Development of multi-locus barcodes for identification of bacterial strains and species in environmental samples using next generation sequencing technologies

Adeola Mujidat Rotimi
Development of multi-locus barcodes for identification of bacterial strains and species in environmental samples using next generation sequencing technologies

by

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Faculty of Natural and Agricultural Sciences

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In the beginning was the Word, and the Word was with God, and the Word was God.

John 1 vs 1
Dedication

This work is dedicated to GOD Almighty and my late mother (Mrs Modupe Ayodele Helen Salawu); may your gentle soul continue to rest in perfect peace
SUBMISSION DECLARATION

I, Adeola Mujidat Rotimi, declare that the thesis, which I hereby submit for the degree Philosophiae Doctor in the Department of Biochemistry, Genetics and Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: _________

DATE: 17th October, 2018
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ACKNOWLEDGEMENTS

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SUMMARY

Metagenomic approaches have revealed the complexity of environmental microbiomes and the advancement in whole genome sequencing showed a significant level of genetic heterogeneity on species level. It has become clear that a superior pattern of bioactivity of bacteria applicable in biotechnology, as well as the enhanced virulence of pathogens, often requires distinguishing between closely related species or sub-species. Current methods for binning of metagenomic reads usually do not allow identification below the genus level and very often, stop at the level of families.

In this work, an attempt was made to improve metagenome binning resolution by creating genome-specific barcodes, based on the core and accessory gene sequences. This protocol was implemented in novel software tools available for use and download from http://bargene.bi.up.ac.za/. The most abundant barcode genes from the core genomes were found to encode for ribosomal proteins, some other central metabolic genes and ABC transporters. The performance of the created metabarcode sequences was evaluated using artificially generated and publicly available metagenomic datasets. Furthermore, a program, Barcoding 2.0, was developed to align reads against barcode sequences and calculate various parameters for scoring the alignment results and individual barcodes. Taxonomic units were identified in metagenomic samples by comparison of the calculated barcode scores to set cut-off values. In the study, it was found that varying sample sizes, i.e. the number of reads in a metagenome and metabarcode lengths had no significant effect on the sensitivity and specificity of the algorithm. Receiver operating characteristics curves were calculated for different taxonomic groups based on the results of identification of the corresponding genomes in artificial metagenomic datasets and the reliability of distinguishing between species of the same genus or family by the program was close to 100%.

The results showed that the novel online tool, BarcodeGenerator (http://bargene.bi.up.ac.za/), was an efficient approach to generating barcode sequences from a set of complete genomes provided by users. Another program, Barcoder 2.0, was made available from the same resource to enable efficient and practical use of metabarcodes for visualisation of distribution of organisms of interest in environmental and clinical samples.
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LIST OF ABBREVIATIONS

AUC- Area under curve

BCG- Bacilli Caimette-Guérin

BLAST- Basic Local Alignment Search Tool

BWA- Burrows-Wheeler alignment

ddNTPs- dideoxynucleotides

DNA- Deoxyribonucleic acid

dNTPs- deoxyribonucleotides

EDGAR- Efficient Database framework for Comparative Genome Analyses using BLAST Score Ratios

eDNA- Environmental deoxyribonucleic acid

HMMs- Hidden Markov models

LSU- Large Subunit

MAC- Mycobacterium avium complex

MDR-TB- Multidrug resistant Mycobacterium tuberculosis

MEGAN- Metagenome Analyser

MLST- Multi-locus sequence typing-

MRSA- Methicillin-resistant Staphylococcus aureus

MUSCLE- Multiple Sequence Comparison by Log-Expectation

NCBI- National Centre for Biotechnology Information

NGS- Next generation sequencing

OTU- Operational taxonomic units

PCR- Polymerase chain reaction
PHP- Hypertext Preprocessor

RDP- Ribosomal Database Project

rMLST- Ribosomal multi-locus sequence typing

SBL- Sequencing by ligation

SBS- Sequencing by synthesis

SEN- Sensitivity

SMRT- Single-molecule real-time

SMS- Single-molecule sequencing

SNPs- Single nucleotide polymorphisms

SPE- Specificity

SRST- Short read sequence typing

wgMLST- Whole genome multi-locus sequence typing
CHAPTER 1: Literature Review

Knowledge of sequences could contribute much to our understanding of living matter. Frederick Sanger

1.1 Sequencing technologies and advance in genomic studies

In 1944, Oswald Theodore proved that deoxyribonucleic acid (DNA) was a genetic material. James D. Watson and Francis Crick demonstrated in 1953 that the double helical strand structure of DNA was made up of four bases, which led to the central dogma of molecular biology (Church and Gilbert, 1984; Liu et al., 2012). The arrangement of nucleic acids in polynucleotide chains contains the genetic information for heritable and biochemical properties of terrestrial life (Heather and Chain, 2016). Hence, knowing the order of sequences is of vital importance in a wide range of uses such as molecular cloning, breeding, finding pathogenic genes, comparative and evolution studies (Liu et al., 2012; Heather and Chain, 2016).

The chain termination or dideoxy sequencing method published by Federick Sanger in 1977 became the gold standard for sequencing for the next 30 years (Sanger et al., 1977; McGinn and Gut, 2013). Dideoxy terminator DNA sequencing was initiated with the use of automated gel electrophoresis (slab gel) and fluorescent terminator chemistry (capillary gel-based systems) (McGinn and Gut, 2013). The introduction of next-generation sequencing (NGS) techniques in 2005 made the effects of the chain termination method more far-reaching, with a distinct increase in the amount of sequencing data produced per instrument (Mardis, 2017). Next-generation sequencing technologies do immense parallel sequencing, which generates millions of fragments of DNA from a single sample that is sequenced in unison (Grada and Weinbrecht, 2013). The massive parallel sequencing enables high-throughput sequencing, which allows a whole genome to be sequenced in less than a day (Grada and Weinbrecht, 2013). The main aim of these sequencing technologies is to decrease the time, effort and cost of whole genome sequencing (WGS) to a level where it can be done on a routine basis for research and clinical applications. At present, there are at least four generations of sequencing technologies that can be categorised by unique features (McGinn and Gut, 2013).
1.1.1 First-generation sequencing: Classical sequencing

In the early 1970s Sanger and his colleague, Coulson, developed enzymatic DNA sequencing, also known as Sanger sequencing, which uses DNA polymerase (Morey et al., 2013). The sequencing method known as ‘plus and minus’ and the sequence of bacteriophage φX174 were also published in 1975 by Sanger and Coulson (Sanger and Coulson, 1975). In 1977, the same authors presented the ‘chain termination’ method, which was less tedious and more efficient than the plus and minus method (Sanger et al., 1977; Morey et al., 2013). The chain termination method makes use of chemical analogues of the deoxyribonucleotides (dNTPs), which are known monomers of DNA strands. The dideoxynucleotides (ddNTPs) cannot form a bond with the 5’ phosphate of the next dNTP because it lacks the 3’ hydroxyl group needed to form a bond (Heather and Chain, 2016). Fragment ladders of sequence are created by enzymatically extending a primer hybridised to a pool of template molecules and introducing specific T, C, G or A terminations along the template (Liu et al., 2012; Heather and Chain, 2016). Series of improvements were made to the chain termination method in the following years, one of which was the non-enzymatic sequencing method developed by Maxam and Gilbert. The non-enzymatic method involves selective fragmentation of the region to be sequenced for each of the nitrogenous bases and the resulting fragment is loaded on a polyacrylamide gel. Radioactive labelling and exposure to film or the introduction of fluorescent dyes into the termination reaction and fluorescent imaging are mostly used for product revelation (Maxam and Gilbert, 1977; Heather and Chain, 2016).

The major disadvantages of first-generation sequencing are: (i) low throughput, due to template preparation; (ii) high background levels, variants that are present at low frequency, such as mosaics, are often difficult to detect; and (iii) in comparison to the new sequencing technologies available, the cost per base is still high (Mardis, 2011; Morey et al., 2013).

1.1.2 Second-generation sequencing: Next-generation sequencing

Next-generation sequencing platforms were able to overcome the limitations associated with classical sequencing. The first commercially available NGS platform was the 454 Roche GS FLX system in 2004, followed by several others, as shown in Table 1 (Mardis, 2017). For assessment of any NGS platforms, the main factors to be considered are cost per base, read and depth, read accuracy, throughput and read length (Mardis, 2017).
Table 1.1: NGS instruments and the year they were introduced; SBS, sequencing by synthesis; SMS, single-molecule sequencing; SBL, sequencing by ligation

<table>
<thead>
<tr>
<th>YEAR</th>
<th>NEXT-GENERATION SEQUENCING INSTRUMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>454 (Roche) pyro-sequencing (SBS)</td>
</tr>
<tr>
<td>2006</td>
<td>Solexa 1G (Illumina) (SBS)</td>
</tr>
<tr>
<td>2007</td>
<td>ABI SOLiD (SBL)</td>
</tr>
<tr>
<td>2008</td>
<td>Helicos Helioscope (SMS)</td>
</tr>
<tr>
<td>2010</td>
<td>Ion Torrent (PGM)</td>
</tr>
<tr>
<td>2010</td>
<td>Pacific Biosciences SMRT (SMS)</td>
</tr>
<tr>
<td>2014</td>
<td>Oxford Nanopore MinION (SMS)</td>
</tr>
<tr>
<td>2015</td>
<td>Qiagen Gene Reader (SBS)</td>
</tr>
</tbody>
</table>

The two main processes involved in all NGS platforms are template preparation and sequencing. Template preparation is further divided into three steps, namely source nucleic acid extraction, library preparation and template amplification (Metzker, 2005, Ambardar et al; 2016). Two basic sequencing methods established so far are sequencing by synthesis (SBS) and sequencing by hybridisation and ligation (SBL), as shown in Figure 1.1 (Ambardar et al., 2016). A polymerase and a signal (a fluorophore/change in ionic concentration) detect the integration of a nucleotide into the elongating strand in the SBS methods. Pyrosequencing, sequencing by reversible termination and sequencing by detection of hydrogen ions are the sequencing chemistries that fall under SBS. In SBL methods, a probe sequence that is bound to a fluorophore hybridises to a DNA fragment, which is then ligated to an adjacent oligonucleotide for imaging. The SBL is the basis of support for the oligonucleotide ligation detection (SOLiD) sequencing technique by Applied Biosystems (Ambardar et al; 2016). The major disadvantage associated with the NGS methods is the short read length needed to be assembled with the aid of a bioinformatics pipeline into the original length template and polymerase chain reaction (PCR) bias introduced by clonal amplification for discovery of the base incorporation signal.
1.1.3 Third-generation sequencing: Insight into the near future

Some researchers have argued that real-time sequencing, single-molecule sequencing (SMS) and divergence from prior technologies should define third-generation sequencing platforms (Schadt et al., 2010; Niedringhaus et al.; 2011; Pareek et al.; 2011; Heather and Chain, 2016). Stephen Quake developed the first SMS technology, which was later commercialised by Helicos BioSciences. It works on the same principle as illumina, excluding the bridge amplification step (Braslavsky et al., 2003; Harris et al., 2008). The DNA template is attached to a planar surface and the property fluorescent reversible terminator dNTPs/virtual terminators are washed over one base at a time and imaged, before cleavage and cycling the next base over (Bowers et al., 2009).

Pacific Biosciences developed the single-molecule real-time (SMRT) sequencing platform, which is the first third-generation sequencing technology to observe a single molecule of DNA polymerase directly as it synthesises a strand of DNA (Van Dijk et al., 2014). Sequencing-by-synthesis is used by SMRT technology and it optically screens fluorescence marked nucleotides as they are merged into individual template molecules (Lee et al., 2016). Read lengths of up to -100,000 bp with a throughput of >8GB/day are produced by the PacBio RS II and the new release of PacBio can increase the throughput by as much as
The Oxford Nanopore MinIon, released in 2014, is the latest third-generation sequencing technology. It is a hand held device that sequences DNA electronically by measuring the minute disruptions to electric current as DNA molecules pass through a nanopore (Loman et al., 2015). The major limitation of this technology is that it is less accurate and its throughput is lower, which has limited its range to sequencing small genomes such as yeast (12 Mbp). However, by using error correction algorithms that are comparable to those available by PacBio reads, the per-nucleotide accuracy of genomes sequenced using the MinION has been measured to be >99.5%. Because of its low cost and small size, it has been used extensively for studies in secluded settings, including the ebola outbreak in West Africa (Quick et al., 2016; Lee et al., 2016).

1.1.4 Fourth-generation sequencing: In situ sequencing

The newly defined fourth-generation in situ sequencing technique makes use of second-generation NGS chemistry to read nucleic acid composition directly in fixed cells and tissues (Mignardi and Nilsson, 2014). Lee et al. (2014) demonstrated in situ sequencing of messenger ribonucleic acid (RNA) for the first time. They used a targeted method to sequence short nucleotide sequences in breast cancer tissue sections. Complementary DNA (cDNA) was first generated in situ and then padlock probes, which are approximately 70-base-long oligonucleotides, were used to encircle a short target sequence of four to six bases (Lee et al., 2014).

Lee and colleagues further described a new technique to generate amplicons in a non-targeted approach in which random hexamers labelled with a sequencing adaptor are used to reverse transcribe RNA molecules in situ. The newly synthesised cDNA self-circularises and is amplified by rolling circle amplification. The amplicons are covalently linked to cellular proteins and can be produced in several different cell types, tissue sections and whole mount embryos.

Though in situ sequencing is still in its infancy, there is a possibility of it being used as a complementary tool to filter clinically important information from the huge amount of data produced by traditional NGS methods (Mignardi and Nilsson, 2014).
1.2 Metagenomics

Metagenomics can be defined as a culture-independent genomic investigation of microbial communities, which has emerged as a potent tool in the field of microbiology over the past two decades (Allan, 2014). Figure 1.2 shows a diagram of a typical metagenome project. The novelty of metagenomics lies in the fact that microbial DNA is isolated directly from the environmental sample, giving access to the whole microbial community, including the majority that has not been cultured in the laboratory. Metagenomics provides genetic information on possibly new biocatalyst, genomic linkages between function and phylogeny for uncultured organisms and evolutionary profiles of community function and structure. Metagenomics can also be supplemented with metatranscriptomic/metaproteomic techniques to define expressed activities (Wilmes and Bond, 2006; Gilbert et al., 2008). Hence, metagenomics can be described as a potent tool for creating new hypotheses for microbial function such as the outstanding discoveries of proterorhodopsin-based photo-heterotrophy or ammonia-oxidising archaea (Beja et al., 2000; Nicol and Schleper 2006).

**Figure 1.1: Diagram of a typical metagenome project (Thomas et al., 2012).**

**1.2.1. Methods and approaches of metagenomics**

Two common methods are used for classification of taxonomic content of environmental samples. The first approach is sequencing of PCR amplified phylogenetic markers such as 16S ribosomal RNA (rRNA). This approach is referred to as amplicon analysis (marker gene metabarcoding). The second approach applies shotgun sequencing whereby all genomic DNA
in a community is sequenced (Handelsman, 2004; Peabody et al., 2015).

Amplicon analysis or maker gene metabarcoding, also known as meta-genetics, is a rapid approach used to acquire a community diversity profile or to fingerprint a community using PCR amplification with universal primers, followed by sequencing of evolutionarily conserved genes such as the 16S rRNA gene (Tringe et al., 2005; Oulas et al., 2015). Amplicon sequencing has been applied in an extensive range of contexts that include among others bacterial metabarcoding, biomonitoring and community functioning analysis (Murray et al., 2015). An environmental sample is collected and the total DNA is extracted from all cells in the sample. A taxonomically informative genomic maker that is common to virtually all organisms of interest is then targeted and amplified by PCR. The resulting amplicons are sequenced and bioinformatically characterised to identify which microbes exist in the sample and at what relative abundance. For bacteria and archaea, the amplicon approach commonly targets the small-subunit rRNA (16S rRNA) locus, which is both taxonomically and phylogenetically an informative marker (Pace et al., 1986; Hugenholtz and Pace, 1996). Amplicon sequencing of the 16S locus has shown a remarkable quantity of microbial diversity on earth (Pace, 1997; Rappé and Giovannoni, 2003; Lozupone and Knight, 2007) and has been used to characterise the biodiversity of microbes from a variety of environments, comprising: (i) human microbiome (Human Microbiome Project Consortium, 2012a; Yatsunenko et al., 2012); (ii) microbiota associated with Arabidopsis thaliana roots (Lundberg et al., 2012); (iii) bacteria of the ocean thermal vent (McCliment et al., 2006); (iv) bacterial communities of hot springs (Bowen De Leon et al., 2013); (v) micro-flora of the Antarctic volcano mineral soils (Soo et al., 2009) and many others. Associating 16S sequence profiles across samples explains how microbial diversity links with and scales across varieties of environmental habitats. Such observations have enabled insight into host-microbe relations and generated hypotheses about microbiota-based disease mechanisms (Turnbaugh et al., 2009; Muegge et al., 2011; Bulgarelli et al., 2012; Smith et al., 2013; Sharpton, 2014). Follow-up microbiota-manipulation research usually confirms these hypotheses (Smith et al., 2013; David et al., 2014; Sharpton, 2014). The most auspicious hypotheses and future planning of experiments tend to derive from the comparisons of microbiota associated with cohorts of hosts of specific genotypes or treatment conditions (Sharpton, 2014). Kuczynski and colleagues gave a thorough review on the use of 16S amplicon sequencing in a microbiota study in 2011.

The Ribosomal Database Project (RDP; http://rdp.cme msu.edu) is one of the tools
specifically created for processing high-volume amplicon sequence data (Cole et al., 2013). The RDP 11.1, released in October 2013, comprises 2,809,406 aligned and annotated bacterial and archaeal small subunit rRNA gene sequences and 62,860 fungal large subunit rRNA gene sequences. The RDP tools provided by classifier and aligner pipelines have been upgraded to work with the fungal collection. Since the use of NGS platforms in characterising environmental microbial populations has increased rapidly in the past years, the sizes of environmental data sets have also increased. The RDP provides tools for browsing and searching the data collections, for taxonomic classification and nearest-neighbour (NN) search, for prime probe testing and for phylogenetic tree building. These new tools have been created with speed capability in mind. The recognised tools have been upgraded to accommodate the current changes of the sequencing technology. Many RDP tools are also made accessible as open-source stand-alone packages (Cole et al., 2013).

Several limitations are associated with the amplicon sequencing approach, which include: (i) the fact that resolving a large portion of the diversity in a community is difficult, given several biases associated with PCR (Hong et al., 2009; Sharpton et al., 2011; Logares et al., 2013; Sharpton, 2014); (ii) amplicon sequencing can produce widely variable estimates of diversity (Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012); (iii) sequencing errors and inaccurately assembled amplicons can produce artificial sequences that are usually difficult to identify (Wylie et al., 2012); (iv) amplicon sequencing mostly only gives insight into the taxonomic composition of microbial community; it is usually difficult to resolve the biological functions linked with these taxa directly using this method; (Langille et al., 2013); and (v) amplicon sequencing is limited to the analysis of taxa for which taxonomically informative genetic markers are known and can be amplified; new or highly diverged microbial species are difficult to study using this method (Acinas et al., 2004; Sharpton, 2014).

The shotgun metagenomic sequencing technique, also known as WGS metagenomics, is an alternative method used to study uncultured microbiota that avoids many limitations of amplicon-based sequencing (Sharpton, 2014). WGS metagenomics has the ability to sequence the bulk of existing genomes within an environmental sample or community fully. This generates a community biodiversity profile that can be further linked with functional composition analysis of known and unknown organism lineages that are the genera or taxa (Tringe et al., 2008; Oulas et al., 2015). The DNA is extracted from cells in a community, but instead of aiming at a specific genomic locus for amplification, the entire DNA is then
clipped into tiny fragments that are autonomously sequenced. This results in DNA reads that correspond to specific genomic locations in numerous genomes available in the sample, including fungi and even multicellular organisms. Some of these reads will be sampled from taxonomically informative loci such as 16S, while others will be sampled from coding sequences that offer insight into the biological roles encoded in the genome (Sharpton, 2014). Shotgun metagenomics has advanced to address the following questions: (i) who is present in an environmental community; (ii) what those present are doing function-wise; and (iii) how these microorganisms interact to sustain a balanced ecological niche. It also offers unrestricted access to functional gene composition information derived from microbial communities residing in practical ecosystems (Oulas et al., 2015). Hence, the present study aims at dealing with WGS metagenomics and the program (Barcoder software tools) created in this work is basically designed to work with WGS reads.

Notwithstanding the advantages linked with shotgun metagenomic sequencing, several limitations have been encountered. Since metagenomic data is relatively intricate and huge, this obfuscates its informatics analysis. It may be problematic to determine the genome from which a read was obtained. Moreover, most communities are so diverse that most genomes are far from being completely signified by the generated reads. Hence two reads from the same gene may not overlap and are thus difficult to compare correctly with a sequence alignment (Schloss and Handelsman, 2008; Sharpton et al., 2011; Sharpton, 2014). When reads do overlap, it is not always obvious if they are from unique or repeated genomic fragments, which can challenge the sequence assembly (Mavromatis et al., 2007; Mende et al., 2012; Sharpton, 2014). Metagenomic analysis usually needs a huge volume of data to identify meaningful results because of the vast amount of genetic information being sampled. This need can cause computational challenges. Providentially bioinformatics software development is rapidly progressing and refining the ease and efficiency of metagenomics analysis (Sharpton, 2014).

Metagenomes can also contain unwanted DNA contaminations, for example the host DNA in samples generated from host-associated microbiota. In some scenarios, host DNA can so engulf the community DNA that complicated molecular approaches must be used to enhance the microbial DNA selectively before sequencing. Molecular and bioinformatics approaches needed to filter the host DNA from metagenomes either before or successive to sequencing of the data are being developed (Schmieder and Edwards, 2011b; Garcia-Garcerà et al., 2013).
Though contamination is a general problem to all environmental sequencing studies, identification and removal of contaminants from metagenomics datasets are specifically challenging (Kunin et al., 2008; Degnan and Ochman, 2012). It can become problematic to determine which reads were generated from which genome and chimeric assemblies are common. A metagenomic contaminant may reduce the coverage of genomes of interest, create chimeras and mislead the analysis of community function. Fortunately, software tools that identify and filter contaminants are made available (Schmieder and Edwards, 2011a). In 2011, Schmieder and Edwards developed DeconSeq, a rich framework for quick, automated identification and elimination of sequence contamination in longer read datasets > 150 bp mean read length. DeconSeq classifies likely contamination sequences, removes redundant hits with similarity to non-contaminants and offers graphical visualisations of the alignment results and classifications. DeconSeq allows scientists to automatically detect and proficiently remove unwanted sequence contamination from their datasets (Schmieder and Edwards, 2011). Other tools available include PRINSEQ (Schmieder and Edwards, 2011), Solexa QA (Cox et al., 2010), FASTX-Toolkit (http://hannonlab.cshl.edu/fastx-toolkit/) and FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/).

WGS metagenomics tends to be more expensive compared to amplicon-based metabarcoding, particularly when complex communities are sequenced or when the host DNA significantly outstrips the microbial DNA (Sharpton, 2014). The express reduction in the cost of sequencing has enhanced the popularity of WGS metagenomics. A rapid increase has been seen in the number of shotgun sequence datasets in the past years (Thomas et al., 2012).

In recent years, WGS metagenomics has been used: (i) to identify novel viruses (Yozwiak et al., 2012); (ii) to characterise the genomic diversity and function of uncultured bacteria (Wrighton et al., 2012); (iii) to identify novel and industrially significant proteins (Godzik, 2011); (iv) to identify metabolic pathways controlled by gut microbiota, which were associated with human health and chronic disease development (Morgan et al., 2012); and (v) to characterise plant and rhizosphere associated microbiota (Delmotte et al., 2009; Bulgarelli et al., 2013).

In 2016 Ranjan et al. performed a microbiome analysis where they compared the advantages of whole genome shotgun versus 16S amplicon sequencing. They studied the human faecal microbiome, accumulating a total of 194.1 x 10⁶ reads from a single sample by means of
multiple sequencing methods and platforms (Ranjan et al., 2016). The 16S amplicon approach has been the technique used most frequently to analyze bacterial microbiomes because of providing several important advantages such as: (i) cost-effectiveness; (ii) data analysis being done by established pipelines; and (iii) the availability of a large body of archived data for reference. Nevertheless, the study by Rajan et al. showed substantial advantages of the WGS approach. WGS identified twice as many species as the 16S-based approach, with $32 \times 10^6$ reads generated. Greater species diversity predicted by WGS was confirmed by calculating the Shannon and Simpson diversity coefficients. The WGS method also identified the presence of viruses, fungi and protozoa in the biotope that was obviously missed by the 16S rRNA approach. Another point being considered was the taxonomic resolution abilities of the 16S versus the WGS approaches (Ranjan et al., 2016). Owing to the high level of conservation of 16S rRNA sequences, the RDP classifier often assigns the 16S amplicon reads maximum to the genus level and fails with the species identification. In contrast, the WGS method can assertively bin the reads to the species level (Rajan et al., 2016); however, this approach requires alignment of the reads against much larger reference databases.

In assessment of taxonomic diversity, marker gene analysis is one of the most computationally effective methods. This procedure involves: (i) aligning metagenomic reads to a database of taxonomically informative sequences such as genes 16S rRNA or internal transcribed spacer (ITS) regions; (ii) identifying those reads that show a reliable sequence similarity to the marker gene; and (iii) using the values of sequence similarity to perform an assignment or binning the read to the appropriate taxonomic unit (Sharpton, 2014).

One advantage of WGS sequencing is that this approach allows identification of multiple protein coding genes hosted by constituent microorganisms. Thus, the functionality of microbiomes can be predicted. Gene prediction can be done on assembled or unassembled metagenomic sequences. Three approaches by which genes are predicted in metagenomes are gene fragment recruitment, protein family classification and de novo gene prediction. However, because of the enormous diversity of bacterial genomes in natural environments, which significantly exceeds the capacity of available sequence databases, the majority of protein coding genes cannot be identified (Wu et al., 2009). One of the most commonly used methods for detecting coding sequences in a metagenome is the use of the fragment recruitment approach to align metagenomic reads against a database of gene sequences. Metagenomic reads displaying a significant sequence similarity along their whole length to
respective gene sequences are considered representative subsequences of the gene. If the identified gene has a functional annotation, this approach to gene prediction can also concurrently offer a functional annotation to the recruited metagenomic sequences (Desai et al., 2012). This approach has been useful in quantifying the genetic diversity of the gut microbiota (Qin et al., 2010) and is commonly used for cataloguing specific genes present in metagenomes. This technique was designed as high-throughput likelihood-based gene identification. It relies on several read mapping algorithms that promptly evaluate to which extent a genomic fragment is similar to database sequence records. However, this comes at the cost of a possibility to return multiple various homolog sequences equally similar to a query read. Hence, this approach is not applicable to metagenomes obtained from communities comprising many unknown microorganisms, which sequences are scarce in public databases, particularly if the identification of new or highly divergent genes is an aim of the project (Sharpton, 2014).

A similar methodology involves the translation of each metagenomic read into six potential protein coding frames and matching each of the resulting peptides to a database of protein sequences by sequence alignment. Alignments can then be analysed to identify those metagenomic sequences that code translated peptides that show homology to proteins in the database. Translation tools such as transeq (Rice et al., 2000) can be used to translate reads before conducting the protein sequence alignment using BLASTP or FASTA algorithms implemented in multiple online tools: USEARCH (Edgar, 2010), RAPsearch (Zhao et al., 2012) and (iii) lastp (Kielbasa et al., 2011). There are also implementations of these algorithms in stand-alone programs: blastx (Altschul et al., 1997), USEARCH with ublast option or lastx (Kielbasa et al., 2011). This gene prediction process is commonly used along with the functional annotation obtained from metadata records associated in databases with homologous protein sequences. Since this method depends on comparing metagenomic sequences to a reference database of known sequences, this approach is not appropriate for the identification of new types of protein. Only diverged homologs of known proteins can be predicted (Sharpton, 2014).

The de novo gene prediction approach can actually detect new genes. Gene prediction models that are capable of estimating diverse functions of microbial genes by analysing DNA sequence signals such as GC bias, codon usage, frequencies oligonucleotide and amino acids words and potential ORF length are used to evaluate the likelihood that a metagenomic read or contig contains a protein coding gene. This approach does not depend on the presence of
similar sequences in reference databases. Hence, these approaches can classify genes in a metagenome that share mutual functions with other microbial genes, but may be extremely diverged from any gene that has been revealed to date (Sharpton, 2014). Tools used for de novo gene prediction include: (i) MetaGene (Noguchi et al., 2006), (ii) Glimmer-MG (Kelly et al., 2012), (iii) MetaGeneMark (Zhu et al., 2010), (iv) FragGeneScan (Rho et al., 2010) and (v) Orphelia (Hoff et al., 2009). Trimble et al. (2012) compared these methods by means of statistical simulations. Their concerts varied as a function of read properties such as length and sequencing error rate, with different approaches producing peak accuracies at diverse property thresholds, which makes it important for researchers to choose the right algorithm for their data carefully. For genome annotation, Yok and Rosen (2011) reported that gene predictions in metagenomes are improved when several approaches are used to the same data and then combined in a consensus approach (Yok and Rosen, 2011). Though these approaches need more time and means to envisage genes, they are usually more discerning than six-frame translation and may reduce the time consumed by the functional annotation of sequences, as fewer pairwise sequence comparisons may be needed (Trimble et al., 2012). In case scenarios where the predicted gene is new relative to the database sequences, it can be challenging to decide whether the gene is an actual one or a false prediction (Sharpton, 2014).

1.2.2 NGS sequencing technology

Sample processing is the major and most important phase in any metagenomic research. Hence, DNA extracted should represent all cells present in the sample and an adequate quantity of high-quality DNA must be acquired for consequent library production and sequencing. Exact procedures are required for each sample type and different robust methods of DNA extraction are presented by different researchers (Venter et al., 2004; Bruke et al., 2009; Delmont et al., 2011). Attempts have also been made to discover microbial diversity from different ecosystems using a single DNA extraction technology to ensure compatibility and a high level of precision.

Different sequencing technologies are now available, though Sanger sequencing is still considered the gold standard for sequencing, because of its low error rate, long read length (>1,500 bp) and large insert sizes. These features will help improve assembly outcomes for shotgun data, which makes Sanger sequencing still appropriate for generating close to complete genomes in low-diversity environments (Goltsman et al., 2009). Of all the NGS technologies, the Roche 454, Illumina and Ion Torrent systems have been used extensively in
metagenomic samples (Mardis, 2008; Metzker, 2010); however, the PacBio technology may replace them in the near future. Since the Roche 454 and Illumina technologies are mostly used in metagenomic research, it is of importance to describe their advantages and limitations in sequencing of metagenomics samples briefly (Oulas et al., 2015).

The chemistry of the 454 pyrosequencer relies on immobilisation of DNA fragments on DNA-capture beads in a water-oil emulsion and then using PCR to amplify the fixed fragments. The beads are placed on a PicoTiterPlate. DNA polymerase is also packed in the plate and pyrosequencing takes place (Ronaghi et al., 1998; Ronaghi, 2001). While Roche 454 pyrosequencing technology is considered highly reliable, it is associated with generation of several types of artefacts, which may affect the metagenomic data analysis and lead to biased results (Rosen et al., 2012). One problem consists in generation of artificial replicates of the same read that may cause an overestimation of species abundance or functional gene abundance in a sample. Amplification errors in the form of single base pair mismatches and improper sequencing of mononucleotide stretches of DNA may cause frame shifts in protein-coding genes (Rothberg and Leamon, 2008). Chimera sequences generated by an undesired end joining of two or more true sequences can also affect the results of metabarcoding based on amplified 16S rRNA with respect to the species richness (Bordin et al., 2013). The 454 pyrosequencing technology produces reads of up to 1 000 bp in length and >1 000 000 reads per run. The comparatively long reads length produced by this technology compared to other NGS technologies makes it more suitable for assembly genomes from shotgun metagenomic datasets and allows for better annotation accuracy (Wommack et al., 2008; Thomas et al., 2012).

