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Predictions and Computational Analysis of Novel Chromosomal Type II Toxin Antitoxin Systems in the Human Oral Microbiome

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School of Science and Engineering

PREDICTION AND COMPUTATIONAL ANALYSIS OF NOVEL CHROMOSOMAL TYPE II TOXIN ANTITOXIN SYSTEMS IN THE HUMAN ORAL MICROBIOME

A Thesis Submitted to

The Biotechnology Master's Program

In partial fulfillment of the requirements for
the degree of Master of Science in Biotechnology

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THESIS DEFENSE

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DEDICATION

I dedicate this work to Allah, my parents Mervat Bazan and Abdel-Raouf Bazan and to patients whom this project could affect their lives by any means.

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ABSTRACT

The American University in Cairo

Prediction and Computational Analysis of Novel Chromosomal Type II Toxin Antitoxin Systems in the Human Oral Microbiome

By: Ashraf A. Bazan
Under supervision of: Dr. Ahmed Abdellatif, Dr. Tamer Salem and Dr. Heba Abostate

The importance of the human oral microbiome is progressively receiving considerable attention in recent research, serving as a model niche for studying microevolution. The impact of horizontal gene transfer by mobile genetic elements in such environment is the driving force for the mosaic nature of the oral microbiome. However, there is a missing link between the molecular systems interactions responsible for the plasticity of the genomes and the adaptations of the oral microbiome to physiological and pathological changes. The mobile chromosomal type II Toxin Antitoxin Systems (TASs). are known for their effective role in dynamic environment adaptation and stress response. In this study, we predicted and analyzed the genetic diversity and evolution of type II TAS in the oral microbiome of an Egyptian, presumably healthy, individual. 16S rRNA sequencing (submitted to GenBank). showed taxonomic analysis and microbial diversity and species abundance in three samples of supragingival plaque, subgingival plaque and buccal mucosa. Two hundred and seventy-eight type II TAS were identified from sequenced chromosomal genomes of the oral microbiome by means of exhaustive sequence and 3D structure homology, Hidden Markov Modelling and manual domain analysis. Gene family assignment were proposed since majority of the genes were previously annotated as hypothetical proteins. TAS network of the oral microbiome showed highly interconnected centralities which entails the extensive cross talk and intra-regulatory nature. Molecular ecology analysis of the type II TAS using diversity indexes confirms both diversity and relative abundance of these systems in the oral microbiome. Molecular evolutionary phylogenetic maximum likelihood analysis of the type II TAS, using modified Whelan And Goldman (WAG) as best fit evolution model, was performed for the predicted toxin antitoxin systems. Further analysis revealed evidence for the persistence of the toxin antitoxin systems throughout the oral microbiome. Molecular allometric analysis confirms uneven persistent distribution of the type II TAS. This comprehensive study of new chromosomal type II toxin antitoxin systems found in the oral microbiome provides insights on plasticity of the human oral microbiome and its adaptation to change in the host environment.

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ABBREVIATIONS

ADP	Adenosine DiPhosphate
AMP	Adenosine MonoPhosphate
BLAST	Basic Local Alignment Search Tool
CAMP	Cyclic Adenosine MonoPhosphate
CDS	CoDing Sequence
CD-search	Conserved Domain Search
CFU/ml	Colony Forming Unit per milli liter
CPR	Candidate Phyla Radiation
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR associated
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDBJ	DNA Data Bank of Japan
DNA	DeoxyriboNucleic Acid
eHOMD	expanded Human Oral Microbiome Database
EMBL	European Molecular Biology Laboratory
GI	Genomic Island
GMO	Genetically Modified Organism
GO	Gene Ontology
HGT	Horizontal Gene Transfer
HMM	Hidden Markov Model
ICE	Integrative Conjugative Element
IRB	Institutional Review Board
IS	Insertion Sequence
MEGA	Molecular Evolutionary Genetic Analysis
MEME	Multiple Expression Motif for Motif Elicitation
MGE	Mobile Genetic Element
NCBI	National Center for Biotechnology Information
ORF	Open Reading Frame
PATRIC	PAThosystems Resource Integration Center

PHI Blast	Pattern Hit Initiated BLAST
PSI Blast	Position Specific Iterated BLAST
PSK	Post Segregation Killing
OTU	Operational Taxonomic Unit
QS	Quorum Sensing
RAST	Rapid Annotation using Subcellular Technology
RM	Restriction Modification
RNA	RiboNucleic Acid
rRNA	ribosomal RNA
SI	Super Integron
sRNAs	small RNA
TA	Toxin Antitoxin
TADB	Toxin Antitoxin DataBase
TALEN	Transcription Activator-Like Effector Nuclease
TAS	Toxin Antitoxin System
TCCRAS	Toxin Counter-electable Cassette Regulated by an Antitoxin Switch
tRNA	Transfer RNA

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1. LITERATURE REVIEW

1.1. Human Oral Microbiome

1.1.1. Introduction on Human Oral Microbiome

Since its inception, microbiology has focused on the human oral “Animalcules” as described by the very first scientist Antonie van Leeuwenhoek to discover microbes (Gest, 2004; Leeuwenhoek Antoni Van, 1677) Since then, it has been of great interest to study the human oral microbiome for reasons of easy accessibility, understanding diseases pathophysiology and studying host – microbe ecology to name a few (Dewhirst et al., 2010) Before the advent of the culture-independent analysis methods, most the studies were focusing on culturable microbes; however, recently, the focus has shifted toward a holistic view of the oral microbiome (Verma, Garg, & Dubey, 2018)

The complex system biology of the oral microbiome includes strikingly important cues for both the diversity and richness of the Operational Taxonomic Units (OTUs) of the regional ecosystem, where its evolutionary dynamics changes promptly to ecological inter and intra-species interactions including that of the host physiology and/or pathology (Krishnan, Chen, & Paster, 2017) Recognizing the extensive form and the non-zero-sum nature of the oral microbiome, the research has been reformed to study the elements of the system differently. Studying the interactions of the microbes alongside the molecular systems of these microbes holds informative and divergent ingenuity to understand short-comings of reductionist approach in understanding clinical diseases etiology and molecular biology (Cross et al., 2018; Edlund et al., 2013, 2015; Pál & Papp, 2017) A prime example for this is the dental caries, where it was believed for centuries that *Streptococcus mutans* is the sole cause of the condition. Currently, it is evident that the instability of the composition of the dental biofilm is what aggravate the periodontitis and dental caries (Bowen, Burne, Wu, & Koo, 2018; McLean, 2014) Another solid example for the holistic approach used in the oral microbiome research is the discovery of the mechanism of action and the evolution patterns of the miropin, a serpin-type suicidal endopeptidase inhibitor, found in the metagenome of the human oral microbiome (Goulas et al., 2017)

This approach has revolutionized the research where our fundamental understanding of the ecology, evolution and clinical diseases is more rationally restructured, yet, incompletely resolved and leave us with questions more than answers.

1.1.2. Ecology of the Human Oral Microbiome

The human oral microbiome is the second largest microbial community right after the gut microbiome. The expanded Human Oral Microbiome Database (eHOMD) contains, as of April 2019, seven hundred and seventy-two different species (Chen et al., 2010; Human Microbiome Project Consortium, 2012; Verma et al., 2018) Although the salivary microbiome is considered broadly stable throughout different states, it is just a collection of planktonic microbes shredded from different sites in the buccal cavity (Belstrøm et al., 2016) The abundance and diversity varies in the oral microbiome of the buccal mucosa, hard palate, masticator mucosa, soft palate, different teeth enamels, supragingival tract, subgingival tract, tongue dorsum, keratinized gingiva and palatine tonsils (Moon & Lee, 2016)

The human oral microbiome has nearly third of the taxa unculturable. The culturable taxa can be broadly categorized into six phyla, namely, *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria and Spirochetes* (Palmer, 2014; Verma et al., 2018) According to the updated eHOMD, the most abundant phylum is the *Firmicutes*, followed by *Bacteroides* (Dewhirst et al., 2010) However, false positives of taxonomic assignments are present due to Operational Taxonomic Units (OTU) similarity bias to the overrepresented *Firmicutes* in the databases used in the analysis. On the genus level, the most common are the *Streptococcus* and *Veillonella*. The major genera found are those of the *Actinomyces, Corynebacterium, Rothia, Capnocytophaga, Prevotella, Haemophilus, Granulicatella and Neisseria*. All of these constitute what is called the core oral microbiome because of their abundance. On the other hand, the variable oral microbiome is formed by the fluctuations of several other genera depending on the niche conditions (Verma et al., 2018; Zaura, Keijser, Huse, & Crielaard, 2009)

There are different niches in the buccal cavity which can be categorized into saliva, dental plaque and buccal mucosa. As mentioned above, whatever shed from the biofilms of the mucosa or plaque would be in the saliva in the plankton form. It has been estimated that there are around 140 million CFU/ml of the saliva. The major constituents of the salivary microbiome are *Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes*, and TM7 (J.

He, Li, Cao, Xue, & Zhou, 2015; Human Microbiome Project Consortium, 2012; Zaura et al., 2009) The microbiome of the dental plaque forms biofilm on the teeth surface (L. Yang et al., 2011) Although the microbial community may vary based on which tooth, the main variation in the dental plaque biofilms is between the supragingival plaque and the subgingival plaque with the supragingival microbiome being more rich and diverse (Filoche, Wong, & Sissons, 2010; Gross et al., 2010; J. He et al., 2015; Keijser et al., 2008) Conversely, the buccal mucosa microbiome has lower diversity profile. The tongue dorsum is being extensively studied for research on halitosis. The characterized microbes of the tongue that are abundant include *Streptococcus salivarius* and *Rothia mucilaginosa* (J. He et al., 2015; Kazor et al., 2003)

In addition to the traditional bacterial phyla, there is a group of bacteria lineages that are extremely small in shape and genome size compared to most bacterial lineages. They are termed Candidate Phyla Radiations (CPRs) (Hug et al., 2016) The CPRs have provocative interspecies interactions that largely shapes the ecology of the oral microbiome. This newly categorized group is huge, consisting of around 15% of the bacterial domain with around 70 different phyla. Interestingly, they have less functional diversity and minimal metabolome, thus leading to the inference of being some sort of parasitic organisms (Danczak et al., 2017) Although they harbor the archaeal enzyme RubisCO that is used in carbon dioxide fixation, they are well-known obligate symbiont dependent on other bacteria (Baker, Bor, Agnello, Shi, & He, 2017; Wrighton et al., 2016) One of the best studied CPRs is the TM7x (Kianoush et al., 2014; Segata et al., 2012; Soro et al., 2014; Zhou et al., 2013). Under different physiological and environmental conditions, it can significantly alter the whole oral habitat ecosystem through its multivariate interactions with other species especially those of the *Actinomyces* (X. He et al., 2015; McLean et al., 2016) The abundance of such organisms increases from around 1% to approximately 21% of all of the microbiome in oral diseases such as periodontitis (B. Liu et al., 2012; Rylev, Bek-Thomsen, Reinholdt, Ennibi, & Kilian, 2011) Besides, they can expressively modify host immune responses, such as, macrophages response, cytokines concentrations and TNF-α inhibition (X. He et al., 2015) Thus, the dynamic ecology of the buccal cavity is more than just the richness, but also, the interactions within and between species.

Indeed, The human oral microbiome is more than just the bacteriome. In fact, there are about more than 100 species of known fungi that resides in the oral cavity (Bandara, Panduwawala, & Samaranayake, 2019; Dupuy et al., 2014; Ghannoum et al., 2010; Peters, Wu, Hayes, & Ahn,

2017) The oral mycobiome has low abundance compared to the oral bacterial communities (Baker et al., 2017); however, the impact should not be underestimated. This is because of two reasons; firstly, Despite the fungal communities low abundance, the larger fungal cell sizes constitutes a larger biomass in the oral ecosystem (Cui, Morris, & Ghedin, 2013) Secondly, hyphens of the fungi formulates backbone for the formation of multi-species biofilm (Baker et al., 2017) The medical impact of the fungi on the host is, clearly, huge. This is evident as clinicians encounters several fungal infections from local candidiasis to lethal systemic infections (Cui et al., 2013; Samaranayake, 2018) The mycobiome has massive inter-kingdom communication that is regarded as keystone species. For example, *Candida albicans* virulence is orchestrated with a handful of bacterial species like *Streptococcus mutan*s, S*treptococcus oralis* and *Fusobacterium nucleatum*. On the other hand, biofilm formation abilities of such bacteria are reformed when interacting with *Candida albicans* (Allison et al., 2016; Baker et al., 2017; Diaz, Strausbaugh, & Dongari-Bagtzoglou, 2014; Janus, Willems, & Krom, 2016) We can infer from the *Candida albicans* interactions the importance of the oral mycobiome ecology (Bandara et al., 2019)

The virome of the oral microbiome is unique for individual hosts (Abeles et al., 2014; Naidu, Robles-Sikisaka, Abeles, Boehm, & Pride, 2014; Pérez-Brocal & Moya, 2018; Pride et al., 2012) Most of the virome are bacteriophages, mostly, the Myoviridae, Podoviridae and Siphoviridae families (Pérez-Brocal & Moya, 2018; Pride et al., 2012) The most abundant eukaryotic virus in the oral cavity is human Herpesviridae followed by Papillomaviridae and Anelloviridae(Baker et al., 2017; Pérez-Brocal & Moya, 2018) Beguilingly to explore, the extensive co-evolution, complex adaptation and exaptation of mobile genetic element is believed to modulate the ecology of the oral microbiome, which results in what has recently been termed "Ecological Diseases." (Hoare, Marsh, & Diaz, 2017)

1.1.3. Association of Human Oral Microbiome and Clinical Diseases

The healthy oral ecosystem is personalized. Nevertheless, it is, also, considered allostatic in the normal physiology of the host. This happens when dynamic interactions among elements of the microbiome and the host attain a homeostasis that can actively adapt to changing conditions in the oral cavity (Zaura & Cate, 2015) Accordingly, dysbiosis of the human oral microbiome is defined as failure of the host or microbial communities to adapt to the changing physiology of the buccal cavity. Dysbiosis of the human oral microbiome is statistically associated with various clinical diseases which raise questions, not only for the causality predicament, but also the fundamental etiology of the diseases and, thereby, possibilities for effective treatment. The diseases could be categorized into two categories: oral diseases and systemic diseases.

Oral disease includes caries, periodontal diseases, mucosal diseases and oral cancer. Systemic diseases include a plethora of gastrointestinal diseases, such as, irritable bowel syndrome, neurological diseases like Alzheimer's disease, endocrine diseases, such as, obesity, immunological diseases, such as, Rheumatoid Arthritis and cardiovascular diseases, such as, atherosclerosis. All of which have significant skew in the microbial communities in the polymicrobial biofilms on different buccal sites suggesting strong association and, surprisingly, causation of molecular etiology. These diseases are reviewed elsewhere as being out of the thesis's scope (Bowen et al., 2018; Cong & Zhang, 2018; L. Gao et al., 2018; Healy & Moran, 2019; Verma et al., 2018; Yangheng Zhang et al., 2018)

1.1.4. Mobile Genetic Elements as Key Members of the Human Oral Microbiome

Vital members of the human oral microbiome, yet understudied in this context, are the Mobile Genetic Elements (MGEs). The human oral microbiome has remarkable interplay among its elements at both the inter-genome and intra-genome levels. The biology of the mobile genetic elements is a key player in understanding the ecology, evolution and host response to the oral microbiome (Koonin Eugene V., 2016) The mobile genetic elements include integrative conjugative elements (ICE), transposons, group I and II introns, phages, prophages, plasmids, Insertion Sequence (IS) elements, Genomic Islands (GI), pathogenicity islands and selfish elements (Frost, Leplae, Summers, & Toussaint, 2005; Koonin Eugene V., 2016)

Most of the virome are bacteriophages living through most phyla of the oral bacteria (Pérez-Brocal & Moya, 2018; Pride et al., 2012) Both lysogenic prophages and lytic phages develop both adaptive and antagonized co-evolutionary red queen dynamics of the host bacteria (Baker et al., 2017; Fernández, Rodríguez, & García, 2018; Wahida, Ritter, & Horz, 2016; Yutin et al., 2018) Recently, there have been evidence of microbiome bacteriophages interacting with human mammalian cells albeit *in vitro* (Kaźmierczak & Dąbrowska, 2018) Beyond phages, the extensive horizontal gene transfer in the oral niche is predominantly central as the oral microbiome is primarily polymicrobial biofilm that facilitates exogenous flow of DNA (Bowen et al., 2018; Pinilla-Redondo, Cyriaque, Jacquiod, Sørensen, & Riber, 2018)

Horizontal Gene Transfer (HGT) provides augmented phenotype diversification upon co-evolution of the oral microbiome. This diversification outnumbers gene duplication leading to extensive co-speciation via elevated fitness cost of independent evolution (Shropshire & Bordenstein, 2016; Treangen & Rocha, 2011) The mosaic plastic nature of the bacterial communities enables release, passage and uptake of DNA – and more recently RNA – within and between species and kingdoms (Smillie et al., 2011) This communication of genotype occurs through different vehicles from secretion systems and integrative conjugative elements to, the newly discovered, bacterial nanotubes, viral-like gene transfer agents and membrane vesicles (Brimacombe, Ding, Johnson, & Beatty, 2015; Broaders, Gahan, & Marchesi, 2013; Dorward, Garon, & Judd, 1989; Dubey & Ben-Yehuda, 2011; Klieve et al., 2005; Koonin, 2016; Marrs, 1974; Wagner et al., 2017) Astonishingly, these transfers occur by unexpected rate and extent that reach estimates of 20% of the rate of point mutations (Vos, Hesselman, Te Beek, van Passel, & Eyre-Walker, 2015) It was calculated that on average there is 43.9 HGT event occur per one microbe in the human microbiome, at the time of the study. Thus, the whole human holobiont is considered a hotspot for HGT (L. Liu et al., 2012; Rosenberg & Zilber-Rosenberg, 2018) A study was conducted on human gut microbiome to analyze the HGT extent. By the compositional methods of calculating signature GC content, they found that up to 6.5% of all Open Reading Frames (ORFs) of all of the microbiome occurred by HGT (Sitaraman, 2018; Tamames & Moya, 2008)

Diving into the effector mechanisms for the host modulation of the HGT, the Cationic Anti-Microbial Peptides (CAMPs) of the innate immune system delivers a constant stress pressure on the microbial communities that collaterally empowers HGT events. This empowers evolutionary pathways required for adaptation (Andersson, Hughes, & Kubicek-Sutherland, 2016; Cullen et al., 2015; Wimley, 2010) Thus, HGT promotes resilience against host perturbations viciously allowing for numerous cascades like persistence until fitness adaptation, virulence to the host and resistance to host xenobiotics with quorum sensing orchestrating such endeavors among the microbiome population (Ferreiro, Crook, Gasparrini, & Dantas, 2018) This gave rise to what is known as microbiome-derived resistome (Adu-Oppong, Gasparrini, & Dantas, 2017, p.; Crofts, Gasparrini, & Dantas, 2017) Different physiology and conditions of the host affects the HGT-mediated evolution of the microbiome such as host diet, heavy metal exposure and antibiotic chemotherapy exposure (Ferreiro et al., 2018) This is a classic example of how elements of ecosystem evolve to escape local fitness optima in the ecological stability landscape. (Pál & Papp, 2017)

On another front, pitching on the HGT among the microbiome, it is noticeable the extensive evolutionary conflicts within individual microbe genome, among members of the same species, between different species, genera and, even, kingdoms (Ferreiro et al., 2018; Pinilla-Redondo et al., 2018) Certain communities of the oral microbiome produce bacteriocin that poses stress on other communities nearby (Burton et al., 2013; Hasannejad Bibalan, Eshaghi, Rohani, Pourshafie, & Talebi, 2017) The dental biofilm members *Streptococcus mutans*, *Streptococcus gordonii* and *Streptococcus salivarius* produce bacteriocins that negatively impact other species but itself. This is due to acquisition of several Integrative Conjugative Element (ICE) that codes for bacteriocin and its immune protein. Co-cultures of the two species mentioned above are capable of communicating and distributing the bacteriocin ICE. However, the other species in the community are susceptible to bacteriocins (Dahmane et al., 2017; Kreth, Merritt, Shi, & Qi, 2005; Mignolet et al., 2018) Another example is the bacteriolytic elements produced by the Type VI Secretion Systems in the ICE harbored by some species of the oral microbiome. This lysis occurs mainly for exogenous genetic uptake essential for competence in adaptive evolution as well as nutrition (Borgeaud, Metzger, Scrignari, & Blokesch, 2015; Coyne, Roelofs, & Comstock, 2016; Finkel & Kolter, 2001)

In the arm race between the microbiome, there are several systems that interplay in the evolutionary conflicts driving adaptation (McLaughlin & Malik, 2017) They are protection mechanisms for the genome if required. Model examples are the Restriction Modification (RM) systems and the CRISPR-Cas systems. A supporting example is the CRISPR-Cas system in periodontal biofilm bacteria *Aggregatibacter actinomycetemcomitans* that increases the transformability and cell competence in the oral microbiome thereby driving diversity (Jorth & Whiteley, 2012) Although both RM and CRISPR-Cas systems are being horizontally transferred, they operate as checkpoints for mobile genetic elements disseminating harmful horizontal gene transfer events to attain genome stability (Furuta, Abe, & Kobayashi, 2010; Kobayashi, 2001) Both are key players in HGT of the human oral microbiome.(Sitaraman, 2018)

The study of mobilome of the oral microbiome is still in its infancy (Martínez, 2018; Sitaraman, 2018) Although there have been several studies on the horizontal gene transfer events in the oral microbiome, several questions remained unanswered, such as, how these mobile genetic elements shape and “quorum-sense” communities as biofilms collaborating virulence and antibiotic resistance genes on the system level (Ferreiro et al., 2018) There are investigations on how phages and exogenous genes are invited or fought against depending on the need or the fitness cost. Studies attempted to explore mobile genetic elements that alleviate conflict molecular systems are insufficient to draw conclusive answers (Fernández et al., 2018) As being understudied in the human oral microbiome, it came clear that one molecular system holds a common role as described in the above questions; it suggests the chromosomal type II toxin antitoxin systems.

1.2. Toxin Antitoxin Systems

1.2.1. Introduction on Toxin Antitoxin Systems

The Toxin Antitoxin (TA) system appeared first in the literature in 1997 by Martin Holčík and V. M. Iyer (Holcík & Iyer, 1997)They categorized the post segregational killing effect of two components gene operons and labeled them as being a Toxin Antitoxin Systems (TAS). The first described TAS operon was in 1985 by Kenn Gerdes and colleagues (K Gerdes, Rasmussen, & Molin, 1986) They described a novel mechanism of plasmid maintenance in the population of *Escherichia coli*. At that time, it was known that the only plasmid maintenance mechanism is the partitioning system, which was less efficient with plasmids of low copy number as the bacterial replication could overwhelm the low copy numbered plasmids leading to limited partitioning. Additionally, several plasmids lack the partitioning system genes (Guynet & de la Cruz, 2011) Although the “Pilot-Fish” – like mechanism could drive the plasmid maintenance in such Partitioning – lacked plasmids, a negative selection hypothesis were believed to take place (Ogura & Hiraga, 1983) Gerdes group discovered the hok/sok system in the R1 plasmid that could apply a conditional negative selection pressure on plasmid-free cells; this is to say, the hok/sok system is killing of the plasmid free segregated cells (K Gerdes, Bech, et al., 1986)

Since then, the Post Segregation Killing (PSK) effect of several genes has been described in different plasmids which lead to their annotation by Holcík group as Toxin Antitoxin System. This is due to their neat molecular mechanism that involves two components: a stable toxic component that is rescued by a continuously expressed cognate antidote (Figure 1-1). Thus, in the progeny of mother cells, the labile antitoxin of lower half-life time than the toxin should be continuously expressed on the plasmid segregated cells, while the stable toxin is inherited through the cytoplasmic partitioning during replication. If the daughter cells are deficient of the plasmid, the antitoxin will be depleted at a higher rate than the toxin leading to killing of the plasmid-less daughter cells.

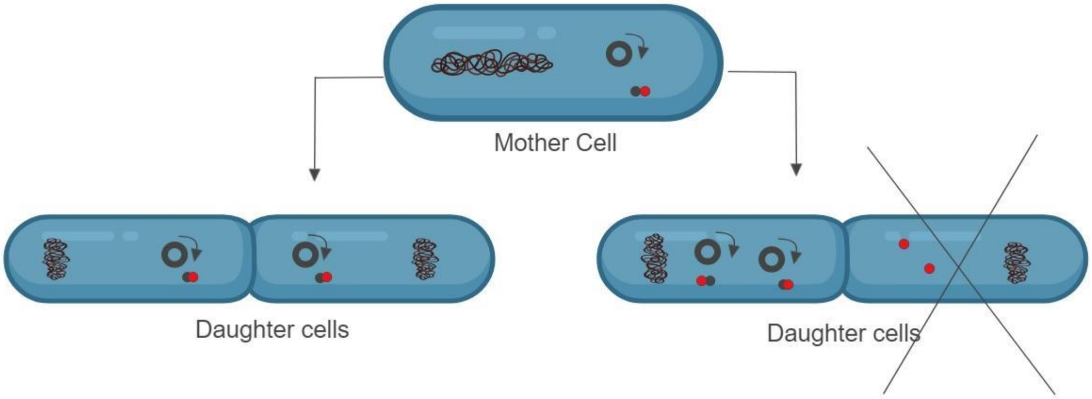

Figure 1-1: A cartoon diagram representing the basic idea of post segregation killing (PSK). of plasmid-less bacterial cells. The toxin (denoted as red dots) is expressed from the plasmid and inherited from the mother to daughters as they have high half-life time. On the contrary, the antitoxin (denoted as black dots) requires continuous expression from the plasmid; that is, the plasmid must be inherited for the daughter cell to survive the inherited toxin.

1.2.2. Classification of the Toxin Antitoxin Systems.

The Toxin Antitoxin Systems could be classified structurally and mechanistically. The common classification of the TAS is that according to the mechanisms of the neutralization of the antitoxin to the toxin. In that manner, the TAS has been recently categorized into seven types (Harms, Brodersen, Mitarai, & Gerdes, 2018a; Walling & Butler, 2019) On the other hand, the TAS families under each type are further classified based on the structural differences. This classification is sensible; as the mechanisms of action of the TAS has prominent association with their evolutionary patterns and abundance throughout the three life domains. Besides, structural homology is minimal between different mechanistic types of the TAS. Although there is a quite debate on the functions of the TAS, certain functions are allocated to certain types of the TAS which makes it more meaningful to classify the TAS according to the mechanisms of neutralization. Holistically, the antitoxins are proteins in case of types II, IV, V, VI and VII. In types I and III, the antitoxin are small RNAs (Goeders & Van Melderen, 2014; Page & Peti, 2016; Song & Wood, 2018a)

1.2.2.1. Type I Toxin Antitoxin Systems.

This type of the TAS has the active toxin in a protein form. However, the antitoxin is a small antisense RNA that binds to the toxin's mRNA and hinder its translation (Fozo, Hemm, & Storz, 2008; Kenn Gerdes & Wagner, 2007) The toxin protein is a small hydrophobic protein with a mechanism that is not-fully understood; however, it is believed to increase the cellular membrane potential through depolarization (K Gerdes, Bech, et al., 1986; Ono, Akimoto, Ono, & Ohnishi, 1986; Weaver et al., 2003) The Type I Toxins have transmembrane domains much like several antimicrobial peptides albeit here the toxic effect is self-destructive (Arnion et al., 2017; Henriques, Melo, & Castanho, 2006; Unoson & Wagner, 2008) As for the antitoxin, the regulation of the toxins is through direct binding to the ribosomal binding site by simple base-pairing. Although this is not the case for the most studied case of Type I Toxin Antitoxin System, the Hok/Sok system, some antitoxins bind to the mRNA of the toxins in other sites blocking standby ribosomes (Fozo et al., 2008; Reif, Löser, & Brantl, 2018) It is evident that the type I Toxin Antitoxin Systems are usually in tandem repeat. The chromosomal TAS in this type are known to be laterally transferred. This could limit its breadth of taxonomic distribution; nonetheless, it is merely apparent to be increasingly abundant (Coray, Wheeler, Heinemann, & Gardner, 2017; Fozo et al., 2010)

1.2.2.2. Type II Toxin Antitoxin Systems.

The Type II Toxin Antitoxin Systems are the most studied of the Toxin Antitoxin Systems. Both the toxin and the antitoxin are protein in nature. The type II toxins are small in size and have a large variety of target molecules. On the other hand, the type II antitoxins neutralize the toxin proteins. They, mainly, possess two domains; (1) DNA Binding Domain and (2) Protein Interacting Domain. The type II antitoxin proteins neutralize the toxin proteins by two main mechanisms; (1) They have direct interaction to the toxin protein through the C – terminal toxin binding domain and (2) the toxin-antitoxin complex exposes the N – terminus of the antitoxin and binds to the promoter of the Toxin Antitoxin Operon repressing the expression of the toxin. The DNA Binding Domain at the N – terminus is also responsible for the vast regulatory roles of the type II antitoxins that will be discussed below. The structure of the antitoxin is readily distorted exposing itself for proteolysis (Harms et al., 2018a; Makarova, Wolf, & Koonin, 2009a; Xia, Bao, Zhang, Linhardt, & Liang, 2019)

The Type II Toxin Antitoxin Systems can be *sensu lato* classified further into families. There are two systems for classification of Type II Toxin Antitoxin Systems. One that is based on toxin protein sequence similarity and the other is based on the domains of the toxin antitoxin systems independent from the proteins they are homologous to (Kenn Gerdes, Christensen, & Løbner-Olesen, 2005; Van Melderen & Saavedra De Bast, 2009)

The classification based on the toxin amino acid similarity is presented in the (Table *1-1*) which embodies the 14 Toxin Antitoxin Systems super families. Eleven of which are of two components, which are the toxin and the antitoxin. They are the super families: ccd, HicBA, HipBA, mazEF(chpA), parD (PemKI), parDE, phd-doc, relBE, vapBC (vag), mosAT and yeeUV. Three of the TAS super families are three component systems, having a regulator beside the toxin and antitoxin genes as will be discussed below in the regulation section. These super families are Omega Epsilon Zeta (ω-ε-ζ), pasABC and paaR-paaA-parE.

It was suggested to categorize the type II toxins super families and Type II antitoxins super families separately because of the extensive cross talk between the members of each super families in “mix and match” phenomenon (Leplae et al., 2011)

Table 1-1: The Type II Toxin Antitoxin Systems Classification according to toxin protein sequence similarity.

TAS SuperFamily	**TAS SubFamily**	**Toxin Component**	**Antitoxin Component**	**Reference***
Ccd	-		COG5302 CcdA	(De Jonge et al., 2010; Wilbaux, Mine, Guérout, Mazel, & Van Melderen, 2007)
		pfam01845 CcdB		
			pfam07362 CcdA	
			COG3609	
HicBA	-		COG4226 HicB	(Jørgensen, Pandey, Jaskolska, & Gerdes, 2009; Makarova, Grishin, & Koonin, 2006)
			pfam05534 HicB	
			COG1598	
		COG1724		
HipBA	-		COG1396 HipB	(Black, Irwin, & Moyed, 1994; Moyed & Bertrand, 1983; Rotem et al., 2010)
		HipA		
mazEF (chpA).	-		COG2002 AbrB	(Aizenman, Engelberg-Kulka, & Glaser, 1996; Engelberg-Kulka et al., 1998; Syed et al., 2011)
			COG2336 MazE	
		COG2337 MazF		
		pfam02452 PemK		
			pfam04014 AbrB	
parD (PemKI).	-	Kid (PemK).		(Kamphuis et al., 2007; Monti et al., 2007)
			Kis (PemI).	
parDE	-	COG3668 ParE		(Easter, Sobecky, & Helinski, 1997;
			COG3609	

TAS SuperFamily	**TAS SubFamily**	**Toxin Component**	**Antitoxin Component**	**Reference***
				Yuan, Yamaichi, & Waldor, 2011)
phd-doc	-	COG3654 Doc		(Garcia-Pino, Sterckx, Vandenbussche, & Loris, 2010; Gazit & Sauer, 1999; Lehnherr, Maguin, Jafri, & Yarmolinsky, 1993)
			COG4118 Phd	
		pfam05012 Doc		
relBE	higBA		cd00093 HTH_XRE	(Arbing et al., 2010; Tian, Ohnishi, Tabuchi, & Terawaki, 1996)
		COG3549 HigB		
		COG3550 HipA		
			COG4691 StbC	
			smart00530 Xre	
		pfam05015		
			pfam01381 HTH_3	
	relBE	COG2026 RelE		(Grønlund & Gerdes, 1999; Han et al., 2011)
			COG2161 StbD	
			pfam04221 RelB	
			COG3609	
		pfam05016		
	yefM-yoeB	YoeB		(Christensen et al., 2004; Yonglong Zhang & Inouye, 2009)
			YefM	

TAS SuperFamily	**TAS SubFamily**	**Toxin Component**	**Antitoxin Component**	**Reference***
	ygiTU (mqsAR).	MqsR (YgiU).		(Kasari, Kurg, Margus, Tenson, & Kaldalu, 2010; Kim et al., 2010)
			MqsA (YgiT).	
	prlF-yhaV	YhaV		(Schmidt et al., 2007)
			PrlF	
VapBC (vag).	-		cd00093 HTH_XRE	(Arcus, McKenzie, Robson, & Cook, 2011; Bloomfield, Whittle, McDonagh, Katz, & Cheetham, 1997; Cooper, Daugherty, Tachdjian, Blum, & Kelly, 2009)
		VapC		
			COG2002 AbrB	
			COG3093 VapI	
			COG4456 VagC	
		pfam01850 PIN		
			pfam04014 AbrB	
			smart00530 Xre	
		COG1848		
			COG3609	
		COG4113		
			COG4423	
			pfam01381 HTH_3	
mosAT	-	cl00973 DUF1814		(Wozniak & Waldor, 2009)
			pfam11459 DUF2893	
yeeUV	-	yeeV		(Brown & Shaw, 2003)
			yeeU	

TAS SuperFamily	**TAS SubFamily**	**Toxin Component**	**Antitoxin Component**	**Reference***
ω-ε-ζ (PezAT).	-	ζ zeta		(Mutschler, Reinstein, & Meinhart, 2010; Zielenkiewicz & Ceglowski, 2005)
			ε epsilon	
pasABC	-	PasB		(Matcher & Rawlings, 2009; Smith & Rawlings, 1998)
			PasA	
paaR-paaA-parE	-	ParE		(Hallez et al., 2010)
			PaaA	

*Representative bibliography is only mentioned in this table.

The other classification system for type II Toxin Antitoxin Systems is according to the TAS domains. This domain-based classification is suggested by Makarova et al. (Makarova et al., 2009a) The reason behind this classification is that it is flexible to accommodate novel Toxin Antitoxin Systems that are predicted computationally, since there are a lot of interplay and cross talk between domains of different TAS super families of the previous classification (Goeders & Van Melderen, 2014) The list of Toxin Antitoxin domains is presented in (Table *1-2*) and it has been concatenated by developers of the Toxin Antitoxin DataBase (TADB) (Xie et al., 2018a)

Table 1-2: Domain-based Classification of the Toxin Antitoxin Systems

TA pair	**T-domain**	**AT-domain**
Xre-HipA	HipA	Xre
Xre-COG5654	COG5654	Xre
Xre-COG2856	COG2856	Xre
Xre-Bro	Bro	Xre
Xre-YgiU	YgiU	Xre
Xre-DUF397	DUF397	Xre
Xre-Fic	Fic	Xre
Xre-PIN	PIN	Xre
Xre-MazF	MazF	Xre
Xre-GNAT	GNAT	Xre
Xre-RelE	RelE	Xre
COG5606-RelE	RelE	COG5606
RHH-RelE	RelE	RHH
AbrB-RelE	RelE	AbrB
PHD-RelE	RelE	PHD
RHH-COG2929	COG2929	RHH
RHH-GNAT	GNAT	RHH
RHH-Fic	Fic	RHH
RHH-PIN	PIN	RHH
RHH-MazF	MazF	RHH
XF1863-MazF	MazF	XF1863
PHD-MazF	MazF	PHD
AbrB-MazF	MazF	AbrB
AbrB-Fic	Fic	AbrB
AbrB-PIN	PIN	AbrB
MerR-PIN	PIN	MerR
COG2442-PIN	PIN	COG2442
COG2886-PIN	PIN	COG2886

TA pair	**T-domain**	**AT-domain**
PHD-PIN	PIN	PHD
PHD-Fic	Fic	PHD
HicB-HicA	HicA	HicB
HEPN-MNT	MNT	HEPN
ArsR-COG3832	COG3832	ArsR
YhfG-Fic	Fic	YhfG
Xre-COG3832	COG3832	Xre
COG2886-RelE	RelE	COG2886
Xre-COG2929	COG2929	Xre
COG5304-COG2929	COG2929	COG5304
PHD-MazFn	MazFn	PHD
COG1753-PIN	PIN	COG1753
COG2880-PIN	PIN	COG2880
PHD-SMa0917	SMa0917	PHD
COG5606-COG4679	COG4679	COG5606
COG5642-COG5654	COG5654	COG5642

1.2.2.3. Type III Toxin Antitoxin Systems.

The third type of the Toxin Antitoxin systems has the antitoxin in form of sRNA that interact with the proteinaceous toxin. What distinct this type of the TAS is their unusual stoichiometry of the RNA-Protein interaction (Goeders, Chai, Chen, Day, & Salmond, 2016) The Type III TAS cassettes are bicistronic operon with the antitoxin composed of several repeats of short nucleotide sequences. The toxin protein processes this sRNA into monomers of these repeats forming dynamic complexes of the toxin interacting with one or more of the antitoxin monomers (Short et al., 2013) Although the half life time of the antitoxin is relatively higher than the other TAS types, the antitoxin can neutralize several toxins via the monomers. Also, the genetic organization of the type III operon contains intra-genetic Rho independent terminator which favours the antitoxin expression over the toxin protein (Rao et al., 2015) Under stress condition, the antitoxin half-life is shorter than the toxin protein; therefore, it survives and out paces the antitoxin interactions in a fashion not fully understood (Goeders et al., 2016)

The most common families of the type III TAS are the TenpIN, CptIN and ToxIN (Blower et al., 2012) In spite of some sequence homology with the type II TAS, the families of type III do not cross talk with each other or with other types of TAS. This could be due to the distinct pseudoknots the sRNA of the antitoxins form that differ in each family of the type III TAS (Short et al., 2013) Besides its function as addiction module, the characteristic role of the type III TAS is the antiviral abortive infection systems. Unlike the CRISPR and the Restriction Modification systems that solely protects the bacterium cell, the abortive infection systems perform “altruistic suicide” to protect the population of the colony (Short, Akusobi, Broadhurst, & Salmond, 2018)

1.2.2.4. Type IV Toxin Antitoxin Systems.

Both the toxin and the antitoxin of this poorly studies type of the TAS are protein in nature. In this type, the antitoxin interacts with the target of the toxin protecting it from the poisonous activity of the toxin. Thus, there is no direct interaction between the toxin and the antitoxin; instead, each independently acts on the target (Masuda, Tan, Awano, Wu, & Inouye, 2012; Zhongling Wen, Pengxia Wang, Chenglong Sun, Yunxue Guo, & Xiaoxue Wang, 2017) The vastly studied example is the cbeA/cbtA system (formerly known as YeeUV system), in which the target is the MreB/FtsZ proteins of the bacterial cytoskeleton. The toxin cbtA stalls the polymerization leading to fatal wobbly cell wall; on the other hand, the antitoxin promotes polymer bundling (Brown & Shaw, 2003)

1.2.2.5. Type V Toxin Antitoxin Systems.

The type V Toxin Antitoxin Systems are discovered in 2012 as Page and Wood group analyzed the GhoST system. It is established as a novel type of Toxin Antitoxin System as the antitoxin mechanism of neutralization is unique. The type V antitoxins are ribonucleases that specifically targets the mRNA of the toxin (Wang et al., 2012)

1.2.2.6. Type VI Toxin Antitoxin Systems.

The ClpXP proteases are potent proteolytic systems that can act on the type VI toxin proteins after being activated by the type VI antitoxin proteins. The antitoxins are, thus, adaptors that affects the toxin proteins indirectly through Clp-facilitated pathway. This indirect inhibition of the antitoxin is the reason to categorize these systems as an atypical type of TAS, namely, type VI toxin antitoxin systems (Aakre, Phung, Huang, & Laub, 2013)

1.2.2.7. Type VII Toxin Antitoxin Systems.

The newly categorized Type VII Toxin Antitoxin Systems have the antitoxin gene codes for an enzyme that spontaneously deactivates the toxin by oxidizing the cysteine amino acid into sulphonic, sulphenic or sulphinic acid derivative (Marimon et al., 2016) This oxidation reduction reaction destabilizes the toxin making its half-life significantly shorter. This constitutes a novel mechanism of toxin neutralization as there is no stable complex formed between the toxin and the antitoxin. That been said had made it recently declared as a novel class of Toxin Antitoxin Systems (Song & Wood, 2018a) Thus far, Marimon and their colleagues characterized the Hha/TomB TAS member of this class leaving it open for further research (Marimon et al., 2016)

1.2.3. Roles of the Chromosomal Toxin Antitoxin Systems

The chromosomal Toxin Antitoxin Systems (TAS) have wide range of molecular functions that translates to few, yet critical, biological functions (Harms et al., 2018a) The driving force for the TASs are the functions of the toxins. The antitoxin role is regulation of the toxins' actions. Although several antitoxin have numerous other roles in the global transcription regulatory networks of the bacterial physiology, the toxins roles are much more diverse (Makarova et al., 2009a; Slayden, Dawson, & Cummings, 2018; Xia et al., 2019)

1.2.3.1. Molecular Functions

Toxins, by definition, are enzymes that compromise cellular normal physiology. They perform this at every step on molecular biology central dogma and, mostly, at very low relative protein concentration (Harms et al., 2018a, 2017; Klein & Klein, 2016)

Nucleases Toxins

The majority of such are nucleases. Under this class, toxins include ribosome-dependent mRNA endonucleases like that of the RelE Superfamily (Christensen & Gerdes, 2003; Pedersen et al., 2003) Also, ribosome-independent mRNA endonucleases such as the MazF and HicA families (Jørgensen et al., 2009; Masuda & Inouye, 2017; Sofos, Xu, Dedic, & Brodersen, 2015; Yonglong Zhang et al., 2003) Also, the tRNA nucleases and rRNA nucleases are common among TAS families, for instance, the PilT N-terminus PIN containing toxins (Winther & Gerdes, 2011) Finally for the nucleases class, there are DNAses present in, for example, RalRA TAS (Guo et al., 2014)

Transferases Toxins

Aside from the nucleases, toxins could alter the post translation machinery of the bacteria; the toxins Death on curing (Doc) and the HipA are kinases that phosphorylates the elongation factor EF-Tu and glutamyl-tRNA synthetase GltX, respectively (Castro-Roa et al., 2013; Germain, Castro-Roa, Zenkin, & Gerdes, 2013) The tRNA targeting acetyl transferases are the main action of the Gcn5-related N-acetyltransferases(GNAT) (Cheverton et al., 2016; Salah Ud-Din, Tikhomirova, & Roujeinikova, 2016; Yeo, 2018) The FicT toxin family are AMP transferases that targets enzymes of DNA replication, namely, topoisomerase and gyrase (Harms et al., 2015) PezT toxins interferes with peptidoglycan biosynthesis through phosphorylation (Mutschler, Gebhardt, Shoeman, & Meinhart, 2011) Lastly, the toxin component of the DarAT TAS is a reversible ADP-ribosyl transferases that act on DNA single strand (Jankevicius, Ariza, Ahel, & Ahel, 2016)

Oxidoreductase Toxins

Even though it is poorly understood, the Hok toxins are depolarizing agents that interrupts the ATP synthesis process at the plasmic membrane (K Gerdes, Bech, et al., 1986; Verstraeten et al., 2015)

Stoichiometric and steric hindrance

Unlike the topoisomerase inhibitor FicT discussed above, the CcdB toxin inhibits the topoisomerase, but through a different mechanism. It poses a steric hinderance upon physical interaction with the FtsZ or the MreB terminating the polymerization step and halting the DNA replication process with a non-enzymatic approach (Bernard & Couturier, 1992; Masuda et al., 2012)

1.2.3.2. Biological Functions

The topic of biological roles of the toxin antitoxin systems is heated subject in the scientific community. It can be classified into dynamic evolution and environment adaptation.

