ORAL MICROBIOME VARIATIONS ASSOCIATED WITH NORMAL, PRE-CANCEROUS AND CANCEROUS ORAL CONDITIONS BASED ON 16S rDNA SEQUENCING AND DENATURING GRADIENT GEL ELECTROPHORESIS

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APRIL 2015

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By

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A dissertation submitted to the Department of Pre-clinical Sciences,
Faculty of Medicine and Health Sciences,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Master of Medical Sciences
April 2015

ABSTRACT

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Mok Shao Feng

The human oral microbiome has been known to show strong association with various oral diseases including oral cancer. This study attempts to characterize the community variations between normal, pre-cancer and cancer associated oral microbiota using 16S rDNA sequencing and denaturing gradient gel electrophoresis (DGGE). Forty normal volunteers, 10 pre-cancer and 13 cancer patients were recruited in this study. Swab samples were collected and bacteria genomic DNA was isolated using a commercial kit in which full length and partial 16S rDNA that contains V6 to V8 regions were amplified and used for clone library sequencing and DGGE analysis respectively. 16S rDNA sequences were processed and analysed using the MOTHUR software and DGGE fingerprints were processed and clustered using BioNumerics 6.2. A core oral microbiome consist of Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes and Actinobacteria at the phylum level while *Streptococcus*, *Veillonella*, *Gemella*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Selenomonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Porphyromonas* and *Lachnoanaerobaculum* at the genus level was identified. Firmicutes and *Streptococcus* were the predominant phylum and genus respectively. Potential

oral microbiome compositions unique to Malaysian subjects were also identified. The DGGE dendogram showed a clustering of oral microbiota according to mix, normal and patient groups. When bacteria communities identified from clone library were analyzed with AMOVA, a significant difference between the normal and the cancer associated oral microbiota but not between the pre-cancer and the other two groups were observed. However, the pre-cancer associated oral microbiome was found to overlap between the normal and cancer groups with a 3D Non-metric Multidimensional Scaling (NMDS) plot. Unique pre-cancer associated oral microbiome profiles and Operational Taxonomic Units (OTUs) were also identified. Several oral microbes were found to have consistent association with a particular oral state and in higher abundance in this study. For instance, *Eubacterium saphenum* was only observed in the normal state, *Megasphaera micronuciformis* in the pre-cancerous state and *Camplylobacter showae* in the cancerous state. *Prevotella melaninogenica* and *P. veroralis* were found in both normal and pre-cancerous states while *Rothia mucilaginosa*, *R. dentocariosa* and *Catonella morbi* were found in both diseased states. Further search done on the GIDEON database indicated a strong alkaline phosphatase and catalase producing phenotypes linked to bacterial groups associated with pre-cancer state and sulfate reducing phenotype linked to bacteria groups associated with cancerous state. These oral microbes maybe potential biomarkers to distinguish between normal, pre-cancer and cancer subjects.

ACKNOWLEDGEMENT

The long days of growing transformed *E. coli* and casting difficult DGGE were certainly tough, but rewarding at the same time, in terms of gaining knowledge and experience and shaping a better research attitude. I owe my deepest gratitude to my supervisor, Assoc. Prof. Dr. Alan Ong Han Kiat, who has given me the opportunity to venture into my field of interest. His patience and guidance greatly assisted me in accomplishing my objectives at my own pace. Many thanks as well to my co-supervisor, Prof. Dr. Yap Sook Fan, who provided me many useful advices during subject recruitment and sampling stage. I would like to thank collaborators from UM-OCRCC, Prof. Dr. Rosnah Binti Mohd Zain, Prof. Dr. Ngeow Wei Cheong, Karen Ng Lee Peng and Wong Gou Ren for their effort in recruiting patient subjects. Special thanks to Dr. Karuthan Chinna for his statistical consultations and Dr. Cheah Yoke Kqueen for his assistant in fingerprint data analysis.

I appreciate the unconditioned love and understanding from my parents and girlfriend that have kept me going throughout my hardships and sleepless nights. I also value the company of all my labmates in which they had added cheerful and happiness reagents into the otherwise stressful lab environment. I would like to extend my gratitude to the staff of faculty general office and IPSR for their speed and hard work in processing all relevant documents pertaining to my project. Lastly, this study would not have been possible without the support from all the volunteers.

APPROVAL SHEET

This dissertation/thesis entitled "**ORAL MICROBIOME VARIATIONS ASSOCIATED WITH NORMAL, PRE-CANCEROUS AND CANCEROUS ORAL CONDITIONS BASED ON 16S rDNA SEQUENCING AND DENATURING GRADIENT GEL ELECTROPHORESIS"** was prepared by MOK SHAO FENG and submitted as partial fulfillment of the requirements for the degree of Master of Medical Sciences at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **MOK SHAO FENG** (ID No: **11UMM05703**) has completed this dissertation entitled "ORAL MICROBIOME VARIATIONS ASSOCIATED WITH NORMAL, PRE-CANCEROUS AND CANCEROUS ORAL CONDITIONS BASED ON 16S rDNA SEQUENCING AND DENATURING GRADIENT GEL ELECTROPHORESIS" under the supervision of Assoc. Prof. Dr. Alan Ong Han Kiat (Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, and Prof. Dr. Yap Sook Fan (Co-Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences.

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DECLARATION

I, Mok Shao Feng, hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(Mok Shao Feng)

Date _____

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LIST OF ABBREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
rDNA	Ribosomal DNA
PCR	Polymerase Chain Reaction
DGGE	Denaturing Gradient Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
NO	Nitrogen Monoxide
ROS	Reactive oxygen species
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
HMP-DACC	Human Microbiome Project Data Analysis and Coordination Center
GIDEON	Global Infectious Disease and Epidemiology Network
NMDS	Non-metric Multidimensional Scaling
AMOVA	Analysis of Molecular Variance

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INTRODUCTION

Cancer is a global burden as it is among the primary causes of worldwide morbidity and mortality with expected increase of new cases in the future (World Health Organization, 2015). Cancer that occurs in and around the mouth is termed oral cancer (Sankaranarayanan et al., 2014) and it has higher prevalence in developing countries (Warnakulasuriya, 2009b). In Malaysia, oral cancer is categorized as one of the head and neck cancer which are detected mostly in the elderly and Indian populations (Omar et al., 2006, Yap et al., 2010, Omar and Tamin, 2011).

Most oral cancer cases are detected at advanced stages due to subtle early symptoms and absence of dependable diagnostic markers (Markopoulos et al., 2010). It is widely recognized that advanced stages of oral tumours produce poor treatment outcomes in contrast to cancer diagnosed at the early stages which can be treated effectively. Hence one of the main motivations of oral cancer research is the development of better diagnostic markers including molecular and serological markers (Gonzalez-Moles et al., 2012). However, these diagnostic tools have been reported to be either not suitable for routine applications (Gonzalez-Moles et al., 2012) or not ready for clinical usage (Markopoulos et al., 2010). Thus histopathological examination remains as the gold standard for oral cancer diagnosis (van Der Waal et al., 2011).

Nonetheless, this gold standard strategy is hampered by subjective visual grading and unpleasant oral biopsy (van Der Waal et al., 2011).

Bacteria could be an alternative biomarker for oral cancer diagnostic purpose since several genera were reported with the ability to specifically bind to tumours after systemic administration (Morrissey et al., 2010). Besides that, the overall community profiles, shown as Denaturing Gradient Gel Electrophoresis (DGGE) fingerprint profile, of the cancer associated salivary microbiota were observed as a distinct cluster separate from the normal group (Pushalkar et al., 2011). In addition, several bacteria species and groups were found to be associated with cancerous clinical samples. For instance, the amount of *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, *Streptococcus mitis* (Mager et al., 2005), *Johnsonella* genus (Pushalkar et al., 2012) as well as Proteobacteria and Actinobacteria phyla (Hooper et al., 2007) were found to be significantly higher in cancerous clinical samples as compared to their normal counterparts. These findings suggested that our human oral microbiome may harbour natural oral cancer biomarkers with potential diagnostic value.

However, there is limited information regarding the pre-cancer associated oral microbiome which otherwise would provide a more comprehensive view on the association between oral cancer and oral microbiome. The pre-cancer stage precedes early cancer stage and comprises a layer of transformed pre-cancerous epithelial cells with asymptomatic abnormalities (Feller et al., 2013). The detection of this pre-cancer stage to

permit early treatment intervention would greatly improve cancer survival rate (Markopoulos et al., 2010, National Institutes of Health, 2010) and quality of life (Rapidis et al., 2009). Hence it would be a valuable effort to investigate the oral microbiome in pre-cancerous condition together with normal and cancer associated oral microbiota.

16S rRNA gene is the gold standard marker which is commonly used to gauge the diversity of environmental bacteria (Harmsen and Karch, 2004) (Wang and Qian, 2009) by analyzing the sequence variations in the hypervariable regions. Clone library sequencing was used to identify the sequence order of the 16S hypervariable region for phylogenentic analysis (Huse et al., 2008, Liu et al., 2008) and taxonomic assignment (Armougom and Raoult, 2009). Besides that, DGGE was also employed to produce fingerprint profiles based on the sequence variations in the 16S hypervariable regions to generate a snapshot of community variations in terms of different oral conditions (Muyzer and Smalla, 1998).

Therefore, the objectives of this study are:

- To isolate genomic bacterial DNA in the oral cavity from subjects with oral squamous cell carcinoma (OSCC), pre-cancerous lesions and those who are cancer free,
- To infer the community structure of the normal, pre-cancer and cancer associated oral microbiome via clone library sequencing and DGGE analysis,
- To identify unique oral microbiome profile associated with pre-cancerous oral condition and,
- To identify microbial groups or species that are associated with pre-cancer and cancer conditions of the oral cavities in Malaysian subjects.

2

LITERATURE REVIEWS

2.1. Oral Cancer

2.1.1. Definition

Oral cancer is a subset of head and neck cancer (National Cancer Institute, 2013) which occurs in the oral cavity such as lip, tongue, gum, mouth floor, palate and other related mouth parts (International Classification of Diseases 10th edition codes C00-06) (World Health Organization, 2010). It is often termed oral squamous cell carcinoma (OSCC) because this cancer type comprises more than 90% of all cases (Werning, 2011a).

2.1.2. Global and Regional Burdens

Although oral cancer was estimated to be the 15th most common cancer globally in 2012, it is in fact one of the most common cancer in parts of South Central Asia and South East Asia, such as Papua New Guinea, Pakistan, Maldives, Sri Lanka and India (Ferlay et al., 2013). Two-third of the cases occur in developing countries (Warnakulasuriya, 2009b) and it could comprise up to 40% of all cancer cases in high risk regions (Cox, 2000).

In Malaysia, oral cancer is the third most common head and neck cancer, affecting the older population and with equal risk for both genders (Yap et al., 2010). It is most common among the Indians regardless of different subsites such as mouth and tongue (Omar et al., 2006, Omar and Tamin, 2011).

2.1.3. Risk Factors

Tobacco smoking and alcoholic drinking are the two known major risk factors of oral cancer (Kirita and Omura, 2015). Tobacco contains the carcinogenic nitrosamines and tobacco burning produces carcinogenic products such as arylamines, polycyclic aromatic, hydro carbons and volatile organics while alcohol decomposition produces the genotoxic compound acetaldehyde (Monograph Working Group, 2009). Although many oral cancer cases were attributed to either smoking or drinking alone, these two lifestyle habits are able to act synergistically (Hashibe et al., 2009, Warnakulasuriya, 2009a) and increase the cancer risk by 30-fold (American Cancer Society, 2009). Betel quid chewing, a common habit in South Asia, South East Asia, China and Taiwan (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004), is another major risk factor of oral cancer (Hashibe et al., 2009, Warnakulasuriya, 2009a, Wen et al., 2010, Johnson et al., 2011) as it contains the carcinogenic Areca nuts, which was classified as Group 1 carcinogen together with betel quid (Monograph Working Group, 2009).

However, the increase of oral cancer prevalence among young adults with less exposure to the mentioned risk factors suggested the interplay of other etiological factors (Campo-Trapero et al., 2008) such as infections. More than 60% of the oral cavities with poor hygiene condition were found to harbour tumours (Holmes Jr et al., 2009) while around 40% of the candidal leukoplakias were found to be malignant transformable (Sanjaya et al., 2011). In addition, several different types of Human Papilloma Virus (HPV) were also found in considerable fraction of the oral cancer patients, ranging from 25.4% to 55.7% (Syrjänen, 2005, Saini et al., 2011).

2.1.4. Field of pre-cancerization

Oral tumours arise from a large area of "pre-cancerized" epithelial cells which are microscopically dysplastic (Slaughter et al., 1953) and genetically abnormal (Braakhuis et al., 2003). This layer of transformed epithelial cells, termed "field of pre-cancerisation", are epigenetically and cytogenetically altered. These changes affect the DNA repair and cell cycle mechanisms leading to higher malignancy potential and additional events of genetic mutations (Feller et al., 2013). This "field of pre-cancerisation" does not only present as a cancer diagnosis challenge due to its subtle symptoms (Jayam, 2010), but also reduces treatment effectiveness as the remnant pre-cancerized cells surrounding the removed tumours are able to develop into independent primary or second primary tumours (Slaughter et al., 1953, Feller and Lemmer, 2012). In addition, this multiple tumour development or local

recurrence within the“field of pre-cancerisation” also complicate cancer prognosis (Feller et al., 2013).

2.1.5. Diagnosis Strategies

The current standard oral cancer diagnosis protocol starts with the detection of suspicious oral lesions via visual oral screening and followed by malignancy confirmation via histopathological biopsy (Werning, 2011b). The sensitivity and specificity of this conventional approach is hampered by the subjective grading and difficulties in detecting subtle symptoms as well as the invasive biopsy and possible excision of benign dysplasias (Gonzalez-Moles et al., 2012). Hence there is a need for more reliable and convenient markers to improve current diagnostic strategy. Unfortunately, there are no available serological markers (Mydlarz et al., 2010) while many potential molecular diagnostic markers are either still in the research phase (Markopoulos et al., 2010) or too complicated for routine application (Gonzalez-Moles et al., 2012).

Besides that, the histopathogical classification of the “field of pre-cancerization” remains subjective (Campo-Trapero et al., 2008) and it was not addressed in many molecular marker studies (Chen et al., 2007, Cheong et al., 2009, Mahdey et al., 2011, Elashoff et al., 2013, Cheng et al., 2014) although many genetic alteration and abnormalities were detected in the “pre-cancerized field”, such as, reduced expression of type 2 chain ABH antigen and B-cell lyphoma 2 and increased expression of epidermal growth factor

receptor (EGFR) and transforming growth factor alpha (TGF-α) mRNA (Angadi et al., 2012).

2.1.6. Treatment Approaches

Surgery is the most common and well established treatment approach but the success rate is influenced by the tumour size, location, infiltration depth and proximity to bone (Shah and Gil, 2009) with poorer outcome for metastatic tumours (Pagedar and Gilbert, 2009). Radiotherapy is another cancer treatment option that can be applied either alone or conjunction with surgery and chemotherapy to early stage or locally advanced tumours (Mazeron et al., 2009). Alternatively, chemotherapy can be administered together with radiotherapy (CT-RT) or as the induction before or adjuvant therapy after another local treatment on locally advanced tumours (Specenier and Vermorken, 2009). Recently, oral cancer management options has been expanded and improved with targeted molecular treatments, especially the EGFR targeting cetuximab, which has resulted in improved survival of patients with locally advanced or recurrent tumours (Lorch et al., 2009, Rapidis et al., 2009). However, advanced stage oral cancer often requires more radical treatments which could lower patient survival and quality of life (Pushalkar et al., 2012) due to repeated toxic therapy (Gonzalez-Moles et al., 2012) and the progression of secondary or multiple tumours with drug resistance (Morrissey et al., 2010). In short, oral cancer treatment outcome depends on early diagnosis.

2.1.7. Present Challenges

Oral cancer is a serious health issue that warrants study focus due to the absence of reliable early diagnostic markers (Bebek et al., 2012), the poor improvement of patient survival rate (Priebe et al., 2008, Warnakulasuriya, 2009b) despite advanced treatments (Deng et al., 2011) and the global increment of 14.2% and 13.9% of estimated incidence and death respectively from 2008 (Jemal et al., 2011) to 2012 (Feller et al., 2013). Hence, early diagnosis of the "pre-cancerized field" is crucial (Gonzalez-Moles et al., 2012) because cancer treatment at early stages not only increases cure rate (Markopoulos et al., 2010) but also improve life quality (Pushalkar et al., 2012) and cancer prognosis (van Der Waal et al., 2011).

2.2. Oral Microbiome

2.2.1. Definition

The human oral microbiome is the community of commensal, symbiotic, and pathogenic microorganisms that live inside the human oral cavity (Lederberg and McCray, 2001) with the ability to influence the oral health condition (Lazarevic et al., 2009). It is one of the major defence mechanisms that protects the human body from other pathogenic bacteria through colonization resistance and host immune response stimulation (Haraldsson, 2005). Its stability disruption could lead to diseases, either

caused by the invasion of pathogens (Blaser, 2008) or the activities of opportunistic commensals (Blaser and Falkow, 2009). Therefore, knowledge about the relationship between the oral microbiome and various oral diseases is important to improve medical diagnosis and treatment (Fábián et al., 2008, Armougom and Raoult, 2009, Nasidze et al., 2009). Previous oral microbiome studies had identified about 750 distinct taxa (Mager et al., 2003, Jenkinson and Lamont, 2005) and up to 10,000 phylotypes (Keijser et al., 2008) that can be found in the oral cavity. Surprisingly, majority of these sequences were confined to few bacterial phyla, such as Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria although there were also small proportions that were classified under Fusobacteria, TM7, Spirochaetes, OD2 and Synergistes (Bik et al., 2010) despite variations of gender, age and geographical locations (Nasidze et al., 2009). This suggested the existence of a core healthy human oral microbiome that is important to maintain the functional stability and homeostasis of the oral ecosystem (Zaura et al., 2009).

2.2.2. Relationship between Oral Microbiome and Oral Diseases

Various studies had found that a subpopulation of the oral microbiome was different between healthy and diseased states. Healthy oral cavity has more homofermentative lactobacilli compared to periodontitis diseased mouth (Kõll-Klais et al., 2005) while a group of "red complex" bacteria, which consist of *Tannerella forsythia*, *Treponema denticola* and *Porphyromonas gingivalis*, is related to periodontal disease (Socransky et al., 1998, Haffajee

et al., 2008). Besides that, the plaque microbiome between healthy and gingivitis subjects was found to be structurally distinct (Huang et al., 2011) and caries-active oral environment harboured more *Streptococcus mutans* compared to healthy counterparts (Li et al., 2005). In addition, the predominant microbiota of the healthy tongue dorsa was found to be different from halitosis tongue condition (Kazor et al., 2003) and there was even a positive correlation between the relative abundance of Leptotrichia and Prevotella with the severity of oral halitosis (Yang et al., 2013).

2.2.3. Relationship between Oral Microbiome and Oral Cancer

Periodontitis was found to increase the risk of head and neck cancer even without the presence of other risk factors such as tobacco and alcohol (Tezal et al., 2009). Besides that, various stages of oral cancer were accompanied by chronic inflammations (Kurago et al., 2008). Several cancer associated bacteria species and groups such as *Treponema denticola*, *Streptococcus mitis*, and *Streptococcus anginosus* were found frequently in esophageal cancer (Narikiyo et al., 2004). Mager et al. (2005) reported that salivary *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *S. mitis* were significantly higher in oral cancer patients while Hooper et al. (2007) found that tumour tissues contained higher amount of Proteobacteria and Actinobacteria as well as saccharolytic and aciduric bacteria group. Besides that, *Johnsonella* genus and Gram positive bacteria were shown to be significantly increased in tumour tissues as compared to non-tumour tissues

(Pushalkar et al., 2012). However, it has yet to be determined whether these association could be etiological factors or are coincident colonization of oral cancer (Chocolatewala et al., 2010).

The human oral microbiome can be influenced by food and drinks during adulthood (Zaura et al., 2009) although the pioneer colonizers may come from the mother (Haraldsson, 2005). The food culture is different between Southeast Asia and western regions (Roman and Russell, 2009), which may have an effect on the oral microbiome. Hence studies that recruit subject groups from different geographical regions may uncover different healthy and diseased oral microbiome communities.

2.2.4. Hypothetic Carcinogenic Mechanism by Oral Bacteria

Three plausible mechanisms of oral bacteria induced oral carcinogenesis were hypothesized. Oral cancer may be caused by chronic bacterial inflammations, bacterial toxins and secondary metabolites or carcinogenic end products of bacterial metabolism.

Bacterial infections stimulate the release of nuclear factor-κB (NF-κB), human epidermal growth factor receptor 2 (HER-2/neu), nitric oxide (NO_2) and reactive oxygen species (ROS) that may contribute to carcinogenesis under prolong exposure (Nath et al., 2010).

The activation of NF-κB upregulates cell cycle control genes and numerous cytokines and downregulates tumour suppressor genes (Nath et al., 2010). The upregulated cell cycle control genes, such as cyclin D1, CDK2 kinase and c-myc, enhance cell proliferation while the overexpression of cytokines, such as IL-1β, IL6, VEGF, promote inflammatory responses and angiogenesis. On the other hand, downregulated tumour suppressor genes, such as p21, p53, pRb and TNF, create immortal cells and enhance tumor growth. This inflammation induced niche environment not only favours the survival of partially transformed cells (Nath et al., 2010), but also allows pathogen survival to prolong the carcinogenic effect of chronic inflammations (Ferrero-Miliani et al., 2007).

Bacterial inflammation also stimulates normal epithelial cells and leukocytes to produce NO and ROS which could cause oxidative damage to the genetic content (Nath et al., 2010) leading to genetic mutations on oncogenes and tumour suppressor genes. This in term could initiate carcinogenesis (Lee Eva and Muller, 2010).

The second hypothesis proposed that bacterial toxins and secondary metabolites could inflict damages on host cells through either direct or indirect mechanisms (Nougayrède et al., 2005). The direct form involves enzymatic attacks on the host cells which cause damage on the host genetic content and DNA repair mechanism while the indirect way involves the induction of chronic inflammation which stimulate the production of inflammatory factors and free radicals.

Oral carcinogenesis may also be due to the bacterial metabolism that produces carcinogenic end products. It was found that carcinogenic acetaldehyde and N-nitroso were elevated in heavy smokers and drinkers. Both carcinogens can be converted from ethanol and nitrosatable compounds through the metabolism of oral microbiota (Chocolatewala et al., 2010).

2.2.5. Potential Medical Value of Cancer Associated Oral Microbiome

The association between oral microbiome and oral cancer can be exploited to answer questions regarding the role of bacteria as biomarker of oral cancer (Mager et al., 2005). Few bacteria genera, such as Clostridium, Bifidobacterium and Salmonella, specifically colonize cancer tumours and certain species were even recoverable throughout the tumour progression phase. This tumour homing ability not only shed light on new potential drug delivery vectors for targeted molecular treatments, but also potential ultilization in tumour imaging diagnosis with genetic engineered light emitting bacteria. Nonetheless, there were clinical safety concerns regarding the pathogenic nature of invasive genera, particularly Clostridium and Salmonella, and the possibility of acquired virulence factors by attenuated bacteria through horizontal gene transfer with native commensals that could induce adverse immune responses or cause diseases (Morrissey et al., 2010). However, the native human oral microbiome is a good source for the identification of safe commensals with oral cancer diagnostic and therapeutic values.

2.3. 16S rRNA Gene

2.3.1. Definitions

16S rRNA gene is a part of the prokaryotic genome that has extremely conserved nucleotide sequences due to its important cellular functions (Armougom and Raoult, 2009). The entire 16S rRNA gene region is about 1,600 bp in length and contains several conserved and variable regions. The variable regions are extremely useful for phylogenetic analysis (Woese and Fox, 1977) while the flanking conserved regions are treated as priming sites of the PCR primers (Li et al., 2005). It is the gold standard marker for microbial taxonomic classification (Harmsen and Karch, 2004). The PCR amplification of the 16S rRNA gene is often termed as 16S PCR for convenience and is commonly used to estimate the microbial diversity and abundance (Wang and Qian, 2009).

2.3.2. Clinical Microbiology Applications

The conventional phenotypic identification of microbial pathogens was time-consuming and hampered by the subjective interpretation of the test results (Stager and Dvis, 1992). These problems were solved by the sensitive and rapid 16S rRNA gene analysis (Sontakke et al., 2009) which gained wide spread popularity including broad clinical applications.

16S rRNA gene analysis is very useful in identifying bacterial pathogens with unusual phenotypic profiles (Woo et al., 2002), rare pathogens with limited information (Lau et al., 2006a, Lau et al., 2006b, Lau et al., 2006c, Woo et al., 2007) and fastidious (Woo et al., 2008), culture-negative or even uncultivable (Le Monnier et al., 2006, Fihman et al., 2007, Welinder-Olsson et al., 2007) as well as novel species.

From the perspective of the patient, the accurate identification of bacterial pathogens enables clinicians to determine the appropriate antibiotics and the respective treatment duration (Jakobsson et al., 2010). With respect to the general population, 16S rRNA gene analysis allows medical researchers to understand the epidemiology, reservoirs, transmission routes, antibiotic resistance threats, treatment plans and outcomes of the disease associated bacteria (Woo et al., 2008).

2.3.3. The Advantages and Challenges

The identification of bacterial pathogens using 16S rRNA gene is advantageous in term of speed, sensitivity and accuracy (Sontakke et al., 2009). Bacterial pathogens can be identified up to their genus level in over 90% of bacteria associated cases and even up to the species level (Drancourt et al., 2000, Mignard and Flandrois, 2006). Such accurate recognition of etiological factors is an important factor for better medical intervention and improved patient recovery rate.

However, there is no single pair of universal primers that are able to detect all reported bacterial phyla (Sontakke et al., 2009), hence the choice of primers requires reviews on previous usage and detectable bacterial groups. In addition, there are considerations with respect to sequence such as the sequence length and quality as well as the species assignment based on similarity search results.

Drancourt et al. (2000) highlighted the need of full length 16S rRNA gene for accurate taxonomic assignments. However, Huse et al. (2008) showed that 454 pyrosequencing tags that encompassed either V3 or V6 regions were able to provide taxonomic assignments equivalent to respective full length sequences by using a Sanger sequence data of gut microbial community. This suggest that partial 16S sequences are as informative as full length 16S if they consist of V3 and V6 regions.

There is no consensus sequence similarity threshold value for bacterial species assignment due to variations of sequence similarities for the identification of new species caused by different evolution rate (Woo et al., 2008). The common sequence divergence values used in previous studies included 0.5%, 1.0% and 3.0% (Armougom and Raoult, 2009) but sequence similarities with more than 97% was commonly used for genus identification (Drancourt et al., 2000).

2.3.4. Suitable 16S Databases

The choice of a suitable 16S rRNA gene database is important because different databases may contain redundant unrelated or limited related reference sequences. GenBank contains huge collections of reference 16S rRNA gene sequences, which include many non-human environmental associated sequences that could hamper the decision of actual identity based on “first hit” or “closest match” (Woo et al., 2008). Such ambiguities in sequence identification are due to the similar level of intra- and interspecific differences caused by the presence of many redundant sequences (Clayton et al., 1995). On the other side, inappropriate choice of database would also hinders accurate sequence assignments due to the limitation of few relevant reference sequences (Drancourt et al., 2000). Therefore, the Human Microbiome Project Data Analysis and Coordination Center (HMP-DACC) was chosen as the reference 16S rRNA gene database in this study because

HMP-DACC consists of curated reference 16S rRNA gene sequences identified from samples originated from the human body.

Other 16S databases, such as Ribosomal Database Project (RDP), SILVA and Greengenes Project, also contain bacterial 16S rRNA gene sequences and can provide additional useful features and tools. RDP contains bacterial and archeal small subunit rRNA sequences as well as fungal large subunit rDNA sequences in complete and incomplete length (Cole et al., 2014). The SILVA database is a collection of non-redundant, curated and aligned small and large subunit rRNA sequences from the domains of Bacteria, Archaea and Eukarya (Quast et al., 2013). Greengenes Project comprises full length bacterial and archeal 16S sequences which were annotated and checked for chimera (DeSantis et al., 2006). All these online databases allow researchers to perform various tasks such as sequences browsing and alignment, probe matching as well as taxonomic identification.

2.3.5. Universal Primers

Any microbiome studies that deal with the general microbial diversity require the use of the universal 16S primers that attach to highly conserved 16S regions (Wang and Qian, 2009). In this study, two pairs of universal 16S primers were selected based on their previous usage in similar studies. The first pair, named D88 and E94, was used to amplify the near full length 16S rRNA gene region of around 1.5 kb which were used for clone library. The

second pair, which was designated as F968-GC and R1401, was used to amplify a short segment of the 16S rRNA gene of about 433bp length which was suitable for DGGE analysis.

2.3.6. Universal 16S Primers for Clone Library

The D88 and E94 primers had been extensively used in various oral microbiome studies, for instance, exploring the healthy oral microbiome (Aas et al., 2005), comparing the oral microbiome between healthy and diseased states (Paster et al., 2001, Kazor et al., 2003) and for the identification of oral cancer associated microbial diversity (Hooper et al., 2007). Nine bacterial phyla were detected with this primer pair which were also identified in other studies using next generation sequencing technique (Keijser et al., 2008, Lazarevic et al., 2009, Zaura et al., 2009). The same bacterial phyla, albeit with variations in the relative abundance at the genus level, were also identified using different primer pairs that target partial 16S rRNA gene (Nasidze et al., 2009).

2.3.7. Universal 16S Primers for DGGE Analysis

F968-GC and R1401 are also termed as DGGE primers because they include GC-clamps to generate PCR amplicons that are suitable for DGGE analysis. The GC clamp, a short GC rich oligo nucleotide of about 40bp in

length that is attached to the 5’ end of F968 primer, ensures the maximum detection of sequence variation between different DNA species, by preventing complete DNA denaturation thus providing the detection of sequence variations at higher melting domain (Myers et al., 1985, Sheffield et al., 1989). The F968-GC / R1401 primer pair was previously used in the study of periodontitis related oral microbiome (Zijnge et al., 2003) and it amplifies the variable regions of V6, V7, V8 and V9. The V6 region was found to be as informative as the full length 16S sequence (Huse et al., 2008) which make F968-GC / R1401 primer pair suitable to generate highly informative partial 16S amplicons for 16S metagenomic studies.

2.4. Clone Library

2.4.1 Definition

A clone library comprises a collection of library of many identical cloned host cells that carry the same recombinant plasmid bearing DNA molecules of interest (Brown, 2010b). The combined use of clone library, PCR and DNA sequencing has revolutionized the field of molecular biology since 1971 (Sambrook and Russell, 2001). These conventional techniques were improved over time and new methods were introduced. Examples of these new improved molecular technique include Gateway Recombinational Cloning (Walhout et al., 2000), Loop-mediated isothermal amplification (LAMP)

(Notomi et al., 2000) and Next Generation sequencing (NGS) (Margulies et al., 2005, Shendure et al., 2005).

2.4.2. Application in Microbiome Studies

Clone library analysis has been used to characterize not only environmental bacterial communities associated with rhizosphere (Trivedi et al., 2012), activated sludge (Sánchez et al., 2011), marine sponges (Erwin et al., 2011), coastline soil (Yousuf et al., 2012), and ocean (Allers et al., 2013), but also the human microbiota of skin (Gao et al., 2007), nasopharynx, oropharynx (Lemon et al., 2010), lung (Zakharkina et al., 2013) as well as oral cavity (Bik et al., 2010).

2.4.3. Advantages and Challenges

The use of clone library supersedes culture based techniques due to its ability to explore the unculturable constitutes of any microbiome with estimated coverage of more than 90% (Feingersch and Beja, 2009), hence providing a more comprehensive representation of any microbial community. The cloning of 16S rRNA gene PCR amplicons not only provide the highest phylogenetic resolution of any microbial community (Sánchez et al., 2011), but also abundant information through colony screening (DeAngelis et al., 2008). In addition, the use of clone library is advantageous over other

molecular methods with its gene isolation potential (Brown, 2010b), which is useful for protein expression and functional analysis (Scanlon et al., 2009). Consequently, many fields, for instance, research (DNA sequencing and genome construction), medicine (recombinant pharmaceutical proteins, disease genes, gene therapy), agriculture (plant genetic engineering) and forensic (identification of crime, kinship and gender), experienced major advancements through the use of this technique (Brown, 2010a).

However, the comprehensive quantitative information of any microbial community can only be obtained after extensive screening of a large clone population (Muyzer et al., 1993) which is time consuming (Džunková et al., 2012) and laborious (Liu et al., 2008). Besides that, the minor constituents of any microbial community represented by only single or few copy clones may not be screened (Kunin et al., 2008). Consequently, clone library is not feasible for in-depth analysis of microbial communities (Morgan et al., 2013) unless the colony screening procedure can be done faster by skipping the identification of redundant clones with identical inserts.

2.4.4. Screening of Clone Library

Any microbial community consists of a predominant constituent which could occupy a large proportion of the respective clone library. The repeating DNA sequencing of different clones carrying the same 16S rRNA gene is redundant and is a cause of waste in terms of time and resources. Restriction

Fragment Length Polymorphism (RFLP) can provide a solution to this problem as an initial clone library screening tool (Zhang et al., 2011b) that classifies the clones according to their unique fingerprint patterns prior to selective sequencing of the few representatives from each RFLP groups. RFLP could have high discriminative power with the usage of frequent cutters, such as MspI and HhaI, which have high frequency of restriction sites in 16S rRNA gene. Besides that, *in silico* study found that double digestions with 2 frequent cutters was able to distinguish between 83% – 96% of any clone libraries (Moyer et al., 1996).

In other words, RFLP technique will help to reduce the workload and resources as well as hasten the process by avoiding the need of redundant bacteria culture and DNA sequencing from clones with identical inserts. Furthermore, meaningful qualitative and quantitative information of any bacteria diversity can be retained. This information is useful for comparison of the community structure in different environments, such as between healthy and disease associated oral microbiota. Such information can help to characterize unique individuals or groups of microbes with potential medical value.

2.5. Denaturing Gel Gradient Electrophoresis (DGGE)

2.5.1. Definition

DGGE is a molecular technique that produces DNA fingerprinting profiles using a chemically created linear denaturing gradient (Myers et al., 1985) that allows the discrimination of PCR amplicons of similar sizes but with different sequence compositions (Li et al., 2005). Sequence variations along the stretch of DNA molecule that affect the GC percentage will produce various melting domains. For instance, low GC content regions lead to low melting domains and vice versa. These assorted melting domains result in different melting behaviour of the PCR amplicons which will denature at different points along the linear denaturing gradient in a manner similar to increasing melting temperature (T_m). The migration rate of the partial PCR amplicons is significantly reduced and hence can be separated from those that remain intact to produce DGGE fingerprints (Muyzer et al., 1993).

2.5.2. Applications in Microbiome Studies

DGGE was initially developed to detect point mutations (Myers et al., 1985, Børresen et al., 1988, Cariello et al., 1988) and heterozygosity (Sheffield et al., 1989). However, it soon became a popular tool to characterize microbial diversity of various environments, such as activated sludge (Sánchez et al., 2011), intestine (Wang et al., 2014), skin (Li et al., 2014) as well as oral

cavity (Zijnge et al., 2003, Li et al., 2005, Rasiah et al., 2005, Ledder et al., 2007). It is a powerful molecular technique that is able to rapidly generate a comprehensive snapshot of any microbial community and display community changes in terms of temporal and spatial distribution (Muyzer and Smalla, 1998). Besides the demonstration of constituent complexities, it can also generate profiles of metabolic activity of any microbial communities (Muyzer and Smalla, 1998).

2.5.3. Advantages and Challenges

DGGE is a faster and less laborious technique compare to clone library to obtain a comprehensive overview of not only any microbial community structure, but also the temporal and spatial shift of bacteria compositions between different samples in a single gel image or dendogram (Muyzer et al., 1993, Gafan et al., 2005). Further, DGGE is able to detect minor constituent of any microbial community with percentage as low as 1% (Muyzer et al., 1993). Although DGGE presents the microbial community in the form of fingerprint profile, it can be complicated by the presence of many different bands. The fingerprint profile complexity may lead to subjective and ambiguous visual analysis, but this can be solved by the inclusion of reference markers (Zijnge et al., 2003, Li et al., 2005) and the assistance of computational analysis (Rademaker and de Bruijn, 1997, Gafan et al., 2005).

The DGGE fingerprints present semi-quantitative information as the relative band intensity represents the relative abundance of a particular species. Each DGGE band carries no immediate bacteria species identify besides the assumption that they represent different microorganisms (Muyzer et al., 1993, Muyzer, 1999, Fromin et al., 2002, Zijnge et al., 2003). Clone library analysis could be a complementing molecular technique to DGGE with its ability to generate both species identity and abundance record. The strength of DGGE technique is best seen as a preliminary screening tool to identify the general changing patterns and to generate initial hypothesis prior to further analysis (Timmis et al., 2010).

3

METHODOLOGY

3.1. Ethical Approval

Ethical approvals for the recruitment of normal volunteers and oral cancer patients were obtained from the UTAR Scientific & Ethical Review Committee (U/SERC/03/2011) and the UM Medical Ethics Committee Faculty of Dentistry respectively (DF OP1208/0056(L)).

3.2. Subject Recruitment

Three groups of subjects, namely normal volunteers, pre-cancer and cancer patients, were recruited in this study according to selection criteria (Table 3.1). Informed consent was obtained from all participants. Normal subjects were recruited at University Tunku Abdul Rahman while pre-cancer and cancer patients were recruited from patients attending the Oral Medicine/Oral Surgery Clinic at the Faculty of Dentistry (coordinated by the Oral Cancer Research and Coordinating Center (OCRCC), University Malaya).

Table 3.1: Selection Criteria

Inclusion Criteria	Groups	Exclusion Criteria
- Malaysian - At least 20 years old - At least 20 teeth	All	- Having malaise, pregnancy or lactating - Infected with HIV - On antibiotic medication within the past 3 months - Had surgery or chemotherapy or radiation treatment within the past 1 month
	Normal	- Having gingival health issues such as ulcers and inflammations
- Diagnosed with pre-cancerous or cancerous lesions	Pre-cancer and Cancer	- Feel discomfort during sampling procedures and withdraw from volunteering

3.3. Sample Collection

The human oral microbiome was sampled using two sterile nylon flocked swabs (Millipore) per individual. The swabs were rubbed against the entire oral cavity (Table 3.2) using the sampling protocol outlined in Appendix A. Swabs were dipped into 400µL of preservation buffer (50mM Tris, pH8.0, 50mM EDTA, 50mM sucrose, 100mM NaCl, 1% SDS) and stored at ambient temperature (Nasidze et al., 2009) prior to DNA extraction.

Table 3.2: Swabs and Selected Oral Regions

Swabs	Oral Regions
1	Tongue, mouth floor and lower gingival region
2	Buccal, soft and hard palate as well as upper gingival region

Swabs have been used to collect oral microbiome samples (Cole et al., 1999, Dewhirst et al., 2010, Diaz et al., 2012) and was shown to have better sampling opportunity for certain cancer patients who have difficulty in producing saliva (Lee and Wong, 2010). The preservation buffer was previously used to preserve salivary microbiome samples but was proven to have equal effect on swab samples.

3.4. Bacterial Genomic DNA Extraction

Bacterial genomic DNA was extracted with GeneMATRIX Swab-Extract DNA Purification Kit (EURx) according to the protocol which was a modification of the manufacturer's protocol (Appendix B). A procedures prior to column centrifugation were modified compensate for the addition of the preservation buffer as the starting material. The changes were increase in the volume of Proteinase K enzyme, Sol S buffer and absolute ethanol and prolongation of the incubation period of Proteinase K digestion.

DNA yield and purity were determined spectrophotometrically with a NanoPhotometer (IMPLEN) while DNA integrity was analysed through 1% agarose gel electrophoresis at 70V for 50 minutes. Genomic DNA was stained with 3X RedSafe (iNtRON) for 30 minutes and visualized with BioSpectrum (UVP). All nucleic acid samples were stored at -80°C prior to downstream molecular processes.

3.5. PCR Optimization

PCR optimization step was carried out for both D88/E94 and F968-GC/R1401 primers (Table 3.3) to determine the best annealing temperature as well as the concentration of DNA template and magnesium ions. DNA template concentration was optimized prior to concurrent optimization of annealing temperature and magnesium concentration. PCR optimization was carried out using the chemical and temperature profiles as shown in Table 3.4 and 3.5 in the Veriti® 96-Well Thermal Cycler (Applied Biosystems). PCR amplicons were subjected to gel electrophoresis using 1% agarose gel at 90V for 30 minutes, stained with 3X RedSafe (iNtRON) for 30 minutes and visualized with BioSpectrum (UVP).

Table 3.3: The Sequence of Universal 16S primers

Primers	Sequences (5' – 3')
D88	GAGAG TTTGA TYMTG GCTCA G
E94	GAAGG AGGTG WTCCA RCCGC A
F968-GC	(CGCCC GCCGC GCCCC GCGCC CGTCC CGCCG CCCCC GCCCG) AACGC GAAGA ACCTT AC
R1401	CGGTG TGTAC AAGAC CC

Table 3.4: PCR Chemical Profiles used for Optimization

PCR Reagents	Concentrations	
	D88/E94	**F968-GC/R1401**
DNA template	0.35ng, 3.5ng, 35ng, 70ng, 350ng	10ng, 35ng, 70ng, 105ng
$MgCl_2$	0.5mM, 1.0mM, 1.5mM	0.5mM, 1.0mM, 1.5mM
Primers	0.50µM each	0.25µM each
dNTPs mixture	0.2mM	
Taq polymerase	1.5U	
$MgCl_2$ free buffer	1X	
Total volume	50µL	

Table 3.5: PCR Temperature Profiles used for Optimization

Stage		Temperature, ºC (Duration, second)	
		D88/E94	**F968-GC/R1401**
Pre-denaturation		95 (480)	94 (480)
PCR Cycles	**Denaturation**	95 (45)	94 (30)
	Annealing	60, 63, 65, 67 (60)	50, 54, 58 (60)
	Extension	72 (60)	90 (60)
Final Extension		72 (1,800)	600 (1,800)
Cycles		25	30

3.6. 16S PCR for Clone Library and DGGE

All 50µL PCR mixture contained 1X $MgCl_2$ free buffer, 0.2mM dNTPs mixture and 1.5U Taq polymerase (Intron Biotechnology) as well as optimized DNA template and magnesium ion concentration.

PCR was done in the Veriti® 96-Well Thermal Cycler (Applied Biosystems). The cycling condition for the primer pair D88/E94 is denaturation at 95ºC for 8 minutes, followed by 25 cycles of denaturation at 95ºC for 45 seconds, annealing at the optimized temperature for 60 seconds and extension at 72ºC for 90 seconds, and a final extension step at 72ºC for 10 minutes. Likewise, cycling condition for the primer pair F968-GC/R1401 is denaturation at 94ºC for 8 minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at the optimized temperature for 60 seconds and extension at 72ºC for 60 seconds, and a final extension step at 72ºC for 30 minutes.

All PCR amplificons were subjected to gel electrophoresis in 1% agarose gel at 90V for 30 minutes, Genomic DNA was stained with 3X RedSafe (iNtRON) for 30 minutes and visualized with BioSpectrum (UVP).

3.7. DGGE Reference Markers

Several colonies with known sequences were chosen as candidate DGGE reference markers base on their different GC percentage and estimated melting temperatures calculated with the online software EndMemo (available at http://www.endmemo.com/bio/gc.php) and Oligo Calc (Kibbe, 2007). DGGE PCR was carried out for several random colonies as described in chapter 3.6 using 1mM of $MgCl_2$ and 10ng of plasmid and annealing temperature of 58ºC. The PCR amplicons were electrophoresed in 8% (w/v) polyacrylamide gel at 100V and 60ºC in Temporal Temperature Gradient Electrophoresis (TTGE system (C.B.S. Scientific) filled with 25L of 1X TAE for a duration that had been previously optimized.

3.8. GC-clamp Selection

This study found that the F968-GC/R1401 primer pair was able to produce double bands from a single clone colony. It was suggested that this observation could be due to the interference from the GC-clamp. Subsequently, the fingerprint profiles of F968-GC/R1401 primer pair attached to two different GC-clamps, which are the original (Zijnge et al., 2003) and the Sheffield GC clamp (Sheffield et al., 1989), were compared with each other in order to choose a better GC clamp. Besides that, Minimum Gibbs Free Energy required to generate secondary structures by the two GC clamps were

calculated with EndMemo (available at http://www.endmemo.com/bio/gc.php) and Oligo Calc (Kibbe, 2007).