Illumina dye sequencing by synthesis starts with the attachment of DNA molecules to primers on a glass slide, followed by amplification to generate local colonies of identical DNA fragments (Mardis, 2008). The production of DNA clusters is accompanied by an addition of fluorescently labelled adenine, cytosine, guanine and thymine terminator bases attached with a blocking group (Bentley et al., 2008). These bases then compete for binding sites on the template DNA to be sequenced and unbound molecules are washed away. A laser is used to excite the dye after each synthesis cycle and a high-tenacity scan of the merged base is done. A chemical deblocking phase enables the removal of the 3’ terminal blocking group together with the dye in a single step that generates a colour light impulse, which is recorded by the system. This procedure is repeated till the full DNA molecule is sequenced. Diverse Illumina sequencing instruments are dedicated to various uses. The
Hiseq2500 has large output of 1 000 GB per run but gives 125 bp reads. The MiSeq has an output of 15 GB and 25 million sequencing reads of 300 bp in length, of which clustered paired-end fragments can be sequenced from both ends, which can be combined so that 600 bp reads can be attained (Bantely et al., 2008; Kircher et al.; 2012; Oulas et al.; 2015). Shorter read lengths generated by Illumina increase the chances of errors during assembly and then the annotation inaccuracies during shotgun metagenomics data analysis (Kircher et al., 2012), while analysing 16S rRNA metabarcodes by Illumina obviates the need for time-consuming and inaccurate removal artifacts generated by Roche 454 pyrosequencing and makes this analysis less error-prone (Werner et al., 2011). The greater coverage provided by Illumina enables a substantial reduction of systematic errors. This benefit and the low cost of sequencing are the defining reasons that have made Illumina the preferred NGS for metagenomics studies (Oulas et al., 2015).

PacBio offers longer read lengths of -10 000 bp compared to other sequencing technologies, therefore having the advantage of addressing issues of annotation and assembly for shotgun metagenomics (Metzker, 2010). The PacBio platform uses a process termed “storing” to perform pair-end read sequencing. Notwithstanding the high read length of PacBio, this technology is limited by higher error rates and low coverage (Metzker, 2010; Oulas et al., 2015). Ion Torrent provides higher quality than 454, particularly when sequencing homopolymers, but at a similar cost of about US$23 per Mb for the Ion Torrent PGM -314 chip. However, given that 454 will eventually stop being supported by life sciences, it is most likely that the former users of 454 pyrosequencing will switch to Ion Torrent sequencing chemistry in view of their similarities, such as the emulsion PCR step (Oulas et al., 2015).

1.2.3 Assembly

The assembly procedure joins collinear metagenomic reads from the same genome into a single contiguous sequence and is suitable for creating longer sequences, which can simplify further bioinformatics analysis and genome comparison. Sometimes, complete or nearly completed genomes of non-cultured microorganisms can be assembled from metagenomic sets of reads (Iverson et al., 2012; Wrighton et al., 2012; Ruby et al., 2013; Sharpton, 2014).

Two approaches are employed for assembly of metagenomic reads: reference-based assembly (co-assembly) and de novo assembly. Software packages used for reference-based assembly are MIRA or AMOS (http://sourceforge.net/projects/amos/) and Newbler (Roche) (Chevreux
The algorithms of these software packages are fast, memory-efficient and can be executed even on laptop computers in a few hours. Reference-based assembly performs better, if the metagenomic dataset sequences are closely related to one or several available reference genomes (Chevreux et al., 1999).

The de novo assembly usually needs stronger computational resources. Hence, a whole class of assembly tools based on the de Bruijn graphs were specially created to handle large amounts of data (Pevzner et al., 2001; Miller et al., 2010). The machine requirements for the de Bruijn assemblers, such as Velvet and Soap, are still considerably higher than those for the reference-based assembly. Usually they require hundreds of gigabytes of memory in a single machine or a computer cluster, and the run time often takes days (Li et al., 2009; Zerbino and Birney, 2008).

1.2.4. Binning and binning algorithms

Binning is defined as a process of assigning DNA sequences to taxon-specific groups, which may represent an individual genome or genomes of several closely related organisms (Thomas et al., 2012). Usually, each sequence is either categorised into a taxonomic group (i.e. operational taxonomic unit (OTU), genus, family) through comparison to some referential data, or clustered into groups of sequences that signify taxonomic groups based on shared characteristics such as sequence or nucleotide composition similarity. Binning plays a key role in metagenomics by (i) providing insight into the presence of groups of unknown organisms, which are difficult to separate and identify; (ii) providing insight into the distinct numbers and types of taxa in the community; and (iii) providing methods of reducing the complexity of data such as post-binning analyses, for example by read assembly, which can be performed independently on each set of the binned reads rather than on the whole population of data (Sharpton, 2014).

Different binning algorithms have been designed, which make use of different types of information in a given DNA sequence (Thomas et al., 2012). The analysis of datasets obtained by shotgun sequencing includes characterisation of the taxonomic and functional diversity of specified environmental micro-flora by analysing DNA fragments originating from genomes of the inhabitant microbes (Mande et al., 2012). Binning techniques available for these types of analyses can be grouped into two categories: taxonomy-dependent and taxonomy-independent (Mande et al., 2012).
1.2.5 Taxonomy-dependent methods

Most of the methods for binning datasets from shotgun sequencing belong to the taxonomy-dependent category, which includes: (i) alignment-based methods, (ii) composition-based methods and (iii) hybrid-based methods (Mande et al., 2012).

1.2.5.1 Alignment-based methods

Most alignment-based methods work by aligning reads to sequences followed by some statistical procedures, such as hidden Markov models (HMMs) for example, which assign the sequences to known taxonomic groups. Algorithms such as BLAST, BLAT or read mapping approaches such as Burrows-Wheeler alignment (BWA) or BOWTIE are usually first used to align individual reads to nucleotide or protein sequences belonging to known genomes in the alignment-based methods (Altschul et al., 1990; Kent et al.; 2002; Langmead et al., 2009; Li and Durbin, 2010; Mande et al., 2012). Collections of reference sequences are usually obtained from public repositories such as Ensembl (http://www.ensembl.org/), DDBJ (http://www.ddbj.nig.ac.jp/), National Centre for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nih.gov/blast/db/), PFAM (http://pfam.sanger.ac.uk), Uniprot (http://www.uniprot.org/), EMBL (http://www.ebi.ac.uk/embl/), NCBI Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and NCBI Refseq (http://www.ncbi.nlm.nih.gov/RefSeq). Analyses of the quality of alignment of the searched sequence against various reference sequences determine taxonomic groups to which the read can be assigned. The MG-RAST server and the CAMERA pipeline make use of this method, where reads are assigned to taxa of microorganisms corresponding to their respective best BLAST hits (Seshadri et al., 2007; Meyer et al., 2008). The major disadvantage of BLAST-based methods is the need for large computing power to align millions of reads against a large number of sequences constituting a reference database (Mande et al., 2012). Since a large fraction of reads from datasets attained from the shotgun sequencing method originated from unidentified taxa, which might be of novel specie/genus/family, this fraction of sequences cannot be assigned to any taxonomic unit. Hence, the MG-RAST server and metagenome analyser (MEGAN) provide an option based on the lowest common ancestor (LCA) to infer taxonomic affiliation at the lowest possible level according to the sequence similarity of the top best hits (Huson et al., 2007; Meyer et al., 2008).

Huson et al. (2007) introduced a novel computational software tool called MEGAN, which is used for analysis of large datasets on personal computer (PC) machines. Basically, this
program was designed for a visualisation of local BLAST outputs, as first of all the set of metagenomic DNA reads should be aligned using BLAST against a local database of reference sequences. The MEGAN software then allows the taxonomic content of the datasets to be explored, using the NCBI taxonomy to summarise and order the result (Huson et al., 2007). The MEGAN algorithm assigns each read to the LCA knob in the taxonomic tree that lies above all the species for which the reads have obtained significant alignment hit values. A rationale for doing this is that the reads that match widely conserved genes with similar hit values should be allocated to high-level taxa unifying all these equally matching groups of organisms. Reads that hit to a specific gene of a particular microorganism are allotted to lesser taxa. The number of reads confirming the presence of a specific taxonomic unit in the sample is also controlled. Hence, the reads can be binned by this approach across all taxonomic levels. The naïve LCA algorithm provides a rapid method for taxonomic binning, which runs at a rate of over 100 million reads and 2 billion alignments per hour on a PC machine (Huson et al., 2007; Huson et al., 2016). In 2016, a new release of the MEGAN software tool was produced and termed ‘MEGAN Community Edition (CE)’. The MEGAN Community Edition (CE) allows interactive analysis and comparison of data, making it possible to explore hundreds of samples and billions of reads. All source code for MEGAN CE is made available at https://gitup.com/danielhuson/megan-ce. MEGAN CE also makes use of the naïve LCA algorithm for taxonomic binning by default (Huson et al., 2016).

MEGAN makes use of bit scores of individual BLAST hit as the main parameter for judging hit significance (Mande et al., 2012). Research has shown that the single-parameter method adversely affects the accuracy of taxonomic assignments in diverse situations, especially taking into account that BLAST hits reflect the accuracy of local alignments (Monzoorul et al., 2009; Ghosh et al., 2010). Methods such as the Sort-ITEMS, DiScRIBinATE, ProViDE MetaPhyler and MARTA have been able to solve this limitation by using, apart from BLAST bit scores, several pre-computed thresholds for other alignment parameters such as percentages of identities of global sequence alignments, numbers of positives and gaps to determine the quality of alignments (Monzoorul et al., 2009; Ghosh et al., 2010; Horton et al., 2010; Gosh et al., 2011; Liu et al., 2010).

The CARMA and AMPHORA tools also make use of HMM-based binning methods (Krause et al., 2008; Wu and Eisen, 2008). Reads are first compared using BLASTx against protein sequences in the PFAM database with CARMA. The program subsequently creates a phylogenetic tree by comparing read alignments to different proteins using HMM-based
A taxonomic dendrogram is eventually inferred for the analysed reads (Krause et al., 2008). For the AMPHORA, reads are first compared using an HMM algorithm against reference sequences representing 31 phylogenetic marker gene families. Subsequently, a phylogenetic tree is created embodying all the reads and the sequences belonging to the best scoring HMM hits. Taxonomic assignments are then attained in a mode similar to CARMA (Wu et al., 2008). Other approaches that make use of HMMs/reference trees for the assignment procedures include: (i) ML TreeMap; (ii) Treephyler; (iii) pplacer; and (iv) papara (Masten et al., 2010; Schreiber et al., 2010; Stark et al., 2010; Berger and Stamatakis, 2011). These approaches also make use of either the Bayesian or maximum-likelihood algorithms to compute confidence scores. Treephyler uses the PFAM database in its workflow, while MLTreeMap compares query sequences using the HMM approach against protein sequences of 40 marker gene families (Schreiber et al., 2010; Stark et al., 2010). The pplacer and papara procedures offer a comprehensive scope of algorithms, which can be used for placing reads into the best scoring insertion edge on a user-specified reference phylogenetic tree (Masten et al., 2010; Berger et al., 2011).

1.2.5.2 Composition-based methods

Compositional binning makes use of the fact that the genomes have conserved nucleotide composition such as a certain GC or abundance distribution of k-mers, which will be reflected in the sequence fragments of the genomes, codon usage and oligonucleotide usage patterns for comparing reads to sequences or models present in reference databases (Mande et al., 2012; Thomas et al., 2012). Composition-based methods differ in the way they characterise, measure and compare compositional properties. Most tools involve an initial preparation phase during which one or more compositional properties of known genomes are used for creating genome-specific reference models or classifiers (Mande et al., 2012). The Phylopythia and NBC classifiers create genome- or clade-specific classifiers with support vector machines (SVMs) and naïve-Bayesian methods to capture and represent oligonucleotide usage forms seen in known taxonomic clades (McHardy et al., 2007; Rosen et al., 2010).

TACOA first creates genome-specific models by analysing tetra- and penta-nucleotide usage forms. A kernelised-NN (k-NN) method is then used to decipher taxonomic assignments of individual reads (Diaz et al., 2009). The Phymm tool represents oligonucleotide usage forms of reference genomes as interpolated Markov models. Reads are usually scored against these
models and a Bayesian method is thereafter used to draw inferences (Brady et al., 2009).

The ClaMS tool creates training models using de Bruijin graphs and Markovian chain algorithms. During the classification stage, a similar procedure is used for creation and comparison of signatures of query reads against pre-computed signatures of the training sequences (Pati et al., 2011). In 2011, Nalbantoglu et al. developed a semi-supervised approach, which performs read binning by creating an index referred to as the relative abundance index, which shows the under-abundance patterns of k-mers in sequences belonging to different known taxonomic clades. The index is then used as a measure to associate a given taxon to a query sequence (Nalbantoglu et al., 2011).

All the approaches described above adopt the idea that each genome can be represented by a single DNA compositional model of genome-signature k-mer frequencies. Some authors criticise this assumption by pointing out a significant level of compositional heterogeneity in genomic loci of several organisms (Cole et al., 1998). From these observations, it was concluded that the representation of each genome by one single composition model may be inappropriate (Mohammed et al., 2011). The INDUS algorithm disregards this hypothesis and characterises each genome in the form of multiple vectors. Each vector captures the form of tetranucleotide frequencies of individual 1-kb segments created by dicing the particular genome. In the assignment process, INDUS makes use of compositional distances between the query read and the closest known set of reference segments for determining an appropriate taxonomic level of assignment for the query. Hence, the final assignment is made to a consensus taxon that matches the next reference segments at or above the known taxonomic level (Mohammed et al., 2011).

1.2.5.3 Hybrid methods

Hybrid binning approaches make use of both alignment- and composition-based strategies for taxonomic classification. SPHINX and PhymmBL are two examples of the hybrid approach (Brady et al., 2009; Mohammed et al., 2011). SPHINX makes use of a two-phase binning algorithm. In the first stage, it compares the composition of a specified read with those of reference sequences. Hence, it speedily finds a subset of clusters of reference sequences that are next in composition to a given read. In the second stage, the taxonomic classification of the query read is inferred by first aligning the query read to reference sequences in the closet cluster and then engaging a similarity-based method such as Sort-ITEMS (Mohammed et al., 2011). PhymmBL combines the composition-based method of Phymm with the alignment-
based stage that involves BLAST to enhance the confidence of taxonomic assignments (Brady et al., 2009).

Taxonomy-dependent binning approaches are usually used to classify sequences constituting metagenomic datasets. However, lengths of metagenomic read, which are mostly dependent on the used sequencing techniques, are observed to be the main factor that determines selection of the binning approach. The alignment-based and composition-based approaches are most suitable for relatively longer lengths, though the composition-based approach is of more benefit in respect of the speed of execution and low memory requirement (Mande et al., 2012). Lykidis et al. (2011) performed a taxonomic characterisation of the terephthalate (TA) wastewater metagenome using a compositional approach (Phylopythia), given that the input was of adequate length (Lykidis et al., 2011). The assembled sequence data contained 37,818 and 14,526 contiguous fragments of intermediate length, the largest fragment being approximately 240 kb and 45 fragments between 24 and 167 kb. Phylopythia helped in identifying specific microbial species that played an important role not only in the degradation of TA but also in maintaining the stability of this distinctive microbial community. However, for lower length sequences providing very weak compositional signals, alignment-based approaches yielded much better performance, as seen in the studies conducted by Gupta et al. (2011) and Belda-Ferre et al. (2012). Sequences with a length of 200-400 bp in both malnourished gut metagenome (Gupta et al., 2011) and oral metagemones (Belda-Ferre et al., 2012) respectively, the hybrid (SPHINX and PhymmBL algorithms) and alignment-based (MEGAN) binning approaches resulted in the best binning outputs.

For most ultra-short sequences, a pre-assembly phase is necessary before performing taxonomic binning. In a comparative study conducted by Qin et al. (2010) on reads from human gut metagenomes, reads of length of < 75 bp generated by the Illumina sequencing technique were first assembled into contigs and then classified using MEGAN.

1.2.6 Taxonomy-independent or read clustering approaches

Approaches under this category include: (i) TETRA; (ii) CompostBin; (iii) AbundanceBin; (iv) variants of Self Organizing Maps (SOMs); and (v) MetaCluster (Teeling et al., 2004; Ultsch and Moerchen, 2005; Chan et al., 2008; Chatterji et al., 2008). The simplest methodology is used by TETRA. In a known sequence dataset, TETRA computes pairwise correlations between tetra-nucleotide usage patterns of all reads. This information is then used for segregation of reads into unique bins (Teeling et al., 2004). The SOMs program
applies a neural network-based method, which involves multidimensional clustering of data points. The results of clustering are then plotted onto a two-dimensional map. Both methods use 4-mer frequencies in their algorithms, as it has been demonstrated in papers that 4-mers provide programs with much better discrimination power compared to any other k-mer patterns (Pride et al., 2003). The CompostBin approach uses frequencies of k-mers of different lengths and then applies a weighted PCA-based approach to lessen the dimensionality of the output plot (Chatterji et al., 2008).

One limitation reported for TETRA was that in a metagenome sample with uneven species distribution there was a tendency to group reads originating from the genome of the most abundant organism into several clusters. However, this limitation has been dealt with using the AbundanceBin approach (Wu and Ye, 2011). The main goal of the AbundanceBin is to apply different clustering parameters for reads with different abundance levels. Though the AbundanceBin works proficiently with samples having extremely diverse abundance levels, this approach created artificial bins when the species distribution in a sample was even. However, in practice environmental samples with even species distributions are very unlikely to occur (Wu and Ye, 2011). The program MetaCluster, developed by Leung and colleagues, attempts to address problems with even species distribution samples by making use of a two-phase approach. Reads are segregated into taxonomically homogenous clusters in the first stage. The second stage, performed by MetaCluster, involves the merging of diverse clusters by generating probabilistic models based on a GC-content analysis of fragments constituting these clusters (Leung et al., 2011).

Most of the taxonomy-independent approaches are more relevant for metagenomes where the numbers of taxonomically classifiable species are very low. Results obtained using taxonomy-independent approaches can also help in downstream processes such as assembly (Mande et al., 2012).

1.2.7 Strategies of validation of binning results

To validate taxonomy-dependent binning approaches, validation should be done using simulated metagenomics datasets and databases. The reads in these datasets should simulate the lengths as well as the sequencing errors characteristic of different sequencing technologies. To ensure confidence of validation, multiple datasets of varying sizes should be tried (Mande et al., 2012). To evaluate the performance of different metagenomics tools, Fidelity of Analysis of Metagenomic Samples (FAMeS) is one of the datasets providing
access to multiple simulated datasets and at present is used as a gold standard (Mavromatis et al., 2007). Datasets of diverse taxonomic complexity usually contain about 100 000 reads, having lengths ranging from 650 to 1000 bp. These reads are sampled from 112 real genome sequencing projects, which are populated with typical sequencing errors associated with the Sanger sequencing technology. It should be noted that this database has no data sets simulating the typical errors of the current popular NGS technologies (Mavromatis et al., 2007; Mande et al., 2012). To simulate NGS reads, software tools such as Metasim and ART have to be used (Richter et al., 2008; Huang et al., 2011).

MetaSim, which is a sequencing simulator of genomic and metagenomic data, can be used to generate a collection of artificial reads from provided genomes that mimic typical errors of Roche 454 or Illumina sequencing technologies. Based on a collection of real genome sequences, the program constructs a metagenome allowing unequal representation of the initial genomes in the resulting data set. By representing various levels of taxonomic nodes of the NCBI taxonomy, binning of different programs can be compared in terms of sensitivity and specificity (Richter et al., 2008).

Huang et al. (2011) developed the program ARTS, which is a set of simulation tools to generate artificial next-generation sequencing reads. ART simulates both single-end and paired-end reads of the three most popular next-generation sequencing technologies: Roche 454, Illumina and SOLiD. This functionality is most important for testing tools developed for processing and analysis of next-generation sequencing data, for example read alignment, de novo assembly and genetic variation detection. ART produces simulated sequencing reads by imitating the sequencing process with built-in technology-specific read error models and base quality value profiles parameterised empirically from large sequencing datasets (Huang et al., 2011).

Simulation of reference databases is another important question to be considered during the assessment of taxonomic-dependent approaches. Simulated databases should be populated by artificial reference sequences showing some level of similarity at different taxonomic levels to the sequences used as input reads. Hence, the simulation is usually done using a leave one scheme, where species are removed from reference databases and validation is performed using reads from this species (Huson et al., 2007; Monzoorul et al., 2009). However, in real metagenomics cases, query reads may also originate from completely unknown taxonomic clades, which cannot be binned at all. So, to incorporate these cases in
the evaluation process, it has been recommended to use the omit one clade strategies, where sequences belonging to an entire clade, such as genus, family, order, class, phylum and above, are removed from the reference database. Sort-ITEMS and DiScRIBinATE make use of this validation strategy (Monzoorul et al., 2009; Gosh et al., 2010).

Four parameters, accuracy, specificity, execution time and required compute power, are used to quantify the binning efficiency of the taxonomy-dependent approaches. The assignment of a read is said to be precise if it is assigned to any taxon that lies in the taxonomic lineage of the source organism read and when assignment specificity is well-defined in terms of the taxonomic levels such as the strain, species, genus, family, order, class, phylum and superkingdom to which the read is assigned. Assignment at the strain level is said to be most precise in cases where the reads originate from an identified strain, sequences of which are present in the reference database, though in most metagenomic case scenarios, the taxon corresponding to the source organism of a read is absent from the reference database (Mande et al., 2012). Different taxonomy-dependent approaches make use of various measures to quantify accuracy and specificity. Methods such as MEGAN and Sort-ITEMS compute the percentage of properly assigned reads at different taxonomic levels and use this information as a measure of sensitivity and specificity (Huson et al., 2007; Monzoorul et al., 2009).

It is of importance to note that a balance exists between the sensitivity and specificity of a method and the requirements for the time of computing. Though composition-based approaches have been shown to outscore alignment-based methods in terms of execution time and computing power, the comparatively lower sensitivity and specificity of these methods compared to alignment-based methods and their limited use with metagenomic datasets containing short reads are still a challenge. However, hybrid binning methods make use of both alignment- and composition-based approaches in order to exploit the relative benefits of both (Teeling et al., 2004; Mohammed et al., 2011).

The efficiency of binning of reads by taxonomy-independent methods is assessed by the following parameters: taxonomic homogeneity of the resultant bins and the number as well as size of bins generated. Hence, in an ideal situation an efficient method should form \( n \) number of taxonomically homogenous bins where \( n \) is the number of species constituting the validation dataset. However, in scenarios where multiple homogenous bins arise from the segregation of reads originating from the same species, such parameters as the normalised mutual information and F-score have to be used (Mande et al., 2012). Sun et al. (2012) gave
a detailed description of the application of these parameters. Different algorithms used by microbiologists were surveyed in this paper and the authors also compared in a large benchmark study seven representative approaches, which address different issues of concern. A novel protocol was introduced, which allowed different algorithms to be compared using the same platform and different criteria to enable a qualitative assessment of the clustering performance of each algorithm. The newly developed program, ESPRIT-TREE, was found to be one of the best algorithms available in terms of computational efficiency and clustering accuracy (Sun et al., 2012).

Well-established tools, such as the primer-E package, allow for various multivariate analyses, which include generation of multivariate and multidimensional scaling plots, similarity analysis (ANOSIM) and identification of species or functions that contribute to differentiation between two samples (SIMPER) (Clarke, 1993). Multivariate statistics are also incorporated in web-based tools called Metastats, which show high-level discriminatory power to distinguish between replicated metagenome datasets originating from the gut microbiota of lean and obese mice (Turnbaugh et al., 2009; White et al. 2009). The ShotgunFunctionalizeR package is also known to provide different statistical procedures for assessing functional differences between samples, both for individual genes and for entire pathways using the R statistical package (Kristiansson et al., 2009).

1.2.8 Annotation of metagenomic reads

Two different initial schemes can be used for the annotation of metagenomes. The first approach is applied when assembly of genomes from metagenomic reads is one of the main objectives of the study and the assembly has produced large contigs. In this case it is preferable to use existing pipelines for genome annotation, such as RAST and IMG, hence the minimum length of contigs required for this method is 30,000 bp or longer. The second approach is applied for annotation of individual reads or short contigs. Tools specifically developed for metagenomic annotation should be used to perform this task (Aziz et al., 2008; Markowitz et al., 2014)

Metagenomic sequence data annotation can be executed in two steps: features of interest such as coding and non-coding genes are identified (feature prediction step) and then putative gene functions can be assigned by homology search in taxonomic neighbours (functional annotation step). The feature prediction step is the process of labelling sequences as genes or genomic elements. Tools such as FragGeneSan, MetaGeneMark, MetaGeneAnnotator and
Orphelia were specifically designed to handle prediction of CDS in metagenomic reads (McHardy et al., 2007; Noguchi et al., 2006; Hoff et al., 2009; Rho, 2010; Yok et al., 2011). At present, estimates show that only 20-50% of metagenomic sequences can be annotated, leaving the question of the importance of the remaining genes unanswered (Gilbert et al., 2010). The annotation is rarely performed de novo. On the contrary, mapping of reads to databases of genes with known functions is preferable. Sequences that cannot be mapped to any known sequence are termed orphan. Orphans constitute the never-ending genetic novelty in microbial metagenomics (Yooseph et al., 2007).

Many databases are available and can be used to annotate metagenomic reads functionally. They commonly come in two varieties: sequence and HMM databases. Searching metagenomic reads through a database of sequences has a tendency to be comparatively quick and may generate more specific hits for the reads that are closely related to sequences in the database, whereas comparing metagenomic reads to an HMM database tends to detect more vaguely related and diverged members of the family, though the precision of identification of very short sequences is not well established. Commonly used sequence databases consist of the SEED annotation system, which is used by MG-RAST and links precise family level functions to higher-order functional subsystems (Overbeek et al., 2014). The KEGG orthology class has also been demonstrated to be particularly valuable, as it maps suitably to KEGG metabolic pathway modules (Kanehisa et al., 2014). The MetaCyc class is alike in that the families are mapped to extensively curated and well-defined metabolic pathways (Caspi et al., 2014). The EggNOG is a database of non-supervised groups of orthologous proteins that incline to be improved frequently, so as to include a huge amount of sequence diversity (Powell et al., 2014). HMM databases suitable for querying metagenomic reads are limited by the Pfam, which uses HMMs to model protein domains (Finn et al., 2013). At present, generation of HMM databases of the full-length and phylogenetically varied protein family is under process. These new databases may be exemplified by Phylofacts (Afrasiabi et al., 2013) and SiftingFamilies, which are also regularly upgraded, like EggNOG (Sharpton et al., 2012; Sharpton, 2014).

1.2.9 Sharing and storage of metagenomic data

Tools such as IMG/MER, CAMERA, MG-RAST and EBI metagenomics, which also incorporate QIIME, offer an integrated environment for: (i) analysis, (ii) management, (iii) storage and (iv) sharing of metagenome projects (Oulas et al., 2015). This requires a
constant, generally accepted annotation scheme to be considered to allow for efficient data exchange, integration, sharing and visualisation between different platforms. This will decrease the necessity for reprocessing of metagenomic datasets further, an assignment that is very costly computationally (Oulas et al., 2015).

The Genomic Standards Consortium is currently investing heavily in a generally acknowledged language that shares ontologies and nomenclatures, thus providing a common standard for exchange of data resulting from the analysis of metagenomic projects. Hence, Minimum Information about Metagenome Sequence (Yilmaz et al., 2011) and Minimum Information about a MARKer Sequence (Field et al., 2011) have been devised, which offer a scheme of standard languages for metadata annotation (Thomas et al., 2012; Oulas et al., 2015).

1.3 Barcoding

A broad range of genetic data about microorganisms of importance has been made accessible with the advancement seen in different NGS platforms. However, it remains a challenge for many researchers to process this enormous quantity of genetic data to resolve practical demands. Genetic barcoding of microorganisms is the first major area where NGS has met the need of applied microbiology (Reva et al., 2014). Kress and Erickson (2008), defined barcodes as 400-800 bp DNA fragments, which serve as explicit specie identifiers. Deoxyribonucleic acid barcoding is an advanced technique for rapid specie identification based on a standard fragment of DNA sequence (Albu et al., 2011). A DNA barcode gene region should fulfil three major conditions: it should (i) have substantial species-level genetic variability and divergence; (ii) have conserved flanking sites for developing universal PCR primers for varied taxonomic use (however, the present NGS technologies have made this requirement obsolete as there is currently no need to care about PCR-based amplification); and (iii) require a short sequence length to enable the present capabilities of DNA extraction and amplification (Kress and Erickson, 2008) (Table 1.2).

In bacteriology, DNA barcoding was initiated using 16S rRNA as taxonomic markers. This was then followed by the use of many other housekeeping gene sequences as possible barcodes (Weisburg et al., 1991; Case et al., 1997). The 16S rRNA is one of the most sequenced DNA fragments used for specie identification, since it is well conserved in eubacteria and Archaea. This permits the creation of universal primers that enclose different informative variable regions (Coenye and Vandamme, 2003). Some of the limitations
associated with barcoding with 16S rRNA include: (i) the gene is too conserved for
discrimination of closely related species; and (ii) the possession of various copies of variable
copies of 16S rRNA by an organism also causes a problem (Kunst and Devine, 1991).
However, a significant factor that made the 16S rRNA gene suitable for phylogenetic
inferences was its resistance to horizontal exchange (Woese and Fox, 1977). The ITS region
of nuclear ribosomal DNA (rDNA) was recommended as genetic makers for eukaryotes
(fungi), while the mitochondrial gene cytochrome c oxidase I (COI) was recognised as a
universal DNA barcode for animals (Hebert et al., 2003; Nilsson et al., 2008).

The main barcoding bodies and resources are Consortium for the Barcode of Life (CBOL),
Quarantine Barcoding of Life (QBOL) and Barcode of Life Datasystem (BOLD)
(Salvolainen et al., 2005; Ali et al., 2014). The CBOL was established in 2004 and has more
than 170 member groups from 50 countries to endorse DNA barcoding as the universal
standard of identification of biological specimens (Miller, 2005). The QBOL aims to obtain
DNA barcode data of significant species of bacteria and other organisms to establish an
analytical tool for quarantine (Bonants et al., 2010). The BOLD workbench supports the
possession, analysis, storage and publication of DNA barcode records. It enables
bioinformatics opening by collecting morphological, molecular and distributional data. The
BOLD could be described as the global beginning of the identification of species, which
allows users to refer to a specialised database such as disease vector species, threatened
species and pathogenic strains (Ball and Armstrong, 2006; Lebonah et al., 2014). It is freely
accessible and enables researchers to carry out neighbour-joining clustering, identification of
taxa using a restructured sequence library and storage of information on the different groups
studied (Amanda and Luciane, 2010).

1.3.1 Advantages of DNA barcoding

Generally, DNA barcoding is designed to benefit population genetics and systemic studies of
habitat-specific bacterial consortia. Barcoding approaches are used for inventory of
biodiversity, routine identification of species of interest in environmental samples and
flagging of atypical specimens for detailed taxonomic research, since there are almost 1.7
million named species and probably another 10 million (excluding bacteria and archaea) that
have not been counted. By contrast to phylogenetics and taxonomy, which aim at inferring
relationships of common ancestry, the objective of molecular barcoding is the identification
of the presence or absence of taxonomic units of interest in selected environmental samples.
or habitats. In population genetics studies, DNA barcoding can provide a first signal of the extent and nature of population divergences; comparative studies of the population diversity in many species and detection and tracing down of microorganisms of interest in the environment (Stoeckle 2003; Hajibabaei et al., 2007).

1.3.2 Challenges of DNA barcoding

Since DNA-based species identification depends on differentiating intraspecific from interspecific genetic variation, the ranges of these types of differences are unidentified and may vary between groups. Attempting to resolve newly diverged species may be difficult, since there is no universal DNA barcode gene, no single gene that is conserved in all domains of life and exhibits enough sequence divergence for species discrimination (Kress and Erickson, 2008). Two dynamics that may also strongly affect how well barcode markers work at species identification and discovery are database design and sequence search strategies. The exact method or algorithm to be used in searching a barcode database has not been thoroughly investigated or debated, particularly as regards a multi-locus DNA barcode (Kress and Erickson, 2008).

Advances in sequencing technologies allowed scaling up the barcoding approaches to study environmental populations of microorganisms by using metagenomic technologies. One of the most popular technologies is metabarcoding, which is built upon massive parallel sequencing of species-specific marker genes (i.e. 16S rRNA) from environmental DNA samples by means of universal primers. Metabarcoding should be clearly distinguished from both metagenomics and phylogenetics, which are common sources of confusion. Metagenomics, in contrast to metabarcoding, relies on WGS approaches, omitting either amplification or cloning of DNA fragments. By contrast to phylogenetics, the aim of metabarcoding is to identify known species in the sample, leaving out questions of phylogenetic relations between them and/or new species proclamation.

