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Henrik Christensen *Editor*

Introduction to Bioinformatics in Microbiology

Second Edition

Springer

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Editor

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Editor
Henrik Christensen
Department of Veterinary and Animal Sciences
University of Copenhagen
Copenhagen, Denmark

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Preface to Second Edition

Bioinformatics is the convergence of two trends in biological research since the 1960s: storage of molecular sequences in computer databases and application of computational algorithms to the analysis of DNA and protein sequences. Both sequence information accumulated in databases and computer capacities have been growing exponentially which explain why bioinformatics has been developing so fast as a scientific field.

Bioinformatics is integrating many scientific fields such as mathematics, computer science, and statistics. Other disciplines involve biochemistry, molecular biology, and physics. The microbiologists formulate the questions and hypothesis to be investigated. The bioinformaticians integrate all information, and bioinformatics has matured during the past two decades to become a scientific field of its own and so has the integration of bioinformatics with microbiology. Related to small and uniform size of most microorganisms, bioinformatics has revolutionized microbiological research by providing new insight into all aspects of microbiology. The analysis of all genomes (microbiome) from all organisms in a sample (microbiota) is designated metagenomics and has not only revolutionized the investigation of microbiology but also human medicine (human microbiome). The analysis and interpretation of the sum of genetic information of a host and its symbiotic microbiota has been designated the hologenome (Rosenberg and Rosenberg 2016. mBio e01395).

The aim of this book is to teach bioinformatics to microbiologists working within biotechnology, biology, veterinary medicine, clinical medicine and with environmental microbiology at graduate as well as post graduate levels and equivalent continuous education. The book can be used for novices as well as scientists with some experience in the field. Some basic concepts have been explained very detailed to provide beginners with the proper understanding which allow the more complicated problems to be understood and the right conclusions drawn.

The book is based on material and experience gained from teaching the PhD course "Bioinformatics for Microbiology" during the past 15 years. Teaching has been performed by introductory lectures followed by computer exercises where participants have worked on cases including their own data. With a background in the course, they have worked out

independent bioinformatics projects with realistic cases at the level of scientific publications.

The idea behind the course and also this book is to use the "free-ware" computer programs without prepaid licenses. Readers will be able to start their own bioinformatics investigations just with a laptop computer and an Internet connection. This book will only be an introduction for most users and they will have to seek to further teaching including self-teaching to continue with the more advanced bioinformatics pipelines as well as writing of bioinformatics programs which will not be covered in this book.

Microorganisms in the context of this book include mainly Prokaryotes and occasionally virus and components of Eukaryotes defined by small size such as fungi and protozoa. The category is not defined by common ancestry but by the need and practice of using common bioinformatical tools. Prokaryotic genes are often organized in operons which are transcribed into polycistronic units, whereas Eukaryotic genes are transcribed as single gene units. Eukaryotic genes are often organized in non-translated introns and translated exons as well as longer untranslated leader and terminal sequences compared to prokaryotes without such an organization. To sum up, these differences make bioinformatical analysis of Prokaryotes more straightforward compared to Eukaryotes. Also, the bioinformatical analysis of "micro" Eukaryotes is often more simple than that of "macro" Eukaryotes.

The new version of the book represents the increasing use of whole genomic sequencing for bioinformatical analysis of microorganisms. The whole genomic sequence of a single strain provides a wide range of possibilities for prediction of functional capabilities. The drawback with such massive technological improvement is that we tend to ignore other important areas of research and information. A parallel can be drawn from the past to the television and more recently to the Internet where drawbacks from the technologies have provided other problems. A problem with the misuse of whole genomic sequences is a neglect to store and characterize microorganisms phenotypically as well to experimentally characterize the function of genes and gene products. Without such information, we will not be able to maintain reliable predictions in the databases.

I acknowledge my colleagues at the Danish Informatics Network in Agricultural Sciences who took the initiative to the first bioinformatics PhD course level training in 1999. I also acknowledge more than 400 PhD level students who have attended the course "Bioinformatics for Microbiology." They have contributed to a continuous development of the course with their personal experience from active research projects, curiosity, and enthusiasm. Some former participants have become co-authors on this book and they in particular are thanked for their work. Finally, I would like to acknowledge editor Silvia Herold and project coordinator Sivachandran Ravanan at Springer Nature for inviting me to write the book and thank them for providing optimal support during the process.

Copenhagen, Denmark Henrik Christensen

Contents

List of Abbreviations

ANI	Average nucleotide identity
ASN.1	Abstract Syntax Notation one
BAC	Bacterial artificial chromosome
BChl	Bacteriochlorophyll
BLAST	Basic Local Alignment Search Tool
BLOSUM	BLOcks SUbstitution Matrix
CAZyme	Carbohydrate Active enZYmes
CC	Clonal complex
CDT	cytolethal distending toxin
DDBJ	DNA Data Bank of Japan
dDDN	digital DDN
DDN	DNA-DNA reassociation
ddNTPs	dideoxynucleotides
DE	Differentially expressed
DLV	Double locus variants
dN	Non-synonymous nucleotide substitutions per non-synonymous site
DRISEE	Duplicate read inferred sequencing error estimation
dS	Synonymous nucleotide substitutions per synonymous site
EBI	European Bioinformatics Institute
ENA	European Nucleotide Archive
FPKM	Fragments per Kilobase gene per Million mapped fragments
GGDC	Genome-to-Genome Distance Calculator
HGT	Horizontal gene transfer
HSP	High-scoring sequence pairs
INSD	International Nucleotide Sequence Databases
INSDC	International Nucleotide Sequence Database Collaboration
ISFET	Ion-sensitive field-effect transistor
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
MIGS	Minimum information about a genome sequence

MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
MS	Mass spectrometry
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
NGS	Next-generation DNA Sequencing
NIG	National Institute of Genetics
ORF	Open reading frames
OTU	Operational taxonomic units
PAM	Percent accepted mutations
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PDB	Protein Data Bank
PERMANOVA	Permutational multivariate analysis of variance
PIR	Protein Information Resource
qPCR	Quantitative PCR
RAST	Rapid Annotation using Subsystem Technology
RBH	Reciprocal Best Hits
RDP	Ribosomal Database Project
RPKM	Read per Kilobases of transcripts per million reads mapped
SGML	Standard Generalized Markup Language
SLV	Single locus variants
SMART	Simple Modular Architecture Research Tool
SNP	Single Nucleotide Polymorphism
SRA	Sequence Read Archive
ST	Sequence Type
TLV	Triple Locus Variants
TPA	Third Party Annotation
TPM	Transcript per million
USS	Uptake signal sequences
wgMLST	Whole-genome MLST
WGS	Whole-genome sequence
XML	Extensible Markup Language
ZMW	Zero-mode waveguides

Introduction

1

What the Book Will Teach You

Henrik Christensen

Contents

What You Will Learn in This Chapter

You will learn why bioinformatics became an independent scientific discipline and why bioinformatics is of particular relevance to microbiology. You will then be provided with the background of this book as well as its major outline. At the end, you will get information about computers, operating systems, and computer programs required to carry out bioinformatics. The book has many practical relevant examples.

H. Christensen (✉)
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk

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1.1 Basics and History of Bioinformatics

Bioinformatics has become a major scientific field within biological sciences. This includes microbiology in particular related to the small and often uniform size of the organisms investigated, which promoted molecular investigation early on. The use of molecular biology including DNA sequencing has been essential to microbiological investigations. The first literature references to bioinformatics date back to the early 1990s, and the term came into common use in the late 1990s. Bioinformatics is mainly the convergence of two trends in biological research: storage of molecular sequences in computer databases and application of computational algorithms to the analysis of DNA and protein sequences (Bogusky 1998). Now, it becomes a routine in most labs not only to sequence single genes but also whole genomes and in many labs to use metagenomics based on 16S rRNA amplicon sequencing. Scientists and students are automatically assumed to know how to analyze this immense information. The book will teach you how to handle this problem top down. The bipartition of bioinformatics between the databases and the computers explains why bioinformatics has grown and is growing as a scientific field—both sequence information accumulated in databases and computer capacities have been and are still growing exponentially.

1.1.1 Bioinformatics Is Integrating Many Scientific Fields

Bioinformatics is integrating many scientific fields (Fig. 1.1), and a major task of the "information" part of bioinformatics relates to the understanding and translation of terms and concepts between these scientific disciplines. It all starts with a biological problem to be solved. In the context of this book, the microbiologist formulates the biological question and hypothesis. For instance, if a microbiologist wants to use an enzyme from seawater for the production of "plastic" (synthetic polymers), a biochemists may need to set up tests to identify the synthetic polymers, which are related to the organism or sample. Computer programs are used that include algorithms formulated by mathematicians and implemented by computer scientists to analyze the data and identify relevant protein sequences that have significant similarity to enzymes already known to be involved in the specific polymerization. The interaction with molecular biologists and physicists is needed to further interpret and annotate the information about the enzyme in the databases. The bioinformatician will have to integrate all information into one concept and know of all scientific disciplines needed. Bioinformaticians often have their background in one of the traditional disciplines such as computer science, biochemistry, physics, or microbiology.

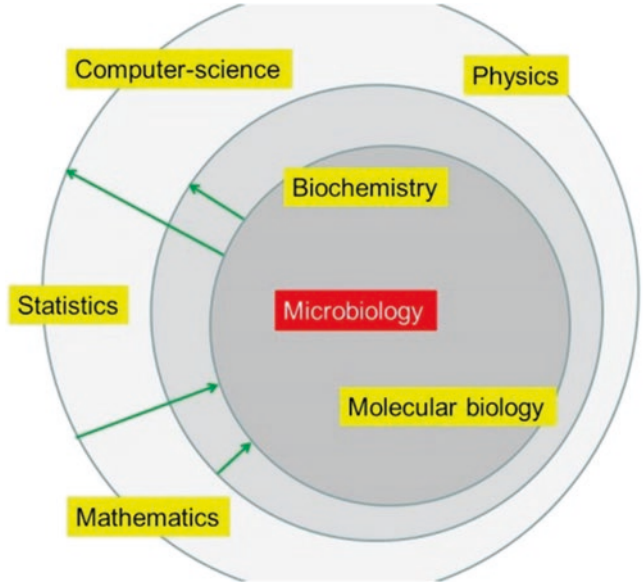

Fig. 1.1 Good communication between the different scientific disciplines is essential for bioinformaticians to do research

1.1.2 Homology

A key biological concept behind bioinformatics is homology. Homologous structures have the same ancestor or are predicted to have the same ancestor. The different bones in a wing of a bird and in a human arm can be compared since they are thought to belong to common structures in a common ancestor even though this common structure is several 100 millions of age. DNA and protein sequences also only can be compared in a meaningful way if they are homologs, that is, if they once belonged to a common ancestor. The criteria of comparative anatomy and physiology "… by tracing the progressive additions to an organ through the animal series from its simples to its most complex structure…" were formulated by Owen (1843) and the terminology of morphological homology later refined and defined by Remane (1952). It is not needed to read this German text unless you love comparative morphology since the comparison of molecular sequences has provided us with tools to model ancestry, which are many times stronger and faster than the comparison of morphologic characteristics. One reason for the success of bioinformatics has been that we are able to model evolution and test homology. The homology concept can be subdivided into orthology and paralogy. These concepts were defined by Walter M. Fitch and have become key terms used in bioinformatics (Fitch 2000). Ortholog genes or proteins are homologs that diverged following speciation events, whereas paralogs (para for parallel) are homologs that diverged as a consequence of gene duplication. In prokaryotes, multiple copies of a gene with the same function are usually not tolerated, and as a consequence, paralogs in the same species will have different functions.

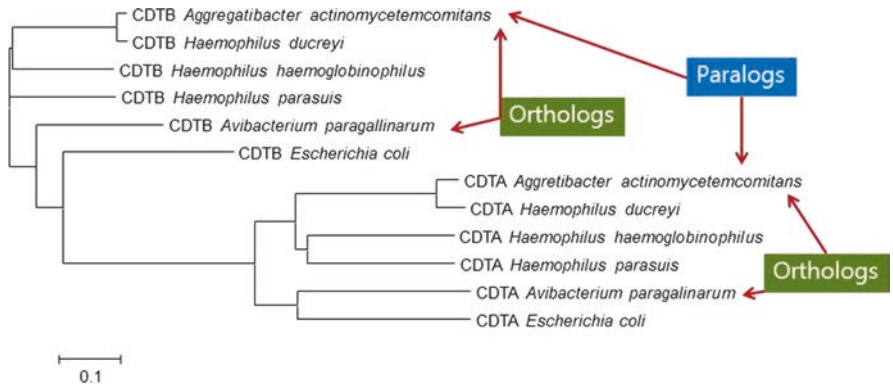

Fig. 1.2 The concepts of orthology and paralogy illustrated by phylogenetic relationships between protein sequences A and B of the cytolethal distending toxin (CDT)

An example is shown in Fig. 1.2 of the phylogeny of the proteins in the toxin cytolethal distending toxin (CDT). CDT is produced by some bacterial pathogens like species of *Campylobacter* and *Haemophilus*. CDT has not been found in gram-positive bacteria. CDT can induce G2/M cell cycle arrest, chromatin fragmentation, cell distention, and nucleus enlargement of eukaryotic cells. CDT is a heterotrimer consisting of CDTA, CDTB, and CDTC protein chains. The three proteins are homologs but distantly related. From the figure, it is seen that all CDTA proteins are related in the different species compared, and they are therefore orthologs. Also, the CDTB proteins are related in different species, and they are also orthologs according to the definition. However, the CDTA and CDTB proteins are more distantly related, and they are paralogs since they are present in the same species but with different functions. The phylogenetic relationships are explained in more detail in Chap. 6.

Another example of the use of phylogeny is for the classification of all prokaryotes based on phylogenetic analysis of the 16S rRNA gene sequences. The 16S rRNA-based phylogeny has changed the classification of all prokaryotes dramatically during the past 2–3 decades and as a consequence also how identification of prokaryotes is performed by sequencing (Chaps. 7 and 8).

1.1.3 Evolution Is Related to Sequence Diversity Caused by Mutations

The observed differences between related DNA sequences are related to mutations. A mutation is a stable inheritable alteration of the genome. There are two types of mutations: point mutations and mutations involving larger fragments of DNA. Point mutations involve only one nucleotide. Point mutations are rare events related to mistakes in DNA replication. If such a mutation is changing the codon of an amino acid (non-synonymous substitution) of a protein, it may lead to a change in the function of the organism or be fatal for the organisms. Other point mutation may not result in the change of an amino acid (synonymous substitution); however, they can or course still be registered at the level of DNA. Point

mutations are distinguished from changes of larger fragments of DNA that may have been exchanged between different organisms, and such changes are referred to as horizontal gene transfer (Chap. 11).

1.2 The Aim, Structure, and Outline of the Book

The aim of the book is to provide readers with a background to carry out bioinformatics investigations within microbiology, which will provide sufficient scientific background to publish their investigations in scientific peer-reviewed journals. The weight on bioinformatics teaching in this book has been understanding, introduction, use of proper controls to confirm results, evaluating the quality of analysis, and graduating the precision of results required. In some cases, only round figures are needed, for example, when the similarity between CDTA and CDTB proteins is compared to each other in Fig. 1.2 since they are so distantly related. In other cases, results need several digits to be significant. For instance, when 16S rRNA gene sequences of bacterial species are compared for the purpose of identification (Chap. 7). Members of the prokaryotes have the main focus in the book since we have mainly worked with this group including the examples provided. The book is still relevant for microbiologists investigating fungi and virus.

Figure 1.3 shows the main topics of bioinformatics treated in this book. This structure has been used in many bioinformatics textbooks. After the current introductory chapter,

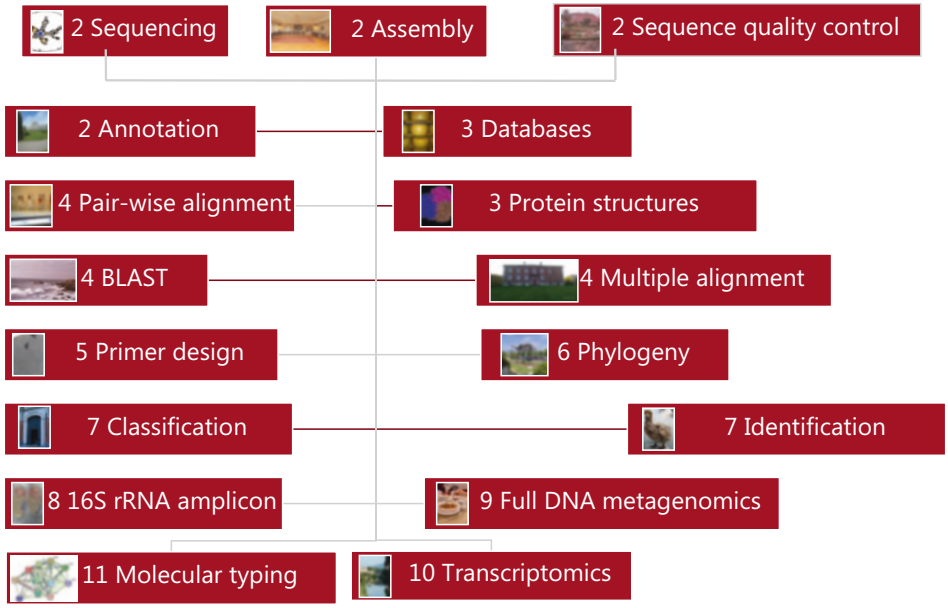

Fig. 1.3 Structure of the book. The topics are illustrated as boxes. The highlighted bricks are most closely related to the actual chapter which will be shown in green

the book starts with sequence assembly and annotation. Annotation is actually a consequence of the databases covered in Chap. 3. Chapter 4 introduces substitution matrices, pair-wise, and multiple alignments used for comparing sequences. Chapter 4 also introduces BLAST, which is used to compare sequences in the databases described in Chap. 3. In Chap. 5, primer design is described. This subject is important both for the generation of new sequences (Chap. 2) and for diagnostic purposes. The chapter includes a distinction between primers used for PCR and oligonucleotides probes used for hybridization proposes. In Chap. 6, phylogeny is introduced, which requires Chap. 4 about the multiple alignment as background. In Chap. 7, sequence-based classification and identification are introduced, which includes backgrounds from all former chapters. The identification of prokaryotes has been dominated by 16S rRNA gene sequence-based identification for the past two decades. 16S rRNA gene sequence-based identification and classification are the background for 16S rRNA amplicon sequencing, which currently is the most frequently used method to characterize prokaryotic communities (Chap. 8). Full DNA metagenomics also called shotgun metagenomics is described in Chap. 9. In Chap. 10, RNA-based transcriptomics is described, which allows an estimation of the relative transcriptional level of different genes. This heavily relies on the databases in Chap. 3. Finally, Chap. 11 describes molecular typing, which is relying both on multilocus gene sequence typing (MLST) and whole genome analysis either by the use of the single nucleotide approach (SNP) or by whole genomic MLST (wgMLST).

The aim is to provide readers with more understanding and inspiration of bioinformatics by reading this book, which they may then use as a stepstone toward further and deeper bioinformatics investigation.

Readers will have to be introduced to microbiology in other relevant text books such as "Brock biology of Microorganisms" edited by Michael Madigan and coworkers (Madigan et al. 2020). Some readers may also need to consult basic text books about biochemistry and molecular biology.

Comparative genomics is a key element to the analysis of whole genomic sequences. The term covers many different types of analysis aiming at different results. Comparative genomics is therefore not treated on its own but included where needed in the different chapters.

1.3 Computers and Operating Systems Required for Bioinformatics

Bioinformatics is practiced with a computer. If you have not worked with bioinformatics before, you need to choose the optimal computer to solve a given problem. For a start, your favorite lab-top will probably do the job. The computer needs to have at least 50 GB free space on the hard disk and a reasonable amount of RAM (ready access memory) where 8 GB is minimum and 16 GB preferred—the more RAM the better. If you do a lot of bioinformatics, it will be beneficial with a desk top computer with 32 GB RAM. For

Fig. 1.4 Computers "hall of fame." A collection of operating systems and computers. SuperUsers is acknowledged for this historical photo (www.superusers.dk)

beginners, operating systems can both be of the "Mac" type provided by Apple® or "Windows" type by Microsoft® (Fig. 1.4). Most of the basics bioinformatics programs will be available for both types. Such programs will also usually run most smoothly on the Linux type of operating systems such as Ubuntu. However, it is only recommended to start on Ubuntu if you have some experience with this operating systems already or else you risk to use most of the time learning Linux instead of learning bioinformatics. Many bioinformatics tools can be performed for free on servers, which can be accessed by the internet. The same holds true for the databases, and a good internet connection is mandatory. Readers of this book will be able to start their own bioinformatics analysis just with a lab-top computer and an internet connection.

To work with Linux (and Ubuntu) on a Windows 10 computer, there are two options. The first is to install Linux Subsystem on Windows: Control panel > Programs and Features > Turn Windows Features on and check Windows Subsystems on Linux. In Microsoft store, search for Linux and select Ubuntu (Sitto and Battistuzzi 2020). The other option is to install Virtual Box and import an image of Ubuntu with the needed environments installed; however, for that, you need to be able to create this image, and it requires some system administrator skills.

1.4 Computer Programs and Pipelines

The majority of the best computer programs for bioinformatics are noncommercial in the way that they are free to use for nonprofit purposes. The weight will therefore be on free programs in this book. Unfortunately, most free programs are with command line inter-

face, which is unfamiliar for most beginners in this field. Especially for sequence assembly, it has been difficult to suggest free programs with a graphical user interface, and in this case, suggestions to use programs with a user fee have been made.

1.5 Activity

How much RAM is in your computer?

Windows 10: Right click on the **Windows start icon**, left click on **System**.

Mac: click on the **Apple icon** in top left corner and click **About this Mac**.

Ubuntu: Left click at the settings **icon (cogwheel)** and left click at **Details (cogwheel)** under System.

Useful links for help.

https://www.biostars.org/ (different pages).

Further Reading

Basic bioinformatics text books

Durbin R, Eddy S, Krogh A, Mitchison G (1999) Biological sequence analysis. Cambridge Univ. Press

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press

Zvelebbil M, Baum JO (2008) Understanding bioinformatics. Garland Science, New York

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Fitch WM (2000) Homology a personal view on some of the problems. Trends Genet 16:227–231

Madigan M, Bender K, Sattley WM, Stahl D (2020) Brock biology of microorganisms, 16th edn. Pearson, New York

Owen R (1843) Classification of animals. In: Lectures on the comparative anatomy and physiology of the Invertebrate animals. Longman, Brown, Green, and Longmans, London

Remane A (1952) Die Grundlagen de natürlichen Systems, der vergleichenden Anatomie und er Phylogenetik. Akad. Verlagsges. Geest & Portig, Leipzig

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DNA Sequence Assembly and Annotation of Genes

2

How to Generate the DNA Sequence and to Predict the Function of Genes

Henrik Christensen and Arshnee Moodley

Contents

H. Christensen (✉) · A. Moodley
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; asm@sund.ku.dk

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What You Will Learn in This Chapter

In this chapter, you will learn about the different sequencing strategies currently available and the pros and cons of the different strategies to help you select the optimal DNA sequencing strategy for your research question. Thereafter, you are introduced to the challenges associated with assembly of DNA sequence data and the different quality criteria needed in the process including how genomes can be closed. The simple FASTA format used for representing DNA sequence is demonstrated, and annotation of the DNA sequence is then introduced.

2.1 DNA Sequencing

DNA sequencing is the determination of the order of nucleotides of parts or whole chromosomes of organisms and virus. DNA sequencing can be done for a single gene or a whole genome or many genomes at a time such as in metagenomics. The first decision to be taken is the selection of the optimal DNA sequencing strategy for your research question in your project. In Tables 2.1 and 2.2, we summarize the different technologies and what they could be used for and their benchmarks. Often, laboratories outsource the sequencing to companies or institutions if they do not have their own in house sequencer, resulting in a longer turnaround time and higher sequencing costs. Therefore, research labs are now investing in smaller, more affordable benchtop next-generation sequencers for more cost-efficient sequencing (Fig. 2.1).

The development of DNA sequencing technology has a long history with multiple leaps occurring within a few decades. In 1973, Maxim and Gilbert published the first ever DNA sequence, 24 bp of the *lacR* binding site. It took two years, meaning one base per month. In 1976, Sanger and Coulson described the chain terminator method, which is based on the inclusion of one of the four radioactive-labeled dideoxynucleotides and when incorporated would stop strand elongation (chain termination), producing fragments of different lengths (Sect. 2.1.1). All four reactions would then be resolved individually by electrophoresis on a polyacrylamide gel (one lane, one dideoxynucleotide base). The X-ray gel picture would resemble a ladder from which the sequence could be read off. Traditional Sanger sequencing has the limitation in that sequencing larger genomes, for example, a 3300 Mb human genome, is rather challenging leading to the development of next-generation DNA sequencing (NGS) or massive parallel sequencing of small DNA fragments. The key difference between Sanger sequencing and NGS is the ability to multiplex.

The first high-throughput sequencing platform that became available was 454 GS20 pyrosequencer marketed by Roche in 2005 (later replaced by model GSFLX). 454 sequencing is no longer widely used as the company has stopped sales of this instrument. However, data generated by this method are available in databases, and from a bioinformatical perspective, it is relevant to know about the platform. This is similar for data derived from an Ion Torrent system, which will be explained further down. When we are

Table 2.1 Selection of DNA sequencing strategies and methods in relation to capacity and cost

Problem	Method	Read length (nt)	Capacity	Cost (related to capacity)
1. Single genes, sequencing of BAC, FOS[a] or plasmid inserts (cloning)	Sanger sequencing of PCR amplicons	500–1000	Low	High
2. Genomes and 16S metagenomics	Illumina	150–600	High	Low
	Ion torrent	400–600	High	Low
3. Single cell sequencing	Single cell dissection, DNA extraction, phi29 amplification and Illumina sequencing	150–251	High	High
4. Closing of single genomes	Pacbio® (Pacific Biosciences)	15,000–100,000	Medium	High
	Nanopore	1,000,000	Medium	High

[a]*BAC* bacterial artificial chromosome, *FOS* fosmid, based on bacterial F-plasmid. Both are used for cloning of large fragments of DNA in the order of 50 kbases

Table 2.2 Comparison of different commonly used sequencing platforms

Platform	Read Length	Amount of data produced	Number of reads	Run time	Accuracy	Cost of instrument (US$)	Cost per Gb (Approx. US$)
Illumina MiSeq v3 600bp	300 bp PE	13.2–15 Gb	44–50 million PE	56 h	99.9%	99,000	110
Illumina HiSeq 2500 v2	250 bp PE	125–150 Gb	600 million PE	60 h	99.9%	690,000	45
Ion Torrent Ion PGM 318	400 bp SE	1–2 Gb	4–5.5 million	7.3 h	98–99%	49,000	450–800
PacBio RS II	Approx. 20 Kb	,500 Mb–1Gb	55,000	4 h	87%	695,000	1000
MinIon	Variable up to 900 Kb	5 Gb	Up to one million	Up to 48 h	88%	1000	500–900

PE Paired end, *SE* Single end, *Gb* Gigabase, *Mb* Megabase, *Kb* Kilobase
Table derived from Goodwin et al. (2016) and https://blog.genohub.com/2017/06/16/pacbio-vs-oxford-nanopore-sequencing/

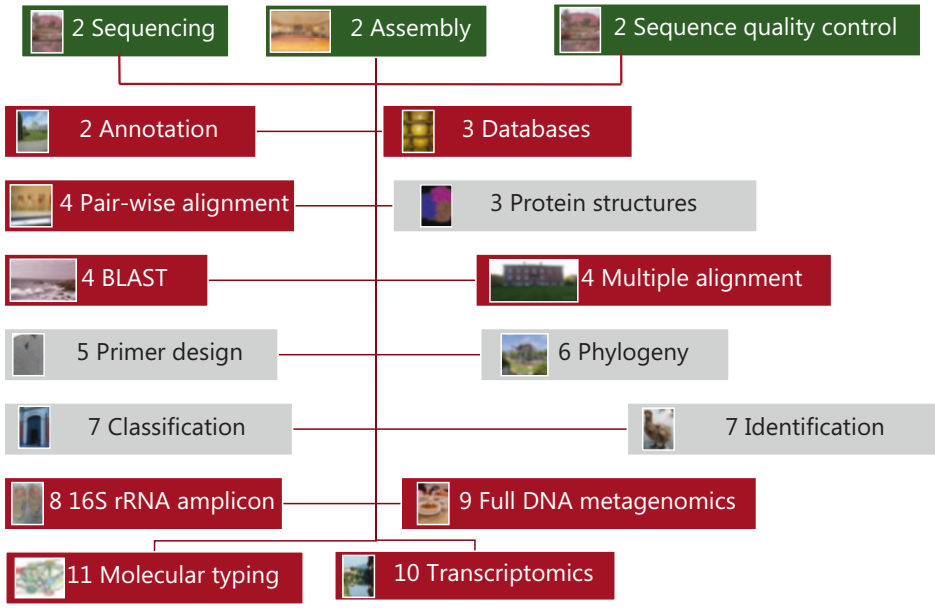

Fig. 2.1 DNA sequence assembly and annotation are related to most other chapters in this book by providing the raw DNA data and information about the location of genes on chromosomes, their functional and taxonomic relationships

dealing with handling of the data, the 454/Ion Torrent-derived data is very similar to Illumina data since sequencing is done by replication of DNA and millions of short DNA reads are generated, which need to be assembled to the final DNA sequence.

2.1.1 Sanger Sequencing

This sequencing method was developed in the 1970s (Sanger et al. 1977) and was the predominant method until the mid-2000s. Now, it is mainly used as a method for sequencing single genes or fragments of DNA. The principle of the method is based on sequencing by replication of DNA and the incorporation of dideoxynucleotides (ddNTPs), which will stop replication randomly when one of the four dideoxynucleotides, ddATP, ddCTP, ddGTP, or ddTTP is incorporated. The position of the stops is determined based on the specific radioactive or fluorescent label of the each ddNTPs to determine the DNA sequence. As mentioned, the initial method used radioactive labeling and polyacrylamide gel electrophoresis, which was very time demanding. By the mid-1980s, automated fluorescence-based Sanger sequencing machines were developed, for example, those by Applied Biosystems. Current methods are based on fluorescent labeling and capillary gel electrophoresis in automatic instruments. Further information of this method can be found in text books (Madigan et al. 2021).

2.1.2 Massive Parallel, Short-Read Sequencing

2.1.2.1 Illumina (Sequencing by Synthesis)

More space will be used to introduce Illumina sequencing since it is the most frequently used platform today. Illumina acquired Solexa GA in 2007 to develop and commercialize genome sequencing technology. The company currently has a number of different sequencers depending on the sequencing capacity needed, ranging from benchtop sequencers to production scale sequencers. Microbiology research labs would invest in one of the benchtop sequencer, which at the moment the most common platform is the MiSeq (Fig. 2.2). Smaller systems such as the MiniSeq and iSeq 100 are also available. These are cheaper than the MiSeq but have a limited sequencing capacity. More information can be found on the company website https://www.illumina.com/systems/sequencing-platforms.html. We will only focus on the MiSeq, but the principle of sequencing is the same across the machines.

The MiSeq is capable of doing small whole genome sequencing, transcriptomics, 16S metagenomics, and DNA-protein interaction analysis (ChIP-Seq). It is possible to multiplex by using unique combinations of specific barcodes and indexes (see Chap. 8 for the use of barcodes and indexes). One of the first steps of sequencing is the fragmentation of DNA. Depending on the library preparation kit used, the DNA can be fragmented manu-

Fig. 2.2 A picture of our Miseq instrument (Illumina)

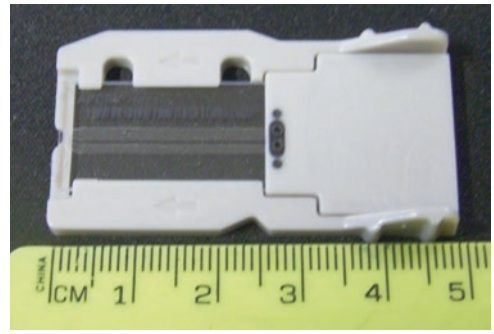

Fig. 2.3 The flow cell from Miseq (Illumina)

ally, for example, using a Covaris instrument or an enzymatic fragmentation, for example, transposon-mediated fragmentation as in the Nextera XT kit. This process is called tagmentation. For sequencing of small genomes, the Nextera XT kit has been widely used since the library preparation protocol is simple. DNA is tagmentation used two transposons that randomly cuts the DNA. During this tagmentation process, the transposon attaches adaptor sequences to the ends of the fragments. These sequences are then used as primer binding sites for the inclusion of indexes and the adaptors that are needed to anchor the DNA to a solid support, which is the surface of the flow cell (Fig. 2.3). Now, the DNA Prep kit has become in use. Our experience has been that DNA from different types of organisms as, for instance, bacteria and virus should not be mixed in the same library preparation.

From each template of DNA, "spots" or "cluster" are generated that are complementary to the original DNA template sequence. This is done by "bridge amplification" or "fold-back" step. This step is crucial to generate sufficient signal to be detected in the sequencing process. Too few spots (< 1200/mm^2) will provide limited utilization of the flow cell, whereas too many (> 1800/mm^2) will generate overlap between spots and compromising the reading of the sequences. Sequencing is then done by the addition of fluorescently labeled nucleotides in the presence of DNA polymerase in cycles. This way a complementary strand is generated, and for each cycle, which includes a wash step, the flow cell is imaged, and the emission from each cluster is recorded. The wavelength and intensity are used to identify the base. This is repeated a number of cycles depending on the flow cell used, for example, V3 600 bp (600 cycles or 2 × 300 cycles for paired end sequencing). The final DNA sequence is then generated by comparing all images as a stack.

Aside from Illumina sequencers, there is the Ion Torrent system by Thermo Fisher Scientific. This is also a benchtop sequencer released in 2010. Similar to Illumina, the technology is based on sequencing by synthesis, but the detection method is different. The Ion Torrent system uses ion semiconductor sequencing technology, which detects hydrogen ions released during DNA polymerization. After DNA amplification, each microwell contains a single species of template DNA. Unmodified nucleotides are washed into the microwells, one dNTP species at a time. When a nucleotide is incorporated into a complementary strand, a covalent bond is formed, and a phosphate and hydrogen ion are released.

This release of a hydrogen ion changes the pH, and this change is detected by an ion-sensitive field-effect transistor (ISFET) ion sensor on the chip. The major benefit of the Ion Torrent is the low cost of the instrument since unmodified nucleotides are used and no optics needed. One of the major limitation to this method is the correct identification of homopolymers, that is, stretches of repeating nucleotides. If homopolymers are present, this means multiple nucleotides are incorporated and hence the release of more hydrogen ions in a single cycle. This results in a greater pH change and a greater electronic signal detected. The system is not able to determine exactly how many nucleotides were included.

2.1.3 DNA Sequencing in Metagenomic and for Single Cell Sequencing

The principle of sequencing DNA extracted for metagenomics and for single cell sequencing is the same as described above for single genomes. Further explanation is given in Chaps. 8 and 9 including the use of index primers to perform Illumina sequencing with multiple samples.

2.1.4 Real-Time, Single Molecule Sequencing

All of the platforms that are capable of massive parallel sequencing (Sect. 2.1.2) requires fragmentation and amplification of the DNA. These steps can introduce errors, and there are sequence-dependent biases. Furthermore, these steps add to the overall turnaround time for a sequencing run. Real-time, single molecule sequencing allows for sequencing of the native DNA, resulting in significantly longer read lengths and sequence information available when the bases are incorporated, that is, information available in real time. The first of this type of technology was developed by Pacific Biosciences, and the PacBio RS system was made available in 2011. The second approach is nanopore sequencing by Oxford Nanopore Technologies, who released the MinIon in 2014.

2.1.4.1 PacBio®

PacBio is built on the ability to optically observe polymerase-mediated synthesis in real time. On a SMRT® cell, a single polymerase is fixed at the bottom of a microwell (also called a zero-mode waveguides, ZMW) with a single DNA template. Four fluorescently labeled nucleotides are introduced. When a labeled nucleotide incorporated, the fluorophore is cleave, and a light pulse corresponding to the incorporated base is produced. Each pulse has its own color intensity, and hence, the type of base is identified. This technology produces long-read lengths, on average >15 Kb, with some reads >100 Kb. These long reads can be used to assist with closing small genomes. Furthermore, PacBio sequencing can be used to span long, repetitive regions and can also be used to determine the methy-

lome of microorganisms (methylated nucleotides) used for so-called epigenetics. The major drawbacks to the technology is the high sequencing costs and high error rate.

2.1.4.2 Nanopore Sequencing

Nanopores are small holes created by pore-forming proteins in a membrane. Specifically, for nanopore sequencing, engineered porin A is used with a single constriction of 1.2 nm (Derrington et al. 2010). The principle of nanopore sequencing is the direct detection of the nucleotide sequence when a single-stranded DNA molecule is driven by a voltage of 180 mV and passes through the nanopore, and a current in pA range is generated by a flow of ions across the flow. When the template is threaded through the pore, a voltage block occurs that affects the current passing through the pore. These changes are characteristic of a particular nucleotide base. A MinIon has 512 nanopores, with each pore capable of sequencing approximately 70 bp/second. The read lengths produced comparable to PacBio. Nanopore sequencing has been brought to the market by Oxford Nanopore Technologies who released the MinIon in 2014. The uniqueness of this system is the extreme portability of the device. The MinIon is a USB sequencer that plugs directly to a computer. Sequencing is cheaper compared to PacBio but had initially the same high error probability drawback. For further reading, see Wang et al. (2021). Our current experience is that a series of MinIon devices can be run on a big gaming computer. Such computer has a GPU that can be used for heavy calculations, and memory and hard disk space is relative cheap. The current challenge with Nanopore sequencing is to load the device with high-quality DNA and to handle the command line-driven analysis of data.

2.2 DNA Sequence Assembly

Base calling is the first step in sequencing where the electronic signal generated in the sequencing machine is separated from random noise and converted to nucleotide information. Then, the nucleotide information needs to be assembled to DNA sequences, which resemble the original DNA sequenced as best as possible. This can either be done de novo without a reference or with a reference if the genome of the organism or virus is well known.

2.2.1 Base Calling and Trimming

Base calling is the conversion of electronic signal generated in the sequencing machine to the sequence of nucleotides in the DNA sequence. First, the signal needs to be quality controlled. Only a certain threshold passes the quality control. The *phred* index (q, Q or qual) defined by Ewing et al. (1998) and for ABI data manipulation (automatic Sanger) is one way of expressing the quality of signal and now has become a standard. Another way is the error probability (p) of the signal that each nucleotide has been determined with. The two, q and p, are related by

$q = -10 \times log\ 10(p)$

An error probability of 0.01 equals *phred* 20 (Q10 = 10%, Q20 = 1%, etc.). In the CLC Genomic Workbench program, p is used. The quality thresholds are used to filter the output from the sequencing machine and are referred to as trimming. Stand-alone trimming programs are available such as Trimmomatic (Bolger et al. 2014).

Denoising

Errors introduced during sequencing comparing the different platforms have been reviewed by Laehnemann et al. (2016). At that time, the Illumina short-read sequencing technology was reported with the lowest error rate and insertion and deletion error particular common on the PacBio platform. The Nanopore platform is considered to have the highest error rate. The current R10.4.1 flow cell has raw read accuracy of >99%. One strategy to correct errors is to remove low frequency k-mers (short sequence of constant length k) considering errors to be rare.

2.2.2 Assembly of DNA Sequences

The output from the DNA sequencing machine is short single "reads" a few hundred nucleotides in length that need to be combined to mimic the original DNA that was subjected to sequencing, which is often several millions in length. After the reads have been trimmed as described above, they need to be assembled. At least an overlap of forward and reverse reads needs to be generated and assembled with Sanger sequencing compared to the original DNA sequence (Table 2.3).

With high-throughput Illumina sequencing, many times more reads need to be combined compared to the original DNA being sequenced. This relates to short-read length and to high error rates with individual nucleotides of the reads.

During assembly, the short DNA reads from the sequencing machine are combined to long DNA strands, which equal the original DNA, which was sequenced and which can be used for gene prediction (annotation below). The goal is always to assemble the longest

Table 2.3 Assembly programs

Program	Principle	Use	Reference
CLC Main Workbench[a]	Overlap-consensus	Single genes	https://www.qiagenbioinformatics.com/products/clc-main-workbench/
Velvet	k-mer and de Bruijn graph	Genomes	Zerbino and Birney (2008)
SPAdes	k-mer and de Bruijn graph	Genomes and single cell sequencing	Nurk et al. (2013)
CLC Genomics Workbench[a]	NI	Genomes and 16S rRNA amplicons	https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/

[a]Requires fee

DNA strand as possible, and such a strand is called a contig (a sequenced contiguous region of DNA). Ideally, a contig should span the entire chromosome.

2.2.2.1 Assembly by Overlap Consensus Methods

Overlap and consensus methods are used for assembly of sequences generated by Sanger sequencing. Sequence reads are compared and matched by principles, which equal pairwise alignments (Chap. 4). Unfortunately, assembly programs are not freely available for Windows or Apple operating systems. Reads generated by Sanger sequencing can be assembled by CLC Main Workbench, which requires a minor fee.

2.2.2.2 Assembly by k-mer Strategy

Short "words" of DNA sequence called k-mers (length k) are used as an intermedial tool to combine the single reads from the sequencing machine to the DNA sequence of the organisms or virus originally sequenced. This method is most relevant to whole genomic sequences and to metagenomic datasets. The assembly of such datasets would not be possible by use of the "overlap" strategy above since it would be too demanding for the computers. This method originated in attempts to sequence by hybridization where the k-mers were used to link specific sequence motifs to hybridization probes (Idury & Waterman 1995). Sequence assembly is based on a so-called sequence graph (Fig. 2.4).

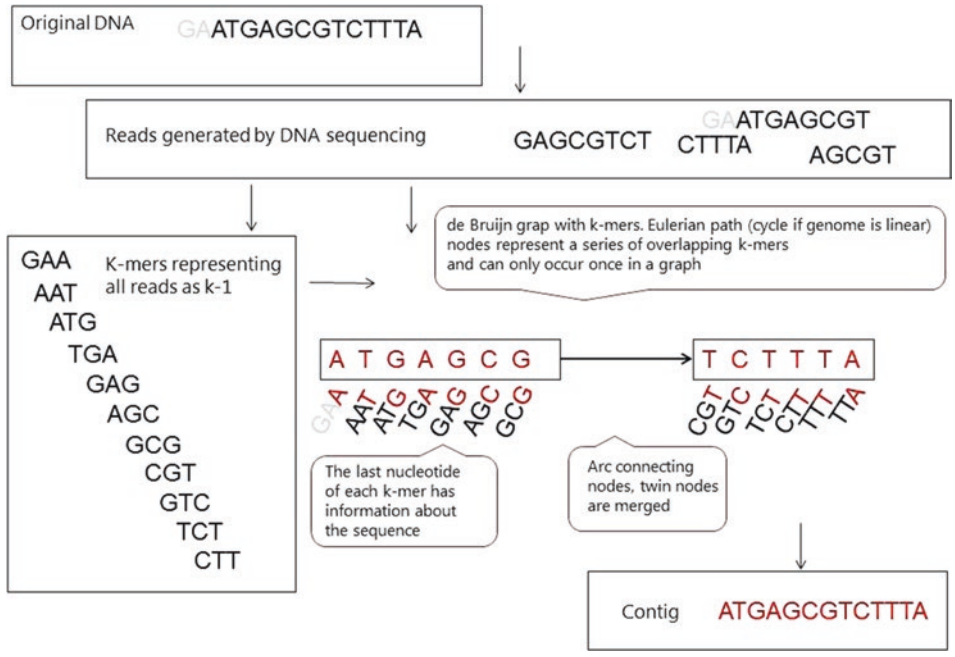

Fig. 2.4 Principles of assembly strategies based on Euler principle using k-mers resulting in a so-called de Bruijn graph. Only short fragments are shown to illustrate the principle. Only assembly of the forward DNA sequence is shown. Linear genomes need to be assembled by finding an Eulerian path. Modified from Compeau et al. (2011)

The k-mers will cover the DNA sequences observed in all of the reads generated by the sequencing machine. All k-mers are shifted one nucleotide to the right (1-tuples) so that their overlap is k−1. A popular k-mer size has been 31 since it fits with four base representation as a 64 bit integer. The idea is then to label a read on the k-mers and to visit all edges only once. This way contigs are formed, and shorter contigs are joined together (Fig. 2.3). The k-mer length often needs optimization accounting for the length of repeats since repeats longer than k-mer length can break up contigs. Too long k-mers will lower the chance they will be found in reads.

Next to the handling of large datasets, the problems of repeats and sequencing errors can also be handled by use of Euler's algorithm and de Bruijn graphs. Errors are handled as "bulges" in the de Bruijn graph (Pevzner et al. 2001). The time to run computer implementation of Euler's algorithm is roughly proportional the number of edges in the de Bruijn graph construction for the particular problem. The de novo assembly can be extended by scaffolding where the information from the paired end reads is used to link contigs (Medvedev et al. 2011). This is important if a repeat is longer than twice the read length since it then becomes difficult to extent up- and downstream regions. Repeats are often longer than read length, and the assembly can then be improved using paired-end read sequencing in the assembly process. In Table 2.3, a comparison of different assembly programs has been made.

Hybrid assemblies are used to combine the high-quality Illumina short-read sequencing with the longer reads but lower-quality reads obtained by Nanopore or PacBio. Unicycler is currently used to generate hybrid assemblies. It starts with short-read sequences and then add long reads to the contig path generated (Wick et al. 2017). Initial assembly of short Illumina reads is based on SPAdes (Nurk et al. 2013). The program needs to be installed on Linux with some helper programs. It can be downloaded from https://github.com/rrwick/Unicycler where further explanation if provided.

2.2.2.3 Quality Criteria of the Final Assembled DNA Sequence

The minimal requirements to assemblies are summarized from Chun et al. (2018).

$$\text{The first important parameter to consider is the average}$$
$$\text{coverage} = \left(\text{number of reads x average read length}\right) / \text{genome size}$$

The genome size will often be provided as the sum of contigs from assembly programs. For taxonomy, more than 50 times coverage is recommended (Chun et al. 2018). For 16S rRNA amplicon sequencing (Chap. 8), Q20 or higher is preferred.

Another important parameter is N_{50}, which is obtained by listing all contigs from the longest to the shortest. The length is then accumulated from the longest to the shortest contig, and when half the genome size is reached, the length of that particular contig is read as N_{50} (Fig. 2.5).

Fig. 2.5 Illustration of the calculation of N_{50}. First the contigs are arranged in decreasing order from longest to shortest. The length of sequence is accumulated and when the accumulation reaches 50% the specific contig is identified and its length recorded. The length of this contig is defined as N_{50}

Comparison of different assemblies can be made with Quast (Gurevich et al. 2013). The "minimum information about a genome sequence" (MIGS) and variant hereof specification (Field et al. 2008) provide an exhaustive list of the information required for genomic sequences including demands to metadata. The standards are maintained by Genomics Standards Consortium (http://www.gensc.org/index.html), which is an open-membership working body. The reads can be compared to the assembled contigs by mapping (Fig. 2.6). This way local coverage can be evaluated. SPADES provides information of coverage for every coverage, whereas output from CLC Genomic Workbench has to be calculated on average all contigs.

CheckM (Parks et al. 2015) is a program used to evaluate the completeness of a final assembled genome by making comparison to a set of reference genomes. FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc) provides different quality parameters for FASTQ files and is available for the major operating systems by way of JAVA. Quality control may also include a contamination check where the assembled prokaryotic genome is compared against an eukaryote often human genome. Such comparison can be performed with KRAKEN (Wood and Salzberg 2014).

Fig. 2.6 The original reads of DNA sequences from the sequencing machine are mapped to the contigs assembled from the reads (Consensus in the figure) and to another related genome (De Novo Assembly) in the figure. Green reads were determined by forward sequencing primers and red reads by reverse sequencing primers. Reads in blue are paired read of both forward and reverse reads. Output from CLC Genomics Workbench (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/)

2.2.2.4 De Novo or with Reference

The assembly process can be made easier if an existing genome of a related strain is used in the process. It is used as a reference. This approach is only possible for well-known species where fully closed genomes are available.

2.3 Closing of Genomes

If repeats in the DNA sequences to be assembles are longer than the read length, the DNA sequences become harder to assemble. With prokaryotes, the problem is often ignored. If the researcher is interested in coding genes to predict the function of the proteins, genome closing is not critical since the majority of functions can be scored without full assembly. Also, whole genomic multilocus sequence typing (wgsMLST) can be done on contigs that are not fully assembled to a full chromosome. The rRNA operons (5–7 kilobases) and tRNA genes are major reasons for lack of full assembly since they are longer or at the same size as most read lengths. Fully closed genomes are preferred as references in single nucleotide polymorphism (SNP) finding further described in Chap. 11. To combine contigs, short-paired reads can be combined with long-paired reads. Long reads can be obtained by the PacBio method with a significant fraction of reads longer than 7 kb (Koren et al. 2013).

2.4 DNA Sequence Formats

The most simple and frequently used format to represent DNA sequences is the FASTA format named after the sequence search program FASTA (Pearson and Lipman 1988). It is a text format with the first line starting with ">" and some information about the sequence. On the following lines follow the DNA sequence written as the order of nucleotides. Files can include one or more sequences. FASTA format files often have the extension .fa, .fna, .fsa, or .fasta. However, the extension is only required for certain programs to recognize the format, and the files can be used without an extension or with .txt as extension if used for text editing. An extension to the FASTA format is the FASTQ, the format where the "Q" is short for quality (Table 2.4). Such files include both DNA sequences and quality scores, and they are suited for high-throughput sequencing (Cock et al. 2010). A quality score is included for each nucleotide in the DNA sequence. The quality score is in the from *phred*: QUAL format. Scores are based on ASCII codes 33–126 for *phred* qualities 0–93 (1 wrong base to $10^{-9.3}$ is extremely accurate).

Table 2.4 The Fastq format

Format	Example
@ (title line)	@SEQ_ID
DNA sequence	GATTTGGGGT
+ shows end of sequence and start of quality	+
Quality lines	"*(+)%0.1–5

2.5 Annotation

Genome annotation is the identification and labeling of all the relevant features of the genomic sequence. At first, this includes the coordinates provided as nucleotide positions where coding regions are predicted. It is mainly a prediction of coding genes; however, other structural genes such as rRNA are also identified. The prediction of protein sequence from DNA sequence is based on the central dogma that information can only be transferred from DNA sequence to protein sequence and not in the opposite direction (Crick 1958). Bioinformatics can be used to predict function of proteins from DNA sequences of around one third of coding genes from bacterial genomes only. For the rest, bioinformatics can be used to provide some ideas of protein function, which can be tested further (Price et al. 2018).

First, the coordinates of candidate genes open reading frames (ORF) are predicted. ORFs are stretches of DNA that can translate to amino acids without stop codons. There are three reading frames of the forward (sense) strand of DNA and three reading frames on the reverse (antisense) (Appendix). Normally, only one out of six will be without stop codons over hundreds of nucleotides, and such a reading frame is a candidate for a coding gene. Furthermore, to predict the function of ORFs, the gene sequences are compared to different databases by a variant of BLAST. The databases include UniProt, Refseq at NCBI, and domain databases such as Pfam (Chap. 3). For some ORFs, a functional prediction cannot be obtained, and they are designated as hypothetical proteins (no matching function in databases).

The most frequent way to annotate prokaryotic genomes is during the de-position of the sequence with NCBI. Here, whole genomic sequences will be automatically annotated. To obtain an independent annotation, the server RAST (rapid annotation with subsystem technology) can be used (Aziz et al. 2008; Overbeek et al. 2014) (Table 2.5). In some cases, the NCBI and RAST annotation will be different for the same ORF. It is related to different priorities for best hits in the databases. To investigate such conflicts further, the researcher needs to compare the specific sequence to different databases (Chap. 3) by use

Table 2.5 Annotation programs

Program	Database	Principle	Reference
BlastKOALA	NCBI RefSeq, GenBank	Single genomes	http://www.kegg.jp/blastkoala/ Kanehisa et al. (2016)
GhostKOALA	NCBI RefSeq, GenBank	Metagenomes	http://www.kegg.jp/ghostkoala/
RAST	SEED, many databases	Subsystem	http://rast.nmpdr.org/ Aziz et al. (2008), Overbeek et al. (2014)
MG-RAST	Many databases	Subsystem	Glass et al. (2010)
Prokka[a]	RefSeq, UniProt, Pfam	Suite of existing programs	Seemann (2014)

[a]Runs on Unix/Linux

of BLAST or similar search tool (Chap. 4). Prokka is a suite of software tools used for annotation (Seemann 2014) (Table 2.5). It is only available in Linux in the command line mode. Annotation output from Prokka is essential in some downstream analysis like Scoary comparing metadata to annotation and core genome analysis for phylogeny (Chap. 11). Prodigal (Hyatt et al. 2010) is used as one of the tools improving annotation.

2.5.1 Elements from Comparative Genomics Aiming Annotation

Venn diagrams based on comparison of orthologous sequences can be performed by using OrthoVenn2 (Xu et al. 2019) (https://orthovenn2.bioinfotoolkits.net/home). A tRNA database is available from http://gtrnadb.ucsc.edu/ and prediction tool described in Thornlow et al. (2020). Prediction of origin of replication can be carried out at https://genskew.csb.univie.ac.at/webskew#usage_webskew.

Benchmarking Universal Single Copy Orthologs (BUSCO) (https://busco.ezlab.org/) (Manni et al. 2021) can be used to test if the annotated sequences of bacteria and fungi are complete. See further information in Seppey et al. (2019).

The output of three most commonly used annotation tools like Prokka, NCBI (Prokaryote Genome Annotation (PGAP)), and RAST each is based on different principles and produces annotations with their independent benefits and limitations. Not so surprising, annotations differ between tools (Brenner 1999). If there is doubt about the annotation of a specific gene/predicted protein, it is always a good idea to compare annotations and also to search against databases.