Dynamic Evolution

The Toxin Antitoxin Systems are known for their Post-Segregational Killing of plasmid-lost daughter cells as discussed above. As for the TAS in the chromosome, several studies had proven its addiction properties for the dynamic portions of the genome; i.e. Chromosomal Mobile

Genetic Elements. Examples for this include stabilization of the prophage CP4So of the *Shewanella oneidensis* (J. Yao et al., 2018), the IncA/C genomic island in *Salmonella* spp. (Huguet, Gonnet, Doublet, & Cloeckaert, 2016) , SsPI-1 pathogenicity island in *Streptococcus suis* (X. Yao et al., 2015), several prophages in *Bacillus subtilis* (Durand, Jahn, Condon, & Brantl, 2012), the integrative conjugative element ICEAfe1 is addicted to the genome of *Acidothiobacillus ferroxidans* (Bustamante, Tello, & Orellana, 2014), the ICE SXT is maintained through the *Vibrio cholera* genome by a TAS (Wozniak & Waldor, 2009) and, finally, a Super Integron (SI). is the first one to be verified that it can be diminished upon loss of the TAS found in the SI set of genes, which means it is indeed addicted to the chromosome (Szekeres, Dauti, Wilde, Mazel, & Rowe-Magnus, 2007) In spite of this, certain discrepancies and concerns on the experimental design for the functional assays of such TAS role (Song & Wood, 2018a)

The addiction can be for the unit gene as a selfish element within the genome of the same bacteria. The TAS maintained elements can be observed along with housekeeping genes localized near the origin of replication. This means that in an intra-genomic conflict, as a gene, it has high selfishness character over other genes of the bacteria (Rankin Daniel J., Turner Leighton A., Heinemann Jack A., & Brown Sam P., 2012) The selfishness of the TAS should not be taken as being only pathogenic to the genome. In such evolution, the bacteria mostly kept the TAS associated with beneficial impact to the bacteria (Ramisetty & Santhosh, 2017)

The TAS have anti-addiction functions. This means that the genome will not accept mobile genetic elements, plasmids or phages much like an immune system for the bacteria like the Restriction Modification (RM) systems and the clustered regularly interspaced short palindromic repeats CRISPR-Cas systems. On the other hand, the mobile genetic element, plasmid or phage get to choose its host. This happens by the incompatibilities of different Toxin Antitoxin Systems families where the cross-talk regulation favors the expression of the toxin for the uninvited MGE (Saavedra De Bast, Mine, & Van Melderen, 2008)

The abortive infection system is an innate immune system for bacteria where the infected individual altruistically suicide for the benefit of the population. This happens through the activation of the toxin of the TAS when the bacteriophage enters the bacteria and before its replication. This makes the TAS, especially Type III and IV, potent abortive infection systems (Dy, Przybilski, Semeijn, Salmond, & Fineran, 2014; Dy, Richter, Salmond, & Fineran, 2014; Goeders et al., 2016)

Environment Adaptation

Under stress conditions and extreme environment, such as therapeutic antibiotic use, the bacterial population can tolerate such environment by keeping certain subpopulation as dormant as possible leaving out any energy expenditure. This phenomenon is known as persistence. The Toxin Antitoxin Systems (TAS) are an integral part in this pathway. With certain concentrations of the toxin, the cell become dormant and quit all translational biosynthesis cellular processes (Ronneau & Helaine, 2019) When the environment conditions return favorable, the antitoxin is expressed again and regulate the toxin actions reversing back the dormant persisters to normal population. This phenomenon is of tremendous importance as this adaptation allows the bacteria to evolve resistance mechanisms; besides, clinically, bacterial eradication from the host is difficult (Harms, Maisonneuve, & Gerdes, 2016; Page & Peti, 2016) Moreover, the persisters population often become heterogenic. These bet hedging phenotypes signals the rest of the population for a quicker response to the stress condition (Veening, Smits, & Kuipers, 2008) As global regulators, the Toxin Antitoxin Systems are implicated with extensive regulations of quorum sensing and biofilm formation (Chan, Espinosa, & Yeo, 2016; Wang & Wood, 2011; Wen, Behiels, & Devreese, 2014) These roles put the Toxin Antitoxins Systems as a clinically interesting target for drug design of novel anti-infective therapies and key molecular system to study to better understand basic science of bacterial communities including that of biotechnological – industrial microbes.

1.2.4. Importance of the Toxin Antitoxin Systems

In the human oral microbiome, the toxin antitoxin systems have a lot of potential in translational medicine. Understanding the biology of the TAS helps in properly understanding the etiologies of oral and systemic diseases. On the other side, understanding the toxin antitoxin systems dynamics on a population level with a rich arena of the oral ecosystem is principal for making use of the TAS in numerous applications from high-yield industry to synthetic biological circuits.

There are numerous applications for the Toxin Antitoxin Systems. Possibly, we can design novel antibacterial without emerged resistance to it, which is a topic, currently, under extensive research. However, we will focus on the biotechnological applications of the Toxin Antitoxin systems as there are several up-to-date reviews on the clinical or chemotherapeutic applications elsewhere in the literature (Bassegoda, Ivanova, Ramon, & Tzanov, 2018; Harms, Brodersen, Mitarai, & Gerdes, 2018b; Khusro, Aarti, Barbabosa-Pliego, & Salem, 2018; van Geelen, Meier, Rehberg, & Kalscheuer, 2018; Q. E. Yang & Walsh, 2017)

A Tool in Cloning

DNA cloning has the problem of low frequency of the DNA insert. In addition to the problem of plasmid loss after several generations in the transformed cells. Therefore, Toxin Antitoxin systems were used as a toolbox in designing kits that enhance the cloning frequency. StabyCloning^{TM} system and Gateway ^{TM} system are examples for kits that used CcdAB TA module for stabilizing the cloned DNA fragment insert (Stieber, Gabant, & Szpirer, 2008; Unterholzner, Poppenberger, & Rozhon, 2013) There are different ways to positively select the insert cloned. The insert could contain a toxin gene that is disrupted upon the insertion of the DNA fragment to be cloned. If the vector has the insert, the toxin will not be activated. Thus, positively selecting for the transformed hosts that have the insert (Bukowski, Rojowska, & Wladyka, 2011) Another strategy is to design the vector to have a fragment of the antitoxin making the antitoxin inactive. However, the insert is ligated with the remaining fragment of the antitoxin that, if inserted successfully in the correct orientation, will produce a complete active antitoxin that will rescue the host cells form an inducible toxin expression (Stieber et al., 2008) The above-mentioned Gateway^{TM} system uses another approach. The recombination sites attB1 and attB2 are to be flanking around the insert to be cloned. The vector should include the recombination sites attP1 and attP2 besides the integrase enzyme and the integration host factor. In the vector the attP1 and attP2 sites flanks the toxin component of the CcdAB system. Here, a powerful selection will occur to the vectors that underwent integrase-mediated homologous recombination; the toxin is replaced by the insert (Stieber et al., 2008)

A Tool for Expression Vectors for Protein Production

There are several major challenges for the protein production and gene expression systems. The plasmid is not stable and/or maintained throughout several generations of the microorganism. This expression vector could be lost due to its fitness cost. Other plasmids that lack the expression of the protein are more robust in their growth and replication than the expression vector, which lead them to be selected over the expression plasmid. The use of antibiotics to place a selective pressure on the plasmids could be an answer. However, it has several drawbacks. The product is at danger of contamination with antibiotics that could have side effects on the consumers. The release of the antibiotic or antibiotic resistance in nature is risky. In large scale production, there is a problem that resistant cells could deactivate the antibiotic in the bioreactor leading to higher chances for competitor plasmids to outgrow the expression plasmid. Accordingly, the Toxin Antitoxin system could be used to avoid these drawbacks and increase the yield of protein production. Similar to its natural role in Post Segregationally Killing (PSK), TA systems could offer marvelous solution for plasmid stability (Pecota, Kim, Wu, Gerdes, & Wood, 1997) Several strategies from this concept are out there. The toxin gene could be engineered in the genome and the antitoxin on the expression vector; that way the plasmid is addicted to the host. One of the most successful systems is the food grade expression system developed in *Bacillus subtilis* in 2016 (S. Yang, Kang, Cao, Du, & Chen, 2016)

Single Protein Production systems

One of the systems to have higher yields out of an expression vector is the design of a Single Protein Production (SPP) system. In *Escherichia coli*, the toxin component of the MazEF TA system is an endoribonuclease enzyme that can act to interfere with the total mRNA of the cell. Specifically, the MazF toxin targets the degradation of the mRNA containing the sequence ACA. The cell growth is arrested; however, the translation machinery was still operating for four days after the induction of the toxin. The protein of interest to be produced is engineered to lack the sequence ACA (ACA-less mRNA). Upon inducing the toxin expression, all mRNA of the host is degraded except for the ACA-less mRNA of the protein of interest. All resources of the cell will be available for this single protein to be expressed in high yields. This system is proved to be superbly successful (Suzuki, Zhang, Liu, Woychik, & Inouye, 2005)

A Tool to Target Cell Ablation in Plants

For developing model systems, a cell ablation tool was developed using the Toxin Antitoxin system. The antitoxin is derived by a promoter that is expressed in all plant tissue except the only one to be ablated. On the contrary, the toxin is downstream of a promoter that is specifically expressed in the tissue to be ablated. This system was proven to be successful (Baldacci-Cresp et al., 2016)

Stable Killer Switch

A synthetic genetic circuit was developed using the Toxin Antitoxin systems as a switch to kill the bacteria *Escherichia coli* in response to a certain stimulus. This killing switch is designed to be evolutionary stable. This tool is extremely powerful; it can be used in different applications. The microbial growth in mammalian gut is controlled by the temperature as a killer switch (Stirling et al., 2017) Another application is a killer switch that is engineered to bacteria that escaped certain path or to control the bacteria for containment. The unwanted bacteria are switched to be killed by a certain stimulus, for example cold shock causing "Cryodeath" (Boettner, 2017) This phenomenon hold humongous potential as the killer switch can be used for bacteria that perform certain therapeutic and/or theranaustics role giving it a license to kill and, then, by the killer switch the bacteria itself is killed, meaning the killing machines are temporally and spatially controllable.

Inducible Cell Lysis Systems in Bio-production

One of the problems that bio-production faces is the release of the protein expressed outside of the cells. Mechanical, chemical or enzymatic cell lysis techniques are commonly used to release the protein for further purification. Here, the researchers could engineer a cell lysis system that is cost-efficient, controllable, reproducible and efficacious. The regulatory system involves the Toxin Antitoxin system that is conditionally inducible to lyse the cells after they complete their role in the bio-reactors (Y. Gao, Feng, Xian, Wang, & Zhao, 2013)

Engineering Resistance to Phage in Bioreactor Cells

The bacteria *Geobacillus thermoglucosidasius* is used in biofuel production. However, the common problem to these bacteria is phage infection that drastically affect the fermentation process. The toxin csaB of the TA system is engineered to promote the resistance to the phage GVE3 that hinder the bacteria from biofuel production, thus, leading to higher viability in the bacterial population (van Zyl, Taylor, & Trindade, 2016)

A Reporter for Identification of Quorum Sensing

The phenomenon of Quorum Sensing (QS) in prokaryotes changed our view of the microorganisms. They are sociable with several communication pathways and signals intraspecies and interspecies. However, not all microorganisms and/or all pathways are discovered. In fact, there is a deep need in detecting new signal pathways in both quorum sensing and quorum quenching as both are potential novel chemotherapies. Identifying the biomolecules involved in these pathways are made easier using a reporter system. The reporter system, here, is *Escherichia coli* that is engineered with CcdAB TA system linked to promoters that can detect the QS families of signals in both Gram-positive and Gram-negative bacteria, namely, acyl homoserine lactone and autoinducer-2 families. The reporter system was validated and were able to detect the QS of 34 species (Weiland-Bräuer, Pinnow, & Schmitz, 2015)

TA Systems and Bio-photonic Imaging

Imaging of bacteria during the disease in real-time manner is important to study pathophysiology and etiology of infectious diseases. This is possible through luminescence optical imaging. The bio-luminescence technology has many benefits to illuminate the sample for real-time optical imaging. The Toxin Antitoxin systems can play an important role in developing this technology. For example, the research group at University of Auckland developed a system using ω–ε–ζ TA system; it stabilized the plasmid containing luciferase reporter genes in Group A *Streptococcus* (GAS) strains. The bioluminescence signals were quantifiable to be analyzed in culture and animal disease models (Loh & Proft, 2013)

TA in Genome Editing

Genome editing revolutionized our approach in biotechnology. Now, no viral vectors or plasmids would remain in the host. The application arena is vast, starting from crop editing bypassing the GMO labelling regulation dilemma to advancing medical and therapeutic tools. With the appearance of tools like CRISPR and TALEN, genome editing became much easier process. However, the off-targeting remains a problematic drawback (Germini et al., 2018)

Although the system works on Gram-positive bacteria only, the genome editing tool toxin counter–selectable cassette regulated by an antitoxin switch (TCCRAS) holds a very promising versatile tool for genetic engineering and synthetic biology. Several approaches could be done with this tool including, large scale deletions, in-frame deletions, point mutations, large scale insertions and gene knock-ins. For example, the researchers successfully engineered Lycopene biosynthesis pathway in *Bacillus subtilis* using TCCRAS claiming minimal off targets. In the TCCRAS tool, the Toxin Antitoxin system RelBE has been adapted to a switch with inducible promoter and the whole system is harbored on plasmid that is integrated in the chromosome (Wu et al., 2018)

1.3. Objectives

The Toxin Antitoxin Systems have huge potentials in both the industry and on the bed side. However, to fully understand the TAS, we should understand them collectively on the system level in bacterial populations, especially those related to the dynamic human host niche with rich HGT events like that of the human oral microbiome. This project aims to study the distribution, abundance and the evolutionary ecology of the chromosomal type II toxin antitoxin systems in the human oral microbiome which requires hitherto predictions of putative toxin antitoxin systems in such microbial community.

2. METHODOLOGY

2.1. Ethical Statement

All of the human sampling in this study has been approved by the Institutional Review Board (IRB) of the American University of Cairo which adequately address the participating subjects of this study in an ethical manner.

2.2. Microbiome Analysis

Three samples were taken from a 26-years-old Egyptian, presumably, healthy individual claiming good oral hygiene. The location of the sampling was in Atlanta, United states of America (33.7590 N 84.3987 W). The samples were taken by gentle rubbing of a sterile cotton-headed tip to the left canine supragingival and subgingival dental plaque. The third sample was for the buccal mucosa neighboring the same canine tooth. The sample was sent to Zymo-Research® Company for targeted 16S rRNA sequencing.

The ZymoBIOMICS® 96 MagBead Kit (Zymo Research, Irvine, CA) was used for total DNA extraction of the three samples using the company's manual protocol. The Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA) was used to prepare the samples for targeted sequencing of the 16S ribosomal RNA gene variable regions of V3-V4 using customized primers. PCR reactions were conducted in real-time PCR machines to control cycles. qPCR fluorescence readings quantified the final PCR products and pooled them together on equal molarity basis. The Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA) cleaned up the final pooled library. Afterwards, the pooled library was quantified with TapeStation® and Qubit®. Illumina® MiSeq™ was used for sequencing the final library prepared. This was done with a v3 reagent kit (600 cycles) with >10% PhiX spike-in.

Two methods were applied for unique amplicon sequences identification and chimera errored sequences; one using vsearch version 1.41.3 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016; Schloss et al., 2009) The other is using the Dada2 pipeline. (Callahan et al., 2016) Then, Sequence data were deposited in NCBI Genbank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2005) with assigned accession number, BioProject number and BioSample number that are mentioned in the results section. Taxonomic assignments were done using three databases as reference, an internal script with Zymo's own curated 16S database, GreenGene database (DeSantis et al., 2006) and SILVA database (Quast et al., 2013) These assignments were done using Uclust from Qiime version 1.9.1(Bolyen et al., 2018) and Mothur version 1.35.1 (Schloss et al., 2009) Composition visualization of box plots and Bray-Curtis dissimilarity heatmaps, alpha-diversity and beta-diversity analyses were performed with Qiime v.1.9.1 (Bolyen et al., 2018) and plotly in R (https://www.rstudio.com/).

2.3. Toxin – Antitoxin Systems Prediction

2.3.1. Retrieval of Genomic Data

From the genera found in the oral microbiome, selected species were chosen for downstream analysis. The genomic data were retrieved from NCBI genome database and RefSeq that showed sequence similarity with 16S rRNA gene as assigned from the SILVA database. The fifty most abundant Operational Taxonomic Units (OTUs). that are more than 97% similarity with the afore-mentioned SILVA database were included in downstream analysis.

2.3.2. Pipeline for Prediction

The database Toxin Antitoxin Database (TADB) is the principal database used in the primary detection of the type II toxin antitoxin systems (Xie et al., 2018b, p. 0) Using the tool TAfinder (http://202.120.12.133/TAfinder/), the genomic data was run through BlastP (McGinnis & Madden, 2004) with e-value threshold of 0.001 against TADB where the Coding Sequences (CDS) of each genome were blasted and results of the toxins and antitoxins were only considered if there are genomic interspace that ranges from being overlapped by 40 bases and up to interspace distance of 300 bases. Another search is done against TADB Hidden Markov Model profiles through HMMER (Finn, Clements, & Eddy, 2011) with e-value threshold of 0.01.

Afterwards, the output of such processes was comprehensively analyzed by Position Specific Iterated Blast (PSI Blast) of minimum six iterations or upon convergence. Additionally, it was concurrently used with Pattern Hit Initiated Blast (PHI Blast) and Reverse PSI Blast using MEME, InterproScan or CD-search to detect distant similarities with highly similar domain architecture (Altschul, Gish, Miller, Myers, & Lipman, 1990; Altschul et al., 1997; Bailey, Williams, Misleh, & Li, 2006; Jones et al., 2014; Marchler-Bauer et al., 2002; Mitchell et al., 2018; Z. Zhang et al., 1998)

Homology modelling of the 3D structure of selected potential TA loci was done using either of I-Tasser or SWISS Model based on the TAS in question (Benkert, Biasini, & Schwede, 2011; Bertoni, Kiefer, Biasini, Bordoli, & Schwede, 2017; Bienert et al., 2017; Guex, Peitsch, & Schwede, 2009; Schwede, Kopp, Guex, & Peitsch, 2003; Waterhouse et al., 2018; J. Yang & Zhang, 2015, 2015) Then, information was analyzed for the structural similarity with BtoxDB database of Toxin Antitoxin Systems structures from Protein Data Bank using flexible jFATCAT algorithm (Barbosa, Garrido, & Marchetto, 2015; Prlić et al., 2010; Ye & Godzik, 2003)

TA loci that directly inferred from above pipeline were manually curated for annotation. For novel TAS declarations, manual curation and literature-based study of each individual potential TA loci along with the assembly of motifs and domains architecture, families and super families were done to declare them or demote them as putative novel Toxin Antitoxin Systems. Toxin Antitoxin genes nomenclature was avoided and only membership to TAS super-families and families classification was employed in this study.

2.4. Toxin Antitoxin Systems Abundance and Diversity

The Toxin Antitoxin Systems (TAS) abundance was calculated in different contexts. Abundance of the TAS in the oral microbiome, relative molecular abundance of the TAS in the pool of genes of the oral microbiome and heatmap of specific abundance pair-wised for TAS in each OTU were visualized and graphed by GraphPad Prism, R studio, and heatmapper program (Babicki et al., 2016; Khan, 2013; Swift, 1997) Circos is used for tabular visualization of TAS domains abundance in OTUs of the oral microbiome (Krzywinski et al., 2009) The diversity of the Toxin Antitoxin Systems is calculated by different indices. These are Berger-Parker index, Margalef index, Simpson indices, Shannon index and Pielou index (Berger & Parker, 1970; E. K. Morris et al., 2014; Shannon, 1948a; Simpson, 1949)

2.5. Toxin Antitoxin Systems Network

Undirected protein – protein interaction network was built for the toxin antitoxin systems using both CytoScape version 3.7.1 and OmicsNet using internal scripts for calculation of necessary centralities of degree, betweenness, eigen vector, transitivity and closeness (Kofia, Isserlin, Buchan, & Bader, 2015; G. Zhou & Xia, 2018, 2019)

2.6. Toxin Antitoxin Systems Evolutionary and Phylogenetic Analysis

Molecular Evolutionary Genetic Analysis (MEGA) version X is the principal tool used to compute phylogenetic analysis of the Toxin Antitoxin Systems predicted in the Oral Microbiome (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) The putative TAS loci were multiple-aligned using MUSCLE accessed from MEGA X. (Edgar, 2004) Then, a best fit model was calculated to be used to produce bootstrapped Maximum Likelihood phylogeny using MEGA X (Felsenstein, 1973; Henderson, 2005) Finally, test of molecular clocks was computed for the TAS phylogeny (Goldman & Whelan, 2000; Tajima, 1989; Whelan & Goldman, 2001) Molecular allometric analysis was done by comparing genome size with number of genes and/or number of TAS loci by linear regression analysis of Pearson’s correlation represented on a scatter plot with linear regression line visualized through GraphPad Prism tool (Swift, 1997)

2.7. Statistics

Detailed statistics are described in place as per the statistical tests’ results inference. The statistical analysis employed in this study tests the null hypothesis and considers significance in rejection and/or failure of rejection by p-value of maximum value 0.05, unless, otherwise specifically stated elsewhere in the thesis document.

3. RESULTS AND DISCUSSION

This project aims to study the chromosomal type II Toxin Antitoxin Systems in the oral microbiome in terms of predictions, abundance, distribution, diversity, network and evolution. The reason for this study in the oral microbiome is because of the dynamics of this rich niche. Recently, studies on the association of such niche with oral and systemic pathologies are extensive and show insufficient understanding of the etiology (Healy & Moran, 2019; Krishnan et al., 2017) Thus, diving into the molecular systems might hold clear insights for the behavior of the microbiome related to these clinical diseases. As a primer to this approach, predictions and distribution of the toxin antitoxin systems in the oral microbiome is the core of this project.

Contrast to the whole systems approach, the chromosomal type II toxin antitoxin systems had been studied comprehensively in isolated species. As of January 2018, the toxin antitoxin systems have been studied in 613 species with a total of 5634 predicted TAS, of which 144 TAS have been validated experimentally (Xie et al., 2018). However, there are limited studies associates specifically with the oral species; example of these include *Aggregatibacter actinomycetemcomitans, Streptococcus mutans, Treponema denticola, Lactobacillus rhamnosus, Bacillus globigii, Streptococcus pneumoniae* and *Haemophilus influenzae* (Ahn & Rice, 2016; Dufour et al., 2018; Krügel et al., 2015; Mitchell et al., 2010; Schneider, Weigel et al., 2018; Sijbrandij et al., 2014; Tikhomirova et al., 2018). Clearly, the toxin antitoxin systems are studied in isolated species leaving space for research in studying TAS roles in systems of multicellular organismal behavior of microbiomes.

In environmental microbiomes, there had been few attempts to study the TAS primarily to understand their roles in stress response, persisters formation, quorum sensing, disseminating antimicrobial resistance genes and virulence determinants (Hõrak & Tamman, 2017). In the abiotic environment, studies on a polluted river called Zenne in Brussels, Belgium showed metagenomic clusters of TAS in proximity to beta-lactamase resistance gene and histidine biosynthesis operon (Vercammen et al., 2013). Another study was conducted on metagenome of the ground water where plasmodial TAS were found spanning multimetal resistance genes (Kothari et al., 2019). For wastewater environments, metagenomic studies on integrons revealed several hypothetical proteins that are implicated with toxin antitoxin systems among other stress related genes (Gatica et al., 2016). Most interestingly, a study was done on the microbiome of decaying wood and soil of a rainforest in Puerto Rico. They studied a special category of the microbial interactions in the microbiome that involves the toxin antitoxin systems, secondary metabolites, fitness genes, quorum sensing and quorum quenching systems. These systems are main players of the complexity nature of the microbiome, for which they specifically termed these as the “sociomicrobiome.” The researchers promote studying other ecosystems based on the same concept (Santiago-Rodriguez, Toranzos, Bayman, Massey, & Cano, 2013).

Aside from the sociomicrobiome, there has been recent studies on the complexity of microbial interactions in biotic environments. A metagenomic study on the marine sponge showed highly developed organismal behavior such as specialization of metabolic pathways, but universal unity in defense interplayed by global regulatory network of toxin antitoxins systems and restriction modification systems (Slaby, Hackl, Horn, Bayer, & Hentschel, 2017). The human microbiome projects that involves TAS analysis are few. In 2019, Danilenko research group had comprehensively studied the Toxin Antitoxin Systems in the gut microbiome for developing software called TAGMA (Klimina et al., 2019). Finally, a comprehensive study on all metagenome of the human microbiome project that is deposited in the ftp website for NCBI before February 2014 was done for CRISPR -Cas systems prediction and analysis. This study collaterally predicted neighboring Toxin Antitoxin Systems in several body niche including the oral microbiome (Zhang, Doak, & Ye, 2014). As obvious, there is a lack in the research of the Toxin Antitoxin Systems in the Human Microbiome.

By searching the literature, from June 2017 till April 2019, by several keywords including logic combinations of “Toxin”, “Antitoxin”, “Oral”, “Microbiome” and “Metagenome” in public databases of “PubMed” (https://www.ncbi.nlm.nih.gov/pubmed/), there is no research journal article that discusses the analysis of the toxin antitoxin systems in the human oral microbiome. Therefore, the importance of this project, which analyze the human oral microbiome of a healthy individual, lies in understanding roles of molecular systems of the oral microbial interactions that influence pathogenesis.

3.1. Human Oral Microbiome Analysis

The analysis of the targeted sequencing of the 16S rRNA gene of three samples taken from the biofilms of the supragingival, subgingival plaques and buccal mucosa. The samples are termed oral 1, oral 2 and oral 3, respectively. They are presented in terms of taxonomy composition and abundance (From Figure *3-1* to Figure *3-3*). This Targeted Locus Study project has been deposited at DDBJ/EMBL/GenBank under the accession KCXV00000000. The version described in this thesis is the first version, KCXV01000000. The BioProject accession number is PRJNA527703 and the BioSample accession number is SAMN11158134. The total OTUs are 89601 sequences. Figure 3-1 and Figure 3-2 are box plots that visualize the abundance percentage of the phyla, genera and species of the oral microbiome. Different colors represent different taxa. The legends for figure colors are omitted for redundancy and simplicity. The heat map represented in (Figure 3-3) denote the abundance of each unique Operational Taxonomic Unit (OTU) of those used in the downstream analysis for TAS predictions. The Microbiome abundance table S1 is in the appendix section.

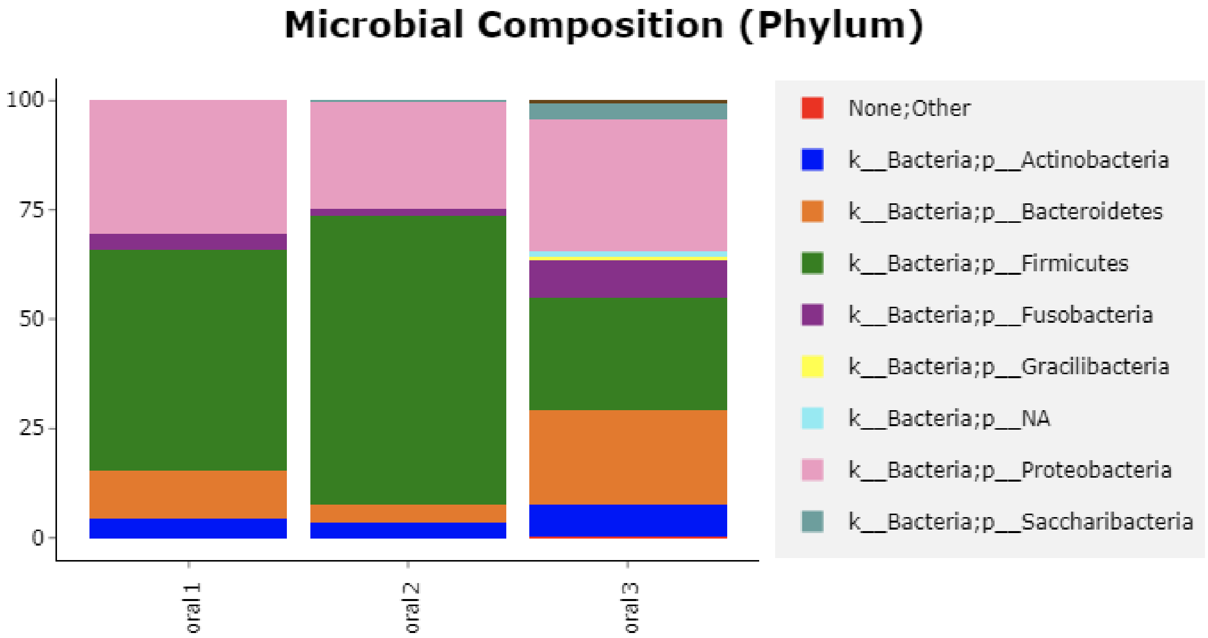

Figure 3-1: The composition of the microbial community in the oral microbiome from three different samples from supragingival, subgingival plaque and buccal mucosa respectively as oral 1, oral 2 and oral 3. The composition on the level of phyla is represented by different colors as represented.

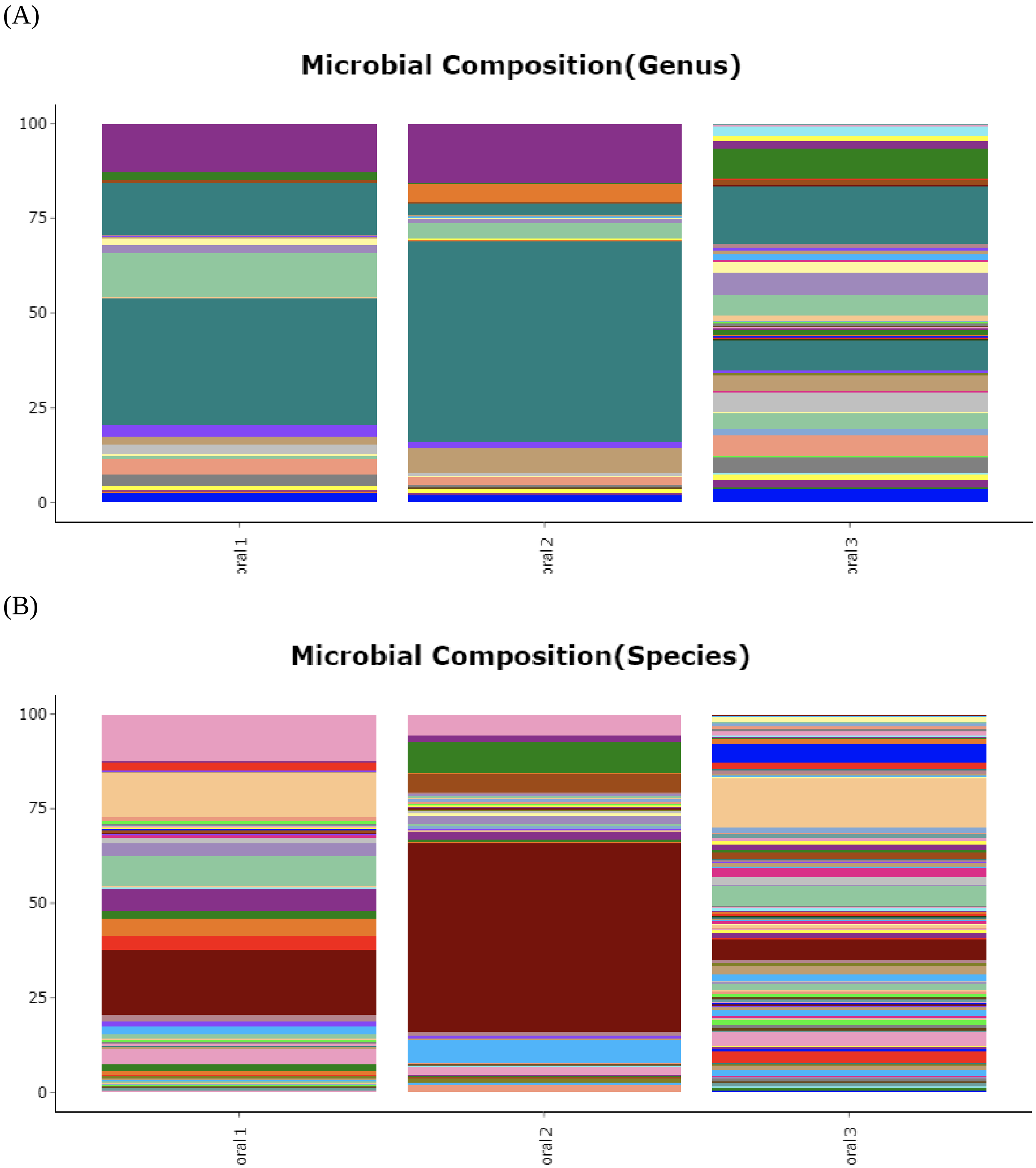

Figure 3-2: The composition of the microbial communities in the oral microbiome from three different samples from supragingival, subgingival plaque and buccal mucosa respectively as oral 1, oral 2 and oral 3. Figure (A) shows the composition on the genus level and figure (B) on the species level with different colors representing abundance. Refer to text for details of the taxonomy.

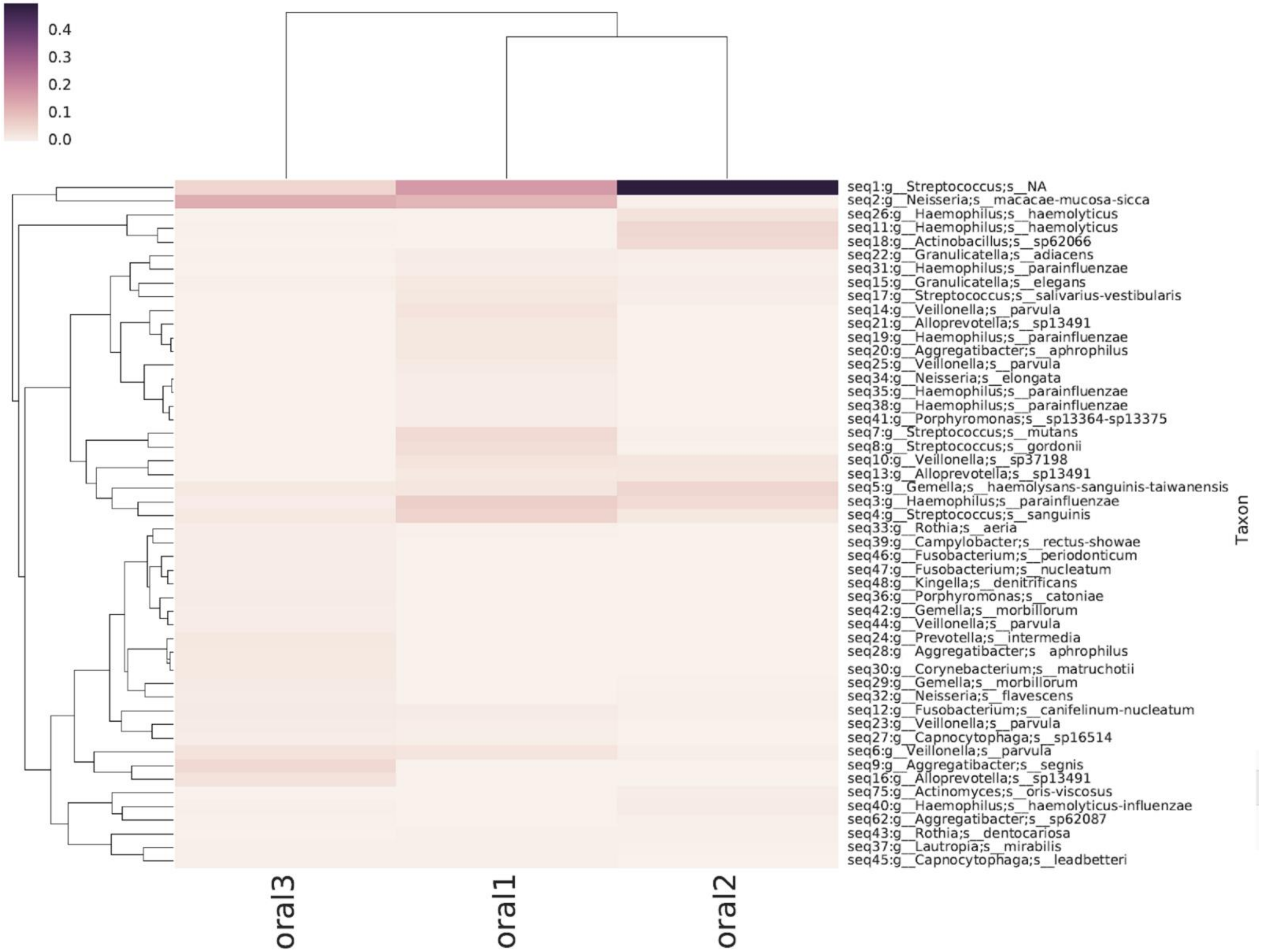

Figure 3-3: Heatmap for Unique Operational Taxonomic Units in three samples from the Oral Microbiome.

Three samples of the oral microbiome were taken from different sites in the oral microbiome. Although these sampling sites were not meant to be studied vis a vis comparing each to another, the analysis is done separately for each sample. These sampling sites are specifically chosen because they are of the thickest and rich biofilms in the oral microbiome compared to other biofilms of the hard palate, soft palate, …etc. (Bowen et al., 2018; Mark Welch, Rossetti, Rieken, Dewhirst, & Borisy, 2016) However, the reason of taking the samples from the canine tooth is just for ease of access.

Much like the literature, the most abundant phyla here in this study for the subgingival and supragingival plaque is the Firmicutes (Escapa et al., 2018; Mark Welch et al., 2016) In the third sample of the buccal mucosa, it showed higher abundance in the Bacteroides in more or less the same as Firmicutes. This coincides with the more abundant phyla of the gut mucosal biofilms which is the Bacteroides (Barko, McMichael, Swanson, & Williams, 2018; Sweeney & Morton, 2013) The most abundant genus in the supragingival and subgingival plaque is the Streptococcus, while that of the buccal mucosa is the Neisseria. It is evident from (Figure 3-2) that the buccal mucosa has higher richness of taxa over the other two samples. In fact, heatmap in (Figure 3-3) shows clustering of the gingival plaques samples together and out-grouping the buccal mucosa.

The diversity of the three samples are observable and quantifiable by different calculations. The diversity within the samples is called alpha diversity. The calculations for this diversity are best visualized by rarefaction curves. The brilliance of these methods lies on the fact that the diversity potential of the samples is assessed accurately. For example, the diversity index for the number of assigned species is measured for a very low number of sequences. Then, a plot for gradually increasing number of sequences is done with measuring the diversity for each point. This increase in diversity, that is due to increase in the observed sequence, is a better measure than just assessing the richness of the assigned taxa for the total sequence because the diversity could increase as a function for increasing observed sequence and not for the innate diversification of the sample. Here, in the rarefaction curves, the innate diversification is observed after attaining plateau of the curve (Gotelli & Colwell, 2001) Figure 3-4 shows the rarefaction curve for the observed assigned taxa for the samples oral 1, oral 2 and oral 3. It shows the oral 3 (buccal mucosa) as the most diverse.

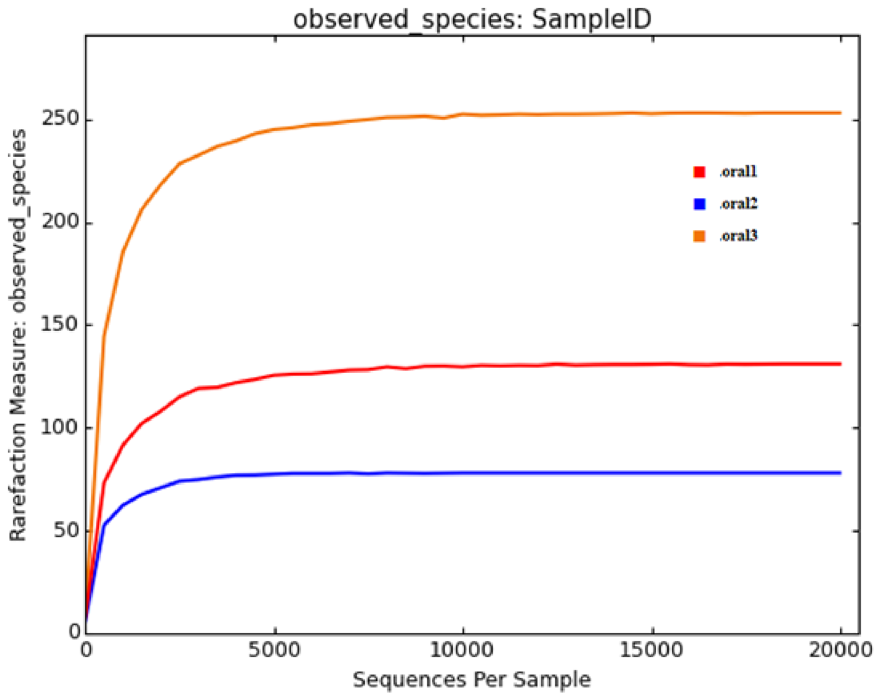

Figure 3-4: represents rarefaction curve based on the observed species.

Other diversity indexes, such as the Shannon (Shannon, 1948b), accurately measures the alpha diversity as observed in (Figure 3-5) (E. K. Morris et al., 2014) This is because they combine the measure of evenness with that of the richness (number of OTUs). The buccal mucosa has the highest diversity (within sample) index compared to the subgingival and supragingival plaque. This is aligning with the literature for the case of healthy patients with good oral hygiene (Moon & Lee, 2016) For the subgingival and supragingival plaque the alpha diversity is lower. Nonetheless, the supragingival plaque (oral 1) has higher within sample diversity compared to the subgingival plaque (oral 2); however, this observation is slightly contradictory to several oral microbiome studies. Aside from the small sample size variations, this could be justified as each individual has slightly different microbiome fingerprint and it been not, at least yet, indicative to any major change in the host physiology or dysbiosis. This might be considered inter-personal microbiome normal dynamics (Hall et al., 2017)

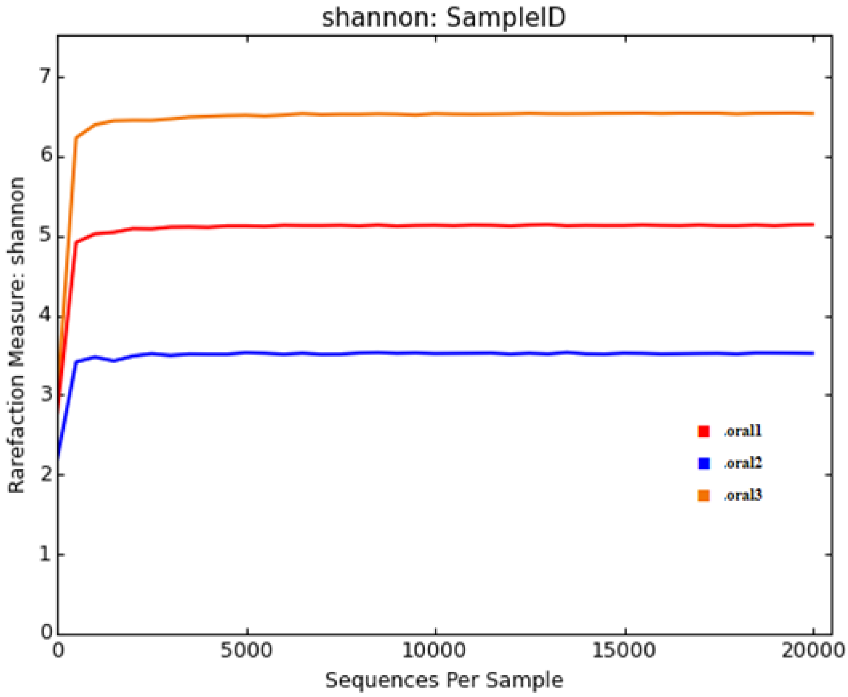

Figure 3-5: Rarefaction curve representing Shanon Index. Upper and lower confidence intervals are not included in this graph.

The beta diversity between the three samples are not of interest of this study as we are not comparing the samples to each other. However, Figure 3-6 is a Principle Component Analysis PCoA based on the ecological pairwise distance between the taxa compositions of the samples calculated by Bray Curtis dissimilarity matrix. The graph is mocked by other points to help visualize the profiles of the microbial community between samples. It is obvious from the figure that the three oral sample are clustered near each other in comparison with the mock points. This analysis is predictable; thus, differential details among the samples are out of the scope for this project.

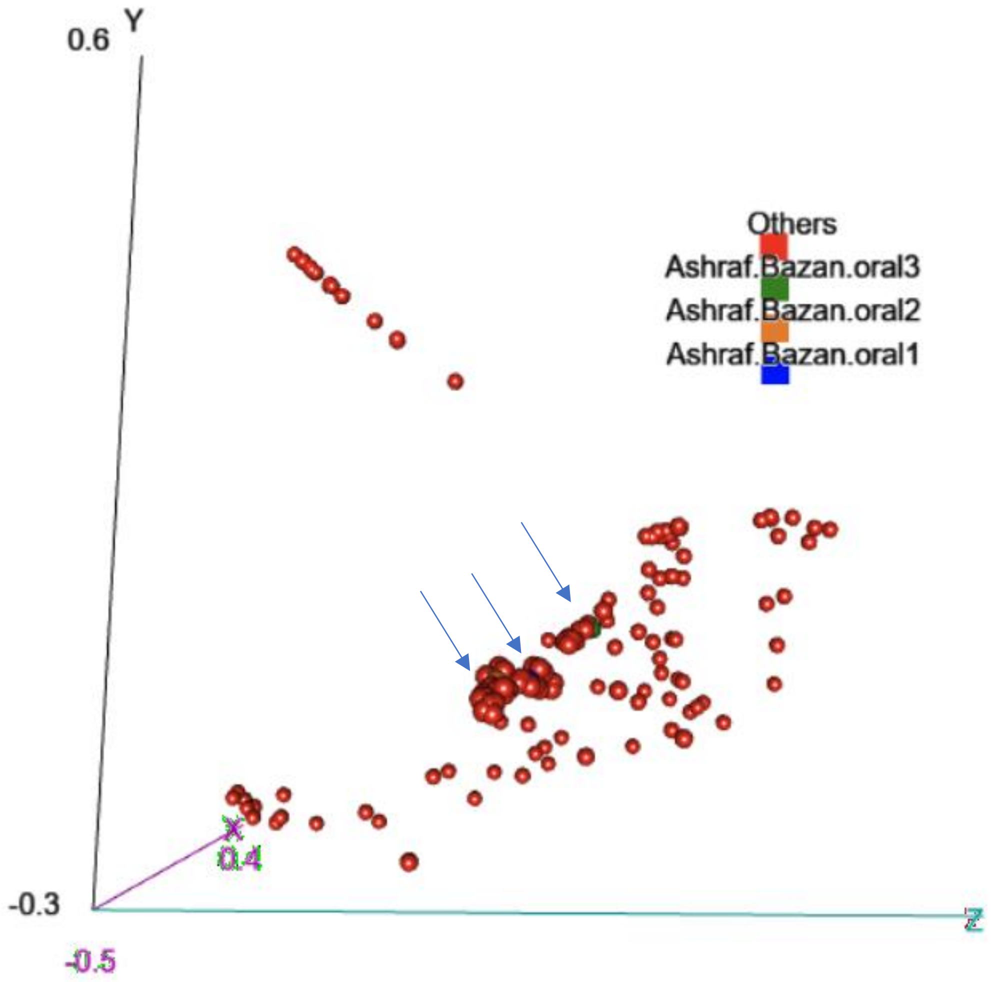

Figure 3-6: Principle Component Analysis to visualize the beta diversity between the three oral samples taken from the oral microbiome. The three arrows points to the three samples (Orange, Green and Blue) among the mock red points that are termed "others".