In the presented work, an idea of multi-locus barcoding is introduced for data mining of metagenomic datasets. It resembles to some extent multi-locus sequence typing (MLST), but in contrast to MLST and metabarcoding it relies on the WGS approach instead of amplification of marker loci. It allows an increase in the number of marker loci to be considered, as there is no need to develop primers for every locus and amplify them separately. In contrast to metagenomics, multi-locus barcoding is not instrumental either for genomic contig assembly or for a functional analysis of bacterial populations. The aim of
multi-locus barcoding remains the same as for other barcoding approaches: species identification in environmental samples and tracing down the strains of interest.

At-a-glance comparison of multi-locus barcoding to metabarcoding is presented in Table 1.2. In subsequent sections of this chapter, a more detailed analysis of multi-locus barcoding is given.

**Table 1.1: Criteria for barcode evaluation**

<table>
<thead>
<tr>
<th>Criteria for Evaluation</th>
<th>Multi-locus Barcoding</th>
<th>Metabarcoding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barcode sequence length</td>
<td>Usually longer than 10 000 bp, up to 200 000 bp.</td>
<td>Length &lt; 400 bp is usually appropriate; however, some authors recommend sequencing the complete 16S rRNA that is around 1500 bp.</td>
</tr>
<tr>
<td>Sensitivity and taxonomic resolution.</td>
<td>Adjustable to different taxonomic levels</td>
<td>16S rRNA is believed to be species-specific, but bacterial genera are usually identified with appropriate statistical reliability.</td>
</tr>
<tr>
<td>Specificity and false positive rate</td>
<td>Specificity increases with the number of individual marker loci in the barcode sequence.</td>
<td>Generation of chimeric 16S rRNA sequences is a common problem for all sequencing technologies based on the massive parallel sequencing by synthesis technologies (Roche 454, Illumina and Ion Torrent).</td>
</tr>
<tr>
<td>Sufficient coverage requirement and biased predictions</td>
<td>This technology assumes further reduction of the sequencing price, as high coverage above 100 is a necessity for this approach.</td>
<td>This approach allows a significant enrichment of sequences of interest by amplification. Coverage above 15 is sufficient for species identification; however, the amplification process based on universal primers is rather biased.</td>
</tr>
<tr>
<td>Applicability for phylogenetic inferences</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Dependence on availability of comprehensive databases</td>
<td>Barcodes are developed “in fly” for user-provided sets of genomes, but the barcodes are applicable to identification of these organisms only.</td>
<td>Metabarcoding allows identification of any organism deposited in the reference database.</td>
</tr>
</tbody>
</table>
1.3.3 Multi-locus barcoding and metabarcoding

Evolving molecular methods show fast advancement with tools used for specie identification in environmental samples (Pochon et al., 2013; Wood et al., 2013). Of interest is DNA barcoding and metabarcoding, which can possibly offer more precise and standardised, high-resolution taxonomic data (Hajibabei et al., 2007; Ji et al., 2013; Zaiko et al., 2015). Metabarcoding is defined as a quick technique of high-throughput, DNA-based identification of species of interest from a complex and degraded sample of environmental DNA or from a bulk group of specimens. Metabarcoding techniques are faced with several limitations, which can obstruct their ability to yield robust, comparable biodiversity estimates (Cristescu, 2014). Limitations associated with metabarcoding are: (i) generation of amplification biases that contribute to errors that can influence biodiversity estimates; this is usually caused by dependency on the intermediate PCR step, which enriches the DNA templates extracted from a bulk sample; (ii) low taxonomic resolution due to a high level of conservation of these sequences; and (iii) generation of chimeric sequences that artificially increase the species richness of the samples (Bik et al., 2012; Ratnasingham and Hebert, 2013).

Though metabarcoding has been labelled as a promising method rendering conventional DNA barcoding irrelevant (conventional DNA barcoding involves the use of a single gene to identify a given species through the comparison of nucleotide sequences in the DNA to that of same gene in other species) (Taylor and Harris, 2012), DNA multi-locus barcoding and metabarcoding are complementary methods and impending biodiversity research would benefit from harmonising the two methods. The two techniques are similar in that they both use DNA-based identification of species, but they have differing assets that are determined by their unique sequencing technologies and precise aims. The multi-locus barcoding method involves aligning DNA reads generated by WGS metagenomics against multiple taxon-specific loci, while metabarcoding involves enormous targeted parallel sequencing of one or several marker genes. Both methods make use of the massive advantages associated with second-generation technology, which enables the production of loads of sequences at a run. However, the ability to interpret the results is highly dependent on both the skill of the experimenter and accessibility of sophisticated computer tools and well-populated databases providing access to reference sequences of interest (Janzen et al., 2005; Ji et al., 2013).

Several international projects have been launched with the aim to barcode multiple live organisms of the earth or specific environments. The DNA barcoding approaches have been
designed to build a link between molecular ecologists and morphological taxonomists by generating reference databases based on verified and curated specimens (Barrett and Hebert 2005; Hebert and Gregory 2005). This alliance can also be extended to metabarcoding techniques by designing metabarcodes within the standardised barcodes. The taxonomic data associated with barcode sequences enables researchers to place OTUs in important evolutionary, physiological and ecological perspectives. With further progressing of barcoding initiatives and better curated specimens of museum assortments, more reference sequences will be provided to link researchers to useful biological information (Janzen et al., 2005; Ji et al., 2013).

1.3.4 DNA barcoding of bacteria

Barcoding of bacterial communities is of enormous importance to resolve several health care, ecologically and epidemiologically associated problems (Reva et al., 2014). The importance of bacterial community barcoding for health care problems has been demonstrated through fingerprinting of both gut micro-flora and the Human Microbiome Project (Eckburg et al., 2005; www.hmpdacc.org/index.php). It was reported that the micro-flora of every individual are distinct owing to the effect of external factors such as dietary specificity, lifestyle, medication and genetic specificity (Zoetendal et al., 1998; Suau et al., 1999; Hayashi et al., 2002; Lay et al., 2005; Mueller et al., 2006; Dicksved et al., 2007; Jernberg et al., 2007; Dethlefsen et al., 2008). Different studies also reported that the micro-flora of an individual may cause predilection to obesity, as well as several other immune and inflammatory diseases, such as diabetes (Larsen et al., 2010; Hullar and Lampe, 2012; Kelly and Mulder, 2012; Shanahan, 2012). The management of an individual course of disease, treatment with specific drugs and selection of the most appropriate therapeutic regimens can also be supervised by barcoding of microbial communities. Profiling of complex communities of potentially pathogenic microorganisms in airways of cystic fibrosis patients may be linked to the inception of disease (Zemanick et al., 2011).

Studies on bacterial pathogens give a representation of how populations of bacteria act as a group, but with inadequate resolution to know how microorganisms act as individuals. Hence, it is important to produce markers, which will allow differentiation between lineages and genetic variants in mixed populations during an extended infection. Signature marked mutagenesis based on insertion of transposon sequences serving as barcodes was presented to be instrumental in the study of the dynamics of bacterial populations. This approach allowed
tracing the fate of every individual mutant in the population (Hensel \textit{et al.}, 1995; Chang and Mekalanos, 1998; Darwin and Miller, 1999; Edelstein \textit{et al.}, 1999; Mecsas \textit{et al.}, 2001).

Several success stories of the application of barcoding and metabarcoding are considered below.

Makarova \textit{et al.} (2012) established a universal DNA barcode based on the elongation factor Tu (\textit{tuf}) gene for phytoplasma identification. They also designed a set of primers, which amplified a 420-444 bp fragment of \textit{tuf} from all 91 strains of phytoplasma (16S rRNA, groups –I through –VII, -IX through –XII, -XV and –XX). Assessment of the neighbour-joining trees constructed from the \textit{tuf} barcode showed that the \textit{tuf} tree was congruent to those based on 16S rRNA but provided greater inter- and intra-group divergence. Hence, they demonstrated that \textit{tuf} sequences can be applicable to the barcoding of phytoplasmas. The \textit{tuf} barcodes performed much better than the 1.2 kbp fragment of the 16S rRNA genes and offered an easy-to-use technique for phytoplasma identification (Makarova \textit{et al.}, 2012).

In another paper, the effect of fire on microbial communities in chaparral soils was tested by means of DNA barcoding. This technique allowed a comparison of microorganisms found in burnt and unburnt soil samples. DNA barcoding was based on analysis of 16S rRNA genes. Two sets of primers were used for PCR, one for bacteria and another for archaea. Purified DNA was then sub-cloned into TA plasmids. Sequencing of 62 plasmids generated an array of DNA data, which was then used to search the GenBank database with the BLASTN program. The generated data revealed that the most abundant microbes found in the unburnt samples were less visible in the burnt samples. Larger diversity of microbes was also observed in the burnt soil samples, meaning that most of the soil archaea microbes might have moved in quickly after the fire; after the community stabilised, these microbes could be displaced (Natalie, 2013).

In oceans, microbial life is responsible for almost all production and mediation of all biogeochemical processes (Sogin \textit{et al.}, 2006). However, the dimension of taxonomic and genetic versatility of these microbiota is poorly understood. Advances in community genomics and metagenomic techniques are leading to valuable insight into the prokaryotic diversity and processes of molecular evolution of ocean-inhabiting bacterial communities (DeLong, 2004; Tyson \textit{et al.}, 2004; Venter \textit{et al.}, 2004; Tringe \textit{et al.}, 2005; DeLong \textit{et al.}, 2006; Leclerc \textit{et al.}, 2007).
Studies exploring the community dynamics of microbes depend heavily on genecentric metagenomic profiling using 16S rRNA and 60 kDa chaperonin protein (cpn60) as marker genes. Links et al. (2012) assessed DNA barcoding techniques based on amplification of 16S rRNA genes and the protein coding cpn60 genes. The cpn60 gene reported as a universal target that outperformed the traditionally used 16S rRNA as a barcode sequence. These authors suggested cpn60 as an ideal barcode for species-level characterisation of bacterial communities. Assembling consensus sequences for barcodes was also reported to be a good method of tracking and identification of new microbes by metagenomics (Links et al., 2012).

Liu et al. (2013) verified whether Mollitrichosiphum, an aphid genus with life cycles on subtropical woody host plants, and Buchnera, the main endosymbiont of aphids, evolved in parallel. Buchnera belongs to the γ subdivision of proteobacteria and is commonly assumed to be present in all aphid species, where it exists in specific cells termed bacteriocytes (Buchner, 1965; Lebonah et al., 2014). The following aphid genes, mitochondrial COI, cytochrome oxidase subunit I and Cytb, cytochrome b: nuclear EF1α, translation elongation factor 1 alpha and two Buchnera genes, namely 16S rDNA and gnd for gluconate-6-phosphate dehydrogenase, were used to reconstitute the phylogenies of these species. The phylogenetic trees of aphids and Buchnera were then compared. It was reported that phylogenetic evidence for the parallel evolution of Mollitrichosiphum and Buchnera at the intraspecific as well as interspecific levels supported the prospect of using endosymbiont genes to analyse the evolutionary history and biogeographical distribution of host organisms. These authors also explored the possibility of the Buchnera gene gnd being used as a barcode marker for aphid identification. This study showed that Buchnera gene gnd was also suitable for barcoding as a marker for aphids, just like the traditional COI barcode (Liu et al., 2013).

1.3.5 Barcoding and multi-locus sequence typing

Since the single-gene technique of DNA barcoding fails to differentiate between closely related organisms on the level of species and sub-species, there is a dire need for the development of more sensitive DNA markers in both medical and biotechnological microbiology (van Belkum et al., 2001; Urwin and Maiden, 2003). Hence, it was hypothesised that the comparison of strains by various gene sequences would provide better resolution to distinguish between closely related organisms. Multi-locus sequence typing was then introduced, which uses PCR amplification and sequencing of small fragments of multiple genes comprising diagnostic signals instead of one barcode gene or whole-gene
sequence data (Maiden et al., 1998). However, the challenge with MLST is that different housekeeping genes and a varying number of polymorphic sites are used in diagnostic protocols of different groups of organisms, which impedes cross-platform evaluation (Urwin and Maiden, 2003). To aid DNA barcoding, numerous potent laboratory information management systems have been presented, including the BIGSdb database, which is now integrated into the PubMLST website (Jolley and Maiden, 2010). The BIGSdb database permits cross-referencing among diverse MLST datasets and makes use of data for genome functionality, epidemiology and evolutionary predictions (Joelly et al., 2012a).

Joelly et al. (2012b) proposed an MLST typing technique called ribosomal MLST (rMLST), which indexes variations in 53 genes encoding bacterial ribosome protein subunits (rps genes) as a way of incorporating microbial genealogy and typing. Grouping provided by rMLST was consistent with the present nomenclature systems independently of the clustering algorithm being used (Joelly et al., 2012b). Moreover, by increasing the analytic sets of polymorphic sites to a larger number of housekeeping genes using high-throughput sequencing techniques, MLST datasets might become universal and useable in several microbial research projects (Reva et al., 2014). A variant MLST technique called short read sequence typing (SRST) was introduced by Inouye et al. (2012). The SRST technique maps Illumina DNA reads against target sequences, which are then spontaneously recovered from the MLST database (http://pubmlst.org). These short reads are initially mapped by the BWA tool and then processed by Samtools (Li et al., 2009; Li and Durbin, 2010). Genometa is another program for effective barcoding of bacterial communities and populations using short Illumina DNA read (Davenport et al., 2012). BOWTIE is used to map reads instead of BLASTN in this program. An extended version of the open source Integrated Genome Browser browser is used to view the alignment (Davenport et al., 2012).

1.4 Research aim and objectives

Aim

The aim of this study was to create a computer system for multi-locus genetic barcoding suitable for identification and tracking down of biotechnological strains in the environment. These software tools provide access to databases of genetic barcodes for program testing and application in biotechnology.
Objectives

- To create and test a novel software tool allowing automatic creation of diagnostic multi-locus metabarcodes (concatenated sequences of marker genes) for user-provided groups of microorganisms. For case studies, the following groups of organisms representing different levels of taxonomic relatedness were used: *Bacillus cereus*, *Escherichia/Shigella*, *Lactobacillus*, *Mycobacteria*, *Streptococcus*, *Salmonella* and *Prochlorococcus*. The composition of each group will be explained in detail in the next chapter.

- To evaluate the performance of the designed metabarcodes in terms of sensitivity and specificity by using publicly available metagenomic datasets and artificially created metagenomic datasets.

- To analyse functional categories of marker genes selected by the program for diagnostic metabarcodes designed for different groups of microorganisms.

- To develop a standardised web-based pipeline (BarcodeGenerator) for metabarcode development for any given group of microorganism.

- To develop a standardised barcoding pipeline implemented in the form of a stand-alone Python program available for download from the BarcodeGenerator Web-site to perform taxonomic binning of metagenomics reads against designed multi-locus barcodes.
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CHAPTER 2 Selection of reference genomes of microorganisms for case studies and design of the BarcodeGenerator: A novel software tool for generation of diagnostic barcode sequences

Abstract

Different microorganisms are of important use in medicine and biotechnology. Despite the diverse bioactivity of these microorganisms, they are almost indistinguishable by phenotype and by the 16S rRNA, which makes it challenging to trace and identify them in nature. In this chapter, a novel software online tool, BarcodeGenerator, was used for the creation of barcode sequences. This allows identification of most suitable marker sequences for DNA-based multi-local barcoding for identification and monitoring of medical and biotechnological strains.

2.1 Introduction

Microorganisms are found everywhere in nature. Different communities of microbes flourish in various environments, ranging from the human gut, the rhizosphere and unreceptive habitat such as acid runoff to geothermal hot springs (Simmons et al., 2008; Walter and Ley, 2011; Philippot et al., 2013; Sharp et al., 2014). Different studies of cultured microbes have shown that they are important constituents of these environments and offer vital ecosystem services (Arrigo, 2005; van der Heijden et al., 2008). Microbiota are referred to as microbes that associate with a macroscopic host organism (Hooper et al., 2012). Full understanding of a macroscopic organism’s composition needs investigation of its microbiota. Regrettably, some microbes are extremely difficult to culture in the laboratory (Sharpton et al., 2014).

Metagenomics is the study of the entire genomic content of a microbial community that bonds the three domains of life: archea, bacteria and eukaryotes (Galagan et al., 2005; Wylie et al., 2012; Chun and Rainey, 2014; Kwong et al., 2015; Land et al., 2015). All DNA and RNA samples may be isolated from a microbial populace, skipping the step of cultivation. Then DNA reads can be obtained by massive parallel sequencing and identified by searching through databases of known reference for binning to known species or assigning them to a cluster of reads of unknown origin (Kulski, 2016). Microbial communities are isolated and studied from different environments, such as the aquatic and terrestrial environments, host-associated ecosystems and various human-engineered systems, such as those involved with food, water, waste production and agriculture (Bashir et al., 2014; Kwong et al., 2015).
Hospitals are no exception; they are a source of pathogenic microorganisms, of which those of most interest develop resistance to commonly used medical antibiotics, such as methicillin-resistant Staphylococcus aureus, multidrug-resistant Mycobacterium tuberculosis and others. Hence, NGS is seen as a vital growing application for epidemiological studies of various pathogens (Grad and Lipsitch, 2014).

Next-generation sequencing and its application in metabarcoding and metagenomics enable researchers to study a complex microbial population without isolation and cultivation of strains. The most common method is the amplification of fragments of 16S rRNA from whole DNA samples using universal primers, followed by massive parallel sequencing of the amplified fragments (Wang and Qian, 2009). Genes encoding 16S rRNA were the earliest metagenomic targets used for identification of different species in environments before the first NGS microbial studies were performed using Roche 454 pyrosequencing and Illumina platforms. New opportunities and the benefits of application of NGS in metabarcoding were demonstrated in numerous publications following the introduction of these technologies: metabarcoding of mining sites and the surface waters of the gulfs, seas, and oceans are but a few projects benefitting from NGS (Gilbert and Dunpont, 2011; Wylie et al., 2012).

However, a limitation of classical 16S rRNA metabarcoding consists in the fact that the many microorganisms are almost indistinguishable by 16S rRNA (Safronova et al., 2012). In general, the level of sensitivity of 16S rRNA metabarcoding is the taxonomic level of genera; above that sensitivity is insufficient to distinguish between microorganisms showing different activities in terms of biotechnology or medical practice.

Kress and Erickson (2008) defined DNA barcoding as a fast and robust technique for species identification based on marker nucleotide sequences (Kress and Erickson, 2008). However, since the single-gene technique of DNA barcoding does not differentiate between closely related species and sub-species, it is of limited importance to develop diagnostic sets of marker sequences for biotechnological and medical microbiology (van Belkum et al., 2001; Urwin and Maiden, 2003; Reva et al., 2014). Hence, it was hypothesised that the comparison of bacterial strains by using multiple gene sequences would give better resolution of their core relationships than a single gene (Maiden et al., 1998). The MLST technique was introduced, which made use of DNA sequences of internal fragments of multiple housekeeping genes for definitive identification of microorganisms (Maiden et al., 1998; Urwin and Maiden, 2003). Various researchers have developed different techniques for MLST, some of which include rMLST, multi-locus sequence analysis (MLSA) and whole
The rMLST technique indexes variations in 53 genes encoding bacterial ribosome protein subunits (rps genes) as a way of incorporating microbial genealogy and typing. Grouping provided by rMLST was consistent with the present nomenclature systems independently of the clustering algorithm being used (Jolley et al., 2012). The MLSA technique is used to obtain better differentiation of species within a genus. Partial sequences of genes coding for housekeeping genes are used to create phylogenetic trees, but can also be used as taxonomic markers in MLSA research. The MLSA technique has also been suggested as a replacement for DNA-DNA hybridisation in species delineation (Glaser and Kämpfer, 2015). The two basic techniques used for species delineation by WGS are wgMLST and single nucleotide polymorphisms (SNPs). In particular, these approaches were used to survey outbreaks of pathogens (Glaser and Kämpfer, 2015). As with the traditional MLST, alleles in wgMLST are either the same as in reference or different, which implies that any nucleotide substitution, insertion or deletion is equivalent to one allele change. In wgMLST, several thousand loci can be matched and estimated distances between them then are used either for species or strain delineation, or to infer phylogenetic relationships by clustering algorithms. For the SNP technique, counts of single nucleotide substitutions are used to deduce phylogenetic relatedness or genetic typing. SNP protocols have been implemented in various software packages (Katz et al., 2017).

Multi-locus sequence typing approaches were also promoted by the advance in NGS. Different software applications have been developed, using various techniques to calculate the sequence types (STs) from the NGS data. However, not all MLST calling applications function as required. Challenges encountered with these programs include (i) computationally inefficient methods; (ii) false positive results; (iii) out-of-date databases; (iv) inability to call alleles from low coverage reads; and (v) variable performance of mixed samples. Hence, there is room for improvement (Page et al., 2017).

The work hypothesis of this study was that several limitations of the traditional metabarcoding and MLST/wgMLST approaches can be overcome by creating a standardised computational approach (BarcodeGenerator) for a dynamic selection of marker genes for multi-locus barcoding depending on research tasks and the taxonomic level of separation specified by users. It is furthermore of interest to investigate functional categories of genes showing the best performance in metabarcoding of different groups of microorganisms. To
evaluate the performance of selected metabarcode sequences, publicly available metagenomic datasets from NCBI and MG-RAST were used.

2.2 Selection of microorganisms for case studies

This project aimed at developing a new software tool for an automated selection of marker genes to distinguish between various microorganisms at different levels of taxonomic relatedness. Different microorganisms were used for case studies in this work: *Bacillus cereus*, *Escherichia* and *Shigella*, *Lactobacillus*, *Mycobacteria*, *Prochlorococcus*, *Salmonella Shewanella* and *Streptococcus*. The strains used represent different species and subspecies, including pathogenic and biotechnological strains.

2.2.1 Bacillus

The genus *Bacillus* comprises rod-shaped, endospore-forming bacteria that belong to the phylum *Firmicute* (Rooney et al., 2009). The species of this genus have an abundant spread in nature and are found in practically every environment (Rooney et al., 2009; Alina et al., 2015). They are actively involved in carbon and nitrogen cycling, while species such as *Bacillus anthracis* and *Bacillus cereus* are known human and livestock pathogens. Since most of these *Bacillus* species are non-pathogenic, they have frequently been used in biotechnological and industrial applications (Bischoff et al., 2006; Price et al., 2007; Rooney et al., 2009). *Bacillus cereus* is an opportunistic pathogen that causes food poisoning, expressed by diarrhoeal or emetic syndromes. *Bacillus cereus* is closely related to *Bacillus anthracis* and the insect pathogen *Bacillus thuringiensis* (Ivanova et al; 2003). *Bacillus anthracis* is used as a biological weapon, while *Bacillus thuringiensis* is used as pesticide. *Bacillus anthracis* and *Bacillus thuringiensis* do have plasmid-borne specific toxins and this fact is usually used to differentiate them from *Bacillus cereus* (Ivanova et al., 2003). Table 2.1 shows the different strains and species of *Bacillus* used in this study.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>NCBI ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> ATCC 14579</td>
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<td><em>Bacillus anthracis</em> str. A0248</td>
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<tr>
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<tr>
<td><em>Bacillus thuringiensis</em> serovar chinensis</td>
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<tr>
<td><em>Bacillus thuringiensis</em> serovar konkukian strain 97-27</td>
<td>NC_005957</td>
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<tr>
<td><em>Bacillus cereus</em> B4264</td>
<td>NC_011725</td>
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<tr>
<td><em>Bacillus thuringiensis</em> str. Al Hakam</td>
<td>NC_008600</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> str. Ames Ancestor</td>
<td>NC_007530</td>
</tr>
</tbody>
</table>
2.2.2 Escherichia coli and Shigella

*Escherichia coli* belongs to the *Enterobacteriaceae* family; it is gram-negative, rod-shaped and oxidase-negative. *E. coli* can be either non-motile or motile, with a petritirichious flagella. It can grow anaerobically or aerobically, preferably at 37°C. It is readily isolated from faecal samples (Croxen et al., 2013), has been described as a known commensal of the gastrointestinal tract in warm-blooded animals and is used as the everyday laboratory mainstay. However, pathogenic *E. coli* has also been reported, which causes human diseases ranging from those affecting the gastrointestinal tract to extra-intestinal sites such as the urinary tract, bloodstream and the central nervous system (Kaper et al., 2004; Croxen and Finlay 2010; Croxen et al., 2013). Though various aetiological agents have been reported as the cause of diarrhoea, pathogenic *E. coli* stands out among others as a major cause. A case control study aimed at understanding the burden of paediatric diarrhoeal disease in Sub-Saharan Africa and South Asia (Levine et al., 2012) reported by the Global Enteric Multi-Centre study showed that entrotoxigenic *E. coli* and *Shigella* are two of the four causative agents for moderate to severe diarrhoea among children in these areas (Kotloff et al., 2013). The main diarrhoeagenic *E. coli* phenotypes are: (i) enteropathogen *E. coli*; (ii) Shiga toxin producing *E. coli*; (iii) *Shigella*/entero-invasive *E. coli*; (iv) enteroaggregative *E. coli*; (v) diffusely adherent *E. coli*; (vi) enterotoxigenic *E. coli*; and (vii) adherent invasive *E. coli* (Croxen et al., 2013).

Entero-invasive *E. coli/Shigella* spp are described as facultative intracellular pathogens and the aetiological agents of bacillary dysentery, also known as shigellosis. *Bacillus dysenteriae*, also called *Shigella*, was first identified in 1897 by Kiyoshi Shiga during an epidemic in
Japan, where it infected more than 91,000 people, causing a mortality rate of more than 20% (Trofa et al., 1999). Fifty years later, EIEC was identified as having similar biochemical, genetic and pathogenic functions as *Shigella* (Lan et al., 2004). *Shigella* is a non-motile, lysine decarboxylase negative microorganism that does not ferment lactose, with the exception of *S. sonnei*, which is a slow lactose fermenter (Schutz and Strockbine, 2005; Strockbine and Maurelli, 2005). Conventionally, *Shigella* is classified based on biochemical, serological and clinical phenotypes and not on the phylogenetic relationship (Ewing 1949; Strockbine and Maurelli, 2005, Kalluri et al., 2004). This comprises 49 sero- and subserotypes that are further clustered into four species: (i) *S. dysenteriae* (sero A, 15 serotypes); (ii) *S. flexneri* (serogroup B, 14 sero- and subserotypes); (iii) *S. boydii* (serogroup C, 19 serotypes) and (iv) *S. sonnei* (serogroup D, one serotype). *S. boydii* was formerly subdivided into 20 serotypes, but the phylogenetic analysis showed that *S. boydii* 13 fits into the *E. albertii* lineage, which is quite distinct from the typical *Shigella* (Croxen et al., 2013).

Table 2.2 shows the strains and species of *Escherichia coli* and *Shigella* used in this study.

**Table 2.2: Strains of *Escherichia coli* and *Shigella* used in this study**

<table>
<thead>
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<td><em>Escherichia coli</em> O157:H7 Str. Sakai</td>
<td>NC_002695</td>
</tr>
</tbody>
</table>
2.2.3 Lactobacillus

*Lactobacillus* is described as the largest genus of the lactic acid bacteria group, which comprises 50 species in total. These are found in the oral, vaginal and intestinal regions of most animals. *Lactobacillus* is used in the production of cheese (Blaiotta et al., 2001), yogurt (Omogbai et al., 2005), bacteriocin (Vuyst and Leroy, 2007) and other products because it produces lactic acid, which prevents the growth of other organisms as well as dropping the pH of food products (Thavasi et al., 2011). *Lactobacillus* has also been reported to be used as probiotics, prebiotics (Teitelbaum and Walker, 2002) and biotherapeutics (Buddington, 2009). Lactic acid bacteria are mostly seen in various natural environments and are characterised by precise *lactobacilli* compositions such as *L. acidophilus* and *L. delbrueckii* spp. *bulgaricus*. *Lactobacillus helveticus* are the classic representatives of the micro-flora of fermented milk products such as yoghurt and kefir, while the *L. casei* group comprising *L. casei*, *L. paracasei* and *L. rhamnosus* can be found in various types of cheese (Bouton et al., 2002; Markiewicz et al., 2010). *Latobacillus delbrueckii* has also been illustrated as a strain producing biosurfactants and crude oil biodegrading compounds (Thavasi et al., 2006). *Lactobacillus* is furthermore known to help prevent infections of the urogenital and intestinal tract. The dominance of *Lactobacillus* in the vagina is linked with a reduced risk of bacterial vaginosis and urinary tract infections. Hence, the instillation of *Lactobacillus* GR-1 and B-54 or RC-14 strains into the vagina has been reported to reduce the risk of urinary tract infections and improve the maintenance of the normal flora (Reid and Burton, 2002). Table 2.3 shows the *Lactobacillus* strains and species used in this study.

**Table 2.3: Strains of Lactobacillus used in this study**

<table>
<thead>
<tr>
<th>STRAINS</th>
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<tbody>
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<td><em>Lactobacillus casei</em> str. Zhang</td>
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<tr>
<td><em>Lactobacillus delbrueckii</em> subsp. bulgaricus ATCC 11842</td>
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Lactobacillus fermentum IFO 3956
Lactobacillus acidophilus 30SC
Lactobacillus salivarius UCC118
Lactobacillus casei BL23
Lactobacillus sanfranciscensis TMW
Lactobacillus reuteri SD2112
Lactobacillus kefiranofaciens ST1
Lactobacillus ruminis NC_004567
Lactobacillus reuteri JCM 1112
Lactobacillus gasseri ATCC 3323
Lactobacillus brevis ATCC 367
Lactobacillus delbruecki subsp. bulgaricus ND02
Lactobacillus reuteri ICM 1112
Lactobacillus buchneri NRRL B-30929
Lactobacillus helveticus DPC 4571
Lactobacillus delbruecki subsp. bulgaricus ATCC BAA-365
Lactobacillus amylovorus GRL 1112
Lactobacillus sakei subsp. sakei 23k chromosome
Lactobacillus plantarum WCFS1
Lactobacillus ruminis ATCC 27782
Lactobacillus plantarum subsp. plantarum ST-III
Lactobacillus johnsonii F19785

2.2.4 Mycobacteria

The family Mycobacteriaceae is made up of only one genus, Mycobacterium. Mycobacterium species are thin, slightly curved to straight bacilli, forming no spores and non-motile. Cells of mycobacteria are 0.2 to 6 µm x 1.0 to 10 µm (Eisenstadt, 1995). The genus is made up of more than 50 species (Wayne et al; 1992). Most mycobacterial species are ubiquitous and can be found in water, soil, food and vegetation. M. bovis infection has been developed by consuming unpasteurised milk. Bacilli Calmette-Guérin, which is a strain of M. bovis, is widely used for immunisation against tuberculosis. It is also administered as an immunotherapeutic agent for the treatment of superficial bladder carcinoma or melanoma. Mycobacterium fortuitum has been described as a normal flora of the skin (Eisenstadt, 1995). Pathogenic isolates of Mycobacterium include (i) M. tuberculosis — the causative agent of human tuberculosis; (ii) M. bovine — the causative agent of bovine tuberculosis; (iii) M. leprae — the causative agent of leprosy; (iv) M. ulcerans, which causes Buruli ulcers and is the third most common form of mycobacterial disease in humans; and (v) M. marinum — the causative agent of fish tank granuloma in humans and granulomatous lesions similar to those of M. tuberculosis in zebra fish (Demangel et al., 2009; Rahman et al., 2014). The non-pathogenic groups are M. gilvum, M. vanbaalenii and M. smegmatis (Raham et al., 2014). Opportunistic pulmonary infections are mostly caused by members of the Mycobacterium
avium complex (MAC) that includes *M. avium* and *M. avium-M. intracellulare*, while Crohn’s disease in humans is suspected to be caused by the third member of the MAC group, *Mycobacterium avium* subsp. *paratuberculosis* (Cook, 2010; Chiodini et al., 2012).

Seven strains of *M. leprae* have been well characterised, namely India2, Thai53, TN, Africa, NHDP63, NHDP98 and Br4923. India2, Thai53 and TN are of SNP type 1, Africa clade is of SNP type 2, NHDP63 and NHDP98 are of SNP type 3 while Br4923 is of SNP type 4 (Monot et al., 2009; Akinola et al., 2013). *Mycobacterium smegmatis* is an aerobic fast-growing non-pathogenic *Mycobacterium*, which has similar features with pathogenic mycobacteria (Cordone et al., 2011). It can adjust to micro-aerobiosis by changing from the active growth to dormant or latent stages. *Mycobacterium smegmatis* is mainly valuable in understanding the cellular processes that are significant to pathogenic mycobacteria such as *M. leprae*, *M. tuberculosis* and *M. avium* subsp. *paratuberculosis* (He and De Buck, 2010; Akinola et al., 2013). Table 2.4 shows the *Mycobacteria* strains and species used in this study.

