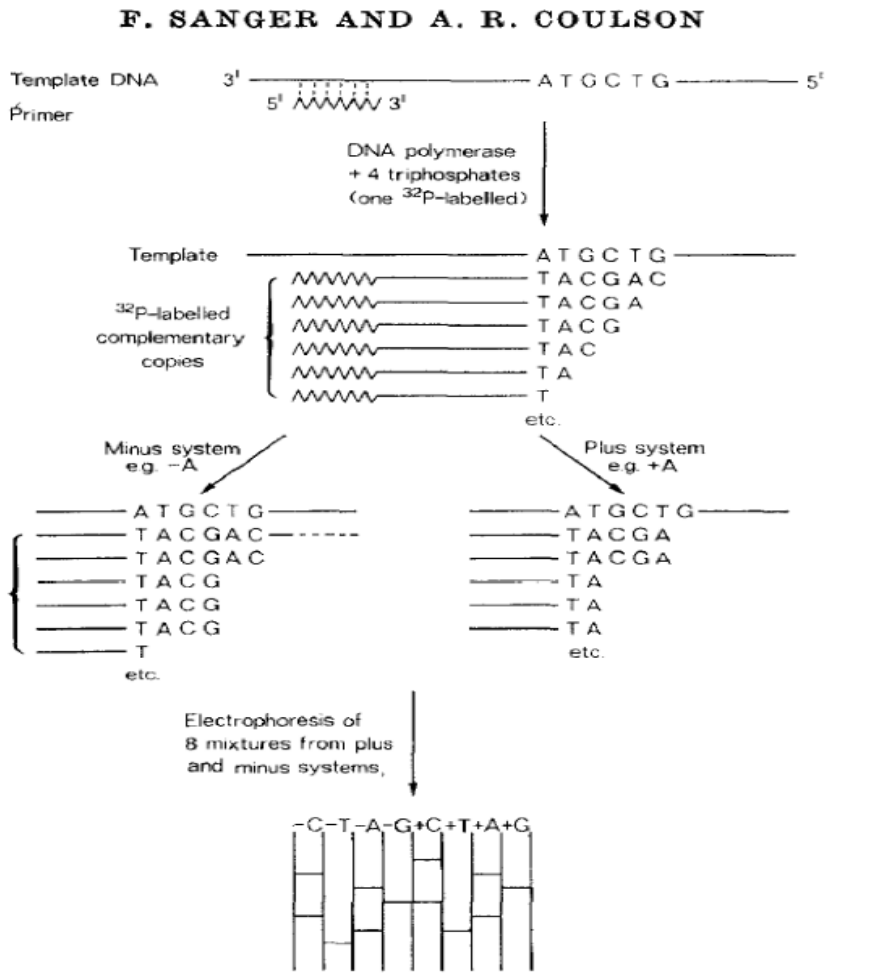


Figure 4. Diagram of Sanger sequencing (Fei, 2014)

This figure represents the Sanger sequencing method using different lengths of DNA molecules. The generated DNA can be used as a template using oligonucleotide primers. The separation of the eight reactions of both of plus and minus systems was achieved using gel electrophoresis.

1.7. Next Generation Sequencing

The toughest challenge scientists faced with the Sanger method was the limited number of samples coupled with the difficulty of a timely preparation and completion of the procedure. Thus, scientists turned their efforts to find an alternative method that eventually gave rise to the so called next generation sequencing technology capable of answering the modern requirements.

Single Molecule Real Time Sequencing (SMRT) is one of the most important modern technologies that has been developed by Pacific Biosciences (Eid et al., 2009). In previous years, SMRT technique was underappreciated, especially when compared to other next generation sequencing techniques such as Illumina and Ion Torrent, due to the many rumors regarding its inaccuracy and complexity. However, SMRT is becoming more and more promising with improvements in precision, speed, production of high and accurate DNA sequences from unamplified molecules and the ability to sequence DNA with small genomes. The SMRT technique depends on labeled nucleotides that generate fluorescent plus. The synthesis of tagged nucleotides can be happened along DNA template molecules (Roberts et al., 2013).

SMRT technique can be carried out on SMRT cells that contain around 150,000 Zero-Mode Waveguide (ZMW) (Levene et al., 2003). The zero-mode waveguide is a nanophotonic structure in the shape of circular holes with a diameter of around tens of nanometers based on a solid and visible metal base containing silicon dioxide substance (Levene et al., 2008). ZMW can be created a small illuminated volume at the lower base of the SMRT cell. During the sequencing process the DNA template is attached and immobilized on the lower surface of the ZMW while the phospholinked nucleotides are inserted into the reaction solution. The four-phospholinked nucleotides (A, G, T, and C) are marked with multiple fluorescent that differs in color. When a nucleotide is held, a production of a light pulse increases the detection volume 1000 times therefore reducing the background noise. Splitting and diffusion of the fluorescent label out of the ZMW area occurs after integration of one of the nucleotides. The process of sequencing is performed in parallel in thousands of ZMW that form a SMRT cell (Figure 5) (Fei, 2014).

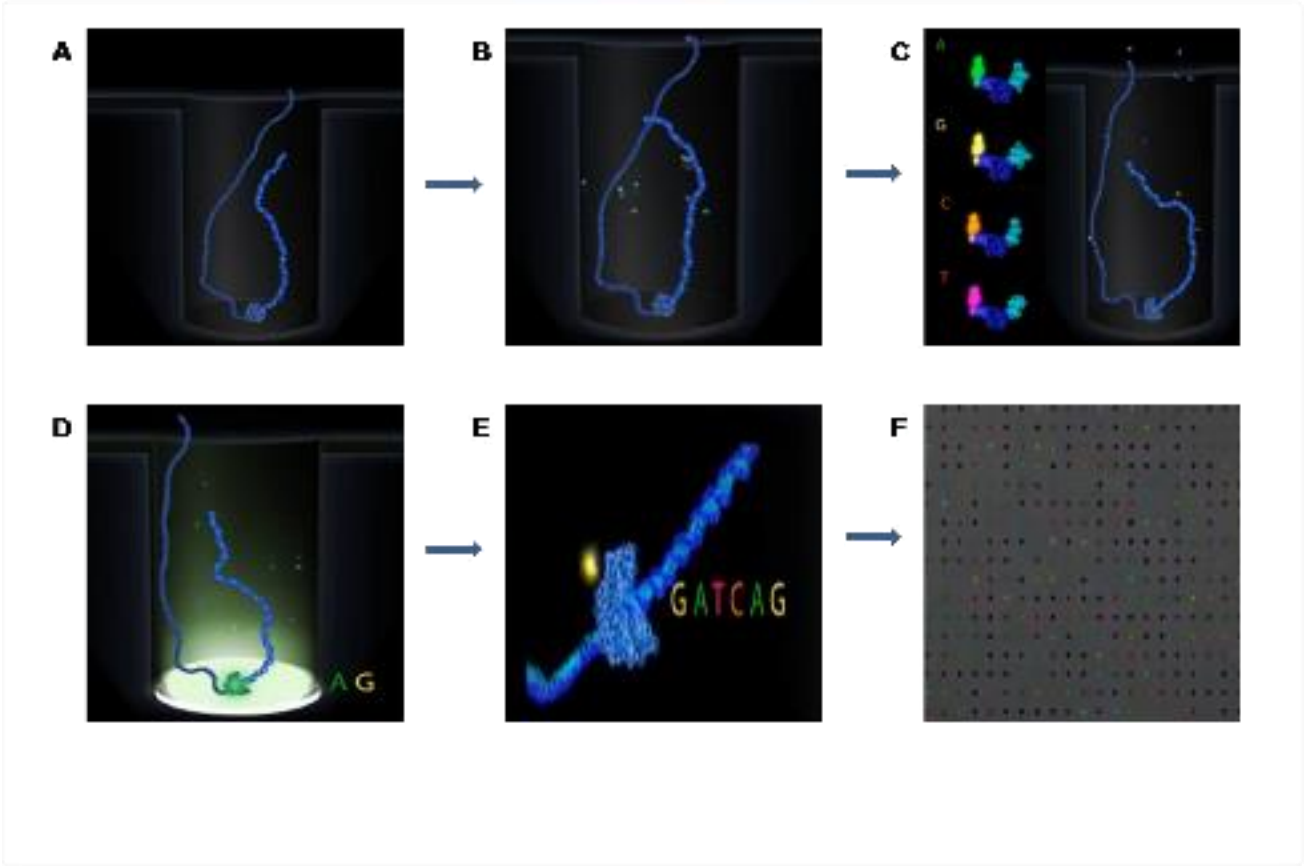


Figure 5. Summary of Single Molecule Real Time Sequencing ((Fei, 2014).) (A) The DNA template is attached with active polymerase and immobilized on the lower surface of the ZMW. (B) The phospholinked nucleotides are inserted into the reaction solution. (C) The four-phospholinked nucleotides (A, G, T, and C) are marked with different colors. (D) Production a light pulse. (E) Splitting and diffusion of the fluorescent label out of the ZMW area occurs after integration of one of the nucleotides. (F)The process of sequencing is performed in parallel to make up thousands of ZMW that form SMRT cell.

1.8. Objective of the study

The species *Bacillus oleronius* has been reported as a potential pathogen associated with demodicosis and as a likely cause of the associated pathology. The aim of this study was based on designing species-specific primers for identification *B. oleronius* in *Demodex* sampled from healthy people.

Strategies:

- Develop a purification method for large amounts of high-quality genomic DNA for single-molecule real time next generation genomic sequencing (SMRT) of *B. oleronius.*
- Confirm that 16S sequence of *B. oleronius* is unsuitable for species descrimination.
- Confirm the *Bacillus* species genetically nearest to *B. oleronius* phylogenetically
- Search for fast evolving protein-coding genes in the *Bacillus subtilus* group as potential marker genes.
- Design group-specific primers for these fast-evolving genes that encompass sequence regions of high divergence.
- Amplify these gene fragments from *B. oleronius* for Sanger sequencing.

CHAPTER TWO

2. MATERIAL AND METHODS

2.1. Preparation of culture media

Nutrient broth powder 6.5 g (Ref. No 84662.0500) was dissolved in 500 mL ionised water dH2O.The mixture was stirred well and sterilized by autoclaving at 121 °C for 3 hours. The media was then stored at room temperature until needed.

2.2. Opening of ampules and rehydration of dried culture

Starter cultures of *B. oleronius* were obtain from (QIAGEN, Germany) and cultured according to the manufacturer's instruction (Appendix 1). The tip of the ampoule was heated in a flame and two to three drops of water were placed onto the hot tip in order to crack the glass. The glass tip was stroked off with forceps and the inner vial containing *B. oleronius* strain was taken out. The cotton plug was removed and kept under sterile conditions. Then, 500 µL of nutrient broth media was added to the inner vial, the plug was replaced and the pellet was allowed to rehydrate for about 30 minutes. Meanwhile, 5 mL of the liquid medium was placed into five clean falcon tubes and 100 µL of the bacterial suspension were added to each of the five falcons and incubated at 30 °C on a shaker at 140 oscillations per minute overnight.

2.3. Extraction of DNA from bacterial culture

Bacillus oleronius was grown overnight on broth media culture .Two protocols and two extraction kits were used for the extraction of DNA from bacterial culture.

2.3.1. DNA purification from Gram –positive bacteria using the Gentra Puregene Yeast/Bact kit.

DNA extraction was carried out by using the Gentra Puregene Yeast/Bact. Kit (No. 84501416) according to the manufacturer's instruction (Appendix 2). 500 µL of the cell culture that was incubated overnight at 30 °C was transferred into a 1.5 mL microcentrifuge tube on ice, followed by centrifugation at 13,000-16,000 rpm for 5 s. The supernatant was discarded and 300 µL of Cell Suspension Solution was added. Then 1.5 µL of Lytic Enzyme Solution was added and mixed by inverting 25 times and then incubated for 30 min at 37 °C, followed by centrifugation at 13,000-16,000 rpm for 1 minute. The supernatant was discarded and then 300 µL of Cell Suspension Solution was added and pipetted up and down to lyse the cells. 1.5 µL RNase A Solution was added and mixed by inverting 25 times and then incubated for 60 minute at 37 °C. 100 µL of Protein Precipitation Solution was added and centrifuged for 3 minute at 13,000-16,000 rpm. 300 µL of isopropanol was pipetted into a clean 1.5 mL microcentrifuge tube and the supernatant was added; the tube was inverted 50 times and centrifuged for 1 minute at 13,000-16,000 rpm. The supernatant was discarded and the tube

was drained by inverting on a clean piece of absorbent paper. 300 µL of 70% ethanol was added and centrifuged again for 1 minute at 13,000-16,000 rpm. The supernatant was discarded and the tube was drained on a clean piece of absorbent paper with allowing drying at room temperature for 10 minute. 100 µL DNA hydration solution was added and the Eppendorf tubes were placed on a thermo block (Techne DRI-Block DB.2A, USA) at 65 ^{0}C for 1 hour and then left overnight at the rotator (IKA- VIBRAX- VXR, Germany).

2.3.2. DNA purification using DNeasy ® Blood & Tissue Kit (50)

DNA extraction was performed by using DNeasy ® Blood & Tissue Kit (50) (cat. no. 69504).The extraction was carried out as following procedure as indicated in the manufacturer's instruction (Appendix 3). In a sterilized microcenterfuge tube, which contained *B. oleronius* was centrifuged for 5 minutes at 190 rpm and 20 µL proteinase K was added. 200 µL proteinase K was added and the sample was placed on the thermo block at 65 ^{0}C for 10 minutes. 200 µL of ethanol was added and the mixture was transferred to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow- through and collection tube were discarded. The spin column was placed in a new micocentrifuge tube and 500 µL Buffer AW1 was added. The sample was centrifuged for 1 minute at 8000 rpm and the flow- through with collection tube was discarded. The spin column was placed again in a new Eppendorf tube and 500 µL Buffer AW2 was added. The column was centrifuged for 3 minutes at 14,000 rpm and the flow- through with collection tube was discarded. The spin column was transferred to a new micocentrifuge tube and 200 µL Buffer AE was added to the centre of the spin column with avoiding any scratching of the filter. The sample was incubated for 1 minute at room temperature (15-25^{0}C) and centrifuged for 1 minute at 8000 rpm. The tube now contained DNA extracted and it was ready for quantification.

2.3.3. DNA purification from Gram –negative bacteria using the Gentra Puregene Yeast/Bact kit

DNA was extracted using also the Gentra Puregene Yeast/Bact kit but following the respective protocol for Gram –negative bacteria (Appendix 4). From the bacterial culture 500 µL were transferred to a 1.5 mL microcentrifuge tube on ice, followed by centrifugation at 13,000-16,000 rpm for 1 minute. The supernatant was discarded by pipetting, and then 300 µL of Cell Suspension Solution was added and the sample was incubated on the thermo block at 80 °C for 60 minute. Subsequently 100 µL of Protein Precipitation Solution was added and centrifuged at 8000 rpm for 2 minute at 5°C. Afterwards, 300 µL of isopropanol was pipetted into a clean 1.5 mL microcentrifuge tube and the supernatant was added. The tube was inverted 50 times and centrifuged for 3 minute at 13,000-16,000 rpm at 4 °C. The supernatant was discarded and the tube was drained by inverting on a clean piece of absorbent paper. Three hundred µL of 70% ethanol was added and centrifuged again for 2 minute at 16,000 rpm AT 4 °C. The supernatant was discarded and the tube was drained on a clean piece of

absorbent paper with allowing to air dry for 10 minute. One hundred µL DNA hydration solution was added and the Eppendorf tubes were placed on the thermo block at 65 ^{0}C for 10 minutes instead of 1 hour and then left overnight at 80°C. Next day the extracted DNA was quantified using the Nanodrop.

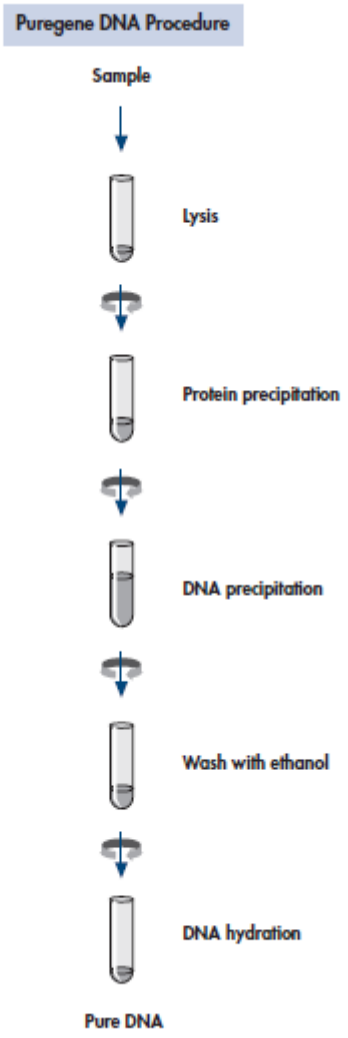


Figure 6. Stages of DNA extraction using Puregene kit

2.4. Gel preparation

A 0.8% agarose gel was prepared as follows: 0.8 g of agarose powder (Batch No. D23374) was added into a clean Erlenmeyer flask and 100 mL of TAE buffer was added and mixed gently. The solution was heated in a microwave for 2-4 minutes. Then, 10 µL of SYBR® Safe View DNA gel stain dye (Lot no. 1459622) was added to the mixture and the gel solution was poured into a gel-casting tray and left for 30 min to set. Next, the gel was gently transferred into an electrophoresis tank with filling the tank with the diluted buffer.

2.5. Loading and running DNA in Agarose gels

In order to evaluate the quality and quantity of genomic DNA, agarose gel analysis was used after finishing the DNA extraction. The samples extracted with the Gentra Puregene Yeast/Bact kit from Gram –negative bacteria were loaded only into the gel with different concentrations. Aliquots of 8 µl [5 µl of DNA sample with 3 µl of loading dye] of DNA extract were loaded into corresponding wells and finally the gel electrophoresis (The PowerPac Basic, Bio-Rad laboratories) was run at 80 volt for 60 min (Appendix 5).

2.6. Re purification the extracted DNA with DNeasy ® Blood & Tissue Kit

The three samples with the highest amount of extracted DNA using the Gram-negative protocol (with concentrations of 176, 140, and 102 µg/µl) were combined and extracted with the DNeasy kit in order to obtain high quality of DNA. For that 300 µl AL buffer without ethanol and 40 µl of proteinase K were added to the mixture and mixed by light vortexing. Then the sample was incubate at 56 ˚C overnight. In the following morning the proteinase K was inactivated at 95 ˚C for 15 minutes. 200 µL of ethanol was added and the mixture was transferred to a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded. The spin column was placed in a new micocentrifuge tube and 500 µL of Buffer AW1 was added. The sample was centrifuged for 1 minute at 8000 rpm and the flow- through with collection tube was once again discarded. The spin column was placed again in a new collection tube and 500 µL of Buffer AW2 was added. The column was centrifuged for 3 minutes at 14,000 rpm and the flow- through with collection tube was discarded. The spin column was transferred to a new eppendrof tube and 200 µL of Buffer AE was added to the centre of the spin column avoiding any scratching of the filter. The sample was incubated for 1 minute at room temperature (15-25^{0}C) and centrifuged for 1 minute at 8000 rpm.

2.7. Design universal primer

In order to design universal primers that can bind to any DNA nucleotide sequence of *Bacillus* species, *rpoB*, *gyrB* and *recA* genes for different *Bacillus* species were downloaded from GenBank (www.ncbi.nlm.nih.gov/GenBank/) within National Centre for Biotechnology Information (NCBI) database. The construction of primers was based on the nucleotide sequences of *rpoB* gene of the 11 *Bacillus* species. On this basis, the *rpoB* sequences (GenBank accession no. in parentheses) from *Bacillus weihenstephanensis* (5840109), *Bacillus thuringiensis* (2853766), *Bacillus subtilis subsp* (936335), *Bacillus pumilus* (5619311), *Bacillus pseudofirmus* (8766702), *Bacillus megaterium* (8984566), *Bacillus licheniformis* (3101115), *Bacillus halodurans* (892766), *Bacillus coagulans* (10763787), *Bacillus clausii* (3201560), *Bacillus cereus* (3024069), *Bacillus atrophaeus* (9893832), *Bacillus anthracis* (2820986), *Bacillus amyloliquefaciens* (9780500) and the *gyrB* sequences from *Bacillus thuringiensis* (9191046), *Bacillus subtilis subsp* (939456), *Bacillus sp* (15383109), *Bacillus pumilus* (5619216), *Bacillus pseudofirmus* (8766585), *Bacillus megaterium* (9115396), *Bacillus licheniformis* (3099964), *Bacillus infantis* (17113005), *Bacillus halodurans* (892561), *Bacillus cytotoxicus* (5344960), *Bacillus coagulans* (10763676), *Bacillus clausii* (3204560), *Bacillus cereus* (3023387), *Bacillus atrophaeus* (9893715), *Bacillus anthracis* (7852267), *Bacillus amyloliquefaciens* (9779917) were aligned using Geneious software (v.5.6) (https://www.geneious.com) and choosing the option ‘’without gaps’’ in order to determine the most conserved regions that would be suitable to design primers (Table 4 and 5) respectively.

Table 4. Sequences of *rpoB* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus weihenstephanensis***	***Bacilli***	**5840109**
2	***Bacillus thuringiensis***	***Bacilli***	**2853766**
3	***Bacillus subtilis subsp***	***Bacilli***	**936335**
4	***Bacillus pumilus***	***Bacilli***	**5619311**
5	***Bacillus pseudofirmus***	***Bacilli***	**8766702**
6	***Bacillus megaterium***	***Bacilli***	**8984566**
7	***Bacillus licheniformis***	***Bacilli***	**3101115**
8	***Bacillus halodurans***	***Bacilli***	**892766**
9	***Bacillus coagulans***	***Bacilli***	**10763787**
10	***Bacillus clausii***	***Bacilli***	**3201560**
11	***Bacillus cereus***	***Bacilli***	**3024069**
12	***Bacillus atrophaeus***	***Bacilli***	**9893832**
13	***Bacillus anthracis***	***Bacilli***	**2820986**
14	***Bacillus amyloliquefaciens***	***Bacilli***	**9780500**

Table 5. Sequences of *gyrB* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	*Bacillus thuringiensis*	*Bacilli*	9191046
2	*Bacillus subtilis subsp*	*Bacilli*	939456
3	*Bacillus sp*	*Bacilli*	15383109
4	*Bacillus pumilus*	*Bacilli*	5619216
5	*Bacillus pseudofirmus*	*Bacilli*	8766585
6	*Bacillus megaterium*	*Bacilli*	9115396
7	*Bacillus licheniformis*	*Bacilli*	3099964
8	*Bacillus infantis*	*Bacilli*	17113005
9	*Bacillus halodurans*	*Bacilli*	892561
10	*Bacillus cytotoxicus*	*Bacilli*	5344960
11	*Bacillus coagulans*	*Bacilli*	10763676
12	*Bacillus clausii*	*Bacilli*	3204560
13	*Bacillus cereus*	*Bacilli*	3023387
14	*Bacillus atrophaeus*	*Bacilli*	9893715
15	*Bacillus anthracis*	*Bacilli*	7852267
16	*Bacillus amyloliquefaciens*	*Bacilli*	9779917

Similarly, the *recA* sequences from *Bacillus weihenstephanensis* (5843762), *Bacillus thuringiensis* (2858232), *Bacillus subtilis subsp* (939497), *Bacillus pumilus* (5620865), *Bacillus pseudofirmus* (8768955), *Bacillus megaterium* (12085437), *Bacillus licheniformis* (3099159), *Bacillus halodurans* (894623), *Bacillus clausii* (3202852), *Bacillus cereus* (1206124), *and Bacillus anthracis* (1087627) were aligned using the previously described method (Table 6).