From a system point of view, we are going to treat the oral microbiome as averaging the abundance and diversity from the three samples for the downstream analysis of the chromosomal type II toxin antitoxin systems analysis.

3.2. Chromosomal Type II Toxin Antitoxin Systems Predictions

The chromosomal toxin antitoxin systems are of a great interest to this study. This is because their exact roles are quite debatable as mentioned in the literature review section of the thesis. On the other hand, the plasmodial toxin antitoxin systems are well established as post segregational killing that addicts the plasmids to the population (Hayes, 2003) They are almost ubiquitous in all plasmids (Schuster & Bertram, 2013) The chromosomal abundance and diversity of the TAS are not properly addressed; studying their distribution patterns could give us insights about their role especially in stress response and adaptation to changing environments in the buccal ecosystem (Shidore, Zeng, & Triplett, 2019)

The type II toxin antitoxin systems are the most studied type of the toxin antitoxin systems; yet, there are a lot of ambiguity in detecting them in the chromosome. This ambiguity in detecting TAS genes are because of the small size of the gene. Another reason is the unusual GC content and codon usage of these genes in comparison with the rest of the context genome. (Makarova, Wolf, & Koonin, 2009b; Song & Wood, 2018b) For example, *ab initio* gene prediction tools, generic homology-based tools, phylogenetic and network functional enrichment tools are all lacking the sensitivity and specificity for detection of the TAS gene sequence pairs due to the miniature size and overlapping nature of the two protein-coding sequences of the TAS in addition to the different codon usage and GC content. Even tools that incorporate metabolic pathways predictions, subsystems technology, Hidden Markov Models and structural homology to the annotation pipelines, with default Open Reading Frame (ORF) calculations, misses most of the TAS identifications. Examples include DAVID, NCBI prokaryotic genome annotation pipeline and PATRIC/RAST annotation server (Aziz et al., 2008; Brettin et al., 2015; Gillespie et al., 2011; Haft et al., 2018; Huang, Sherman, & Lempicki, 2009a, 2009b; Tatusova et al., 2016) These specific examples for annotation tools expressively announced the exclusion of such genes. However, such tools could identify uncomprehensive minority annotations, especially plasmodial TAS (Makarova, Wolf, & Koonin, 2019) Conclusive discussions for such problems occur in nearly most of the research that aims at identifying genes that codes for bacterial immune response like, CRISPR and RM systems (Koonin & Makarova, 2013; Makarova et al., 2011; Makarova, Wolf, & Koonin, 2013; Mruk & Kobayashi, 2014) Clearly, there is a need for specific tools and pipelines for chromosomal TAS detections.

There are specialized tools that are tailored to address the problem of missing ORFs. One tool, that is now obsolete, is called RASTA. This stands for Rapid Automated Scan for Toxins and Antitoxins (Sevin & Barloy-Hubler, 2007) Another tool, that “replaced” this one is called TAfinder. This one is currently supported and inhouse the most comprehensive database for the Toxin Antitoxin Systems (Xie et al., 2018a, p. 2) This tool uses specific default parameters for BlastP and HMMer pipelines that are tailored for detecting the TAS and they use the comprehensive database of TADB which minimizes the distance similarities with other genes. Therefore, there is a problem which is detecting novel TAS genes that are not yet in the TADB, still display domain architecture and structure homology with that of the toxin and antitoxins in the TADB. These novel TAS are vastly derived from the existing knowledgebase of the domains and related proteins to the TAS genes that waits to be mined into (Makarova et al., 2009b) Thus, an exhaustive survey that is manually bio-curated is required to expand the putative TAS database and unleash trends in their distribution in the human oral microbiome.

The genomic data retrieved for the analysis are not the full metagenome in the oral microbiome. OTUs that assigned to species that share 97% similarity with the 16S rRNA are only included to decrease inaccurate data as the genomic sequence is obtained from NCBI RefSeq database and not from the direct genomes of the samples (Haft et al., 2018) Whenever there is unassembled genomic sequence, the downstream analysis is applied on each contig of the project. To avoid duplicate detections of TAS from the contigs that are possibly overlapping, manually screened TAS genes of exact sequences are removed from contigs of the same taxa. As there are 89601 OTUs, there are some that are extremely scarce and minimally abundant in the oral microbiome; they are omitted from TAS analysis for the unlikelihood of changing the abundance of TAS genes. The OTUs used in the analysis are presented in (Table 3-1) and their abundance is visualized in (Figure 3-3) as a clustered heatmap.

Table 3-1 The taxa that are involved in the down-stream analysis of the Toxin Antitoxin Systems

UTO	**Accession or Assembly Number**
Capnocytophaga leadbetteri	CP022384.1
Lautropia mirabilis	LR134378.1
Rothia dentocariosa	CP002280.1
Haemophilus influenzae	LS483480.1
Actinomyces oris	CP014232.1
Aggregatibacter segnis	LS483443.1
Capnocytophaga ochracea	CP001632.1
Fusobacterium canifelinum	NZ_RQYY00000000.1
Neisseria flavescens	LAEI00000000.1
Corynebacterium matruchotii	ACEB00000000.1
Prevotella intermedia	CP030094.1
Gemella morbillorum	LS483440.1
Kingella denitrificans	AEWV00000000.1
Fusobacterium nucleatum	AE009951.2
Fusobacterium periodonticum	CP028108.1
Campylobacter showae	UWOJ00000000.1
Rothia aeria	AP017895.1
Streptococcus sanguinis	LS483346.1
Gemella sanguinis	PNGT00000000.1
Veillonella atypica	CP020566.1
Streptococcus gordonii	LS483375.1
Streptococcus mutans	LS483349.1
Porphyromonas catoniae	AMEQ00000000.1
Neisseria elongata	CP031255.1
Aggregatibacter aphrophilus	LS483485.1
Alloprevotella rava	ACZK00000000.1
Veillonella parvula	CP019721.1
Streptococcus salivarius	CP013216.1

UTO	**Accession or Assembly Number**
Granulicatella elegans	NZ_KI391971.1
Haemophilus parainfluenzae	FQ312002.1
Granulicatella adiacens	NZ_ACKZ00000000.1
Actinobacillus minor	ACQL01
Haemophilus haemolyticus	LS483458.1
Neisseria mucosa	CP028150.1
Streptococcus mitis	NC_013853.1

The chromosomal type II Toxin Antitoxin Systems predicted are classified according to the Type II Toxin Antitoxin Systems super-families and families classification described and referenced before in (Table 1-1). If the putative novel TAS do not fall into the classification in (Table 1-1), the domain-based classification is used as described in (Table 1-2). Although commonly in the literature, the putative novel TAS are assigned to be named, the nomenclature of the novel TAS in this project are avoided due to the recency of the detections and the common nebulous nature of the nomenclature as proposed by experts in the biology of the toxin antitoxin systems (Song & Wood, 2018b) In general, the putative predictions of the toxin antitoxin systems are considered novel in the scientific community; however, the computational detections of the TAS are not *bona fide* due to the lack of experimental validation and *in vivo* functional assay.

The chromosomal type II toxin antitoxin systems in the oral microbiome are 278 gene sequences (i.e. 139 pair of TAS) that are predicted by the pipeline described in the methods. They are denoted in (Table 3-2). This includes manual review of the InterPro domains of each sequence, Gene Ontologies GO predictions, PSI Blast hits after convergence and of minimum 6 iterations, structural homology in abstruse cases. The family classification serves as gene-like style of declaring novel genes. For example, all predicted genes that have the RelB annotation are declared as novel RelB-like gene. In case the classification deems a domain, the declaration of the gene will be as domain-containing gene. For example, all predicted genes with HTH annotation are declared as novel HTH-domain-containing gene. Genes that are annotated in the RefSeq genome differently, yet showed homology throughout all stages of the pipeline, are kept as TAS genes with the original domain declaration. Some of the taxa has no TAS predicted; they are not tabulated in (Table 3-2).

Table 3-2: The predicted chromosomal type II toxin antitoxin systems in the oral microbiome.

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
Capnocytophaga leadbetteri	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	CGC53_RS02185	470181..470486	MNT
	0	AT	CGC53_RS02180	469856..470191	HEPN
	TA_2	T	CGC53_RS03910	910662..911189	START
	0	AT	CGC53_RS03905	909922..910602	HTH
	TA_3	T	CGC53_RS04105	952696..952992	YoeB
	0	AT	CGC53_RS04100	952451..952708	RHH
	TA_4	T	CGC53_RS10070	2256679..2257134	VapC
	0	AT	CGC53_RS10065	2256471..2256698	RelB
Rothia dentocariosa	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	HMPREF0733_10180	201492..201863	VapC
	0	AT	HMPREF0733_10181	201860..202057	VapB
	TA_2	T	HMPREF0733_10355	377568..378707	GNAT
	0	AT	HMPREF0733_10354	376739..377443	HTH
	TA_3	T	HMPREF0733_10590	644678..645097	VapC
	0	AT	HMPREF0733_10591	645101..645406	Phd
	TA_4	T	HMPREF0733_10761	816485..816787	MNT
	0	AT	HMPREF0733_10760	816135..816488	HEPN
	TA_5	T	HMPREF0733_11193	1280854..1282620	GNAT
	0	AT	HMPREF0733_11192	1280138..1280851	AcrR
	TA_6	T	HMPREF0733_11254	1364766..1365056	MNT
	0	AT	HMPREF0733_11255	1365056..1365391	HEPN
	TA_7	T	HMPREF0733_11511	1660539..1661009	HTH
	0	AT	HMPREF0733_11512	1661006..1661326	HTH
	TA_8	T	HMPREF0733_11698	1882625..1882885	YoeB

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	0	AT	HMPREF0733_11697	1882368..1882622	Phd
Haemophilus influenzae	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC13377_00047	48268..48666	VapC
	0	AT	NCTC13377_00046	48035..48268	MazE
	TA_2	T	NCTC13377_00076	76885..77175	RelE
	0	AT	NCTC13377_00075	76641..76895	Phd
	TA_3	T	NCTC13377_00298	300005..300313	RelE
	0	AT	NCTC13377_00299	300313..300609	RelB
	TA_4	T	NCTC13377_00347	349919..350239	HipA
	0	AT	NCTC13377_00346	349623..349931	HTH
	TA_5	T	NCTC13377_00352	358344..358703	RelE
	0	AT	NCTC13377_00353	358696..358992	HTH
	TA_6	T	NCTC13377_01012	997694..998038	MNT
	0	AT	NCTC13377_01011	997261..997692	HEPN
	TA_7	T	NCTC13377_01294	1293131..1293838	HipA
	0	AT	NCTC13377_01295	1293938..1294603	HTH
	TA_8	T	NCTC13377_01416	1396539..1397246	HipA
	0	AT	NCTC13377_01417	1397346..1398011	HTH
	TA_9	T	NCTC13377_01430	1403404..1403709	HigB
	0	AT	NCTC13377_01429	1403114..1403407	HigA
	TA_10	T	NCTC13377_01437	1406854..1407105	RelE
	0	AT	NCTC13377_01436	1406523..1406870	HTH
	TA_11	T	NCTC13377_01632	1614045..1614350	RelE
	0	AT	NCTC13377_01631	1613711..1614034	HTH
Actinomyces oris	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	AXE84_01550	344964..345341	Fido

Organism	TA no.	T/A	Locus Tag	Location	Manually Curated Gene Class
	0	AT	AXE84_01545	344750..344953	RelB
	TA_2	T	AXE84_02440	545051..545683	GNAT
	0	AT	AXE84_02445	545810..546946	LsrR
	TA_3	T	AXE84_02825	658137..658391	YoeB
	0	AT	AXE84_02830	658391..658642	Phd
	TA_4	T	AXE84_02845	661089..662333	HipA
	0	AT	AXE84_02850	662330..662617	HTH
	TA_5	T	AXE84_03195	747284..747661	VapC
	0	AT	AXE84_03190	747090..747287	VapB
	TA_6	T	AXE84_03905	913152..913553	VapC
	0	AT	AXE84_03900	912928..913155	CcdA
	TA_7	T	AXE84_03930	917777..918169	VapC
	0	AT	AXE84_03935	918166..918387	VapB
	TA_8	T	AXE84_04220	991997..992584	VapC
	0	AT	AXE84_04225	992588..993073	MerR
	TA_9	T	AXE84_04425	1037940..1038410	MNT
	0	AT	AXE84_04420	1037554..1037943	HEPN
	TA_10	T	AXE84_06645	1623445..1623798	RelE
	0	AT	AXE84_06640	1623125..1623448	HTH
	TA_11	T	AXE84_06935	1712733..1713014	RelE
	0	AT	AXE84_06940	1713001..1713312	HTH
	TA_12	T	AXE84_06970	1718075..1719739	GNAT
	0	AT	AXE84_06965	1717239..1717979	AcrR
	TA_13	T	AXE84_08665	2160245..2160514	YoeB
	0	AT	AXE84_08660	2160009..2160251	Phd
	TA_14	T	AXE84_09955	2478807..2479232	START

Organism	TA no.	T/A	Locus Tag	Location	Manually Curated Gene Class
	0	AT	AXE84_09960	2479229..2479789	MarR
	TA_15	T	AXE84_11560	2864573..2866099	GNAT
	0	AT	AXE84_11555	2864007..2864576	AcrR
	TA_16	T	AXE84_11985	2963479..2963826	MazF
	0	AT	AXE84_11990	2963813..2964052	RelB
Aggregatibacter segnis	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10977_00366	368843..369205	RelE
	0	AT	NCTC10977_00367	369202..369579	HTH
	TA_2	T	NCTC10977_00853	824328..825641	MNT
	0	AT	NCTC10977_00852	823368..824225	ParD
	TA_3	T	NCTC10977_01792	1838998..1840278	HipA
	0	AT	NCTC10977_01791	1838720..1838998	HTH
Capnocytophaga ochracea	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	Coch_0735	889703..889999	HigB
	0	AT	Coch_0736	890004..890237	HTH
	TA_2	T	Coch_0780	939775..940101	Fido
	0	AT	Coch_0779	939063..939773	Phd
	TA_3	T	Coch_0815	978517..978822	RelE
	0	AT	Coch_0816	978825..979049	RelB
	TA_4	T	Coch_0841	1003635..1003955	RelE
	0	AT	Coch_0840	1003367..1003642	RHH
	TA_5	T	Coch_0997	1170527..1170850	RelE
	0	AT	Coch_0998	1170843..1171100	RelB
	TA_6	T	Coch_1196	1403828..1404136	RelE
	0	AT	Coch_1197	1404140..1404382	RelB
	TA_7	T	Coch_1229	1435589..1436116	START

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	0	AT	Coch_1228	1434881..1435531	HxlR
Gemella morbillorum	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11323_00623	628213..628653	ImmA/IrrE
	0	AT	NCTC11323_00624	628643..629044	HTH
Fusobacterium nucleatum	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	FN1664	162255..162590	RelE
	0	AT	FN1665	162655..162954	HTH
	TA_2	T	FN1998	500269..500451	HipA
	0	AT	FN1997	499898..500218	HTH
	TA_3	T	FN2046	551001..551450	GNAT
	0	AT	FN2045	550464..550892	PerR
	TA_4	T	FN2066	576417..576833	ImmA/IrrE
	0	AT	FN2065	575990..576457	HTH
	TA_5	T	FN0056	692832..693311	GNAT
	0	AT	FN0055	692298..692804	GNAT
	TA_6	T	FN0211	837364..837630	RelE
	0	AT	FN0210	837138..837356	RelB
	TA_7	T	FN0497	1141273..1141545	RelE
	0	AT	FN0496	1141050..1141271	RelB
	TA_8	T	FN1100	1744317..1744583	RelE
	0	AT	FN1099	1744105..1744332	RelB
	TA_9	T	FN1294	1952607..1953158	GNAT
	0	AT	FN1295	1953248..1953655	GNAT
Fusobacterium periodonticum	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	C4N17_08000	1625268..1625546	YoeB
	0	AT	C4N17_08005	1625540..1625779	Phd

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	TA_2	T	C4N17_09110	1824443..1824727	RelE
	0	AT	C4N17_09115	1824714..1824953	RelB
	TA_3	T	C4N17_09580	1921236..1921721	GNAT
	0	AT	C4N17_09585	1921750..1922253	GNAT
	TA_4	T	C4N17_09585	1921750..1922253	GNAT
	0	AT	C4N17_09595	1922516..1922770	YefM
	TA_5	T	C4N17_09590	1922263..1922523	YoeB
	0	AT	C4N17_09595	1922516..1922770	Phd
	TA_6	T	C4N17_10050	2023458..2023649	HicA
	0	AT	C4N17_10045	2022996..2023421	HicB
	TA_7	T	C4N17_10615	2118410..2119150	ImmA/IrrE
	0	AT	C4N17_10610	2118037..2118423	HTH
	TA_8	T	C4N17_10875	2153007..2153279	RelE
	0	AT	C4N17_10880	2153281..2153544	RelB
	TA_9	T	C4N17_11255	2235387..2236526	HipA
	0	AT	C4N17_11250	2234505..2235152	AcrR
	TA_10	T	C4N17_11255	2235387..2236526	HipA
	0	AT	C4N17_11260	2236513..2236833	HTH
	TA_11	T	C4N17_12205	2431810..2431992	HicA
	0	AT	C4N17_12210	2432030..2432428	HicB
	TA_12	T	C4N17_12260	2440858..2441268	ImmA/IrrE
	0	AT	C4N17_12265	2441228..2441695	HTH
Rothia aeria	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	RA11412_0178	154222..154647	VapC
	0	AT	RA11412_0179	154651..154956	Phd
	TA_2	T	RA11412_0821	741232..742998	GNAT

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	0	AT	RA11412_0820	740558..741229	AcrR
	TA_3	T	RA11412_1191	1091799..1092269	HTH
	0	AT	RA11412_1192	1092266..1092721	HTH
	TA_4	T	RA11412_2432	2261884..2262381	GNAT
	0	AT	RA11412_2431	2261591..2261884	RHH
	TA_5	T	RA11412_2432	2261884..2262381	GNAT
	0	AT	RA11412_2433	2262592..2263131	HTH
	TA_6	T	RA11412_2436	2264486..2264653	VapC
	0	AT	RA11412_2437	2264851..2265048	VapB
Streptococcus sanguinis	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11085_00324	309578..309856	RelE
	0	AT	NCTC11085_00323	309322..309585	RelB
	TA_2	T	NCTC11085_00917	948027..948443	GNAT
	0	AT	NCTC11085_00918	948617..949228	AcrR
	TA_3	T	NCTC11085_01051	1097626..1098483	GNAT
	0	AT	NCTC11085_01052	1098603..1099505	LysR
	TA_4	T	NCTC11085_01635	1689503..1690072	GNAT
	0	AT	NCTC11085_01633	1688763..1689299	HTH
	TA_5	T	NCTC11085_02225	2284014..2284886	ImmA/IrrE
	0	AT	NCTC11085_02226	2284886..2285248	HTH
Veillonella atypica	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	B7L28_00880	227561..227965	VapC
	0	AT	B7L28_00875	227301..227564	Phd
	TA_2	T	B7L28_00985	253813..255006	GNAT
	0	AT	B7L28_00990	255064..255951	LysR
	TA_3	T	B7L28_01445	340243..340506	RelE

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	0	AT	B7L28_01440	340015..340239	RelB
	TA_4	T	B7L28_02060	465243..465509	RelE
	0	AT	B7L28_02055	465032..465253	RelB
	TA_5	T	B7L28_09065	1992530..1992907	HicA
	0	AT	B7L28_09060	1992342..1992518	HicB
	TA_6	T	B7L28_09085	1997335..1997610	RelE
	0	AT	B7L28_09080	1997064..1997345	RelB
	TA_7	T	B7L28_09285	2039161..2040165	Fido
	0	AT	B7L28_09290	2040218..2041081	HTH
Streptococcus gordonii	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC3165_00371	383729..384496	PezT
	0	AT	NCTC3165_00370	383253..383729	HTH
	TA_2	T	NCTC3165_00373	385018..385389	Fido
	0	AT	NCTC3165_00372	384764..385021	Phd
	TA_3	T	NCTC3165_01298	1313648..1314064	GNAT
	0	AT	NCTC3165_01297	1312863..1313474	AcrR
	TA_4	T	NCTC3165_01704	1732532..1733392	ImmA/IrrE
	0	AT	NCTC3165_01703	1731839..1732522	HTH
Streptococcus mutans	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10449_00169	173400..173732	MazF
	0	AT	NCTC10449_00170	173719..174024	RelB
	TA_2	T	NCTC10449_00182	183970..184224	YoeB
	0	AT	NCTC10449_00183	184217..184483	YefM
	TA_3	T	NCTC10449_00189	187863..188072	RelE
	0	AT	NCTC10449_00190	188142..188414	RelB
	TA_4	T	NCTC10449_00193	190470..191264	ImmA/IrrE

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	0	AT	NCTC10449_00192	190044..190406	HTH
	TA_5	T	NCTC10449_00202	200685..201017	MazF
	0	AT	NCTC10449_00201	200446..200691	MazE
	TA_6	T	NCTC10449_00413	401966..402157	VapC
	0	AT	NCTC10449_00412	401677..401982	MazE
	TA_7	T	NCTC10449_00760	741130..741321	HicA
	0	AT	NCTC10449_00761	741339..741716	HicB
Neisseria elongata	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	DV445_RS02635	524666..525214	GNAT
	0	AT	DV445_RS02640	525321..525806	HTH
	TA_2	T	DV445_RS05995	1188449..1189306	Bro
	0	AT	DV445_RS06000	1189372..1190073	HTH
	TA_3	T	DV445_RS12620	2480707..2481126	VapC
	0	AT	DV445_RS12615	2480471..2480710	RHH
	TA_4	T	DV445_RS12655	2491470..2491742	RelE
	0	AT	DV445_RS12660	2491732..2491923	RHH
Aggregatibacter aphrophilus	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC11096_01165	1141294..1142574	HipA
	0	AT	NCTC11096_01166	1142574..1142852	HTH
	TA_2	T	NCTC11096_02169	2251436..2252749	MNT
	0	AT	NCTC11096_02170	2252853..2253710	ParD
Streptococcus salivarius	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	HSISS4_00658	705771..706136	HigB
	0	AT	HSISS4_00659	706126..706419	HigA
	TA_2	T	HSISS4_00840	910234..911448	FmhB
	0	AT	HSISS4_00841	911450..912259	HTH

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
	TA_3	T	HSISS4_01146	1242750..1243268	GNAT
	0	AT	HSISS4_01147	1243285..1244328	HTH
	TA_4	T	HSISS4_01298	1438024..1439409	GNAT
	0	AT	HSISS4_01299	1439435..1439845	MarR
	TA_5	T	HSISS4_01524	1722127..1722381	YoeB
	0	AT	HSISS4_01525	1722385..1722639	Phd
Granulicatella elegans	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	ORF1_138	164191..165234	Fido
	0	AT	ORF1_139	165451..166302	HTH
	TA_2	T	ORF1_182	211296..211862	GNAT
	0	AT	ORF1_183	211872..212714	AraC
	TA_3	T	ORF1_377	431367..431636	GNAT
	0	AT	ORF1_376	430909..431334	Rrf2
	TA_4	T	ORF1_630	693298..693477	HicA
	0	AT	ORF1_631	693514..693969	HicB
	TA_5	T	ORF1_835	907257..907529	RelE
	0	AT	ORF1_834	906979..907254	RelB
	TA_6	T	ORF1_854	919944..920765	Fido
	0	AT	ORF1_853	919487..919753	HTH
	TA_7	T	ORF1_928	998474..998653	HicA
	0	AT	ORF1_927	997982..998437	HicB
	TA_8	T	ORF1_1375	1460943..1461380	GNAT
	0	AT	ORF1_1374	1460493..1460750	Fido
	TA_9	T	ORF1_1491	1589708..1589899	HicA
	0	AT	ORF1_1490	1589299..1589676	HicB
	TA_no.	T/A	Locus_tag	Location	

Organism	**TA no.**	**T/A**	**Locus Tag**	**Location**	**Manually Curated Gene Class**
Haemophilus parainfluenzae	TA_1	T	PARA_11550	1159230..1159496	RelE
	0	AT	PARA_11560	1159480..1159824	RelB
Actinobacillus minor	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	AM305_00230	39922..40356	VapC
	0	AT	AM305_00225	39686..39925	MazE
Haemophilus haemolyticus	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NCTC10839_00505	499226..499486	HipA
	0	AT	NCTC10839_00504	498930..499238	HTH
	TA_2	T	NCTC10839_01017	1007834..1008127	MNT
	0	AT	NCTC10839_01018	1008111..1008530	HEPN
	TA_3	T	NCTC10839_01455	1463601..1463855	HicA
	0	AT	NCTC10839_01454	1463105..1463587	HicB
	TA_4	T	NCTC10839_01455	1463601..1463855	HicA
	0	AT	NCTC10839_01456	1463929..1464594	HTH
	TA_5	T	NCTC10839_01587	1597372..1598652	HipA
	0	AT	NCTC10839_01586	1597094..1597372	HTH
	TA_6	T	NCTC10839_01662	1690384..1690746	MazF
	0	AT	NCTC10839_01661	1690121..1690390	MazE
Neisseria mucosa	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	NM96_RS06400	1295082..1295564	START
	0	AT	NM96_RS06405	1295628..1296032	HTH
	TA_2	T	NM96_RS10690	2119236..2119961	Aat
	0	AT	NM96_RS10695	2120154..2120651	Fur
Streptococcus mitis	TA_no.	T/A	Locus_tag	Location	
	TA_1	T	smi_0100	106784..107131	RelE
	0	AT	smi_0099	106507..106794	RelB

Organism	TA no.	T/A	Locus Tag	Location	Manually Curated Gene Class
	TA_2	T	smi_0438	414401..414754	MazF
	0	AT	smi_0439	414738..414953	MazE
	TA_3	T	smi_0611	553127..553477	RelE
	0	AT	smi_0610	552850..553137	RelB
	TA_4	T	smi_0698	647243..647530	RelE
	0	AT	smi_0697	646896..647201	RelB
	TA_5	T	smi_1101	1089095..1089865	PezT
	0	AT	smi_1102	1089865..1090341	HTH
	TA_6	T	smi_1200	1192110..1192673	GNAT
	0	AT	smi_1199	1191656..1192144	GNAT
	TA_7	T	smi_1262	1265315..1265629	MNT
	0	AT	smi_1261	1264960..1265334	HEPN
	TA_8	T	smi_1262	1265315..1265629	MNT
	0	AT	smi_1263	1265676..1265936	RelB
	TA_9	T	smi_1273	1270051..1270410	RelE
	0	AT	smi_1272	1269690..1270061	RelB

The distribution of the type II toxin antitoxin systems on the chromosomes of the genome of the oral microbiome is segregated and shows non-uniform distribution as shown from Figure 3-7 till Figure 3-29. This distribution could imply that the TAS genes are displaying a specific function and not randomly occurred. One reason could be that several TAS shows proximity to the Origin of Replication which accentuates its selfishness character in the intra-genome (Melderen & Bast, 2009; Ramisetty & Santhosh, 2017) The intragenomic existence of the Toxin Antitoxin module is not necessary a conflict. The inclusiveness of the gene does not contradict fitness of the bacterial host (Gardner & Úbeda, 2017; McLaughlin & Malik, 2017; Rankin Daniel J. et al., 2012) The TA gene promotes maintenance of fitness factors, virulence and resistance for xenobiotics and phages. Thus, the TAS evolution is not best described as having Red Queen evolutionary dynamics where the co-evolution of selfish entities is only attributed to attack or defend the host in an arm-race manner (McLaughlin & Malik, 2017) It is thought that TAS are skewing to the Black Queen evolutionary dynamics as they provide fitness characters with reduced fitness cost as they persist near the origin of replication of the bacterial chromosome. Although this require further investigations, this proximity to origin of replication ensures the quality expression of the fitness genes in demand that could be released to the surrounding population. On the other hand, the bacterium can be dependent on costly fitness genes present in the ecosystem but not invited by the antiaddiction role of the TAS modules. This leakiness and adaptive dependency are the main pillars of the Black Queen Hypothesis (Cairns et al., 2018; Goormaghtigh et al., 2018; Kang, Kim, Jin, & Lee, 2018; J. J. Morris, 2015; J. J. Morris, Lenski, & Zinser, 2012)

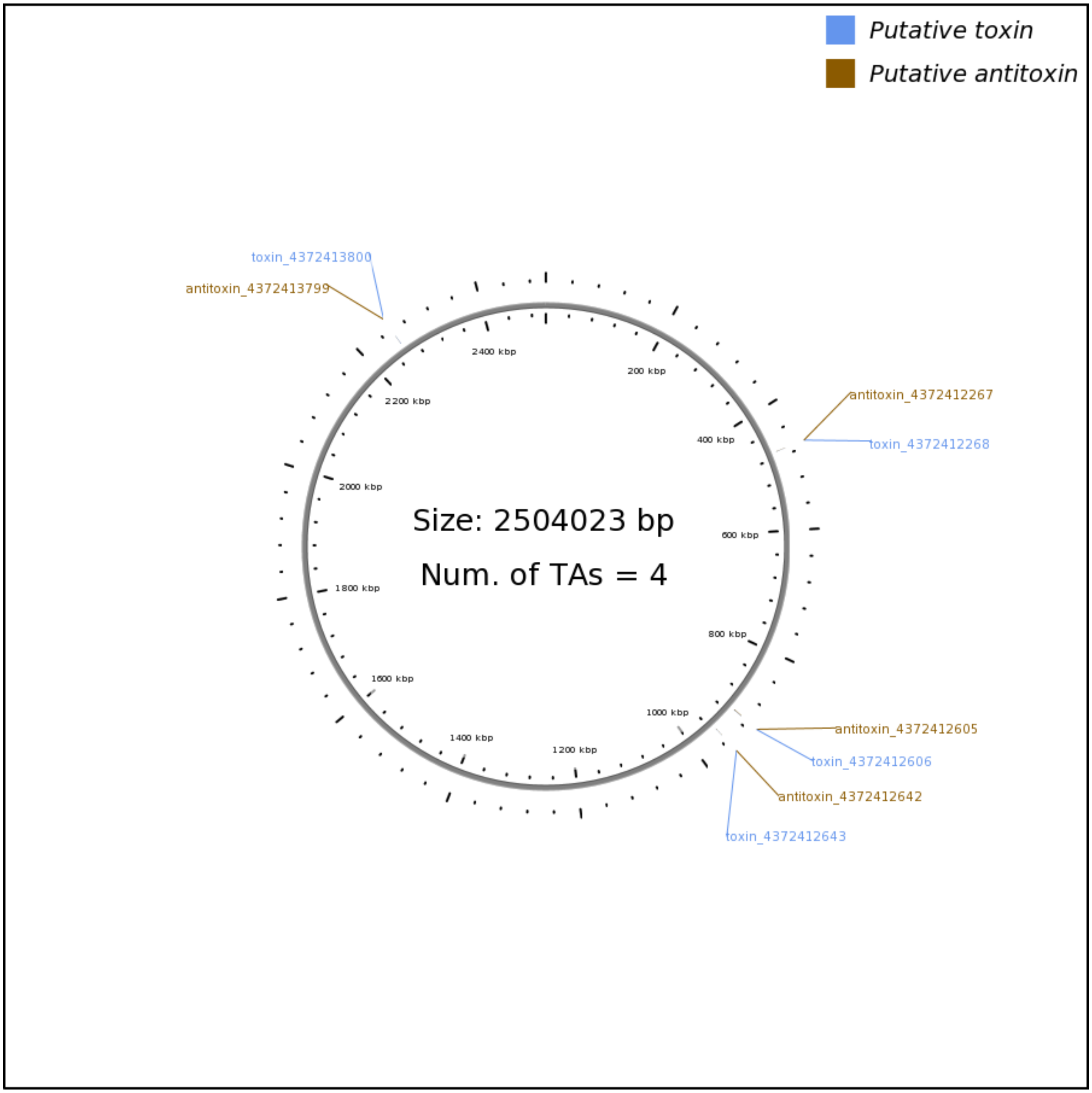

Figure 3-7: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Capnocytophaga leadbetteri* bacteria in the oral microbiome.

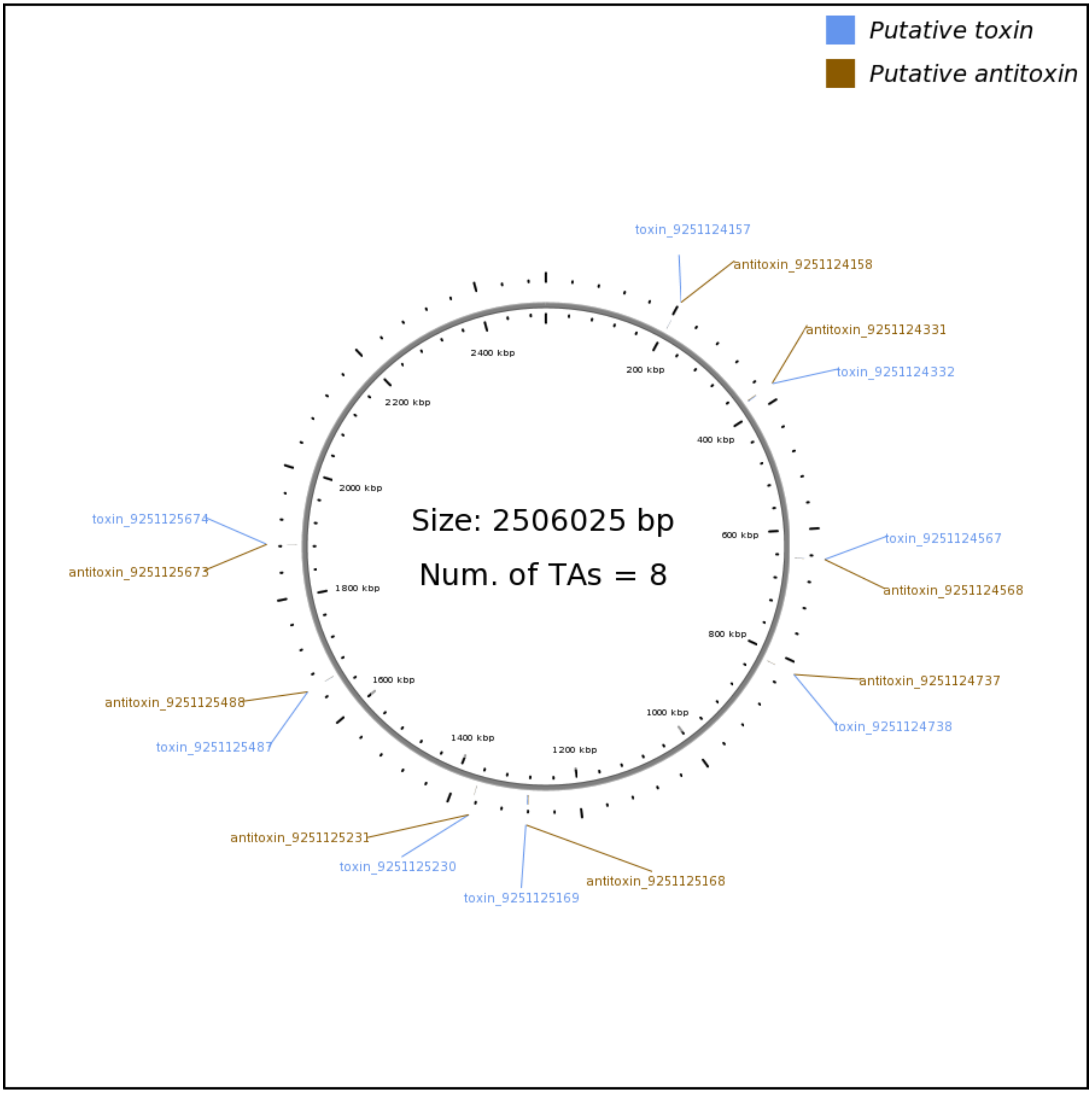

Figure 3-8: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Rothia dentocariosa* bacteria in the oral microbiome.

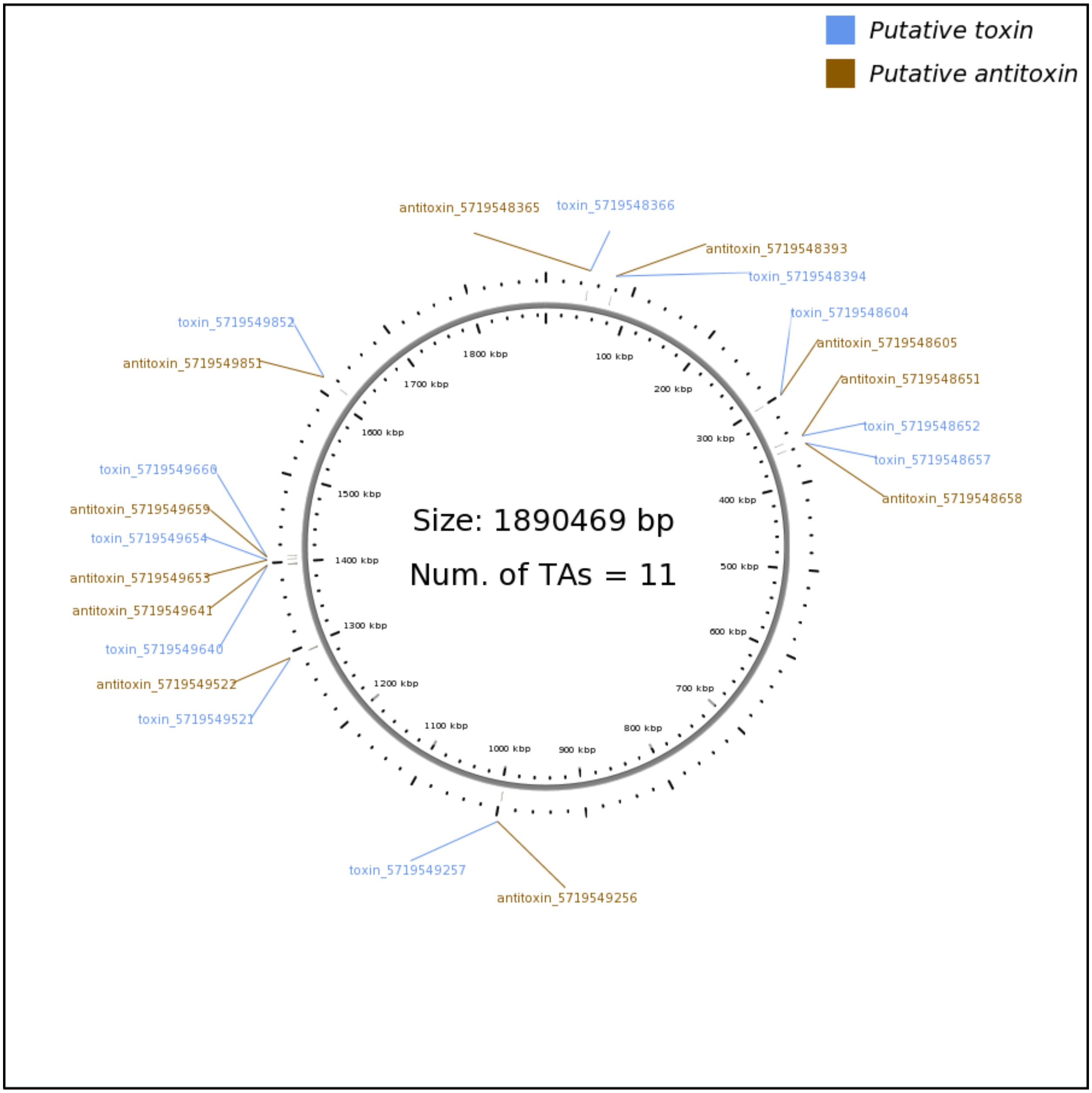

Figure 3-9: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus influenzae* bacteria in the oral microbiome.

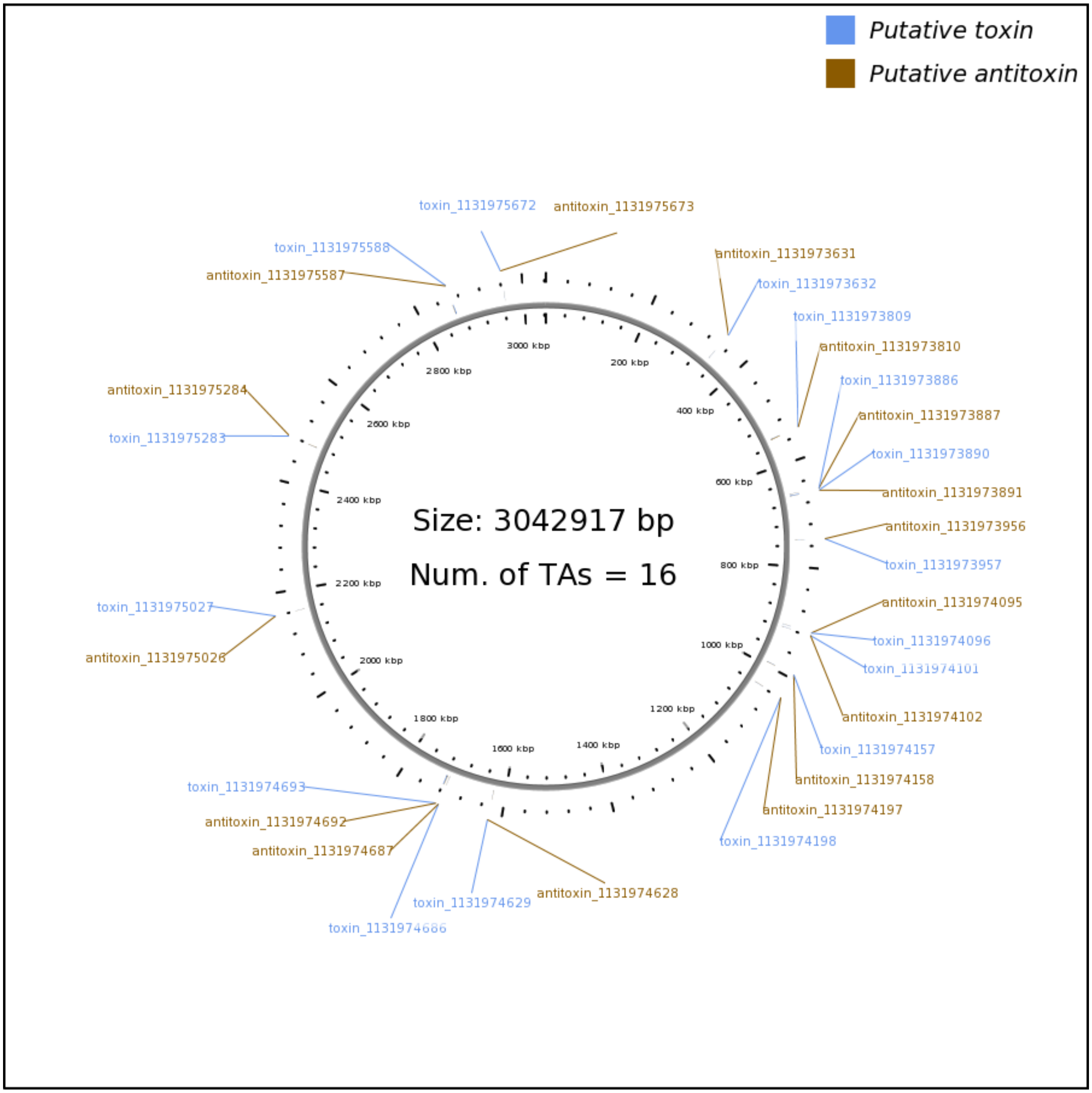

Figure 3-10: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Actinomyces oris* bacteria in the oral microbiome.