2.6 Activities

2.6.1 Annotation at RAST

You need to register to get an account http://rast.nmpdr.org/. The online program performs ORF finding and annotation and returns GenBank formatted files as well as other outputs. The optimal function is with fully assembled genomes, but it can also work with unassembled contigs where you just include all contigs in one file.

When logged on RAST select: Your jobs | Upload new job and select the file with the contigs in FASTA. Select genetic code “Bacteria” and “11” if the genome is from a bacterium.

Leave the next page with default settings. Press submit. Now you can follow the job at “Your jobs.” It usually takes overnight to finish. The three main output files are the GenBank with the annotations and the Amino-Acid FASTA and the Nucleic-Acid FASTA with amino acid and nucleotide sequences of all predicted genes, respectively.

Further Reading

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Seppey M, Manni M, Zdobnov EM (2019) BUSCO: assessing genome assembly and annotation completeness. Methods Mol Biol 1962:227–245. https://doi.org/10.1007/978-1-4939-9173-0_14

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Wood DE, Salzberg SL (2014) Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 15(3):R46. https://doi.org/10.1186/gb-2014-15-3-r46

Xu L, Dong Z, Fang L, Luo Y, Wei Z, Guo H, Zhang G, Gu YQ, Coleman-Derr D, Xia Q, Wang Y (2019) OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. Nucleic Acids Res 47(W1):W52–W58

Zerbino DR, Birney E (2008) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18:821–829

Databases and Protein Structures

3

Henrik Christensen and Lisbeth de Vries

Contents

H. Christensen (✉)
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk

L. de Vries
Københavns Professionshøjskole, København, Denmark

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What You Will Learn in This Chapter

Bioinformatics databases store biological information from life science research. It is an important topic for understanding of the majority of other chapters in this book. In this chapter, you will learn how to access the bioinformatics databases and extract information from them including benefits and drawbacks with primary versus secondary databases. You will be guided for best choices of databases and how to make sequences and related molecular material accessible to the scientific community by deposition of such material with the databases. You will be introduced to tools to visualize protein structures and to compare protein structures, and a brief introduction to proteomics is provided.

3.1 Introduction to Bioinformatics Databases

Bioinformatics databases contain biological data from scientific experiments, most importantly DNA and protein sequences and protein structures. In addition to this core level, databases of published literature, computational analysis of primary data, and metadata are also important. Bioinformatics databases are usually affiliated with query facilities as well as data analysis tools. Bioinformatics databases are important for most other fields of bioinformatics and relate to most other chapters of the book (Fig. 3.1).

We expect the databases to represent high diversity of information as well as data formats that can be handled by most computers. The databases need to be reliable and updated and the documentation for the databases easily accessed. These have to comply with guiding principles for scientific data management and stewardship (Findable, Accessible, Interoperable and Reusable, FAIR) (Wilkinson et al. 2019).

Documentation for the databases can be found in the journal *Nucleic Acid Research* (https://academic.oup.com/nar/issue/51/D1) where the first issue each year publishes papers dealing with new developments in the bioinformatics databases. We will refer to these papers and recommend the readers to refer to the papers when they include bioinformatics databases in their scientific publications.

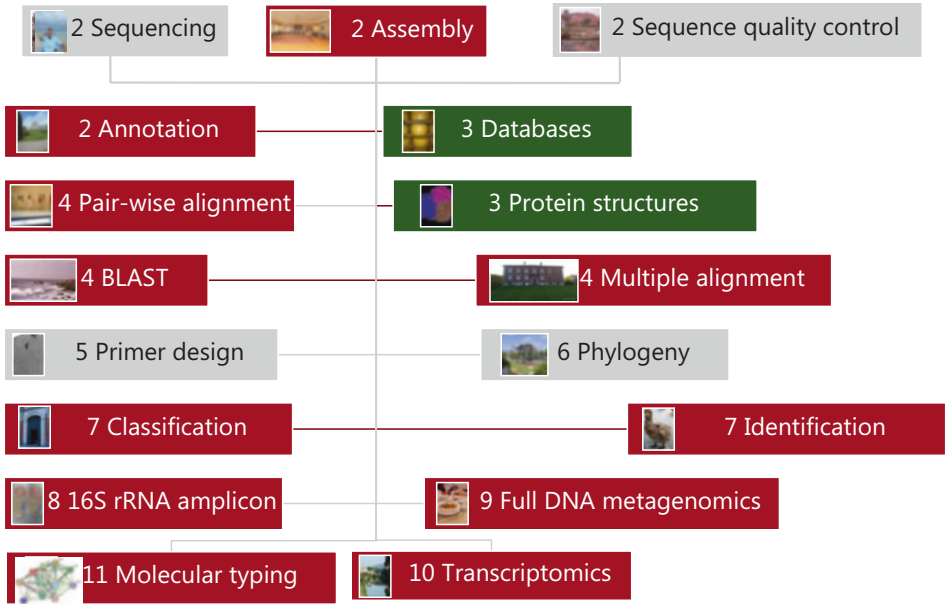


Fig. 3.1 Relation of this chapter to the other chapters in the book

Table 3.1 Major bioinformatics institutions

Abbreviation	Institution and activity	URL and reference
NCBI	National center for biotechnology information. Maintains the most important and frequently used bioinformatics databases such as GenBank and PubMed	https://www.ncbi.nlm.nih.gov/ Sayers et al. (2023a)
UniProt	Universal Protein Resource Maintains secondary protein databases, most important Swiss-Prot	http://www.uniprot.org/ UniProt (2018)
EMBL-EBI	European Bioinformatics Institute Bioinformatics databases, research and training	https://www.ebi.ac.uk/ Cook et al. (2018)
NIG	National Institute of Genetics hosts DDBJ (DNA databank of Japan). DDBJ provides databases and analysis services from life science researches and advances science	https://www.nig.ac.jp/nig/ Tanizawa et al. (2023)

Some databases present parallel information, and each user has a special preference for a certain set of databases, and in this respect, the weight and priority in this chapter reflect author's personal choices and not any ranking of databases.

Bioinformatics databases are hosted by bioinformatics institutions who provide the resources to maintain and curate the databases (Table 3.1). The major bioinformatics databases are available on the internet with free access from bioinformatics institutes. For example, GenBank is hosted with NCBI (Sayers et al. 2023b) and ENA with EBI-EMBL (Cook et al. 2018) (Tables 3.1, 3.2). The bioinformatics institutions are providing the cura-

Table 3.2 Bioinformatics sequence and protein structure databases

Name	Type[a]	Content	Established and hosted at	URL
GenBank	P	DNA sequences[b]	Established in 1982 and hosted at NCBI since 1992	https://www.ncbi.nlm.nih.gov/ Sayers et al. (2023b)
ENA (European nucleotide archive)	P	DNA sequences[b]	Established in 1982 and hosted at EMBL-EBI since 1994	https://www.ebi.ac.uk/ena
DDBJ (DNA Data Bank Japan)	P	DNA sequences[b]	Hosted at NIG since 1987	http://www.ddbj.nig.ac.jp/ Tanizawa et al. (2023)
SRA (sequence read archive)	P	DNA sequences	NCBI	https://www.ncbi.nlm.nih.gov/ Katz et al. (2022)
RefSeq	S	DNA sequences	NCBI	https://www.ncbi.nlm.nih.gov/ Sayers et al. (2023a)
Swiss-Prot	S	Protein sequences	Established in 1986 and since 2003 included with UniProt	http://www.uniprot.org/ UniProt (2023)
PDB (protein data bank)	PS	3D structures of proteins	Established in 1971 and hosted at Research Collaboratory for Structural Bioinformatics (RCSB)	https://www.rcsb.org/ Burley et al. (2023)

[a]P, primary; S, secondary databases
[b]And their protein translations

tion of the databases and maintaining the servers needed to host the large datasets and to make data accessible for the scientific society. NCBI is located and maintained from the United States, whereas EBI-EMBL is located in the United Kingdom and maintained mainly by European funding. DDBJ is located in Japan and maintained by NIG (Kodama et al. 2018).

The first bioinformatics database was established in 1965 (PIR) (Barker et al. 1993), and PIR later became associated with UniProt (Uniprot 2018). The majority of other databases were established during the 1980s (Table 3.2).

3.1.1 Data Formats Used with Bioinformatics Databases

From the information technically point of view, databases can be classified based on the database file structure, for example, as flat file, object-oriented, and relational databases. A flat file database is an ordered collection of similar files in some standard format. A flat file database is made useful by ordering the files and indexing them, which makes them searchable (Gibas and Jamback 2001). These files are in ASCII text file format (https://www.asciitable.com/). The ASCII format translates characters to numbers that can be read into computers. The ASCII format can be read both by humans and computers. Many popular sequence databases, including GenBank, are flat file databases.

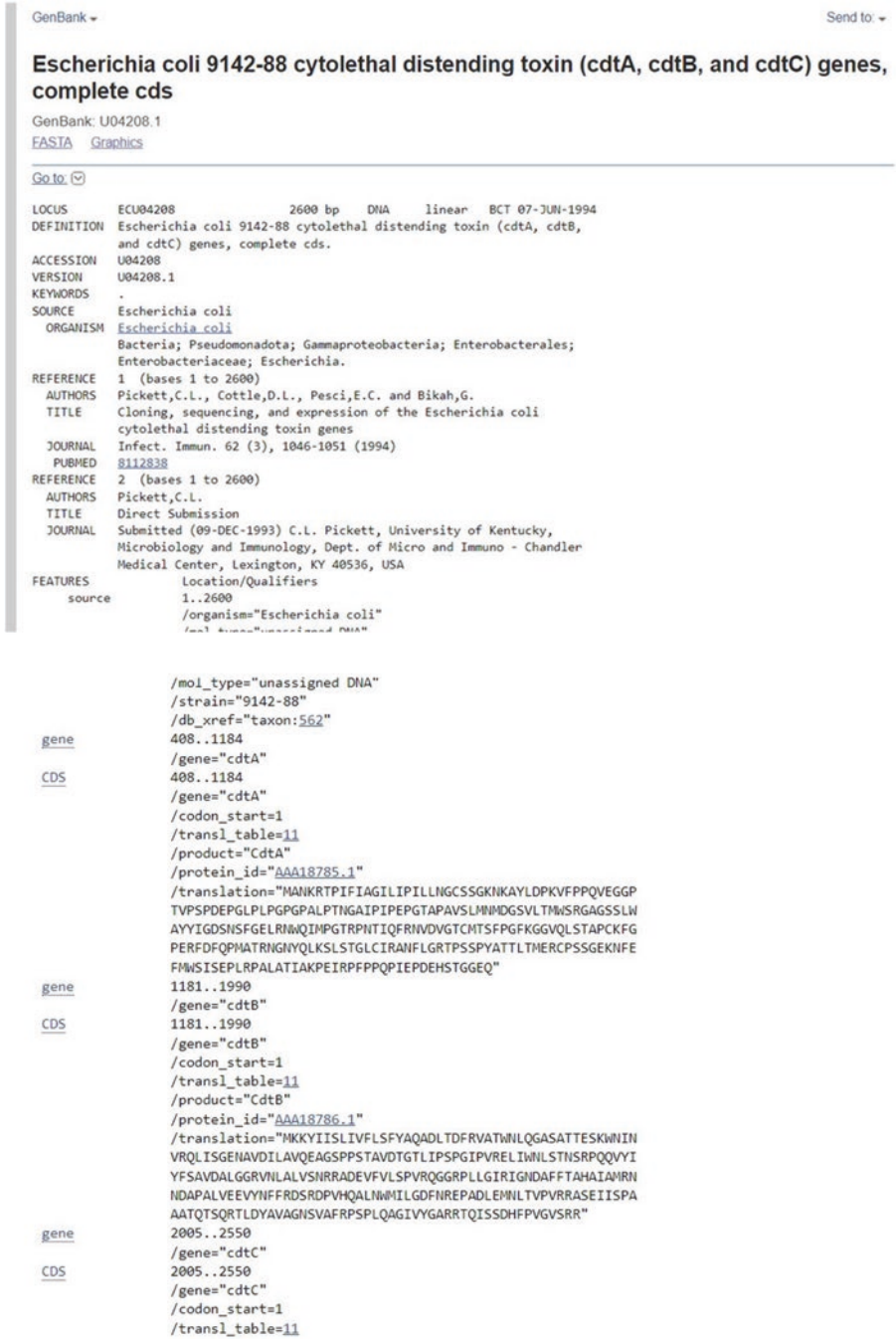
For relational databases, information is stored in a collection of tables, which makes it possibly to extract specific type of information across the database content. For example, from the PBD database, you can extract only information about the secondary structure for a specific type of proteins (Gibas and Jamback 2001).

Object-oriented databases are more complex. They handle data as object (instead of tables), which enables the storage of various information types in different formats, from simple text formats to images and videos (Gibas and Jamback 2001).

3.2 Organization of Databases and Bioinformatics Institutions

The International Nucleotide Sequence Databases (INSD), DDBJ, EMBL, and GenBank in short DDBJ/EMBL/GenBank) are organized in International Nucleotide Sequence Database Collaboration (INSDC) (https://www.insdc.org/). The three databases collaborate to enable access to DNA data in standardized formats for the scientific community worldwide (Arita et al. 2021). The online content of the databases is exchanged on a daily basis. To do that, the officially supported XML format of INSDC, the INSDSeq, is used (see Sect. 3.3.8).

The nucleotide databases DDBJ, EMBL, and GenBank are automatically translated to the protein level if the DNA sequences are coding. These protein translations are available along with the DNA sequences from the databases (Fig. 3.2).



GenBank ▾ Send to: ▾

Escherichia coli 9142-88 cytolethal distending toxin (cdtA, cdtB, and cdtC) genes, complete cds

GenBank: U04208.1

FASTA Graphics

Go to: ⊙

```
LOCUS       ECU04208               2600 bp    DNA     linear   BCT 07-JUN-1994
DEFINITION  Escherichia coli 9142-88 cytolethal distending toxin (cdtA, cdtB,
            and cdtC) genes, complete cds.
ACCESSION   U04208
VERSION     U04208.1
KEYWORDS    .
SOURCE      Escherichia coli
  ORGANISM  Escherichia coli
            Bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales;
            Enterobacteriaceae; Escherichia.
REFERENCE   1  (bases 1 to 2600)
  AUTHORS   Pickett,C.L., Cottle,D.L., Pesci,E.C. and Bikah,G.
  TITLE     Cloning, sequencing, and expression of the Escherichia coli
            cytolethal distending toxin genes
  JOURNAL   Infect. Immun. 62 (3), 1046-1051 (1994)
   PUBMED   8112838
REFERENCE   2  (bases 1 to 2600)
  AUTHORS   Pickett,C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-DEC-1993) C.L. Pickett, University of Kentucky,
            Microbiology and Immunology, Dept. of Micro and Immuno - Chandler
            Medical Center, Lexington, KY 40536, USA
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     CDS             1181..1990
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                     YFSAVDALGGRVNLALVSNRRADEVFVLSPVRQGGRPLLGIRIGNDAFFTAHAIAMRN
                     NDAPALVEEVYNFFRDSRDPVHQALNWMILGDFNREPADLEMNLTVPVRRASEIISPA
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                     /gene="cdtC"
     CDS             2005..2550
                     /gene="cdtC"
                     /codon_start=1
                     /transl_table=11
```

Fig. 3.2 The GenBank format. Note only the start of the entry and a part of the protein section are shown. Readers can easily look at the full record at https://www.ncbi.nlm.nih.gov/nuccore/u04208

3.3 Major Bioinformatics Databases

3.3.1 GenBank

The best known bioinformatics database is GenBank (Fig. 3.2). This is a primary database for DNA sequences. Primary DNA sequence databases are sometimes called "nucleotide" databases and the even shortened as "nt." GenBank is distributed from NCBI as flat file and ASN.1 (Abstract Syntax Notation) file formats (https://www.ncbi.nlm.nih.gov/genbank/) (see Sect. 3.3.8).

From GenBank, all translations to protein are in the GenPept database. The well-recognized GenBank flat file format is shown in Fig. 3.2. Each GenBank record contain three parts: the first part includes information about the record (e.g., type of molecule, submission date, the unique accession number, keywords, source and references), and the second part contains sequence annotations such as biological features, for example, genes, coding sequences (CDS), base counts, and origin. The main characteristics of this format is that it is easy to read both for humans and computers, and information fields are easy to understand (Fig. 3.2).

GenBank can be searched by keyword including accession number via text-based query directly at the root of NCBI (https://www.ncbi.nlm.nih.gov/) or at the nucleotide section (GenBank) where annotations associated with a sequence entry are searched (https://www.ncbi.nlm.nih.gov/nucleotide/).

Sequence similarity search can also be carried out via the algorithm BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi, where a query sequence (DNA or protein) is compared to the sequence database (see Chap. 4 for more details). Data can be retrieved from GenBank in several file formats including the GenBank file record and FASTA file format (see also Activities 3.8.1 and 3.8.2).

3.3.2 The European Nucleotide Archive (ENA)

EMBL was the original primary database for DNA sequences hosted with EBI-EMBL. Now, EMBL is included with ENA (Fig. 3.3). The translations of DNA sequences in EMBL to proteins were called TrEMBL (Cook et al. 2018). TrEMBL was developed specifically for comparison to the Swiss-Prot database (see below). The original EMBL format was using two letter codes for all information fields (Fig. 3.4), which was harder to read than the GenBank format. The original two-letter format is sometimes used. However, the information fields in ENA are now also self-explanatory like GenBank (Silvester et al. 2018).

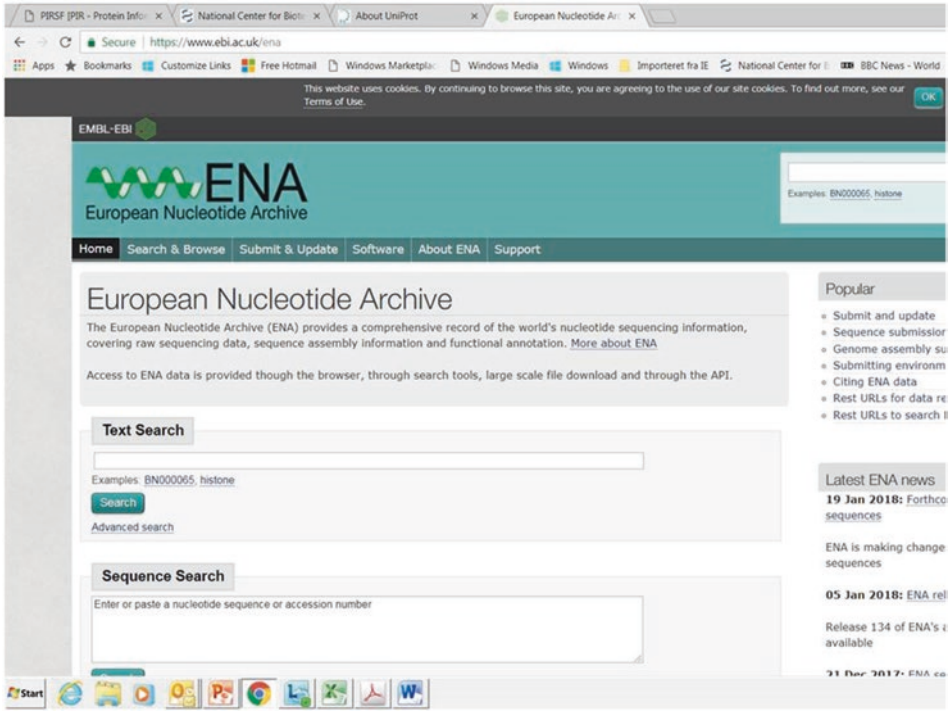

Fig. 3.3 The homepage of the European nucleotide archive (ENA) homepage. Text search by key-work including accession number can be carried out

3.3.3 Swiss-Prot and UniProt

The aim of Swiss-Prot was to include all information from one protein from one species in one entry. Swiss-Prot was included in UniProt in 2002 (in full UniProt Knowledge database) (UniProt 2018), but the context of the Swiss-Prot database has survived.

Protein Information Resource (PIR) was established in 1965 by Margaret O. Dayhoff. The first IT version became available in 1972. The database has been included with UniProt since 2003. PIR is currently focusing on protein information comparisons (https://proteininformationresource.org/).

Really well-known and well-annotated proteins are referred to as "Swiss-Prot" in the UniProt database, and they are also recognized as "reviewer" and labelled with a golden star in UniProt. Sometimes, the Swiss-Prot section is labelled "sp" for short, and this is used at NCBI where proteins from Swiss-Prot also can be looked up. Whenever possible, one should look for information about proteins in the Swiss-Prot section of UniProt (Fig. 3.5).

```
ID U04208; SV 1; linear; unassigned DNA; STD; PRO; 2600 BP.
AC U04208;
DT 31-DEC-1993 (Rel. 38, Created)
DT 04-MAR-2000 (Rel. 63, Last updated, Version 4)
DE Escherichia coli 9142-88 cytolethal distending toxin (cdtA, cdtB, and cdtC)
DE genes, complete cds.
OS Escherichia coli
OC Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales;
OC Enterobacteriaceae; Escherichia.
RN [1] RP 1-2600
RX PUBMED; 8112838.
RA Pickett C.L., Cottle D.L., Pesci E.C., Bikah G.;
RT "Cloning, sequencing, and expression of the Escherichia coli cytolethal
RT distending toxin genes"; RL Infect Immun 62(3):1046-1051(1994).
RN [2] RP 1-2600 RA Pickett C.L.;
RT ;
RL Submitted (09-DEC-1993) to the INSDC. RL C.L. Pickett, University of
Kentucky, Microbiology and Immunology, Dept. of RL Micro and Immuno - Chandler
Medical Center, Lexington,
KY 40536, USA
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FT SAVDALGGRVNLALVSNRRADEVFVLSPVRQGGRPLLGIRIGNDAFFTAHAIAMRNNDA
FT PALVEEVYNFFRDSRDPVHQALNWMILGDFNREPADLEMNLTVPVRRASEIISPAAATQ
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FT PQSHLWMLNPLNTESPII" XX SQ Sequence 2600 BP; 834 A; 555 C; 508 G; 703 T;
0 other;
attaacaaat tacaacaaag atcacattaa ataaaaattg acaattgacc tgtagttcat 60
catttgtaaa ttcatgattt atatcaatca cgctttgtgt tcggagtaag cttataaatt 120
```

Fig. 3.4 EMBL format for acc. U04208 (https://www.ebi.ac.uk/ena/data/view/U04208&display=text). To simplify, some of the reference, cross-reference, and sequence sections have been omitted on the figure

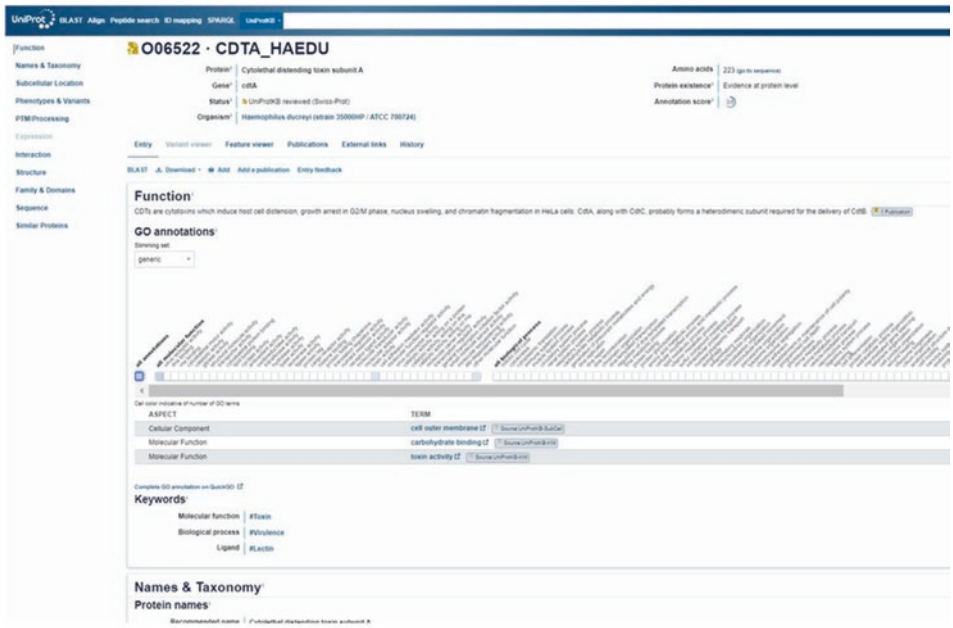

Fig. 3.5 An entry in the UniProt database showing that the protein is in Swiss-Prot (reviewed, note the golden star). The full entry can easily be viewed from https://www.uniprot.org/uniprotkb/O06522/entry (UniProt 2023)

The information about a protein in UniProt can be a bit overwhelming at first sight. This is because all information is actually here gathered about the protein. To guide readers, the information fields are shown in Table 3.3.

3.3.4 Genomics Databases

NCBI registers genomic sequences from single isolates according to biosample and bioproject in the format SAMN00000000 and PRJNA000000, respectively. A genome that is not fully assembled will get an accession number in the form AAAAAA00000000. For fully closed genomic sequences, a GenBank format DNA sequence accession number will be provided in the form AB123456 (see also Activity 3.8.4.2).

The Genomes Online Database (GOLD) (https://gold.jgi.doe.gov/) is an independent resource archiving microbial genomes (Mukherjee et al. 2023).

3.3.5 Raw Sequence Read Datasets

Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/) (Katz et al. 2022) (Table 3.2) is a primary database for raw sequence reads hosted with NCBI. Here, whole

Table 3.3 Major information fields used in an UniProt protein entry

Information field	Content	Example
1. Function	Short summary of biological function	"CdtB exhibits a DNA-nicking endonuclease activity .."
2. Names and taxonomy	Protein names, gene names, organism names	*Escherichia coli*
3. Subcellular location	Localized to the membrane or cytosol	"Secreted"
4. Phenotype and variants	Effect of mutagenesis on function	Mutagenesis H154A will not cause cell cycle arrest in HeLa cells
5. PTM/ processing	How the molecule is processed	Signal peptide at position 1-18
6. Structure	Graphical representation of the secondary structure. Links to 3D models. Note, only models with PDB numbers have been experimentally determined	2F1N link
7. Sequence	The protein sequence	MKKYIISLIV FLSF…
8. Family and domains	Links to family and domain databases	"View protein in Pfam"
9. Interaction	Other proteins that the protein is associated with in vivo	Heterotrimer of 3 subunits, CdtA, CdtB, and CdtC

The CdtB protein has been used as example (accession number Q46669 from UniProt). Note that you can make a custom arrangement of the information fields at UniProt. For instance, if your main interest is STRUCTURE, this field can be shown on the top. Numbers of information fields are arbitrary

raw datasets from Illumina sequencing of whole genomes (Chap. 2) and 16S rRNA metagenomes (Chap. 8) and full DNA metagenomes (Chap. 9) can be stored and downloaded. A similar facility is provided by European Nucleotide Archive (ENA) (Table 3.2).

3.3.6 Other Databases

The following databases are all more specialized (Table 3.4). CAZyme is a database of carbohydrate-active enzymes (André et al. 2016). This database separates enzymes in glycoside hydrolase (GH) families.

Kyoto Encyclopedia of Genes and Genomes (KEGG) has the main objective to establish links from collective sets of genes in the genome to high-level function of the cell and organism (Kanehisa et al. 2016). Annotation can be performed in KEGG (Chap. 2). For a long time, KEGG GENES database was created from NCBI's RefSeq database. From 2014 on, KEGG GENES also includes genomes from GenBank. KEGG has a section devoted to antimicrobial resistance. The benefit of KEGG is that it focus on modules and

Table 3.4 Specialized databases

Name	Content and use	URL
KEGG	Metabolic pathway database	https://www.genome.jp/kegg/ Kanehisa et al. (2023)
iPath3.0	Metabolic pathways analysis and visualization	https://pathways.embl.de/ Darzi et al. (2018)
EcoCyc	Metabolic database dedicated to *Escherichia coli*	https://ecocyc.org/ Keseler et al. (2017)
CAZyme	Database of carbohydrate active enzymes	http://www.cazy.org/ André et al. (2014)
COG	Sorting of proteins: Cluster of ortholog proteins	(https://www.ncbi.nlm.nih.gov/research/cog-project/) Tatusov et al. (2003)
Essential genes	Genes that are indispensable for the survival of organisms	http://www.essentialgene.org/ Gao et al. (2015)
Silva	rRNA gene sequence database all three domains of life	https://www.arb-silva.de/ Yilmaz et al. (2014)
RDP	16S rRNA gene sequence database of prokaryotes and fungi	Cole et al. (2014)
GreenGenes	16S rRNA gene database and quality control	https://greengenes.secondgenome.com/ DeSantis et al. (2006), McDonald et al. (2012)
tRNA	Compilation of tRNA sequences	http://trna.bioinf.uni-leipzig.de/DataOutput/ Jühling et al. (2009)
rrnDB	Comparison of number of rRNA loci in prokaryotes	https://rrndb.umms.med.umich.edu/ Stoddard et al. (2015)
OperonFinder	Operon predictor	https://www.iitg.ac.in/spkanaujia/operonfinder.html

pathways directed against functionality, the drawback that it excludes poorly annotated functions. The 16S rRNA gene sequence databases are further used in Chap. 8.

3.3.7 Primary and Secondary Bioinformatics Databases

From the biological point of view, the most important categorization of databases is into primary and secondary databases, which refers to the type and source of stored data. Primary databases, such as GenBank and ENA, are also called archives or repositories, and they take information directly from the individual researcher, and data are owned by the submitter with privileges to change data. The benefit is fast publication of new entries in the databases and high diversity. The drawbacks of this system are that information occasionally can be wrong, for instance, if the researcher should accidentally have used

the wrong coding table (Chap. 2) or wrong names for organisms (Chap. 7) or wrong information fields.

Users should always be aware of errors in the databases. It is recommended to contact the authors listed with the specific entry of database linked to the dataset if errors are suspected. Such errors will be passed on to all other databases that are linked to the dataset if they are not corrected. Another problem with the primary databases is the tendency for redundancy (repeating information) since two scientists could in principle submit the same sequence for the same gene for the same organism independently of each other. Example 3.9 illustrates the problem with the gray zone between redundant information, variations in genome assemblies, and maybe also errors. When searching primary databases, users should always be critical of the information, especially if the data is not associated with a published reference.

The secondary databases (e.g., Swiss-Prot and PDB) are curated, and they perform a quality control and sorting of information before the information is made accessible to the public. These databases have better chances of reducing redundancy. They can also bypass the submitters of entries in the primary databases, which are no longer updated. This is important in case the submitter changes affiliation or no longer is active in science.

A variant of the secondary database is the third-party annotation (TPA). This is a secondary database mainly of nucleotide sequences. To avoid shortcomings with primary data and bypass submitter privileges, the database has released the submitter burdens of updating data. The best known example is RefSeq at NCBI, which is used a lot with genomic sequences and providing a backbone to a range of DNA metagenomics studies (Chap. 9).

3.3.8 Data Formats in Bioinformatics Databases

FASTA is the most simple format introduced in Chap. 1 (Sect. 2.4). This format is not very suitable for arranging information in the bioinformatics databases since the information next to the sequence is not structured and can only be very brief. Therefore, more special data formats are used.

ASN.1 (Abstract Syntax Notation one) is a format suitable for transferring data between different computers and computer system. If you select this format for a sequence in GenBank (try the link: https://www.ncbi.nlm.nih.gov/nuccore/U04208.1?report=asn1&log$=seqview), it can be read but will not give much meaning to the human reader but to the computer.

A very precise way of representing database information is the XML format. Extensible markup language (XML) is derived from Standard Generalized Markup Language (SGML). All information of a document is annotated in the format <tag>text</tag>. The INSDC variant of XML is used. For instance, the sequence in a GenBank file in INSDSq format is between tags <Seq-data> and <\Seq-data>, and the accession number in a GenBank file in INSDSq format is between tags <Seq-id> and <\Seq-id>.

3.4 Accession Numbers

All sequences in the databases are recognized by accession numbers. They are the unique identifiers for all information. Already the format of an accession number tells about its origin (Table 3.5). Nucleotide sequences in primary DNA database like GenBank have the format of two letters and six numbers. Their translated protein sequences have the format of three letters and five numbers. All GenBank entries had a GI number (GenBank unique number for each sequence 1,234,567); however, this system was stopped in September 2016, and now, only older sequences in GenBank can be recognized by this number. New WP numbers were introduced from 2013 for proteins. Nonredundant protein sequences will only be given one WP number (Clark et al. 2016). To get information about proteins included in WP numbers, use the number as link and see at **Identical proteins** where all the accession numbers included with the WP number are listed.

Table 3.5 Accession numbers

Database	Section	Accession number format	Example
International Nucleotide Sequence Database Collaboration (GenBank, EMBL, DDBJ)	GenBank	Common to DNA X12345 (until 1999) (1 + 5), AB123456 (1999–) (2 + 6)	U04208
	Protein	P12345 (until 1999) (1 + 5), ABC12345 (1999–) (3 + 5)	O06522
	RefSeq	Accession no. 2 + 6 with underscore NT_123456 genomic DNA NM_123456 mRNAs NP_123456 protein	
	Swiss-prot	Primary accession number on the form P12345 but also 6 digit combinations of numbers and letters: NNNN_YYYYY The Ns represent the protein name and the Ys the organism.	O06522 CDTA_HAEDU
PDB		1N1N (capital letters and numbers without fixed order)	2F1N
NCBI	Genomes	Incomplete NNNNNN12345678 Complete AB123456 (or A12345 and NNNN12345678 old number series)	LXWV01000000 L42024

3.5 Protein Structure Databases and Predictions

This section provides an introduction to databases of protein structures and how structures can be downloaded and visualized from the databases. Prediction of structures based on a query protein sequences will be described.

One of the beautiful synergistic achievements in bioinformatics is that the function of a protein can be predicted by a rather low identity of proteins over rather short length of comparison and rather low similarity to protein structures. As a rule of the thumb, the function of a protein can be predicted if it is at least 40% similar in its primary structure to known proteins in the database. In the range of 20–40% similarity, only folds can be predicted but not function. Homology modeling based on 3D structure performed with Swiss-Prot or similar (Table 3.6) can predict 3D structures for proteins if they are at least 25% identical in a pair-wise alignment of at least 100 amino acid (Petsko and Ringe 2004).

With respect to the prokaryotes, we are interested in predicting if a protein is secreted to the extracellular space, if it is part of the cell wall, or if it is located in the cytoplasm of

Table 3.6 Prediction and comparison tools for protein structures

Tool	Function	URL
PRED	Predicting and discriminating beta-barrel outer membrane proteins	http://bioinformatics.biol.uoa.gr/PRED-TMBB/ Bagos et al. (2004)
Dali	Comparison of structures	http://ekhidna2.biocenter.helsinki.fi/dali Holm (2022)
Phyre2	For comparison of models. Visualization in CLC Main Workbench using superimpose function	http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index Kelley et al. (2015)
SignalP	Determination of signal peptide cleavage sites	https://services.healthtech.dtu.dk/services/SignalP-6.0/ Petersen et al. (2011)
PSORT	Protein localization sites	https://psort.hgc.jp/ Nakai and Horton (1999)
PHOBIUS	Transmembrane topology and signal peptide predictor	https://phobius.sbc.su.se/ Käll et al. (2004)
OMPdb	Beta-barrel membrane proteins located in the outer membrane of bacteria with a gram-negative cell wall structure	http://www.ompdb.org/ Tsirigos et al. (2011)
SWISS-MODEL	Homology modeling of protein structures	https://swissmodel.expasy.org/ Waterhouse et al. (2018)
Swiss-PdbViewer (aka DeepView)	User-friendly interface to analyze several proteins at the same time including superimposed functionality	https://spdbv.unil.ch/
PyMOL	The ultimate tool for protein modeling	https://pymol.org/2/

the prokaryote. A special group of very important proteins form pores in the prokaryotic cell and occupy important positions in the periplasmic space of bacteria with a gram-negative cell wall structure. Both function and location of uncharacterized proteins can be predicted bioinformatically by comparison to specialized protein structure databases.

3.5.1 Primary and Secondary Structures

The primary structure of a protein is simply the amino acid sequence. The secondary structure can mainly be separated in α-helixes and β-sheets (Fig. 3.6); however, also the "loop" and "turn" regions linking α-helixes and β-sheets are important. Wool contains α-helices and silk β-sheets. In the prokaryotic world, proteins rich in α-helices may be transmembrane; however, the beta barrel structure predominated by β-sheets may also be transmembrane. An example of a protein with β-sheets is the cytolethal distending toxin (Cdt) (Fig. 3.6). It is a tripartite complex (A, B, C) that is required for the CDT activity

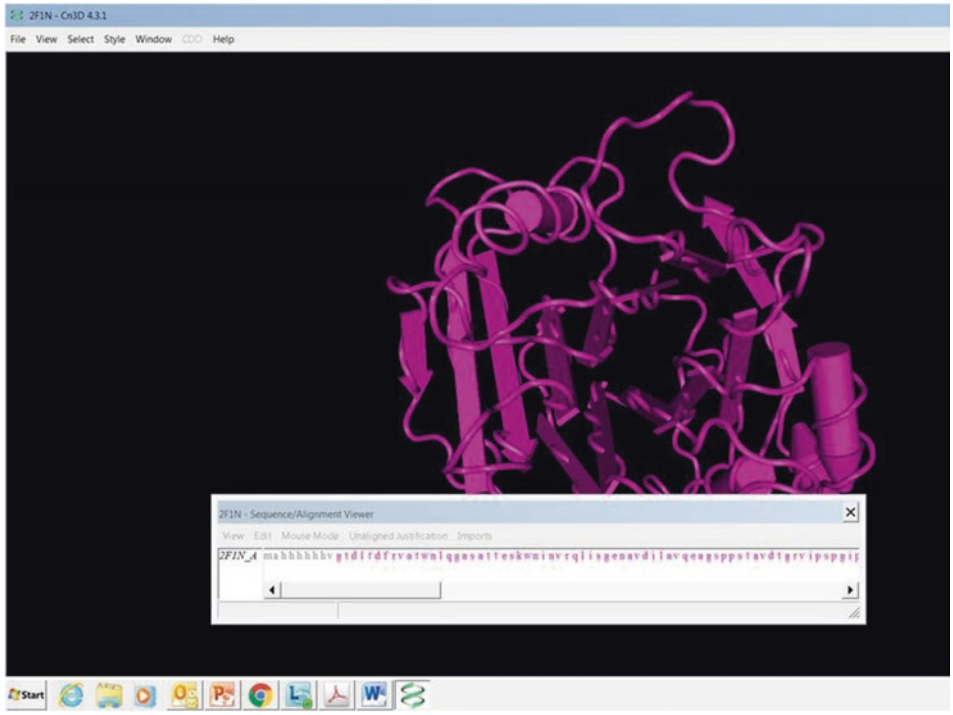

Fig. 3.6 The 3D structure of the CdtB protein (acc. no. 2F1N) visualized with Cn3D viewer (Activity 3.8.3). The flat arrows illustrate regions with β-sheets and the "pencils" regions with α-helices (STRUCTURE [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004 – [cited 2018 04 17]. Available from: https://www.ncbi.nlm.nih.gov/structure/)

(Pickett and Whitehouse 1999). The holoprotein (consisting of proteins A, B, and C) induces G2/M cell cycle arrest, chromatin fragmentation, cell distention, and nucleus enlargement. CDTA and CDTC probably form a heterodimeric subunit required for the delivery of CDTB. CDTB exhibits a DNA-nicking endonuclease activity and very probably causes DNA damage in intoxicated cells.

The M-protein is used as an example of a protein with mainly α-helix secondary structure (Ghosh 2018). The α-helices are here exposed on the surface of the streptococci, and the variable N-terminal may interact with the immune system of the host. Two α-helices are held wound together to coil coiled holoproteins. A coil–coil structure is formed as a super twisted helix. The two α-helices will have a periodicity of seven amino acids with a certain combination of hydrophobic amino acids allowing two adjacent hydrophobic amino acids to bury their hydrophobic nature from the surrounding solvent.

3.5.2 Domain Prediction and Databases

Domains are compact units of proteins that may behave independently and be associated with certain functions. Motifs are conserved regions of proteins, which may be part of domains (Fig. 3.7). On the figure, the three types of predictors are illustrated.

3.5.2.1 Single Motif

PROSITE (https://prosite.expasy.org/) is based on so-called regular expressions to define biologically significant protein patterns and profiles. A regular expression is a sequence of characters that define a *search pattern*.

A regular expression is, for instance:

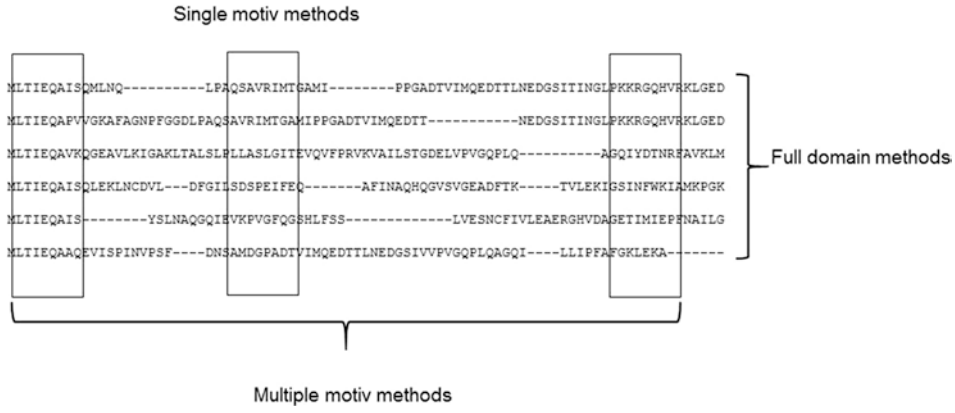

Fig. 3.7 Databases and prediction tool of protein motifs, domains, and families (figure redrawn from Higgs and Attwood 2005)

Y-x-[NQH]-K-[DE]-[IVA]-F-[LM]-R-[ED], which is the heat shock hsp90 proteins family signature. The code is the single symbol code for amino acids and positions where variation is allowed is labelled with "x" and positions where only a subset of amino acids possible are labelled within brackets. In the PROSITE database, an entry will look like this (note the EMBL coding, see Fig. 3.4).

ID CUTINASE_1; PATTERN.

AC PS00155;

DT APR-1990 (CREATED); NOV-1997 (DATA UPDATE); MAR-2005 (INFO UPDATE).

DE Cutinase, serine active site.

PA P-x-[STA]-x-[LIV]-[IVT]-x-[GS]-G-Y-S-[QL]-G.

Epitope prediction is an important task for the prediction of interactions of proteins with the immune system. The paper by Soria-Guerra et al. (2015) compares different tools. Surface-exposed epitopes are often associated with loops and turn located between α-helices and β-sheets.

3.5.2.2 Multiple Motifs

BLOCKS (Henikoff and Henikoff 1992) is based on multiple alignments of conserved regions.

3.5.2.3 Full Domain

Pfam is primarily based on UniProt reference proteomes (Finn et al. 2016). A representative subset of matching sequences is aligned to make a seed alignment. It is used to construct a profile-hidden Markov model (HMM) using the HMMER software (http://hmmer.org). From 2023, Pfam is hosted with InterPro (next section).

Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) is for identification and annotation of protein domains based on manually curated models. Prediction is also here based on hidden Markov modeling (HMM) (Letunic and Bork 2018).

3.5.2.4 Mixing Different Methods

InterPro (https://www.ebi.ac.uk/interpro/) (Paysan-Lafosse et al. 2023) is family and domain database linking information in PROSITE, ProDOM, CDD, Pfam, and seven additional domain databases. InterProScan is the underlying software that allows users to search with protein and DNA sequences against InterPro's predictive models.

Conserved Domain Database (CDD) is linking information from Pfam and SMART (Fig. 3.8). CDD is automatically invoked when a protein BLAST search is activated at NCBI (Chap. 4). The results from CDD is found in the top of the Graphics Summary in the BLAST output.

More general protein interaction networks can be predicted with STRING (https://string-db.org/) (Szklarczyk et al. 2023), which integrates functional protein associations.

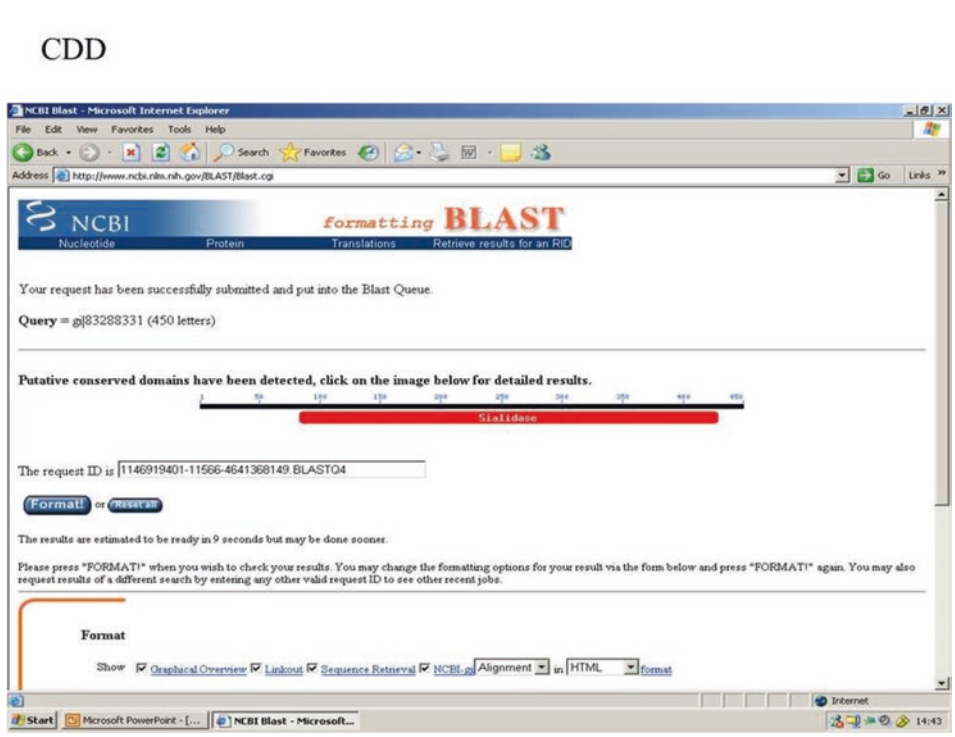

Fig. 3.8 CDD domain prediction invoked in BLASTp. The search resulted in a sialidase domain being predicted (BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004. Available from: https://www.ncbi.nlm.nih.gov/Blast.cgi)

3.5.3 Protein 3D Structure

The 3D structure of a protein is representing the full folding pattern of a protein. Three-dimensional structures are needed to analyze the location of active sites where amino acid substitutions will have an effect on the function of the protein. The 3D structure is also needed to investigate interactions between proteins and other molecules.

The 3D structure of a protein is an exact measurement based on X-ray crystallography or NMR spectroscopy. X-ray crystallography can only be performed on crystals of proteins. For proteins to form crystals, small ions are sometimes needed (ligands). Three-dimensional structures for proteins that cannot be crystallized need to be determined by NMR. The database for the 3D structures is PDB (Table 3.2) (Fig. 3.9).

An example of a 3D structure has been given for a protein with predominantly β-sheets (CdtB) (Fig. 3.8), and the M-protein from *Staphylococcus aureus* has been used to represent a structure predominantly with α-helices.

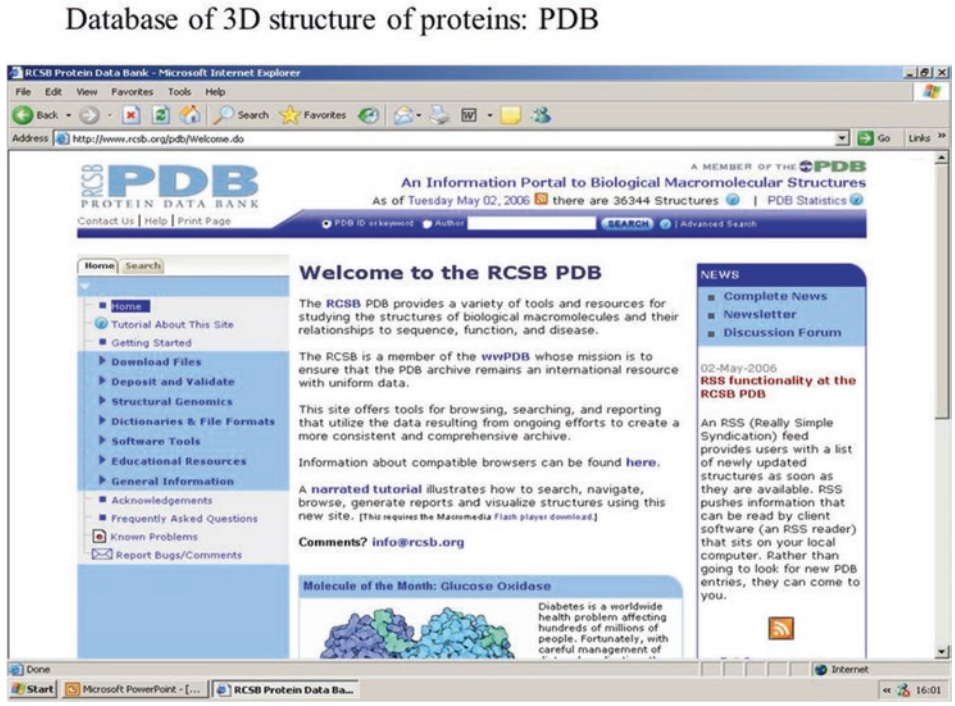

Fig. 3.9 The portal of Protein Data Bank (PDB) hosted by Research Collaboratory for Structural Bioinformatics (RCSB). PDB is a worldwide repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids (https://www.rcsb.org/)

The resolution of the 3D structure is very important. High resolution is best. With 1 Å resolution, the atoms are visible; however, 30 times more data are needed compared to 3 Å resolution. With 3 Å resolution, folds can be seen, and with 2 Å, side chains are resolved.

Comparison of 3D structures can be done in Phyre2 or Swiss-Model (Table 3.6). The folding pattern is theoretically determined by the phi (φ) and psi (ψ) angles. The peptide bound between amino acids in protein chains is planar (180°) (rarely 0°), and the φ (N–C) and ψ (C–C) angles determine the folding. The deviation between expected and observed folding can be visualized in a Ramachandra plot (Fig. 3.10). The upper left field represents φ and ψ angles of β-sheets, whereas the middle left section represents α-helixes. The variants observed in the right quartets may be the amino acid glycine, which is not associated with any particular folding pattern. Amino acids at other positions in the plot represent observed variants not fitting with the theoretical expectations.

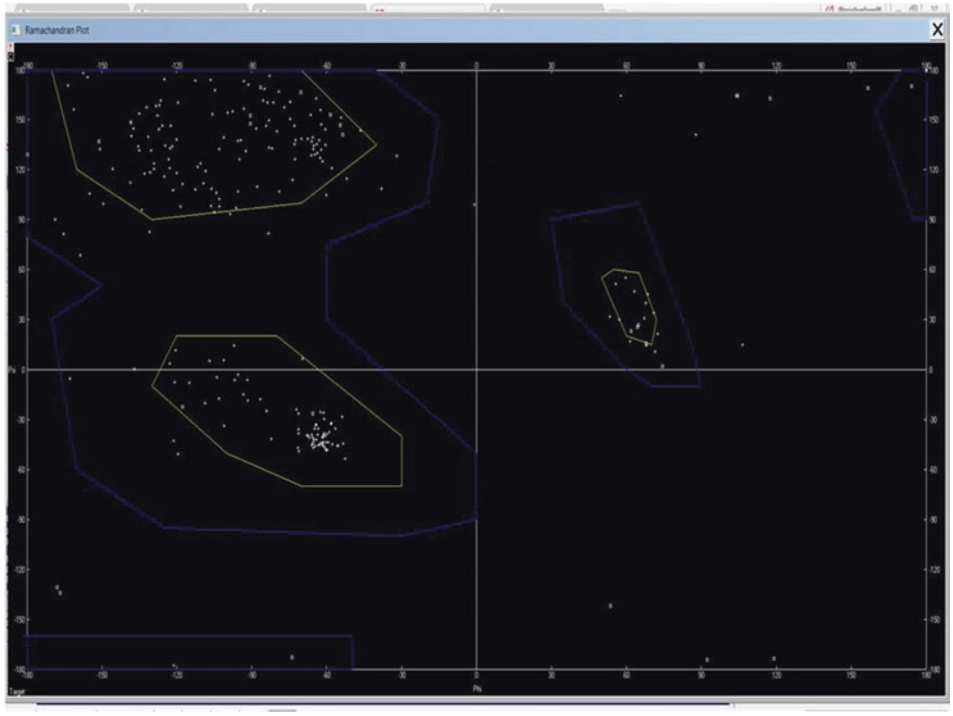

Fig. 3.10 Ramachandra plot is a comparison between observed and expected phi and psi angles. The plot was obtained from SPDB (Activity 3.8.5) (https://spdbv.vital-it.ch/)

3.6 Overview of Proteomics Databases and Servers

Proteomics is dealing with the prediction of proteins based on measurements of mass-to-charge ratios. Proteins need to be degraded to peptides before they can be analyzed by mass spectrometry (MS). Trypsin is the preferred enzyme to degrade proteins to peptides resulting in molecular weights of 300–1500 Daltons. Liquid chromatography (LS) is used to fractionate the protein extract for the analysis. The double MS (MS/(MS) is needed to obtain sufficient resolution of for precise determination of mass-to-charge ratios (m/z). Disulfide bridges are normally ignored in proteomics. The majority of proteins in cells are conserved between types and tissues. Only a minor fraction of proteins varies and changes with disease. The membrane-bound proteins are the most difficult to identify since they cannot be extracted free from their matrix (the membrane). The drawbacks with proteomics are that all proteins cannot be identified. One group of proteins cannot be degraded

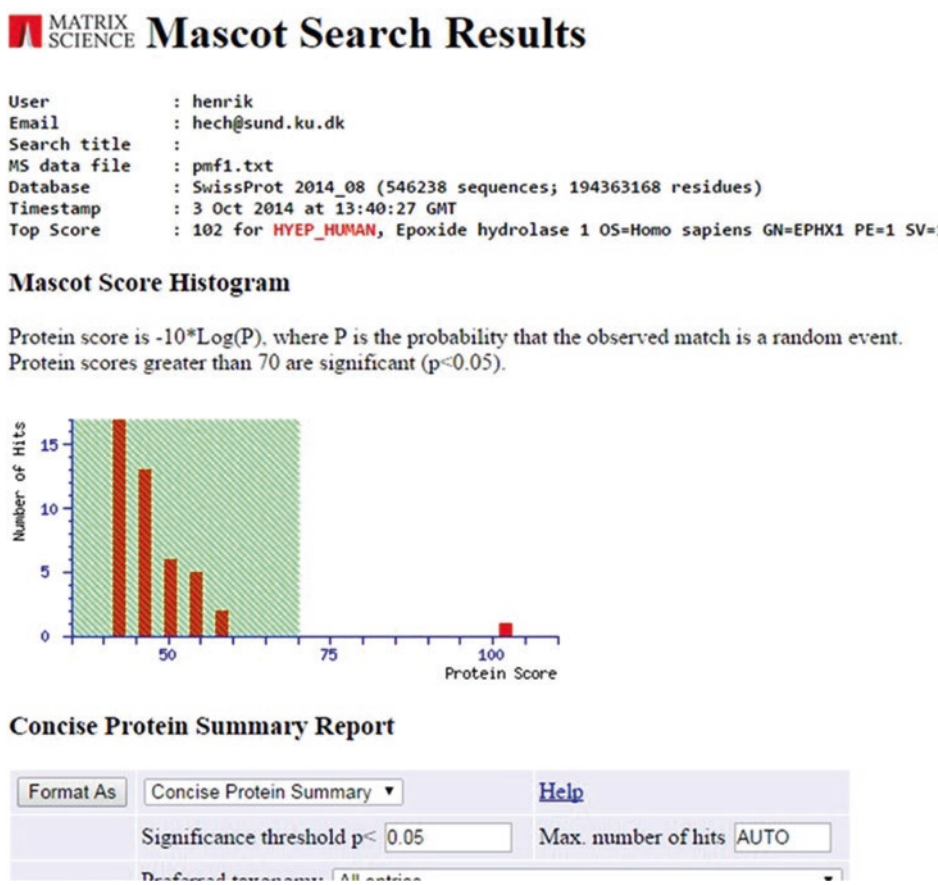

Fig. 3.11 Mascot proteomics search tool (http://www.matrixscience.com/)

to peptides. Another drawback is that the state of the art requires highly advanced hardware and bioinformatics that can acquire full images of samples and full storage of the image that can afterward be analyzed in different ways. For these reasons, proteomics is even more complicated than "genomics." Prediction of proteins is done with Mascot (Fig. 3.11). In this program, the *m/z* coordinates from an analysis are held up against the reviewed part of UniProt (3.3.3). MaxQuant is a free program used for meta-proteomics (Fig. 3.12).