**Table 2.0.4: Strains of Mycobacteria used in this study**

<table>
<thead>
<tr>
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### 2.2.5 Prochlorococcus

*Prochlorococcus* is described as the minutest (< 1 µm) in diameter and most abundant (3 x 10^27 cells) photosynthetic microorganism on planet earth. *Prochlorococcus* is a unicellular marine cyanobacterium, which is found throughout the euphotic zone of open ocean between 45° N and 40° S, where it carries out a significant portion of global photosynthesis (Partensky et al., 1999; Flombaum et al., 2013; Biller et al., 2014). The genome of *Prochlorococcus* is
the smallest of any known free-living photosynthetic cells, ranging from 1.6 to 2.7 Mbp (Kettler et al., 2007). Though the core set of genes present is shared by all strains, a notable diversity in gene content was reported among isolates. The Prochlorococcus group has an open pan-genome such that each newly sequenced genome typically contains various novel genes never identified before (Kettler et al., 2007). Research on the genomic and metagenomic features has provided wide understanding of the features of ocean ecosystems (Rocap et al., 2003; Martiny et al., 2009; Coleman and Chisholm, 2010), microbial evolution (Zhaxybayeva et al., 2009; Baumdicker et al., 2012) and the intrinsic relationships between genotype, phenotypic and ecological variations in marine populations (Moore et al., 1998; Zinser et al., 2007; Kashtan et al., 2014). Using Prochlorococcus genomes as reference sequences has also been tremendously valuable for interpreting marine metagenomic and metatranscriptomic datasets (Venter et al., 2004; Friaz-Lopex et al., 2008; Poretsky et al., 2009; Rusch et al., 2010; Biller et al., 2014).

Prochlorococcus isolates can be subdivided into taxonomically and ecologically distinguishable lineages based on their adaptation to general environmental settings such as high-light (HL) versus low-light (LL) (Moore et al., 1998) or temperate regimes (Johnson et al., 2006). Across the globe, warm surface water is mostly dominated by a particular clade of Prochlorococcus, namely eMIT9312. The eMIT9312 clade has a high rate of sequence divergence while upholding notable conservation both in gene content and synten, notwithstanding worldwide distribution that spans huge gradients in the bioavailable concentrations of the macronutrients nitrogen (N) and phosphorous (P). Table 2.5 shows the strains of Prochlorococcus marinus used in this study.

Table 2.5: Strains of Prochlorococcus marinus used in this study

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<td>Prochlorococcus marinus str. MIT 9313</td>
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2.2.6 Salmonella

The genus *Salmonella* belongs to the *Enterobacteriaceae* family. *Salmonella* are rod-shaped facultative anaerobes. The genus *Salmonella* is divided into typhoidal serotypes *Salmonella enterica* var. Typhi (*S. typhi*); and *Salmonella enterica* var. Paratyphi (*S. paratyphi A*), as well as multiple non-typhoidal *Salmonella* serotypes usually called the NTS serotypes (Feasey et al., 2012). In developed countries, non-typhoidal *Salmonella* are mostly the causative agents of self-limiting diarrhoea in people, while bloodstream or focal infections are usually uncommon (Laupland et al., 2010) and mostly occur in individuals with particular risk factors (Gordon, 2008). However, in sub-Saharan Africa, non-typhoidal *Salmonella* are the most common bloodstream isolates in children and adults presenting with fever (Gilks et al., 1990; Gordon et al., 2008; Reddy et al., 2010) and are usually linked to a mortality rate of 20-25% (Feasey et al., 2012).

Whole-genome sequencing of pathogens, immunological trials and characterisation of bacteria-host interactions at the cellular, humoral and mucosal level helped to generate a comprehensive view on the evolution and emergence of this pathogen (Feasey et al., 2012). *Salmonella typhimurium* or *Salmonella enterica* var Enteritidis (*S. enteritidis*), which are non-typhoidal *Salmonella*, have been reported to be the major cause of disease across Africa (Feasey et al., 2010). Researchers have also reported disease outbreaks associated with the following serotypes: (i) *Salmonella enterica* var Isangi (*S. isangi*) in South Africa (Wadula et al., 2006); (ii) *Salmonella enterica* var concord (*S. concord*) in Ethiopia (Beyene et al., 2011); and (iii) *Salmonella enterica* var Stanleyville (*S. stanleyville*) and *Salmonella enterica* var Dublin (*S. dublin*) in Mali (Tennant et al., 2010). Non-typhoidal *Salmonella* has been established as a major HIV-related pathogen in sub-Saharan African adults (Gilks et al., 1990). While the non-typhoidal *Salmonella* have a broad range of hosts among humans and animals, the typhoidal serotypes *S. typhi* and *S. paratyphi A* are totally host-constrained to people, causing invasive disease in immune-competent individuals (Feasey et al., 2012).

Table 2.6 shows strains and species of *Salmonella* used in this study.

**Table 2.6: Strains of Salmonella used in this study**

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<td><em>Salmonella enterica</em> subsp.enterica serovar Schwarzengrund str.CVM19633</td>
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<td><em>Salmonella enterica</em> subsp.enterica serovar Typhimurium str.14028S</td>
<td>NC_016856</td>
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### 2.2.7 Shewanella

*Shewanella* are facultative anaerobes, Gram-negative gamma-proteobacteria found in varied kinds of environments, but mostly in marine sediments and frequently in association with fish (Ivanova *et al.*, 2004; Dikow 2011; Wright *et al.*, 2016). *Shewanella* species signify a vital family of dissimilatory metal-reducing bacteria, which enables the transmission of metabolically produced electrons from a cell interior to external electron acceptors such as solid metal oxides during anaerobic respiration (Fredrickson *et al.*, 2008; Wang *et al.*, 2011). The metal-reducing capability of *Shewanella* has been credited mostly to a set of surface-linked Mtr/Omc proteins, such as three outer membrane decaheme c-type cytochromes, MtrC, MtrF and OmcA; two periplasmic decaheme cytochromes, Mtr and MtrD; and two outer membrane non-cytochrome proteins, MtrB and MtrE (Meyers *et al.*, 1997; Beliaev *et al.*, 1998; Pitts *et al.*, 2003; Wang *et al.*, 2011). These proteins are known to work jointly to transmit electrons towards the outside of the cell outer membrane and are highly conserved across the *Shewanella* genus. At the cell interface, at least two unique extracellular electron transfer (EET) pathways have been identified, namely the direct charge transfer from the cell surface and the use of self-secreted soluble redox mediators (Beliaev *et al.*, 2001; Pitts *et al.*, 2003). The unique EET systems of *Shewanella* have been comprehensively studied in various biotechnological applications such as bioremediation (Hau and Gralnick, 2007), heavy metal detoxification (Liu *et al.*, 2002; Hau and Gralnick, 2007) and electricity production in microbial fuel cells (Bretscher *et al.*, 2007; Wang *et al.*, 2011).
*Shewanella oneidensis* MR-1 is a recognised dissimilatory metal-reducing bacterium with a distinct respiration design. It has modular electron transport pathways and a huge number of terminal reductases to reduce ferric oxides, manganese oxides, nitrate fumarate, sulphur oxyanions, dimethyl sulphoxide and trimetlyamine oxide (Heidelberg *et al.*, 2002; Fredrickson *et al.*, 2008; Li *et al.*, 2013). The study of *S. oneidensis* genome suggests that there is a vastly varied electron transport system comprising 42 putative *c*-type cytochromes essential in the reduction of chromate, cobalt (iii), vanadium (v) and uranium (vi) salts and oxides (Meyers *et al.*, 2004; Hau *et al.*, 2008; Belchik *et al.*, 2011; Li *et al.*, 2013). Flavin mononucleotide and riboflavin secreted by *S. oneidensis* MR-1 have also been reported to increase the bioreduction of extracellular electron acceptors (Canstein *et al.*, 2008; Marsili *et al.*, 2008; Liu *et al.*, 2013).

Wright and colleagues (2016) tested a number of *Shewanella* strains for their manganese oxidising capacity in aerobic conditions (Wright *et al.*, 2016). *Shewanella loihica* strain PV-4 was reported as the strongest oxidiser, producing oxides at a rate of 20.3 mg/litre/day and oxidising Mn(II) in concentrations of up to 9 mM. Analysis of compounds produced by *S. loihica* PV-4 and another strong oxidiser, *S. putrefaciens* CN-32, identified finely grained nanosize oxide particles with an identical Mn oxidation state of 3.86. By contrast, the strain *S. oneidensis* MR-1 was the weakest oxidiser of all tested *Shewanella* (Wright *et al.*, 2016). Table 2.7 shows the strains and species of *Shewanella* used in this study.

### Table 2.7: Shows strains of *Shewanella* used in this study

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<thead>
<tr>
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</tbody>
</table>
2.2.8 Streptococcus

The name *Streptococcus* originated from the Greek word “strepto”, which means twisted, and “coccus”, which means spherical. Over 100 species of *Streptococcus* have now been identified. According to the Lancefield system, streptococci were grouped by the carbohydrate composition of cell walls (Nobbs *et al.*, 2009). These substances are group-specific antigens, which belong to three different classes of chemical compounds: polysaccharides determining the groups A, B, C, E, F and G of Staphylococci, teichoic acids determining the groups D and N and lipoteichoic acid characteristic for the group H (Nobbs *et al.*, 2009). Streptococci can also be classified based on the 16S rRNA gene sequences (Kilian, 2005). The pyogenic group is made up of *S. pyogenes*, which is Lancefield group A; *S. agalactiae* and *S. uberis*, which are group B; *S. dysgalactiae* (group C, G or L) and *S. equi* (group C) (Nobbs *et al.*, 2009). Several groups are common among isolates from human oral and nasopharynx cavities, which include *S. oralis*, *S. mitis*, *S. gordon* and *S. pneumonia*. Because of intensive horizontal gene exchange between these organisms, phylogenetic relations between these species remain unclear (Humtsoe *et al.*, 2005; Bergmann and Hammerschmidt, 2006). This has led to the introduction of the anginosus and salivarius groups, which comprise mostly human and animal oral cavity isolates, and also the bovis group. Many species isolated from oral cavities of humans and animals remain unclassified: *S. mutans* and *S. soborinus* (human isolates); *S. downei* from macaques; *S. ratei* from rats and *S. criceti* from hamsters (Nobbs *et al.*, 2009). It is known that these bacteria are linked to the development of dental caries. Oral cavity microbes are usually referred to as viridans streptococci because of the greenish pigmentation produced by these bacteria when grown on blood agar. This reaction is often termed alpha-haemolysis and is suggestive of the presence of hydrogen peroxide production (Nobbs *et al.*, 2009).

*Streptococcus pneumonia* is a Gram-positive coccus and a member of the lactic acid bacteria, which has been described as a foremost source of morbidity and mortality worldwide. The World Health Organisation (WHO) reported that approximately 1 million children die of pneumococcal disease every year in third-world countries (Hoskin *et al.*, 2001; WHO/UNICEF, 2005; WHO, 2007). Pneumococcal infections have been reported to be the foremost cause of death from vaccine-preventable illnesses in children younger than five
years (CDC, 2006). Invasive diseases caused by pneumococci include meningitis and pneumonia associated with bacteraemia and emphysema. The risk factors for developing invasive pneumococcal disease (IPD) include age, with the highest risk of incidence among young children less than two years old and also elderly people older than 65 years; ethnicity and geographic location with the ability to attend care centres being the main factor, as well as associated chronic sickness (Fletcher et al., 2006; WHO, 2007; Isaacman et al., 2010).

*Streptococcus pyogenes*, otherwise known as group A streptococcus (GAS) can cause minor human infections such as pharyngitis and impetigo, and also serious systemic infections such as necrotising fasciitis and streptococcal toxic shock syndrome. Furthermore, recurrent GAS infections can activate auto-immune diseases such as acute poststreptococcal glomerulonephritis, acute rheumatic fever and rheumatic heart diseases (Walker et al., 2014).

*Streptococcus agalactiae*, also known as Lancefield’s group B streptococcus, is a Gram-positive coccus that causes septicaemia and meningoencephalitis in various species of marine and freshwater fish globally (Eldar et al., 1995; Evans et al., 2002; Barony et al., 2017). *Streptococcus agalactiae* is also known to cause septicaemia and meningitis in the newborn (Bohnsack et al., 2008) and has been described in other animals, such as guinea pigs, camels, cats, dolphin, horses and frogs as well (Johri et al., 2006). This disease has been described as a key hindrance to the growth of Brazilian aquaculture because it causes high occurrences of disease in Nile tilapia, which is the most frequently farmed fish in Brazil (Mian et al., 2009; Barony et al., 2017).

*Streptococcus suis* comprises an intricate population made up of heterogenous strains (Feng et al., 2009), which can be classified into 35 serotypes based on the composition of capsule antigens (Wertheim et al., 2009). *Streptococcus suis* signifies a health problem in the swine industry globally. It has been described as an evolving zoonotic pathogen that causes severe human infections, clinically presenting with different diseases or syndromes such as meningitis, septicaemia and arthritis (Feng et al., 2014). Table 2.8 shows the strains and species of *Streptococcus* used in this study.

**Table 2.8: Strains of Streptococcus used in this study**

<table>
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</tr>
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<tr>
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<tr>
<td>Streptococcus mutans N22025</td>
<td>NC_013928</td>
</tr>
<tr>
<td>Streptococcus pyogenes NZ131</td>
<td>NC_011375</td>
</tr>
<tr>
<td>Streptococcus gordonii Str. Challis substr. CH1</td>
<td>NC_009785</td>
</tr>
<tr>
<td>Streptococcus pneumoniae TIGR4</td>
<td>NC_003028</td>
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<td>Streptococcus equi subsp. zooepidemicus</td>
<td>NC_012470</td>
</tr>
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<td>Streptococcus mutans UA159</td>
<td>NC_004330</td>
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<td>Streptococcus pseudopneumonia IS7493</td>
<td>NC_015875</td>
</tr>
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<td>Streptococcus pyogenes MGAS10394</td>
<td>NC_006086</td>
</tr>
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<td>Streptococcus pneumonia R6</td>
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<td>NC_012471</td>
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<tr>
<td>Streptococcus suis</td>
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<tr>
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<tr>
<td>Streptococcus pyogenes MGAS8232</td>
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<td>NC_015291</td>
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<td>Streptococcus pasteuriens ATCC 43144</td>
<td>NC_015600</td>
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<tr>
<td>Streptococcus thermophilus LMG 18311</td>
<td>NC_006448</td>
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<tr>
<td>Streptococcus mitis B6</td>
<td>NC_013853</td>
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</table>
2.3 Design and implementation of a computer algorithm for the generation of diagnostic barcode sequences

The basic principles of selection of barcode sequences were explained in detail in a previous publication by Reva et al. (2014) and developed further in this work. The sequence alignment was performed by the MUSCLE algorithm (Edgar et al., 2014). Orthology prediction was done by reciprocal BLASTP implemented in an in-house Python 2.7 script. All the programs for this work were written on Python 2.5 and made accessible at the website http://bargene.bi.up.ac.za/ through a PHP framework.

2.3.1 Input data

To generate a set of barcode sequences, the user should upload corresponding genome sequences in GenBank or FASTA format in a single archived file. A minimum of three genomes is required. The maximum size of the file to be uploaded should be < 500 MB. The proportion of accessory genes required should be selected alongside the desired length needed for the barcode sequences to be created. Then the program algorithm identifies taxa-specific genes and generates diagnostic barcodes as explained below. Schematically, the program algorithm is shown in Figure 2.1. The algorithm was implemented as a Python 2.5 program integrated into a PHP framework creating a Web-based user interface (see Chapter 4).

Figure 2.1: Shows how barcode sequences are generated from the BarcodeGenerator
2.3.2 Orthology prediction

Orthology prediction is performed by a reciprocal BLASTP alignment of all coding sequences predicted in all user-submitted genomes against one another. Two genes were considered as orthologs, if they produced reciprocally the best hit alignments against each other with e-values ≤ 0.0001 and BLAST score ≥ 75. A special group of accessory genes found exclusively in one given genome was marked as unique genes.

2.3.3 Identification of barcode genes

Core and non-unique accessory genes were grouped into clusters of orthologous genes (COG) and further processed by sequence alignment using the MUSCLE algorithm (Edgar et al., 2010). First, protein sequences were aligned and these alignments were used to reconstruct accurate codon alignments of DNA sequences of corresponding protein-coding genes. Analysis of frequencies of nucleotide and amino acid substitutions in the alignments allows assigning COGs to different categories representing different dynamics and involvement of these genes in evolutionary processes. The following statistical parameters were calculated by the program:

- Percentage of amino acid substitutions (sense mutations) of a total number of nucleotide substitutions in a given pair of orthologous genes;
- Diversity between protein sequences that was calculated as 1 − identity;
- Prevalence of positive matches in a pair-wise protein alignment, which includes identical matches and conserved amino acid substitutions, over identities. The value was calculated as (positives – identities)/identities. Conserved pairs of amino acids were identified using the PAM250 matrix of frequencies of amino acid substitutions; and
- The frequency of nucleotide substitutions per 100 bp stretches of DNA, calculated for each pair of aligned DNA (codon) sequences.

The rationale of this analysis was that the different rates of accumulation of nucleotide and amino acid substitutions may depict different categories of genes, i.e. evolutionary conserved genes, genes under strong positive selection and highly variable genes. The distribution of identified COG in a 3D plot is automatically calculated by the program BarcodeGenerator for every pair of submitted genomes and then these values are summarised for every COG and
The COGs from the analysis can be grouped into several categories: conserved; positively selected and highly variable genes. The conserved genes under moderate positive selection (highlighted in Figure 2.2 in brown) were proved to be suitable for barcoding (Reva et al., 2014). Appropriateness of COGs for barcoding was scored as $X \times (1 - X) \times (1 - Y) / (Z + 1)$, where $X$, $Y$ and $Z$ are values of the respective axes in Figure 2.2. Clusters of COG are ordered by these scores from large to small and then nucleotide sequences of the genes from highly scored COGs are concatenated into barcode
sequences until the requested length of barcodes is achieved. The chosen barcode genes provide a sufficient number of nucleotide substitutions to distinguish between the organisms of interest, yet they are sufficiently conserved to ensure correct orthology prediction.

Users may request the addition of a portion of accessory genes to barcode sequences to improve the sensitivity of the barcodes. In this case, the program first identifies two genomes in the dataset provided, which share the smallest number of accessory genes with each other, but share these genes with other genomes in the group. Then the program selects the accessory genes from these two genomes giving preference to longer genes shared by a bigger number of genomes of the group. An example of the selection of 50 accessory genes from 10 genomes of the genus *Shewanella* is shown in Figure 2.3. First, it was identified that the genomes NC_004347 (*S. oneidensis*) and NC_010334 (*S. halifaxensis*) possess a great number of accessory genes but do not share them with each other. Orthologous genes, depicted by blue bars in Figure 2.3, were found in other genomes: NC_008345 (*S. frigidmarina*), NC_008700 (*S. amazonensis*), NC_009052 (*S. baltica* OS155), NC_009997 (*S. baltica* OS195), NC_011663 (*S. baltica* OS223), NC_009438 (*S. putrefaciens*) and NC_010506 (*S. woodyi*). Genes from this selection were used to replace the core genes in the created barcodes to fit the requested length and core/accessory gene proportion in the resulting barcode sequences. To avoid overrepresentation of accessory genes in the two reference genomes (in Figure 2.3 these genomes are NC_004347 and NC_010334, which accessory genes are depicted by red bars), these genes in barcodes were partly replaced with unique genes identified in these genomes, provided that the length of these genes was above 300 bp. Unique genes were used also to fill in the barcode sequence of the genome NC_008750 (*Shewanella* sp. W3-18-1), which did not share accessory genes with any other genomes in this group. If there are no accessory genes suitable for barcode sequences, barcodes made of the core genes will be returned despite the user request.
Figure 2.3: Selection of 50 accessory genes for barcodes to distinguish between \textit{Shewanella} genomes. Sharing of accessory genes is depicted by red and blue bars.

2.3.4 Output data

BarcodeGenerator generates three output files: (i) the core gene plot graphical output, which is a scalable vector graphics (SVG) file shown in Figure 2.2, (ii) the barcode information, which is a text report and (iii) the barcode sequences generated in FASTA format. This information is sent to the e-mail address of the user entered on the website. All groups of microorganisms introduced at the beginning of this chapter were used for case studies, which involved generation of barcode sequences of different lengths and with different proportions of core and accessory genes. All these barcodes and supporting information were made available through the project website at http://seqword.bi.up.ac.za/barcoder_help_download/barcodes/index.html. This page is shown in Figure 2.4.
Figure 2.4: Web-page with the list of barcode sequences generated for this project for testing and evaluation of the developed software tools.

Additional information about every barcode sequence, including the plot of COG distribution and the list of genes selected for each barcode sequence, is accessible by clicking the links “info” next to each barcode in the list shown in Figure 2.4. The information page for a selected barcode is shown in Figure 2.5.

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>Average length</th>
<th>Info &amp; download</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
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<td>info download [92 kb]</td>
</tr>
<tr>
<td></td>
<td>25 kbp</td>
<td>info download [211 kb]</td>
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<tr>
<td></td>
<td>75 kbp</td>
<td>info download [596 kb]</td>
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<td></td>
<td>100 kbp</td>
<td>info download [781 kb]</td>
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<tr>
<td></td>
<td>200 kbp</td>
<td>info download [1500 kb]</td>
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<tr>
<td></td>
<td>250 kbp</td>
<td>info download [1863 kb]</td>
</tr>
<tr>
<td>Escherichia and Shigella</td>
<td>10 kbp</td>
<td>info download [92 kb]</td>
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<td></td>
<td>25 kbp</td>
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<td>Lactobacillus</td>
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<tr>
<td></td>
<td>250 kbp</td>
<td>info download [1035 kb]</td>
</tr>
</tbody>
</table>
Figure 2.5: Information page about barcode sequences generated for the *Bacillus cereus* group.

On all pages of the project web portal all bacterial and gene names are hyperlinked to the corresponding web resources of the NCBI database to allow access to all possible additional information about the subject.

2.3.5 Identification of categories of core and accessory genes automatically selected by the BarcodeGenerator for diagnostic barcodes

It was of interest to investigate which categories of genes were selected for barcodes in different groups of organisms by the above-mentioned algorithm. Among the core genes selected for barcodes, the most abundant group was the genes encoding for ribosomal
proteins (Table 2.9 and Figure 2.6). Ribosomal proteins are considered to facilitate the folding of the rRNA and the maintenance of an ideal configuration, which both speed up protein synthesis and accuracy (Wool, 1996). They are also known to have extraribosomal functions involved in replication, translation, RNA processing, DNA repair and autogenous regulation of translation (Wool, 1996). Most importantly, the ribosomal proteins are regarded as the best markers for studying the phylogenetic relationship because they are universal and are made up of highly conserved as well as variable domains (Patwardhan et al., 2014). This finding is in agreement with many publications reporting ribosomal proteins as the most suitable taxonomic and phylogenetic markers used in rMLST (Jolley et al., 2012b; Glaeser and Kämpfer, 2015). Ribosomal proteins comprised up to 15% of the sequences selected for barcodes by the program BarcodeGenerator. Other genes belonged to purine and pyrimidine biosynthetic pathways, ATP-binding cassette (ABC) transporters, tRNA synthetases and amido-transferases, various oxidoreductases, acyl carrier proteins and several other functional categories.

**Table 2.9: Functional categories of genes selected from the core part of genomes for barcode sequences in different groups of microorganisms used as case studies.**

<table>
<thead>
<tr>
<th>TYPES OF GENES</th>
<th>NUMBER OF GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein</td>
<td>3137</td>
</tr>
<tr>
<td>Purine biosynthesis</td>
<td>450</td>
</tr>
<tr>
<td>ABC transporter ATP-binding protein</td>
<td>242</td>
</tr>
<tr>
<td>Aspartyl/Glutamyl-tRNA amido-transferase</td>
<td>155</td>
</tr>
<tr>
<td>Oxidoreductase</td>
<td>142</td>
</tr>
<tr>
<td>Cysteinyl-tRNA synthetase</td>
<td>100</td>
</tr>
<tr>
<td>Acyl carrier protein</td>
<td>98</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>97</td>
</tr>
<tr>
<td>Cytochrome c-type biogenesis protein</td>
<td>96</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>94</td>
</tr>
<tr>
<td>others</td>
<td>17126</td>
</tr>
</tbody>
</table>
Figure 2.6: Pie chart showing the different functional categories of genes selected from the core genes

Synthesis of purine rings plays a principal metabolic role in all cells. The products that are AMP and GMP offer bases for DNA and RNA, as well as for a quantity of important coenzymes such as NAD, NADP, FAD and signalling molecules such as cAMP (Smith and Atkins, 2002). Purine and pyrimidine nucleotides are synthesised in vivo at an amount constant with physiological requirements. The intracellular mechanism senses and controls the pool amounts of nucleotide triphosphates, which increase during growth and tissue regeneration when cells are speedily dividing. Three processes contribute to purine nucleotide biosynthesis: (i) synthesis from an amphibolic intermediate, which is de novo synthesis; (ii) phosphoribosylation of purines and (iii) phosphorylation of purine nucleosides (Rodwell, 2003).

The ABC transporters are essential membrane proteins that effectively transport all necessary molecules across the lipid membrane against the concentration gradient, using the energy
obtained from the hydrolysis of ATP to ADP. The ABC transporters are found in almost all living organisms and are responsible for a large variety of processes. The ABC domain is also seen in proteins that may couple ATP hydrolysis to function other than transport, such as DNA repair (Doolittle et al., 1986; Linton and Higgins, 1998; Moussatova et al., 2008). Specialised ABC transporters also transport various choices of substrates such as ions, sugars or amino acids to larger compounds, like antibiotics, drugs, lipids and oligopeptides. They also take part in the uptake of nutrients or secretion of toxins in bacteria, as well as conferring multidrug resistance on bacterial cells by pumping diverse drugs and antibiotics into extracellular spaces (Moussatova et al., 2008).

Aminoacyl-tRNA synthetases (ARSs) are made up of an ancient family of enzymes that is found in virtually all cells from the three main kingdoms of life. They are known to catalyse the esterification reactions that bond amino acids with cognate tRNAs bearing the right anticodon triplet to confirm the precise transfer of information directed by the genetic code (Schimmel, 1987; Yao and Fox, 2012). The aminocylation reaction takes place in a two-phase process in which amino acids are first activated by ATP, forming an intermediate aminoacyl adenylate, and then transferred to the 3’-end of the tRNA to form the aminoacyl-tRNA end product (Ibba and Soll, 2000; Yao and Fox, 2012). All ARSs are made up of catalytic and anticodon recognition domains to catalyse aminocacylation reactions exactly for their cognate amino acids. Hence, to enable translational fidelity and sustain usual cellular function, various ARSs have developed editing activities to hydrolyse the inactivated amino acids or mischarged tRNAs and avoid insertion of incorrect amino acids during protein synthesis (Schimmel, 2008).

Oxidoreductases are made up of a huge group of enzymes, which catalyses biological oxidation-reduction reactions (May, 1999). Oxidoreductases make use of the integration of various cofactors such as haeme, flavin and metal ions to catalyse redox reactions. During these reactions, they make use of various electron acceptors and a huge amount of electron-donating substrates generating several products of industrial interest (Gygli and van Berkel, 2015). Several bacteria are made up of proton-translocating membrane-bound nicotinamide adenine dinucleotide (NADH)-quinone oxidoreductases, which show important genetic, spectral and kinetic resemblance with their mitochondrial equivalents (Sled et al., 1993). The mitochondrial NADH:ubiquinone oxidoreductase (complex I, coupling site I) plays an important role in the oxidation of NADH, the reducing product of cellular metabolism, by the
respiratory chain. However, complex I remains the most complex and least understood energy-transducing proto-motive device of the respiratory chain (Sled et al., 1993).

Amidotransferase is a class of enzymes that makes use of the ammonia obtained from the hydrolysis of glutamine for consequent chemical reactions catalysed by the same enzyme. The ammonia intermediate does not dissociate into solution during the chemical alteration (Raushel et al., 1999). A detailed example of the structure and mechanism shown by this group of enzymes is provided by carbamoyl phosphate synthetase. Carbamoyl phosphate synthetase is isolated from *E. coli* as a heterodimeric protein. The smaller of the two subunits is used to catalyse the hydrolysis of glutamine to glutamate and ammonia, while the larger subunit catalyses the formation of carbamoyl phosphate using 2 mol of ATP, bicarbonate and ammonia. Kinetic research has led to a suggested chemical mechanism for this enzyme that needs carboxy phosphate, ammonia and carbamate as kinetically competent reaction intermediates. The amidotransferase part of the CPS best illustrates how protein synthesis can stimulate the capture and use of ammonia obtained from the hydrolysis of glutamine (Raushel et al., 1999).

Acyl carrier proteins (ACPs) perform a major role in *de novo* fatty acid synthesis. Fatty acid synthases (FASs) can be grouped into two unique classes: (i) type I is made up of a single large multifunction polypeptide, which is mostly seen in mammals, fungi and some bacteria; and (ii) type II FASs are mostly seen in archaea, bacteria and plants and are usually categorised by the involvement of distinct mono-functional enzymes for fatty acid synthesis. Acyl carrier proteins exist as a separate domain (Jenke-Kodama et al., 2005; Hung et al., 2017). The *de novo* biosynthesis of FAs takes place through a conserved set of reactions, which are carried out during the elongation cycle (Smith and Sherman, 2008). Acyl carrier proteins are major constituents, which covalently bind all fatty acyl intermediates. During the initial phase, ACPs attach a phosphopantetheine group from CoA on a serine residue of ACP in a conserved Asp-Ser-Leu motif to form holo-ACP (Mofid et al., 2002; Hung et al., 2017). The first substrate of FASs, malonyl-CoA, is moved to ACP and the acetyl-CoA unit; the C2 is expanded to a butyrl group, the C4. The synthetic cycle is then reiterated multiple times depending on when the saturated C16 or C18 acyl-ACPs are generated for utilisation in membrane biosynthesis (Chan and Vogel, 2010).
Thymidine kinase (TK), is one of the major enzymes in the pyrimidine salvage pathway, which catalyses the phosphorylation of thymidine to thymidine 5’-monophosphate. The physiological importance of TK is shown by its extraordinary action in cells, which is involved in active DNA synthesis by the evolution of an elaborate feedback structure to control it (Saito and Tomioka, 1984).

Cytochromes (cysts) are pervasive haemoproteins that are major constituents of the energy transduction pathways and important for cellular processes ranging from chemical energy (ATP) production to planned cell death, also known as apoptosis (Moore and Pethigrew, 1990; Jiang and Wang; 2004; Bertini et al., 2006; Verissimo and Daldal, 2014). Cytochrome c biogenesis is a complex process taking place in almost all organisms and enables the covalent ligation of haeme to an apocyst c. It depends on key cellular functions such as protein translocation followed by post-translational modification extracytoplasmic protein folding and degradation, redox homeostasis, metal cofactor acquisition and insertion into target proteins. Different maturation processes, the systems I to IV sharing similar characteristics, were recognised (Kranz et al., 2009; Sanders et al., 2010; Stevens et al., 2011). In the first system, all apocyts c are synthesised in the cytoplasm and translocated through the sec pathway (Natale et al., 2008; Facey and Kuhn, 2010) across a lipid bilayer into a cellular section where they mature and function. This section is usually on the positive (p) side of an energy-transducing membrane such as the bacterial periplasmic space with the exception of the cyst b6f complex cyst c, also termed Cx or Cn, which is formed on the negative (n) side of the thylakoid membranes (de Vitry, 2011). In the second system, biosynthesis and transport of haeme and translocation of apocysts occur through a unique and autonomous process, which is coordinated spatially and temporally to minimise the cytotoxic effects of haeme and proteolytic degradation of apocyts c (Goldman et al., 1996; Moore and Helmann 2005). For the third system both the haeme iron atom and the apocyst c haeme-binding motif Cys thiol groups need to be reduced for thioether bond formation (Kranz et al., 1998; Sanders et al., 2010). For the fourth system, devoted chaperons and enzymes are needed for ligation of haeme to the apocyts c in a stereo-specific configuration. Mature cyst c are assembled into their respective cyst c complexes following their biogenesis (Verissimo and Daldal, 2014).