3.9. DGGE Optimizations

Optimization of few DGGE parameters was carried out prior to sample electrophoresis. These parameters were chemical gradient, electrophoresis duration and the establishment of DGGE reference marker.

3.9.1. Chemical Gradient

A suitable DGGE chemical gradient was determined based on the approximate initial and final stages of the melting curve produced by F968-GC/R1401 PCR amplicons. The melting curve begins when a single DGGE PCR amplicon band starts to separate into multiple bands as they migrate differently at increasing denaturant concentration. The melting curve ends when all these separated bands migrate at the same rate because the entire amplicon region, with exception of the GC clamp region, is fully denatured at a certain high denaturant concentration. A mixture of 175µl of DGGE PCR amplicons and 35µl of 6X loading dye were loaded into a single well and electrophoresed in 8% (w/v) perpendicular polyacrylamide gel with 1X TAE at 100V and 60ºC for 10h in the TTGE system (C.B.S. Scientific). The initial chemical gradient range tested was from 10% to 90%.

3.9.2. Electrophoresis Duration

F968-GC/R1401 PCR amplicons were electrophoresed for different durations including 12h, 14h, 16h, 18h and 20h and the banding resolution of each run were compared. The DGGE PCR amplicons were electrophoresed in 8% (w/v) parallel polyacrylamide gel with optimized chemical gradient and 1X TAE at 100V and 60ºC. A mixture of 24µl of DGGE PCR amplicons and 6X loading dye was loaded into five wells with 2 hours interval between each sample loading.

3.10. DGGE Analysis

F968-GC/R1401 PCR amplicons were analyzed in 8% (w/v) polyacrylamide gel (Calbiochem) with chemical gradient ranging from 40% to 60% (100% consists of 40ml formamide and 42g urea) created using the Gradient Maker and Mini Pump (C.B.S. Scientific). The polyacrylamide gel was electrophoresed with 1X TAE (1st Base) at 100V and 60ºC for 18h in the TTGE System (C.B.S. Scientific). Each well was loaded with 24µl of PCR amplicons mixed with 6X loading dye at 1:5 ratio. DGGE reference markers were also loaded into every first, middle and last lane of all polyacrylamide gels. The gel was post-stained with Gel Red (Biotium) for 30 minutes and digitally captured and recorded with BioSpectrum (UVP) with consistent parameters (Table 3.6).

Table 3.6: Parameters of BioSpectrum (UVP) for the recording of DGGE gel images

Parameters	Options
Aperture	1.2
Zoom	50%
Focus	6.3%
Post processing option	Dark frame subtraction
Capture binning	1 X 1
AutoExpose option	Best

3.11. BioNumerics Analysis

The software BioNumerics version 6.2 (Applied Maths) was used to process and analyze all DGGE fingerprints.

The DGGE fingerprints were first normalized according to the DGGE reference markers by assigning and matching them across all the DGGE gel images. The normalized DGGE fingerprints were then further fined tuned by match making plausible identical bands, adding missing bands and deleting false positive contaminants with reference to raw DGGE fingerprints.

Then, a DGGE dendogram was generated by the unweighted pair-group method (UPGMA) based on the Dice coefficient similarity between the

DGGE fingerprints. Reliable clusters with cophenetic correlation of at least 80% were identified.

3.12. 16S Clone Library Construction and Colony Screening

16S PCR amplicons generated with D88/E94 primers were purified with MEGAquick-spin (iNtRON) prior to clone library step. Purified 16S PCR amplicons were cloned into pSTBlue-1 AccepTor Vector (Novagen) which was subsequently introduced into NovaBlue Singles™ Competent Cells according to the manufacturer's protocol. The transformed cells were then plated onto ampicillin (nacalai tesque) supplemented LB agar plates (50µg/ml) and incubated overnight at 37°C.

About sixty colonies from each clone library were screened for the correct insert size of approximately 1.5kb by colony PCR using T7 and SP6 primers. An aliquot (5µl) of the colony PCR amplicons with the correct insert size were double digested with MspI and HhaI for 1 hour at 37ºC (Thermo Scientific) to determine the colony diversity (Zhang et al., 2011a). The digested fragments were electrophoresed in 3% NuSieve 3:1 agarose (Lonza) at 120V until the xylene cyanol FF dye reaches the fourth row of the casting tray. The gel was post-stained with Gel Red (Biotium) for 30 minutes and digitally recorded with BioSpectrum (UVP).

The recorded RFLP patterns were analysed visually to group similar patterns. Colonies with similar RFLP patterns were grouped together and the amount of colonies per RFLP group was used as abundance data (Zhang et al., 2011a). Up to ten representative colonies were selected for DNA sequencing from each distinct RFLP group.

3.13. Bacteria Culture, Plasmid Extraction and Partial 16S Sequencing

Selected bacteria colonies were cultured in 5ml LB broth (Pronadisa) with 50µg/ml ampicillin (nacalai tesque) for 16 to 18 hours with shaking at 250rpm. Plasmid was extracted using the High-Speed Plasmid Mini Kit (Geneaid) according to the manufacturer's protocol and then sent to Bioneer Inc. for partial 16S sequencing. The partial sequences of around 750bp in length starting from the R1401 primer site consist of V6, V7 and V8 variable regions and are sufficient for phylogenetic and statistical analysis because V6 have been proven to be equally informative as the full length 16S sequence (Huse et al., 2008).

3.14. Sequence Identification and Nucleotide Accession Number

16S primer sequences were trimmed off while sequences with good quality were selected using the Sequence Scanner 1.0 (Applied Biosystem). These sequences were searched against the Human Microbiome Project Data

Analysis and Coordination Center (HMP-DACC) which is available at http://www.hmpdacc.org/resources/blast.php. Representative sequences were assigned to bacteria species with the highest bit score and lowest E-values. All sequences were deposited in GenBank with accession numbers from KP294530 to KP294905 (Table C.1).

3.15. Clone Library Analysis

3.15.1 Sequence Processing

All representative sequences were processed using the MOTHUR software (Schloss et al., 2009) following a modified SOP. Firstly, sequences were grouped together into fasta format with Mega 6.0 (Tamura et al., 2013). Representative sequences were duplicated according to abundance record of their respective RFLP groups. Then, sequence alignment and filtration were done using the following commands with default parameters; 1. unique.seqs, 2. align.seqs, 3. make.group, 4. make.table, 5. summary.seqs, 6. screen.seqs, 7. summary.seqs, 8. filter.seqs, 9. unique.seqs, with the exception of the screen.seqs command with customized "minlength" and "maxlength" parameters of "722bp". Next, possible chimera and contaminants of mitochondria and chloroplast sequences were checked prior to sequence clustering at 0.03 cut-off value and taxonomic classification through alignment against SILVA-based bacterial reference alignment (Schloss et al., 2009).

Distance matrix was calculated using the following commands with default parameters; 1. dist.seqs, 2.cluster, 3. make.shared and 4. count.

3.15.2. Phylogenetic Analysis

Several 16S reference sequences from NCBI Nucleotide database were included in this phylogenetic analysis based on bacteria identification obtained from HMP-DACC. In addition, reference sequences were also retrieved based on NCBI BLAST results (Altschul et al., 1997) for representative sequences with different HMP-DACC and SILVA assignments. All the reference and representative sequences were aligned by ClustalW and phylogenetic tree was generated by Neighbour Joining method using Mega 6.0 software (Tamura et al., 2013).

3.15.3. Biodiversity Measurements

Rarefaction curve is commonly used to compare species richness to sampling effort (Simberloff, 1978) while library coverage is used to demonstrate how all sequences in a given sample can be represented by the obtained sequences (Good, 1953b). They were calculated using the "rarefaction.single" and "collect.single" commands respectively with the MOTHUR software where the latter command also include the calculation of the Shannon and inverse Simpson indexes. Shannon index were converted to

effective species number (Table 3.7) while the value of inverse Simpson index remained unchanged since it is the effective species number of itself (Jost, 2007).

Table 3.7: Formulae

Calculations	**Formula**	**Symbol descriptions**
Good's coverage	(1-(n/N))100	n is the singletons amount while N is the total amount of OTUs
Shannon Index	$-\Sigma p_i \ln p_i$	p_i is the ratio of specific OTUs
Effective species number of Shannon index	$e^{D_{Shannon}}$	$D_{Shannon}$ is the Shannon index
Simpson Index	Σp_i^2	p_i is the ratio of specific OTUs
Effective species number of Simpson index or Inverse Simpson index	$1/D_{Simpson}$	$D_{Simpson}$ is the Shannon index

3.15.4. The Relative Abundance of Common, Shared and Unique OTUs

The relative abundance of the common OTUs, at the genera and phyla levels, that overlapped across all subject groups were tabulated and graphed with Microsoft Excel. Besides that, shared OTUs that overlapped across any two subject groups and unique OTUs that were only found in any particular subject group were also tabulated with Microsoft Excel.

3.15.5. AMOVA and NMDS Ordination

Individual clone libraries were organized based on the presence or absence of RFLP groups for NMDS ordination and AMOVA. Representative sequences were included regardless of subject group origins and abundance record. NMDS ordination was used to illustrate the similarities between different community structures (Ramette, 2007). The thetaYC generated distance matrix from 80 sub-sampled sequence libraries was used with the "nmds" command for the NMDS plot calculation. NMDS was generated in R language environment using rgl packages (R Core Team, 2013). The significance of difference of genetic diversity between pooled and within two or more communities was tested with AMOVA (Schloss, 2008) using the MOTHUR software.

4

RESULTS

4.1. Subject Recruitment

A total of 40 normal subjects, 10 pre-cancer and 13 cancer patients were recruited in this study (Table 4.1, 4.2 and 4.3). Each group consisted of Malays, Chinese and Indians of both gender. The male to female ratio of the normal group was 1:1 while there were slightly more female subjects in both patient groups. The average age of the normal group which was around 41 years old, was lower than that of the patient groups which was about 56 years old. There were higher percentage of smokers and betel quid chewers in the pre-cancer and cancer groups compared to the normal counterpart. However, only pre-cancer subjects were found to have higher frequency of drinking habit (Table 4.4). There were no further division of pre-cancer and cancer subjects according to lesion types and histopathological grades due to the focus of this study on the microbiome variations between normal, pre-cancer and cancer groups.

Table 4.1: Distribution of Normal Subjects According to Ethnicity and Gender

Ethnicity	Malay		Chinese		Indian		Sub-
Gender	M	F	M	F	M	F	Total
21-29	2		3	4		3	12
30-39		1	4	2			7
40-49	1	1	2	3	1		8
50-59	2	1	1	1	1	2	8
60-69			2	1			3
≥70			1	1			2
Sub-total	8		25		7		40

Table 4.2: Distribution of Pre-cancer Subjects According to Ethnicity and Gender

Ethnicity	Malay		Chinese		Indian		Sub-
Gender	M	F	M	F	M	F	Total
21-29							0
30-39		1					1
40-49				3			3
50-59	1	1			1		3
60-69						1	1
≥70				1		1	2
Sub-total	3		4		3		10

Table 4.3: Distribution of Cancer Subjects According to Ethnicity and Gender

Ethnicity	Malay		Chinese		Indian		Sub-Total
Gender	**M**	**F**	**M**	**F**	**M**	**F**	
21-29							1
30-39			1	1			1
40-49	1	1					2
50-59				1	1		4
60-69			3	1		1	5
≥70				1		1	2
Sub-total	2		8		3		13

Table 4.4: Distribution of Demographic Data of All Groups

Demographic Information	Normal	Pre-cancer	Cancer
Male to female ratio	1:1	3:11	1:1
Average age	41	55	58
Smoking	7.5%	30.0%	30.0%
Betel quid chewing	2.5%	20.0%	15.0%
Drinking	5.0%	30.0%	7.0%

4.2. DNA Extraction

Bacterial genomic DNA was successfully extracted from all 63 subjects with average DNA concentration and purity in absorbance ratio of 58.64ng/µl and 1.72 respectively (Table D.1, D.2 and D.3).

4.3. Primer Optimization

The optimum DNA template concentration was 35ng for D88/E94 and 10ng for F968-GC/R1401. Magnesium ions concentration of 0.5mM was found to be optimal for both primer pairs. The optimum annealing temperature was 63ºC for D88/E94 and 54ºC for F968-GC/R1401. The complete chemical and temperature profiles of both primers are listed in the following table.

Table 4.5: PCR Chemical Profiles of D88/E94 and F968-GC/R1401

PCR Reagents	Final Concentrations	
	D88/E94	**F968-GC/R1401**
DNA template	35ng	10ng
$MgCl_2$ (25mM)	0.5mM	0.5mM
Primers (0.5uM)	0.50µM each	0.25µM each
dNTPs mixture (10mM)	0.2mM	
Taq polymerase (5U/ul)	1.5U	
$MgCl_2$ free buffer (10X)	1X	
Total volume	50µL	

Table 4.6: PCR Temperature Profiles of D88/E94 and F968-GC/R1401

Stage		Temperature, °C (Duration, second)	
		D88/E94	**F968-GC/R1401**
Pre-denaturation		95 (480)	94 (480)
PCR Cycles	**Denaturation**	95 (45)	94 (30)
	Annealing	63 (60)	54 (60)
	Extension	72 (60)	72 (60)
Final Extension		72 (1,800)	72 (1,800)
Cycles		25	30

4.4. DGGE

DGGE was used to produce fingerprint profiles of oral microbiota associated with normal, pre-cancerous and cancerous conditions in this study. These profiles were compared to identify the clustering pattern of general oral microbiome community with respect to different subject groups. A suitable marker and GC clamp, as well as optimum DGGE parameters were determined to allow a more accurate identification and interpretation of fingerprint profiles. Computational analysis was employed to ensure consistent and accurate dendogram clustering.

4.4.1. DGGE Reference Marker

A standard DNA ladder is not suitable for DGGE since DGGE separates PCR amplicons based on variation of melting domains instead of the length of amplicons. Instead an internal standard was derived from the clone colonies inserts in this study since the DGGE PCR amplicon region was included within the full 16S insert. The DGGE PCR amplicons were produced by replacing the bacterial genomic DNA with clone colonies as the PCR template.

At the initial stage, DGGE PCR amplicons that showed distinct bands were chosen (Figure 4.1A & B) and mixed at equal ratio to produce a temporary reference marker (Figure 4.1C, D & E). Then other DGGE PCR amplicons that showed distinct bands beyond the range of the temporary marker (Figure 4.1C, D & E) were chosen and mixed together with selected DGGE PCR amplicons at equal ratio to produce the final DGGE reference marker with comprehensive cover range (Figure 4.1F).

All DGGE PCR amplicons were produced from clone colonies with known sequences hence information about individual bands of the DGGE reference markers was available as shown in Table 4.7.

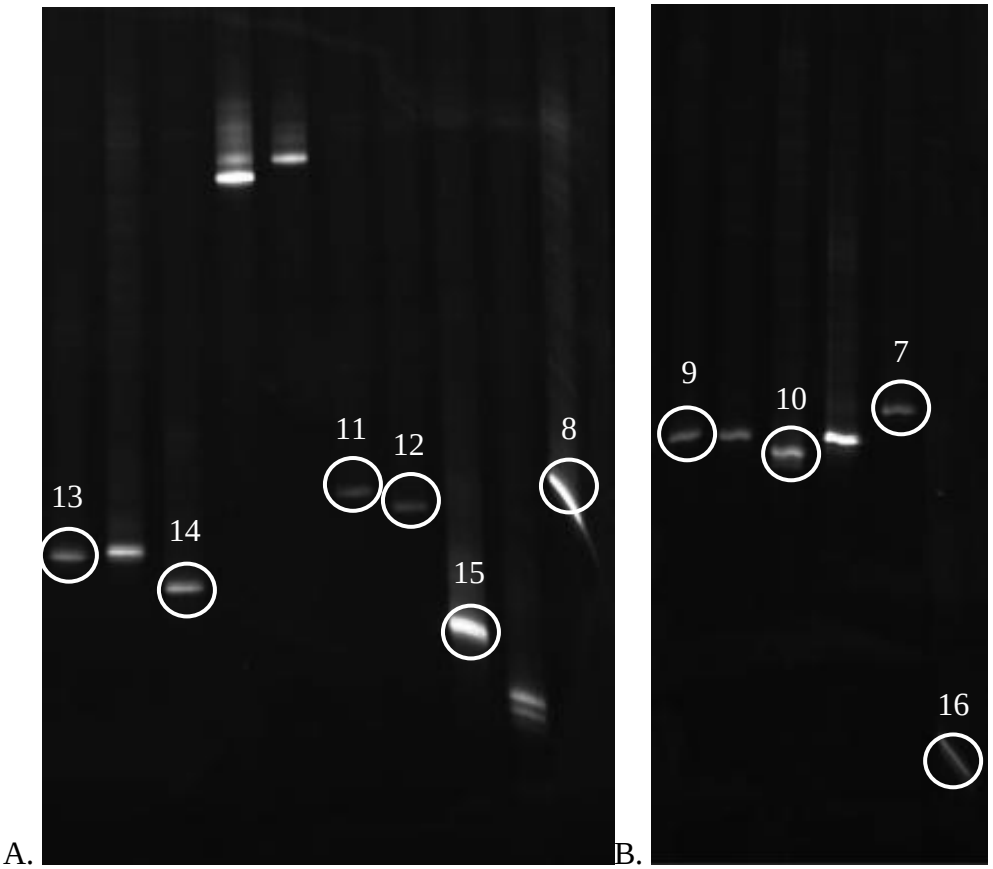
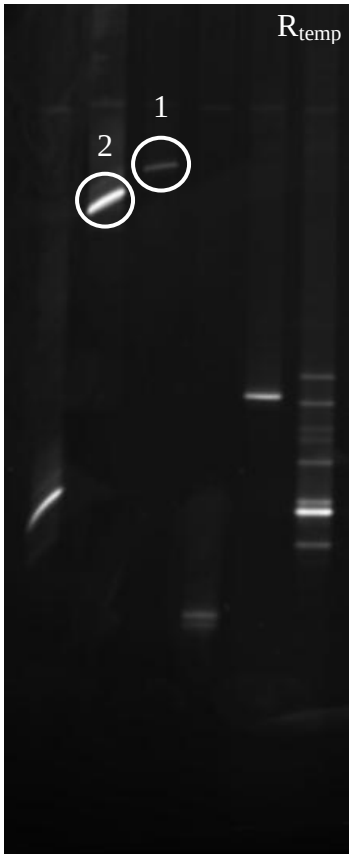
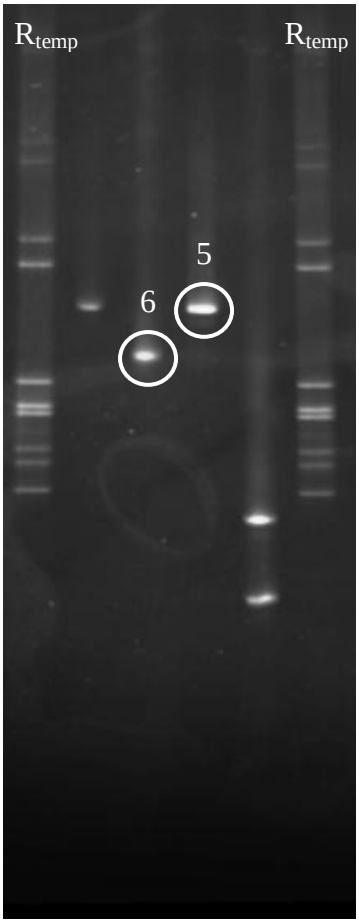


Figure 4.1: The generation of DGGE reference marker (F) through the selection of DGGE amplicons that showed distinct migration distance from each other. These amplicons were produced from clone colonies with known 16S rRNA gene sequences. The information of selected DGGE amplicons (shown in circles in A to E) are tabulated (Table 4.7). The symbol R_{temp} is Temporary Reference Marker while R_f is Finalized Reference Marker.

C. 

D. 

E. 

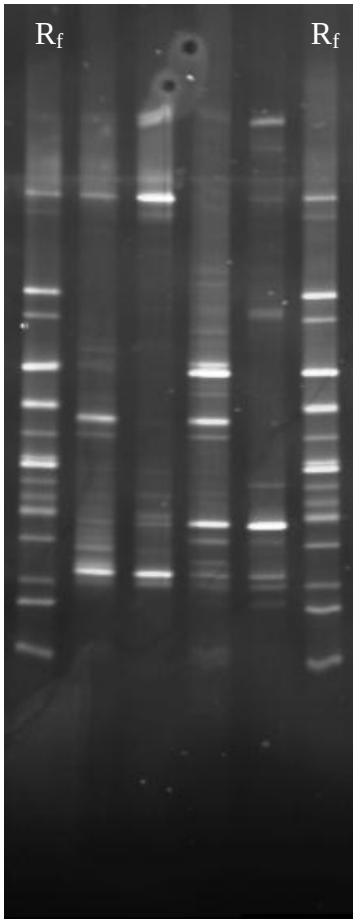
F. 

Table 4.7: Information on Individual Bands of the DGGE Reference Marker

Marker No.	Bacteria Genera	Approximate Length, bp	GC %	T_m, ºC
1	*Gemella*	440	48.18	83.1
2	*Gemella*	440	48.81	83.2
3	*Oribacterium*	433	50.35	84.0
4	*Oribacterium*	433	50.58	84.1
5	*Haemophilus*	434	52.07	84.7
6	*Haemophilus*	434	51.38	84.4
7	*Veillonella*	435	52.41	84.8
8	*Actinomyces*	438	54.00	85.5
9	*Veillonella*	435	52.87	85.0
10	*Veillonella*	436	52.75	85.0
11	*Granulicatella*	433	52.19	84.7
12	*Granulicatella*	433	52.42	84.8
13	*Streptococcus*	433	53.58	85.3
14	*Neisseria*	433	54.73	85.8
15	*Actinomyces*	435	55.86	86.3
16	*Rothia*	435	55.17	86.0

4.4.2. Sheffield GC clamp

F968-GC/R1401 attached with the Sheffield GC clamp produced sharper and brighter bands as compared to the primer with original GC clamp (Figure 4.2). Besides that, Sheffield GC clamp attached primers had lower amount of secondary structures which require higher energy to form as compare to primers with old GC clamp (Table 4.8). Subsequently, F968-GC was attached to Sheffield GC clamp to produce all DGGE fingerprints.

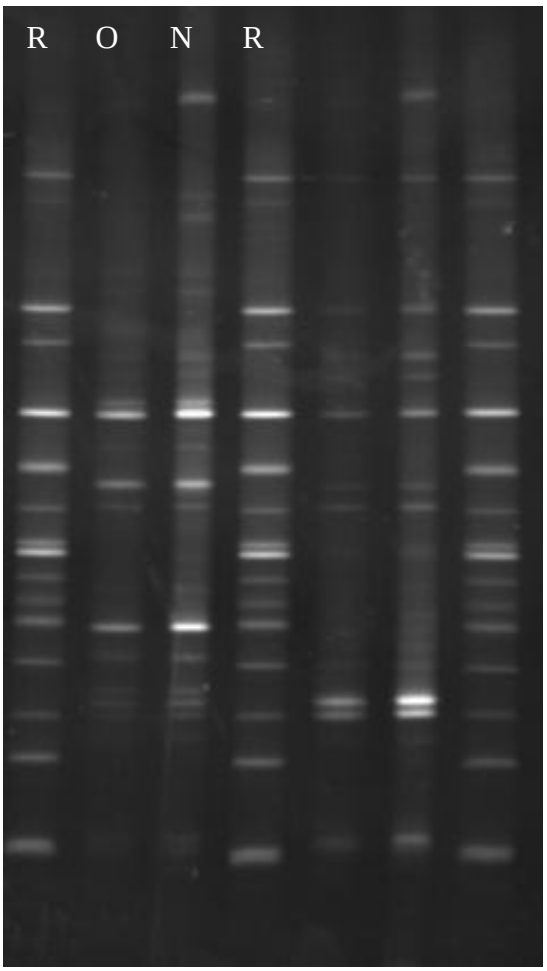


Figure 4.2: The comparison of DGGE amplicons produced with original GC clamp (O) and Sheffield (1989) GC clamp (N). R is the DGGE reference marker.

Table 4.8: Secondary structures and associated minimum Gibbs Free Energy (in bracket) according to NetPrimer (available at http://www.premierbiosoft.com/netprimer/index.html)

GC clamps	Hairpins	Self-anneal
Existing	22 (-25.71 kcal/mol)	20 (-68.06 kcal/mol)
Sheffield	15 (-5.16 kcal/mol)	10 (-10.36 kcal/mol)

4.4.3. Extension Time of PCR

Smearing and multiple bands were observed when the final PCR extension was 5 minutes (Figure 4.3A & B) even though the PCR template was from a single clone colony. These artifactual bands were eliminated when the final PCR extension duration was prolonged to 30 minutes (Figure 4.3C). Subsequently, the final PCR extension duration for PCR DGGE application was extended to 30 minutes.

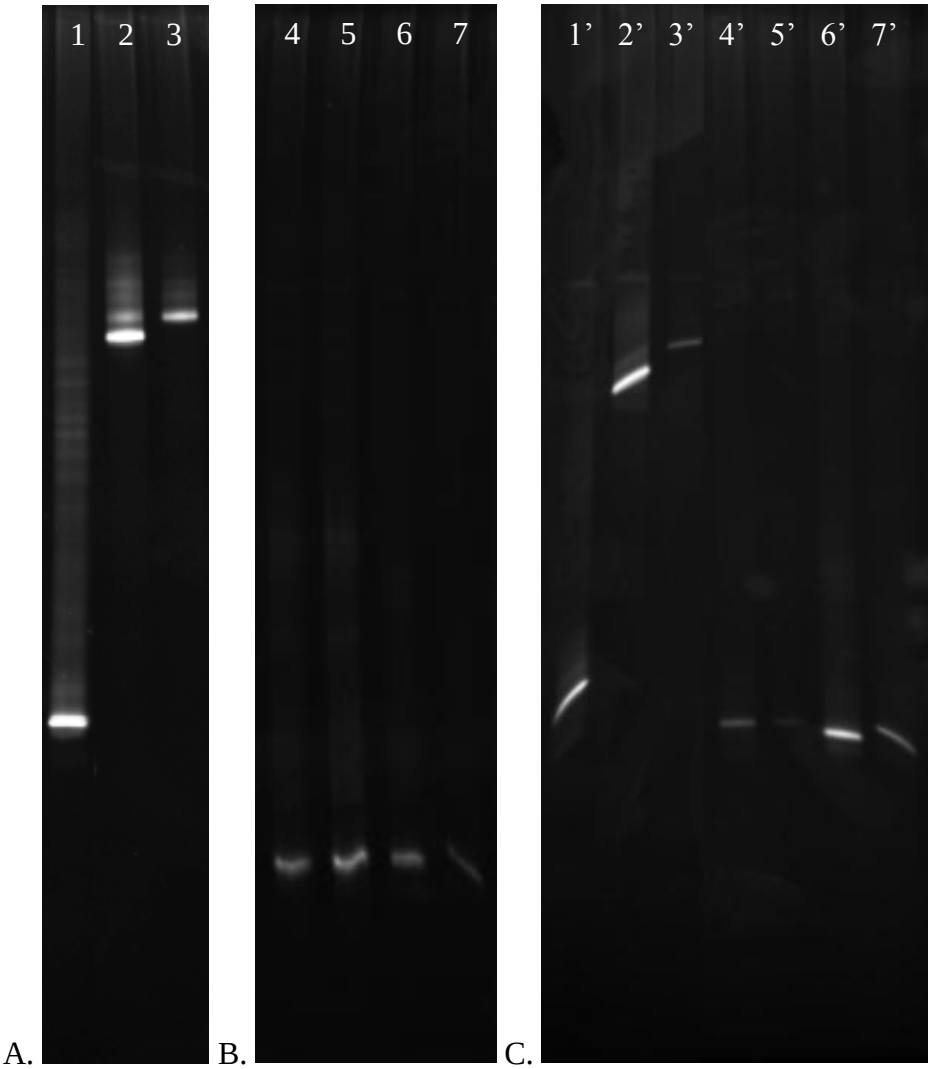


Figure 4.3 A & B: PCR amplicons produced with 5 minutes of final extension.

C. PCR amplicons produced with 30 minutes of final extension

4.4.4. Denaturing Gradient Optimization

The initial melting curve produced from the chemical gradient range of 10% to 90% suggested that the potential optimum range lies between 30% to 60% (Figure 4.4A). Subsequent narrowing of chemical gradient to 30% to 60% produced a melting curve that began at around 32% and levelled off at around 60% (Figure 4.4B). However, further fine tuning was required for the chemical gradient between 32% and 60% due to poor band resolution (Figure 4.5A). Subsequently the final chemical gradient was narrowed to a range of between 40% and 60% which produced better band separation (Figure 4.5B).

A.

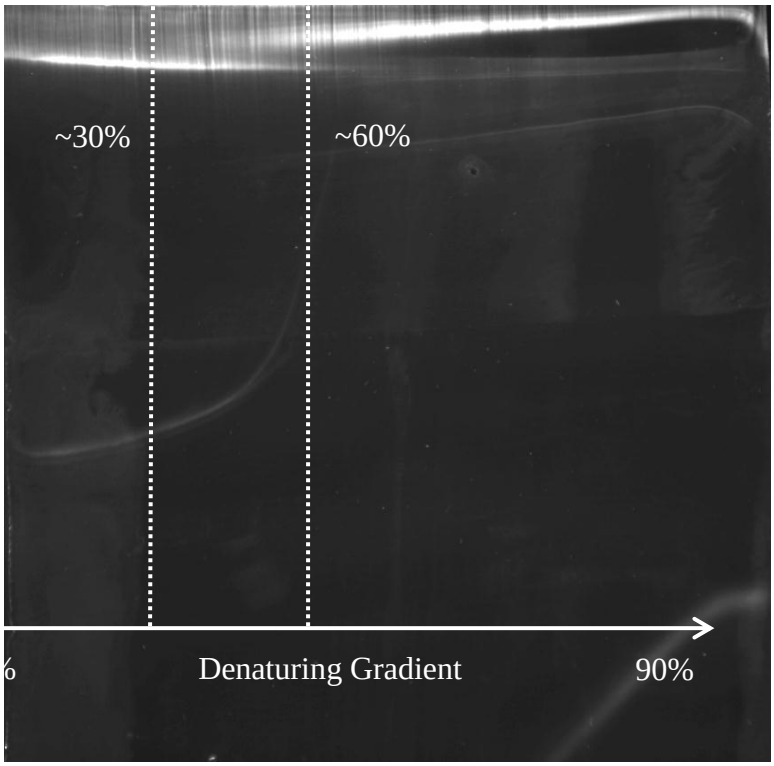
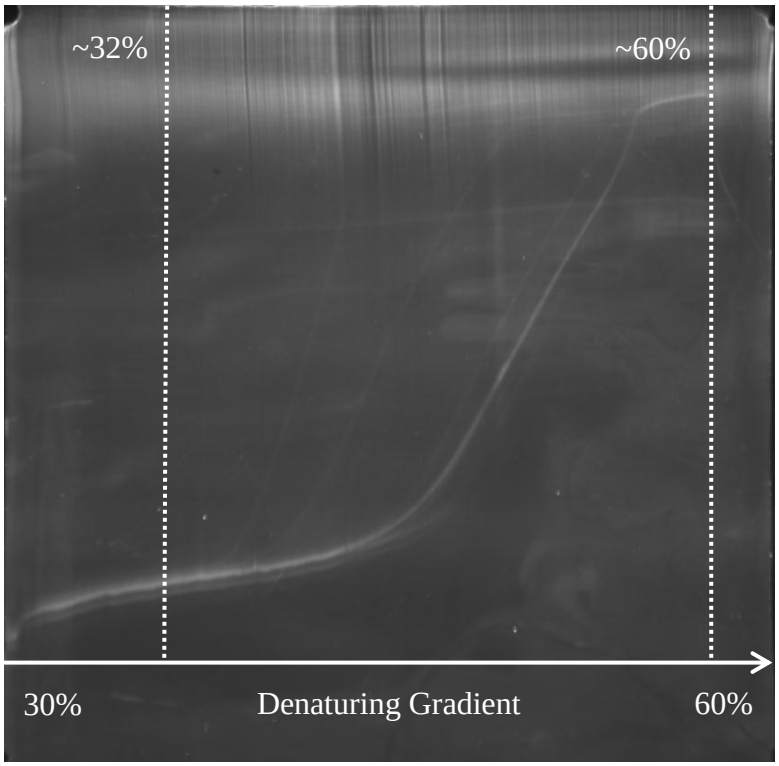


Figure 4.4A & B: Optimization of denaturing gradient with the increasing denaturant concentration (solid arrow) and the potential starting and final denaturant concentration (dotted lines). The denaturant gradient of this DGGE is perpendicular to the electric field.

B. 

4.4.5. Optimization of Electrophoresis Duration

The maximum band resolution was achieved with 18 hours of electrophoresis run time. A longer period of run did not produce further noticeable band separation (Figure 4.5B).

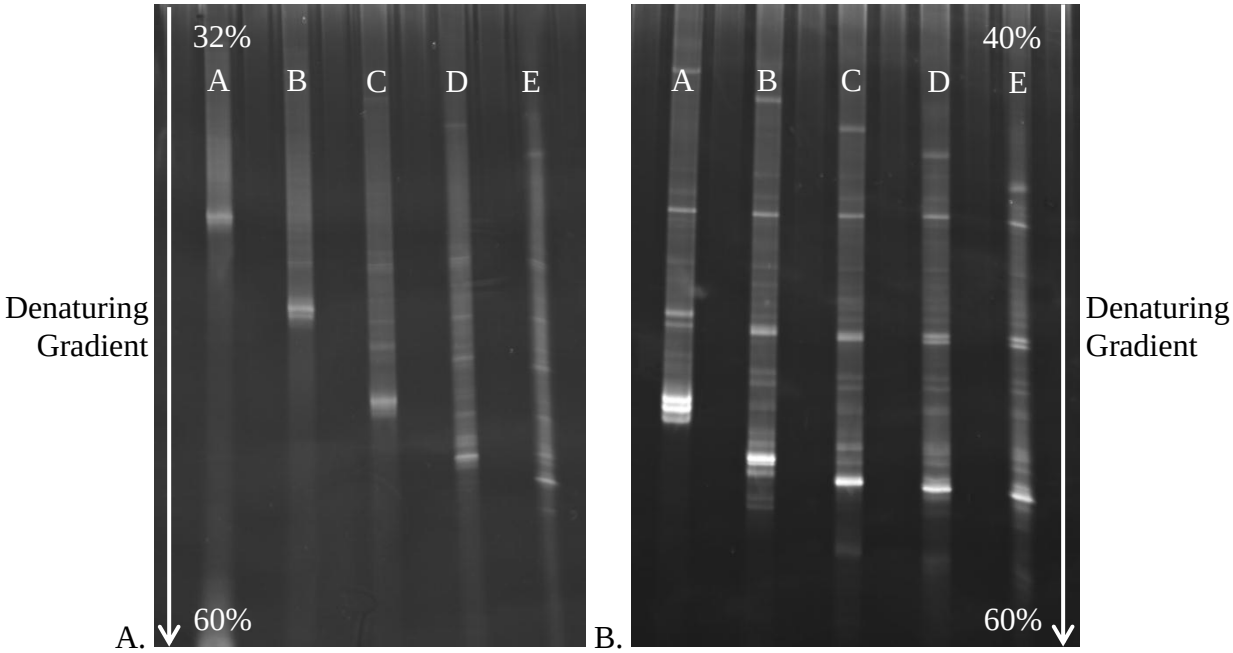


Figure 4.5A & B: Optimization of electrophoresis duration with five different time points; A: 12 hours; B: 14 hours; C: 16 hours; D: 18 hours; E: 20 hours.

4.4.6. DGGE Dendogram

Ten DGGE fingerprint clusters with ≥80% cophenetic correlation were identified (Figure 4.6). When DGGE fingerprints were clustered based on oral cancer status, four normal clusters, one patient cluster and five mixed clusters which consisted of normal, pre-cancer and cancer subjects were observed (Figure 4.6). The similarity scores between the DGGE profiles were tabulated in Table E.1 while all raw DGGE gel images used for the generation of DGGE dendogram can be found from Figure E.1 to E.7.

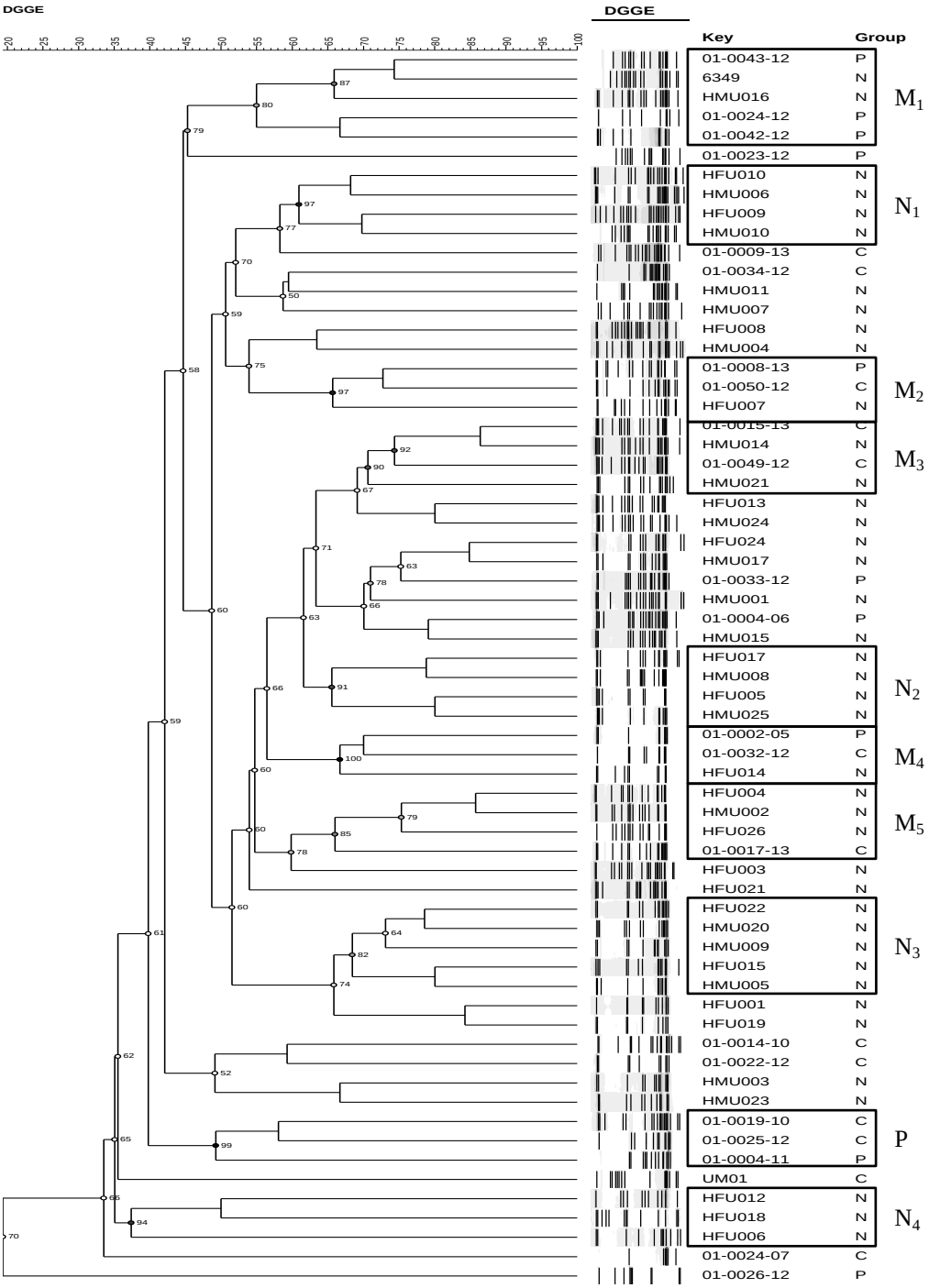


Figure 4.6: UPGMA dendogram with symbols N as normal, P as pre-cancer and C as cancer group respectively. DGGE clusters with at least 80% cophenetic correlation were identified and given symbols such as, N for normal cluster, P for patient cluster and M for mix cluster consists of normal, pre-cancer and cancer groups. The numeric values denote the order of the respective cluster types.

4.5. Clone Library

Clone library analysis was used in this study to provide bacteria identification and abundance data to further characterize the oral microbiome community associated with normal, pre-cancer and cancer conditions. Clone library data yield information such as bacteria diversity, unique and shared OTUs as well as community structure similarities and variations.

4.5.1. Biodiversity Measurements

The rarefaction curves (Figure 4.7) reach a plateau phase after 500 colonies and the Good's coverage (Table 4.9) was around 97% for all three groups. The effective species number of both Shannon and Simpson indexes were highest for the cancer group, followed by pre-cancer and finally the normal group (Table 4.9). The species richness was the highest in the pre-cancer group and similar between the normal and cancer group (Table 4.9).

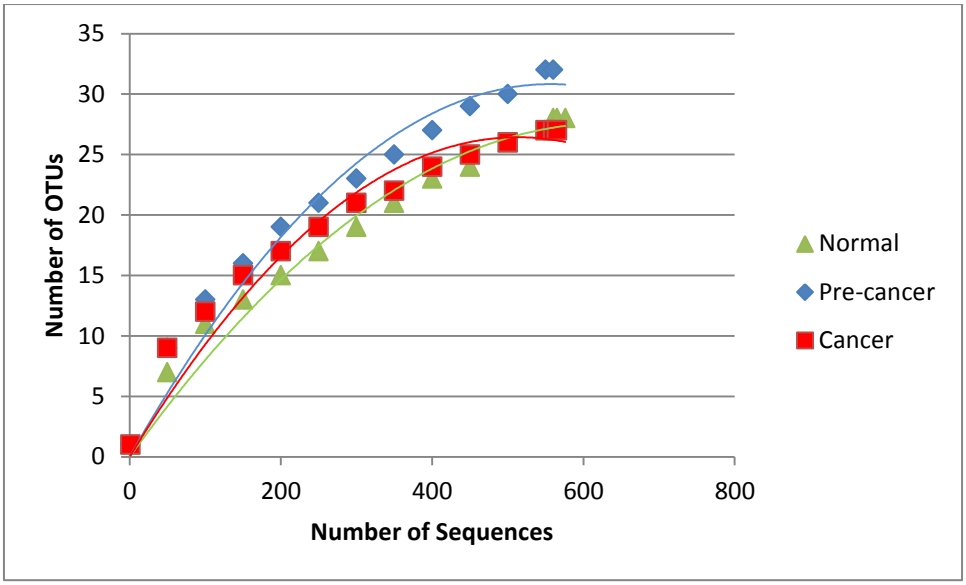


Figure 4.7: Rarefaction curve of normal, pre-cancer and cancer groups

Table 4.9: Good's coverage, species richness and effective species number of Shannon and Simpson indexes

Groups	Good's coverage, %	Species Richness	Effective Species number	
			Shannon Index	Simpson Index
Normal	97.22	29	3.67	2.15
Pre-cancer	96.96	35	4.96	2.66
Cancer	98.05	28	4.99	2.72

4.5.2. Bacterial Profiles of The Oral Cavity in Normal, Pre-cancer and Cancer Subjects

A total of five bacteria phyla were detected in all subject groups, with Firmicutes as the predominant phylum (84% - 94%) followed by Proteobacteria (3% - 12%) and three other smaller phyla namely Fusobacteria (0.7% - 1.6%), Bacteroidetes (0.5% - 1.4%) and Actinobacteria (0.4% - 0.9%) (Figure 4.8). The relative abundance of Firmicutes was the highest in the cancer group followed by the pre-cancer and finally the normal group. A reverse order was observed for Proteobacteria (Figure 4.8). Fusobacteria had higher relative abundance in normal and cancer groups while Bacteroidetes and Actinobacteria had higher relative abundance in pre-cancer and cancer groups (Figure 4.8).

A total of 12 bacterial genera were found across all subject groups with *Streptococcus* being the most dominant genus (Figure 4.9). *Veillonella*, *Gemella*, *Granulicatella* and *Neisseria* form the major part of the oral microbiome with a total relative abundance of between 27.3% to 32.2%. The minor proportion of the oral microbiome consisted of *Haemophilus*, *Selenomonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Porphyromonas* and *Lachnoanaerobaculum* with a total relative abundance of less than 5% (Figure 4.9).