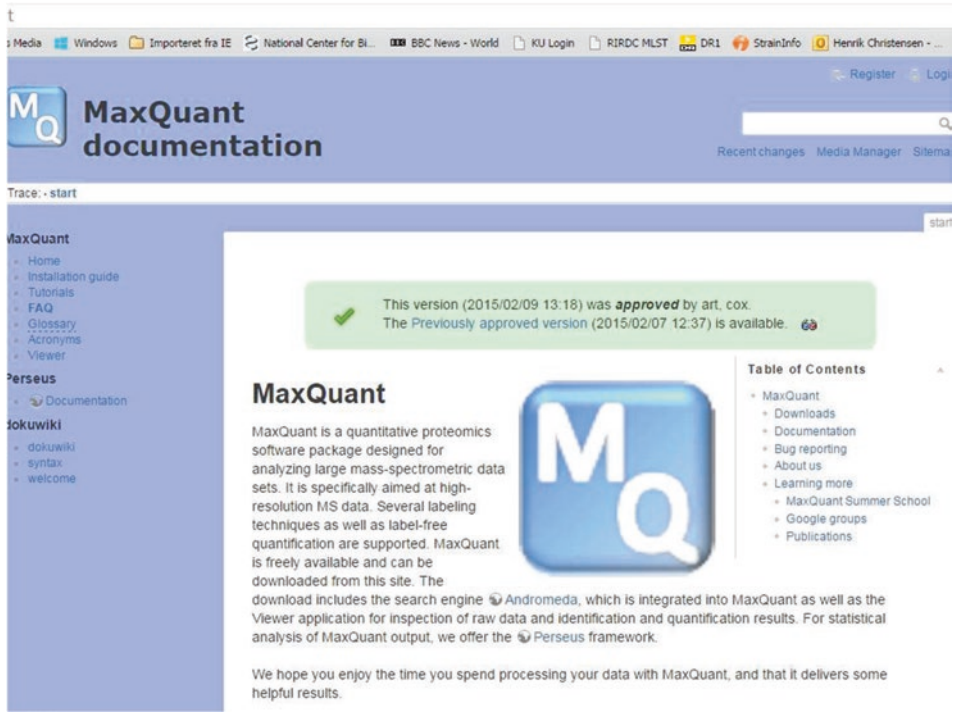

Fig. 3.12 Maxquant (Cox). Freeware used for shotgun proteomics. Includes Perseus module for statistics and Andromeda search engine based on Mascot (Fig. 3.11) (https://www.biochem.mpg.de/6304115/maxquant)

3.7 Help to Databases

The major bioinformatics databases maintain updated online "handbooks" (Table 3.7).

Table 3.7 Help to databases

Database	Subject	URL
Instructions to Swiss-prot	The Swiss-Prot manual, here you will find all documentation	http://www.expasy.org/sprot/userman.html
Instructions to NCBI	The NCBI handbook	https://www.ncbi.nlm.nih.gov/books/NBK143764/
Overview of all databases	First issue of Nucleic Acids Research each or previous years	https://academic.oup.com/nar/article/51/D1/D1/6964796

3.8 Activities

3.8.1 Download a Sequence from NCBI

We will download the DNA sequence for the *cdtB* gene encoding the CdtB protein mentioned in Sect. 3.5.1.

Open GenBank (NCBI) from your browser with the URL: http://www.ncbi.nlm.nih.gov/,

Choose "Nucleotide" in the **Search window** and write the accession number **U04208** in the window after "for" and press **Search**. As a standard, the result is shown in GenBank format.

Convert the format to FASTA by selecting FASTA (left top corner).

Save the file on your computer by clicking **Send to:** (top right corner), select **File** in the **Choose Destination** window, and select **FASTA** in the **Format window** and **Create File**.

Open the file by **Word-pad,** check that it is in FASTA format (Sect. 2.4) and save it on the computer with the acc. no. as name and filename extension, fasta or fna (e.g., U04208.fasta).

You actually downloaded the DNA sequence of *cdtA*, *cdtB*, and *cdtC* genes since they are located in polycistrons. If you only want to download the DNA sequence of the CDS of one gene, then press the CDS link when you are in the GenBank format and then press the FASTA link in the lower right corner, then only the DNA sequence of the CDS will be downloaded.

Batch Download If you need many DNA sequences to download, all acc. no. can be written in the search field at NCBI just separated by space, and all can be downloaded at once. However, in this case, you will get the full sequence of all entries and not only the CDSs.

3.8.2 Download a Genome from NCBI

To access genomic sequences of prokaryotes at NCBI: (https://www.ncbi.nlm.nih.gov/), select **Genome** in right panel and then **Microbe** in the middle column and then **Browse microbial genomes** to the left.

Insert the organism's name: **Christensenella minuta** under **Genome information by organism** and press **Search**.

In the column **Replicons,** use acc. no. CP029256 as link and open the GenBank record. This information can be downloaded as just described at 3.8.1 for a record with a few genes.

Try to open the file with a text editor to control that is what you expected.

You should see a typical GenBank format just with much more information than the one on Fig. 3.2.

At **Graphics**, representation of annotated genes is provided by arrows. You can zoom in. The view can be saved as PDF format from download.

3.8.3 Deposition of Sequence with GenBank

3.8.3.1 Procedure for Single DNA Sequences

Before you deposit a DNA sequence, you need to be 100% certain about the unbiased nature of the sequence and have all metadata available for the sequence. The tool BankIt will be most easy to start with. You need to register as user by following the link: https://www.ncbi.nlm.nih.gov/WebSub/

Sequences are submitted by interactive online approach.

Do not upload the translation independently. The program will automatically translate the DNA sequence. For partial sequences, mark 5′ partial and 3′ partial.

3.8.3.2 Genomic Sequence

The following instruction is based on one bacterial strain with the genome assembled into contigs with CLC or similar program listed in Table 2.2 (Chap. 2).

You should prepare the contigs in a text file and control that the sequences are not including Ns. This means that they should be assembled without ambiguities.

You edit all description lines (first line of FASTA format with the > sign) to >contx [organism = NNN] [strain = XY] where x is the contig number, NNN the species, and XY the strain number.

The replace function in WordPad can be used for that. Alternatively, if your genome has many contigs or you have many genomes, you can use a format function in CLC Genomics Workbench for this: In **CLC** select **Help|Batch rename.**

You then upload from https://submit.ncbi.nlm.nih.gov/subs/genome/.

The NCBI-specific identifiers **Bioproject** and **Biosample** numbers are registered with NCBI during upload. At the end, you will get a genome accession number in the format NNNN12345678 (Table 3.5).

3.8.4 Protein Structure Prediction with Swiss Model and SPDBV

To predict the structure of a protein, we can use SWISS-MODEL found at (https://swiss-model.expasy.org/) (Arnold et al. 2006).

Start modeling by upload of the protein sequence. Try the protein with acc. no. OOF68275. See Activity 3.1 of how to download. Select "**Build Model**." When the analysis is done, usually the top result is the best prediction. However, sometimes, a protein can be split up on many 3D models. This can, for instance, happen with a long autotransporter protein. The outputs show the best hit to a structure already known.

The bars show how good the prediction of structure has been through the sequence based on different tools. This information can be used if you are interested in specific regions of the sequence, for example, conformations of regions involved in substrate binding.

You can download the predicted model (sequences with .pdb extension) by at **"Model"** selecting **SPDBv format** or **pdb format** and save the model.

Use Vast to convert pdb to a structure that can be visualized in Cn3D (Activity 3.3) (https://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html).

As an alternative, you can "**search for template**" if you have no clue about the structure or specify a model if you want to test the similarity to that one.

The models can be opened with the **SPDBV_4.10_PC**. You need to install the program on the PC first.

http://swiss-pdb-viewer.software.informer.com/download/

This happens sooner than expected, and the panel is rather narrow and the file opened from **File|Open**. Tools to move, turn, and zoom in on the structure from the narrow panel are available.

It is possible to look at the **"Ramachandran plot"** from **"Select|All|Wind"** (Fig. 3.10).

3.9 Example Illustrating Variable Information and Redundancy of a Primary Genomic Database and Comparison to Some Other Information in the Book

The *E. coli* strain ATCC 25922 is commonly used as a reference used for testing of antibiotic susceptibility and similar control measures. It is quite natural that the genomic sequence is available from NCBI. It is possible to download ten independent genomic sequences of this strain (Table 3.8). Three genomes are fully closed, whereas the remaining seven are on contig form (see Chap. 2). Further comparison is referring to the information about assemblies from NCBI (see Chap. 2). For the closed genomes from 1 (CP037449), up to four plasmids (CP117235) have been annotated. It is unclear if this difference is related to real differences in plasmid content between different subcultures of the strain or if the plasmids have not been included in the genomic sequence. For the genomes not closed, plasmids have not been annotated.

The GGDC index is expected to be 0.0000 for genomes of the same strain; however, to a surprise, this index differs except for two genomes. Note also the difference in total genome length (variation up to 3.5%)—again, we compare genomes of the same strain and not species!

Table 3.8 Different version of the genomic sequence of the *E. coli* strain ATCC 25922

Accession nr.	Contigs	Total genome length including plasmids	GGDC difference to CP009072 (for method, see Chap. 7)[a]
ASHD01	135	5,113,276	0.0009
CP009072	1 + 2 contigs as plasmids	5,203,425	Reference
CP032085	1 + 3 contigs as plasmids	5,312,633	0.0000
CP037449	1 + 1 contigs as plasmids	5,129,120	0.0051
CP117235	1 + 4 contigs as plasmids	5,209,984	0.0005
JADEVB01	88	5,033,099	0.0006
JAPTQO01	60	5,178,906	0.0002
LRDO01	156	5,122,028	0.0002
MTFU01	83	5,146,680	0.0002
QYKP01	280	5,174,269	0.0001

[a]Formula 1 used for closed genomes and formula 2 for non-closed

Also for *Pasteurella multocida*, variation between assembled genomes of the same strain is observed with 0.0001–0.0002 differences in GGDC (strain X73 acc. no. AMBO01, LUDA01, CM001580; strain ATCC 43137 acc. no. CP008918, LT906458; strain P1933 acc. no. ARNY01, JAMJVV); however, a fourth strain (ATCC 15742) did not show differences between three assemblies (acc. no. CM001518, LUDB01, AMBQ01 (0.0000 GGDC)).

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Pairwise Alignment, Multiple Alignment, and BLAST

4

Henrik Christensen and John Elmerdahl Olsen

Contents

H. Christensen (✉)
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk

J. E. Olsen
National Food institute, Technical University of Denmark, Lyngby, Denmark
e-mail: jkovo@food.dtu.dk

H. Christensen (ed.), *Introduction to Bioinformatics in Microbiology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-031-45293-2_4

What You Will Learn in This Chapter

Pairwise alignments and multiple alignments are the basic tools to compare sequences. BLAST is the most frequently used bioinformatics program to compare your own sequence (query sequence) to all sequences in a database (subject sequences) based on local pairwise alignments. The outcome of the BLAST analysis provides qualitative information about homologous sequences and can quantify the identity of the query sequence to the sequences in the database. You will learn about multiple alignments and how to construct them. Multiple alignments are used for a range of applications described in the other chapters of the book.

4.1 The Pairwise Alignment Problem

A pairwise alignment is a model of the homology between two sequences considering all nucleotides or amino acids and all deletion and insertions.

Two sequences to be compared could, for instance, be the ones below:

GCAGTAGCATGACGATAG
GCGGTAGCATGATAC

A pairwise alignment requires a model for the evolutionary steps that led to the differences observed. First, we should test if they are homologous. Such a test can be done by BLAST (see Sect. 4.3), and it will tell if the sequences are from the same gene. Once this has been established, we need to model evolution to construct the pairwise alignment. The simplest assumptions are that identical nucleotide pairs and that identical amino acids pair. Further, that different amino acids with similar physiochemical properties form pairs.

For most comparisons, it is preferred to base pairwise alignments on the amino acid sequences especially if the sequences are very divergent. Pairwise nucleotide comparisons are preferred for closely related sequences only.

Gaps are used to show that an amino acid or nucleotide is without a match in the other sequence and the gaps represent insertions or deletions in an evolutionary context. Most evolutionary modeling assumes that it is more difficult to form gaps than to form a mismatches and that it is more difficult to form a gap than to extent one already formed. Scoring systems are used to separate matches (high score) from mismatches (low score) and gaps (very low score). For proteins, scoring matrices (see Sect. 4.1.2.1) are used. At the end, scores between amino acids or nucleotides are summarized over the whole pairwise alignment, and penalties for gaps are subtracted. The result is a unit score for the multiple alignment given the parameters used. Given the same sequences are compared, the pairwise alignment with the highest total score is preferred compared to one with a lower score.

When the pairwise alignment has been constructed, the identity and similarity can be quantified. The identity is the number of nucleotides or amino acids matching in two sequences compared at all positions between the two sequences. Similarity is a further comparison also considering different types of nucleotides or amino acids as well as the gaps.

An alignment of the sequences above, and a possible scenario leading to the differences between them, is shown in Fig. 4.1.

The principles of pairwise alignments are used for most multiple alignment programs and for BLAST. The three tools are fundamental to carry out most bioinformatics (Fig. 4.2).

4.1.1 Global or Local Pairwise Alignments?

Before a pairwise alignment is constructed, it needs to be decided if it should be global or local. A pairwise alignment is global if it is known that the sequences are homologous in their full length. In this situation, it is sound to align both sequences in their full length. If preliminary evidence has showed that the genes are encoding for the same proteins and the full length of the gene has been sequenced, a global alignment can be constructed. A local alignment is needed if it is known that one sequence is shorter than the other and that it cannot be related to the other in its full length. This can happen if the DNA sequence of one gene has not been determined in the full length or if the domain structure of the proteins that the genes encode for is rather divergent.

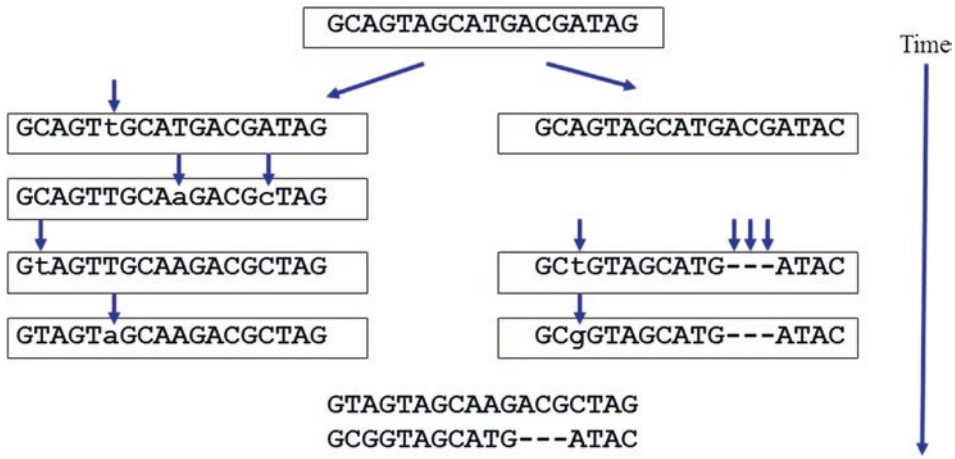

Fig. 4.1 Hypothetical example of a pairwise alignment of two sequences if their evolution was known. The first sequence is the ancestor sequence, which then diverged into two new lineages. In the sequence to the left, five-point mutations occurred over time, and in the one to the right, two-point mutations plus a deletion of three nucleotides happened probably resulting in the loss of an amino acid. With the exact knowledge of the position of the gap, we can construct the pairwise alignment at the bottom without any assumptions needed

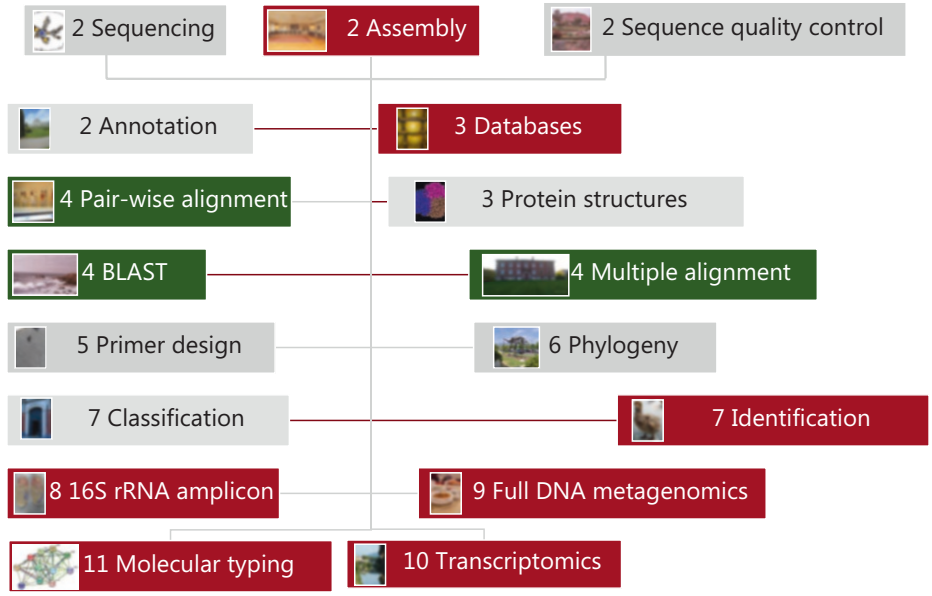

Fig. 4.2 Pairwise and multiple alignments as well as BLAST introduced in this chapter provide the background for most other chapters in this book

4.1.2 Substitution Matrices

Substitution matrices are used to model the probability of mutual amino acid or nucleotide substitutions in sequences. Amino acids or nucleotides with a lower probability of changing are given more weight in the comparisons compared to those with a higher probability of changing. The changes are assumed to occur according to a Markov model where changes only depend on the current nucleotide or amino acid observed and not on past changes (Fig. 4.3). This model is very important to understand in full. A comparison can be made to a human being only acting based on the immediate desire or need. A certain degree of hunger will lead to taking a meal, whereas past events only have minor influence on the decision like meals taken the day before. This way a Markov chain is without memory, and it seems difficult to use the Markov model to trace evolution back in time like done in phylogeny. However, this is actually possible using the assumption about irreversible changes of nucleotides or amino acids since the same probabilities can be used both to model the future and to go back in time.

4.1.2.1 Amino Acid Substitution Matrices

Amino acids have different biochemical and physical properties that influence their relative replaceability during evolution (Table 4.1). The probability of replacement has been related to the physiochemical properties of the amino acids in the way that hydrophobic amino acids are easier replaced by other hydrophobic amino acids again compared to other

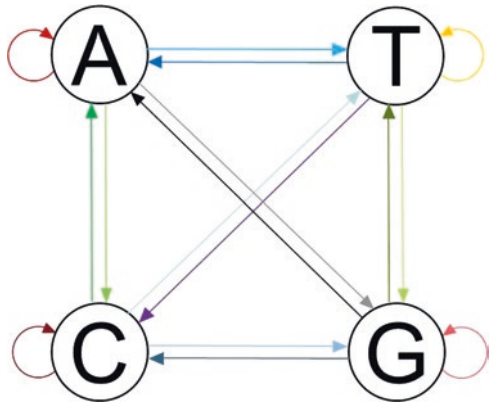

Fig. 4.3 Markow substitution model of nucleotides. The consequence of the Markow model is that only depends on the observed nucleotide or amino acid and not on the past changes. The arrows with different colors indicate that potentially 16 different probabilities exist for changes (Unrestricted model on Table 4.3) (Modified from Durbin et al. 1999)

Table 4.1 Physiochemical properties of amino acids

Amino acid	Hydrophobic	Positive	Negative	Polar	Charged	Small	Tiny	Aliphatic	Aromatic
Ile	+							+	
Leu	+							+	
Val	+					+		+	
Cys				+		+			
Ala	+					+	+	+	
Gly						+	+	+	
Met	+								
Phe	+								+
Tyr				+					+
Trp				+					+
His		+		+					+
Lys		+			+				
Arg		+			+				
Glu			+		+				
Gln				+					
Asp			+		+				
Asn				+		+			
Ser				+		+	+		
Thr				+		+			
Pro	+					+			

Scoring values of the BLOSUM50 Matrix

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	5																			
R	-2	7																		
N	-1	-1	7																	
D	-2	-2	2	8																
C	-1	-4	-2	-4	13															
Q	-1	1	0	0	-3	7														
E	-1	0	0	2	-3	2	6													
G	0	-3	0	-1	-3	-2	-3	8												
H	-2	0	1	-1	-3	1	0	-2	10											
I	-1	-4	-3	-4	-2	-3	-4	-4	-4	5										
L	-2	-3	-4	-4	-2	-2	-3	-4	-3	2	5									
K	-1	3	0	-1	-3	2	1	-2	0	-3	-3	6								
M	-1	-2	-2	-4	-2	0	-2	-3	-1	2	3	-2	7							
F	-3	-3	-4	-5	-2	-4	-3	-4	-1	0	1	-4	0	8						
P	-1	-3	-2	-1	-4	-1	-1	-2	-2	-3	-4	-1	-3	-4	10					
S	1	-1	1	0	-1	0	-1	0	-1	-3	-3	0	-2	-3	-1	5				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	2	5			
W	-3	-3	-4	-5	-5	-1	-3	-3	-3	-3	-2	-3	-1	1	-4	-4	-3	15		
Y	-2	-1	-2	-3	-3	-1	-2	-3	2	-1	-1	-2	0	4	-3	-2	-2	2	8	
V	0	-3	-3	-4	-1	-3	-3	-4	-4	4	1	-3	1	-1	-3	-2	0	-3	-1	5

Fig. 4.4 The BLOSUM50 scoring matrix. Note that low scores both can be 0 and with a minus sign. Low scoring pairs occur with high frequencies in sequences. Jesper Larsen is acknowledged for the figure

types such as charged amino acids. The substitution matrices reflect these properties as seen from Fig. 4.4. Here, the score between the two hydrophobic amino acids isoleucine and histamine is 5, whereas the score between isoleucine and the positive charged arginine is only −4. Some amino acids belong to more groups, for instance, histidine, which is both hydrophobic, positively charged, polar, and aromatic.

The percent accepted mutation (PAM) matrices are based on global alignments of closely related proteins. The number at the end of a PAM matrix refers to the number of steps required for a given percent change. PAM1 corresponds to 99% identity (1% change) between the aligned sequences. This means that PAM112 will recognize more distantly related sequences (40% identity) than PAM23 (80% identity). PAM matrices are based on global alignments and therefore best suited for global pairwise alignments used in evolutionary studies (Table 4.2).

Blocks substitution matrix (BLOSUM) is based on local alignments. The number at the end of a BLOSUM matrix refers to the minimum percent identity allowed in the set of aligned sequences used to build the matrix. This means that BLOSUM50 will recognize more distantly related sequences than BLOSUM80 (Table 4.2). Other matrices have been published aiming at refining amino acid alignments and the more recently by including improved statistics for estimating exchangeability between amino acids (Gonnet et al. 1992; Jones et al. 1992; Le and Gascuel 2008).

Table 4.2 Practical rules for use of PAM and BLOSUM amino acid substitution matrices

Application	Default	Related sequences	Distant sequences
Search	BLOSUM62	BLOSUM80	BLOSUM45
Evolution	PAM100	PAM50	PAM250

Table 4.3 The 16 different types of nucleotide pairs that can be observed between two sequences (modified from Nei and Kumar 2000)

Class	Nucleotide	Pair		
Identical nucleotides	AA	TT	CC	GG
Transition-type pair	AG	GA	TC	CT
Transversion-type pair	AT	TA	AC	CA
	TG	GT	CG	GC

Explanations of the background for the BLOSUM and PAM and other matrices are given by Pearson (2013). The recommendation is to use the "deep scoring matrices" for full-length protein sequence comparisons and only to compare the domain level with "shallower" scoring matrices.

4.1.2.2 Nucleotide Substitution Matrices

The simplest model is the Jukes & Cantor model where equal probabilities for the substitution of all four nucleotides are assumed (Table 4.4). Substitution matrices for nucleotides give higher weight to transitions (G to A or C to T and vice versa) than to transversions (G to C, G to T, A to C, A to T and vice versa) (Table 4.3). This is mainly related to the larger size of the purine compared to the pyrimidine nucleotides. The transversion/transition bias is included in the models of HKY, Kimura, Tamura-Nei, Tamura, and the General reversible model. An additional account for the frequency of nucleotides is included in models of Tamura-Nei, the Equal input, and the General reversible models. These frequencies are the observed frequencies of nucleotides under comparison. The unrestricted model allows different probabilities of changes for all 12 combinations of nucleotides (Table 4.4).

4.1.3 Gaps

Gaps are used to model insertions or deletions in sequences. If arbitrarily many gaps are inserted, this can lead to high scoring alignments of nonhomologous sequences. To abolish this effect, gaps are penalized when alignments are constructed to obtain relatively few gaps and separate penalties used for gap opening and gap elongation. In the linear model, the penalty Υ is proportional to the number of gaps (g) given the penalty for one gap d:

Linear gap penalty score:

$$\Upsilon(g) = -gd$$

Table 4.4 Models of nucleotide substitutions

	A	T	C	G	A	T	C	G
	Jukes & Cantor				HKY			
A	–	α	α	α	–	βg_T	βg_C	αg_G
T	α	–	α	α	βg_A	–	αg_C	βg_G
C	α	α	–	α	βg_A	αg_T	–	βg_G
G	α	α	α	–	αg_A	βg_T	βg_C	–
	Kimura				Tamura-Nei			
A	–	β	β	α	–	βg_T	βg_C	$\alpha_1 g_G$
T	β	–	α	β	βg_A	–	$\alpha_2 g_C$	βg_G
C	β	α	–	β	βg_A	$\alpha_2 g_T$	–	βg_G
G	α	β	β	–	$\alpha_1 g_A$	βg_T	βg_C	–
	Equal input				General reversible			
A	–	αg_T	αg_C	αg_G	–	$a g_T$	$b g_C$	$c g_G$
T	αg_A	–	αg_C	αg_G	$a g_A$	–	$d g_C$	$e g_G$
C	αg_A	αg_T	–	αg_G	$b g_A$	$d g_T$	–	$f g_G$
G	αg_A	αg_T	αg_C	–	$c g_A$	$e g_T$	$f g_C$	–
	Tamura				Unrestricted			
A	–	$\beta\Theta_2$	$\beta\Theta_1$	$\alpha\Theta_1$	–	a_{12}	a_{13}	a_{14}
T	$\beta\Theta_2$	–	$\alpha\Theta_1$	$\beta\Theta_1$	a_{21}	–	a_{23}	a_{24}
C	$\beta\Theta_2$	$\alpha\Theta_2$	–	$\beta\Theta_1$	a_{31}	a_{32}	–	a_{34}
G	$\alpha\Theta_2$	$\beta\Theta_2$	$\beta\Theta_1$	–	a_{41}	a_{42}	a_{43}	–

g_A, g_T, g_C, and g_G are the nucleotide frequencies, $\Theta_1 = g_C + g_G$, $\Theta_2 = g_A + g_T$. The nucleotide frequencies either can be estimated from the data compared or set as fixed parameters
a_{ij} is the substitution rate from the nucleotide in the i-th row to the nucleotide in the j-th column
Modified from Nei and Kumar (2000)

If the penalty of one gap is 8 and there are three gaps, the total penalty will be - 24.

To better model biological events where many gaps often occur in a row, the affine penalty score model is used where the cost is higher for inserting the first gap than to extent this gap to lengths of two and more:

$$\Upsilon(g) = -d - (g-1)e$$

Where Υ (g) = gap penalty score of gap length g, d = gap opening penalty and e = gap extension penalty. For a gap of length 3 with a penalty of opening the gap of 8 and 4 of extending the gap with two more, the total penalty will be −16.

4.1.4 Dynamic Programming

Dynamic programming is a way to compute the pairwise alignment. If all possibilities for the pairwise alignment of two sequences should be tested, there would be around 1059 possibilities for two nucleotide sequences of 100 in length. This number is approximately

the same as the number of molecules in the Milky Way, and even the largest computer would not be able to handle the problem. There is also no need to consider possibilities without relevance, for instance, that one nucleotide in one sequence would pair only with gaps in the other. For a more general comparison of dynamic programming with hidden Markov models, see: https://youtu.be/aELVQo6eRzk.

To reduce the computing effort but still to perform a careful analysis, dynamic programming is used. The computer is only testing relevant comparisons (high scores) and keeping in memory the highest ones already calculated. The most relevant dynamic programming algorithms for comparison of global and local pairwise alignments were published be Needleman and Wunsch (1970) and Smith et al. (1981), respectively.

The Needleman and Wunsch algorithm is used when it is known that the sequences represent exactly a gene or protein in full length (few amino acids or nucleotide differences are tolerated), and it is used to build global pairwise alignments.

The Smith and Waterman algorithm is used with partial sequences or with sequences of unknown length to build local alignments. For both algorithms, the adjustment of gap penalty functions depends on previous knowledge about domain structures, repeats, and other properties. Both algorithms and their implementations in computer programs can be used for both amino acid and nucleotide sequences.

4.1.4.1 Needleman and Wunsch

The score function in this model is illustrated in Fig. 4.5. This function is used on all comparisons between the two sequences. Here, we will use the example with the two sequences:

Sequence 1: HEAGAWGHEE
Sequence 2: PAWHEAE

Figure 4.6 shows one sequence (1) on the top of the table and sequence 2 as a vertical column. First, an alignment path matrix is created. For each cell, F(i,j) is calculated based on the score function in Fig. 4.5. This matrix allows a stepwise calculation of score values.

$$F(i,j) = \max \begin{cases} F(i-1, j-1) + s(i,j) \\ F(i-1, j) - d \\ F(i, j-1) - d \end{cases}$$

Fig. 4.5 For the Needleman and Wunsch algorithm, the score function F is defined as the maximum of the three expressions. F(i−1, j−1) is the score of the previous diagonal cell in the matrix, which is added the score for a match between an amino acid pair in the current cell. F(i−1, j) is the score for the previous cell in the column subtracted the penalty of a gap (d), and F(i, j−1) is the equivalent score for the previous cell in the row subtracted the gap penalty. See Fig. 4.7 for an example of this calculation

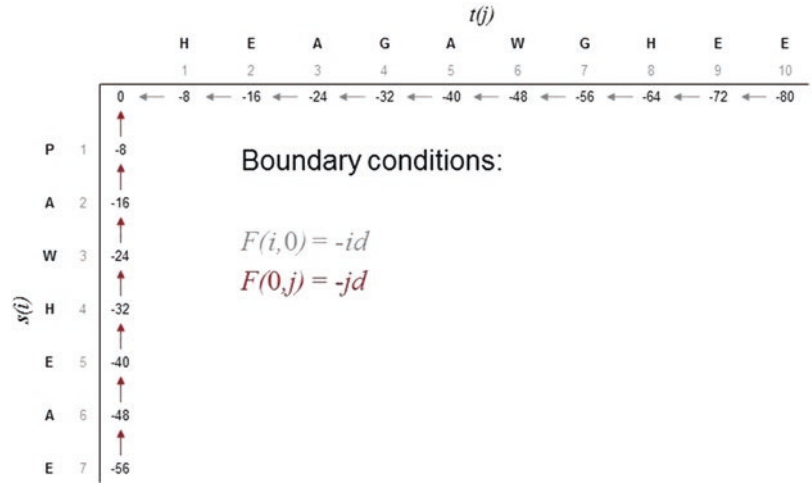

Fig. 4.6 Pairwise alignment with the Needleman and Wunsch algorithm showing the boundary conditions calculated. Here, both of the sequences are paired only with gaps resulting in the lowest negative score. Jesper Larsen is acknowledged for the figure

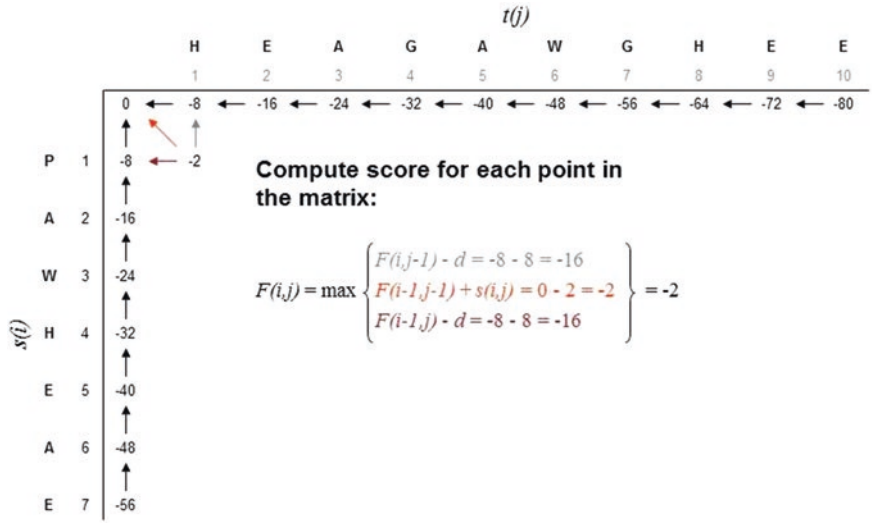

Fig. 4.7 Pairwise alignment with the Needleman and Wunsch algorithm. Here, the first real position of the matrix is calculated. The three possibilities, H pairing with a gap, P pairing with a gap, or H pairing with P, are considered. The one with the highest score: H pairing with P (−2) (see Fig. 4.4) gives the maximum score and is preferred. Jesper Larsen is acknowledged for the figure

The score of the best alignment is calculated between the initial segment $x_{1...i}$ of x up to x_i and the initial segment $y_{1...j}$ of y up to y_j. The algorithm function, F(i,j), is built recursively beginning with F(0,0) = 0 (Figs. 4.6, 4.7, 4.8). When the matrix is filled, backtracking is started (evaluation of the optimal path).

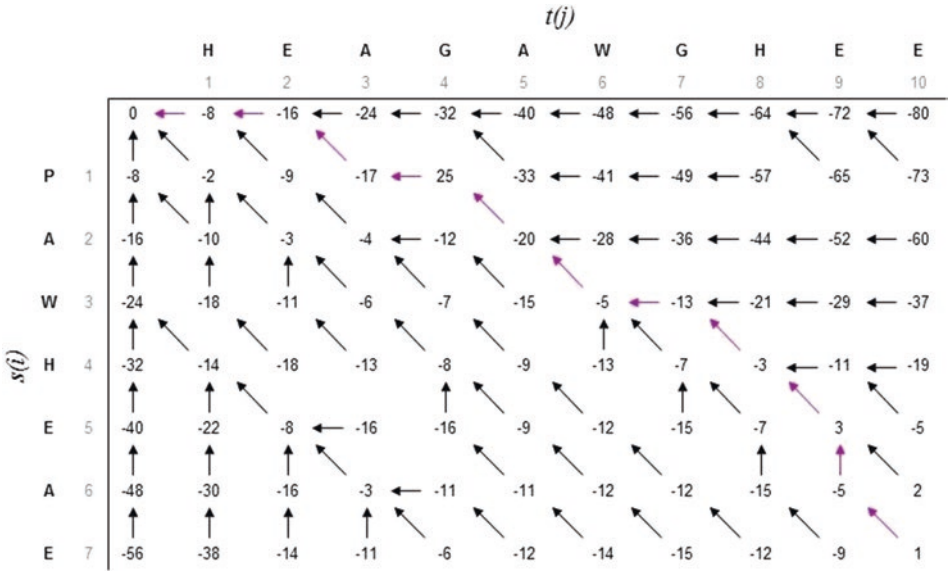

Fig. 4.8 Pairwise alignment with the Needleman and Wunsch algorithm. Here, the maximum score has been inserted for all cells in the matrix and backtracking from the bottom right corner has been performed following the path, which maximizes the score. After the score of 1 for the cells to the lowest right, −5 is chosen since it was marked as a path to 1 in the forward tracking. The higher score of 2 cannot be selected since it was not marked in the forward tracking. Jesper Larsen is acknowledged for the figure

The scoring parameters are given by the BLOSUM 50 matrix (Fig. 4.4) and a linear gap penalty of d = −8. We will align the whole sequence length of the sequences, meaning that we need to form a global alignment and use the Needleman and Wunsch algorithm. The procedure can be followed on Figs. 4.6, 4.7, 4.8, 4.9.

A real example of the use of the Needleman and Wunsch algorithm is shown on Fig. 4.10. Here, realistic long sequences are aligned by the algorithm implemented as the **needle** program in the EMBOSS package (Rice et al. 2000).

4.1.4.2 Smith and Waterman

Now, we will compute a pairwise alignment of the sequences using the Smith and Waterman algorithm. There are two differences in the algorithm compared to Needleman and Wunsch. The first is that the alignment can start/end anywhere in the matrix and the other that backtracking is started at the highest value rather than in the lower right corner. Backtracking is terminated as soon as zero is encountered. The score function is shown in Fig. 4.11.

We will align the same two short model protein sequences with Smith and Waterman:

Sequence 1: HEAGAWGHEE

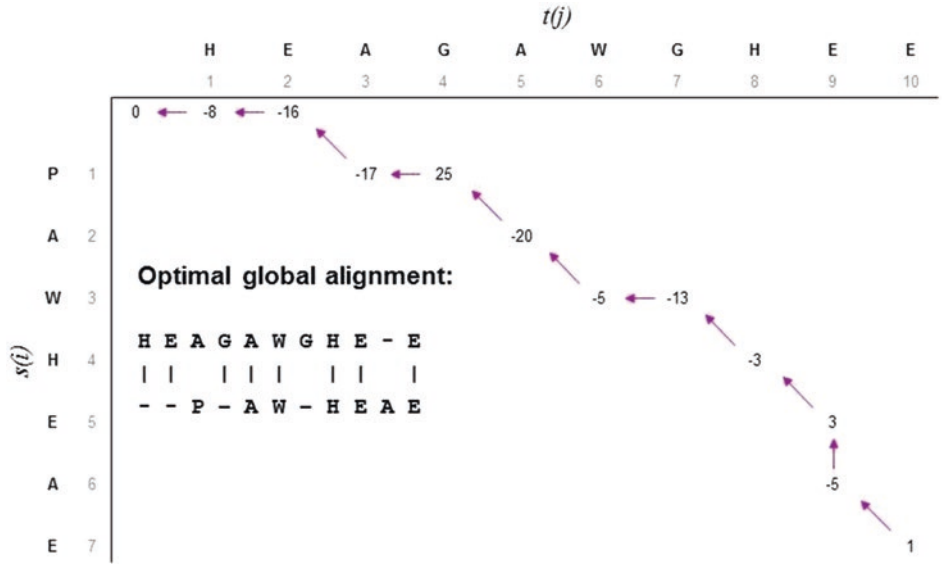

Fig. 4.9 Pairwise alignment with the Needleman and Wunsch algorithm. The optimal path for backtracking is shown again (Fig. 4.8), and the pairwise alignment has been built. Note the combination of H and P considered in Fig. 4.7 has not been selected since the backtrack path did not pass through this cell. The total score for the multiple alignment is 1 (bottom left corner). Jesper Larsen is acknowledged for the figure

Sequence 2: PAWHEAE

And use the same parameters of BLOSUM50 and a linear gap penalty of d = −8.

$$F(i,j) = \max \begin{cases} 0 \\ F(i-1,j-1) + s(i,j) \\ F(i-1,j) - d \\ F(i,J-1) - d \end{cases}$$

Using the score function in Fig. 4.11 results in 0 in most cells when the matrix is filled in the forward direction (Fig. 4.12). The reason is that the three other possibilities in Fig. 4.11 result in negative values, and we then have to use 0. Backtracking starts with the highest score (28) and terminates when 0 is reached. Note that an alternative path is also possible. However, the maximum score of this one is lower (21), and therefore, the first is chosen to construct the final pairwise alignment (Fig. 4.13). Similar to the example with Needleman and Wunsch (Fig. 4.10), a more realistic example is shown in Fig. 4.14 with longer sequences. The sequences are the same that we used in Fig. 4.10, and we see little difference between the two pairwise alignments probably because they were of nearly the same length.

```
########################################
# Program: needle
# Rundate: Thu Feb 12 14:49:37 2004
# Align_format: srspair
# Report_file: outfile.align
########################################
#=======================================
#
# Aligned_sequences: 2
# 1: AAA85484.1
# 2: AAA85485.1
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 163
# Identity:  113/163 (69.3%)
# Similarity:  137/163 (84.0%)
# Gaps:  1/163 ( 0.6%)
# Score: 607.0
#
#
#=======================================

AAA85484.1      1           MKFFAVLALCIVGAIAHPLTSDEAALVKSSWAQVKHNEVDILYTVFKAYP 50
                            ||||||||||:|||||.||::||||:||||||.||||||||||..||.|||
AAA85485.1      1           MKFFAVLALCVVGAIASPLSADEAAIVKSSWDQVKHNEVDILAAVFAAYP 50

AAA85484.1      51          DIQARFPQFAGKDLDTIKTSGQFATHAIRIVSFLSELIALSGSESNLSAI 100
                            ||||:|||||||||.:||.:..||||||||||.:|:|:|||::||||:
AAA85485.1      51          DIQAKFPQFAGKDLASIKDTAAFATHAIRIVSFFTEVISLSGNQANLSAV 100

AAA85484.1      101         YGLISKMGTDHKNRGITQTQFNKFRTALVSYISSNVAWGDNVAAAWTHAL 150
                            |.|:||:|.|||.|||:..||.:||||||||:.::|:|||||||||.|||
AAA85485.1      101         YALVSKLGVDHKARGISAAQFGEFRTALVSYLQAHVSWGDNVAAAWNHAL 150

AAA85484.1      151         DNVYTAVFQIVTA 163
                            ||.|....:.:.
AAA85485.1      151         DNTYAVALKSLE 162
```

Fig. 4.10 Pairwise alignment with the Needleman and Wunsch algorithm. A real example with sequences of realistic length has been computed using implementation of the algorithm in EMBOSS as the needle program (see Activity 4.6.1 for details about this program). Similarity is calculated only based on "positives," which means the scores in the BLOSUM62 matrix, which are positive (signatures | and:)

$$F(i,j) = \max \begin{cases} 0 \\ F(i-1, j-1) + s(i,j) \\ F(i-1, j) - d \\ F(i, j-1) - d \end{cases}$$

Fig. 4.11 Score function for Smith and Waterman. An extra possibility of 0 can be selected in addition to the three in Fig. 4.5. The 0 is chosen if the other three are negative. This way cells are marked as 0 to account for different length of sequences in the local alignment (Fig. 4.12)

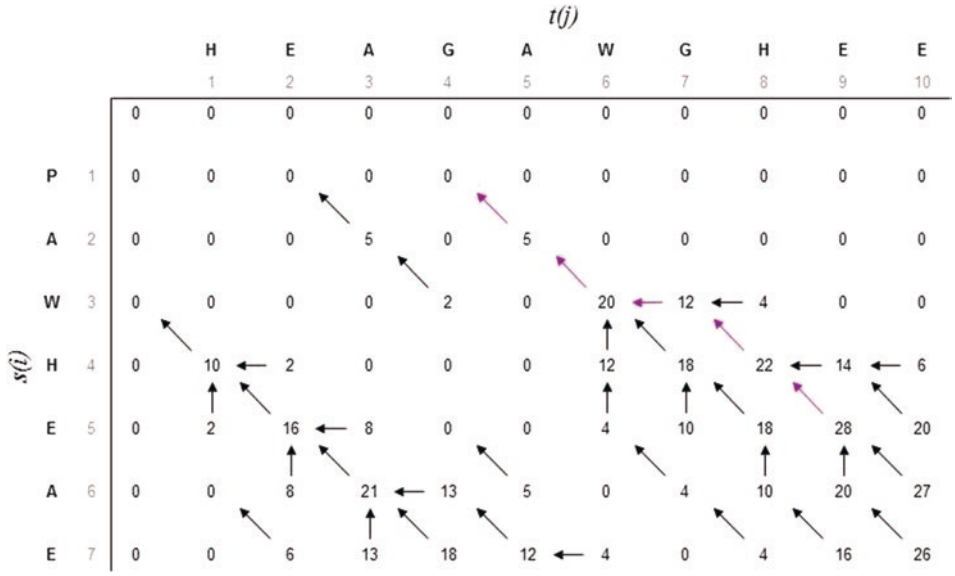

Fig. 4.12 Smith and Waterman alignment. Here, the matrix has been filled with combinations based on the score function in Fig. 4.11. Backtracking started from the highest score in the matrix (28) and continued to join the highest possibilities until 0 was reached. Jesper Larsen is acknowledged for the figure

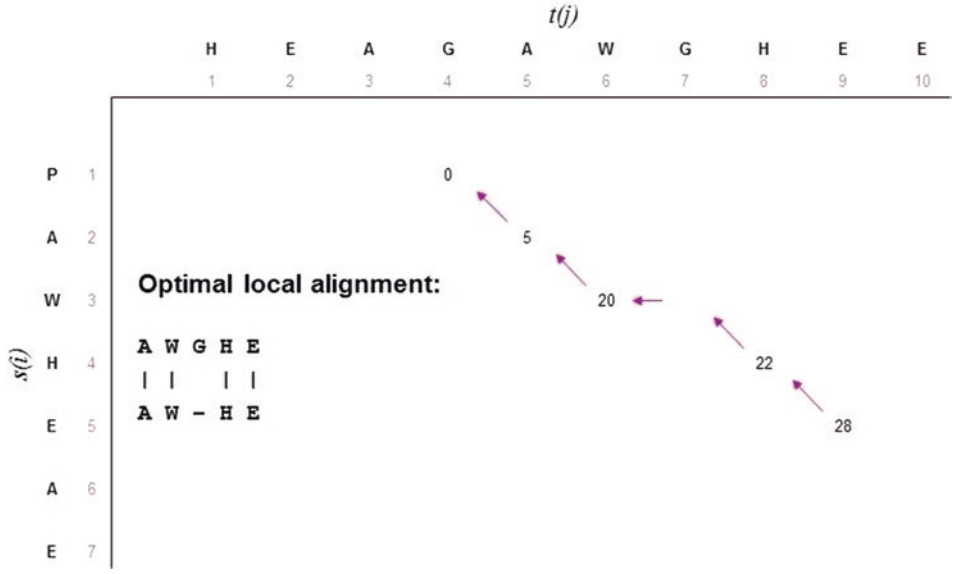

Fig. 4.13 Smith and Waterman algorithm. Here, the backtrack path is shown, and the resulting pairwise alignment has been constructed. Note the difference to global alignment generated by the Needleman and Wunsch algorithm in Fig. 4.9. The total score for the pairwise alignment is 28. Jesper Larsen is acknowledged for the figure

```
########################################
# Program: water
# Rundate: Fri Feb 13 13:11:09 2004
# Align_format: srspair
# Report_file: outfile.align
########################################
#=======================================
#
# Aligned_sequences: 2
# 1: AAA85484.1
# 2: AAA85485.1
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 159
# Identity: 113/159 (71.1%)
# Similarity: 136/159 (85.5%)
# Gaps: 0/159 ( 0.0%)
# Score: 609.0
#
#
#=======================================

AAA85484.1         1 MKFFAVLALCIVGAIAHPLTSDEAALVKSSWAQVKHNEVDILYTVFKAYP 50
                     ||||||||||:|||||.||::||||:|||||.||||||||||..||.|||
AAA85485.1         1 MKFFAVLALCVVGAIASPLSADEAAIVKSSWDQVKHNEVDILAAVFAAYP 50

AAA85484.1        51 DIQARFPQFAGKDLDTIKTSGQFATHATRIVSFLSELIALSGSESNLSAI 100
                     ||||:|||||||||.:||.:..||||||||||.:|:|:|:|||:::||||:
AAA85485.1        51 DIQAKFPQFAGKDLASIKDTAAFATHATRIVSFFTEVISLSGNQANLSAV 100

AAA85484.1       101 YGLISKMGTDHKNRGITQTQFNKFRIALVSYISSNVAWGDNVAAAWTHAL 150
                     |.|:||:|.|||.|||:..||.:|||||||||:.::|:|||||||||.|||
AAA85485.1       101 YALVSKLGVDHKARGISAAQFGEFRIALVSYLQAHVSWGDNVAAAWNHAL 150

AAA85484.1       151 DNVYTAVFQ 159
                     ||.|....:
AAA85485.1       151 DNTYAVALK 159


#---------------------------------------
#---------------------------------------
```

Fig. 4.14 Pairwise alignment with the Smith and Waterman algorithm. A real example with sequences of realistic length has been computed using the water program of EMBOSS, which has implemented the Smith and Waterman algorithm (see Activity 4.6.2 for details about this program). Note that there are only small differences to the pairwise alignment generated by Needleman and Wunsch (Fig. 4.10). It is related to the comparable length of the two sequences. Similarity is calculated only based on "positives," which means the scores in the BLOSUM62 matrix, which are positive (signatures | and:)

4.2 Multiple Alignment

A multiple alignment is the simultaneous alignment of three or more nucleic acid or amino acid sequences. The procedure involves the insertion of gaps in the sequences so as to maximize the overall similarity (Higgins and Sharp 1988). Multiple alignments are rarely used for their own sake but are usually created for another purpose—for instance, primer design (Chap. 5) or analysis of phylogeny (Chap. 6). Users select a favorite program or program package and try to optimize program settings for that.

To start the construction of a multiple alignment, we will use the same criteria as for the pairwise alignment problem. We will assume that they shared ancestors (homologous). We need to model evolution the way that every column represents only orthologous amino acids or nucleotides that have evolved from a common ancestor. The simplest assumptions are again that identical nucleotides or identical amino acids pair, that different amino acids with similar physiochemical properties pair, that it is more difficult to form a gap than to form a mismatch, and that it is more difficult to form a gap than to extent one already formed. The scoring between nucleotides and amino acids is based on the system above for pairwise alignment (Sect. 4.1.2).

4.2.1 Clustal

To form a multiple alignment of the Clustal type, first pairwise alignments between sequences 1–2, 1–3, 1–4, 1–5, 2–3, 2–4, 2–5 …0.4–5 are formed (Fig. 4.15). This is the progressive alignment part of the process, and there are (n(n−1))/2 combinations where n is the number of sequences. In this example with five sequences, there are ten combinations. The next step is to construct a "guide tree" (Fig. 4.16) uniting the pairs with highest score. The final step is to construct the multiple alignment from the guide tree, which is called profile alignment (Fig. 4.17). The Clustal type of multiple alignment is therefore performing progressive profile alignment. Clustal has been called "a quick and dirty version of Feng and Dolittle (1987)" where "only residues that are part of the matches of a given length (k-tuple matches) are scored" (Higgins and Sharp 1988).

The developments in the Clustal programs was started with Clustal (1988) (Higgins and Sharp 1988) and continued with Clustal V (five) (1992), Clustal W (weights) (1994), and Clustal X 2.0 (2007) (Larkin et al. 2007). ClustalX is the implementation of the program for PC (Figs. 4.18, 4.19) and is further described in activity 4.4.3. Clustal Omega (http://www.clustal.org/omega/) is the most recent version of the program for multiple alignment of very large protein datasets (Sievers et al. 2011).