Analysis of the functions of genes selected by the program for diagnostic barcodes demonstrated that all these genes are involved in central indispensable metabolic processes of
all microorganisms. It guarantees that all these genes always constitute the core part of bacterial genomes; they are sufficiently conserved to be unambiguously identified in metagenomic reads but owing to accumulation of random and positively selected mutations, these genes provide sufficient signals to distinguish between species and sub-species of microorganisms. This analysis confirmed that the program can properly identify and select the genes that are suitable for diagnostic barcodes.

Among accessory genes, the most frequently selected were IS1 and IS2 transposases, membrane proteins, transcriptional regulators and capsular polysaccharide biosynthesis proteins (Table 2.10 and Figure 2.7).

Membrane proteins play a major role in identifying and transmitting outside signals into cells, thereby enabling them to network and respond to their environment in a detailed way. There are two major groups of membrane proteins: (i) those that span the membrane through secondary structures and (ii) those that span it as β barrels. The β barrels groups are usually found in the outer membranes of bacteria, mitochondria and chloroplasts, whereas the helical group is usually found in every other place, making them the most abundant group. The in vivo folding of the two classes of proteins is totally different (Bowie, 2004) and usually embroils a discrete cellular mechanism to catalyse the process (Fleming, 2014; Cymer et al., 2015).

Aziz et al. (2010) reported that transposases are the most profuse genes in both completely sequenced genomes and environmental metagenomes and are also the most abundant in metagenomes. Transposase genes are known to encrypt DNA binding enzymes, mostly members of the polynucleotidyl transferase superfamily, which catalyses the cut and paste reactions, thereby enhancing the movement of DNA segments to new sites (Rice and Baker, 2001; Aziz et al., 2010). These move double-stranded DNA (dsDNA) directly by excision and insertion and may be linked with insertion sequences (ISs), but most frequently they catalyse their own mobilisation (Crucio and Derbyshire, 2003; Aziz et al., 2010). Insertion sequences make up a significant part of most bacterial genomes. More than 500 different ISs have been identified and many are still being revealed (Mahillon and Chandler, 1998). The DNA ISs IS1, IS2 and IS3 are natural components of E. coli and K12 chromosome, where they are available in several duplicates (Brahma et al., 1982). The IS1 was one of the first bacterial ISs to be isolated and identified (Mahillon and Chandler, 1998). The original
examples were obtained from F’lac-proB plasmid (IS1216K) and the multiple drug resistance plasmid R100 (IS1216R) (Ohtsubo and Ohtsubo, 1978; Mahillon and Chandler, 1998).

The regulation of gene expression is mainly facilitated by proteins termed ‘transcription factors (TFs), which identify and bind precise nucleotide sequences and affect the transcription of neighbouring genes (Chalancon and Babu, 2013). Transcription factors are described as DNA binding proteins that bind to precise regions and the cis-regulatory elements in the promoter regions of certain genes and finally have an impact on gene expression. In addition to a DNA binding domain that identifies the DNA, most TFs also contain extra-regulatory domains such as small molecule-binding domains and enzyme domains that respond to a signal such as a small molecule (Chalancon and Babu, 2003).

Capsular polysaccharides are usually found on the outermost surface of a varied array of bacteria and are sometimes associated to the cell surface through covalent attachments to phospholipids or a lipid A molecule. Capsular polysaccharides are well hydrated and are normally made up of more than 95% water. They have repeating single monosaccharide units that are linked by glycosidic linkages (Taylor and Roberts, 2005). The ability of S. pneumoniae to regulate CPS production might be a significant factor responsible for its survival in various host environments. The utmost expression of CPS is important for systemic virulence, because of its antiphagocytic properties. Intrusive illnesses are consistently followed by asymptomatic colonisation of the nasopharynx and the thickness of the capsule may have an impact on the degree of exposure of additional significant pneumococcal surface structures, such as adhesins that are needed during this early colonisation stage (Morona et al., 2004).

The gene galT of E. coli codes for the enzyme galactose-1-phosphate uridyltransferase. The galT gene is involved in the metabolism of galactose and it catalyses the reversible conversion of UDP-glucose and galactose-1-phosphate to UDP-galactose and glucose-1-phosphate through an uridylated enzyme intermediate (McCorvie and Timson, 2011; McCorvie et al., 2013).

Permeases are defined as membrane proteins that transduce free energy stored in electrochemical ion gradient into a concentration gradient (Abramson et al., 2003). The E. coli lactose permease is one of the most studied members of the main superfamily of
transporters. The molecule is made up of N- and C-terminal domains, with each having six transmembrane helices, symmetrically structured within the permease. A huge internal hydrophilic cavity open to the cytoplasmic side shows the innermost conformation of the transporter. The structure with a bound lactose homolog β-D-galactopyranosyl-1-thio-β-D-galactose shows the sugar binding site of the cavity and residues that play key roles in substrate identification and proton translocation are recognised (Abramson et al., 2003).

Diacetyl is an essential aroma compound and plays a key role in the flavour of dairy products. Usually *L. lactis* undergoes homolactic fermentation and most of the dominant intermediate pyruvate is converted to lactate, a reaction catalysed by lactate dehydrogenase (LDH) with the oxidation of NADH to NAD$^+$ for maintaining a redox balance (Neves et al., 2005). In aerobic situations the activities of α-acetolactate synthase, i.e ALS and NADH oxidase (NOX), are highly increased (Bassit et al., 1993; Guo et al., 2012). Alpha-acetolactate synthase catalyses the pyruvate to acetolactate. After a decarboxylation process, α acetolactate is then converted to acetoin and diacetyl. Reoxidation of NADH by NOX usually replaces the role of LDH in the regeneration of NAD$^+$, leaving room for the accumulation of the two aroma compounds (Lopez de Felipe, 2000; Guo et al., 2012). Hence, in the presence of Oxygen, *L. lactis* shows the metabolic shift from homolactic to mixed-acid product formation comprising lactate, acetate and CO$_2$, which makes diacetyl accumulation restricted (Guo et al., 2012). Hence, various methods to enhance diacetyl production in *L. lactis* have been established, such as the overexpression of *als* and *nox-2* and the inactivation of the *ldh* and α-acetolactate decarboxylase (*aldB*) genes. Therefore, excessive pyruvate was channelled to diacetyl through ALS while the flux pyruvate to lactate was almost eradicated (Guo et al., 2012).

Hence, the accessory genes selected by the program for barcode sequences belonged to two categories: selfish mobile genetic elements infecting bacteria and functional genes, which provided bacteria with biosynthetic capacities important in specific habitats, or for a molecular redress of surface compounds to avoid the immune response of host organisms.
Table 2.10: Shows the different types of genes selected among the accessory genes for barcode sequences in the different groups of microorganisms used as case studies

<table>
<thead>
<tr>
<th>Types of genes</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane protein</td>
<td>159</td>
</tr>
<tr>
<td>Insertion element IS2 transposase</td>
<td>145</td>
</tr>
<tr>
<td>Transcriptional regulator</td>
<td>123</td>
</tr>
<tr>
<td>Capsular polysaccharide biosynthesis</td>
<td>60</td>
</tr>
<tr>
<td>Transposase ORF A</td>
<td>55</td>
</tr>
<tr>
<td>Galactose-1-phosphate uridylyltransferase</td>
<td>50</td>
</tr>
<tr>
<td>Premoase</td>
<td>48</td>
</tr>
<tr>
<td>Cytochrome c-type biogenesis protein</td>
<td>48</td>
</tr>
<tr>
<td>Alpha-acetolactate decarboxylase</td>
<td>46</td>
</tr>
<tr>
<td>Others</td>
<td>3583</td>
</tr>
</tbody>
</table>

Figure 2.7: Pie chart showing the different classes of genes selected for the accessory genes

2.4 Conclusion

Advances in present-day sequencing technologies have made it affordable to sequence and compare whole genomes of related microorganisms in the infancy of clonal segregation and speciation. Hence, there is a need for new computation techniques for mining an enormous
quantity of data generated by next-generation sequencing technologies. It is also of importance to identify and highlight marker sequences most suitable for the strains of interest and their biological activity. Multi-locus barcoding is a promising method for dependable identification of strains of closely related bacteria in environmental samples.

The aim of this work was to create an interactive computational service for the identification of the most suitable marker sequence for DNA-based multi-barcoding. The BarcodeGenerator is a novel software tool available for use at http://bargene.bi.up.ac.za/. The program BarcodeGenerator creates a specific barcode sequence based on the core and accessory gene provided by the user. The program then returns a link with the generated barcode sequences in FASTA format, information on the genes selected for barcodes and a graphical file in SVG format. The researcher also investigated which categories of genes were selected for barcodes in different groups of organisms by the above-mentioned algorithm. Among the core genes selected for barcodes, the most abundant group was the genes encoding for ribosomal proteins. The next question to address is how efficient the developed barcodes are in binning metagenomic reads to distinguish between closely related organisms. Development of the program for binning DNA reads against multi-locus barcodes and statistical validation of the results of the binning will be covered in the following chapters.
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Abstract

Metagenomic approaches have revealed the complexity of environmental microbiomes and advancement in WGS has led to a significant level of genetic heterogeneity on the species level. It has become clear that a superior pattern of bioactivity of bacteria applicable in biotechnology, as well as the enhanced virulence of pathogens, often requires researchers to distinguish between closely related species or sub-species. Current methods for binning of metagenomic reads usually do not allow identification below the genus level and very often stop at the level of families. In this chapter, an attempt was made to improve metagenome binning resolution using the Barcoding 2.0 program to align reads against barcode sequences and calculate various parameters for scoring the alignment results and individual barcodes. Taxonomic units were identified in metagenomic samples by comparison of the calculated barcode scores to set cut-off values.

3.1 Introduction

Metagenomics can be defined as a technique used for the direct investigation of genomes that contribute to an environmental sample (Handlesman et al., 1998; Thomas et al., 2012). Over the years, the field of metagenomics has transformed from sequencing of cloned DNA fragments using Sanger technology to direct sequencing of DNA without heterologous cloning (Tyson et al., 2004; Gilbert et al., 2008; Desai et al., 2012). Metagenomics offers access to the functional gene composition of microbial communities, which enables a wider depiction than phylogenetic surveys, and a strong tool for creating new hypotheses of microbial functions, such as the discovery of proteorhodopsin (Beja et al., 2000; Gilbert et al., 2008; Desai et al., 2012).

Advances in sequencing technologies have provided researchers with the ability to describe the microbial composition of environmental or clinical samples with exceptional resolutions promptly. A wealth of genetic data has become available owing to these approaches, providing new understanding of environmental and human microbial ecology (Hong et al., 2012). The reduction in the cost of sequencing has also rapidly enhanced the development of
sequence-based metagenomics. The number of metagenome sequence datasets has increased dramatically in the past few years (Thomas et al., 2012). Hence, metagenomics researchers have to analyse huge short-read datasets using tools designed for long reads and more specifically for clonal datasets (Desai et al., 2012).

Binning is generally referred as a method used for grouping reads or contigs and assigning them to OTUs. Normally, each sequence is either classified into a taxonomic group such as OTU, genus or family through association to some referential data, or clustered into groups of sequences that denote taxonomic groups centred on common characteristics such as the GC content (Sharpton, 2014). Binning plays a key part in the analysis of metagenomes, such as: (i) depending on the approach used, binning can give understanding of the presence of new genomes that are challenging to identify; (ii) it can be used to provide better insight into the unique numbers and kinds of taxa in a given community; and (iii) binning can decrease the intricacy of data, as used in post-binning analysis in assembly that can be carried out autonomously on each set of the binned reads rather than on the whole population of data (Sharpton, 2014). There are three common types of binning algorithms, namely sequence composition, sequence similarity and fragment recruitment. Sequence compositional binning uses metagenome sequence characteristics such as tetramer frequency to cluster or classify sequences into taxonomic groups. Some of these approaches, like PhyloPithia, analyse whole genome sequences ahead of time to train classifiers that stratify sequences into taxonomic groups (McHardy et al., 2007; Patil et al., 2011), while other approaches, such as emergent self-organising maps, use sequence characteristics to cluster metagenomic reads into unique classes without demanding a reference database and can be used to classify earlier unidentified organisms (Dick et al., 2009; Sharpton, 2014). Unlike composition-based methods, sequence similarity approaches need larger computational resources, as every read is normally aligned to a big volume of sequences. Sequence similarity-based approaches give better annotation accuracy and resolution compared to compositional binning. The MEGAN tool is one of the most commonly used sequence similarity methods using BLAST to compare reads to a database of sequences that are annotated with NCBI taxonomy (Huson et al., 2011). The fragment recruitment method identifies reads that show almost matching alignments to genome sequences, such as mapping and screen reads based on genomes to which they map. However, there are at present few tools that can handle both mapping of reads to a database of genomes and the calculation of genome abundance. One such tool is
Genometa, which provides users with a graphical user interface (Davenport et al., 2012; Sharpton, 2014).

However, most methods used for binning of metagenomic reads do not allow identification below the genus level and very often stop on the level of bacterial families (Thomas et al., 2012). In this work, an attempt was made to improve the metagenome binning resolution by using the novel Barcoding 2.0 program, which is available from http://bargene.bi.up.ac.za/. The program Barcoding 2.0 is a command-line program on Python 2.5/2.7 designed to align metagenome reads (Roche 454 and Illumina) against taxon-specific barcode sequences generated by the online program BarcodeGenerator (chapter 2).

3.2 Methods and research design

Command-line program Barcoding 2.0 is available for download from the Barcoder web portal. To validate the program, MetaSim software was used to generate collections of artificial reads simulating metagenome data sets (Richter et al., 2008). Sequence alignment was performed by MUSCLE algorithm (Edgar et al., 2004). Orthology prediction was done by reciprocal BLASTP implemented by an in-house Python 2.5/2.7 script. For data visualisation, matplotlib 1.5.1 Python module (https://matplotlib.org/1.5.1/index.html) was used. All the programs for this work were written on Python 2.5 (compatible with Python 2.7) and made accessible at the website http://bargene.bi.up.ac.za/ through a PHP framework.

3.3 Program implementation

Barcode sequences generated by BarcodeGenerator can be used for identification of species of interest in environmental metagenome samples sequenced by Roche 454 or Illumina technologies. Barcoding 2.0 is an application written in Python 2.5 (compatible with Python 2.7) with a command-line user interface made available for downloading from the BarcodeGenerator website (http://bargene.bi.up.ac.za/). The program uses BLASTN to align reads against the generated barcode sequences and then calculates several parameters for scoring the results of the BLASTN alignment and individual barcodes. First, read alignment records with BLASTN scores below an estimated $S'$ score cut-off value are filtered out. The cut-off $S'$ is calculated by equation 1:

$$S' = S + \frac{L - S}{3(L - S)} - 10 \times \left( \ln \left( \frac{2S + 100}{L + 100} \right) - 1 \right)$$

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where $S$ – an average BLASTN score of all aligned reads; $L$ – an average length of reads; and $N$ – number of aligned reads.

The program then calculates the alignment specificity ($a_{specificity}$) of read alignments (equation 2) by estimating the number of metagenomics reads ($N_{aligned \_ reads}$) that were successfully aligned against the given number of barcode sequences ($N_{barcodes}$) and the total number of BLASTN matches ($N_{matches}$):

$$a_{specificity} = 1 - \frac{N_{matches} - N_{aligned \_ reads}}{N_{aligned \_ reads} \times (N_{barcodes} - 1)}. \quad (2)$$

Values of specificity vary in the range from 0 to 1. The value of 0 indicates no specificity, i.e. every read in a given metagenome was aligned against every barcode sequence in the set. The value of 1 reports no overlap between reads aligned to different barcodes – maximal specificity.

Thereafter the program calculates the specificity of every read ($r_{specificity}$):

$$r_{specificity} = \frac{(N_{barcodes} - N_{read \_ aligned \_ barcodes})}{(N_{barcodes} - 1)}. \quad (3)$$

It can be seen from equation 3 that if one read was aligned against all barcodes, its specificity is 0; and if the read was aligned only against one barcode, its specificity is 1.

Then the program calculates two scores, ReadScore1 and ReadScore2, for every aligned read per barcode by equations 4 and 5, respectively:

$$ReadScore1 = \frac{BLASTN \_ score \_ read \_ length}{N_{reads}} \times \frac{r_{specificity} + EXP(r_{specificity} \times r_{vicinity}) + 1}{r_{specificity} + EXP(r_{vicinity}) + 1}. \quad (4)$$

$$ReadScore2 = a_{specificity} \frac{N_{read \_ barcode}}{N_{reads}} \times \frac{BLASTN \_ score \_ read \_ length \times r_{specificity} + 1.5(r_{specificity} \times r_{vicinity}) + 1}{r_{specificity} + 1.5(r_{vicinity}) + 1}. \quad (5)$$

It should be emphasised that $ReadScore2$ is barcode-specific, i.e. reads aligned to several barcodes will have different $ReadScore2$ values but the same value of $ReadScore1$. In
equations 4 and 5, the coefficient $r_{\text{vicinity}}$ was calculated for every read to avoid downgrading those reads, which were aligned to several barcodes of closely related organisms. First, a matrix of Jaccard distances is calculated for the set of barcodes, where the distance between two barcodes is $1 - \frac{\text{number_of_common_reads}}{\text{total_number_of_reads}}$. If one read was aligned to several barcodes, the parameter $r_{\text{vicinity}}$ for this read is calculated as $10 \times \frac{\text{max_barcode_subset_distance}}{\text{max_matrix_distance}}$. Values of $r_{\text{vicinity}}$ are in the range of 0 to 10. If the read is specifically aligned against only one barcode, its $r_{\text{vicinity}}$ is 0. If the read is aligned against several barcodes of closely related organisms, the parameters $r_{\text{vicinity}}$ will be small and the read will be scored high. However, if the read is promiscuously aligned against many unrelated barcodes, the parameters $r_{\text{vicinity}}$ will be high and the read will be scored low.

After scoring all the aligned reads, the program calculates scores for every barcode to identify corresponding species in the metagenome sample. Scores $\text{BarcodeScore}1$ and $\text{BarcodeScore}2$ (equation 6) are calculated from $\text{ReadScore}1$ (equation 4) and $\text{ReadScore}2$ (equations 5) respectively and are independent of the lengths of barcode sequences.

$$\text{BarcodeScore}_i = \frac{1 + \sum_i \text{ReadScore}}{1 + \frac{3 \times \text{BarcodeLength}_i \times \sum \text{BLASTN_score}}{4 \times \sum_i \text{BarcodeLength}}} - 1 \quad (6)$$

After downloading the program archive file from the project website, the file has to be unzipped to a local directory. The structure of internal folders of the program is shown in Fig. 3.1. Diagnostic barcode sequences generated by the program BarcodeGenerator (chapter 2) should be copied to the folder input as FASTA files. In the example in Fig. 3.1 this is the file $\text{Lactobacillus_barcodes.fasta}$. Metagenome reads should be stored in FASTA files in a subfolder within the folder input. In the given example, the folder metadata was created, which contains multiple metagenome files. All these metagenome datasets will be analysed for the presence of species of interest in a single program run with individual reports for each metagenome file. Optionally, a phylogenetic tree file may be provided as an input (file $\text{Lactobacillus.tre}$ in Fig. 3.1). Optimally, this tree file should contain information about all organisms indicating which barcodes are stored in the the barcode sequence file; however, the phylogenetic tree may comprise only part of these organisms and contain more organisms, which were not barcoded. Obviously, names of barcode sequences must correspond to the names in the phylogenetic tree. The tree dendrogram, if provided, will be included in the
graphical report file. The program can be run on computers with Python 2.5/2.7 installed. To run the program, the user has to double-click the file run.py in the top folder of the program. A command line window will appear, as shown in Figure 3.1.

![Figure 3.1: Folders of the program Barcoder unzipped to a local directory.](image1)

By default the barcode file bacillus.fasta is set in the command-line window (Figure 3.2). This file is provided as an example with the program download. To change the input file, use the option <I+Enter> and enter the name of the barcode file to use. Use the option <F+Enter> to change the name of the input tree file, if the file is available. If there is no such file, use <R+Enter> to clear this option. The folder with metagenome files may be changed using the option <D+Enter+new_folder_name+Enter>. The option <S> allows setting of species identification stringency that will be explained below in this chapter. The command-line window ready to run with the example files is shown in Figure 3.3.

![Figure 3.2: An initial command-line window of the program Barcoding 2.](image2)
Figure 3.3: Setting of program run options in the command line program interface.

Keyboard combination <Y-Enter> will run the program for execution with the set parameters. Advanced users can run this program with the same settings from the command prompt line shown in Figure 3.4.
3.3.1 Barcoding program workflow and identification of optimal program run parameters

An overview of the program workflow is shown in Figure 3.5.

The program aligns metagenome reads against barcode sequences and then performs statistical analysis as explained above by equations 1-6. In the next step, the program generates graphical and text output files. The text output file contains a list of barcoded genomes with assigned scores. The graphical SVG file presents these scores in the form of a phylogenetic tree.
histogram. If a phylogenetic tree is provided, barcode bars in this graph are distributed along the corresponding nodes of the phylogenetic tree.

The program uses cut-off values of the barcode scores (see equation 6) to evaluate the results of identification of barcoded organisms in metagenomic samples. To validate the program and identify optimal settings of cut-off values, an artificial metagenome was created comprising DNA reads generated by the program MetaSim from several reference genomes (Table 3.1).

Table 3.1: Composition of the artificial metagenomic dataset generated by MetaSim from reference *Shewanella*, *Escherichia*, *Shigella*, *Lacobacillus* and *Mycobacterium* genomes.

<table>
<thead>
<tr>
<th>Reference genomes</th>
<th>Number of reads (200-500bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Shewanella</strong></td>
<td></td>
</tr>
<tr>
<td>S. <em>amazonensis</em> SB2B</td>
<td></td>
</tr>
<tr>
<td>S. <em>frigidmarina</em> NCIMB 400</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella</em> sp. MR-4</td>
<td></td>
</tr>
<tr>
<td><strong>Escherichia/Shigella</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC8739</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> BL21</td>
<td></td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em> Sd197</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td></td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> TMW1</td>
<td></td>
</tr>
<tr>
<td><em>L. plantarum</em> WCFS1</td>
<td></td>
</tr>
<tr>
<td><em>L. fermentum</em> IFO3956</td>
<td></td>
</tr>
<tr>
<td><strong>Mycobacterium</strong></td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em> Env77</td>
<td></td>
</tr>
<tr>
<td><em>M. abscessus</em> ATCC 19977</td>
<td></td>
</tr>
</tbody>
</table>

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Values for BarcodeScore1 and BarcodeScore2, which are dependent on the percentage of reads in a metagenome, are shown in Fig. 3.6A and B, respectively. BarcodeScore1 was more sensitive to the presence of specific reads in metagenomes. It may be appropriate for a quantitative identification of taxa, while BarcodeScore2 reflects the abundance of specific reads in metagenomes better.

![Figure 3.6: Distribution of calculated values for A) BarcodeScore1 and B) BarcodeScore2 based on the percentage of genome specific reads in artificial metagenomes. Whisker lines depict the minimal, maximal and median values; grey bars show middle quartiles and the open cycles indicate the average values.](image)

Taxonomic units are identified in metagenomic samples by comparison of the calculated barcode scores to pre-computed cut-off values. True positives (TP) would be the genomes that were used for preparation of the artificial metagenomes and correctly identified by the program. Those genomes were false negative (FN), which the program failed to identify. False identification of other genomes represented in a set of barcodes leads to false positives (FP), but if excluded from the program output, they are true negatives (TN). To evaluate the barcoding performance with different cut-off values, parameters of sensitivity, specificity and the ratio of TPs over false predictions TP/(FP + FN), were calculated.

The distribution of values for TP/(FP + FN) calculated for a matrix of combinations of BarcodeScore1 and BarcodeScore2 cut-offs is shown in Table 3.2 and Figure 3.7. The highest proportion of TPs over false predictions was achieved for the pair of cut-offs BarcodeScore1 = 2.5 and BarcodeScore2 = 1. However, in the program the cut-off values
BarcodeScore1 = 2.3 and BarcodeScore2 = 0.5 were set by default as the relaxed mode to allow for higher sensitivity in case of an increase in the number of false positives. The setting BarcodeScore1 = 2.5 and BarcodeScore2 = 1.0 is available as the stringent mode. Switching between the relaxed and stringent modes is performed by using the option <S> in the command line interface (see Fig. 3.3).

Table 3.2: TP / (FP + FN) values calculated for a matrix of combinations of BarcodeScore1 and BarcodeScore2 cut-offs. Combinations of pairs of score cut-off values for the relaxed and stringent operation modes are highlighted.

<table>
<thead>
<tr>
<th>BarcodeScore 2 cutoff values</th>
<th>BarcodeScore 1 cutoff values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.59</td>
</tr>
<tr>
<td>0.6</td>
<td>0.64</td>
</tr>
<tr>
<td>0.7</td>
<td>0.70</td>
</tr>
<tr>
<td>0.8</td>
<td>0.74</td>
</tr>
<tr>
<td>0.9</td>
<td>0.81</td>
</tr>
<tr>
<td>1.0</td>
<td>0.84</td>
</tr>
<tr>
<td>1.1</td>
<td>0.79</td>
</tr>
<tr>
<td>1.2</td>
<td>0.76</td>
</tr>
<tr>
<td>1.3</td>
<td>0.77</td>
</tr>
<tr>
<td>1.4</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Figure 3.7 Surface plotting of the distribution of TP / (FP + FN) values calculated for different pairs of cut-off values of the BarcodeScore 1 and 2.
The barcoding program in the relaxed and stringent modes was used for processing of artificial metagenomes of different sample sizes on the generated barcodes of different lengths. It was found that the sample size (number of reads in a metagenome) had no effect on the sensitivity and specificity of the algorithm in the range from 10,000 to 500,000 (Table 3.3, Figure 3.8A and calculated receiver operating characteristics (ROC) diagrams are shown in Appendix 1. In all these experiments, metagenomic datasets of different size were aligned against barcodes of the same sequence length of 50,000 bp. However, in this range of values, the percentage of TPs grew with the sample size proportionally with the number of FPs. This is illustrated in a series of output files calculated for the artificial metagenomes aligned against *Shewanella* barcode sequences, as displayed in Figure 3.10. This series of figures demonstrates an increasing number of identified genomes (green bars in the figures), both TPs and FPs, with the increase in the sample size. The artificial metagenome comprised fragments generated from three *Shewanella* genomes: *Shewanella* sp. MR-4 [NC_008321] — 15% of reads of the metagenome), *S. frigidimarina* NCIMB 400 [NC_008345] — 10% and *S. amazonensis* SB2B [NC_008700] — 5%. In Figure 3.10A, the size of the metagenome was 10,000 reads. The strains NC_008321 and NC_008345 were reliably identified (green bars), while the minority strain NC_008700 was putatively detected (orange bar), together with two other strains, NC_008577 and NC_008700, which were not present in the metagenome (FPs). Figure 3.10B shows the results of barcoding, when the size of the metagenome was increased to 50,000 reads. In this case, all three strains of *Shewanella* included in the metagenome were identified, together with two FPs. In Figures 3.10C and D the size of the metagenome was progressively increased, which caused an increase in identification scores for both TP and FP predictions. False positive predictions may result from close phylogenetic relatedness between barcoded strains, which will be discussed below in this chapter and in chapter 5.

The ratio TP / (FP + FN) was generally higher in smaller metagenomes (see Table 3.3, Figure 3.8A).
Table 3.3: Influence of metagenome sample size on the program performance

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Operation mode</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>TP / (FP + FN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>relaxed</td>
<td>0.95</td>
<td>0.72</td>
<td>0.98</td>
<td>1.60</td>
</tr>
<tr>
<td>50000</td>
<td>relaxed</td>
<td>0.97</td>
<td>0.81</td>
<td>0.96</td>
<td>1.50</td>
</tr>
<tr>
<td>100000</td>
<td>relaxed</td>
<td>0.97</td>
<td>0.81</td>
<td>0.92</td>
<td>0.90</td>
</tr>
<tr>
<td>300000</td>
<td>relaxed</td>
<td>0.98</td>
<td>0.91</td>
<td>0.86</td>
<td>0.66</td>
</tr>
<tr>
<td>500000</td>
<td>relaxed</td>
<td>0.99</td>
<td>0.91</td>
<td>0.81</td>
<td>0.47</td>
</tr>
<tr>
<td>10000</td>
<td>stringent</td>
<td>0.94</td>
<td>0.54</td>
<td>0.97</td>
<td>0.85</td>
</tr>
<tr>
<td>50000</td>
<td>stringent</td>
<td>0.97</td>
<td>0.82</td>
<td>0.96</td>
<td>1.50</td>
</tr>
<tr>
<td>100000</td>
<td>stringent</td>
<td>0.96</td>
<td>0.73</td>
<td>0.95</td>
<td>1.0</td>
</tr>
<tr>
<td>300000</td>
<td>stringent</td>
<td>0.97</td>
<td>0.82</td>
<td>0.94</td>
<td>1.13</td>
</tr>
<tr>
<td>500000</td>
<td>stringent</td>
<td>0.98</td>
<td>0.82</td>
<td>0.94</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Figure 3.8: Influence of the A) metagenome sample size and B) length of barcode sequence on the program performance.
Figure 3.9: Results of Shewanella strain identification in artificial metagenomes of different sizes: A) 10,000; B) 50,000; C) 100,000 and D) 300,000 reads. The strains Shewanella sp. MR-4 [NC_008321], S. frigidimarina NCIMB 400 [NC_008345] and S. amazonensis SB2B [NC_008700] comprised 15%, 10% and 5% of the total number of reads, respectively. Identification of other barcoded strains was considered as false-positives.

In another set of experiments, the influence of the barcode sequence lengths on barcoding accuracy was examined. It was found that the specificity and sensitivity were constant and independent on lengths of barcode sequences in the range from 10 to 250 kbp (Table 3.4, Figure 3.8B and see also calculated ROC diagrams in Appendix 2). However, the ratio TP/(FP + FN) was optimal when the barcode sequences were in the range from 100 to 200 kbp. Shorter barcodes reduced the number of TPs, as many reads remained unidentified and longer barcodes increased the number of FP predictions. The influence of the barcode sequence length on the program performance was tested on artificial metagenomic datasets with 500,000 randomly generated reads.
Table 3.4: The influence of the length of barcode sequence on the program performance

<table>
<thead>
<tr>
<th>Barcode length (kbp)</th>
<th>Operation mode</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>TP / (FP + FN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>relaxed</td>
<td>0.89</td>
<td>0.73</td>
<td>0.88</td>
<td>0.57</td>
</tr>
<tr>
<td>25</td>
<td>relaxed</td>
<td>0.94</td>
<td>0.91</td>
<td>0.86</td>
<td>0.71</td>
</tr>
<tr>
<td>75</td>
<td>relaxed</td>
<td>0.93</td>
<td>0.82</td>
<td>0.89</td>
<td>0.75</td>
</tr>
<tr>
<td>100</td>
<td>relaxed</td>
<td>0.94</td>
<td>0.82</td>
<td>0.89</td>
<td>0.75</td>
</tr>
<tr>
<td>150</td>
<td>relaxed</td>
<td>0.93</td>
<td>0.82</td>
<td>0.89</td>
<td>0.75</td>
</tr>
<tr>
<td>200</td>
<td>relaxed</td>
<td>0.89</td>
<td>0.82</td>
<td>0.90</td>
<td>0.82</td>
</tr>
<tr>
<td>250</td>
<td>relaxed</td>
<td>0.92</td>
<td>0.78</td>
<td>0.9</td>
<td>0.64</td>
</tr>
<tr>
<td>10</td>
<td>stringent</td>
<td>0.89</td>
<td>0.73</td>
<td>0.91</td>
<td>0.73</td>
</tr>
<tr>
<td>25</td>
<td>stringent</td>
<td>0.93</td>
<td>0.82</td>
<td>0.91</td>
<td>0.90</td>
</tr>
<tr>
<td>75</td>
<td>stringent</td>
<td>0.93</td>
<td>0.82</td>
<td>0.92</td>
<td>1.0</td>
</tr>
<tr>
<td>100</td>
<td>stringent</td>
<td>0.93</td>
<td>0.82</td>
<td>0.95</td>
<td>1.28</td>
</tr>
<tr>
<td>150</td>
<td>stringent</td>
<td>0.93</td>
<td>0.82</td>
<td>0.95</td>
<td>1.29</td>
</tr>
<tr>
<td>200</td>
<td>stringent</td>
<td>0.88</td>
<td>0.82</td>
<td>0.95</td>
<td>1.29</td>
</tr>
<tr>
<td>250</td>
<td>stringent</td>
<td>0.91</td>
<td>0.78</td>
<td>0.93</td>
<td>0.87</td>
</tr>
</tbody>
</table>

3.3.2 Program performance on different groups of microorganisms

Program performance was affected by the level of taxonomic relatedness between barcoded organisms. Receiver operating characteristics curves were calculated for different taxonomic groups based on the results of identification of corresponding genomes in artificial metagenomic datasets (Table 3.5 and Figure 3.12). In addition to sensitivity and specificity parameters, the area under curve was calculated, which is considered a performance measure of diagnostic tools. Distinguishing between species of the same genus or family by the program was close to optimal. However, it was problematic for the program to differentiate between representatives of different lineages of *Escherichia* and *Shigella* (Figure 3.13). It was assumed that the addition of accessory genes in barcodes may improve the diagnostic performance. Comparison of identification results when the barcodes of the
Escherichia/Shigella group of the same length (150 000 bp) with different proportions of core and accessory genes were used is shown in Figure 3.14. It was found that an increase of accessory genes in barcodes hampered distinguishing between closely related organisms even more, compared to barcodes based solely on core genes. It may be explained by related organisms exchanging frequently mobile elements in a random fashion, which impedes proper differentiation between them. However, including species-specific accessory genes may improve identification on higher taxonomic levels.