The relative abundance of *Streptococcus* was highest in the normal group compared to the other two patient groups while the reverse was observed for *Gemella* and *Granulicatella* (Figure 4.9). *Veillonella* was most abundant in the normal group, followed by the pre-cancer and finally the cancer group while the reverse was observed for *Neisseria* with the highest abundance in the cancer group (Figure 4.9). Within the minor group, bacterial genera that have a higher than 1% were *Haemophilus* in normal and pre-cancer groups, *Selenomonas* in the normal group and *Prevotella* in the pre-cancer group (Figure 4.9). The relative abundance of other minor genera such as *Fusobacterium*, *Leptotrichia*, *Porphyromonas* and *Lachnoanaerobaculum* were less than 1% (Figure 4.9).

There were many shared and unique OTUs besides the common microbiome constitution (Table 4.10). The number of shared OTUs was the highest between the pre-cancer and patient groups (23 shared OTUs), followed by the normal and the pre-cancer groups (9 shared OTU) and lastly the normal and cancer groups (4 shared OTUs) (Table 4.10). The number of unique OTUs was the highest in the pre-cancer group (16 unique OTUs) followed by the normal group (12 unique OTUs) and lastly the cancer group (11 unique OTUs) (Table 4.10).

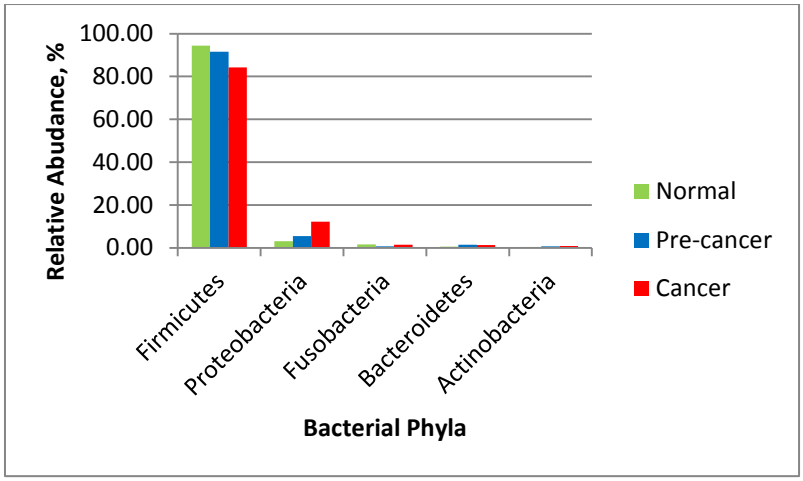


Figure 4.8: The relative abundance of 5 major bacteria phyla in normal, pre-cancer and cancer groups

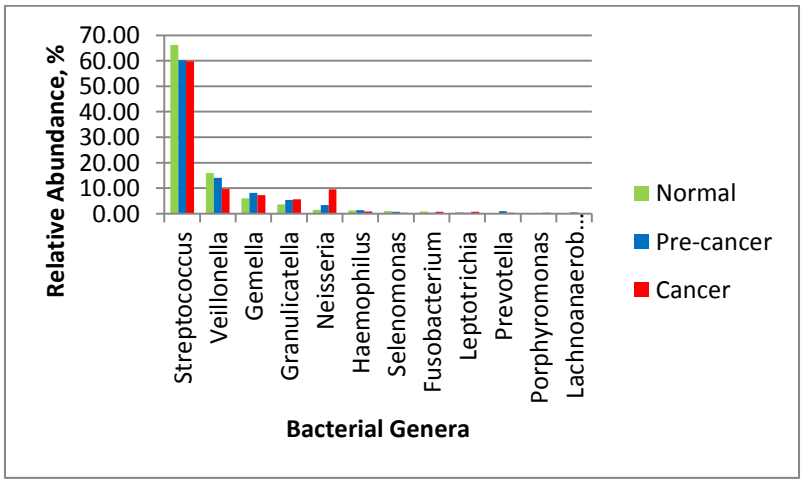


Figure 4.9: The relative abundance of common bacteria genera that were found in all subject groups

Table 4.10: Shared and Unique Bacterial OTUs that were found in the Normal, Pre-cancer and Cancer Groups

Groups	OTU	Bacterial Identities	Counts
N	23	*Eubacterium saphenum*	2
	40	*Lactobacillus iners*	1
	37	*Selenomonas sputigena*	1
	46	*Actinomyces odontolyticus*	1
	27	*Lautropia mirabilis*	1
	26	*Stomatobaculum longum*	2
	34	*Capnocytophaga leadbetteri*	1
	36	*Lachnoanaerobaculum saburreum*	1
	48	*Actinomyces sp. oral taxon 175*	1
	32	Ruminococcaceae (Family)	1
N-P	17	*Prevotella melaninogenica, P. veroralis*	4
	22	*Oribacterium sinus*	2
	25	*Selenomonas noxia*	3
P	15	*Megasphaera micronuciformis*	5
	47	*Prevotella nigrescens*	1
	43	*Eubacterium infirmum*	1
	28	*Lactobacillus fermentum*	1
	45	*Peptostreptococcus stomatis*	1
	50	*Lactobacillus salivarius*	1
	29	*Prevotella multiformis*	1
	38	*Actinomyces urogenitalis*	1
	33	*Bacteroides clarus*	1

Groups	OTU	Bacterial Identities	Counts
P	53	*Rothia aeria*	1
	41	*Peptococcus niger*	1
	52	Veillonellaceae (Family)	1
P-C	14	*Rothia mucilaginosa, R. dentocariosa*	6
	39	*Catonella morbi*	2
	22	*Prevotella pallens*	2
	08	*Haemophilus haemolyticus, H. paraphrohaemolyticus, Aggregatibacter segnis*	9
	13	*Abiotrophia defective*	4
C	24	*Campylobacter showae*	3
	21	*Capnocytophaga sputigena*	3
	44	*Prevotella salivae*	1
	42	*Parvimonas micra*	1
	49	*Corynebacterium matruchotii*	1
	51	Ruminococcaceae (family)	1
	35	Clostridiales (order)	1
N-C	19	*Campylobacter gracilis*	4

Symbols: Shared: N-P – normal and pre-cancer groups; P-C – pre-cancer and cancer groups; N-C – normal and cancer groups; Unique: N – normal group; P – pre-cancer group; C – cancer group

4.5.3. Association between Bacteria Phylogenetic Groups and Oral Conditions

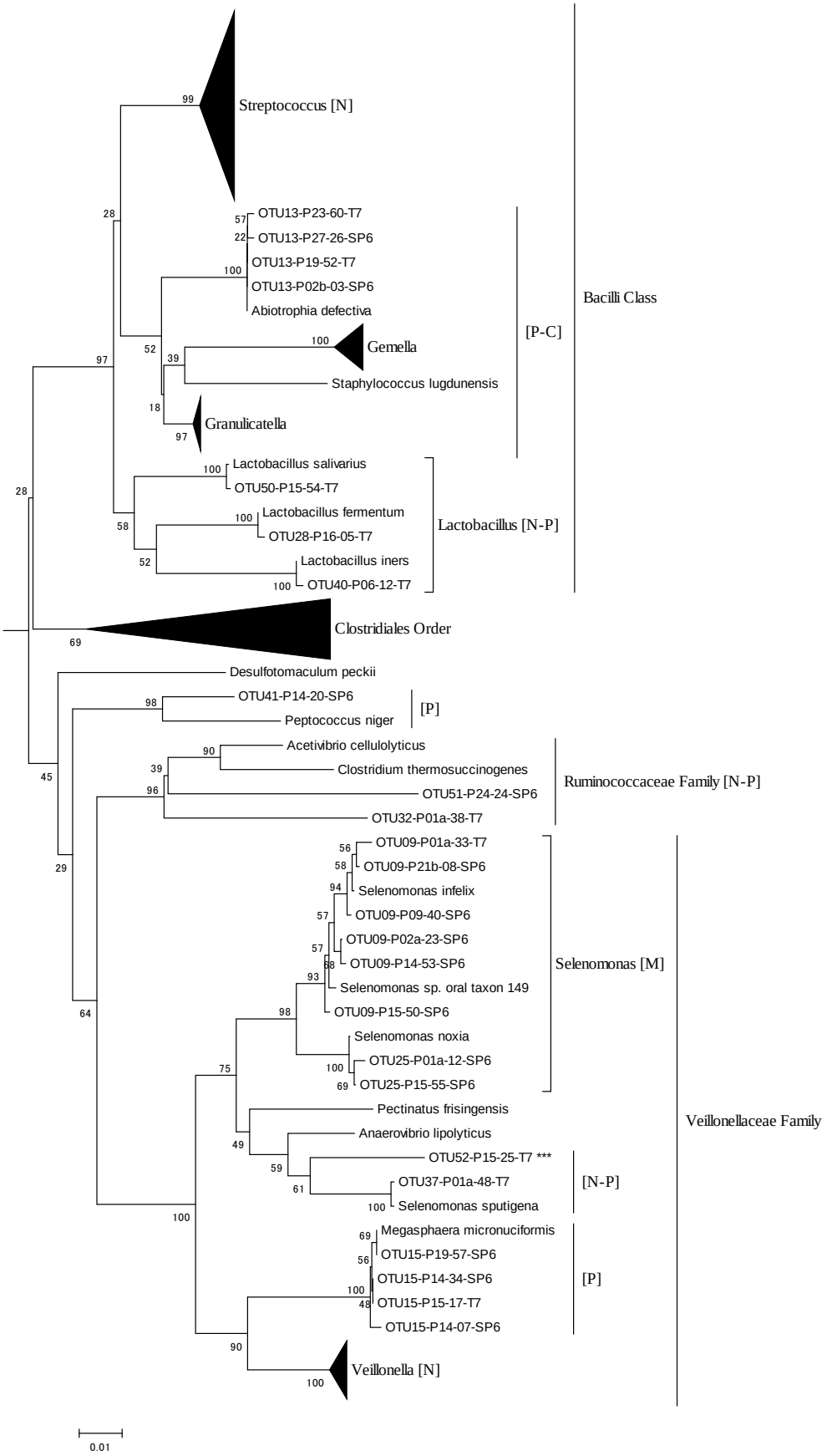
Figure 4.10a to 4.10f show the relationship between oral bacteria OTUs and their closest 16S reference sequences which can be categorised into five bacteria phyla including Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria and Bacteroidetes. Four unclassified OTUs were identified since they did not cluster with any closest reference sequences. Among the four unclassified OTUs, two were closely related to Ruminococcaceae (Figure 4.10a), one was closely related to Clostridiales (Figure 4.10b) and one was closely related to Veillonellaceae (Figure 4.10a). The phylogenetic trees also revealed bacteria groups that were associated with normal and diseased oral clinical samples and they were described further in following section.

4.5.3.1. Bacteria Groups Related to the Normal Oral Cavity

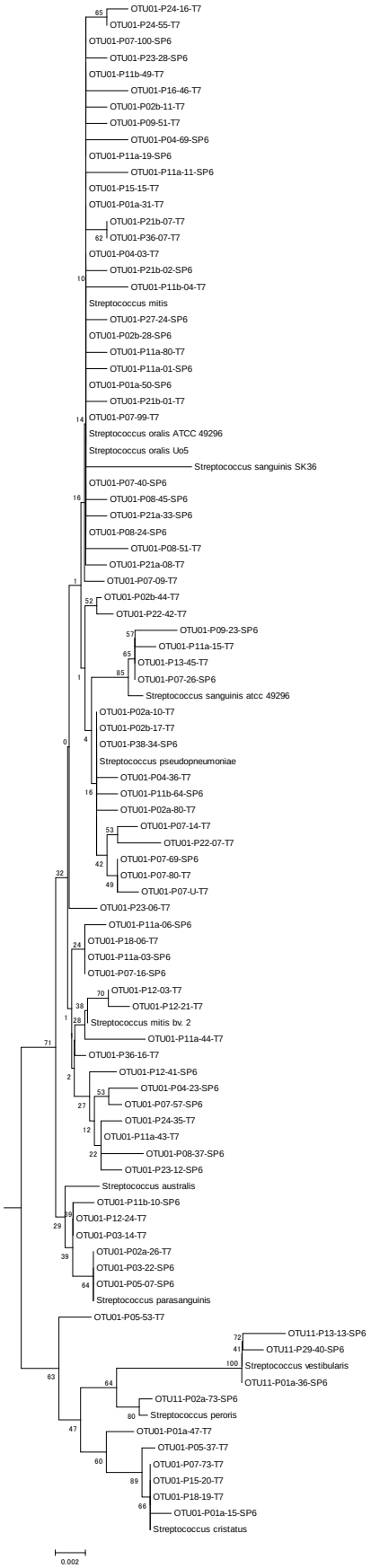
Bacteria groups that are associated with the normal oral condition were classified as normal. These bacteria groups were mainly identified in the Firmicutes phyla and they included the predominant *Streptococcus* and *Veillonella* that had higher relative abundance in the normal oral cavity, and *Lachnospiraceae* that was detected only in the normal condition (Figure 4.10a and b).

4.5.3.2. Bacteria Groups Related to Oral Cancer and Pre-cancer

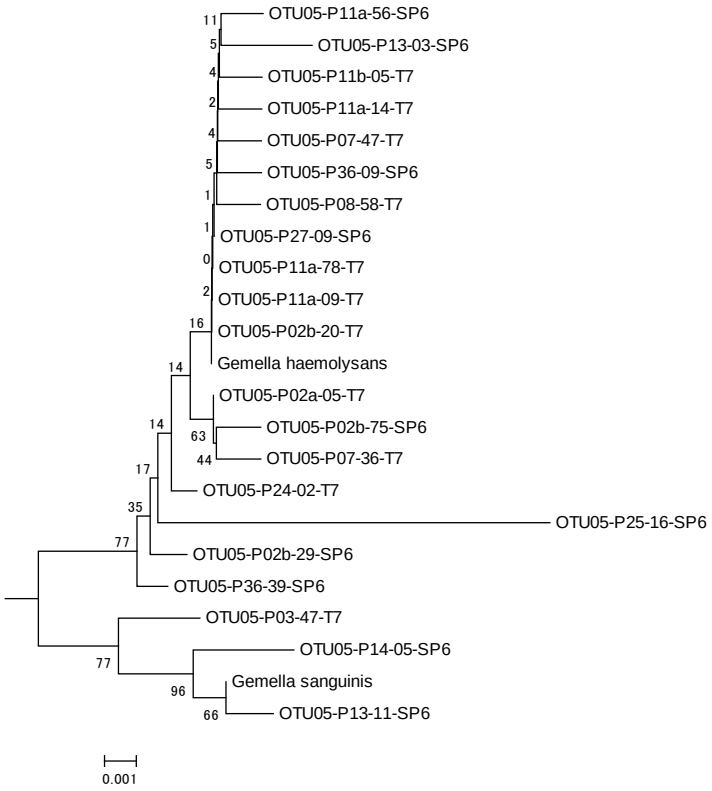
Bacteria groups that have association to oral pre-cancer and / or cancer were classified as disease groups and they can be found across all bacteria phyla except Fusobacteria (Figure 4.10d). However, different bacteria phyla were found to contain different proportion of certain bacteria groups with association to different combinations of oral cancer and pre-cancer. For instance, Firmicutes and Bacteroidetes were found to have more pre-cancer related bacteria groups (Figure 4.10f) while Proteobacteria was found to have more cancer related bacteria groups (Figure 4.10e). Around half of the bacteria groups in Actinobacteria were found to be associated with both diseased conditions.



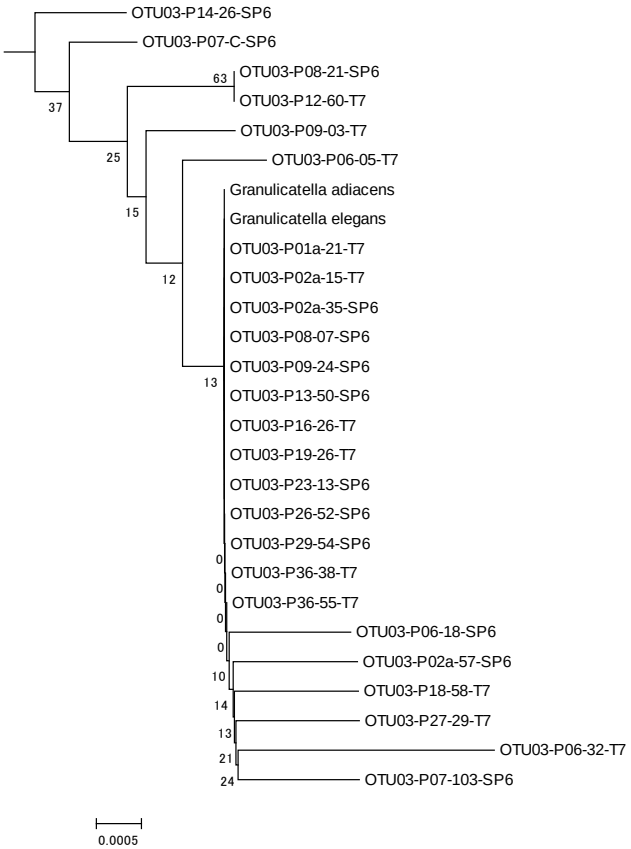
a. Firmicutes Phylum



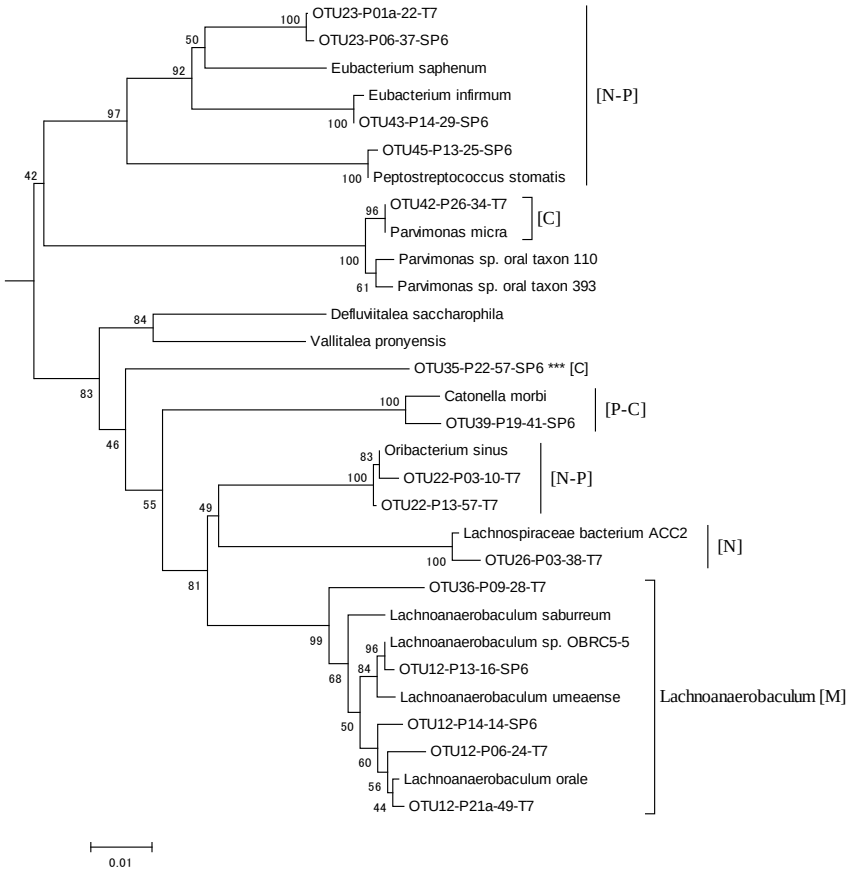
b. *Streptococcus* Genus of the Firmicutes



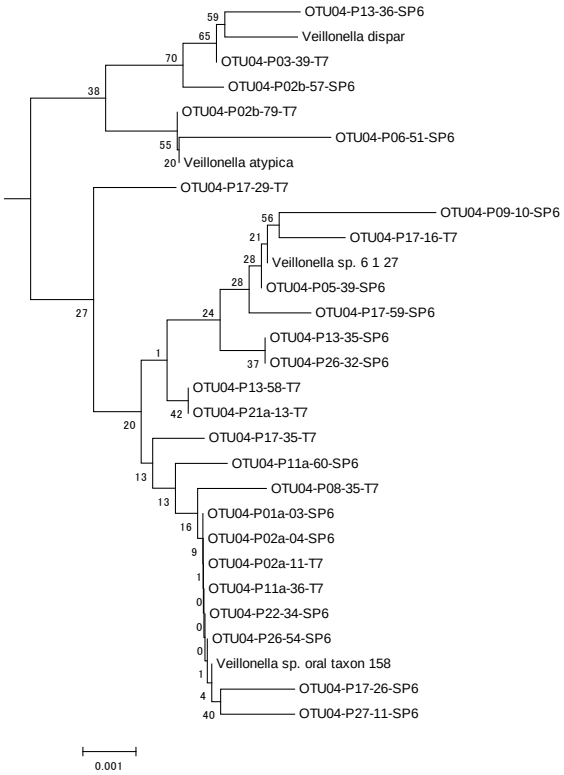
c. *Gemella* Genus of the Firmicutes



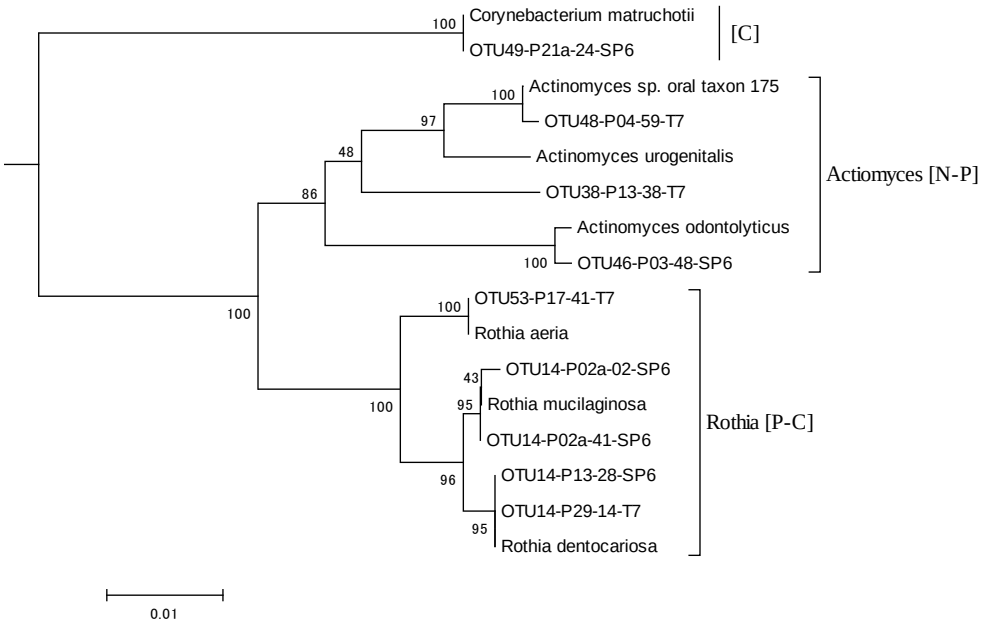
d. *Granulicatella* Genus of the Firmicutes



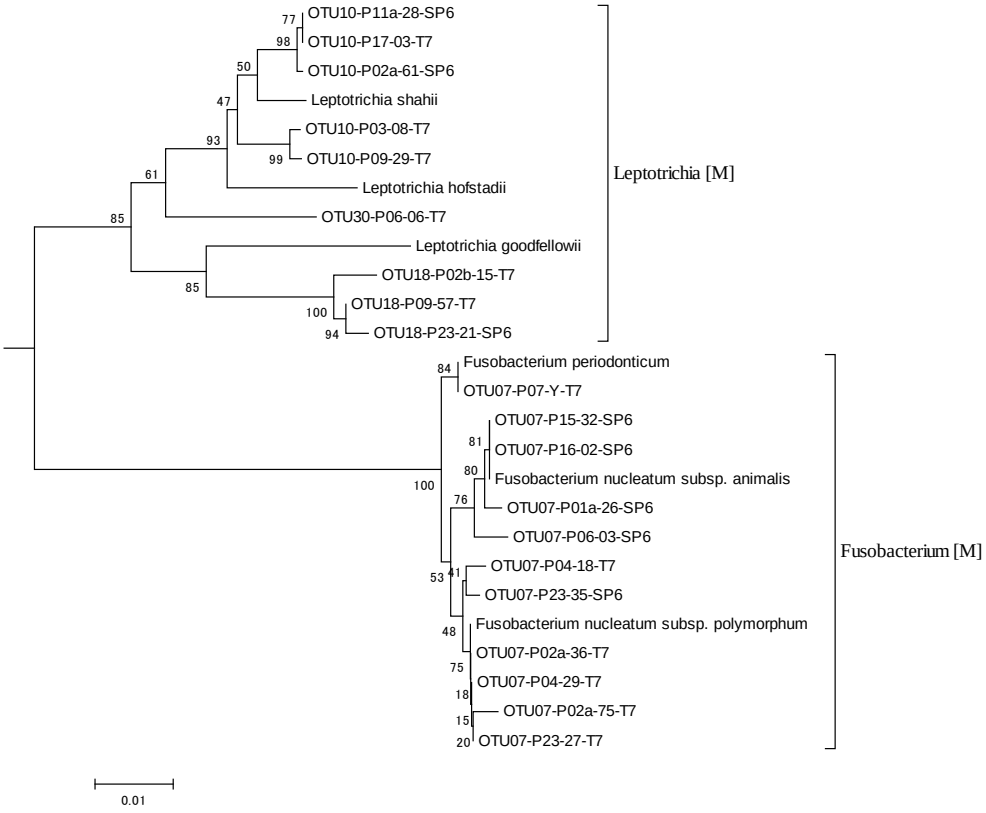
e. Clostridiales Order of the Firmicutes



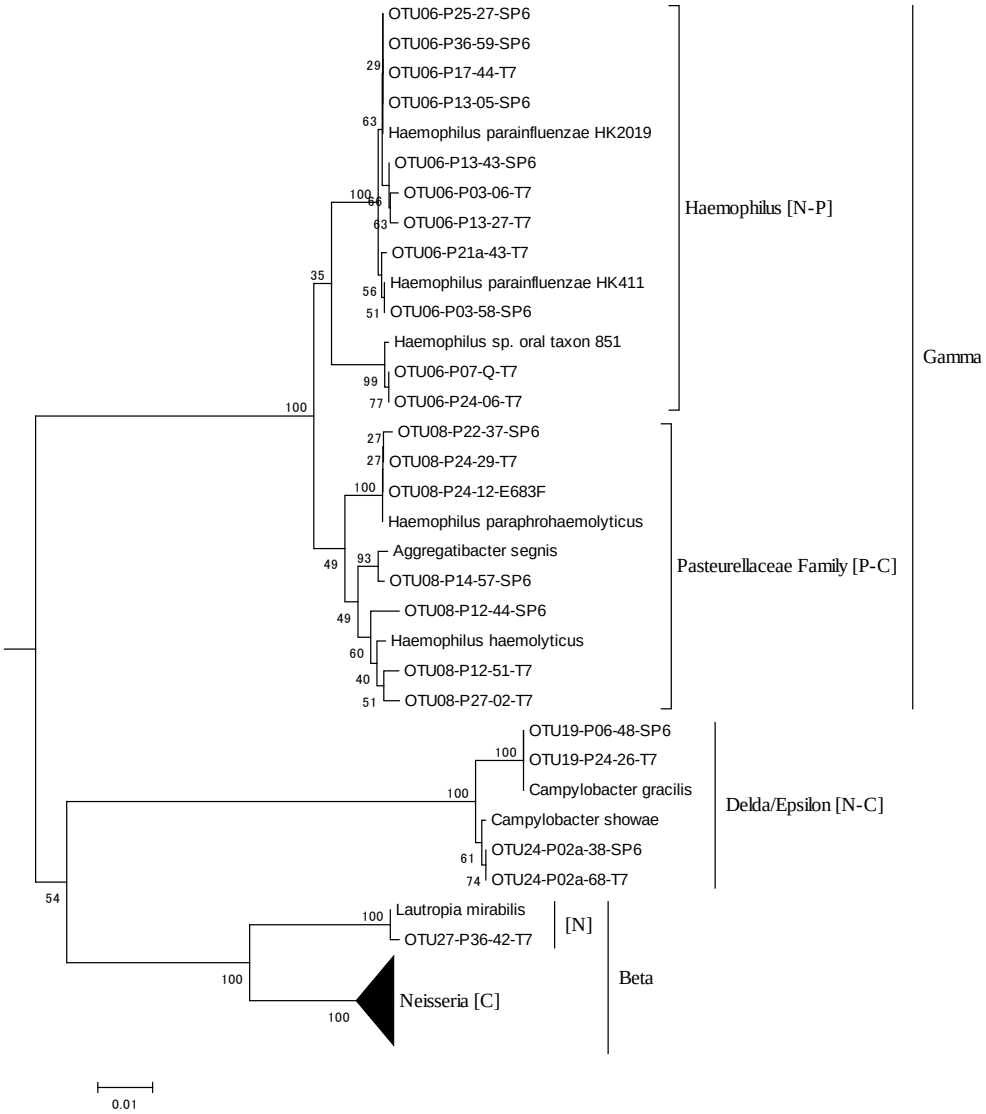
f. *Veillonella* Genus of the Firmicutes



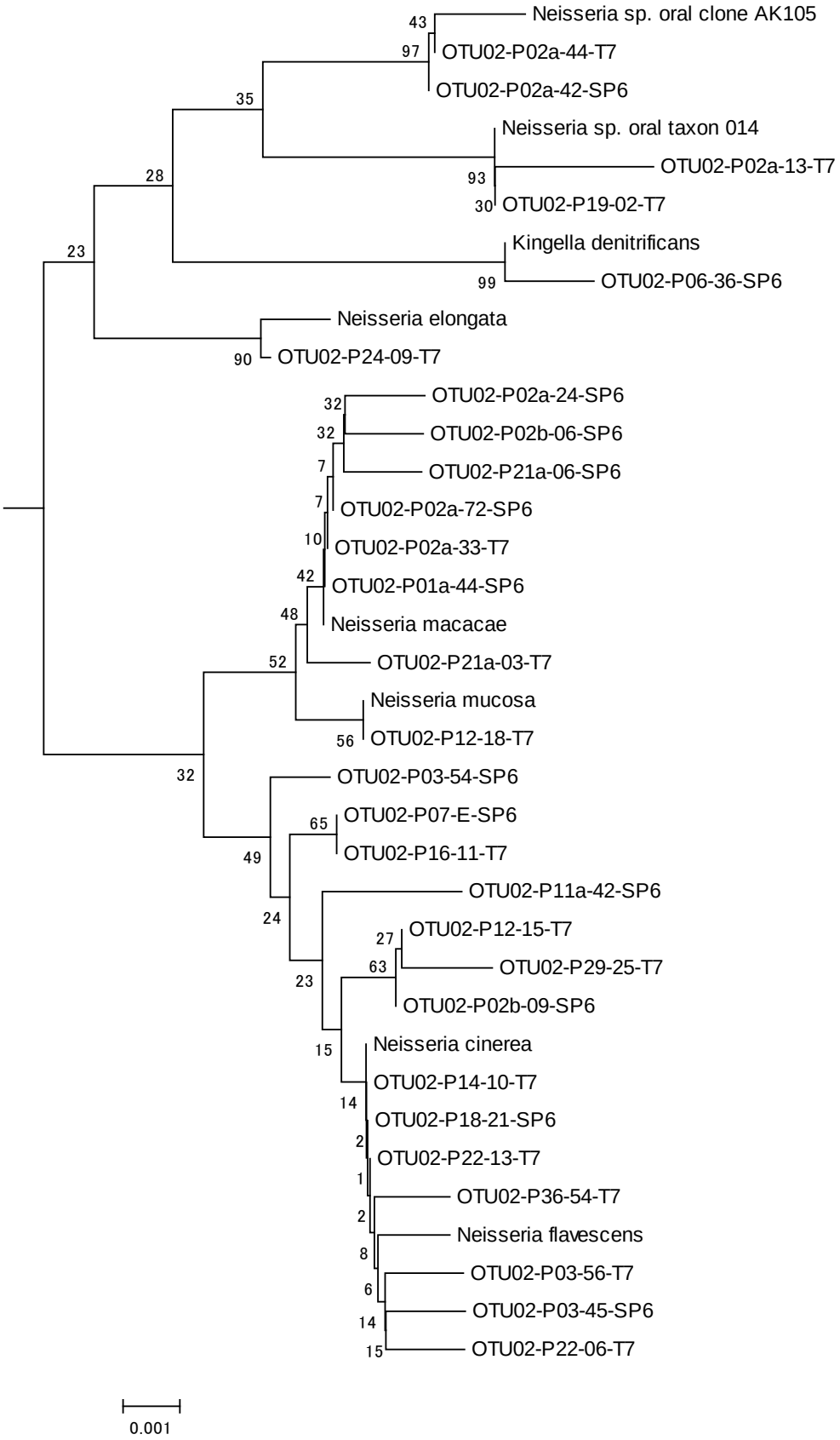
g. Actinobacteria Phylum



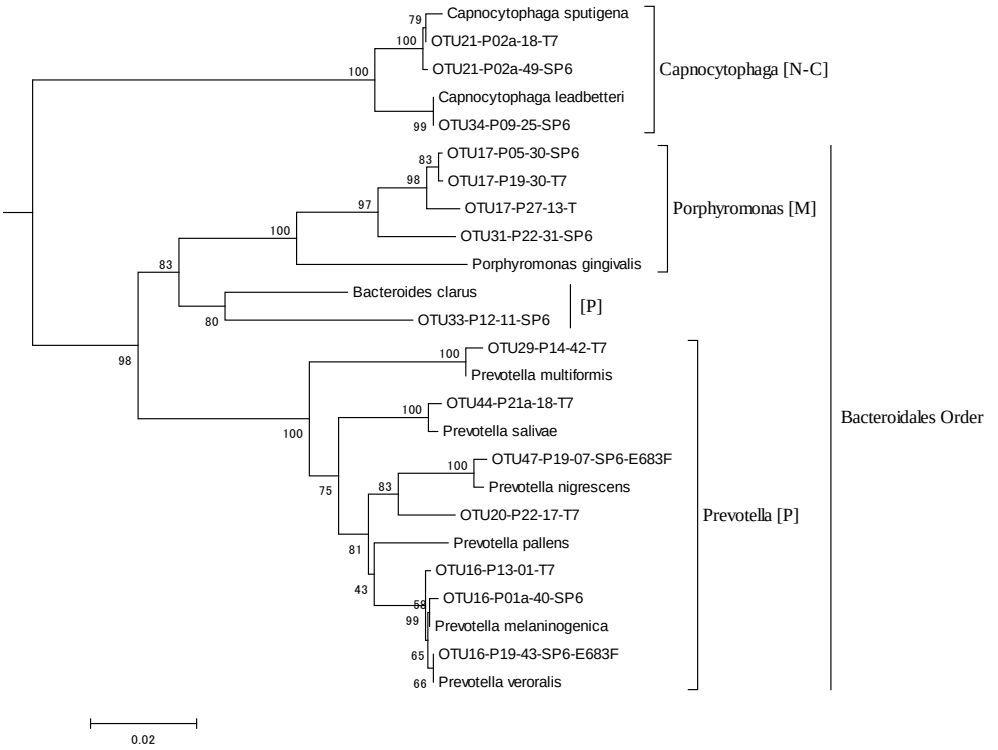
h. Fusobacteria Phylum



i. Proteobacteria Phylum



j. *Neisseria* Genus of Proteobacteria



k. Bacteroidetes Phylum

Figure 4.10: Phylogenetic relationships of 16S clone library and reference sequences analyzed by neighbor-joining (NJ) method. This NJ tree was generated based on ClustalW alignment with 1000 bootstraps. The 16S reference sequences were selected based on top results with lowest E-values from HMP-DACC and NCBI 16S nucleotide databases. The sequences were clustered into five bacteria phyla which were Firmicutes (4a, b, d, d, e and f), Actinobacteria (4g), Fusobacteria (4h), Proteobacteria (4i and j) and Bacteroidetes (4k). Bacteria groups associated with samples from normal individuals were denoted as [N], normal and pre-cancer subjects as [N-P], pre-cancer subjects as [P], pre-cancer and cancer subjects as [P-C], cancer subjects as [C], normal and cancer subjects as [N-C] and all three groups as [M].

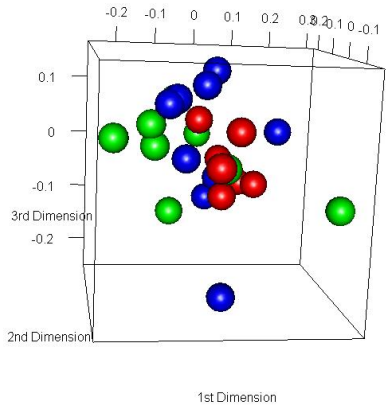
4.5.4. Community Structure Similarities

Oral microbiome communities associated with normal, pre-cancer and cancer cases were compared against each other using AMOVA and NMDS to quantify the degree of similarity between them. Three-dimentional NMDS plot showed that the bacteria community groups associated with pre-cancer subjects were sandwiched between that of normal and cancer subjects (Figure 4.11a, b and c). The 0.12 stress level indicated that the three-dimensional NMDS plot was a good representation of the similarities of the different oral microbiome communities. AMOVA results (Table 4.11) indicated that the significant genetic variations between all subject groups were due to the genetic variations between normal and cancer groups. However, the genetic structure of bacteria diversity in the pre-cancer group was not significantly different from that of normal or cancer groups.

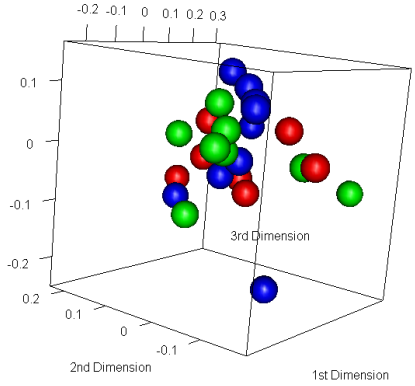
Table 4.11: AMOVA based on V6-V9 regions of 16S rRNA gene which spans 751bp

Groups	Source of variations	SS	Df	MS	Fs	p-value
N-P-C	Among	0.1445	2	0.0723	3.0470	<0.05
	Within	0.4980	21	0.0237		
	Total	0.6426	23			
N-P	Among	0.0335	1	0.0335	1.4775	0.261
	Within	0.3403	15	0.0227		
	Total	0.3739	16			
P-C	Among	0.0414	1	0.0414	1.5798	0.227
	Within	0.3668	14	0.0262		
	Total	0.4082	15			
N-C	Among	0.1491	1	0.1491	6.7081	<0.05
	Within	0.2890	13	0.0222		
	Total	0.4381	14			

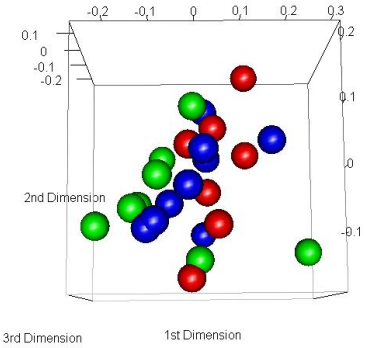
Symbols: SS: Sum of Squares; Df: Degrees of Freedom; MS: Mean Squares; Fs: F test; N-P-C: normal, pre-cancer and cancer groups; N-P: normal and pre-cancer groups; P-C: pre-cancer and cancer groups; N-C: normal and cancer groups



a.



b.



c.

Figure 4.11: Three-dimensional NMDS Plot with normal associated communities symbolized as green spheres, pre-cancer associated communities symbolized as blue spheres and cancer associated communities symbolized as red spheres. The stress level of 0.12 indicates a good representation in three dimension plot. 4a is front view; 4b is side view while 4c is top view.

5

DISCUSSIONS

5.1. Technical Challenges of DGGE in Oral Microbiome Studies

We found that DGGE analysis of the oral microbiome using F968-GC/R1401 primers with GC clamp (Nübel et al., 1996, Zijnge et al., 2003, Signoretto et al., 2009) reduces PCR amplification due to the formation of secondary structures (Table 4.8). It was also observed that the standard final PCR extension of 5 minutes produced artifactual bands (Figure 4.3a & b). Reduced efficiency of PCR amplification caused fuzzy pattern as shown in Figure 4.2. This subsequently increased challenges for downstream analysis, especially due to the production of complex fingerprint patterns. For example, artifactual bands could lead to diversity overestimation due to inclusion of additional bands during downstream analysis (Janse et al., 2004). These challenges were overcome in two ways. Firstly, the use of Sheffield GC clamp attached to the 5' end of F968-GC was shown to reduce the formation of secondary structures and produced sharper bands and clearer patterns (Figure 4.2). Prolongation of the duration of the final PCR extension to at least 30 minutes prevented the formations of artifactual bands; this allowed complete elongation of the PCR amplicons to occur (Janse et al., 2004).

5.2. General Characteristics of the Study Subjects

All three subject groups comprised all the three major ethic groups of Malaysia. There were small variations of male to female ratio and average age between normal and patient groups. The major difference between them is the higher occurrence of risk habits, such as smoking, betel quid chewing and drinking, in the pre-cancer and cancer groups compared to the normal counterpart (Table 4.4). Nonetheless, no DGGE groups with at least 80% cophenetic correlation were found to cluster according to ethnicity, gender or risk habits.

5.3. The Relationship between Oral Cancer and the Overall Oral Microbiome

DGGE profiles of individual oral microbiome generated in this study were clustered according to their similarity distance with BioNumerics version 6.2 to produce DGGE dendogram as shown in Figure 4.6. This dendogram allowed us to have an instant overview of the degree of similarity between oral microbiome community structures of different oral conditions. The oral microbiome community structure of the normal, pre-cancer and cancer groups can be similar to each other due to the presence of multiple mix DGGE clusters. This finding can be explained by the concept of core oral microbiome where different oral cavities have a common oral microbiome to maintain the micro ecology functional stability and homeostasis (Zaura et al., 2009).

Nonetheless, the presence of normal and patient DGGE clusters (Figure 4.6) suggests that there are variations between the normal and cancer associated oral microbiome. Such variations, especially the presence of a patient associated DGGE cluster, suggested that there were presence of patient associated oral bacteria community members, hence sequence based analysis was warranted to shed light on their identities.

5.4. Clone Library Colony Screening

The rarefaction curve was used to determine whether the sample amount is sufficient to represent its total bacterial diversity under study (Hurlbert, 1971, Heck et al., 1975) while the Good's coverage was used to estimate the degree of sample completeness (Good, 1953a). The level off rarefaction curves (Figure 4.7) indicated that the amount of colonies screened were sufficient to represent the normal, pre-cancer and cancer clone libraries while the Good's coverage of around 97% (Table 4.9) suggested that the sequencing of between 30 and 52 of additional colonies, that have new unrecorded RFLP, would only yield one additional OTU.

5.5. The Core Human Oral Microbiome

All five bacterial phyla (Figure 4.8) were consistently reported as among the main oral microbiome components in both healthy (Bik et al., 2010)

and diseased states such as oral cancer (Pushalkar et al., 2012), halitosis (Kazor et al., 2003) and periodontitis (Paster et al., 2001). Most bacterial genera that were commonly identified in all three oral conditions were also reported in other cancer associated oral microbiome studies (Hooper et al., 2007, Pushalkar et al., 2011, Pushalkar et al., 2012) with the exception of *Lachnoanaerobaculum* (Figure 4.9). Although *Lachnoanaerobaculum* was first isolated from human saliva (Hedberg et al., 2012) and it was previously identified as *Clostridiales bacterium* by HMP-DACC. This genus was listed in the Human Oral Microbiome Database (HOMD) and includes species previously known as *Eubacterium saburreum* (Prevot) and *Lachnospiraceae bacterium oral taxon 082* (Human Oral Microbiome Database). This observation of consistent oral bacteria taxa suggests the presence of a core oral microbiome at both genus and phylum levels across different oral states.