Table 6. Sequences of *recA* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus weihenstephanensis***	***Bacilli***	**5843762**
2	***Bacillus thuringiensis***	***Bacilli***	**2858232**
3	***Bacillus subtilis subsp***	***Bacilli***	**939497**
4	***Bacillus pumilus***	***Bacilli***	**5620865**
5	***Bacillus pseudofirmus***	***Bacilli***	**8768955**
6	***Bacillus megaterium***	***Bacilli***	**12085437**
7	***Bacillus licheniformis***	***Bacilli***	**3099159**
8	***Bacillus halodurans***	***Bacilli***	**894623**
9	***Bacillus clausii***	***Bacilli***	**3202852**
10	***Bacillus cereus***	***Bacilli***	**1206124**
11	***Bacillus anthracis***	***Bacilli***	**1087627**

The nucleotides sequences of the genes retrieved from GenBank were aligned using Genious program, 27 regions were identified in the *rpoB* gene as being an appropriately conserved regions for design universal primers (Table 14). Some positions with conserved regions were suitable for designing reverse and forward primers while some of these regions were not.

Table 7. The most conserved regions of *rpoB* that are suitable for design sets of universal primers

Location
26 - 45
1516 - 1535
1525 - 1544
1549 - 1568
1928 - 1946
2299 - 2318
2539 - 2558
2782 - 2801
2794 - 2813
2830 - 2850
3169 - 3188
3205 - 3244
3208 - 3227
3211 - 3230
3238 - 3257
3244- 3263

For identification the most important areas of the alignments to design universal primers, the most highly conserved regions were chosen. According to these regions, the forward and reverse primers with about 20 base pairs nucleotides long were designed using the Geneious software. The degenerated primers were designed based on the difference of the nucleotides sequences along the alignments by using the International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes (Table 8).

Table 8. IUPAC ambiguity codes

Code	Description
M	AC
R	AG
W	AT
S	CG
Y	CT
K	GT
V	ACG
H	ACT
D	AGT
B	CGT
N	ACGT

The candidate primers were checked to ensure suitability for work in PCR. The melting temperatures of the primers, GC content, secondary structure (hairpins) and dimer formation were checked by using the Geneious software and OligoAnalyzer 3.1 tool (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/).

2.8. Designing group- specific primers

In order to design specific primers for *bacillus oleronius*, fast evolving protein-coding genes such as *rpoB*, *gyrB*, *recA*, ITS, 16SrRNA, and *ytcP* genes were chosen in the *Bacillus subtilus* groups. The Individual primers were analyzed with the help of the programme Primer 3 version 4.0.0 (Koressaar and Remm, 2007; Untergrasser et al., 2012) and primer pairs with the help of the programme PriDimerCheck (Shen et al., 2010). The specificity of primer pairs was tested with Primer-BLAST (Ye et al., 2012).

Using NCBI database, the sex genes *rpoB*, *gyrB* and *recA*, ITS, *ytcP* and 16sRNA genes for different *Bacillus* species were downloaded as shown in Table 9, 10, 11, 12 and 13 respectively.

Table 9. Sequences of *recA* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus subtilis***	***Bacilli***	**936335**
2	***Bacillus licheniformis***	***Bacilli***	**3101115**
3	***Bacillus atrophaeus***	***Bacilli***	**9893832**
4	***Bacillus amyloliquefaciens***	***Bacilli***	**9780500**

recA (DNA recombination/repair protein)
(Kwon et al., 2009)
Complete gene ~ 1,050 bp; fragment 908 bp.

Bsg recA-1F **5-ATG AGT GAT CGT CAG GCA GCC-3′**

Bsg recA-908R **5-GCR TTT TCA CGG CCY TGD CC -3′**

Table 10. Sequences of *rpoB* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus subtilis***	***Bacilli***	**939456**
2	***Bacillus pumilus***	***Bacilli***	**5619216**
3	***Bacillus licheniformis***	***Bacilli***	**3099964**
4	***Bacillus atrophaeus***	***Bacilli***	**9893832**
5	***Bacillus amyloliquefaciens***	***Bacilli***	**9780500**
6	***Bacillus aquimaris***	***Bacilli***	**JF812006.1**

rpoB (RNA polymerase β subunit)

(Blackwood et al., 2004; Adékambi et al., 2009; Ki et al., 2009; Deperrois-Lafarge and Meheut, 2012)

Complete gene ~ 3,600 bp.

Bsg rpoB-1537F **CGKATGTGTCCGATTGAAAC**

Bsg rpoB-2440R **GAATATCRCGRGTGATYTCTTC**

RC GAAGAAATCACTCGTGATATTC

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CGTATGTGTCCGATTGAAAC	20	54.85	45.00	3.00	3.00
Reverse primer	GAATATCACGGGTGATCTCTTC	22	56.15	45.45	8.00	5.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:rpoB-1537 vs RP:rpoB-2440 Matches 31 = 5 alignment score= 0	5' CGtATGTGTCCGATTGAAAC 3' |||.....|| 3' CTTCTcTAGTGgGCaCTATAAG 5'	-0.83kcal/mol

Table 11. Sequences of *gyrB* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus subtilis***	***Bacilli***	**939497**
2	***Bacillus pumilus***	***Bacilli***	**5620865**
3	***Bacillus licheniformis***	***Bacilli***	**3099159**
4	***Bacillus amyloliquefaciens***	***Bacilli***	**9779917**
5	***Bacillus atrophaeus***	***Bacilli***	**9893715**
6	***Bacillus endophyticus***	***Bacilli***	**NZ-ALIM01000035**

gyrB (gyrase B subunit)

(Wang et al., 2007; Huang et al., 2012; Liu et al., 2013)

Complete gene 1,920 bp, fragment 1,045 bp.

gyrB used for species-specific detection *B. licheniformis* (Huang et al., 2012).

Huang

Specific primers at 479-497 and 1091-1073 position.

Fragment 1,028 bp

GACGGAAGCGGCTATAAAGT

Bsg gyrB-328F **GACGGHRGCGGHTATAAAGT**

GGCAAAATGGCTTGGAAATG

Bsg gyrB-1355R **GGYRRAATVGCYTGGAAATG**

RC CATTTCCARGCBATTYYRCC

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GACGGAAGCGGCTATAAAGT	20	57.78	50.00	5.00	2.00
Reverse primer	GGCAAAATGGCTTGGAAATG	20	56.43	45.00	3.00	0.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:gyrB-328F vs RP:gyrB-1355 Matches 29 = 2 alignment score= -6	5' GACGGAAGCGGCTATAAAGT 3' .....||. 3' GTAAAGGTTCGGTAAAACGG 5'	-0.08kcal/mol

Table 12. Sequences of *pyrE* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus subtilis***	***Bacilli***	**936335**
2	***Bacillus licheniformis***	***Bacilli***	**3101115**
3	***Bacillus atrophaeus***	***Bacilli***	**9893832**
4	***Bacillus amyloliquefaciens***	***Bacilli***	**9780500**
5	***Bacillus pumilus***	***Bacilli***	**5620722**

pyrE (orotate phosphoribosyltransferase)

(Liu et al., 2013)

Complete gene ~ 640 bp.

(Liu et al., 2013)

Liu

pyrBF AGACCGTTTCTTCCATCCA

pyrBR CACCTATTACAAATCAAAGC

57 ˚C annealing

Fragement 332 bp.

CCgTTtACaTGGGCgAGCGG

Bsg pyrE-64F CCKTTYACRTGGGCRAGCGG

GCTTCAAGaACaCTgCcTCC

Bsg pyrE-395R GCTTCAAGMACRCTKCYTCC

GGAgGaAGcGTtCTTGAAGC

GGARGMAGYGTKCTTGAAGC

Primer pair not synthesized due to lack of time.

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCGTTTACATGGGCGAGCGG	20	64.11	65.00	4.00	1.00
Reverse primer	GCTTCAAGAACACTGCCTCC	20	59.12	55.00	5.00	0.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bsg pyrE- vs RP:Bsg pyrE- Matches 21 = 5 alignment score= -8	5' CCgTTtACaTGGGCgAGCGG 3' ..|.....|.....||| 3' CCTcCgTCaCAaGAACTTCG 5'	-1.18kcal/mol

Table 13. Sequences of *ytcP* gene retrieved from GenBank, species (scientific name), class and GenBank Accession Number (AN).

No	Scientific name	Class	GenBank AN
1	***Bacillus subtilis***	***Bacilli***	**939456**
2	***Bacillus pumilus***	***Bacilli***	**5619216**
3	***Bacillus licheniformis***	***Bacilli***	**3099964**
4	***Bacillus atrophaeus***	***Bacilli***	**9893715**

ytcP (putative ABC transporter permease protein)

(Kwon et al., 2009)

Complete gene ~ 880 bp, fragment 750 bp.

	ATAAACGGATAGACAAGCAG
Bsg ytcP-50F	**ATA AAY GGA TAS ACR AGC AG**
Bsg ytcP-798R	**GTD CTK CCG TTC ATY CAY GT**
	GTaCTtCCGTTCATcCAtGT
RC	ACA TGA ATG AAC GGA AGT AC
RC	ACR TGR ATG AAC GGM AGH AC

F 53 ˚C, R 55 ˚C, annealing 50 ˚C or higher

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:ytcP 50F vs RP:ytcP 798R Matches 25 = 10 alignment score= 6	5' ATAAACGGATAGACAAGCAG 3' ||.|||||...|| 3' TGTACTTACTTGCCTTCATG 5'	-2.60kcal/mol

Genes associated with pathogenesis encoding hemolysin (*hbl*), cytotoxin K (*cytK*) and cereulide (*ces*) have been used (Martinez-Blanch et al., 2011). However, *B. oleronius* is not here considered a primary pathogen.

ITS (intergenic transcribed spacer, 16S-23S intergenic spacer region (ISR))

(Xu and Cote, 2003; Martinez-Blanch et al., 2011)

Complete region 417-795 bp, fragment same range.

Bsg 16SF-ITS **5-TCG CTA GTA ATC GCG GAT AGG C-3′**

Bsg 23SR-ITS **5-GCA TAT CGG TGT TMG TCC CGT CC-3′**

16S (small ribosomal subunit), Complete gene ~ 1550 bp.

(Szkaradkiewicz et al., 2012)

16S, 299 bp

BO1 **266-285** **AACGGCTCACCAAGGCGACG**

BO2 **564-545** **TCCGGACAACGCTTGCCACC**

RC GGTGGCAAGCGTTGTCCGGA

60 ˚C annealing

Fragments

Bo 16S-202FaL – Bo 16S-668R 467 bp

Bo 16S-202FaL – Bo 16S-684R 483 bp

Bo 16S-202FaL **AAC TTT TTT CTT CGC ATG ARG GAG AAT TG**

Bo 16S-202FbL **AAC TTT TTT CTT CGC ATG AAG AAG AAT TG** completely specific

F 4N, R 4N different to closest sequence of *Amphibacillus tropicus* strain W19, 30N1-4, PCSB2, HPC 637

Bo 16S-202FcL **AAC TTT TTT CTT CGC ATG AAG GGG AAT TG** completely specific,

F 2N, R 4N different to closest sequence of *Amphibacillus tropicus* strain HR63

>NR_025192.1 Amphibacillus tropicus strain Z-7792 16S ribosomal RNA gene, partial sequence

```
product length = 478
Forward primer  1    AACTTTTTTCTTCGCATGAAGGGG  24
Template        146  ........C...............  169

Reverse primer  1    CTCCCAGTTTCCAATGGCCGCTTG  24
Template        623  TCT.............A...N...  600
```

Bo 16S-668R **GCC GCT TGC GGT TGA GCC GCA AGA**

RC TCTTGCGGCTCAACCGCAAGCGGC

Product 463 bp

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAGGGAG	24	60.02	41.67	4.00	2.00
Reverse primer	GCCGCTTGCGGTTGAGCCGCAAGA	24	72.54	66.67	13.00	11.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-66 Matches 25 = 8 alignment score= -6	5' AACTTTTTTCTTCGCATGAGGGAG 3' .....|....||||.|.||. 3' AGAACGCCGAGTTGGCGTTCGCCG 5'	-2.99kcal/mol

Bo 16S-684R **CTC CCA GTT TCC AAT GGC CGC TTG**

RC CAAGCGGCCATTGGAAACTGGGAG

Product 479 bp

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAAGGAG	24	58.48	37.50	4.00	2.00
Reverse primer	CTCCCAGTTTCCAATGGCCGCTTG	24	66.48	58.33	5.00	4.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-68 Matches 15 = 5 alignment score= -6	5' AACTTTTTTCTTCGCATGAGGGAG 3' ....||.....|||. 3' GTTCGCCGGTAACCTTTGACCCTC 5'	-1.49kcal/mol

Bo 16S-202FaL

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAGGGAGAATTG	29	63.07	37.93	6.00	2.00
Reverse primer	CTCCCAGTTTCCAATGGCCGCTTG	24	66.48	58.33	5.00	4.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-68 Matches 43 = 4 alignment score= -1	5' AACTTTTTTCTTCGCATGAGGGAGAATTG 3' ||||..... 3' GTTCGCCGGTAACCTTTGACCCTC 5'	-1.12kcal/mol

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAAGGAGAATTG	29	61.77	34.48	6.00	2.00
Reverse primer	CTCCCAGTTTCCAATGGCCGCTTG	24	66.48	58.33	5.00	4.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-68 Matches 43 = 4 alignment score= -1	5' AACTTTTTTCTTCGCATGAAGGAGAATTG 3' ||||..... 3' GTTCGCCGGTAACCTTTGACCCTC 5'	-1.12kcal/mol

Bo 16S-202FbL

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAAGAAGAATTG	29	60.48	31.03	10.00	6.00
Reverse primer	CTCCCAGTTTCCAATGGCCGCTTG	24	66.48	58.33	5.00	4.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-68 Matches 43 = 4 alignment score= -1	5' AACTTTTTTCTTCGCATGAAGAAGAATTG 3' |||..... 3' GTTCGCCGGTAACCTTTGACCCTC 5'	-1.12kcal/mol

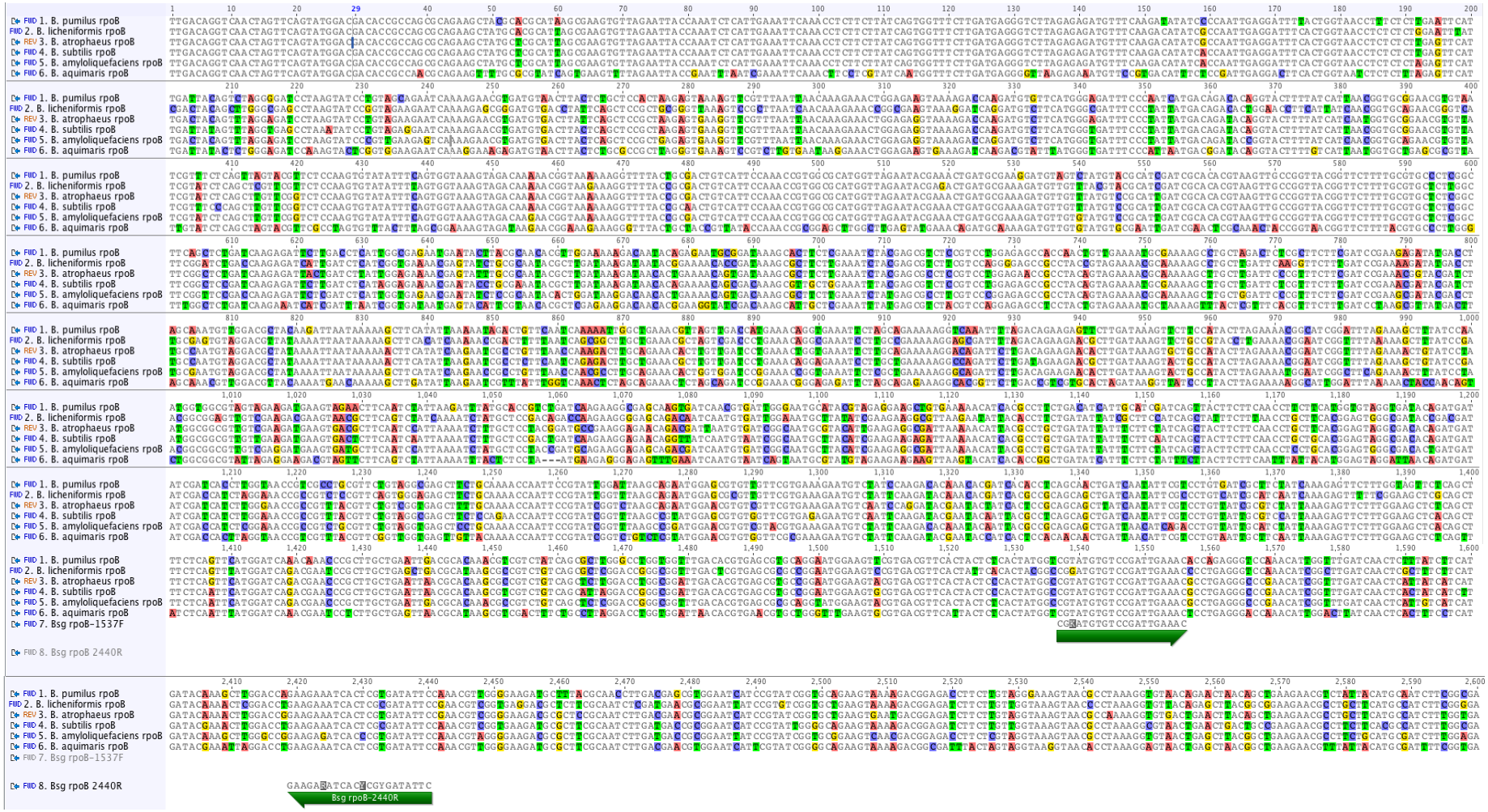
Bo 16S-202FcL

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AACTTTTTTCTTCGCATGAAGGGGAATTG	29	63.47	37.93	4.00	2.00
Reverse primer	CTCCCAGTTTCCAATGGCCGCTTG	24	66.48	58.33	5.00	4.00

Sequence information	Possible Primer Dimer	ΔG(37°C)
FP:Bo 16S-20 vs RP:Bo 16S-68 Matches 11 = 6 alignment score= 0	5' AACTTTTTTCTTCGCATGAAGGGGAATTG 3' .|||.||...|. 3' GTTCGCCGGTAACCTTTGACCCTC 5'	-1.62kcal/mol

All subsequent primer pairs were designed to be selective for *subtilis*/*pumilus* group. Alignments all the sequences were performed with the help of the programme Geineious R6, version 6.1.7 (Biomatters, Auckland, New Zealand) (Figure).

Figure 7: Translation alignment and primer annotation of *recA* sequences of *Bacillus subtilis* group species. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour. The most diverse region of the gene is at the very 3' end, between 950 and 1050, out of reach for primer design.