Table 3.5: shows the ROC result calculated for different taxonomic groups

<table>
<thead>
<tr>
<th>Group of micro-organisms</th>
<th>Operation mode</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>TP / (FP + FN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecol_Shig</td>
<td>relaxed</td>
<td>0.74</td>
<td>0.47</td>
<td>0.87</td>
<td>0.19</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>relaxed</td>
<td>1</td>
<td>1</td>
<td>0.96</td>
<td>3</td>
</tr>
<tr>
<td>Mycobacteria</td>
<td>relaxed</td>
<td>1</td>
<td>0.9</td>
<td>0.95</td>
<td>1.8</td>
</tr>
<tr>
<td>Shewanella</td>
<td>relaxed</td>
<td>1</td>
<td>1</td>
<td>0.89</td>
<td>1.5</td>
</tr>
<tr>
<td>Ecol_Shig</td>
<td>stringent</td>
<td>0.7</td>
<td>0.33</td>
<td>0.98</td>
<td>0.33</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>stringent</td>
<td>1</td>
<td>0.93</td>
<td>0.96</td>
<td>2.33</td>
</tr>
<tr>
<td>Mycobacteria</td>
<td>stringent</td>
<td>1</td>
<td>0.9</td>
<td>0.94</td>
<td>1.8</td>
</tr>
<tr>
<td>Shewanella</td>
<td>stringent</td>
<td>1</td>
<td>0.87</td>
<td>0.9</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Figure 3.10: Histogram for the taxonomic relatedness between organisms used as case study
Figure 3.11: ROC diagrams of barcoding of genomes on different taxonomic levels. The following parameters were calculated: AUC – area under the curve; SEN – sensitivity; and SPE – specificity.

Figure 3.12: ROC diagrams of barcoding of genomes of the *Escherichia / Shigella* group by barcodes with different contribution of accessory genes. The following parameters were calculated: AUC – area under the curve; SEN – sensitivity; and SPE – specificity.
3.4 CONCLUSION

In this chapter, a novel command-line program, Barcoding 2.0, was used for binning of metagenomic reads against barcode sequences generated with the BarcodeGenerator. The program MetaSim, which is a sequencing simulator, was used to generate collections of DNA reads from chosen bacterial genomes to design artificial metagenomic datasets with known species composition and species abundance. Metagenomes of different sample sizes (of 10 000, 50 000, 100 000, 300 000 and 500 000 bp) were generated by random selection of DNA fragments of a specified range of length from the selected reference organisms to simulate next-generation sequencing.

The program (Barcoding 2.0) uses BLASTN to align reads against barcode sequences and then calculates scores for the BLASTN alignment and individual barcodes. After scoring all the aligned reads, the program calculates scores for every barcode to identify organisms present in metagenome samples. Taxonomic units are identified by comparison of calculated barcode scores to standard cut-off values set by default.

The researcher also performed two experiments using varying metagenomes of different sample size and barcode sequences of different lengths. In the first experiment, metagenomic datasets of varying sizes of 10 000 to 500 000 reads were aligned against barcodes of the same length (50 kbp). It was found that the sample size (the number of reads in a metagenome) has no effect on the sensitivity or specificity of the algorithm. In this range of values, the percentage of TPs increased with the sample size proportionally to the number of false positives. The ratio of TPs over false prediction was higher in smaller metagenomes. Furthermore, when varying lengths of barcode sequences (10 to 250 kbp) were used for aligning an artificial metagenomic dataset of 500 000 bp, the sensitivity and specificity remained unchangeable. However, the ratio of the TPs over FNs was optimal when the barcode sequences were in the range from 100 to 200 kbp.

Receiver operating characteristic curves of the algorithm performance were calculated for all experiments with artificial metagenomics datasets. Distinguishing between species of the same genus or family by the program was close to perfect, but in distinguishing between strains of Escherichia coli and Shigella the program fared worse. Closely related organisms could be identified better when barcodes were based solely on core genes.
Hence, Barcoding 2.0 enables efficient and practical use of metabarcodes for visualisation of distribution of organisms of interest in environmental and clinical samples. Barcoding 2.0 is available for download from the same source as the BarcodeGenerator (http://bargene.bi.up.ac.za/).
References


CHAPTER 4: Barcoder web interface and case study of barcode-guided species detection

Abstract

In this chapter the Barcoder software tools are discussed and a case study of barcode-guided species is provided. A detailed explanation is provided on using the Barcoder software tools. The web page/web application, Barcoder, is available at http://bargene.bi.up.ac.za.

4.1 Introduction

The Barcoder web application serves as an interactive computational service for identification of the most suitable marker sequences for DNA for multi-local barcoding. In this chapter the researcher discusses in detail how the Barcoder web application works. For framework data visualisation, matplotlib 1.5.1 Python module (https://matplotlib.org/1.5.1/index.html) was used. This web application was made accessible at the webpage http://bargene.bi.up.ac.za/ through a PHP

4.2 BarcodeGenerator

This webpage provides users with online access to the program BarcodeGenerator, which creates diagnostic barcodes based on the genome sequences of species of interest submitted by users (Figure 4.1). The computational algorithm implemented in this program was described in detail in Chapter 2. The program BarcodeGenerator allows for the creation of barcode sequences based on a given set of genomes. It compares all pairs of genomes and selects barcodes (DNA sequences) from core and accessory genes, depending on the program run parameters. The program allows addition of accessory genes, which are believed to be genome-specific and may improve the sensitivity of the barcode sequences. However, in Chapter 3 it was demonstrated that the addition of accessory genes to barcode sequences of closely related organisms (the Escherichia-Shigella group was considered) may worsen the sensitivity of barcoding owing to random sharing of horizontally transferred genes by these organisms. The researcher may suggest the use of accessory genes to distinguish between closely related species, but not sub-species or lineages of the same species.

To generate a set of barcode sequences, the user has to upload corresponding genome sequences in GenBank format in a single archived file. The archived file has to have a minimum of three sequence files, but the maximum file size has to be below 500 MB.
Uploading of the input file is performed by using a corresponding key in the web interface (Figure 4.1). The user may then change the proportion of accessory genes in the generated barcode sequences and request the approximate length of barcode sequences. The project name is entered alongside the e-mail address, which will be used to provide the user with links to output files with generated barcode sequences and other supporting information.

Figure 4.1: The screenshot of the BarcodeGenerator Web-interface

By default, the program will look in provided genomes for genes most suitable for identification (barcoding) of these organisms. Several alternative algorithms were implemented and may be chosen from the drop-box ‘Select Mode of Operation’:

- Darwinian – select genes under highest pressure of the positive Darwinian selection;
- Conserved – select the most conserved genes in the given genomes;
• Hotspotted – select the most variable genes in the given genomes.

Figure 4.2 shows examples of gene selections by these different algorithms from the same input set of Thermotoga genomes.

Figure 4.2: Graphical outputs of the program BarcodeGenerator generated for A) Barcode; B) Darwinian; C) Conserved and D) hotspotted algorithms. Selected clusters of orthologous genes are shown in brown colour.

The difference between these algorithms consists only in the way the score is calculated for different clusters of orthologous genes:

• Barcode

\[ Score = \frac{X(1 - X)(1 - Y)}{(Z + 1)} \]  \hspace{1cm} (Eq. 4.1)

• Darwinian

\[ Score = \begin{cases} 
if \ Z \leq 0.3 &rightarrow \ Score = \frac{X}{(Z + 1)} \\
else &rightarrow \ Score = 0 
\end{cases} \]  \hspace{1cm} (Eq. 4.2)
Conserved

\[ \text{Score} = \frac{1}{(X + 1)(Y + 1)(Z + 1)} \]  \hspace{1cm} (Eq. 4.3)

Hotspotted

\[ \text{Score} = (X + 1)(Y + 1)(Z + 1). \]  \hspace{1cm} (Eq. 4.4)

In all these equations, X, Y and Z are the values of the corresponding axes in Figure 4.2; i.e. X is the percentage of sense mutations in alignments of sequences of orthologous genes; Y is \(1 - \text{identities of alignments}\); Z is \((\text{positives} - \text{identities})/\text{identities}\).

In this work, only the barcode algorithm was considered in detail.

### 4.2.1 Local version of BarcodeGenerator

The local version of the program BarcodeGenerator is a command-line program that can be run on Python 2.7 or Python 2.5. It was designed to select the most appropriate genes for genetic barcoding and generate barcode sequences that can be used for the analysis and visualisation of metagenomic datasets by using another program, Barcoding 2.0, provided from the same web page.

To use the local version of BarcodeGenerator, the user has to download an archived ZIP file of BarcodeGenerator to the local computer and unzip it. When all the archive content has been extracted, the following folders will appear in the computer, as shown in Figure 4.3: \textit{bin, gbk\_examples, input, lib and output}. The desired GenBank files of the organisms needed to be barcoded are then copied into the \textit{input} folder. To run the program, the user double-clicks on the file \textit{run.py} in the top-level folder of the program. The command-line interface of the BarcodeGenerator is shown in Figure 4.4.
Figure 4.3: Unzipped folder structure of the local version of BarcodeGenerator.

Figure 4.4: Command line interface of BarcodeGenerator

Option P is used to change the project name. A new folder title by the project name will be created in the folder output where all the resulting output files of the current program run will be stored. The operation mode (option M) by default is barcode to generate barcode sequences. The program may also allow selection of genes of the following categories: (i) Darwinian (orthologous genes under positive selection); and (ii) conserved (the most conserved genes and hotspotted (orthologous genes with the highest number of random mutations) as discussed above (see Figure 4.2). Option A is used to specify the percentage of accessory genes in the barcode sequences. This option is available only with the barcode mode of operation. Option L is used to set an average length of generated barcode sequences in bp. When Y is typed and the Enter key pressed, the program starts showing the progress bar. The program generates three output files and stores them to the folder with the project
name in output folder. These files are: (i) graphical core gene plot SVG file, as those shown in Figure 4.2; (ii) barcode info-file in text format; and (iii) generated barcode sequences in FASTA format.

4.3 Barcoding 2.0 command line interface for metagenome analysis and visualisation

Barcoding 2.0, provided with a command-line user interface, is available for download from the Barcoder web page. The command-line program Barcoding 2.0 can be used for binning of reads of WGS metagenomes (Figure 4.5). The program Barcoding 2.0 is a command-line program in Python 2.5/2.7 designed to align metagenomic reads of Roche 454 and Illumina against taxon-specific barcode sequences generated by the online program BarcodeGenerator. The program performs a BLASTN alignment of reads against the barcode sequences and scores every barcode in the set, as explained in Chapter 3.

The user needs to download the zip file Barcoding 2.0 to a computer and unzip it. The unzipped file is made up of the following folders: (i) bin, (ii) db, (iii) input, (iv) lib and (v) output (Figure 4.6). The user then copies the following files into the input folder: (i) FASTA file with barcode sequences and named as barcodes.fas; and (ii) FASTA files of metagenomic reads of one or several metagenomes stored in a new folder in the folder input. Optionally, the user can copy to the input folder to a phylogenetic tree in phylip/Newick format to align barcoded taxa against the phylogenetic tree. Taxonomic units in the tree file MUST have the same names in the barcode file. A minimum of three barcoded taxa should be present in the tree file; however, the total number of barcodes may differ from the total number of taxonomic units in the tree file. Taxonomic units not found among barcodes will be ignored and the barcode sequences not represented in the tree file will be grouped outside the tree in the graphical output file. The user can then run the program by clicking on the file run.py in the top-level folder of the program.
Figure 4.5: Command line interface of Barcoding 2.0 with the argument setting by default.

Figure 4.6: Folders of the program Barcoding 2.0 unzipped to a local directory.

The name given in the *Query file* option (Figure 4.5) is the name of the barcode file and the option *Database name* indicates the name of the subfolder containing FASTA files of metagenomic reads. These settings should correspond to real names in the *input* folder, otherwise these names can be changed using keys I and D, respectively. The option C is used to choose the stringency parameter between relaxed and stringent. The stringent mode corresponds to cut-off values 2.5 and 1 of BarcodeScore1 and BarcodeScore2, while in the relaxed mode these values are 2.3 and 0.5 (see discussion in Chapter 3, Table 3.3 and Figure 3.16 for detail). If the user has a phylogenetic tree file (*phylotree.tre* for example), it should
be copied and placed in the input folder. The user can type F and press the Enter key; the program will then allow the user to enter the name of the tree file, as shown in Figure 4.7.

Figure 4.7: Command line interface when the user wants to enter a phylogenetic tree file.

If the program does not find the indicated file in the input folder, no dendogram setting is returned. However, if it does exist, the name will appear in the option set, as shown in Figure 4.8.

Figure 4.8: Command line interface of Barcoding 2.0 with the phylogenetic tree file.
When the user types Y and presses the Enter key, the program shows the progress made. The program generates the graphical file in SVG format and a text file. An example of identification of *Lactobacillus* species by generated barcode sequences in the phyllosphere 9673 metagenome publicly available from MG-RAST database is shown in Figure 4.9. The green columns indicate strains, which are likely to be present in the metagenome. The height of the columns depicts values of BarcodeScore2 (see equations 4-6, Chapter 3). The phylogenetic tree beneath the plot was generated by the SWPhylo program (http://swphylo.bi.up.ac.za/) by whole genome sequence comparison. Table 4.1 shows the text file generated.

![Graphical file for Lactobacillus.](image)

**Figure 4.9:** Graphical file for *Lactobacillus*.

**Table 4.1:** Shows the screenshot text file generated for Lactobacillus.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Count CorrScore</th>
<th>Score Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC_008530</td>
<td>11 0.000000 0.000000</td>
<td></td>
</tr>
<tr>
<td>NC_006814</td>
<td>19 0.000000 0.063199</td>
<td></td>
</tr>
<tr>
<td>NC_007576</td>
<td>16 0.000000 0.561481</td>
<td></td>
</tr>
<tr>
<td>NC_008497</td>
<td>13 0.000000 0.693096</td>
<td></td>
</tr>
<tr>
<td>NC_019610</td>
<td>54 3.223211 8.460976</td>
<td></td>
</tr>
<tr>
<td>NC_013199</td>
<td>24 0.091129 0.615798</td>
<td></td>
</tr>
<tr>
<td>NC_015551</td>
<td>10 0.062169 0.610268</td>
<td></td>
</tr>
<tr>
<td>NC_008513</td>
<td>9   0.000000 0.023401</td>
<td></td>
</tr>
<tr>
<td>NC_014724</td>
<td>8   0.000000 0.030435</td>
<td></td>
</tr>
<tr>
<td>NC_014727</td>
<td>24 1.000000 2.427307</td>
<td></td>
</tr>
<tr>
<td>NC_014334</td>
<td>14 0.128455 1.302963</td>
<td></td>
</tr>
<tr>
<td>NC_010080</td>
<td>11 0.000000 0.172568</td>
<td></td>
</tr>
<tr>
<td>NC_015978</td>
<td>12 0.000000 0.563240</td>
<td></td>
</tr>
<tr>
<td>NC_015575</td>
<td>26 0.284323 1.995125</td>
<td></td>
</tr>
<tr>
<td>NC_015579</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>NC_005629</td>
<td>10 0.000000 0.000000</td>
<td></td>
</tr>
</tbody>
</table>
4.4 Help and downloads

All the necessary help and information needed are found on the webpage http://seqword.bi.up.ac.za/barcoder_help_download/index.html. In the webpage, the readme.html provides information about the Barcoder software tools (BarcodeGenerator and Barcoding 2.0). Lists of instructions are also provided on how to use the Barcoder software tools.

4.4.1 Downloads

Downloads available on the webpage include: (i) the program Barcoding 2.0 (320 MB); (ii) example of input files of bacterial genomes to test the BarcodeGenerator; (iii) diagnostic barcodes created during the project; and (iv) examples of artificial metagenomes created for this project and discussed in Chapter 3. The program Barcoding 2.0 with the command-line user interface is available for download from the Barcoder webpage. The command-line program Barcoding 2.0 can be used for binning of reads of WGS metagenomes as explained earlier. Also available for download is an archived input file example with eight genbank (GBK) files of Bacillus genomes (Bacillus amyloliquefaciens NC_014551, Bacillus clausii NC_006582, Bacillus coagulans NC_015634, Bacillus halodurans NC_002570, Bacillus licheniforms NC_006322, Bacillus pumilus NC_009848, Bacillus subtilis NC_000964 and Bacillus velezensis NC_009725. This file was prepared as an example to test the BarcodeGenerator. To use the input file of the eight Bacillus genomes the user has to: (i) download the file example.zip to a computer; (ii) go to the Webpage http://bargene.bi.up.ac.za/; (iii) click the key Browse and select this file on the local computer; (iv) click the key Upload files and barcode; and (v) wait for a message to come to the user’s e-mail with links showing: (i) the Core Gene plot (svg); (ii) barcode information; and (iii) barcode sequences in FASTA format. Examples of the expected output files are also available for viewing on the web-page http://seqword.bi.up.ac.za/barcoder_help_download/example/example.html.

The generated barcode sequences of different length for all organisms used in the case studies of this project (Bacillus cereus, Escherichia and Shigella, Lactobacillus, Mycobacteria, Prochlorococcus, Salmonella, Shewanella, Streptococcus) were made available for download at http://seqword.bi.up.ac.za/barcoder_help_download/barcodes/index.html, as shown in Table 4.2.
Table 4.2: Different taxonomic groups for which barcode sequences were created and made available for download

<table>
<thead>
<tr>
<th>Taxonomic group</th>
<th>Average length</th>
<th>Info &amp; downloads</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus cereus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10kbp</td>
<td></td>
<td>Info download (92kb)</td>
</tr>
<tr>
<td>25kbp</td>
<td></td>
<td>Info download (211kb)</td>
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<tr>
<td>75kbp</td>
<td></td>
<td>Info download (596kb)</td>
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<tr>
<td>100kbp</td>
<td></td>
<td>Info download (781kb)</td>
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<tr>
<td>150kbp</td>
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<td>Info download (781kb)</td>
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<tr>
<td>200kbp</td>
<td></td>
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<tr>
<td>250kbp</td>
<td></td>
<td>Info download (1863kb)</td>
</tr>
<tr>
<td><strong>Escherichia and Shigella</strong></td>
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<td>200kbp</td>
<td></td>
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<td>Info download (2702kb)</td>
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<tr>
<td><strong>Lactobacillus</strong></td>
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<td></td>
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<td>10kbp</td>
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<td>Info download (101kb)</td>
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<tr>
<td>250kbp</td>
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<tr>
<td><strong>Mycobacteria</strong></td>
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</tr>
<tr>
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<td></td>
<td>Info download (1035kb)</td>
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<tr>
<td><strong>Prochlorococcus</strong></td>
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<td></td>
</tr>
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<td>10kbp</td>
<td></td>
<td>Info download (56kb)</td>
</tr>
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<td>75kbp</td>
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<td>Info download (539kb)</td>
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<tr>
<td>100kbp</td>
<td></td>
<td>Info download (705kb)</td>
</tr>
<tr>
<td>150kbp</td>
<td></td>
<td>Info download (1057kb)</td>
</tr>
<tr>
<td>200kbp</td>
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<td>Info download (1407kb)</td>
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<tr>
<td>250kbp</td>
<td></td>
<td>Info download (1746kb)</td>
</tr>
<tr>
<td><strong>Shewanella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10kbp</td>
<td></td>
<td>Info download (97kb)</td>
</tr>
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<td>25kbp</td>
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<td>Info download (206kb)</td>
</tr>
<tr>
<td>75kbp</td>
<td></td>
<td>Info download (541kb)</td>
</tr>
<tr>
<td>100kbp</td>
<td></td>
<td>Info download (711kb)</td>
</tr>
</tbody>
</table>
In the information section for each taxonomic group, the graphical output of the diagnostic barcode generated for each length is provided (Figure 4.10). Other information, such as the original genome, locus tag, annotation and location in the barcode of core/accessory genes, is provided (Figure 4.11). The information section is also linked to the NCBI. The NCBI offers an enormous collection of online resources for biological information and data, comprising the: (i) GenBank, (ii) nucleic acid sequence database; (iii) PubMed database of citations; and (iv) abstracts for published life science journals (NCBI Resource Coordinators, 2016). Over the years, the quantity and diversity of data that the NCBI sustains have expanded immensely and the data can commonly be divided into six groups: (i) Literature; (ii) Health; (iii) Genomes; (iv) Genes; (v) Proteins; and (vi) Chemicals (NCBI Resource Coordinators, 2016). The Entrez system (Schuler et al., 2016) of the NCBI offers access to varied groups of 37 databases that together contain 2.1 billion records. Since the information section is linked to the NCBI, more detailed information about each genome is provided (Figure 4.12).

In the download section, the average length (10, 25, 75, 100, 150, 200, 250 kbp) of each barcode sequence in the different taxonomic group is available for download.
Figure 4.10: Graphical output of clusters of orthologous genes selected for diagnostic barcodes generated for the group *Bacillus cereus* with an average length of 10 kbp.

<table>
<thead>
<tr>
<th>Genome</th>
<th>Locus tag</th>
<th>Annotation</th>
<th>Location in barcode</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC2924</td>
<td></td>
<td>cytoplasmic protein (rev) [3907023..3907493]</td>
<td>[1-471] Core</td>
</tr>
<tr>
<td>BC3159</td>
<td></td>
<td>NAD-dependent epimerase/dehydratase /</td>
<td>[522-2378] Core</td>
</tr>
<tr>
<td>BC0131</td>
<td></td>
<td>rplC,50S ribosomal protein L3 (dir) [126823..127455]</td>
<td>[2429-3061] Core</td>
</tr>
<tr>
<td>BC3130</td>
<td></td>
<td>hypothetical protein (rev) [3100771..3101796]</td>
<td>[3112-4137] Core</td>
</tr>
<tr>
<td>BC0117</td>
<td></td>
<td>rpK,50S ribosomal protein L11 (dir) [110486..110911]</td>
<td>[4188-4613] Core</td>
</tr>
<tr>
<td>BC3160</td>
<td></td>
<td>TetR family transcriptional regulator</td>
<td>[4664-5248] Core</td>
</tr>
<tr>
<td>BC1002</td>
<td></td>
<td>anti-sigma B factor antagonist (dir) [983659..983997]</td>
<td>[5299-5637] Core</td>
</tr>
<tr>
<td>BC2156</td>
<td></td>
<td>hypothetical protein (dir) [2099430..2099813]</td>
<td>[5688-6071] Core</td>
</tr>
<tr>
<td>BC0450</td>
<td></td>
<td>protein tyrosine phosphatase (dir) [439787..440251]</td>
<td>[6122-6586] Core</td>
</tr>
<tr>
<td>BC2637</td>
<td></td>
<td>hypothetical protein (rev) [2603031..2603225]</td>
<td>[6696-6941] Accessory</td>
</tr>
<tr>
<td>BC5124</td>
<td></td>
<td>hypothetical protein (rev) [5025633..5027765]</td>
<td>[7246-9429] Accessory</td>
</tr>
<tr>
<td>BC3019</td>
<td></td>
<td>hypothetical protein (rev) [2981452..2981655]</td>
<td>[11917-12171] Accessory</td>
</tr>
<tr>
<td>BC1875</td>
<td></td>
<td>Phage protein (dir) [1827729..1827866]</td>
<td>[14013-15551] Accessory</td>
</tr>
<tr>
<td>BC2976</td>
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<td>hypothetical protein (dir) [2934856..2935068]</td>
<td>[18931-19194] Accessory</td>
</tr>
<tr>
<td>BC0943</td>
<td></td>
<td>hypothetical protein (dir) [927449..927979]</td>
<td>[22837-23418] Accessory</td>
</tr>
</tbody>
</table>

Figure 4.11: Information provided for each genome used to generate diagnostic barcode sequences available for download.
Figure 4.12: Screenshot example of NCBI page linked to each genome used to generate barcode sequences.

Also available for download are the sets of artificial metagenomic reads (200-500 bp) generated by the program MetaSim. Contents of artificial metagenomes provided on the Web-page http://seqword.bi.up.ac.za/barcoder_help_download/artificial_metagenomes/index.html are shown in Table 4.3.

Table 4.3: Contents of the artificial metagenomes

<table>
<thead>
<tr>
<th>ARTIFICIAL METAGENONES</th>
<th>GenBank Accession Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shewanella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. amazonensis</em> SB2B</td>
<td>NC_008700</td>
<td>5%</td>
</tr>
<tr>
<td><em>S. frigidimarina</em> NCIMB400</td>
<td>NC_008345</td>
<td>10%</td>
</tr>
<tr>
<td><em>Shewanella sp.</em> MR4</td>
<td>NC_008321</td>
<td>15%</td>
</tr>
<tr>
<td><strong>Escherichia/Shigella</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> ATCC8739</td>
<td>NC_010468</td>
<td>5%</td>
</tr>
<tr>
<td><em>E. coli</em> BL21</td>
<td>NC_012947</td>
<td>10%</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em> Sd197</td>
<td>NC_007606</td>
<td>15%</td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. sanfranciscensis</em> TMW1</td>
<td>NC_015978</td>
<td>5%</td>
</tr>
<tr>
<td><em>L. plantarum</em> NCFS1</td>
<td>NC_004567</td>
<td>10%</td>
</tr>
<tr>
<td><em>L. fermentum</em> IF03956</td>
<td>NC_010610</td>
<td>10%</td>
</tr>
<tr>
<td><strong>Mycobacterium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em> Env77</td>
<td>NC_008595</td>
<td>5%</td>
</tr>
<tr>
<td><em>M. abscessus</em> ATCC 1977</td>
<td>NC_010397</td>
<td>10%</td>
</tr>
</tbody>
</table>
4.4 SeqWord project

The Barcoder software tools are part of the SeqWord project (genome linguistic approaches for comparative genomics, phylogenomics and mobilomics). The SeqWord project addresses the development of an integrated research environment for data mining in DNA sequences by using genome linguistics. SeqWord projects are non-commercial academic software tools and web applications, which were developed with the support of the National Research Foundation of South Africa (NRF). The principal investigator of the SeqWord project is Prof. Oleg Reva. All tools in the SeqWord project were created by post-graduate students. Other tools available in the SeqWord project (http://seqword.bi.up.ac.za/) are Genome browser, Genomic Island Sniffer, Sniffer GI Browser, GI Databases, Interactive GI maps, SWPhylo, GenomeBarcoder, OligoDBViewer, MetaLingvo and LingvoCom.

4.6 Conclusion

This web interface provides users with online access to the program BarcodeGenerator, which creates diagnostic barcodes based on the genome sequences of species of interest submitted by users. The program also allows the addition of a genome-specific accessory to improve the sensitivity of the barcode sequences. Hence the BarcodeGenerator is an efficient approach for generating diagnostic barcode sequences. The BarcodeGenerator also has a local version, which is a command-line program. It was designed to select the most appropriate genes for genetic barcoding and generate barcode sequences, which can be used for the analysis and visualisation of metagenomic datasets by using the program Barcoding 2. Barcoding 2.0 is another program available from the same resources that enables efficient and practical use of metabarcodes for visualisation of distribution of organisms of interest in environmental and clinical metagenomic samples. The program Barcoding 2.0 is a command-line program written in Python 2.7 and designed to align metagenomic reads generated by Roche 454 and/or Illumina against taxon-specific barcode sequences generated by the program BarcodeGenerator (locally or through the web interface). The Barcoder software tools (BarcodeGenerator and Barcoding 2.0) are available for download at http://bargene.bi.up.ac.za/. For framework data visualisation, matplotlib 1.5.1 Python module (https://matplotlib.org/1.5.1/index.html) was used. All the programs for this work are compatible with Python 2.5/2.7 and are made accessible at the website http://bargene.bi.up.ac.za/ through a PHP interface.
CHAPTER 5 Evaluation of the program Barcoding 2.0 by binning real metagenomic reads

Abstract

In this chapter the researcher gives an in-depth explanation of case studies of DNA reads of different metagenomics datasets from the MG-RAST database used together with barcode sequences generated for selected groups of microorganisms discussed in Chapter 2.

5.1 Introduction

Advancement in technology has made it possible for the genome sequencing project to move from the study of single genomes to the investigation of genomes in the community. Metagenomics allows culture-independent and sequence-based studies of microbial communities (Chan et al., 2008). Metagenomics projects usually start by using shotgun WGS on environmental samples to conduct: (i) sequence reads; (ii) assembly of sequence reads; (iii) gene prediction; and (iv) functional annotation and metabolic pathway construction (Chan et al., 2008).

An important step in metagenomics is called “binning”. The binning process sorts sequence fragments (either original reads generated by sequencers or assembled contigs) of various species obtained from WGS sequencing into phylogenetically related bins or groups (Mavromatis et al., 2007). Normally, each sequence is either classified into a taxonomic group such as OTU, genus or family through association to some referential data, or clustered into groups of sequences that denote taxonomic groups centred on common characteristics such as the GC content (Sharpton et al., 2014). Binning plays a key part in the analysis of metagenomes: (i) depending on the approach used, binning can give understanding into the presence of new genomes that are challenging to identify; (ii) it can be used to provide better insight into the unique numbers and kinds of taxa in a given community; and (iii) it can decrease the intricacy of data, as used in post-binning analysis such as assembly, which can be done autonomously on each set of the binned reads rather than on the whole population of data (Sharpton, 2014). Most of the present binning techniques involve assigning of sequence fragments by comparing sequence similarity or sequence composition with already sequenced
genomes that are still far from comprehensive (Chan et al., 2008). Hence, most methods used for binning of metagenomic reads do not allow identification below the genus level and very often stop on the level of bacterial families (Thomas et al., 2012).

In this chapter, the researcher discusses the results of different case studies where DNA reads of different metagenomic datasets from MG-RAST database were aligned with BLASTN using the novel Barcoding 2.0 program (Chapter 3) against taxon-specific barcode sequences generated by the online program BarcodeGenerator (Chapter 2).

5.2 Program implementation

Barcode sequences generated by BarcodeGenerator can be used for identification of species of interest in environmental metagenome samples sequenced by Roche 454 or Illumina technologies. Barcoding 2.0 is an application written in Python 2.5 (also compatible with Python 2.7) with a command-line user interface made available for downloading from the BarcodeGenerator website (http://bargene.bi.up.ac.za/). The program uses BLASTN to align reads against the generated barcode sequences and then calculates several parameters for scoring the results of the BLASTN alignment and individual barcodes.

5.3 Identification of barcoded sequences in real metagenomes

An attempt was made to test the barcode sequences created by the program BarcodeGenerator for various genomes of bacteria of industrial, medicinal and ecological importance on real metagenomic datasets available from NCBI and MG-RAST. The metagenomes used are divided into three groups: (i) symbiotic microbiomes (canine gut, human gut, mammalian blood, termite gut and cow gut); (ii) soil and rhizosphere microbiomes (desert soil, grassland, forest rhizosphere, phyllosphere, rain forest, soybean rhizosphere); and (iii) environmental microbiomes (anthropogenic estuarine, sludge, hydrothermal vent and mediterian bathypelagic).