5.6. Unique and Consistent Membership of Normal and Cancer Associated Oral Microbiome

We observed that the relative abundance of the core oral microbiome, at both phylum and genus levels, varies across different studies. The Fermicutes is predominant in the normal oral microbiome in this study with over 94% relative abundance while one of the minor phyla, Proteobacteria, only accounted around 3% relative abundance. In contrast, a less dominating Firmicutes phylum was reported with relative abundance as low as 28% (Lazarevic et al., 2009) while the relative abundance of Proteobacteria could

be as high as 28% (Nasidze et al., 2009). Further, the relative abundance of many of the bacteria genera also varied across different studies with the exception of *Gemella* and *Porphyromonas* (Table 5.1). We found higher relative abundance of *Granulicatella* and *Neisseria* in the pre-cancerous and cancerous clinical samples while they were reported previously with higher relative abundance in the normal oral cavities (Table 5.1). On the other hand, *Haemophilus* was found with higher relative abundance in the normal and pre-cancer subject groups contrary to previous reports of higher relative abundance in the cancer subject group only (Table 5.1).

We suggest that the variations of relative abundance identified in this study could be unique to the oral microbiome of the Malaysian subjects. Our subjects were from the Malaysia population while previous studies recruited subjects from the western population such as United States and England (Table 5.1). The food culture between these two populations was different (Roman and Russell, 2009) and the host dietary patterns could be influential on the adult oral microbiome community structure (Sanjaya et al., 2011). Hence it is possible to find differences in the oral microbiome profiles between current and previous studies. A study from the Congo reported that the frequency of several oral bacterial genera of their population was different from other countries (Nasidze et al., 2009). Our results suggested that *Granulicatella* and *Neisseria* could be associated with diseased oral conditions while *Haemophilus* could be associated with normal and pre-cancerous oral conditions in Malaysian subjects. However, further validations with larger sample size are necessary to confirm this finding.

On the other hand, the consistency in relative abundance despite the differences in sample types and populations suggested strong associations of *Gemella* and *Porphyromonas* to their respective oral environments. The affiliation of *Gemella* to oral cavities in pre-cancerous and cancerous conditions could be due to its characteristic as opportunistic pathogens in immunocompromised patients (Repetto et al., 2012) while *Porphyromonas* could be a steady oral microbiome composition since members of this genus were found to be oral commensals of human and animal origins (Summanen et al., 2009).

Table 5.1: Comparison of the relative abundance of common oral bacteria genera in different subject groups between the current and previous studies

Common oral bacteria genera	Subject Group with the Highest Relative Abundance			
	Current Study	Pushalkar et al., 2011	Pushalkar et al., 2012	Hooper at al., 2007
Streptococcus	N	C	C	N
Veillonella	N	≈	N	≈
Gemella	P-C	C	C	N/A
Granulicatella	P-C	N	N	N
Neisseria	C	N	N	≈
Haemophilus	N-P	C	N/A	N/A
Selenomonas	≈	N/A	N	≈
Fusobacterium	≈	≈	N	C
Leptotrichia	≈	N	N	≈
Prevotella	P	C	N	C
Porphyromonas	≈	C	≈	≈
Lachnoanaerobaculum	≈	N/A	N/A	N/A

Symbols: N – normal group; P – pre-cancer group; C – cancer group; ≈ – relative abundance was similar between groups; N/A – no available relative abundance information

5.7. The Influence of Oral Cancer and Pre-cancer on the Oral Microbiome

The diversity was higher in pre-cancer and cancer associated oral microbiota since both groups were recorded with higher effective species number than the normal group (Table 4.9). The high diversity level of the cancer group was due to increased species evenness since both cancer and normal groups shared similar species richness, whereas the high diversity level of the pre-cancer group was attributed to increased species evenness and richness (Table 4.9). Consequently, the increasing order of the diversity indexes from normal to pre-cancer and cancer associated oral microbiota (Table 4.9) indicates that the diseased oral cavity states promote a more diverse oral microbiome. This finding suggests that pre-cancer and cancer affiliated oral microenvironments harbour microbial communities that consist of reduced dominant members. This can be seen as reduced relative abundance that could contribute to the increased species evenness, and increased level of potential opportunistic pathogens that could accounted for the increased species richness. However, such a finding is different from those previously reported, where bacteria communities with less diversity level were observed in diseased clinical conditions as compared to their normal counterparts (Pushalkar et al., 2012, Yang et al., 2013). One possibility is that this is due to the different geographical origins of target subject groups. Alternatively, this could be unique to the Malaysia population, although studies using a larger sample size are necessary to support this conclusion.

The significant difference of AMOVA between the normal and cancer groups (Table 4.11) was in agreement with the result reported by Pushalkar et al. (2011) who found a major separation of DGGE oral microbiota fingerprints into normal and cancer clusters. On the other hand, the overlapping of pre-cancer associated bacteria communities between the normal and cancer groups (Figure 4.11) suggests that pre-cancer group contained community memberships that can be found in both normal and cancer groups. Indeed, there were also considerable amount of shared OTUs between pre-cancer and the other groups (Table 4.10). These overlapping community memberships can be the plausible factor of the insignificant difference found between the pre-cancer and the other two groups (Table 4.11). Besides that, this overlapping membership of pre-cancer associated bacteria communities with that of normal and cancer groups suggest that a possible shift of oral microbiome community at a subpopulation level may occur in line with the state of cancer progression, which could be useful to differentiate oral microbial communities of normal, pre-cancer and cancer conditions.

AMOVA was recalculated and NMDS was re-plotted for the clone libraries with DGGE profiles that form normal and patient DGGE clusters (Figure 4.6). NMDS plot showed a separation between normal and patient groups (Figure 5.1) which was in agreement with DGGE dendogram where there was distinct separation of normal and patient clusters. However the AMOVA showed no significant difference between these two groups. This could be due to the small clone library as the normal group consists of only four clone libraries while the cancer group consist of three clone libraries.

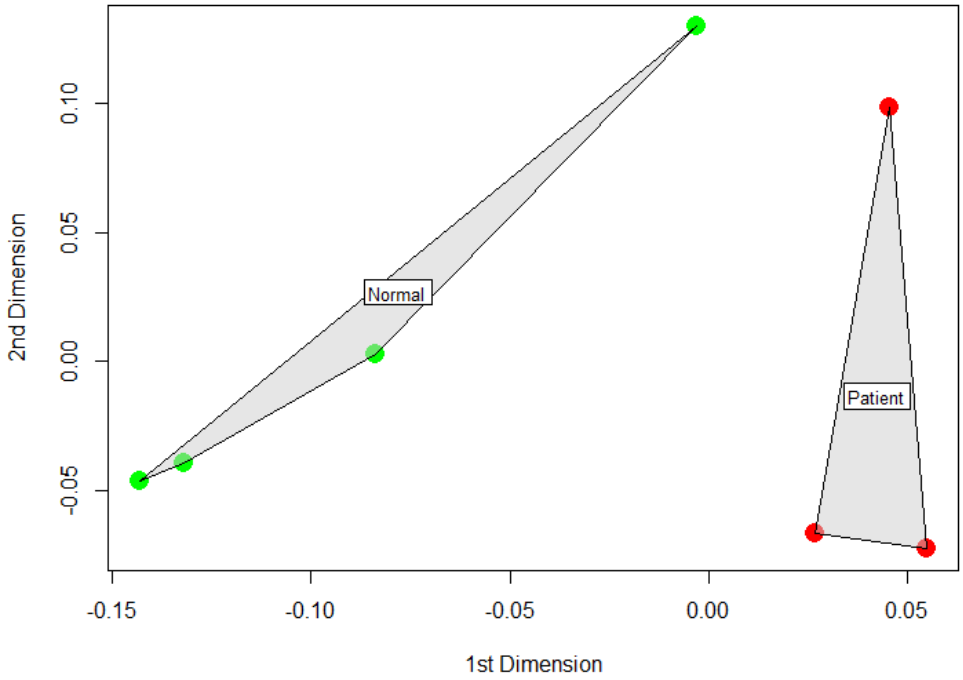


Figure 5.1: Two-dimensional NMDS Plot with normal associated communities symbolized as green spheres and patient associated communities symbolized as red spheres. The stress level of 0.15 indicates a good representation in two dimension plot.

Table 5.2: AMOVA of Normal and Patient Clone Libraries based DGGE Clustering Patterns

Groups	Source of variations	SS	Df	MS	Fs	p-value
N-P	Among	0.0548	1	0.0548	2.8529	0.054
	Within	0.0961	5	0.0192		
	Total	0.1509	6			

Symbols: SS: Sum of Squares; Df: Degrees of Freedom; MS: Mean Squares; Fs: F test; N-P: normal and patient groups

5.8. Pre-cancer Associated Oral Microbiome

The oral microbiome associated with pre-cancerous oral clinical samples was included in this study, in contrast to many previous studies in which the focus was on oral cancer (Mager et al., 2005, Hooper et al., 2007, Pushalkar et al., 2011, Pushalkar et al., 2012). This study presents a preliminary picture on the diversity and community structure of the pre-cancer associated oral microbiome.

We found that the oral microbiota affiliated to pre-cancer patients consist of the core oral microbiome of twelve common bacteria genera (Figure 4.9). Pre-cancer associated oral microbiome was found to harbour the highest number of unique OTUs, with a highest amount of OTUs closely related to *M. micronuciformis* (Figure 4.9). Besides that, *Prevotella* was found to have the highest relative abundance in the pre-cancer clinical samples compared to the normal and cancer clinical samples, a finding that was not reported previously (Table 5.1). *Prevotella* species such as *P. melaninogenica*, *P. veroralis* and *P. nigrescens* have previously been reported to be elevated in or present only in the cancerous clinical samples (Mager et al., 2005, Hooper et al., 2007, Pushalkar et al., 2011) (Table 5.3). Hence, we propose that *M. micronuciformis* and members of *Prevotella* genus could be unique attributes of the pre-cancerous associated oral microbiome.

In addition, the pre-cancer associated oral microbiota had diversity indexes and NMDS positions in-between that of the normal and cancer

counterparts. Based on these analyses, oral microbiome may have a transitional structure between the normal and the cancerous condition of the oral cavity with the pre-cancer associated bacteria community as the intermediate state. However, this proposal could be only validated with a longitudinal study involving long term monitoring of oral cancer progression. Nonetheless, the pre-cancer associated oral microbiome shows a closer relationship to the cancer counterpart since a higher amount of shared OTUs was observed between pre-cancer and cancer groups compared to that of between normal and pre-cancer groups (Figure 4.9).

5.9. Oral Microbes with Potential Diagnostic and Therapeutic Values

Several oral microbes were linked to particular oral health conditions as indicated by their relatively high count number (Table 4.10) and consistent association to particular clinical oral sample between studies (Table 5.3). Among them are *E. saphenum* (normal), *M. micronuciformis* (pre-cancer), *C. showae* (cancer), *P. melaninogenica* and *P. veroralis* (normal and pre-cancer) as well as *R. mucilaginosa*, *R. dentocariosa* and *C. morbi* (pre-cancer and cancer).

E. saphenum, *C. morbi* and *C. showae* have been reported to be related to periodontitis (Han et al., 2005, Colombo et al., 2012) while *M. micronuciformis* was first isolated from abscess samples (Marchandin et al., 2003) and was found to be associated with immunocompromised HIV infected

patients as an opportunist (Dang et al., 2012). In addition to periodontitis, *C. showae* was found to aggregate with tumour associated *Fusobacterium nucleatum* in the colon cancer (Warren et al., 2013). However, this study found the presence of *F. nucleatum* in all subject groups. *P. melaninogenica* are commonly isolated from healthy gingival crevice (Marsh et al., 2009) while *P. veroralis* has only known to be isolated from the human oral cavity according to Global Infectious Disease and Epidemiology Network (GIDEON) (www.gideononline.com). *R. mucilaginosa* and *R. dentocariosa* are commensals of the human oral cavity and pharynx (Whitman et al., 2012). *P. melaninogenica*, a causal agent of periodontitis (Mane et al., 2009), was found to be a potential salivary marker for early oral cancer detection (Mager et al., 2005). It was reported to be also associated with other oral diseases such as gingivitis, noma (Huyghe et al., 2013), caries (Tanner et al., 2011) and endodontitis (Hsiao et al., 2012). *R. mucilaginosa*, which was associated with malodour (Hartley et al., 1996), was found to be an emerging opportunistic pathogen due to frequent detection in immunocompromised hosts (Yamane et al., 2010). *R. dentocariosa* was found to be associated with dental caries & periodontitis (Groves and Allen, 1996) although it is a rare clinical infection (Ricaurte et al., 2001).

The elevated amount of these oral microbes in either pre-cancer and cancer conditions could be due to the ability of these oral microbes to survive in specialized niche environment (Chocolatewala et al., 2010) or bind specifically to tumour tissues (Mager et al., 2005), although the exact mechanism behind such association is yet to be known. Nonetheless, the

higher abundant of these oral microbes in the cancer oral conditions, as compared to the healthy counterpart, indicates that they may have potential medical values as diagnosis and therapeutic tools. Such hypothesis would requires further validation with more number of cancer subjects to confirm the consistent association between these oral microbes, pre-cancer and cancer oral cavities.

Those oral bacteria associated with pre-cancer of the oral cavity such as *P. melaninogenica*, *P. veroralis*, *R. mucilaginosa*, *R. dentocariosa*, *C. morbi* and especially *M. micronuciformis* may be of potential diagnostic value. The ability to diagnose pre-cancerous lesions, which precede early oral cancer stage, would lead to better treatment outcome and increase survival rate (American Cancer Society, 2009, Markopoulos et al., 2010, National Institutes of Health, 2010). Besides that, it has been suggested that cancer associated *C. showae*, *R. mucilaginosa*, *R. dentocariosa* and *C. morbi* could be further studied to explore therapeutic potentials such as cancer prognosis (Grice and Segre, 2012) and targeted treatment (Morrissey et al., 2010).

The higher fraction of pre-cancer associated bacteria genera in Bacteroidetes (Figure 4.10) was in accordance with the higher amount of OTUs closely related to *M. micronuciformis* and *Prevotella* species as seen in Table 4.10. Besides that, the presence of many cancer associated bacteria genera in Proteobacteria (Figure 4.10) was supported by the higher count number of OTUs related to *C. showae* as seen in Table 4.10. Hence Bacteroidetes and Proteobacteria could be good sources for the identification

of oral microbes with cancer diagnostic and therapeutic values respectively. Bacteria groups belonging to Actinobacteria phylum could also be further studied for their potential as diagnostic aids and their therapeutic applications since they were identified in both diseased states.

Table 5.3: Phenotypes of Unique and Shared Bacteria Species Associated with Normal, Pre-cancer and Cancer Groups

Bacteria Species	Previous Association to Oral Clinical Samples	Phenotypes[e]		
		Alkaline Phosphatase Positive	Catalase Positive	Sulfate Reduction
Normal				
Eubacterium saphenum	normal saliva[a]			
Lactobacillus iners	NT tissues[d]			
Selenomonas sputigena	NT[d]			
Actinomyces odontolyticus	cancer saliva[a]			
Lautropia mirabilis	isolated from mouth of HIV infected patient[f]			
Stomatobaculum logum	isolated from oral cavity[e]			
Capnocytophaga leadbetteri	isolated from oral cavity[e]			
Lachnoanaerobaculum saburreum	isolated from dental plaque[e]			Y

Bacteria Species	Previous Association to Oral Clinical Samples	Phenotypes[e]		
		Alkaline Phosphatase Positive	Catalase Positive	Sulfate Reduction
Normal – Pre-cancer				
Prevotella melaninogenica,	T_b; NT_d; normal saliva[c]	Y		
Prevotella veroralis				
Oribacterium sinus	N/A			
Selenomonas noxia	N/A			
Pre-cancer				
Megasphaera micronuciformis	T_d		Y	
Prevotella nigrescens	cancer saliva[a,c]	Y		
Eubacterium infirmum	T_d			
Lactobacillus fermentum	T_d			
Peptostreptococcus stomatis	T_d			

Bacteria Species	Previous Association to Oral Clinical Samples	Phenotypes[e]		
		Alkaline Phosphatase Positive	Catalase Positive	Sulfate Reduction
Lactobacillus salivarius	oral probiotic[h]			
Prevotella multiformis	periodontitis[i]	Y		
Actinomyces urogenitalis	N/A	(V)		
Bacteroides clarus	N/A			
Rothia aeria	N/A		Y	
Peptococcus niger	N/A		Y	Y
Pre-cancer – Cancer				
Rothia mucilaginosa,	cancer saliva[c]		(V)	Y
Rothia dentocariosa				
Catonella morbi	T[d]			
Prevotella pallens	NT tissues[d]	Y		

Bacteria Species	Previous Association to Oral Clinical Samples	Phenotypes[e]		
		Alkaline Phosphatase Positive	Catalase Positive	Sulfate Reduction
Aggregatibacter segnis	isolated from saliva of HIV infected patients[g]	Y	Y	
Haemophilus paraphrohaemolyticus	N/A		Y	
Haemophilus haemolyticus	N/A	Y	Y	(V)
Abiotrophia defectiva	N/A			
Cancer				
Camplylobacter showae	T_d		Y	Y
Capnocytophaga sputigena	N/A	Y		
Parvimonas micra	NT_d	Y		(V)
Prevotella salivae	NT_d	Y		
Corynebacterium matruchotii	N/A		Y	

Bacteria Species	Previous Association to Oral Clinical Samples	Phenotypes[e]		
		Alkaline Phosphatase Positive	Catalase Positive	Sulfate Reduction
Normal – Cancer				
Campylobacter gracilis	oral lichen planus[j]			

Symbols: a – Mager et al., 2005; b – Hooper et al. 2007; c – Pushalkar et al., 2011; d – Pushalkar et al., 2012; e - http://web.gideononline.com/web/microbiology/pathogen_index.php?type=bacteria#; f – Rossman et al., 1998; g – Li et al., 2014; h – Iwamoto et al., 2010; i – Sakamoto et al., 2005; j - Seckin Ertugrul et al., 2013; T – tumout tissues; NT – non-tumour tissues; N/A – not available; Y – more than 90% of the strains are positive; (V) – between 10% and 90% of the strains are positive

5.10. Potential Association between Bacterial Phenotypes and Pre-cancerous and Cancerous Oral Conditions

It was proposed that oral cancer could be can be induced by carcinogenic substances as the result of bacterial metabolism or activities (Warnakulasuriya et al., 2008, Chocolatewala et al., 2010). This prompted the search of the phenotypic identities of OTUs that demonstrate association with either only one or two oral clinical samples (Table 5.3). All phenotypic characteristics were retrieved from an online database, Global Infectious Disease and Epidemiology Network (GIDEON) (www.gideononline.com) and three noteworthy phenotypes were identified based on their occurrence in particular oral clinical states as shown in Table 5.3. Phenotypes including alkaline phosphatase and catalase producing as well as sulfate reducing activities were found to be infrequent in the normal oral samples whereas the majority of the OTUs in groups with pre-cancer and cancer were identified to have one or more of these phenotypic characteristics. Several OTUs associated with the pre-cancer and cancer groups were found to be positive for alkaline phosphatase and catalase while the sulfate reducing phenotype was mostly identified in OTUs associated with oral cancer. Hence, alkaline phosphatase and catalase positive phenotypes were shown to be associated with both pre-cancer and cancer of the oral cavity while sulfate reducing phenotype was associated with oral cancer only. Alkaline phosphatase was found in higher amount not only in various cancerous conditions such as lung, liver, ovarian and testicular cancer (Fishman et al., 1968, Warnock and Reisman, 1969,

Chung et al., 2010) but also in pre-cancerous states (Warnock and Reisman, 1969) but its cancer associated biological function remains unclear. Cancer tissues have higher oxidative stress compared to their normal counterpart (Gewirtz et al., 2007) and perhaps this creates a niche for the survival of catalase producing microbes. Sulfate reducing bacteria have been suggested as a key player in colon cancer due to the production of genotoxin hydrogen sulfide (H_2S) but their mechanism in the oral cancer environment remains elusive (Medani et al., 2011).

6

CONCLUSIONS

A core human oral microbiome, in terms of common bacterial phyla and genera, was observed in oral samples of all three types of subjects (normal, pre-cancer and cancer patients). However, we also found evidence of possible oral microbiome compositions unique to Malaysian subjects as it was different from those previously reported which were of western origin. The normal and cancer associated general oral microbiome were significantly distinct from each other while the community membership of the pre-cancer associated oral microbiome was found to overlap between the normal and cancer oral microbiota. With the pre-cancer associated oral microbiome and OTUs which were identified in this study, a more inclusive comparison provided details on this group of bacteria that may be potential early indicators of oral cancer. We also identified several OTUs associated with normal and cancer oral clinical samples. The normal associated OTUs is an indication of presence of members of the core oral microbiome while the cancer associated OTUs could have potential as markers for cancer prognosis or therapeutic but more studies are warranted to explore such possibilities. Further, it is suggested that the alkaline phosphatase and catalase positive phenotypes are possibly related to pre-cancerous and cancerous oral conditions while the sulfate reducing phenotype is potentially related to cancerous oral condition based on the observation that majority of the OTUs with these phenotypic characteristics

were identified in the respective oral conditions. It is also noted that Bacteroidetes, Proteobacteria and Actinobacteria phyla could be good sources for the identification of potential pre-cancer and cancer associated bio-markers as a high fraction of these bacteria groups are associated with either pre-cancerous and/or cancerous samples. However, the diagnostic or therapeutic values of these potential biomarkers require further validations using higher number of pre-cancerous and cancerous samples.

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APPENDIX

APPENDIX A

Swab Sampling Protocol

1. Assign unique sample tag number to each volunteer.
2. Label the 1.5ml centrifuge tube that contains 400µL of preservation buffer (50mM Tris, pH8.0, 50mM EDTA, 50mM sucrose, 100mM NaCl, 1% SDS) with designated sample tag number.
3. Remove swab from its respective tube.
4. Swab each selected oral region 5 to 6 times with following procedures:
   a. For tongue sampling; move swab in anterior to posterior direction on dorsum and lateral tongue while applying slight pressure.
   b. For buccal and palate sampling; move swab in circular motion over the central part while applying slight pressure.
   c. For mouth floor and gingival sampling: move swab along mucosa layer while rotating swab with slight pressure.
5. Cut out and dip the swab tip into the pre-labeled 1.5ml centrifuge tube and keep it at either ambient temperature or 4ºC.

APPENDIX B

Modified Protocol of GeneMATRIX Swab-Extract DNA Purification Kit (EURx)

Note: elements of modification were underlined.

1. 40µl of Buffer S was added onto the spin-column and was kept until centrifugation steps.
2. 400µl of Lyse S buffer and 20µl of Proteinase K were mixed into swab samples and incubated overnight at 56ºC with shaking.
3. 800µl of Sol S buffer was added and mix thoroughly which was then incubated for 10 minutes at 70ºC.
4. 400µl of absolute ethanol was added and mixed thoroughly where the swab samples were subsequently centrifuged for 2 minutes at 14,000 rpm.
5. 600µl of the lysate was transfer to the spin-column and centrifuged for 1 minute at 12,000 rpm. The eluted lysate was discarded.
6. Step 5 was repeated until all lysate had passed through the column and the final centrifugation duration and speed were 2 minutes and 12,000 rpm respectively.
7. 500µl of Wash SX1 buffer was added onto the spin-column and centrifuged for 1 min at 12,000 rpm. The eluted lysate was discarded.
8. 500µl of Wash SX2 buffer was added onto the spin-column and centrifuged for 2 minutes at 12,000 rpm. The eluted lysate was discarded.
9. 50µl of Elution buffer (10 mM Tris-HCl, pH 8.5) was added onto the spin-column and incubated for 5 minutes at room temperature which was then centrifuged for 1 minute at 12,000 rpm.
10. Eluted nucleic acid samples were stored at -80ºC.

APPENDIX C

Genbank Accession Number

Table C.1: Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P01a-03-SP6	KP294530
OralMicrobiome.sqn P01a-09-T7	KP294531
OralMicrobiome.sqn P01a-12-SP6	KP294532
OralMicrobiome.sqn P01a-15-SP6	KP294533
OralMicrobiome.sqn P01a-21-T7	KP294534
OralMicrobiome.sqn P01a-22-T7	KP294535
OralMicrobiome.sqn P01a-26-SP6	KP294536
OralMicrobiome.sqn P01a-31-T7	KP294537
OralMicrobiome.sqn P01a-33-T7	KP294538
OralMicrobiome.sqn P01a-36-SP6	KP294539
OralMicrobiome.sqn P01a-38-T7	KP294540
OralMicrobiome.sqn P01a-40-SP6	KP294541
OralMicrobiome.sqn P01a-44-SP6	KP294542
OralMicrobiome.sqn P01a-47-T7	KP294543
OralMicrobiome.sqn P01a-48-T7	KP294544
OralMicrobiome.sqn P01a-50-SP6	KP294545
OralMicrobiome.sqn P02a-01-SP6	KP294546
OralMicrobiome.sqn P02a-02-SP6	KP294547
OralMicrobiome.sqn P02a-04-SP6	KP294548
OralMicrobiome.sqn P02a-05-T7	KP294549
OralMicrobiome.sqn P02a-06-T7	KP294550
OralMicrobiome.sqn P02a-10-T7	KP294551
OralMicrobiome.sqn P02a-11-T7	KP294552
OralMicrobiome.sqn P02a-13-T7	KP294553
OralMicrobiome.sqn P02a-15-T7	KP294554
OralMicrobiome.sqn P02a-18-T7	KP294555
OralMicrobiome.sqn P02a-23-SP6	KP294556
OralMicrobiome.sqn P02a-24-SP6	KP294557
OralMicrobiome.sqn P02a-26-T7	KP294558

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P02a-28-T7	KP294559
OralMicrobiome.sqn P02a-31-T7	KP294560
OralMicrobiome.sqn P02a-33-T7	KP294561
OralMicrobiome.sqn P02a-35-SP6	KP294562
OralMicrobiome.sqn P02a-36-T7	KP294563
OralMicrobiome.sqn P02a-38-SP6	KP294564
OralMicrobiome.sqn P02a-39-SP6	KP294565
OralMicrobiome.sqn P02a-41-SP6	KP294566
OralMicrobiome.sqn P02a-42-SP6	KP294567
OralMicrobiome.sqn P02a-44-T7	KP294568
OralMicrobiome.sqn P02a-49-SP6	KP294569
OralMicrobiome.sqn P02a-57-SP6	KP294570
OralMicrobiome.sqn P02a-60-SP6	KP294571
OralMicrobiome.sqn P02a-61-SP6	KP294572
OralMicrobiome.sqn P02a-62-SP6	KP294573
OralMicrobiome.sqn P02a-68-T7	KP294574
OralMicrobiome.sqn P02a-72-SP6	KP294575
OralMicrobiome.sqn P02a-73-SP6	KP294576
OralMicrobiome.sqn P02a-75-T7	KP294577
OralMicrobiome.sqn P02a-77-T7	KP294578
OralMicrobiome.sqn P02a-80-T7	KP294579
OralMicrobiome.sqn P02b-03-SP6	KP294580
OralMicrobiome.sqn P02b-06-SP6	KP294581
OralMicrobiome.sqn P02b-09-SP6	KP294582
OralMicrobiome.sqn P02b-11-T7	KP294583
OralMicrobiome.sqn P02b-14-T7	KP294584
OralMicrobiome.sqn P02b-15-T7	KP294585
OralMicrobiome.sqn P02b-17-T7	KP294586
OralMicrobiome.sqn P02b-20-T7	KP294587
OralMicrobiome.sqn P02b-27-T7	KP294588
OralMicrobiome.sqn P02b-28-SP6	KP294589
OralMicrobiome.sqn P02b-29-SP6	KP294590
OralMicrobiome.sqn P02b-40-SP6	KP294591

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P02b-44-T7	KP294592
OralMicrobiome.sqn P02b-52-T7	KP294593
OralMicrobiome.sqn P02b-57-SP6	KP294594
OralMicrobiome.sqn P02b-63-SP6	KP294595
OralMicrobiome.sqn P02b-73-T7	KP294596
OralMicrobiome.sqn P02b-75-SP6	KP294597
OralMicrobiome.sqn P02b-79-T7	KP294598
OralMicrobiome.sqn P03-06-T7	KP294599
OralMicrobiome.sqn P03-08-T7	KP294600
OralMicrobiome.sqn P03-10-T7	KP294601
OralMicrobiome.sqn P03-14-T7	KP294602
OralMicrobiome.sqn P03-22-SP6	KP294603
OralMicrobiome.sqn P03-25-T7	KP294604
OralMicrobiome.sqn P03-38-T7	KP294605
OralMicrobiome.sqn P03-39-T7	KP294606
OralMicrobiome.sqn P03-45-SP6	KP294607
OralMicrobiome.sqn P03-47-T7	KP294608
OralMicrobiome.sqn P03-48-SP6	KP294609
OralMicrobiome.sqn P03-54-SP6	KP294610
OralMicrobiome.sqn P03-56-T7	KP294611
OralMicrobiome.sqn P03-58-SP6	KP294612
OralMicrobiome.sqn P04-03-T7	KP294613
OralMicrobiome.sqn P04-18-T7	KP294614
OralMicrobiome.sqn P04-23-SP6	KP294615
OralMicrobiome.sqn P04-29-T7	KP294616
OralMicrobiome.sqn P04-36-T7	KP294617
OralMicrobiome.sqn P04-59-T7	KP294618
OralMicrobiome.sqn P04-64-T7	KP294619
OralMicrobiome.sqn P04-69-SP6	KP294620
OralMicrobiome.sqn P05-03-SP6	KP294621
OralMicrobiome.sqn P05-07-SP6	KP294622
OralMicrobiome.sqn P05-17-SP6	KP294623
OralMicrobiome.sqn P05-23-T7	KP294624

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P05-30-SP6	KP294625
OralMicrobiome.sqn P05-33-SP6	KP294626
OralMicrobiome.sqn P05-35-SP6	KP294627
OralMicrobiome.sqn P05-37-T7	KP294628
OralMicrobiome.sqn P05-39-SP6	KP294629
OralMicrobiome.sqn P05-51-SP6	KP294630
OralMicrobiome.sqn P05-53-T7	KP294631
OralMicrobiome.sqn P06-03-SP6	KP294632
OralMicrobiome.sqn P06-05-T7	KP294633
OralMicrobiome.sqn P06-06-T7	KP294634
OralMicrobiome.sqn P06-12-T7	KP294635
OralMicrobiome.sqn P06-18-SP6	KP294636
OralMicrobiome.sqn P06-24-T7	KP294637
OralMicrobiome.sqn P06-32-T7	KP294638
OralMicrobiome.sqn P06-36-SP6	KP294639
OralMicrobiome.sqn P06-37-SP6	KP294640
OralMicrobiome.sqn P06-48-SP6	KP294641
OralMicrobiome.sqn P06-51-SP6	KP294642
OralMicrobiome.sqn P07-09-T7	KP294643
OralMicrobiome.sqn P07-100-SP6	KP294644
OralMicrobiome.sqn P07-103-SP6	KP294645
OralMicrobiome.sqn P07-14-T7	KP294646
OralMicrobiome.sqn P07-16-SP6	KP294647
OralMicrobiome.sqn P07-24-SP6	KP294648
OralMicrobiome.sqn P07-26-SP6	KP294649
OralMicrobiome.sqn P07-36-T7	KP294650
OralMicrobiome.sqn P07-37-T7	KP294651
OralMicrobiome.sqn P07-40-SP6	KP294652
OralMicrobiome.sqn P07-43-T7	KP294653
OralMicrobiome.sqn P07-47-T7	KP294654
OralMicrobiome.sqn P07-57-SP6	KP294655
OralMicrobiome.sqn P07-69-SP6	KP294656
OralMicrobiome.sqn P07-73-T7	KP294657

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P07-80-T7	KP294658
OralMicrobiome.sqn P07-84-T7	KP294659
OralMicrobiome.sqn P07-88-T7	KP294660
OralMicrobiome.sqn P07-94-T7	KP294661
OralMicrobiome.sqn P07-98-SP6	KP294662
OralMicrobiome.sqn P07-99-T7	KP294663
OralMicrobiome.sqn P07-C-SP6	KP294664
OralMicrobiome.sqn P07-E-SP6	KP294665
OralMicrobiome.sqn P07-P-T7	KP294666
OralMicrobiome.sqn P07-Q-T7	KP294667
OralMicrobiome.sqn P07-U-T7	KP294668
OralMicrobiome.sqn P07-W-T7	KP294669
OralMicrobiome.sqn P07-Y-T7	KP294670
OralMicrobiome.sqn P08-07-SP6	KP294671
OralMicrobiome.sqn P08-20-T7	KP294672
OralMicrobiome.sqn P08-21-SP6	KP294673
OralMicrobiome.sqn P08-24-SP6	KP294674
OralMicrobiome.sqn P08-35-T7	KP294675
OralMicrobiome.sqn P08-37-SP6	KP294676
OralMicrobiome.sqn P08-42-T7	KP294677
OralMicrobiome.sqn P08-45-SP6	KP294678
OralMicrobiome.sqn P08-51-T7	KP294679
OralMicrobiome.sqn P08-53-SP6	KP294680
OralMicrobiome.sqn P08-58-T7	KP294681
OralMicrobiome.sqn P09-03-T7	KP294682
OralMicrobiome.sqn P09-10-SP6	KP294683
OralMicrobiome.sqn P09-23-SP6	KP294684
OralMicrobiome.sqn P09-24-SP6	KP294685
OralMicrobiome.sqn P09-25-SP6	KP294686
OralMicrobiome.sqn P09-28-T7	KP294687
OralMicrobiome.sqn P09-29-T7	KP294688
OralMicrobiome.sqn P09-40-SP6	KP294689
OralMicrobiome.sqn P09-45-T7	KP294690

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P09-51-T7	KP294691
OralMicrobiome.sqn P09-57-T7	KP294692
OralMicrobiome.sqn P11a-01-SP6	KP294693
OralMicrobiome.sqn P11a-03-SP6	KP294694
OralMicrobiome.sqn P11a-05-SP6	KP294695
OralMicrobiome.sqn P11a-06-SP6	KP294696
OralMicrobiome.sqn P11a-09-T7	KP294697
OralMicrobiome.sqn P11a-11-SP6	KP294698
OralMicrobiome.sqn P11a-14-T7	KP294699
OralMicrobiome.sqn P11a-15-T7	KP294700
OralMicrobiome.sqn P11a-19-SP6	KP294701
OralMicrobiome.sqn P11a-21-T7	KP294702
OralMicrobiome.sqn P11a-28-SP6	KP294703
OralMicrobiome.sqn P11a-36-T7	KP294704
OralMicrobiome.sqn P11a-42-SP6	KP294705
OralMicrobiome.sqn P11a-43-T7	KP294706
OralMicrobiome.sqn P11a-44-T7	KP294707
OralMicrobiome.sqn P11a-56-SP6	KP294708
OralMicrobiome.sqn P11a-60-SP6	KP294709
OralMicrobiome.sqn P11a-69-T7	KP294710
OralMicrobiome.sqn P11a-78-T7	KP294711
OralMicrobiome.sqn P11a-80-T7	KP294712
OralMicrobiome.sqn P11b-04-T7	KP294713
OralMicrobiome.sqn P11b-05-T7	KP294714
OralMicrobiome.sqn P11b-10-SP6	KP294715
OralMicrobiome.sqn P11b-16-SP6	KP294716
OralMicrobiome.sqn P11b-39-SP6	KP294717
OralMicrobiome.sqn P11b-49-T7	KP294718
OralMicrobiome.sqn P11b-55-T7	KP294719
OralMicrobiome.sqn P11b-64-SP6	KP294720
OralMicrobiome.sqn P11b-71-T7	KP294721
OralMicrobiome.sqn P12-03-T7	KP294722
OralMicrobiome.sqn P12-11-SP6	KP294723

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P12-15-T7	KP294724
OralMicrobiome.sqn P12-18-T7	KP294725
OralMicrobiome.sqn P12-21-T7	KP294726
OralMicrobiome.sqn P12-23-SP6	KP294727
OralMicrobiome.sqn P12-24-T7	KP294728
OralMicrobiome.sqn P12-41-SP6	KP294729
OralMicrobiome.sqn P12-44-SP6	KP294730
OralMicrobiome.sqn P12-51-T7	KP294731
OralMicrobiome.sqn P12-60-T7	KP294732
OralMicrobiome.sqn P13-01-T7	KP294733
OralMicrobiome.sqn P13-03-SP6	KP294734
OralMicrobiome.sqn P13-05-SP6	KP294735
OralMicrobiome.sqn P13-11-SP6	KP294736
OralMicrobiome.sqn P13-13-SP6	KP294737
OralMicrobiome.sqn P13-16-SP6	KP294738
OralMicrobiome.sqn P13-23-SP6	KP294739
OralMicrobiome.sqn P13-25-SP6	KP294740
OralMicrobiome.sqn P13-27-T7	KP294741
OralMicrobiome.sqn P13-28-SP6	KP294742
OralMicrobiome.sqn P13-31-SP6	KP294743
OralMicrobiome.sqn P13-35-SP6	KP294744
OralMicrobiome.sqn P13-36-SP6	KP294745
OralMicrobiome.sqn P13-38-T7	KP294746
OralMicrobiome.sqn P13-42-SP6	KP294747
OralMicrobiome.sqn P13-43-SP6	KP294748
OralMicrobiome.sqn P13-45-T7	KP294749
OralMicrobiome.sqn P13-50-SP6	KP294750
OralMicrobiome.sqn P13-52-T7	KP294751
OralMicrobiome.sqn P13-57-T7	KP294752
OralMicrobiome.sqn P13-58-T7	KP294753
OralMicrobiome.sqn P14-05-SP6	KP294754
OralMicrobiome.sqn P14-07-SP6	KP294755
OralMicrobiome.sqn P14-10-T7	KP294756

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P14-14-SP6	KP294757
OralMicrobiome.sqn P14-17-SP6	KP294758
OralMicrobiome.sqn P14-20-SP6	KP294759
OralMicrobiome.sqn P14-22-T7	KP294760
OralMicrobiome.sqn P14-26-SP6	KP294761
OralMicrobiome.sqn P14-29-SP6	KP294762
OralMicrobiome.sqn P14-34-SP6	KP294763
OralMicrobiome.sqn P14-42-T7	KP294764
OralMicrobiome.sqn P14-52-SP6	KP294765
OralMicrobiome.sqn P14-53-SP6	KP294766
OralMicrobiome.sqn P14-57-SP6	KP294767
OralMicrobiome.sqn P15-15-T7	KP294768
OralMicrobiome.sqn P15-17-T7	KP294769
OralMicrobiome.sqn P15-20-T7	KP294770
OralMicrobiome.sqn P15-21-T7	KP294771
OralMicrobiome.sqn P15-25-T7	KP294772
OralMicrobiome.sqn P15-32-SP6	KP294773
OralMicrobiome.sqn P15-50-SP6	KP294774
OralMicrobiome.sqn P15-54-T7	KP294775
OralMicrobiome.sqn P15-55-SP6	KP294776
OralMicrobiome.sqn P16-02-SP6	KP294777
OralMicrobiome.sqn P16-05-T7	KP294778
OralMicrobiome.sqn P16-09-SP6	KP294779
OralMicrobiome.sqn P16-11-T7	KP294780
OralMicrobiome.sqn P16-13-SP6	KP294781
OralMicrobiome.sqn P16-23-SP6	KP294782
OralMicrobiome.sqn P16-26-T7	KP294783
OralMicrobiome.sqn P16-37-SP6	KP294784
OralMicrobiome.sqn P16-46-T7	KP294785
OralMicrobiome.sqn P17-03-T7	KP294786
OralMicrobiome.sqn P17-16-T7	KP294787
OralMicrobiome.sqn P17-26-SP6	KP294788
OralMicrobiome.sqn P17-29-T7	KP294789

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P17-35-T7	KP294790
OralMicrobiome.sqn P17-41-T7	KP294791
OralMicrobiome.sqn P17-44-T7	KP294792
OralMicrobiome.sqn P17-51-SP6	KP294793
OralMicrobiome.sqn P17-56-T7	KP294794
OralMicrobiome.sqn P17-59-SP6	KP294795
OralMicrobiome.sqn P18-05-SP6	KP294796
OralMicrobiome.sqn P18-06-T7	KP294797
OralMicrobiome.sqn P18-19-T7	KP294798
OralMicrobiome.sqn P18-21-SP6	KP294799
OralMicrobiome.sqn P18-55-SP6	KP294800
OralMicrobiome.sqn P18-58-T7	KP294801
OralMicrobiome.sqn P19-02-T7	KP294802
OralMicrobiome.sqn P19-07-SP6	KP294803
OralMicrobiome.sqn P19-26-T7	KP294804
OralMicrobiome.sqn P19-30-T7	KP294805
OralMicrobiome.sqn P19-41-SP6	KP294806
OralMicrobiome.sqn P19-43-SP6	KP294807
OralMicrobiome.sqn P19-52-T7	KP294808
OralMicrobiome.sqn P19-57-SP6	KP294809
OralMicrobiome.sqn P21a-03-T7	KP294810
OralMicrobiome.sqn P21a-06-SP6	KP294811
OralMicrobiome.sqn P21a-08-T7	KP294812
OralMicrobiome.sqn P21a-13-T7	KP294813
OralMicrobiome.sqn P21a-18-T7	KP294814
OralMicrobiome.sqn P21a-24-SP6	KP294815
OralMicrobiome.sqn P21a-30-SP6	KP294816
OralMicrobiome.sqn P21a-33-SP6	KP294817
OralMicrobiome.sqn P21a-43-T7	KP294818
OralMicrobiome.sqn P21a-49-T7	KP294819
OralMicrobiome.sqn P21b-01-T7	KP294820
OralMicrobiome.sqn P21b-02-SP6	KP294821
OralMicrobiome.sqn P21b-07-T7	KP294822

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P21b-08-SP6	KP294823
OralMicrobiome.sqn P22-06-T7	KP294824
OralMicrobiome.sqn P22-07-T7	KP294825
OralMicrobiome.sqn P22-08-SP6	KP294826
OralMicrobiome.sqn P22-11-SP6	KP294827
OralMicrobiome.sqn P22-13-T7	KP294828
OralMicrobiome.sqn P22-17-T7	KP294829
OralMicrobiome.sqn P22-18-SP6	KP294830
OralMicrobiome.sqn P22-31-SP6	KP294831
OralMicrobiome.sqn P22-34-SP6	KP294832
OralMicrobiome.sqn P22-37-SP6	KP294833
OralMicrobiome.sqn P22-42-T7	KP294834
OralMicrobiome.sqn P22-45-SP6	KP294835
OralMicrobiome.sqn P22-55-T7	KP294836
OralMicrobiome.sqn P22-57-SP6	KP294837
OralMicrobiome.sqn P23-01-T7	KP294838
OralMicrobiome.sqn P23-06-T7	KP294839
OralMicrobiome.sqn P23-12-SP6	KP294840
OralMicrobiome.sqn P23-13-SP6	KP294841
OralMicrobiome.sqn P23-19-T7	KP294842
OralMicrobiome.sqn P23-20-SP6	KP294843
OralMicrobiome.sqn P23-21-SP6	KP294844
OralMicrobiome.sqn P23-27-T7	KP294845
OralMicrobiome.sqn P23-28-SP6	KP294846
OralMicrobiome.sqn P23-35-SP6	KP294847
OralMicrobiome.sqn P23-46-SP6	KP294848
OralMicrobiome.sqn P23-51-T7	KP294849
OralMicrobiome.sqn P23-60-T7	KP294850
OralMicrobiome.sqn P24-01-SP6	KP294851
OralMicrobiome.sqn P24-02-T7	KP294852
OralMicrobiome.sqn P24-04-T7	KP294853
OralMicrobiome.sqn P24-06-T7	KP294854
OralMicrobiome.sqn P24-09-T7	KP294855