FWD 1. B. pumilus rpoB
FWD 2. B. licheniformis rpoB
REV 3. B. atrophaeus rpoB
FWD 4. B. subtilis rpoB
FWD 5. B. amyloliquefaciens rpoB
FWD 6. B. aquimaris rpoB
FWD 7. Bsg rpoB-1537F
FWD 8. Bsg rpoB 2440R
Bsg rpoB-2440R

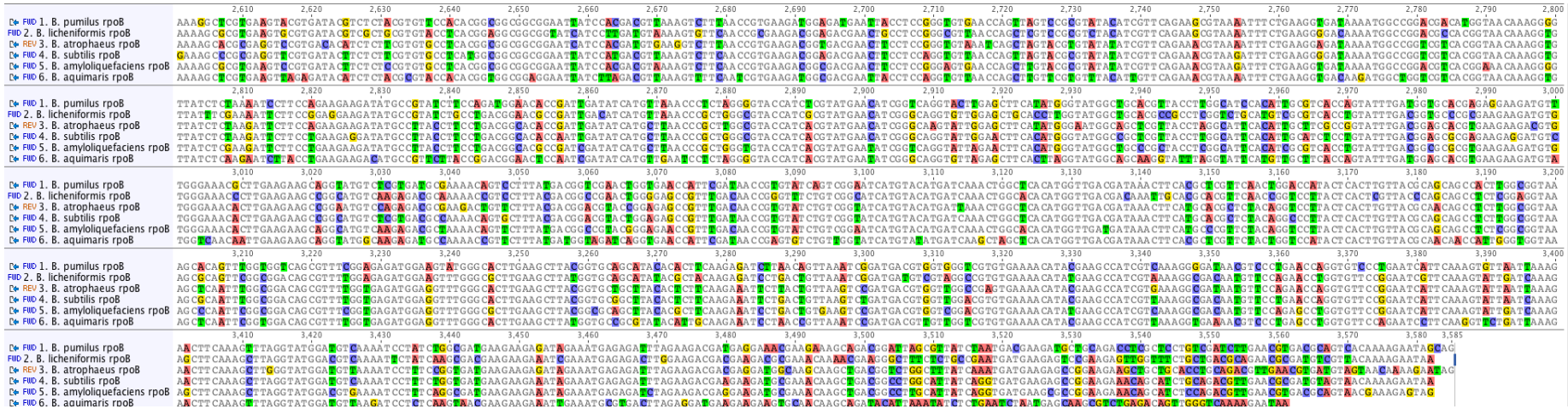
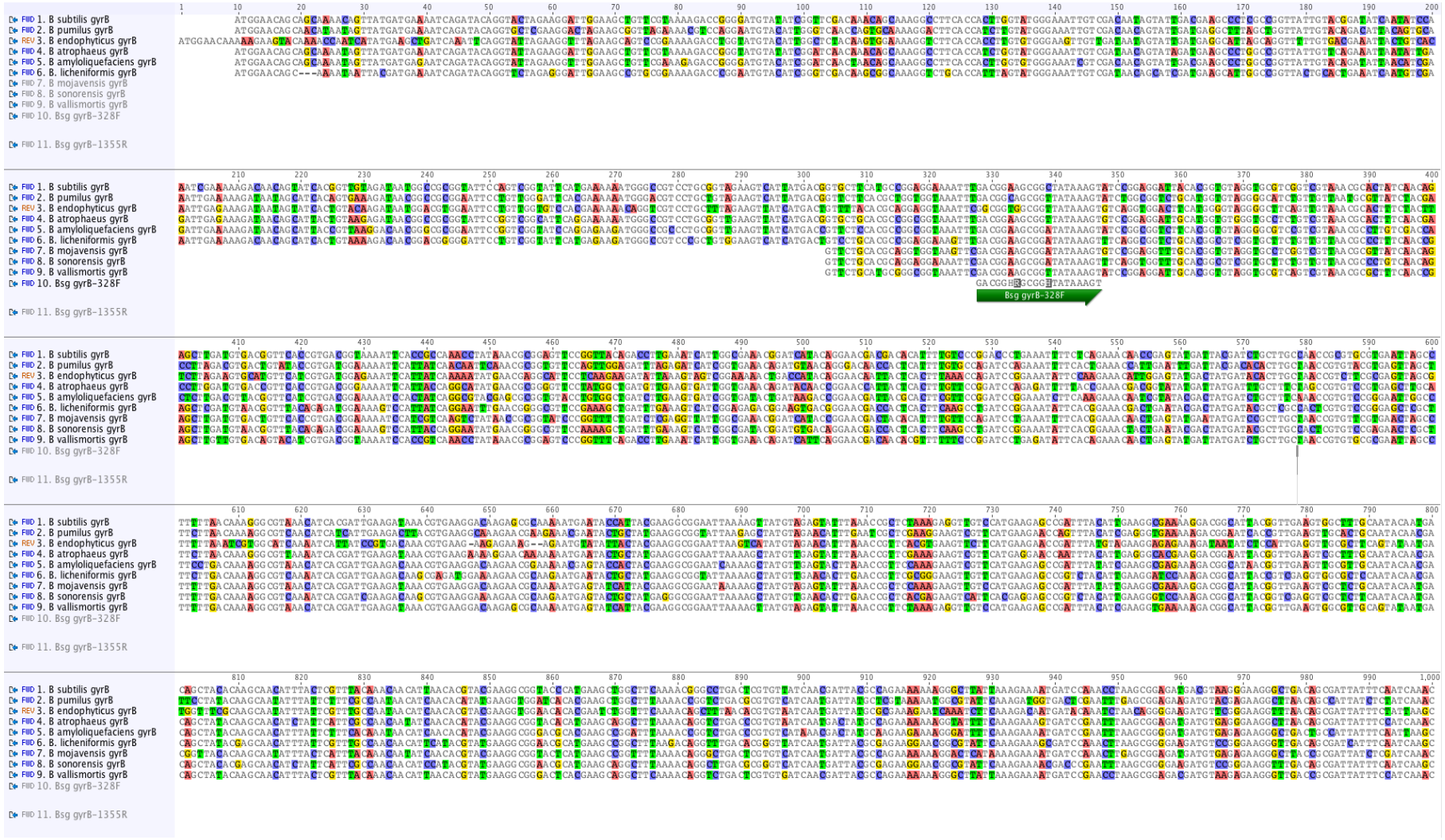


Figure 8: Manually modified translation alignment and primer annotation of *rpoB* sequences of *Bacillus subtilis* group species. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour. The most diverse region of the gene is at the very 3' end, between 3,500 and 3,085, out of reach for primer design; however, the gene has at the 5' prime end more variable regions that can be exploited.


1. B subtilis gyrB
2. B pumilus gyrB
3. B endophyticus gyrB
4. B atrophaeus gyrB
5. B amyloliquefaciens gyrB
6. B. licheniformis gyrB
7. B mojavensis gyrB
8. B sonorensis gyrB
9. B vallismortis gyrB
10. Bsg gyrB-328F
11. Bsg gyrB-1355R
Bsg gyrB-328F

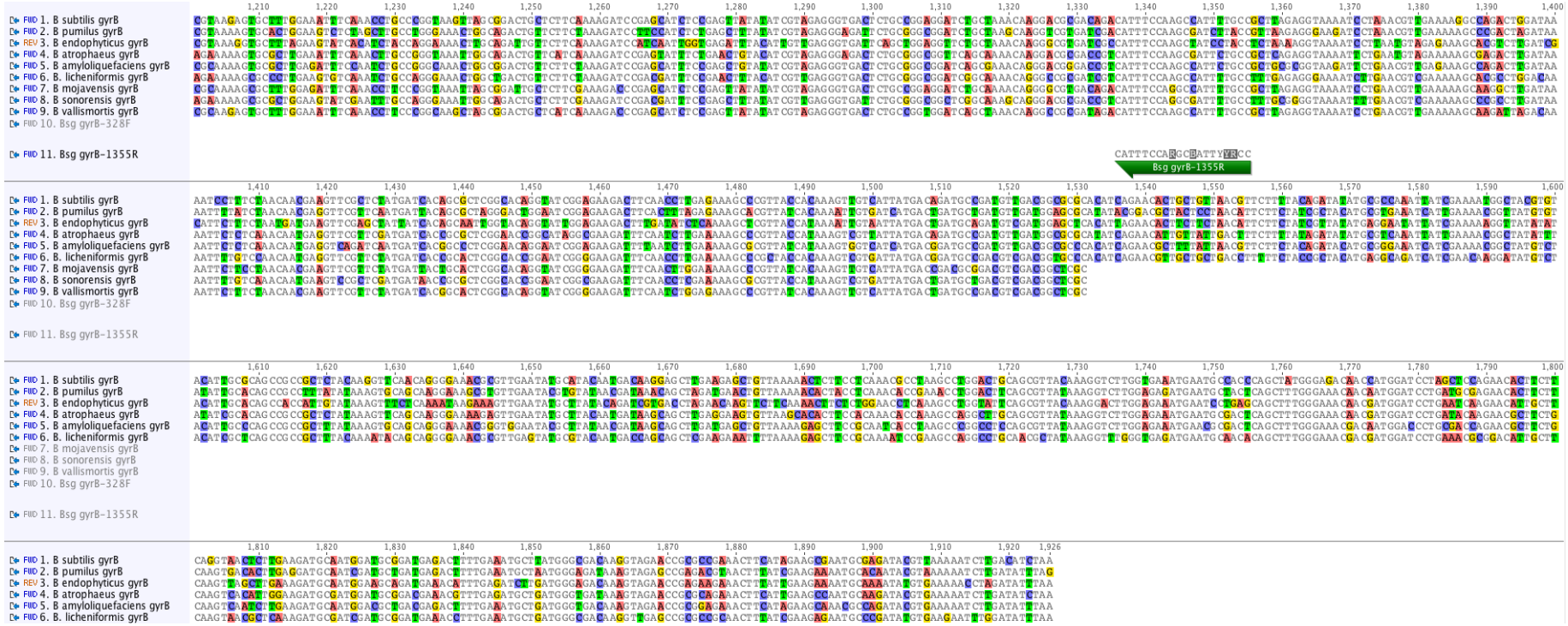


Figure 9: Geneious global multiple alignment and primer annotation of *gyrB* sequences of *Bacillus subtilis* group species. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour.

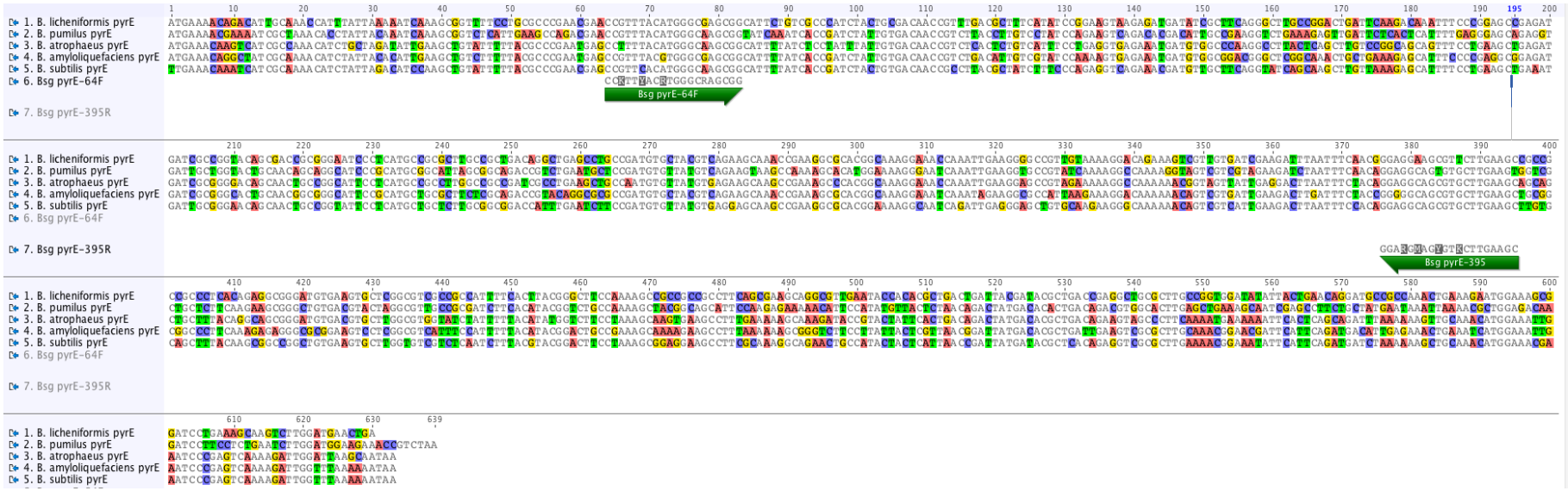


Figure 10: Geneious global multiple alignment and primer annotation of *pyrE* sequences of *Bacillus subtilis* group species. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour.

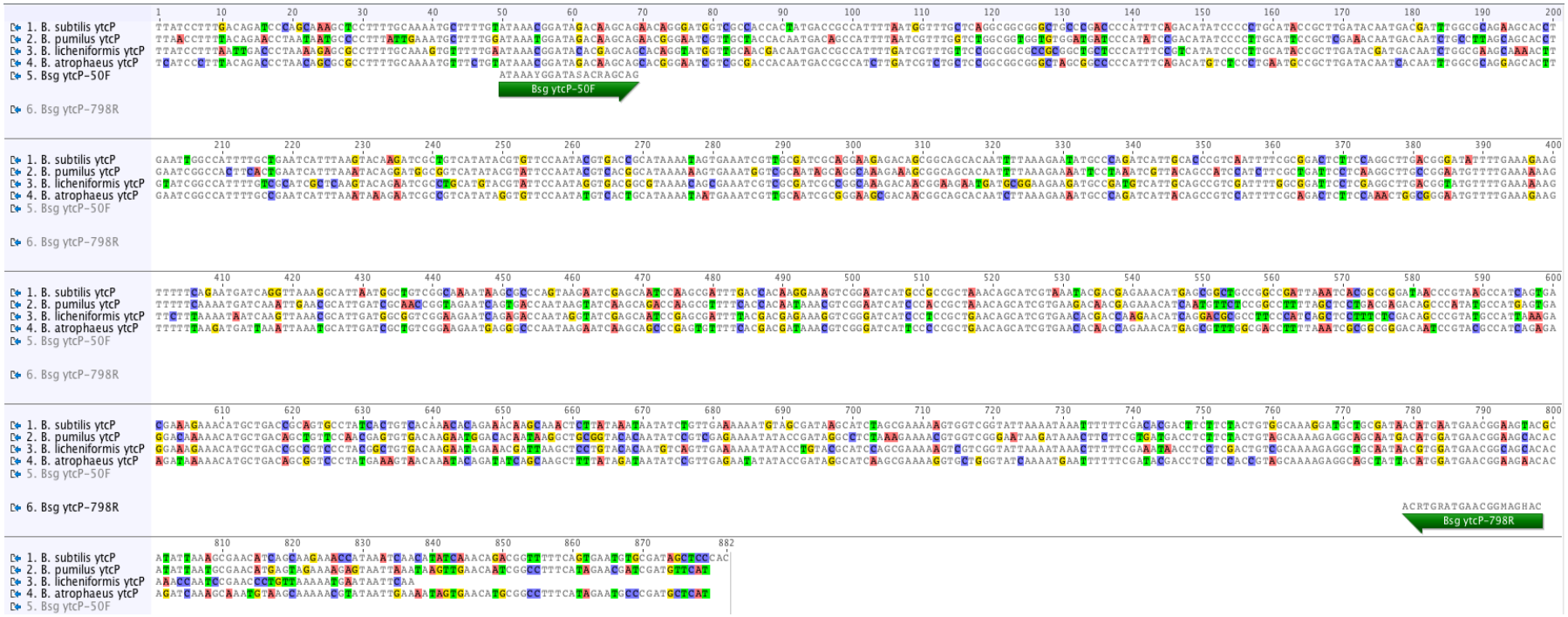


Figure 11: Translation alignment and primer annotation of *ytcP* sequences of *Bacillus subtilis* group species. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour.


1. Amphibacillus tropicus 16S
2. B. oleronius DSM9356 16S
3. B. oleronius ATCC 700005 16S
4. B. sporothermodurans 16S
5. B. carboniphilus 16S
6. B. aquimaris 16S
7. B. pumilus 16S
8. B. endophyticus 16S
9. B. subtilis 16S
10. B.vallismortis 16S
11. B. sonorensis 16S
12. B. mojavensis 16S
13. B. licheniformis 16S
14. B. atrophaeus 16S
15. B. amyloliquefaciens 16S
16. Bo 16S-202FcL
17. Bo 16S-202FbL
18. Bo 16S-202FaL
19. BO1
Bo 16S-202FcL
Bo 16S-202FbL
Bo 16S-202FaL
BO1
1. Amphibacillus tropicus 16S
2. B. oleronius DSM9356 16S
3. B. oleronius ATCC 700005 16S
4. B. sporothermodurans 16S
5. B. carboniphilus 16S
6. B. aquimaris 16S
7. B. pumilus 16S
8. B. endophyticus 16S
9. B. subtilis 16S
10. B.vallismortis 16S
11. B. sonorensis 16S
12. B. mojavensis 16S
13. B. licheniformis 16S
14. B. atrophaeus 16S
15. B. amyloliquefaciens 16S
16. BO2
BO2 RC
1. Amphibacillus tropicus 16S
2. B. oleronius DSM9356 16S
3. B. oleronius ATCC 700005 16S
4. B. sporothermodurans 16S
5. B. carboniphilus 16S
6. B. aquimaris 16S
7. B. pumilus 16S
8. B. endophyticus 16S
9. B. subtilis 16S
10. B.vallismortis 16S
11. B. sonorensis 16S
12. B. mojavensis 16S
13. B. licheniformis 16S
14. B. atrophaeus 16S
15. B. amyloliquefaciens 16S
16. Bo 16S-684R
17. Bo 16S-668R
18. BO2
Bo 16S-684R
Bo 16S-668R

Figure 12: Geneious global multiple alignment and primer annotation of 16S sequences of *Bacillus subtilis* group species and *Amphibacillus tropicus*. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour. Only the available full-length sequences of *B. oleronius* are shown, for detailed variation, see Figure 13. BO1 and BO2 are primers designed by Szkaradkiewicz et al., 2012, as *B. oleronius*-specific, all other primers are designed in this project. The Figure shows that the published primers are not species-specific, the newly designed primers are species-specific according to all available sequences in GenBank up to September 2014.

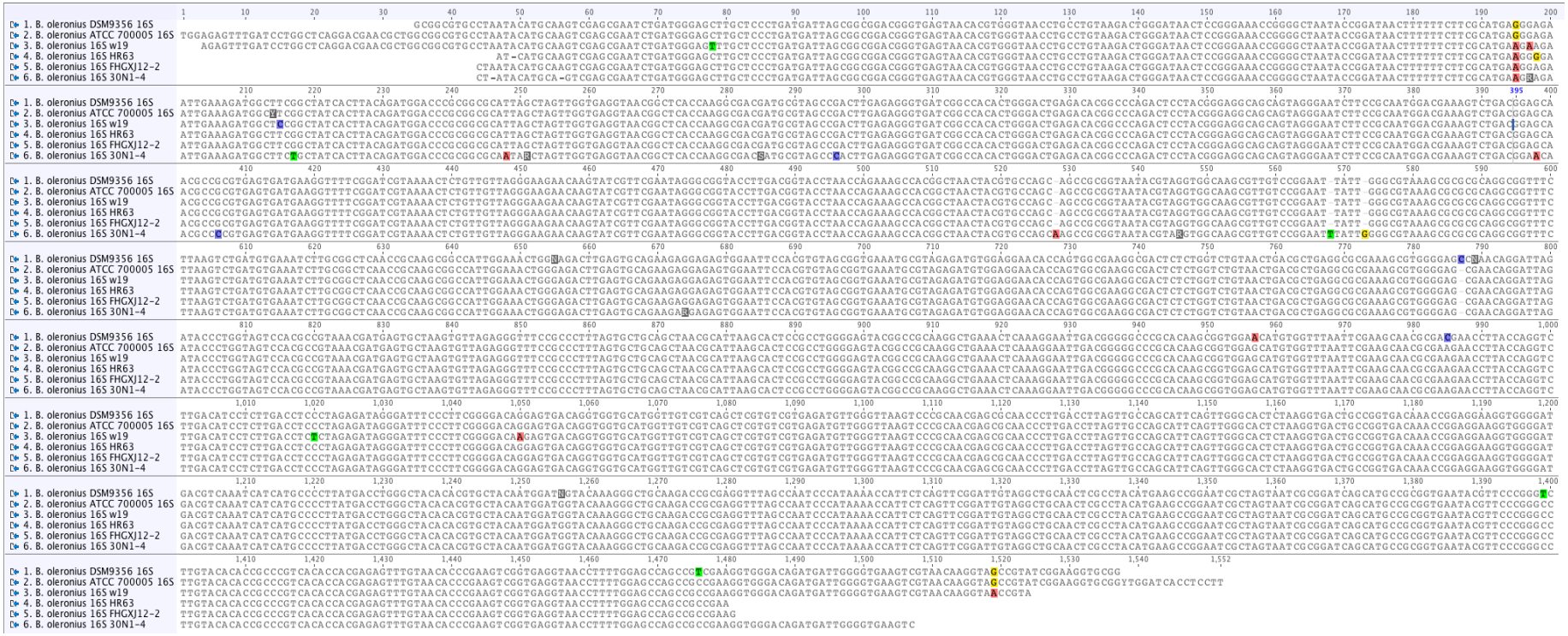


Figure 13: Geneious global multiple alignment of 16S sequences of *Bacillus oleronius* strains based on the analysis of a total of 30 strains and isolates covering the primer design regions. Only one representative of each 16S allele is shown plus the European (DSM 9356) and American (ATCC 70 005) full-length type strain sequences. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour. The Figure shows the unusual high intraspecies variation in 16S sequences that has to be catered for in primer design. There seems to be an evolutionary hotspot in the region between 190 and 200.

2.9. Preparation primers

Primers were purchased from Eurofins Genomics company. sterile and purified water was added to hidrate the lyophilezed primers in order to achaive a concentration of 100pmol/ µL as specified in the manufactures instructions.

2.10. Testing the all designed primers using Polymerase Chain Reaction (PCR)

PCR technique developed for the first time in 1983 by Kary Mullis (Rahman et al., 2013). PCR amplification was performed using the GoTaq® Flexi Master Mix (Promega) and Eppendorf Mastercycler PCR machine (Authorized Thermal cycler). Reactions were prepared according to the following parameters.

Table 14. Components of the PCR reaction mixture

Components	For 1 sample (µl
Buffer (GoTaq® Green M.M)	4.4
MgCl2	2.2
Primer F	1
Primer R	1
dNTPs	0.5
DNA template	5
dH2O	7.9
Taq polymerase	0.1
Total	20

Table 15. Thermal cycling conditions for PCR amplification

Step	Temperature	Time	Number cycles
Initial Denaturation	**95 °C**	**2 minutes**	**1 cycle**
denaturation	**95°C**	**1 minute**	**35 cycles**
Annealing	**50-61°C***	**1 minute**	
Extension	**72°C**	**1 minute**	
Final extension	**72°C**	**5 minutes**	**1 cycle**
Store	**4°C**	**Indefinite**	**1 cycle**

*annealing temperatures varied from 50°C to 61°C depending on primer sets

2.11. DNA electrophoresis samples loading

After the PCR, all the primers were ran in a 1% agarose gel (prepared as previously described) and aliquots of PCR product were loaded as 8 µl [5 µl of DNA sample with 3 µl of loading dye]. The first and the last well in the gel were loaded with1Kb DNA Ladder (Promega) and the PCR products were loaded starting from the second well. The second well was loaded with (UNF forward and UNA reverse primers), the third well with (UNC forward and UNX reverse), the forth well with (UNB forward and UNH reverse), the fifth well with (BO1 and BO2 primers), the sixth well with (Bo 16S-202FaL and Bo 16S-668R), the seventh well with (Bo 16S-202FaL and Bo 16S-684R), the eighth well with (Bo 16S-202FbL and Bo 16S-668R), the ninth well with (Bo 16S-202FbL and Bo 16S-684R), the tenth well with (Bo 16S-202FcL and Bo 16S-668R), the eleventh well with (Bo 16S-202FcL and Bo 16S-684R), the twelfth well with (Bsg recA-1F and Bsg recA-908R), the thirteenth well with (Bsg rpoB-1537F and Bsg rpoB-2440R), the fourteenth well with (Bsg gyrB-328F and Bsg gyrB-1355F), the fifteenth well with (Bsg 16SF-ITS and Bsg 23SR-ITS), the sixteenth well with (Bsg ytcP 50F and Bsg ytcP 798R) and the last well with the Ladder respectively. Next the gel was ran at 120 V for approximately 30 min followed by the acquisition of a photograph (Figure 15).

2.12. Testing the designed primers for the 16SRNA using Polymerase Chain Reaction (PCR)

PCR amplification of 16SRNA was attempted using DNA from the following strains (*Bacillus sporothermodurans, Bacillus carboniphilus, Bacillus licheniformis, Bacillus aquimaris, Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus subtilis* and *Amphibacillus tropicus*) as negative controls and Bacillus oleonius as positive control. In this experiment different melting temperatures for the new primers were tested. Firstly, the melting temperature (Tm) that used for the primer sets in this experiment was 50 °C (Table). Then the Tm for the primer sets was varied to different melting temperatures (Table 7, 8 and 9).