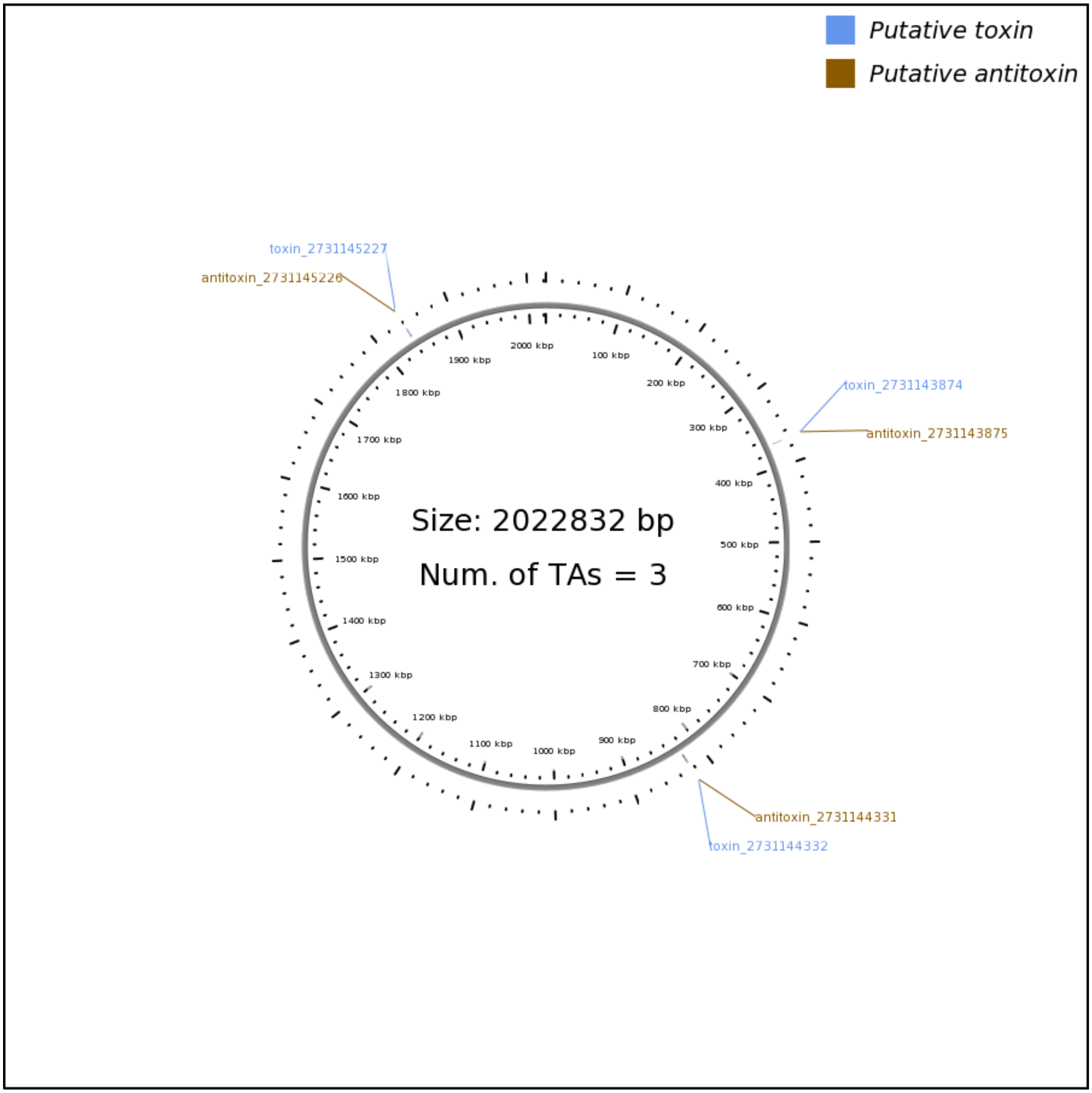

Figure 3-11 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Aggregatibacter segnis* bacteria in the oral microbiome.

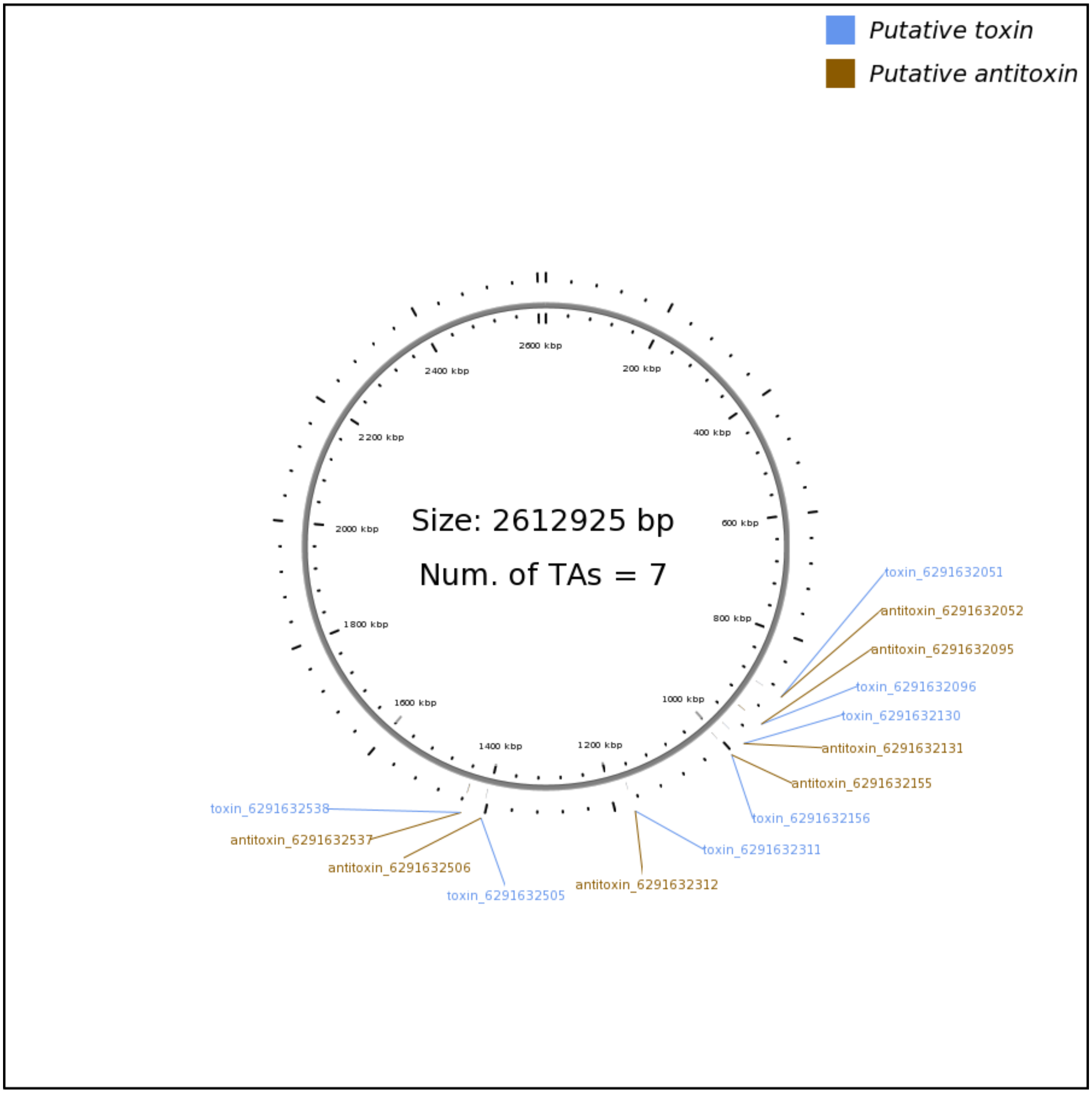

Figure 3-12 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Capnocytophaga ochracea* bacteria in the oral microbiome.

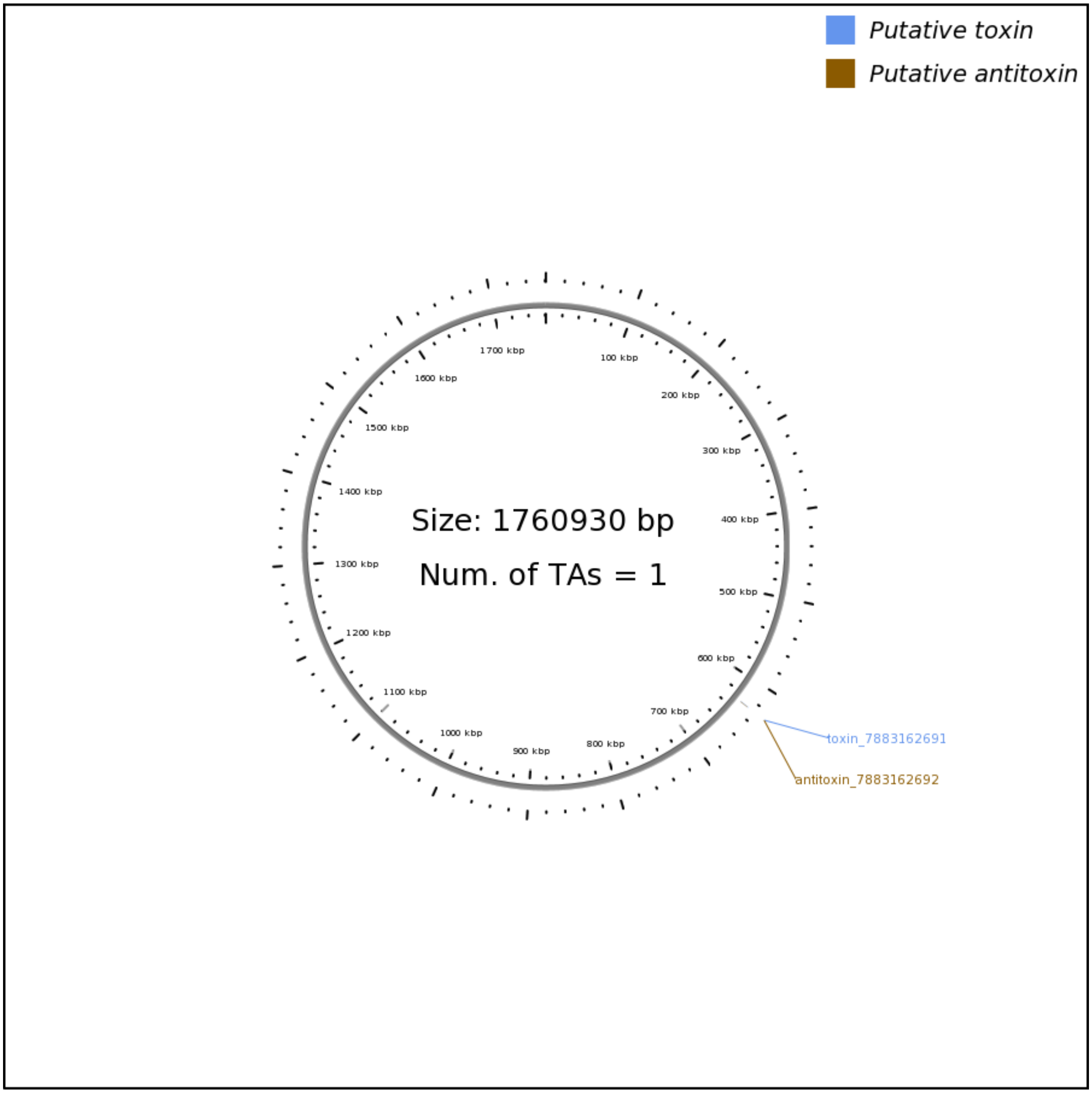

Figure 3-13: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Gemella morbillorum* bacteria in the oral microbiome.

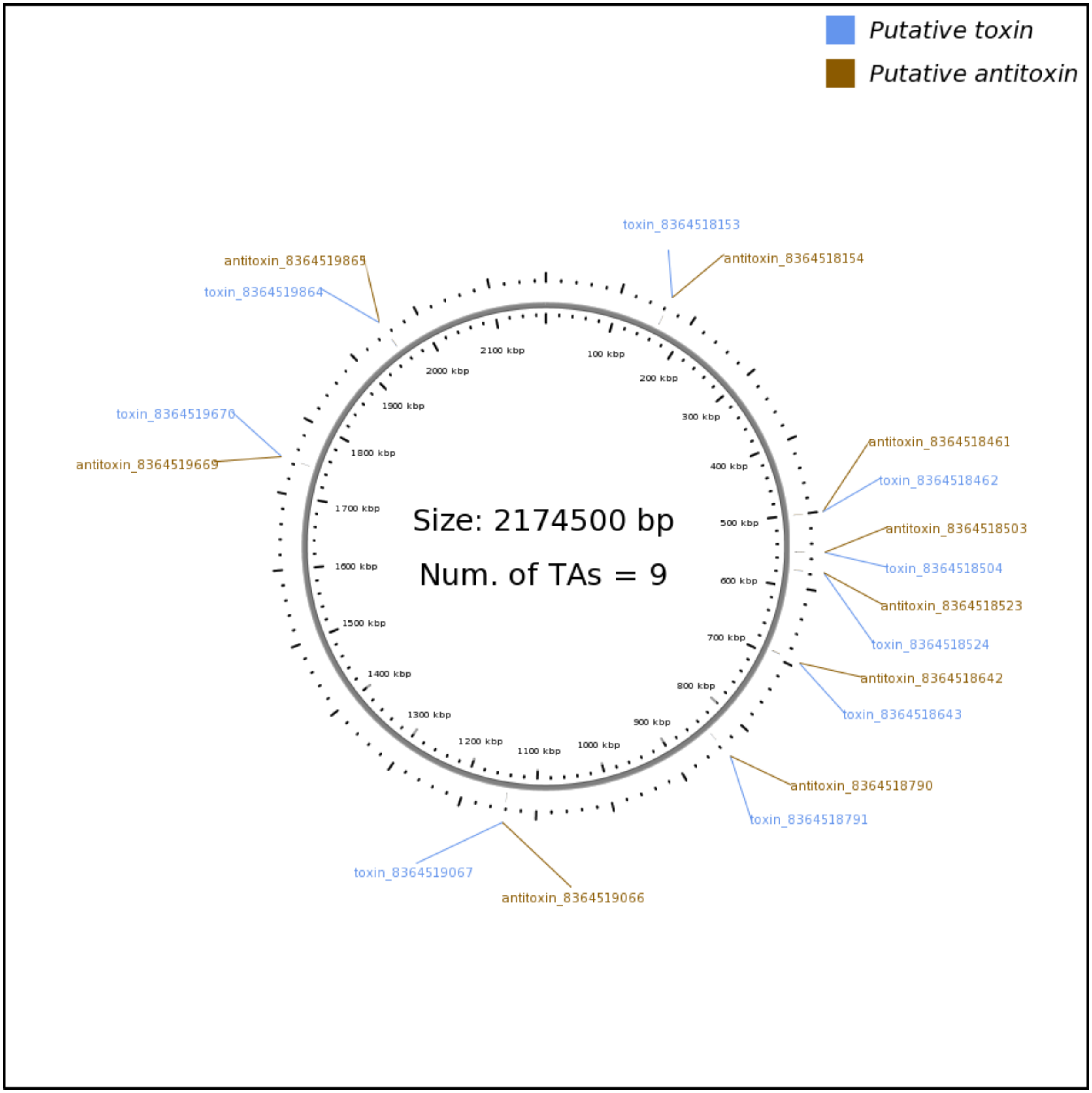

Figure 3-14: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Fusobacterium nucleatum* bacteria in the oral microbiome.

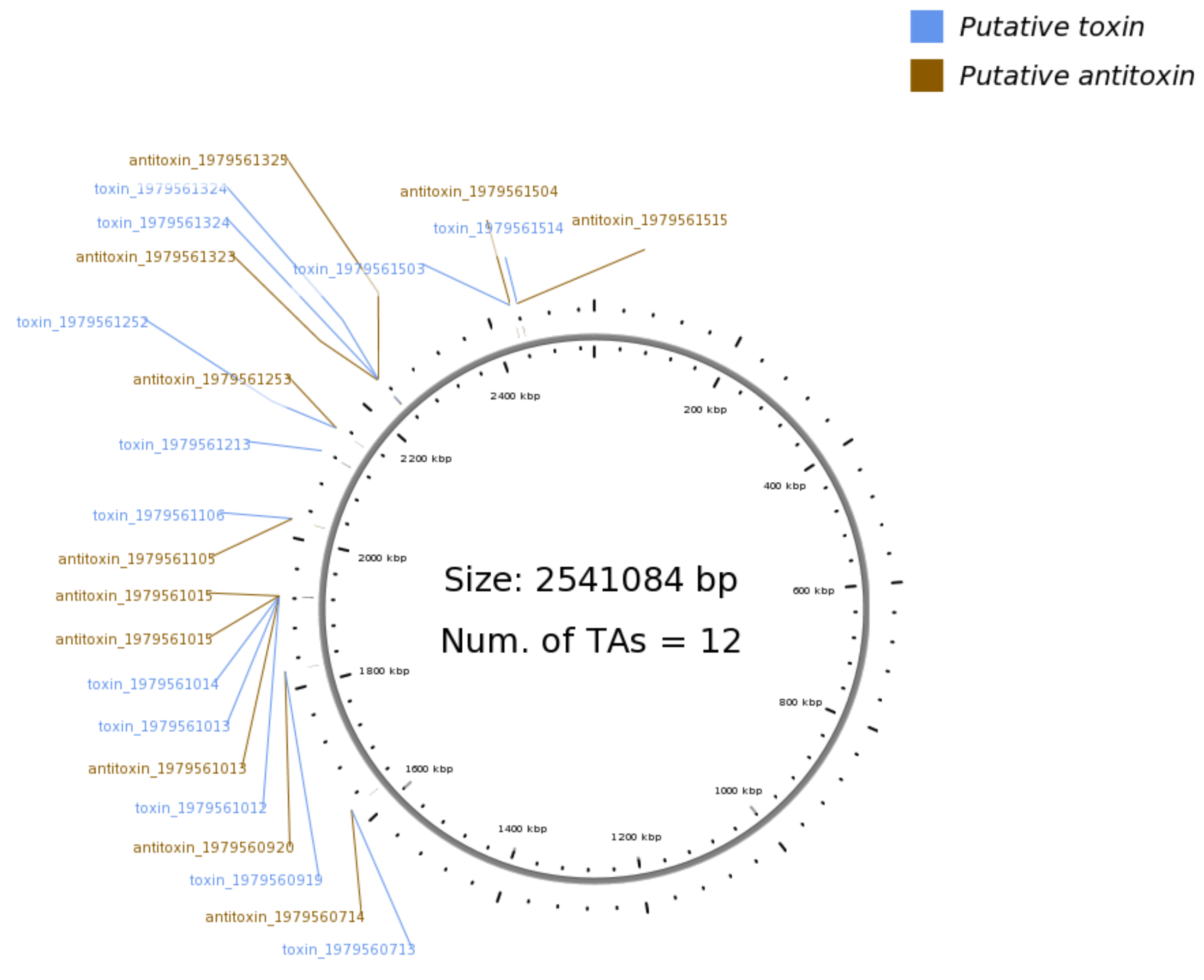

Figure 3-15: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Fusobacterium periodonticum* bacteria in the oral microbiome.

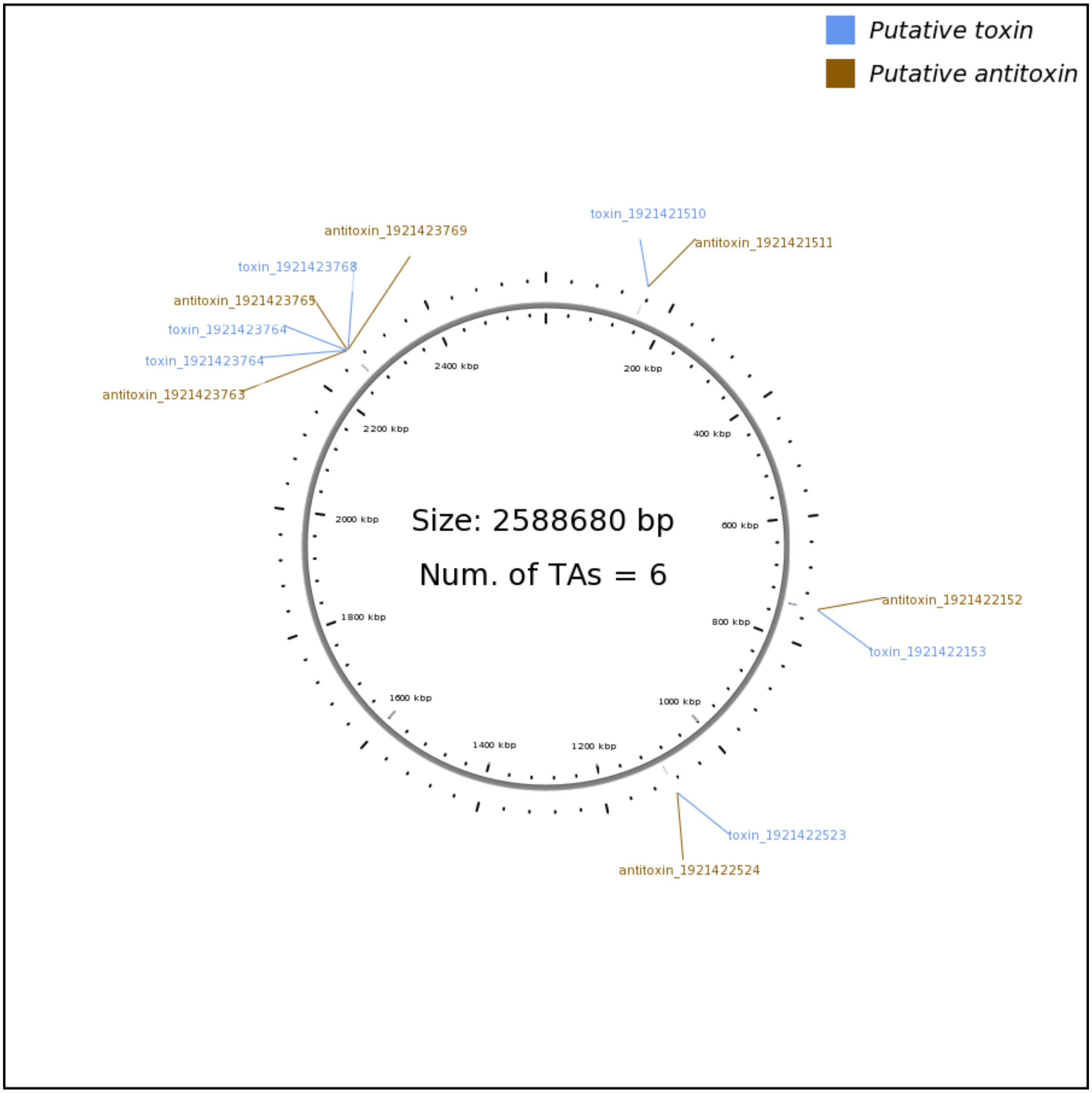

Figure 3-16: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Rothia aeria* bacteria in the oral microbiome.

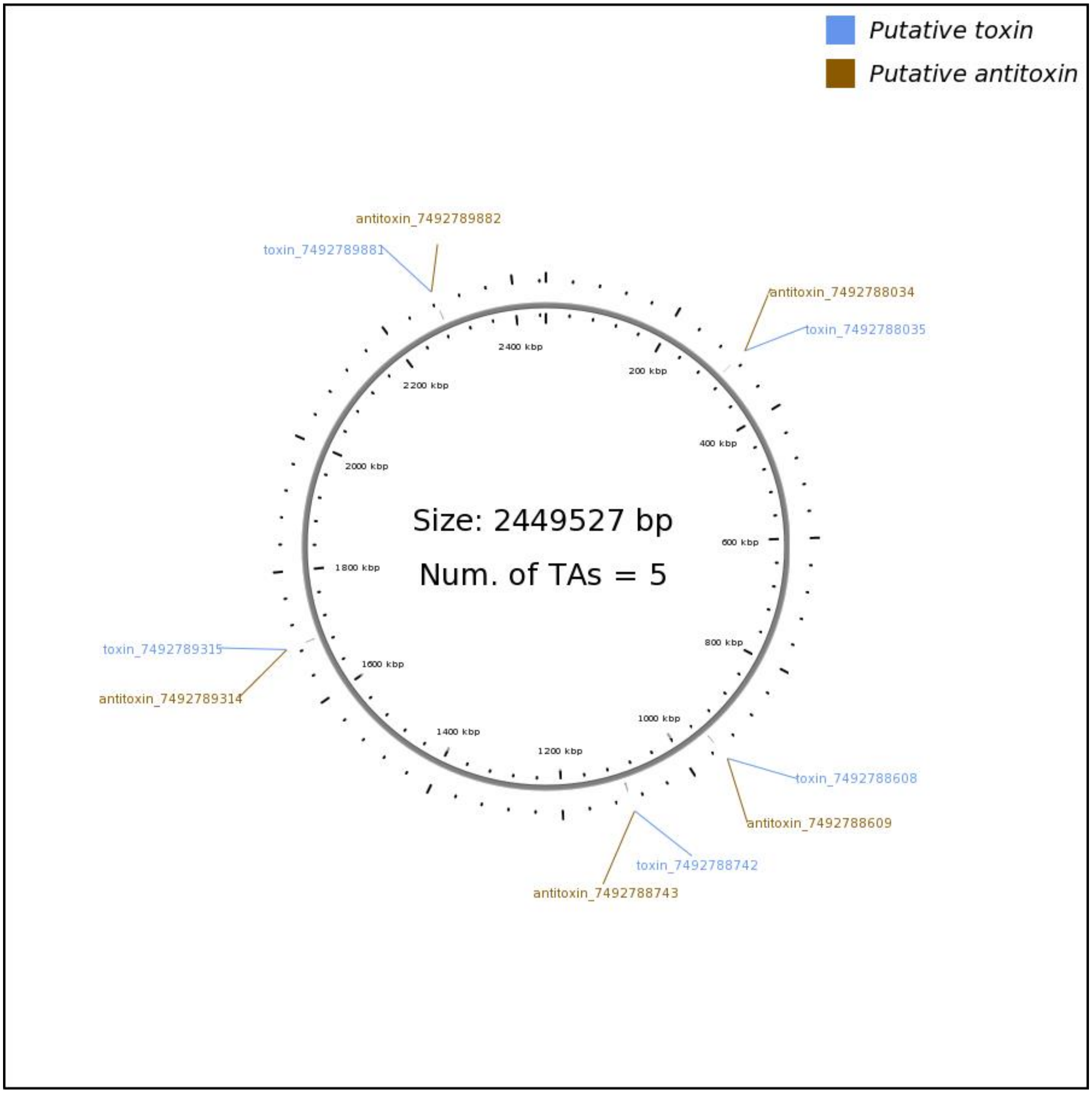

Figure 3-17: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus sanguinis* bacteria in the oral microbiome.

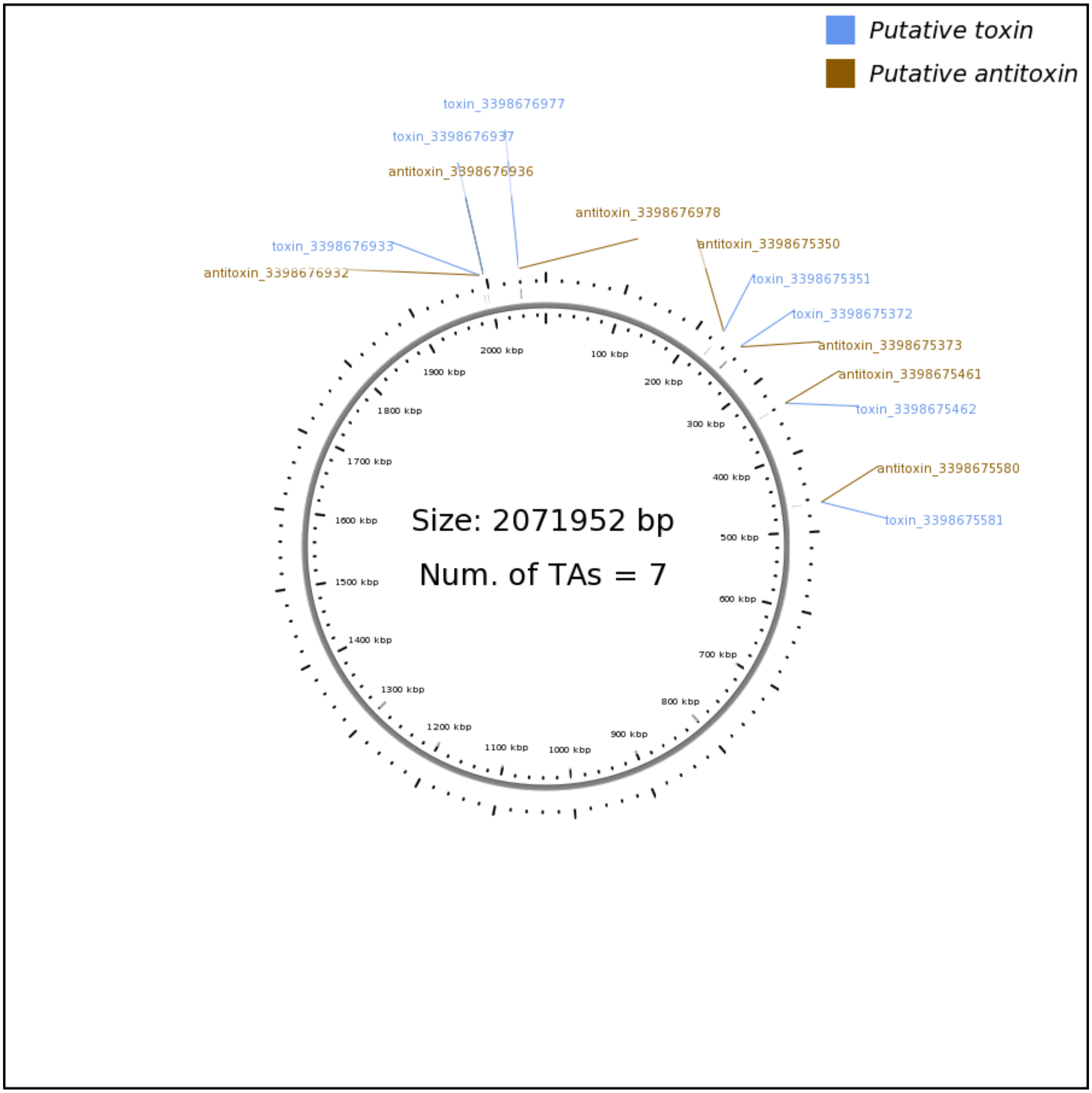

Figure 3-18: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Veillonella atypica* bacteria in the oral microbiome.

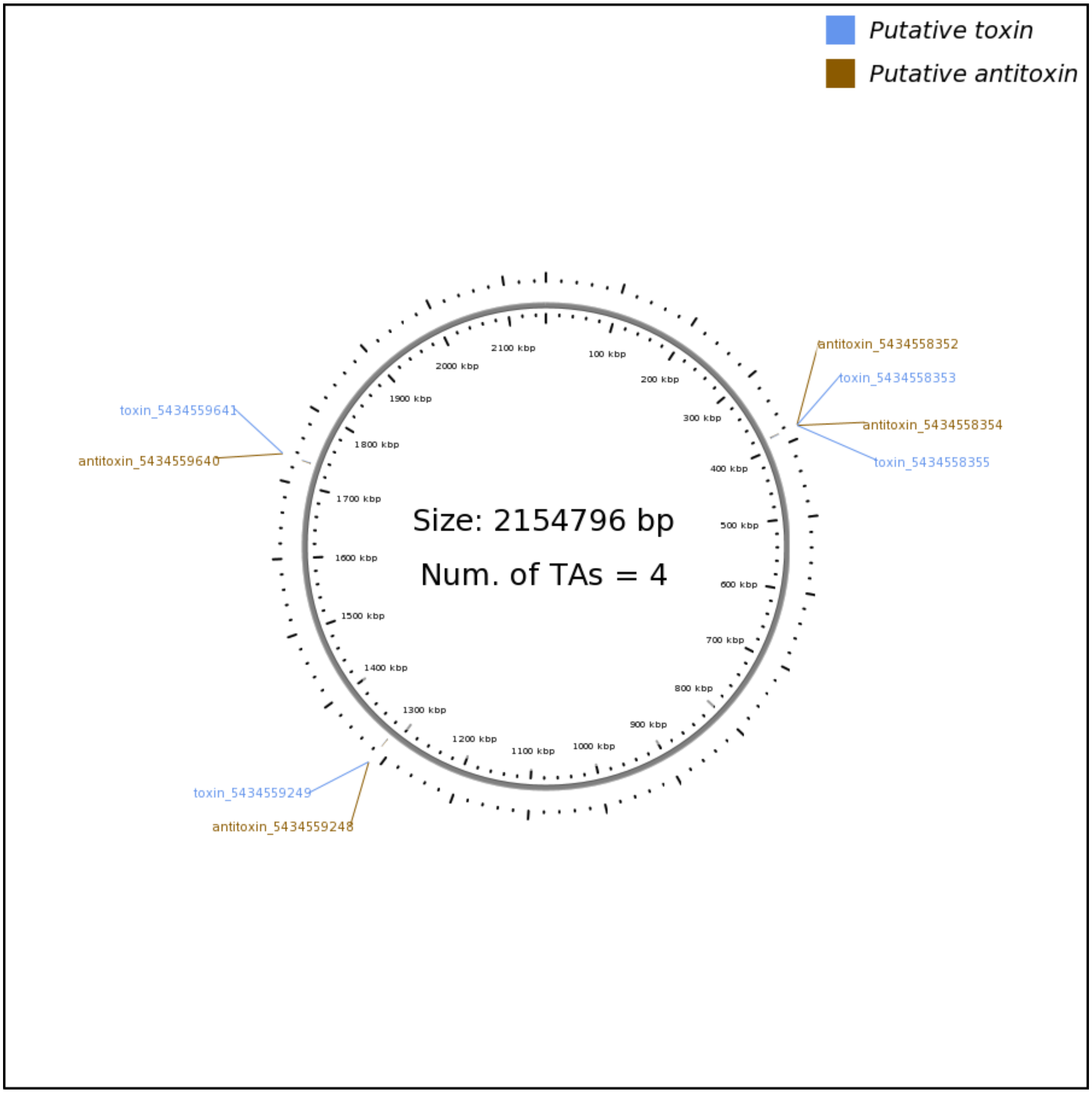

Figure 3-19: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus gordonii* bacteria in the oral microbiome.

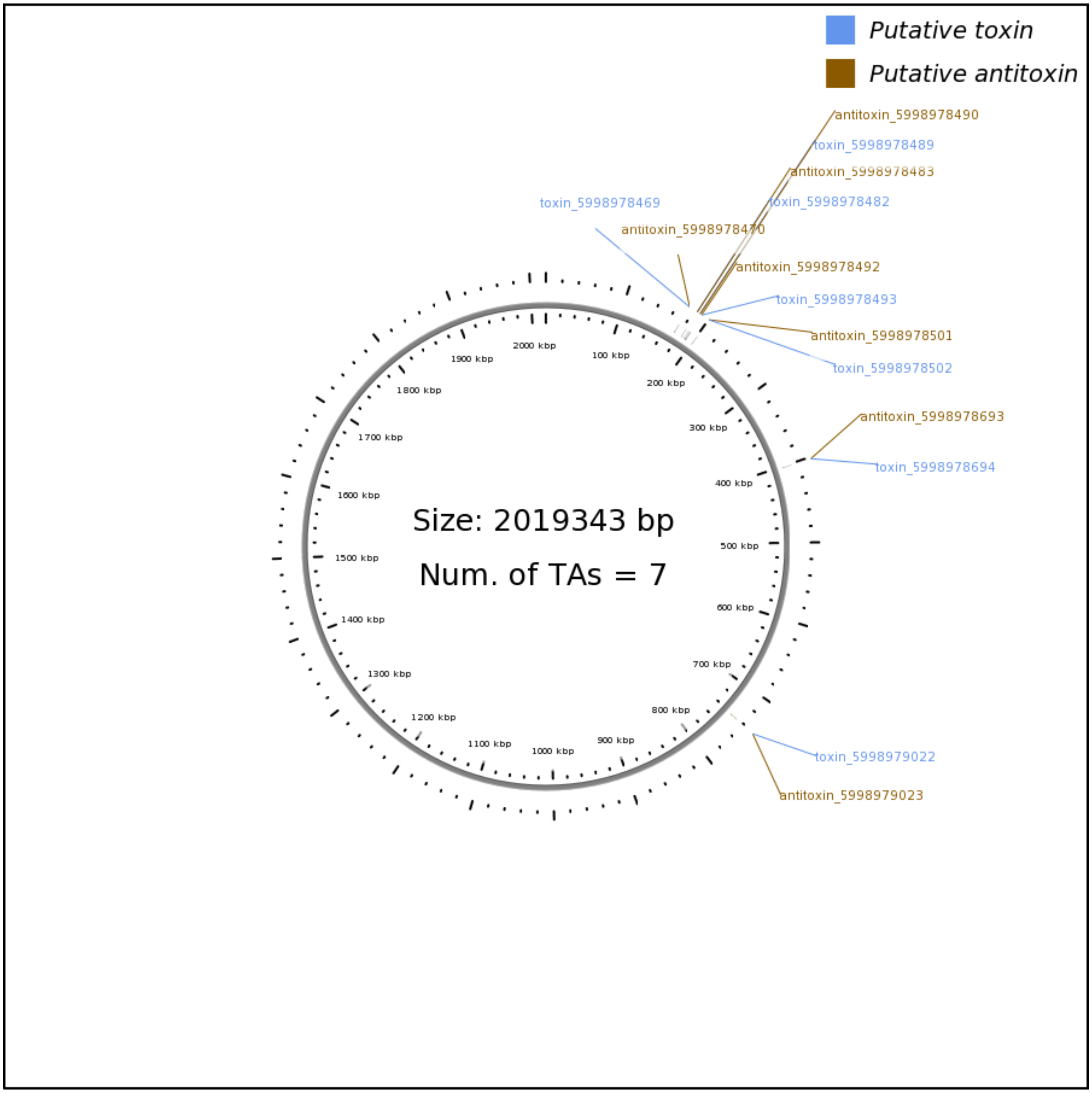

Figure 3-20: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus mutans* bacteria in the oral microbiome.

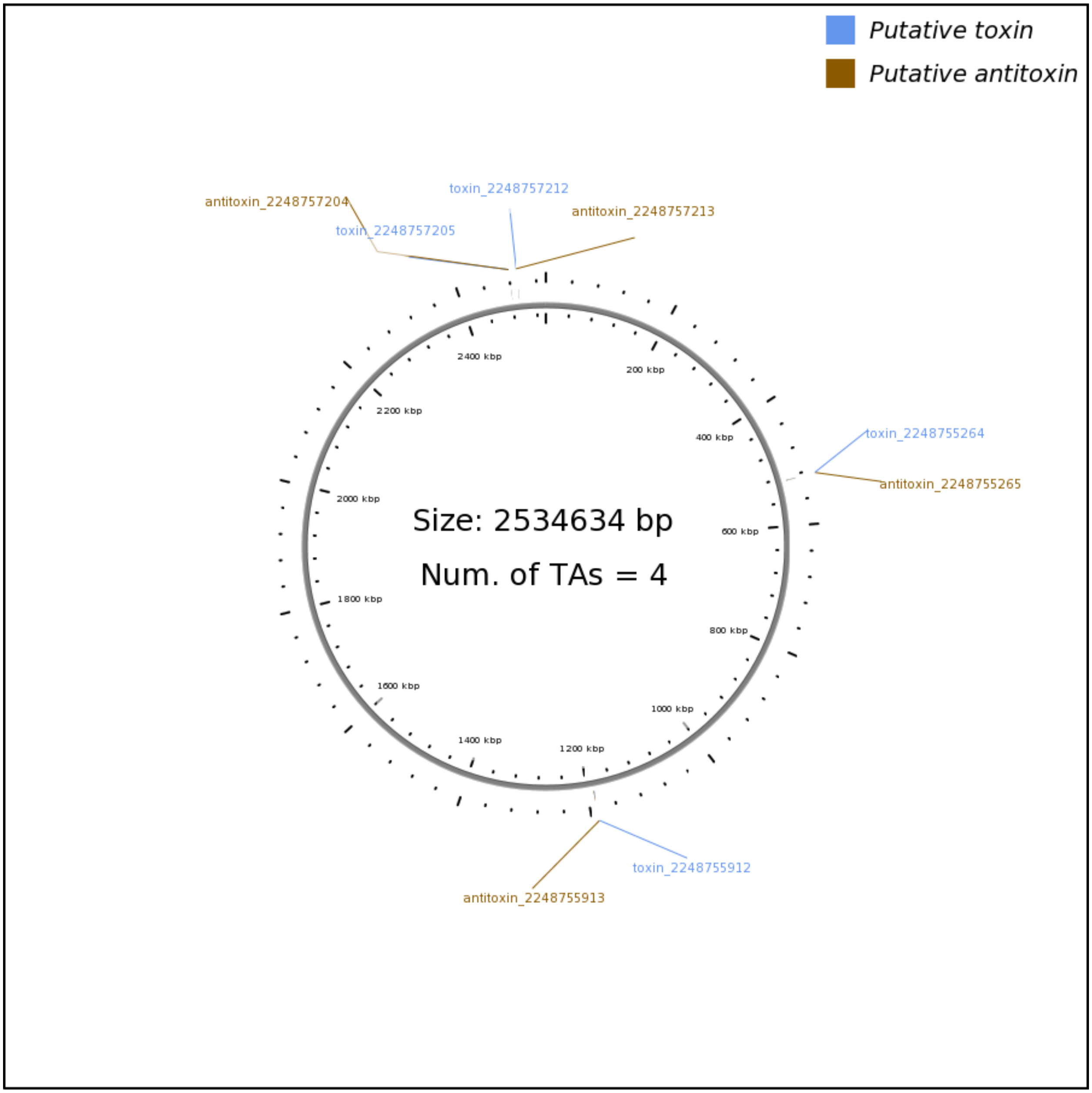

Figure 3-21: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Neisseria elongata* bacteria in the oral microbiome.

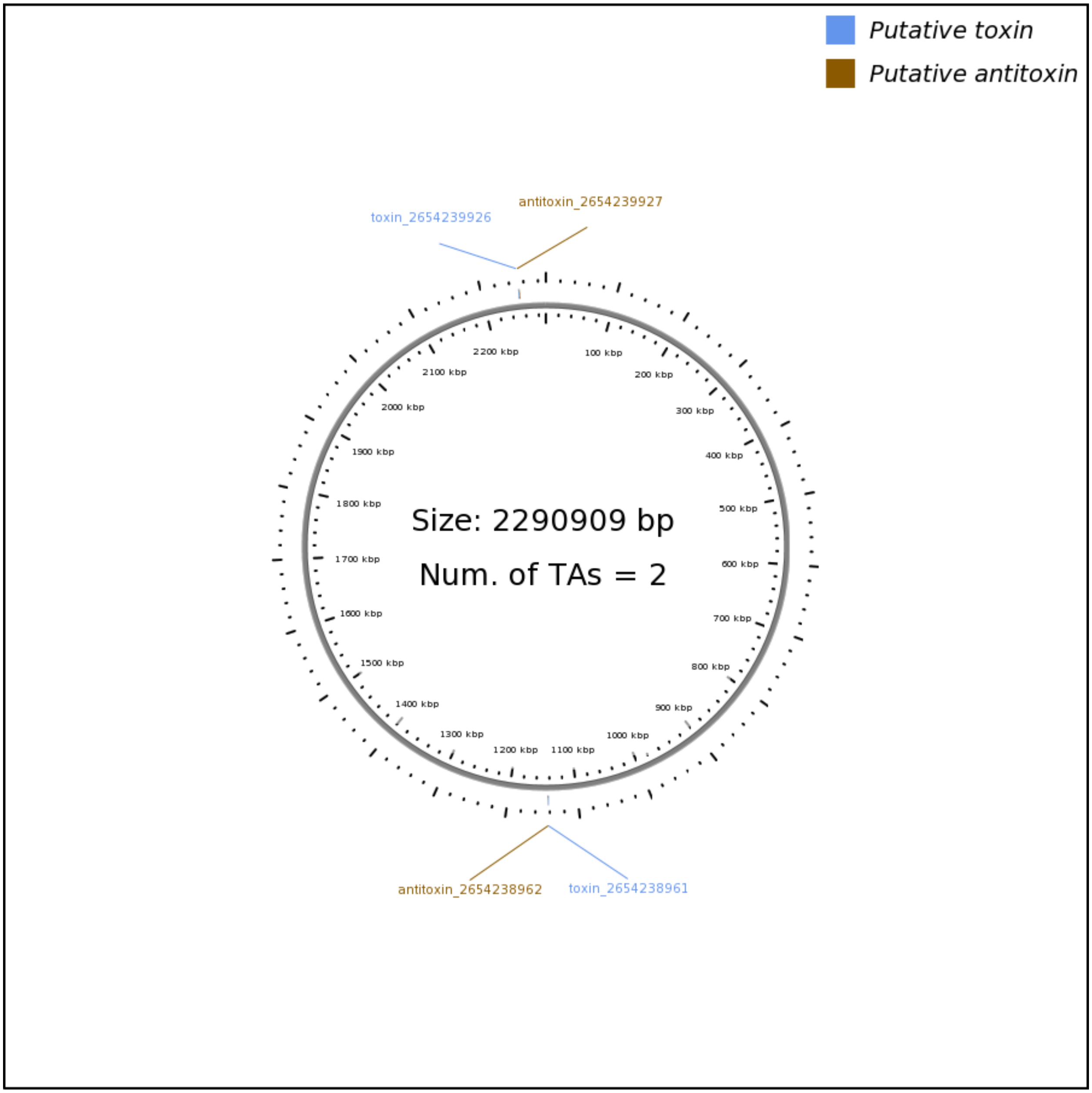

Figure 3-22: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Aggregatibacter aphrophilus* bacteria in the oral microbiome.