ClustalX/W produces global alignments, which include benefits and drawbacks inhered from then pairwise alignment part included in the construction of the alignment. Domain structures of proteins can be optimized by.

```
 CLUSTAL W (1.82) Multiple Sequence Alignments


Sequence format is Pearson
Sequence 1: 001             238 bp
Sequence 2: 002             228 bp
Sequence 3: 005             227 bp
Sequence 4: 018             227 bp
Sequence 5: 120             228 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  98
Sequences (1:3) Aligned. Score:  86
Sequences (1:4) Aligned. Score:  75
Sequences (1:5) Aligned. Score:  80
Sequences (2:3) Aligned. Score:  86
Sequences (2:4) Aligned. Score:  76
Sequences (2:5) Aligned. Score:  81
Sequences (3:4) Aligned. Score:  76
Sequences (3:5) Aligned. Score:  81
Sequences (4:5) Aligned. Score:  76
Guide tree        file created:   [/ebi/extserv/clustalw-work/interactive/clustalw-20040304-09194253.dnd]
Start of Multiple Alignment
There are 4 groups
Aligning...
Group 1: Sequences:   2      Score:4197
Group 2: Sequences:   3      Score:3797
Group 3: Sequences:   4      Score:3662
Group 4: Sequences:   5      Score:3496
Alignment Score 10389
CLUSTAL-Alignment file created  [/ebi/extserv/clustalw-work/interactive/clustalw-20040304-09194253.aln]
```

Fig. 4.15 Output from CLUSTALW showing the pairwise combinations and the grouping of sequences based on the guide tree (Fig. 4.16)

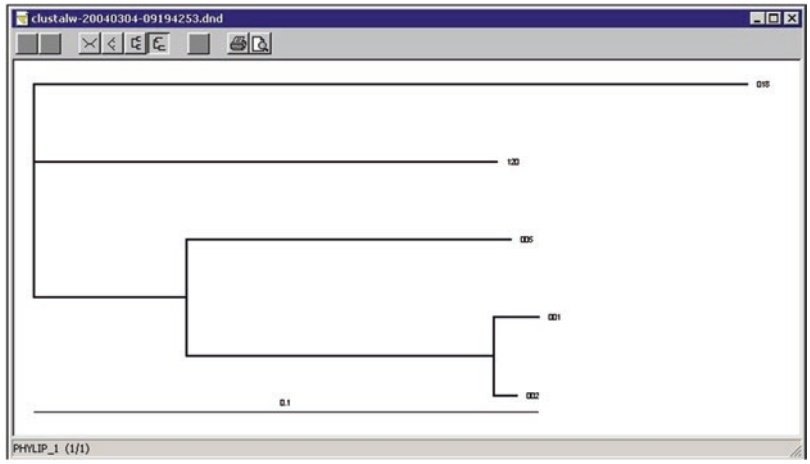

Fig. 4.16 The guide tree of ClustalW, which is used by the program to perform the full multiple alignment

ClustalW/X (Fig. 4.20). ClustalW/X invokes different "hidden" functions during alignment and tend to cluster gaps and take hydrophobic/hydrophobic properties into account. An additional benefit with the ClustalX program is that it allows an identity matrix to be generated. After the multiple alignment has been generated under the menu "Trees" and

```
CLUSTAL W (1.82) multiple sequence alignment


001             AAAAAGAAAAGTATATGTTACATTAATATTACAATGTAATTATTTTGTTTAATTTCCCTA 60
002             AAAAAGAAAAGTATATGTTACATTAATATTACAATGTAATTATTTTGTTTAATTTCCCTA 60
005             AAAACAAAAAGTATATGTTACATTAATATTACAATGTAATTATTTTGTTTAATTTCCCTA 60
120             AAAATGAAAAGTATATGTTACATTGGTTTTACAATGTAATTATTTTGTTTAATTTCCCTA 60
018             AAAATGAAAAGTAAATATTACATTAATTTTACAATGTAATTATTTCGTTTATTTTCTCTA 60
                ****  ******* ** *******  * ****************** ***** **** ***

001             CATTTTGTATAACTTTAAAACACTCCTTTTTCTCTTCTGATTATATAAAAGACAAAAAAT 120
002             CATTTTGTATAACTTTAAAACACTCCTTTTTCTCTTCTGATTATATAAAAGACAAAAAAT 120
005             CATTTTGTATAACTTTAAAACATTTAATTTTATCTTCTGGCTATACAAAAGACAAAAAAT 120
120             CATTTTGTATAACTTTAAAACATTTTATTTTCCAGTCTGTACACATAAAAGGCAAAAAAT 120
018             CATTTTGTGTAACTTTAAAGCATTTAATTTTACAACTATTTTATATAAAAGATAAAAAAT 120
                ******** ********** ** *   ****           * * *****  *******

001             ACAATTTAAGCTACAAAAAACAACAAAAAACAACAAAAAACACGACAATAAGATCGAGTA 180
002             ACAATTTAAGCTAAAAAAAACAACAAAAAATA----------CGACAATAAGATCGAGTA 170
005             ATAATTAAAGCTACAAAAA-TAACAAAAACAA----------CTGCACTTATATCAAAAT 169
120             ATAATTAAAGCCACAAAAG-TAATAAAAAGTA----------CTGTGGTCTGATCATATA 169
018             TCAACTAAATCCATAAAAA-CAATAAAAAATC----------CTGTGGTAAGAGCAGTTG 169
                  ** * ** * * ****   ** *****             *     *   * *

001             ATGATTATATTATGTTATAATTTTTGACCTAATTTAGAATAATTATCGAGTGCAAATT 238
002             ATGATTATATTATGCTATAATTTTTGACCTAATTTAGAATAATTATCGAGTGCAAATT 228
005             GTGGTTATATTATGCTATAATTTTGGGTCTAATTTAGAATAATTATCGAGTGCAAATT 227
120             ATGAATACATTATGCTATAATCAGCCCCCTAATTTAGAATAATTATCGAGTGCAAAAT 227
018             GTTAATGTATTATGCTAAAATTTTTATTCTAATCTAGAATCATTATCGAGTGTGAATT 227
                 *   *  ****** ** ***       ***** ****** ***********  ** *
```

Fig. 4.17 The final multiple alignment made with CLUSTALW

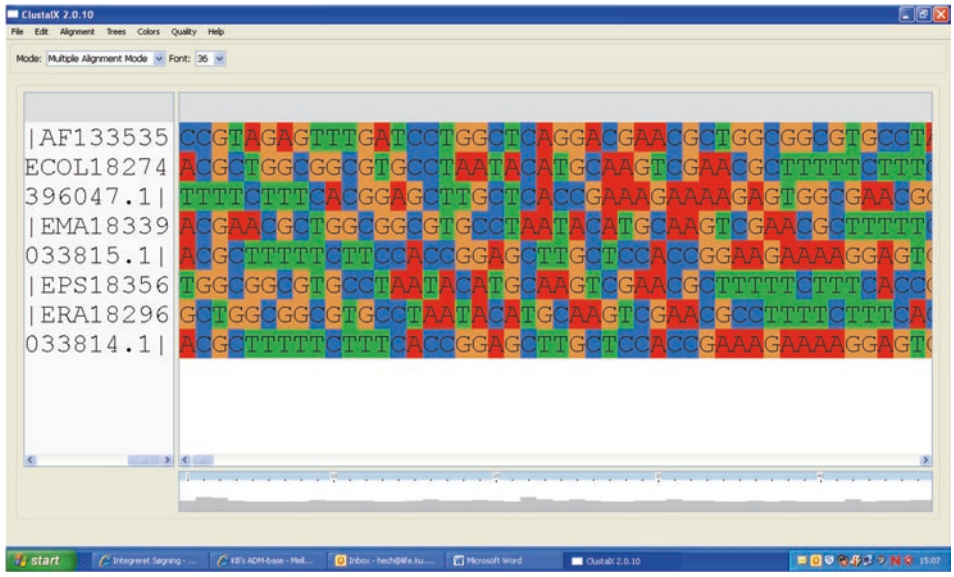

Fig. 4.18 CLUSTALX (Larkin et al. 2007) showing the sequences not yet aligned. This is seem from the unordered arrangement of the nucleotides in the columns

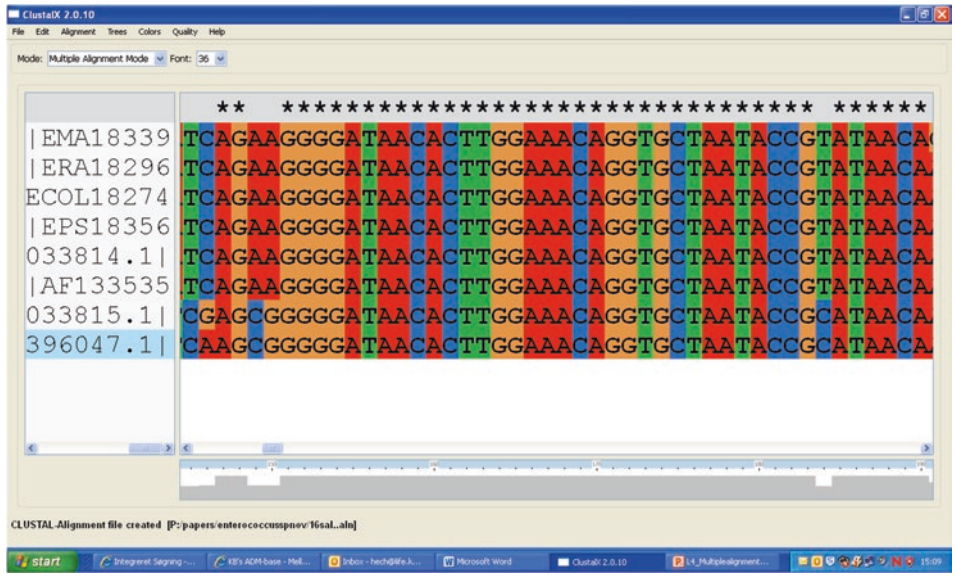

Fig. 4.19 CLUSTALX (Larkin et al. 2007) showing aligned sequences where most of the columns show only one type of nucleotide

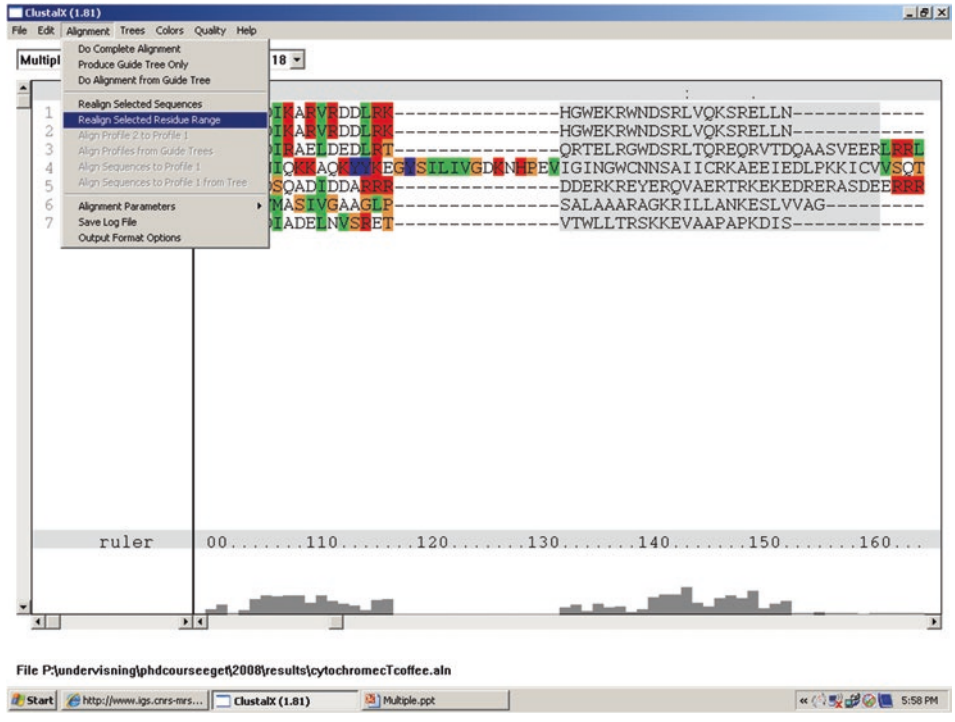

Fig. 4.20 CLUSTALX (Larkin et al. 2007) showing optimization by realignment of a region of the multiple alignment to improve local regions

submenu "Output format Options," one can mark "% identity matrix." The matrix will then be generated by running the "Trees | Draw tree" command.

An inherent problem with the progressive approach used in ClustalX/W is that mistakes made in the initial alignment cannot be corrected later. To account for this and other problems, other programs have been constructed.

4.2.2 Other Multiple Alignment Programs

Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar 2004) (https://drive5.com/muscle5/) includes no guide tree. MUSCLE is claimed both to achieve better average accuracy and better speed than ClustalW2 or T-Coffee (Edgar 2004). The main focus is on protein multiple alignments, but it works also on DNA. The principle is based on K-mer comparison between sequences. K-mers are contiguous subsequence of short length (k-tuple). Related sequences tend to have more k-mers in common than expected by chance. The program can be used from MEGA11 (Tamura et al. 2011) (https://www.megasoftware.net/) used in Chaps. 6 and 11. Like the following programs, T-Coffee and MAFFT have options to adjust alignment parameters for a range of applications.

T-Coffee (http://tcoffee.crg.cat/) (Notredame et al. 2000) incorporates a "tree-based consistency objective function for alignment evaluation." The benefit should be high accuracy. The program comes in a range of flavors suitable for different applications.

MAFFT (Yamada et al. 2016; Katoh et al. 2019 and other papers) (https://mafft.cbrc.jp/alignment/software/) is also a multiple alignment package that includes applications for very large datasets.

COBALT is a progressive constraint-based alignment program (Papadopoulos and Agarwala 2007). Constraints are from Conserved Domain Database (CDD) (Chap. 3) and PROSITE motif databases.

Multiple alignment programs can be compared with sets of reference sequence (BAliBASE) (Thompson et al. 1999).

The ARB package has been developed to account for the secondary structures in 16S rRNA sequence (Ludwig et al. 2004) (http://www.arb-home.de/). It has been used to construct precomputed multiple alignments in the SILVA database (www.arb-silva-de), which is further considered in Chap. 8. Folding patterns in 16S rRNA genes can be predicted with Mfold (http://www.unafold.org/mfold/applications/rna-folding-form-v2.php) (Zuker and Jacobson 1998).

Trimming should only be done with limited datasets of closely related organisms. Trimming can be done in BioEdit (Fig. 4.21) (see also Sect. 5.6). For Mac users, Jalview can be used (https://www.jalview.org) (Waterhouse et al. 2009).

Graphic output of multiple alignments can be made in Boxshade (http://arete.ibb.waw.pl/PL/html/boxshade.html) (in Polish) (Fig. 4.22).

For whole genome alignments, we need to account for patchy distribution of matching regions, and we cannot use the strategies for single genome alignments. Strategies using a

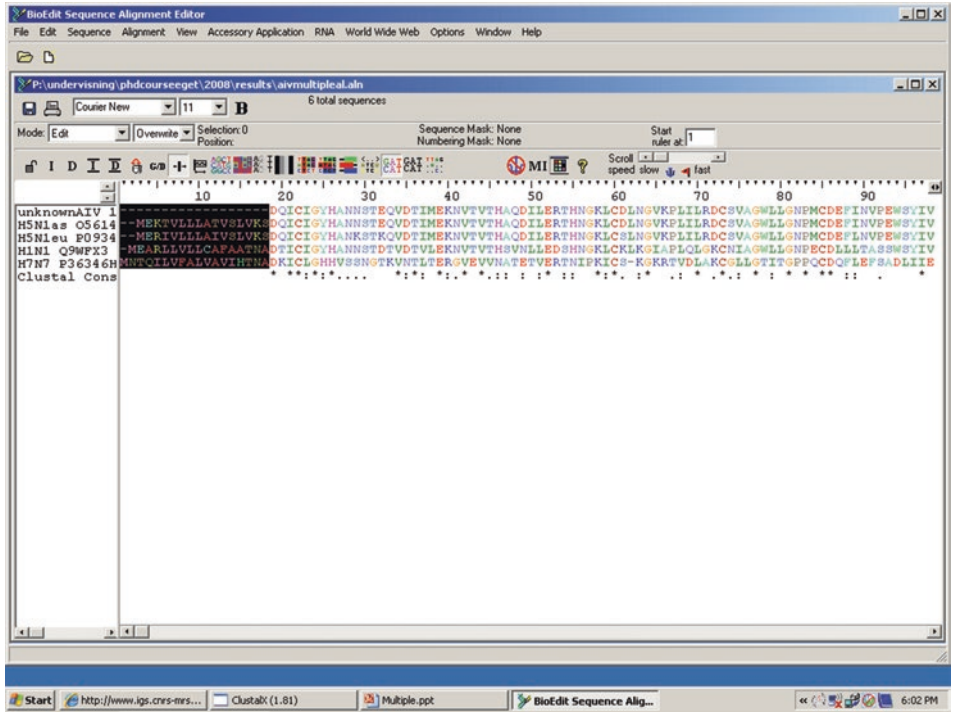

Fig. 4.21 BIOEDIT (Hall 1999) (https://bioedit.software.informer.com/download/) used to trim a region of a multiple alignment with many gaps inserted as a consequence of lack of data (short sequences). The box marked in black was deleted

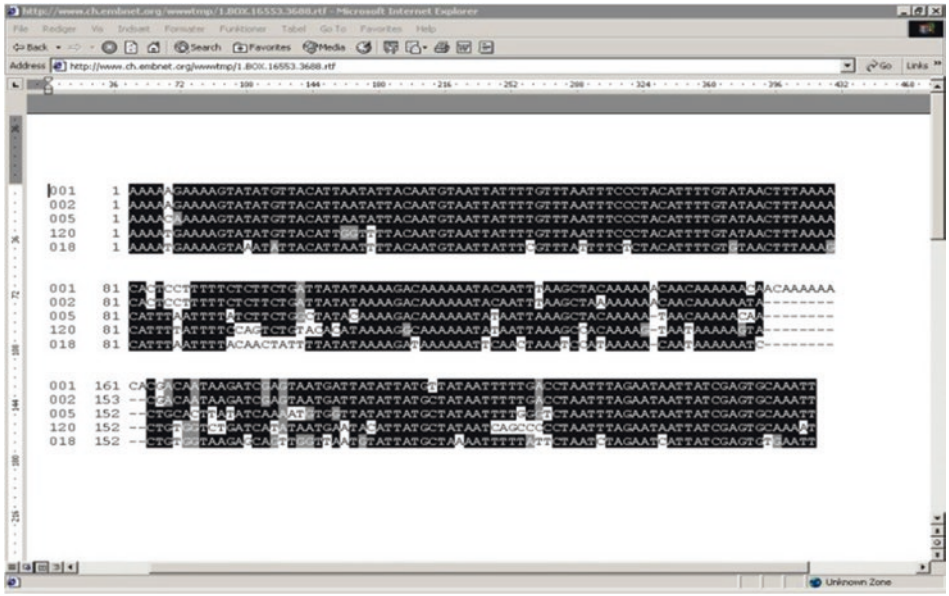

Fig. 4.22 Output from BOXSHADE (http://arete.ibb.waw.pl/PL/html/boxshade.html). This output can be included for presentation in a publication

suffix tree can solve the problem. One strategy is to create maximal unique match (MUM) decomposition of the two genomes to be aligned. A MUM is a subsequence occurring exactly once in each of the two genomes. The strategy (MUMmer) for alignment in all includes suffix tree construction, sorting and extraction of the longest increasing subsequences and generation of Smit-Waterman alignments for all the MUMs (Delcher et al. 1999).

4.3 BLAST

Basic Local Alignment Search Tool (BLAST) was originally described by Altschul et al. (1990), and this tool enabled fast search in the electronic nucleotide and protein databases during the 1990s when the internet was invented. Later, an improved algorithm was described allowing gaps to be inserted during the analysis (Altschul et al. 1997). Both versions of BLAST are in use as the "ungapped" (1990) and "gapped" (1997), respectively.

BLAST is based on a heuristics search algorithm, which is able to search through the ever-growing sizes of databases. BLAST can be compared to the search tool Google in popularity within the bioinformatical community including the formation of the verb "to blast." BLAST is based on the initial identification of the user query sequence by short pieces of sequence (words). These words are compared to the database with all the sequences (subjects). If the word is identified in a sequence of the database, the match of the word can be extended and will form a high scoring pair (HSP). When no further extension is possible, the result is returned as a hit list to the used with indication of similarity between the query and the closest related subjects in the database. The "gapped" version of BLAST is most frequently used, and its "two hit method" requires two nonoverlapping "words" on the same diagonal with a distance of A before an extension is invoked. Gapped BLAST should only require 1/3 computer time compared to ungapped version because of less extensions of the words. BLAST uses dynamic programming to extend residues in both directions, and BLAST is most suitable for finding of subject DNA and protein sequences in databases that match with a reasonable similarity and a comparable length to the query. BLAST is good for sequences of at least 100 in length that are well represented in the databases. BLAST is not suitable to make really precise determination of similarity between sequences, and pairwise alignment programs should instead be used for that (Sect. 4.1). BLAST is not suitable for sequence-based identification of bacterial species since there are simply too many sequences in GenBank and the type strains are not always clearly labeled (Chap. 7). BLAST is not suitable for databases beyond a certain size and cannot be used to search very large metagenomics databases (Chap. 9). In principle, the database search of BLAST is based on local alignments. It needs to be local since we are never certain if our query sequence will be represented by a homolog in the database with similar length. BLAST is most often used on the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi); however, the BLAST program can also be installed on your local computer and used with you own database.

4.3.1 NCBI BLAST

The most common way of performing a BLAST search is to access the program at NCBI and search in their databases. A search can be performed by a DNA or protein sequence in FASTA format, by uploading a sequence file in FASTA format or by inserting an accession number as AB490809 shown in Fig. 4.23 if the sequence is already included with GenBank. In the example shown on Fig. 4.23, default setting has been selected for a BLAST search with a DNA sequence. Under **Program Selection** and **Optimize for different versions of BLAST** can be selected. In this case, **megablast**, **discontigous megablast,** and **blastn** can be selected. The three versions differ by the scores given to matches and their way of penalizing gaps. For shorter sequences, **blastn** should be used.

When the search is terminated, you will see the familiar graphical overview in (Fig. 4.24)—the colored section with horizontal bars.

In the next section comes the descriptions with one line for each subject, which gives a more detailed information about the subject sequences providing **Score**, **Query cover**, **E value,** and **accession number**. These parameters will be explained in the following.

The hit list is arranged by decreasing **Score**. In the example, you see that hits are sorted by decreasing score. The identities are also decreasing but not systematically since the score is not always directly related to the identity. The longer the match between two sequences, the higher the score given the same identity. E values cannot be directly seen here since they are very small and they are just shown as "0.0."

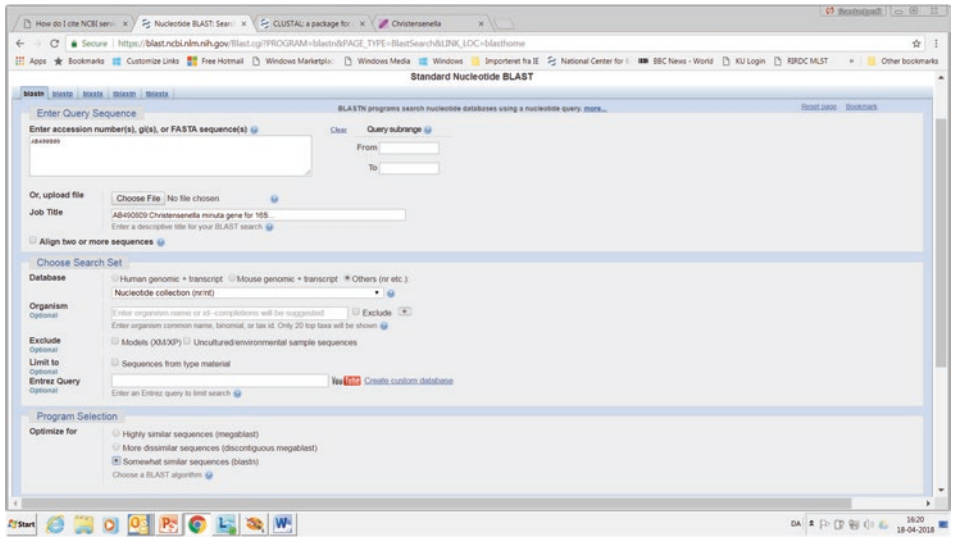

Fig. 4.23 BLAST search (Altschul et al. 1990, 1997). BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 2018 04 17]. Available from: https://blast.ncbi.nlm.nih.gov/Blast.cgi

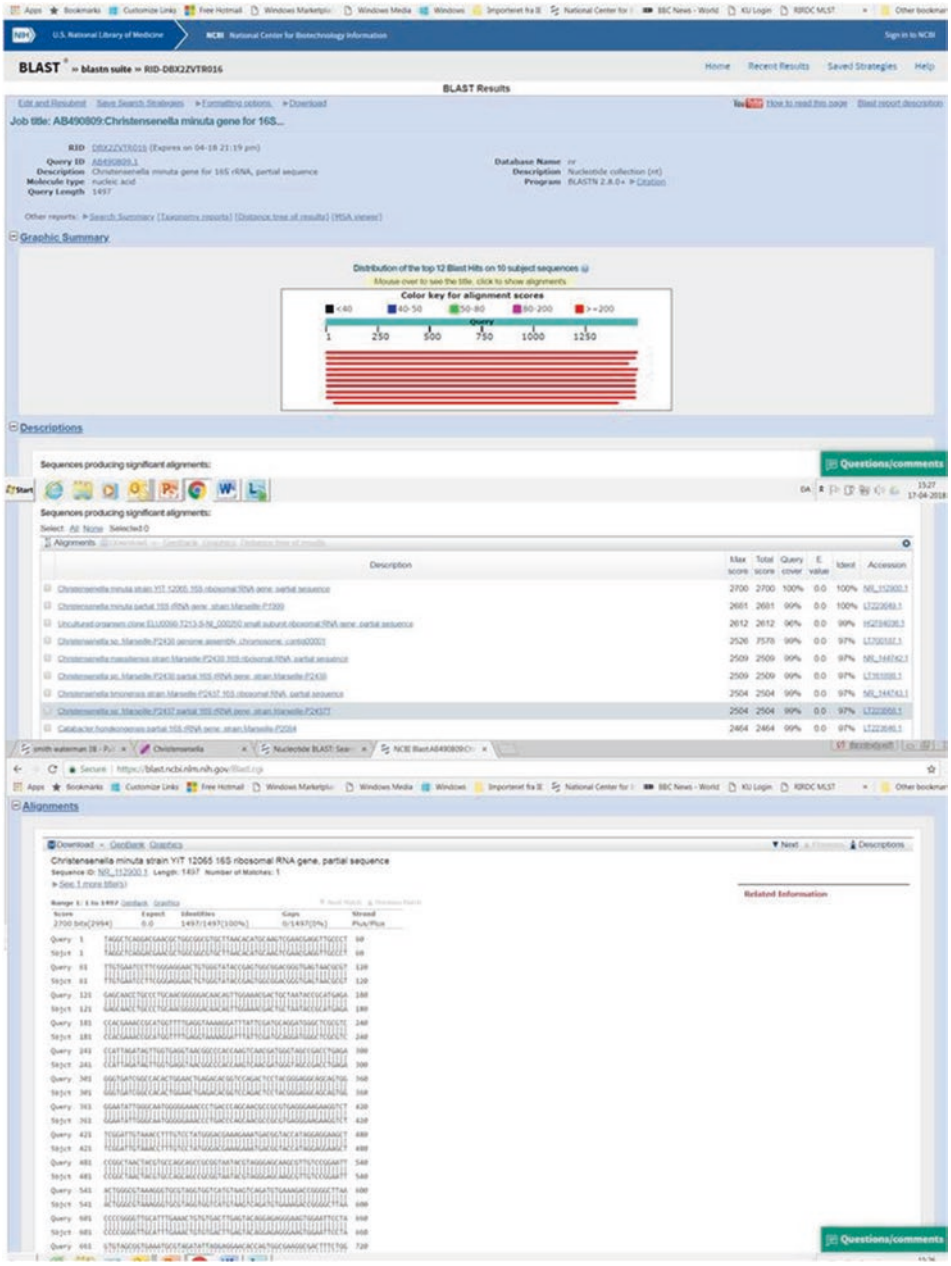

Fig. 4.24 BLAST (Altschul et al. 1990, 1997) output showing three screenshots of the output from BLAST search: **Graphic Summary**, **Description,** and **Alignments**, respectively. The number of Max target sequences was reduced to 10 in the Alignment parameters section to show it all on one page (BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004 – [cited 2018 04 17]. Available from: https://www.ncbi.nlm.nih.gov/Blast.cgi)

In the alignment section, the pairwise alignments are shown. This is the most detailed information of comparison between your query sequence and the subjects in the database. The parameters from the description section are available, and additional information of gaps and visual location of both gaps and mismatches can be observed.

4.3.2 Ortholog Detection

Sequences are orthologs if they are homologs and have the same function in different species. The paralogs are also homologs but have been duplicated during evolution, and they have got divergent functions in the same species, and the sequences are often quite divergent. BLAST can be used to sort the orthologs from the paralogs. The principle of reciprocal best hits (RBH) is used as explained in Moreno-Hagelsieb and Latimer (2008) "Two genes residing in two different genomes are deemed orthologs if their protein products find each other as the best hit in the opposite genome" (Moreno-Hagelsieb and Latimer 2008). When BLAST search is done against different organisms, the orthologs will often have the highest scores and the paralogs lower scores. Further investigation of orthologs and paralogs can be done by phylogeny (Chap. 6).

4.3.3 BLAST2 sequences

BLAST2 sequences is a version of NCBI BLAST that allows pairwise comparisons on server or on stand-alone BLAST installed on your PC. The program is available on both DNA and protein level, and it can be used to build your own database for many purposes by including many sequences in the second window (Fig. 4.25). For instance, if the query DNA or protein sequence is included with the first search window and a range of genomes included in the second search, you can identify the query sequence in the whole genomic sequences including genomes not yet deposited with the databases. The same approach can be used to build a small MLST database (Chap. 11). BLAST2 sequences are started from the ordinary BLAST page NCBI by marking "Align two or more sequences." It can be used for fast draft comparisons between two sequences, for example, if you want to look up the location of a primer or gene on a genome. This is your "Swiss knife" always available if you just can access the internet. Like for an ordinary knife, be careful with BLAST2 sequences! If you need real precise similarities between sequences, they should be obtained by the pairwise alignment programs described Sect. 4.1.

4.3.4 Statistics

Evaluation of the results in the BLAST output is based on the Karlin and Altschul formula $E = k \times m \times N \times e^{-\lambda s}$ (Karlin and Altschul 1990) where m is letters in query meaning the number of nucleotides or amino acids, N is the total letters in database, and S is the actual

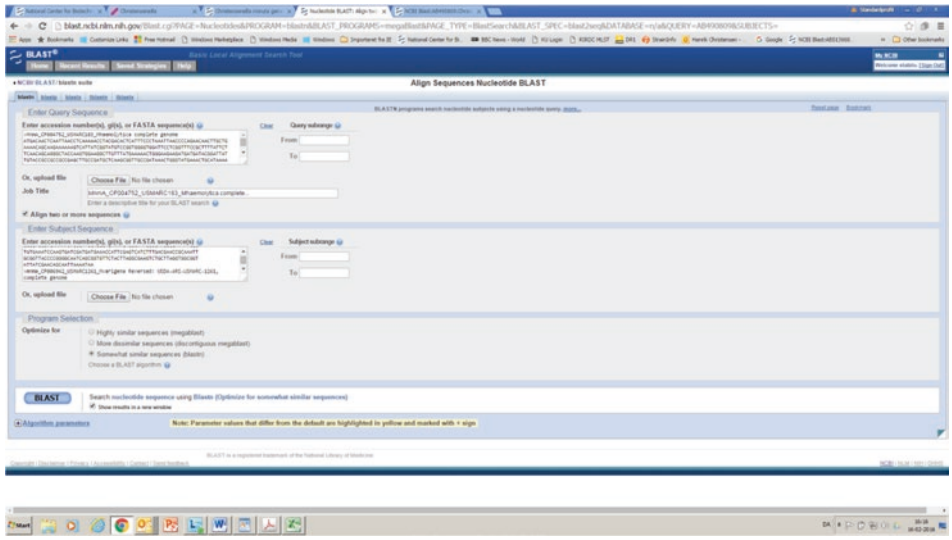

Fig. 4.25 BLAST 2 sequences. (Altschul et al. 1990, 1997). BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 2018 04 17]. Available from: https://www.ncbi.nlm.nih.gov/Blast.cgi

score. For amino acids, S is defined by the BLOSUM matrix. S is scaled to bit score to better fit the computer. The bit score = $((\lambda \times S) - \ln \kappa)/\ln 2$. In the pairwise section (Fig. 4.24 above), you can see the raw score in parenthesis along with the bit score. The constants κ and λ depending on the database. E reflects that we find the sequence in the database by chance (chance for false positives). E is this way related to the size of the database. The smaller E, the less likely is it found by chance, and the more unique is the sequence—"The smaller E the better." For really "unique" sequences, E is so small that it cannot be represented on the computer; this is the reason for "0.0" with the output.

All parameters related to the search can be found in **Search Summary** (Fig. 4.26). Here, you can find parameters used for calculating the statistics in the Karlin and Altschul formula, and you can understand everything about the BLAST output if you compare these parameters to the statistics just explained.

Note that parameters for ungapped blast (Altschul et al. 1990) differ from gapped-blast (Altschul et al. 1997) (Fig. 4.26), and λ and κ are different for gapped BLAST compared to ungapped.

4.3.5 Variants of BLAST

A BLAST search can be based on a query DNA sequence that the program translates to protein and compares to all protein sequence in the database (BLASTx). In this case, the search takes more time, and it should only be done when it is absolute needed (rarely). For

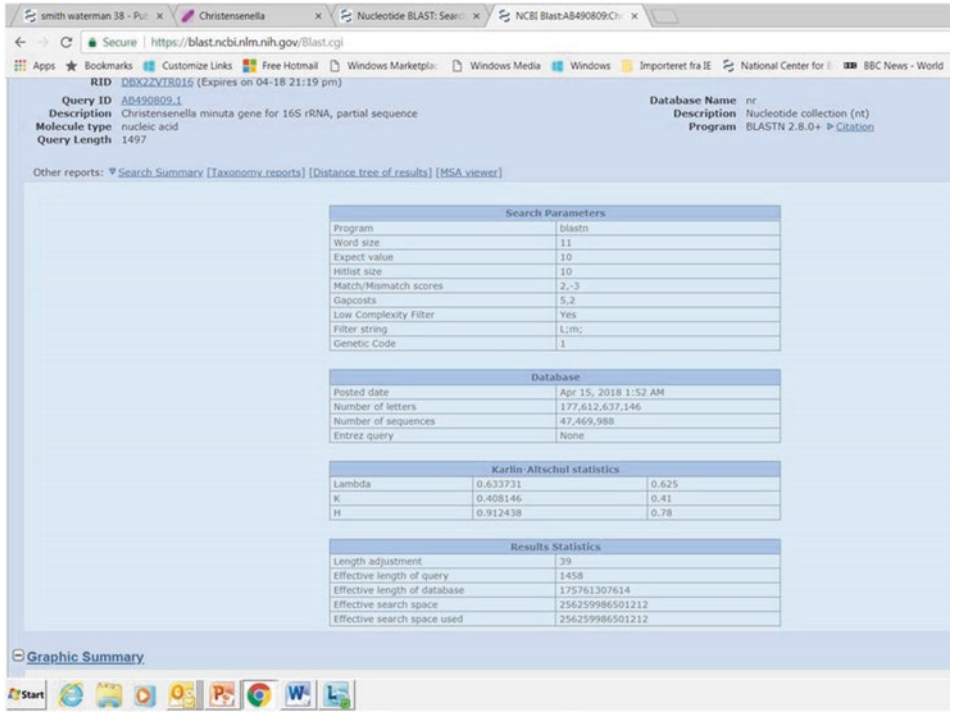

Fig. 4.26 The Search Summary section of the output showing all parameters used for the search (Altschul et al. 1990, 1997). BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 2018 04 17]. Available from: https://www.ncbi.nlm.nih.gov/Blast.cgi

such comparison, the codon table should be defined according to the type of organisms. Prokaryotes are using codon table 11 (Appendix). tBLASTn searches a translated nucleotide databases using a protein query. tBLASTx searches a translated nucleotide query against the translated nucleotide databases.

For BLASTp, it is sometimes convenient to select Swiss-Prot or ref_seq_protein if you want a really precise information about the hits. For protein searches, note the additional options PSI-, PHI-, and delta BLAST (Fig. 4.27), and they can be used to build up a profile used to search for query proteins, which are rather distantly related to the subject proteins in the database.

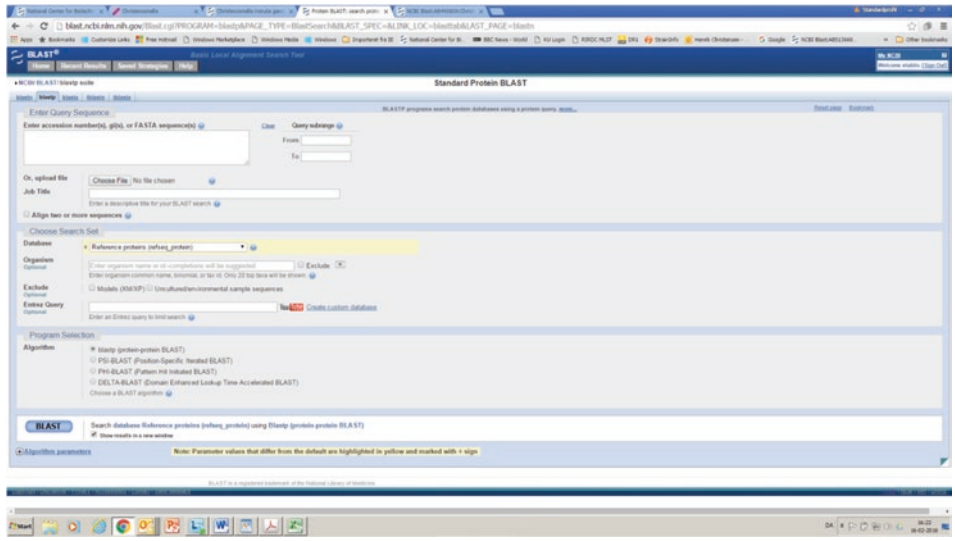

Fig. 4.27 Protein BLAST, BLASTp, note the additional options PSI- PHI- and delta BLAST (Altschul et al. 1990, 1997). (Lipman 1997) BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 2018 04 17]. Available from: https://www.ncbi.nlm.nih.gov/Blast.cgi

4.4 Activities

4.4.1 Pairwise Alignment

We will construct local and global pairwise alignments by the use of the programs "water" and "needle," respectively, available as EMBOSS programs (Rice et al. 2000) on the EBI server. The protein sequences AAA85484 and AAA85485 can be downloaded from NCBI as described in Chap. 3 (see Sect. 3.3.1 including Activity 3.1).

Decide if you want to use "needle" for global or "water" for local alignment.

Open the FASTA format files and paste them in the windows of the relevant EMBOSS program on the server and select protein or DNA:

https://www.ebi.ac.uk/Tools/psa/emboss_needle/

https://www.ebi.ac.uk/Tools/psa/emboss_water/

4.4.2 Multiple Alignment with ClustalX

From http://www.clustal.org/download/current/ download clustalx-2.1-win.msi, install and locate the icon to the desktop. Open the program by double-clicking on the icon. **File | Load sequences** and select the sequences in FASTA format from the location on your

computer. You need to edit the input file yourself. The sequences that you want to use as input should be in one file only with all sequences in FASTA format:

>sequence1
ATGACGATAC…
>sequence2
GATAGATAGACS…
etc.

Select **Alignment | Do complete Alignment | ok.**

In the lower left corner, you can follow how the program works.

Scroll through the alignment when it is completed.

At the bottom, the bars show the degree of conservation of columns. Above the alignment, the signature * shows columns with conserved nucleotides (or amino acids) with the same properties.

4.4.3 BLAST

We will use this activity to learn how to perform an ordinary BLAST search in GenBank with a DNA sequence. However, we also learn some more about BLAST in this activity. We will perform a search with the sequence with acc. no. AB490809 from GenBank.

First perform a BLAST search at NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi. On the graphics, select **nucleotide BLAST** and insert the acc. no. AB490809 in the window and mark "**somewhat similar sequences blastn,**" mark "**show results in a new window**" and press the blue **BLAST** bottom. Recently, we have seen a request from the NCBI-BLAST server to restrict the search to reduce the demand for CPU capacity. For the current example, rRNA/ITS databases can be selected instead of Standard nr.

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Primer Design

5

Design of Oligonucleotide PCR Primers and Hybridization Probes

Henrik Christensen and John Elmerdahl Olsen

Contents

H. Christensen (✉) · J. E. Olsen
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; jeo@sund.ku.dk

H. Christensen (ed.), *Introduction to Bioinformatics in Microbiology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-031-45293-2_5

What You Will Learn in This Chapter

You will learn to identify different situations where primer design is necessary and to separate exploratory applications from diagnostic applications. In some situations, de novo design of primers is needed; in others, it will be relevant to test primers already published. You will learn about the basic rules for primer design and how to evaluate sequence differences and T_m, and you will be presented for a list of primer design programs. In the activity, you will train primer design based on the single DNA sequence (exploratory) as well as design for PCR primers for diagnostic purpose.

5.1 Background for Oligonucleotide Design

Computer programs are available allowing users with limited background knowledge to design oligonucleotides. Most programs are available for free and easy to use from convenient web interfaces. A minimum theoretical background is needed to obtain meaningful results from such analysis. With a limited investment in time including an overview of the different programs available, users can obtain much better performance even without the need to go into details of computer science and algorithms of programs. The aim of the current chapter is to provide an updated overview of the design of oligonucleotides within a bioinformatical framework including presentation of relevant software (Fig. 5.1). The in silico design of oligonucleotides can lead to tremendous savings of time and money in the development of oligonucleotide tests; however, it can never replace experimental verification since it is not possible fully to predict oligonucleotide probe specificity in silico.

Oligonucleotides are in short referred to as "probes" when used for hybridization and "primers" when used for PCR. The design of oligonucleotides will be described both with reference to primers and probes since most researchers use both PCR and hybridization technology. Demands to both probes and primers are formation of stable duplexes with their target sequences and low self-complementarity. In other respects, the design of oligonucleotides is different depending on function either in hybridization or PCR, respectively. For many applications, probes and primers have already been designed and are available from publications and databases.

5.1.1 Practical Approach to Oligonucleotide Design Whether of Exploratory Nature or for Diagnostic Purpose

The use of oligonucleotides can broadly be identified in regard to exploratory projects such as the design of PCR primers to amplify DNA based on a single DNA sequence or

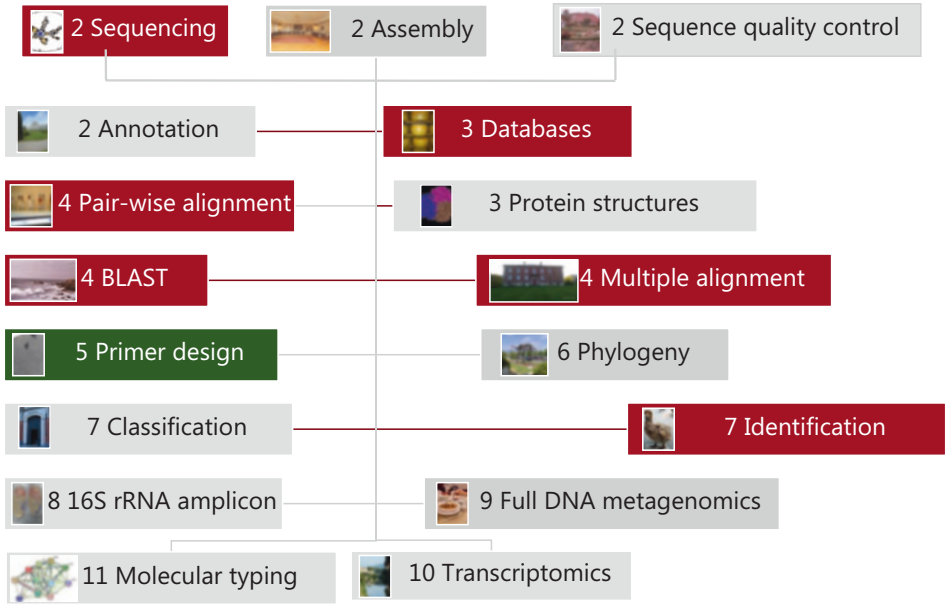

Fig. 5.1 Relation of this chapter to the other chapters in the book. Primer design relates both to the generation of sequence, to the multiple alignments of sequences, and to databases, identification, and BLAST

Table 5.1 Strategies for the design of oligonucleotide PCR primers and hybridization probes

Task	Action
1. Identification of target sequences and literature	Tables/files of information are generated based on database search (Chap. 3)
2. Identification of conserved and homologous regions	Multiple alignments or/and phylogenetic analysis (Chaps. 4 and 6)
3. Generation of sequence	Experimental sequencing (Chap. 2)
4. Identification of oligonucleotide candidates	Primer search programs (Table 5.3)
5. Verification	Experimental tests including positive and negative controls

diagnostic purpose where the specific amplification of only one or a group of sequences is intended, whereas others are not wanted. For most research projects involving the design of oligonucleotide probes, the work is performed in five steps (Table 5.1). First, a database search is performed with respect to target gene(s) and organism(s). The DNA sequences for design of oligonucleotides are mostly downloaded from the International Nucleotide Sequence Database Collaboration (GenBank/EBI/DDBJ; Sayers et al. 2023a, b; Arita et al., 2021; Tanizawa et al., 2023). For instance, GenBank is searched with BLAST (Altschul et al. 1997). Sequences of importance can be downloaded as described in Chap. 3. The second step is to compare the selected sequences by multiple alignments and phy-

logenetic analysis. As a third step, it needs to be considered if more DNA sequences need to be generated to cover the target sequences being investigated. First at step 4, the real sequence comparison allows search for oligonucleotides with the software to be described below (Sect. 5.7). The oligonucleotides can further be tested against the database(s) by BLAST to confirm target sequences. The final step is to set up and try the oligonucleotides for real.

It is important to be able to design new probes and primers for three reasons:

(i) New diagnostic problems are faced.
(ii) Improved knowledge of the organisms including new varieties and genes need to be accounted for.
(iii) Natural genetic variation occurs in the organisms under investigation especially virus.

The main function of oligonucleotides is to "prime" PCR reactions in the way that they hybridize to single-stranded DNA and prime the replication of DNA by the action of Taq-polymerase (Fig. 5.2).

Fig. 5.2 Hot well from Yellowstone National Park, the harsh environment where *Thermus aquaticus* was isolated. From this bacterium the thermostable polymerase was isolated that is essential to perform PCR

The other main function of is with in situ hybridization. Hybridization is also used for microarrays; however, in this case, oligonucleotide probes are usually fixed to a solid support and used unlabeled, and the test DNA is labeled.

5.1.1.1 Exploratory Applications

Exploratory applications include procedures to amplify DNA for sequencing only requiring PCR primers of low specificity and similar tools to manipulate DNA such as PCR primers used for cloning. PCR primers for use with multilocus sequence typing of bacteria (Chap. 11) are also in this category.

Degenerate primers and probes include different nucleotides at some positions of the sequence. Degenerate primers and probes are needed if the target DNA sequences include an unknown diversity, for instance, if the protein sequence is known and then underlying DNA sequence is known to include codon ambiguities.

The exploratory applications also include PCR primers for cloning with recognition sites for restriction enzymes. Other PCR primers are used to "prime" the reverse transcription of mRNA and viral RNA to DNA.

5.1.1.2 Diagnostics Applications

Oligonucleotides designed for this application are typically used for diagnostics of microbial pathogens of animals and plants where high specificity is needed. Oligonucleotide probes are used for detection of specific DNA target sequences by hybridization when labeled by radioactivity, fluorescence, or other handles facilitating detection.

PCR detection is achieved by overproduction of target DNA sequence, in modern diagnostic assays often combined with fluorescent detection achieved in real time (RT)-PCR. RT-PCR is not different to ordinary PCR when it comes to oligonucleotide design except for various types of detection chemistries (Sharkey et al. 2004; Wong and Medrano 2005).

The primary goal of PCR primer design for diagnostic purpose has been formulated to obtain a balance between specificity of amplification and efficiency of amplification (Dieffenbach et al. 1995). Specificity is the tendency for a primer to hybridize to its intended target and not to other targets. In other words, specificity is defined as the absence of reaction with negative targets. The same principle exists for hybridization. With PCR, the efficiency is the ease that the expected product is produced. Often, a compromise has to be made between specificity and efficiency. For clinical applications, "bedside" specificity might be increased at the cost of efficiency to avoid false-positive results that might result in the wrong treatment of patients (Dieffenbach et al. 1995; Hyndman and Mitsuhashi 2003; Grunenwald 2003).

5.2 General Rules for Design of Oligonucleotides

Both PCR and hybridization techniques are based on the hybridization of an oligonucleotide to a complementary target DNA sequence. Hybridization is the formation in vitro of specific double-stranded nucleic acid molecules from two complementary single-stranded

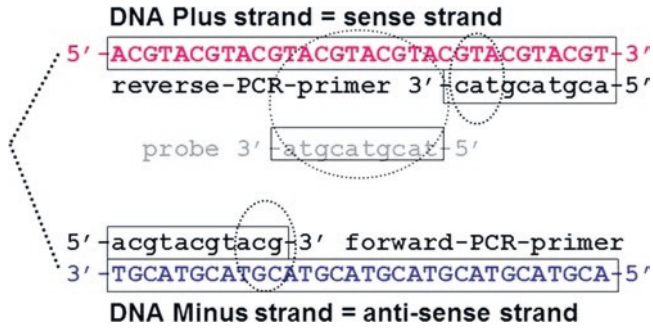

Fig. 5.3 Sketch of orientation of PCR oligonucleotide primer and hybridization probes in relation to DNA target. For PCR, the primer for forward amplification has the same sequence as the sense strand of template DNA and binds to the antisense DNA strand. The primer for reverse PCR amplification has a sequence that is both reverse and complementary to the sense DNA strains. Probes for hybridization are oriented the same way as the antisense DNA strand.

molecules under defined physical and chemical conditions. Figure 5.3 shows the orientation of PCR primers and hybridization probes with respect to target double-stranded DNA. As a rule of thumb, the forward primer is always oriented as the sense DNA strand, whereas the reverse primer is both complementary and reverse to the sense DNA strand. Probes are oriented as the antisense DNA strand.

With four possibilities (A, C, G, T) for selection of nucleotides at each position of the sequence, 4^{18} (~10^{11}) possibilities exist for the design of an 18 nucleotide oligonucleotide. For this reason, very stringent criteria are needed to select the best oligonucleotide for a specific purpose. However, hybridization, PCR amplification, and probe binding are also possible with a few mismatches under some conditions, and it is not only the sequence but also the conditions that define the outcome.

The ability of an oligonucleotide to serve as a PCR primer is dependent on the kinetics of:

(i) Association and dissociation of probe or primer-template duplexes at the annealing and extension temperatures
(ii) The effects on duplex stability of mismatched bases and their location
(iii) The efficiency that the polymerase can recognize and extent a mismatched duplex

The ability of an oligonucleotide to serve as a hybridization probe depends on the kinetics of:

(i) Association and dissociation of probe-template duplexes at the hybridization temperature
(ii) The effects on duplex stability of mismatched bases and their location

In conclusion, for the design of oligonucleotides, the sequence comparison between oligonucleotide and template is most important. It is also important to calculate T_m (Sect.

5.4) to estimate the annealing temperature in PCR and hybridizations since the actual hybridization temperature in the end will determine the outcome of the experiment. With multiplex PCR, many probes and primers, respectively, have to function together at the same temperature, and for this reason, they need to be designed within a common threshold level of T_m.

5.2.1 Lengths of PCR Primers and Products

PCR primers should normally be between 18 and 24 nt (Table 5.2). The length depends on the GC content. For primers of the same length, the T_m will increase with higher GC content related to the three hydrogen bonds in the GC pair compared to only two in the AT pair. To match the T_m of both forward and reverse primers, the length of a primer with high GC content needs to be shorter compared to one with less GCs. PCR primers of 15 nucleotides or shorter are only used for arbitrary or random short priming in the mapping of simple genomes. Their sequence is so short that the chance that they will be complementary to a DNA sequence in a genome is very high. For ordinary PCR, product ranges from approximately 200 to 1500 bp are preferred to allow resolution on ordinary agarose gels. For real-time PCR (RT-PCR), products are usually shorter (< 150 bp). Guidelines for RT-PCR are found in Huggett et al. (2013).

5.2.2 Lengths of Oligonucleotide Hybridization Probes

Hybridization probes have been applied with lengths from around 20 nt up to several hundreds of nt. For microarray experiments, the usual size is 25 nt, but probes of 50–70 nt are also used on certain applications (Gibson and Muse 2004). Generally, the longer the probe, the more mismatches are needed to separate target from nontarget sequences.