Table 16. Different melting temperatures for the new primers sets used in this study

Set	Primer	°C	Amplification
1	BO 16S-202FaL & BO 16S-684R	50	Unspecific
		59	Unspecific
		61	Specific
2	BO 16S-202FaL & BO 16S-668R	50	Unspecific
		59	Specific
3	BO 16S-202FbL & BO 16S-684R	50	Unspecific
		57.5	Unspecific
		59	Unspecific
		60	Unspecific
4	BO 16S-202FcL & BO 16S-668R	60	Unspecific
		61	Unspecific
5	BO 16S-202FcL & BO 16S-684R	60	Unspecific
		61	Unspecific

2.13. The phylogenetic tree of *Bacillus oleronius*

Bayesian analyses were done using MrBayes version 3.2.1 (Ronquist et al., 2012). As an evolutionary model GTR + I + Γ was chosen, which stands for General Time Reversal with a part of the sequence taken as invariable and four categories of Gamma to estimate rate variation in the other part of the sequence and unconstrained branch length as priors. The Markov chain Monte Carlo (MCMC) analysis was carried out using two independent runs with four chains each for 10,000,000 generations. Samples were taken every 5,000 generations and split frequencies calculated, a burn-in of 1,000,000 generations was applied, resulting in 1,801 trees per independent run.

For the alignment of *B. oleronius* strains only sequences longer than 1,256 nucleotides were considered; the full length of the 16S rDNA of the type strain is 1,548 bp. Structural alignment of the ribosomal sequences were accomplished with the help of Silva (high resolution ribosomal RNA databases) release 119 (24.07.2014) (Quast et al., 2013). Sequence length, sequence quality and pintail value indicative of anomalies and chimera were taken into account (Ashelford et al., 2005).

CHAPTER THREE

3. RESULTS

3.1. Comparison of DNA isolation kits

In order to determine the success of DNA extraction and to assess the quality and quantity of DNA, the total DNA concentrations obtained with the three different DNA extraction protocols (Gentra Puregene Yeast/Bact kit Gram positive protocol; Gentra Puregene Yeast/Bact kit Gram negative protocol; DNeasy ® Blood & Tissue) were measured spectrophotometry (NanoDrop 1000, Thermo Scientific) as shown in Table 17.

The results of the extracted DNA using the protocol for Gram positive bacteria yielded a low concentration of DNA in all samples (Table 17) and the extraction results with DNeasy ® Blood & Tissue Kit produced the lowest quantity of DNA (DNeasy 1, 2, 3 and 4). The samples extracted using the protocol for Gram negative bacteria yielded the highest concentrations with only one sample presenting low concentration (36.72ng/µL) (Table 17) compared to the extractions using the Gentra Puregene Yeast/Bact kit for positive bacteria and DNeasy ® Blood & Tissue Kit. However, the 260/280 ratios of samples extracted with the Gentra Puregene Yeast/Bact kit for positive bacteria indicated that the purity and quality of the extracted DNA was very low. Of the samples extracted with the Gram positive protocol, twenty-four extracts had low concentration of DNA of which 17 samples had A260/A280 ratios below 1.8 and 7 samples had A260/A280 ratios above 1.8. Similarly, for the samples extracted with the negative protocol, of the 9 samples two had A260/A280 ratios above 1.8 and 7 samples had A260/A280 ratios below 1.8. In contrast, the A260/A280 ratios for the DNeasy ® Blood & Tissue Kit samples (DNeasy 1 to 4) were higher than 1.8 (Table 17).

Regarding the 260/230 ratios, that are used to assess the purity of nucleic acid, the mean 260/230 ratios obtained with the three different DNA extraction protocols (Gentra Puregene Yeast/Bact kit Gram positive protocol; Gentra Puregene Yeast/Bact kit Gram negative protocol; DNeasy ® Blood & Tissue) was lower than the acceptable range (2.0- 2.2) as shown in table 17.

Table 17. Determination of DNA quantity and quality of samples extracted using DNeasy ® Blood & Tissue and the Gentra Puregene Yeast/Bact kit and measured with NanoDrop 1000 (Thermo Scientific).

Positive 1 to 24 represent the DNA quantity of samples extracted using the Gentra Puregene Yeast/Bact for positive bacteria. Negative 1 to 9 represent the DNA quantity of samples extracted using the Gentra Puregene Yeast/Bact for negative bacteria. And finally, DNeasy 1 to 4 represent the DNA quantity of samples extracted using DNeasy ® Blood & Tissue kit. Re-purification sample refers to the highest amount of extracted DNA using the Gram-negative protocol that was combined and extracted using the DNeasy ® Blood & Tissue kit.

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
positive1	Default	01/10/2014	12:24 ã	26.38	0.528	0.301	1.75	1.26	50.00	230	0.419	0.135
positive2	Default	01/10/2014	12:25 ã	27.34	0.547	0.315	1.74	1.77	50.00	230	0.309	0.051
positive3	Default	01/10/2014	12:25 ã	20.69	0.414	0.403	1.03	-10.01	50.00	230	-0.041	-0.161
positive4	Default	01/10/2014	12:26 ã	40.62	0.812	0.554	1.47	0.54	50.00	230	1.495	2.136
positive5	Default	01/10/2014	12:27 ã	39.88	0.798	0.574	1.39	0.52	50.00	230	1.537	1.438
positive6	Default	01/10/2014	12:28 ã	38.99	0.780	0.466	1.67	0.93	50.00	230	0.838	0.603
positive7	Default	01/10/2014	12:29 ã	23.91	0.478	0.303	1.58	0.66	50.00	230	0.726	0.478
positive8	Default	01/10/2014	12:30 ã	17.68	0.354	0.233	1.52	0.55	50.00	230	0.647	0.186
positive9	Default	01/10/2014	12:30 ã	20.98	0.420	0.377	1.11	0.52	50.00	230	0.799	14.155
positive10	Default	01/10/2014	12:31 ã	41.75	0.835	0.515	1.62	0.82	50.00	230	1.014	0.736
positive11	Default	01/10/2014	12:32 ã	12.16	0.243	0.144	1.69	1.12	50.00	230	0.216	0.203
positive12	Default	01/10/2014	12:32 ã	18.22	0.364	0.211	1.72	0.19	50.00	230	1.917	0.364
positive13	Default	01/10/2014	12:33 ã	12.02	0.240	0.155	1.56	0.62	50.00	230	0.391	0.252
positive14	Default	01/10/2014	12:34 ã	8.48	0.170	0.084	2.03	0.48	50.00	230	0.350	0.122
positive15	Default	01/10/2014	12:34 ã	12.36	0.247	0.144	1.72	0.50	50.00	230	0.496	0.313
positive16	Default	01/10/2014	12:35 ã	55.26	1.105	0.605	1.83	1.28	50.00	230	0.860	0.493
positive17	Default	01/10/2014	12:36 ã	60.74	1.215	0.693	1.75	1.34	50.00	230	0.906	0.681
positive18	Default	01/10/2014	12:37 ã	18.48	0.370	0.182	2.03	0.80	50.00	230	0.464	0.037
positive19	Default	01/10/2014	12:37 ã	4.70	0.094	0.043	2.17	0.25	50.00	230	0.381	0.018
positive20	Default	01/10/2014	12:38 ã	1.88	0.038	0.025	1.51	0.23	50.00	230	0.164	-0.001
positive21	Default	01/10/2014	12:39 ã	11.65	0.233	0.140	1.66	0.94	50.00	230	0.249	0.119
Positive22	Default	01/10/2014	12:40 ã	5.07	0.101	0.047	2.16	0.23	50.00	230	0.434	0.047
Positive23	Default	01/10/2014	12:41 ã	34.50	0.690	0.372	1.86	0.61	50.00	230	1.124	0.473
Positive24	Default	01/10/2014	12:41 ã	27.89	0.558	0.309	1.80	0.52	50.00	230	1.063	0.420
Negative1	Default	01/10/2014	12:42 ã	124.25	2.485	1.807	1.38	0.68	50.00	230	3.650	6.795
Negative2	Default	01/10/2014	12:43 ã	155.30	3.106	3.485	0.89	0.75	50.00	230	4.136	12.801
Negative3	Default	01/10/2014	12:44 ã	64.76	1.295	0.801	1.62	0.69	50.00	230	1.870	1.331
Negative4	Default	01/10/2014	12:45 ã	212.77	4.255	2.202	1.93	1.82	50.00	230	2.341	0.849
Negative5	Default	01/10/2014	12:47 ã	399.02	7.980	4.963	1.61	0.86	50.00	230	9.249	14.223
Negative6	Default	01/10/2014	12:47 ã	271.74	5.435	2.788	1.95	1.86	50.00	230	2.923	1.267
Negative7	Default	01/10/2014	12:48 ã	36.72	0.734	0.436	1.69	0.98	50.00	230	0.748	0.510
Negative8	Default	01/10/2014	12:52 ã	138.64	2.773	1.980	1.40	0.72	50.00	230	3.866	5.966
Negative9	Default	01/10/2014	12:52 ã	147.88	2.958	2.210	1.34	0.68	50.00	230	4.345	8.937
DNeasy1	Default	01/10/2014	12:54 ã	0.94	0.019	0.005	3.83	0.09	50.00	230	0.221	0.027
DNeasy2	Default	01/10/2014	12:55 ã	5.37	0.107	0.060	1.80	0.43	50.00	230	0.253	0.110
DNeasy3	Default	01/10/2014	12:55 ã	4.97	0.099	0.057	1.73	0.26	50.00	230	0.379	0.090
DNeasy4	Default	01/10/2014	12:56 ã	4.63	0.093	0.045	2.07	0.16	50.00	230	0.590	0.076
Repurification	Default	01/10/2014	12:58 ã	13.92	0.278	0.149	1.87	1.91	50.00	230	0.146	0.067

3.2. Gel preparation

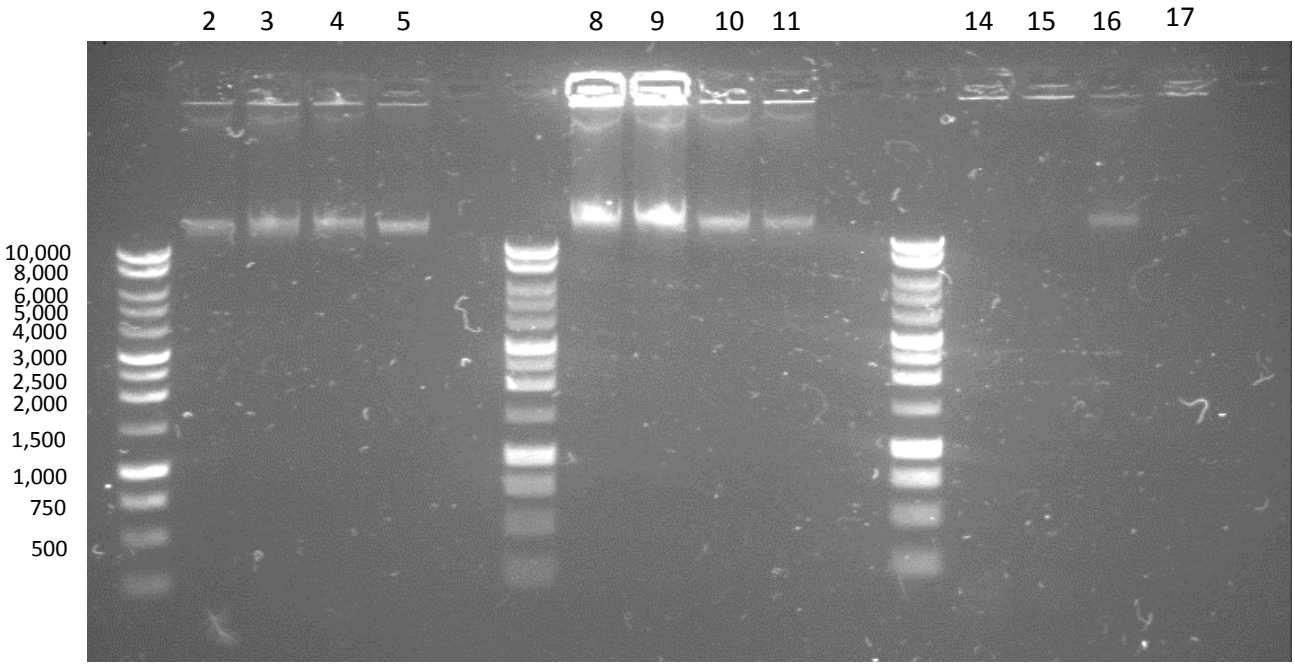


Figure 14. Agarose gel electrophoresis of 12 DNA samples extracted using the Gentra Puregene Yeast/Bact kit for negative bacteria. Lane 1, 7 and 13 1kb DNA Ladder, Lane 2, 3, 4, 5, 8, 9, 10, 11, 14, 15, 16, 17, DNA samples of different concentrations (217, 240, 282, 39, 176, 140, 102, 114, 53, 54, 47, 61 ng/µL respectively).

This figure represents the result after agarose gel electrophoresis of DNA extractions. After separation, the DNA fragments are detectable as obviously clear bands. Most the samples looked intact and undegraded. The first four lanes were separated on the agarose gel clearly and no smear or degradation was detected. Because of using different concentrations of DNA, it was noticeable that the DNA fragments were different in size along the agarose gel. As shown in this figure, the middle lanes contained most of the DNA and the bands looked more intensive while the last lanes showed no bands only one faint band was observed in the lane 16.

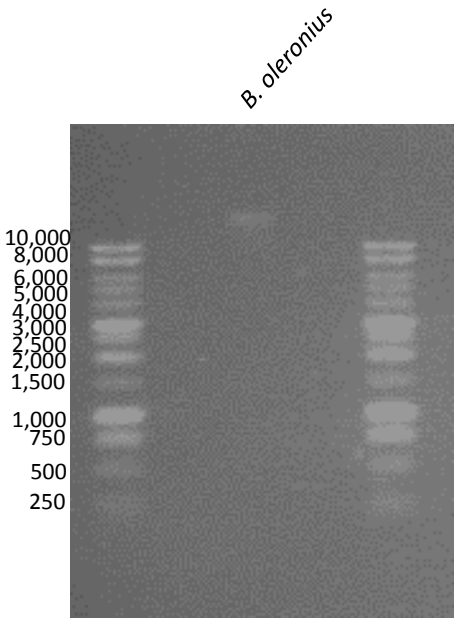


Figure 15. Agarose gel electrophoresis of *B. oleronius* sample extracted from the Gentra Puregene Yeast/Bact kit for negative bacteria and re purified with DNeasy ® Blood & Tissue Kit.

Figure 15 shows a band of *B. oleronius* resulting from the re-purification method, where three samples extracted with the Gentra Puregene Yeast/Bact kit for negative bacteria were combined and re-extracted using DNeasy ® Blood & Tissue Kit. The band clearly shows that the resulting extraction produced a very low amount of DNA.

Table 18. Sequencing universal primers used in this study

Gene	Primer name	Sequence 5'→3'	Tm [^{0}C]	GC content
rpoB	UNF	GARGTNCGWGACGTBCACTA	58.7	53.3 %
rpoB	UNA	TGGGHGCRAACATGCARCG	59.5	59.6 %
rpoB	UNC	TTCARTAYGGACGHCACC	54.4	51.8 %
rpoB	UNX	ARGTNCGWGACGTBCACTA	55.9	50.8 %
rpoB	UNB	GGGHGCRAACATGCARCGB	61.0	63.1 %
rpoB	UNH	GNGARATGGARGTWTGGGC	57.7	55.3 %

3.3. Design group specific primers

Table 19. Sequencing group specific primers used in this study

Gene name	Primer name	Primer sequence 5'→3'	Tm °C	GC content %	Vol. for 100pmol/µL
16 rRNA	BO1	5'-AACGGCTCACCAAGGCGACG-3'	63.5	65%	303
16 rRNA	BO2	5'-TCCGGACAACGCTTGCCACC-3'	63.5	65%	300
16 rRNA	BO 16S-202FaL	AACTTTTTTCTTCGCATGARGGAGAATTG	61.7	36.2%	350
16 rRNA	BO 16S-202FbL	AACTTTTTTCTTCGCATGAAGAAGAATTG	59.6	31%	344
16 rRNA	BO 16S-202FcL	AACTTTTTTCTTCGCATGAAGGGGAATTG	62.4	37.9%	333
16 rRNA	BO 16S-668R	5'-GCCGCTTGCGGTTGAGCCGCAAGA-3'	69.5	66.7%	273
16 rRNA	BO 16S-684R	5'-CTCCCAGTTTCCAATGGCCGCTTG-3'	66.1	58.3%	347
recA	Bsg recA-1F	5'-ATGAGTGATCGTCAGGCAGCC-3'	61.8	57.1%	330
recA	Bsg recA-908R	5'-GCRTTTTCACGGCCYTGDCC -3'	62.1	61.6%	349

rpoB	Bsg rpoB-1537F	5'-CGRATGTGTCCGATTGAAAC-3'	56.3	47.5%	293
rpoB	Bsg rpoB-2440R	5'-GAATATCRCGRGTGATYTCTTC-3'	57.5	43.2%	302
gyrB	Bsg gyrB-328F	5'-GACGGHRGCGGHTATAAAGT-3'	57.6	50.8%	313
gyrB	Bsg gyrB-1355R	5'-GGYRRAATVGCYTGGAAATG-3'	56.6	48.3%	231
ITS	Bsg 16SF-ITS	5'-TCGCTAGTAATCGCGGATAGGC-3'	62.1	54.5%	268
ITS	Bsg 23SR-ITS	5'-GCATATCGGTGTTMGTCCCGTCC-3'	65.1	58.7%	253
ytcP	Bsg ytcP 50F	5'-ATAAAYGGATASACRAGAG-3'	53.2	40%	252
ytcP	Bsg ytcP 798R	5'-GTDCTKCCGTTCATYCAYGT-3'	57.0	49.1%	374

3.4. Polymerase Chain Reaction (PCR)

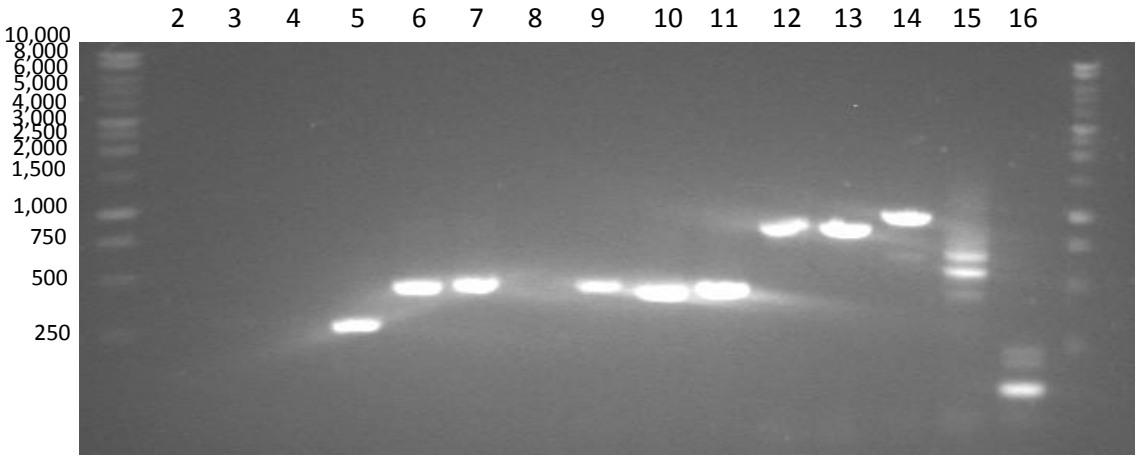


Figure 16. PCR amplification of DNA extracted from 14 samples of *B. oleronius* using universal primers and species-specific primers. Lane 1 and 17 represent 1 kb Ladder marker, lane 2 (UNF forward and UNA reverse), lane 3 (UNC forward and UNX reverse), lane 4 (UNB forward and UNH reverse), lane 5 (BO1 and BO2 primers), lane 6 (Bo 16S-202FaL and Bo 16S-668R), lane 7 (Bo 16S-202FaL and Bo 16S-684R), lane 8 (Bo 16S-202FbL and Bo 16S-668R), lane 9 (Bo 16S-202FbL and Bo 16S-684R), lane 10 (Bo 16S-202FcL and Bo 16S-668R), lane 11 (Bo 16S-202FcL and Bo 16S-684R), lane 12 (Bsg recA-1F and Bsg recA-908R), lane 13 (Bsg rpoB-1537F and Bsg rpoB-2440R), lane 14 (Bsg gyrB-328F and Bsg gyrB-1355F), lane 15 (Bsg 16SF-ITS and Bsg 23SR-ITS), lane 16 (Bsg ytcP 50F and Bsg ytcP 798R). PCR products were electrophoresed on agarose gel (0.8%).

As seen in this figure 11 out of 15 primer sets produced bands. The first four lanes denote universal primers and these were designed to amplify *rpoB* gene only while the species-specific primers were started from lane 5 to 16 to amplify various genes (Table 19). Lane 2, 3 and 4 were amplified using primer sets UNF forward and UNA reverse, UNC forward and UNX reverse, UNB forward and UNH reverse, respectively. Lane 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16 were amplified using species-specific primer sets (BO1 and BO2 primers, Bo 16S-202FaL and Bo 16S-668R, Bo 16S-202FaL and Bo 16S-684R, Bo 16S-202FbL and Bo 16S-668R, Bo 16S-202FbL and Bo 16S-684R, Bo 16S-202FcL and Bo 16S-668R, Bo 16S-202FcL and Bo 16S-684R, Bsg recA-1F and Bsg recA-908R, Bsg rpoB-1537F and Bsg rpoB-2440R, Bsg gyrB-328F and Bsg gyrB-1355F, Bsg 16SF-ITS and Bsg 23SR-ITS, Bsg ytcP 50F and Bsg ytcP 798R) respectively. PCR amplified product yielded clear bands with different intensities on the gel in all the lanes except lane 2, 3, 4, and 8.

No bands were detected in lanes 2, 3 and 4 and 8 corresponding to the lack of amplification product using primers UNF forward and UNA reverse), (UNC forward and UNX reverse), (UNB forward and UNH reverse), (Bo 16S-202FbL and Bo 16S-668R) respectively.

It is worth mentioned that the three last lanes produce more than one band. The primer set (Bsg gyrB-328F and Bsg gyrB-1355F) in lane 14 produce several products of size (700, 600 and 450bp) and the primer sets (Bsg 16SF-ITS and Bsg 23SR-ITS) in lane 15 produce three bands of sizes (700, 500 and 400bp) while the primer set (Bsg ytcP 50F and Bsg ytcP 798R) in lane 16 produce three bands of size (300, 250 and 100bp).