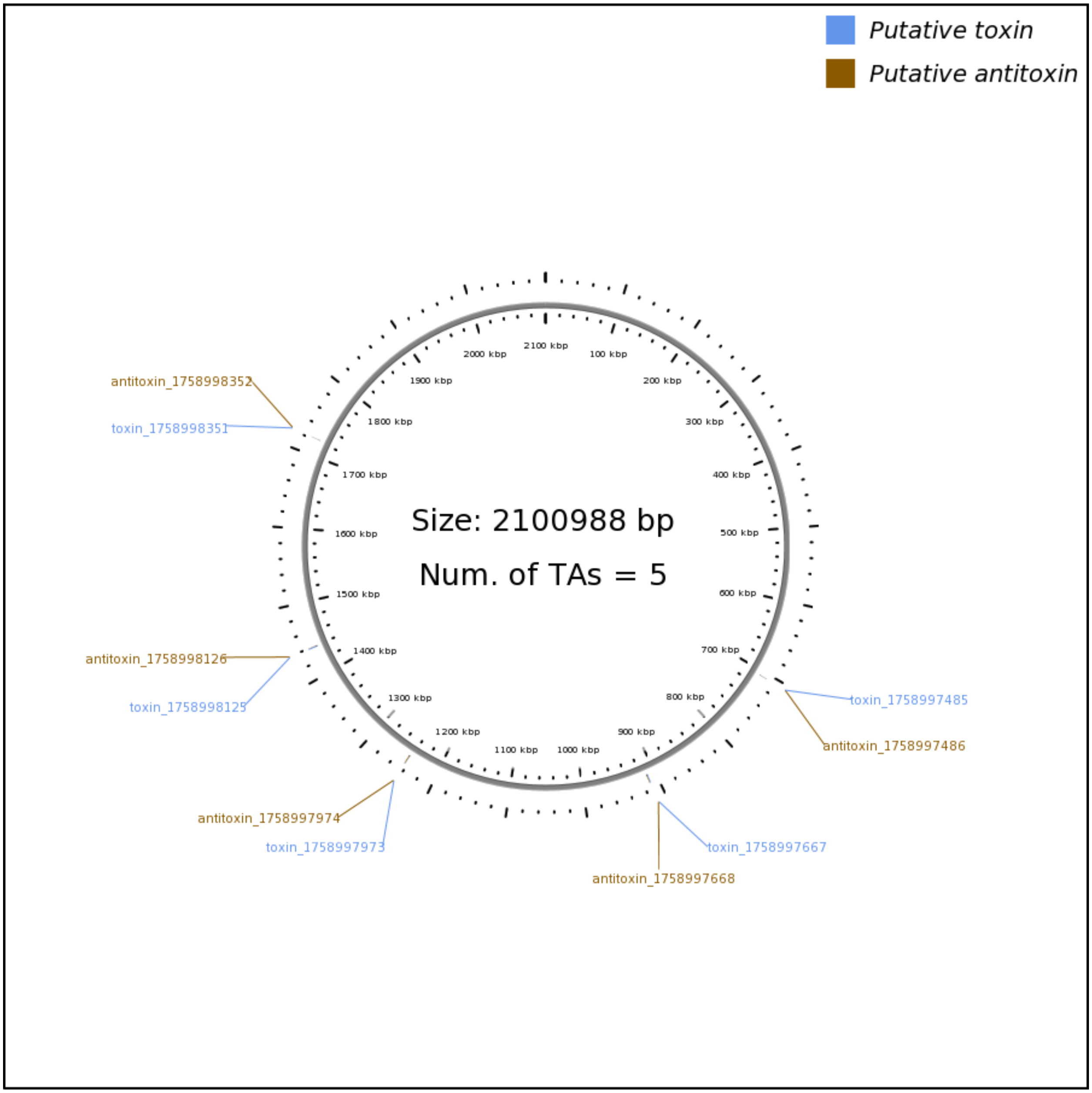

Figure 3-23: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus salivarius* bacteria in the oral microbiome.

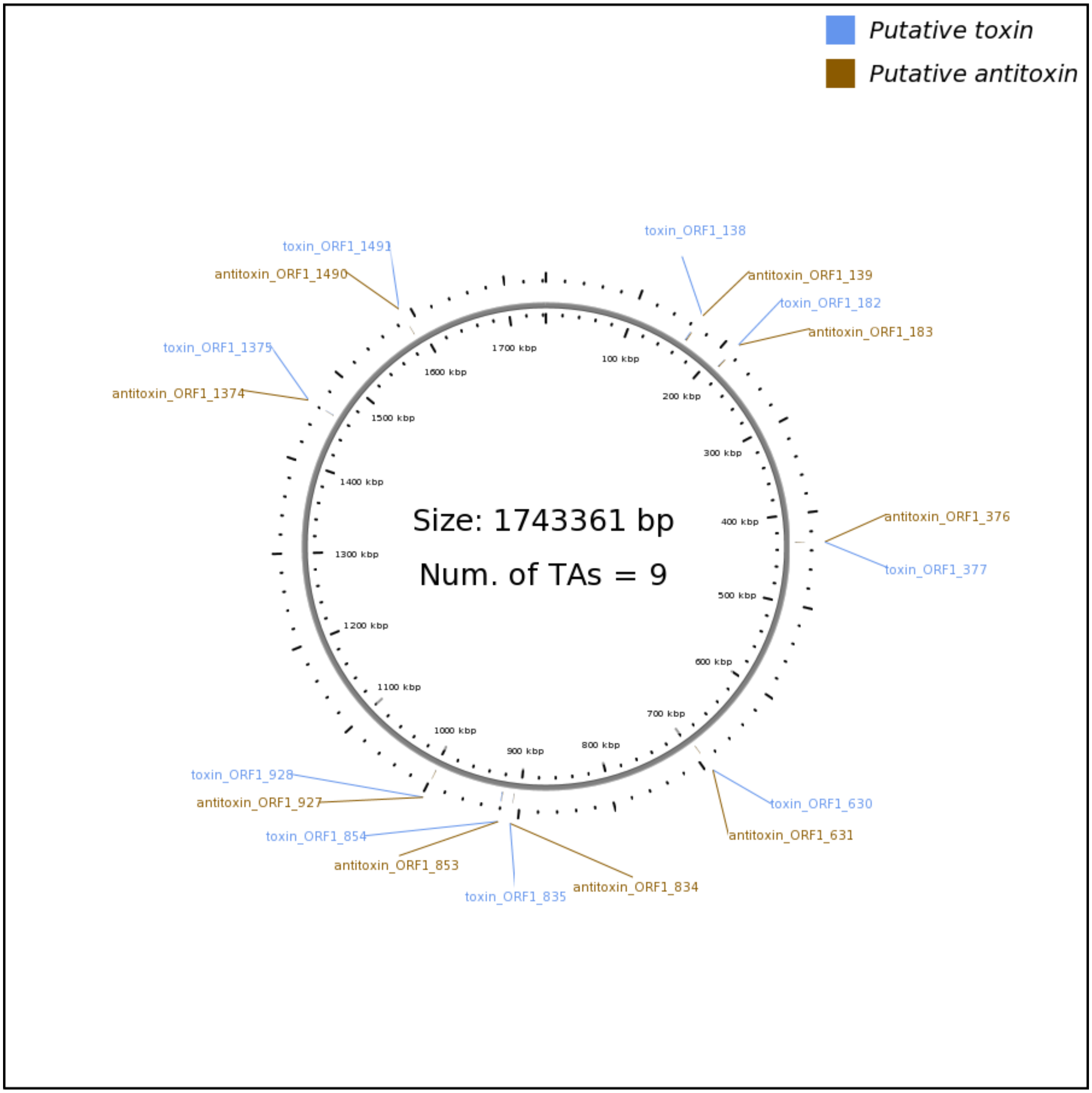

Figure 3-24: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Granulicatella elegans* bacteria in the oral microbiome.

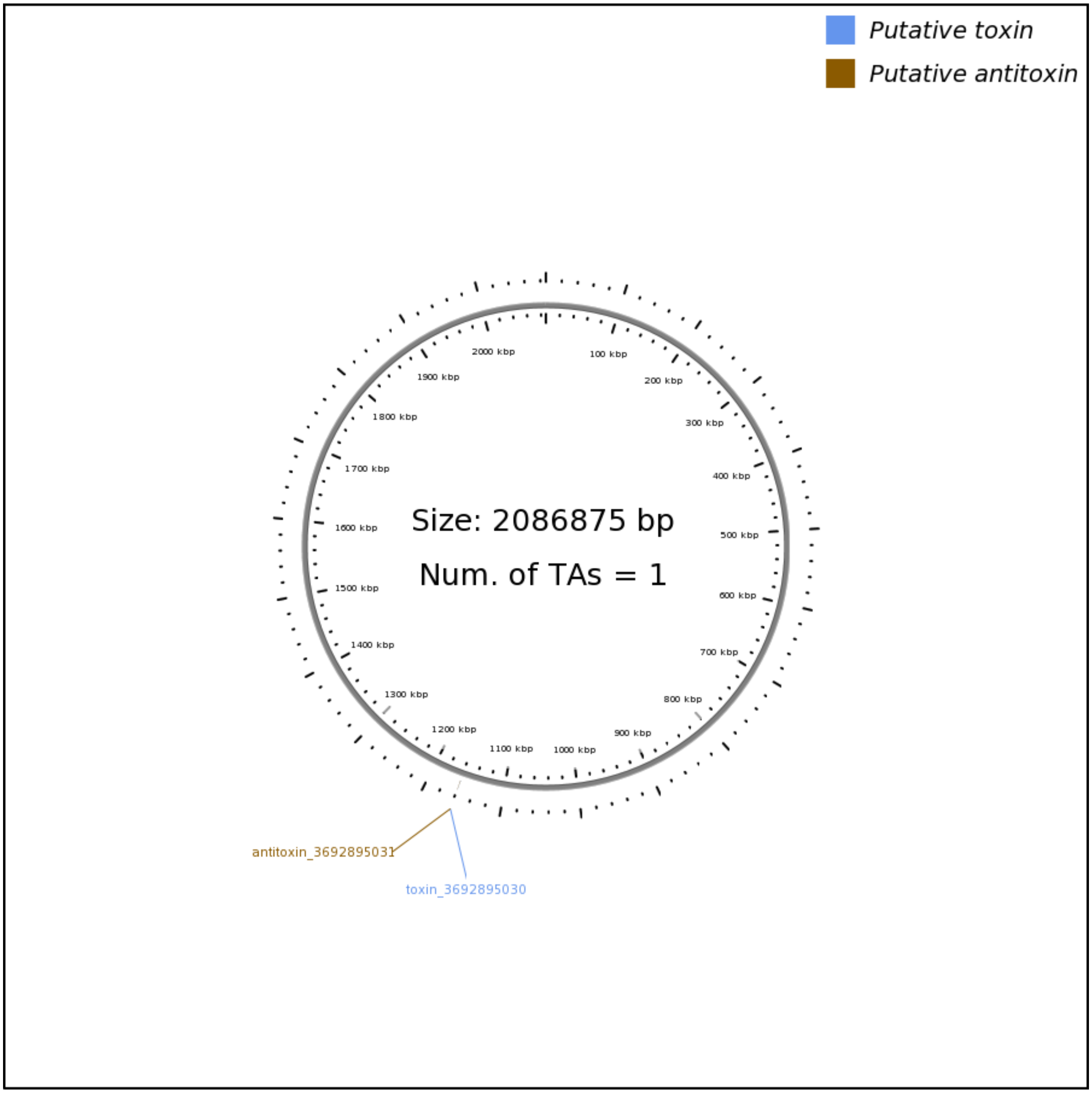

Figure 3-25: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus parainfluenzae* bacteria in the oral microbiome.

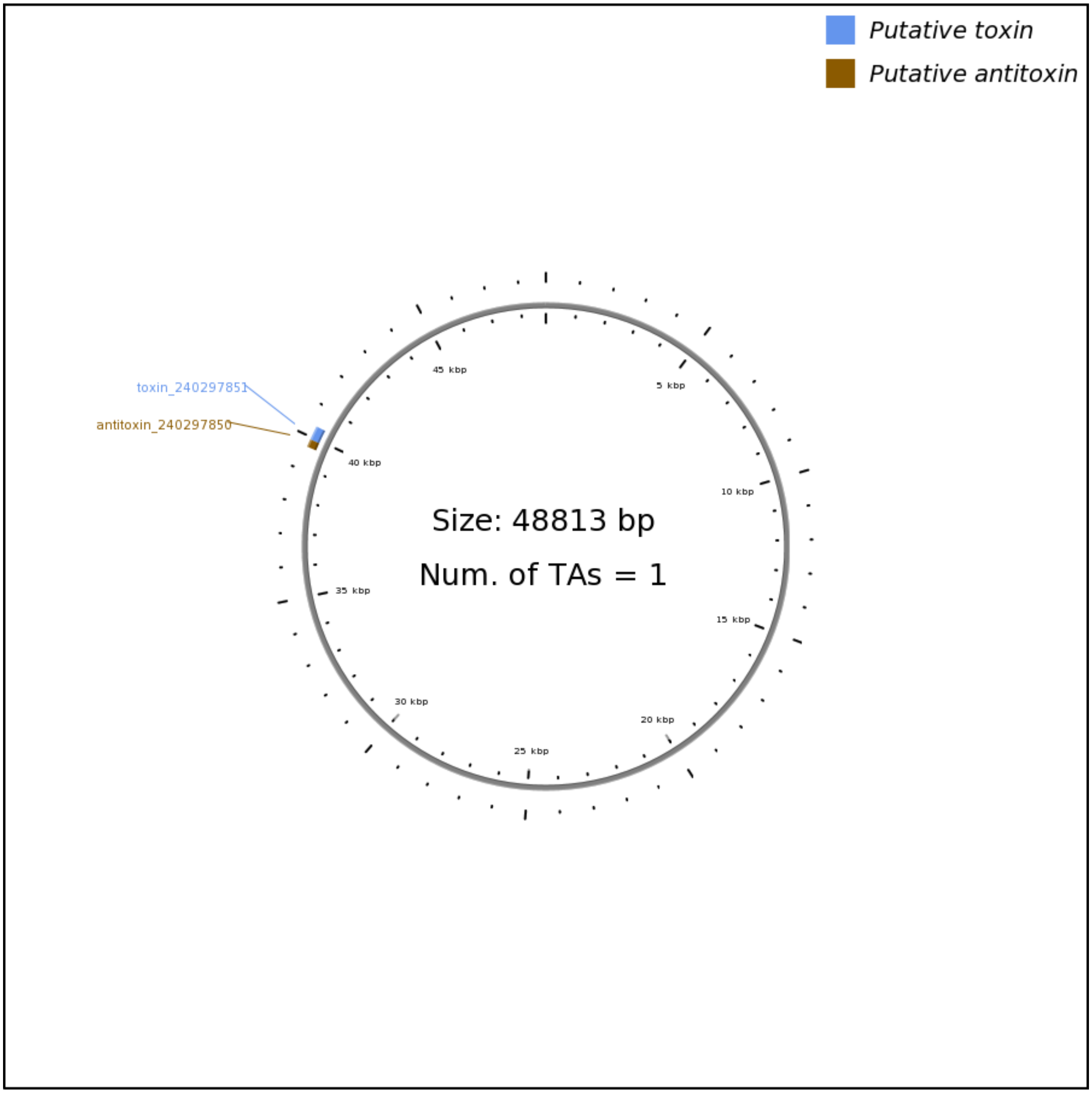

Figure 3-26 Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Actinobacillus minor* bacteria in the oral microbiome.

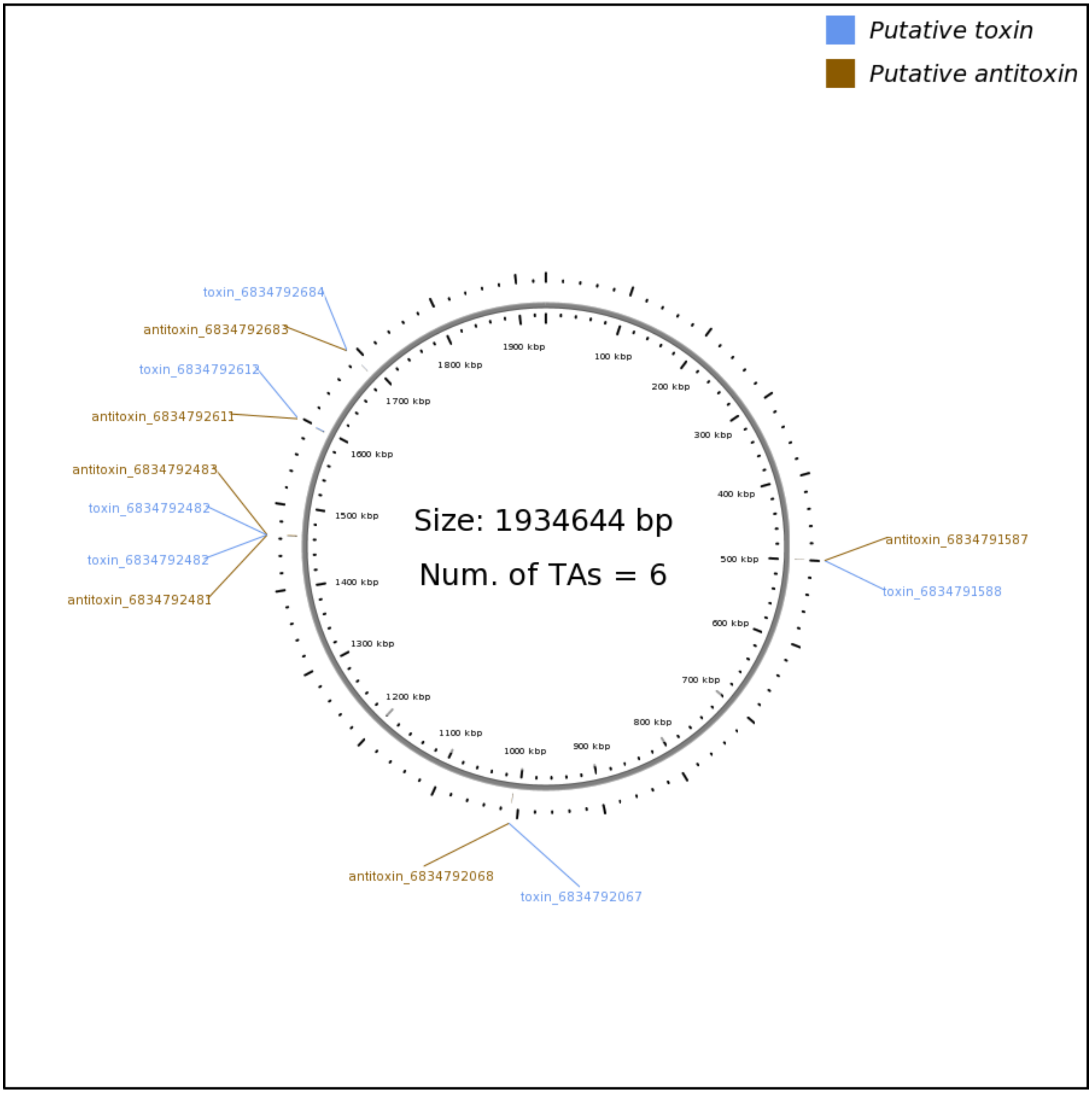

Figure 3-27: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Haemophilus haemolyticus* bacteria in the oral microbiome.

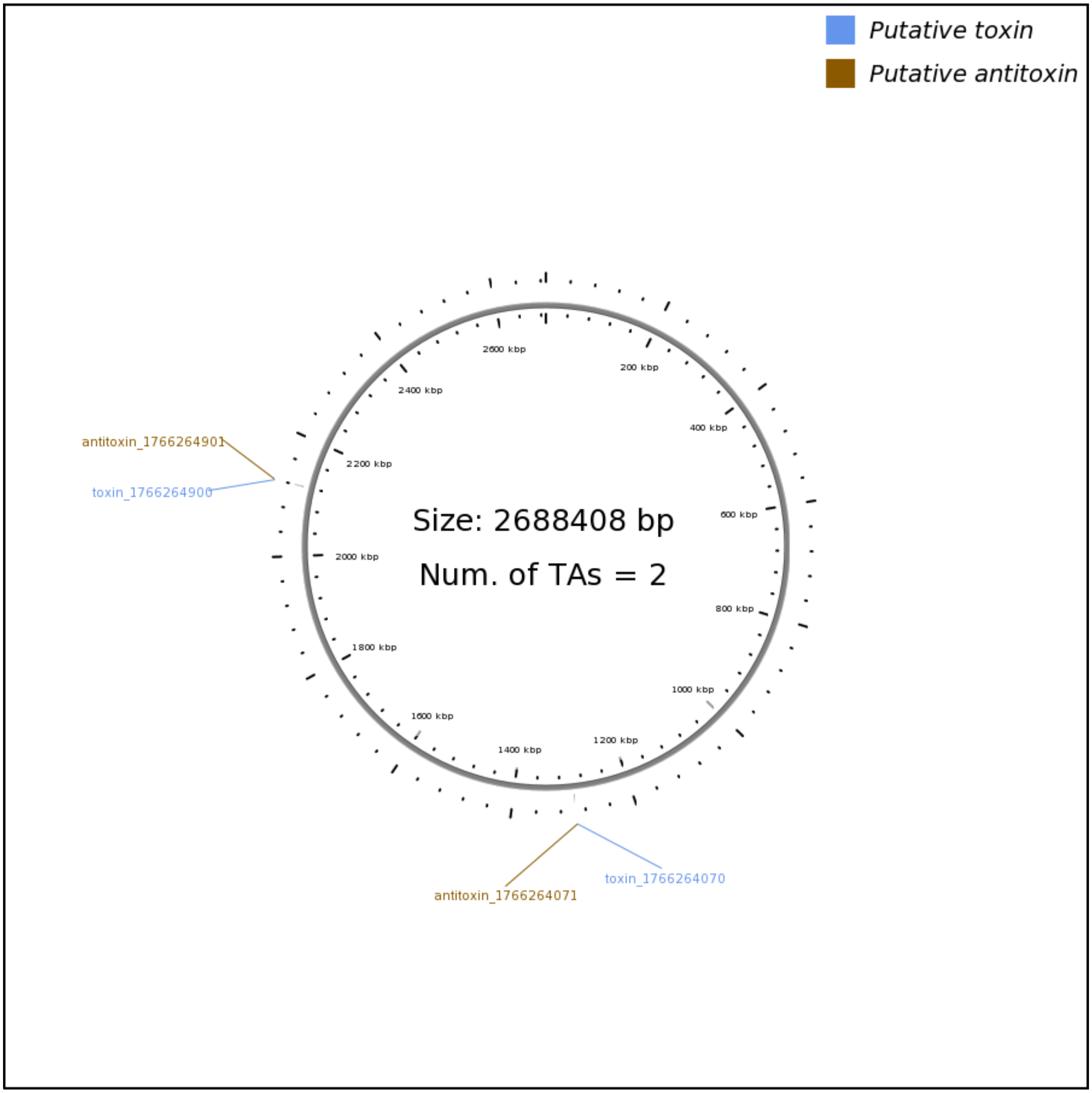

Figure 3-28: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Neisseria mucosa* bacteria in the oral microbiome.

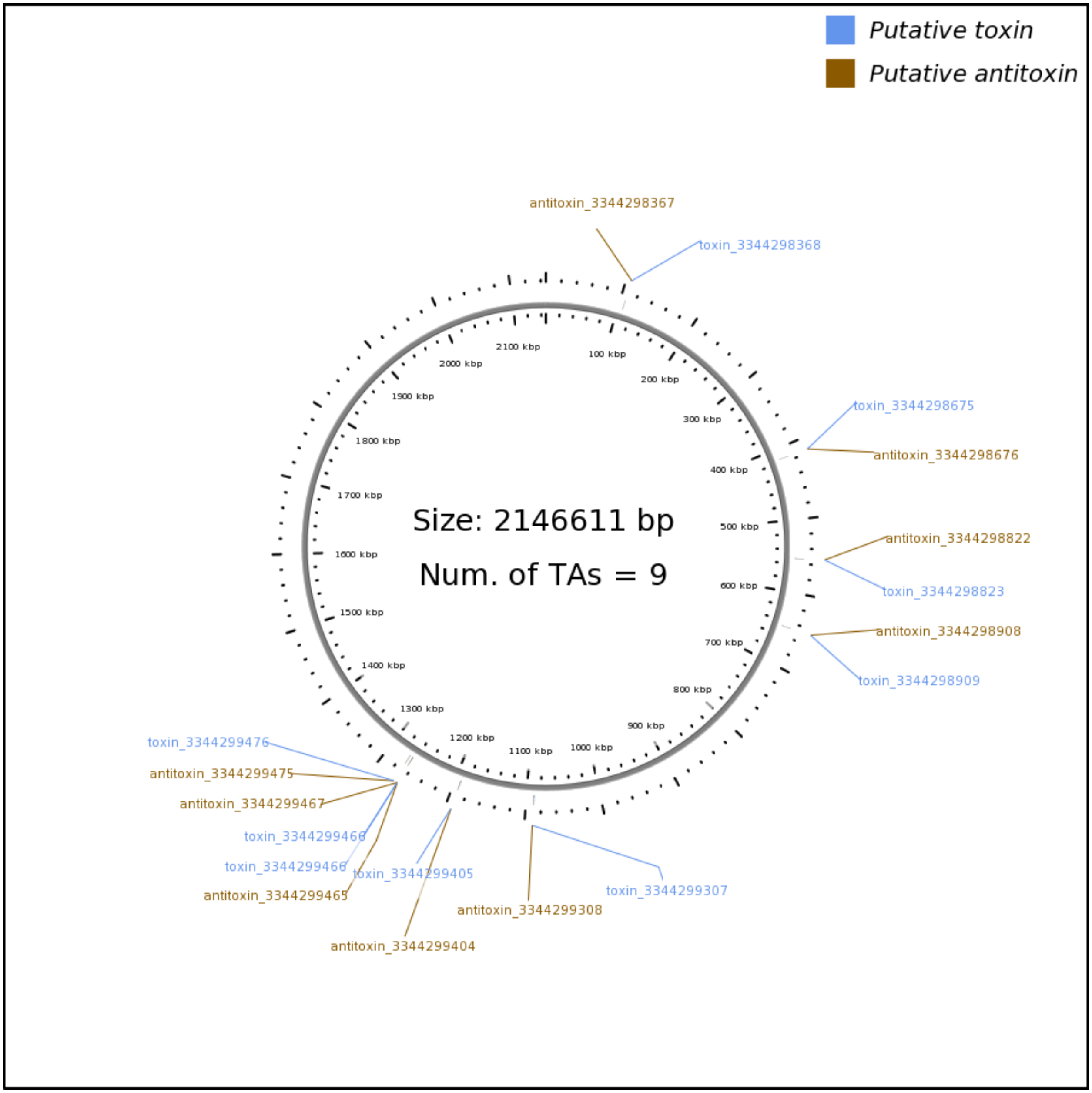

Figure 3-29: Cartoon to visualize intragenomic distances between toxin antitoxin systems in *Streptococcus mitis* bacteria in the oral microbiome.

3.3. Toxin Antitoxin Systems Diversity, Abundance and Interaction Network

The abundance of the chromosomal type II toxin antitoxin systems is important to study. (Harms, Brodersen, Mitarai, & Gerdes, 2018c) Their unique character of altruistic selfishness has impact on the TAS distribution and abundance. (Melderen & Bast, 2009; Ramisetty & Santhosh, 2017) These become imperative as the TAS alters the plasticity of the genome and would affect it depending on the host environment and immune response. The abundance of the taxa used from the oral microbiome for the toxin antitoxin systems analysis is visualized from Figure 3-30 to Figure 3-33.

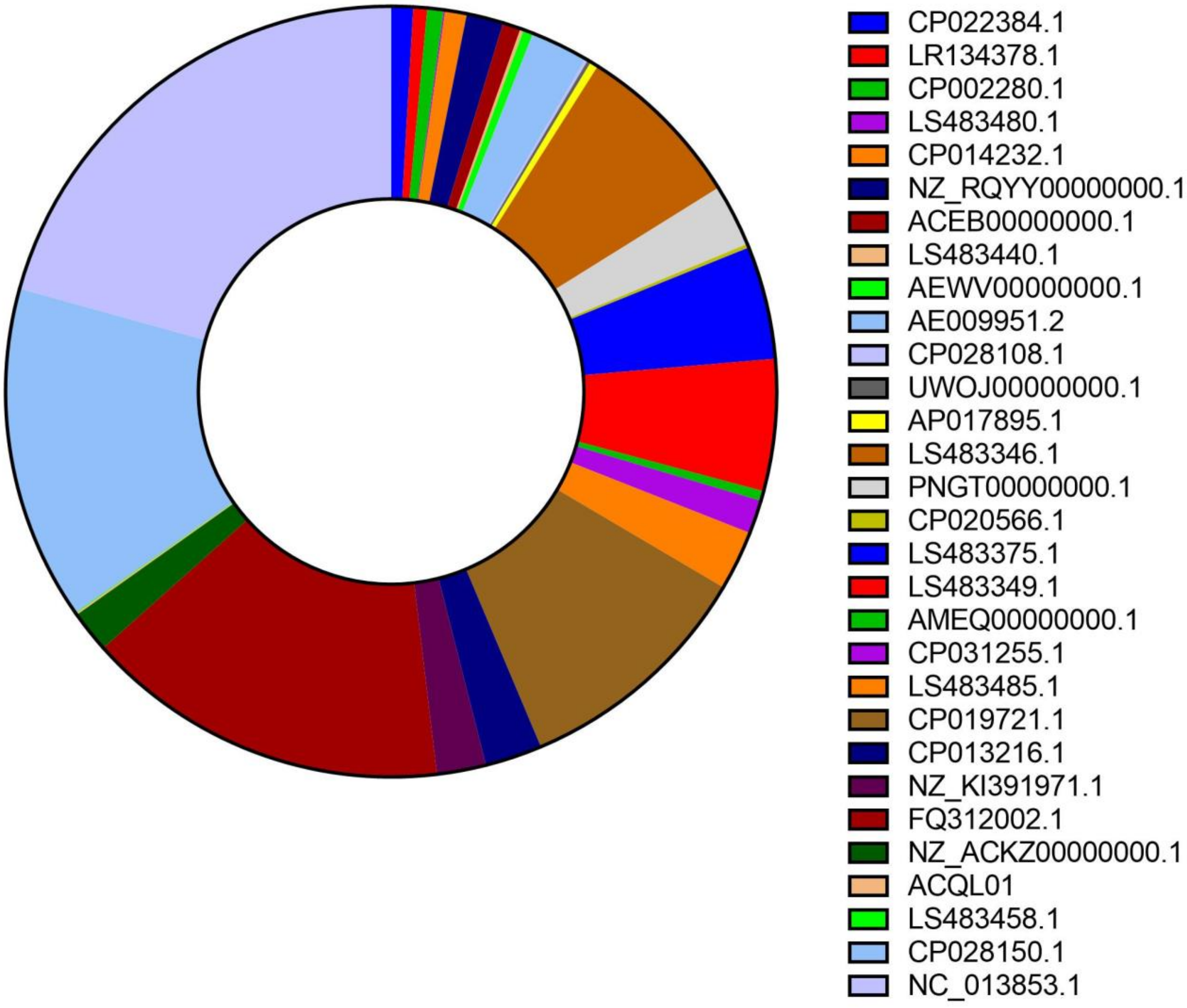

Figure 3-30: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 1. The total of the abundance of the taxa in this figure represents 81.854% of the total microbiome in sample 1.

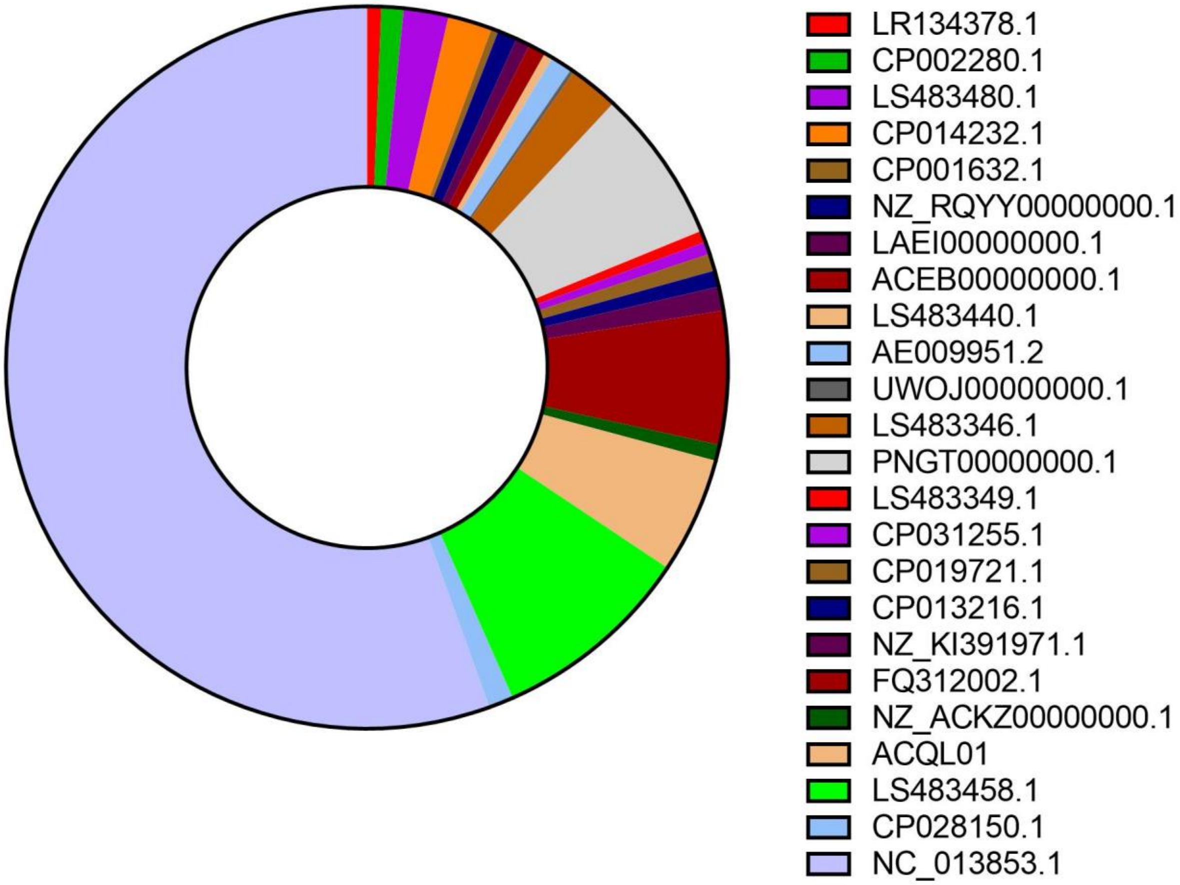

Figure 3-31: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 2. The total of the abundance of the taxa in this figure represents 89.7507% of the total microbiome in sample 2.

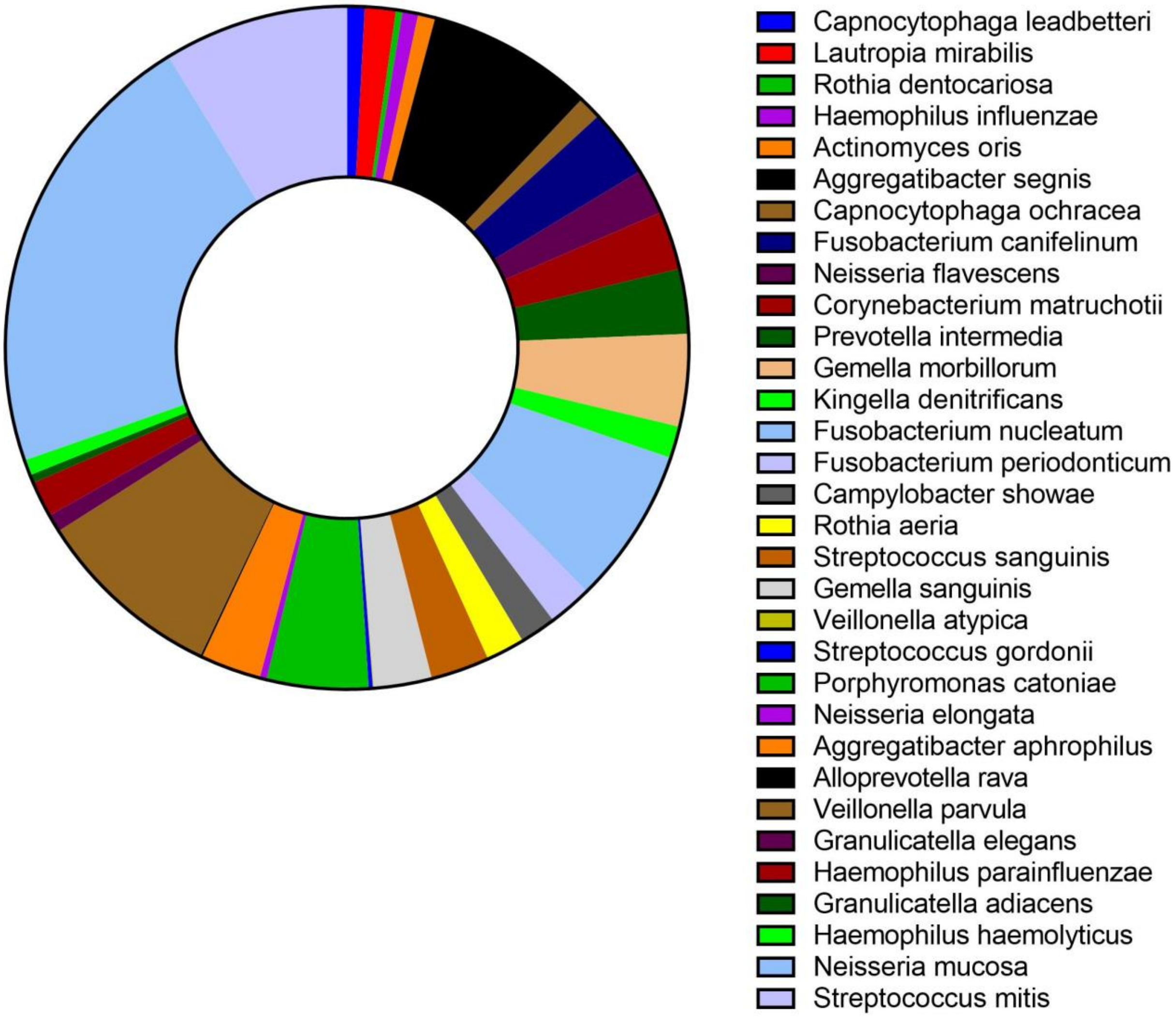

Figure 3-32: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis from Sample 3. The total of the abundance of the taxa in this figure represents 61.1886% of the total microbiome in sample 3.

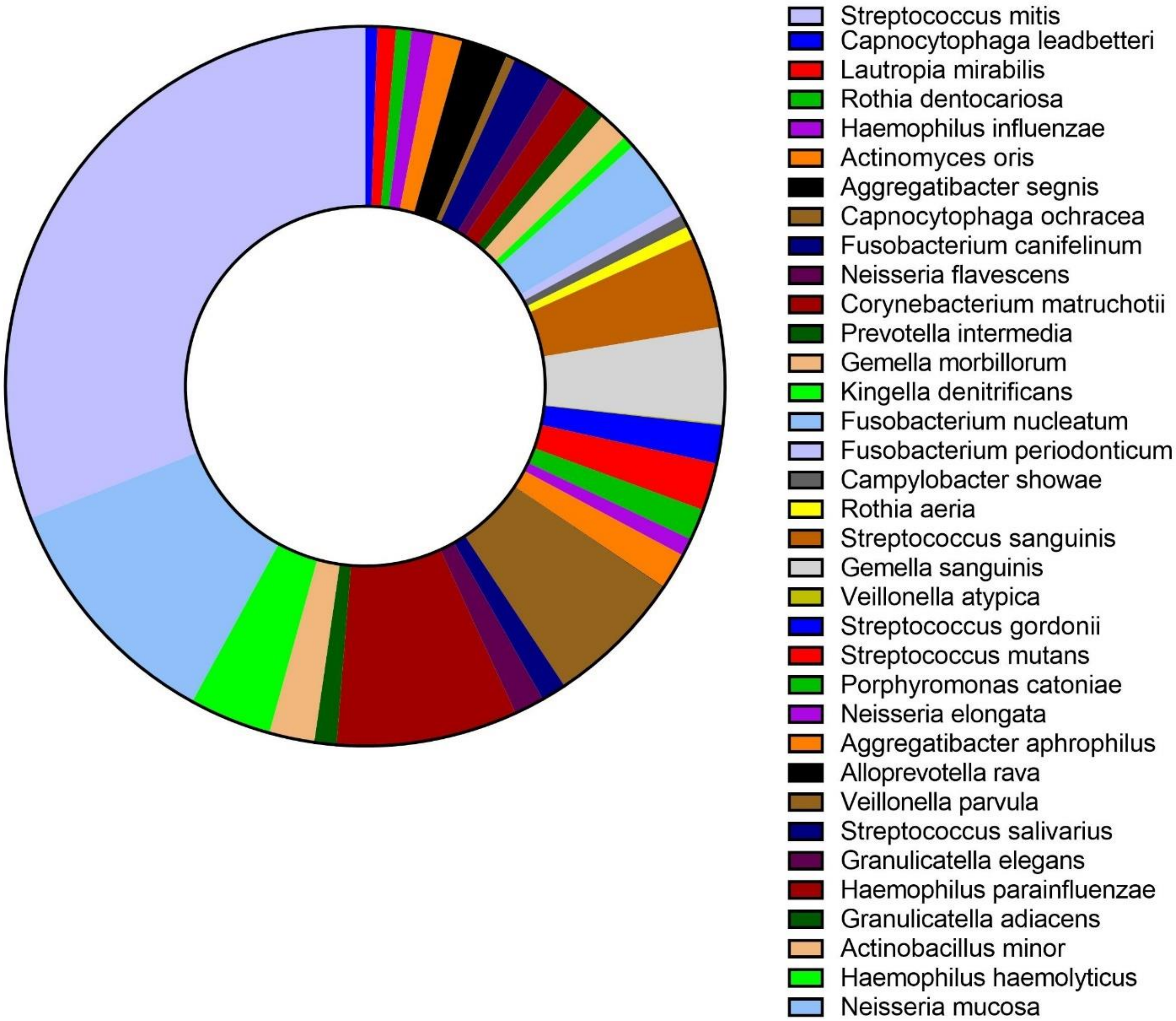

Figure 3-33: describe the abundance of the OTUs used for the Toxin Antitoxin Systems analysis. The total of the abundance of the taxa in this figure represents 77.5978% of the total microbiome.

To calculate the abundance of the TAS in the oral microbiome, it has to be corrected for the abundance of the taxa in the microbiome. This is calculated by multiplying the TAS proportion by the OTUs abundance in the oral microbiome samples as shown in Table 3-3 and graphed in figures from Figure 3-37 to Figure 3-40. Another abundance study for the TAS genes is to account for the genome size and number of coding sequences (CDS) per taxa. This molecular abundance study treats the TAS as a molecular system as a unit of interest. This calculation has been applied, previously in many research studies, for prophages abundance calculations as molecular entity as compared to the context of the coding genes they are infecting (Nadeem & Wahl, 2017). These data are tabulated and visualized in Table 3-4 and from Figure *3-41* till Figure 3-44.

The frequency of the chromosomal type II Toxin Antitoxin Systems in the human oral microbiome is graphed from Figure 3-34 to Figure *3-36*. It shows the highest frequency to the HTH-domain containing genes followed by RelE-like toxins. The RelE toxins are the most frequent type II TAS in the chromosomes in the database TADB (Xie et al., 2018a) However, the highly abundant HTH-domain containing proteins we observed in this project could have this higher frequency because they are a collection of different proteins that cannot be segmented through the domain and/or structural analysis applied.

A heatmap that is constructed based on the pairwise distance between frequencies of each Toxin Antitoxin family predicted in each Operational Taxonomic Unit of the taxa used in the analysis from the human oral microbiome (Figure 3-45). Another interesting tool in visualization of the toxin antitoxin systems frequencies in the oral microbiome is the Circos visualization tool (Figure 3-46). A directed Protein-Protein Interaction Network is constructed by CytoScape based on the degree centrality of the Toxin Antitoxin Systems interactions (Figure 3-47). The edges thickness corresponds to the frequencies of the interaction. The closeness and degree centralities for this network are charted in Table 3-5. The molecular diversity of the type II toxin antitoxin systems predicted from the oral microbiome is recorded in Table 3-6.

Table 3-3: denotes the count and corrected abundance of the Toxin Antitoxin Systems as described in the text.

Operational Taxonomic Unit	**Accession or Assembly Number**	**TAS Count**	**TAS %**	**Corrected Abundance of TAS in Oral 1**	**Corrected Abundance of TAS in Oral 2**	**Corrected Abundance of TAS in Oral 3**
Capnocytophaga leadbetteri	CP022384.1	4	2.877697842	0.000215926	0	0.000145064
Lautropia mirabilis	LR134378.1	0	0	0	0	0
Rothia dentocariosa	CP002280.1	8	5.755395683	0.000320858	0.000513028	0.000108798
Haemophilus influenzae	LS483480.1	11	7.913669065	3.8475E-05	0.001426798	0.000359967
Actinomyces oris	CP014232.1	16	11.51079137	0.000854378	0.002036623	0.000568921
Aggregatibacter segnis	LS483443.1	3	2.158273381	0	0	0.001041228
Capnocytophaga ochracea	CP001632.1	7	5.035971223	0	0.00014568	0.000337159
Fusobacterium canifelinum	NZ_RQYY00000000.1	0	0	0	0	0
Neisseria flavescens	LAEI00000000.1	0	0	0	0	0

Operational Taxonomic Unit	**Accession or Assembly Number**	**TAS Count**	**TAS %**	**Corrected Abundance of TAS in Oral 1**	**Corrected Abundance of TAS in Oral 2**	**Corrected Abundance of TAS in Oral 3**
Corynebacterium matruchotii	ACEB00000000.1	0	0	0	0	0
Prevotella intermedia	CP030094.1	0	0	0	0	0
Gemella morbillorum	LS483440.1	1	0.71942446	8.16137E-06	2.54094E-05	0.000192379
Kingella denitrificans	AEWV00000000.1	0	0	0	0	0
Fusobacterium nucleatum	AE009951.2	9	6.474820144	0.001277021	0.000559733	0.002936262
Fusobacterium periodonticum	CP028108.1	12	8.633093525	8.11473E-05	0	0.001093076
Campylobacter showae	UWOJ00000000.1	0	0	0	0	0
Rothia aeria	AP017895.1	6	4.316546763	0.000116824	0	0.00048024
Streptococcus sanguinis	LS483346.1	5	3.597122302	0.002098637	0.000715093	0.000611278

Operational Taxonomic Unit	Accession or Assembly Number	TAS Count	TAS %	Corrected Abundance of TAS in Oral 1	Corrected Abundance of TAS in Oral 2	Corrected Abundance of TAS in Oral 3
Gemella sanguinis	PNGT00000000.1	0	0	0	0	0
Veillonella atypica	CP020566.1	7	5.035971223	6.61071E-05	0	1.18997E-05
Streptococcus gordonii	LS483375.1	4	2.877697842	0.001116009	0	3.39993E-05
Streptococcus mutans	LS483349.1	7	5.035971223	0.002277021	0.00023546	0
Porphyromonas catoniae	AMEQ00000000.1	0	0	0	0	0
Neisseria elongata	CP031255.1	4	2.877697842	0.000331118	0.000144229	5.60988E-05
Aggregatibacter aphrophilus	LS483485.1	2	1.438848921	0.000294276	0	0.000251878
Alloprevotella rava	ACZK00000000.1	0	0	0	0	0
Veillonella parvula	CP019721.1	0	0	0	0	0

Operational Taxonomic Unit	**Accession or Assembly Number**	**TAS Count**	**TAS %**	**Corrected Abundance of TAS in Oral 1**	**Corrected Abundance of TAS in Oral 2**	**Corrected Abundance of TAS in Oral 3**
Streptococcus salivarius	CP013216.1	5	3.597122302	0.000704792	0.000237154	0
Granulicatella elegans	NZ_KI391971.1	9	6.474820144	0.001091291	0.000622893	0.000332768
Haemophilus parainfluenzae	FQ312002.1	1	0.71942446	0.000901481	0.000385255	7.47984E-05
Granulicatella adiacens	NZ_ACKZ00000000.1	0	0	0	0	0
Actinobacillus minor	ACQL01	1	0.71942446	4.54705E-06	0.000335888	0
Haemophilus haemolyticus	LS483458.1	6	4.316546763	1.18923E-05	0.00349488	0.000194646
Neisseria mucosa	CP028150.1	2	1.438848921	0.001651627	0.000143261	0.001901409
Streptococcus mitis	NC_013853.1	9	6.474820144	0.010968527	0.032255403	0.00346665
Total	Total	139	100	0.024430118	0.043276785	0.014198518

Table 3-4: denotes the molecular abundance of the Toxin Antitoxin Systems in relation to the gene counts.