5.3.1 Metagenome analyser

Since this is the first version of the Barcoding 2.0 program released, to validate the researcher’s results and to determine how well the Barcoding 2.0 performed, the researcher first performed a BLASTN alignment of various metagenomic reads used in the case studies against a local copy of the NCBI nt database using the blastn for Linux implementation of the
alignment program installed on the computer server. The MEGAN 4.70.4 program was then used to estimate and interactively explore the taxonomical content of the dataset, using the NCBI taxonomy to summarise and order the results. MEGAN uses a simple algorithm that reads standard BLASTN output files and assigns each read to the LCA of the set of taxa that it hits in comparison. Hence, species-specific sequences are assigned to the taxa near the leaves of the NCBI tree, whereas widely conserved sequences are assigned to high order taxa closer to the root (Huson et al., 2007). As discussed in Chapter 3, the 50-100 000 nucleotide long barcodes gave the best results. For all case studies the researcher used 100 000-nucleotide-long barcodes generated by the BarcodeGenerator. Results obtained from the MEGAN program are discussed in further detail below.

5.3.1.1 Canine and cow intestinal microbiomes

All mammals are populated by groups of organisms vital to the typical form and function of the host. Regarding cellular composition, genetic diversity and metabolic capacity, the host mammal should be regarded as a multispecies hybrid organism made up of host and microbial cells functioning in vibrant and symbiotic symmetry (Turnbaugh et al., 2007; Shreiner et al., 2015; Barko et al., 2018). The gastrointestinal microbiome is a varied conglomerate of bacteria, archaea, fungi, protozoa and viruses that occupy the gut of mammals. Research in humans and mammals has associated the microbiome in a series of physiologic processes that are important to host health, including energy homeostasis, metabolism, gut epithelial health, immunologic activity and neurobehavioral progress. The microbial genomes confer metabolic competences above those of the host organism alone, making the gut microbiome a dynamic contributor in host physiology (Barko et al., 2018).

Figure 5.1 shows the results obtained for the MEGAN binning of reads of canine gut. From the figure one can see the different groups of bacteria that could possibly be identified in the canine metagenome. From the top of the phylogenetic tree, groups include the Bacteriodetes/Chlorobi group, Chylamydiae/Verrucomicrobia group, Fibrobacteres/Acidobacteria group and Proteobacteria group. The canine gut metagenomic dataset was selected for this case study as representative of rich symbiotic gut micro-flora enriched with many organisms, which were used in previous steps to generate diagnostic barcodes by the program BarcodeGenerator.
Figure 5.1: MEGAN analysis of reads from the canine gut metagenome.
5.3.1.2 Phyllosphere

The phyllosphere in the aerial surface of plants is an essential and pervasive habitat for bacteria (Vorholt, 2012). It is appraised on a universal scale that the phyllosphere spans more than $10^8$ km$^2$ and serves as home to approximately $10^{26}$ bacterial cells (Lindow and Brandl, 2003). Leaf-related bacteria epitomise a widespread and primeval symbiosis that can affect host growth and function in various ways, including the production of growth-promoting nutrients and hormones (Reed et al., 2010) and protection of hosts against pathogen infection (Innerenbner et al., 2011). The phyllosphere bacteria can influence plant biogeography and the ecosystem function through their influence on plant performance under different environmental conditions (Kembel et al., 2012).

Figure 5.2 shows the results obtained for the MEGAN binning of reads of the phyllosphere. From the figure one can see the different groups of bacteria that could possibly be identified in the phyllosphere metagenome. From the top of the phylogenetic tree the groups include (i) Actinobacteria; (ii) Armatimonadete; (iii) Bacteroidetes; (iv) Verrucomicrobia; (v) Thermomicrobia; (vi) Cyanobacteria; (vii) Acidobacteria; (viii) Firmicutes; (ix) Planctomycetes; (x) Alphaproteobacteria; (xi) different subdivisions of proteobacteria; (xii) Mollicutes; and (xii) unclassified groups of bacteria. This metagenome was selected for the case studies as representative of species-rich environmental micro-flora to validate diagnostic barcodes prepared for the identification of *Mycobacteria* and *Shewanella*. 

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5.3.1.3 Grassland

The significance of the soil microbiome in the cycling of important nutrients such as carbon and nitrogen is well understood. However, because of the microbiome’s complexity, little is understood about how climate will affect the diversity, abundance and structure of the community (Shaver et al., 2000). In grassland soils, experimental warming has been shown to increase bacterial biomass in winter and spring (Belay-Tedla et al., 2009; Sheik et al., 2011); nevertheless, warming affected bacterial biomass negatively and 16S rRNA gene abundance in summertime and early fall correspondingly, signifying that warming may have a seasonal effect on soil moisture (Castro et al., 2010; Sheik et al., 2011).

This metagenomic dataset was selected for the case studies as an example of species-rich micro-flora associated with a plant rhizosphere, first of all to test the identification of environmental Mycobacteria.
5.3.1.4 Hydrothermal vent

The greatly varying chemical conditions present in different places above and below the sea floor at deep-sea hydrothermal vents and the often very steep gradients between different conditions generate a wide range of geochemical niches and potential energy sources for microorganisms (Fisher et al., 2000). Primary production by chemolithoautotrophs sustains not only the heterotroph components in the microbial ecosystem, but also the animal communities through either symbioses or free-living bacteria that form the base of food webs (Fisher et al., 2000). The pathways of inorganic carbon metabolism used for primary production by hydrothermal vent microbes are very diverse, which may reflect the diversity of physical and chemical microhabitats they occupy (Fisher et al., 2000).

This metagenome was used as an example of an environment with a relatively limited number of specific bacterial species. Figure 5.3 shows the results obtained for the MEGAN binning of reads of the hydrothermal vent. From the figure one can see the different groups of bacteria that could possibly be identified in the hydrothermal vent metagenome, though not as rich and diverse as the canine gut and phyllosphere metagenome. From the top of the phylogenetic tree there are groups that include: (i) Actinobacteria; (ii) Bacteroidetes; (iii) Cyanobacteria; (iv) proteobacteria; and (v) mollicutes.
Figure 5.3: MEGAN analysis of reads of the hydrothermal vent metagenome.

5.3.1.5 Mammalian blood

Mammalian blood is believed to be sterile, except in cases of bacteremia and contamination, when microorganisms are isolated in mammalian blood. Hence, MEGAN analysis for mammalian blood in Figure 5.4 showed very few microorganisms. In this study the researcher used mammalian blood as a form of negative control.
5.3.1.6 Other metagenomes used in this study

In addition to metagenomes described above, subsets of several other metagenomic datasets available from the MG-RAST database were used to evaluate the diagnostic barcodes generated for this study (Chapter 3), which were made available from the project web-site at http://seqword.bi.up.ac.za/barcoder_help_download/barcodes/index.html. A description of all the metagenomic datasets used is given in Table 5.1.

Table 5.1: Samples of metagenomic datasets from MG-RAST database used in this study.

<table>
<thead>
<tr>
<th>Name of metagenome</th>
<th>Number of reads</th>
<th>Average length of reads in bp</th>
<th>MG-RAST sample number or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYMBIOTIC MICROBIOMES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canine gut</td>
<td>583,523</td>
<td>400</td>
<td>Swanson et al., 2010</td>
</tr>
<tr>
<td>Human gut</td>
<td>500,000</td>
<td>1,365</td>
<td>mgs79383</td>
</tr>
<tr>
<td>Mammalian Blood</td>
<td>92,948</td>
<td>1,915</td>
<td>mgs81295</td>
</tr>
<tr>
<td>Termite gut</td>
<td>99,776</td>
<td>856</td>
<td>Singh et al., 2015</td>
</tr>
<tr>
<td>Cow gut</td>
<td>264,849</td>
<td>100</td>
<td>mgs17404</td>
</tr>
<tr>
<td>SOIL AND RHIZOSPHERE MICROBIOMES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desert soil</td>
<td>85,549</td>
<td>65</td>
<td>mgs64929</td>
</tr>
<tr>
<td>Grassland</td>
<td>976,268</td>
<td>374</td>
<td>Delmont et al., 2012</td>
</tr>
<tr>
<td>Forest rhizosphere</td>
<td>561,526</td>
<td>148</td>
<td>mgs50708</td>
</tr>
<tr>
<td>METAGENOMES</td>
<td>BARCODES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phyllosphere</strong></td>
<td>1,026,982</td>
<td>386</td>
<td></td>
</tr>
<tr>
<td><strong>Rain Forest</strong></td>
<td>782,404</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td><strong>Soybean rhizosphere</strong></td>
<td>151,054</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td><strong>ENVIRONMENTAL MICROBIOMES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthropogenic Estuarine</td>
<td>526,919</td>
<td>358</td>
<td></td>
</tr>
<tr>
<td>Sludge</td>
<td>96,563</td>
<td>1,056</td>
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<tr>
<td>Hydrothermal Vent</td>
<td>293,065</td>
<td>1,008</td>
<td></td>
</tr>
<tr>
<td>Mediterranean Bathypelagic</td>
<td>9,047</td>
<td>797</td>
<td></td>
</tr>
</tbody>
</table>

### 5.3.2 BARCODING 2.0

In the next step, an attempt was made to test the barcode sequences created by the program BarcodeGenerator for selected genomes of various bacteria of industrial, medicinal and ecological importance on real metagenomic datasets obtained from the MG-RAST database.

The results of identification of bacterial taxa by aligning reads against diagnostic barcode sequences by means of the program Barcoding 2.0 are shown in Table 5.2. The identification is depicted by (++), meaning that at least one organism was reliably identified by BarcodingScore1 and BarcodingScore2, as indicated on the output graphs by green bars; (+) indicates that at least one organism was identified with the BarcodingScore1 above 1, which is depicted by an orange bar; (+/-) means at least one organism was identified with BarcodingScore1 below 1 and the (-) sign means that no organisms were identified in the sample. The results obtained for each taxonomic group are discussed below.

**Table 5.2: Results obtained with Barcoding 2.0 program for different metagenomes.**

<table>
<thead>
<tr>
<th>METAGENOMES</th>
<th>BARCODES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SYMBIOTIC MICROBIOMES</strong></td>
<td></td>
</tr>
<tr>
<td>Canine gut</td>
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</tr>
<tr>
<td>Human gut</td>
<td>+/-</td>
</tr>
<tr>
<td>Mammalian Blood</td>
<td>-</td>
</tr>
<tr>
<td>Termite gut</td>
<td>-</td>
</tr>
<tr>
<td>Cow gut</td>
<td>-</td>
</tr>
<tr>
<td><strong>SOIL AND RHIZOSPHERE</strong></td>
<td></td>
</tr>
</tbody>
</table>

156
<table>
<thead>
<tr>
<th>MICROBIOMES</th>
<th>Desert soil</th>
<th>Grassland</th>
<th>Forest rhizosphere</th>
<th>Phyllosphere</th>
<th>Rain Forest</th>
<th>Soybean rhizosphere</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>—</td>
<td>+/—</td>
<td>+</td>
<td>+/—</td>
<td>—</td>
<td>—</td>
</tr>
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<td></td>
<td>+/—</td>
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<td>+/—</td>
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<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ENVIRONMENTAL MICROBIOMES</th>
<th>Anthropogenic</th>
<th>Estuariane</th>
<th>Sludge</th>
<th>Hydrothermal Vent</th>
<th>Mediterrane</th>
<th>Bathypelagic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>—</td>
<td>+/—</td>
<td>++</td>
<td>+/—</td>
<td>—</td>
<td>—</td>
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<td>+/—</td>
<td>+</td>
<td>+/—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

++ - at least one organism is reliably identified (green bar);
+ - at least one organism is identified with the score above 1 (orange bar);
+/— - at least one organism is identified with the score below 1 (orange bar);
— - no organism was identified (all short red bars);

5.3.2.1 Bacillus cereus

*Bacillus cereus* is closely related to *Bacillus anthracis* and the insect pathogen *Bacillus thuringiensis* (Ivanova et al; 2003). *Bacillus anthracis* are dangerous zoonotic pathogens while *Bacillus thuringiensis* are used in pesticides. *Bacillus anthracis* and *Bacillus thuringiensis* do contain plasmid borne-specific toxins and this is usually used to differentiate them from *Bacillus cereus* (Ivanova et al., 2003).

In symbiotic microbiomes *Bacillus cereus* was only identified in canine gut (+) and in human gut (+/-). For soil and rhizosphere microbiomes *Bacillus cereus* was identified in grassland (+/-), the forest rhizosphere (+/-) and phyllosphere (+/-). In the environmental microbiomes, *Bacillus cereus* was only identified in a hydrothermal vent (+/-). This indicates that these bacteria are widely distributed in nature but are not abundant in selected habitats.
5.3.2.2 **Escherichia coli/Shigella**

*Escherichia coli* are a known commensal of the gastrointestinal tract of warm-blooded animals and are used as the everyday laboratory mainstay. However, pathogenic *E. coli* has also been reported, which causes human diseases ranging from disorders of the gastrointestinal tract to ones affecting extra-intestinal sites such as the urinary tract, bloodstream and the central nervous system (Kaper *et al.*, 2004; Croxen and Finlay 2010; Croxen *et al.*, 2013). Though various aetiological agents have been reported as the cause of diarrhoea, pathogenic *E. coli* stands out among others as a major cause. Entero-invasive *E.coli/Shigella* spp are described as facultative intracellular pathogens and the aetiological agents of bacillary dysentery, also known as shigellosis. *Bacillus dysenteriae*, also called *Shigella*, was first identified in 1897 by Kiyoshi Shiga during an epidemic in Japan, where it infected more than 91 000 people, causing a mortality rate of more than 20% (Trofa *et al.*, 1999).

*Escherichia coli/Shigella* was identified in all the symbiotic microbiomes with the exception of mammalian blood, which is very much anticipated, except in cases of bacteraemia or contamination. In soil and rhizosphere microbiomes, *Escherichia coli/Shigella* was identified in all the microbiomes with the exception of desert soil. However, *Escherichia coli/Shigella* is not usually isolated from desert soils. *Escherichia coli/Shigella* was identified in all environmental microbiomes. However, most of the identifications of *Escherichia coli/Shigella* in the metagenomes had scores below 1, depicted by a short orange bar (+/-), meaning they were not abundant in any of these habitats.

5.3.2.3 **Lactobacillus**

Lactic acid bacteria were mostly seen in various natural environments and were represented by precise lactobacilli compositions such as *L. acidophilus, L. delbrueckii* spp. *bulgaricus*. *Lactobacillus helveticus* are the classic representatives of the micro-flora of fermented milk products such as yoghurt and kefir, while the *L. casei* group, comprising *L. casei, L. paracasei* and *L. rhamnosus*, can be found in various types of cheese (Bouton *et al.*, 2002; Markiewicz *et al.*, 2010). *Lactobacillus delbrueckii* has also been illustrated as a strain producing biosurfactants and crude oil biodegrading compounds (Thavasi *et al.*, 2006). *Lactobacillus* is known to help prevent infections of the urogenital and intestinal tracts as
well. The dominance of *Lactobacillus* in the vagina is linked with a reduced risk of bacterial vaginosis and urinary tract infections (Reid and Burton, 2002).

*Lactobacillus* species were reliably identified (++) in each of the metagenomes used in this work, except for mammalian blood and Mediterranean bathypelagic.

### 5.3.2.4 Mycobacteria

Most mycobacterial species are ubiquitous and can be found in water, soil, food and vegetation. *M. bovis* infection has been developed by consuming unpasteurised milk. Bacilli Calmette-Guérin, which is a strain of *M. bovis*, is widely used for immunisation against tuberculosis. It is also administered as an immunotherapeutic agent for the treatment of superficial bladder carcinoma or melanoma. *Mycobacterium fortuitum* has been described as a normal flora of the skin (Eisenstadt, 1995). Pathogenic isolates of *Mycobacterium* include (i) *M. tuberculosis* — the causative agent of human tuberculosis; (ii) *M. bovis* — the causative agent of bovine tuberculosis; (iii) *M. leprae* — the causative agent of leprosy; (iv) *M. ulcerans*, which causes Buruli ulcers and is the third most common form of mycobacterial disease in humans; and (v) *M. marinum* — the causative agent of fish tank granuloma in humans and granulomatous lesions similar to those of *M. tuberculosis* in zebra fish (Demangel *et al.*, 2009; Rahman *et al.*, 2014). The non-pathogenic groups are *M. gilvum*, *M. vanbaalenii* and *M. smegmatis* (Raham *et al.*, 2014). Opportunistic pulmonary infections are mostly caused by members of the MAC that includes *M. avium* and *M. avium-M. intracellularare*, while Crohn’s disease in humans is suspected to be caused by the third member of the MAC group, *Mycobacterium avium* subsp. *paratuberculosis* (Cook, 2010; Chiodini *et al.*, 2012).

In the symbiotic microbiomes, *Mycobacteria* were identified in human gut (+/-) and cow gut (+/-), which was to be expected, for in the soil and rhizosphere microbiomes *Mycobacteria* were identified in all microbiomes; in environmental microbiomes *Mycobacteria* were identified in the anthropogenic estuarine (+/-) and sludge (+) environments.
5.3.2.5 Prochlorococcus

*Prochlorococcus* is a unicellular marine cyanobacterium, which is found throughout the euphotic zone of open ocean between 45°N and 40°S, where it carries out a significant portion of global photosynthesis (Partensky *et al.*, 1999; Flombauum *et al.*, 2013; Biller *et al.*, 2014). The genome of *Prochlorococcus* is the smallest of any known free-living photosynthetic cells, ranging from 1.6 to 2.7 Mbp (Kettler *et al.*, 2007). Though the core set of genes present is shared by all strains, notable diversity in the gene content was reported among isolates. The *Prochlorococcus* group has an open pan-genome, such that each newly sequenced genome typically contains various novel genes never identified before (Kettler *et al.*, 2007).

In the symbiotic microbiomes, *Prochlorococcus* was identified with a score above 1 with an orange bar (+) for canine gut/cow gut and human gut and (+/-) in termite gut, which was rather unexpected for these microbiota. Signature sequences of *Prochlorococcus* were also found in the soil and rhizosphere microbiomes, grassland, phyllosphere and rain forest had at least one organism reliably identified with a green bar (++); the forest rhizosphere returned a (+) identification, while for desert soil the result was (+/-). For environmental microbiomes, a hydrothermal vent showed a (+) identification, while the anthropogenic estuarine environment and sludge showed a (+/-) identification.

5.3.2.6 Salmonella

Whole genome sequencing of pathogens, immunological trials and characterisation of bacteria-host interactions at the cellular, humoral and mucosal level helped to generate a comprehensive view of the evolution and emergence of pathogens (Feasey *et al.*, 2012). *Salmonella typhimurium* or *Salmonella enterica* var Enteritidis (*S. enteritidis*), which are non-typhoidal *Salmonella*, have been reported to be the major cause of disease across Africa (Feasey *et al.*, 2012). Researchers have also reported disease outbreaks associated with the following serotypes: (i) *Salmonella enterica* var Isangi (*S. isangi*) in South Africa (Wadula *et al.*, 2006); (ii) *Salmonella enterica* var concord (*S. concord*) in Ethiopia (Beyene *et al.*, 2011); and (iii) *Salmonella enterica* var Stanleyville (*S. stanleyville*) and *Salmonella enterica* var Dublin (*S. dublin*) in Mali (Tennant *et al.*, 2010). Non-typhoidal *Salmonella* have been established as a major HIV-related pathogen in sub-Saharan African adults (Gilks *et al.*, 1990). While non-typhoidal *Salmonella* have a broad range of hosts among humans and
animals, the typhoidal serotypes *S. typhi* and *S. paratyphi A* are totally host-constrained to people, causing invasive disease in immune-competent individuals (Feasey *et al*., 2012).

In this work, *Salmonella* was in the canine gut (+) micro-flora representing symbiotic microbiomes. In the soil and rhizosphere microbiomes, *Salmonella* strains were identified in grassland (++)). Weak signals of the presence of *Salmonella* were also seen in the forest rhizosphere (+/-), phyllosphere (+/-), rain forest (+/-) and soybean rhizosphere (+/-) metagenomes. *Salmonella* was not identified in desert soil. In the environmental microbiomes, *Salmonella* was identified in the sludge metagenome (+) and probably in the anthropogenic estuarine (+/-) environment.

5.3.2.7 *Shewanella*

*Shewanella* genus microorganisms are facultative anaerobes, Gram-negative gamma-Proteobacteria found in various environments, but mostly in marine sediments and frequently in association with fish (Ivanova *et al*., 2004; Dikow 2011; Wright *et al*., 2016). *Shewanella* species signify a vital family of dissimilatory metal-reducing bacteria, which enables the transmission of metabolically produced electrons from a cell interior to external electron acceptors such as solid metal oxides during anaerobic respiration (Fredrickson *et al*., 2008; Wang *et al*., 2011).

Weak signals of the presence of *Shewanella* were unexpectedly recorded in this study in symbiotic human gut, termite gut and cow gut with a (+/-). In the soil and rhizosphere metagenome, the rain forest and phyllosphere had a (++) identification, grassland (+), forest rhizosphere (+/-), desert soil (+/-) and soybean rhizosphere (+/-). Again unexpectedly, in the environmental microbiomes *Shewanella* was identified in the anthropogenic estuarine and hydrothermal vent with a weak signal (+/-).

5.3.2.8 *Streptococcus*

The genus *Streptococcus* currently comprises more than 100 recognised species and the number of these species is expected to rise with the increasing availability of next-generation sequencing technologies (Spellerberg and Brandt, 2015). *Streptococcal* bacteria are linked to the development of dental caries. Oral cavity microbes are usually referred to as viridans
streptococci because of the greenish pigmentation produced by these bacteria when grown on blood agar. This reaction is often termed alpha-haemolysis and is suggestive of the presence of hydrogen peroxide production (Nobbs et al., 2009).

*Streptococcus pneumonia* is a Gram-positive coccus and a member of the lactic acid bacteria, which has been described as one of the foremost sources of morbidity and mortality worldwide. The WHO reported that approximately 1 million children die of pneumococcal disease every year in third-world countries (Hoskin et al., 2001; WHO/UNICEF, 2005; WHO, 2007). Pneumococcal infections have been reported to be the foremost cause of death from vaccine-preventable illnesses in children younger than five years (CDC, 2006). Invasive diseases caused by pneumococci include meningitis and pneumonia associated with bacteraemia and emphysema. The risk factors for developing invasive IPD include age, with the highest risk of incidence among young children less than two years old and elderly people older than 65 years; ethnicity and geographic location, with the ability to attend care centres being the main factor; as well as associated chronic sickness (Fletcher et al., 2006; WHO, 2007; Isaacman et al., 2010).

*Streptococcus* was well identified in symbiotic microbiomes in this study, with most having a (+) and (++) identification. However, the mammalian blood metagenome, which is believed to be sterile, also showed a (++) sign, which could be possible in cases of bacteraemia and contamination. In the soil and rhizosphere microbiomes, most identification was of the weak (+/-), signifying that at least one organism had been identified with a score below 1, with an orange bar. *Streptococcus* was not identified in desert soil and the soybean rhizosphere. In environmental microbiomes, *Streptococcus* was well identified in anthropogenic estuarine (++), hydrothermal vent (++) and sludge (+) environments.

In Table 5.3, the researcher looked at the total number of reads in some of the metagenomes (canine gut, grassland, phyllosphere and hydrothermal vent) used, how many of those reads were binned to species of interest using BLASTN alignment against the *nt* NCBI database and to which extent these results corresponded with the barcoding results of this study. The total number of reads in the canine gut metagenome used in this study was 99 125, of which 83 were aligned by BLASTN to *Bacillus cereus*, 138 to *Escherichia coli* and *Shigella*, 544 to *Lactobacillus*, 18 to *Mycobacteria*, 0 to *Prochlorococcus*, 10 to *Salmonella*, 0 to *Shewanella* and 256 to *Streptococcus* (see Figure 5.1). Alignment of these reads against the diagnostic
barcodes by the program Barcoding 2.0 confirmed their presence in *Bacillus cereus*,
*E. coli/ Shigella*, *Lactobacillus*, *Salmonella* and *Streptococcus*, and also the absence of
*Shewanella* in this metagenome. However, *Mycobacteria* were not identified in canine gut
micro-flora by the program, despite the presence of mycobacterial reads identified by the
program MEGAN (Figure 5.1). The reason for this is that all mycobacterial reads binned by
MEGAN were assigned to the species *M. terrae*, which was not present among diagnostic
barcodes generated for the group Mycobacteria. The Barcoding program identified some
signals of *Prochlorococcus*. Indeed, the BLASTN search identified 133 reads of
Cyanobacteria in canine gut microflora, of which 37 were binned to *Chroococcales*;
however, MEGAN did not resolve the taxonomy of these reads to the species level. One has
to conclude that the presence of *Prochlorococcus* species in the canine gut was not
confirmed.

For the soil and rhizosphere metagenome, the grassland metagenome used yielded a total of
134 368 reads; 24 of them were aligned to *Bacillus cereus*, seven to *Escherichia
coli/Shigella*, 23 to *Lactobacillus*, 4 796 to *Mycobacteria*, five to *Prochlorococcus*, 13 to
*Salmonella*, 19 to *Shewanella* and seven to *Streptococcus*. In total 4 907 reads were binned
to microorganisms of interest used in the case studies. The phyllosphere metagenome yielded
a total of 1 933 702 reads, of which nine aligned to *Bacillus cereus*, 13 to *Escherichia
coli/Shigella*, 40 to *Lactobacillus*, 4 024 to *Mycobacteria*, 29 to *Prochlorococcus*, 29 to
*Salmonella*, 60 to *Shewanella* and 34 to *Streptococcus*. In total 4 221 reads were assigned to
the microorganisms of interest. Mycobacteria were the most abundant among bacterial taxa
barcoded in this study; however, they were not scored high by the program Barcoding. The
reason for this may be that the genes selected for barcodes were too conserved among
mycobacterial species and aligning of reads among multiple barcodes would diminish the
final score. The current version of the program does not allow any direct control of the level
of sequence similarity between diagnostic barcode sequences at the time of their generation
by the program BarcodeGenerator. This function will be added to the next version of the
program to improve the sensitivity of diagnostic barcodes.

Barcoding of other taxonomic groups confirmed the binning results obtained by MEGAN;
however, the barcode scoring did not correlate with the numbers of reads binned to these taxa
by the program MEGAN. This may be explained by the fact that MEGAN usually does not
allow resolving of taxonomy below the genus level and the barcode sequences generated for these case studies were species- and strain-specific.

In the environmental microbiomes, the hydrothermal vent metagenome comprised 11,326 reads, 28 of which were aligned to *Bacillus cereus*, 0 to *Escherichia coli/Shigella*, 14 to *Lactobacillus*, 0 to *Mycobacteria*, 19 to *Prochlorococcus*, 0 to *Salmonella*, 15 to *Shewanella* and 11 to *Streptococcus*. In total 87 reads were assigned by MEGAN to the microorganisms of interest. Very unexpected were strong signals of *E. coli* and *Streptococcus* returns by the program Barcoding for this rather exotic environment, where none of these microorganisms could be expected. Binning of the reads by BLASTN against the *nt* database confirmed the presence of *Streptococcus*, but not *E. coli*. This discrepancy can be explained by contamination of the sample with a small amount of *E. coli* DNA from humans working with these samples. Barcodes are sensitive and can identify the presence of this DNA, while MEGAN reports taxonomic units only if the number of assigned reads is above a certain cutoff value.

A stronger scoring of Shewanella barcodes could be expected for this marine environment. MEGAN has identified DNA reads generated from *S. violacea*, which was barcoded for this study. Low scoring may be explained by the same problem that was reported for *Mycobacteria* – a high level of sequence similarity between the generated diagnostic barcodes that reduces the specificity of the barcodes.

Table 5.3: Shows the total number of reads for some of the metagenomes used and the results obtained.

<table>
<thead>
<tr>
<th>METAGENOMES</th>
<th>B. cereus</th>
<th>L. acidophilus</th>
<th>Lactobacillus</th>
<th>Mycobacteria</th>
<th>Prochlorococcus</th>
<th>Salmonella</th>
<th>Shewanella</th>
<th>Streptococcus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine gut</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+ S. enetica - 18</td>
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<td>+</td>
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<tr>
<td></td>
<td>83</td>
<td>138</td>
<td>544</td>
<td></td>
<td>0</td>
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<tr>
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<td>+/−</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++ S. baltica - 19</td>
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<td>4796</td>
<td>5</td>
<td>13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Phyllosphere</td>
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<td>+/−</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+/− S. anginosus</td>
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<td>+/−</td>
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<tr>
<td></td>
<td>9</td>
<td>13</td>
<td>40</td>
<td>4024</td>
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In the following sections, the consistency of identification of different barcoded taxonomic groups by Barcoding 2.0 was determined.

5.4. Consistency of identification of taxonomic groups in real metagenomes

The following study was conducted with the aim to validate identification of species and predict the species content of metagenomic samples.

5.4.1 Analysis of Lactobacillus in different metagenomes

5.4.1.1 Gut micro-flora

Twenty-eight strains from the Lactobacillus group representing different species and subspecies, including commercial and biotechnological potential strains, were used to generate genetic barcodes for Lactobacillus (Chapter 2). When DNA reads from the canine gut metagenomic datasets obtained from the MG-RAST database were aligned with BLASTN using the Barcoding 2.0 program, strong signals were obtained for strains of the following Lactobacillus species (Figure 5.5A): (i) Lactobacillus salivarius [strain UCC118 (NC-007929)] (ii) Lactobacillus delbruecki subsp. bulgaricus [strain ATCC BAA-365 (NC-008529)] (iii) Lactobacillus fermentum [strain IFO 3956 (NC-010610)] (iv) Lactobacillus casei [strain. Zhang (NC-014334)] (v) Lactobacillus brevis [strain ATCC 367 (NC-008497)] (vi) Lactobacillus reuteri [strain JCM 1112 (NC-010609)] (vii) Lactobacillus sanfranciscensis [strain TMW (NC-015978)] and (viii) Lactobacillus kefiranofaciens [strain ZW3 (NC-015602)]. This is in agreement with other studies, which show the abundance of L. acidophilus, L. rhamnosus, L. salivarius, L. fermentum and L. reuteri in canine gut (Pasupathy et al., 2001; Beasley et al., 2006; McCoy and Gilliland, 2007). Lactobacillus fermentum has been researched to help prevent and treat urogenital infections and to be effective in inhibiting the growth of harmful bacteria in the canine body (Beasley et al., 2006). Lactobacillus reuteri has been studied in dogs and cats; it is known to inhibit the growth of harmful bacteria as well as support the production of natural antibiotic-like substances (McCoy and Gilliland, 2007). Lactobacillus salivarus produces a large quantity of lactic acid, which helps to stop the growth of Helicobacter pylori, hence reducing the inflammation and risk of dogs with peptic ulcers and irritable bowel syndrome (Beasley et al., 2006).
However, the metagenome analyser MEGAN identified 25 different species of *Lactobacillus* (Figure 5.6) in the canine gut, including those not identified by Barcoding 2. The researcher believes that a subsequent version of Barcoding 2.0 released would identify many other species/strains that was not picked up by this version.

Compared to canine gut, *Lactobacillus amylovorus* [strain GRL 1112 (NC-014724)] yielded a very strong signal with a green bar (Figure 5.5B). *Lactobacillus amylovorus* is a widely abundant specie of *Lactobacillus* found in the intestines of piglets and cows. It is known to have various probiotic properties, such as antimicrobial activity against enteric pathogens (Kant *et al.*, 2011). *Lactobacillus amylovorus* has also been isolated in the bovine uterus, possessing immunomodulatory properties of endometrial cells (Gärtner *et al.*, 2015). Some species of *Lactobacillus* were identified in both canine and cow gut: (i) *Lactobacillus salivarius* [strain UCC118 (NC-007929)]; (ii) *Lactobacillus gasseri* [strain ATCC 33323 (NC-008530)]; (iii) *Lactobacillus fermentum* [strain IFO 3956 (NC-010610)]; (iv) *Lactobacillus casei* [strain Zhang (NC-014334)]; and (v) *Lactobacillus reuteri* [strain JCM 1112 (NC-010609)].