3.5. Testing the designed primers for the 16SRNA using Polymerase Chain Reaction (PCR)

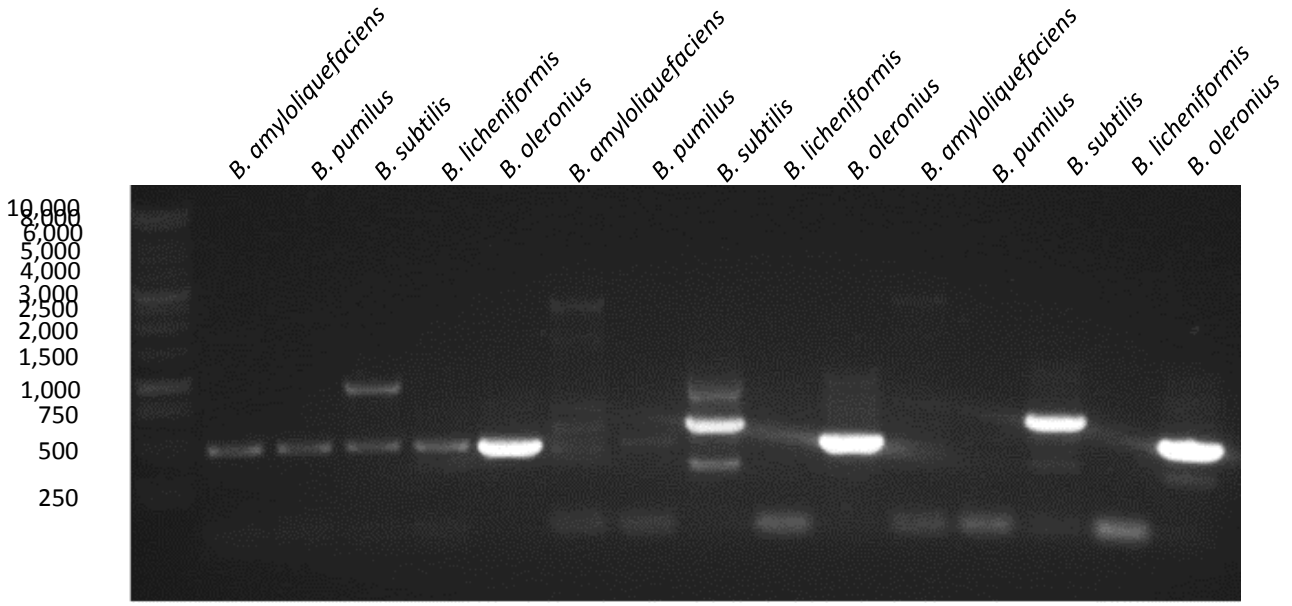


Figure 17. Agarose gel electrophoresis of PCR amplification products obtained from different primer sets. Lane 1 represent 1 kb Ladder marker, lane 2 (*B. amyloliquefaciens*), lane 3 (*B. pumilus*), lane 4 (*B. subtilis*), lane 5 (*B. licheniformis*), lane 6 (*B. oleronius*) using primer set (Bo 16S-202FaL and Bo 16S-668R), lane 7 (*B. amyloliquefaciens*), lane 8 (*B. pumilus*), lane 9 (B. subtilis), lane 10 (*B. licheniformis*), lane 11 (*B. oleronius*) using primer set (Bo 16S-202FaL and Bo 16S-684R), lane 12 (*B. amyloliquefaciens*), lane 13 (*B. pumilus*), lane 14 (*B. subtilis*), lane 15 (*B. licheniformis*), lane 16 (*B. oleronius*) using primer set (Bo 16S-202FbL and Bo 16S-684R).

It can be cleary seen that all the primers sets in this figure amplified non- specific DNA fragments. Bright bands of fragment size 467bp for primer set (Bo 16S-202FaL and Bo 16S-668R) and 467bp for primer set (Bo 16S-202FbL and Bo 16S-684R) have been detected for *B. oleronius*. The PCR amplification has been performed using low annealing temperature of (Ta) 50°C.

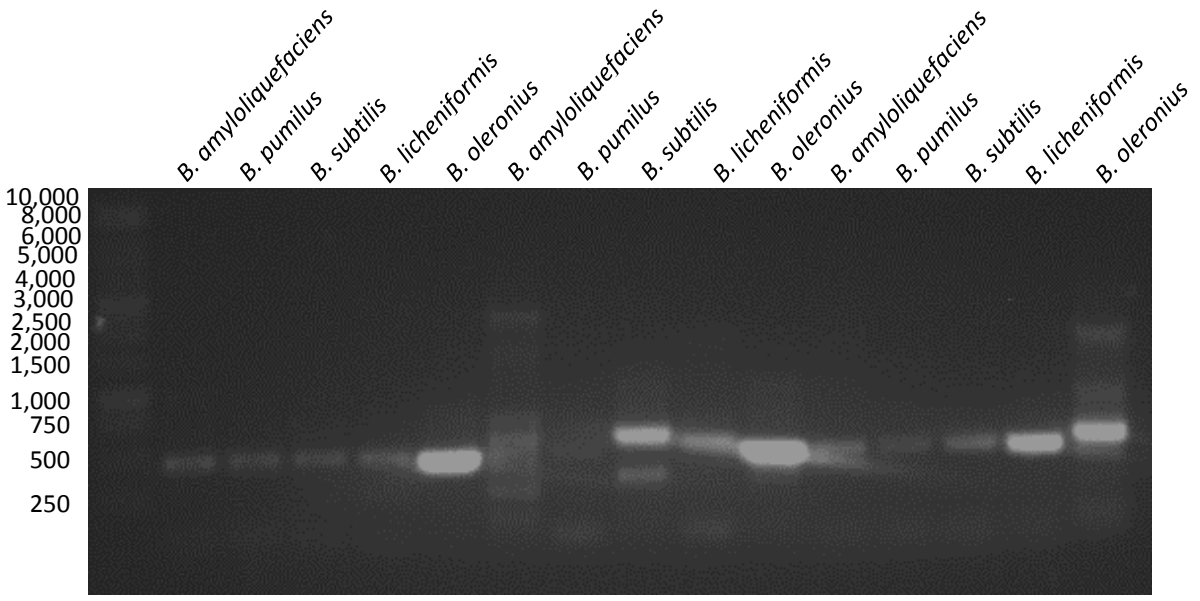


Figure 18. Agarose gel electrophoresis of PCR amplification products obtained different primer sets. Lane 1 represent 1 kb Ladder marker, lane 2 (*B. amyloliquefaciens*), lane 3 (*B. pumilus*), lane 4 (*B. subtilis*), lane 5 (*B. licheniformis*), lane 6 (*B. oleronius*) using primer set (Bo 16S-202FcL and Bo 16S-668R), lane 7 (*B. amyloliquefaciens*), lane 8 (*B. pumilus*), lane 9 (B. subtilis), lane 10 (*B. licheniformis*), lane 11 (*B. oleronius*) using primer set (Bo 16S-202FcL and Bo 16S-684R), lane 12 (*B. amyloliquefaciens*), lane 13 (*B. pumilus*), lane 14 (*B. subtilis*), lane 15 (*B. licheniformis*), lane 16 (*B. oleronius*) using primer set (Bo 16S-202FaL and Bo 16S-668R).

This fighure show that all primers sets amplified non- specific DNA fragments. Bright bands of 500 bp were detected for *B. oleronius*. The PCR amplification was performed using low annealing temperature of 50°C.

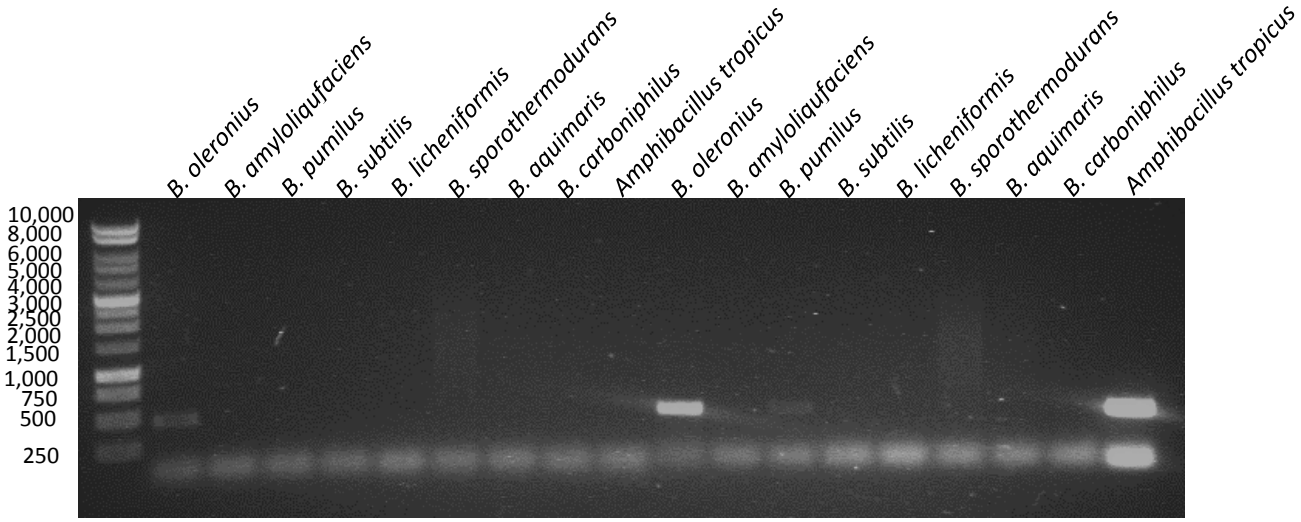


Figure 19. Agarose gel electrophoresis of PCR amplification products obtained from different primer sets. Lane 1 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B. sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*), lane 10 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FaL and Bo 16S-668R), lane 11 (*B. oleronius*), lane 12 (*B. amyloliquefaciens*), lane 13 (*B. pumilus*), lane 14 (*B. subtilis*), lane 15 (*B. licheniformis*), lane 16 (*B. sporothermodurans*), lane 17 (B. aquimaris), lane 18 (*B. carboniphalius*) and lane 19 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FaL and Bo 16S-668R).

This figure represents the effect of increasing the annealing temperature from 50°C to 59°C on PCR specificity. The first primer set (Bo 16S-202FaL and Bo 16S-668R) in the first lanes (2, 3, 4, 5, 6, 7, 8, 9 and 10 respectively) produced a bright band of 467bp for *B. oleronius* only (lane 2) while the second primer set (Bo 16S-202FaL and Bo 16S-668R) amplified a 483bp fragment of both of *B. oleronius* and *Amphibacillus. tropicus.*

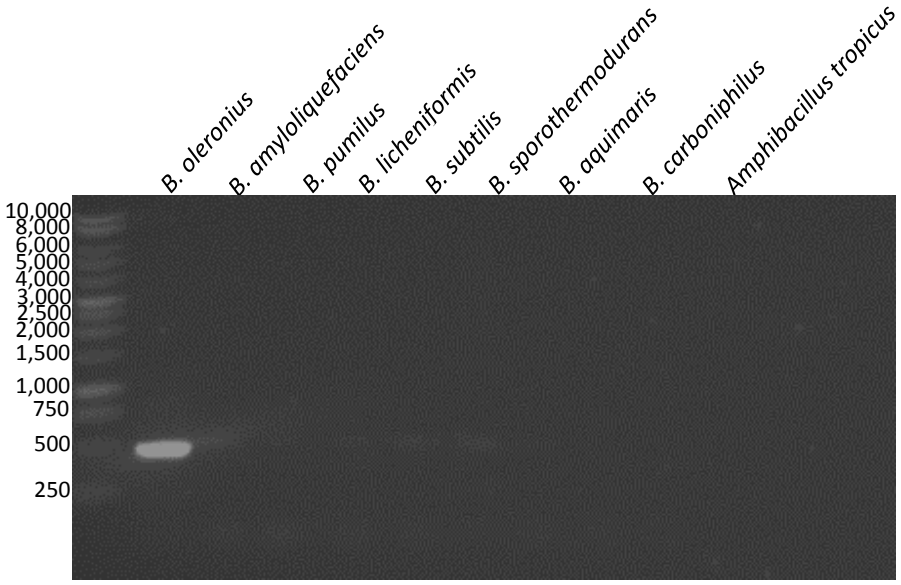


Figure 20. Agarose gel electrophoresis of PCR amplification products using primer set (Bo 16S-202FbL and Bo 16S-684R). Lane 1 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*) and lane 10 (*Amphibacillus. tropicus*).

In figure 20 it can be seen that the primer set (Bo 16S-202FbL and Bo 16S-684R) were bound nonspecifically since there was amplification for *B. oleronius* and of some of the negative controls *B. amyloliquefaciens*, *B. pumilus*, *B. subtilis*, *B. licheniformis* and *B. sporothermodurans*. The PCR amplification in this experiment was performed using an annealing temperature of 57.5°C.

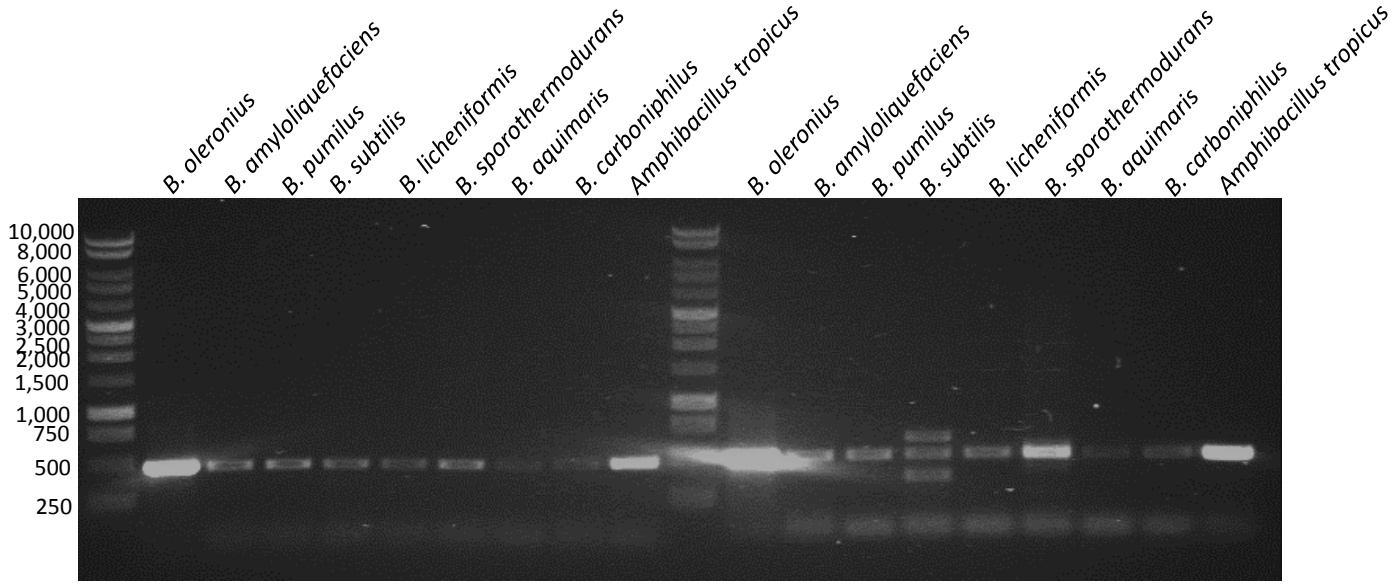


Figure 21. Agarose gel electrophoresis of PCR amplification products obtained different primer sets. Lane 1 and 11 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*), lane 10 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FcL and Bo 16S-668R), lane 12 (*B. oleronius*), lane 13 (*B. amyloliquefaciens*), lane 14 (*B. pumilus*), lane 15 (*B. subtilis*), lane 16 (*B. licheniformis*), lane 17 (*B.sporothermodurans*), lane 18 (B. aquimaris), lane 19 (*B. carboniphalius*) and lane 20 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FcL and Bo 16S-684R).

It can be cleary seen that all the primers sets (Bo 16S-202FcL and Bo 16S-668R) and (Bo 16S-202FcL and Bo 16S-684R) in this figure amplified non-specific DNA fragments as well as to *B. oleronius* showing various bands on agarose gel. The PCR amplification in this experiment was performed using an annealing temperature of 60°C.

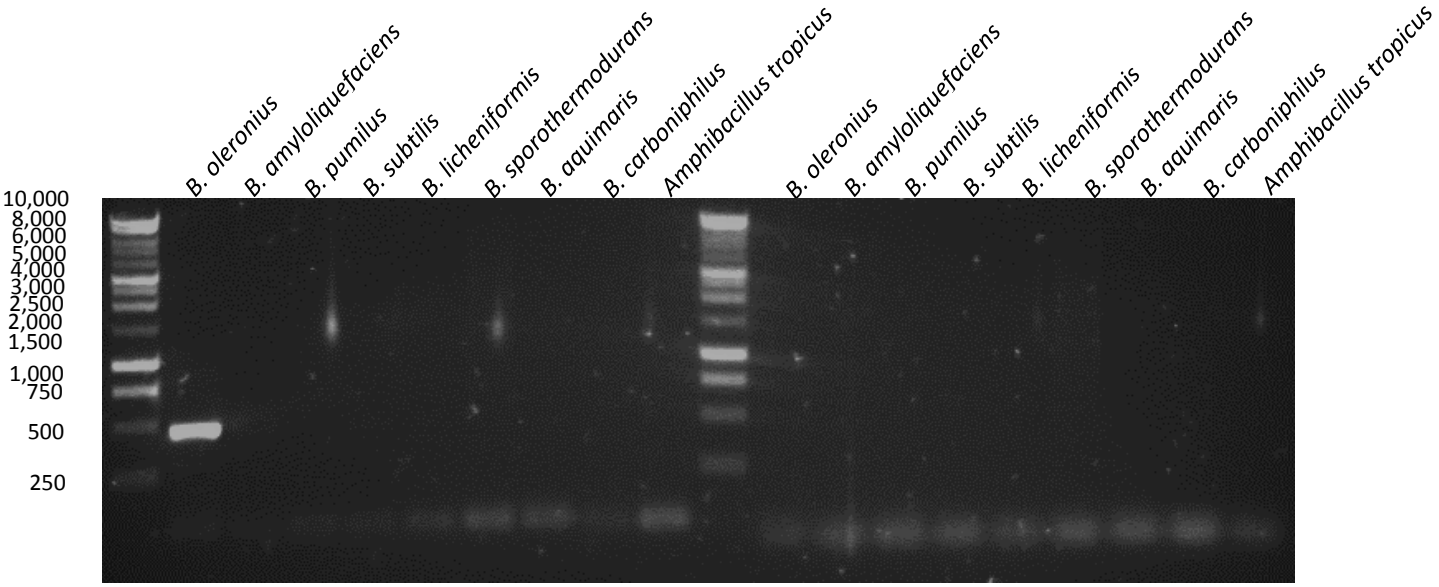


Figure 22. Agarose gel electrophoresis of PCR amplification products obtained different primer sets. Lane 1 and 11 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*), lane 10 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FaL and Bo 16S-684R), lane 12 (*B. oleronius*), lane 13 (*B. amyloliquefaciens*), lane 14 (*B. pumilus*), lane 15 (*B. subtilis*), lane 16 (*B. licheniformis*), lane 17 (*B.sporothermodurans*), lane 18 (B. aquimaris), lane 19 (*B. carboniphalius*) and lane 20 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FcL and Bo 16S-668R).

This figure shows that increasing the annealing temperature from 59°C to 61°C for primer set (Bo 16S-202FaL and Bo 16S-684R) in the first lanes (2, 3, 4, 5, 6, 7, 8, 9, 10) specifically amplified a 483bp fragments of *B. oleronius*. The second primer set (Bo 16S-202FcL and Bo 16S-668R) from lane 12 to 20 showed that these primers did not amplified anything and that changing the temperature to 61°C did not result in any amplification (specific or non-specific).

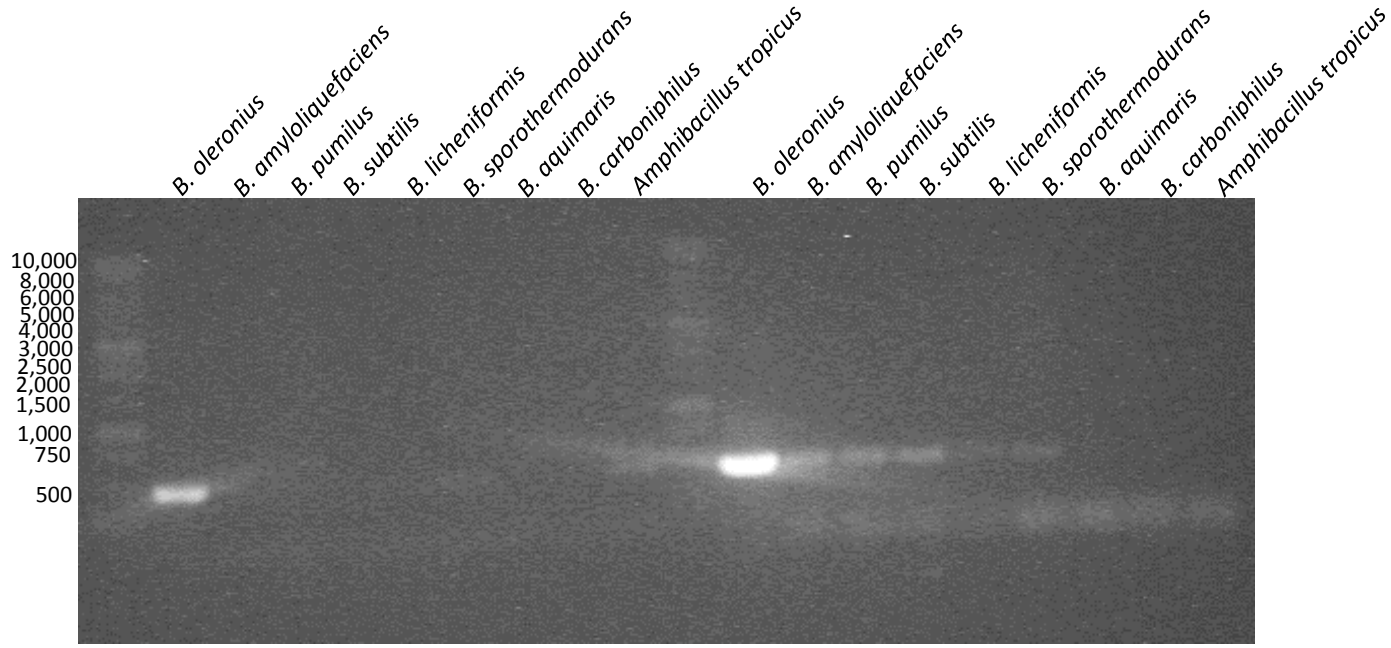


Figure 23. Agarose gel electrophoresis of PCR amplification products obtained different primer sets. Lane 1 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*), lane 10 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FcL and Bo 16S-684R), lane 11 ladder marker, lane 12 (*B. oleronius*), lane 13 (*B. amyloliquefaciens*), lane 14 (*B. pumilus*), lane 15 (*B. subtilis*), lane 16 (*B. licheniformis*), lane 17 (*B. sporothermodurans*), lane 18 (*B. aquimaris*), lane 19 (*B. carboniphalius*) and lane 20 (*Amphibacillus. tropicus*) using primer set (Bo 16S-202FcL and Bo 16S-668R).