Table 5.2 Rules-of-thump for design of oligonucleotide PCR primers and hybridization probes

Application	Rules
All	Purines and pyrimidines should be equally distributed and long stretches of identical nucleotides avoided. The GC content should be similar to or higher than the target DNA sequence. The medium length is 18 nt. The hybridization temperature including annealing temperature should be 5°C lower than T_m
PCR primers	Primer lengths of 18 and up to 24 nt are preferred with T_m of 54°C or higher. Perfect base pairing between the 3′ end of the primer and the template is necessary for maximal specificity and the last five to six nucleotides at the 3′ end of the primer must contain minimal mismatches
Hybridization probes	At least one or two nucleotide differences are needed for separation of target from nontarget sequences. Mismatches near the end of probes are less destabilizing than internal mismatches For design of oligonucleotide probes targeting rRNA, probes should bind along one or the other side of hairpins

5.3 Sequence Comparison

For comparison of sequence matches between primer and template, sequence comparison can be performed in two fundamentally different ways. Either sequences can be compared as so-called strings and evaluated in relation to scores between nucleotide pairs (Sect. 5.3.1). This procedure was shown for local pair-wise sequence comparison (Chap. 4). Alternatively, sequences can be compared thermodynamically in relation to their changes in free energy upon forming duplexes; however, this method is only useful for shorter oligonucleotides (Sect. 5.3.2).

5.3.1 String Comparison by Score

Sequences are matched pair-wise by alignments and alignments compared by scores. Scores are defined in favor of canonical base pairing (G–C, A–T) and the alignment with the highest score chosen. The method is by intuition simple in that complementary nucleotide pairs are favored in the comparison. For pair-wise comparison between oligonucleotide and template, precise algorithms are used, whereas for database comparisons, less precise but greedier algorithms are implemented in programs like BLAST.

5.3.2 Nearest Neighbor Comparisons of Duplex Stability

The nearest neighbor calculation is based on the fact that the change in Gibbs free energy (ΔG) of the probe or probe-template complex can be calculated from the enthalpy (ΔH) and entropy (ΔS) at a given temperature (T) as $\Delta G = \Delta H – T\Delta S$. If ΔG for the oligonucleotide binding to itself is negative, the primer should be redesigned by extension/removal of 5′ or 3′ positions in order to reduce self-complementarity. A nearest neighbor pair is defined by two neighboring nucleotides in a sequence. For example, in the probe sequence 5′-ACGT-3′, pairs to be considered for binding to a complementary sequence as a template will be AC, CG, and GT. The calculation can either be used for selection of oligonucleotides by comparison of all nearest neighbor pairs formed between probe and template or for evaluation of probe self-complementarity including loops and for calculation of T_m (Sect. 5.4.3). Considerations are taken to free-energy change in relation to helix initiation associated with forming the first base pair in the duplex, the sum of free energies for the subsequent pairs, and correction for self-complementary (Owczarzy et al. 2008). The parameters ΔG, ΔH, and ΔS were empirically determined for all nearest neighbors of DNA by Breslauer et al. (1986) and further refinements performed as reviewed by SantaLucia (1998). These parameters are only used for implementation in computer programs.

5.3.3 Design of Primers for PCR and "Kwok's Rules"

In 1989, Kwok et al. published a set of rules for the design of PCR primers based on their experience with PCR amplification of HIV sequences. The principles determined have found general use known as "Kwok's" rules. The discrimination of different PCR targets is related to the lack of 3′ to 5′ exonuclease activity of Taq DNA polymerase resulting in extension of mismatched 3′ termini at a lower rate compared to complementary termini (Lawyer et al. 1989).

The conclusion of the work of Kwok et al. (1990) was that extension is most dependent on the outermost 3′ base-pairing, less on second and third last pair, and even less on the other pairs. Figure 5.4 summarizes some conclusions from the Kwok paper. The experi-

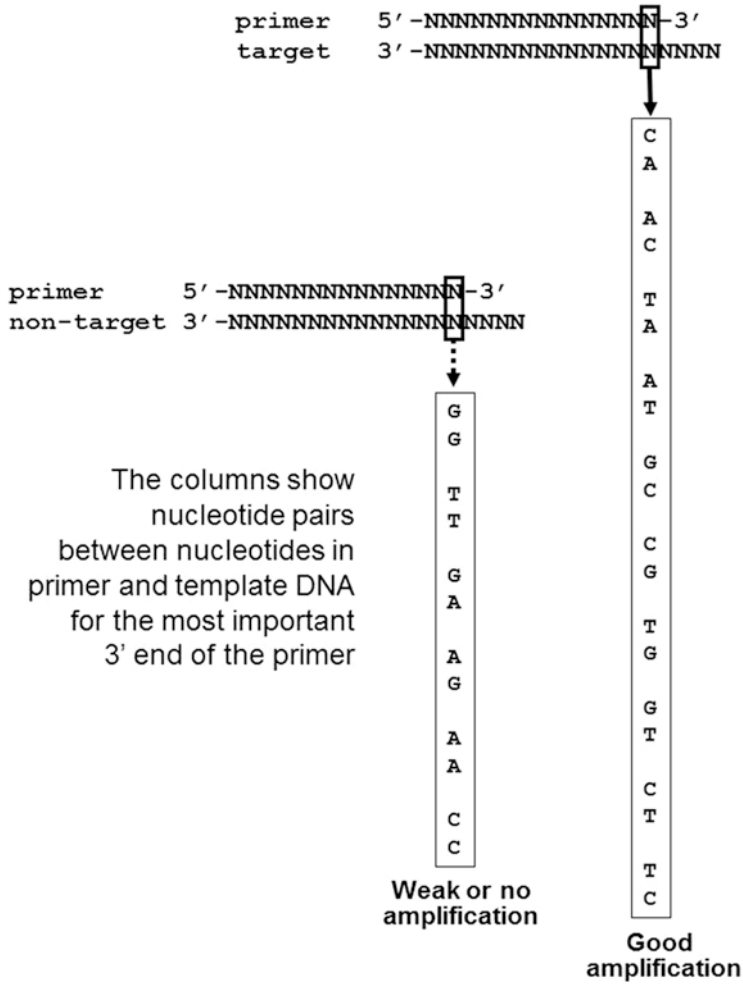

Fig. 5.4 Prediction of amplication from interactions between PCR oligonucleotide primer at the 3′ end and template (based on Kwok et al. 1990). Low degree of mismatch is assumed between oligonucleotide and template for the remaining part of sequence

ments with PCR were performed at 55°C annealing temperature and are therefore comparable to most PCR conditions. The conclusion is that if the aim of the design of primers is to obtain specific detection of certain target sequences without amplification other nontargets, G–C-pairs in 3′ end will in most cases allow discrimination, but A–T pairs will not. It might come as a surprise that amplification also is possible from noncanonical pairs as, for example, G–T. G–T pairs should be avoided in the 3′ end since they are more stable than other types of mismatches (Kwok et al. 1995) (see also Newton et al. 1989).

5.3.4 Design of Probes for Hybridization

In parallel to the rules just outlined for PCR, it is also important to account for different sorts of noncanonical base pairing in hybridization experiments. For DNA oligonucleotide hybridization, the pairs G–T and G–A are involved in weak binding, and the pairs G–A, G–G, C–A, A–A, G–T, T–T, and C–T are destabilizing in decreasing order (Ikuta et al. 1987; Pozhitkov et al. 2006) (Fig. 5.5). In correspondence, the stability of RNA/DNA duplexes on microarrays is

TG, TU, TC > GU, AC, CC, CU, AA, AG > CA > GG, GA (Pozhitkov et al. 2006).

These observed interactions are mostly a consequence of pyrimidine–pyrimidine mismatches being more stable than purine–purine mismatches related to higher steric hindrance for binding of the latter (Pozhitkov et al. 2006). In addition, the G–U pair is stable in RNA compared to the more unstable G–T pair in DNA. For microarrays, oligonucleotides bound at the 3′ end and mismatches nearest the 5′ end are less destabilizing than those in the middle of the oligonucleotide (Szostak et al. 1979; Urakawa et al. 2002; Pozhitkov et al. 2006).

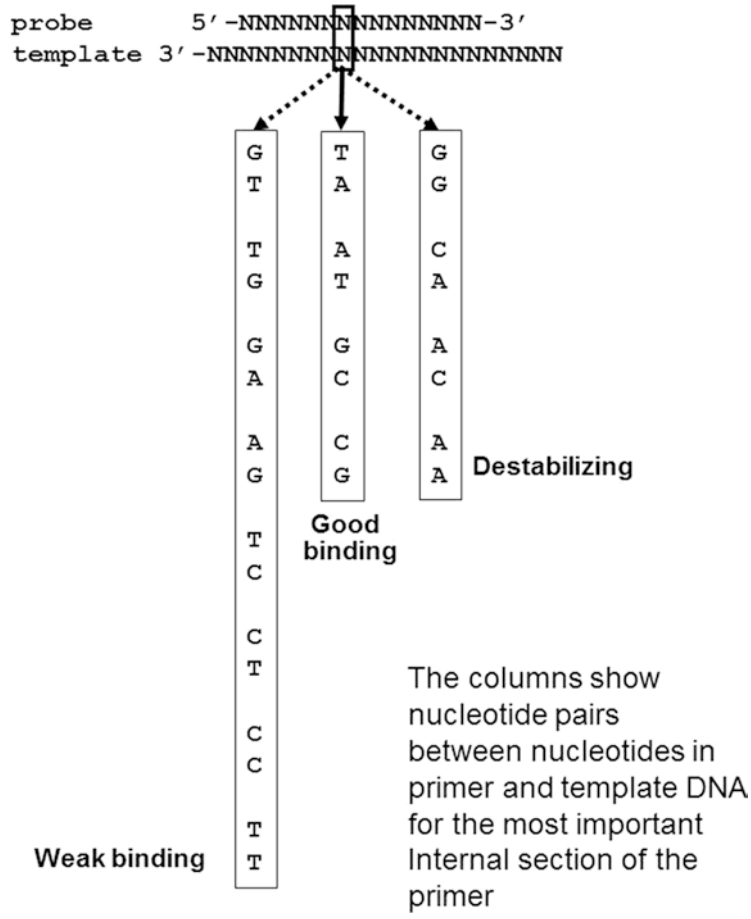

Fig. 5.5 Prediction of binding in hybridization between probe and template. A low degree of mismatch formation is assumed between oligonucleotide and template for the remaining part of the sequence

5.4 T_m Calculations

The nucleic acid melting temperature, T_m, is defined as the midpoint in the transition from helix to random coil measured as change in optical density. The original definition implied almost equal proportions of complementary strands. For oligonucleotides, concentrations are orders of magnitude higher than template concentration and the actual dissociation temperature, T_d, where 50% of oligonucleotides are bound to template out of the maximum possible might be different from T_m and depend on the concentration of the DNA strands. The thermodynamic approach can also be used for calculation of T_m (Sect. 5.4.3). Alternatively, T_m is determined by a formula (Sect. 5.4.1).

5.4.1 Estimation of T_m by Formula

The crudest way to estimate T_m is used with primers shorter than 20 nt. The formula, $T_m = 4$ (G + C + 2(A–T), can be used (Suggs et al. 1981). However, the nearest neighbor method (see below) was found four times more accurate (Rychlik and Rhoads 1989). An empiric derived formula for calculation of T_m was given by Schildkraut (1965) for unsheared DNA fragments. This formula has been modified for short DNA fragments. For oligonucleotides of 10–50 nt, $T_m = 81.5 + 16.6\log M + 0.41(G + C\%) – (820/(\text{length of probe})$ (Lathe 1985). For probes longer than 50 nt, $T_m = 81.5°C + 16.6\log M + 0.41(G + C\%) – (500/\text{length of probe})$ (Meinkoth and Wahl 1984). In these formulas, M is the concentration of monovalent cations (N^+ and K^+). Typical values for PCR are 50 mM KCl where K^+ is by far the dominant cation. For 1 × SSC, the concentration of monovalent cations is 195 mM.

5.4.2 Formamide Considerations

For practical purpose, formamide can be included during hybridization to decrease the actual temperature required. It is possible to adjust T_m by application of a gradient of formamide concentrations rather than to use a set of different temperatures during incubation. Another reason to include formamide is that temperatures higher than 50–60°C might damage tissue samples with in situ hybridization as well as reagents, materials, and equipment. The common rule has been that 1% formamide reduces T_m by 0.72°C (McConaughy et al. 1969). However, recently, it was found that 1% formamide reduces T_m by only 0.6°C in oligonucleotide microarrays (Urakawa et al. 2002). Unfortunately, health hazards associated with formamide have limited its use.

5.4.3 Estimation of T_m by Nearest Neighbor Prediction

The composition of the nucleotide sequence has been found to influence T_m more that either length of oligonucleotide or base composition. This is the basis for use of the nearest neighbor models for calculations of T_m. As mentioned, nearest neighbor calculations are based on the former mentioned stability of base pairs only depending on the immediate up- and downstream neighbors. As an example of such calculation used in the computer programs, $T_d = \Delta H/(\Delta S + \text{RlnC}) + 16.6\log 10\ [M]$, where $T_m = T_d$ for PCR and T_m is less than 7.6°C of T_d for filter hybridization (Rychlik and Rhoads 1989). R is the universal gas constant (1.99 cal K^{-1} mol^{-1}). C is the molar concentration of all strands when oligonucleotides are self-complementary and is replaced by C/4 for noncomplementary oligo-template interactions (Borer et al. 1974).

Formula and nearest neighbor calculations have been combined. The combination was used by Rychlik et al. (1990) for calculation of annealing temperatures (T_a). T_a was calcu-

lated as T_a = 0.3 T_m primer +0.7 T_m product – 14.9. T_m of primer was calculated by the nearest neighbor model and T_m of product by formula. The calculation of T_a was compared to empirically determined T_a in PCR reactions, and they were found to be in good agreement.

5.5 Special Applications

5.5.1 Exploratory Applications

5.5.1.1 Degenerate Primers and Probes

If the purpose is to detect homologous sequences of a particular type, a high degree of variation often must be tolerated, and for this purpose, degenerate oligonucleotides are designed. A primer sequence is degenerate if some of its positions have several possible bases. The degeneracy of the primer is the number of unique sequence combination it contains. For example, the degeneracy of the primer GGSABA is six where S means "strong" either G or C and B can be either C, G, or T resulting in 1 × 1 × 2 × 1 × 3 × 1 = 6 possibilities (GGGACA, GGGAGA, GGGATA, GGCACA, GGCAGA, GGCATA). The IUB codes are shown in the Appendix. Two strategies exist for designing degenerate primers and probes. Either two or more different nucleotides can be incorporated at specific positions of the oligonucleotide at the time of synthesis, or different batches of oligonucleotide primers can be mixed after synthesis. A third possibility is to combine the two strategies. Inosine can be incorporated as an "inert" nucleotide forming base-pairs with all four nucleotides. Degeneracies are usually incorporated to account for codon variation at second and third positions. Knowledge of codon bias can be used to limit the degeneracy needed (Kwok et al. 1995). A procedure for this strategy is described with the CODEHOP program (Table 5.3). A consequence of the introduction of ambiguous nucleotides is that the actual concentration of the important nucleotides at the 3′ end will decrease. Efficient PCR amplification (detectable on conventional agarose gel) will probably not work with less than 0.1 μM final conc. of primer, and, for example, four ambiguous positions will decrease the actual conc. With 2 × 2 × 2 × 2 = 32 then requires 3.2 μM for each of the primers to work.

5.5.1.2 Nested PCRs

In nested PCR, the sample is first amplified with an "outer" primer set and a subsample of this product then amplified with the "inner" primer set. The inner primer set is designed to be the specific one. In designing primers for this purpose, primer-dimer formation between outer and inner pairs should be tested for and avoided (primer-dimer formation is the ability of two oligonucleotides to base-pair often allowing initiation of short PCR products). The product obtained with the inner pair should be short (< 300 bp). The method can be used when the concentration of template is low or the knowledge of the target sequence is limited and amplification cannot be achieved in other ways (Dieffenback et al. 1995). Programs for the design of nested PCRs include PrimerPremier (Table 5.3).

Table 5.3 Programs listed according to application

Program	Description	URL and reference
General purpose		
Amplifx2.1	PCR design program for MacOS X	https://macdownload.informer.com/amplifx/
FastPCR	PCR primer design including multiplex PCR	http://primerdigital.com/fastpcr.html
Netprimer	Numerous parameters of single PCR primers	http://www.premierbiosoft.com/netprimer/index.html
Oligo	Nearest neighbor calculations of secondary structures and T_m	https://www.oligo.net/ Rychlik and Rhoads (1989)
OligoAnalyzer	Nearest neighbor parameters. Hair-pin and primer-dimer analysis	https://eu.idtdna.com/pages/tools Owczarzy et al. (2008)
OligoCalc	Physical properties of oligonucleotides, self-complementarity and hairpin loop formation	http://biotools.nubic.northwestern.edu/OligoCalc.html Kibbe (2007)
OligoFaktory	DNA microarrays, primers for PCR, siRNAs	http://www.bioinformatics.org/oligofaktory/
Pride and Genome pride	PCR and microarray	http://pride.molgen.mpg.de/ (Staden package)
Prifi	Search for primers in multiple alignments	https://services.birc.au.dk/prifi/ Fredslund et al. (2005)
Primegens	Primer design	http://primegens.org/ Xu et al. (2002)
Primer3	The most frequently used program see also Activity 5.1	http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm Rozen and Skaletsky (2000)
Primer3Plus	Simplified version of Primer3	http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/
Primer Design Assistant (PDA)	Primer design with all parameters	http://dbb.nhri.org.tw/primer/ Chen et al. (2003)
PrimerPremier	PCR primer design, multiplex, degeneracies and nested PCRs	http://www.premierbiosoft.com/primerdesign/index.html
PrimerQuest	PCR and hybridization probes. Based on Primer3	https://eu.idtdna.com/PrimerQuest/Home/Index
Primer Search (EMBOSS)	Matches between primers-pairs and DNA template by string comparisons	http://emboss.sourceforge.net/ Rice et al. (2000)
Primo Oligo	Calculation of Tm	http://www.changbioscience.com/primo/oligo.html
Web Primer	PCR primer design	https://www.yeastgenome.org/cgi-bin/web-primer

Table 5.3 (continued)

Program	Description	URL and reference
SciTools	A series of tools	http://www.idtdna.com/SciTools/Scitools.aspx Owczarzy et al. (2008)
Special appliations		
ARB	Design of rRNA targeted probes	http://www.arb-home.de Ludwig et al. (2004).
ArrayDesigner	Oligo-arrays, cDNA arrays and SNP arrays	http://www.premierbiosoft.com/dnamicroarray/index.html
Assembly PCR oligo maker	PCR-based construction of long DNA molecules for RNA molecules by T7 RNA polymerase	http://www.yorku.ca/pjohnson/AssemblyPCRoligomaker.html
Beacon designer™	Real-time PCR	http://www.premierbiosoft.com/molecular_beacons/index.html
BLOCKMAKER and CODEHOP	Degenerate primers to genes of proteins	https://4virology.net/virology-ca-tools/j-codehop/ Rose et al. (2003)
Expeditor	QTL design	https://www.animalgenome.org/cgi-bin/expeditor/expeditor2
Genefisher2	Degenerate PCR primers based on multiple aligned sequences	https://bibiserv.cebitec.uni-bielefeld.de/genefisher2/
MFOLD	Evaluates probes	http://www.unafold.org/mfold/applications/rna-folding-form-v2.php
OligoPicker	microarray design of oligonucleotides	https://pga.mgh.harvard.edu/oligopicker/index.html
Pira-PCR	SNP	http://primer1.soton.ac.uk/primer2.html Ke et al. (2001)
PrimerD	Degenerate primer pairs	http://mblab.wustl.edu/software.html#primerdLink
Primer exmplore	LAMP primers	http://primerexplorer.jp/e/
PrimerX	Site-directed mutagenesis	http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi
ProDesign	Oligonucleotide design for microarray	http://wwwlabs.uhnresearch.ca/tillier/ProDesign/ProDesign.html Feng and Tillier (2007)

5.5.2 Diagnostic Applications

5.5.2.1 Primers for Multiplex PCR

Multiplex PCR is preferred in order to conserve reagents (template) as well as reducing preparation and analysis time compared to conventional PCR. With mixtures of primers with different sequences, the prediction of T_m becomes difficult since one value might only apply to each of the primers included. The programs FastPCR and PrimerPremier

(Table 5.3) should be able to handle the design of multiplex PCR primers. Experimental testing will be needed to confirm the choice of T_m.

5.5.2.2 SNP Analysis

Single nucleotide polymorphisms (SNP) are differences between individual nucleotides within the same gene, which may affect virulence or other properties between isolates of microorganisms. PCRs can be designed to target SNPs. For this, the SNPs need to be located in the 3′ of the primer binding to the template. The programs pira-PCR can be used to identify SNPs based on PCR tests (Table 5.3). In order to increase specificity, mismatches may be introduced according to Newton et al. (1989).

5.6 Data Formats

The FASTA format is used most frequently for sequence manipulation (Pearson and Lipman 1988) (see Chap. 2). This format is simply a text-file starting with a ">" sign and followed by the name of the sequence on the first line. On the second and following lines, the nucleotide string is listed without any additional characters such as numbers or spaces. Some programs will only consider the ten first characters of the name or identifier on the first line. Sequences are usually downloaded in FASTA format from the databases.

For certain programs, the sequences included with multiple alignments need to be trimmed to the same length before they can be further analyzed for oligonucleotide design. First, a multiple alignment is prepared, for example, by ClustalX or ClustalW (Larkin et al. 2007) and saved in the *.aln format (Chap. 4). The program BIOEDIT (Hall, 1999) can be used to trim sequences to the same length. BIOEDIT is installed from the URL (https://bioedit.software.informer.com/download/).

"File" and "Open" are activated in BIOEDIT and the file in *.aln format selected. The "Edit-mode" is chosen and regions with gaps identified by "mousing-over" column-wise and deleted when the region is marked. The consensus line at the bottom is not to be touched. The file is then saved by "File" and "Save as" with the choice of FASTA format. The file is reopened with ClustalX or ClustalW and realigned and saved in appropriate format.

Important points to consider using BLAST for evaluation of primer and probes are to use the lowest word size (7) and to turn off dust filters and low complexity filters. Even with these precautions, BLAST searchers in databases with oligonucleotides cannot always be expected to identify all sequences with perfect match. This is related to the short sequence length.

5.7 Programs

A list of relevant computer programs for primer and probe design is shown in Table 5.3. For these programs, we have briefly tested that the links have been open and that program installation has worked; however, full functionality has not been tested exhaustively. Many programs are online installations on servers assessed by internet as described yearly with the server issue of the journal, Nucleic Acids Research (https://academic.oup.com/nar/issue/51/W1).

These resources can be consulted to fix broken links as well as to search for new programs.

5.8 Activities

5.8.1 Exploratory Primers with Primer3 for Recognition of Single DNA Sequences

The purpose is to amplify DNA from a strain of avian influenza virus. Primer3 is used at https://bioinfo.ut.ee/primer3-0.4.0/ (Untergasser et al. 2007, 2012). For this activity, we will use the DNA sequence of the hemagglutinin (HA) gene of strain 1734 with serotype H5N1 isolated from duck in Fujian in 2005 with GenBank/EBI/DDBJ acc. no. DQ095629. Download the sequence from NCBI or similar database as described in Chap. 3. Cut and paste the DNA sequence into the window. You first have to select "**Pick left primer**" and "**Pick right primer**" and at Product size ranges delete all ranges except for 100–300 and leave other settings as defaults. Press "**Pick Primers**." Two primers are suggested with a predicted PCR product of 203 bp. Inspect the location and orientation of the primers (marked with >>>>> on output). Four more sets of primers are further suggested.

5.8.2 Diagnostic Primers with PrimerBLAST

The program is provided by NCBI to select specific PCR primers for one taxon and avoid amplification of others.

This server application at NCBI is based on Primer3 and BLAST. Open the program from: http://www.ncbi.nlm.nih.gov/tools/primer-blast/

For this example, we will design specific PCR primers for the serotype H7N2 of influenza virus. Download GenBank/EBI/DDBJ acc. no. **U20461** in FASTA format (Chap. 2). Include the target sequence by "**paste**" in the window or "**Choose file**" or simply write the GenBank acc. no. in the yellow field.

In the section **Primer Pair Specificity Checking Parameters** at,

Search mode change **Automatic** to **User guided**.

At **Organisms,** select family or the specific organisms you are working with (for Prokaryotes the genus name). In this case, **Influenzavirus A**.

At **Database** select **nr.**

Mark **Show results in new window.**

Press **Get primers.**

When the search is completed, look at the list to remove unwanted targets and to include more similar targets of the same type (H7N2) (Your PCR template is highly similar to the following sequence(s) from the search database. To increase the chance of finding specific primers …. …..). In this case, you can mark the 9 H7N2 sequences (CY006029, CY067681, AB302789 ….. KX130809).

Mark **Show results in a new window.**

Then, proceed pressing **Submit.**

Review the list and consider if a specific primer pair can be designed (it should look like Fig. 5.6). Are all primers of **Products of intended targets** for **Primer pair 1** matching the type H7N2? Are all **Products of unintended targets** forming mismatches that are expected not to result in generation of product? (look at Kwok's rules Fig. 5.4).

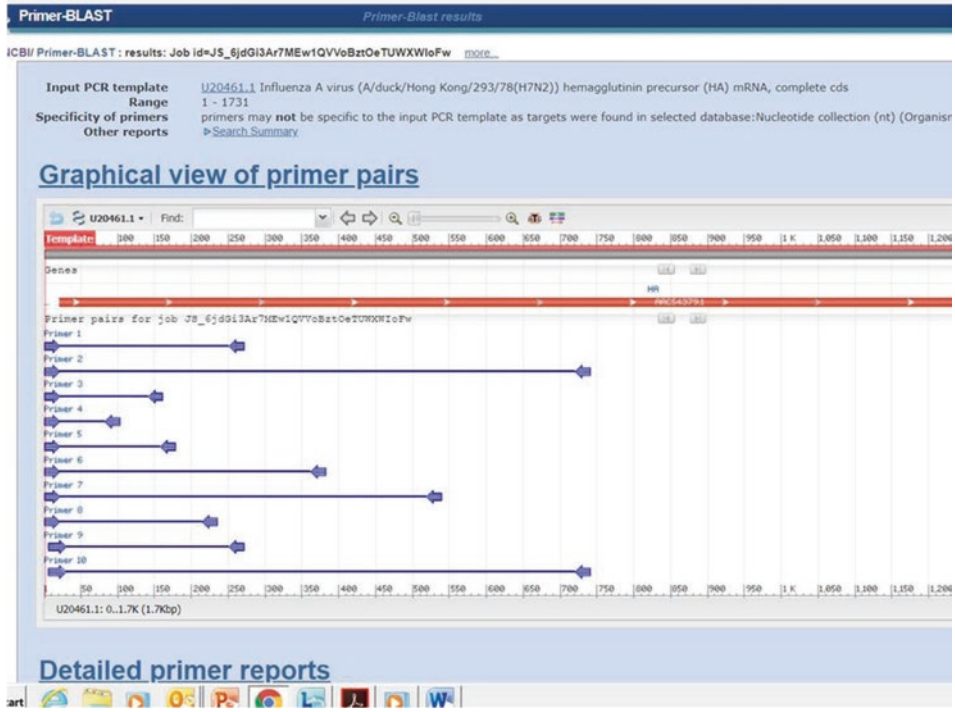

Fig. 5.6 Output from primerBLAST as expected from Activity 5.8.2. You see the graphical view of suggested primers for specific amplification of the hemagglutinin gene of the H7N2 type of type A influenza virus. BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 25 18 04]. Available from: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Fig. 5.7 Output from primerBLAST as expected from Activity 5.8.2. You see the graphical view of suggested primers for specific amplification of the hemagglutinin gene of the H7N2 type of type A influenza virus. BLAST [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004—[cited 25 18 04]. Available from: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Note that this is an automatic prediction, and you need to judge if the suggestions would be useful in real PCR tests. Among the unintended targets (see also Fig. 5.7), the forward primer is matching, and mismatches are only observed on the reverse primers and even for them it is questionable if they will actually not lead to PCR amplification in a real-time PCR since the C-T mismatch in the 5′ is end is expected to result in amplification (Fig. 5.4).

Further Reading

Introduction to practical work with PCR as well to the historical background is found in Sambrook and Russell (2001):

Sambrook and Russell (2001) Molecular Cloning. CSHL Press, A laboratory manual

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Introduction to Phylogenetic Analysis of Molecular Sequence Data

6

Henrico Christensen and John Elmer Dahl Olsen

Contents

H. Christensen (✉) · J. E. D. Olsen
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; jeo@sund.ku.dk

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What You Will Learn in This Chapter

You will learn how to read a phylogenetic tree and evaluate the nature of the data that are suitable for phylogenetic analysis. The models required for phylogenetic analysis such as the substitution matrix, the tree shape, and the weights that can be put on different positions in the multiple alignments are introduced. You are then presented for the different types of phylogenetic methods in order to learn their basic principles and the program packages from where they can be used. You are guided to evaluate the strengths of phylogenetic trees and to optimize model parameters. In the activity, you will learn how to construct a neighbor joining phylogenetic tree on your own computer.

6.1 Background

Phylogeny (*Phylo-* from Greek, family, tribe; *-geny*, ancestry) has become one of the most fundamental bioinformatical tools. The definition of phylogeny is that it is a model of the relationships between organisms, genes, proteins, or other structures based on common ancestry. Lamarck (1809) was first to present an evolutionary tree. The tree was based on bifurcating lines connecting different invertebrate animal groups as well as vertebrates such as fish, birds, mammals, and subdivisions within the mammals. Darwin (1859) used a hierarchical treelike structure to illustrate common ancestry among organisms, alternating processes of variation and selection, and geological stratification of organisms. The hierarchical tree and Darwin's conceptual "tree of life" were used by Häckel (1875) graphically to illustrate the relationships between organisms. Hennig (1950, 1966) developed concepts statistically to analyze biological data in a phylogenetic perspective.

Phylogenetic analysis will only make sense if the characters compared are homologous. DNA and protein sequences are homologous if they share common ancestry. The justification for homology based on DNA sequence data comparison has promoted phylogenetic analysis tremendously. Other reasons to the frequent use of phylogenetic analysis are the easy access to molecular sequencing and access to sequence data through the internet and that hierarchical data representation and dichotomies fit well into construction of computer programs.

Phylogeny *sensu stricto* is dealing with the ancestral relationships of species. Currently, "phylogeny" is also used about numerous biological relationships between populations, genes, and groups of organisms. Data expected to include phylogenetic information are DNA or protein sequences of homologous genes and morphological characters shown to be homologous, while most restriction patterns (PFGE, AFLP, ribotyping) and most DNA hybridization data (microarray, Southern blot, etc.) are data with dubious phylogenetic information. The term phylogenomic analysis covers comparison between full genomic sequences by phylogenetic analysis. Phylogenomics has also been used to predict the function of uncharacterized genes based on genes with known functions (Eisen 1998); however, the term will not be used in that meaning here.

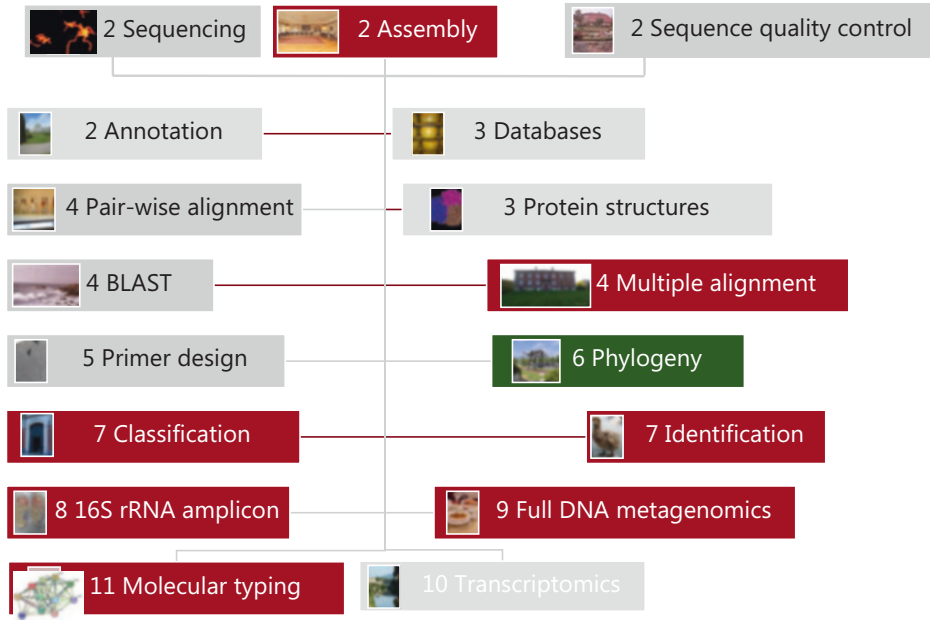

Fig. 6.1 Relation of this chapter to the other chapters in the book. Phylogeny is based on multiple alignments of sequences, and it can be used for the classification. Phylogenetic analysis is included as a tool in many other applications such as 16S rRNA amplicon sequencing, full DNA metagenomics, and molecular typing

Phylogenetic analysis involving molecular sequence comparisons has at least four uses, which are central to a range of downstream bioinformatics analysis (Fig. 6.1):

- Classification (taxonomy)
- Grouping of genes, proteins, and other molecular sequences including noncoding sequences
- Epidemiological investigations
- Analysis of parallel evolution between host and parasite

6.2 Understanding the Phylogenetic Tree

In its most simple form, a phylogenetic tree is represented by straight lines joined as bifurcations. The free ends of lines represent observed sequences (or any other character compared), and the junctions between lines (nodes) represent the hypothetical ancestors for the observed sequences (Fig. 6.2). The lengths of lines to each sequence reflect sequence divergence; however, they can also be of equal length and only show the relationships between species.

The tree can either be on a radial form or on a dendrogram form (Fig. 6.3). On the dendrogram form, all lines from the radial tree are represented vertically, and the horizon-

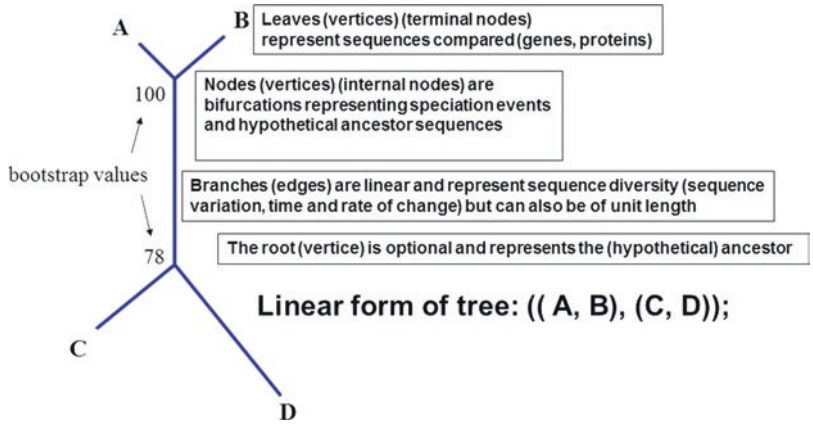

Fig. 6.2 Phylogeny linked to its graphic representation the "tree." A, B, C, and D represent some kinds of homologous character that can be compared. In the current context of bioinformatics, they can be DNA or protein sequences. The linear formula for the tree shown below is in Newick format (named after http://www.newicks.com/). This is the simplest way to instruct a computer program to handle a phylogenetic tree

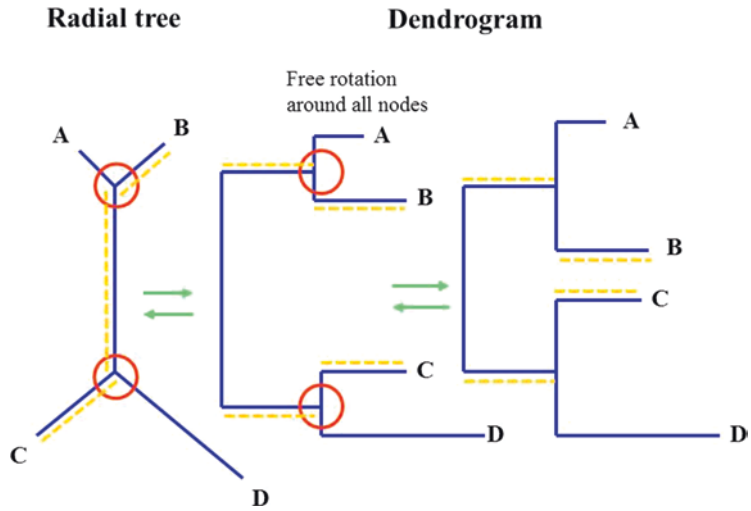

Fig. 6.3 Illustration of free rotation around nodes and visualization as radial tree or dendrogram. All three unrooted trees show exactly the same information. The branch lengths are proportional to sequence divergence or other similar characters. In the radial tree, all branch lengths are related to the sequence diversity. In the dendrogram, it is only the horizontal lines that are related to sequence divergence. The vertical lines are inserted to show how the horizontal branches are related. The calculation of the distance from A to C is illustrated by the stippled orange line, and it is seen that only the lengths of the horizontal lines counts in the dendrogram

tal lines included have the purpose only of showing the relationships between the horizontal branches. A radial tree can always be represented as a dendrogram with the same information and vice versa (Fig. 6.3).

To get the most obvious information out of a dendrogram, it needs to be rooted with the out-group. The out-group is a branch selected to be unrelated to all other branches of the tree meaning that it is a branch with a free end, which is remarkable longer than the other branches. The root is selected as the vertical line at the most far left of the tree. The root is selected as the starting point of the dendrogram (Fig. 6.4). In the example shown on Fig. 6.4, the internal branch is rotated 180°, which will bring the longest branch to the root.

In the strict sense, a root is deliberately selected as a branch with a free end without any label (see Figs. 6.5 and 6.6); however, in practice, all methods demonstrated in the following are producing unrooted trees, and it will not make sense further to consider true rooted trees in this chapter.

The most important information that can be read from a phylogenetic tree is the location of monophyletic groups (Fig. 6.5). A monophyletic group (clade) is characterized by common descent of all members (at least two) and by all members sharing a common branch. A phylogenetic tree contains many different monophyletic groups. Only some will have biological meaning. Paraphyletic groups have two ancestors, and groups are poly-

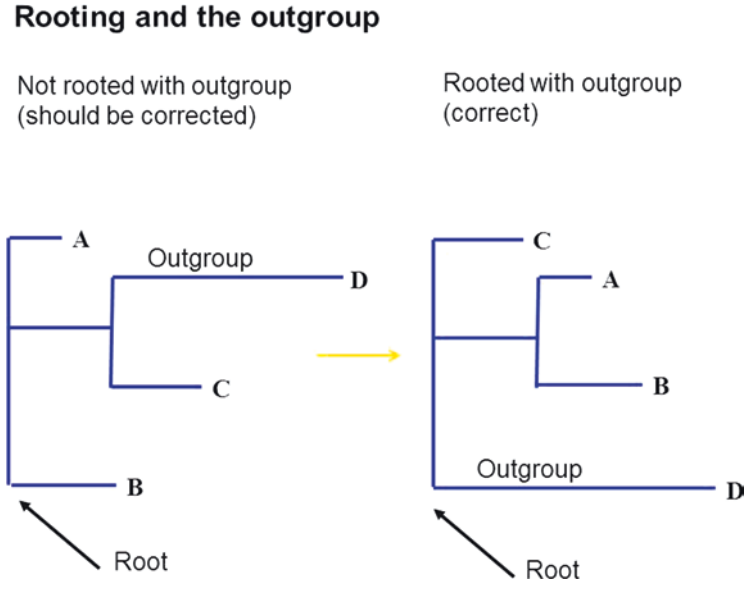

Fig. 6.4 Illustration of rooting with the out-group. The internal branch is mirrored placing the longest branch leading to D at the root. Note that the information obtained from the tree before and after rooting is the same. The rooting with the out-group is only performed to improve the reading of the tree

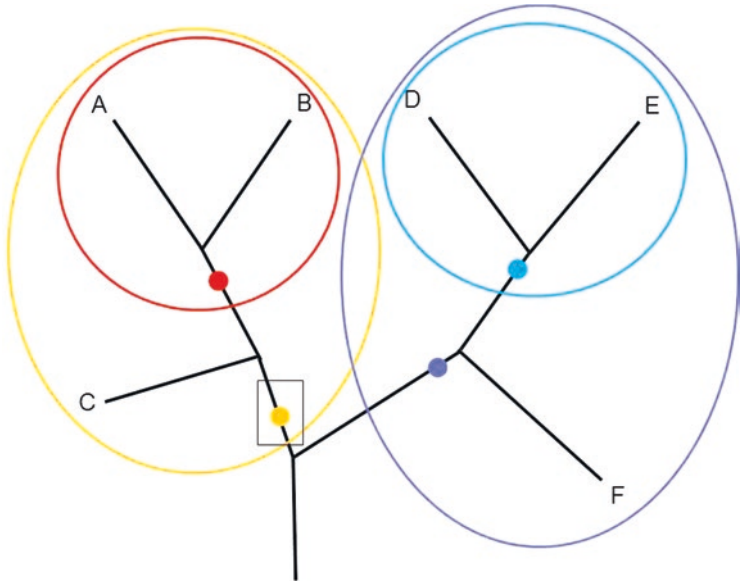

Fig. 6.5 Definition of monophyletic groups (clades). A monophyletic group (clade) is characterized by common descent of all members (at least two) and by all members sharing one and only common branch dot). This tree includes four monophyletic groups marked in different colors and for each with the common branch in the same color. Note that this tree is rooted. The monophyletic group concept applies both to rooted and unrooted trees

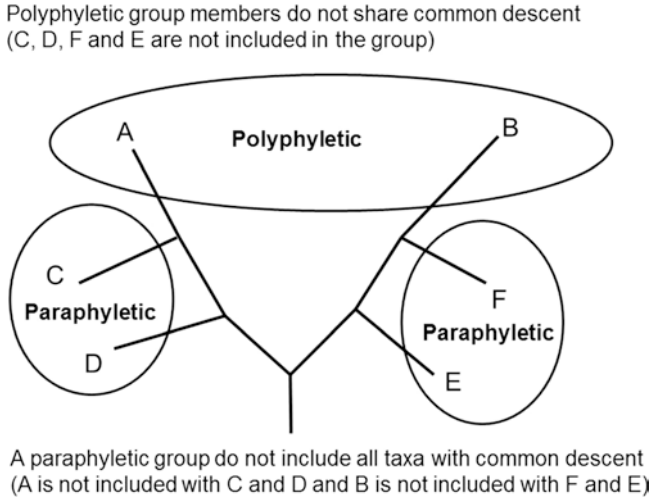

Fig. 6.6 Para- and polyphyletic trees. Note that this tree is rooted. The para- and polyphyletic group concepts apply both to rooted and unrooted trees. Like for the monophyletic groups in Fig. 6.5, many para- and polyphyletic groups can be identified in a phylogenetic tree; however, only a few examples are given above since these groups are less interesting compared to the monophyletic

phyletic if they have more than two ancestors. Ancestral taxa tend to be paraphyletic since they are very difficult to resolve. Polyphyletic groups are without a common ancestor, and polyphyletic groups are normally just considered for their negative nature.

6.3 Assumptions About Data in Order to Perform Phylogenetic Analysis

A list of nine conditions can be made about the demands to data to make a phylogenetic tree (Table 6.1). All nine assumptions will probably never be satisfied, or it cannot be tested if they all are satisfied. The two first about homology and multiple alignment are obligatory. It will only make sense to compare homologous sequences based on a good multiple alignment.

If some conditions cannot be met in order to construct a phylogeny a network and be made. A network is characterized by trifurcations instead of bifurcations. The program,

Table 6.1 Assumptions about data

No.	Assumption	Tests
1	Sequences compared are homologous	The homology of molecules can normally be verified by high scoring pairwise alignments, for example, by BLAST or by analysis of shared domains in relation to proteins
2	Positional homology can be established	Sequences can be aligned (multiple alignment)
3	Sequences have been selected in a way that they are representative for the units they are meant to represent (species, genes, proteins)	More sampling with stratified representation of groups at the same level should give the same result
4	Independent sampling of the units in 3. such as species and genes have been performed	Better stratified representation of groups at the same level should give the same result
5	Sequences compared have evolved independently whether they represent species, genes, or proteins	Comparison of other related species, genes or proteins should give the same result
6	Nucleotide or amino acids positions have evolved independently	Comparison of other related genes should give the same result
7	Events are rare	Comparison of more conserved sequences or conserved regions of sequences compared should give the same result
8	Same rate of evolution in all sequences (some methods)	Further testing of model parameters
9	Long-branch attraction can be handled (some methods). Long-branch attraction is if the phylogeny includes very short and very long branches, then the risk for the methods to reverse long branches on short branches increases	Reduced mixing of short and long branches should give the same result

SplitsTrees (Huson 1998) (https://software-ab.cs.uni-tuebingen.de/download/splitstree4/welcome.html), can be used to analyze the data. It is used on data that are related by a network. If a tree of the seven species A-G is compared and a "split" between F and B evaluated: ACDF|BEG: "to satisfy a phylogeny the distances across the split should be pairwise higher than on each side if this is not possible it must be a network." A weakness of the method is that it is distance matrix-based and cannot account for all different positions of the alignment individually. The main reason to form network structures is weakly defined, but some are related to horizontal gene transfer (HGT). Horizontal gene transfer is a violation of condition 3 in Table 6.1. HGT will be reflected in a network structure, but a network structure will not always have been caused by HGT. PADRE is another tool for analysis of reticulate evolution (https://www.uea.ac.uk/groups-and-centres/computational-biology/software/padre) (Lott et al. 2009)

6.4 Phylogenetic Model Parameters

6.4.1 The Tree Structure

When we see a tree, we often take it for granted. However, in phylogenetic analysis, the tree is only one out of many model parameters that we try to optimize given our data—the multiple alignment. We want to identify the tree that best reflects our data. For some phylogenetic methods, this means we can test different trees against our data. It is shown in the right part of the model on Fig. 6.7. There are two problems with the statistical analysis of tree structures. The first is that the statistical distribution of phylogenetic trees is very complex and cannot be compared to known statistical distributions like the normal or gamma distributions. Another problem is that there are unrealistic high numbers of trees to compare (Felsenstein 2004). The solution most often chosen is heuristic tree search where only the trees with the highest probability to represent the data are compared.

6.4.2 Substitution Matrix and Evolutionary Models

The probability of changing one nucleic acid or one amino acid to another is defined by comparing the actual data to a substitution matrix. For this reason, the substitution matrix should be selected to represent the data closely. Substitution matrices were described in Chap. 4. For DNA, an equal probability for exchange of nucleotides is assumed by the Jukes and Cantor matrix, whereas compensation for a transition/transversion bias is possible by other substitutions models. For protein, the Point Accepted Mutations (PAM) matrix is most suitable for evolutionary and phylogenetic analysis. With maximum parsimony and maximum likelihood methods described below, the substitution matrix is incor-

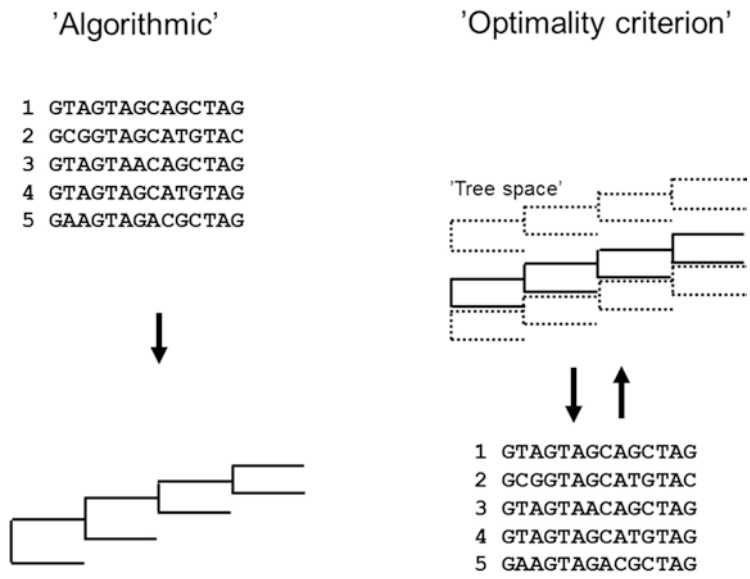

Fig. 6.7 Phylogenetic methods. To make a tree from the multiple alignment or to fit a tree to the multiple alignment

porated into the methods, whereas for the distance matrix/neighbor joining method, calculation of the distance matrix is always the first step later followed by the construction of the phylogenetic tree.

6.4.3 Weighting of Characters

The weighting of characters is sometimes included as an additional model parameter. Weighting of characters is done with the different columns of the multiple alignment, and such weighting is performed to place less weight on characters or nucleotides or amino acids with frequent changes. Different weights w_1, w_2, w_3, …, w_n are placed on n positions of the multiple alignment. For example, with codon positions in DNA, the weights w_1, = 3, $w_2 = 4$ and $w_3 = 1$ can be added accounting for higher variation in third codon position compared to first and second.

6.5 Phylogenetic Methods

Algorithmic (numeric) methods such as neighbor joining analysis work by simply "calculating" a tree bases on the data. Only one tree is generated. A specific sequence of steps (algorithm) is defined that leads to the tree (Fig. 6.7). The alternative procedure used in maximum parsimony, maximum likelihood, and Bayesian methods is based on a so-called

optimality criterion. An optimality criterion (described by an objective function) is a procedure for evaluating a given tree in relation to the data (Fig. 6.7).

The interpretation of a phylogeny according to the second approach is not always unequivocal. Especially, for complex phylogenies, many trees can be considered to reflect the phylogeny given the data. The trees accepted according to certain statistical limits can be interpreted as the tree "space" given the data (Fig. 6.7).

6.5.1 Maximum Parsimony

The maximum parsimony tree represents the minimum number of nucleotide or amino acid changes given the data (multiple alignment) (Table 6.2). This method was the first phylogenetic method to be used with molecular sequences (Eck and Dayhoff 1966). Two fundamentally different ways of comparing data by parsimony exist. One is based on the separation of data into shared and derived characters and use of only the derived common characters (synapomorphs) for parsimony analysis (Hennig 1966). This procedure cannot

Table 6.2 Phylogenetic methods used with molecular sequence data

Methods	Principle	Benefit	Drawback	Use and limitations	Options for extension
Neighbor Joining	Algorithmic	Simple and fast in relation to computational power	Only one tree	Routine draft phylogeny	Bootstrap
Maximum parsimony	Optimality criteria	Includes all informative positions of alignment and more trees are compared based on statistical criterion	More trees can be "equally parsimonious"	Routine phylogeny of closely related sequences	Bootstrap
Maximum likelihood	Optimality criteria	Includes all informative positions of alignment and more trees are compared based on statistical and probabilistic (likelihood criterion)	With complex phylogenies the "maximum likelihood" tree will never be found	Routine phylogeny with fewer sequences	Bootstrap and likelihood ratio tests
Bayesian (Mr. Bayes)	Optimality criteria	Includes all informative positions of alignment and more trees are compared based on statistical and probabilistic (Bayesian) criterion)	Very complex phylogenies are not fully resolved	For complex phylogenies but not too complex	

be used with molecular sequence data because differences between ancestral and derived changed are normally unknown. The alternative choice with these data is to relax initial assumptions about the direction of character changes.

The most strict parsimony method is Camin snd Sokal (1965) parsimony. The ancestral state must be known, and only single character changes in one direction are allowed. The method can be used to make a phylogeny of small DNA deletions that are not assumed to revert but cannot be used with sequences per se. Dollo parsimony refers to Dollo's law: "A complex character once attained cannot be attained in that form again" (evolution is irreversible). Only unidirectional changes are allowed although reversals are possible but minimized. This method can be used with restriction sites in DNA but not sequences. The Fitch-Wagner algorithm allows fully reversible changes and for this reason can be used with sequences. It is implemented as DNAPARS and DNAPROT in the PHYLIP package (see below Tables 6.2 and 6.3) as well as other programs.

Most parsimony methods work by "post order tree traversal" meaning that a tree is evaluated in relation to the data starting with the branch tips and moving toward the base through all nodes.

6.5.2 Distance Matrix/Neighbor Joining

The first distance matrix method was published by Fitch and Margoliash (1967). Neighbor joining (Saito and Nei 1987) belongs to the clustering methods originally developed for numeric taxonomy (Sneath and Sokal 1973) (Table 6.3). It is now one of the most simple and most frequently used routine methods. First, a distance matrix is calculated based on pairwise comparison of all sequences with each other. Then the neighbor joining algorithm constructs the tree. Clustering by the neighbor joining algorithm does not need a molecular clock (ultra-metric data). Neighbor joining analysis is used for draft phylogenies. Neighbor joining analysis can be performed directly from ClustalX (see below in Activity 6.9.1).

6.5.3 Maximum Likelihood

The likelihood is the probability of the parameters (tree structure, substitution model) given the data (multiple alignment), and the tree is this way part of the model maximizing the likelihood in relation to data (Tables 6.2 and 6.3). Maximum likelihood analysis of phylogeny was first proposed by Cavalli-Sforza and Edwards (1967). It was later used on molecular sequences by Joseph Felsenstein and implemented in the PHYLIP package as the DNAML program. An extension including many scripts is available as the fastDNAml program (Olsen et al. 1994) (https://www.life.illinois.edu/gary/programs/fastDNAml/) (Table 6.3).