Operational Taxonomic Units	**Accession or Assembly Number**	**Genome Size**	**Gene Count**	**Molecular Abundance of TAS in Oral 1**	**Molecular Abundance of TAS in Oral 2**	**Molecular Abundance of TAS in Oral 3**	**Molecular Abundance of TAS in the Oral Microbiome**
Capnocytophaga leadbetteri	CP022384.1	2,504,023	2,253	1.33217E-05	0	8.94977E-06	0.001775411
Lautropia mirabilis	LR134378.1	3,172,010	2,595	0	0	0	0
Rothia dentocariosa	CP002280.1	2,492,820	2,217	2.0117E-05	3.21655E-05	6.82132E-06	0.00360848
Haemophilus influenzae	LS483480.1	1,846,600	1,863	2.87065E-06	0.000106455	2.68575E-05	0.005904455
Actinomyces oris	CP014232.1	3,104,690	2,459	4.82955E-05	0.000115124	3.21594E-05	0.00650671
Aggregatibacter segnis	LS483443.1	1,965,660	1,896	0	0	7.63347E-05	0.001582278
Capnocytophaga ochracea	CP001632.1	2,658,650	2,171	0	9.32731E-06	2.15869E-05	0.003224321
Fusobacterium canifelinum	NZ_RQYY00000000.1	2,224,460	2,204	0	0	0	0

Operational Taxonomic Units	**Accession or Assembly Number**	**Genome Size**	**Gene Count**	**Molecular Abundance of TAS in Oral 1**	**Molecular Abundance of TAS in Oral 2**	**Molecular Abundance of TAS in Oral 3**	**Molecular Abundance of TAS in the Oral Microbiome**
Neisseria flavescens	LAEI00000000.1	2,291,790	1,994	0	0	0	0
Corynebacterium matruchotii	ACEB00000000.1	2,867,410	3,144	0	0	0	0
Prevotella intermedia	CP030094.1	2,777,800	1,762	0	0	0	0
Gemella morbillorum	LS483440.1	1,756,930	1,656	6.85042E-07	2.13279E-06	1.61478E-05	0.000603865
Kingella denitrificans	AEWV00000000.1	2,220,450	2,522	0	0	0	0
Fusobacterium nucleatum	AE009951.2	2,408,530	2,067	8.58761E-05	3.76405E-05	0.000197455	0.004354136
Fusobacterium periodonticum	CP028108.1	2,477,380	2,540	4.44074E-06	0	5.9818E-05	0.004724409
Campylobacter showae	UWOJ00000000.1	2,213,800	2,392	0	0	0	0
Rothia aeria	AP017895.1	2,603,190	2,799	5.80156E-06	0	2.3849E-05	0.002143623

Operational Taxonomic Units	**Accession or Assembly Number**	**Genome Size**	**Gene Count**	**Molecular Abundance of TAS in Oral 1**	**Molecular Abundance of TAS in Oral 2**	**Molecular Abundance of TAS in Oral 3**	**Molecular Abundance of TAS in the Oral Microbiome**
Streptococcus sanguinis	LS483346.1	2,361,220	2,314	0.000126063	4.2955E-05	3.6719E-05	0.002160761
Gemella sanguinis	PNGT00000000.1	1,836,490	1,893	0	0	0	0
Veillonella atypica	CP020566.1	2,071,950	1,887	4.86957E-06	0	8.76558E-07	0.003709592
Streptococcus gordonii	LS483375.1	2,190,540	2,051	7.56339E-05	0	2.30419E-06	0.001950268
Streptococcus mutans	LS483349.1	1,962,000	1,885	0.000167908	1.73629E-05	0	0.003713528
Porphyromonas catoniae	AMEQ00000000.1	2,070,830	1,853	0	0	0	0
Neisseria elongata	CP031255.1	2,397,850	2,574	1.78809E-05	7.78856E-06	3.02942E-06	0.001554002
Aggregatibacter aphrophilus	LS483485.1	2,332,870	2,097	1.95061E-05	0	1.66958E-05	0.000953743

Operational Taxonomic Units	**Accession or Assembly Number**	**Genome Size**	**Gene Count**	**Molecular Abundance of TAS in Oral 1**	**Molecular Abundance of TAS in Oral 2**	**Molecular Abundance of TAS in Oral 3**	**Molecular Abundance of TAS in the Oral Microbiome**
Alloprevotella rava	ACZK00000000.1	2,593,870	2,177	0	0	0	0
Veillonella parvula	CP019721.1	2,142,200	2,036	0	0	0	0
Streptococcus salivarius	CP013216.1	2,236,020	1,903	5.14798E-05	1.73224E-05	0	0.00262743
Granulicatella elegans	NZ_KI391971.1	1,743,360	1,629	9.31182E-05	5.31505E-05	2.83946E-05	0.005524862
Haemophilus parainfluenzae	FQ312002.1	2,074,140	1,993	6.2873E-05	2.68692E-05	5.21675E-06	0.000501756
Granulicatella adiacens	NZ_ACKZ00000000.1	1,947,170	1,889	0	0	0	0
Actinobacillus minor	ACQL01	2,276,210	2,411	2.62148E-07	1.93648E-05	0	0.000414766
Haemophilus haemolyticus	LS483458.1	1,914,630	1,774	9.31807E-07	0.000273838	1.52513E-05	0.003382187

Operational Taxonomic Units	**Accession or Assembly Number**	**Genome Size**	**Gene Count**	**Molecular Abundance of TAS in Oral 1**	**Molecular Abundance of TAS in Oral 2**	**Molecular Abundance of TAS in Oral 3**	**Molecular Abundance of TAS in the Oral Microbiome**
Neisseria mucosa	CP028150.1	2,492,650	2,734	8.39708E-05	7.28355E-06	9.667E-05	0.000731529
Streptococcus mitis	NC_013853.1	1,989,190	2,002	0.000761551	0.002239511	0.000240691	0.004495504
Total	Accession or Assembly Number	80,219,383	75,636	0.001647457	0.00300829	0.000915829	0.066147616

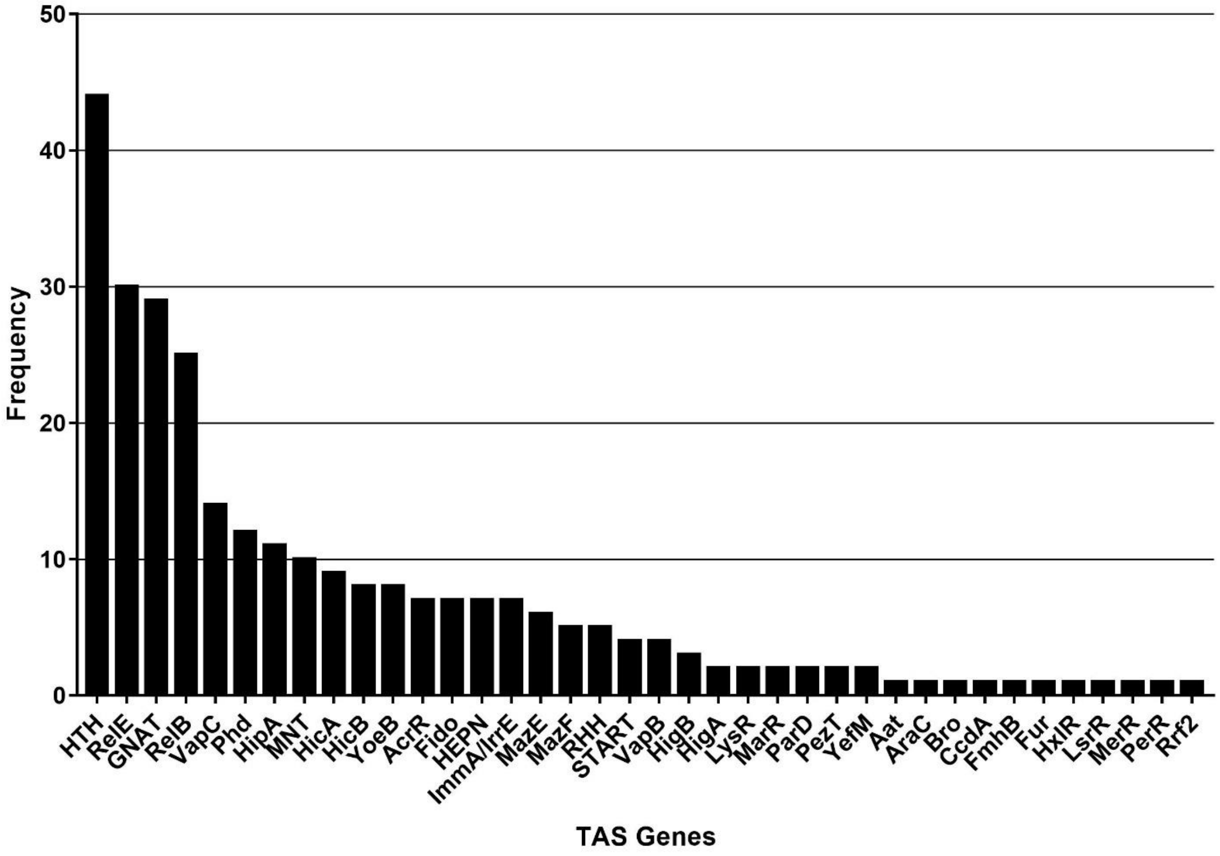

Figure 3-34: represents a histogram to visualize the frequencies of the chromosomal type II toxin antitoxin systems predicted in the human oral microbiome.

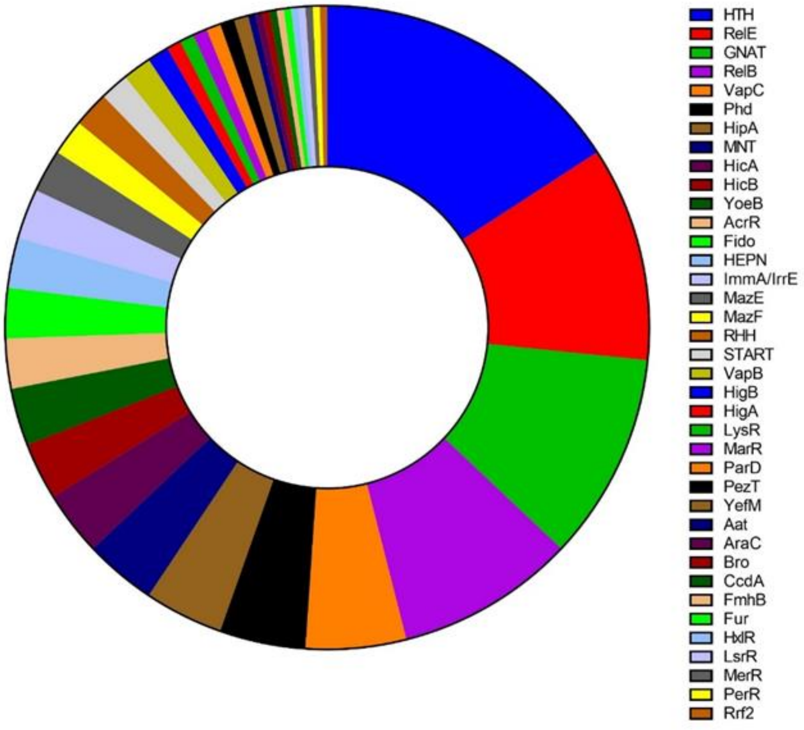

Figure 3-35: A pie chart that visualizes the frequencies of the Toxin Antitoxin Systems families predicted in the human oral microbiome.

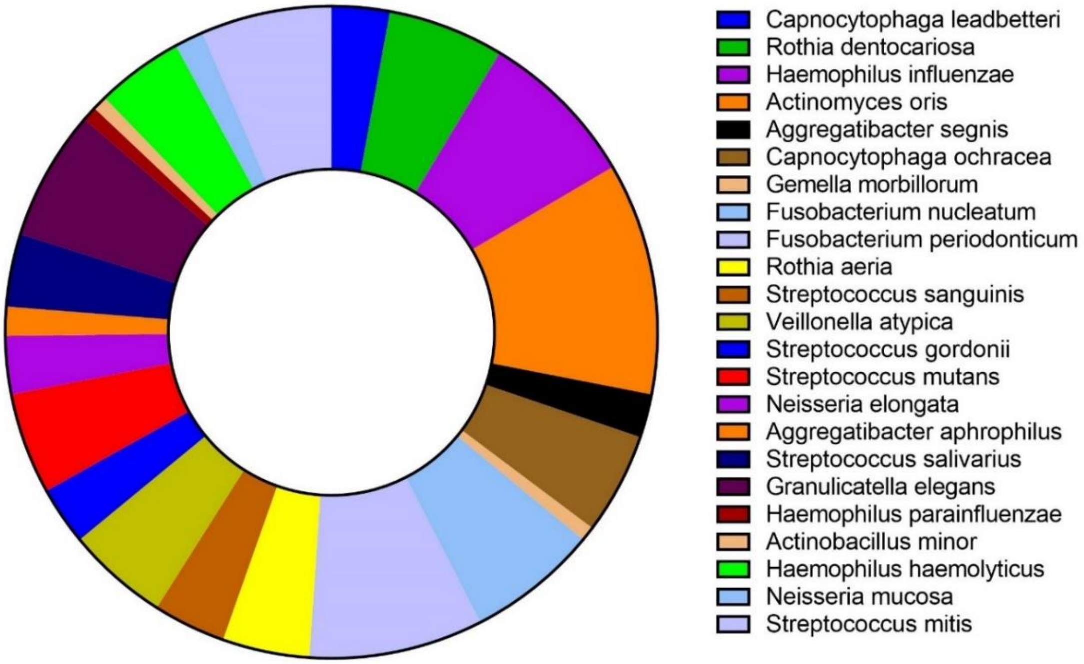

Figure 3-36: A pie chart shows the percentages of each family of the type II Toxin Antitoxin Systems in each of the Operational Taxonomic Units used in this study from the human oral microbiome.

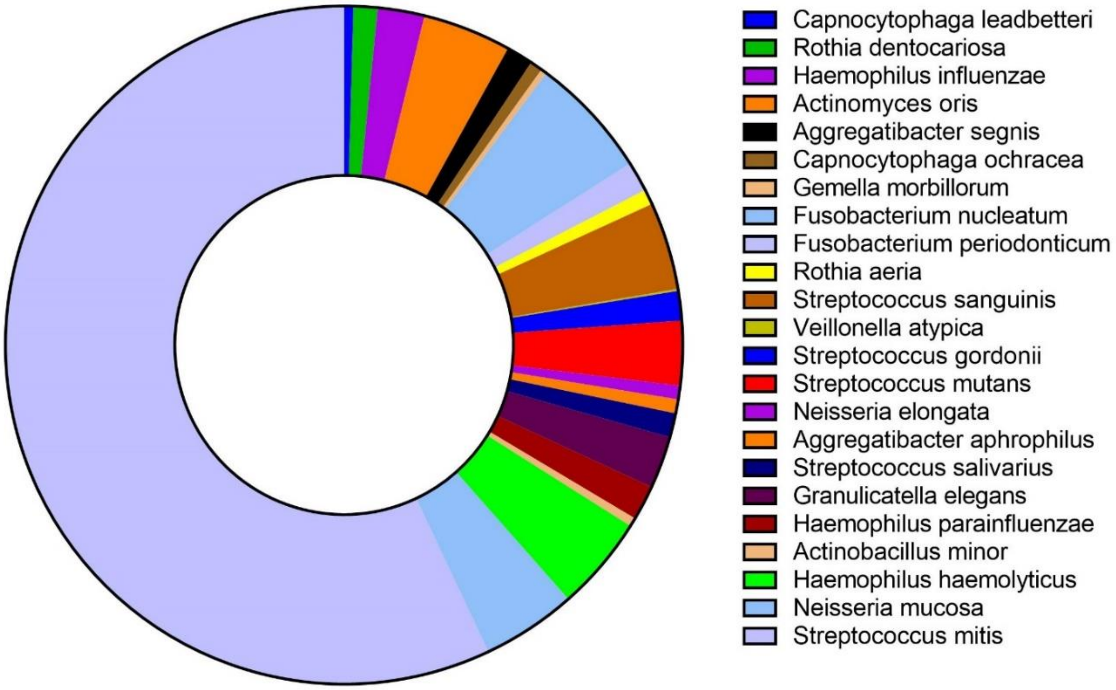

Figure 3-37: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon that are used for the analysis from the human oral microbiome.

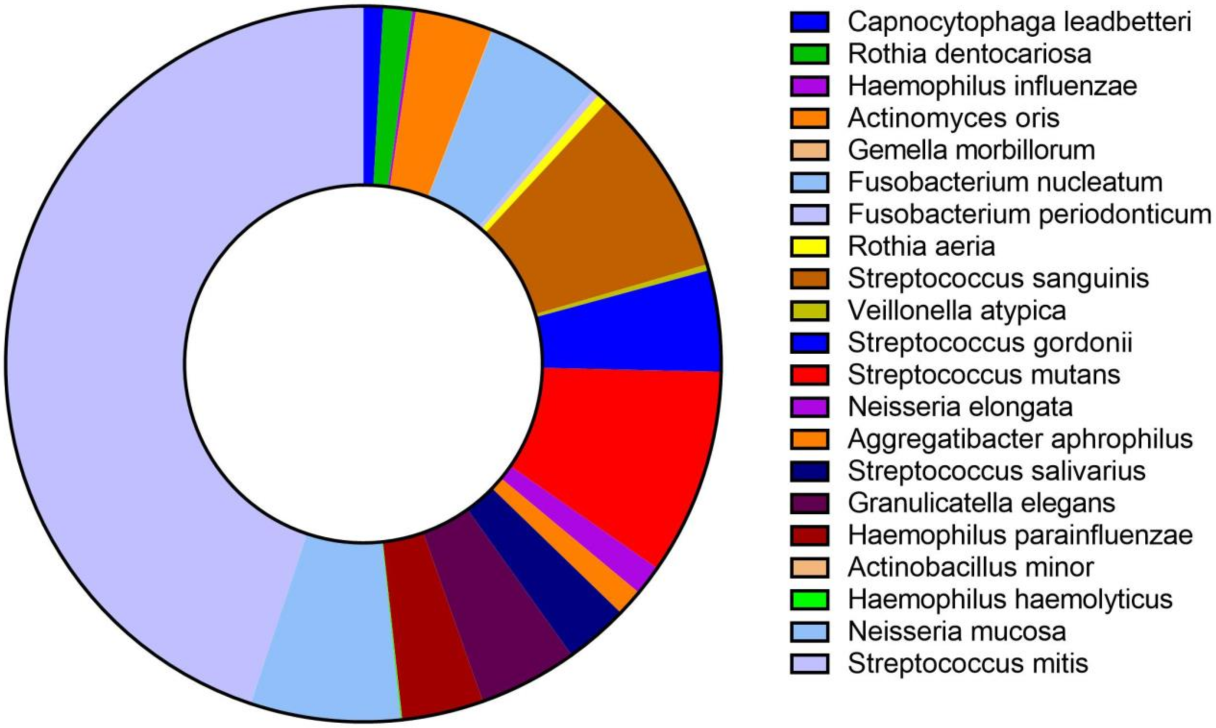

Figure 3-38: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 1 sample that are used for the analysis from the human oral microbiome.

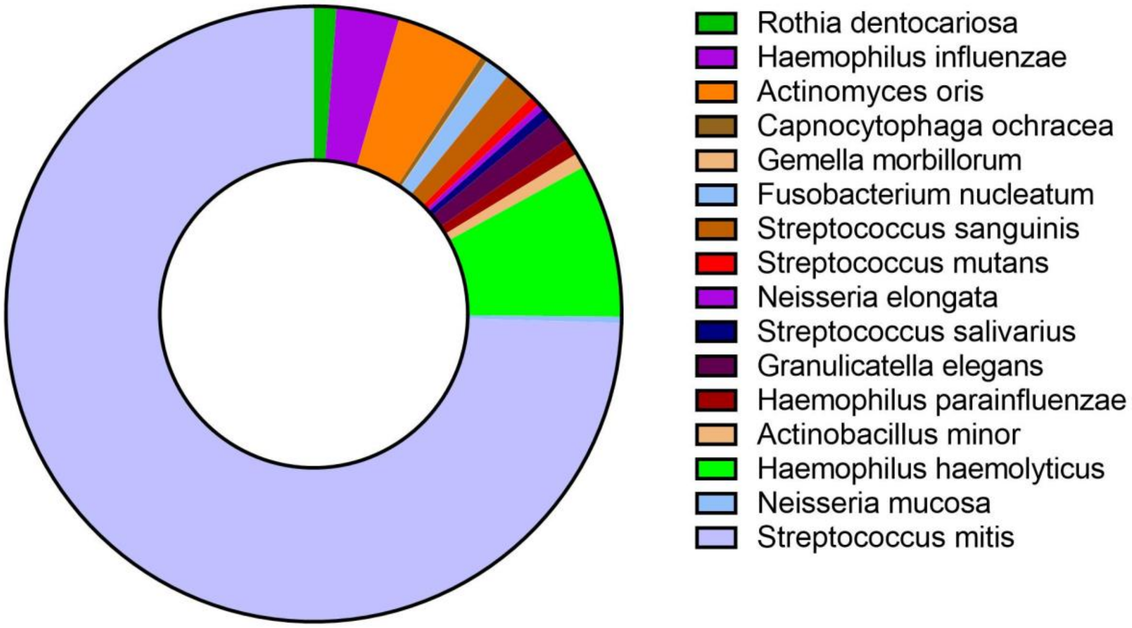

Figure 3-39: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 2 sample that are used for the analysis from the human oral microbiome.

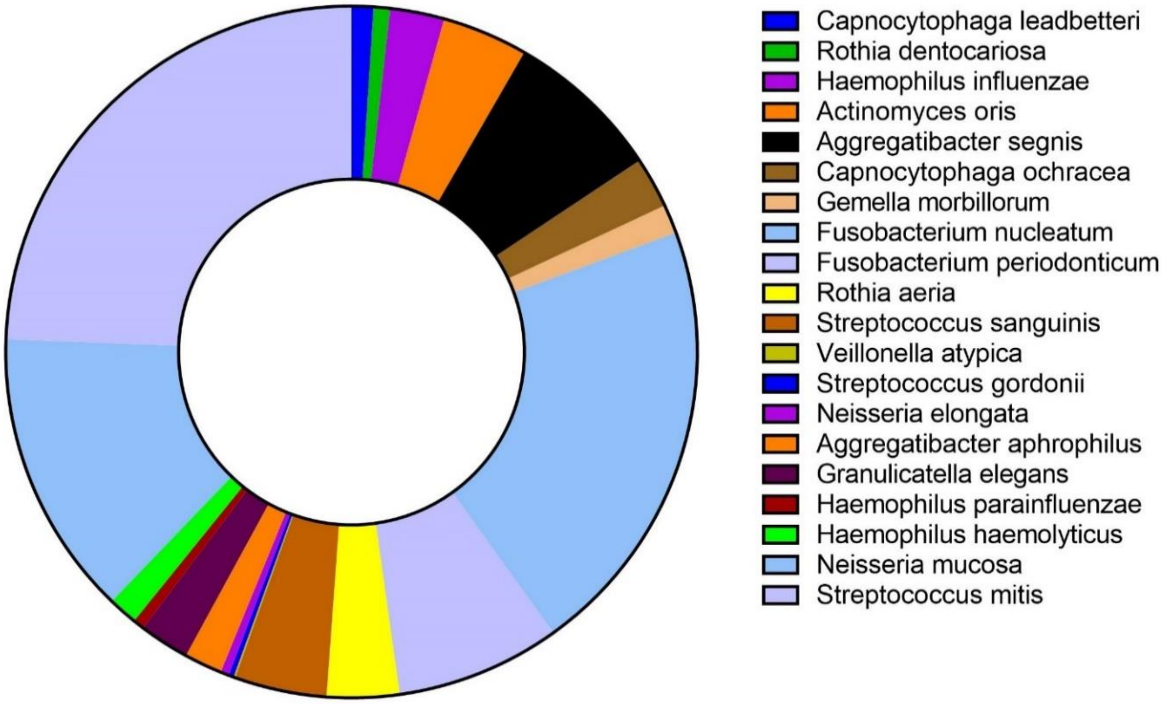

Figure 3-40: A pie chart visualizes the corrected abundance of the type II toxin antitoxin systems as represented by each taxon in oral 3 sample that are used for the analysis from the oral microbiome.

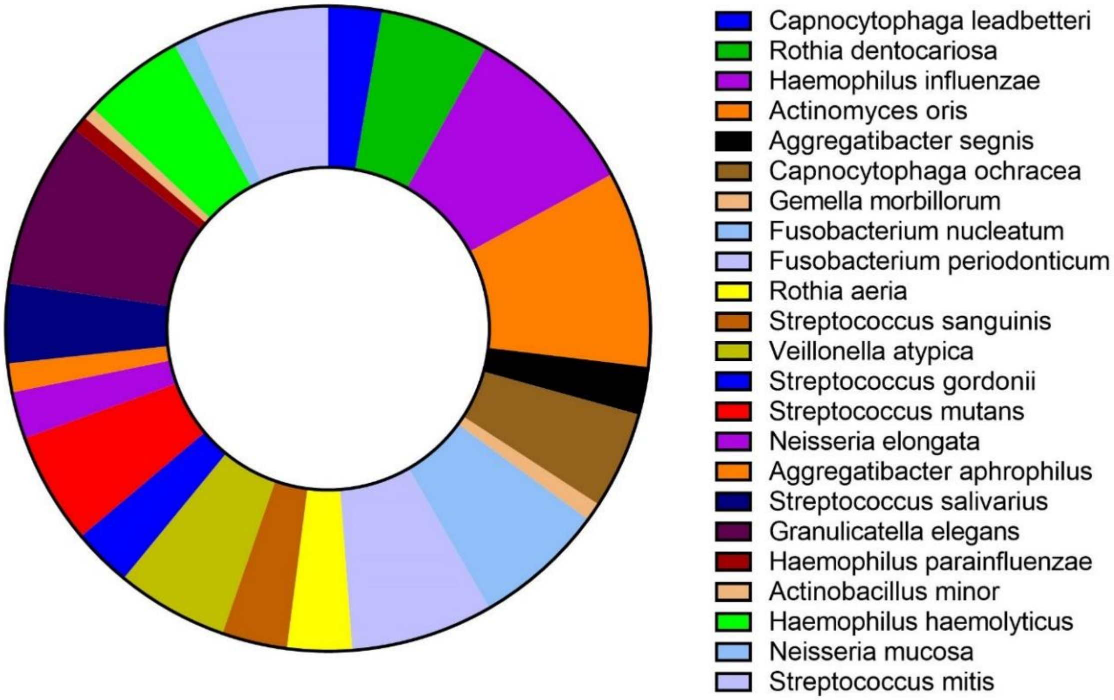

Figure 3-41: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon that are used for the analysis from the human oral microbiome.

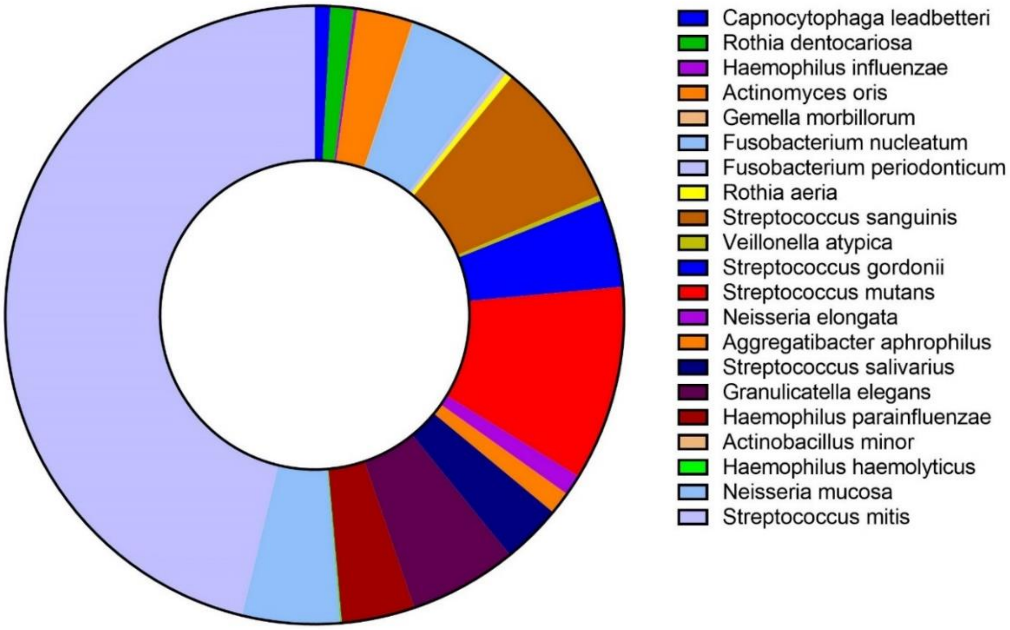

Figure 3-42: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 1 sample that are used for the analysis from the human oral microbiome.

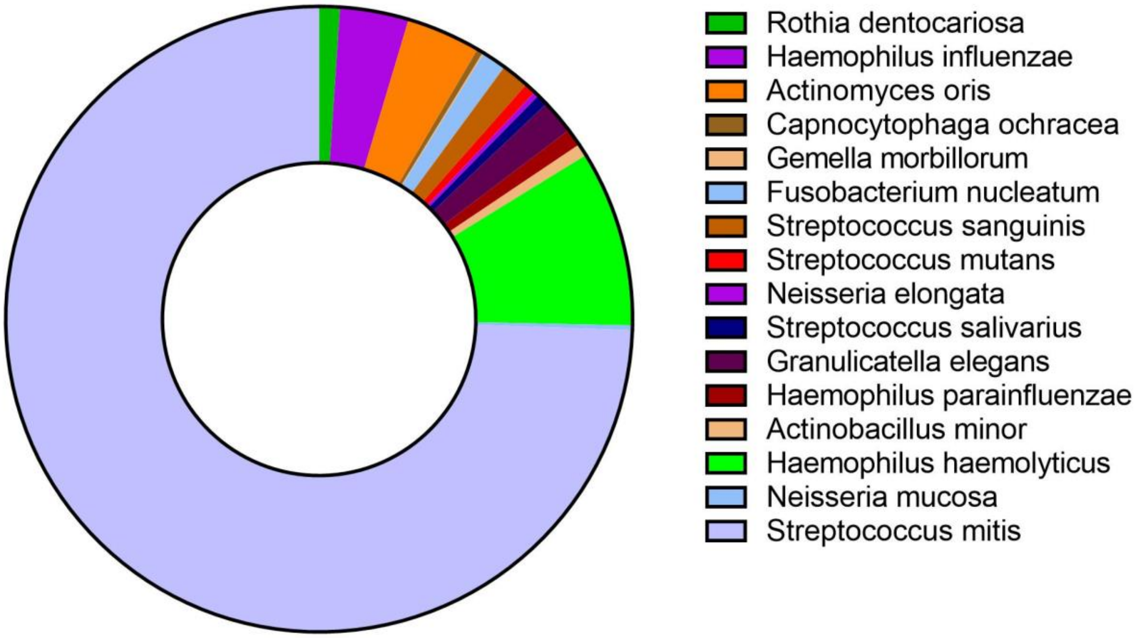

Figure 3-43 A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 2 sample that are used for the analysis from the human oral microbiome.

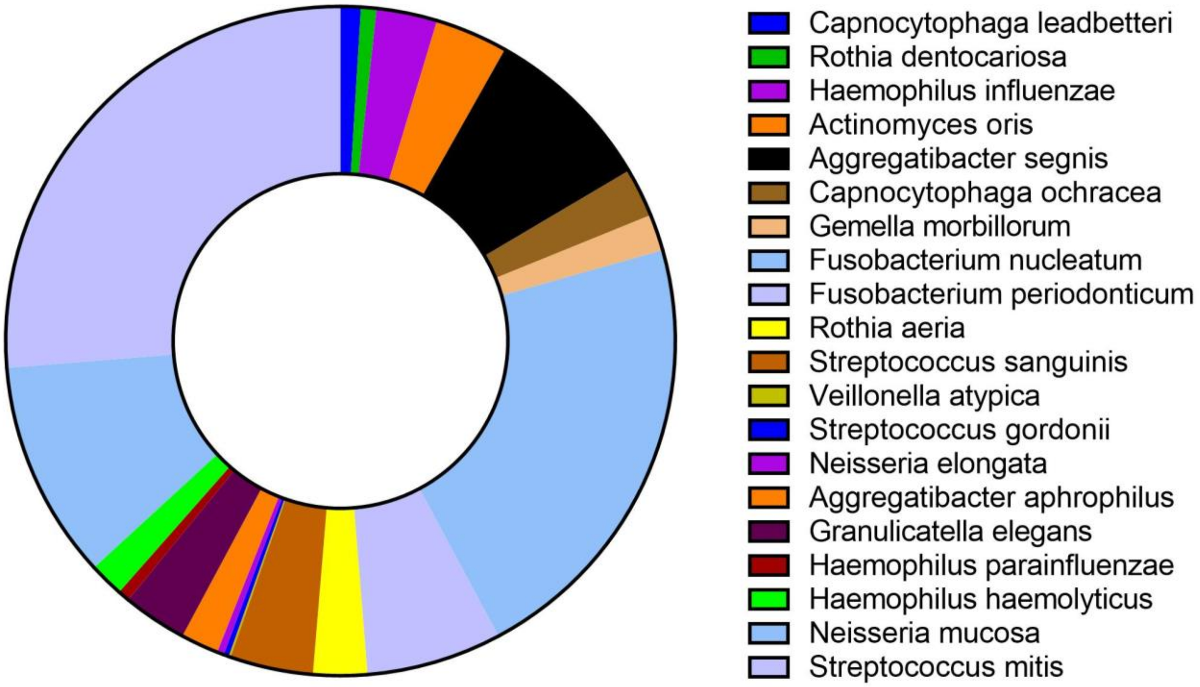

Figure 3-44: A pie chart visualizes the molecular abundance of the type II toxin antitoxin systems as represented by each taxon from oral 3 sample that are used for the analysis from the human oral microbiome.

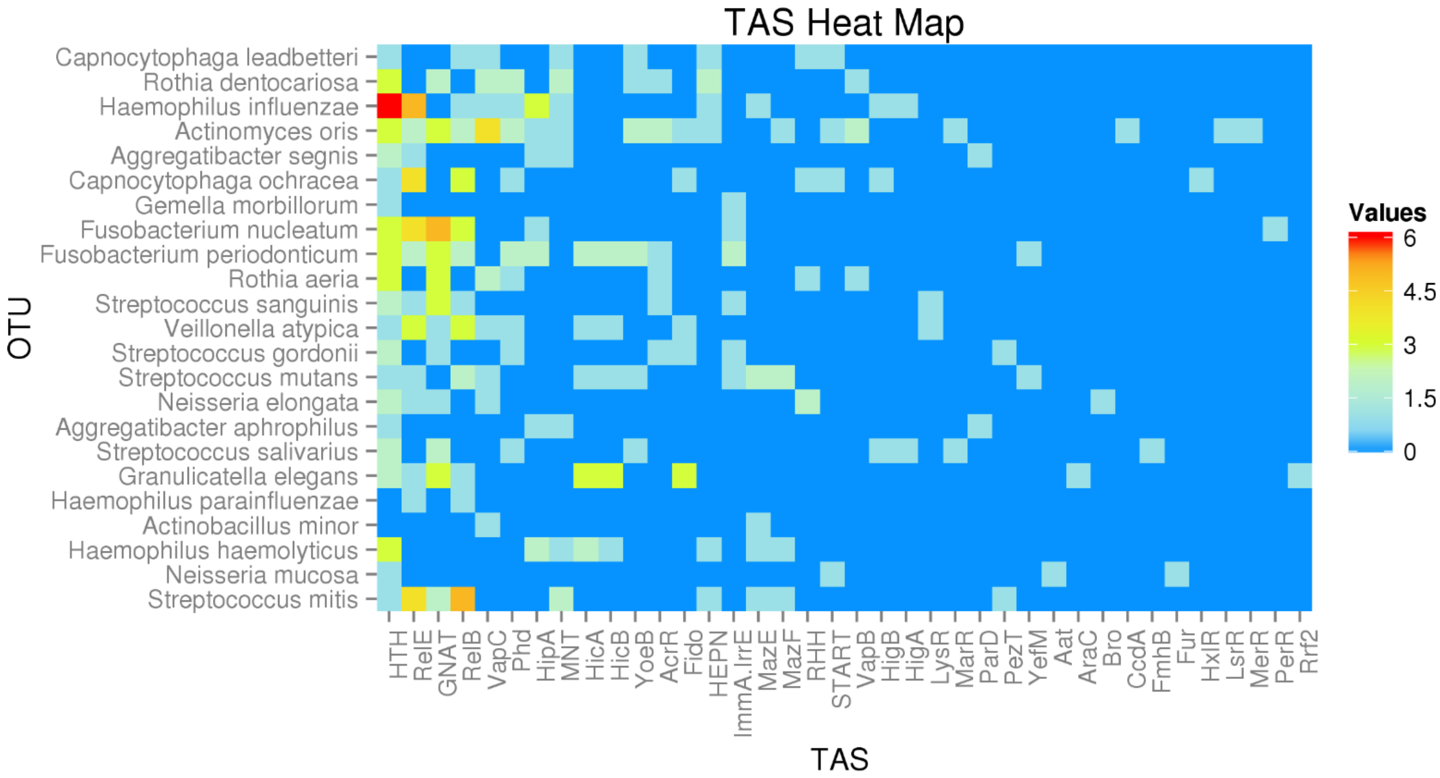

Figure 3-45: A heatmap visualizes the frequencies of the Type II Toxin Antitoxin Systems predicted in each Operational Taxonomic Unit for the taxa used in the analysis for the human oral microbiome.

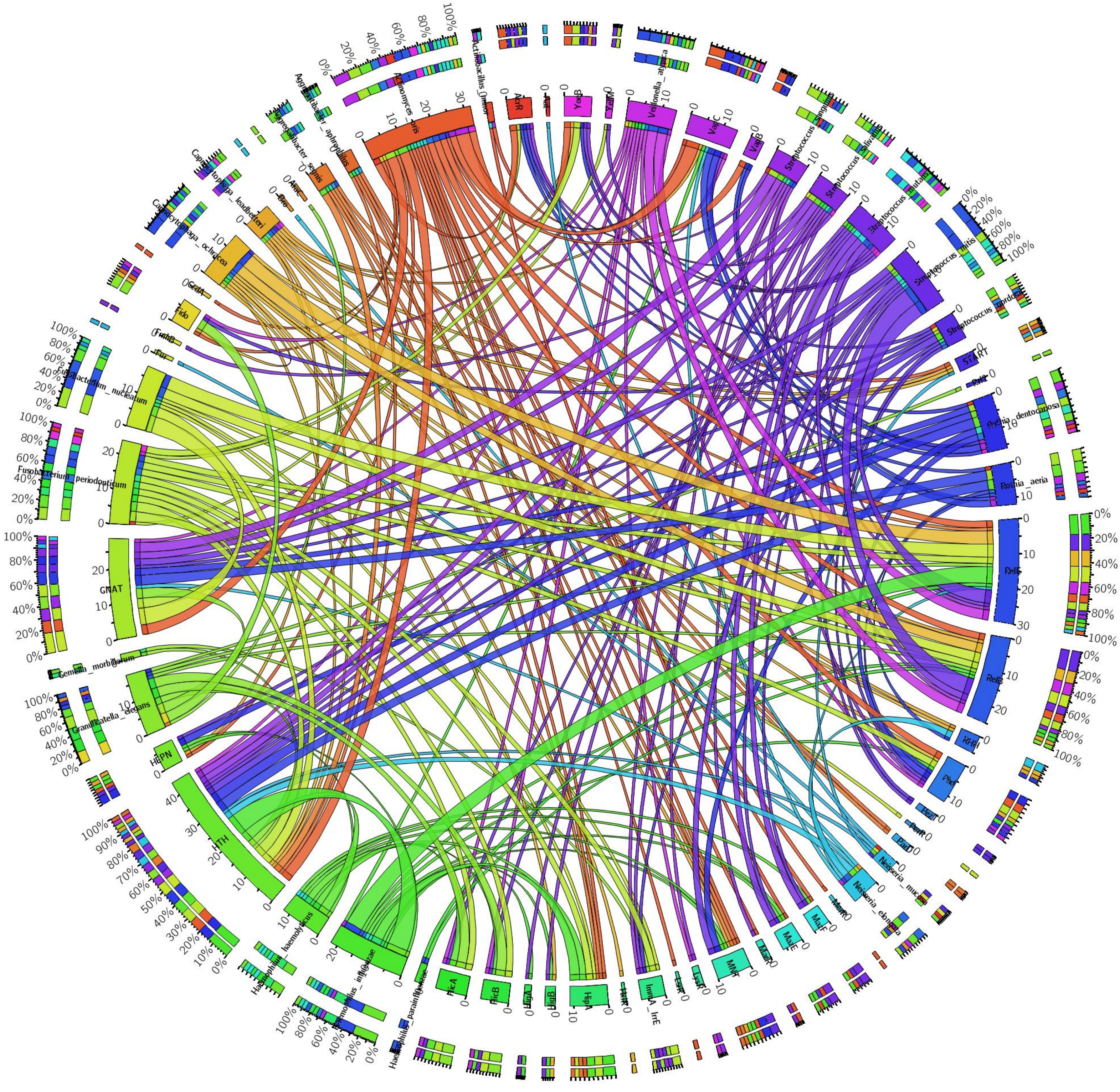

Figure 3-46: A Circos map that visualizes the frequencies of the Type II Toxin Antitoxin Systems predicted in each Operational Taxonomic Unit for the taxa used in the analysis for the human oral microbiome. The bands connect the toxin-antitoxin genes with their respective species. Thickness of the bands represents the frequency.

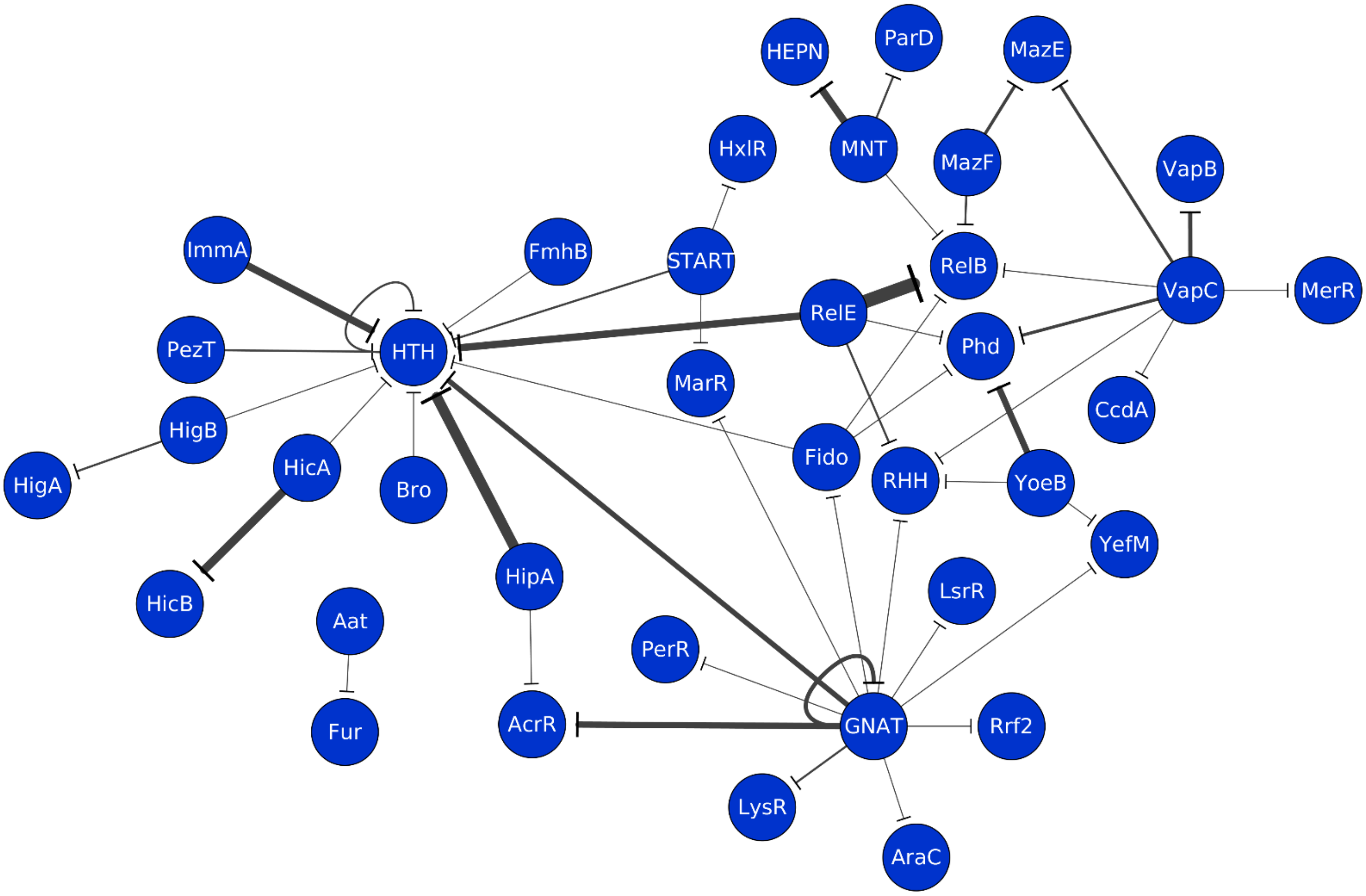

Figure 3-47: A directed protein-protein interaction network visualizes cross talk of the chromosomal Type II Toxin Antitoxin Systems that are predicted in the human oral microbiome. The edges thickness corresponds to the frequencies of the toxin-antitoxin interactions.