The result in this figure is similar to the one in Figure 22. Although, for the first primer set (Bo 16S-202FcL and Bo 16S-684R) only one band seems to be visible, there was in fact unspecific amplification for both *B. sporothermodurans* and *Amphibacillus. tropicus.* There are two very faint bands of 500bp. The second primer set (Bo 16S-202FcL and Bo 16S-668R) clearly amplified nonspecifically since there was amplification for *B. oleronius* and to some of the bacterial species (*B. amyloliquefaciens*), (*B. pumilus*), (*B. subtilis*), (*B. licheniformis*), (*B. sporothermodurans*), (*B. aquimaris*), (*B. carboniphalius*) and (*Amphibacillus. tropicus*). The PCR amplification in this experiment was performed using an annealing temperature of 61°C.

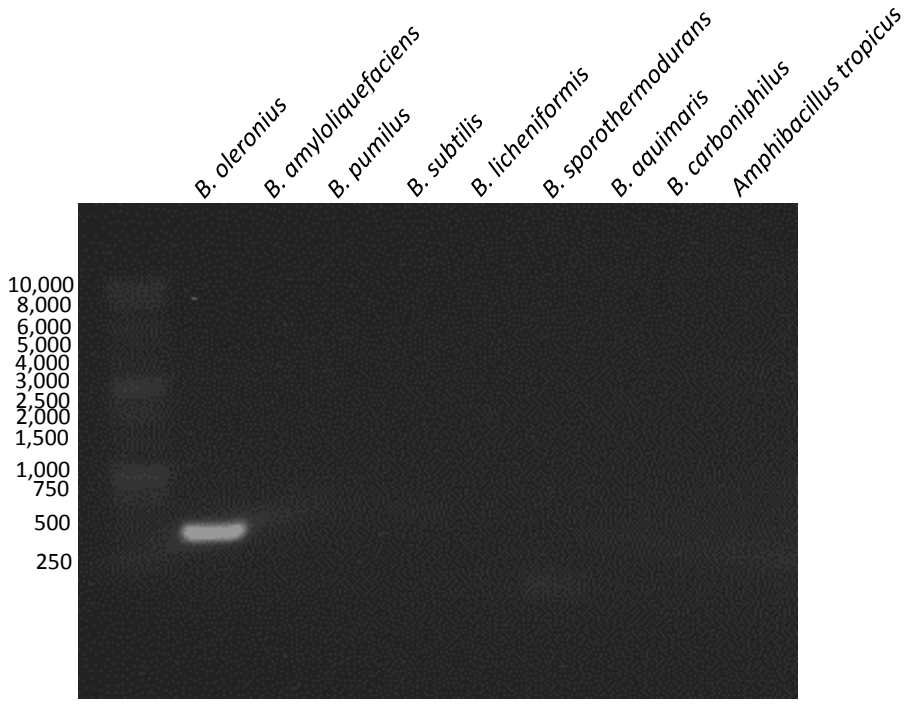


Figure 24. Agarose gel electrophoresis of PCR amplification products using primer set (Bo 16S-202FbL and Bo 16S-684R). Lane 1 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*) and lane 10 (*Amphibacillus. tropicus*).

It can be unmistakably seen that for the primer set (Bo 16S-202FbL and Bo 16S-684R) changing the temperature from 57.5°C to 61°C improved its specificity. However, extremely faint signals were also detected for *B. subtilis* and *B.sporothermodurans,* and therefore this primer set cannot be considered specific.

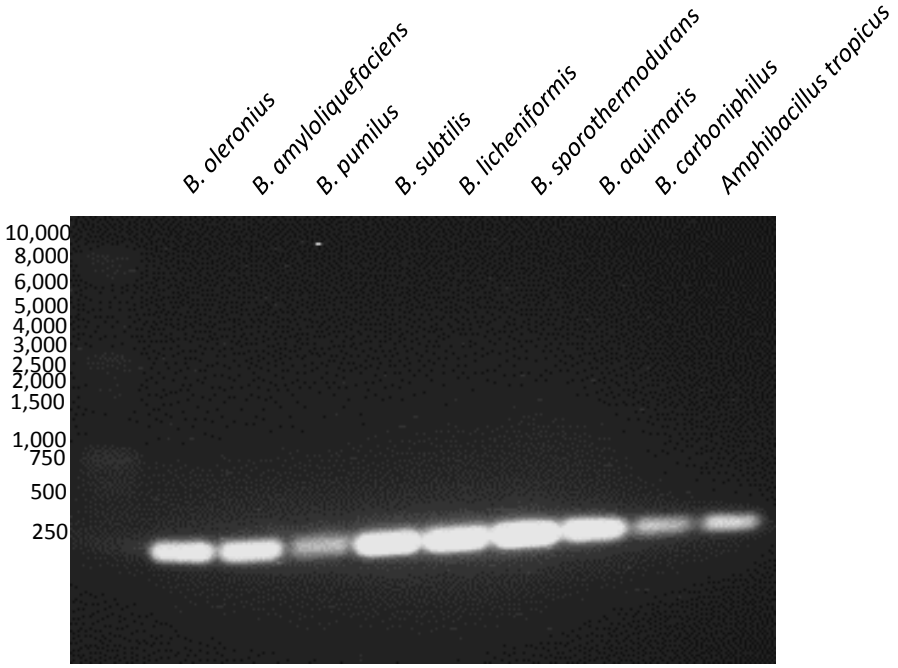


Figure 25. Agarose gel electrophoresis of PCR amplification products using primer set (BO1 and BO2) published by Szkaradkiewicz et al., 2012. Lane 1 represent 1 kb Ladder marker, lane 2 (*B. oleronius*), lane 3 (*B. amyloliquefaciens*), lane 4 (*B. pumilus*), lane 5 (*B. subtilis*), lane 6 (*B. licheniformis*), lane 7 (*B.sporothermodurans*), lane 8 (*B. aquimaris*), lane 9 (*B. carboniphalius*) and lane 10 (*Amphibacillus. tropicus*).

This figure shows the bands that resulted from the PCR amplification using primer set (BO1 and BO2) published by Szkaradkiewicz et al., 2012 who claimed that these were species-specific for *B. oleronius.* However, the result in this figure shows the opposite where it can be seen that these primers amplified non-specifically fragments of varying sizes of all bacillus species.

3.6. Phylogenetic analysis

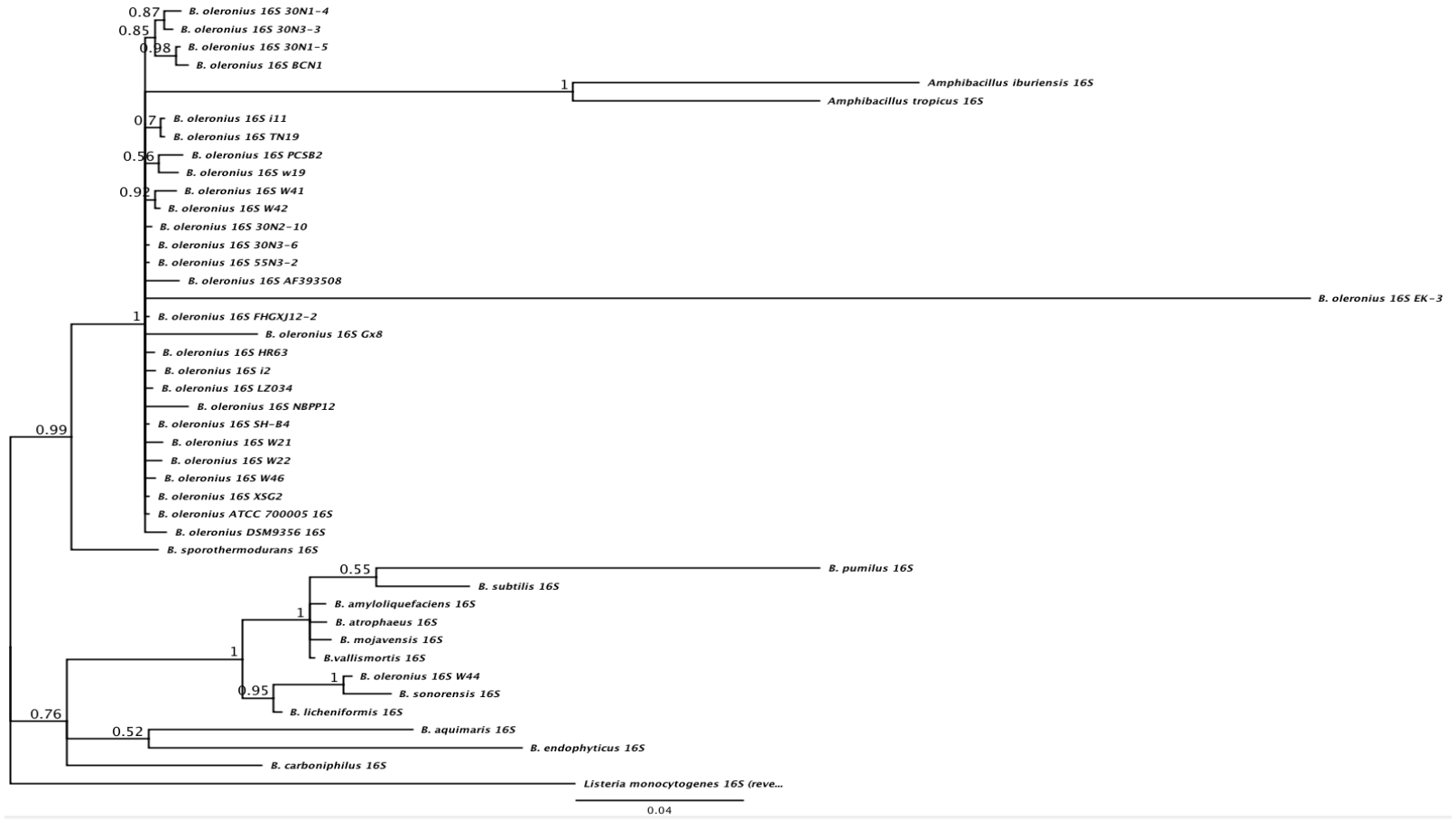


Figure 27: Phylogenetic tree of 16S rDNA of *Bacillus oleronius* strains and species of the *Bacillus subtilis* group. Geneious alignment and Bayesian analysis with *Listeria monocytogenes* as outgroup. Posterior probability is given at nodes. Scale bare denotes substitutions per nucleotide. An average standard deviation of split frequencies of 0.020625 was reached. Mean likelihood score for the tree is – lnL = 7,589.752 (for run 1: – lnL = 7,589.564 ± 0.357; run 2: – lnL = 7,589.939 ± 0.395).

The tree shows that all but one strain of *B. oleronius* form a single clade with maximum support. ATCC and DSM are type strains of *B. oleronius*. The genetically closest species to *B. oleronius* is *B. sporothermodurans*. Two *Amphibacillus* species arise from within the *B. oleronius* clade. *B. oleronius* strain EK-3 shows a great difference to other *B. oleronius* strains. Strain W44 clusters with maximum support with *B. sonorensis.*

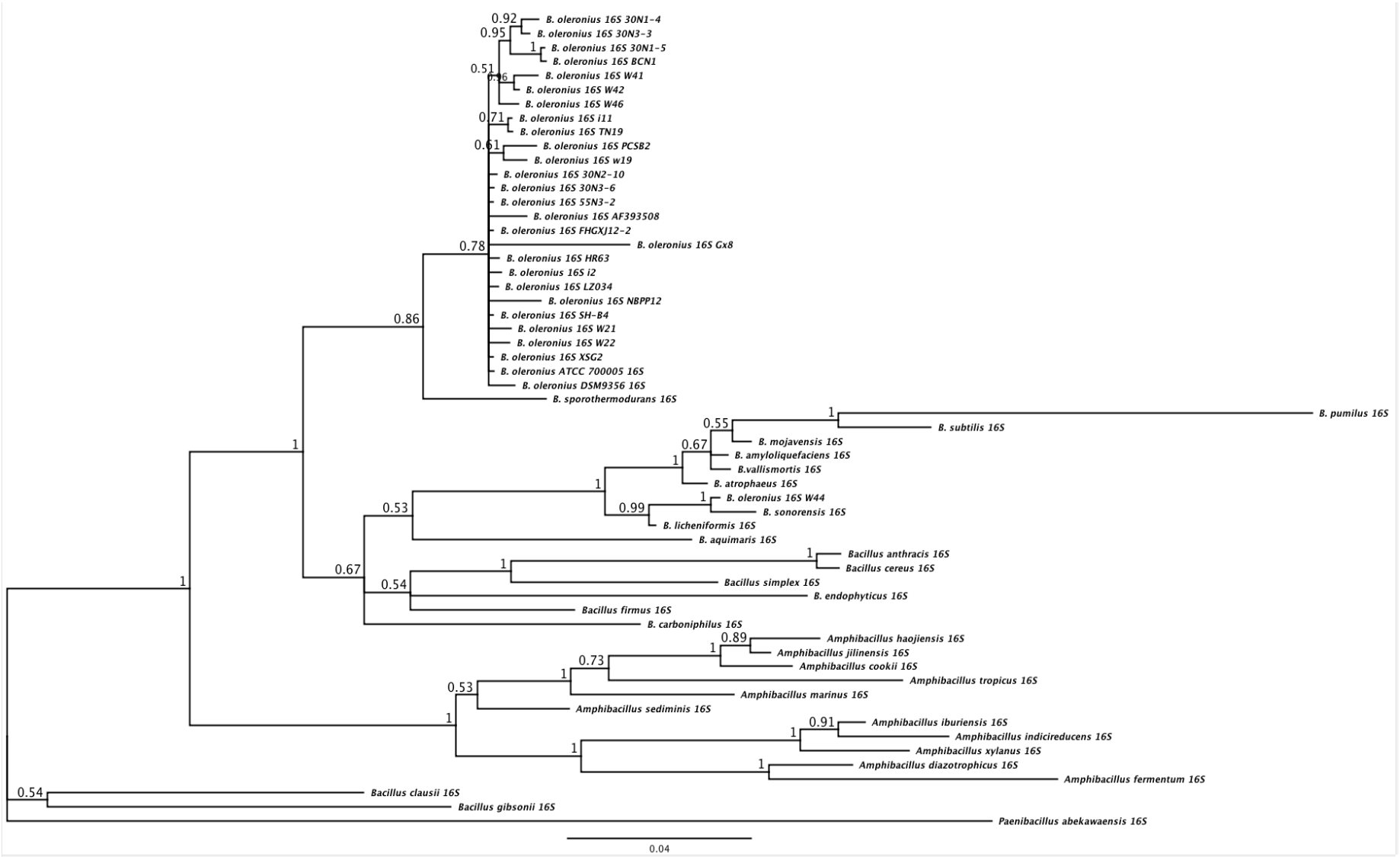

B. oleronius 16S 30N1-4
B. oleronius 16S 30N3-3
B. oleronius 16S 30N1-5
B. oleronius 16S BCN1
B. oleronius 16S W41
B. oleronius 16S W42
B. oleronius 16S W46
B. oleronius 16S i11
B. oleronius 16S TN19
B. oleronius 16S PCSB2
B. oleronius 16S w19
B. oleronius 16S 30N2-10
B. oleronius 16S 30N3-6
B. oleronius 16S 55N3-2
B. oleronius 16S AF393508
B. oleronius 16S FHGXJ12-2
B. oleronius 16S Gx8
B. oleronius 16S HR63
B. oleronius 16S i2
B. oleronius 16S LZ034
B. oleronius 16S NBPP12
B. oleronius 16S SH-B4
B. oleronius 16S W21
B. oleronius 16S W22
B. oleronius 16S XSG2
B. oleronius ATCC 700005 16S
B. oleronius DSM9356 16S
B. sporothermodurans 16S
B. pumilus 16S
B. subtilis 16S
B. mojavensis 16S
B. amyloliquefaciens 16S
B.vallismortis 16S
B. atrophaeus 16S
B. oleronius 16S W44
B. sonorensis 16S
B. licheniformis 16S
B. aquimaris 16S
Bacillus anthracis 16S
Bacillus cereus 16S
Bacillus simplex 16S
B. endophyticus 16S
Bacillus firmus 16S
B. carboniphilus 16S
Amphibacillus haojiensis 16S
Amphibacillus jilinensis 16S
Amphibacillus cookii 16S
Amphibacillus tropicus 16S
Amphibacillus marinus 16S
Amphibacillus sediminis 16S
Amphibacillus iburiensis 16S
Amphibacillus indicireducens 16S
Amphibacillus xylanus 16S
Amphibacillus diazotrophicus 16S
Amphibacillus fermentum 16S
Bacillus clausii 16S
Bacillus gibsonii 16S
Paenibacillus abekawaensis 16S
0.04

Figure 28: Phylogenetic tree of 16S rDNA of *Bacillus oleronius* strains and *Bacillus* species. Geneious alignment and Bayesian analysis with *Paenibacillus abekawaensis* as outgroup. Posterior probability is given at nodes. Scale bare denotes substitutions per nucleotide. An average standard deviation of split frequencies of 0.010495 was reached. Mean likelihood score for the tree is – lnL = 9,225.912 (for run 1: – lnL = 9,226.751 ± 0.404; run 2: – lnL = 9,225.074 ± 0.421).

The tree shows that the strains of *B. oleronius* are monophyletic. ATCC and DSM are type strains of *B. oleronius*. The genetically closest species to *B. oleronius* is still *B. sporothermodurans*. *Amphibacillus* species are not part of the *B. oleronius* clade. The genus *Bacillus* is paraphyletic, meaning that *Bacillus* species have more than one common ancestor. The support for some species like *B. endophyticus* and *B. carboniphilus* considered to be part of *B. subtilis* group is missing. Equally, the support that *B. oleronius* and *B. sporothermodurans* are part of a *B. subtilis* clade is missing.

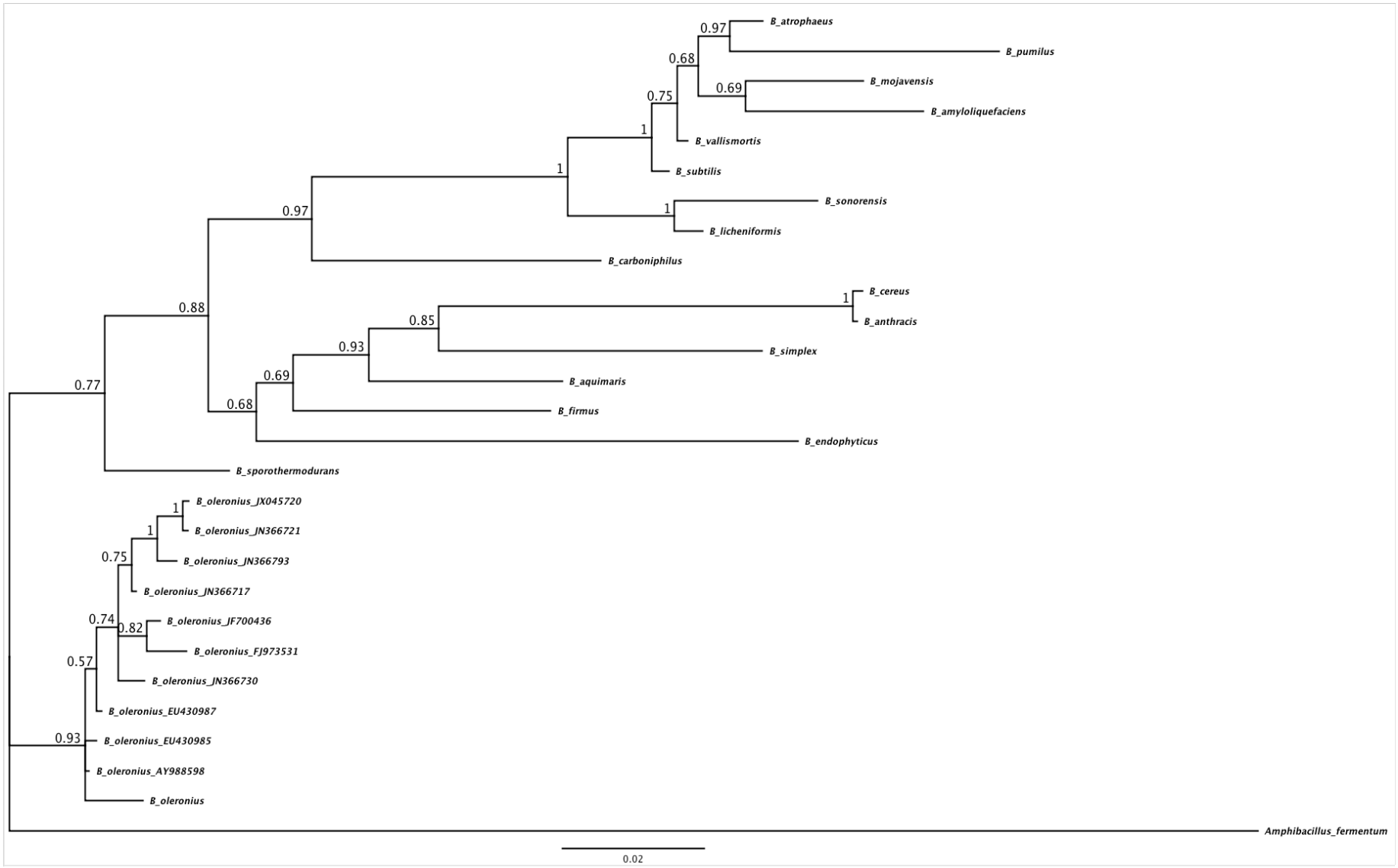
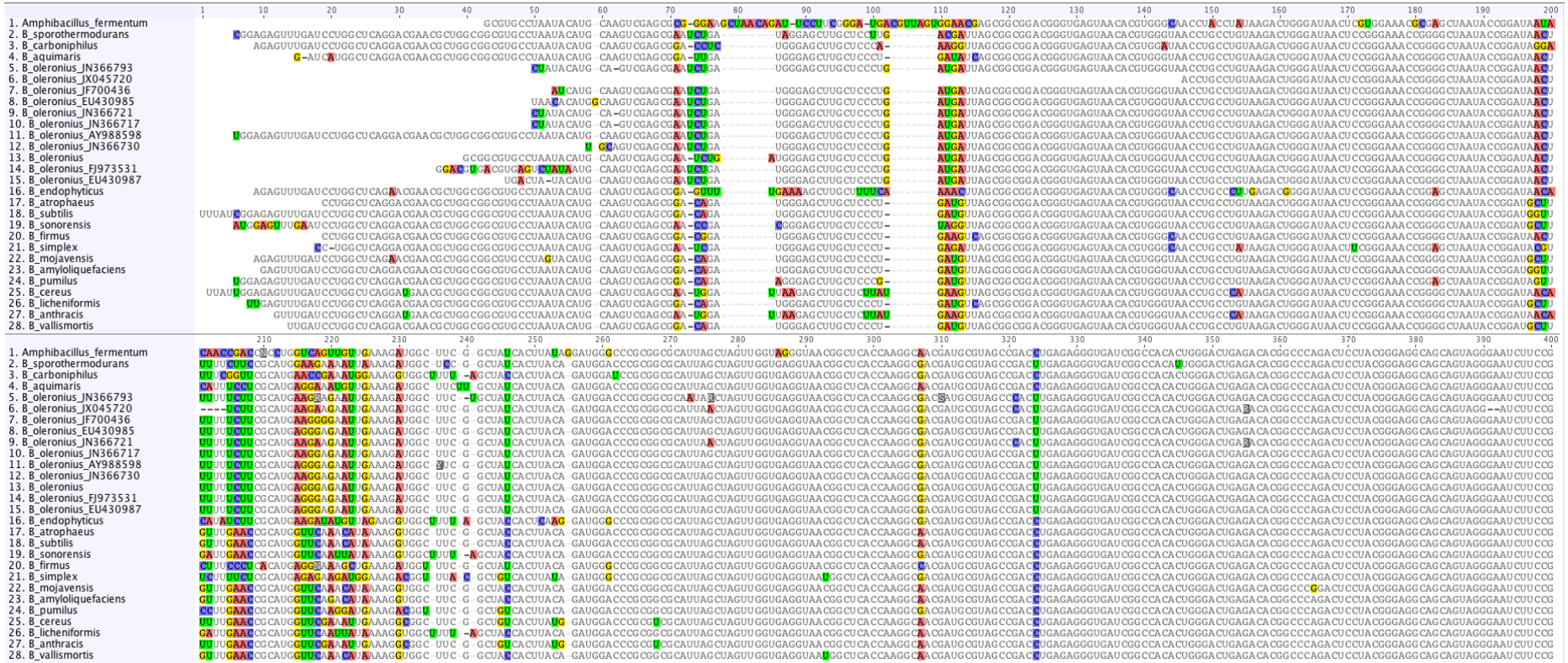