Table 6.3 Most commonly used programs and program packages

Purpose	Program/ package	URL, references and notes
Neighbor joining, maximum parsimony and others	PHYLIP	http://evolution.genetics.washington.edu/phylip.html The use of the package is free with open source code. PC version for use with DOS is available. The programs on the package can also be used from server
Maximum likelihood and maximum likelihood ratio test	fastDNAml	https://www.life.illinois.edu/gary/programs/fastDNAml/ Olsen et al. (1994)
Maximum likelihood and maximum likelihood ratio test	PhyML	http://www.atgc-montpellier.fr/phyml/ Guindon et al. (2010)
Neighbor joining, maximum parsimony, maximum likelihood	MEGA11	https://www.megasoftware.net/ (Tamura et al. 2021). For simple analysis of phylogeny and population genetics, the use of MEGA is described in the book of Nei and Kumar (2000). Older versions like MEGA7 may be more suitable for certain applications than the most recent.
Maximum parsimony advanced and others	PAUP	http://paup.phylosolutions.com/get-paup/ Source code hidden but see DNAPARS and PROTPARS in PHYLIP. Runs best on MAC. Without preliminary knowledge, the most easy way to start using PAUP is to modify one of the examples included when the program is downloaded
Maximum likelihood advanced applications linked to the	PAML	http://abacus.gene.ucl.ac.uk/software/paml.html
specialized population genetics program	HyPhy	http://hyphy.org/w/index.php/Download
Maximum likelihood with large datasets	FastTree	http://www.microbesonline.org/fasttree/ Price et al. (2010)
	RAxML	https://github.com/stamatak/standard-RAxML Stamatakis (2014)
Interactive Tree of life, an online tool for phylogenetic tree display and ab	iTOL	(https://itol.embl.de/) Letunic and Bork (2007)
Tree modifications	Macclade	http://macclade.org/macclade.html

For a comprehensive list of all packages and programs, see (https://evolution.genetics.washington.edu/phylip/software.html)

For up to approximately 25 sequences, it is possible for the tree search algorithm to find the tree that will give the highest lnL. The can be verified by the loop script with fastDNAml. For more sequences, it becomes difficult to justify if the tree with the highest lnL is achieved. One solution is to accept a cloud of trees with likelihood above a certain

threshold. Another way to come around the problem is to use Baysian inference (Sect. 6.5.4).

The easiest way to run maximum likelihood analysis is to use the PhyML program implemented on the server at http://www.atgc-montpellier.fr/phyml/ (Guindon et al. 2010). RAxML is another implementation of maximum likelihood found at https://github.com/stamatak/standard-RAxML (Stamatakis 2014). For large datasets up to a million of sequences, FastTree (Price et al. 2010) (Table 6.3) can be used. It infers approximately maximum likelihood phylogenetic tress from multiple alignments of DNA or protein sequences.

6.5.4 Bayesian (Mr. Bayes) Inference of Phylogeny

This method is recommended to complex phylogenies not resolved fully by maximum likelihood (Table 6.2). The method is based on the Bayesian statistical principle and is nicknamed Mr. Bayes. The method starts with the best guess of a tree to calculate the prior probability. Trees are simulated by Markov Chain Monte Carlo (MCMC), and all the best trees are kept. The posterior probabilities for the branches in all these best trees are then written on to the final tree. These numbers are true probabilities in a statistical sense saying, for instance, that the branch leading to taxon 1 has 93% probability. The program is available from http://mrbayes.sourceforge.net/. At this website, a very good and comprehensive manual can found (Table 6.3). The installation and execution of the program is not always running so smoothly related to conflicts between versions of the program and versions of Windows. To solve this and similar problems in bioinformatics, great patience is needed.

6.6 Comparison of Phylogenetic Methods

Phylogenies are in most cases hypothesis about evolution, and appropriate approaches to test phylogenetic methods are few. One can either use simulated data to define a phylogeny and then compare methods against this unbiased control, or one can use real evolution of organisms with fast evolution as, for example, virus. Exact phylogenies for organisms with really well-defined palaeontologic evidence may also be used.

A phylogenetic method is consistent for an evolutionary model if the results obtained by the method converge on the correct tree as the data become infinite. A phylogenetic method has high efficiency if it quickly converges on the correct solution as more data are applied to the problem. A phylogenetic method is robust if it converges on the correct solution even with violations of the assumptions about the evolutionary model (Hillis 1995). Given the assumptions about data have been optimally handled (Table 6.1), we will of course prefer a phylogenetic method, which is consistent, efficient, and robust.

Identical sequences should be avoided since it will only extent the time of computation and not add any new information to the analysis. For complex phylogenies, all taxa should be included since it will be very difficult to judge which ones to exclude. A symmetric topology is said to indicate a balance between speciation and extinction where as a more comp-like topology should indicate either very high speciation or very high extinction rates.

Probably, mainly for Prokaryotes, a standard operating procedure for phylogenetic inference (SOPPI) using rRNA marker genes has been described by Peplies et al. (2008).

6.6.1 Bootstrap

Bootstrap analysis is a permutation test. The aim of this tests is to show how well supported the branches are by the data. The bootstrap estimates the sampling distribution of a group of sequences by generating new samples by drawing with replacement from the original data (Fig. 6.8). The alternative jackknife analysis computes the statistic of interest

The original data are simulated by drawing columns randomly with replacement 100 times (or a higher number). The phylogenetic analysis is repeated for each of the simulated multiple alignments and the number of branched common in all 100 trees summarized on the original tree

Original data	1 replicate	2 replicate	3 replicate
Species 1	AGGA	AAGA	GGAA
Species 2	ACGT	AACT	CGTT
Species 3	ACGT	AACT	CGTT
Species 4	ACTT	AACT	CTTT
Species 5	CCGT	CCCT	CGTT
linear form	(2,3)4)5)1;	(2,3)4)5)1;	(2,3)5)4)1;

All 100 trees are compared and the frequency of the same branches in the simulated tree are labelled on to the original tree

Fig. 6.8 A simple example with five short sequences (species) of only four nucleotides in length to show how bootstrap analysis works. Three replicates of the original multiple alignments are shown (the original multiple alignment is not shown). The simulated multiple alignments are each constructed by drawing (with replacement) one column at a time from the original multiple alignments. For each simulated multiple alignments, a phylogenetic tree has been constructed. The frequency that each branch occurs in the simulated trees is the bootstrap value. The bootstrap value can then labeled on the same branch in the original tree

for all combinations of the data where one (or more) of the original data points is removed. It will not be further described here.

For the bootstrap, the original multiple alignment data are simulated by drawing columns randomly with replacement 100 or 1000 times. The phylogenetic analysis is repeated for each of the 100 or 1000 simulated alignments (Fig. 6.8) and the number of branches common in all 100 or 1000 trees summarized in a consensus tree. These values are then compared to and written on the original tree.

The result of the bootstrap is a support for a particular internal branch in the original tree. As a consequence, the out-group receives no bootstrap since it is not an internal branch but a terminal. If reviewers who are not familiar with phylogenetic analysis request a bootstrap value to the out-group, you can use two out-group taxa, then their common branch will receive an out-group. Unfortunately, this contradicts the general rule that there should be one and only one out-group of appropriate distance and relatedness to the taxa investigated for monophyly. The bootstrap values cannot be evaluated in a strictly probabilistic sense since it is just a parameter indicating the robustness of the branches in the tree. Bootstrap values at 90% or higher are very "good," those within 50–89% are acceptable, and those below 50% are not considered. Some branches may not receive a bootstrap value at all. This can happen if this branch has not been simulated in the permutation test. With closely related sequence, another problem can be rather low bootstrap values simply because the simulating imposes random noise into the data.

6.7 Whole Genome Phylogeny

6.7.1 Core Phylogeny

The core genome phylogeny can be constructed using the Linux program Roary to identify core genes of the genomes to be compared. The core genes are then used for construction of a phylogenetic tree. The analysis is run on the output from annotation in gff format, which can be obtained from the annotation program Prokka, and the command will look like: "roary *.gff." Additional options to add can be:

-e -n "create a multiFASTA alignment of core genes",
-p 20 "number of threads, here we choose 20"
-f "output directory name"
-r "makes R plots"

If the annotation files are in a common directory, the command can typically be run there. The analysis will require the installation of Prokka for annotation and Roary in a Linux environment and requires some more skills compared to most of the other tools described in the book.

6.7.2 Genome Distance Phylogeny

The GGDC distances described in Chap. 7 for classification and identification can be used for phylogenetic inference (Henz et al. 2005). In principle, this is a distance matrix that can be analyzed by neighbor joining or similar algorithm described in the previous. This strategy is most suitable for closely related organisms such as species.

SNP-based phylogeny has been described in Sect. 11.3.2 since it is the most relevant for closely related strains to be compared.

6.8 Data Formats

The most common format is the classical PHYLIP format:

Example of PHYLIP interleaved format:

```
 4 20
Species001aggcgctagc
Species002agtagctagc
Species003agtccctagc
Species004agtcgttagc
aggcgctagc
aggcgctagc
aggcgctagc
aggcgctagc
```

On the first line are a number of species (four in this example) and sequence length (20 in this example). On the next line, the sequences are listed. Each sequence starts with a name no longer than ten characters. The sequence starts on position 11. Longer sequences continue in blocks separated by a blank line-shift but without the species name. In the classic PHYLIP format, all blank spaces after position 11 should be removed. The alternative is PHYLIP, non-interleaved where sequence after sequence is listed with line breaks. Remember to add "I" on the first line.

Example of PHYLIP non-interleaved:

```
4 20 I
Species001aggcgctagc
aggcgctagc
Species002agtagctagc
aggcgctagc
Species003agtccctagc
aggcgctagc
Species004agtcgttagc
aggcgctagc
```

For more advanced use of the PHYLIP format, different options in the form of a capital letter can be added on the first line and further instructions given on the second. For example, J for jumple on first line changes the order that the lines in the alignment are read into the phylogenetic analysis.

Another common format is the NEXUS format originally used with PAUP (Table 6.3) but now also required for many other phylogeny programs including Mr. Bayes.

The format looks like this:

```
    #NEXUS
    BEGIN DATA;
    dimensions ntax=11 nchar=1495;
    format datatype=dna interleave=yes gap=-;
    matrix
    HinfluNPxx
AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTA
ACACATG
    HinflKW20
AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTT
AACACATG
```

If there are problems getting a "new" phylogeny program to work, one can first try to match an implementation of the program to the correct operating system (Windows, Mac, Linux). The next step will be to look at the data format and try to optimize. It is always a good idea to run a small test dataset and even better to run example datasets included with the program. Two main problems often realized with data formats are that the output of the multiple alignments is interleaved, but the programs only read non-interleaved and the other that the output of multiple alignments is with line-breaks but the programs only works without line breaks. Different solutions can be tried. In ClustalW/X, it is possible to specify different output formats. For instance, in ClustalX, this can be specified under **Alignment** (top menu bar) and then selecting **Output Format Options**. Here is Clustal shown as default; however, for phylogenetic applications, PHYLIP, NEXUS, and FASTA are also relevant.

The final hard way is "manual" editing. Search and replace (space with nothing) from text editor. Lines can be joined with the UNIX text editor, vi. If these procedures are to be used more frequently, it should be considered to write scripts for editing.

6.9 Phylogenetic Program Packages

Several hundreds of packages are available, but only a few are widely used (Table 6.3). The classical package, PHYLIP, has been used for decades. It includes programs for variants of parsimony analysis, distance matrix analysis, tools to make consensus trees, and maximum likelihood analysis. For more advance, maximum likelihood analysis packets like fastDNAml, PhyML, FastTree, and RAxML are available. PAUP is the classical pack-

age for parsimony analysis, and MEGA11 can both perform the main phylogenetic analysis and analysis needed in population genetics.

6.10 Activities

6.10.1 Neighbor Joining Phylogeny

Install ClustalX on your PC from (http://www.clustal.org/download/current/).

download clustalx-2.1-win.msi, install and locate the icon to the desktop.

Mac users should use the big file (12 Mb).

Do multiple alignments with **ClustalX** (if not already done in Chap. 4).

Open the program by double-click on the icon.

File | Load sequences and select the sequences in FASTA format from the location on your computer. The sequences that you want to use as input should be in one file only with all sequences in FASTA format:

>sequence1.
ATGACGATAC…
>sequence2.
GATAGATAGACS…
etc.

Alignment | Do complete Alignment | ok.

Choose Trees | Draw Tree and then **Trees | Bootstrap N-J tree**. In both cases, confirm by "ok" if output files are stored where you expect. The output is automatically saved in the same folder as the input file, and this folder should have write access (if you define 1000 bootstrap replicates (default) the output will be numbers of 1000 and not %).

To draw the tree, you need to install **MEGA11**.

From https://www.megasoftware.net/, press **Download** at **Windows** (or what operating system you prefer). Write the required information and download to PC and install the program and locate the icon to the desktop.

Open **MEGA11** by the icon.

User Tree | Display Newick Trees browse to the directory where you stored the ClustalX input/output and choose the file with extension "***.phb**" (or any other extension). At this step, the tree should be graphically presented with bootstrap values.

Mark "**Place root on branch**" tool to the left and select an out-group.

Manipulate the tree by "**Swap**" or "**Flip**" to root the tree with the out-group and to bring the rest of tree in shape. Often, rotation around a branch horizontally can give better order. It is also possible to adjust the graphics such as line thickness and fonts in **View | Options**.

Save the tree from **Image | Save as EMF**. The tree can be inserted into PowerPoint or Word by **Insert | Picture** and select the file in emf-format. In PowerPoint, you can manipulate text and lines in the tree further: mark the picture with the tree inserted, right click

and select ungroup and then again ungroup and then you should be able to move text boxes and lines as well as to edit then text.

Note In the example above, ClustalX is used for multiple alignments and construction of the tree, whereas MEGA11 is only used to construct the tree. MEGA11 can also be used for the whole analysis including multiple alignment and tree construction.

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Sequence-Based Classification and Identification

7

Henrik Christensen and John Elmerdahl Olsen

Contents

H. Christensen (✉) · J. E. Olsen
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; jeo@sund.ku.dk

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What You Will Learn in This Chapter

You will be introduced to the classification of prokaryotes from species to the higher levels of class and phyla. You will learn that the 16S rRNA gene sequence comparison including phylogenetic analysis provides the main information for classification. DNA–DNA hybridization predicted in silico from whole genomic sequences is mainly used for classification of species. Now, whole genomic sequences (WGS) are increasingly used for classification and identification. In the activity, you will learn to identify an isolate by 16S rRNA sequence in the EzBioCloud server and by whole genomic sequencing in Type Strain Genome Server (TYGS).

7.1 Introduction

Bioinformatics is needed for classification and identification since all prokaryotes have been classified based on the phylogeny of the 16S rRNA gene sequence. This gene sequence has also to great extent been the golden standard for identification. Now, whole genomic sequences (WGS) are increasingly used for classification and identification. The analysis of 16S rRNA gene sequences and also WGS sequences involves bioinformatics described in many other chapters of this book (Fig. 7.1).

The phenotypic characteristics have for more than a century been used for classification. New species still need to be documented with differences to existing species. Attempts are being made to predict the phenotype from the WGS. Unfortunately, only few phenotypic characteristics can be precisely predicted from the gene sequences.

Identification is part of taxonomy that associates a bacterial strain with a species name (Fig. 7.2). The traditional phenotypic biochemical and physiological methods used for identification of bacteria have serious limitations with respect to accuracy, and they are very time-consuming. Although fast high-throughput versions of these methods are available, there are still problems with the accurate identification of prokaryotes including some human pathogens and food contaminants. Especially prokaryotes of veterinary importance have been underrepresented in the databases of the fast phenotypic identification systems.

The comparison of 16S rRNA gene sequences has been the first choice to identify isolates that are problematic to identify by other means. Other gene sequences, for instance, of the *rpoB* gene have often been the choice to identify problematic isolates belonging to species with narrow 16S rRNA gene sequence relationships (Adékambi et al. 2009; Korczak et al. 2004; Case et al. 2007). Now, WGS is increasingly used for identification, for example, TYGS (see Sect. 7.2.2.2).

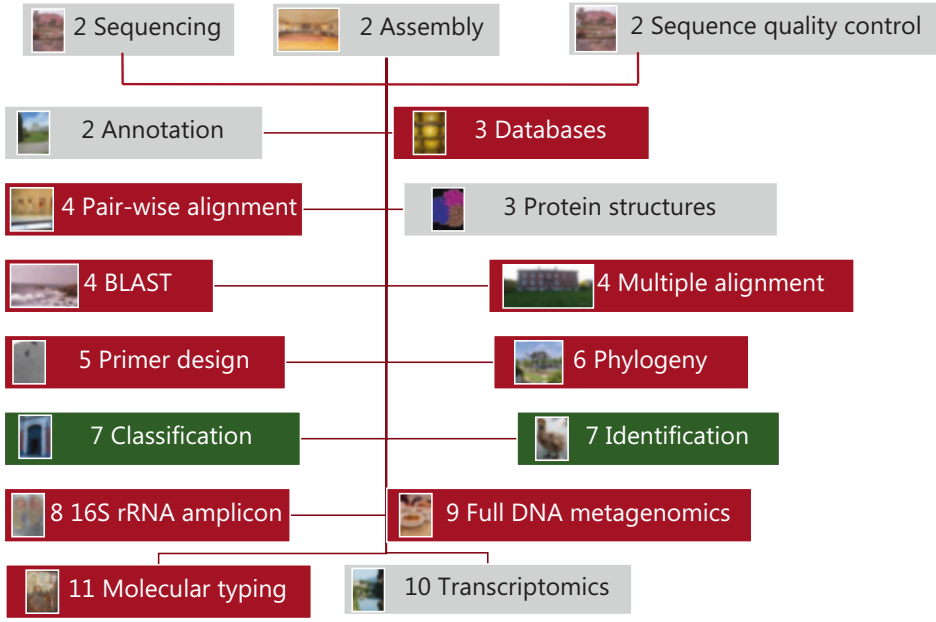

Fig. 7.1 Relationships between sequence-based classification and identification and the other bioinformatics disciplines. DNA sequence-based classification involves assembly of sequences, multiple alignments, phylogeny, and search in the databases. 16S rRNA-based identification also involves 16S rRNA amplicon sequencing described in Chap. 8. Full DNA metagenomics both include information from the 16S rRNA gene sequence and from all other mainly coding genes

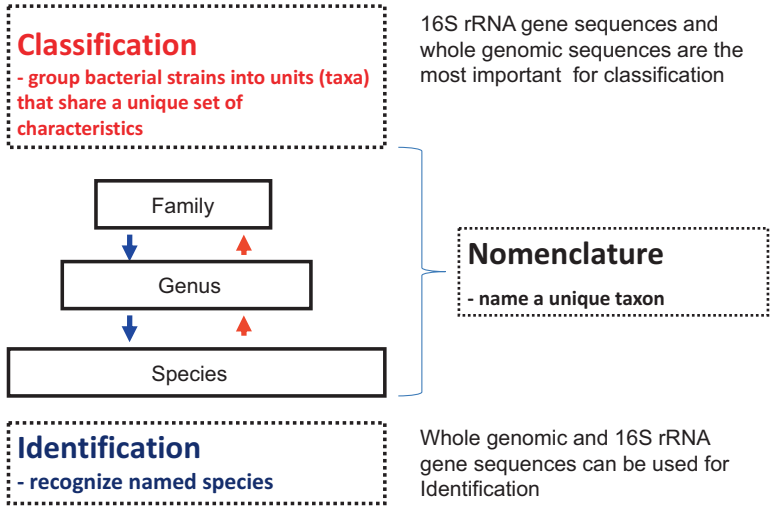

Fig. 7.2 Taxonomy of prokaryotes

7.2 Classification of Prokaryotes

7.2.1 Classification Based on 16S rRNA Gene Sequence Comparison

16S rRNA sequence comparison is the primary character to classify bacteria at species and genus level as well as into the higher taxonomic categories (family, order, class, phylum). The reason for the choice of the 16S rRNA gene is that it forms the backbone of the ribosomes, and ribosomes are of only one type in all prokaryotes and homologous to 18S rRNA in the rest. The comparison of the 16S rRNA sequence can therefore be used to model the natural relationships and evolution of bacteria. rRNA can be sequenced on gene level (DNA) and allows separation of all bacteria to genus and (species) level. For most, there is less than 97% 16S rRNA gene sequence similarity between species and less than 95% 16S rRNA gene sequence similarity between genera. Some species are closely related than 97% 16S rRNA gene sequence similarity, and for them, other genetic methods such as DNA-DNA hybridization need to be performed to confirm species status in regard to classification.

The traditional starting point for 16S rRNA gene sequencing is a colony of an isolate from an agar plate transferred under sterile conditions to the tube with the PCR mix. For some bacteria, DNA needs to be purified before the PCR. The traditional starting point is PCR amplification of the 16S rRNA gene with conserved primers that in principle can be used for all bacteria (Table 7.1). Members of Archaea are so different in their 16S rRNA gene sequences because they need other primer sets. Some of the PCR product is used for agarose gel electrophoresis to confirm that a PCR product has been generated and that fragments are of expected size (Table 7.1). The rest of the DNA in the PCR tube is purified to reduce the content of PCR primers and send to a sequencing company with the sequencing primers (Table 7.1) (Edwards et al. 1989), or you can do it yourself if equipment is available. The DNA sequence can be assembled using relevant computer program (see Chap. 2). It is important to assemble the consensus sequence from reads made both by forward and reverse sequencing primers. When the consensus sequence is generated, it is stored in text format and used as a query for comparison to all "subjects" in a database and be used further for multiple alignments and phylogeny (Chaps. 4 and 6). Now, the 16S rRNA gene sequences are more often extracted from the WGS rather than gene sequences determined by traditional Sanger sequencing.

Full-length 16S rRNA sequence can often not be extracted from the genomic sequence if it has not been fully closed. The 16S rRNA gene is in multiple copies, which limit the full assembly of the WGS. Read mapping (Chap. 2) to a known 16S rRNA gene sequence will often be an alternative possibility if the whole 16S rRNA gene sequence cannot be extracted in full. WGS generated by PacBio and Nanopore should provide full-length 16S rRNA gene sequences on the genomic level.

Table 7.1 Primers for 16S rRNA PCR and sequencing (Sanger)

Primer	Application	Sequence 5′–3′	Reference
The classical set for Bacteria			
8–27f[a]	PCR and sequencing	AGAGTTTGATCCTGGCTNAG	Edwards et al. (1989), Weisburg et al. (1991)
1390–1408r	PCR and sequencing	TGACGGGCGGTGTGTACAA	Lane et al. (1985)
518–536r	Sequencing	GTATTACCGCGGCTGCTGG	Lane et al. (1985)
785–805r	Sequencing	GACTACCNGGGTATCTAATCC	Dewhirst et al. (1992)
785–805f	Sequencing	GGATTAGATACCCNGGTAGTC	
Alternatives for bacteria			
5f	PCR and sequencing	TTGGAGAGTTTGATCCTGGCTC	Simmon et al. (2006)
810r	Sequencing	GGCGTGGACTTCCAGGGTATCT	Simmon et al. (2006)
1194f	Sequencing	ACGTCATCCCCACCTTCCTC	Simmon et al. (2006)
rD1r	PCR and sequencing	AAGGAGGTGATCCAGCC	Edwards et al. (1989), Weisburg et al. (1991)
16SUNIr	PCR and sequencing	GTGTGACGGGCGGTGTGTAC	Lane et al. (1985), Kuhnert et al. (2002)
Archaea			
SDArch0333aS15f	PCR and sequencing	TCCAGGCCCTACGGG	Lepp et al. (2004)
SDArch0958aA19r	PCR and sequencing	YCCGGCGTTGAMTCCAATT	Barns et al. (1994), DeLong (1992)
SDArch1378aA20r	PCR and sequencing	TGTGTGCAAGGAGCAGGGAC	Lepp et al. (2004)

[a]Numbering follows *E. coli rrnB* (NCBI/DDBJ/EBI acc. no. J01696); f, forward; r, reverse

7.2.2 From DNA–DNA Hybridization of Total DNA to WGS for Classification

The way a mixture of denatured DNA preparations from two different bacterial strains hybridize can be used to classify the strains at species level. The closer relationship is, the more easily hybridization will be. The degree of DNA–DNA hybridization (DDH) is measured by heating the DNA preparations for two strains to be compared. Heating will denature the double-stranded DNA to single-stranded DNA. Then the DNA preparations are mixed in equal amounts and slowly cooled, and the DNA will then start to re-nature to double-stranded DNA. The DNA of the mixture of the two strains will re-nature less perfectly compared to DNA from the same strain. The DNA–DNA hybridization between strains of the same species will be more than 70%, whereas the DNA–DNA hybridization between strains of the different species will be less than 70%. A DNA–DNA hybridization (DDH) below 70% separates strains of different species (Wayne et al. 1987). DNA–DNA

hybridization or alternative methods are used as a control to classify species when their 16S rRNA gene sequence similarity is higher than 97%. Traditional DNA hybridization is time-consuming and costly, and alternative procedures such as WGS have been used.

WGS comparison has become the new method to estimate DDH. For species level separation of strains, WGS-based comparisons based on overall genome relatedness indices (OGRI) have replaced the laborious DNA–DNA hybridization methodology. The two different tools average nucleotide identity (ANI) and Genome-to-Genome Distance Comparison (GGDC) are both based on different types of basic local alignment search tool (BLAST) (Altschul et al. 1990) or BLAST like calculations.

7.2.2.1 ANI

The use of WGS comparison to separate species was first formulated as ANI (Konstantinidis and Tiedje 2005a, b) and later modified by Goris et al. (2007). For practical applications, an ANI range of 93–96% should "be treated cautiously" (Rosselló-Móra and Amann 2015), and species should only be separated based on this very narrow interval if DDH data are supported by other very stable phenotypic characteristics. Early on ANI was found correlated with traditional DDH with 95% ANI corresponding to 70% DDH for species level separation (Goris et al. 2007). The ANI algorithm is "cutting" a WGS sequence up randomly in fragments of 1020 nt and blasting all fragments of this query to the other subject genome, which is on the assembled form, and then only the pairs with more that 70% coverage and more than 30% identity are considered. For all such pairs, the mean identity is calculated as ANI. Following this approach, ANIs obtained by reversing query and subject WGSs sometimes diverged, and an average was needed. To overcome this hurdle, the ANI variant OrthoANI (Lee et al. 2016) replaced the ANI of Goris et al. (2007). OrthoANI allows simultaneous evaluation of comparisons of sequences in both directions. This is facilitated by randomly cutting both genomes up in 1020 nt segments and performing pairwise identity comparisons between all pairs with more than 35% coverage. The threshold of 35% is set to ascertain that the fragments are homologs. OrthoANI is then calculated as the average of all the identities. The word "ortho" refers in this context to the match obtained between fragments in both genomes. The correlation between ANI and OrthoANI was excellent with only 0.1% higher OrthoANI compared to ANI (Lee et al. 2016; Yoon et al. 2017b). In OrthoANI, BLAST was substituted with the quicker search algorithm USEARCH (Edgar 2010) to increase search speed without losing accuracy. ANI can be performed online from EzBioCloud: https://www.ezbiocloud.net/tools/ani.

JSpecies (http://www.imedea.uib.es/jspecies/download.html) (Richter and Rosello-Mora 2009) has facilities for ANI using both BLAST and Mummer approaches (Table 7.2). The Mummer approach is only for closed genomes. With the BLAST approach, non-closed genomes can be used; however, the use of more than 50% of the genome is recommended.

Table 7.2 Bioinformatics tools to predict DNA–DNA hybridization based on WGS

Name	Content	Reference	URL
JSpecies	ANI, MUMmer	Richter and Rosello-Mora (2009), Goris et al. (2007)	http://imedea.uib-csic.es/jspecies/
	OrthoANIu	Yoon et al. (2017b)	https://www.ezbiocloud.net/tools/ani
GGDC	GGDC	Auch et al. (2010a, b)	http://ggdc.dsmz.de/

ANI can be calculated on EDGAR 3.0 (https://edgar3.computational.bio.uni-giessen.de/cgi-bin/edgar_login.cgi) (Dieckmann et al. 2021). This pipeline also offers tools for many other comparative genomics applications.

7.2.2.2 GGDC

Genome blast distance phylogeny (GBDP) was introduced by Henz et al. (2005) and was further developed to Genome-to-Genome Distance Calculator (GGDC), which is often referred to as digital DNA–DNA hybridization (dDDH). GGDC is mainly based on comparisons of HSPs (short for high scoring sequence pairs, high scoring segment pairs or high speed products), which is a parameter in BLAST. GGDC is used from http://ggdc.dsmz.de/ggdc.php# (Auch et al. 2010a, b; Meier-Kolthoff et al. 2013). The GGDC distances were transformed to DDH by log generalized linear models (Meier-Kolthoff et al. 2013).

WGSs to be compared are uploaded as FASTA format in contigs or as fully closed genomes, and the results are returned by email.

Different formulas are used:

Formula 1: d_0 = 1 – (total HSP length from both genomes/total length of both genomes).
Formula 2: d_4 = 1 – ((2 × sum of identities of base pairs of all HSP pairs)/total HSP length for both genomes).
Formula 3: d_6 = 1 – ((2 × sum of identities of base pairs of all HSP pairs/total length of both genomes).

Formula 1 and Formula 3 include total length of both genomes in the nominator and therefore require the fully closed WGS. This requirement is relaxed with Formula 2, and it can be used with genomes only available on contig form. The program automatically estimates DDH from the three indices d_0, d_4, and d_6 described with the formulas above.

GGDC has recently been complemented with a tool for species level identification, TYGS (https://tygs.dsmz.de/) (Meier-Kolthoff and Göker 2019). In principle, the same formulas are used as with GGDC. The benefit with TYGS is that the query WGS is automatically compared to WGS of all type strains of prokaryotes.

Rounding up, the benefits of ANI and GGDC are evaluations of species relationships based on WGSs. The drawback with both methods is that they are based on shared sequences and that insertions and deletions of genes in principle are not included in the

Table 7.3 Proposed minimal standards for use of genomic taxa for taxonomy of prokaryotes (Chun et al. 2018)

Parameter	Minimum requirements to documentation in scientific publications
GC content	Needs to be stated
Genome size	Needs to be stated
Number of contigs	Needs to be stated, limit depends on taxon
N_{50}	Needs to be stated, limit depends on taxon
Coverage	More than 50
Deposition of sequence	Assembled DNA sequence
Number of conserved genes for phylogeny	More than 30

comparisons. At species level, ANI seems more relaxed than GGDC to link strains to the same species: for example, with *Canicola haemoglobinophilus*, strain CCUG 16472 was linked to the type strain with 94.75% ANI, which indicates species level relationship; however, GGDC was only 57.2%, which is below the species limit of 70% (Christensen et al. 2021). Species of *Streptomyces* share 99.99% ANI (Ciufo et al. 2018) compared to the threshold of 95% ANI for species level separation. For such species, identification will rather become an identification to the strain level, which will be treated in Sect. 7.6.

The GC content is of taxonomic value in the way that species of the same genus normally will not differ more than 5% in GC content. It is standard to determine the GC content for the type strains of the type species of a genus. The GC content can be determined based on the WGS, which is easier and more precise than previous biochemical methods like HPLC or buoyant density centrifugation methods (Kim et al. 2015) (Table 7.3).

Minimal standards have been formulated for the requirements to the quality of WGS to be used for taxonomic studies (Table 7.3). The coverage used to determine the assembled sequence should be a minimum of 50 (Chun et al. 2018). Information about genome size, GC content, number of contigs, and N_{50} need to be provided in publications including this type of information. It is a prerequisite that the assembled genomic sequence is deposited with a public database (Chap. 3).

7.2.3 Classification Based on Core Genome Analysis and Multilocus Sequence Analysis (MLSA)

For classification based on phylogenetic relationships, many conserved genes can be used to complement the 16S rRNA analysis. The concept was introduced with MLST (Chap. 11).

In principle, all shared gene sequences between taxa investigated can be used to construct core gene phylogenies. Recently, certain subsets of the core genes have been published. From EzBioCloud, a set of 92 conserved proteins are recommended for core genome analysis. They include mainly protein involved in translations such as tRNA synthesis as well as ribosomal proteins.

For *Pasteurellaceae*, we have systematically recorded protein sequences present in all species, and currently, a set of 40 conserved protein sequences is used (Christensen and Bisgaard 2018).

Multilocus sequence analysis (MLSA) is based on DNA sequences of conserved genes, which are then concatenated meaning that they are joined end by end. MLSA has been used for control of 16S rRNA classification during the past two decades based on up to ten genes. The increased availability of WGS has allowed the comparison of full gene sequences of in principle all conserved genes or protein sequences of a taxonomic unit. For whole genomic MLSA, a lower limit of 31 genes or protein has been proposed (Table 7.3). MLSA for classification is used on species level and higher taxonomic levels. In Chap. 11, we will see that MLSA also can be used below the species level for typing.

7.3 Classification of the Taxonomic Hierarchy

7.3.1 Classification of Species

The species represent the main taxonomic unit in classification. Species include prokaryotes with 16S rRNA gene sequence similarities higher than 97.0%. If 16S rRNA gene sequence similarity higher than 97% between different species is found, then DNA–DNA hybridization or comparable methods have been used to confirm the nature of such species since members of a species also need to be related with more than 70% DNA reassociation (Tindall et al. 2010). WGS comparison to group species at the genotypic level is now replacing experimental DNA–DNA hybridization (see Sect. 7.2.2).

7.3.2 Classification of Genera

The rules and recommendations are less strict compared to species. As mentioned, usually less than 95% 16S rRNA gene sequence similarity has been determined between genera (Yarza et al. 2008). In meta-genomic analysis based on partial 16S rRNA gene sequence, a 96% cutoff has been practiced (Ley et al. 2008). In parallel to ANI for nucleotides, there is also an ANI based on protein comparison (Qin et al. 2014). The percentage of conserved proteins (POCP) formulated by Qin et al. (2014) has been used to verify the classification at genus level. Different genera should have less than half of their proteins in common according to that definition.

7.3.3 Classification of Families, Orders, Classes, and Phyla

The rules and recommendations are again less strict for the higher *hierarchical* units of families, orders, classes, and phyla. Sometimes families have been created to find a home for a new genus, which again has created a new order. Analysis of 16S rRNA gene

Phylum	[*Pseodomonadota*]
Class	[*Gammaproteobacteria*]
Order	[*Pseudomonadales*]
Family	[*Pseudomonadaceae*]
Genus	[*Pseudomonas*]
Species	[*Pseudomonas aeruginosa*
Subspecies	

Fig. 7.3 Taxonomic categories of prokaryotes. The ones covered by the International Code of Nomenclature of Prokaryotes (ICSP) are valid publishes names written in italics font, and they are universal in all scientific publishing. For new categories, the naming should follow the genus (ICNP rule 8) (Oren et al. 2023). An example with *Pseudomonas* is included in brackets

sequenced showed a minimum of 87.5% identity between type strains of type species of type genera of different families (Yarza et al. 2008) (Fig. 7.3). There is no 16S rRNA gene sequence limits; however, families, orders, classes, and phyla should be monophyletic (see Chap. 6).

Genome Taxonomy Database (GTDB) has been implemented for classification of families, orders, classes, and phyla based on WGS comparison (https://gtdb.ecogenomic.org/) (Parks et al. 2022). This classification has been implemented in Bergey's Manual of Systematics of Archaea and Bacteria.

Names of families, orders, class, and phylum are covered by the International Code of Nomenclature of Prokaryotes. For new names of families, classes, and orders, the type of taxon has to carry the genus names. This has not been made retrospective in order not to cause too much confusion (Oren et al. 2015, 2016).

7.4 Rules for the Naming of a New Prokaryote

The way prokaryotes (bacteria and archaea) are named is a consequence of International Code of Nomenclature of Prokaryotes (ICNP) (Oren et al. 2023). There are rules for naming of prokaryotes but not for their classification. Names published in International Journal of Systematic and Evolutionary Microbiology (IJSEM) automatically become validly published, whereas names validly published outside IJSEM can be become validly published if accepted for the Validation List (VL) in IJSEM. All scientific publishers follow this nomenclature, and they are used as identifiers in databases including those dealing with bioinformatics.

Very few species are homotypic synonyms (two different names can be used for the same species). *Klebsiella aerogenes* and *Enterobacter aerogenes* have the same type strain

and therefore are homotypic synonyms, and as a consequence, either name can in principle be used. Users of the names seem to favor

Enterobacter aerogenes with 89,800 hits in Google Scholar (https://scholar.google.dk/) compared to only 43,400 for the other. However, the correct name is *K. aerogenes* since it has priority (Tindall et al. 2017). Each species has its own circumscription, and one can use the name that fits the circumscription one finds most correct. A heterotypic synonym is if two species are so closely related that they from the point of classification belong to one species. In this case, the name first published normally will have priority.

Prokaryotic scientific names are put in italics font since they are derived from Latin or classic Greek. They are binary combinations of a genus followed by a single specific epithet. The taxonomy of prokaryotes was originally derived from botanical rules; however, independent rules have existed for bacteria for nearly 100 years (Arahal et al. 2023). For older names, only those names included with the Lists of Bacterial Names in 1980 (Skerman et al. 1980) are valid. Names after 1980 are on List of Prokaryotic names with Standing in Nomenclature (LPSN) (https://lpsn.dsmz.de/) (Meier-Kolthoff et al. 2022).

7.4.1 Bacterial Species Names Are Linked to the Type Strain

The type strain is one and only one well-characterized strain from the species. To secure loss and access to the strain, it needs to be deposited in at least two culture collections with public access. Such cultures are copies of the same strain but have different strain designations in the form of a letter and number code. The type strain is a "name bearer," and it follows the species even when it is transferred to a new genus. For example, when *Streptococcus faecalis* (type strain ATCC 19433) was transferred to *Enterococcus faecalis* (type strain ATCC 19433), the type strain was unchanged. The type strain may have lost virulence properties due to repeated transfers in culture collections, and it is mostly only a good reference for taxonomic purposes. The type strain should always be included as a reference for classification and identification.

7.4.1.1 Example of an Old Bacterial Name that Never Changed

Staphylococcus aureus was validly published by Rosenbach (1884), and it is the type species of the genus *Staphylococcus*. The name is valid since it was included with Approved Lists of Bacterial Names (Skerman et al. 1980). The type strain is ATCC 12600 = ATCC 12600-U = CCM 885 = CCUG 1800 = CIP 65.8 = DSM 20231 = HAMBI 66 = NCAIM B.01065 = NCCB 72047 = NCTC 8532. The different numbers reflect the deposition of the same strain in different culture collections with public access (LPSN).

7.4.1.2 Example of Taxon with Many Reclassifications and Changes in Genus Name

Brachyspira hyodysenteriae was reclassified from *Treponema* by Ochiai et al. (1997). It was originally named *Treponema hyodysenteriae* (Harris et al. 1972). This name is

its basonym, meaning first name given by Harris et al. (1972). The name was included with the Approved Lists in 1980 (Skerman et al. 1980). In between, it was named *Serpula hyodysenteriae* by Stanton et al. (1991) since it was reclassified from *Treponema hyodysenteriae*. This name had to be changed one year later to *Serpulina hyodysenteriae* by Stanton (1992) since *Serpula* is illegitimate being used for a fungal genus Serpula. In between, *Brachyspira* had been named by Hovind-Hougen et al. (1982) and had to be used instead of *Serpulina* since *Brachyspira* was an earlier synonym of *Serpulina*. Despite of the changes, the type strain of the species was always the same: B78 = ATCC 27164 = CCUG 46668 = NCTC 13041 (LPSN) (Meier-Kolthoff et al. 2022).

7.5 The Benefits of Sequence-Based Identification

The comparison at DNA sequence level is definitive. DNA sequences can be compared on global scale and handled as electronic public information that for many applications substitute the analysis of live strains. Besides the applications of DNA sequences for identification to the species level, DNA sequence-based identification can be useful for molecular epidemiology and population genetics (see Chap. 11), and annotations of DNA sequences can be used to predict phenotypic properties.

7.5.1 16S rRNA Sequence-Based Identification, Step-by-Step

The principles for sequencing of the 16S rRNA gene were outlined in Sect. 7.2.1. When the 16S rRNA gene sequence has been determined, comparison in EzBioCloud (Yoon et al. 2017a) (https://www.ezbiocloud.net/) allows identification by comparison to all type strains of bacterial species with a validated published name (Activity 7.7). Often, such comparison is more precise than comparison in GenBank by BLAST search since the status of strains used to generate the sequence in GenBank is often not known. A phylogenetic 16S rRNA sequence-based analysis can be needed if similarity is obtained to more closely related species (see Chap. 6). The combination of database search and phylogenetic analysis can be used to improve the identification of an isolate to species and genus level. Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOF MS) has recently become an alternative method for fast and cheap identification of bacteria. 16S rRNA sequence identification is as a golden standard to make the MALDI-TOF database. Current limitations are with limited databases and limited resolution between some species. Future developments are expected with respect to improved resolution by using peptides generated from proteins and tandem mass spectrometry (Karlsson et al. 2015).

7.5.2 16S rRNA-Based Identification Without Culture

In the late 1980s, it became possible to characterize prokaryotes from their natural habitat without culture. The rRNA was extracted, reverse transcribed to DNA, cloned, and sequenced (Ward et al. 1990). Later, PCR was used to amplify the 16S rRNA genes from environmental DNA, and it was then cloned and sequenced. Recently, high-throughput sequencing technology has been combined with 16S rRNA target regions V1–V3 to deep sequence environmental samples (see Chap. 8). The technology is part of metagenomics and probably the most rapidly evolving field of microbiology. The current drawback with 16S rRNA-targeted metagenomics is the short-read length providing low accuracy for species level identification.

7.5.3 Sequence-Based Identification of Fungi

Sequence-based identification of fungi differs from prokaryotes in the way that type strains are not defined so clearly like for prokaryotes and full-length WGS comparisons are not used as standard. WGS comparisons are used for phylogenomics of fungi (Li et al. 2021). Names of fungi can be checked in MycoBank database (https://www.mycobank.org/).

Traditionally, some conserved marker genes have been used for sequence-based identification. For instance, for species of *Trichoderma* ITS, translation elongation factor 1 alfa (*tef1*) and RNA polymerase B subunit II (*rpb2*) have been used (Cai and Druzhinina 2021). Westerdijk Fungal Diversith Institute provides links to various databases where pairwise sequence-based identification can be performed online (https://wi.knaw.nl/Pairwise_alignment). Further details about the choice of genes for identification of fungi can be found in Houbraken et al. (2021).

7.5.4 Sequence-Based Identification of Protists

Protists are included in a polyphyletic group of eukaryotes and are probably the most diverse group of organisms despite their common morphology as unicellular eukaryotes. The protist database has various facilities mainly centered on the 18S rRNA gene sequence (Guillou et al. 2013) (https://pr2-database.org/).

7.6 Strain Identification by WGS Analysis

Experimental and applied microbiology require controls to confirm strain identity. WGS has become the primary method for confirmation and identification of strains in microbiology. Identification of an isolate to the strain level is needed to confirm the use of the correct strain in laboratory experiments. Confirmation of production organisms is needed in bio-

technology production. Add to this legal reason to confirm strain identity in regard to licenses, patents, and working environment safety.

A strain is made up of the descendants of a single isolation in pure culture, and only a minimum of genetic variation (mutations) can be expected and tolerated between different sub-cultivations. For instance, on the average of one single nucleotide polymorphisms (SNP) was identified every 3 months in the WGS when DNA was extracted from a frozen stock culture of *Listeria monocytogenes* (Kwong et al. 2016). In the past, a range of DNA fingerprinting methods has been used for strain level confirmation (summarized by Tindall et al. 2010)—now almost all have been replaced by WGS.

Details about the cultivation of bacterial strains including procedures to make them grow and to obtain purity have been reviewed including an argumentation for the use of DNA-based methods (Pinevich et al. 2018). It is important to refer to the original reference strain by culture collection number and not to use parallel numbers from other culture collections holding the strain since misplacements and contamination of strains can in theory occur.

WGS analysis is now part of the methodology implemented in most microbiological laboratories. If the sequencing equipment is not directly available, WGS analysis can be outsourced at an affordable cost. The quality criteria used to evaluate WGSs as well as the use of WGSs for characterization of strains used for taxonomy, biotechnological production, and probiotics have been outlined (Chun et al. 2018; EFSA 2021). The raw data generated by sequencing of DNA can with some effort be assembled to fully closed genomes if sequencing has been performed on Nanopore or PacBio platforms. However, if sequences have been generated by Illumina short-read technology, the assembly will end with a number of contigs. The following evaluation will therefore at some places deal with fully closed genomes and make a distinction to WGSs only available on contig form even though both represent WGSs.

7.6.1 SNP

SNP analysis is the most sensitive method for strain level identification. In principle, all nucleotide differences between two or more genomes can be scored. However, the concept is only operational with closely related genomes and used in regard to minor genetic changes (point mutations). To exclude insertions and deletions, nucleotide (nt) changes are only considered as SNPs if they are spaced at least 10. For the prokaryotic WGSs only assembled as draft genomes with a number of contigs, a lot of SNPs would be scored just as noise in non-assembled or purely assembled regions. For this reason, sequence reads of strains to be compared are mapped to a reference sequence, and this way indirectly com-

pared. For instance, if we want the SNP distance between WGSs of strains A and B, we first compare A to a reference strain R and then B to the same reference strain R and then accumulate all SNPs between A and R and B and R, which will be the SNP distance. The reference is best chosen as a fully closed WGS; however, if not available, the best WGS on contig form with a low number of contigs can substitute. The reference should be closely related to the strains being compared.

7.6.2 OGRI for Strain Level Conformation and Identification

OGRI was developed for species level identification, and the concept is not defined for strain level identification. The following simulations illustrate how the indices also relate to minor genetic differences between WGSs of strains. The simulation was based on *E. coli* K12 strain MG1655 genome acc. no. NC_000913, which is fully closed (length 4,641,652 bp). Pairwise comparisons were made between the original genome sequence and modified versions either made by shuffling or deleting parts of the original WGS. Shuffling was done by complete randomization of the given nucleotides in blocks of 980 nt (simulating gene length) and performed with EMBOSS (Rice et al. 2000). The resulting shuffled "genes" had similarities of 30–40% to the original. Deletion was done in blocks of 980 nt also at the size of single genes. ANI was run from https://www.ezbiocloud.net/tools/ani. GGDC was done on http://ggdc.dsmz.de/ggdc.php#.

ANI was sensitive to deletions down to the level of two genes, whereas one deleted gene will not be detected (Table 7.4). Theoretically, this should be impossible since deleted genes would not be considered in the calculation of shared genes; however, the random cutting up in 1020 nt fragments, which is included in the ANI program, is probably out of phase with the simulated deletions introduced, and the index will in practice be affected by insertions and deletions as well. ANI was insensitive to a few gene substitutions (up to 10 at least) (Table 7.5). This may be related to the relative high identity of shuffled genes of 30–40% where such genes probably are included in the ANI calculation as shared genes. GGDC was sensitive to both deletions and gene substitutions at single gene level when formula 2 was used (Tables 7.4 and 7.5).

Table 7.4 Simulation of OGRI based on deleted gene sequences of *E. coli* K12

No. genes deleted in one genome	% ANI	GGDC formula 2
1	100	0.0001
2	99.98	0.0002
3	99.98	0.0003
4	99.98	0.0004
5	99.97	0.0005

Table 7.5 Simulation of OGRI based on shuffled gene sequences of *E. coli* K12

No. genes shuffled in one genome	% ANI	GGDC formula 2
1	100	0.0002
2	100	0.0004
3	100	0.0006
4	100	0.0008
5	100	0.0011
10	100	0.0021

7.6.3 OGRI for Fungi

Both ANI and GGDC were used to evaluate a new species of yeast by Čadež et al. (2021) although other thresholds were used compared to bacteria (see further references in the paper). The use of OGRI for conformation of strain identity for fungi may be considered (Houbraken et al. 2021).

7.6.4 Identification of *E. coli* K12

Hundreds or maybe thousands of mutant variants have been constructed and used in laboratory experiments and for biotech production since the original isolation of strain K12 in 1922. Despite of many modifications, strain K12 can be recognized by some conserved mutations either with a reference to WGS (e.g., NC_000913 used above) or on single gene level. Characteristic frameshift mutations are present in the *rph* and *ilvG* genes (Lawther et al. 1982; Jensen 1993). Another signature mutation is found in the O-antigen gene *rfb* cluster (Liu and Reeves 1994; Stevenson et al. 1994), and a PCR has been developed for this *rfb* mutation (Kuhnert et al. 1995).

7.7 Activity

7.7.1 16S rRNA Gene Sequence-Based Identification

Sometimes you end up with a bacterial isolate that is difficult to identify by phenotypic means, or you do not believe in the result from the identification by a kit. You will use fPCR to amplify the 16S rRNA gene and sequence the DNA. After assembly of the sequence, you want to perform a database search to obtain the most accurate identification. Traditional nucleotide BLAST in, for example, GenBank, can be performed. The problem is that so many sequences are deposited, and you cannot always rely on the species name associated a sequence. The optimal reference is the sequence of the type strain. However, it is not always so easy to locate in a BLAST output. To overcome this, a server has been set up by Professor Chun and his team where a search is performed against 16S rRNA

sequences of type strains only. For the activity, we will download a 16S rRNA gene sequence from NCBI and assume it is unknown and recently determined in the lab. Download the sequence with acc. no. KX858032 from NCBI like described in Chap. 3. Use EzBioCloud (https://www.ezbiocloud.net/identify) to identify the sequence. You need to register for a free account. Press **Identify single sequence** and paste the sequence in FASTA format into the window, leave other parameters default and **Next**, and then **Submit** when the sequence has been verified.

What is the top hit taxon and similarity %? also click at "+" in the **Task column** to see alternative less likely identifications. In this activity, the expected result is 95.7% similarity to the closest related species, which is lower than the species threshold. Based only on the 16S rRNA-based identification, this strain would represent a new species. However, higher similarity is shown to a genomic sequence. If the genome (acc. no. MLHN01) is looked up in NCBI, it represents another strains of the unnamed species.

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16S rRNA Amplicon Sequencing

8

Henrik Christensen, Jasmine Andersson,
Steffen Lynge Jørgensen, and Josef Korbinian Vogt

Contents

H. Christensen (✉)
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk

J. Andersson
Eye Department, Copenhagen University Hospitals, Rigshospitalet, København, Denmark
e-mail: anna.jasmine.andersson.01@regionh.dk

S. L. Jørgensen
Næstved Hospital, Næstved, Denmark

J. K. Vogt
National Food institute, Technical University of Denmark, Lyngby, Denmark
e-mail: jkovo@food.dtu.dk

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What You Will Learn in This Chapter

The experimental setup to use of 16S rRNA amplicon sequencing will be introduced. The different steps in the bioinformatical analysis will be explained with background in the major pipelines Mothur and QIIME2. We have extracted examples from Mothur in the text, and we have chosen to demonstrate QIIME2 in more detail in Activity 8.10. You will be able to get a good start with the analysis of 16S rRNA amplicon data with QIIME2.

8.1 Use of 16S rRNA Amplicon Sequencing, Generation of Data, and Bioinformatics Pipelines

8.1.1 Use of 16S rRNA Amplicon Sequencing and Generation of Raw Data

The 16S rRNA gene sequence is used to classify all prokaryotes as described in Chap. 7, and 16S rRNA amplicon sequencing is an application of the 16S rRNA sequence-based classification concept to characterize biodiversity and to investigate the ecological characteristics of all sample types. The benefit of 16S rRNA amplicon sequencing is that comparison can be made to the extremely detailed and well-curated taxonomic databases. The 16S rRNA amplicon approach to metagenomic analysis started in 2007–2008 by a synergy between the use of high-throughput pyrosequencing analysis by the "454" methodology, bar coding, and the existing framework of 16S rRNA classification of the prokaryotes. The

brilliant idea was to amplify a variable region of the 16S rRNA gene from the whole microbiome of a sample and then to barcode samples allowing sequencing of multiple samples in one run, and this way to reduce the cost with the "454" method. The theoretical background for this concept was first described by Liu et al. (2007), and later, the use was published from the group of professor Rob Knight (Liu et al. 2008; Hamady et al. 2008; McKenna et al. 2008). The combination of high throughput related to "454," and later Illumina sequencing, relatively low cost related to the use of barcoding and the high information gained by coupling to the huge 16S rRNA sequence taxonomy databases have revolutionized microbiology, and the technique is now in use in most labs dealing with the metagenomics characterization of diverse environments—from environmental samples to the intestinal microbiome (Fig. 8.1).

The procedures used for DNA extraction are dependent on the type of sample material. For the extraction of DNA from fecal samples and similar complex organic matrices, we can recommend MO BIO PowerMag® Soil DNA Isolation Kit (Qiagen) and FastDNA Spin Kit (MP Biomedicals). Several comparisons of DNA extraction procedures have been published. Chapagain et al. (2019) reported that the Promega-Maxwell DNA isolation kit resulted in the lowest variation between samples. We refer the reader to Anderson et al. (2016) for an overview all lab work related to 16S rRNA amplicon sequencing. Further descriptions of sample preparation and the procedures for DNA sequencing will unfortunately not be described here since the focus will be on the bioinformatical analysis.

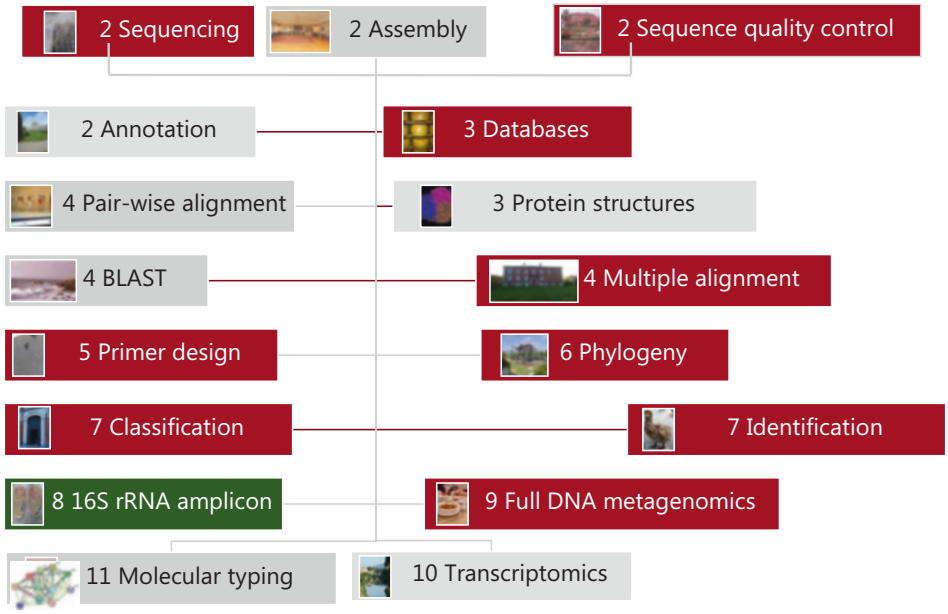


Fig. 8.1 16S rRNA amplicon sequencing relates to nearly all other topics in the book

Table 8.1 Primers for 16S rRNA metagenomics[a]

Target	Forward 5′–3′	Reverse 5′–3′	Size[c]	Reference
V3[b]	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	194	Lane (1991), Danzeisen et al. (2011)
V1–V3	AGAGTTTGATCCTGG	ATTACCGCGGCTGCTGG	527	Lane (1991), Danzeisen et al. (2011)
V3–V4	GGAGGCAGCAGTRRGGAAT	CTACCTGGGTATCTAATCC	457	Nossa et al. (2010)
V4–V5	GTGYCAGCMGCCGCGGTA	CCCGYCAATTCMTTTRAGT	413	Tang et al. (2014)

[a]The paper of Soergel et al. (2012) presents an exhaustive list of primer combinations and comparisons
[b]Information on V1–V9 (Ashelford et al. 2005)
[c]Referring to acc. no. J01695 of *E. coli rrnB*

Mock samples can be included in the analysis. They are constructed from known prokaryotic isolates pooled at even concentrations. The mock sample is included as a control sample in downstream analysis (Fouhy et al. 2016).