In human gut metagenome *Lactobacillus casei* [strain Zhang (NC-014334) and *Lactobacillus sanfranciscensis* [strain TMW (NC-015978)] showed very strong signals with green bars (Figure 5.5C). A study by Zhang *et al.* in 2017 proved that *L. casei zhang* or vitamin k12 could significantly alleviate the intestinal tumour burden in mice (Zhang *et al.*, 2017). *Lactobacillus sanfranciscensis* is the main bacterium and probably the most recently used species in the production of traditionally fermented sourdoughs. *Lactobacillus sanfranciscensis* contributes to dough rheology and flavour properties owing to solid acidification by an optimised carbohydrate metabolism and the liberation of precursors of volatile compounds by a proteolytic system and the catabolism of specific amino acid (Vogel *et al.*, 2011). Other *Lactobacillus* species that showed strong signals in orange in the human gut were: (i) *Lactobacillus crispatus* [strain ST1 (NC-014106)]; (ii) *Lactobacillus salivarius* [strain UCC118 (NC-007929)]; (iii) *Lactobacillus fermentum* [strain IFO 3956 (NC-010610)]; (iv) *Lactobacillus buchneri* [strain NRRL B-30929 (NC-015428)]; (v) *Lactobacillus rhamnosus* [strain Le 705 (NC-013199)]; (vi) *Lactobacillus casei* [strain BL23 (NC-010999)]; (vii) *Lactobacillus sakei* subsp. sakei 23k chromosome (NC-007576); (viii) *Lactobacillus reuteri* [strain JCM 1112 (NC-010609)]; and (ix) *Lactobacillus johnsonii* [strain F19785 (NC-013504)], while *Lactobacillus plantarum* [strain WCFS1 (NC-004567)]
yielded a strong signal with a score above 1 with an orange bar. In termite gut *Lactobacillus casei* str. Zhang (NC-014334) produced strong signals with green bars and scores above 1 (Figure 5.5D).

In this study, *Lactobacillus casei* str. Zhang (NC-014334) was identified in the four metagenomes (canine, cow, human and termite) used. All species of *Lactobacillus* identified in the gut micro-flora represented strains and species that were of commercial and biotechnological importance. The performance of Barcoding 2.0 was average in the identification of different *Lactobacillus* species/strains in the four metagenomes used for gut micro-flora in this study.

![Figure 5.5: Lactobacillus specie profile in: (A) canine gut (B) cow gut (C) human gut and (d) termite gut.](image)

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5.4.1.2 Plant-associated micro-flora

Barcoding 2.0 performed averagely in identifying the species and strains of *Lactobacillus* in plant-associated micro-flora. In plant-associated micro-flora, the *Lactobacillus* specie profile was quite similar for all metagenomes used in this study. However, the *Lactobacillus* specie profile for desert soil was slightly different, as *Lactobacillus* species are not commonly isolated in desert soils. Studies from across the world show that desert soil typically contains a number of ubiquitous phyla, which include actinobacteria, bacteriodetes and proteobacteria (Channal *et al.*, 2006; Makhalanyane *et al.*, 2015). This explains why *Lactobacillus* specie profiles in desert soil were mostly orange bars with scores above 1 (Figure 5.7A).

*Lactobacillus* species with stronger signals with scores above 1 and green bars were mostly identified in the grassland forest, forest rhizosphere, phyllosphere, rain forest and soybean rhizosphere. Strains of *Lactobacillus* species with strong signals in the grassland metagenome include: (i) *Lactobacillus amylovorus* GRL 1112 (NC-014724); (ii) *Lactobacillus buchneri* NRRL B-30929 (NC-015428); (iii) *Lactobacillus casei* BL23 (NC-010999); and (iv) *Lactobacillus sakei* subsp. sakei 23k chromosome (NC-007576).
*Lactobacillus amylovorus* is known to show features of a common homofermentative *Lactobacillus* species, such as the production of enormous quantities of lactic acid and small amounts of acetic acid, but no gas from glucose. *Lactobacillus amylovorus* is one of the main S-layer-carrying *Lactobacillus* species in pigs. It shows strong adherence to pig intestinal epithelial cells and is of interest because of its potential probiotic properties (Kant *et al*., 2011). Since most pigs do readily graze on grassland, this probably explains why *Lactobacillus amylovorus* GRL 1112 (NC-014724) was clearly identified in the grassland metagenome used in this study.

*Lactobacillus buchneri* is a specie relevant for commercial silage, bioethanol and vegetable fermentations (Briner and Barrangou, 2014). Under anaerobic conditions *Lactobacillus buchneri* is known to use lactic acid to ferment cucumber (Franco *et al*., 2012). *Lactobacillus casei* is a specie of *Lactobacillus* that is used in several foods, agricultural and industrial fermentations. This leaves room for genetic manipulation of *L. casei*, which can be undertaken to understand their physiological and biochemical properties and allows for the progress of industrial strains (Welker *et al*., 2014). *Lactobacillus sakei* is a psychrotrophic lactic acid bacterium found naturally on fresh meat and fish. *Lactobacillus sakei* strain 23k identified in the grassland metagenome in this study was originally isolated from French sausage with specific reference to survival aspects and competition with other meat-borne bacteria. *Lactobacillus sakei* is generally mostly used in the manufacture of fermented meats and has biotechnological potential in biopreservation and food safety (Chaillou *et al*., 2005) (Figure 5.9B).

For other metagenomes (forest rhizosphere, phyllosphere, rain forest and soybean-rhizosphere, Figure 5.7C - Figure 5.7F) used for plant-associated micro-flora, the specie composition for *Lactobacillus* specie was similar to that of the grassland metagenome. All species/strains of *Lactobacillus* identified in the plant-associated micro-flora metagenome in this study are of biotechnological and commercial importance.
Figure 5.7: *Lactobacillus* specie profile in: (A) desert soil (B) grass land (C) forest rhizosphere (D) phyllosphere (E) rain forest and (F) soybean -rhizosphere.
5.4.1.3 Environmental micro-flora

The composition of estuary water is known to be very complex and to vary depending on the degree of anthropogenic inference (Edet et al., 2018). Strong signals were obtained for *Lactobacillus salivarius* [strain UCC118 (NC-007929)] and *Lactobacillus sanfranciscensis* [strain TMW (NC-015978)] in the anthropogenic-estuarine metagenome in this study (Figure 5.8A). *Lactobacillus salivarius* helps to inhibit the growth of *H. pylori*, hence reducing the associated inflammation and risk for dogs with peptic ulcers and irritable bowel syndrome (Beasely et al., 2006), while *Lactobacillus sanfranciscensis* has been described as probably the best adapted specie and is also regarded as the autochthonous key organism of sourdough microbiota (Vogel et al., 2011). *Lactobacillus* specie composition was similar for the anthropogenic-estuarine metagenome and the hydrothermal vent; a strong signal was also obtained in the hydrothermal vent for *Lactobacillus sanfranciscensis* (Figure 5.8B).

The *Lactobacillus* specie composition for the sludge metagenome was slightly different from that of the anthropogenic-estuarine and the hydrothermal vent (Figure 5.8C). Strong signals were obtained for the following strains of *Lactobacillus* species in the sludge: *L. gasseri* [strain ATCC 33323 (NC-008530)], *L. amylovorus* [strain GRL 1112 (NC-014724)] and *L. acidophilus* [strain 30s (NC-015214)] (Figure 5.8C). *Lactobacillus acidophilus* can be isolated everywhere, both in humans and dogs. It is known to have the ability to cling to the intestinal wall without harming it. *Lactobacillus acidophilus* is a stable for any probiotic supplement (Pasupathy et al., 2001), while *L. gasseri* is another specie of *Lactobacillus* widely used as a probiotic for fermented products (Tada et al., 2017).
**5.4.2 Analysis of Mycobacteria in the phyllosphere and grassland**

Sixteen strains from the *Mycobacteria* group representing different species and subspecies, including commercial and biotechnological potential strains, were used to generate genetic barcodes for *Mycobacteria* (Chapter 2). When DNA reads from the phyllosphere and grassland metagenomic datasets obtained from the MG-RAST database were aligned with BLASTN using the Barcoding 2.0 program, the *Mycobacteria* species profile obtained in the phyllosphere and grassland were quite similar to the same species of *Mycobacteria*. This is, however, not surprising, as these two metagenomes are somewhat similar. The phyllosphere represents the interface between the above-ground parts of the air. A conservative estimate shows that roughly 1 billion square kilometres of the worldwide leaf surfaces host more than $10^{26}$ bacteria, which are the most abundant colonizers of the habitat (Delmotte *et al.*, 2009). Grasslands are among the largest ecosystems in the world; their area is estimated at 52.5 million square kilometres or 40.5 percent of the terrestrial area, excluding Greenland and Antarctica.
Most species of Mycobacteria are ubiquitous and can be found in water, soil, food and vegetation (Eisenstadt, 1995). Signals were obtained for the following strains of Mycobacteria species in grassland and the phyllosphere metagenome: (i) Mycobacterium vanbaalenii [strain PYR-1 (NC-008726)]; (ii) Mycobacterium sp. [strain JDM601 (NC-015576)]; (iii) Mycobacterium abscessus (NC-010397); (iv) Mycobacterium marinum M (NC-010612); (v) Mycobacterium intracellulare MOTT-02 (NC-016947); (vi) Mycobacterium sp. MOTT36Y (NC-017904); (vii) Mycobacterium avium 104 (NC-008595); (viii) Mycobacterium avium subsp. paratuberculosis k-10 (NC-002944); (ix) Mycobacterium sp. MCS (NC-008146); (x) Mycobacterium sp. KMS (NC-008705); and (xi) Mycobacterium sp. JLS (NC-009077) (Figure 5.9).

![Figure 5.9: Mycobacteria specie profile in the (A) grassland and (B) phyllosphere metagenome.](image)

However, the metagenome analyser MEGAN identified 29 different species of the Mycobacteria phyllosphere and 30 in the grassland (Figure 5.10A/5.10B), including those not identified by Barcoding 2. The researcher believes that a subsequent version of Barcoding 2.0 released would identify many other species/stains that were not picked up by this version.
Figure 5.10: MEGAN analysis showing the different species of Mycobacteria in A) phyllosphere and B) grassland metagenome.
5.4.3. Identification of *Streptococcus* in various metagenomes

5.4.3.1 Analysis of *Streptococcus* in symbiotic microbiomes

The gut metagenome is rich with diverse groups of microorganisms. Hence, it was interesting to investigate if the species of *Streptococcus* seen in human gut, cow gut and canine gut were similar, using the Barcoding 2.0 program. In this study very strong signals were obtained of *Streptococcus equi* subsp. zooepidemicus (NC-012470) and *Streptococcus oralis* Uo5 (NC-015291) in human gut. *Streptococcus equi* subsp. zooepidemicus (NC-012470) infection is uncommon in humans. However, a case-control study by Bordes-Benítez *et al.* in 2006 proved that the consumption of inadequately pasteurised cheese of a specific brand was associated with *Streptococcus equi* subsp. zooepidemicus disease (Bordes-Benítez *et al.*, 2006). *Streptococcus oralis* is a commensal specie of the human oral cavity and belongs to the Mitis group of *Streptococci* ((Reichmann *et al.*, 2011). *Streptococcus oralis* Uo5 is a known high-level penicillin- and multiple-antibiotic-resistant isolate from Hungary. It is competent for genetic transformation under laboratory conditions. Hence, the comparative and functional genomics of *Streptococcus oralis* Uo5 will be of importance in understanding the evolution of pathogenesis among the Mitis *Streptococci* and their potential to engage in interspecies gene transfer (Reichmann *et al.*, 2011). Eleven species of *Streptococcus* were picked up in canine gut by the MEGAN (Figure 5.12)

In the cow metagenome, *Streptococcus pyogenes* [strain M1 GAS (NC-002737)], *Streptococcus pyogenes* [strain MGAS8232 (NC-003485)] and *Streptococcus macedonicus* [strain ACA-DC (NC-016749)] yielded strong signals with green bars and scores above 1 (Figure 5.11B). *Streptococcus pyogenes*, also referred to as GAS, for harbouring Lancefield group A antigen, is a clinically important human pathogen commonly associated with skin or throat infections, but can also cause life-threatening situations including sepsis, streptococcal toxic shock syndrome and necrotising fasciitis (Ibrahim *et al.*, 2011). Physical contact between man and cow probably explains why it was identified in the cow metagenome used in this study. *Streptococcus macedonicus* belongs to the *Streptococcus bovis/Streptococcus equinus* complex (SBSEC) and is mostly isolated from fermented foods, mainly of dairy origin. Members of the SBSEC have been implicated in human endocarditis and colon cancer (Papadimitriou *et al.*, 2014). The *Streptococcus* specie composition in human gut and the canine metagenome was quite similar. However, in the canine metagenome, the majority of *Streptococcus* species identified had strong signals with orange bars and scores above 1.
In mammalian blood *Streptococcus equi* subsp. zooepidemicus (NC-012470) was very strongly dominant with green colours and scores of 1. However, the researcher believes that this was a form of contamination (Figure 5.11C).

Figure 5.11: *Streptococcus* specie profile in (A) human gut (B) cow gut (C) mammalian blood and (D) canine gut.
5.4.3.2 Analysis of *Streptococcus* in environmental metagenomes

The composition of estuary water is known to be very complex and to vary depending on the degree of anthropogenic inference (Edet *et al.*, 2018). In the anthropogenic estuarine environment *Streptococcus pyogenes* [strain MGAS10750 (NC-008024)] yielded a very strong signal with a green bar and score above 1. *Streptococcus pyogenes* is a clinically important human pathogen commonly associated with skin or throat infections, but can also cause life-threatening situations including sepsis, streptococcal toxic shock syndrome and necrotising fasciitis (Ibrahim *et al.*, 2011). In the hydrothermal vent metagenome a strong signal was obtained for *Streptococcus equi* subsp. zooepidemicus (NC-012470). However, the researcher believes that the identification of *Streptococcus equi* subsp. zooepidemicus (NC-012470) in the hydrothermal vent was a form of contamination. Moreover, *Streptococcus* is not a known hydrothermal microorganism. The *Streptococcus* specie composition for the anthropogenic estuarine and sludge environments was similar. In the sludge, signals were obtained for: (i) *Streptococcus pneumonia* [strain G54 (NC-0011072)]; (ii) *Streptococcus pyogenes* [strain MGAS10750 (NC-008024)]; (iii) NC-002737; (iv) *Streptococcus pyogenes* [strain MGAS8232 (NC-003485)]; (v) *Streptococcus parauberis* KCTC 11537 (NC-015558); (vi) *Streptococcus macedonicus* [strain ACA-DC (NC-016749)]; and (vii) *Streptococcus gordonii* Str. Challis substr. CH1 (NC-009785) with orange bars and scores above 1 (Figure 5.13).
Figure 5.13: *Streptococcus* specie profile in: (A) anthropogenic estuarine (B) hydrothermal vent and (C) Sludge.

5.4.4 Analysis of *Escherichia coli/Shigella* in the hydrothermal vent metagenome.

Thirty-seven strains from the *Escherichia coli/Shigella* group representing different species and subspecies, including commercial and biotechnological potential strains, were used to generate genetic barcodes for *Escherichia coli/Shigella* (Chapter 2). When DNA reads from the hydrothermal vent metagenomic datasets obtained from the MG-RAST database were aligned with BLASTN using the Barcoding 2.0 program, strong signals were obtained for strains of the following *Escherichia coli/Shigella* species shown in Figure 5.14: (i)
Escherichia coli [strain BW2952 (NC-012759)]; (ii) Escherichia coli O127:H6 [strain E2348/69 (NC-011601)]; (iii) Escherichia coli [strain 536 (NC-008253)]; (iv) Escherichia coli [strain IA139 (NC-011750)]; and (v) Shigella boydii [strain Sb227 (NC-007613)] as the most dominant species in the hydrothermal vent, with green bars and scores above 1. Species of Escherichia coli and Shigella are mostly isolated in clinical samples from patients with diarrhoea and show high resistance to antibiotics (Nguyen et al., 2005). Thermophiles are the most common microorganisms seen in hydrothermal vents. The presence of Escherichia coli/Shigella in the hydrothermal vent could be due to pollution from the human gastrointestinal tract.

5.4.5 Analysis of Shewanella in the phyllosphere and rain forest

Shewanella is a mostly aquatic microorganism. It may, however, be isolated or carried by insects, worms or amphibians to areas such as the phyllosphere and rain forest. Shewanella was clearly identified in the phyllosphere and rain forest in this study using the Barcoding 2.0 program. Shewanella baltica [strain OS195 (NC-00997)] and Shewanella violacea [strain DSS12 (NC-014012)] were identified in the phyllosphere and rain forest metagenome with green bars. However, Shewanella violacea [strain DSS12 (NC-014012)] was seen in a very
small proportion of the rain forest (Figure 5.15). *Shewanella* specie composition was different in the hydrothermal vent compared to the phyllosphere and rain forest. MEGAN analysis picked up three species of *Shewanella* in the phyllosphere, namely *Shewanella amazonensis*, *Shewanella baltica* and *Shewanella lohica* (Figure 5.16)

**Figure 5.15: Shewanella species profile in the phyllosphere and rain forest metagenome.**

**Figure 5.16: MEGAN analysis showing species of Shewanella in the phyllosphere.**

5.4.6 Analysis of *Prochlorococcus* in the gut and environmental metagenomes

*Prochlorococcus* are well known marine cyanobacteria. Barcode sequences were created for 12 strains of *Prochlorococcus marinus* (Chapter 2). When DNA reads from cow gut metagenomic datasets obtained from the MG-RAST database were aligned with BLASTN using the Barcoding 2.0 program, strong signals were obtained for *Prochlorococcus marinus*
str.MIT 9301 (NC-009091) in the cow gut. *Prochlorococcus marinus* is the dominant photosynthetic organism in the ocean. It is usually seen in two major ecological forms: HL-adapted genotypes in the upper part of the water column and LL-adapted genotypes at the bottom of the illuminated layer (Dufresne et al., 2003). The grazing of cows in grassland areas close to streams and oceans probably explains why *Prochlorococcus marinus* str.MIT 9301 (NC-009091) was identified in the cow metagenome. *Prochlorococcus* specie composition in canine gut was very different compared to cow gut, as more *Prochlorococcus marinus* strains were identified: (i) *Prochlorococcus marinus* str. NATL2A (NC-007335); (ii) *Prochlorococcus marinus* str. MIT 9313 (NC-005071); (iii) *Prochlorococcus marinus* subsp. marinus str. CCMP1375 (NC-005042); (iv) *Prochlorococcus marinus* str. MIT 9312 (NC-007577); and (v) *Prochlorococcus marinus* str. MIT 9215 (NC-009840). Wild canine animals do feed on everything (vegetable matter, rotten fruit and semi-digested contents of their prey’s stomach) and also drink water from streams and oceans, hence this explains why different strains of *Prochlorococcus marinus* were identified in canine gut.

The specie composition of *Prochlorococcus* in the grassland, phyllosphere and rhizosphere metagenome was similar; *Prochlorococcus marinus* str.MIT 9301 (NC-009091) yielded strong signals in the three metagenomes. These metagenomes are similar and close to the streams and oceans where *Prochlorococcus* are mostly found (Figure 5.17 and Figure 5.18).

![Figure 5.17: Prochlorococcus specie profile in the cow and canine gut metagenome.](image)
Figure 5.18: Prochlorococcus specie profile in: (A) hydrothermal vent (B) grassland (C) phyllosphere and (D) rain forest.

5.5 Conclusion

In this chapter, the novel command-line program Barcoding 2.0 (Chapter 3) was used for binning of metagenomic reads of different metagenomes obtained from MG-RAST.

The MEGAN program was first used to estimate and interactively explore the taxonomical content of the dataset used, by using the NCBI taxonomy to summarise and order the results.
The results from the MEGAN analysis gave researchers an idea of how well the novel program Barcoding 2.0 performed.

An attempt was then made to test the barcode sequences created by the program BarcodeGenerator (Chapter 2) for selected genomes of various bacteria used as case studies on real metagenomic datasets obtained from MG-RAST using the Barcoding 2.0 program. Compared to the MEGAN program, Barcoding 2.0 performed averagely in the identification of microorganisms. The microorganisms identified by Barcoding 2.0 were of biotechnological and commercial importance. However, the researcher believes that newer versions of Barcoding 2.0 released in the near future will perform much better.
References


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CHAPTER 6 General conclusion

6.1 Summary

Bacteria and archae (prokaryotes) make up a substantial percentage of the living biomass on earth and help to sustain the geochemical element cycles: an enormously complicated, planetary scale metabolic network (Strous et al., 2012). Prokaryotes form intricate ecological communities comprising an assembly of different species and only a small quota of these species has been cultivated in the laboratory, has been studied experimentally and has a known genome sequence (Strous et al., 2012).

Hence, with the express development of next-generation sequencing methods, metagenomics, also known as environmental metagenomics, has emerged as a thrilling research area that allows the analysis of the microbial environment which humans live (Leung et al., 2011). The DNA fragments of a metagenomics project are usually from several genomes and most of the genome sequences are unidentified. A vital phase in metagenomic analysis is grouping DNA fragments from similar species together, which is referred to as “binning” (Mavromatis et al., 2007), to determine the microbe distribution of the sample and classify species within the sample. Subject to different research requirements, the binning process could be done on different taxonomic levels ranging from kingdom to species (Leung et al., 2011).

Moreover, the choice of binning methods for any metagenomic dataset is steered by the length of reads that make up the datasets. The performance of binning methods centred on the comparison of compositional characteristics of sequences is reliable only for longer sequences, having sufficient lengths to stem a robust compositional signature (Dutta et al., 2014). Sequence alignment-centred binning methods perform better for an extensive range of read lengths. Of all the NGS technologies, Roche 454, Illumina and Ion Torrent systems are most frequently used for metagenomic samples (Mardis 2010; Metzker 2010). The Illumina sequencers offer excellent sequencing throughput and result in read lengths of about 100-200 bp; Roche-454 sequencing technology produces relatively longer reads around 400-600 bp, with suggestively lower throughput (Dutta et al., 2014). Recently, Roche 454 became obsolete and gave way to new technologies: PacBio, MinION and Oxford Nanopore, which produce long reads up to 20 Gbp. However, public databases still contain many metagenomic datasets generated by older technologies.

The aim of this study was to create multi-locus genetic barcodes for identification and
tracking down of biotechnological and/or pathogenic strains in the environment and
development of software tools and databases for design and utilisation of genetic barcodes for
application in biotechnology and medicine. To achieve this aim, an attempt was made to
improve the metagenome binning resolution by creating genome-specific barcodes based on
larger selections of core and accessory gene sequences. This protocol was implemented in
novel software tools available for use and downloaded from http://bargene.bi.up.ac.za/
BarcodeGenerator is a novel online software tool, which for any given set of genomes would
compare all pairs of genes in the genomes and select the most appropriate COG for
diagnostic barcodes. The appropriateness of COG for barcoding is estimated by an analysis
of alignments of respective DNA and protein sequences, as described in detail in Chapter 2.
It was assumed that barcodes have to comprise important core genes under pressure of
positive evolutionary selections, which should be distinguishable on the level of species and
subspecies, but sufficiently conserved to allow unambiguous identification. To improve the
sensitivity of the barcode sequences created, accessory genes that are genome-specific may
be added to the barcode sequences. Hence, the program allows the addition of accessory
genes to constitute the barcodes in user-defined proportions. The program was implemented
in the form of a web application that allows uploading of genome sequences of organisms of
interest and then returns a link to the user’s e-mail address to the generated barcode
sequences in FASTA format, information on the genes selected for barcodes and a graphical
file in SVG format. BarcodeGenerator was used to create barcode sequences for different
microorganisms used as case studies in this work; all of them were made available from the
project website (http://seqword.bi.up.ac.za/barcoder_help_download/index.html). The strains
used in the case studies represent different species and subspecies, including pathogenic and
biotechnological strains.

To check the consistency of the selection of marker genes, the researcher also investigated
the evaluation of ontology terms of genes selected for barcodes in different groups of
microorganisms by the BarcodeGenerator. Among the core genes selected for barcodes, the
most abundant groups was the gene-encoding ribosomal proteins, enzymes of purine and
pyrimidine biosynthetic pathways, ABC transporters, tRNA synthetases and
amidotransferases, various oxidoreductases, acyl carrier proteins and several other functional
categories. Constituents of the central metabolic pathways were expected to be among the
conserved genes involved in bacterial speciation and suitable for barcoding. For example,
ribosomal proteins comprised up to 15% of the sequences selected for barcodes by the
program BarcodeGenerator. This finding was in agreement with many publications reporting ribosomal proteins as the most suitable taxonomic and phylogenetic markers used in rMLST (Jolley et al., 2012; Glaeser and Kämpfer, 2015).

Among accessory genes selected for barcodes, the most frequently selected ones were IS1 and IS2 transposases and Orf2/OrfB genes, Ynhf-type membrane proteins, phage-related transcriptional regulators and capsular polysaccharide biosynthesis proteins and other mobile elements abundant in bacterial populations. The case study with closely related organisms of *Escherichia* and *Shigella* demonstrated that including accessory genes in barcodes worsens the specificity of the methods, as the mobile elements were shared by all these microorganisms in a random fashion. Accessory genes may improve the sensitivity of the methods when more diverse organisms are to be distinguished, as mobile genetic elements are mostly clade- and species-specific.

Barcode sequences mined by BarcodeGenerator can be used for the identification of species of interest in Roche 454 or Illumina metagenomics datasets. Barcoding 2.0 is an application written in Python 2.5/Python 2.7 with a command-line user interface made available for downloading from the BarcodeGenerator website (http://bargene.bi.up.ac.za/). The program uses BLASTN to align reads against the generated barcode sequences and then calculates several parameters for scoring the results of the BLASTN alignment and individual barcodes. After scoring all the aligned reads, the program calculates scores for every barcode to identify organisms present in metagenome samples. Taxonomic units are identified by comparison of calculated barcode scores to standard cut-off values set by default. This approach may not be applicable for the analysis of metagenomes generated by PacBio and Oxford Nanopore technologies owing to the high rate of sequencing errors and computational inefficacy of BLAST alignment of long reads.

With the aim to determine whether the length of barcode sequences and the number of reads in a metagenomic dataset influence the sensitivity and specificity of the method, artificial metagenomes of different sizes with a pre-defined composition of reads generated from several reference microorganisms were aligned against barcodes of various lengths. In the first experiment, metagenomic datasets of varying sizes from 10 000 to 500 000 reads were aligned against barcodes of the same length (50 kbp). The researcher found that the sample size (the number of reads in a metagenome) has basically no effect on the sensitivity or
specificity of the algorithm in the given range of sizes (see Chapter 3 for more details). In this range of values, the percentage of TPs increased with the sample size proportional to the number of false positives. The ratio of TPs over false prediction was higher in smaller metagenomes. When varying lengths of barcode sequences (10 to 250 kbp) were aligned against an artificial metagenomic dataset of 500 000 reads, the sensitivity and specificity also remained unchanged. However, the ratio of TPs over FNs was optimal when the barcode sequences were in the range from 100 to 200 kbp. All generated barcodes and artificial metagenomic datasets were made available for download from the project website (http://seqword.bi.up.ac.za/barcoder_help_download/index.html).

Receiver operating characteristic curves of the algorithm performance were calculated for different microorganisms used in the artificial metagenomics datasets. Distinguishing between species of the same genus or family by the program was close to perfect, but the program performed worse in distinguishing between strains of *Escherichia coli* and *Shigella*. Closely related organisms could be identified better when barcodes were based solely on core genes.

The web interface provides users with an online access to the program BarcodeGenerator, which creates diagnostic barcodes based on the genome sequences of species of interest submitted by users. To generate a set of barcode sequences, the user uploads the corresponding genome sequences in GenBank format in a single archived ZIP, TAR or GZ file (maximum file size is 500 MB). For barcode generation, the Barcode mode set by default is used as the mode of operation. The user then selects the proportion of accessory genes in the generated barcode sequences and the approximate length of the barcode sequences. The project name is entered alongside an e-mail address, which is used to receive a link to the file with the generated barcode sequences. Having uploaded the input file, the program starts generating barcode sequences according to the program run parameters set by the user. A local version of the program BarcodeGenerator with a command line interface was also made available for advanced users at http://seqword.bi.up.ac.za/barcoder_help_download/barcodegenerator.html.

To perform metagenomic read binning against the generated diagnostic barcode, another command-line program, Barcoding 2.0, written in Python 2.5/Python 2.7, was designed and made available for downloading from http://bargene.bi.up.ac.za/. The command-line
program Barcoding 2.0 can be used for binning reads of WGS metagenomes. The program Barcoding 2.0 is a command-line program written in Python 2.7 that aligns metagenomic reads of Roche 454 and/or Illumina against taxon-specific barcode sequences generated by the online program BarcodeGenerator. The program performs BLASTN alignment of reads against barcode sequences and then scores every barcode in a set and every taxonomic unit represented by a corresponding diagnostic barcode. The program workflow and the scores calculated by the program were explained in detail in Chapter 3.

Also available for download from the website are: (i) examples of all generated barcode sequences for all organisms used as case studies; (ii) graphical output of the diagnostic barcode generated for each length (10, 25, 75, 100, 150, 200, 250 kbp); (iii) artificial metagenomes created with the Metasim program with the supporting information regarding the contents of the artificial metagenomes; and (iv) hyperlinks to NCBI resources to provide more detailed information about each barcoded organism and genes selected for generated barcodes. All the programs for this work were written on Python 2.5/Python 2.7 and made accessible at the website http://bargene.bi.up.ac.za/ through a PHP.

An attempt was then made to evaluate the barcode sequences created by the program BarcodeGenerator (Chapter 2) for selected genomes in real metagenomic datasets obtained from MG-RAST database using the Barcoding 2.0 program. However, since this is the first version of the Barcoding 2.0 program released, to validate the results and to determine how well Barcoding 2.0 performed, the researcher first performed a BLASTN alignment of various metagenomic reads used in the case studies against a local copy of the NCBI nt database using the BLASTN for Linux implementation of the alignment program installed on the computer server. The MEGAN 4.70.4 program was then used to estimate and interactively explore the taxonomical content of the dataset, using the NCBI taxonomy to summarise and order the results. MEGAN uses a simple algorithm that reads standard BLASTN output files and assigns each read to the LCA of the set of taxa that it hits in comparison. Hence, species-specific sequences are assigned to the taxa near the leaves of the NCBI tree, whereas widely conserved sequences are assigned to high order taxa closer to the root (Huson et al., 2007). Compared to MEGAN, Barcoding 2.0 also performed averagely in identification of microorganisms. All species/strains identified by Barcoding 2.0 represented strains and species that were of commercial and biotechnological importance.

The results from this work showed that the novel online tool BarcodeGenerator
(http://bargene.bi.up.ac.za/) is an efficient approach to generating barcode sequences from a set of complete genomes provided by users. The Barcoding 2.0 program made available from the same resource enabled efficient and practical use of metabarcodes for visualisation of distribution of organisms of interest in environmental and clinical samples.

6.2 Conclusion

In this work, the researcher created an interactive web application and software tools for identification of the most suitable marker sequences for DNA-based multi-local barcoding. The basic idea was to allow the selection and use of different marker genes for identification of organisms of interest on different taxonomic levels in environmental samples. The program BarcodeGenerator, available online at http://bargene.bi.up.ac.za, creates genome-specific barcodes based on the core and accessory genes for genome sequences provided by users. Another command-line application, Barcoder 2.0, available for download from the same website, performs binning of metagenomics reads against generated barcodes and visualises the results. It should be noted that these software tools were developed exclusively for metabarcoding, i.e. for identification of strains and species of interest in environmental samples by binning of metagenomics reads, but not for phylogenetic inferences. However, the program Barcoder 2.0 allows the alignment of identified organisms along phylogenetic trees generated by other programs and provided in PHYLIP/Newick format together with other input files.

This type of research is unique, useful and necessary because:

(i) Research on bacterial DNA barcoding is yet very limited and still in its infancy.
(ii) There are no standard interactive computational services for the identification of the most suitable marker sequences for DNA-based multi-local barcoding.
(iii) Most methods used for binning metagenomic reads do not allow identification below the genus level and very often stop on the level of bacterial families.
(iv) There are many bacterial and fungal cultures that have shown significant enzymatic, antibacterial and hormonal activities, which may be of importance for the medical, biotechnological and agricultural industries.
References


Research output

This research has been presented in local and international conferences and published in peer reviewed scientific journal

Publications:


Presentations:

- Rotimi Adeola and Oleg Reva (2016) Genetic barcoding and metabarcoding in Biotechnology. Presented at SASBi (South African Society for Bioinformatics) (Oral Presentation)

- Rotimi Adeola and Oleg Reva (2016) Genetic barcoding and metabarcoding in Biotechnology. Presented at the GRI Symposium University of Pretoria (Oral Presentation)


Appendix 1

ROC diagrams calculated for artificial metagenomic datasets of different sizes for 50,000 bp long barcode sequences. The following parameters were calculated: AUC – area under the curve; SEN – sensitivity; and SPE – specificity.
Appendix 2

ROC diagrams calculated for artificial metagenomic datasets of 500,000 randomly generated reads with barcode sequences of different lengths. The following parameters were calculated: AUC – area under the curve; SEN – sensitivity; and SPE – specificity.