Table 3-5: The closeness and degree centralities of the protein-protein interaction network depicted in Figure 3-47.

Toxin Antitoxin System	**Degree**	**Closeness**
HTH	44	0.000794
RelE	30	0.000893
GNAT	29	0.001239
RelB	25	0.000794
VapC	14	0.000985
Phd	12	0.000794
HipA	11	0.00084
MNT	10	0.000866
HicA	9	0.00084
HicB	8	0.000794
YoeB	8	0.000866
AcrR	7	0.000794
Fido	7	0.000866
HEPN	7	0.000794
ImmA/IrrE	7	0.000816
MazE	6	0.000794
MazF	5	0.00084
RHH	5	0.000794
START	4	0.000866
VapB	4	0.000794
HigB	3	0.00084
HigA	2	0.000794
LysR	2	0.000794
MarR	2	0.000794
ParD	2	0.000794
PezT	2	0.000816
YefM	2	0.000794
AraC	1	0.000794

Toxin Antitoxin System	**Degree**	**Closeness**
Bro	1	0.000816
CcdA	1	0.000794
FmhB	1	0.000816
HxlR	1	0.000794
LsrR	1	0.000794
MerR	1	0.000794
PerR	1	0.000794
Rrf2	1	0.000794

Table 3-6: The molecular diversity of the type II toxin antitoxin systems predicted in the oral microbiome. TAS stands for Toxin Antitoxin Systems. OM stands for Oral Microbiome.

Diversity Index	**Berger-Parker index**	**Margalef index**	**Simpson index λ**	**Simpson index D**	**Simpson index Dr**	**Shannon index**	**Pielou index**
	d	dMa	λ	D	Dr	He	J Je
TAS in OM	6.318181818	6.57471412	0.066903878	0.933096122	14.94681677	3.038007498	0.835171282

3.4. Evolutionary and Phylogenetic Analysis

During population bottlenecks in dynamic environments, like the oral microbiome, the stress response and Horizontal Gene Transfer play critical roles (Koonin, 2016; L. Liu et al., 2012; Pinilla-Redondo et al., 2018) The fact that one genetic system has both capabilities for stress response and modulating horizontal transfer is quite unique from the evolutionary point of view. To expand, the protein-protein interactions is believed to be evolved from promiscuous intermediates that enables it for the “mixing and matching” phenomenon among different families and super-families of the toxins and the antitoxins (Aakre et al., 2015; Gillis & Pavlidis, 2012; Goeders & Van Melderen, 2014) This is visualized by making a phylogenetic analysis of all the toxin and antitoxin families (see appendix). The results show a mosaic nature where nearly different families of different species are not clustered in a clade. By evaluating the evolutionary grades, it emphasizes the para-speciation style of the oral microbiome (Flintoft, 2013) This means that the genetic drift is prominent where genetic variations are extensive which drives the exaptation and provide background for adaptation (Harms et al., 2017) Under extreme stress, natural selection is the driving force (Martinez Jose L., 2009) However, in benevolent quasi stressful nature, the genetic drift mediated by the altruistic selfish systems is probably the main drive force for evolution. For example, neither the HTH-containing genes and GNAT containing genes are not clustered together nor the species are clustered together. Moreover, the divergence timing of the phylogenetic analysis was close, yet showed different evolutionary rates as measured by the molecular clock test. The tests of molecular clocks using the Maximum Likelihood method showed lnL Parameters With Clock equals to -104601.156 297 43.851 and Without Clock equals to -102026.798 573 16.3. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P = 0.000E+000). This demote the notion of lateral gene transfer of the chromosomal type II toxin antitoxin systems as agreed in the literature (Harms et al., 2018c)

Molecular allometry analysis is an analysis to compare the genome size, coding genes and the Toxin Antitoxin Systems (Figure 3-48 and Figure 3-49). The results demonstrate the persistence and maintenance of the TAS throughout all genome sizes despite the natural decline in the number of coding genes as the genome size decreases. The impact of the chromosomal type II toxin antitoxin systems in the evolution of the oral microbiome is one of the questions that need further investigations to make us appreciate the vast functionality of the TAS.

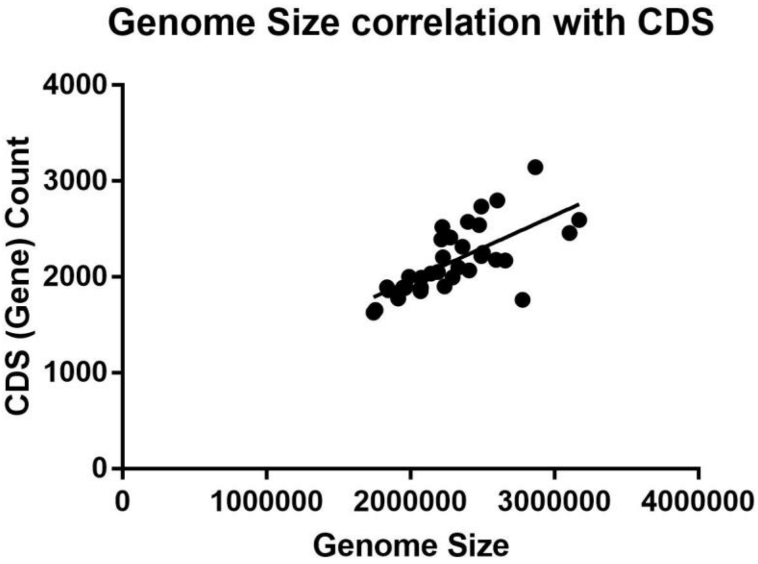

Figure 3-48: represents Pearson's correlation with regression analysis for the genome size for all genomes used in this study against the number of gene coding sequences for each respective genome. The analysis has R square value of 0.4729. The line equation is Y = 0.0006787*X + 605.4. The deviation from zero is significant with P value <0.0001. CDS stands for CoDing Sequences.

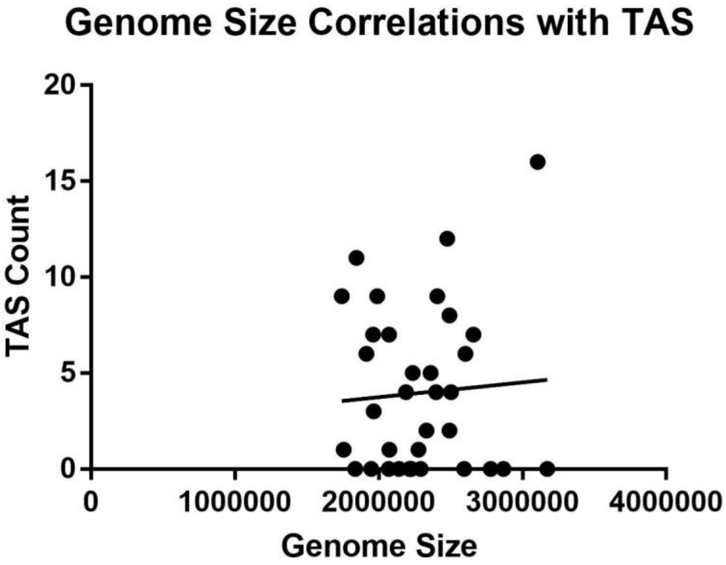

Figure 3-49: represents Pearson's correlation with regression analysis for the genome size for all genomes used in this study against the number of predicted chromosomal type II toxin antitoxin systems for each respective genome. The analysis has R square value of 0.00418. The line equation is Y = 7.743e-007*X + 2.197. The deviation from zero is insignificant with p value equals to 0.7121 implying a slope of near zero value of 7.743e-007 ± 2.08e-006. TAS stands for Toxin Antitoxin Systems.

4. CONCLUSION

The predictions of potentially novel chromosomal type II toxin antitoxin systems in the oral microbiome had opened questions more than just resolved ones. There have been 278 sequences that are revealed that are putatively functional due to the similarity in the domain architecture. This highlights the need in better functional prediction tools that can sense small protein coding genes. The distribution of the toxin antitoxin systems throughout the species in the oral microbiome is magnitude. Uneven intra-genomic and inter-genomic distribution as well as abundance are evident all over the microbial communities in the buccal cavity. The cross-talk network of the toxin antitoxin systems is, probably, shaping the co-evolutions that affects the bacterial adaptations.

Experimental validations for the predicted novel toxin antitoxin systems is a logical step. Yet, the functional analysis assays are difficult. The ectopic expression of proteins affects the fitness of the host bacterium. Thus, it is always debatable to assess the cytotoxicity of the protein that is attenuated by another co-expressed protein. The research group that first experimentally tested the toxin antitoxin systems published recent paper that antagonizes their methodology expressing this challenge in the functional assay (Song & Wood, 2018b)

On the other hand, this strikingly valuable system is a core for designing genetic networks of synthetic organisms. This could be applied in the post-antibiotic era where clinician could use organisms of beneficial characters addicted as a tool for competing against pathogens instead of the chemotherapy (Rugbjerg, Sarup-Lytzen, Nagy, & Sommer, 2018) The overall value for such endeavor is the ice-berg of understanding how the nature hold the answer when it comes to surviving communicable, as well as non-communicable, diseases.

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APPENDIX

S1 table: This table contains the abundance data for all unique Operational Taxonomic Units assigned in the oral microbiome analysis

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
Other	Other	0.000356535126813	0.0	0.00295368620038
g__Actinomyces	s__cardiffensis	0.0	0.0	0.000295368620038
g__Actinomyces	s__dentalis-orihominis	0.0	0.0	0.00277646502836
g__Actinomyces	s__georgiae	0.0	0.0	0.00498188405797
g__Actinomyces	s__gerencseriae	0.000842719390649	0.0	0.0
g__Actinomyces	s__israelii	0.0	0.0	0.00167375551355
g__Actinomyces	s__massiliensis	0.00106960538044	0.0	0.000984562066793
g__Actinomyces	s__meyeri-odontolyticus	0.00149096507576	0.0	0.0
g__Actinomyces	s__naeslundii	0.00764929908435	0.0	0.013134057971
g__Actinomyces	s__naeslundii-oris	0.00312778543068	0.0	0.00354442344045
g__Actinomyces	s__odontolyticus	0.0	0.0	0.00604521109011
g__Actinomyces	s__oris	0.00429462766388	0.0	0.00112240075614
g__Actinomyces	s__oris-viscosus	0.0	0.0176931615594	0.0

Genus	Species	Oral 1	Oral 2	Oral 3
g__Actinomyces	s__sp4769	0.00405153553197	0.0	0.0
g__Actinomyces	s__viscosus	0.00118304837533	0.0	0.0
g__NA	s__sp4816	0.00330605299408	0.0	0.0
g__Bifidobacterium	s__adolescentis	0.0	0.0	0.000315059861374
g__Bifidobacterium	s__angulatum	0.0	0.0	0.000275677378702
g__Bifidobacterium	s__ruminantium	0.0	0.000370009082041	0.000275677378702
g__Corynebacterium	s__durum	0.000567214974475	0.00148003632816	0.00500157529931
g__Corynebacterium	s__matruchotii	0.00619074629284	0.00689562380168	0.0169738500315
g__Rothia	s__aeria	0.00270642573535	0.0	0.0111255513548
g__Rothia	s__dentocariosa	0.00557491289199	0.00891385515826	0.00189035916824
g__Rothia	s__mucilaginosa	0.000939956243416	0.0	0.00112240075614
g__Propionibacterium	s__propionicum	0.000972368527672	0.00151367351744	0.00057104599874
g__Propionibacterium	s__sp7795	0.0	0.0	0.000649810964083
g__Atopobium	s__parvulum	0.0	0.0	0.00151622558286
g__Olsenella	s__uli	0.0	0.0	0.000492281033396

Genus	Species	Oral 1	Oral 2	Oral 3
g__Bacteroides	s__fragilis	0.0	0.00104275286757	0.000866414618778
g__Porphyromonas	s__catoniae	0.0033708775626	0.0	0.0293990233144
g__Porphyromonas	s__endodontalis	0.0	0.0	0.00679347826087
g__Porphyromonas	s__sp13364-sp13375	0.0100316019771	0.0	0.0
g__Porphyromonas	s__sp13375	0.0153148043108	0.00450738336305	0.0
g__Porphyromonas	s__sp13380	0.0	0.00322917017054	0.00275677378702
g__Tannerella	s__forsythia	0.0	0.000807292542635	0.00515910522999
g__Alloprevotella	s__rava	0.0	0.0	0.000551354757404
g__Alloprevotella	s__sp13491	0.0416497852686	0.0213596151906	0.0371179899181
g__Alloprevotella	s__sp13512-sp13517	0.00191232477109	0.0	0.00250078764965
g__Alloprevotella	s__sp13514	0.0	0.0	0.00864445494644
g__Alloprevotella	s__tannerae	0.0	0.0	0.00385948330183
g__NA	s__sp13865	0.0	0.0	0.0145124448645
g__NA	s__sp13879	0.0	0.0	0.00127993068683
g__NA	s__sp13918	0.0	0.0	0.000964870825457

Genus	Species	Oral 1	Oral 2	Oral 3
g__NA	s__sp13920	0.0	0.0	0.00047258979206
g__NA	s__sp13921	0.0	0.0	0.00133900441084
g__Paraprevotella	s__clara	0.0	0.0	0.00108301827347
g__Prevotella	s__baroniae	0.0	0.0	0.000551354757404
g__Prevotella	s__copri	0.0	0.0	0.000708884688091
g__Prevotella	s__histicola-jejuni	0.0	0.0	0.00452898550725
g__Prevotella	s__intermedia	0.0	0.0	0.0187460617517
g__Prevotella	s__loescheii	0.000518596548092	0.0	0.00578922495274
g__Prevotella	s__marshii	0.0	0.0	0.000492281033396
g__Prevotella	s__melaninogenica	0.000421359695325	0.0	0.00222511027095
g__Prevotella	s__micans	0.0	0.0	0.00122085696282
g__Prevotella	s__nanceiensis	0.00341949598898	0.000740018164082	0.000393824826717
g__Prevotella	s__nigrescens	0.000226885989799	0.0	0.00224480151229
g__Prevotella	s__oris	0.0	0.0	0.000275677378702
g__Prevotella	s__pallens	0.000907543959161	0.0	0.0

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__Prevotella	s__pleuritidis	0.0	0.0	0.000531663516068
g__Prevotella	s__saccharolytica	0.0	0.0	0.00232356647763
g__Prevotella	s__shahii	0.0	0.0	0.000669502205419
g__Prevotella	s__veroralis	0.000502390405964	0.0	0.0
g__NA	s__sp14445	0.0	0.0	0.00033475110271
g__Bergeyella	s__cardium	0.000421359695325	0.0	0.000630119722747
g__Bergeyella	s__sp16466	0.0	0.000672743785529	0.0
g__Bergeyella	s__sp16471	0.00468357507495	0.000874566921188	0.00220541902962
g__Capnocytophaga	s__gingivalis	0.00243092131918	0.0	0.0054347826087
g__Capnocytophaga	s__granulosa	0.000324122842557	0.00380100238824	0.00878229363579
g__Capnocytophaga	s__haemolytica	0.000502390405964	0.0	0.0
g__Capnocytophaga	s__leadbetteri	0.0075034438052	0.0	0.00504095778198
g__Capnocytophaga	s__ochracea	0.0	0.00289279827778	0.00669502205419
g__Capnocytophaga	s__sp16491	0.000469978121708	0.0	0.00120116572149
g__Capnocytophaga	s__sp16511	0.00333846527834	0.000437283460594	0.00527725267801

Genus	Species	Oral 1	Oral 2	Oral 3
g__Capnocytophaga	s__sp16514	0.0094805931448	0.0	0.0147684310019
g__Capnocytophaga	s__sp16515	0.000372741268941	0.0	0.00456836798992
g__NA	s__sp19416	0.0	0.0	0.000492281033396
g__NA	s__sp19423	0.0	0.0	0.00120116572149
g__Olivibacter	s__jilunii	0.0	0.000538195028423	0.000275677378702
g__Gemella	s__haemolysans-sanguinis-taiwanensis	0.0214083137509	0.0618251538901	0.0168163201008
g__Gemella	s__morbillorum	0.00113442994895	0.00353190487403	0.0267407057341
g__Abiotrophia	s__sp28088	0.0	0.0	0.00504095778198
g__Granulicatella	s__adiacens	0.0143748480674	0.0064247031518	0.00238264020164
g__Granulicatella	s__elegans	0.016854387813	0.00962023613307	0.00513941398866
g__Lactobacillus	s__fermentum	0.0	0.00121093881395	0.000492281033396
g__Streptococcus	s__NA	0.169402803663	0.498166773184	0.0535404851922
g__Streptococcus	s__anginosus	0.0	0.0	0.000767958412098
g__Streptococcus	s__anginosus-constellatus-intermedius	0.000502390405964	0.0	0.000886105860113

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__Streptococcus	s__gordonii	0.038781298112	0.0	0.00118147448015
g__Streptococcus	s__intermedius	0.000324122842557	0.0	0.0
g__Streptococcus	s__mutans	0.0452151365367	0.00467556930943	0.0
g__Streptococcus	s__salivarius-vestibularis	0.0195932258326	0.00659288909819	0.0
g__Streptococcus	s__sanguinis	0.0583421116603	0.0198795788624	0.0169935412728
g__Streptococcus	s__sinensis	0.0	0.0	0.00474558916194
g__Parvimonas	s__micra	0.0	0.0	0.0020872715816
g__Mogibacterium	s__neglectum	0.0	0.0	0.0013193131695
g__NA	s__sp31630	0.0	0.0	0.000846723377442
g__NA	s__sp31635	0.0	0.0	0.00023629489603
g__NA	s__sp31682	0.0	0.0	0.000374133585381
g__Butyrivibrio	s__sp32116	0.0	0.0	0.000787649653434
g__Catonella	s__morbi	0.000307916700429	0.0	0.00342627599244
g__Johnsonella	s__ignava	0.0	0.000874566921188	0.0
g__Johnsonella	s__sp32278	0.0	0.00104275286757	0.00511972274732

Genus	Species	Oral 1	Oral 2	Oral 3
g__Johnsonella	s__sp32283	0.0	0.0	0.000492281033396
g__Johnsonella	s__sp32285	0.0	0.000706380974806	0.0
g__Lachnoanaerobaculum	s__saburreum	0.0	0.000740018164082	0.00277646502836
g__Lachnoanaerobaculum	s__sp32292	0.0	0.0	0.00161468178954
g__Lachnoanaerobaculum	s__sp32293	0.0	0.0	0.00704946439824
g__Lachnoanaerobaculum	s__umeaense	0.000599627258731	0.0	0.00324905482042
g__NA	s__rectale	0.0	0.00131185038178	0.000708884688091
g__NA	s__sp33423	0.0	0.0	0.000295368620038
g__Oribacterium	s__sp33063	0.0	0.0005718322177	0.0
g__NA	s__sp30555	0.000259298274046	0.0	0.0
g__Peptococcus	s__sp34118	0.0	0.0	0.00448960302457
g__Filifactor	s__alocis	0.0	0.0	0.00057104599874
g__Peptoclostridium	s__sp34347	0.0	0.0	0.00212665406427
g__Peptoclostridium	s__sp34351	0.0	0.0	0.00206758034026
g__Peptostreptococcus	s__anaerobius-stomatis	0.0	0.0	0.00490311909263

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__NA	s__sp35348	0.0	0.0	0.0018115942029
g__NA	s__sp35352	0.0	0.0	0.00112240075614
g__Solobacterium	s__moorei	0.0	0.0	0.00108301827347
g__Dialister	s__invisus	0.0	0.00107639005685	0.00224480151229
g__Selenomonas	s__artemidis	0.000275504416174	0.0	0.00232356647763
g__Selenomonas	s__infelix	0.000551008832347	0.0	0.00567107750473
g__Selenomonas	s__noxia	0.0	0.0	0.00218572778828
g__Selenomonas	s__sp37070	0.0	0.0	0.00116178323882
g__Selenomonas	s__sp37070-sp37072	0.0	0.0	0.00175252047889
g__Selenomonas	s__sp37072	0.0	0.0	0.000433207309389
g__Veillonella	s__atypica	0.00131269751236	0.0	0.0
g__Veillonella	s__atypica-parvula	0.0	0.0	0.00023629489603
g__Veillonella	s__denticariosi	0.0	0.00581923374483	0.0
g__Veillonella	s__denticariosi-parvula-tobetsuensis	0.00341949598898	0.0	0.0

Genus	Species	Oral 1	Oral 2	Oral 3
g__Veillonella	s__parvula	0.079847662264	0.0068619866124	0.054347826087
g__Veillonella	s__sp37198	0.0337411879102	0.022267819301	0.000827032136106
g__Veillonella	s__tobetsuensis	0.0	0.00581923374483	0.00248109640832
g__Fusobacterium	s__canifelinum-nucleatum	0.0126245847176	0.00773655353359	0.0197503150599
g__Fusobacterium	s__nucleatum	0.00709829025201	0.000908204110465	0.0239248582231
g__Fusobacterium	s__nucleatum-periodonticum	0.0	0.0	0.00167375551355
g__Fusobacterium	s__periodonticum	0.000939956243416	0.0	0.0109877126654
g__Fusobacterium	s__sp37444	0.0	0.0022873288708	0.0
g__Leptotrichia	s__buccalis	0.00218782918726	0.0	0.00527725267801
g__Leptotrichia	s__buccalis-hofstadii	0.0	0.0	0.00322936357908
g__Leptotrichia	s__hofstadii	0.0	0.0	0.00157529930687
g__Leptotrichia	s__shahii	0.00372741268941	0.00225369168152	0.00185097668557
g__Leptotrichia	s__sp37510	0.000194473705534	0.0	0.0
g__Leptotrichia	s__sp37518	0.0083137509116	0.00174913384238	0.0179584120983
g__Leptotrichia	s__trevisanii	0.000875131674905	0.0	0.0

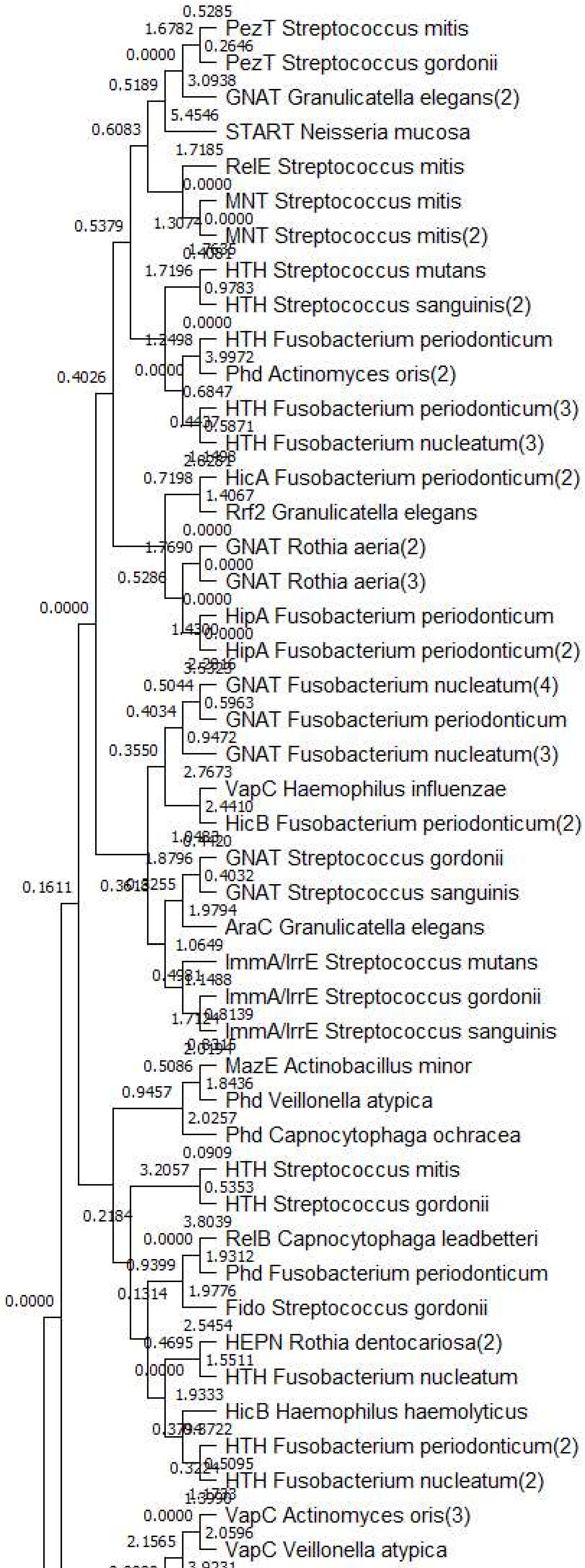
Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__Leptotrichia	s__wadei	0.000956162385544	0.0	0.0
g__Streptobacillus	s__hongkongensis	0.0	0.000908204110465	0.0
g__NA	s__sp38230	0.0	0.0	0.00555293005671
g__NA	s__sp19816	0.000518596548092	0.000504557839147	0.0146699747952
g__Lautropia	s__mirabilis	0.00478081192772	0.00568468498772	0.00897920604915
g__Comamonas	s__sp48939	0.000583421116603	0.0	0.00124054820416
g__Eikenella	s__corrodens	0.000567214974475	0.0	0.00580891619408
g__Kingella	s__denitrificans	0.00338708370472	0.0	0.00921550094518
g__Kingella	s__oralis	0.00262539502471	0.000773655353359	0.000984562066793
g__Neisseria	s__bacilliformis	0.000972368527672	0.0	0.0
g__Neisseria	s__cinerea	0.00847581233287	0.00319553298126	0.000610428481411
g__Neisseria	s__elongata	0.0115063609108	0.00501194120219	0.00194943289225
g__Neisseria	s__flavescens	0.0	0.00575195936628	0.0131734404537
g__Neisseria	s__macacae-mucosa-sicca	0.11431812657	0.00370009082041	0.132147920605

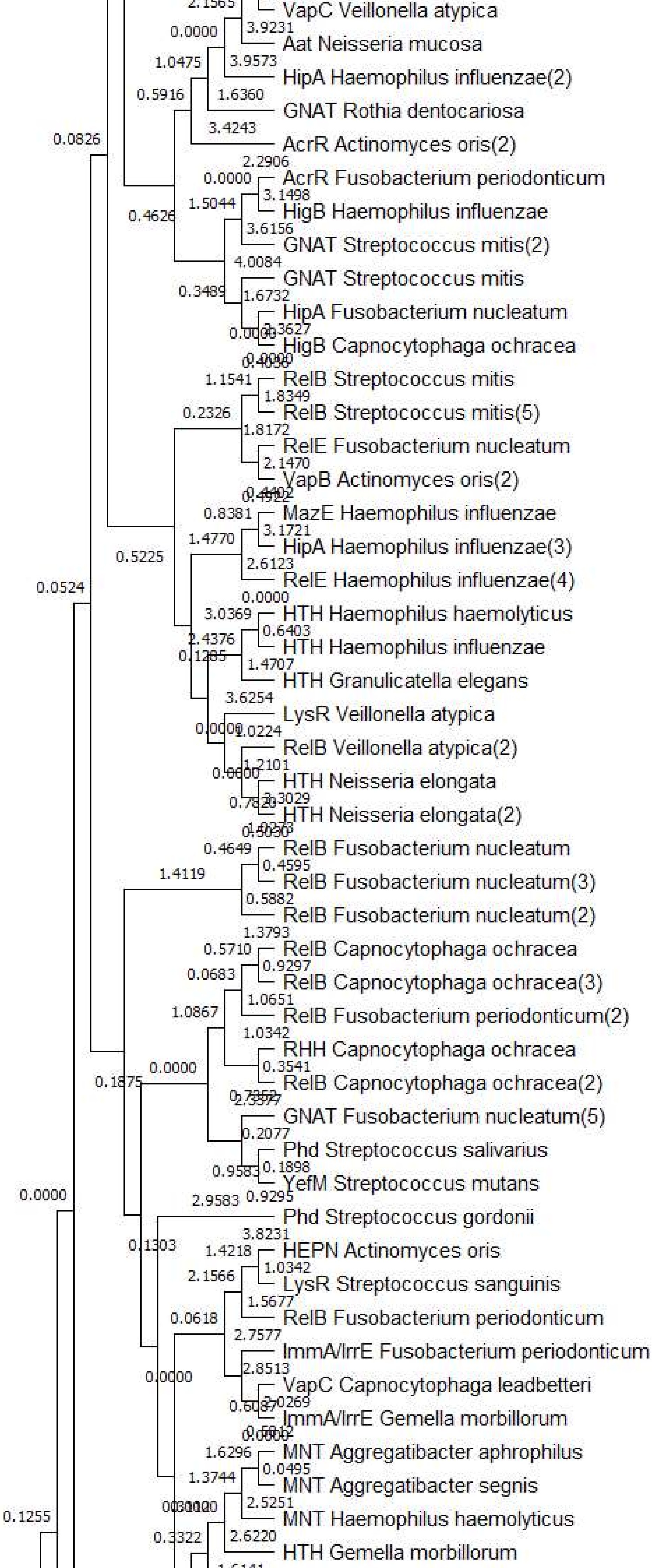
Genus	Species	Oral 1	Oral 2	Oral 3
g__Neisseria	s__mucosa-perflava-subflava	0.000469978121708	0.00625651720542	0.0
g__Neisseria	s__oralis	0.000486184263836	0.00669380066602	0.00129962192817
g__Neisseria	s__shayeganii	0.0	0.0	0.00364287964713
g__Neisseria	s__sp49957	0.000648245685115	0.0	0.0
g__Simonsiella	s__muelleri	0.0	0.0	0.000255986137366
g__Bilophila	s__wadsworthia	0.0	0.0	0.000413516068053
g__Campylobacter	s__concisus	0.003549145126	0.0	0.00419423440454
g__Campylobacter	s__curvus	0.0	0.0	0.000315059861374
g__Campylobacter	s__gracilis	0.00108581152257	0.0	0.000767958412098
g__Campylobacter	s__rectus-showae	0.00118304837533	0.00151367351744	0.0104757403907
g__Cardiobacterium	s__hominis	0.000664451827243	0.0	0.00163437303088
g__Cardiobacterium	s__valvarum	0.000340328984685	0.0	0.00023629489603
g__Escherichia-Shigella	s__coli	0.0	0.000908204110465	0.000630119722747
g__Actinobacillus	s__sp62066	0.000632039542987	0.0466884187157	0.0

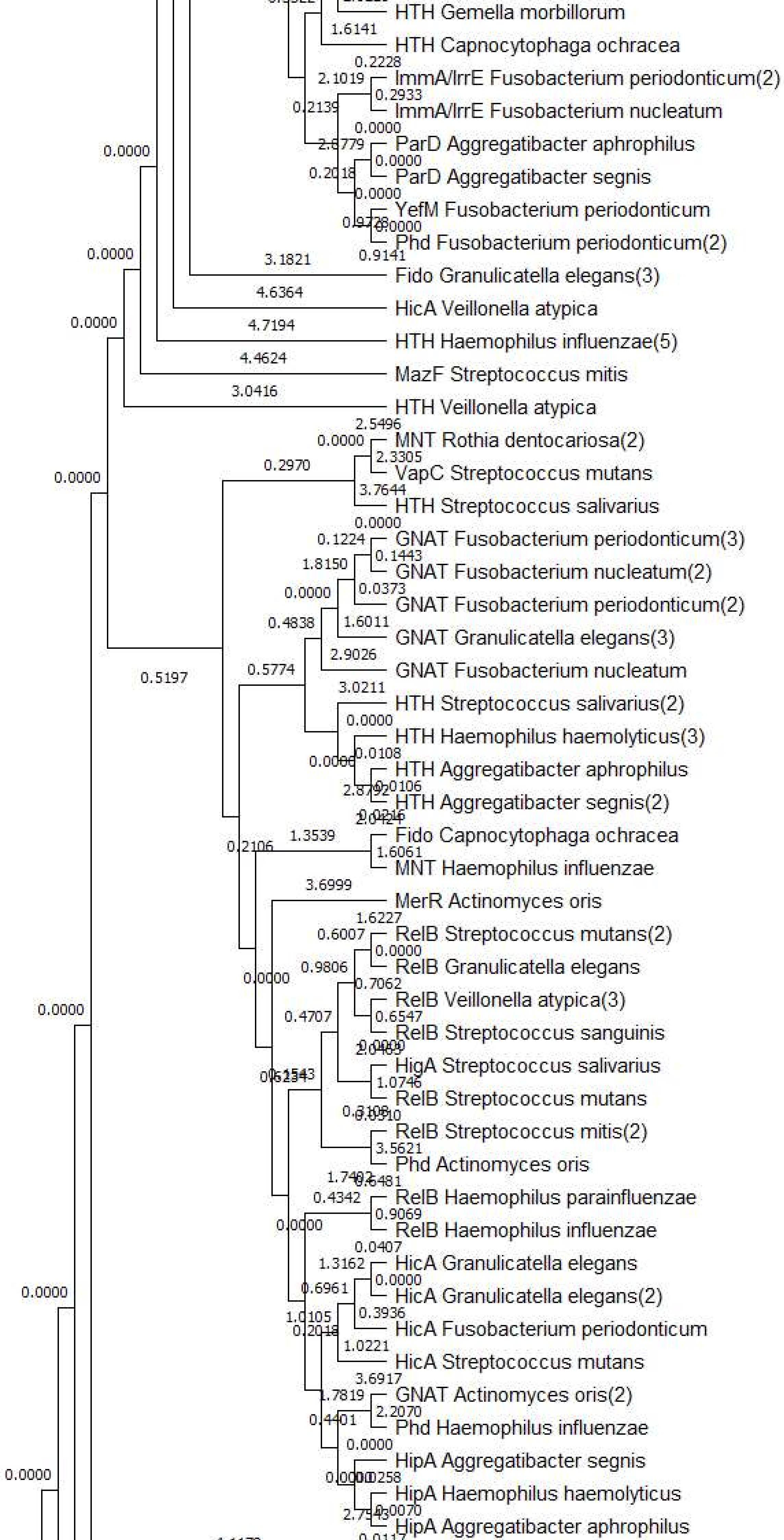
Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__Aggregatibacter	s__aphrophilus	0.0204521513654	0.0	0.0175055135476
g__Aggregatibacter	s__segnis	0.0	0.0	0.0482435412728
g__Aggregatibacter	s__sp62087	0.000340328984685	0.00578559655555	0.0120510396975
g__Haemophilus	s__haemolyticus	0.000275504416174	0.0809647145884	0.00450929426591
g__Haemophilus	s__haemolyticus-influenzae	0.000486184263836	0.0180295334522	0.00454867674858
g__Haemophilus	s__haemolyticus-quentini	0.000372741268941	0.0	0.0
g__Haemophilus	s__influenzae	0.0	0.0	0.00137838689351
g__Haemophilus	s__parainfluenzae	0.125305890933	0.0535504053281	0.0103969754253
g__Haemophilus	s__pittmaniae	0.0	0.000504557839147	0.00206758034026
g__*Saccharimonas	s__sp65946	0.0	0.0	0.000669502205419
g__*Saccharimonas	s__sp65955	0.0	0.0	0.00409577819786
g__*Saccharimonas	s__sp65958	0.0	0.0	0.000964870825457
g__*Saccharimonas	s__sp65962	0.0	0.0	0.00823093887839
g__NA	s__sp65941	0.000469978121708	0.0	0.00427299936988
g__NA	s__sp66013	0.0	0.0	0.00240233144297

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__NA	s__sp66034	0.000210679847662	0.0	0.00133900441084
g__NA	s__sp66108	0.0	0.00117730162468	0.0
g__NA	s__sp66127	0.0	0.0	0.0143549149338
g__NA	s__sp66606	0.0	0.0	0.00163437303088
g__NA	s__sp66623	0.0	0.000672743785529	0.0
g__NA	s__sp66675	0.0	0.0	0.00102394454946
g__NA	s__sp66707	0.0	0.0	0.000315059861374
g__Treponema	s__denticola	0.0	0.0	0.00057104599874
g__Treponema	s__maltophilum	0.0	0.0	0.000374133585381
g__Treponema	s__medium	0.0	0.0	0.00057104599874
g__Treponema	s__medium-vincentii	0.0	0.0	0.000551354757404
g__Treponema	s__socranskii	0.0	0.0	0.00151622558286
g__Treponema	s__sp66800	0.0	0.0	0.000295368620038
g__Treponema	s__vincentii	0.0	0.0	0.00135869565217
g__Fretibacterium	s__sp67092	0.0	0.0	0.000354442344045

Genus	**Species**	**Oral 1**	**Oral 2**	**Oral 3**
g__NA	s__sp67855	0.0	0.0	0.000393824826717


0.5285
1.6782
PezT Streptococcus mitis
0.2646
0.0000
PezT Streptococcus gordonii
0.5189
3.0938
GNAT Granulicatella elegans(2)
5.4546
0.6083
START Neisseria mucosa
1.7185
RelE Streptococcus mitis
0.0000
MNT Streptococcus mitis
0.5379
1.3074
0.0000
MNT Streptococcus mitis(2)
1.7196
HTH Streptococcus mutans
0.9783
HTH Streptococcus sanguinis(2)
0.0000
1.2498
HTH Fusobacterium periodonticum
0.4026
3.9972
0.0000
Phd Actinomyces oris(2)
0.6847
HTH Fusobacterium periodonticum(3)
0.4437
0.5871
HTH Fusobacterium nucleatum(3)
0.7198
HicA Fusobacterium periodonticum(2)
1.4067
Rrf2 Granulicatella elegans
0.0000
1.7690
GNAT Rothia aeria(2)
0.0000
0.5286
GNAT Rothia aeria(3)
0.0000
0.0000
HipA Fusobacterium periodonticum
1.4300
0.0000
HipA Fusobacterium periodonticum(2)
0.5044
GNAT Fusobacterium nucleatum(4)
0.5963
0.4034
GNAT Fusobacterium periodonticum
0.9472
0.3550
GNAT Fusobacterium nucleatum(3)
2.7673
VapC Haemophilus influenzae
2.4410
HicB Fusobacterium periodonticum(2)
1.8796
GNAT Streptococcus gordonii
0.4032
0.1611
GNAT Streptococcus sanguinis
1.9794
AraC Granulicatella elegans
1.0649
ImmA/IrrE Streptococcus mutans
1.1488
ImmA/IrrE Streptococcus gordonii
0.8139
1.7124
ImmA/IrrE Streptococcus sanguinis
0.5086
MazE Actinobacillus minor
1.8436
0.9457
Phd Veillonella atypica
2.0257
Phd Capnocytophaga ochracea
0.0909
3.2057
HTH Streptococcus mitis
0.5353
HTH Streptococcus gordonii
0.2184
3.8039
0.0000
RelB Capnocytophaga leadbetteri
1.9312
0.9399
Phd Fusobacterium periodonticum
1.9776
0.1314
0.0000
Fido Streptococcus gordonii
2.5454
0.4695
HEPN Rothia dentocariosa(2)
1.5511
0.0000
HTH Fusobacterium nucleatum
1.9333
HicB Haemophilus haemolyticus
HTH Fusobacterium periodonticum(2)
0.5095
HTH Fusobacterium nucleatum(2)
0.0000
VapC Actinomyces oris(3)
2.0596
2.1565
VapC Veillonella atypica


2.1565
VapC Veillonella atypica
0.0000
3.9231
Aat Neisseria mucosa
1.0475
3.9573
HipA Haemophilus influenzae(2)
0.5916
1.6360
GNAT Rothia dentocariosa
0.0826
3.4243
AcrR Actinomyces oris(2)
2.2906
0.0000
AcrR Fusobacterium periodonticum
3.1498
1.5044
0.4626
HigB Haemophilus influenzae
3.6156
GNAT Streptococcus mitis(2)
4.0084
GNAT Streptococcus mitis
0.3489
1.6732
HipA Fusobacterium nucleatum
0.0000
2.3627
HigB Capnocytophaga ochracea
0.4036
0.0000
1.1541
RelB Streptococcus mitis
1.8349
0.2326
RelB Streptococcus mitis(5)
1.8172
RelE Fusobacterium nucleatum
2.1470
VapB Actinomyces oris(2)
0.4402
0.4922
0.8381
MazE Haemophilus influenzae
3.1721
1.4770
HipA Haemophilus influenzae(3)
0.5225
2.6123
0.0524
RelE Haemophilus influenzae(4)
0.0000
3.0369
HTH Haemophilus haemolyticus
0.6403
2.4376
HTH Haemophilus influenzae
0.1285
1.4707
HTH Granulicatella elegans
3.6254
LysR Veillonella atypica
0.0000
1.0224
RelB Veillonella atypica(2)
1.2101
0.0000
HTH Neisseria elongata
2.3029
0.7820
HTH Neisseria elongata(2)
1.0273
0.5030
0.4649
RelB Fusobacterium nucleatum
0.4595
1.4119
RelB Fusobacterium nucleatum(3)
0.5882
RelB Fusobacterium nucleatum(2)
1.3793
0.5710
RelB Capnocytophaga ochracea
0.9297
0.0683
RelB Capnocytophaga ochracea(3)
1.0867
1.0651
RelB Fusobacterium periodonticum(2)
1.0342
RHH Capnocytophaga ochracea
0.0000
0.3541
0.1875
RelB Capnocytophaga ochracea(2)
0.7352
2.3377
GNAT Fusobacterium nucleatum(5)
0.2077
Phd Streptococcus salivarius
0.9583
0.1898
YefM Streptococcus mutans
0.0000
0.9295
2.9583
Phd Streptococcus gordonii
0.1303
3.8231
1.4218
HEPN Actinomyces oris
1.0342
2.1566
LysR Streptococcus sanguinis
1.5677
0.0618
RelB Fusobacterium periodonticum
2.7577
ImmA/IrrE Fusobacterium periodonticum
0.0000
2.8513
VapC Capnocytophaga leadbetteri
0.6037
2.0269
ImmA/IrrE Gemella morbillorum
0.5812
0.0000
1.6296
MNT Aggregatibacter aphrophilus
0.0495
1.3744
MNT Aggregatibacter segnis
0.0000
0.3100
2.5251
0.1255
MNT Haemophilus haemolyticus
0.3322
2.6220
HTH Gemella morbillorum
1.6141