The primers used for PCR amplification depend on the desired degree of variability and length of product. Usually, the higher variability of the 16S rRNA gene is near the 5′ end (Table 8.1). Comparison of primer sets was provided in Klindworth et al. (2013). Primers are constructed with adaptors of ca. 50 bp, which are needed to attach the DNA amplicons to the solid support of the flow cell (Fig. 8.2). In addition, barcodes on the 16S rRNA amplicons (multiplexing) are needed in order to include many samples on one flow cell. The final product is called the 16S rRNA amplicon sequencing library. Forward adaptors each with a unique index combined with similar reverse adaptors will give 96 unique indexes meaning that up to 96 independent samples can be sequenced at one time. Barcoding is further outlined in Hamady et al. (2008). Actually, Miseq allows up to 400 16S rRNA amplicon libraries (50,000 reads per sample) in a single sequencing run. As a rule of thumb, there should at least be 50,000 reads per sample and more than 100 reads per bacterium of interest. The main limitation with MiSeq sequencing is the short sequence length of the 16S rRNA gene. Attempts have been made to sequence nearly full-length 16S rRNA genes with PacBio to improve the information content (Schloss et al. 2016; Wagner et al. 2016; Karst et al. 2018).

This chapter focuses on the characterization of the prokaryotes. The same concept can be used with microfungi and other eukaryotic microorganisms; however, other primers need to be used for the initial PCR amplification, and the databases are more difficult to access including the “mask” to align the de-replicated reads.

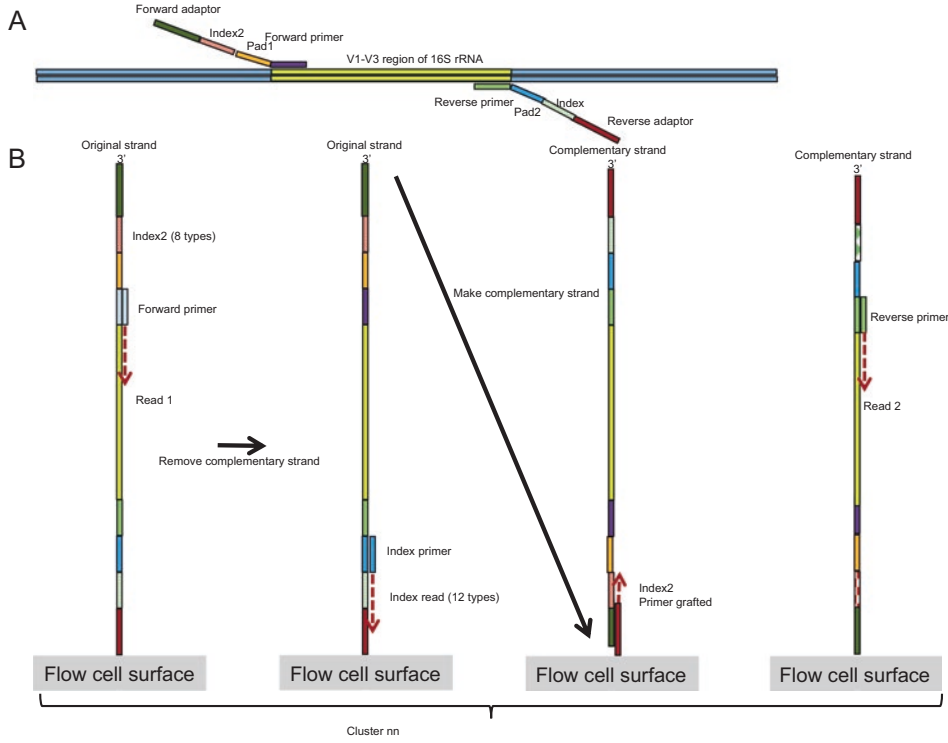


Fig. 8.2 Use of adaptor (fixing the sequence to the flow cell) and index primers (separation of different samples) for Illumina platform sequencing of a genomic DNA fragment, which includes the V1–3 region of 16S rRNA gene. **a** Shows now adaptor, and index primers are added to a fragment by PCR. **b** Shows how the fragment (library) is attached to the solid support (flow cell surface) and sequencing proceeds in forward direction and how the fragment afterward is prepared for reverse sequencing (paired end sequencing)

8.1.2 Bioinformatical Pipelines to Analyze Data

The microbiome pipelines allow users to analyze raw DNA sequence data, produce results, apply statistics, and visualize results for publications. A common theme in the analysis of 16S rRNA amplicon sequence data is that all samples can be analyzed at one time as well as the differences between samples can be compared. The two options are possible thanks to the use of the barcoding of all reads analyzed. A recent review of current pipelines has been provided by Liu et al. (2021).

Mothur (Schloss et al. 2009) (https://www.mothur.org/) was developed to satisfy the need to analyze high-throughput sequence datasets in a modular way and to speed up the existing algorithms. The program is written in C + +. The most simple way to use the package is from the command (dos) prompt of Windows PC. All material is included in one folder including the raw data and program. The program is started with the Mothur

icon. The commands can then be copied from the SOP available from the website and full analysis of 16S rRNA amplicon libraries carried out in a series of steps. The program keeps track on all read files and sample types in the "Stability.files." You need to edit this file with a text editor to get started.

Quantitative insights into microbial ecology (QIIME) (pronounced chime) (Caporaso et al. 2010) (https://qiime2.org/) is a pipeline for analysis of high-throughput sequence data in microbiological ecology studies. From January 1, 2018, QIIME2 is the supported version of this pipeline. QIIME2 is based on the computer language Python and is set up with a series of modules, which allow the user to handle, manipulate, and analyze the large multiplexed high-throughput datasets. Package managers like Conda and Miniconda are installed to deploy metagenomics pipelines.

In this chapter, we will mainly refer to Mothur in the text and work with QIIME2 in the activity. Other equally important pipelines are available such as USEARCH (Edgar 2010) and UPARSE (Edgar 2013). The latter includes quality filtering, trimming, merging of identical reads (dereplication), and clustering. VSEARCH (Rognes et al. 2016) is an open-source version of USEARCH. Sometimes modules from pipelines are combined. QIIME2 makes it possible to easily run VSEARCH via plugins.

CLC Genomics Workbench (Quiagen) provides a module for 16S rRNA microbiome analysis. It is recommended for users without experience. The program will carry out a standard analysis and is a good way to start and save time in the steep learning that is required in this field. Unfortunately, the software is licensed and relatively expensive; however, the cost probably pays off in time. The Microbial Genomics Module needs to be added to the CLC Genomics Workbench package. The Help function will provide guidance. The module is accessed from the top menu bar. The following steps should be followed starting with raw data: trim, merge paired reads, trim to fixed length, filter samples based on number of reads, OTU clustering, α-diversity, and β-diversity. Finally, PCoA analysis is offered. The software allows the user to perform a standard 16S rRNA microbiome analysis with a set of samples. For further use, readers are recommended to contact the company's hotline.

8.2 Data Analysis

8.2.1 Quality Trim by Sequence

Denoising is quality filtering of individual reads to remove low-quality reads, and it can also include removal of chimeras (this will be discussed in Sect. 8.3), foreign DNA, and individual bases with low-quality scores. In UPARSE, a limit of Phred score (see Sect. 2.2.1) was set at 16 (Edgar 2013). Trimming is invoked if two or more adjacent low-quality base calls are identified. If some paired reads are longer than the expected size based on the PCR primers (Table 8.1), the sequences that are too long are excluded for analysis (**scree.seqs command, maxlength=**) in Mothur.

At this step, some reads will be completely removed for further analysis, and others will be trimmed in length removing the low-quality regions.

8.2.2 Pairing of Reads

At this initial step, forward and reverse paired reads are paired to contigs. With M**othur,** this is done with the "**make.contigs**" command. USEARCH also includes this procedure. A feature table is generated.

8.3 Removal of Chimeras and DNA from Other Domains or Life

Chimeras are biased merged read sequences from two origins. These sequences can artificially increase the diversity of the samples and need to be identified and removed during the bioinformatics analysis. In addition to the tools included in Mothur and QIIME2 pipelines, chimeras can be removed by UCHIME (Edgar et al. 2011). Filters can be included to remove eukaryotic DNA from mitochondria and chloroplasts and DNA from Archaea if one is only interested in bacteria: (**remove.lineage** in **Mothur**).

8.4 Grouping of Reads into OTUs

Dereplication is the grouping of identical reads (**unique.seqs** in Mothur). UPARSE also includes this procedure. Singletons were recommended to be discarded (Edgar 2013).

Reads are grouped into OTUs with a 97% similarity threshold (**cluster.split** in **Mothur**). The 97% limit is based on the 16S rRNA threshold between prokaryotic species (Chap. 7). OTU clustering is also used to improve precision of the analysis since the error of sequencing can be as high as 0.1% per nucleotide in a single read.

An alternative to OTU dereplication is divisive amplicon denoising algorithm (DADA). This algorithm groups reads according to statistical modeling identifying a most probable central sequence (Callahan et al. 2016). Amplicon sequence variants (ASVs) is an alternative term.

The outcome of OTU or ASV analysis is a reduced dataset, which can be analyzed downstream. The frequency of the representation of OTUs or ASVs can be included in a features table.

8.5 Alignment of OTUs and Association of OTUs with Taxonomic Units

First, a multiple alignment (Chap. 4) is performed to further reduce noise and prepare the dataset for phylogenetic analysis. The multiple alignments will include a mask that allows comparison with the specific formats of the databases. Three main databases, SILVA (Quast et al. 2013), **Greengenes** (DeSantis et al. 2006; McDonald et al. 2012), and RDP (Cole et al. 2014), are commonly used.

The SOP of Mothur will guide you through how to align to the SILVA database. **In QIIME2, MAFFT** and FastTree are used for multiple alignment and phylogenetic analysis, respectively. Greengenes is used as the standard database for alignment as described in Activity 8.10. The QIIME2 tutorial also explains the eventual need to train a classifier tailored for sample preparation and sequencing parameters, including the primers that were used for amplification as well as the length of the sequenced reads.

Variations between databases have been found to associate OTUs with taxonomic units. The highest assignment success on genus level was obtained with MiDAS-SILVA, whereas Greengenes came second, and RDP was least efficient. However, MiDAS-SILVA required more computing time compared to RDP (Popp et al. 2017).

Unidentified OTUs in the databases are not identified further. The short length of sequence makes further characterization of such unassigned OTUs very difficult.

8.6 α-(Within Group) and β-Diversity (Between Groups) Comparison

Before these indices can be calculated, normalization is needed. The numbers of sequences in all samples are normalized to include the same number. This number is usually set as the number of sequences in the samples with the lowest number.

For α-diversity analysis, different comparisons can be used. In QIIME2, the choices are Faith's phylogenetic diversity (Faith 1992), Pielou's evenness, observed OTUs, and Shannon's diversity (further explained in Activity 8.10, Fig. 8.16). Pielou's evenness is a measure for how much the different species in a sample resembles in terms of numbers, that is, a low value (scale 0–1) meaning less evenness and more dominant species and vice versa. Qualitative measurements of community richness are Faith's phylogenetic diversity, where the phylogenetic tree is incorporated, and observed OTUs. In Shannon's diversity index for quantitative measure, both richness and evenness are taken into account.

For β-diversity analysis, UniFrac can be used in both Mothur and QIIME2. Unifrac requires a phylogenetic tree to have been constructed, which is why this module is included in both pipelines. UniFrac is based on comparison of the branch length fraction of distances of shared and unshared branch lengths in the phylogenetic tree between the compared samples. An Unifrac distance of zero suggests that the compared samples are identical. There are two types of Unifrac distance metrics—unweighted and weighted. Unweighted Unifrac measures the presence and absence of taxa/OTUs, whereas weighted Unifrac also incorporates the abundances of taxa/OTUs. Bray-Curtis calculates the dissimilarity in the samples composition but is not based on phylogeny.

8.6.1 Rarefaction Analysis

This analysis is performed to see if some samples are represented with low sequencing depths. Based on the analysis, a cutoff read depth is chosen for the following alpha-diversity analysis. Samples below a certain threshold are not included in further analysis (Figs. 8.3, 8.4, 8.16).

The chosen cutoff read depth should be at the state when the slope of the diversity curve approaches 0, which indicates that the maximum diversity is represented at the given read depth.

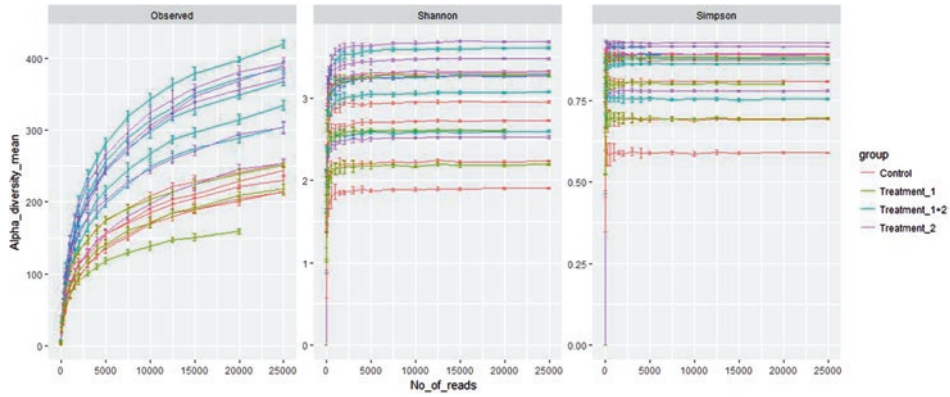


Fig. 8.3 Rarefaction curves for all samples from four different groups, using three alpha-diversity metrics: Observed richness, Shannon diversity index and the Simpson diversity index. All plots are visualized at 25,000 read depth

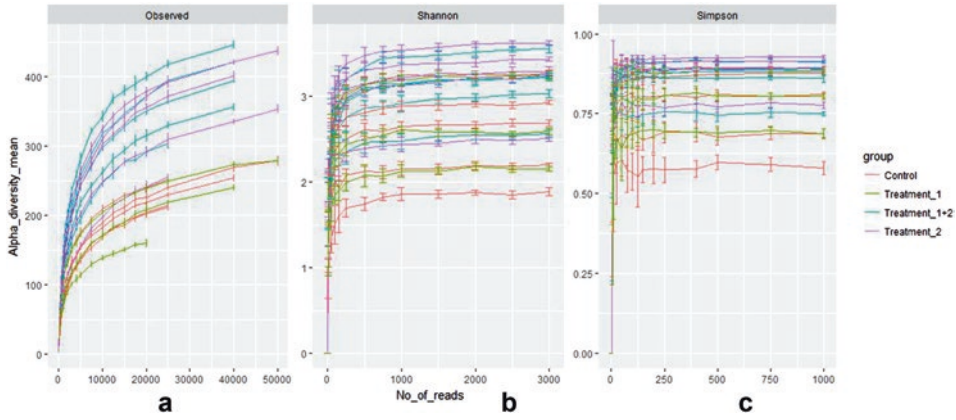


Fig. 8.4 Rarefaction curves with different maximum read depth to demonstrate the slope of the curves ((**a**) Observed; (**b**) Shannon; (**c**) Simpson)

The Phyloseq R package (McMurdie and Holmes 2012) can produce rarefaction curves with different α-diversity metric units, including observed number of OTUs, Shannon diversity index, Simpson diversity index, and Chao1 (Fig. 8.3).

Initial analysis using rarefaction curves can give a visual overview and comparison of the quality of the samples. Samples that demonstrate significantly low sequence depth or α-diversity can be identified and excluded if necessary.

Figure 8.4 demonstrates the rarefaction curves of four different microbiota treatments, using three different α-diversity metric units: observed no of OTUs, Shannon diversity index, and Simpson diversity index.

The figure demonstrates that the samples have different maximum read depth, with the lowest at approximately 20,000 reads and the highest exceeding 50,000 reads. Hence, the highest cutoff value can be 20,000 read depth if all samples are included.

The different α-diversity metrics demonstrates slopes reaching 0 at different read depths. Figure 8.4 visualizes the same dataset, however with different read depths depending on the α-diversity metric.

While the observed richness (Fig. 8.4a) never reaches a maximum, both Shannon diversity index curve and Simpson diversity index curve (Fig. 8.4b and c) reach asymptotes at read depths <1500 reads.

Both Shannon diversity index and Simpson diversity index demonstrate that overall α-diversity does not significantly change by increasing the read depth after 1500 reads. However, one might suggest that the full α-diversity is not reached even at a read depth of 50,000 reads due to the stump observed richness curves, which demonstrates an increase in identified OTUs when increase read depth.

Similar results can also be visualized when producing boxplots of the alpha-diversity at different read depths. The Ampvis2 package can produce boxplots at different read depths and alpha-diversity metrics (Fig. 8.5).

Both the rarefaction curves and boxplots demonstrate that increasing the read depth only increase the observed richness but not the overall α-Shannon or Simpson diversity. Hence, these results suggest that all samples are suitable to be included in the further analysis.

These findings suggest that the significant OTUs are already represented at a read depth of 1500–2500 reads, and only OTUs, which are only identified in very low and insignificant numbers, are added at higher read depths. Especially the *Control* and *Treatment 1* samples indicate a lower observed richness compared to the *Treatment 2* and *Treatment 1 + 2*. Additionally, the Simpson diversity index, which is more weighted on evenness, indicates a more uneven diversity in the *Control* and *Treatment 1*. This could be due to an overrepresentation and high abundance of a few OTUs in the *Control* and *Treatment 1* samples.

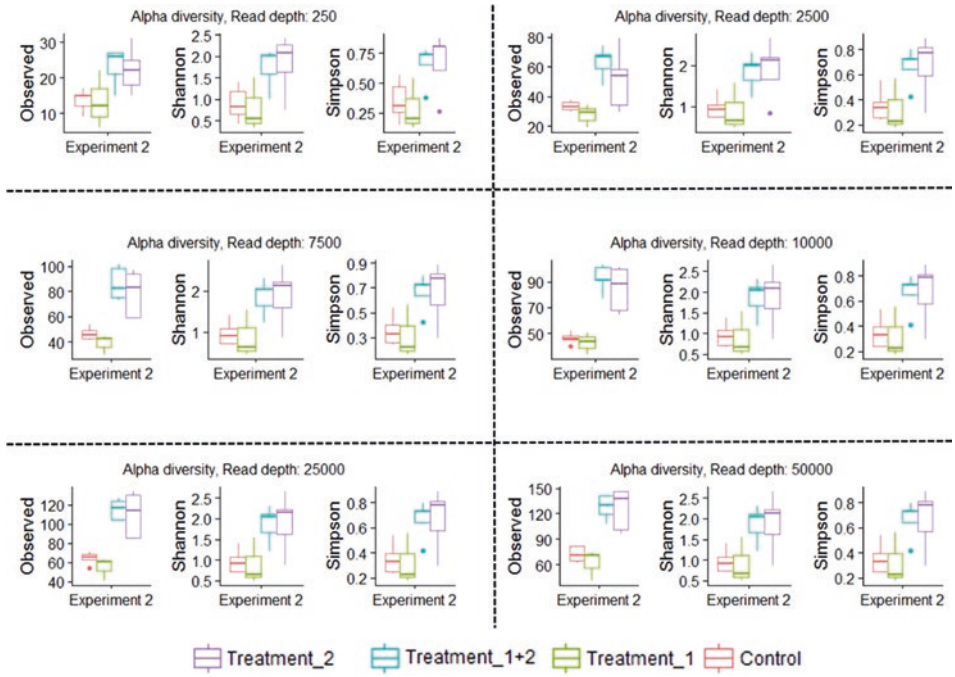


Fig. 8.5 Boxplots of the α-diversity represented by three α-diversity metrics: observed richness, Shannon diversity index, and the Simpson diversity index

8.7 Taxonomic Comparisons

The number of OTUs for each best taxonomic match will provide the raw data for this analysis (**classify.otu** in **Mothur**). The output file from Mothur can be opened in Microsoft Excel.

8.7.1 Generation and Interpretation of Heatmaps and Boxplots

Heatmaps and boxplots (Figs. 8.6, 8.7) can generate an overview of the samples taxonomic makeup. Simple heatmaps of the most abundant OTUs can be useful to identify if there are OTUs with very high abundance and for presenting results. The Ampvis2 package can produce heatmaps and boxplots that present a chosen number of OTUs at different taxonomic levels.

Similar to the heatmaps, boxplots are excellent to demonstrate the scale of the predominant OTUs in a sample set.

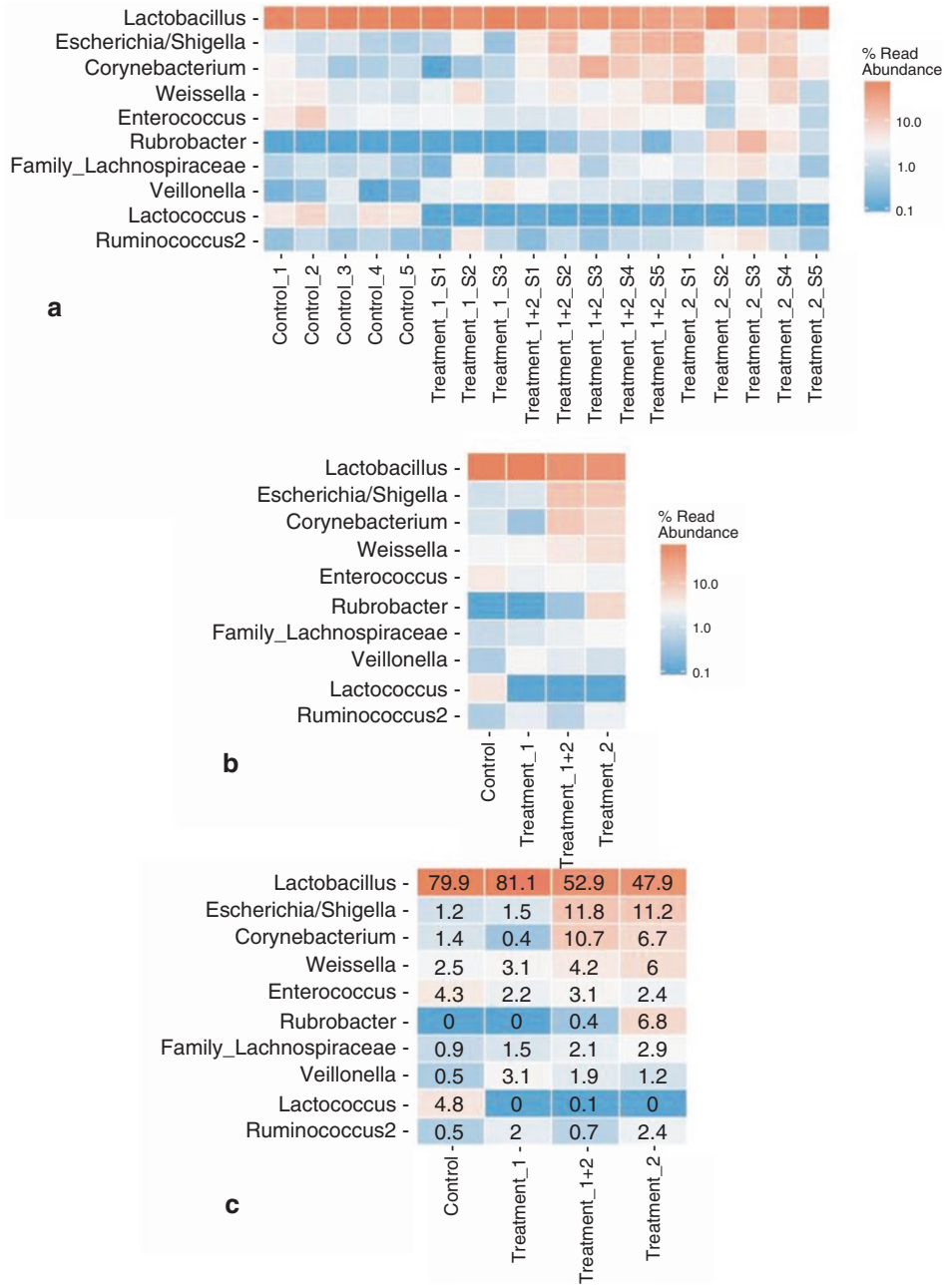


Fig. 8.6 Heatmap of the ten most abundant genera in the analyzed sample set. All three heatmaps are based on the same dataset and presented in three different ways. (**a**) Includes all samples, with the % read abundance represented by the color temperature of the cell. (**b**) Each treatment group has been summed up, and the average is represented in the heatmap. (**c**) Similar to **b**, but with the average % read abundance presented in each cell

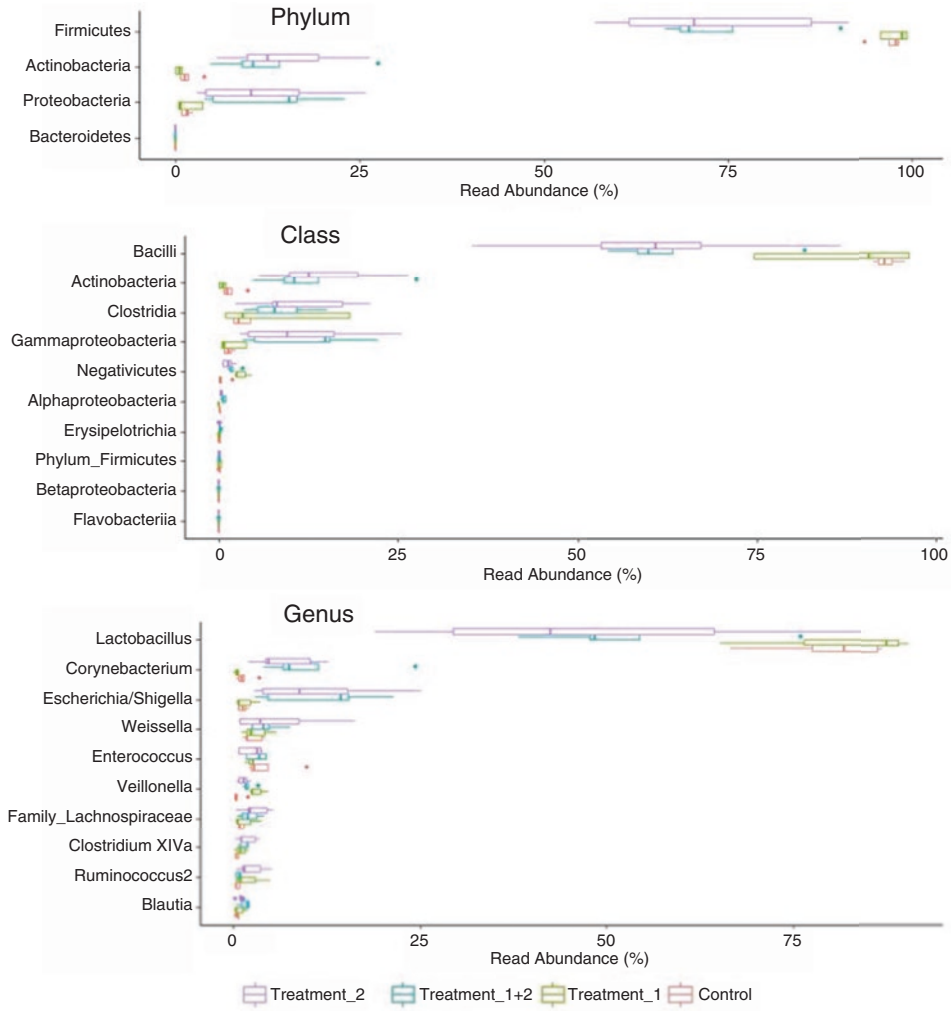


Fig. 8.7 Boxplots of the most predominant OTUs on phylum, class, and genera level in four different groups. All boxplots are based on the average of the samples of the respective group

Both heatmaps and boxplots can give an overview of the composition of OTUs and the predominant groups, as well as an initial idea of the microbiota similarity of the samples within the group as well as between the groups.

As demonstrated in both boxplots and heatmaps, all four treatment groups demonstrate high abundance of Firmicutes, especially *Bacilli* and specifically *Lactobacillus*. However, especially the Control and Treatment 1 groups demonstrate very high abundance of *Lactobacillus*, which agrees with the indication of overrepresentation of few OTUs in the observed richness alpha-diversity from the rarefaction curves and α-diversity boxplots (Figs. 8.3, 8.4). These results further underline that increasing the cutoff read depth would

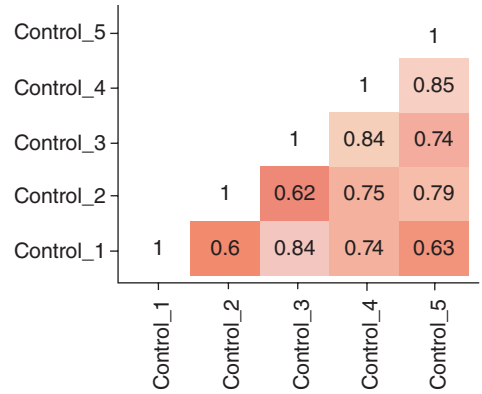


Fig. 8.8 Comparison of the five control samples based on Bray-Curtis similarity distance (1 = 100% similar, 0 = 100% dissimilar)

not improve the analysis in any way, as the only OTUs with insignificant abundance would be added to the analysis.

Both boxplots and heatmaps suggest a more similar microbiota composition in Treatment_2 and Treatment_1 + 2 compared to the control and the Treatment_1 groups. These results confer with the results from the α-diversity boxplots, which demonstrated signs of similar α-diversity in the Treatment_2 and Treatment_1 + 2 and the control and Treatment_1 groups (Figs. 8.3, 8.4).

Before β-diversity analysis such as PCoA (Sect. 8.8), initial comparison of the similarity/dissimilarity of the samples within each group, based on non-metric or semi-metric distance, can give an indication of how uniform the samples of the group are. The Ampvis2extra package offers a simple β-diversity comparison analysis using Bray-Curtis similarity distances (Fig. 8.8).

8.8 Principal Coordinates Analysis (PCoA)

Principal coordinate analysis (PCoA) is a tool to explore 16S rRNA amplicon sequence data visually by reducing a multidimensional sample vector (for 16S data, the feature counts for a sample) to principle components (PCs). PC can be understood as a combination of features capturing a certain degree of variance within all samples. By visualizing with a PCoA plot, sample clustering can be observed, that is, similarities or dissimilarities of the data (Fig. 8.9). QIIME2 offers plotting samples in three dimensions. In the QIIME2 activity, later in this chapter, some of the diversity analysis results are shown as PCoA (Figs. 8.14, 8.15).

Similar to the initial α-diversity analysis as well as the heatmaps and boxplots; the *Treatment_2* and *Treatment_1 + 2* demonstrates a similar microbiota composition, with overlaps in PC1, PC2, and PC3. Meanwhile, the *control* and *Treatment_1* groups also demonstrate an overlap in both PC1 and PC3.

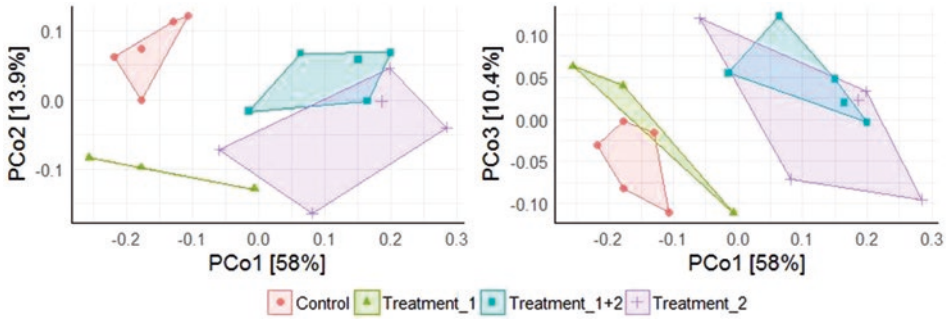


Fig. 8.9 PCoA of the samples from all four treatment groups, visualized by the first three PCs. To the left, PC1 and PC2 visualize 71.9% of the analysis model. To the right, PC1 and PC3 visualize 68.4% of the model

Collectively, these results indicate that Treatment 1 induces a similar microbiota as the control group. Meanwhile, Treatment 2 induces distinct microbiota, different from the control and Treatment 1. Both Treatment 1 and Treatment 2 simultaneously result in a microbiota similar to Treatment 2 alone, suggesting that Treatment 1 has no or a very weak effect on the microbiota.

To verify some of the conclusions drawn above, a permutational multivariate analysis of variance (*PERMANOVA*) is needed. This is a nonparametric multivariate statistical test, and it is used to compare groups of objects and test the null hypothesis that the centroids and dispersion of the groups as defined by measure space are equivalent for all groups.

8.9 Prediction of Function

It is possible to some extent to predict information about function based on comparison to taxonomic profiles. This is a shortcut to full DNA microbiome analysis (Chap. 9). The drawbacks are that the same functions can often be undertaken by different and even diverse taxonomic groups. For activated sludge, a species taxonomic prediction of function is possible at: (https://www.midasfieldguide.org/guide).

PICRUSt (http://picrust.github.io/picrust/) predicts abundance of gene families in host associated and environmental communities (Langille et al. 2013). Alternative tools are available such as FAPROTAX, BugBase, and Tax4Fun (see overview in Liu et al. 2021).

8.10 Activity QIIME2

QIIME is one of the major software packages for 16S rRNA microbiome analysis as described in Sect. 8.1.2. QIIME1 was replaced with QIIME2 in 2018 (current version: (https://docs.qiime2.org/2023.5/), and it is the version to be installed when working with

QIIME. There are continuously updated versions of QIIME2, with improvements of the pipeline. The user needs to be aware of the version as it has to be specified while working with QIIME2. The developers recommend the users to work with the latest version available.

There is an official forum for QIIME2 where users can address questions about different problems encountered. It is a good idea to start searching this forum for questions and problems already discussed and solved before adding a new subject.

There are several tutorials on QIIME's website addressing various aspects of 16S rRNA microbiome analysis. We would recommend starting with the "Moving Pictures" tutorial. "Moving Pictures" was a time series study of the microbial communities of different anatomical sites of humans. This dataset is used as a case for the tutorial. The manual is best suited for Mac OS, and we recommend that you get access to a new Mac computer with a lot of RAM before starting. The subset of the Moving Pictures dataset includes only five time points and four anatomical sites totaling 20 samples. The tutorial will take you through most relevant analysis for 16S rRNA amplicon sequencing including alternative approaches for some analysis steps. You can use the tutorial to learn and understand 16S rRNA amplicon sequencing.

The commands used in this book can change depending on the version (current version 2023.5). All commands can, however, be found on the version specific documentation site.

8.10.1 Installation

If you try this for the first time, it is recommended to do exactly as the manual says even though the names for directories seem a little odd. You can copy the whole command strings from the QIIME2 tutorial including the \ that will join lines on the command prompt. You can get the command prompt from **Go|Utilities** select **Terminal. App**. You can copy commands from the tutorial by using copy paste functions.

Open the manual https://docs.qiime2.org/2023.5/ and select **Getting started** and **Install QIIME2**. Select Natively **install QIIME2 and install Miniconda, and then QIIME2** within a **miniconda** environment. Select **macOS**. From the command prompt, run **source activate qiime2–2023.5** (or actual version).

If you are a Windows user, install MobaXTerm (serves as your terminal) and then use the manual Installing QIIME2 using virtual Machines.

8.10.2 Running QIIME2

First time you start QIIME 2, you will have to open the terminal window and write or copy paste **source activate qiime2–2023.5** (or actual version). Then continue to do **exactly** as the tutorial says: **mkdir qiime2-moving-pictures-tutorial** and then the command **cd**

qiime2-moving-pictures-tutorial. It will create the directory "qiime2-moving-pictures-tutorial" and place you in that directory.

Next time you start QIIME2, you will have to open the terminal window and write or copy paste **source activate qiime2–2023.5** (or actual version). Then continue with the **cd qiime2-moving-pictures-tutorial**.

There are three choices for downloading data in general in the tutorials. "Browser" clicking on the link and download data. "wget" and "curl" are utilities to download data conveniently via a terminal. Copy paste the "wget" or"curl" command to your command prompt, and the data is saved in your directory.

Download URL: https://docs.qiime2.org/2023.5/tutorials/moving-pictures/

Save as: sample-metadata.tsv

OR

wget-O "sample-metadata.tsv""https://data.qiime2.org/2018.2/tutorials/moving--pictures/sample_metadata.tsv

OR

curl -sL "https://data.qiime2.org/2023.5/tutorials/moving-pictures/sample_metadata.tsv"> "sample-metadata.tsv"8.3. Download metadata

The metadata file contains information about the samples that are necessary for further analysis. For example, the categories included in the tutorial are Sample-ID, BarcodeSequence, and BodySite. If you analyze your own samples, this file has to be created to fit your own analysis. The file has to be in TSV format (tab separated values) and saved in your working directory. For more information about this topic see "Metadata in QIIME2" tutorial.

Choose one of the three alternatives for downloading the sample metadata. For the rest of this exercise, the curl command will be used.

8.10.3 Download Data

First, you need to make a sub-directory **emp-single-end-sequences** to the one you are standing in (qiime2-moving-pictures-tutorial). This is done by the same procedure as used in Sect. 8.10.2:

mkdir emp-single-end-sequences

Now, you download the data from the QIIME2 homepage to this directory by the command:

curl -sL "https://data.qiime2.org/2023.5/tutorials/moving-pictures/emp-single--end-sequences/sequences.fastq.gz"> "emp-single-end-sequences/sequences.fastq.gz"

At this step, it can be a good idea to check if the files are located in your working directory.

cd emp-single-end-sequences

ls –l

Here, you should see the two files **barcodes.fastq.gz** at a size of around 3,783,785 bytes and **sequences.fastq.gz** of 25,303,756 bytes. Remember to navigate back to the qiime2-moving-pictures-tutorial directory by the command **cd ..** .

8.10.4 Change Data Format to QIIME2 Artifacts

The imported data is multiplexed and has to be converted into "QIIME2artifacts" before continuing the analysis.

qiime tools import --type EMPSingleEndSequences --input-path emp-single-end-sequences --output-path emp-single-end-sequences.qza

8.10.5 Demultiplexing Sequences

Demultiplexing is the step where the barcodes of all the single-end-reads are associated with their samples as described above.

qiime demux emp-single --m-barcodes-file sample-metadata.tsv --m-barcodes-column BarcodeSequence --o-per-sample-sequences demux.qza

Create a summary of the distribution of results from demultiplexing. The important information is the summary of the sequence quality at each base in the reads (Interactive Quality Plot in demux.qzv artifact).

qiime demux summarize --i-data demux.qza --o-visualization demux.qzv

See the results with command.

qiime tools view demux.qzv

This command will invoke the browser and show the result in a browser window (**Firefox**). The graphics can be downloaded in PDF format. As the manual elaborates, you can visualize all similar graphics output by the command:

qiime tools view new_file.qzv

where new_file means that the file names will change for different outputs for the different analysis.

If your own project data is already demultiplexed, refer to Sect. 8.10.14.1 to proceed.

When working with paired-end reads, it is a good idea to merge the forward and reverse read, refer to Sect. 8.10.14.2.

8.10.6 Denoising

Denoising is quality filtering of individual reads to remove low-quality reads. In QIIME2, one can choose to use DADA2 or Deblur. Here, DADA2 is used.

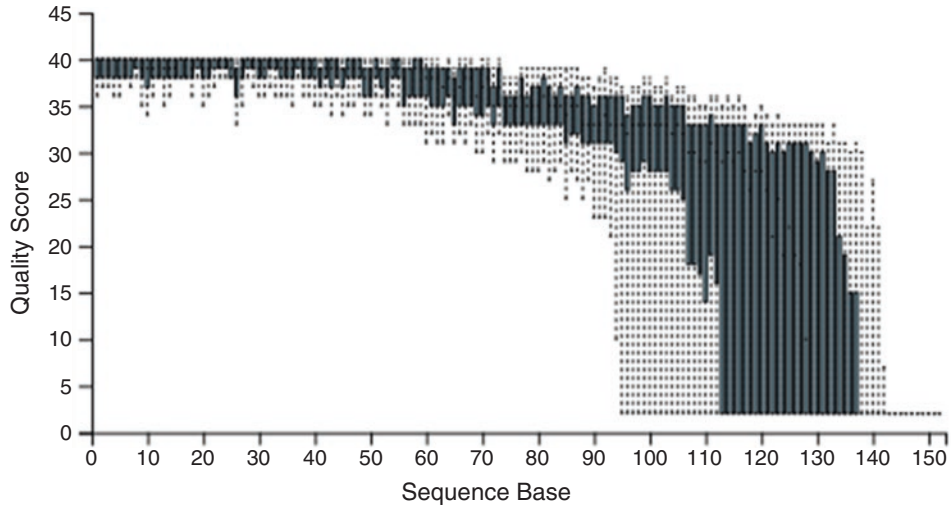


Fig. 8.10 Sequence quality control of the DNA sequences in DADA2. https://qiime2.org/

qiime dada2 denoise-single --i-demultiplexed-seqs demux.qza --p-trim-left 0 --p-trunc-len 120 --o-representative-sequences rep-seqs-dada2.qza --o-table table-dada2.qza

Then, rename the two files created with the command:

mv rep-seqs-dada2.qza rep-seqs.qza

mv table-dada2.qza table.qza

In this example, all reads are truncated to 120 nucleotides. The dataset is from the early 2010s when reads were shorter than the current 250 nucleotides. The truncation value of 120 is chosen based on the quality scores that dropped at this base number. To choose this value, inspect the demux.qzv artifact with the command **qiime tools view demux.qzv,** and in this visualization, view the Interactive Quality Plot (Fig. 8.10).

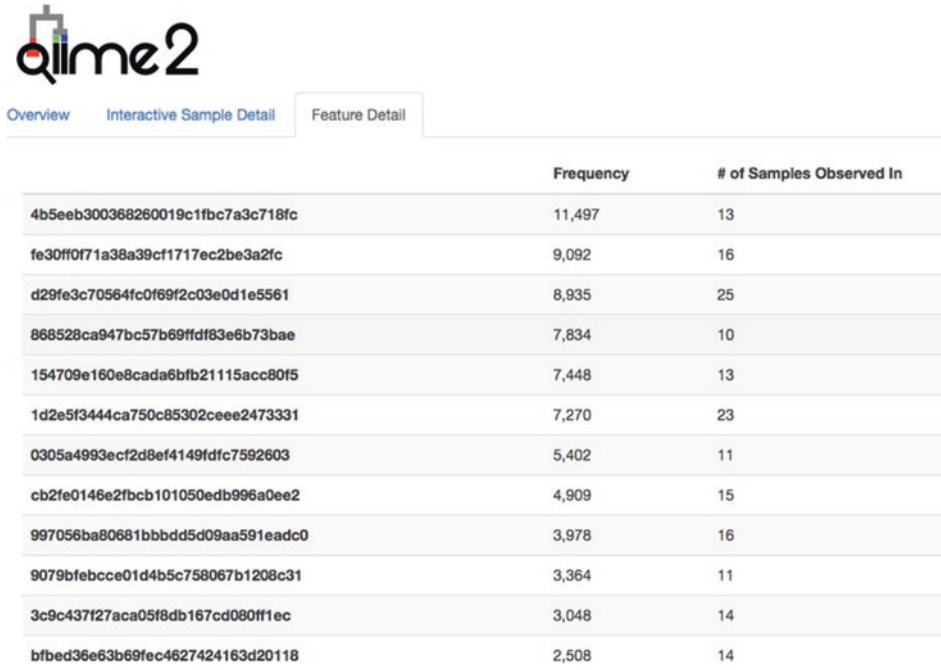
8.10.7 Visualization Summaries of the Data

In the previous exercise, you created FeatureTable (table.qza) and FeatureData (rep-seqs.qza) artifacts. Now, you want to view the content in these files. To do this, use the command below.

qiime feature-table summarize --i-table table.qza--o-visualization table.qzv--m-sample-metadata-file sample-metadata.tsv

qiime feature-table tabulate-seqs --i-data rep-seqs.qza --o-visualization rep-seqs.qzv

The output files are **table.qzv and rep-seqs.qzv** that can be viewed with **qiime tools view** as described earlier.



	Frequency	# of Samples Observed In
4b5eeb300368260019c1fbc7a3c718fc	11,497	13
fe30ff0f71a38a39cf1717ec2be3a2fc	9,092	16
d29fe3c70564fc0f69f2c03e0d1e5561	8,935	25
868528ca947bc57b69ffdf83e6b73bae	7,834	10
154709e160e8cada6bfb21115acc80f5	7,448	13
1d2e5f3444ca750c85302ceee2473331	7,270	23
0305a4993ecf2d8ef4149fdfc7592603	5,402	11
cb2fe0146e2fbcb101050edb996a0ee2	4,909	15
997056ba80681bbbdd5d09aa591eadc0	3,978	16
9079bfebcce01d4b5c758067b1208c31	3,364	11
3c9c437f27aca05f8db167cd080ff1ec	3,048	14
bfbed36e63b69fec4627424163d20118	2,508	14

Fig. 8.11 Feature detail from the artifact table.qzv shown below with feature IDs (OTUs) and their corresponding frequency and samples observed in. https://qiime2.org/

In table.qzv, there are three main summaries: overview, interactive sample detail, and feature detail (Fig. 8.11).

8.10.8 Phylogenetic Tree

The α- and β-diversity metrics such as Faith's Phylogenetic Diversity and Unifrac uses the phylogenetic tree, and therefore, this tree has to be created before these diversity metrics can be performed.

qiime alignment mafft --i-sequences rep-seqs.qza --o-alignment aligned-rep-seqs.qza

qiime alignment mask --i-alignment aligned-rep-seqs.qza --o-masked-alignment masked-aligned-rep-seqs,qza

qiime phylogeny fasttree --i-alignment masked-aligned-rep-seqs.qza --o-tree unrooted-tree.qza

qiime phylogeny midpoint-root --i-tree unrooted-tree.qza --o-rooted-tree rooted-tree.qza

For the next exercise, the **rooted-tree.qza** artifact is used.

8.10.9 α- and β-Diversity Analyses

For the different diversity metrics calculations, you have to choose the value for the **p-sampling depth** by viewing the **table.qzv** artifact (interactive sample detail). The number of reads in each sample has to be normalized, that is, rarefied. This is done by the command **--p-sampling-depth**. It can be difficult to choose this value. The chosen sampling depth should reflect the balance between capturing the alpha-diversity in your samples with high number of reads but low enough to not exclude too many samples with low depths. In this example, 1109 was used as threshold.

qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza--i-table table.qza --p-sampling-depth 1109--m-metadata-file sample-metadata.tsv --output-dir core-metrics-results

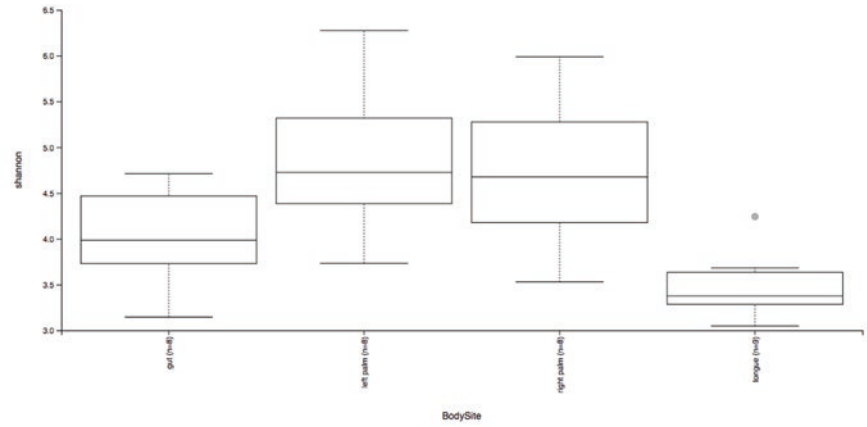
A new directory (core-metrics-results) is created, and it contains all the necessary files you need to explore for the α- and β-diversity (Figs. 8.12 and 8.13).

Shannon's diversity for category BodySites from the tutorial is shown as example (Fig. 8.12). The statistical test used in the boxplots for the different α-diversity metrics is nonparametric Kruskal Wallis test, with p-value at 0.05 considered as statistical significant. Shannon's diversity indexes were significant for BodySites ($p = 0.001$). The highest Shannon value, that is, greater diversity, was observed for palms when compared to tongue and gut. However, the gut/right palm comparison was not significant.

For β-diversity, weighted Unifrac and Bray-Curtis dissimilarity was used as example (Fig. 8.13). Bray-Curtis is a non-phylogenetic method using the abundance information of OTUs, where the distance metric is calculated by the species difference of abundance divided by total abundance in both samples. The PCoA plot shows relatively stable microbial communities during the time period (Figs. 8.14 and 8.15). PERMANOVA is used as significance test with *p*-value at 0.05 as statistical significant.

8.10.10 Alpha Rarefaction Plotting

This step in the analysis process is to explore if you have sequenced deep enough, that is, captured the diversity in the samples. The rarefaction curve demonstrates that the sequencing depth (1109) was sufficient for almost all samples (Fig. 8.16). The sample with the highest sequence count was not sequenced deep enough as the curve is not plateauing as the rest of the samples. So for this sample, the richness would be underestimated for the top curve in red Fig. 8.16.



Kruskal-Wallis (all groups)

	Result
H	16.028817587641115
p-value	0.0011186613628180366

Kruskal-Wallis (pairwise)

Download CSV

		H	p-value	q-value
Group 1	Group 2			
gut (n=8)	left palm (n=8)	3.981618	0.045999	0.068999
	right palm (n=8)	2.481618	0.115184	0.138220
	tongue (n=9)	4.898148	0.026886	0.053771
left palm (n=8)	right palm (n=8)	0.099265	0.752714	0.752714
	tongue (n=9)	10.703704	0.001069	0.006415
right palm (n=8)	tongue (n=9)	8.898148	0.002855	0.008564

Fig. 8.12 Box plot for Shannon's diversity index for category BodySite from the tutorial "Moving Pictures" in QIIME2. https://qiime2.org/

8.10.11 Taxonomic Analysis

In this step, the bacterial DNA is annotated to the Greengenes database using the classifier (gg-13-8-99-515-806-nb-classifier.qza). The rep-seqs.qza artifact is used here, and it contains the feature IDs and direct link to the NCBI database.