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P24-12-E683F	KP294856
OralMicrobiome.sqn P24-16-T7	KP294857
OralMicrobiome.sqn P24-24-SP6	KP294858
OralMicrobiome.sqn P24-26-T7	KP294859
OralMicrobiome.sqn P24-29-T7	KP294860
OralMicrobiome.sqn P24-35-T7	KP294861
OralMicrobiome.sqn P24-55-T7	KP294862
OralMicrobiome.sqn P25-04-SP6	KP294863
OralMicrobiome.sqn P25-09-T7	KP294864
OralMicrobiome.sqn P25-14-SP6	KP294865
OralMicrobiome.sqn P25-16-SP6	KP294866
OralMicrobiome.sqn P25-27-SP6	KP294867
OralMicrobiome.sqn P25-45-SP6	KP294868
OralMicrobiome.sqn P26-15-T7	KP294869
OralMicrobiome.sqn P26-17-SP6	KP294870
OralMicrobiome.sqn P26-28-SP6	KP294871
OralMicrobiome.sqn P26-32-SP6	KP294872
OralMicrobiome.sqn P26-34-T7	KP294873
OralMicrobiome.sqn P26-37-T7	KP294874
OralMicrobiome.sqn P26-51-T7	KP294875
OralMicrobiome.sqn P26-52-SP6	KP294876
OralMicrobiome.sqn P26-54-SP6	KP294877
OralMicrobiome.sqn P27-02-T7	KP294878
OralMicrobiome.sqn P27-09-SP6	KP294879
OralMicrobiome.sqn P27-11-SP6	KP294880
OralMicrobiome.sqn P27-13-T7	KP294881
OralMicrobiome.sqn P27-22-T7	KP294882
OralMicrobiome.sqn P27-24-SP6	KP294883
OralMicrobiome.sqn P27-26-SP6	KP294884
OralMicrobiome.sqn P27-29-T7	KP294885
OralMicrobiome.sqn P27-51-SP6	KP294886
OralMicrobiome.sqn P29-14-T7	KP294887
OralMicrobiome.sqn P29-25-T7	KP294888

Table C.1 (continue): Deposited Sequences with Their Respective Accession Number

OTUs	Genbank Accession Number
OralMicrobiome.sqn P29-40-SP6	KP294889
OralMicrobiome.sqn P29-52-SP6	KP294890
OralMicrobiome.sqn P29-54-SP6	KP294891
OralMicrobiome.sqn P36-04-T7	KP294892
OralMicrobiome.sqn P36-07-T7	KP294893
OralMicrobiome.sqn P36-09-SP6	KP294894
OralMicrobiome.sqn P36-16-T7	KP294895
OralMicrobiome.sqn P36-38-T7	KP294896
OralMicrobiome.sqn P36-39-SP6	KP294897
OralMicrobiome.sqn P36-42-T7	KP294898
OralMicrobiome.sqn P36-46-T7	KP294899
OralMicrobiome.sqn P36-52-SP6	KP294900
OralMicrobiome.sqn P36-54-T7	KP294901
OralMicrobiome.sqn P36-55-T7	KP294902
OralMicrobiome.sqn P36-59-SP6	KP294903
OralMicrobiome.sqn P38-06-T7	KP294904
OralMicrobiome.sqn P38-34-SP6	KP294905

APPENDIX D

A260/A280 Ratio and DNA Concentration

Table D.1: Triplicate Readings of A260/A280 Ratio and DNA Concentration of Normal Subjects

Label	A260/A280				Concentration, ng/ul			
	1	2	3	Average	1	2	3	Average
6349	1.694	1.743	1.714	1.72	30.5	30.5	30.0	30.33
H/F/U/001	1.780	1.789	1.787	1.79	81.0	80.5	79.5	80.33
H/F/U/003	1.770	1.780	1.730	1.76	118.0	118.0	117.0	117.67
H/F/U/004	1.681	1.702	1.702	1.70	39.5	40.0	40.0	39.83
H/F/U/005	1.844	1.871	1.812	1.84	29.5	29.0	29.0	29.17
H/F/U/006	1.813	1.836	1.824	1.82	68.0	67.0	67.5	67.50
H/F/U/007	1.733	1.689	1.700	1.71	52.0	51.5	51.0	51.50
H/F/U/008	1.701	1.687	1.662	1.68	57.0	56.5	56.5	56.67
H/F/U/009	1.846	1.830	1.840	1.84	168.0	166.0	166.0	166.67
H/F/U/010	1.910	1.886	1.885	1.89	74.50	74.50	73.50	74.17
H/F/U/012	1.717	1.717	1.696	1.71	39.5	39.5	39.0	39.33
H/F/U/013	1.644	1.658	1.636	1.65	37.0	36.5	36.0	36.50
H/F/U/014	1.694	1.640	1.653	1.66	41.5	41.0	40.5	41.00
H/F/U/015	1.732	1.753	1.750	1.75	71.0	71.0	70.0	70.67
H/F/U/017	1.652	1.636	1.565	1.62	19.0	18.0	18.0	18.33
H/F/U/018	1.754	1.711	1.689	1.72	39.5	38.5	38.0	38.67
H/F/U/019	1.767	1.775	1.753	1.77	79.5	79.0	78.0	78.83
H/F/U/021	1.853	1.824	1.848	1.84	31.5	31.0	30.5	31.00
H/F/U/022	1.774	1.755	1.750	1.76	47.0	46.5	45.5	46.33
H/F/U/024	1.754	1.737	1.768	1.75	50.0	49.5	49.5	49.67
H/F/U/026	1.476	1.470	1.455	1.47	46.5	48.3	48.0	47.60
H/M/U/001	1.798	1.816	1.818	1.81	89.0	89.0	90.0	89.33
H/M/U/002	1.521	1.521	1.521	1.52	36.5	36.5	36.5	36.50
H/M/U/003	1.789	1.781	1.772	1.78	102.0	102.0	101.0	101.67
H/M/U/004	1.616	1.616	1.616	1.62	59.0	59.0	59.0	59.00
H/M/U/005	1.897	1.897	1.864	1.89	82.5	82.5	82.0	82.33
H/M/U/006	1.603	1.565	1.574	1.58	54.5	54.0	53.5	54.00

Table D.1 (continue): Triplicate Readings of A260/A280 Ratio and DNA Concentration of Normal Subjects

Label	A260/A280				Concentration, ng/ul			
	1	2	3	Average	1	2	3	Average
H/M/U/007	1.684	1.671	1.658	1.67	66.5	66.0	65.5	66.00
H/M/U/008	1.655	1.621	1.643	1.64	24.0	23.5	23.0	23.50
H/M/U/009	1.755	1.736	1.750	1.75	46.5	46.0	45.5	46.00
H/M/U/010	1.661	1.661	1.655	1.66	49.0	49.0	48.0	48.67
H/M/U/011	1.645	1.667	1.655	1.66	25.5	25.0	24.0	24.83
H/M/U/014	1.804	1.813	1.802	1.81	83.0	82.5	82.0	82.50
H/M/U/015	1.627	1.600	1.608	1.61	61.0	60.0	59.5	60.17
H/M/U/016	1.755	1.755	1.750	1.75	43.0	43.0	43.0	43.00
H/M/U/017	1.775	1.757	1.767	1.77	90.5	90.5	90.0	90.33
H/M/U/020	1.812	1.797	1.781	1.80	58.0	57.5	57.0	57.50
H/M/U/021	1.733	1.700	1.759	1.73	26.0	25.5	25.5	25.67
H/M/U/023	1.795	1.767	1.767	1.78	39.5	38.5	38.0	38.67
H/M/U/024	1.600	1.600	1.567	1.59	24.0	24.0	23.5	23.83
H/M/U/025	1.786	1.786	1.750	1.77	25.0	25.0	24.5	24.83

Table D.2: Triplicate Readings of A260/A280 Ratio and DNA Concentration of Pre-cancer Subjects

Label	A260/A280				Concentration, ng/ul			
	1	2	3	Average	1	2	3	Average
01-0002-05	1.753	1.753	1.750	1.75	67.5	67.5	66.5	67.17
01-0004-06	1.857	1.757	1.770	1.79	32.5	32.5	32.0	32.33
01-0004-11	1.821	1.857	1.889	1.86	25.5	26.0	25.5	25.67
01-0008-13-2	1.806	1.792	1.792	1.80	65.0	64.5	64.5	64.67
01-0023-12	1.745	1.723	1.702	1.72	41.0	40.0	40.0	40.33
01-0024-12	1.758	1.758	1.758	1.76	116.0	116.0	116.0	116.00
01-0026-12	1.805	1.793	1.793	1.80	78.5	78.0	78.0	78.17
01-0033-12	1.834	1.833	1.821	1.83	138.0	138.0	138.0	138.00
01-0042-12-2	1.667	1.653	1.653	1.66	60.0	59.5	59.5	59.67
01-0043-12	1.840	1.811	1.819	1.82	86.5	86.0	85.5	86.00

Table D.3: Triplicate Readings of A260/A280 Ratio and DNA Concentration of Cancer Subjects

Label	A260/A280				Concentration, ng/ul			
	1	2	3	Average	1	2	3	Average
01-0009-13-1	1.753	1.762	1.771	1.76	74.5	74.0	73.5	74.00
01-0014-10	1.407	1.444	1.370	1.41	19.0	19.5	18.5	19.00
01-0015-13-1	1.700	1.654	1.667	1.67	42.5	43.0	42.5	42.67
01-0017-13-1	1.754	1.738	1.723	1.74	228.0	226.0	224.0	226.00
01-0019-10	1.857	1.821	1.789	1.82	52.0	51.0	51.0	51.33
01-0022-12	1.673	1.686	1.654	1.67	43.5	43.0	43.0	43.17
01-0024-07	1.429	1.400	1.371	1.40	25.0	24.5	24.0	24.50
01-0025-12	1.763	1.737	1.737	1.75	33.5	33.0	33.0	33.17
01-0032-12-1	1.618	1.636	1.630	1.63	44.5	45.0	44.0	44.50
01-0034-12	1.676	1.676	1.647	1.67	28.5	28.5	28.0	28.33
01-0049-12	1.826	1.826	1.864	1.84	84.0	84.0	82.0	83.33
01-0050-12-1	1.775	1.744	1.756	1.76	79.0	78.5	79.0	78.83
UM01 / 01-0001-11	1.605	1.622	1.553	1.59	30.5	30.0	29.5	30.00

APPENDIX E
DGGE Similarity Scores

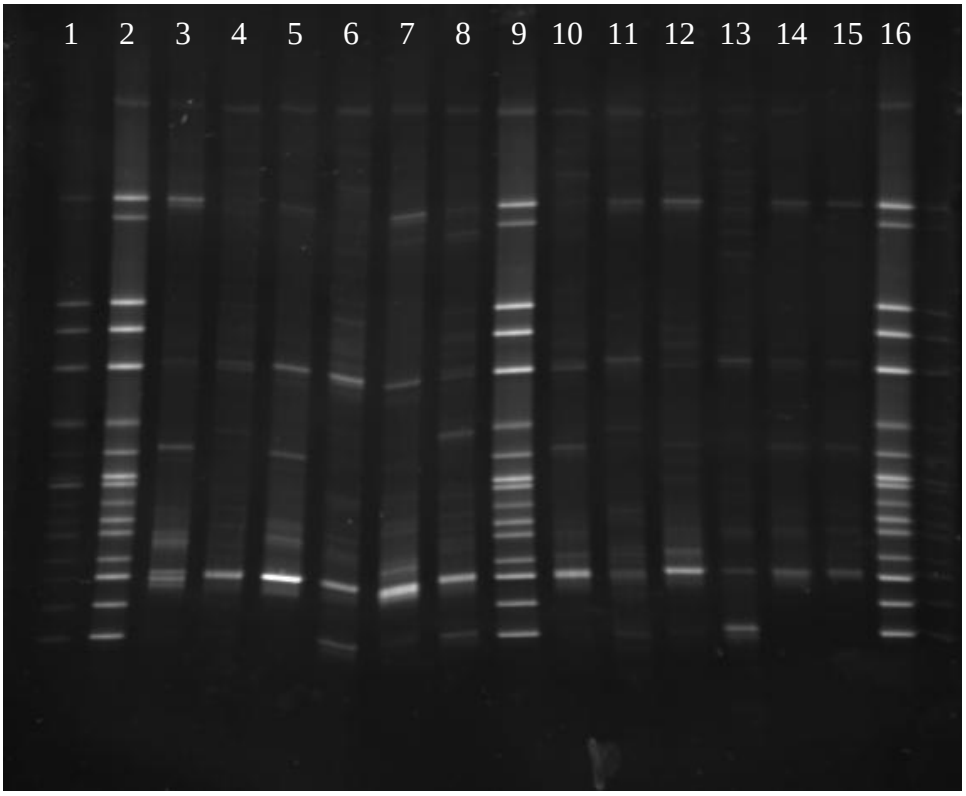
Table E.1: Similarity Scores between the DGGE profiles

01-0043-12	100																																			
6349	74.29	100																																		
HMU016	68.57	63.16	100																																	
01-0024-12	48	57.15	64.29	100																																
01-0042-12	57.15	45.17	58.07	66.67	100																															
01-0023-12	50	51.43	57.15	32.01	35.72	100																														
HFU010	43.25	45	40	33.33	42.43	48.65	100																													
HMU006	46.15	42.86	47.62	37.5	45.72	35.9	68.19	100																												
HFU009	55.01	51.17	41.87	30.31	50	45	62.23	59.58	100																											
HMU010	45.72	47.37	36.85	21.43	32.26	45.72	60	61.91	69.77	100																										
01-0009-13	36.85	34.15	43.91	25.81	41.18	47.37	65.12	53.33	60.87	53.66	100																									
01-0034-12	51.43	36.85	57.9	42.86	45.17	45.72	45	47.62	46.51	42.11	48.78	100																								
HMU011	52.95	48.65	54.06	29.63	53.33	41.18	51.29	58.54	57.15	59.46	40	59.46	100																							
HMU007	40	47.37	52.63	35.72	45.17	45.72	55.01	52.39	60.47	57.9	58.54	57.9	59.46	100																						
HFU008	54.06	50	35	40	36.37	43.25	52.39	45.46	44.45	55.01	46.51	35	30.77	40	100																					
HMU004	61.11	41.03	61.54	48.28	62.5	55.56	53.66	51.17	59.09	56.41	47.62	51.29	47.37	51.29	63.42	100																				
01-0008-13	42.43	27.78	33.33	15.39	34.49	36.37	57.9	55.01	43.91	50	41.03	44.45	45.72	50	47.37	59.46	100																			
01-0050-12	43.75	34.29	57.15	40	57.15	37.5	54.06	61.54	40	45.72	52.63	57.15	64.71	45.72	43.25	66.67	72.73	100																		
HFU007	42.43	44.45	55.56	30.77	41.38	48.49	57.9	65	43.91	66.67	51.29	44.45	62.86	55.56	52.63	54.06	64.71	66.67	100																	
01-0015-13	48.65	45	65	26.67	36.37	54.06	42.86	59.09	48.89	50	46.51	50	51.29	60	61.91	58.54	47.37	54.06	57.9	100																
HMU014	51.29	57.15	61.91	37.5	45.72	51.29	54.55	65.22	63.83	52.39	62.23	47.62	58.54	66.67	54.55	55.81	50	56.41	65	86.37	100															
01-0049-12	59.46	60	60	26.67	54.55	43.25	42.86	50	44.45	55.01	55.81	50	56.41	55.01	42.86	53.66	47.37	43.25	57.9	71.43	77.27	100														
HMU021	72.73	61.11	61.11	53.85	62.07	54.55	36.85	55.01	39.03	50	41.03	55.56	45.72	61.11	52.63	64.87	52.95	60.61	52.95	68.43	75	68.43	100													
HFU013	38.89	41.03	61.54	20.69	43.75	55.56	43.91	46.51	31.82	51.29	38.1	46.15	57.9	46.15	58.54	60	54.06	55.56	48.65	82.93	69.77	68.29	59.46	100												
HMU024	77.78	66.67	71.8	55.17	68.75	55.56	43.91	41.87	40.91	46.15	38.1	51.29	52.63	56.41	58.54	65	54.06	50	64.87	68.29	65.12	63.42	75.68	80.01	100											
HFU024	37.5	40	62.86	32.01	50	37.5	59.46	56.41	45	40	52.63	57.15	47.06	57.15	43.25	50	48.49	62.5	48.49	64.87	61.54	59.46	54.55	61.11	50	100										
HMU017	60.61	50	72.23	46.15	55.17	42.43	42.11	55.01	39.03	38.89	41.03	66.67	57.15	50	42.11	48.65	47.06	60.61	41.18	63.16	70	68.43	64.71	70.27	64.87	84.85	100									
01-0033-12	63.16	58.54	63.42	45.17	52.95	57.9	41.87	48.89	47.83	34.15	54.55	73.17	50	48.78	55.81	57.15	46.15	57.9	46.15	60.47	57.78	65.12	71.8	66.67	66.67	68.43	82.05	100								
HMU001	41.03	52.39	52.39	31.25	45.72	35.9	59.09	52.17	51.07	52.39	53.33	61.91	43.91	66.67	54.55	60.47	50	51.29	45	63.64	60.87	63.64	55.01	60.47	60.47	71.8	70	71.11	100							
01-0004-06	60	51.17	65.12	36.37	44.45	50	48.89	59.58	41.67	51.17	43.48	60.47	52.39	51.17	48.89	54.55	58.54	60	43.91	71.11	63.83	57.78	63.42	72.73	72.73	70	82.93	73.91	68.09	100						
HMU015	57.15	52.63	63.16	21.43	38.71	45.72	55.01	57.15	41.87	42.11	53.66	52.63	54.06	52.63	45	41.03	50	57.15	50	50	61.91	60	66.67	66.67	66.67	68.57	66.67	63.42	66.67	79.07	100					
HFU017	43.75	51.43	62.86	40	57.15	31.25	43.25	56.41	45	22.86	47.37	57.15	47.06	45.72	43.25	50	42.43	62.5	36.37	54.06	66.67	54.06	60.61	61.11	61.11	68.75	78.79	68.43	56.41	65	62.86	100				
HMU008	42.43	44.45	50	23.08	41.38	36.37	42.11	55.01	43.91	27.78	46.15	50	51.43	50	47.37	43.25	41.18	54.55	41.18	63.16	65	52.63	64.71	64.87	48.65	60.61	70.59	66.67	50	58.54	61.11	78.79	100			
HFU005	35.72	32.26	58.07	28.57	41.67	42.86	30.31	45.72	22.23	25.81	41.18	38.71	33.33	38.71	30.31	43.75	41.38	50	48.28	66.67	62.86	60.61	55.17	56.25	62.5	64.29	75.87	58.83	51.43	61.11	51.61	64.29	68.97	100		
HMU025	55.17	43.75	56.25	45.46	72	34.49	35.3	55.56	37.84	31.25	34.29	50	51.61	31.25	29.41	42.43	40	62.07	46.67	52.95	66.67	70.59	73.33	54.55	60.61	68.97	80.01	62.86	50	64.87	37.5	68.97	60	80.01	100	
01-0002-05	46.15	34.49	55.17	42.11	63.64	30.77	38.71	60.61	29.41	34.49	31.25	48.28	42.86	41.38	38.71	53.33	44.45	61.54	37.04	51.61	54.55	51.61	59.26	46.67	46.67	61.54	66.67	56.25	48.49	58.83	41.38	69.23	59.26	63.64	69.57	100
01-0032-12	53.85	41.38	62.07	31.58	54.55	46.15	38.71	48.49	23.53	41.38	37.5	62.07	50	48.28	25.81	53.33	37.04	46.15	37.04	51.61	48.49	51.61	59.26	40	53.33	69.23	66.67	62.5	60.61	58.83	48.28	53.85	51.85	63.64	69.57	70
HFU014	37.04	40	33.33	30	52.17	44.45	50	58.83	34.29	53.33	42.43	46.67	55.17	60	50	45.17	50	51.85	64.29	62.5	58.83	56.25	57.15	58.07	51.61	51.85	64.29	54.55	58.83	51.43	40	51.85	57.15	60.87	58.33	66.67
HFU004	36.37	33.33	44.45	30.77	34.49	42.43	47.37	60	43.91	50	41.03	33.33	57.15	44.45	42.11	48.65	52.95	54.55	52.95	57.9	65	52.63	35.3	64.87	43.25	36.37	47.06	41.03	40	48.78	44.45	42.43	64.71	48.28	46.67	51.85
HMU002	58.83	37.84	43.25	29.63	46.67	47.06	41.03	58.54	47.62	54.06	50	43.25	44.45	48.65	51.29	52.63	57.15	47.06	57.15	71.8	68.29	61.54	40	68.43	47.37	64.71	62.86	55.01	63.42	61.91	64.87	58.83	68.57	60	45.17	50
HFU026	66.67	61.11	66.67	30.77	55.17	66.67	36.85	45	29.27	61.11	41.03	44.45	45.72	50	68.43	54.06	47.06	48.49	58.83	73.69	65	52.63	58.83	64.87	75.68	54.55	58.83	61.54	55.01	63.42	55.56	54.55	58.83	55.17	53.33	51.85
01-0017-13	54.55	44.45	61.11	38.47	62.07	48.49	47.37	60	48.78	44.45	35.9	55.56	57.15	61.11	57.9	54.06	52.95	48.49	58.83	73.69	65	63.16	64.71	70.27	54.06	66.67	64.71	71.8	60	68.29	55.56	60.61	70.59	48.28	53.33	66.67
HFU003	31.58	29.27	39.03	19.36	23.53	42.11	60.47	57.78	52.17	58.54	59.09	39.03	50	43.91	51.17	47.62	61.54	42.11	51.29	51.17	48.89	46.51	46.15	57.15	42.86	36.85	41.03	40.91	35.56	47.83	48.78	36.85	41.03	29.41	28.57	37.5
HFU021	34.29	42.11	52.63	28.57	38.71	28.57	50	47.62	51.17	36.85	53.66	47.37	54.06	57.9	50	41.03	44.45	45.72	50	60	61.91	55.01	50	61.54	46.15	57.15	61.11	58.54	66.67	51.17	57.9	51.43	61.11	51.61	43.75	48.28
HFU022	40	36.37	48.49	26.09	46.15	40	51.43	54.06	47.37	36.37	55.56	54.55	56.25	54.55	51.43	58.83	45.17	66.67	45.17	62.86	64.87	57.15	51.61	64.71	41.18	73.33	64.52	72.23	59.46	57.9	54.55	73.33	70.97	46.15	44.45	50
HMU020	40	54.55	60.61	34.79	53.85	40	40	48.65	47.37	42.43	44.45	48.49	56.25	54.55	57.15	41.18	51.61	60	58.07	68.57	64.87	62.86	77.42	70.59	58.83	60	58.07	61.11	54.06	63.16	48.49	60	70.97	53.85	59.26	58.33
HMU009	35.72	32.26	45.17	9.53	33.33	28.57	48.49	45.72	50	45.17	52.95	58.07	53.33	51.61	48.49	56.25	55.17	71.43	48.28	54.55	51.43	48.49	41.38	62.5	37.5	64.29	55.17	47.06	51.43	38.89	51.61	57.15	62.07	50	40	54.55
HFU015	40	42.43	48.49	52.17	46.15	26.67	45.72	54.06	52.63	42.43	61.11	36.37	37.5	42.43	51.43	47.06	45.17	46.67	38.71	51.43	64.87	68.57	51.61	47.06	35.3	53.33	45.17	50	48.65	47.37	48.49	66.67	64.52	46.15	44.45	50
HMU005	37.04	26.67	46.67	20	60.87	29.63	50	47.06	51.43	40	42.43	40	48.28	53.33	37.5	51.61	57.15	51.85	42.86	56.25	58.83	68.75	64.29	51.61	45.17	59.26	64.29	54.55	47.06	51.43	53.33	66.67	64.29	52.17	66.67	66.67
HFU001	46.15	27.59	41.38	31.58	27.27	30.77	38.71	36.37	47.06	34.49	43.75	27.59	35.72	41.38	45.17	40	37.04	46.15	44.45	38.71	42.43	38.71	44.45	40	26.67	46.15	51.85	43.75	42.43	41.18	34.49	38.47	51.85	36.37	43.48	50
HFU019	40	28.57	35.72	33.33	28.57	24	46.67	43.75	42.43	28.57	32.26	28.57	37.04	42.86	53.33	41.38	38.47	56.01	38.47	46.67	50	53.33	46.15	48.28	27.59	48	46.15	45.17	43.75	42.43	42.86	56.01	53.85	38.1	36.37	63.16
01-0014-10	30.31	33.33	50	53.85	41.38	36.37	42.11	40	39.03	33.33	41.03	44.45	34.29	50	26.32	43.25	41.18	36.37	35.3	36.85	45	47.37	35.3	32.43	48.65	24.25	29.41	46.15	40	29.27	27.78	24.25	17.65	27.59	40	37.04
01-0022-12	46.15	34.49	55.17	63.16	54.55	30.77	38.71	48.49	35.3	48.28	43.75	41.38	42.86	55.17	38.71	60	37.04	46.15	51.85	45.17	48.49	58.07	59.26	46.67	53.33	38.47	51.85	56.25	42.43	41.18	48.28	46.15	44.45	45.46	52.17	60
HMU003	42.86	25.81	45.17	38.1	50	21.43	36.37	45.72	44.45	45.17	58.83	51.61	53.33	51.61	42.43	56.25	27.59	50	48.28	42.43	51.43	54.55	55.17	43.75	37.5	50	62.07	58.83	57.15	50	64.52	71.43	55.17	50	48	63.64
HMU023	35.72	32.26	38.71	47.62	41.67	28.57	42.43	34.29	44.45	38.71	52.95	45.17	33.33	38.71	36.37	37.5	27.59	21.43	34.49	24.25	40	42.43	34.49	25	50	28.57	41.38	47.06	34.29	33.33	38.71	35.72	34.49	33.33	48	27.27
01-0019-10	50	41.03	51.29	48.28	43.75	44.45	39.03	51.17	59.09	30.77	47.62	41.03	57.9	56.41	14.64	30	27.03	38.89	37.84	39.03	51.17	48.78	48.65	45	45	44.45	43.25	47.62	46.51	50	51.29	61.11	43.25	43.75	48.49	46.67
01-0025-12	29.63	26.67	33.33	30	52.17	29.63	50	29.41	45.72	40	48.49	46.67	41.38	46.67	18.75	38.71	28.57	44.45	28.57	31.25	41.18	43.75	42.86	32.26	32.26	44.45	42.86	42.43	47.06	45.72	40	37.04	28.57	34.79	33.33	38.1
01-0004-11	41.38	37.5	50	36.37	24	34.49	47.06	38.89	43.25	31.25	45.72	50	38.71	37.5	29.41	42.43	26.67	48.28	26.67	35.3	38.89	35.3	46.67	36.37	30.31	55.17	46.67	57.15	44.45	48.65	31.25	41.38	46.67	32.01	38.47	34.79
UM01	42.43	55.56	44.45	38.47	41.38	42.43	36.85	45	29.27	44.45	25.65	33.33	45.72	33.33	52.63	48.65	35.3	36.37	52.95	47.37	45	36.85	41.18	32.43	54.06	24.25	29.41	41.03	35	34.15	27.78	30.31	29.41	34.49	33.33	44.45
HFU012	37.04	26.67	20	30	26.09	37.04	37.5	41.18	45.72	40	42.43	20	41.38	33.33	25	32.26	42.86	44.45	42.86	43.75	41.18	37.5	21.43	45.17	12.91	29.63	35.72	24.25	29.41	34.29	46.67	51.85	50	34.79	8.33	19.05
HFU018	34.49	37.5	25	36.37	24	13.79	29.41	33.33	43.25	31.25	22.86	18.75	38.71	50	47.06	42.43	40	41.38	33.33	47.06	38.89	35.3	33.33	48.49	42.43	34.49	40	40	38.89	32.43	31.25	55.17	40	32.01	30.77	34.79
HFU006	21.43	38.71	38.71	38.1	16.67	28.57	30.31	34.29	44.45	12.91	41.18	25.81	40	25.81	24.25	25	34.49	42.86	27.59	36.37	40	36.37	41.38	37.5	25	42.86	27.59	47.06	28.57	27.78	38.71	42.86	41.38	25	24	27.27
01-0024-07	54.55	40	40	40	22.23	36.37	37.04	41.38	40	40	28.57	32.01	50	32.01	29.63	38.47	34.79	54.55	43.48	29.63	27.59	29.63	34.79	30.77	30.77	36.37	34.79	28.57	20.69	33.33	16	27.27	34.79	33.33	31.58	50
01-0026-12	15.39	20.69	20.69	31.58	18.19	38.47	19.36	18.19	29.41	20.69	31.25	20.69	14.29	27.59	19.36	26.67	22.23	7.69	29.63	25.81	24.25	19.36	22.23	33.33	33.33	15.39	14.82	25	24.25	23.53	27.59	7.69	7.41	18.19	17.39	10

Table E.1 (continue): Similarity Scores between the DGGE profiles

01-0043-12																													
6349																													
HMU016																													
01-0024-12																													
01-0042-12																													
01-0023-12																													
HFU010																													
HMU006																													
HFU009																													
HMU010																													
01-0009-13																													
01-0034-12																													
HMU011																													
HMU007																													
HFU008																													
HMU004																													
01-0008-13																													
01-0050-12																													
HFU007																													
01-0015-13																													
HMU014																													
01-0049-12																													
HMU021																													
HFU013																													
HMU024																													
HFU024																													
HMU017																													
01-0033-12																													
HMU001																													
01-0004-06																													
HMU015																													
HFU017																													
HMU008																													
HFU005																													
HMU025																													
01-0002-05																													
01-0032-12																													
HFU014	100																												
HFU004	57.15	100																											
HMU002	68.97	85.72	100																										
HFU026	64.29	70.59	80.01	100																									
01-0017-13	64.29	58.83	68.57	70.59	100																								
HFU003	48.49	71.8	65	51.29	51.29	100																							
HFU021	53.33	55.56	48.65	44.45	61.11	53.66	100																						
HFU022	56.01	51.61	56.25	45.17	64.52	50	60.61	100																					
HMU020	56.01	45.17	43.75	51.61	58.07	33.33	48.49	78.57	100																				
HMU009	69.57	62.07	53.33	41.38	55.17	41.18	64.52	76.93	69.23	100																			
HFU015	56.01	51.61	43.75	32.26	51.61	38.89	48.49	71.43	64.29	69.23	100																		
HMU005	63.64	57.15	55.17	42.86	64.29	36.37	46.67	72	64	69.57	80.01	100																	
HFU001	38.1	44.45	35.72	37.04	37.04	37.5	41.38	66.67	58.33	63.64	58.33	57.15	100																
HFU019	50	38.47	44.45	38.47	46.15	32.26	35.72	78.26	69.57	66.67	69.57	70	84.21	100															
01-0014-10	28.57	29.41	22.86	17.65	41.18	25.65	33.33	32.26	38.71	27.59	58.07	28.57	29.63	23.08	100														
01-0022-12	57.15	37.04	42.86	51.85	59.26	31.25	48.28	58.33	50	45.46	58.33	57.15	50	52.63	59.26	100													
HMU003	52.17	41.38	46.67	34.49	41.38	47.06	51.61	76.93	53.85	58.33	53.85	60.87	63.64	57.15	34.49	63.64	100												
HMU023	17.39	20.69	33.33	20.69	20.69	23.53	32.26	38.47	46.15	25	46.15	17.39	45.46	28.57	62.07	36.37	66.67	100											
01-0019-10	32.26	37.84	31.58	27.03	32.43	23.81	35.9	41.18	52.95	31.25	52.95	38.71	33.33	34.49	48.65	33.33	43.75	62.5	100										
01-0025-12	36.37	35.72	27.59	21.43	35.72	30.31	26.67	40	40	43.48	40	36.37	28.57	30	42.86	38.1	34.79	34.79	58.07	100									
01-0004-11	25	20	32.26	26.67	46.67	40	37.5	59.26	51.85	48	29.63	33.33	52.17	45.46	40	34.79	48	40	48.49	50	100								
UM01	42.86	41.18	45.72	58.83	41.18	35.9	27.78	19.36	32.26	27.59	19.36	21.43	22.23	23.08	29.41	29.63	27.59	6.9	37.84	21.43	13.33	100							
HFU012	36.37	64.29	48.28	35.72	35.72	48.49	33.33	48	32.01	43.48	48	54.55	57.15	50	21.43	19.05	43.48	34.79	38.71	18.19	25	21.43	100						
HFU018	50	26.67	25.81	33.33	40	22.86	43.75	37.04	51.85	40	44.45	50	43.48	54.55	20	26.09	40	16	30.31	16.67	7.69	33.33	50	100					
HFU006	26.09	27.59	26.67	13.79	41.38	23.53	38.71	53.85	46.15	41.67	53.85	43.48	36.37	47.62	48.28	36.37	41.67	33.33	56.25	17.39	32.01	20.69	34.79	40	100				
01-0024-07	35.3	34.79	25	26.09	34.79	21.43	24	50	50	44.45	20	23.53	25	40	26.09	37.5	33.33	11.11	30.77	35.3	42.11	34.79	11.77	21.05	22.23	100			
01-0026-12	28.57	14.82	21.43	29.63	14.82	25	13.79	0	25	0	16.67	0	0	0	51.85	20	9.09	27.27	26.67	19.05	26.09	14.82	9.53	8.7	9.09	12.5	100		

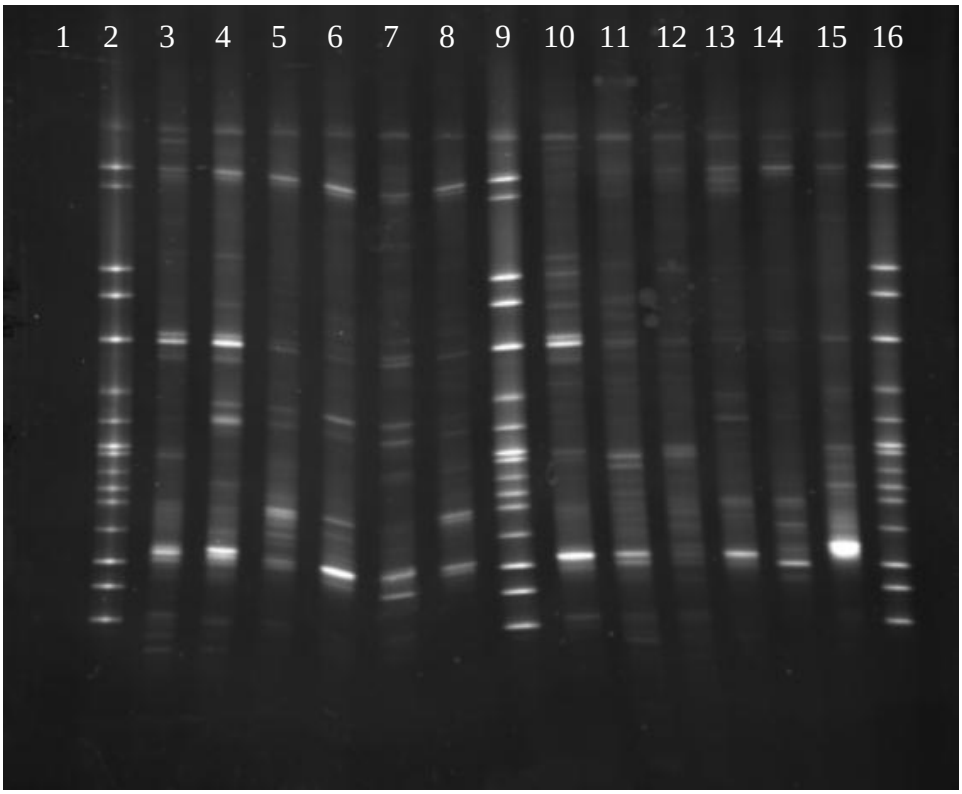
Figure E.1: DGGE Fingerprints
E.1.



Lanes	Samples
1	*Reference Marker*
2	*Reference Marker*
3	HMU003
4	HMU007
5	HMU009
6	HMU010
7	Saliva01
8	Saliva02

Lanes	Samples
9	*Reference Marker*
10	HFU001
11	HFU006
12	HFU012
13	HFU018
14	HFU019
15	HFU019
16	*Reference Marker*

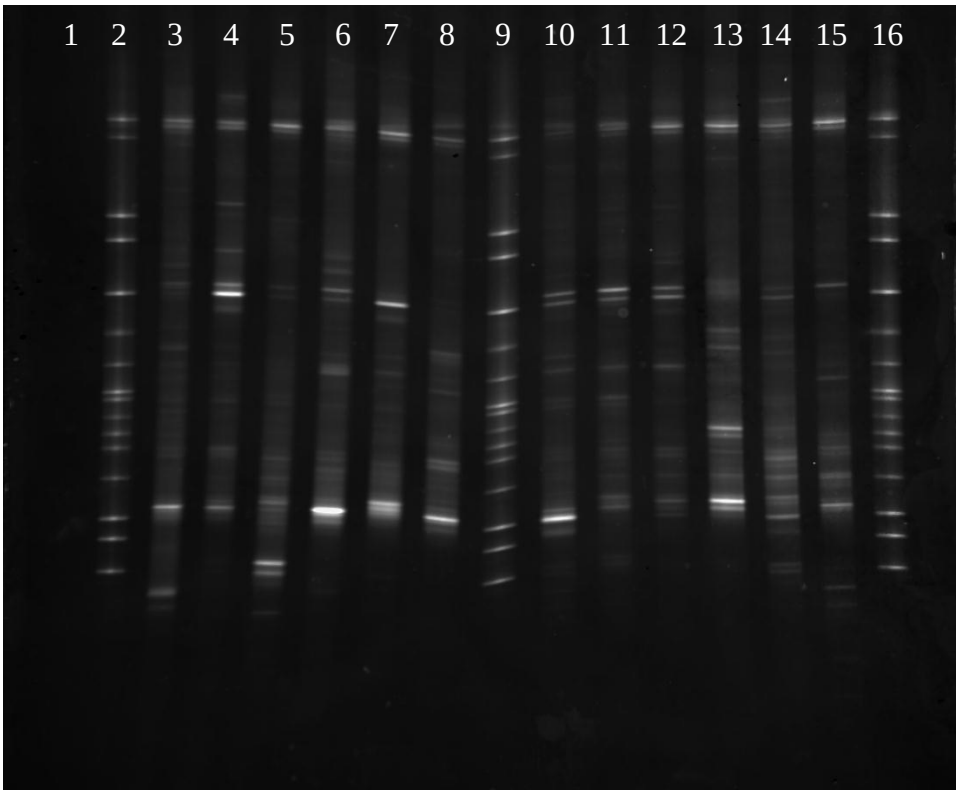
E.2.



Lanes	Samples
1	Negative control
2	*Reference Marker*
3	HMU004
4	HMU016
5	Saliva03
6	HMU020
7	HMU021
8	HMU023

Lanes	Samples
9	*Reference Marker*
10	HFU008
11	HFU009
12	HFU010
13	HFU015
14	HFU022
15	HFU027 (X)
16	*Reference Marker*

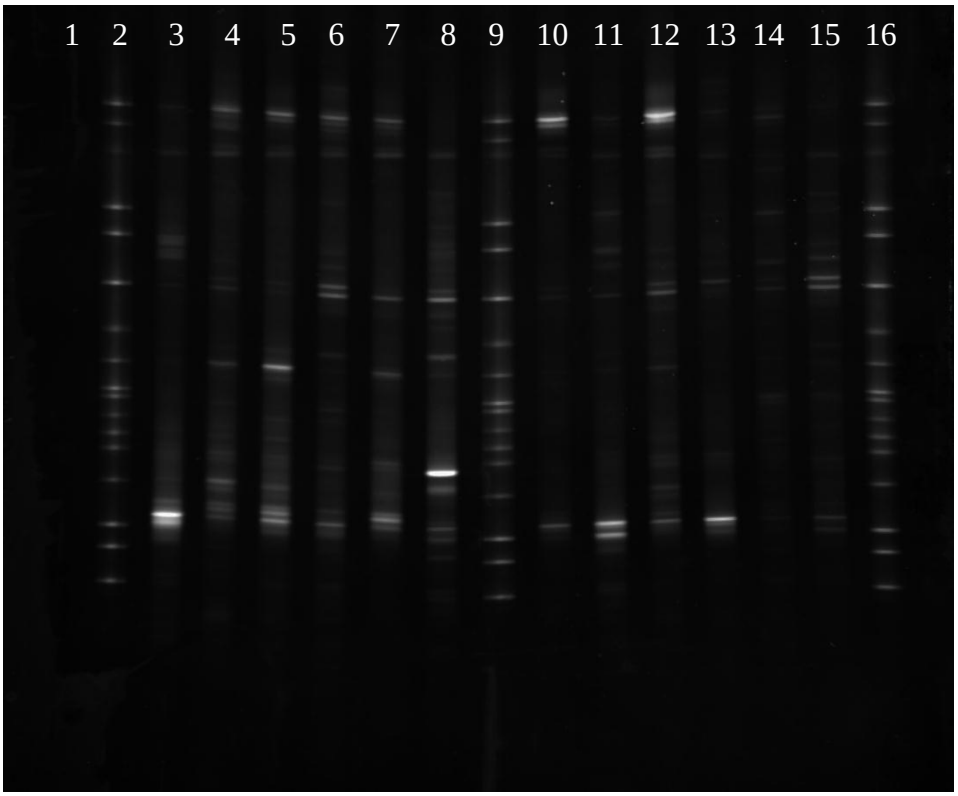
E.3.



Lanes	Samples
1	Negative control
2	*Reference Marker*
3	HMU001
4	HMU002
5	HMU006
6	HMU014
7	HMU015
8	Saliva01

Lanes	Samples
9	*Reference Marker*
10	Saliva02
11	HFU003
12	HFU004
13	HFU021
14	Saliva03
15	HFU024
16	*Reference Marker*

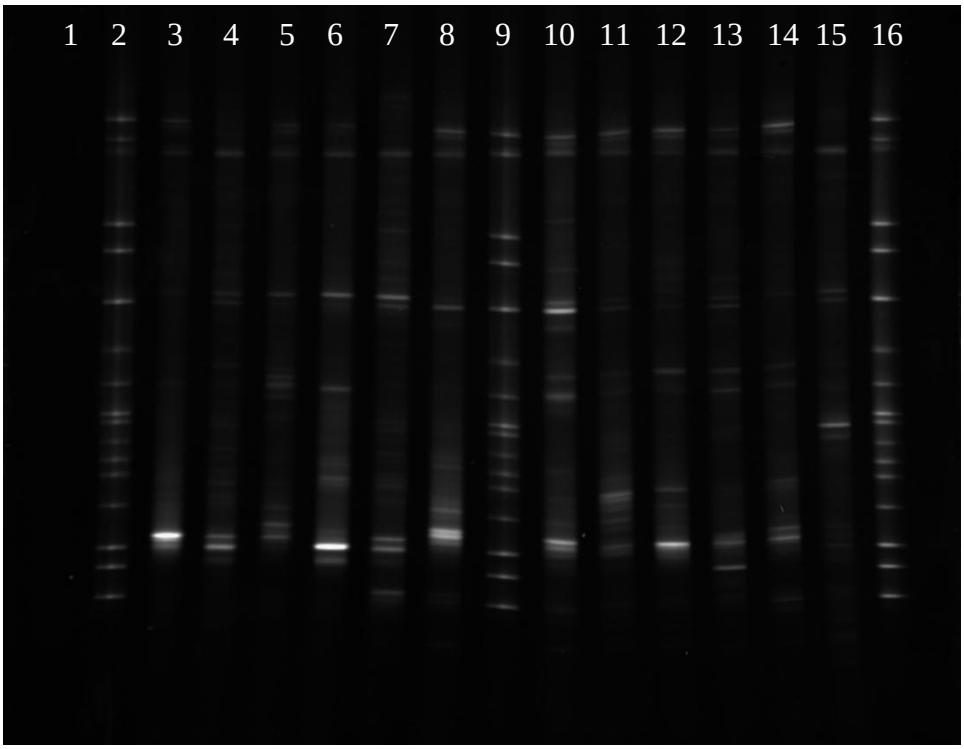
E.4.