HTH Gemella morbillorum
1.6141
HTH Capnocytophaga ochracea
0.2228
2.1019
ImmA/IrrE Fusobacterium periodonticum(2)
0.2933
0.2139
ImmA/IrrE Fusobacterium nucleatum
0.0000
0.0000
2.6779
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0.0000
0.2018
ParD Aggregatibacter segnis
0.0000
YefM Fusobacterium periodonticum
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0.0000
0.0000
Phd Fusobacterium periodonticum(2)
0.9141
3.1821
Fido Granulicatella elegans(3)
4.6364
HicA Veillonella atypica
0.0000
4.7194
HTH Haemophilus influenzae(5)
4.4624
MazF Streptococcus mitis
3.0416
HTH Veillonella atypica
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MNT Rothia dentocariosa(2)
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1.3539
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MerR Actinomyces oris
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RelB Streptococcus sanguinis
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RelB Streptococcus mutans
0.3108
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RelB Streptococcus mitis(2)
3.5621
Phd Actinomyces oris
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0.4342
RelB Haemophilus parainfluenzae
0.9069
0.0000
RelB Haemophilus influenzae
0.0407
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0.0000
0.6961
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0.0000
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1.0221
HicA Streptococcus mutans
3.6917
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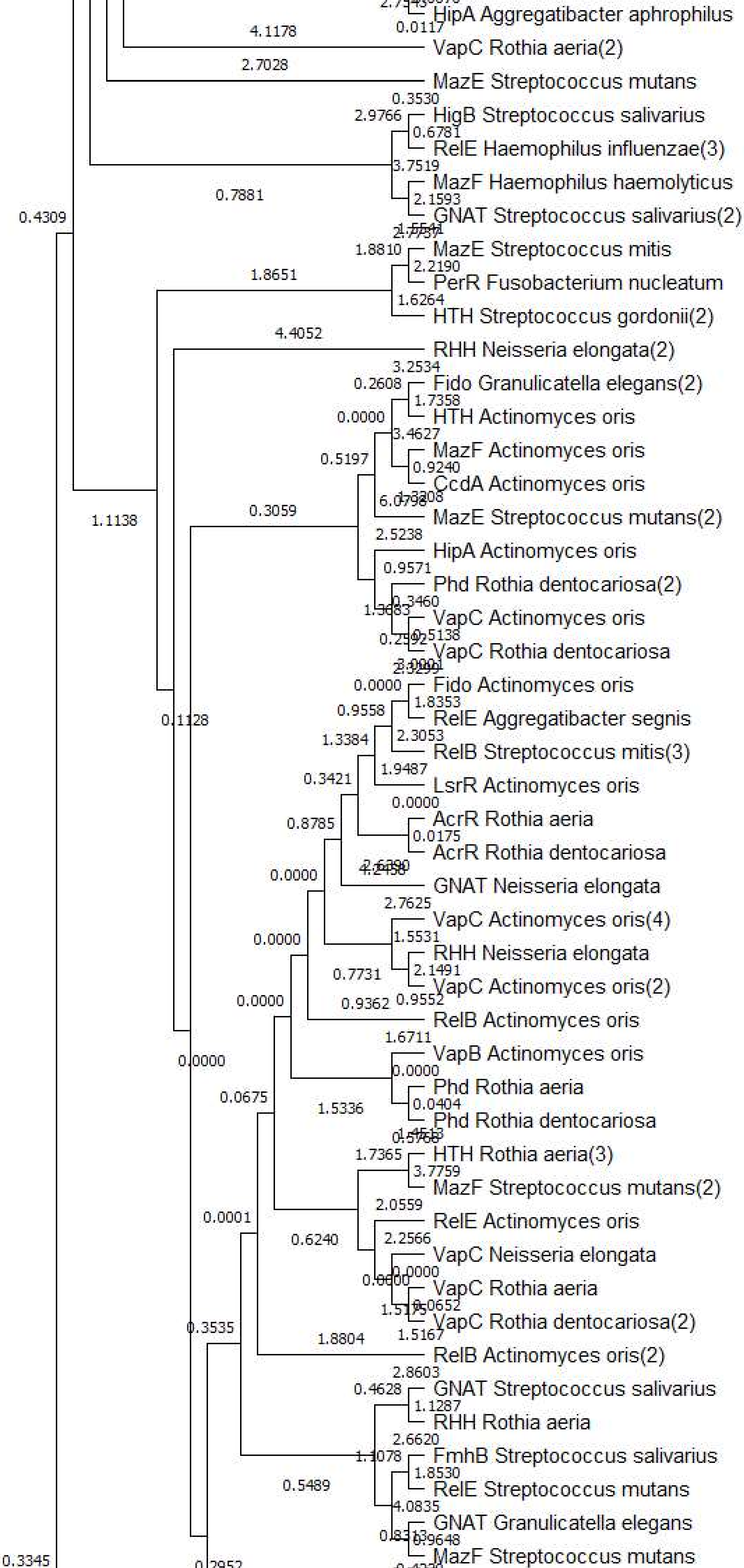
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MazF Haemophilus haemolyticus
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0.4309
GNAT Streptococcus salivarius(2)
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MazE Streptococcus mitis
2.2190
1.8651
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4.4052
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3.2534
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2.5238
HipA Actinomyces oris
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Phd Rothia dentocariosa(2)
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VapC Actinomyces oris
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VapC Rothia dentocariosa
0.0000
Fido Actinomyces oris
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RelE Aggregatibacter segnis
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1.3384
RelB Streptococcus mitis(3)
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AcrR Rothia aeria
0.8785
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0.0000
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2.7625
VapC Actinomyces oris(4)
0.0000
1.5531
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VapB Actinomyces oris
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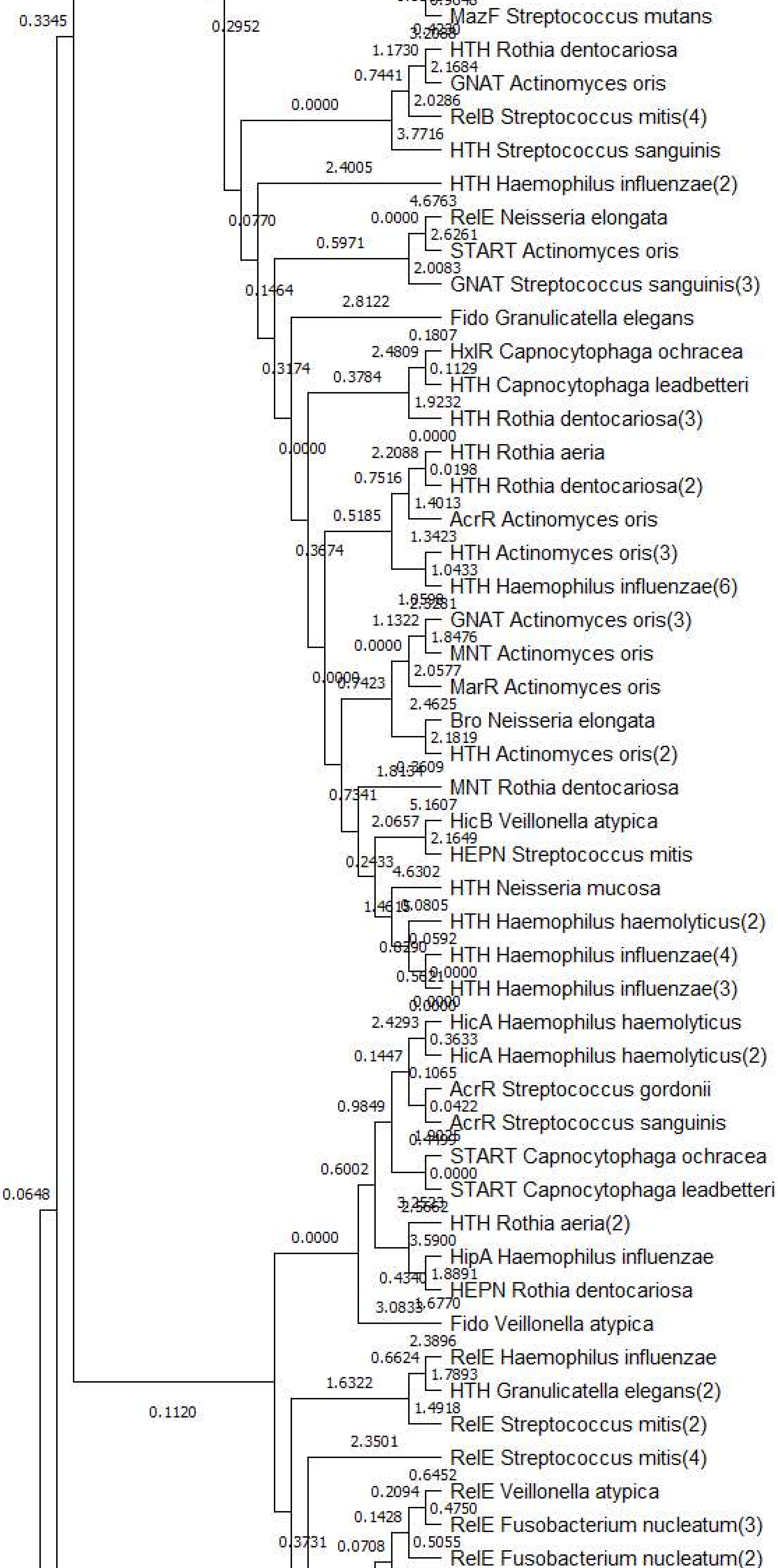
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HTH Capnocytophaga leadbetteri
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HTH Haemophilus influenzae(6)
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HTH Neisseria mucosa
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HTH Haemophilus influenzae(4)
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HicA Haemophilus haemolyticus(2)
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AcrR Streptococcus sanguinis
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0.0000
START Capnocytophaga leadbetteri
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1.4918
RelE Streptococcus mitis(2)
2.3501
RelE Streptococcus mitis(4)
0.6452
0.2094
RelE Veillonella atypica
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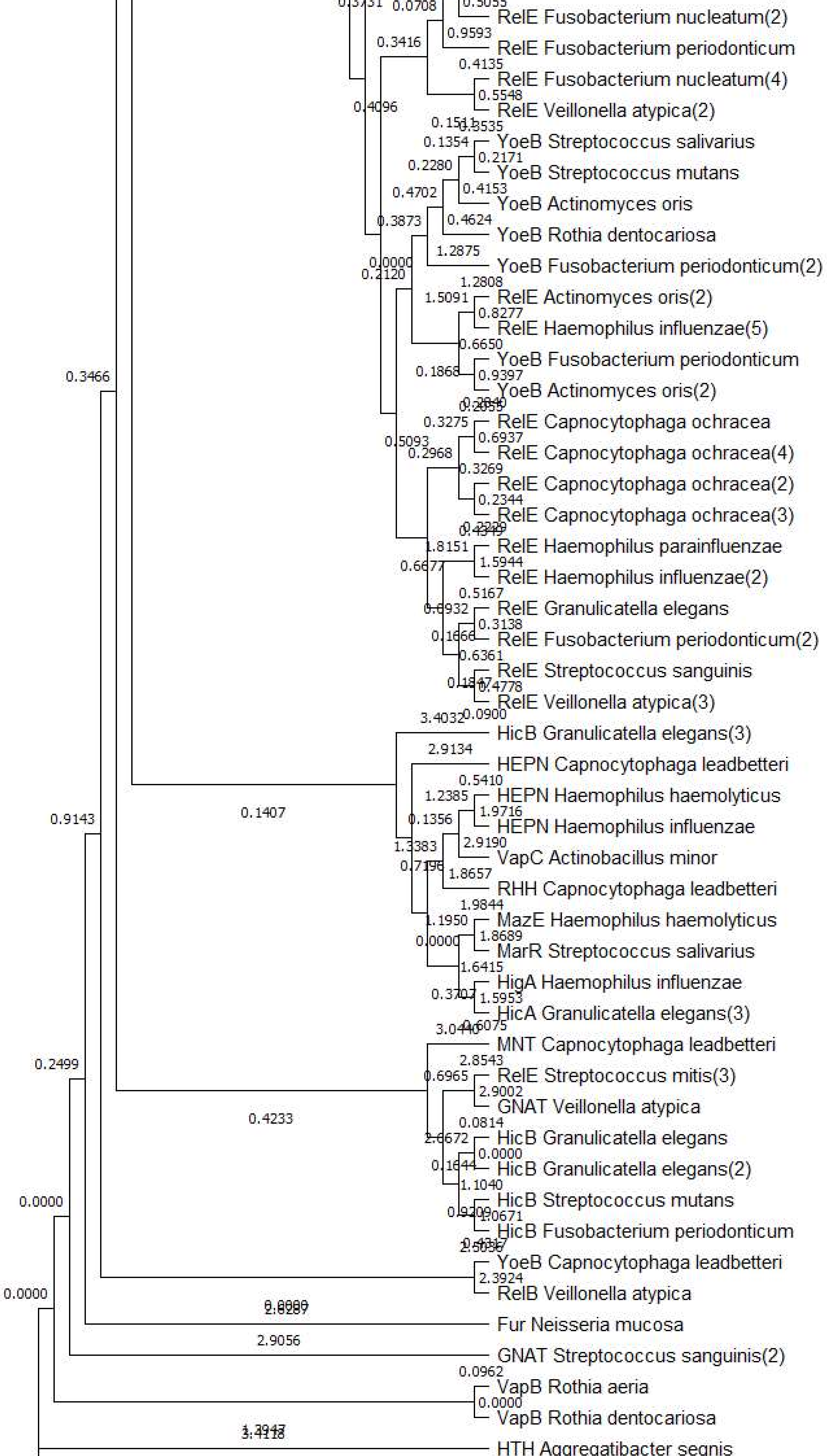
RelE Fusobacterium nucleatum(2)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RelE Fusobacterium nucleatum(4)
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RelE Veillonella atypica(2)
0.1511
0.3535
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YoeB Streptococcus salivarius
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0.2171
YoeB Streptococcus mutans
0.4702
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YoeB Fusobacterium periodonticum(2)
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1.2808
1.5091
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RelE Haemophilus influenzae(5)
0.6650
0.3466
YoeB Fusobacterium periodonticum
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0.9397
YoeB Actinomyces oris(2)
0.2840
0.2055
0.3275
RelE Capnocytophaga ochracea
0.5093
0.6937
0.2968
RelE Capnocytophaga ochracea(4)
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RelE Capnocytophaga ochracea(2)
0.2344
RelE Capnocytophaga ochracea(3)
0.2229
0.4349
1.8151
RelE Haemophilus parainfluenzae
0.6677
1.5944
RelE Haemophilus influenzae(2)
0.5167
0.0932
RelE Granulicatella elegans
0.3138
0.1666
RelE Fusobacterium periodonticum(2)
0.6361
RelE Streptococcus sanguinis
0.1847
0.4778
RelE Veillonella atypica(3)
3.4032
0.0900
HicB Granulicatella elegans(3)
2.9134
HEPN Capnocytophaga leadbetteri
0.5410
1.2385
HEPN Haemophilus haemolyticus
0.9143
0.1356
1.9716
0.1407
HEPN Haemophilus influenzae
2.9190
1.3383
VapC Actinobacillus minor
0.7198
1.8657
RHH Capnocytophaga leadbetteri
1.9844
1.1950
MazE Haemophilus haemolyticus
0.0000
1.8689
MarR Streptococcus salivarius
1.6415
HigA Haemophilus influenzae
0.3707
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HicA Granulicatella elegans(3)
3.0440
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0.2499
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0.4233
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0.0814
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HicB Granulicatella elegans
0.0000
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HicB Granulicatella elegans(2)
0.0000
1.1040
HicB Streptococcus mutans
0.9209
1.0671
HicB Fusobacterium periodonticum
0.4317
2.5056
YoeB Capnocytophaga leadbetteri
2.3924
0.0000
RelB Veillonella atypica
0.0000
2.6287
Fur Neisseria mucosa
2.9056
GNAT Streptococcus sanguinis(2)
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VapB Rothia aeria
0.0000
VapB Rothia dentocariosa
1.2947
3.4118
HTH Aggregatibacter segnis

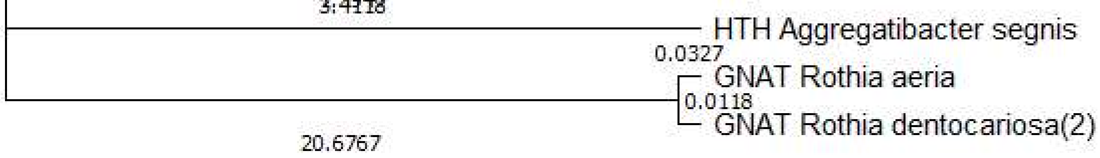
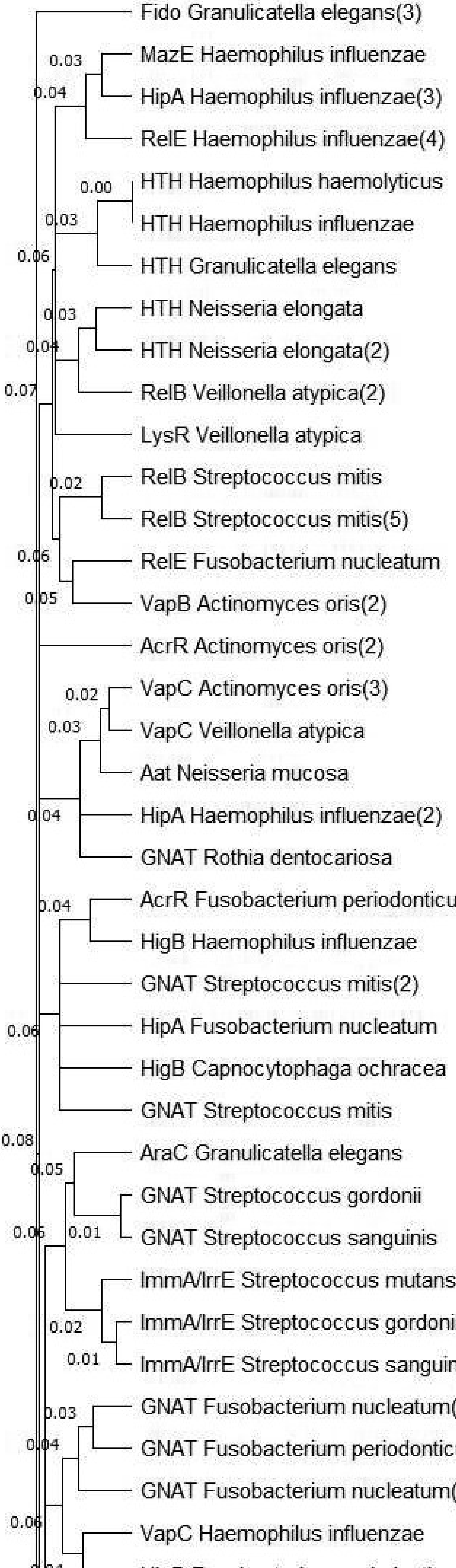

3.4118
HTH Aggregatibacter segnis
0.0327
GNAT Rothia aeria
0.0118
GNAT Rothia dentocariosa(2)
20.6767

Figure. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model [1]. The tree with the highest log likelihood (-103010.66) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 13.2539)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 277 amino acid sequences. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2].

1. Whelan, S. and Goldman, N. (**2001**). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Molecular Biology and Evolution* **18**:691-699.
2. Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (**2018**). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.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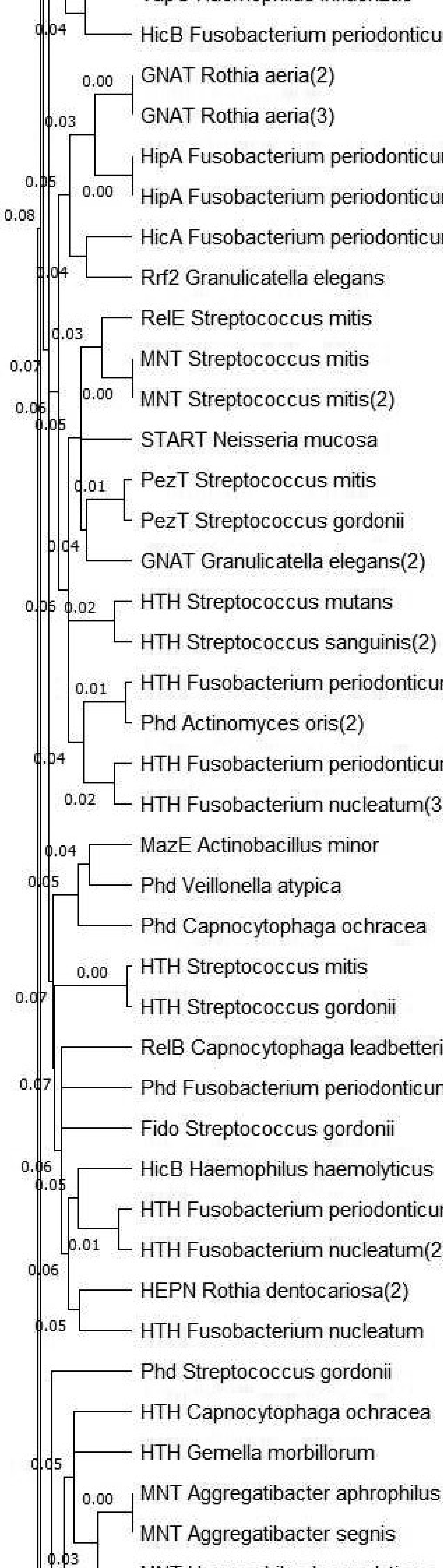
0.03
MazE Haemophilus influenzae
0.04
HipA Haemophilus influenzae(3)
RelE Haemophilus influenzae(4)
0.00
HTH Haemophilus haemolyticus
0.03
HTH Haemophilus influenzae
0.06
HTH Granulicatella elegans
0.03
HTH Neisseria elongata
0.04
HTH Neisseria elongata(2)
0.07
RelB Veillonella atypica(2)
LysR Veillonella atypica
0.02
RelB Streptococcus mitis
RelB Streptococcus mitis(5)
0.06
RelE Fusobacterium nucleatum
0.05
VapB Actinomyces oris(2)
AcrR Actinomyces oris(2)
0.02
VapC Actinomyces oris(3)
0.03
VapC Veillonella atypica
Aat Neisseria mucosa
0.04
HipA Haemophilus influenzae(2)
GNAT Rothia dentocariosa
0.04
AcrR Fusobacterium periodonticu
HigB Haemophilus influenzae
GNAT Streptococcus mitis(2)
0.06
HipA Fusobacterium nucleatum
HigB Capnocytophaga ochracea
GNAT Streptococcus mitis
0.08
AraC Granulicatella elegans
0.05
GNAT Streptococcus gordonii
0.06
0.01
GNAT Streptococcus sanguinis
ImmA/IrrE Streptococcus mutans
0.02
ImmA/IrrE Streptococcus gordoni
0.01
ImmA/IrrE Streptococcus sanguin
0.03
GNAT Fusobacterium nucleatum(
0.04
GNAT Fusobacterium periodontic
GNAT Fusobacterium nucleatum(
0.06
VapC Haemophilus influenzae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0.05
0.00
HipA Fusobacterium periodonticu
0.08
HicA Fusobacterium periodonticu
0.04
Rrf2 Granulicatella elegans
RelE Streptococcus mitis
0.03
MNT Streptococcus mitis
0.07
0.00
MNT Streptococcus mitis(2)
0.06
0.05
START Neisseria mucosa
0.01
PezT Streptococcus mitis
PezT Streptococcus gordonii
0.04
GNAT Granulicatella elegans(2)
HTH Streptococcus mutans
0.06
0.02
HTH Streptococcus sanguinis(2)
0.01
HTH Fusobacterium periodonticu
Phd Actinomyces oris(2)
0.04
HTH Fusobacterium periodonticu
0.02
HTH Fusobacterium nucleatum(3
0.04
MazE Actinobacillus minor
0.05
Phd Veillonella atypica
Phd Capnocytophaga ochracea
0.00
HTH Streptococcus mitis
0.07
HTH Streptococcus gordonii
RelB Capnocytophaga leadbetteri
0.07
Phd Fusobacterium periodonticu
Fido Streptococcus gordonii
0.06
HicB Haemophilus haemolyticus
0.05
HTH Fusobacterium periodonticu
0.01
HTH Fusobacterium nucleatum(2
0.06
HEPN Rothia dentocariosa(2)
0.05
HTH Fusobacterium nucleatum
Phd Streptococcus gordonii
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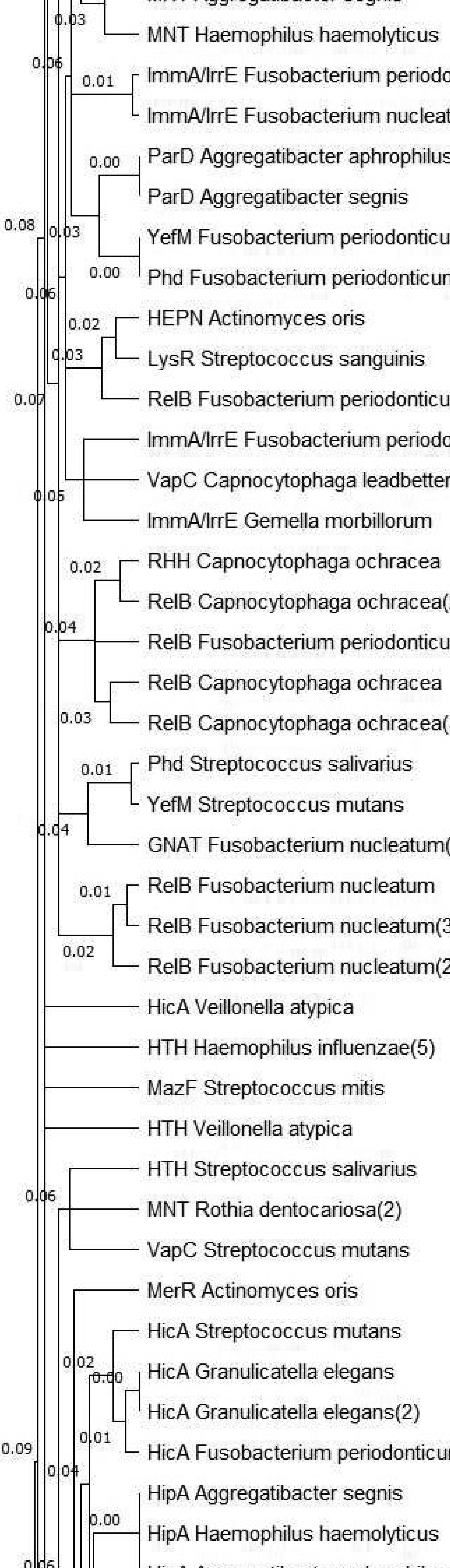
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ImmA/IrrE Fusobacterium nucleat
0.00
ParD Aggregatibacter aphrophilus
ParD Aggregatibacter segnis
0.08
0.03
YefM Fusobacterium periodonticu
0.00
Phd Fusobacterium periodonticum
0.06
0.02
HEPN Actinomyces oris
0.03
LysR Streptococcus sanguinis
0.07
RelB Fusobacterium periodonticu
ImmA/IrrE Fusobacterium periodo
VapC Capnocytophaga leadbetter
0.05
ImmA/IrrE Gemella morbillorum
0.02
RHH Capnocytophaga ochracea
RelB Capnocytophaga ochracea(
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RelB Fusobacterium periodonticu
RelB Capnocytophaga ochracea
0.03
RelB Capnocytophaga ochracea(
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Phd Streptococcus salivarius
YefM Streptococcus mutans
0.04
GNAT Fusobacterium nucleatum(
0.01
RelB Fusobacterium nucleatum
RelB Fusobacterium nucleatum(3
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RelB Fusobacterium nucleatum(2
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HTH Haemophilus influenzae(5)
MazF Streptococcus mitis
HTH Veillonella atypica
HTH Streptococcus salivarius
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MNT Rothia dentocariosa(2)
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MerR Actinomyces oris
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HicA Granulicatella elegans
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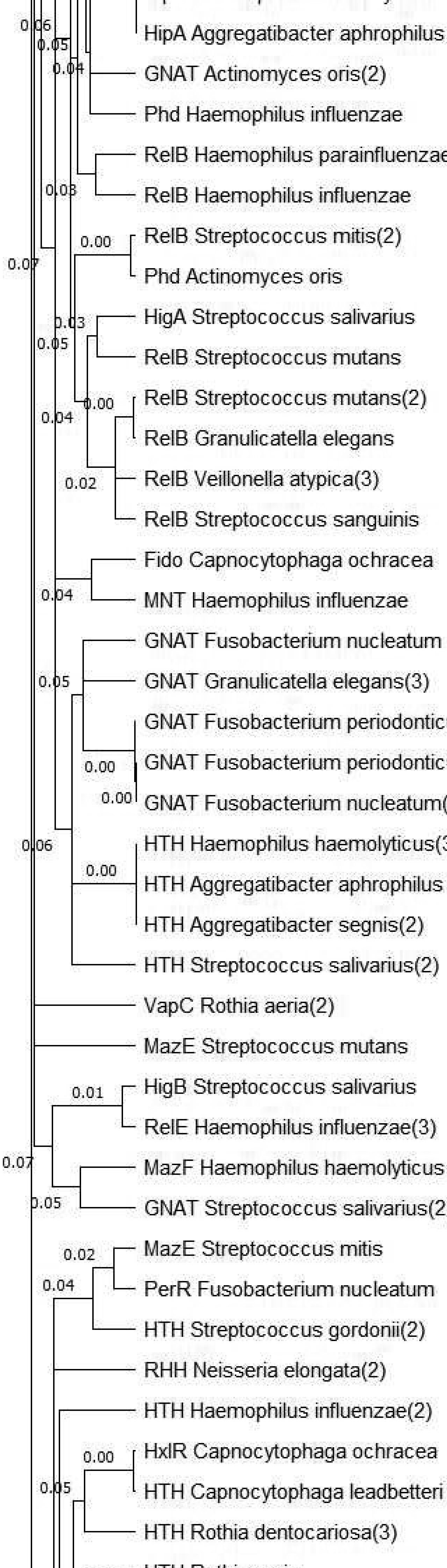
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HigA Streptococcus salivarius
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RelB Streptococcus mutans
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RelB Streptococcus mutans(2)
0.04
RelB Granulicatella elegans
0.02
RelB Veillonella atypica(3)
RelB Streptococcus sanguinis
Fido Capnocytophaga ochracea
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GNAT Fusobacterium nucleatum
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GNAT Fusobacterium periodontic
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HTH Streptococcus salivarius(2)
VapC Rothia aeria(2)
MazE Streptococcus mutans
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HigB Streptococcus salivarius
RelE Haemophilus influenzae(3)
0.07
MazF Haemophilus haemolyticus
0.05
GNAT Streptococcus salivarius(2
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MazE Streptococcus mitis
0.04
PerR Fusobacterium nucleatum
HTH Streptococcus gordonii(2)
RHH Neisseria elongata(2)
HTH Haemophilus influenzae(2)
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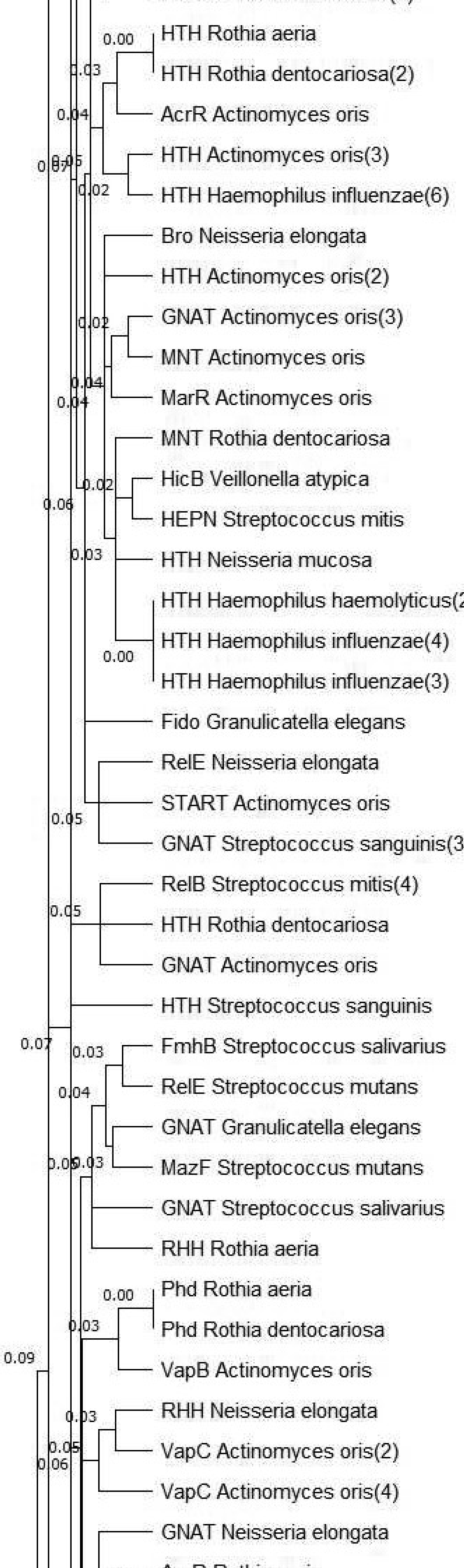
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HTH Haemophilus influenzae(6)
Bro Neisseria elongata
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GNAT Actinomyces oris(3)
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MarR Actinomyces oris
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HicB Veillonella atypica
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HTH Haemophilus haemolyticus(2
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START Actinomyces oris
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RelB Streptococcus mitis(4)
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HTH Streptococcus sanguinis
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FmhB Streptococcus salivarius
RelE Streptococcus mutans
0.04
GNAT Granulicatella elegans
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Phd Rothia aeria
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Phd Rothia dentocariosa
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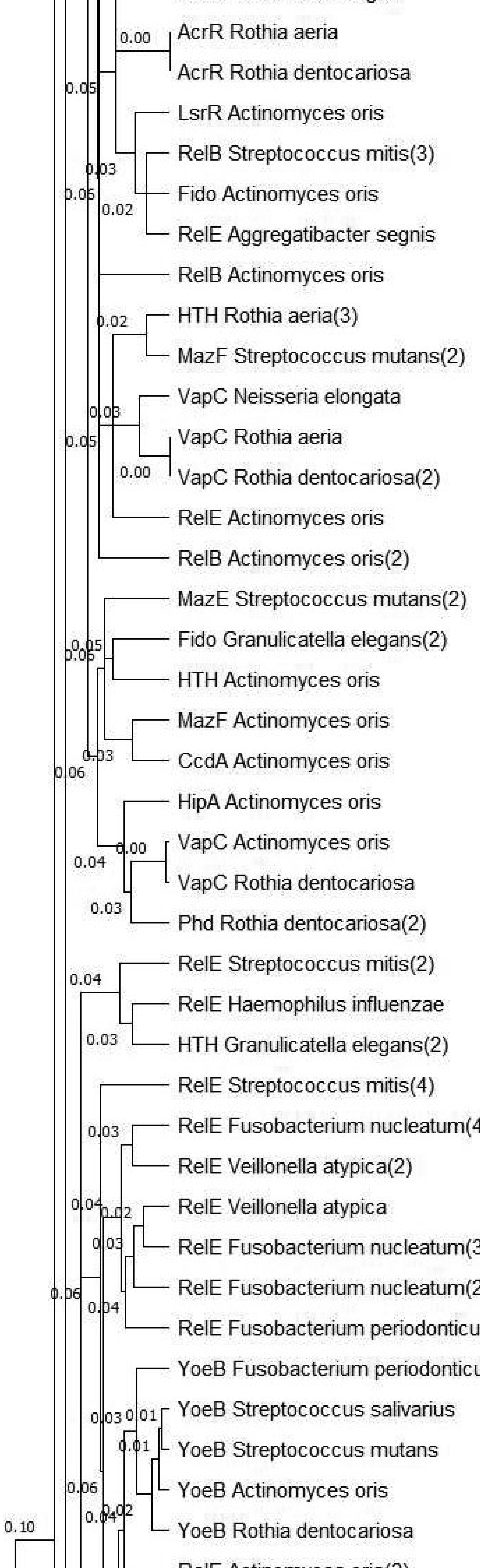
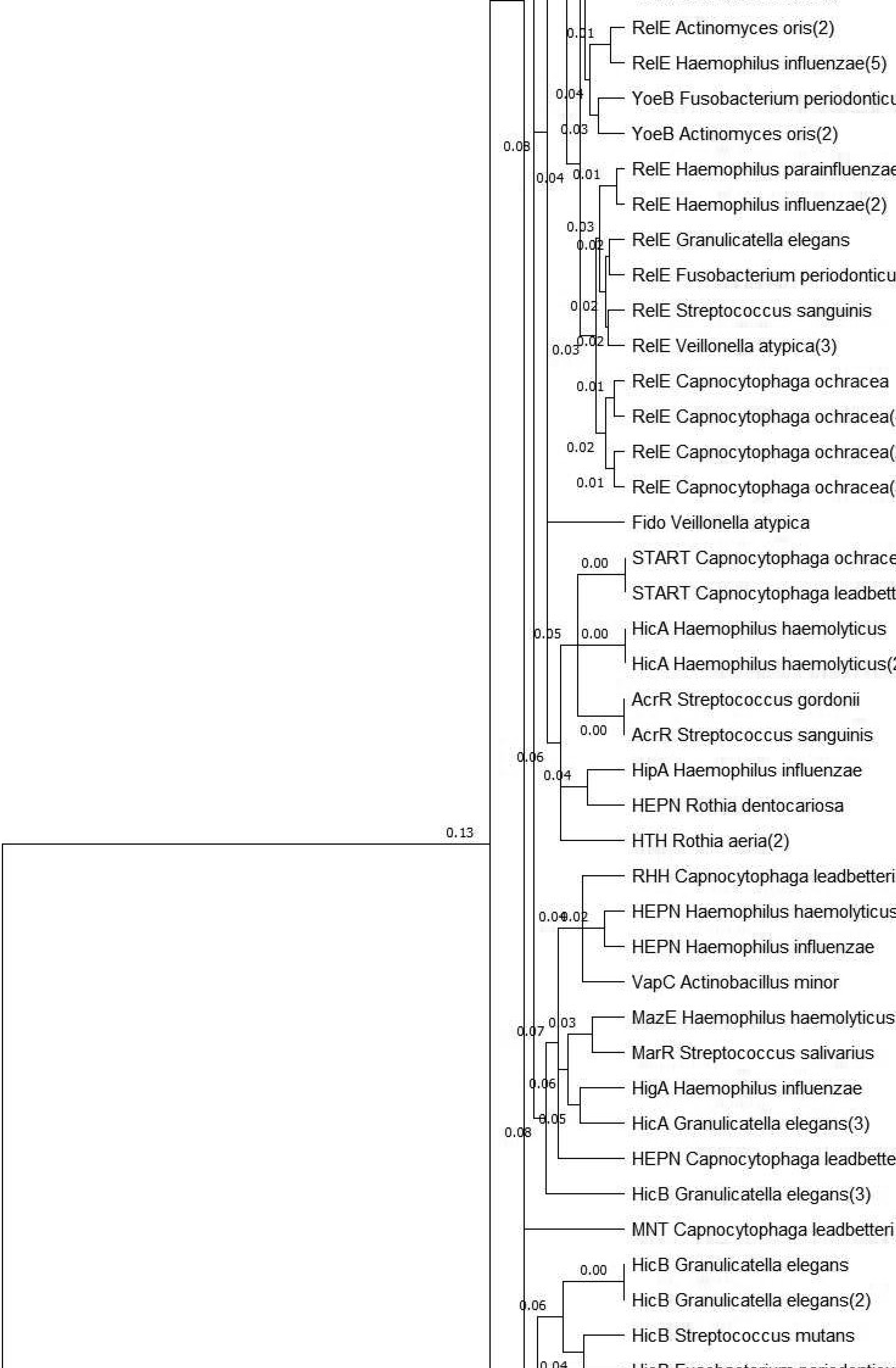
VapB Actinomyces oris
0.03
RHH Neisseria elongata
0.05
0.06
VapC Actinomyces oris(2)
VapC Actinomyces oris(4)
GNAT Neisseria elongata


0.00
AcrR Rothia aeria
0.05
AcrR Rothia dentocariosa
LsrR Actinomyces oris
0.03
RelB Streptococcus mitis(3)
0.06
Fido Actinomyces oris
0.02
RelE Aggregatibacter segnis
RelB Actinomyces oris
0.02
HTH Rothia aeria(3)
MazF Streptococcus mutans(2)
VapC Neisseria elongata
0.03
VapC Rothia aeria
0.05
0.00
VapC Rothia dentocariosa(2)
RelE Actinomyces oris
RelB Actinomyces oris(2)
MazE Streptococcus mutans(2)
Fido Granulicatella elegans(2)
0.05
0.06
HTH Actinomyces oris
MazF Actinomyces oris
0.03
CcdA Actinomyces oris
0.06
HipA Actinomyces oris
0.00
VapC Actinomyces oris
0.04
VapC Rothia dentocariosa
0.03
Phd Rothia dentocariosa(2)
RelE Streptococcus mitis(2)
0.04
RelE Haemophilus influenzae
0.03
HTH Granulicatella elegans(2)
RelE Streptococcus mitis(4)
RelE Fusobacterium nucleatum(4
0.03
RelE Veillonella atypica(2)
RelE Veillonella atypica
0.04
0.02
0.03
RelE Fusobacterium nucleatum(3
RelE Fusobacterium nucleatum(2
0.06
0.04
RelE Fusobacterium periodonticu
YoeB Fusobacterium periodonticu
YoeB Streptococcus salivarius
0.03
0.01
YoeB Streptococcus mutans
0.01
YoeB Actinomyces oris
0.06
0.02
YoeB Rothia dentocariosa
0.10
0.04


0.01
RelE Actinomyces oris(2)
RelE Haemophilus influenzae(5)
0.04
YoeB Fusobacterium periodonticu
0.03
YoeB Actinomyces oris(2)
0.08
0.04
0.01
RelE Haemophilus parainfluenzae
RelE Haemophilus influenzae(2)
0.03
0.02
RelE Granulicatella elegans
RelE Fusobacterium periodonticu
0.02
RelE Streptococcus sanguinis
0.03
0.02
RelE Veillonella atypica(3)
0.01
RelE Capnocytophaga ochracea
RelE Capnocytophaga ochracea(
0.02
RelE Capnocytophaga ochracea(
0.01
RelE Capnocytophaga ochracea(
Fido Veillonella atypica
0.00
START Capnocytophaga ochrace
START Capnocytophaga leadbett
0.05
0.00
HicA Haemophilus haemolyticus
HicA Haemophilus haemolyticus(2
AcrR Streptococcus gordonii
0.00
AcrR Streptococcus sanguinis
0.06
0.04
HipA Haemophilus influenzae
HEPN Rothia dentocariosa
0.13
HTH Rothia aeria(2)
RHH Capnocytophaga leadbetteri
0.04
0.02
HEPN Haemophilus haemolyticus
HEPN Haemophilus influenzae
VapC Actinobacillus minor
0.07
0.03
MazE Haemophilus haemolyticus
MarR Streptococcus salivarius
0.06
HigA Haemophilus influenzae
0.05
HicA Granulicatella elegans(3)
0.08
HEPN Capnocytophaga leadbette
HicB Granulicatella elegans(3)
MNT Capnocytophaga leadbetteri
0.00
HicB Granulicatella elegans
0.06
HicB Granulicatella elegans(2)
HicB Streptococcus mutans
0.04

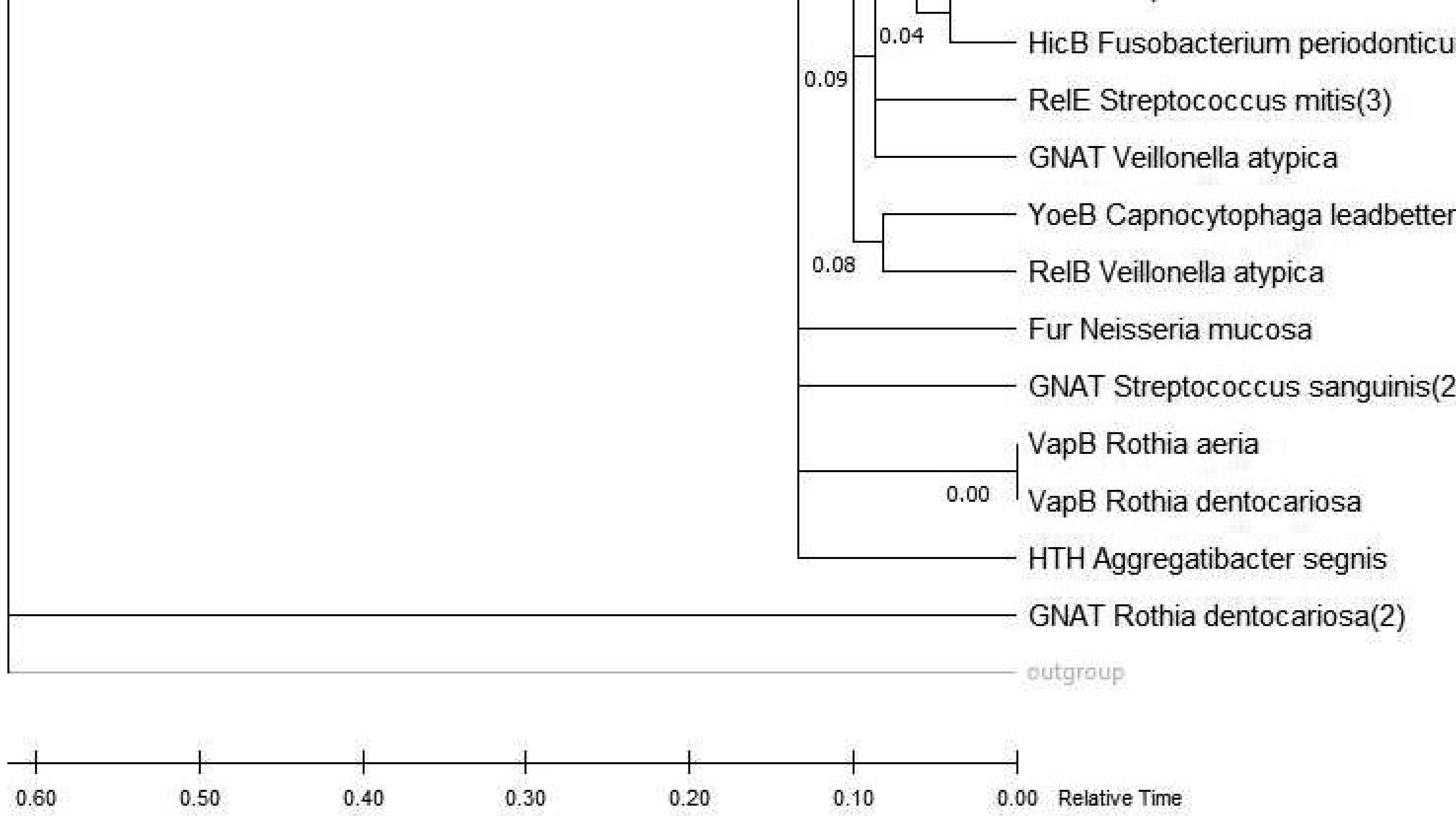

0.04
0.09
HicB Fusobacterium periodonticu
RelE Streptococcus mitis(3)
GNAT Veillonella atypica
YoeB Capnocytophaga leadbetter
0.08
RelB Veillonella atypica
Fur Neisseria mucosa
GNAT Streptococcus sanguinis(2
VapB Rothia aeria
0.00
VapB Rothia dentocariosa
HTH Aggregatibacter segnis
GNAT Rothia dentocariosa(2)
outgroup
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Relative Time

Figure. Timetree analysis using the RelTime method

A timetree inferred using the Reltime method [1][2] and the Whelan And Goldman + Freq. model [3]. The estimated log likelihood value is -103039.01. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+*G*, parameter = 24.5210)). The analysis involved 277 amino acid sequences. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

1. Tamura K., Battistuzzi FU, Billing-Ross P, Murillo O, Filipski A, and Kumar S. (**2012**). Estimating Divergence Times in Large Molecular Phylogenies. *Proceedings of the National Academy of Sciences* 109:19333-19338.
2. Tamura K., Qiqing T., and Kumar S. (**2018**). Theoretical Foundation of the RelTime Method for Estimating Divergence Times from Variable Evolutionary Rates. *Molecular Biology and Evolution* 35: 1770-1782.
3. Whelan, S. and Goldman, N. (**2001**). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Molecular Biology and Evolution* **18**:691-699.
4. Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (**2018**). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.

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