B_atrophaeus
B_pumilus
B_mojavensis
B_amyloliquefaciens
B_vallismortis
B_subtilis
B_sonorensis
B_licheniformis
B_carboniphilus
B_cereus
B_anthracis
B_simplex
B_aquimaris
B_firmus
B_endophyticus
B_sporothermodurans
B_oleronius_JX045720
B_oleronius_JN366721
B_oleronius_JN366793
B_oleronius_JN366717
B_oleronius_JF700436
B_oleronius_FJ973531
B_oleronius_JN366730
B_oleronius_EU430987
B_oleronius_EU430985
B_oleronius_AY988598
B_oleronius
Amphibacillus_fermentum
0.97
0.68
0.69
0.75
1
1
0.97
1
0.88
0.85
1
0.93
0.69
0.77
0.68
1
1
0.75
0.74
0.82
0.57
0.93
0.02

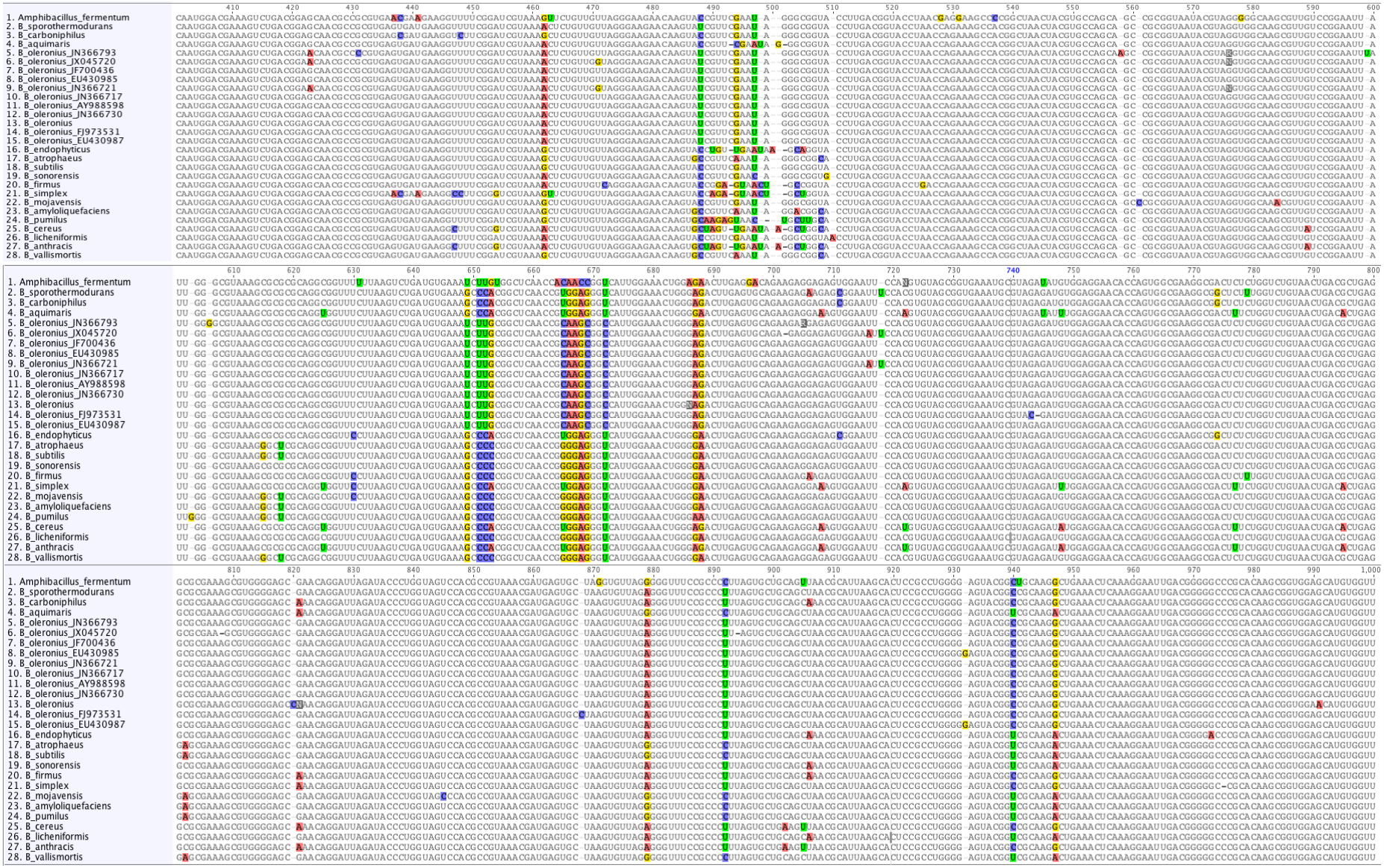
Figure 29: Phylogenetic tree of 16S rDNA of selected *Bacillus oleronius* strains and *Bacillus* species. Alignment based on ribosomal secondary structure and Bayesian analysis with *Amphibacillus fermentum* as outgroup. Posterior probability is given at nodes. Scale bare denotes substitutions per nucleotide. An average standard deviation of split frequencies of 0.006467 was reached; a value below 0.05 indicates a good analysis, a value below 0.01 indicates an excellent analysis. Mean likelihood score for the tree is – lnL = 5,775.038 (for run 1: – lnL = 5,775.188 ± 0.18; run 2: – lnL = 5,774.889 ± 0.169).

The tree shows that the strains of *B. oleronius* are monophyletic. The *B. oleronius* clade is next to all other *Bacillus* species. A genetically closest species to *B. oleronius* cannot be unambiguously assigned. *B. endophyticus, B. sporothermodurans* and *B. aquimaris* considered to be part of *B. subtilis* group is less probable. Equally, the support that *B. oleronius* is part of a *B. subtilis* clade is less probable. *Amphibacillus fermentum* is an excellent outgroup for this analysis. 16S sequences of *Bacillus* species do not contain sufficient informative sites to resolve *Bacillus* phylogenies.

Table 20: Sequences with quality indicators used for structural alignment.

AF418603	*Amphibacillus fermentum*	1454			
AB735985	*Bacillus amyloliquefaciens*	1573			
AF176321	*Bacillus anthracis*	1544			
JQ799058	*Bacillus aquimaris*	1508			
DQ993677	*Bacillus atrophaeus*	1536			
KC494304	*Bacillus carboniphilus*	1511			
AY224379	*Bacillus cereus*	1554			
GQ903415	*Bacillus endophyticus*	1516			
AJ717384	*Bacillus firmus*	1537			
AY052767	*Bacillus licheniformis*	1547			
AY189750	*Bacillus mojavensis*	1530			
AY988598	*Bacillus oleronius*	1548			
EU430985	*Bacillus oleronius*	1466			
EU430987	*Bacillus oleronius*	1476			
FJ973531	*Bacillus oleronius*	1518			
JF700436	*Bacillus oleronius*	1429			
JN366717	*Bacillus oleronius*	1461			
JN366721	*Bacillus oleronius*	1459			
JN366730	*Bacillus oleronius*	1447			
JN366793	*Bacillus oleronius*	1461			
JX045720	*Bacillus oleronius*	1256			
X82492	*Bacillus oleronius*	1500			
AB048252	*Bacillus pumilus*	1548			
AJ439078	*Bacillus simplex*	1522			
DQ993679	*Bacillus sonorensis*	1549			


1. Amphibacillus_fermentum
2. B_sporothermodurans
3. B_carboniphilus
4. B_aquimaris
5. B_oleronius_JN366793
6. B_oleronius_JX045720
7. B_oleronius_JF700436
8. B_oleronius_EU430985
9. B_oleronius_JN366721
10. B_oleronius_JN366717
11. B_oleronius_AY988598
12. B_oleronius_JN366730
13. B_oleronius
14. B_oleronius_FJ973531
15. B_oleronius_EU430987
16. B_endophyticus
17. B_atrophaeus
18. B_subtilis
19. B_sonorensis
20. B_firmus
21. B_simplex
22. B_mojavensis
23. B_amyloliquefaciens
24. B_pumilus
25. B_cereus
26. B_licheniformis
27. B_anthracis
28. B_vallismortis


1. Amphibacillus_fermentum
2. B_sporothermodurans
3. B_carboniphilus
4. B_aquimaris
5. B_oleronius_JN366793
6. B_oleronius_JX045720
7. B_oleronius_JF700436
8. B_oleronius_EU430985
9. B_oleronius_JN366721
10. B_oleronius_JN366717
11. B_oleronius_AY988598
12. B_oleronius_JN366730
13. B_oleronius
14. B_oleronius_FJ973531
15. B_oleronius_EU430987
16. B_endophyticus
17. B_atrophaeus
18. B_subtilis
19. B_sonorensis
20. B_firmus
21. B_simplex
22. B_mojavensis
23. B_amyloliquefaciens
24. B_pumilus
25. B_cereus
26. B_licheniformis
27. B_anthracis
28. B_vallismortis

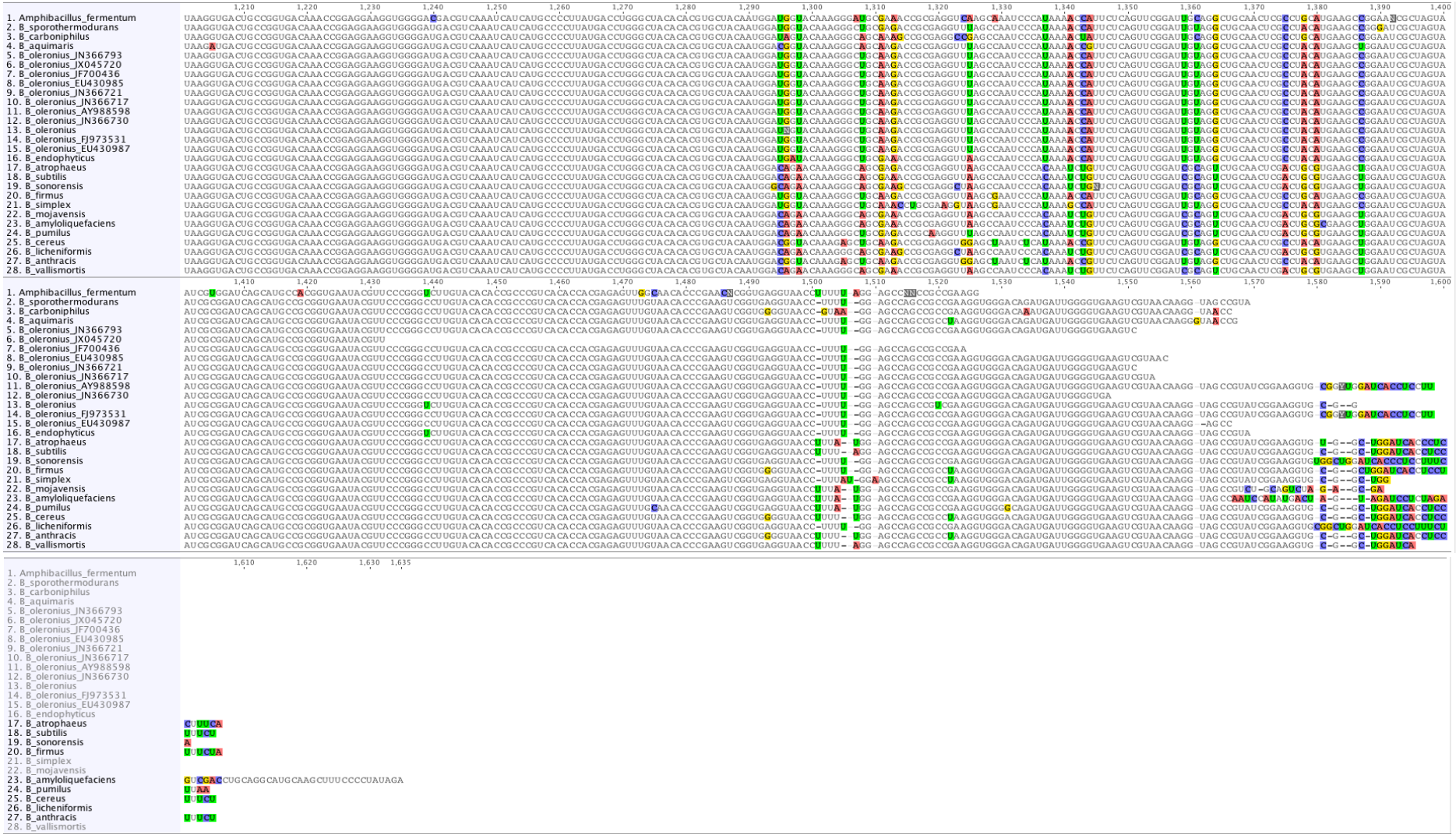
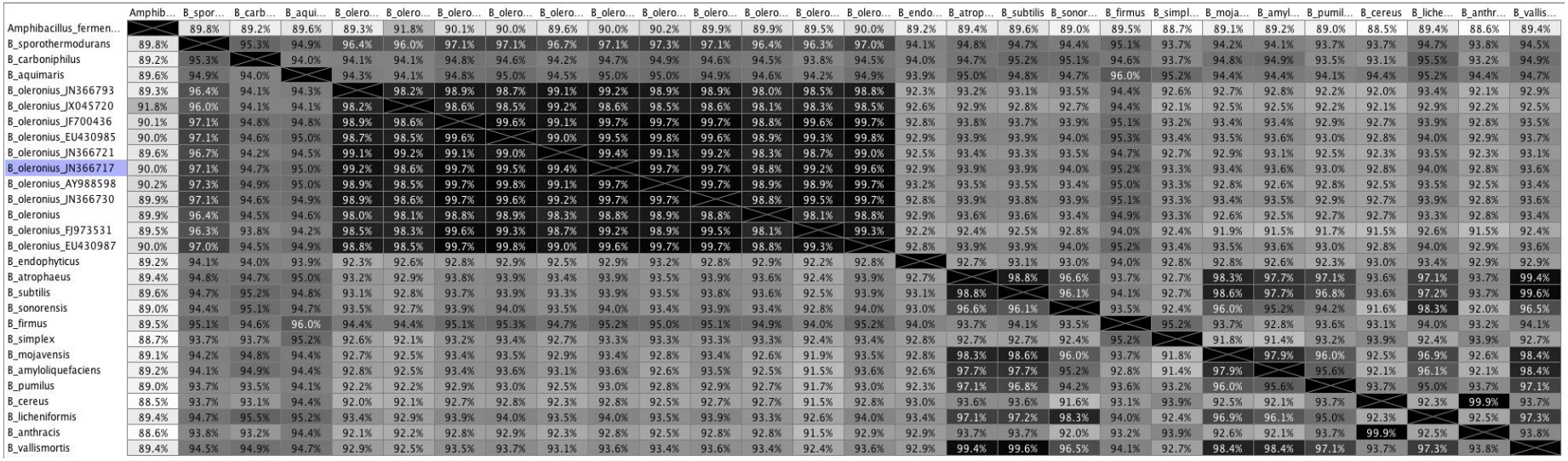


Figure 30: Structural alignment of 16S sequences of *B. oleronius* strains, *Bacillus* species and *Amphibacillus fermentum*. Variation in sequence is highlighted in grey and disagreement to consensus is highlighted in colour.



	Amphib…	B_spor…	B_carb…	B_aqui…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_olero…	B_endo…	B_atrop…	B_subtilis	B_sonor…	B_firmus	B_simpl…	B_moja…	B_amyl…	B_pumil…	B_cereus	B_liche…	B_anthr…	B_vallis…
Amphibacillus_fermen…		89.8%	89.2%	89.6%	89.3%	91.8%	90.1%	90.0%	89.6%	90.0%	90.2%	89.9%	89.9%	89.5%	90.0%	89.2%	89.4%	89.6%	89.0%	89.5%	88.7%	89.1%	89.2%	89.0%	88.5%	89.4%	88.6%	89.4%
B_sporothermodurans	89.8%		95.3%	94.9%	96.4%	96.0%	97.1%	97.1%	96.7%	97.1%	97.3%	97.1%	96.4%	96.3%	97.0%	94.1%	94.8%	94.7%	94.4%	95.1%	93.7%	94.2%	94.1%	93.7%	93.7%	94.7%	93.8%	94.5%
B_carboniphilus	89.2%	95.3%		94.0%	94.1%	94.1%	94.8%	94.6%	94.2%	94.7%	94.9%	94.6%	94.5%	93.8%	94.5%	94.0%	94.7%	95.2%	95.1%	94.6%	93.7%	94.8%	94.9%	93.5%	93.1%	95.5%	93.2%	94.9%
B_aquimaris	89.6%	94.9%	94.0%		94.3%	94.1%	94.8%	95.0%	94.5%	95.0%	95.0%	94.9%	94.6%	94.2%	94.9%	93.9%	95.0%	94.8%	94.7%	96.0%	95.2%	94.4%	94.4%	94.1%	94.4%	95.2%	94.4%	94.7%
B_oleronius_JN366793	89.3%	96.4%	94.1%	94.3%		98.2%	98.9%	98.7%	99.1%	99.2%	98.9%	98.9%	98.0%	98.5%	98.8%	92.3%	93.2%	93.1%	93.5%	94.4%	92.6%	92.7%	92.8%	92.2%	92.0%	93.4%	92.1%	92.9%
B_oleronius_JX045720	91.8%	96.0%	94.1%	94.1%	98.2%		98.6%	98.5%	99.2%	98.6%	98.5%	98.6%	98.1%	98.3%	98.5%	92.6%	92.9%	92.8%	92.7%	94.4%	92.1%	92.5%	92.5%	92.2%	92.1%	92.9%	92.2%	92.5%
B_oleronius_JF700436	90.1%	97.1%	94.8%	94.8%	98.9%	98.6%		99.6%	99.1%	99.7%	99.7%	99.7%	98.8%	99.6%	99.7%	92.8%	93.8%	93.7%	93.9%	95.1%	93.2%	93.4%	93.4%	92.9%	92.7%	93.9%	92.8%	93.5%
B_oleronius_EU430985	90.0%	97.1%	94.6%	95.0%	98.7%	98.5%	99.6%		99.0%	99.5%	99.8%	99.6%	98.9%	99.3%	99.8%	92.9%	93.9%	93.9%	94.0%	95.3%	93.4%	93.5%	93.6%	93.0%	92.8%	94.0%	92.9%	93.7%
B_oleronius_JN366721	89.6%	96.7%	94.2%	94.5%	99.1%	99.2%	99.1%	99.0%		99.4%	99.1%	99.2%	98.3%	98.7%	99.0%	92.5%	93.4%	93.3%	93.5%	94.7%	92.7%	92.9%	93.1%	92.5%	92.3%	93.5%	92.3%	93.1%
B_oleronius_JN366717	90.0%	97.1%	94.7%	95.0%	99.2%	98.6%	99.7%	99.5%	99.4%		99.7%	99.7%	98.8%	99.2%	99.6%	92.9%	93.9%	93.9%	94.0%	95.2%	93.3%	93.4%	93.6%	93.0%	92.8%	94.0%	92.8%	93.6%
B_oleronius_AY988598	90.2%	97.3%	94.9%	95.0%	98.9%	98.5%	99.7%	99.8%	99.1%	99.7%		99.7%	98.9%	98.9%	99.7%	93.2%	93.5%	93.5%	93.4%	95.0%	93.3%	92.8%	92.6%	92.8%	92.5%	93.5%	92.5%	93.4%
B_oleronius_JN366730	89.9%	97.1%	94.6%	94.9%	98.9%	98.6%	99.7%	99.6%	99.2%	99.7%	99.7%		98.8%	99.5%	99.7%	92.8%	93.9%	93.8%	93.9%	95.1%	93.3%	93.4%	93.5%	92.9%	92.7%	93.9%	92.8%	93.6%
B_oleronius	89.9%	96.4%	94.5%	94.6%	98.0%	98.1%	98.8%	98.9%	98.3%	98.8%	98.9%	98.8%		98.1%	98.8%	92.9%	93.6%	93.6%	93.4%	94.9%	93.3%	92.6%	92.5%	92.7%	92.7%	93.3%	92.8%	93.4%
B_oleronius_FJ973531	89.5%	96.3%	93.8%	94.2%	98.5%	98.3%	99.6%	99.3%	98.7%	99.2%	98.9%	99.5%	98.1%		99.3%	92.2%	92.4%	92.5%	92.8%	94.0%	92.4%	91.9%	91.5%	91.7%	91.5%	92.6%	91.5%	92.4%
B_oleronius_EU430987	90.0%	97.0%	94.5%	94.9%	98.8%	98.5%	99.7%	99.8%	99.0%	99.6%	99.7%	99.7%	98.8%	99.3%		92.8%	93.9%	93.9%	94.0%	95.2%	93.4%	93.5%	93.6%	93.0%	92.8%	94.0%	92.9%	93.6%
B_endophyticus	89.2%	94.1%	94.0%	93.9%	92.3%	92.6%	92.8%	92.9%	92.5%	92.9%	93.2%	92.8%	92.9%	92.2%	92.8%		92.7%	93.1%	93.0%	94.0%	92.8%	92.8%	92.6%	92.3%	93.0%	93.4%	92.9%	92.9%
B_atrophaeus	89.4%	94.8%	94.7%	95.0%	93.2%	92.9%	93.8%	93.9%	93.4%	93.9%	93.5%	93.9%	93.6%	92.4%	93.9%	92.7%		98.8%	96.6%	93.7%	92.7%	98.3%	97.7%	97.1%	93.6%	97.1%	93.7%	99.4%
B_subtilis	89.6%	94.7%	95.2%	94.8%	93.1%	92.8%	93.7%	93.9%	93.3%	93.9%	93.5%	93.8%	93.6%	92.5%	93.9%	93.1%	98.8%		96.1%	94.1%	92.7%	98.6%	97.7%	96.8%	93.6%	97.2%	93.7%	99.6%
B_sonorensis	89.0%	94.4%	95.1%	94.7%	93.5%	92.7%	93.9%	94.0%	93.5%	94.0%	93.4%	93.9%	93.4%	92.8%	94.0%	93.0%	96.6%	96.1%		93.5%	92.4%	96.0%	95.2%	94.2%	91.6%	98.3%	92.0%	96.5%
B_firmus	89.5%	95.1%	94.6%	96.0%	94.4%	94.4%	95.1%	95.3%	94.7%	95.2%	95.0%	95.1%	94.9%	94.0%	95.2%	94.0%	93.7%	94.1%	93.5%		95.2%	93.7%	92.8%	93.6%	93.1%	94.0%	93.2%	94.1%
B_simplex	88.7%	93.7%	93.7%	95.2%	92.6%	92.1%	93.2%	93.4%	92.7%	93.3%	93.3%	93.3%	93.3%	92.4%	93.4%	92.8%	92.7%	92.7%	92.4%	95.2%		91.8%	91.4%	93.2%	93.9%	92.4%	93.9%	92.7%
B_mojavensis	89.1%	94.2%	94.8%	94.4%	92.7%	92.5%	93.4%	93.5%	92.9%	93.4%	92.8%	93.4%	92.6%	91.9%	93.5%	92.8%	98.3%	98.6%	96.0%	93.7%	91.8%		97.9%	96.0%	92.5%	96.9%	92.6%	98.4%
B_amyloliquefaciens	89.2%	94.1%	94.9%	94.4%	92.8%	92.5%	93.4%	93.6%	93.1%	93.6%	92.6%	93.5%	92.5%	91.5%	93.6%	92.6%	97.7%	97.7%	95.2%	92.8%	91.4%	97.9%		95.6%	92.1%	96.1%	92.1%	98.4%
B_pumilus	89.0%	93.7%	93.5%	94.1%	92.2%	92.2%	92.9%	93.0%	92.5%	93.0%	92.8%	92.9%	92.7%	91.7%	93.0%	92.3%	97.1%	96.8%	94.2%	93.6%	93.2%	96.0%	95.6%		93.7%	95.0%	93.7%	97.1%
B_cereus	88.5%	93.7%	93.1%	94.4%	92.0%	92.1%	92.7%	92.8%	92.3%	92.8%	92.5%	92.7%	92.7%	91.5%	92.8%	93.0%	93.6%	93.6%	91.6%	93.1%	93.9%	92.5%	92.1%	93.7%		92.3%	99.9%	93.7%
B_licheniformis	89.4%	94.7%	95.5%	95.2%	93.4%	92.9%	93.9%	94.0%	93.5%	94.0%	93.5%	93.9%	93.3%	92.6%	94.0%	93.4%	97.1%	97.2%	98.3%	94.0%	92.4%	96.9%	96.1%	95.0%	92.3%		92.5%	97.3%
B_anthracis	88.6%	93.8%	93.2%	94.4%	92.1%	92.2%	92.8%	92.9%	92.3%	92.8%	92.5%	92.8%	92.8%	91.5%	92.9%	92.9%	93.7%	93.7%	92.0%	93.2%	93.9%	92.6%	92.1%	93.7%	99.9%	92.5%		93.8%
B_vallismortis	89.4%	94.5%	94.9%	94.7%	92.9%	92.5%	93.5%	93.7%	93.1%	93.6%	93.4%	93.6%	93.4%	92.4%	93.6%	92.9%	99.4%	99.6%	96.5%	94.1%	92.7%	98.4%	98.4%	97.1%	93.7%	97.3%	93.8%	