Lanes	Samples
1	Negative control
2	*Reference Marker*
3	HMU011
4	Saliva04
5	HMU017
6	HMU024
7	HMU025
8	6349

Lanes	Samples
9	*Reference Marker*
10	HFU005
11	HFU007
12	HFU013
13	HFU014
14	Saliva05
15	HFU026
16	*Reference Marker*

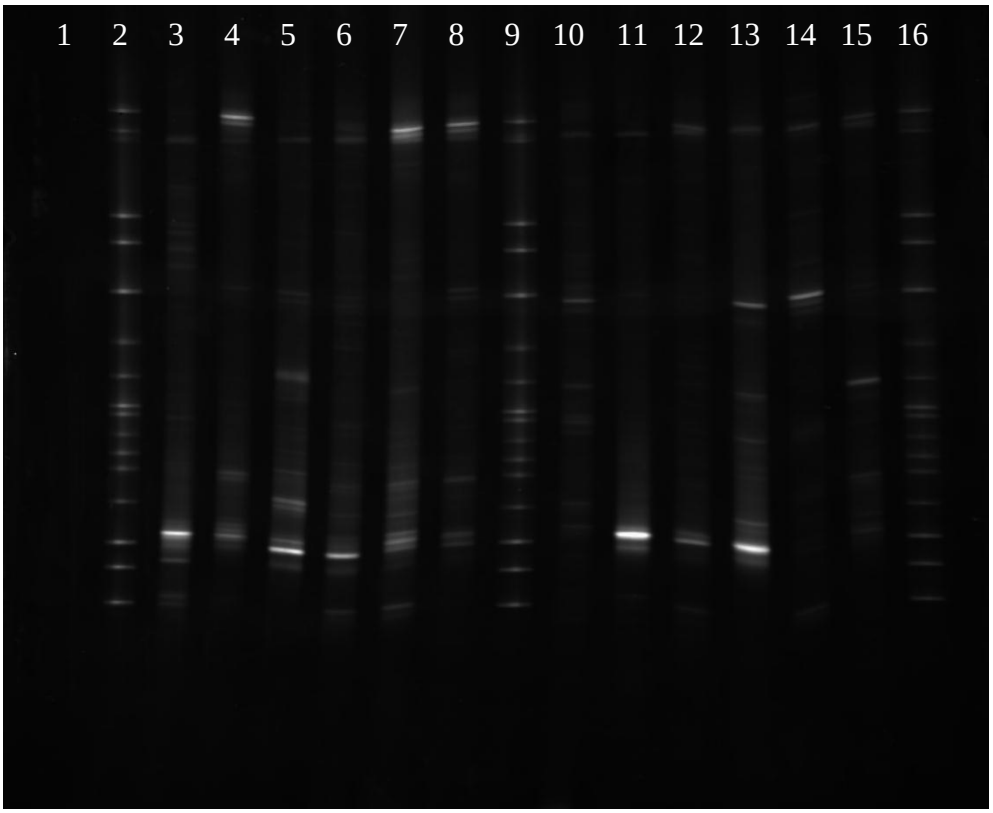
E.5.



Lanes	Samples
1	Negative control
2	*Reference Marker*
3	Saliva06
4	HMU007
5	HMU008
6	HMU009
7	HMU010
8	Saliva07

Lanes	Samples
9	*Reference Marker*
10	HMU016
11	Saliva08
12	HMU020
13	HMU021
14	HFU017
15	Saliva09
16	*Reference Marker*

E.6.



Lanes	Samples
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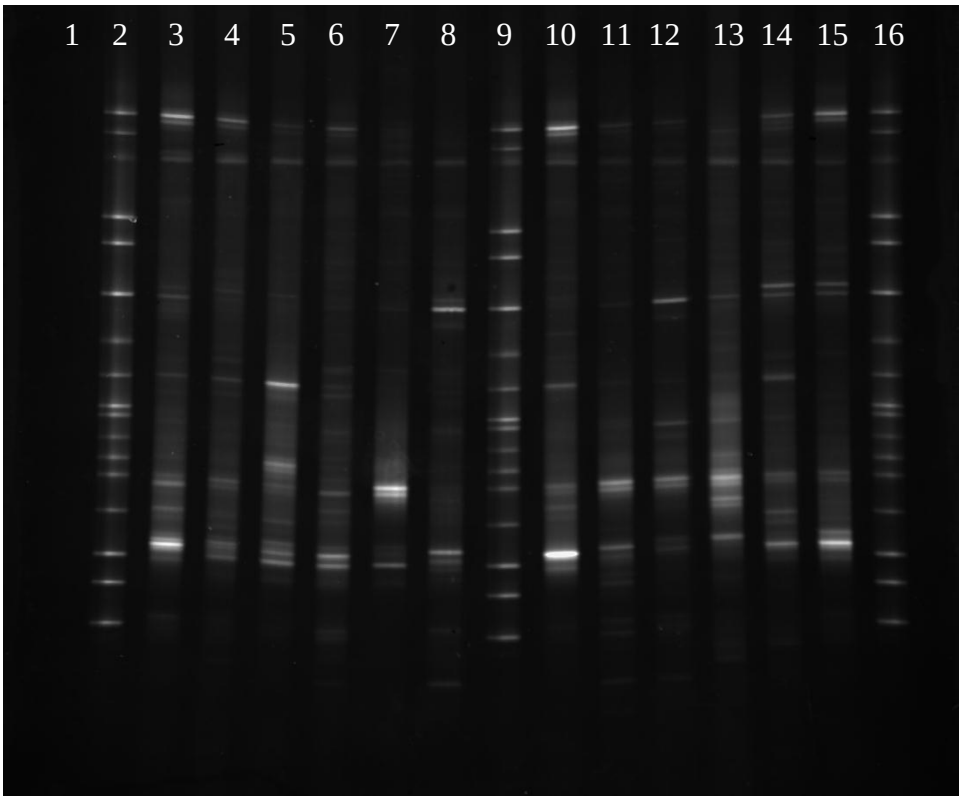
2	*Reference Marker*
3	UM01/OS
4	01-0002-05
5	01-0004-11
6	01-0014-10
7	01-0019-10
8	01-0022-12

Lanes	Samples
9	*Reference Marker*
10	01-0023-12
11	01-0024-07
12	01-0024-12
13	01-0025-12
14	01-0026-12
15	01-0032-12
16	*Reference Marker*

E.7.



Lanes	Samples
1	Negative control
2	*Reference Marker*
3	01-0004-06
4	01-0033-12
5	01-0034-12
6	Saliva10
7	01-0042-12
8	01-0043-12

Lanes	Samples
9	*Reference Marker*
10	01-0049-12
11	01-0050-12
12	01-0008-13
13	01-0009-13
14	01-0015-13
15	01-0017-13
16	*Reference Marker*

APPENDIX F
Sequence Alignment of all Reported OTUs

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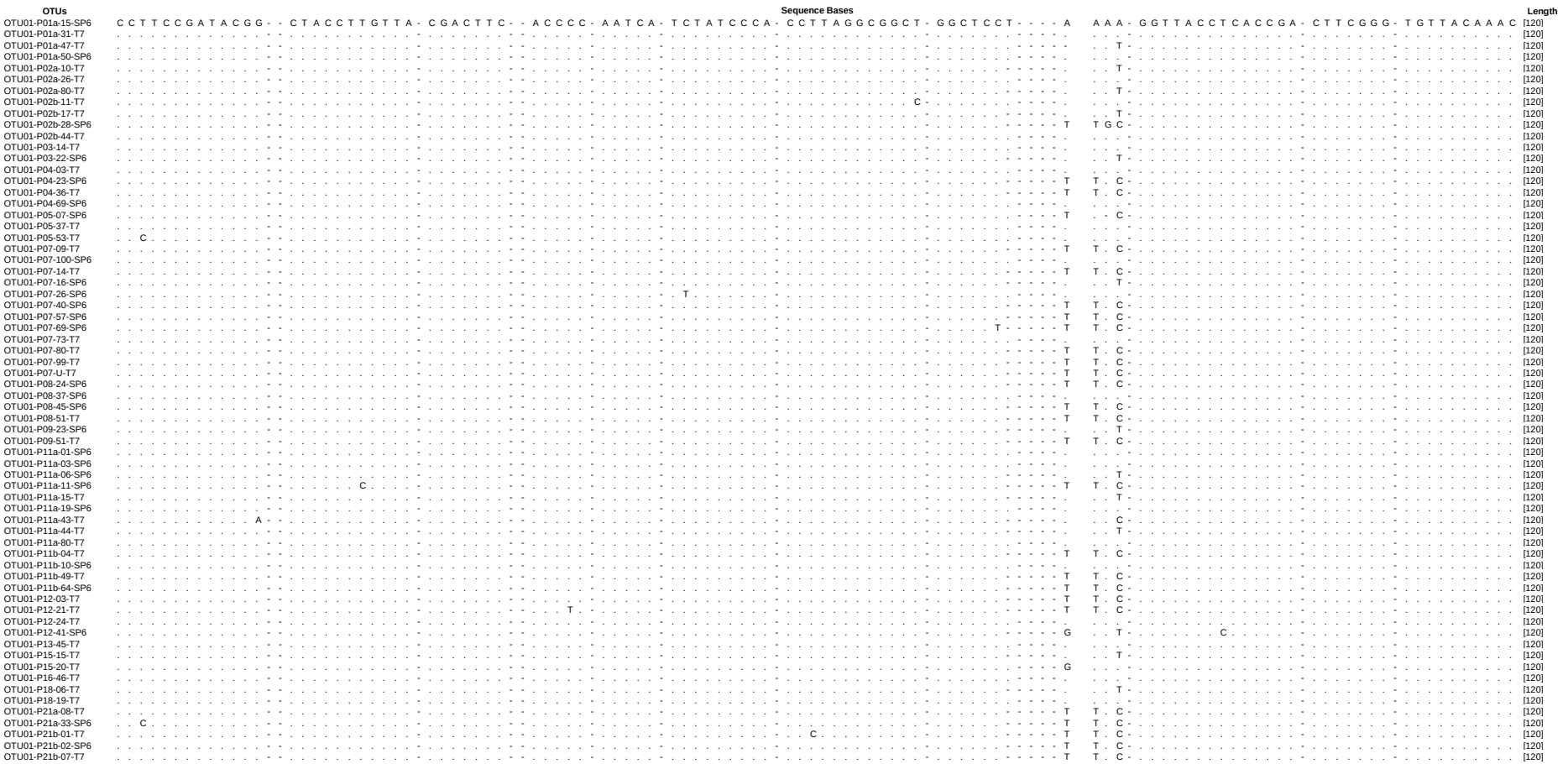

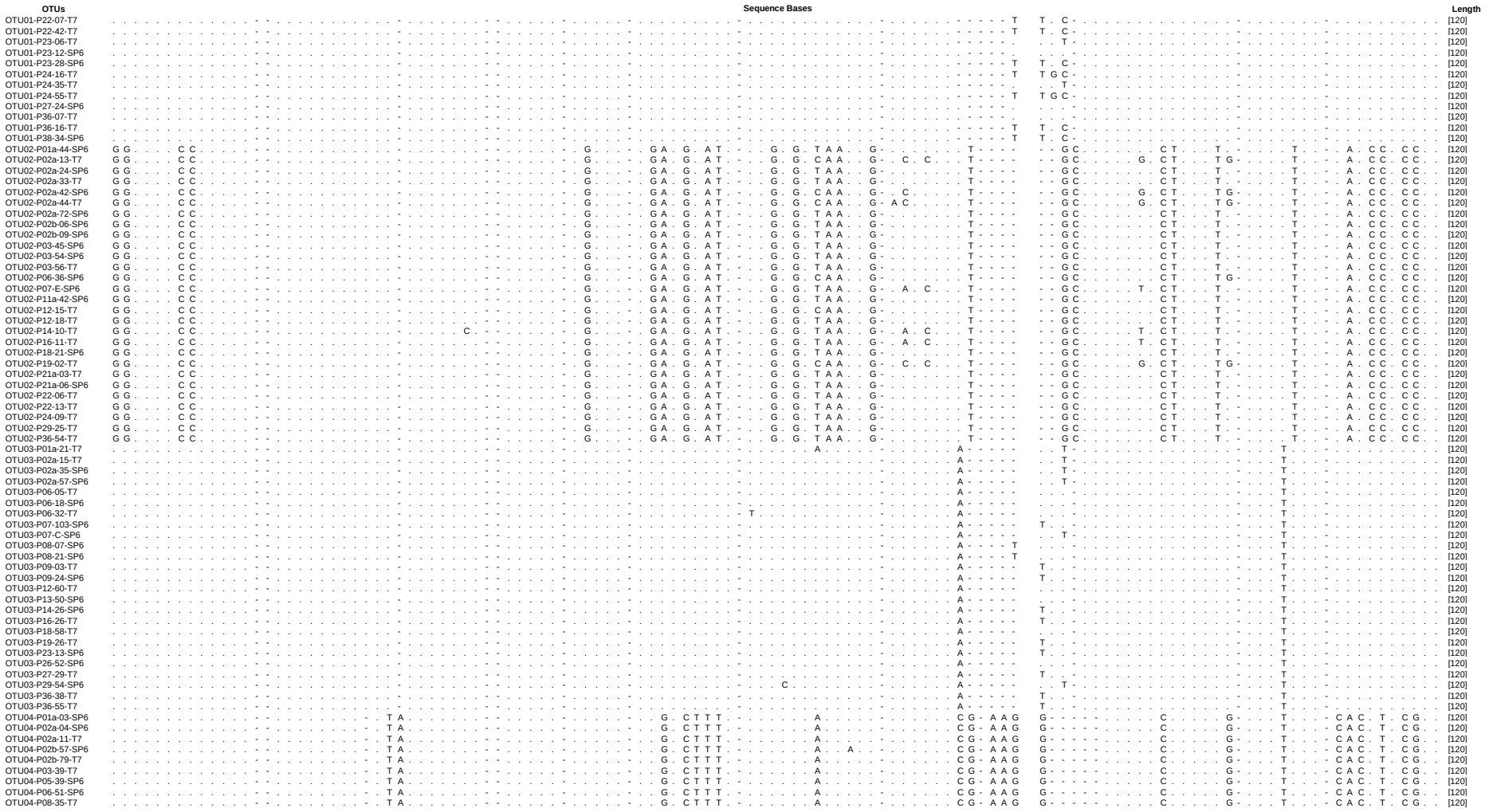
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OTU01-P23-06-T7 [120]
OTU01-P23-12-SP6 [120]
OTU01-P23-28-SP6 [120]
OTU01-P24-16-T7 [120]
OTU01-P24-35-T7 [120]
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OTU01-P36-07-T7 [120]
OTU01-P36-16-T7 [120]
OTU01-P38-34-SP6 [120]
OTU02-P01a-44-SP6 [120]
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OTU02-P02a-24-SP6 [120]
OTU02-P02a-33-T7 [120]
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OTU02-P07-E-SP6 [120]
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OTU02-P29-25-T7 [120]
OTU02-P36-54-T7 [120]
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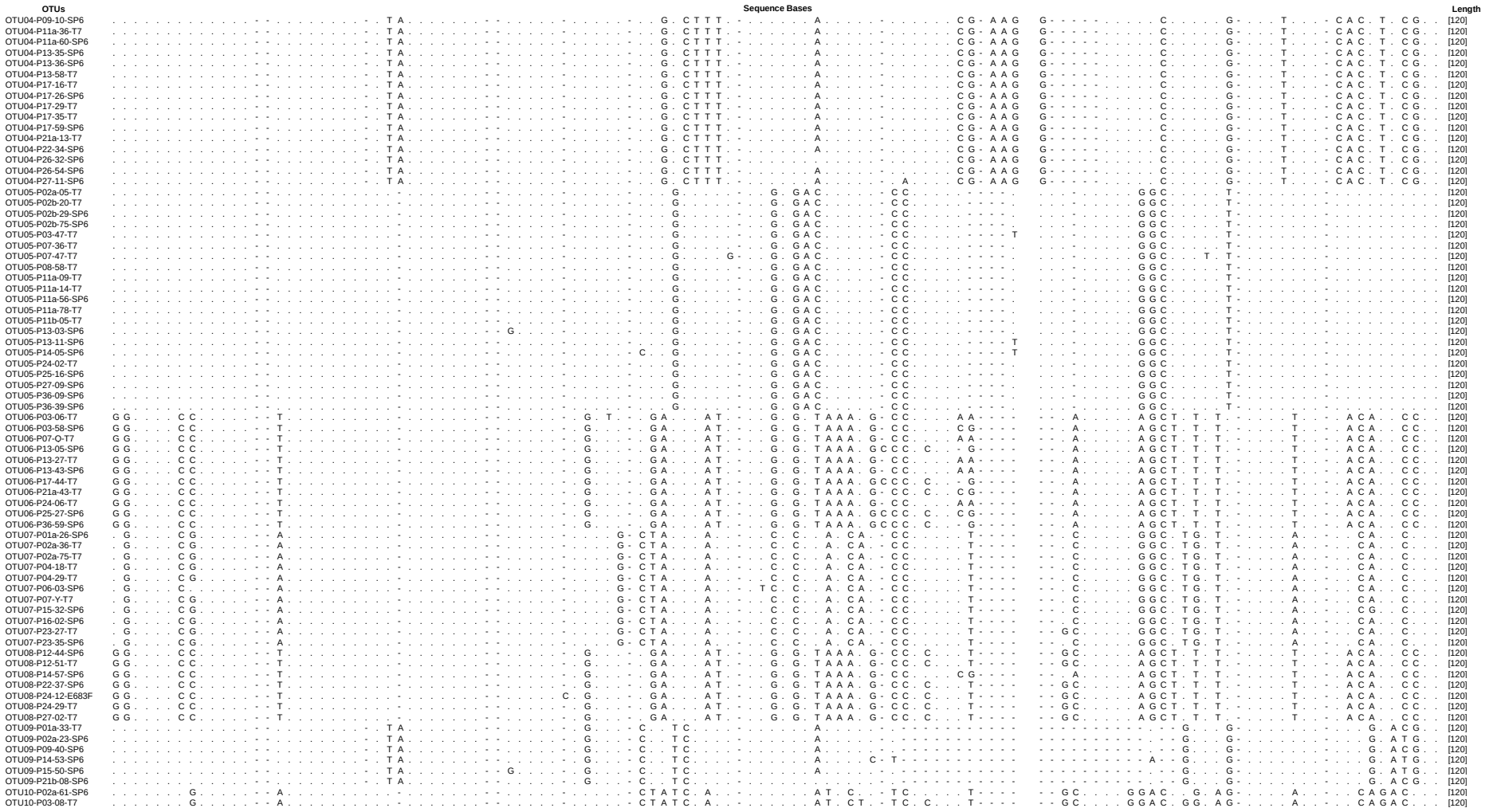
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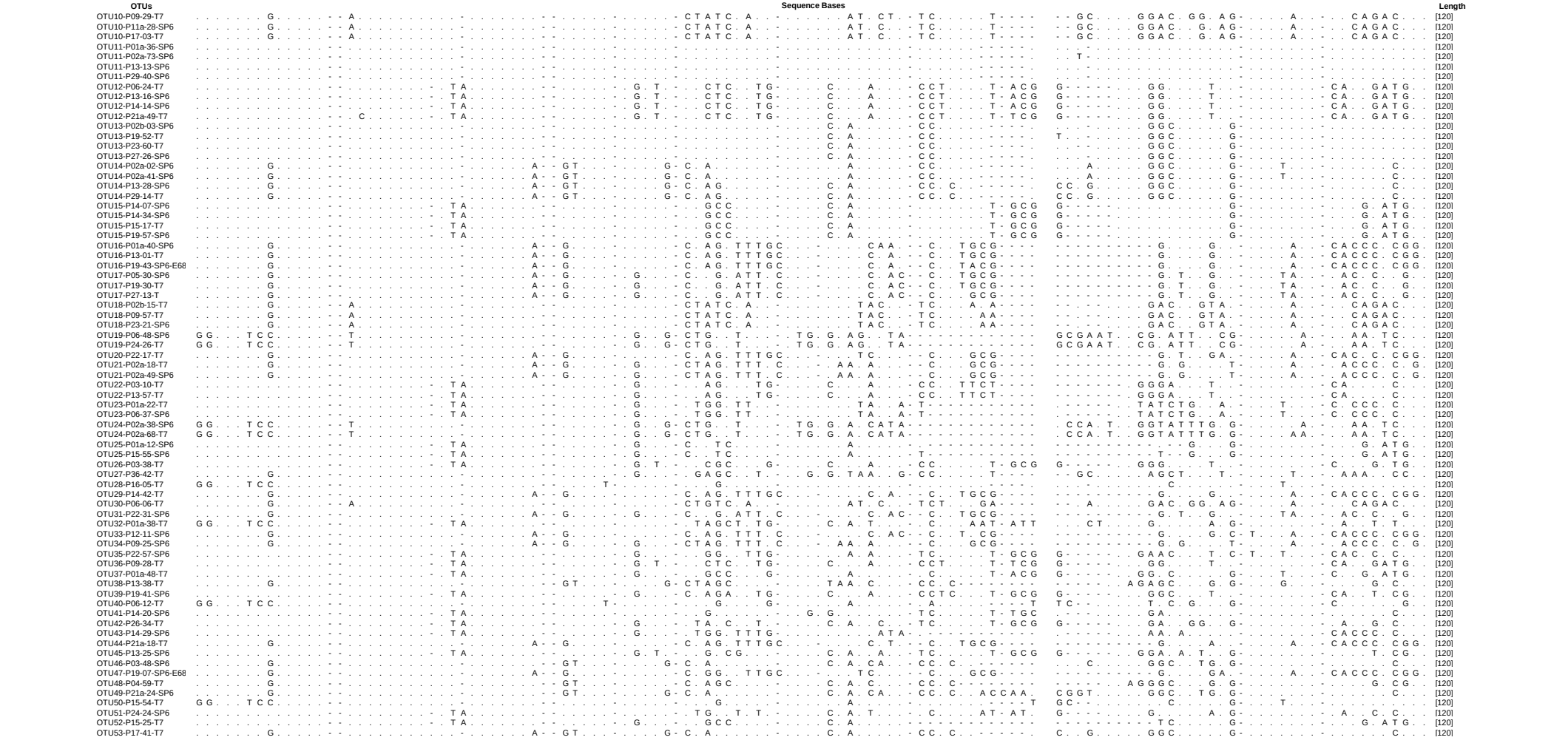
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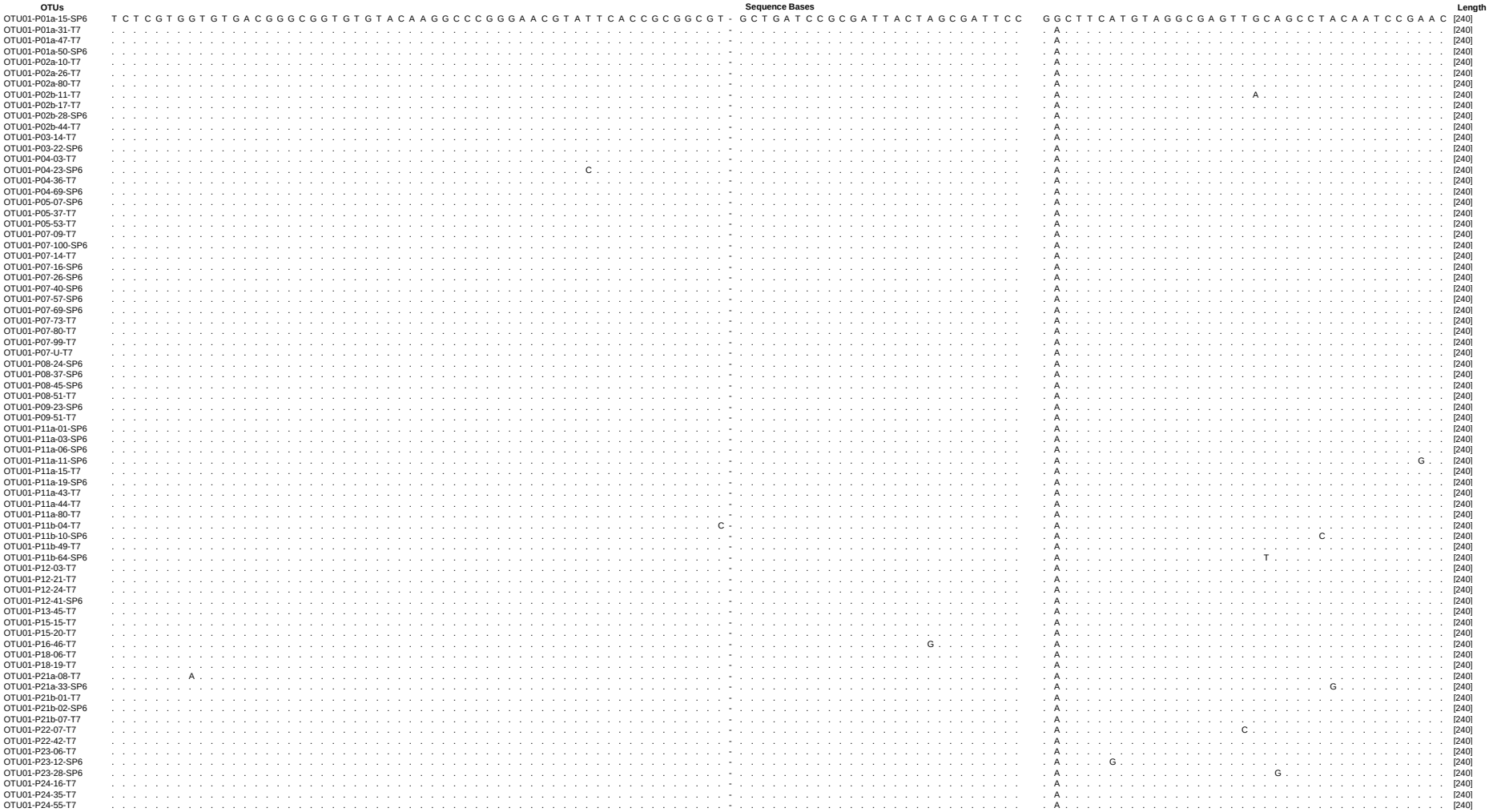
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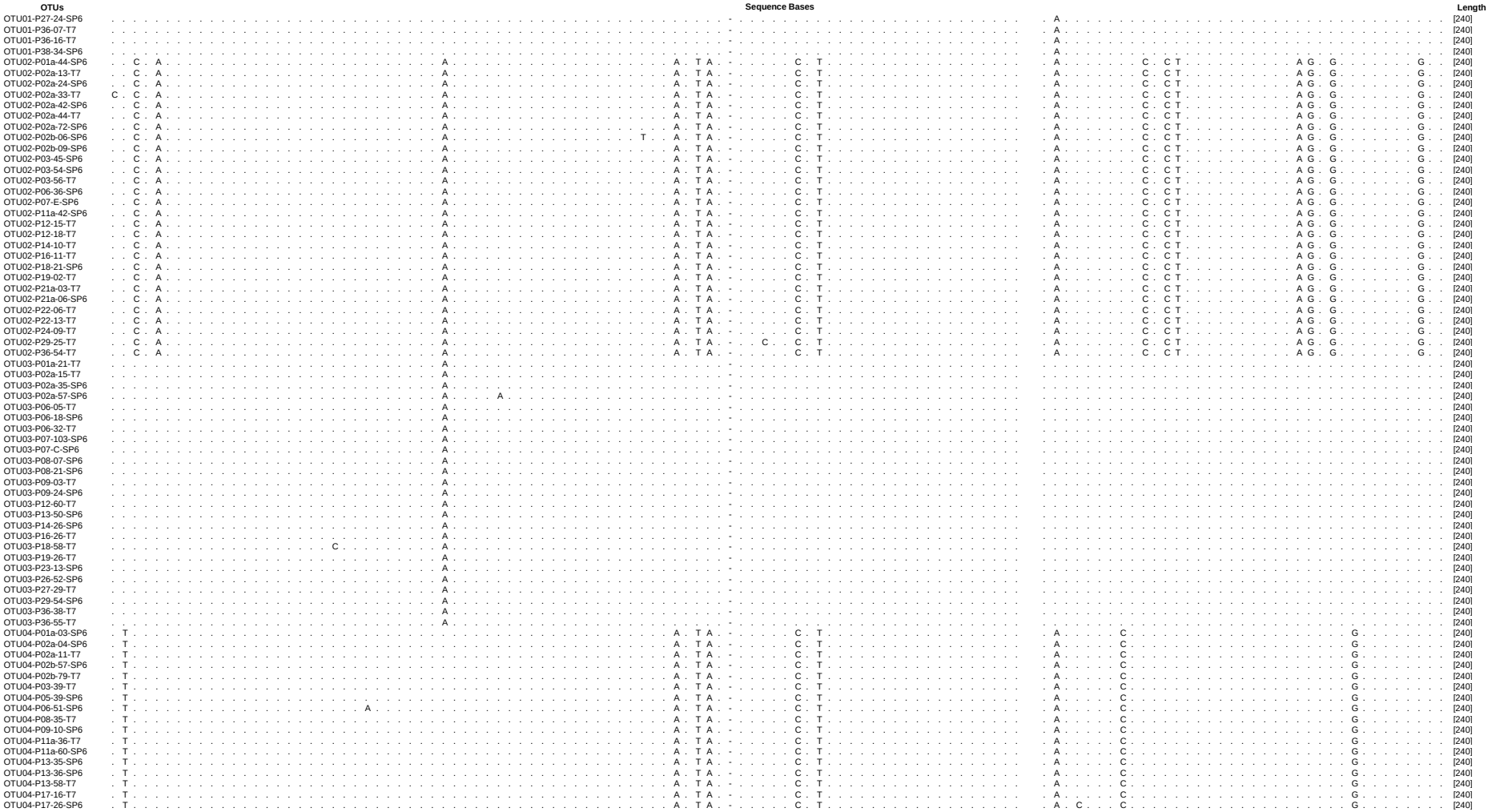
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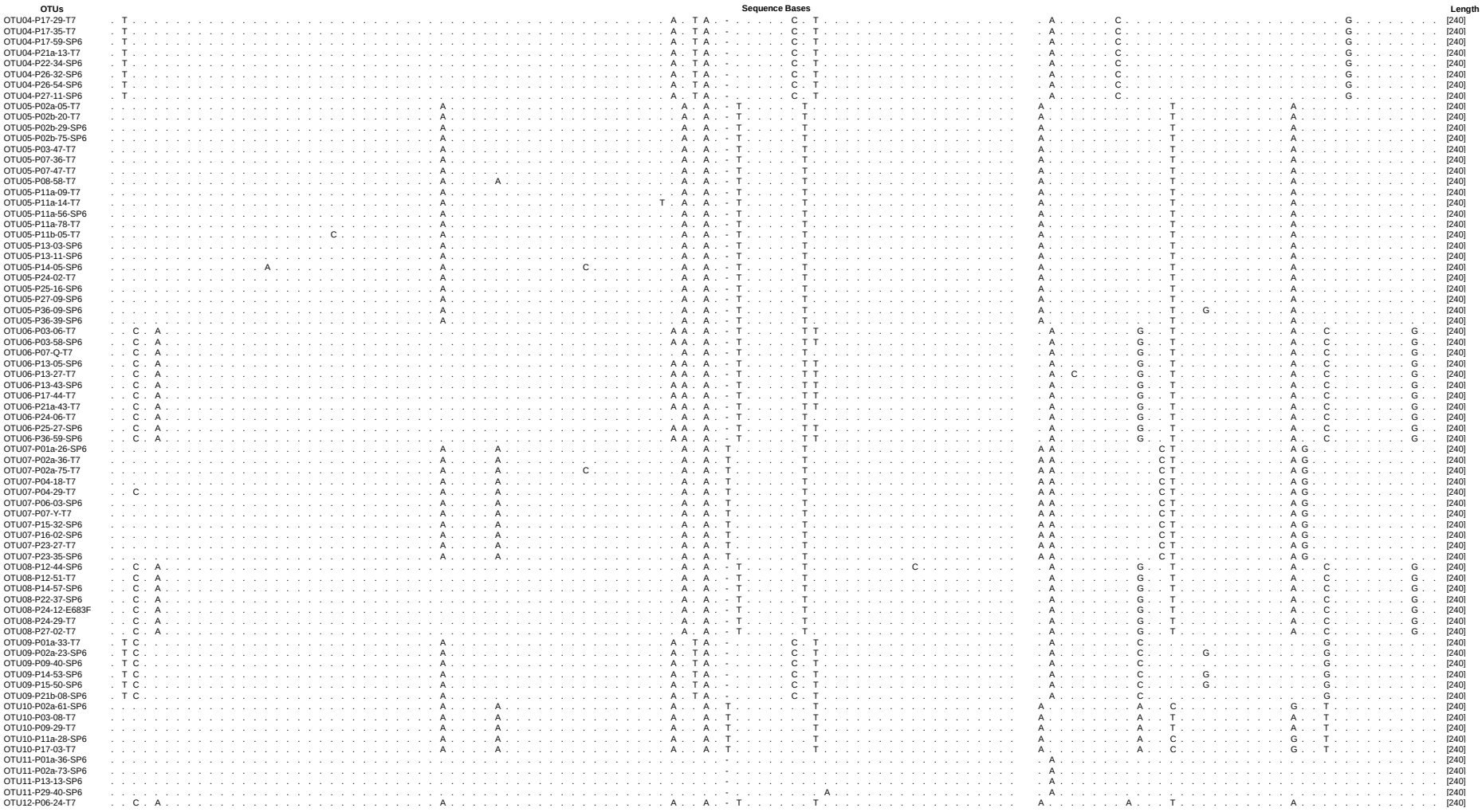
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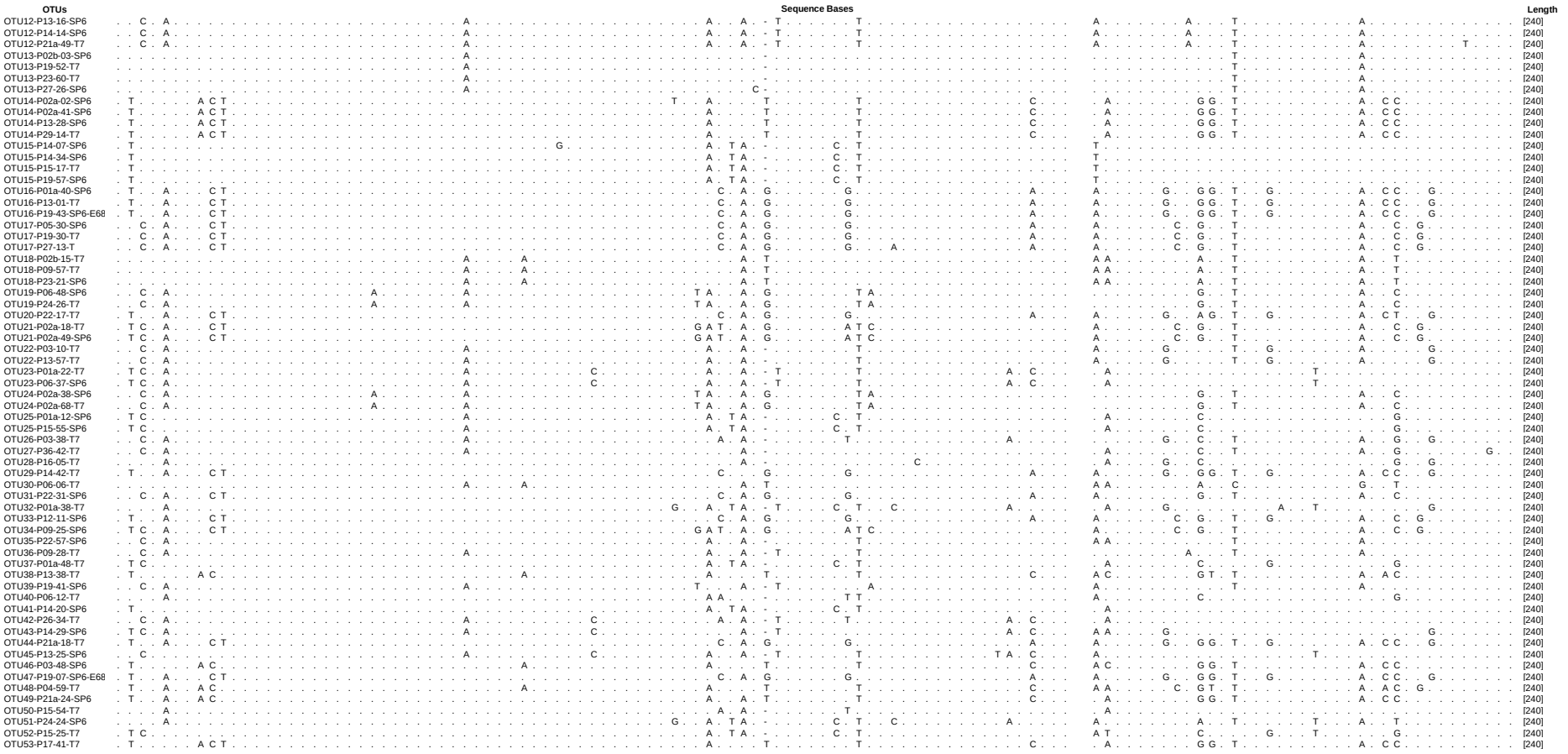
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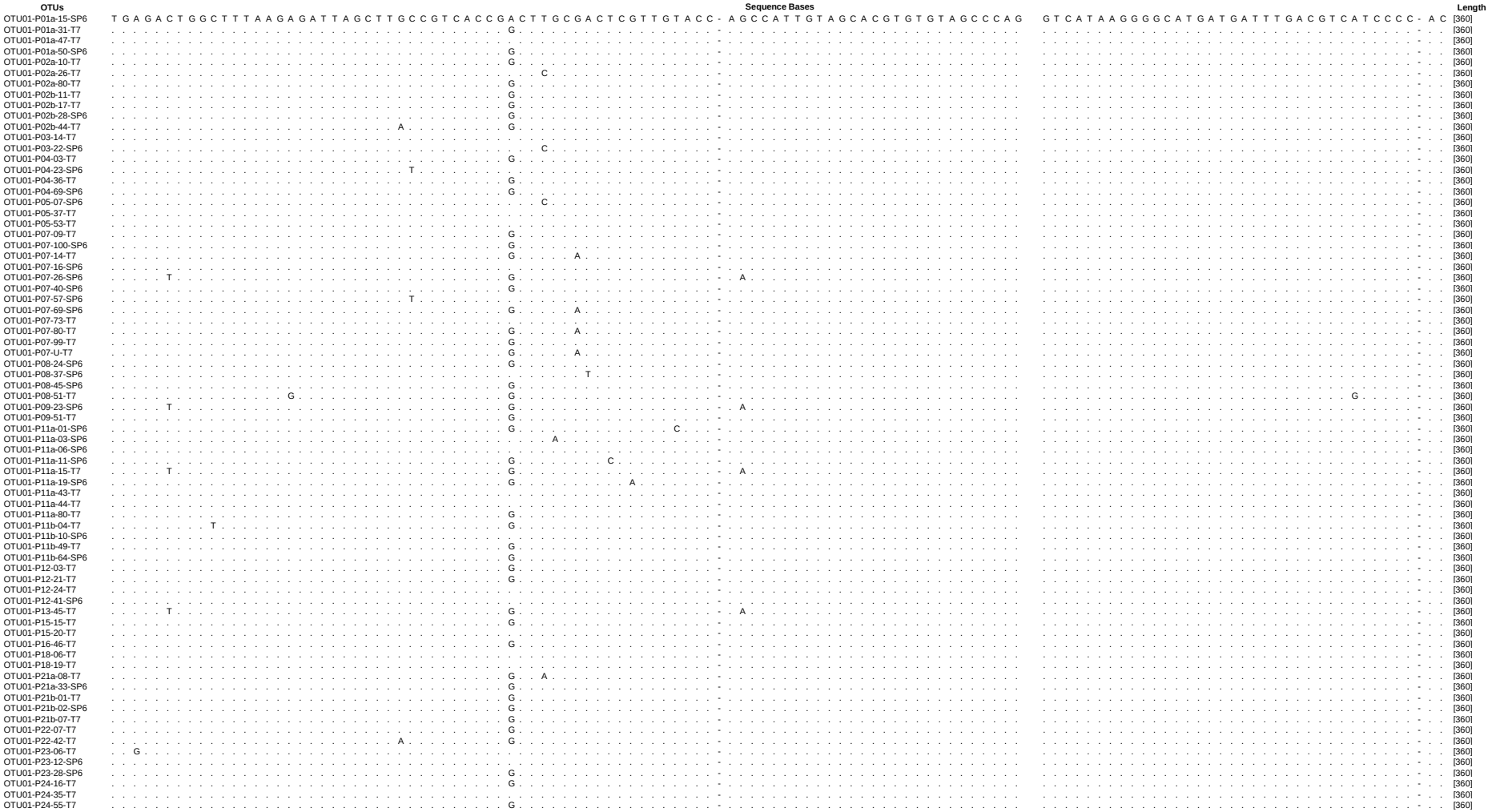
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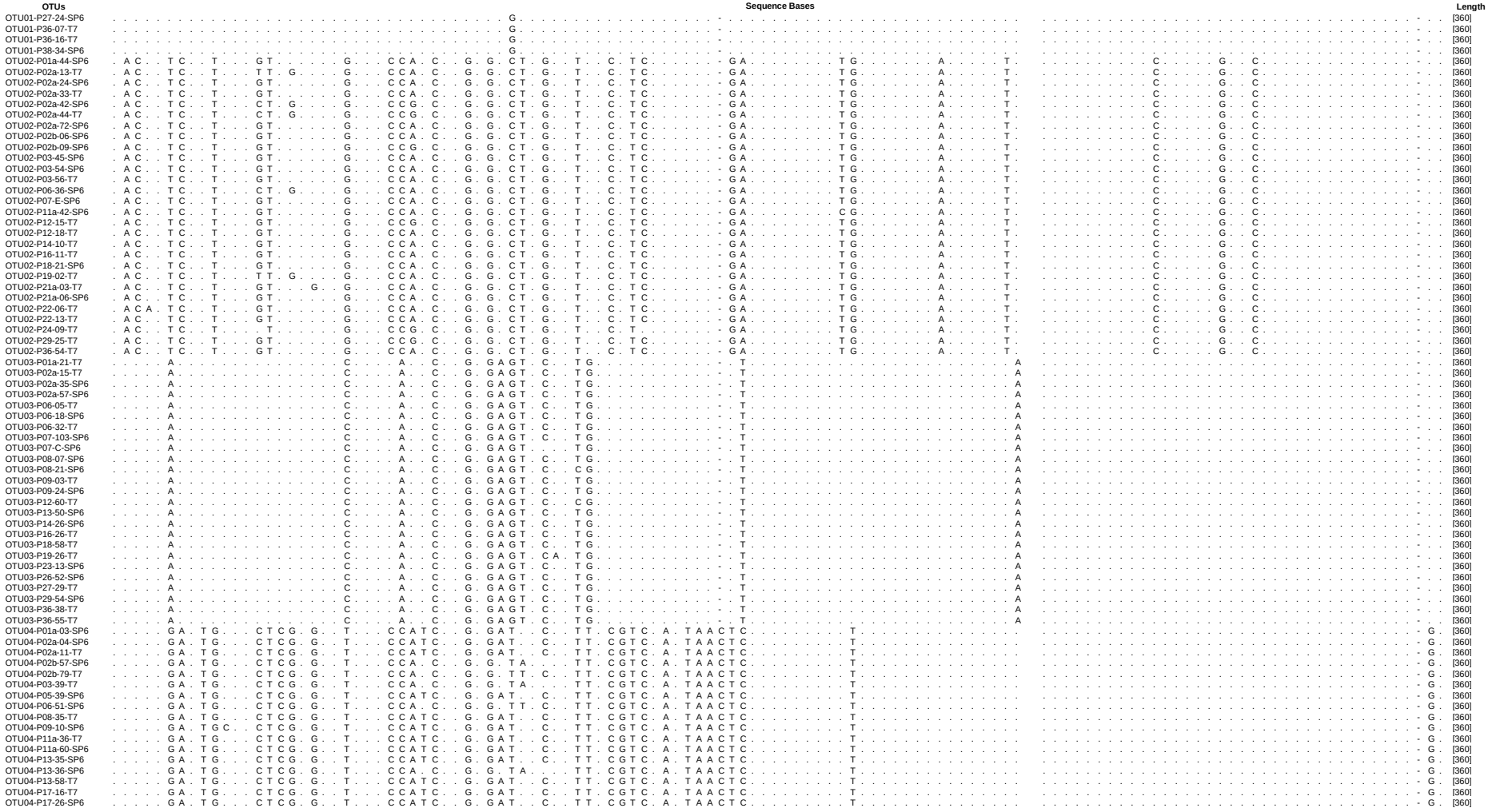
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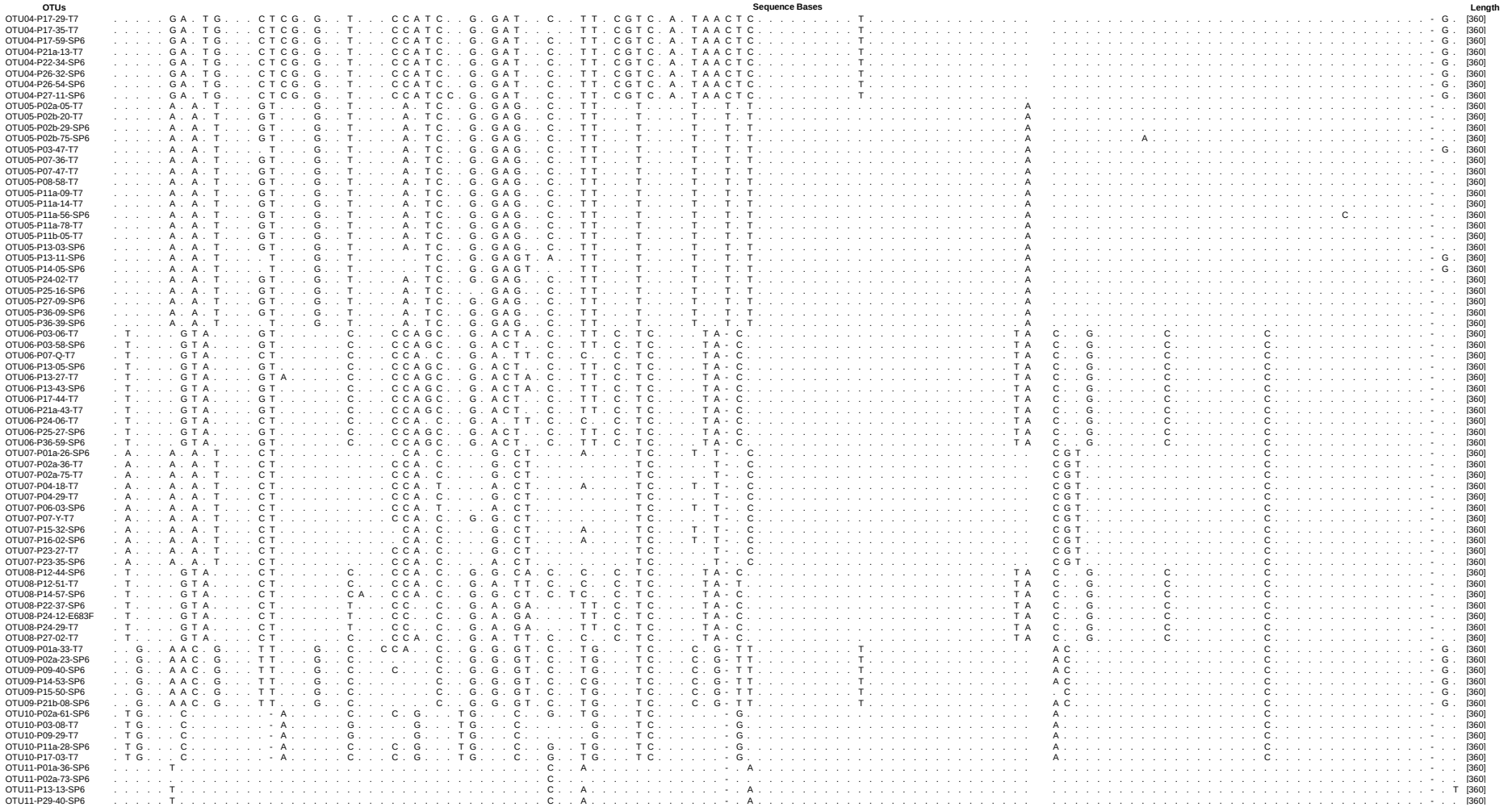
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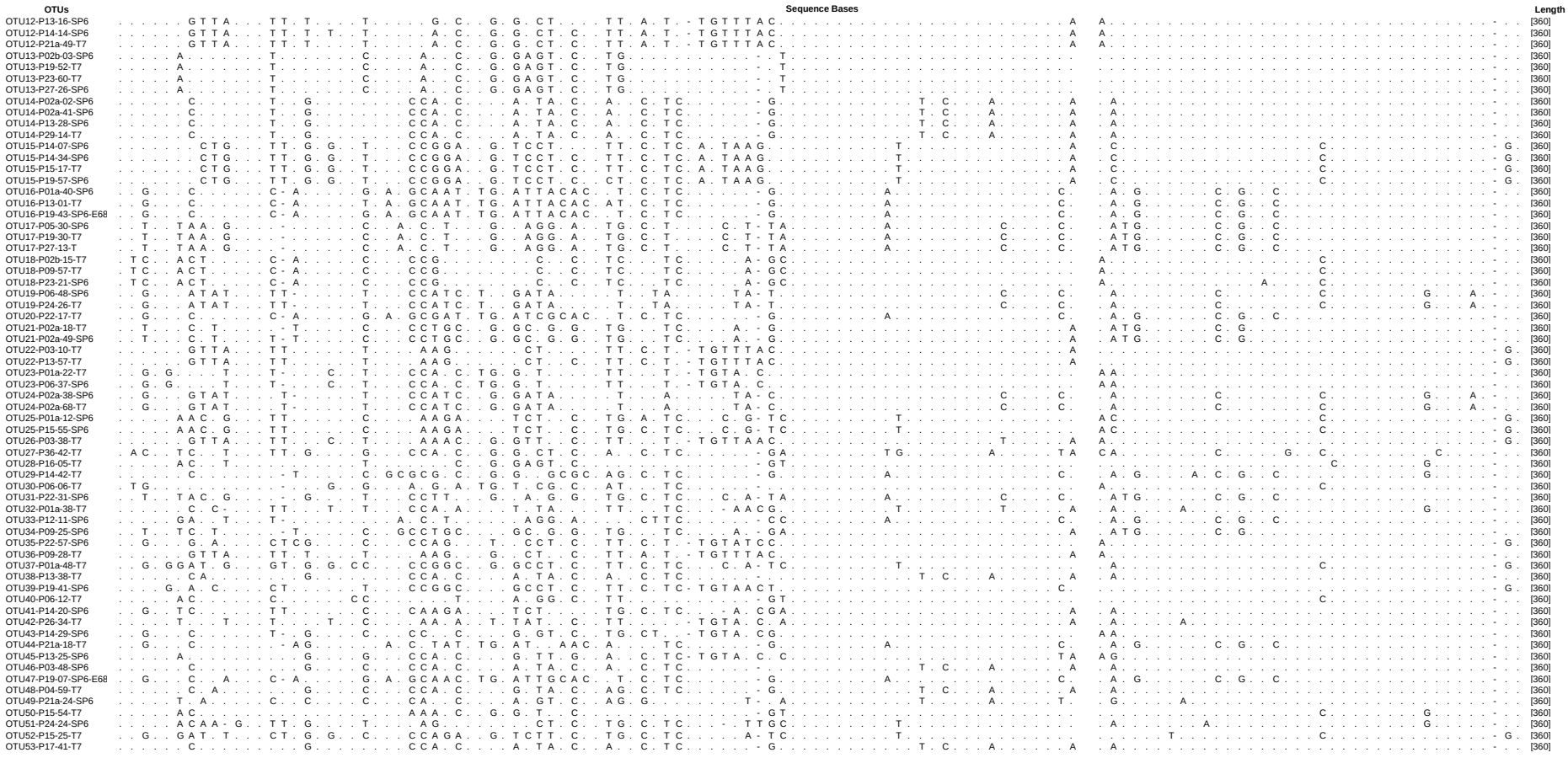
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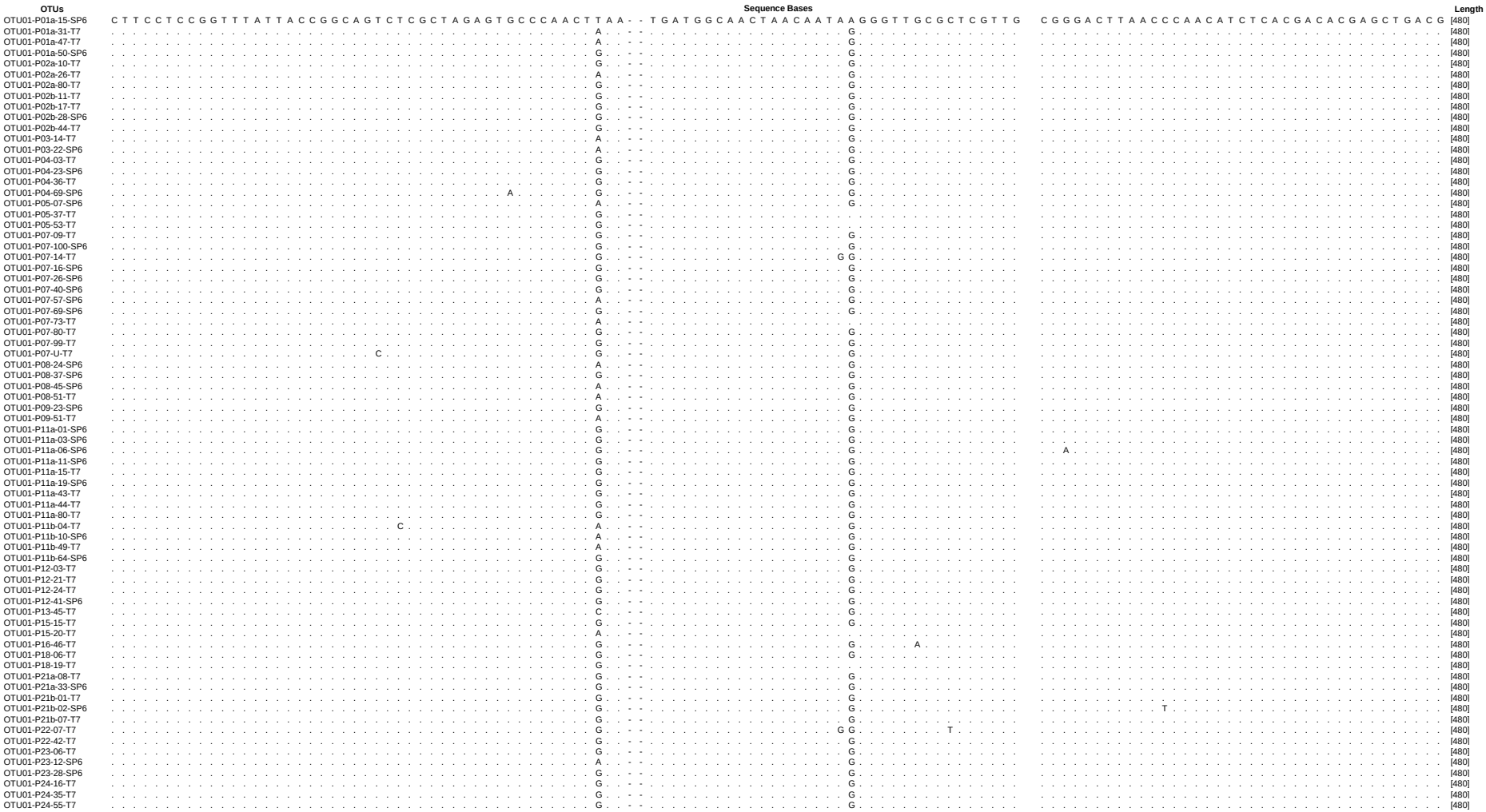
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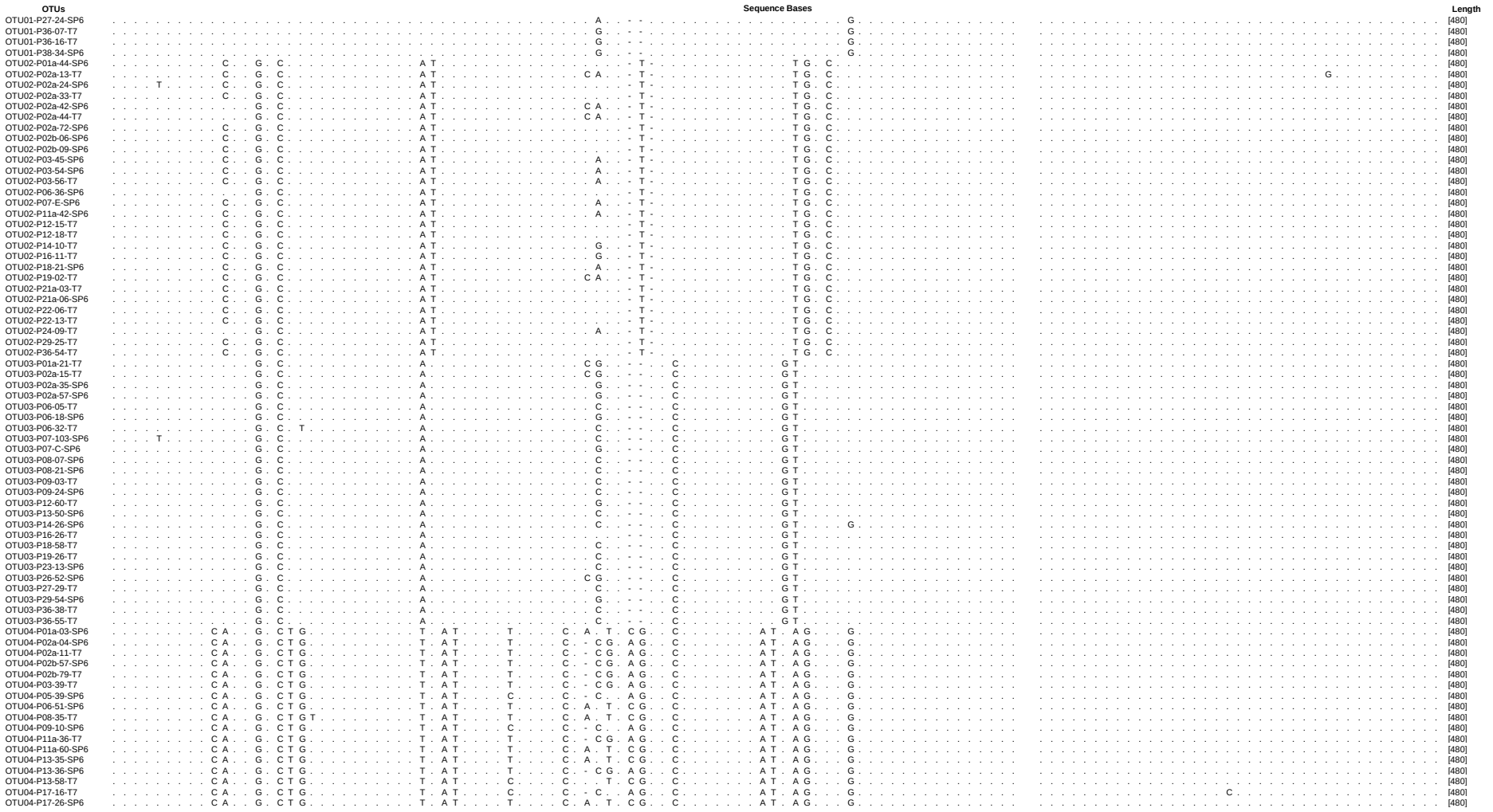
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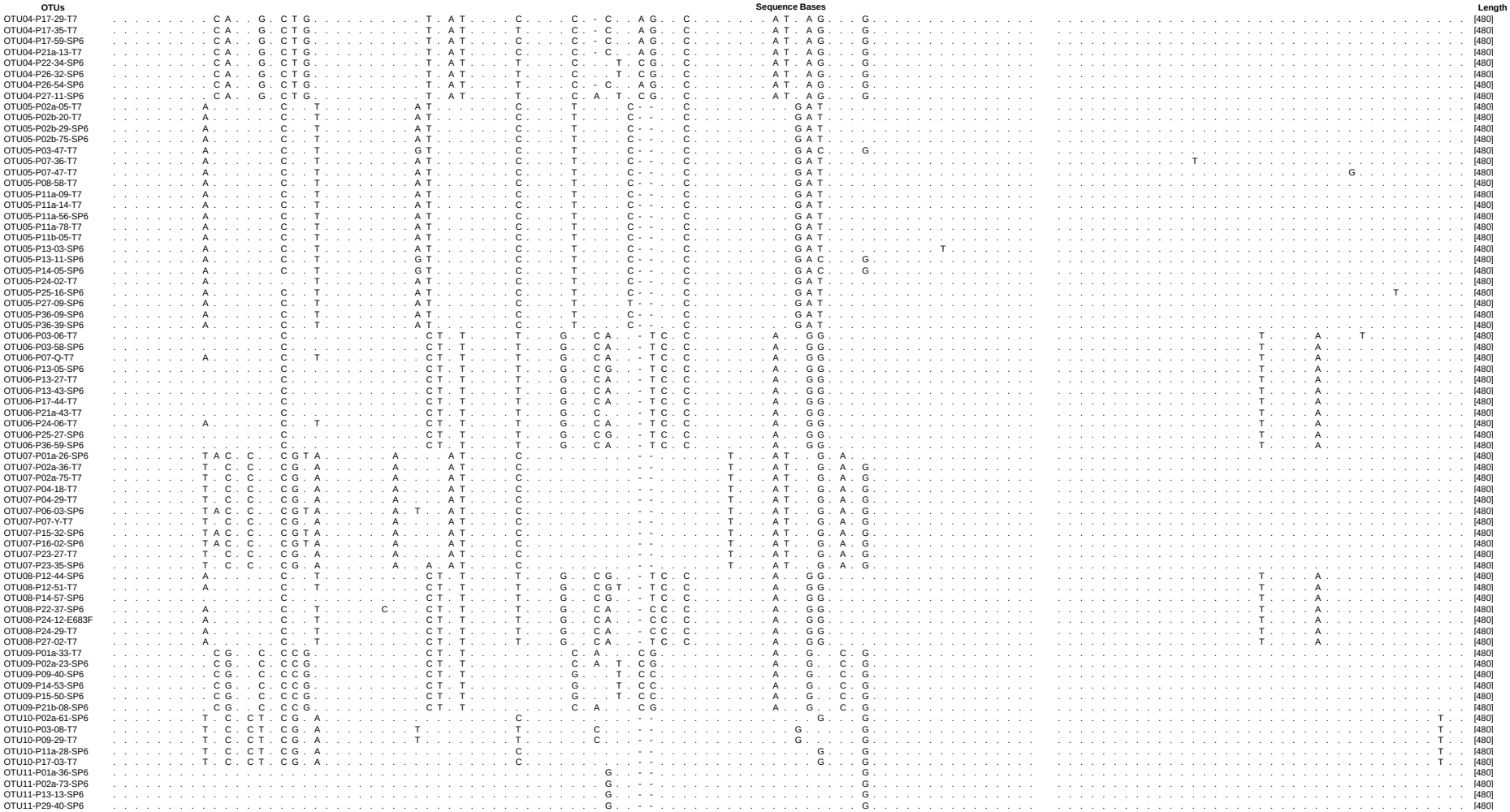
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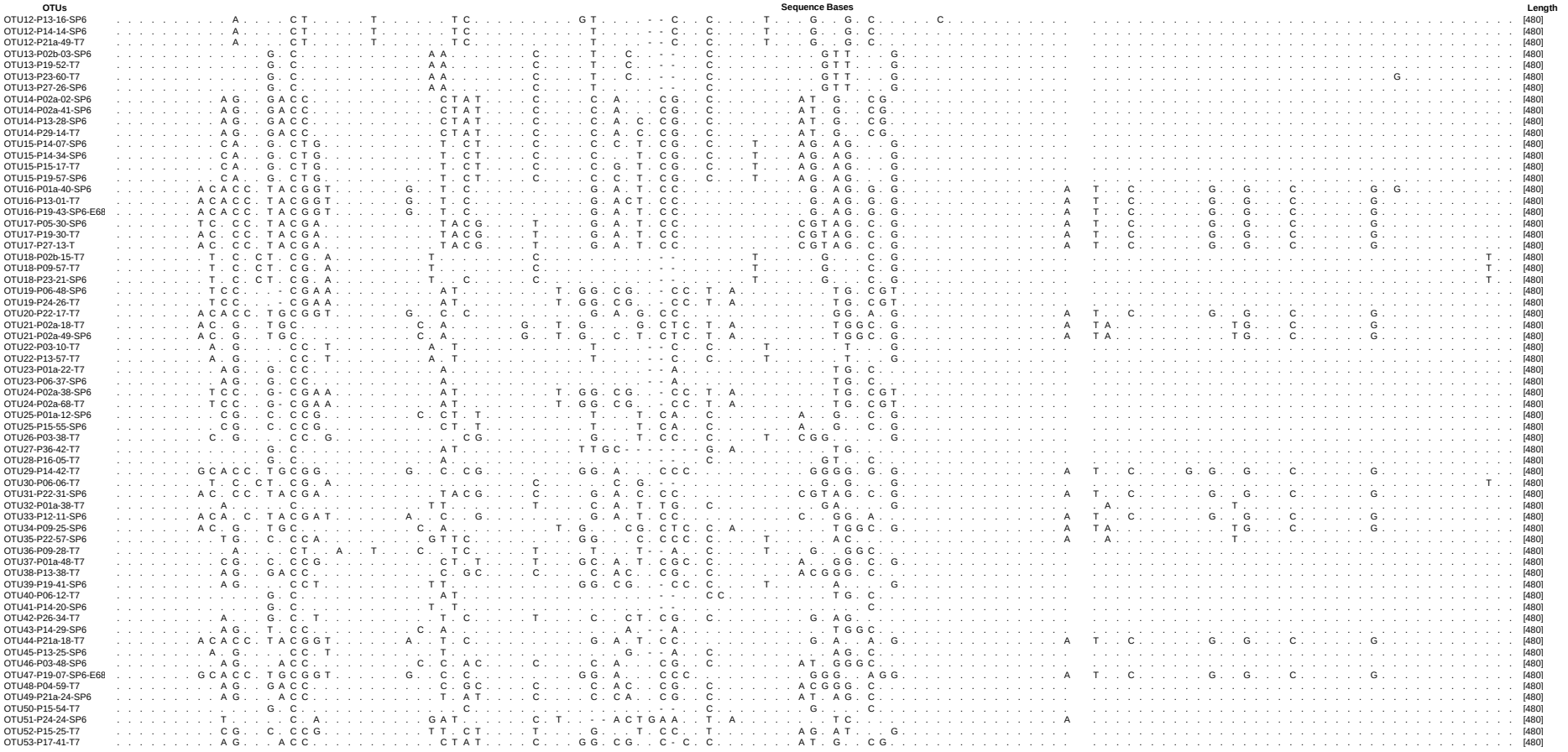
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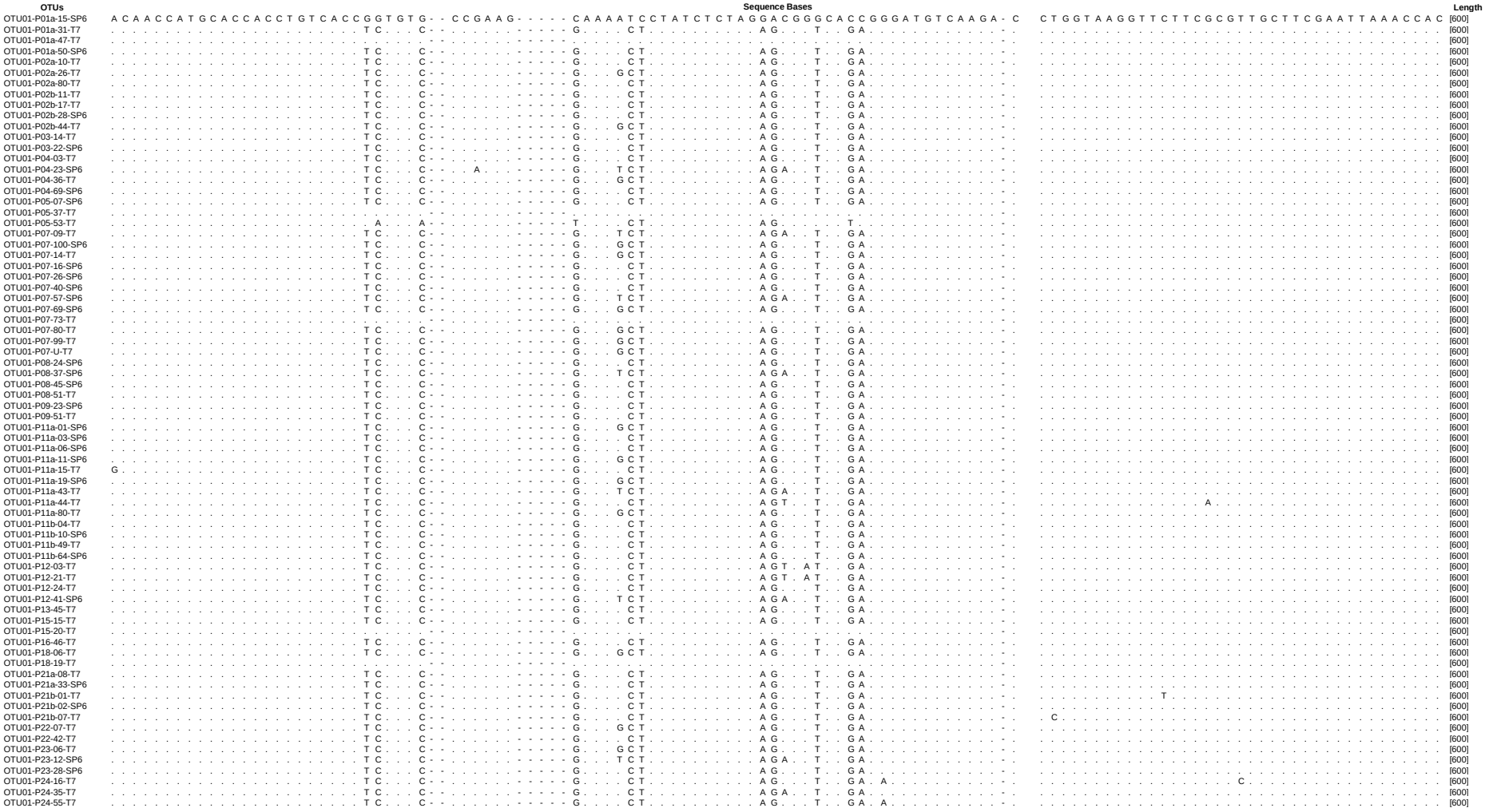
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OTU01-P07-57-SP6 T C C - G T C T A G A T G A [600]
OTU01-P07-69-SP6 T C C - G G C T A G T G A [600]
OTU01-P07-73-T7 [600]
OTU01-P07-80-T7 T C C - G G C T A G T G A [600]
OTU01-P07-99-T7 T C C - G G C T A G T G A [600]
OTU01-P07-U-T7 T C C - G G C T A G T G A [600]
OTU01-P08-24-SP6 T C C - G C T A G T G A [600]
OTU01-P08-37-SP6 T C C - G T C T A G A T G A [600]
OTU01-P08-45-SP6 T C C - G C T A G T G A [600]
OTU01-P08-51-T7 T C C - G C T A G T G A [600]
OTU01-P09-23-SP6 T C C - G C T A G T G A [600]
OTU01-P09-51-T7 T C C - G C T A G T G A [600]
OTU01-P11a-01-SP6 T C C - G G C T A G T G A [600]
OTU01-P11a-03-SP6 T C C - G C T A G T G A [600]
OTU01-P11a-06-SP6 T C C - G C T A G T G A [600]
OTU01-P11a-11-SP6 T C C - G G C T A G T G A [600]
OTU01-P11a-15-T7 G T C C - G C T A G T G A [600]
OTU01-P11a-19-SP6 T C C - G G C T A G T G A [600]
OTU01-P11a-43-T7 T C C - G T C T A G A T G A [600]
OTU01-P11a-44-T7 T C C - G C T A G T T G A A [600]
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OTU01-P12-03-T7 T C C - G C T A G T A T G A [600]
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OTU01-P12-24-T7 T C C - G C T A G T G A [600]
OTU01-P12-41-SP6 T C C - G T C T A G A T G A [600]
OTU01-P13-45-T7 T C C - G C T A G T G A [600]
OTU01-P15-15-T7 T C C - G C T A G T G A [600]
OTU01-P15-20-T7 [600]
OTU01-P16-46-T7 T C C - G C T A G T G A [600]
OTU01-P18-06-T7 T C C - G G C T A G T G A [600]
OTU01-P18-19-T7 [600]
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OTU01-P21a-33-SP6 T C C - G C T A G T G A [600]
OTU01-P21b-01-T7 T C C - G C T A G T G A T [600]
OTU01-P21b-02-SP6 T C C - G C T A G T G A [600]
OTU01-P21b-07-T7 T C C - G C T A G T G A C [600]
OTU01-P22-07-T7 T C C - G G C T A G T G A [600]
OTU01-P22-42-T7 T C C - G C T A G T G A [600]
OTU01-P23-06-T7 T C C - G G C T A G T G A [600]
OTU01-P23-12-SP6 T C C - G T C T A G A T G A [600]
OTU01-P23-28-SP6 T C C - G C T A G T G A [600]
OTU01-P24-16-T7 T C C - G C T A G T G A A C [600]
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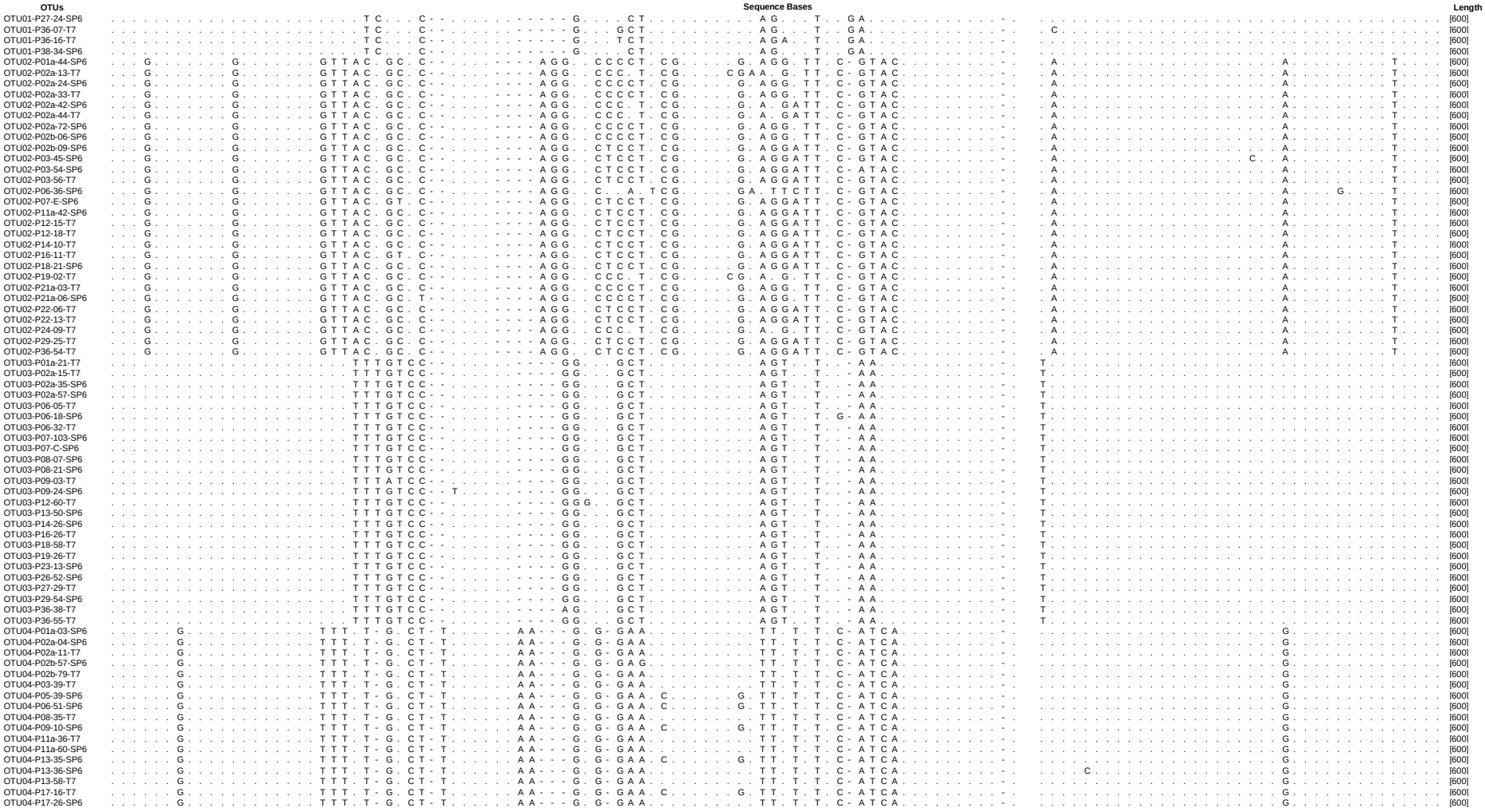
OTUs
Sequence Bases
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OTU01-P38-34-SP6 [600]
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OTU02-P02a-13-T7 [600]
OTU02-P02a-24-SP6 [600]
OTU02-P02a-33-T7 [600]
OTU02-P02a-42-SP6 [600]
OTU02-P02a-44-T7 [600]
OTU02-P02a-72-SP6 [600]
OTU02-P02b-06-SP6 [600]
OTU02-P02b-09-SP6 [600]
OTU02-P03-45-SP6 [600]
OTU02-P03-54-SP6 [600]
OTU02-P03-56-T7 [600]
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OTU02-P07-E-SP6 [600]
OTU02-P11a-42-SP6 [600]
OTU02-P12-15-T7 [600]
OTU02-P12-18-T7 [600]
OTU02-P14-10-T7 [600]
OTU02-P16-11-T7 [600]
OTU02-P18-21-SP6 [600]
OTU02-P19-02-T7 [600]
OTU02-P21a-03-T7 [600]
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OTU02-P22-06-T7 [600]
OTU02-P22-13-T7 [600]
OTU02-P24-09-T7 [600]
OTU02-P29-25-T7 [600]
OTU02-P36-54-T7 [600]
OTU03-P01a-21-T7 [600]
OTU03-P02a-15-T7 [600]
OTU03-P02a-35-SP6 [600]
OTU03-P02a-57-SP6 [600]
OTU03-P06-05-T7 [600]
OTU03-P06-18-SP6 [600]
OTU03-P06-32-T7 [600]
OTU03-P07-103-SP6 [600]
OTU03-P07-C-SP6 [600]
OTU03-P08-07-SP6 [600]
OTU03-P08-21-SP6 [600]
OTU03-P09-03-T7 [600]
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OTU03-P13-50-SP6 [600]
OTU03-P14-26-SP6 [600]
OTU03-P16-26-T7 [600]
OTU03-P18-58-T7 [600]
OTU03-P19-26-T7 [600]
OTU03-P23-13-SP6 [600]
OTU03-P26-52-SP6 [600]
OTU03-P27-29-T7 [600]
OTU03-P29-54-SP6 [600]
OTU03-P36-38-T7 [600]
OTU03-P36-55-T7 [600]
OTU04-P01a-03-SP6 [600]
OTU04-P02a-04-SP6 [600]
OTU04-P02a-11-T7 [600]
OTU04-P02b-57-SP6 [600]
OTU04-P02b-79-T7 [600]
OTU04-P03-39-T7 [600]
OTU04-P05-39-SP6 [600]
OTU04-P06-51-SP6 [600]
OTU04-P08-35-T7 [600]
OTU04-P09-10-SP6 [600]
OTU04-P11a-36-T7 [600]
OTU04-P11a-60-SP6 [600]
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OTU04-P17-16-T7 [600]
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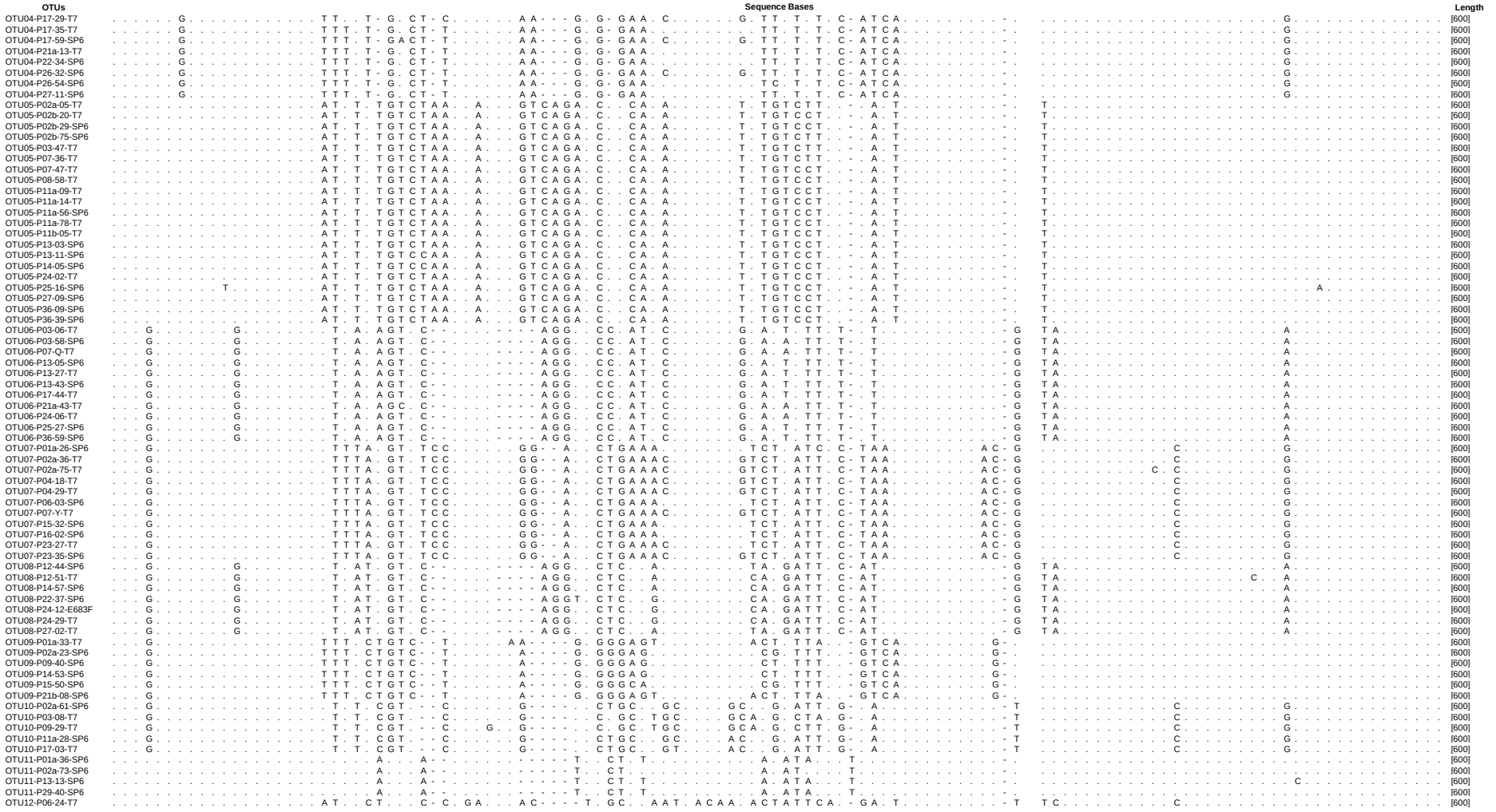
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Sequence Bases
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OTU04-P22-34-SP6 [600]
OTU04-P26-32-SP6 [600]
OTU04-P26-54-SP6 [600]
OTU04-P27-11-SP6 [600]
OTU05-P02a-05-T7 [600]
OTU05-P02b-20-T7 [600]
OTU05-P02b-29-SP6 [600]
OTU05-P02b-75-SP6 [600]
OTU05-P03-47-T7 [600]
OTU05-P07-36-T7 [600]
OTU05-P07-47-T7 [600]
OTU05-P08-58-T7 [600]
OTU05-P11a-09-T7 [600]
OTU05-P11a-14-T7 [600]
OTU05-P11a-56-SP6 [600]
OTU05-P11a-78-T7 [600]
OTU05-P11b-05-T7 [600]
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OTU06-P03-58-SP6 [600]
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OTU06-P13-27-T7 [600]
OTU06-P13-43-SP6 [600]
OTU06-P17-44-T7 [600]
OTU06-P21a-43-T7 [600]
OTU06-P24-06-T7 [600]
OTU06-P25-27-SP6 [600]
OTU06-P36-59-SP6 [600]
OTU07-P01a-26-SP6 [600]
OTU07-P02a-36-T7 [600]
OTU07-P02a-75-T7 [600]
OTU07-P04-18-T7 [600]
OTU07-P04-29-T7 [600]
OTU07-P06-03-SP6 [600]
OTU07-P07-Y-T7 [600]
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OTU07-P16-02-SP6 [600]
OTU07-P23-27-T7 [600]
OTU07-P23-35-SP6 [600]
OTU08-P12-44-SP6 [600]
OTU08-P12-51-T7 [600]
OTU08-P14-57-SP6 [600]
OTU08-P22-37-SP6 [600]
OTU08-P24-12-E683F [600]
OTU08-P24-29-T7 [600]
OTU08-P27-02-T7 [600]
OTU09-P01a-33-T7 [600]
OTU09-P02a-23-SP6 [600]
OTU09-P09-40-SP6 [600]
OTU09-P14-53-SP6 [600]
OTU09-P15-50-SP6 [600]
OTU09-P21b-08-SP6 [600]
OTU10-P02a-61-SP6 [600]
OTU10-P03-08-T7 [600]
OTU10-P09-29-T7 [600]
OTU10-P11a-28-SP6 [600]
OTU10-P17-03-T7 [600]
OTU11-P01a-36-SP6 [600]
OTU11-P02a-73-SP6 [600]
OTU11-P13-13-SP6 [600]
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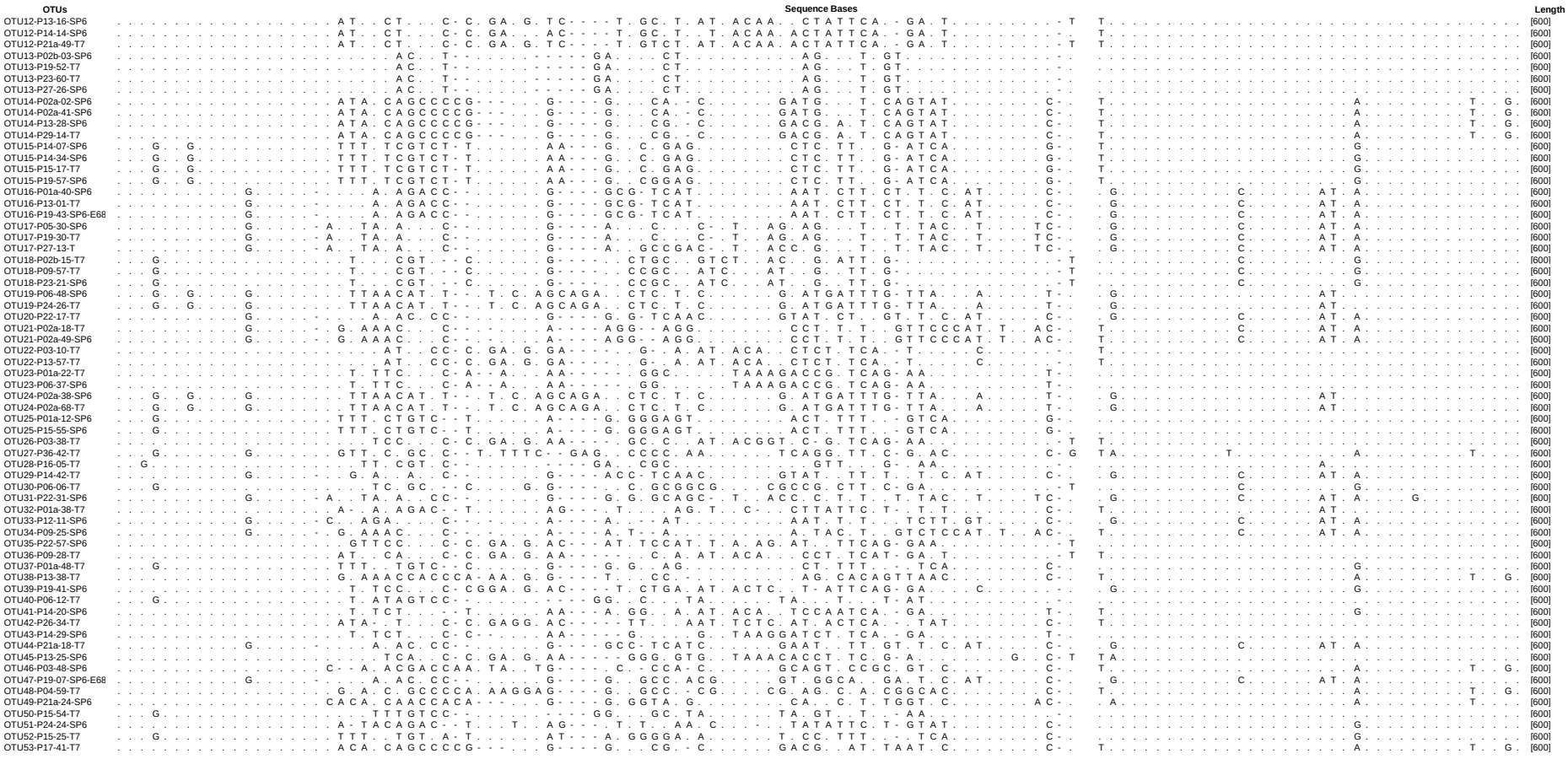
OTU12-P06-24-T7 [600]