Figure 31: Genetic distance matrix of structural alignment of 16S sequences of *B. oleronius* strains, *Bacillus* species and *Amphibacillus fermentum*. Black and white heatmap and percentage identical nucleotides.

CHAPTER FOURTH

Discussion and conclusion

4.1. Discussion

The purpose of this work was to create novel species-specific primers for *B. oleronius* in order to prove that primers published by Szkaradkiewicz et al., 2012, unlike the authors claim, are not truly specific for *B. oleronius*. Two extraction kits were evaluated in this study to identify which would be the best purification method for obtaining large amounts of high-quality genomic DNA for single-molecule real time next generation genomic sequencing (SMRT) of *B. oleronius.* Several studies indicated that all methods of DNA extraction differ from each other, especially in their ability to extract sufficient and pure quantities of DNA, making it difficult to find an optimal method to extract DNA for all kinds of bacteria (McOrist et al., 2002). Many differences have been observed in obtaining a sufficient DNA yield using two DNA extraction kits in this study. Throughout the period of the study, the process of DNA extraction turned out to take a long time and various modifications were used to extract the DNA of *B. oleronius*.

Generally, In order to assess the quality and purity of the DNA samples it would be necessary to measure the absorbance at 280 nm. The absorbance ratios (A260 ⁄A280) and (A260/A23) can be used to evaluate the purity of DNA and nucleic acid respectively. If the average ratio (A260 ⁄A280) was above 0.8 this meant the samples were pure and if the ratio was less than 0.8 that indicated presence of contamination such as proteins, phenol, salt, polysaccharide or other kinds of contaminants that can inhibit the PCR amplifications and thus its efficiency (Manchester,1995). The results using Gentra Puregene Yeast/Bact kit Gram positive protocol yielded low amounts of DNA with low ratio (A260 ⁄A280) which indicate that most the samples were contaminated. The Gram positive protocol was used several times in order to get high quality DNA but the results showed that this protocol was not suitable for extraction *B. oleronius* considering the low quantity and poor quality of DNA but the results showed that this protocol was not in terms of the poor quantity and low quality of DNA extracted. Accordingly, it was necessary to use the protocol for Gram negative bacteria as an alternative method. The results showed that this protocol was acceptable in term of obtaining high amounts of DNA but at the same time the extracted DNA was not pure enough since the quantification showed that most the samples had low (A260 ⁄A280) ratio indicating the presence of contaminants. For all extraction methods the (A260/A230) ratios presented low

values (Table 17). This could be related to problems during extraction procedure and even to the presence of residues during culturing.

DNeasy ® Blood & Tissue kit was the last choice in this study as it commonly used to get pure and high-quality of genomic DNA. Based on the experiment, the amount of DNA obtained using the DNeasy ® Blood & Tissue Kit was the lowest and was not enough for sequencing. However, the 260/280 ratios using this kit were very low indicating that the DNA was free from contaminants. All the purification methods use for extraction of *B. oleronius* failed to extract sufficiently pure and high-quality genomic DNA for sequencing. The main reason for the lack of high quantity of DNA is thought to be due to the fact that *B. oleronius* is a Gram-positive bacterium that as a species is defined as being Gram-negative. This poses a challenge for the extraction of DNA. It was noted that short incubation of samples in the heater for 10 minute at 65 °C instead of 1 hour and re-centrifugation of the DNA during the step of protein precipitation increased the chance of getting higher amount of DNA. The process of extraction pure and high quality amounts of DNA can be considered a big challenge however, it is possible to make amendments and changes to some of the protocols. Despite time consuming, the adjustments seemed to be worthwhile.

The results of the nearest neighbours (related Bacillus species) phylogenetically to B*. oleronius* showed that *B. oleronius* strain EK-3 behaved differently from all other *B. oleronius* strains in alignment experiments. The strain showed in a BLAST analysis 4 separate parts of maximum similarity suggesting that during PCR amplification one or more chimera had formed or that during sequence assembly errors were made. The sequence of this strain was considered not reliable. *B. oleronius* strain W44 is considered a misidentification based on the tree in Figure 27. *Amphibacillus* species were included in the phylogenetic analysis because the hyper variable region of the *B. oleronius* sequence matched *Amphibacillus* sequences. The placement of *Amphibacillus* species within the *B. oleronius* clade needs further investigation with a larger taxon sampling. *Listeria monocytogenes* has been used as an outgroup by Maughan and Van der Auwera (2011) in their phylogenetic analysis of the family Bacillaceae. In Figure 27, the branch length for *L. monocytogenes* seems short for an outgroup species for *Bacillus* investigations. Bacterial species normally show a divergence of 1.5 % in the 16S sequence between sister species, allowing the design of species-specific primers. Several *Bacillus* species have either identical or near identical 16S sequences.

As it has been mentioned previously, the aim of this study was to design species-specific primers for *B. oleronius* and through this study it was possible to refute the idea which states that 16S sequence of *B. oleronius* is suitable for species discrimination and to confirm that the published primers designed by Szkaradkiewicz et al., 2012 are not species-specific while the

newly designed primers in this study are species-specific. The primer set BO1 (5'-AACGGCTCACCAAGGCGACG-3') and BO2 (5'-TCCGGACAACGCTTGCCACC-3') designed by Szkaradkiewicz et al., 2012 were tested in this study and they amplified all the *Bacillus* species tested including *B. oleronius*. This ultimately shows that these primers were not specific for *B. oleronius* and in fact they work as universal primers (Figure 25).

In contrast, the primer sets Bo16S-202FaL (AACTTTTTTCTTCGCATGARGGAGAATTG) with Bo16S-684R (5'-CTCCCAGTTTCCAATGGCCGCTTG-3') and Bo16S-202FaL (AACTTTTTTCTTCGCATGARGGAGAATTG) with Bo16S-668R (5'-GCCGCTTGCGGTTGAGCCGCAAGA-3') designed in this project for the 16SRNA showed to be species-specific since they only amplify *B. oleronius* fragments with the expected size of 483 bp and 467 bp respectively (Figure 22 and 19).

Regarding to the universal primers used for amplification of the *rpoB* gene the results showed that these primers did not work correctly because no band was detected (Figure 16).The reasons for these results are thought to be due to wrong design or the use of unsuitable annealing temperature. Optimizing the appropriate annealing temperatures (T_a) for the primers is correlated to the success of the PCR, especially during the early stages of the amplification (Verkuil et al., 2010). In this experiment the PCR has been performed using a range of annealing temperatures. Initially, the PCR was set up using all the newly primers designed for 16SRNA on 50 °C. Once PCR amplification was completed it was noted that all the primers amplified the negative controls. This was due to the use of a lower annealing temperature for all the primers. When the annealing temperature becomes too low the amplification becomes non-specific (Figure 17and 18). When the PCR was performed on 50°C all the primers annealed randomly and amplified all the bacterial species. At the same time when the annealing temperatures become too high this lead to a more precise annealing and ultimately to obtain the desired products (Rychlik et al., 1990).Therefore, it is very important to choose suitable annealing temperatures in an average of 5 °C lower than the melting temperature (T_m) of the primer.

The results in (Figure 22) show that the effect of increasing the annealing temperature for the primers set Bo16S-202FaL and Bo16S-668R from 59 °C to 61 °C lead to a specific annealing and amplification of the desired product (*B. oleronius*) while ignoring the other species indicating that these primers are species-specific to *B. oleronius*. Some primer sets such as (Bo16S-202FbL with Bo16S-684R), (Bo16S-202FcL with Bo16S-668R) and (Bo16S-202FcL with Bo16S-684R) did not show any change when the annealing temperature had increased showing that these primers are not species-specific (Figure 23 and 24).

4.2. Conclusion

The development of a purification method for large amounts of high-quality genomic DNA for single-molecule real time next generation genomic sequencing (SMRT) of *B. oleronius* was challenging because *B. oleronius* is Gram-positive that as a species is defined as being Gram-negative. The Gentra Puregene Yeast/Bact kit Gram positive protocol, Gentra Puregene Yeast/Bact kit Gram negative protocol and DNeasy ® Blood & Tissue kit were used for DNA extraction. Each kit has its advantages and disadvantages in terms of getting high-quality and quantity genomic DNA. The two Puregene kits failed to get a pure DNA due to the presence the contaminants in most of the samples while the DNeasy ® Blood & Tissue kit yielded a pure DNA but in extremely low concentration. Therefore, it is very important to find a new solution to get rid of the contaminants during the process of DNA extraction in order to achieve a high grade DNA extract of *B. oleronius*

In this study species-specific primers *B. oleronius* were designed for the first time. The primers were designed for the 16SRNA gene using Genious software. The primers designed by Szkaradkiewicz et al., 2012 were used in this study to confirm that these primers were not in fact species-specific as claimed by the authors. They claimed that *Demodex* was infect only with *B. oleronius* and that this species of bacteria was responsible for demodecosis. When in fact they cannot be sure that the mites only have *B. oleronius* because the primers are not specific. The new primers designed in this project have succeeded to amplify only *B. oleronius* and can therefore be used as species specific. These results have opened a door for more future applications and identification of *Bacillus* species.

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Appendix

Appendix 1. Opening of ampoules

1. Check list

In addition to the items mentioned above you will need the following: broken glass disposal, sterile, water, Pasteur pipettes and forceps. Work in microbiologically controlled conditions

2. Heat the tip of the ampoule with a flame

Usually only 5-15 seconds suffice (though it may take longer if the flame is too weak).Make sure that only the tip is being heated in order to prevent a loss of viability of the preserved material. The inner cotton plug should not become brown (this indicates excessive heating).

3. Break the tip of the ampoule

Place 1-4 drops of sterile water onto the hot tip to crack the glass. If no breakage occurs, repeat the previous step trying to increase the heating of the tip (however excessive heating is not desirable either since it may damage the strain).

4. Remove glass fragments

Wear protective goggles if you think that small glass fragments may reach your eyes (for instance if you are not working at a vertical flow bench).Carefully strike off the glass tip with an appropriate tool (e. g. forceps).Lift the cotton plug with the forceps so that it can be more easily handled in coming steps.

Rehydration of strain and inoculation

1. Rehydration

Add 0.2-0.3 mL of recommended broth using a Pasteur pipette. Carefully suspend the cells until the appearance of the liquid is homogeneous. For better mixing the suspension can be gently loaded into the pipette several times trying not to form air bubbles or foam.

2. Inoculation

Use several drops of the suspension to inoculate an agar Petri plate and/or a slant and transfer the rest to a tube with 5-10 mL of the recommended liquid medium. Some cells may show a prolonged lag period; therefore incubate the tubes and plate for several days before discarding them as non-viable.

3. Incubation

Incubate under the conditions specified for the strain in the delivery note or our on line catalogue (www.cect.org). Before discarding sterilize all the remains of the original ampoule Please note that some strains have a long lag phase and may need to be incubated up to two weeks or more.

Appendix 2. DNA Purification from Gram-Positive Bacteria Using the Gentra Puregene Yeast/Bact. Kit

This protocol is for purification of genomic DNA from fresh or frozen samples of 0.5 mL Gram-positive bacterial cultures using the Gentra Puregene Yeast/Bact. Kit.

Things to do before starting:

■ Preheat water baths to 37°C for use in steps 6 and 10, 65°C for use in step 22, and 80°C for use in step 9 of the procedure.

■ Frozen bacterial samples should be thawed and equilibrated to room temperature (15–25°C) before beginning the procedure.

Procedure

1. Prepare an overnight culture.

2. Transfer 500 µl of the cell culture (containing approx. 0.5–1.5 x 109 cells) to a

1.5 mL microcentrifuge tube on ice.

3. Centrifuge for 5 s at 13,000–16,000 x g to pellet cells.

Longer centrifuge times may be necessary for some species to form a tight pellet.

4. Carefully discard the supernatant by pipetting or pouring.

5. Add 300 µl Cell Suspension Solution, and pipet up and down.

6. Add 1.5 µl Lytic Enzyme Solution, and mix by inverting 25 times. Incubate for

30 min at 37°C.

7. Centrifuge for 1 min at 13,000–16,000 x g to pellet cells.

8. Carefully discard the supernatant with a pipet.

9. Add 300 µl Cell Lysis Solution, and pipet up and down to lyse the cells.

An incubation for 5 min at 80°C may be necessary to lyse cells of some species.

10. Add 1.5 µl RNase A Solution, and mix by inverting 25 times. Incubate for 15–60 min at 37°C.

11. Incubate for 1 min on ice to quickly cool the sample.

12. Add 100 µl Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.

Note: For species with high polysaccharide content, incubate the sample on ice for 15–60 min.

13. Centrifuge for 3 min at 13,000–16,000 x g.

The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

14. Pipet 300 µl isopropanol into a clean 1.5 mL microcentrifuge tube and add the supernatant from the previous step by pouring carefully.

Be sure the protein pellet is not dislodged during pouring.

15. Mix by inverting gently 50 times.

16. Centrifuge for 1 min at 13,000–16,000 x g.

The DNA will be visible as a small white pellet.

17. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

18. Add 300 µl of 70% ethanol and invert several times to wash the DNA pellet.

19. Centrifuge for 1 min at 13,000–16,000 x g.

20. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min.

The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

21. Add 100 µl DNA Hydration Solution and vortex 5 s at medium speed to mix.

22. Incubate at 65°C for 1 h to dissolve the DNA.

23. Incubate at room temperature (15–25°C) overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

Appendix 3. Additional Purification of Gentra Puregene samples using DNA the DNeasy Blood & Tissue Kit:

1. Combine the three samples with the highest amount of gel DNA of the middle purification (90 µl each of 176, 140, and 102 µg/µl), totalling 270 µl.
2. Add 300 µl AL buffer without ethanol and mix by light vortexing
3. Add 40 µl of proteinase K mix by light vortexing
4. Incubate at 56 ˚C overnight
5. Inactivate proteinase K at 95 ˚C for 15 minutes
6. Centrifuge the sample for 5 minutes at 190 rpm
7. Add 20 µl proteinase K
8. Add 300 µl ethanol (96-100 %) to the sample, and mix thoroughly by vortexing.

It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column. This precipitate does not interfere with the DNeasy procedure.

9. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 mL collection tube (provided). Centrifuge at _6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.
10. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µL Buffer AW1, and centrifuge for 1 min at _6000 x g (8000 rpm). Discard flow-through and collection tube.
11. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µL Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.
12. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

13. Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL microcentrifuge tube (not provided), and pipet 200 µL Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at _6000 x g (8000 rpm) to elute. Elution with 100 µL (instead of 200 µL) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

14. **Recommended: For maximum DNA yield, repeat elution once as described in step 13.** This step leads to increased overall DNA yield. A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

Note: Do not elute more than 200 µL into a 1.5 mL microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Appendix 4. Protocol: DNA Purification from Gram-Negative Bacteria Using the Gentra Puregene Yeast/Bact. Kit

This protocol is for purification of genomic DNA from fresh or frozen samples of 0.5 mL Gram-negative bacterial cultures using the Gentra Puregene Yeast/Bact. Kit.

Things to do before starting:

Preheat water baths to 37°C for use in step 6, 65°C for use in step 18, and 80°C for use in step 5 of the procedure.

Gram-negative bacterial cultures can be used either fresh or frozen. Typically, an overnight culture contains 1–3 x 109 cells/mL. Due to the small genome size of Gram negative bacteria, up to 3 x 109 cells may be used for the protocol. Thus, culture can either be used directly, or, if necessary, concentrated by centrifuging. To concentrate, pellet 1 mL of overnight culture at 13,000–16,000 x g for 1 min. Remove the supernatant, leaving 200 µL residual fluid. Thoroughly suspend the pellet in the residual fluid by pipetting up and down 10 times. Place the sample on ice for immediate use or store frozen at –80°C.

Frozen bacterial samples should be thawed and equilibrated to room temperature (15–25°C) before beginning the procedure.

Procedure

1. Prepare an overnight culture and transfer 500 µL of the culture (containing approximately 0.5–1.5 x 109 cells) to a 1.5 mL microcentrifuge tube on ice.

2. Centrifuge for 5 s at 13,000–16,000 x g to pellet cells.

3. Carefully discard the supernatant by pipetting or pouring.

4. Add 300 µL Cell Lysis Solution, and mix by pipetting up and down. Incubate sample at 80°C for 5 min to lyse the cells. Samples are stable in Cell Lysis Solution for at least 2 years at room temperature (15–25°C).

5. Add 1.5 µL RNase A Solution, and mix by inverting 25 times. Incubate for 15–60 min at 37°C.

6. Incubate for 1 min on ice to quickly cool the sample.

7. Add 100 µL Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.

8. Centrifuge for 3 min at 13,000–16,000 x g.

The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

9. Pipet 300 µL isopropanol into a clean 1.5 mL microcentrifuge tube and add the supernatant from the previous step by pouring carefully.

Be sure the protein pellet is not dislodged during pouring.

10. Mix by inverting gently 50 times.

11. Centrifuge for 1 min at 13,000–16,000 x g.

The DNA will be visible as a small white pellet.

12. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

13. Add 300 µL of 70% ethanol and invert several times to wash the DNA pellet.

14. Centrifuge for 1 min at 13,000–16,000 x g.

15. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min.

The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

16. Add 100 µL DNA Hydration Solution and vortex for 5 s at medium speed to mix.

17. Incubate at 65°C for 1 h to dissolve the DNA.

18. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

Appendix 5. Preparation an Agarose gel and running buffer

1. Pour a 50 mL aliquot of 10X Tris acetate electrophoresis buffer (TAE) stock solution into a 1 Litre- measuring cylinder and make up to 1L by adding 950 mL of deionized water. This will create a 1L stock of 0.5X TAE buffer.
2. Assemble a gel casting mold by attaching the red rubber stoppers to the two open sides of a casting tray. Place the completed assembly on a flat surface. A good idea is then to test the casting tray for leaks. Do not pouring a small aliquot of 0.5X TAE buffer into the mold and observing for ‘leaks’
3. Weight out 0.8g of agarose into a clean 250mL bottle, add 150mL of your 0.5X TAE buffer and gently ‘swirl’ to mix the agarose/TAE slurry.
4. Heat the slurry in a microwave until the agarose fully dissolves. Be careful, not to let the slurry boil over. About 4 minutes on medium- high power should be sufficient.
5. When the solution has cooled sufficiently, approximately 55 °C add 15 µL of SafeView DNA stain dye (you will be given this) to the gel mixture. Gently swirl to mix in the dye, and pour the gel solution into the gel casting tray. If needed, remove any bubbles that may have formed with a clean pipette tip.
6. Place a gel comb into one of the slots at the top of the gel mold and leave the agarose gel to set. Take care not to disturb the gel until it has set. The gel is set when it appears milky white and translucent.
7. Once set, carefully remove the rubber stoppers from both ends of the mold
8. Transfer the whole gel try into an electrophoresis tank and fill the tank to just under the max fill level with 0.5X TAE buffer.
9. Remove the comb from the gel, taking care not to break the wells. A demonstrator will help you to do this if required.
10. Your gel now is ready to be loaded.