OTUs
Sequence Bases
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OTU13-P02b-03-SP6 [600]
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OTU13-P23-60-T7 [600]
OTU13-P27-26-SP6 [600]
OTU14-P02a-02-SP6 [600]
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OTU14-P13-28-SP6 [600]
OTU14-P29-14-T7 [600]
OTU15-P14-07-SP6 [600]
OTU15-P14-34-SP6 [600]
OTU15-P15-17-T7 [600]
OTU15-P19-57-SP6 [600]
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OTU16-P19-43-SP6-E68 [600]
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OTU17-P27-13-T [600]
OTU18-P02b-15-T7 [600]
OTU18-P09-57-T7 [600]
OTU18-P23-21-SP6 [600]
OTU19-P06-48-SP6 [600]
OTU19-P24-26-T7 [600]
OTU20-P22-17-T7 [600]
OTU21-P02a-18-T7 [600]
OTU21-P02a-49-SP6 [600]
OTU22-P03-10-T7 [600]
OTU22-P13-57-T7 [600]
OTU23-P01a-22-T7 [600]
OTU23-P06-37-SP6 [600]
OTU24-P02a-38-SP6 [600]
OTU24-P02a-68-T7 [600]
OTU25-P01a-12-SP6 [600]
OTU25-P15-55-SP6 [600]
OTU26-P03-38-T7 [600]
OTU27-P36-42-T7 [600]
OTU28-P16-05-T7 [600]
OTU29-P14-42-T7 [600]
OTU30-P06-06-T7 [600]
OTU31-P22-31-SP6 [600]
OTU32-P01a-38-T7 [600]
OTU33-P12-11-SP6 [600]
OTU34-P09-25-SP6 [600]
OTU35-P22-57-SP6 [600]
OTU36-P09-28-T7 [600]
OTU37-P01a-48-T7 [600]
OTU38-P13-38-T7 [600]
OTU39-P19-41-SP6 [600]
OTU40-P06-12-T7 [600]
OTU41-P14-20-SP6 [600]
OTU42-P26-34-T7 [600]
OTU43-P14-29-SP6 [600]
OTU44-P21a-18-T7 [600]
OTU45-P13-25-SP6 [600]
OTU46-P03-48-SP6 [600]
OTU47-P19-07-SP6-E68 [600]
OTU48-P04-59-T7 [600]
OTU49-P21a-24-SP6 [600]
OTU50-P15-54-T7 [600]
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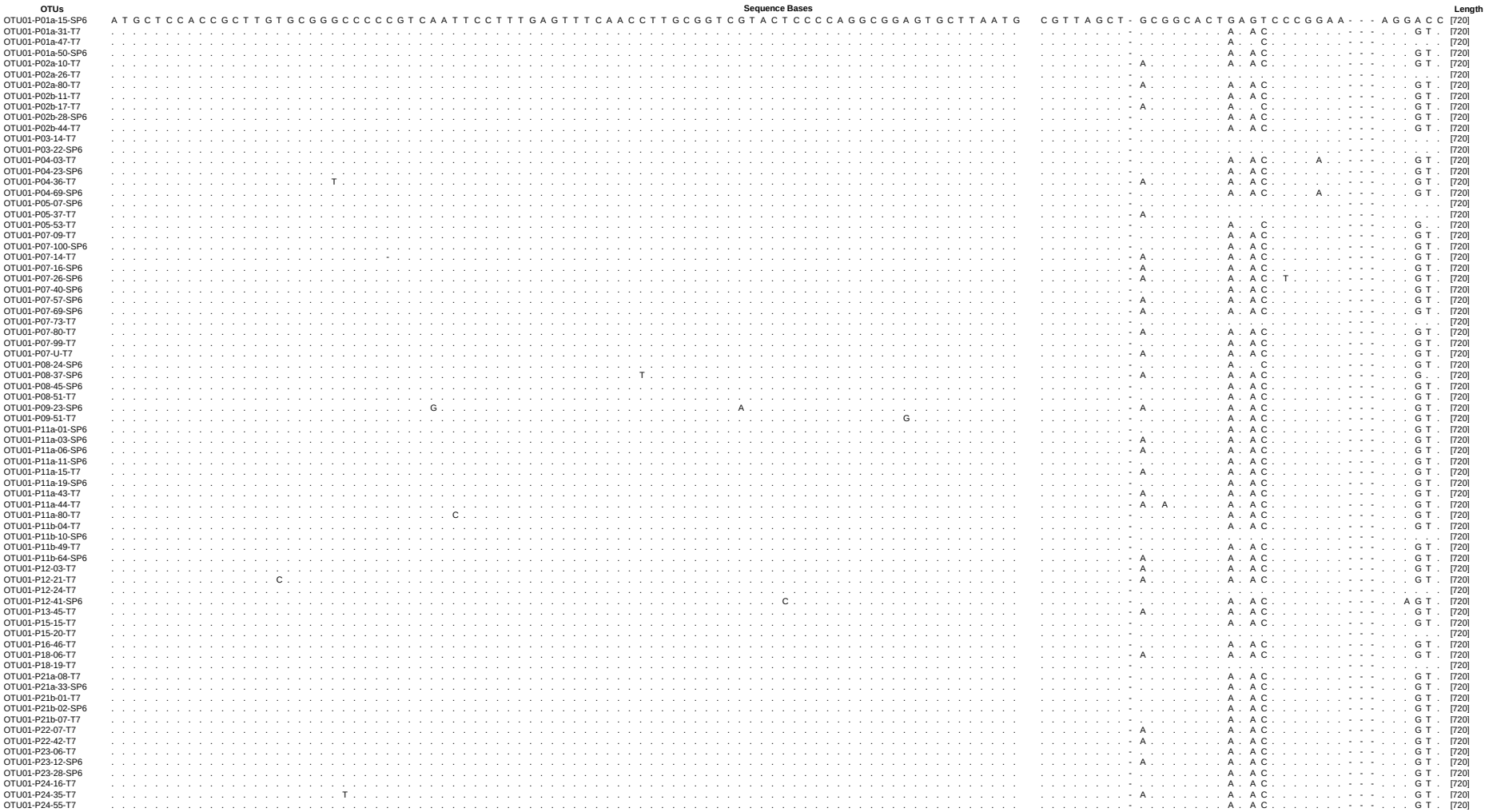
OTU52-P15-25-T7 [600]
OTU53-P17-41-T7 [600]


OTUs
Sequence Bases
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OTU01-P01a-50-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P02a-10-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P02a-26-T7 . . . . . - . . . . . [720]
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OTU01-P02b-11-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
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OTU01-P02b-28-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P02b-44-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P03-14-T7 . . . . . - . . . . . [720]
OTU01-P03-22-SP6 . . . . . - . . . . . [720]
OTU01-P04-03-T7 . . . . . - . . . . . A . A C . . . . A . . . . . G T . [720]
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OTU01-P04-69-SP6 . . . . . - . . . . . A . A C . . . . A . . . . . G T . [720]
OTU01-P05-07-SP6 . . . . . - . . . . . [720]
OTU01-P05-37-T7 . . . . . - A . . . . . [720]
OTU01-P05-53-T7 . . . . . - . . . . . A . . C . . . . . G . . [720]
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OTU01-P07-26-SP6 . . . . . - A . . . . A . A C . T . . . . . G T . [720]
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OTU01-P07-69-SP6 . . . . . - A . . . . A . A C . . . . . G T . [720]
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OTU01-P07-99-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P07-U-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P08-24-SP6 . . . . . - . . . . . A . . C . . . . . G T . [720]
OTU01-P08-37-SP6 . . . . . T . . . . . - A . . . . A . A C . . . . . G . . [720]
OTU01-P08-45-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
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OTU01-P11a-03-SP6 . . . . . - A . . . . A . A C . . . . . G T . [720]
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OTU01-P11a-11-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
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OTU01-P11a-19-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
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OTU01-P12-03-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P12-21-T7 . . . . . C . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P12-24-T7 . . . . . - . . . . . [720]
OTU01-P12-41-SP6 . . . . . C . . . . . - . . . . . A . A C . . . . . A G T . [720]
OTU01-P13-45-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P15-15-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P15-20-T7 . . . . . - . . . . . [720]
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OTU01-P21a-33-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
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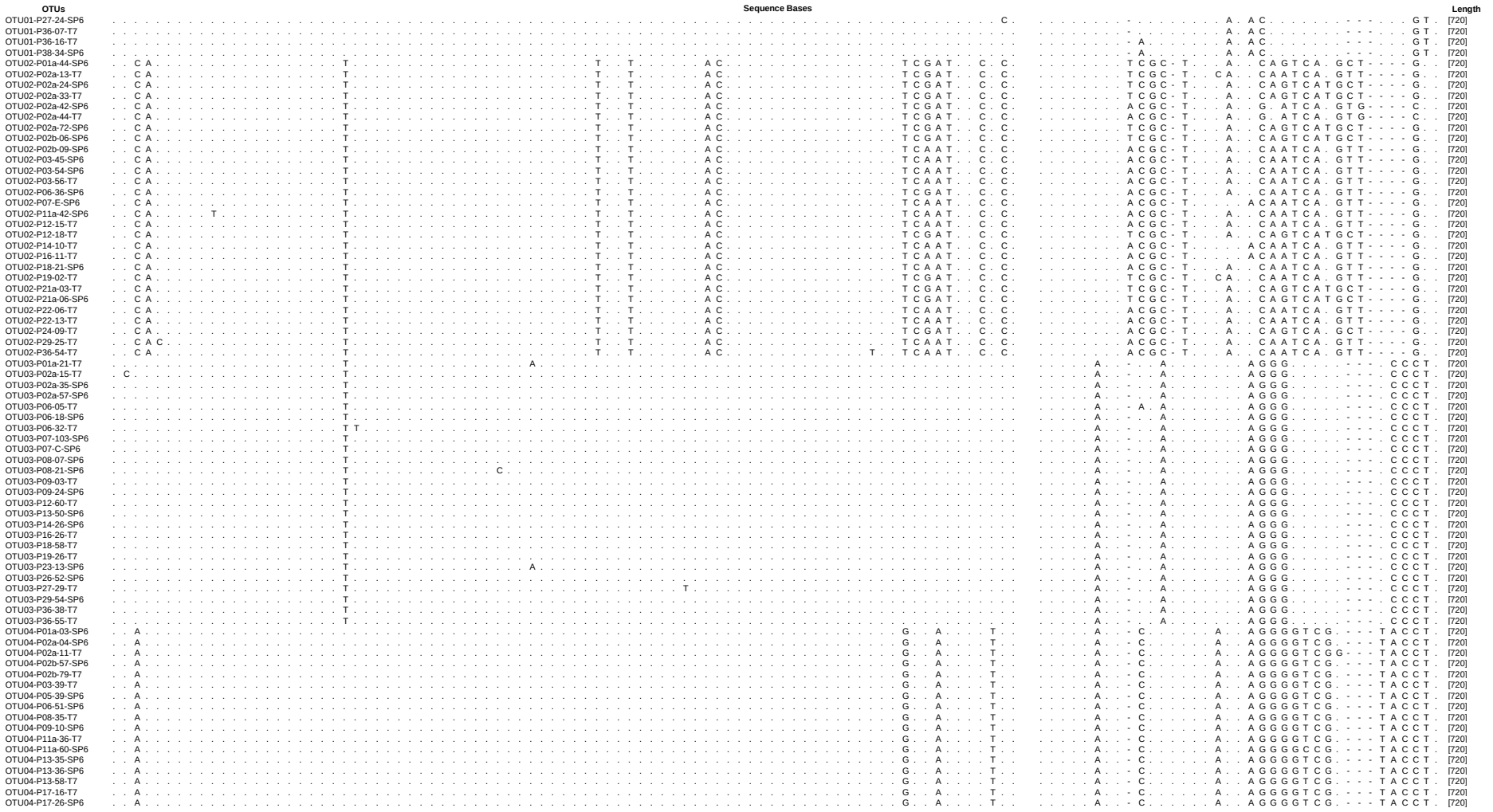
OTU01-P21b-02-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P21b-07-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P22-07-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P22-42-T7 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P23-06-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P23-12-SP6 . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P23-28-SP6 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P24-16-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]
OTU01-P24-35-T7 . . . . . T . . . . . - A . . . . A . A C . . . . . G T . [720]
OTU01-P24-55-T7 . . . . . - . . . . . A . A C . . . . . G T . [720]


OTUs
Sequence Bases
Length
OTU01-P27-24-SP6 [720]
OTU01-P36-07-T7 [720]
OTU01-P36-16-T7 [720]
OTU01-P38-34-SP6 [720]
OTU02-P01a-44-SP6 [720]
OTU02-P02a-13-T7 [720]
OTU02-P02a-24-SP6 [720]
OTU02-P02a-33-T7 [720]
OTU02-P02a-42-SP6 [720]
OTU02-P02a-44-T7 [720]
OTU02-P02a-72-SP6 [720]
OTU02-P02b-06-SP6 [720]
OTU02-P02b-09-SP6 [720]
OTU02-P03-45-SP6 [720]
OTU02-P03-54-SP6 [720]
OTU02-P03-56-T7 [720]
OTU02-P06-36-SP6 [720]
OTU02-P07-E-SP6 [720]
OTU02-P11a-42-SP6 [720]
OTU02-P12-15-T7 [720]
OTU02-P12-18-T7 [720]
OTU02-P14-10-T7 [720]
OTU02-P16-11-T7 [720]
OTU02-P18-21-SP6 [720]
OTU02-P19-02-T7 [720]
OTU02-P21a-03-T7 [720]
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OTU02-P22-06-T7 [720]
OTU02-P22-13-T7 [720]
OTU02-P24-09-T7 [720]
OTU02-P29-25-T7 [720]
OTU02-P36-54-T7 [720]
OTU03-P01a-21-T7 [720]
OTU03-P02a-15-T7 [720]
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OTU03-P02a-57-SP6 [720]
OTU03-P06-05-T7 [720]
OTU03-P06-18-SP6 [720]
OTU03-P06-32-T7 [720]
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OTU03-P08-21-SP6 [720]
OTU03-P09-03-T7 [720]
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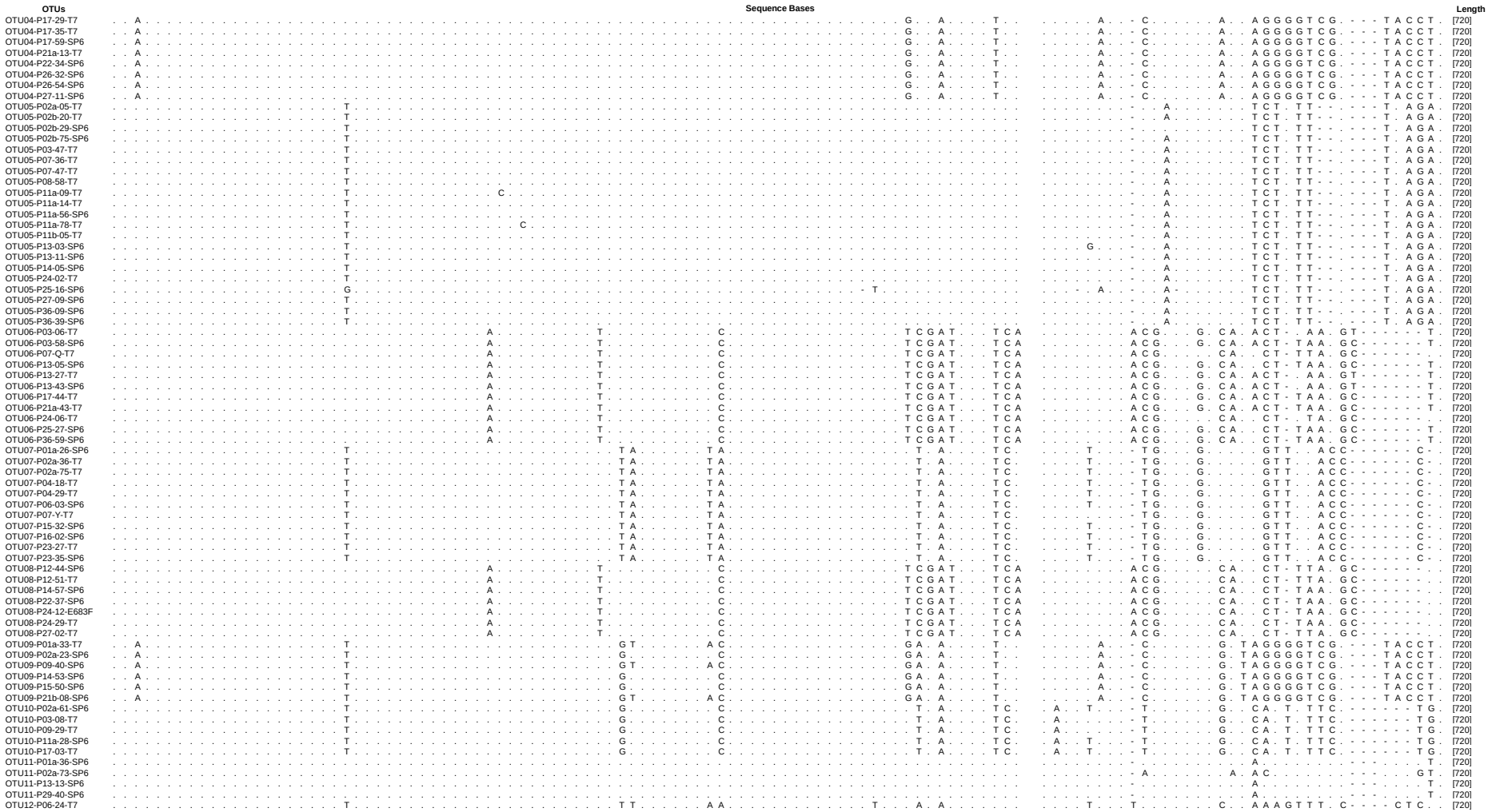
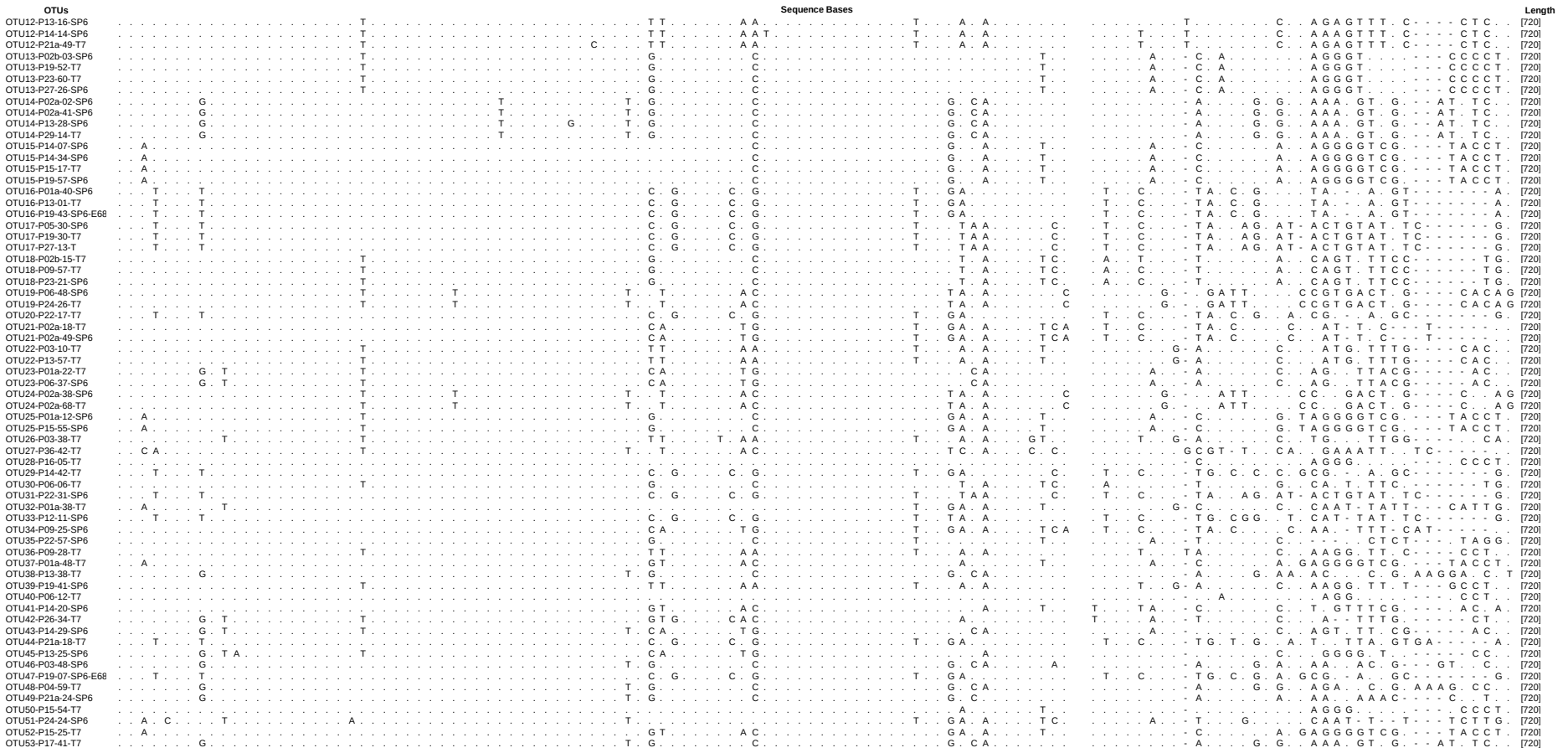
OTU03-P12-60-T7 [720]
OTU03-P13-50-SP6 [720]
OTU03-P14-26-SP6 [720]
OTU03-P16-26-T7 [720]
OTU03-P18-58-T7 [720]
OTU03-P19-26-T7 [720]
OTU03-P23-13-SP6 [720]
OTU03-P26-52-SP6 [720]
OTU03-P27-29-T7 [720]
OTU03-P29-54-SP6 [720]
OTU03-P36-38-T7 [720]
OTU03-P36-55-T7 [720]
OTU04-P01a-03-SP6 [720]
OTU04-P02a-04-SP6 [720]
OTU04-P02a-11-T7 [720]
OTU04-P02b-57-SP6 [720]
OTU04-P02b-79-T7 [720]
OTU04-P03-39-T7 [720]
OTU04-P05-39-SP6 [720]
OTU04-P06-51-SP6 [720]
OTU04-P08-35-T7 [720]
OTU04-P09-10-SP6 [720]
OTU04-P11a-36-T7 [720]
OTU04-P11a-60-SP6 [720]
OTU04-P13-35-SP6 [720]
OTU04-P13-36-SP6 [720]
OTU04-P13-58-T7 [720]
OTU04-P17-16-T7 [720]
OTU04-P17-26-SP6 [720]


OTUs
Sequence Bases
Length
OTU04-P17-29-T7 [720]
OTU04-P17-35-T7 [720]
OTU04-P17-59-SP6 [720]
OTU04-P21a-13-T7 [720]
OTU04-P22-34-SP6 [720]
OTU04-P26-32-SP6 [720]
OTU04-P26-54-SP6 [720]
OTU04-P27-11-SP6 [720]
OTU05-P02a-05-T7 [720]
OTU05-P02b-20-T7 [720]
OTU05-P02b-29-SP6 [720]
OTU05-P02b-75-SP6 [720]
OTU05-P03-47-T7 [720]
OTU05-P07-36-T7 [720]
OTU05-P07-47-T7 [720]
OTU05-P08-58-T7 [720]
OTU05-P11a-09-T7 [720]
OTU05-P11a-14-T7 [720]
OTU05-P11a-56-SP6 [720]
OTU05-P11a-78-T7 [720]
OTU05-P11b-05-T7 [720]
OTU05-P13-03-SP6 [720]
OTU05-P13-11-SP6 [720]
OTU05-P14-05-SP6 [720]
OTU05-P24-02-T7 [720]
OTU05-P25-16-SP6 [720]
OTU05-P27-09-SP6 [720]
OTU05-P36-09-SP6 [720]
OTU05-P36-39-SP6 [720]
OTU06-P03-06-T7 [720]
OTU06-P03-58-SP6 [720]
OTU06-P07-Q-T7 [720]
OTU06-P13-05-SP6 [720]
OTU06-P13-27-T7 [720]
OTU06-P13-43-SP6 [720]
OTU06-P17-44-T7 [720]
OTU06-P21a-43-T7 [720]
OTU06-P24-06-T7 [720]
OTU06-P25-27-SP6 [720]
OTU06-P36-59-SP6 [720]
OTU07-P01a-26-SP6 [720]
OTU07-P02a-36-T7 [720]
OTU07-P02a-75-T7 [720]
OTU07-P04-18-T7 [720]
OTU07-P04-29-T7 [720]
OTU07-P06-03-SP6 [720]
OTU07-P07-Y-T7 [720]
OTU07-P15-32-SP6 [720]
OTU07-P16-02-SP6 [720]
OTU07-P23-27-T7 [720]
OTU07-P23-35-SP6 [720]
OTU08-P12-44-SP6 [720]
OTU08-P12-51-T7 [720]
OTU08-P14-57-SP6 [720]
OTU08-P22-37-SP6 [720]
OTU08-P24-12-E683F [720]
OTU08-P24-29-T7 [720]
OTU08-P27-02-T7 [720]
OTU09-P01a-33-T7 [720]
OTU09-P02a-23-SP6 [720]
OTU09-P09-40-SP6 [720]
OTU09-P14-53-SP6 [720]
OTU09-P15-50-SP6 [720]
OTU09-P21b-08-SP6 [720]
OTU10-P02a-61-SP6 [720]
OTU10-P03-08-T7 [720]
OTU10-P09-29-T7 [720]
OTU10-P11a-28-SP6 [720]
OTU10-P17-03-T7 [720]
OTU11-P01a-36-SP6 [720]
OTU11-P02a-73-SP6 [720]
OTU11-P13-13-SP6 [720]
OTU11-P29-40-SP6 [720]
OTU12-P06-24-T7 [720]


OTUs
Sequence Bases
Length
OTU12-P13-16-SP6 [720]
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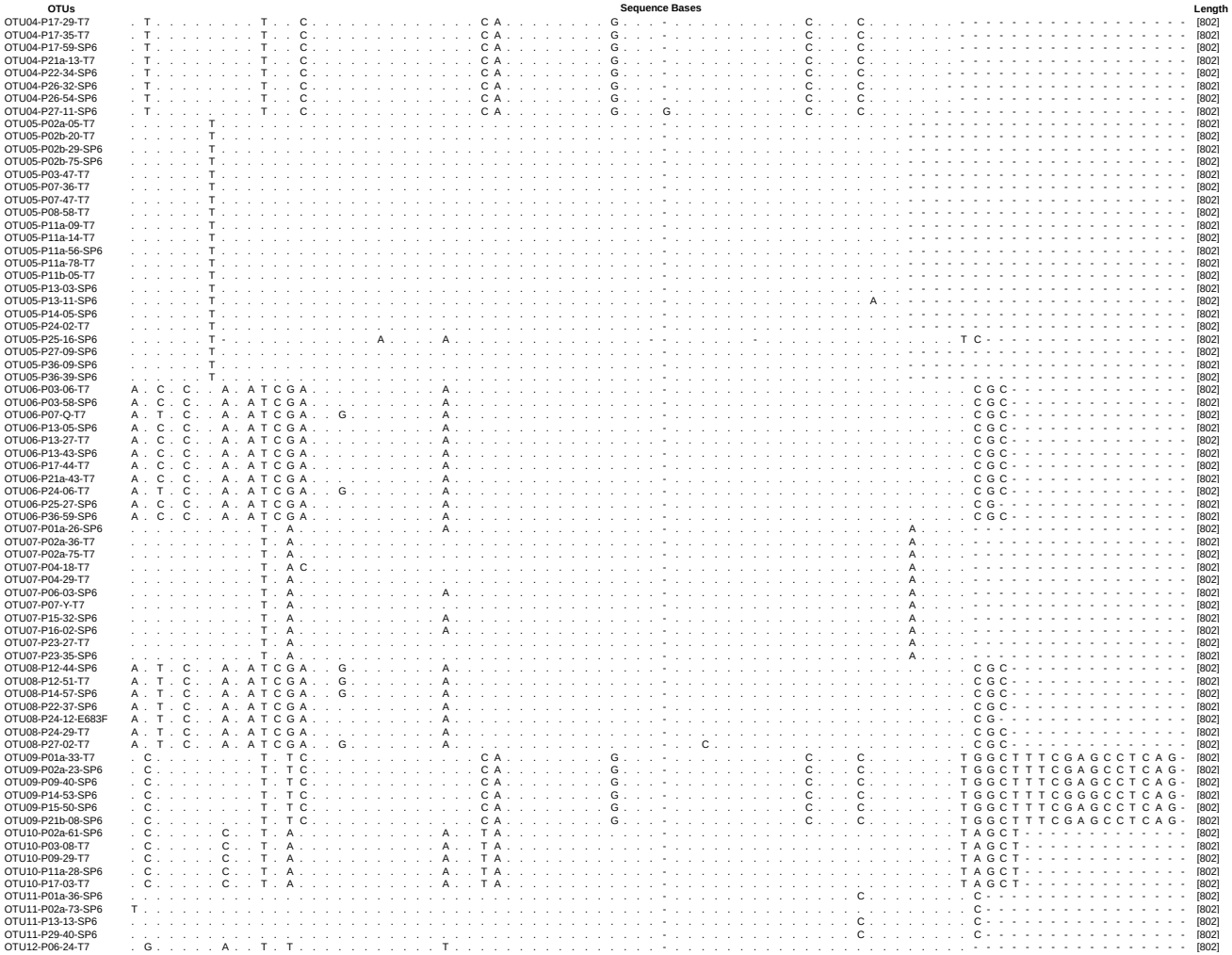
OTU12-P21a-49-T7 [720]
OTU13-P02b-03-SP6 [720]
OTU13-P19-52-T7 [720]
OTU13-P23-60-T7 [720]
OTU13-P27-26-SP6 [720]
OTU14-P02a-02-SP6 [720]
OTU14-P02a-41-SP6 [720]
OTU14-P13-28-SP6 [720]
OTU14-P29-14-T7 [720]
OTU15-P14-07-SP6 [720]
OTU15-P14-34-SP6 [720]
OTU15-P15-17-T7 [720]
OTU15-P19-57-SP6 [720]
OTU16-P01a-40-SP6 [720]
OTU16-P13-01-T7 [720]
OTU16-P19-43-SP6-E68 [720]
OTU17-P05-30-SP6 [720]
OTU17-P19-30-T7 [720]
OTU17-P27-13-T [720]
OTU18-P02b-15-T7 [720]
OTU18-P09-57-T7 [720]
OTU18-P23-21-SP6 [720]
OTU19-P06-48-SP6 [720]
OTU19-P24-26-T7 [720]
OTU20-P22-17-T7 [720]
OTU21-P02a-18-T7 [720]
OTU21-P02a-49-SP6 [720]
OTU22-P03-10-T7 [720]
OTU22-P13-57-T7 [720]
OTU23-P01a-22-T7 [720]
OTU23-P06-37-SP6 [720]
OTU24-P02a-38-SP6 [720]
OTU24-P02a-68-T7 [720]
OTU25-P01a-12-SP6 [720]
OTU25-P15-55-SP6 [720]
OTU26-P03-38-T7 [720]
OTU27-P36-42-T7 [720]
OTU28-P16-05-T7 [720]
OTU29-P14-42-T7 [720]
OTU30-P06-06-T7 [720]
OTU31-P22-31-SP6 [720]
OTU32-P01a-38-T7 [720]
OTU33-P12-11-SP6 [720]
OTU34-P09-25-SP6 [720]
OTU35-P22-57-SP6 [720]
OTU36-P09-28-T7 [720]
OTU37-P01a-48-T7 [720]
OTU38-P13-38-T7 [720]
OTU39-P19-41-SP6 [720]
OTU40-P06-12-T7 [720]
OTU41-P14-20-SP6 [720]
OTU42-P26-34-T7 [720]
OTU43-P14-29-SP6 [720]
OTU44-P21a-18-T7 [720]
OTU45-P13-25-SP6 [720]
OTU46-P03-48-SP6 [720]
OTU47-P19-07-SP6-E68 [720]
OTU48-P04-59-T7 [720]
OTU49-P21a-24-SP6 [720]
OTU50-P15-54-T7 [720]
OTU51-P24-24-SP6 [720]
OTU52-P15-25-T7 [720]
OTU53-P17-41-T7 [720]

OTUs	Sequence Bases	Length
OTU01-P01a-15-SP6	C A A C A C C T A G C A C T C A T C G T T T A C G G C G T G G A C T A C C A G G G - T A T C T A A T C C T G T T T G C T C C C C A - - - - - - - - - - - - - - - - -	[802]
OTU01-P01a-31-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P01a-47-T7	T . . . . - . . . . C . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P01a-50-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P02a-10-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P02a-26-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P02a-80-T7	T . . T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P02b-11-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P02b-17-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P02b-28-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P02b-44-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P03-14-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P03-22-SP6	. . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P04-03-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P04-23-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P04-36-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P04-69-SP6	T . . . . G . . . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P05-07-SP6	. . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P05-37-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P05-53-T7	T . . . . - . . . . C . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P07-09-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-100-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P07-14-T7	T . . . . - . . . . C G - - - - - - - - - - - - - - -	[802]
OTU01-P07-16-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-26-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P07-40-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-57-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-69-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-73-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P07-80-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-99-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P07-U-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P08-24-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P08-37-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P08-45-SP6	T . . . . . A . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P08-51-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P09-23-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P09-51-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-01-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-03-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-06-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-11-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-15-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-19-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-43-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-44-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11a-80-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P11b-04-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11b-10-SP6	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P11b-49-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P11b-64-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P12-03-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P12-21-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P12-24-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P12-41-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P13-45-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P15-15-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P15-20-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P16-46-T7	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P18-06-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P18-19-T7	. . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P21a-08-T7	T . . . . - . . . . T . . . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P21a-33-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P21b-01-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P21b-02-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P21b-07-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P22-07-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P22-42-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P23-06-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P23-12-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P23-28-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P24-16-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P24-35-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P24-55-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]

OTUs	Sequence Bases	Length
OTU01-P27-24-SP6	T . . . . - . . . . - - - - - - - - - - - - - - - - -	[802]
OTU01-P36-07-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - - -	[802]
OTU01-P36-16-T7	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU01-P38-34-SP6	T . . . . - . . . . C - - - - - - - - - - - - - - - -	[802]
OTU02-P01a-44-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-13-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-24-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-33-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-42-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-44-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02a-72-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02b-06-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P02b-09-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P03-45-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P03-54-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P03-56-T7	. . . . . G . . G A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P06-36-SP6	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P07-E-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P11a-42-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P12-15-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P12-18-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P14-10-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P16-11-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P18-21-SP6	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P19-02-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P21a-03-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P21a-06-SP6	. . . . . G . . . A T C G A . . . . G . A . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P22-06-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P22-13-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P24-09-T7	. . . . . G . . . A T C G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P29-25-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU02-P36-54-T7	. . . . . G . . . A T T G A . . . . G . . . . - . . . . A . . . . C G - - - - - - - - - - - - - - -	[802]
OTU03-P01a-21-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P02a-15-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P02a-35-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P02a-57-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P06-05-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P06-18-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P06-32-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P07-103-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P07-C-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P08-07-SP6	. . . . . . T . . . . - . . . . A . . . . - - - - - - - - - - - - - - - - -	[802]
OTU03-P08-21-SP6	. . . . . . T . . . . - . . . . A . . . . - - - - - - - - - - - - - - - - -	[802]
OTU03-P09-03-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P09-24-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P12-60-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P13-50-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P14-26-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P16-26-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P18-58-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P19-26-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P23-13-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P26-52-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P27-29-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P29-54-SP6	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P36-38-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU03-P36-55-T7	. . . . . . T . . . . - . . . . A . . . . C - - - - - - - - - - - - - - - -	[802]
OTU04-P01a-03-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P02a-04-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P02a-11-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P02b-57-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P02b-79-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P03-39-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P05-39-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P06-51-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P08-35-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P09-10-SP6	. T . . . . T . . C . . . . A . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P11a-36-T7	. T . . . . T . . C . . . . C A . . . . G . . . G . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P11a-60-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P13-35-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P13-36-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P13-58-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]
OTU04-P17-16-T7	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . - - - - - - - - - - - - - - - - - -	[802]
OTU04-P17-26-SP6	. T . . . . T . . C . . . . C A . . . . G . . . - . . . . C . . . C . . . . . . - - - - - - - - - - - - - - - - - - -	[802]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OTU04-P22-34-SP6 [802]
OTU04-P26-32-SP6 [802]
OTU04-P26-54-SP6 [802]
OTU04-P27-11-SP6 [802]
OTU05-P02a-05-T7 [802]
OTU05-P02b-20-T7 [802]
OTU05-P02b-29-SP6 [802]
OTU05-P02b-75-SP6 [802]
OTU05-P03-47-T7 [802]
OTU05-P07-36-T7 [802]
OTU05-P07-47-T7 [802]
OTU05-P08-58-T7 [802]
OTU05-P11a-09-T7 [802]
OTU05-P11a-14-T7 [802]
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OTU05-P11b-05-T7 [802]
OTU05-P13-03-SP6 [802]
OTU05-P13-11-SP6 [802]
OTU05-P14-05-SP6 [802]
OTU05-P24-02-T7 [802]
OTU05-P25-16-SP6 [802]
OTU05-P27-09-SP6 [802]
OTU05-P36-09-SP6 [802]
OTU05-P36-39-SP6 [802]
OTU06-P03-06-T7 [802]
OTU06-P03-58-SP6 [802]
OTU06-P07-Q-T7 [802]
OTU06-P13-05-SP6 [802]
OTU06-P13-27-T7 [802]
OTU06-P13-43-SP6 [802]
OTU06-P17-44-T7 [802]
OTU06-P21a-43-T7 [802]
OTU06-P24-06-T7 [802]
OTU06-P25-27-SP6 [802]
OTU06-P36-59-SP6 [802]
OTU07-P01a-26-SP6 [802]
OTU07-P02a-36-T7 [802]
OTU07-P02a-75-T7 [802]
OTU07-P04-18-T7 [802]
OTU07-P04-29-T7 [802]
OTU07-P06-03-SP6 [802]
OTU07-P07-Y-T7 [802]
OTU07-P15-32-SP6 [802]
OTU07-P16-02-SP6 [802]
OTU07-P23-27-T7 [802]
OTU07-P23-35-SP6 [802]
OTU08-P12-44-SP6 [802]
OTU08-P12-51-T7 [802]
OTU08-P14-57-SP6 [802]
OTU08-P22-37-SP6 [802]
OTU08-P24-12-E683F [802]
OTU08-P24-29-T7 [802]
OTU08-P27-02-T7 [802]
OTU09-P01a-33-T7 [802]
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OTU09-P09-40-SP6 [802]
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OTU09-P15-50-SP6 [802]
OTU09-P21b-08-SP6 [802]
OTU10-P02a-61-SP6 [802]
OTU10-P03-08-T7 [802]
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OTU10-P17-03-T7 [802]
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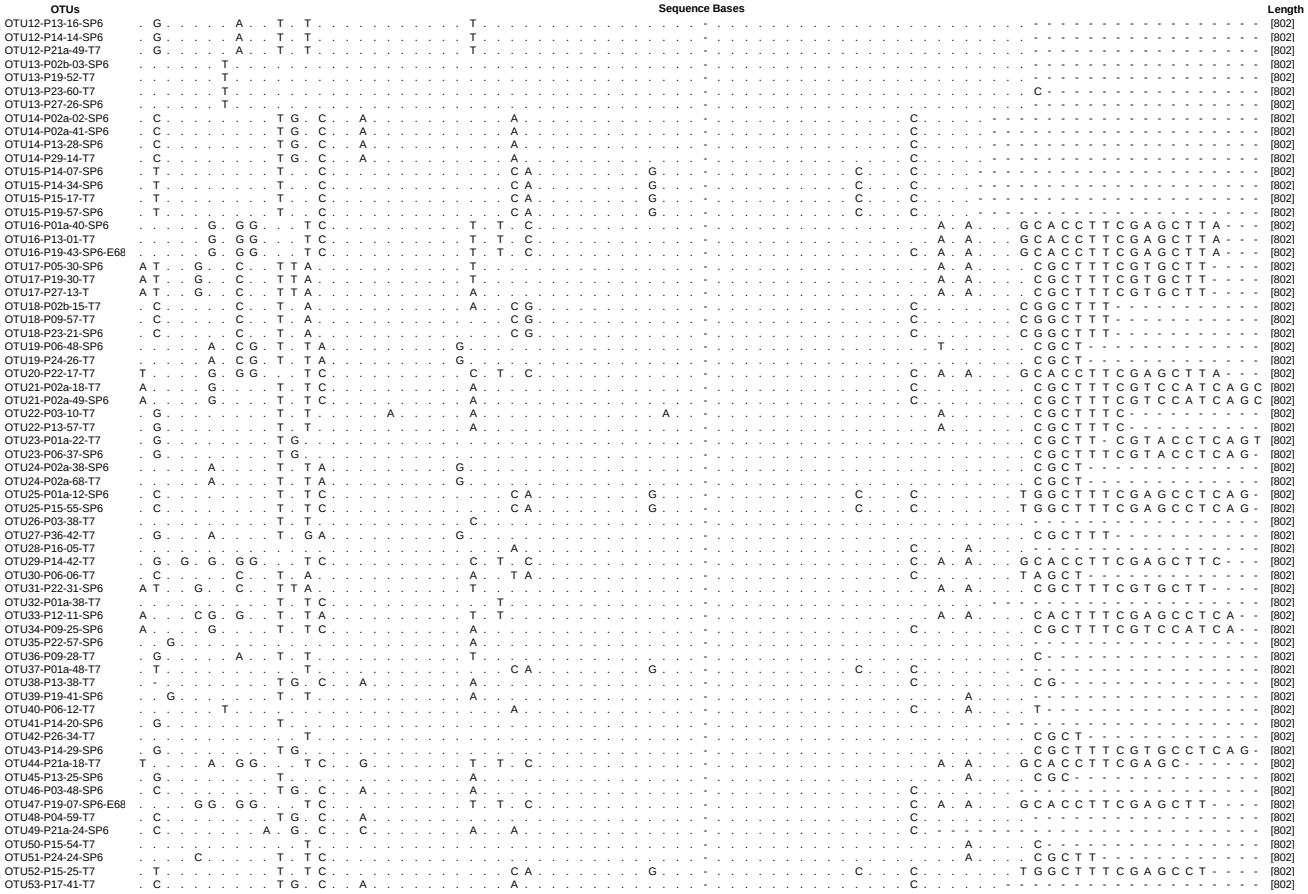
OTU11-P29-40-SP6 [802]
OTU12-P06-24-T7 [802]


OTUs
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OTU13-P23-60-T7 [802]
OTU13-P27-26-SP6 [802]
OTU14-P02a-02-SP6 [802]
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OTU15-P14-34-SP6 [802]
OTU15-P15-17-T7 [802]
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OTU18-P09-57-T7 [802]
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OTU27-P36-42-T7 [802]
OTU28-P16-05-T7 [802]
OTU29-P14-42-T7 [802]
OTU30-P06-06-T7 [802]
OTU31-P22-31-SP6 [802]
OTU32-P01a-38-T7 [802]
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OTU35-P22-57-SP6 [802]
OTU36-P09-28-T7 [802]
OTU37-P01a-48-T7 [802]
OTU38-P13-38-T7 [802]
OTU39-P19-41-SP6 [802]
OTU40-P06-12-T7 [802]
OTU41-P14-20-SP6 [802]
OTU42-P26-34-T7 [802]
OTU43-P14-29-SP6 [802]
OTU44-P21a-18-T7 [802]
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OTU46-P03-48-SP6 [802]
OTU47-P19-07-SP6-E68 [802]
OTU48-P04-59-T7 [802]
OTU49-P21a-24-SP6 [802]
OTU50-P15-54-T7 [802]
OTU51-P24-24-SP6 [802]
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