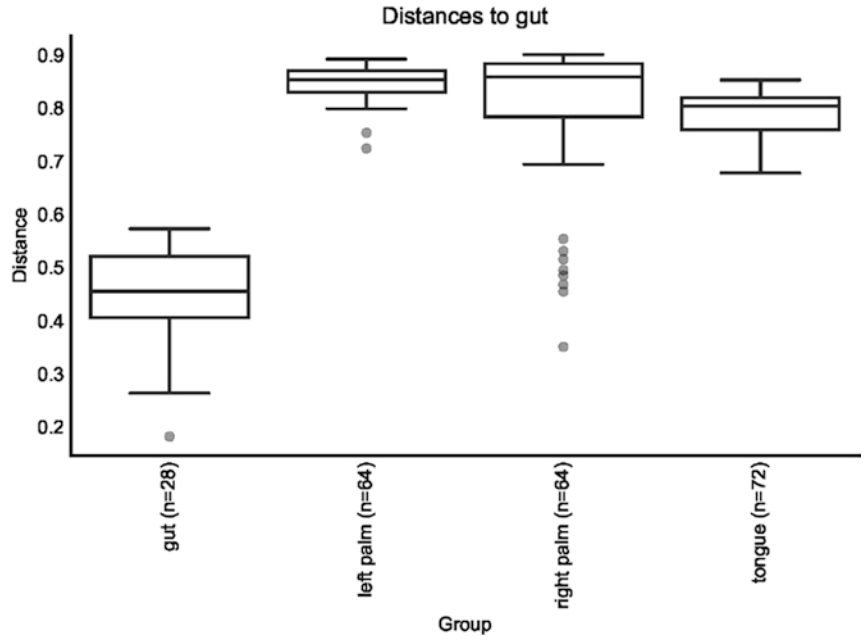
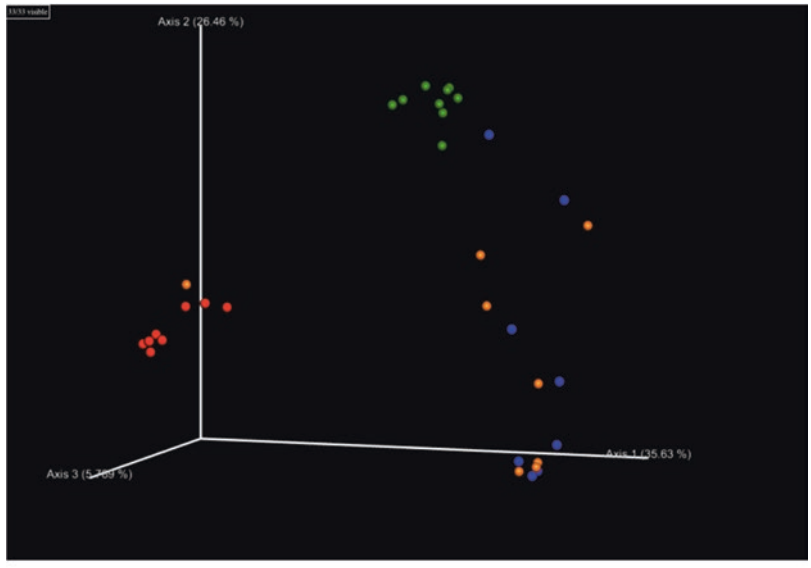


Fig. 8.13 Box plot for β-diversity with weighted Unifrac as output example for category BodySites from the "Moving Pictures" tutorial in QIIME2. PERMANOVA was used as statistical significance test (https://qiime2.org/)



Colors red=gut, blue=left palm, orange=right palm, green=tongue

Fig. 8.14 Plots of PCoA based on Unifrac distance matrices of microbial communities in gut, left palm, right palm, and tongue from the "Moving Pictures" tutorial in QIIME2. https://qiime2.org/

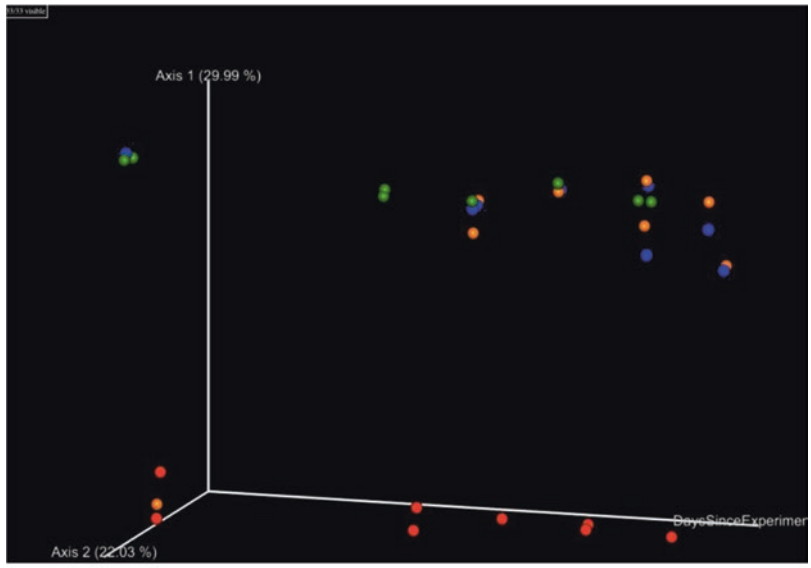


Fig. 8.15 Plots of PCoA based on Bray-Curtis dissimilarity of microbial communities in gut, left palm, right palm, and tongue during DaysSinceExperimentStart. "Moving Pictures" tutorial in QIIME2 (https://qiime2.org/)

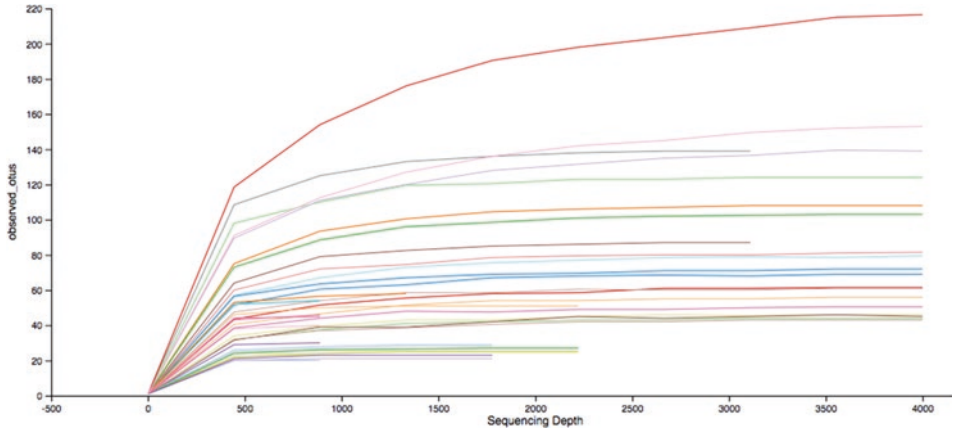


Fig. 8.16 Alpha rarefaction curve from the "Moving Pictures" tutorial in QIIME2. https://qiime2.org/

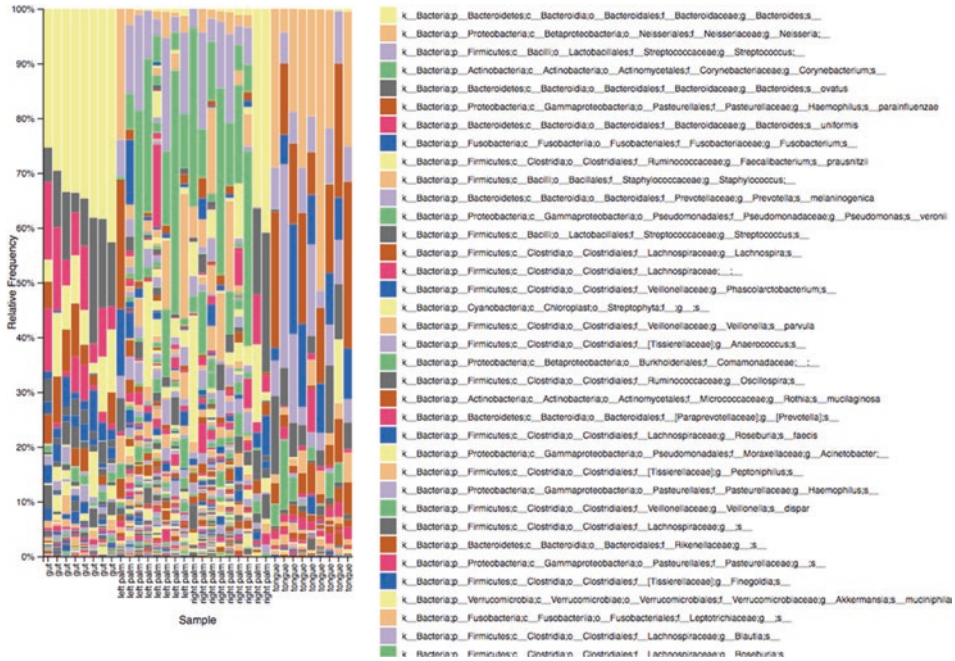


Fig. 8.17 Taxa bar plot with classified bacteria and their relative abundance in the samples for category BodySites. Example from the "Moving pictures" tutorial in QIIME2 (https://qiime2.org/)

To improve taxonomic classification, creating your own classifier might be beneficial. To do that, please follow the instructions in the tutorial "**Training feature classifiers with q2-feature-classifier**" in Sect. 8.10.14.3.

Results from this analysis are shown in Fig. 8.17 (genus level).

8.10.12 Exporting Data

QIIME2 is a good tool to start analyzing your data. However, to further explore and visualize your data, we recommend you to export relevant files from QIIME2.

To do this, you have to convert the QIIME2 artifacts into formats that are suited for export and visualization in other programs. Use the "Exporting data tutorial" in QIIME2 to export your files, for example, exporting the feature table (table.qza):

qiime tools export table.qza –output-dir exported-table

Note: you might also have to convert your BIOM table to TSV format for convenience.

biom convert exported/feature-table.biom --o-feature-table.tsv --to-tsv

8.10.13 Filtering Data

The tutorial "filtering data" in QIIME2 explains how to filter feature tables, sequences, and distance matrices, forexample, if you want to modify a taxa bar plot (Fig. 8.10). The q2-taxa plugin sorts the taxa as you want to display the results.

An alternative for this step is to convert the **taxonomy.qza** file to TSV format (see above) and import into your favorite statistics program (e.g., R).

8.10.14 Good to Know When Working with QIIME2

8.10.14.1 Already Demultiplexed Data

Your project data might already be demultiplexed and still paired. Use the "Importing data" tutorial and create a "Fastq manifest" file in CSV format and then follow the instructions for single-end reads or paired-end reads. The final output files are: paired-end-demux.qza (or single-end-demux.qza for single-end reads). Paired-end reads with PHRED offset 33 were chosen as the shown example from the tutorial.

The manifest file must be in CSV format with the header line *sample-id, absolute-filepath, direction*. There can only be one line per sample-id as shown below:

sample-id,absolute-filepath,direction
sample-1,$PWD/some/filepath/sample1_R1.fastq,forward
sample-1,$PWD/some/filepath/sample1_R2.fastq,reverse

Import the manifest file (e.g., pe-33-manifest) you have created with command:

qiime tools import --type 'SampleData[SequencesWithQuality]' --input-path se-33-manifest --output-path single-end-demux.qza --source-format SingleEndFastqManifestPhred33

When you have converted the manifest file to a QIIME artifact, visualize it with:

qiime demux summarize --i-data paired-end-demux.qza --o-visualization paired-end-demux.qzv

If you use Deblur instead of DADA2 continue to the step merging reads (refer to Sect. 8.15.2), otherwise follow the instructions in "Sequence and quality control and feature table construction" for "Moving Pictures."

8.10.14.2 Merging of Paired-End Reads

The sequence quality control can be performed wither with DADA2 or Deblur. If you use Deblur, the forward and reverse reads have to be merged first as it only works for single-end reads. The plugin vsearch join-pairs is used for this step of the analysis (https://docs.qiime2.org/2018.2/plugins/available/vsearch/). The commands for join pairs are shown below.

qiime vsearch join-pairs --i-demultiplexed-seqs paired-end-demux.qza --o-joined-sequences demux-joined.qza

qiime demux summarize --i-data demux-joined.qza --o-visualization demux-joined.qzv

Now, you can run the **demux-joined.qza** through the sequence quality control with Deblur (refer to "Moving Pictures" tutorial in QIIME2).

8.10.14.3 Train Your Classifier

The tutorial "Training feature identifiers with q2-feature-classifier" guides you through the steps that create the specific classifier for your dataset (https://docs.qiime2.org/2023.5/tutorials/feature-classifier/). The elements required are Greengenes reference sequences, your own rep-seqs.qza, primer sequences used for your dataset, and the length of the reads (value chosen in Deblur's quality check).

8.10.14.4 Plugins

If you cannot find the desired functions in the tutorials provided, view the available plugins (https://docs.qiime2.org/2023.5/plugins/). For example, heatmaps are often used for visualization of the count data. In QIIME2, there is a plugin called feature-table (with heatmap) for this purpose. Bar plots generate an overview of the sample taxonomic makeup. These plots can, however, be difficult to read on lower taxonomic levels. The plugin "feature-table" in QIIME2 can be used to plot heatmaps from the features tables.

8.10.14.5 Decontamination

Contamination disrupts the accuracy of microbiome studies and is a known problem in this field. Upon until recently, there was no standard statistical method to handle these contaminants in the bioinformatics analysis. Davis et al. (2018) published a study about a new available open-source R-package, decontam, that they created for the purpose to identify and remove the contaminants in marker gene (especially low biomass samples) and metagenomics (Davis et al. 2018). Download it from (https://github.com/benjjneb/decontam).

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Full Shotgun DNA Metagenomics

9

Henrik Christensen and John Elmerdahl Olsen

Contents

What You Will Learn

Full DNA metagenomics includes the ultimate way to sequence all DNA of a sample and to obtain all information of the microbiome. The microbiome can be analyzed with respect to taxonomic comparison and to functional characteristics. We will mainly focus on the pipeline MG-RAST to demonstrate different steps in the analysis. MG-RAST can also be used to archive data, to download data, and to share data with the scientific community.

H. Christensen (✉) · J. E. Olsen
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; jeo@sund.ku.dk

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9.1 Background

Full DNA metagenomics is the sequencing of all DNA from a sample followed by assembly of DNA sequence reads and annotation and assignment of sequence information to known organisms and functions. The assembly attempts to reconstruct genome fragments to draft genomes. The technique involves most of the other subjects described in the book (Fig. 9.1).

The first real shotgun sequencing project was performed by Tyson et al. (2004). A very simple microbial community (with respect to diversity) of acid mine drainage was investigated (Fig. 9.2). The scientists managed to assemble two major bacterial genomes, Leptospirillum groups II and III (Bacteria) and a third composed of populations of Ferroplasma type II (Archaea). The study was based on 103,462 reads obtained by Sanger sequencing of plasmid inserts.

Another outstanding study was the in silico assembly of the genome of the organism *Candidatus* Chloracidobacterium thermophilum, an aerobic phototrophic acidobacterium (Bryant et al. 2007). The metagenomic data from the phototrophic microbial mats of an alkaline siliceous hot spring in Yellowstone National Park allowed the assembly of this distinctive bacteriochlorophyll (BChl)-synthesizing, phototrophic bacterium without any previous isolation and cultivation.

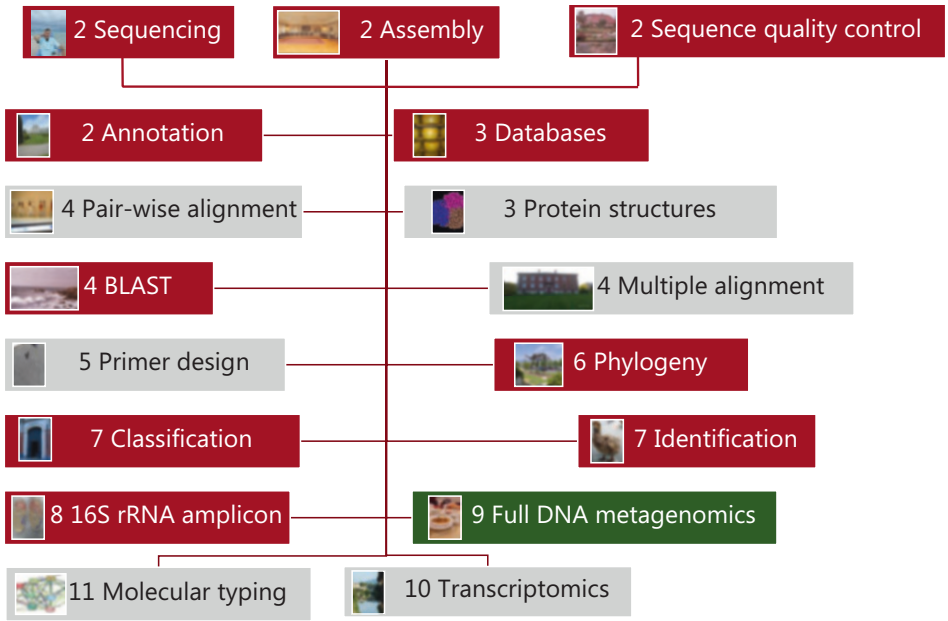

Fig. 9.1 Full DNA metagenomics is related to nearly all other chapters in the book

Fig. 9.2 Picture of acid mine discharge. This type of sample was used for the first full DNA metagenome study of Tyson et al. (2004). US Geological Survey http://wwwbrr.cr.usgs.gov/projects/GWC_chemtherm/ironmtn.htm, Public Domain, https://commons.wikimedia.org/w/index.php?curid=2085336

The study of Patro et al. (2016) represents a more recent application of metagenomics where different commercial probiotic products were analyzed for their contents of specific strains. Most metagenomics studies probably represent the human gastrointestinal microbiome (2751 publications in PubMed from 2005 until now).

The full DNA metagenomics concept has early on been used to diagnose infections caused by microorganisms in relation to human and animal medicine (Nakamura et al. 2008). In that investigation, *Campylobacter jejuni* was identified in a patient with diarrhea. A similar investigation of *Shigella* was reported more recently (Liu et al. 2018). A full DNA metagenomic approach to clinical diagnostics will overcome the problems with non-culturable bacteria that are problematic to identify by other methods. The level of detection is depending on the sequencing "depth" meaning the sequenced coverage of an expected target. This relationship is not yet standardized but has to be evaluated from case to case. More recent use of the Nanopore technology has allowed the surveillance of human pathogens online and animal pathogens in situ (Fomsgaard et al. 2023).

Also in food production, full DNA metaganome has been tested for detection of *Escherichia coli*, *Salmonella enterica*, and *Clostridium botulinum* (Yang et al. 2016). However, it was recognized that the technique was impractical for routine use.

The benefit of full DNA metagenomics is that the general diversity of all organisms can be investigated. The most serious drawback with the full DNA metagenomic technique is the limitations of the databases in particular with respect to taxonomy. Some pipelines are not able to extract 16S rRNA sequence information and provide search in the 16S rRNA databases, which is further limiting the analysis. Another drawback is the higher cost of sequencing the full DNA metagenome compared to 16S rRNA amplicon sequencing.

9.2 Sequencing Strategies and Data Types

For experimental design and DNA extraction in general, we refer to other publications. Here, as well as for 16S rRNA amplicon sequencing (Chap. 8), we will refer to the MO BIO PowerMag® Soil DNA Isolation Kit (Qiagen) kit that has been used for DNA extraction. For the investigation of microbial communities, the output of DNA after extraction for sequencing should include at least 50% microbial DNA.

For sequencing strategies, see Table 9.1. In the past, before high-throughput sequencing was invented, full DNA shotgun sequencing of cloned libraries was used. Figure 9.3 shows an example of such analysis where a dataset of the microbiome of mother and child was generated by sequencing of fosmid libraries. The taxonomic groups were predicted from by searching Genbank.

End sequencing was a shortcut to the analysis of the cloned libraries of BAC (bacterial artificial chromosome) or fosmid libraries. Conserved primers complementary to the cloning vector are used to sequence the 5′ and 3′ ends of the inserts in the vectors. Such a sequence can be used to determine the type of organisms inserted and maybe to locate the insert on a fully sequenced organism. The information generated from the end reads have been used to select clones for full sequencing.

The benefit of full DNA shotgun sequencing with cloned libraries is that the cloned libraries can be kept and reanalyzed compared to the approach of using extracted DNA without cloning where the possibility for reanalysis ends when the DNA tube is empty. However, the sequencing capacity with cloned libraries is very limited compared to sequencing of DNA directly by high throughput without cloning.

Full DNA shotgun high-throughput sequencing without cloning is now the preferred method for sequencing. Sequencing platforms from Illumina and Oxford Nanopore are the

Table 9.1 Sequencing strategies in full DNA metagenomics

Strategy	Outline	Benefit	Drawback	Application
Full DNA shotgun	All DNA of a sample is high-throughput sequenced	High information content	Costly and analysis demanding	Main method
Full DNA with cloning	All DNA of a sample is shotgun cloned and sequenced	High information content, libraries can be kept	Extremely costly and demanding to analysis	Used before high-throughput sequencing. Now only used for special applications where libraries are needed
Full DNA with cloning and end sequencing	All DNA of a sample is shotgun cloned but clones are only end sequenced	Less work demanding compared to sequencing of full libraries	Lower information content (only end reads of clones)	Used for initial screening of full libraries

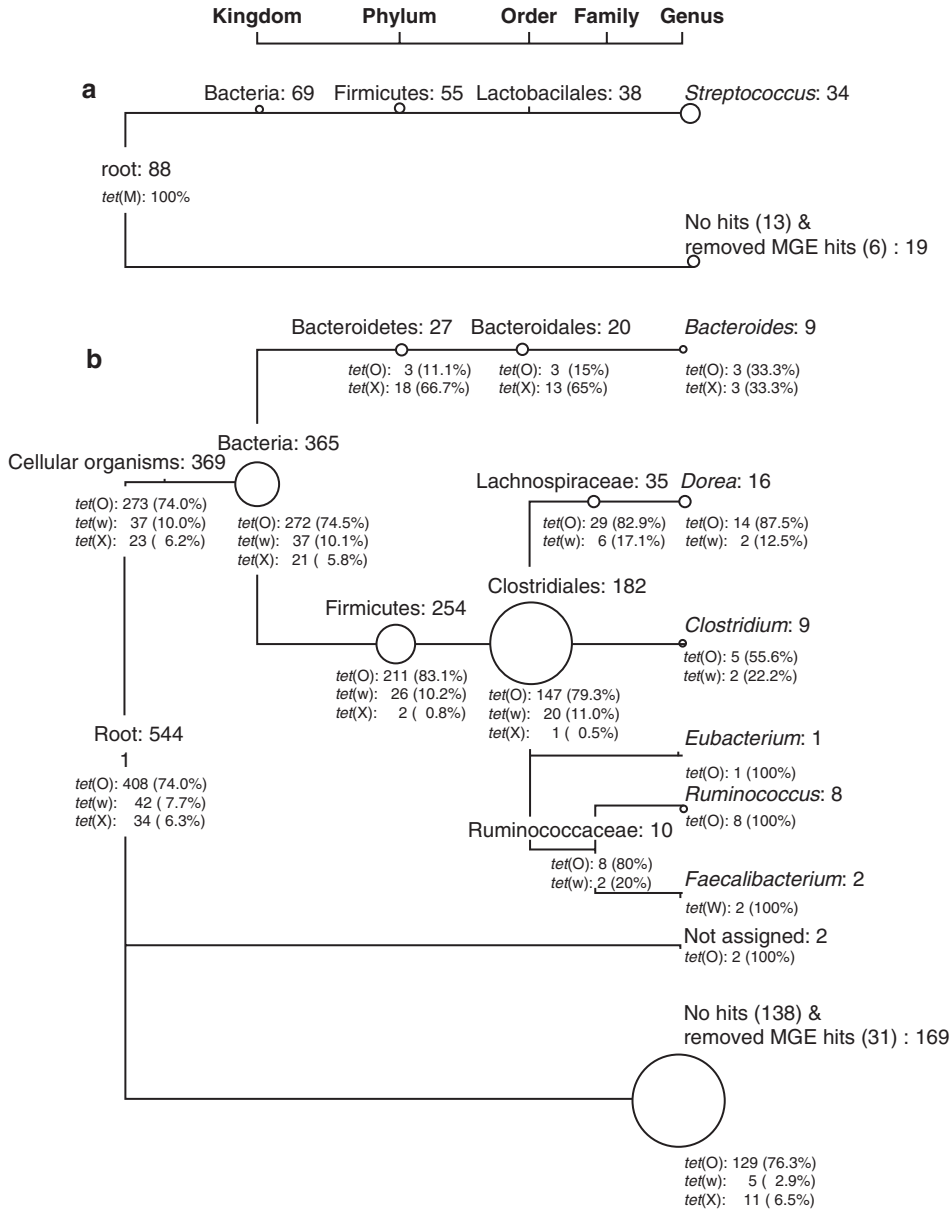

Fig. 9.3 Microbial diversity of tetracycline resistance fosmid clones in infant (**a**) and mother (**b**). The MEGAN (Huson et al. 2016) (Table 9.2) tree is collapsed at genus level and summarizes the numbers of reads assigned at different taxonomical levels with the size of the node proportional to the number of reads assigned to the specific node. For each taxon level, the number of reads assigned to different tetracycline resistance genes is shown (from de Vries et al. 2011) (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0021644)

most frequently used. DNA extracts from samples can be processed with the Nextera XT DNA sample Prep Kit (Illumina). The same principles of barcoding and indexing samples as used for 16S rRNA amplicon sequencing (Chap. 8) are used. Paired-end sequencing can, for instance, be performed on the MiSeq platform (Illumina).

9.3 Analysis of Full DNA Shotgun Sequence Data

A recent review of current pipelines has been provided by Liu et al. (2021). After sequencing, the raw reads need to be de-multiplexed (to associate barcodes with samples) and converted to FASTAQ format files. This step can be done on the MiSeq sequencing platform. Further handling of data requires a bioinformatics pipeline. It is recommended to perform a control for completeness and contamination by CheckM (Parks et al. 2015) (http://ecogenomics.github.io/CheckM/) or similar program.

Removal of host DNA from raw reads requires procedures to map raw reads to host DNA for filtering the reads out (Bowtie 2) (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml) (Langmead and Salzberg 2012). An alternative is Trimmomatic (Bolger et al. 2014) used in combination with Bowtie 2 in the KneadData pipeline (https://huttenhower.sph.harvard.edu/kneaddata/)

Quality guidelines have been published for metagenome-assembled genomes (Bowers et al. 2017). According to the paper, there are a range of criteria but no strict definition or guidelines to assemble genomes from metagenomes. Like for whole genomes from cultured bacterial isolates, key parameters are total assembly size, contig N_{50}, and maximum contig length. Sequences should be assembled to the longest possible contigs (Chap. 2). The assembly may involve assembly of scaffolds (genome frame).

For high-throughput sequencing data, the initial steps in the bioinformatical analysis with quality control and filtering are the same as described for 16S rRNA amplicon sequencing (Chap. 8). Full DNA metagenomics differs from 16S rRNA amplicon sequencing in that open reading frames (ORF) are predicted to encode for proteins. ORFs are compared to the existing databases (Chap. 3). The database search can link information about organism (taxonomy) with function. This way taxonomy is predicted from the ORF in the databases.

The pipelines QIIME2 and Morthur described in relation to 16S rRNA amplicon sequencing (Chap. 8) can be used; however, for full DNA shotgun sequencing, other pipelines are also available (Table 9.2). MEGAN was introduced by Huson et al. (2007) linking taxonomy with function (Fig. 9.3). The platform has been updated (Huson et al. 2016). MEGAN assigns taxonomic relationships by comparison to NCBI taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy) using the lowest common ancestor (LCA) algorithm. Comparison can be made to the most common databases for functional identification.

Kraken 2 performs k-mer matching to NCBI databases (RefSeq or nonredundant) using lowest common ancestor (LCA) algorithms to perform taxonomic classification.

Table 9.2 Bioinformatics pipelines for analyzing full DNA shotgun data

Name	Functions	URL	Reference
EBI Metagenomics	Analysis and archiving of metagenomic data. Phylogenetic diversity as functional and metabolic potential of a sample	https://www.ebi.ac.uk/metagenomics	Richardson et al. (2023)
GhostKOALA	Metagenomic annotation in the KEGG database	https://www.kegg.jp/ghostkoala/	Kanehisa et al. (2016)
IGC	Dedicated human microbiome analysis	https://db.cngb.org/microbiome/genecatalog/genecatalog_human/	Li et al. (2014)
Kraken	Default database is NCBI RefSeq complete genome database. To investigate environments with well-known target organisms	https://ccb.jhu.edu/software/kraken2/	Wood and Salzberg (2014), Lu et al. (2022)
MEGAHIT	Assembly of large metagenomics datasets	https://github.com/voutcn/megahit	Li et al. (2016)
MEGAN	Linking taxonomy with function	https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/informatik/lehrstuehle/algorithms-in-bioinformatics/software/megan6/	Huson et al. (2016)
MetaPhlAn	Human Microbiome Project	http://huttenhower.sph.harvard.edu/metaphlan	Truong et al. (2015)
Morthur	Analysis of data	https://www.mothur.org/	Schloss et al. (2009)
MG-RAST	Automatic quality control, storage of data, annotation, and comparative analysis of samples on a simple interface	https://www.mg-rast.org/	Meyer et al. (2008)
QIIME2	Analysis of data	https://qiime2.org/	Caparoso et al. (2010), Bolyen et al. (2019)

MEGAHIT can be used to assemble large metagenomics datasets (Li et al. 2016). It is based on a modified de Brujin strategy for assembly and is typically installed on a server. The program was many times faster the SPAdes with test dataset.

A variant of SPAdes, metaSPAdes (Nurk et al. 2017), can also be used for assembly in parallel to SPAdes for whole genomic sequences described in Chap. 2.

For annotation of assembled metagenomics data, PROKKA can be used as also described in Chap. 2.

Metagenomic datasets are comprehensive and typically ranges from 6 to 9 GB for individual samples and extents to 30–300 GB if data are contaminated with host DNA (Liu et al. 2021). A collection of pipeline scripts for amplicon and full DNA metagenome can be found at https://github.com/YongxinLiu/Liu2020ProteinCell.

9.4 MG-RAST

The focus in this chapter will be on MG-RAST (metagenoics RAST) (Meyer et al. 2008) since it is user-friendly. MG-RAST can automatically perform assessment of sequence quality and annotation with respect to multiple databases based on uploaded of raw metagenomic sequence data (Keegan et al. 2016). In MG-RAST, preprocessing including removal of low-quality reads, dereplication (merging of identical reads), DRISEE, screening and removal of host-specific sequences including human, gene calling, protein sequence clustering, protein identification, annotation mapping, and abundance profiling are done in the pipeline. DRISEE (duplicate read inferred sequencing error estimation) accounts for artificial overrepresentation of certain genes (PCR artifact). Protein sequence clustering is clusters of proteins build on 90% similarity. This is done to simplify the computational analysis.

MG-RAST has several databases available and, for instance, can functional annotation be done against a multisource protein database M5nr (MD5 based on nonredundant protein databases) (Fig. 9.4). Subsystems under SEED (see description of RAST in Sect. 2.5) can be used for further functional annotation. (Keegan et al. 2016) (Fig. 9.5). The search in the databases is performed by BLAST (see Chap. 4) or BLAT (Kent 2002). If 16S rRNA sequences can be extracted from the dataset, the databases Silva, Greengenes, or RDP can be used for taxonomic identification like described in Chap. 8. The abundance plot shows the abundances of specific taxonomic categories (Figs. 9.6 and 9.7). MG-RAST is able to calculate α-diversity (Fig. 9.8).

MG-RAST allows both taxonomy prediction based on the approach in Chap. 8 based on the rRNA databases SILVA, Greenegenes, and RDP as well as the prediction achieved from the ORF. The databases used for the ORF are very much limited with respect to organism diversity, and for data where a high degree of non-cultured prokaryotes are expected, the 16S rRNA function in MG-RAST will probably improve the taxonomic prediction.

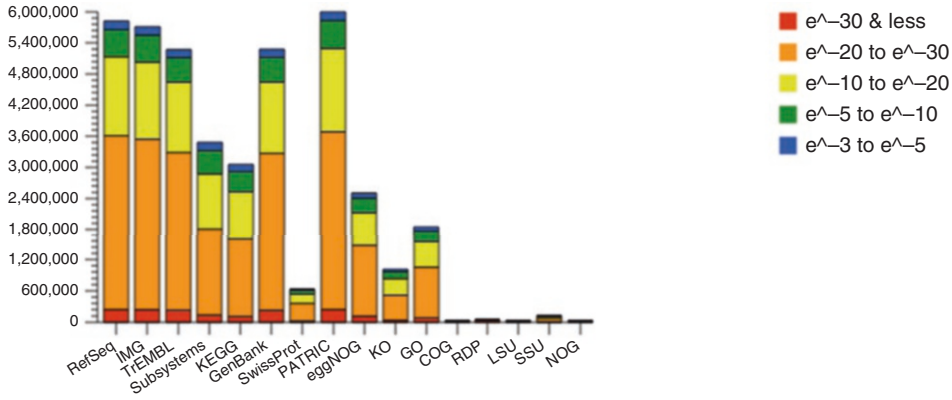

Fig. 9.4 Distribution of database hits based on the MG-RAST (Meyer et al. 2008). An example is shown based on the acc. no. mgm4629274.3 reported in Young et al. (2016) (https://www.mg-rast.org/). The sample is from cat feces, and the figure shows how many annotated sequence reads that are found in the different databases. The annotated reads are finding most hits in the databases PATRIC (part of MG-RAST), RefSeq, IMG, TrEMBL, and GenBank. The expected value e indicates the chance that the sequence is present in the database (see explanation of e in Sect. 4.3.4)

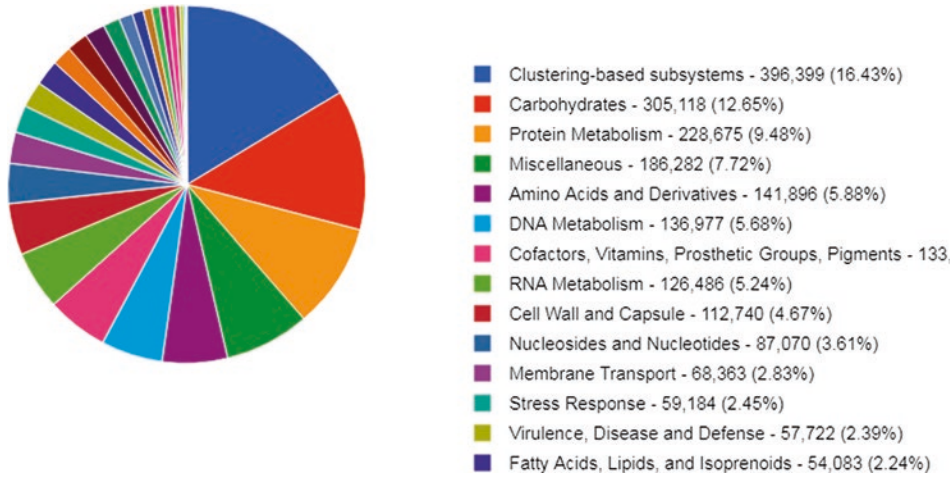

Fig. 9.5 Distribution of subsystem in MG-RAST (Meyer et al. 2008) using acc. no. mgm4629274.3 reported in Young et al. (2016) (https://www.mg-rast.org/) as an example. The sample is from cat feces, and the figure shows the distribution of annotated sequence reads on the functional protein subsystem categories in the databases of MG-RAST

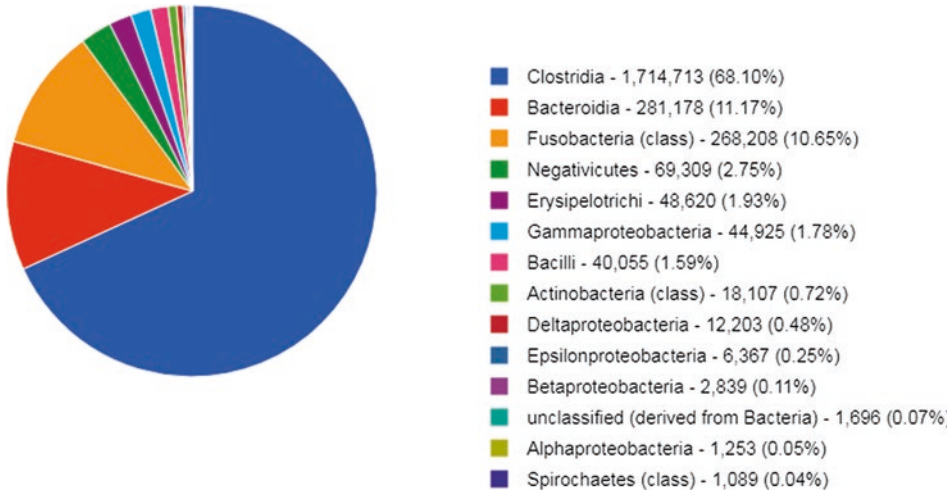

Fig. 9.6 Distribution of taxonomic hits based on the MG-RAST (Meyer et al. 2008) based on an example with acc. no. mgm4629274.3 reported in Young et al. (2016) (https://www.mg-rast.org/). The sample is from cat feces, and the figure shows the distribution of sequence reads on class level of prokaryotes

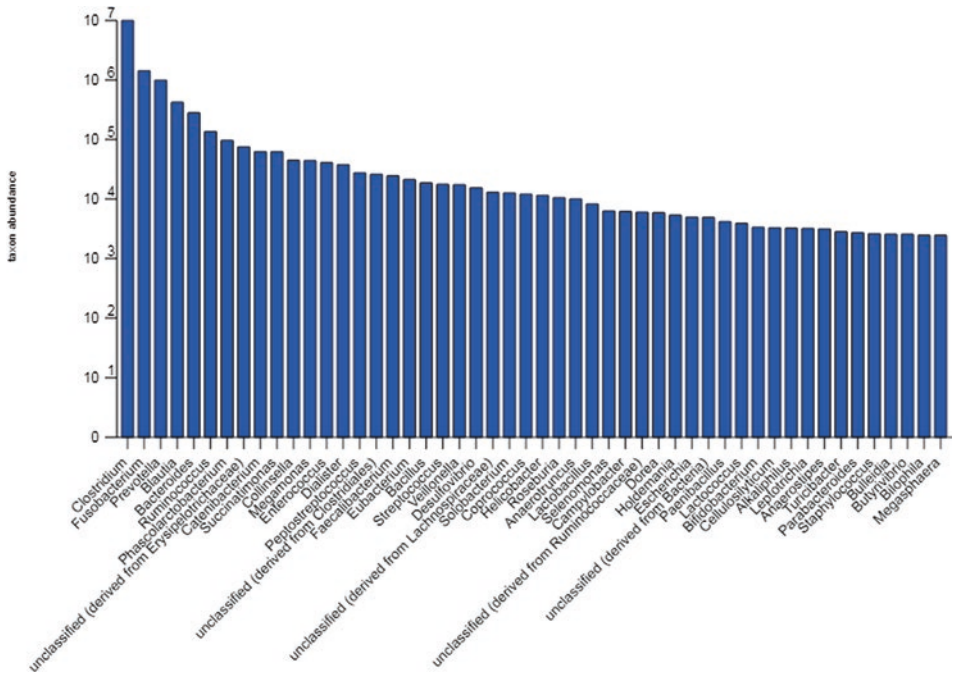

Fig. 9.7 Distribution of taxonomic hits at genus level based on the MG-RAST (Meyer et al. 2008) using acc. no. mgm4629274.3 reported in Young et al. (2016) (https://www.mg-rast.org/) as an example. The sample is from cat feces, and the figure shows the abundance of sequence reads with respect to genera of prokaryotes

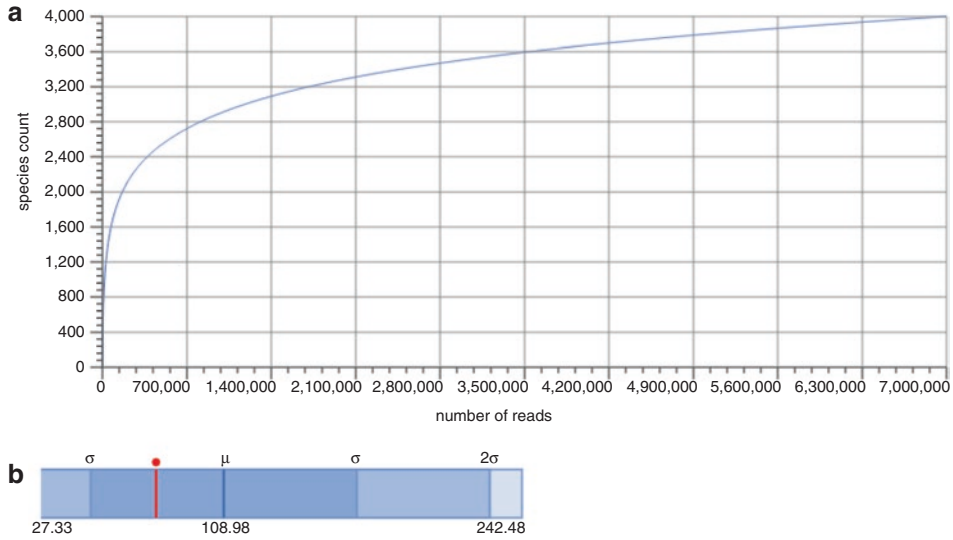

Fig. 9.8 Rarefaction plot (**a**) and α-diversity (**b**) based on the MG-RAST analysis (Meyer et al. 2008) of the sample with acc. no. mgm4629274.3 reported in Young et al. (2016). The sample is from cat feces, and the plot in A shows the total number of distinct species annotations as a function of the number of sequences sampled. The more flat the curve, the less likely it is to find additional species. When this type of curve levels off, a sufficient number of reads have been sequenced for a given sample. The plot in B shows that the α-diversity is 79 (red spot) in this example meaning that the 79 different species have been estimated. The min, max, and mean values are shown, with the standard deviation ranges (σ and 2σ) (https://www.mg-rast.org/).

Alpha-diversity evaluates species richness in each sample as shown in Fig. 9.8 and explained in Chap. 8.

Beta diversity compares diversity between samples. First, distances between samples are calculated, and then eigenvector ordination-based methods are used to group data by principal component methods. Statistical differences between taxonomic units identified can be evaluated by permutational multivariate analysis of variance (PERMANOVA), for instance, in vegan. Relative abundance of taxonomic units can further be considered.

9.5 Upload and Analyze on MG-RAST

To prepare a dataset for analysis on MG-RAST, we will use the data from Patro et al. (2016) as an example. The study compared the content of ten commercial probiotics products. Sequences were generated by Miseq (Illumina) and deposited under PRJNA291686. Raw reads are downloaded from SRA (SRR128530- SRR128558). First, a metadata file was generated (Figs. 9.9 and 9.10).

When the metadata file has been approved on the server (Figs. 9.9 and 9.10), the data can be uploaded (Figs. 9.11, 9.12, 9.13, 9.14) and will then be quality controlled. When

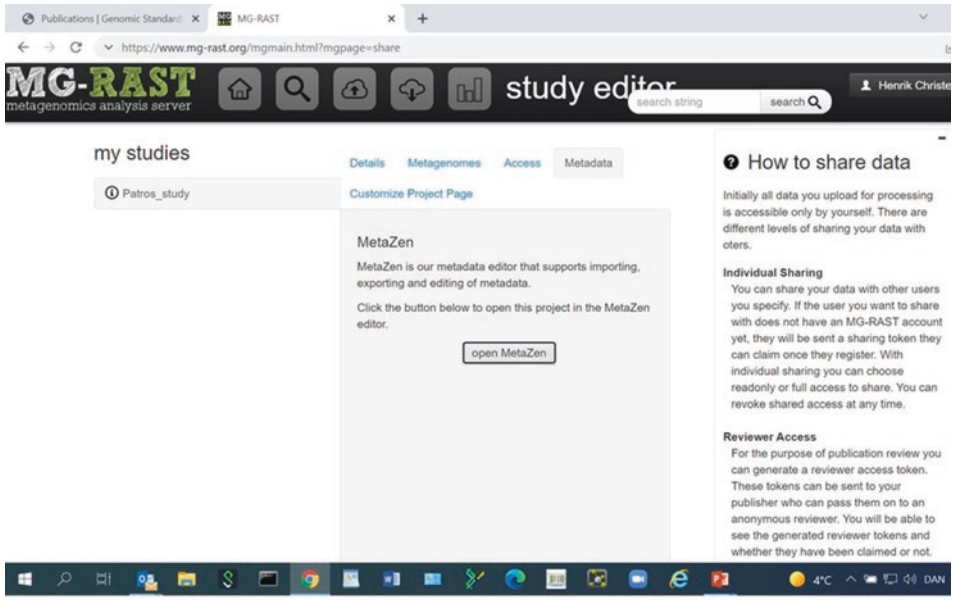

Fig. 9.9 MG-RAST. Start generation of meta-data in MetaZen

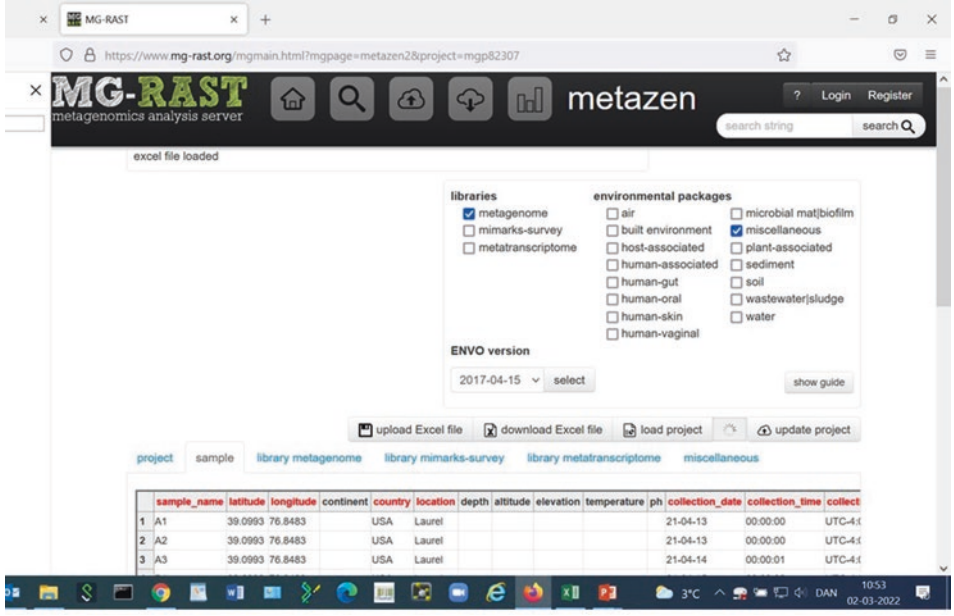

Fig. 9.10 MG-RAST. Further definition of sample material in meta-data

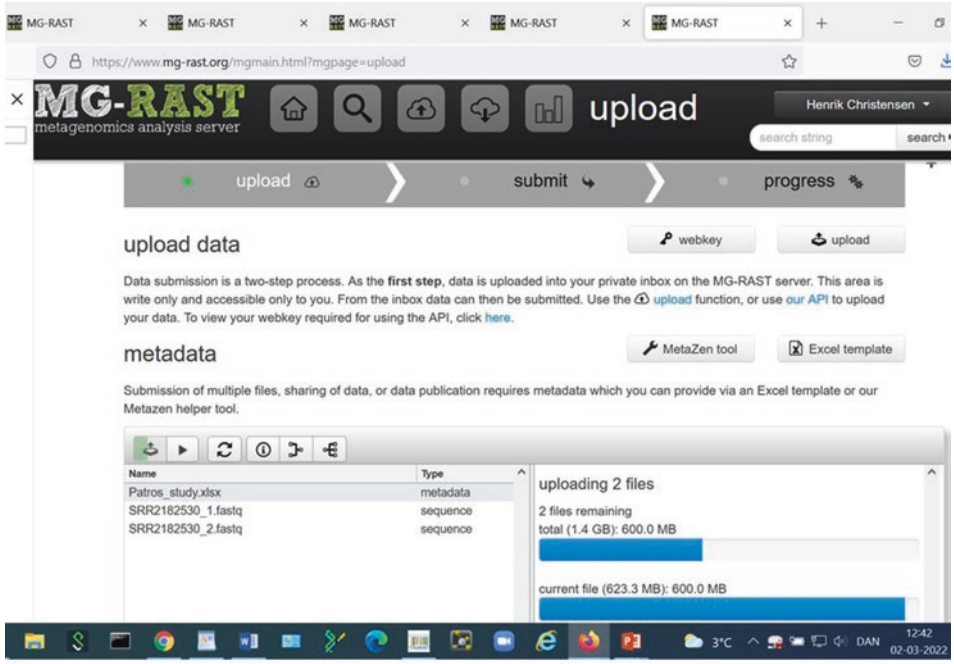

Fig. 9.11 MG-RAST. Upload of data after meta-data has been approved

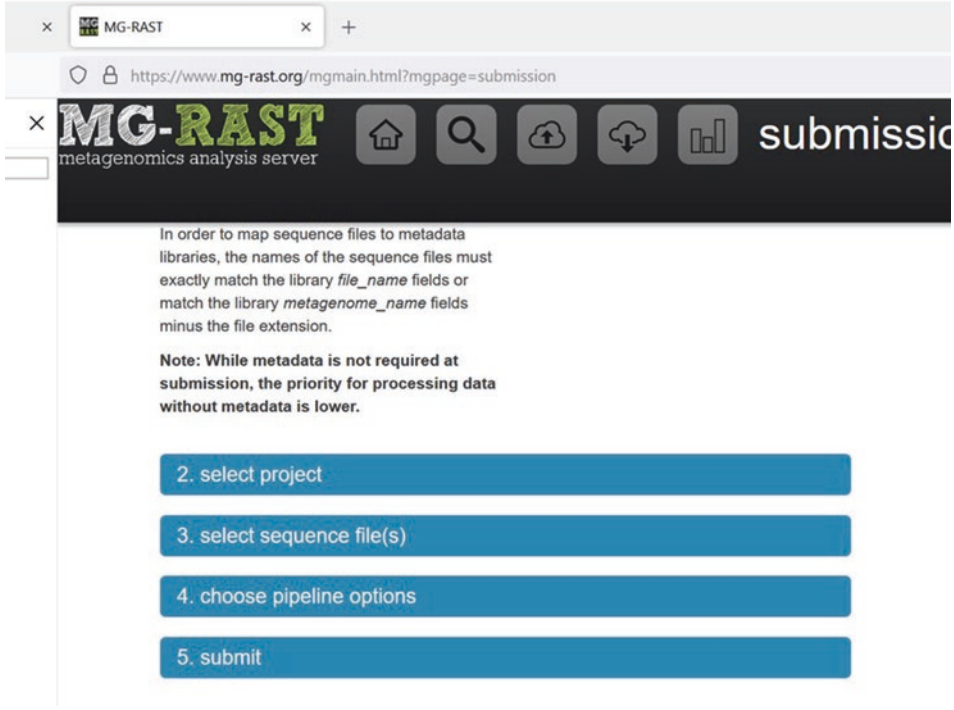

Fig. 9.12 MG-RAST. Overview of steps in analysis pipeline

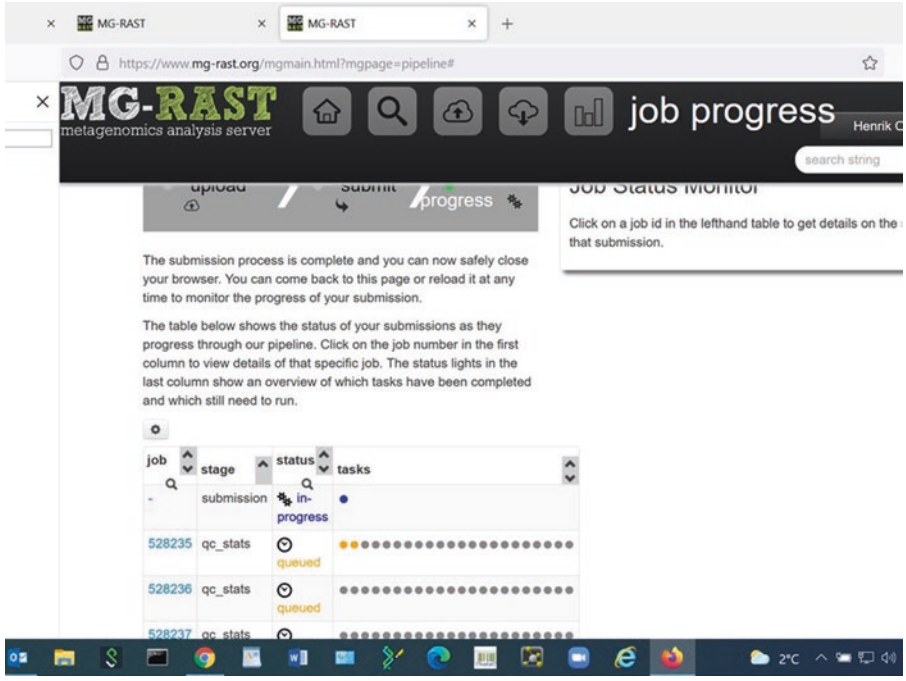

Fig. 9.13 MG-RAST. Analysis is processed

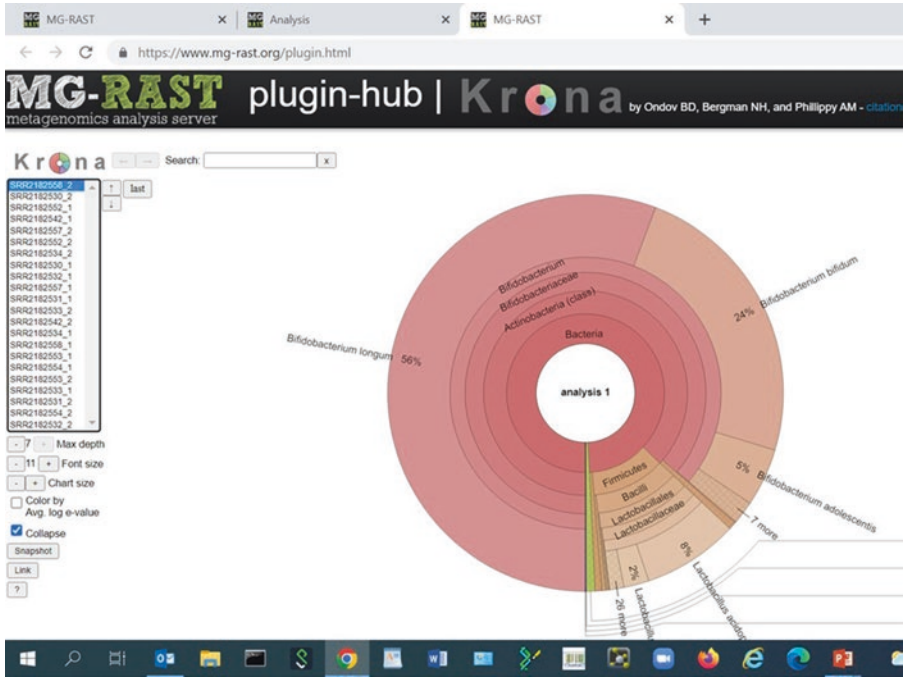

Fig. 9.14 MG-RAST. One of the outputs of results. The Krona plot

metadata are approved and sequence data quality controlled, the analysis can begin like described in Sect. 9.4 including Krona plots (Figs. 9.11, 9.12, 9.13, 9.14) (https://hpc.nih.gov/examples/krona_taxonomy.html).

9.6 Activities

9.6.1 Full DNA Metagenome–Shotgun DNA Metagenomics

We will use data reported in Young et al. (2016), which are available from MG-RAST. This is a metagenomics study of feces from 20 cats. The 20 datasets are listed in the paper, and we will only look at the first dataset mgm4629274.3. This is the MG-RAST acc. no. and you can view a lot of information about the dataset in MG-RAST.

Open MG-RAST from https://www.mg-rast.org/ (Fig. 9.15). Type in the number mgm4629274.3 in the search field, then press the search button. Note that some of the metadata seem odd (temperate broadleaf and mixed forest biome). It is just because we expect something about cats. These metadata are referring to the geographic location of the cats including the natural vegetation type.

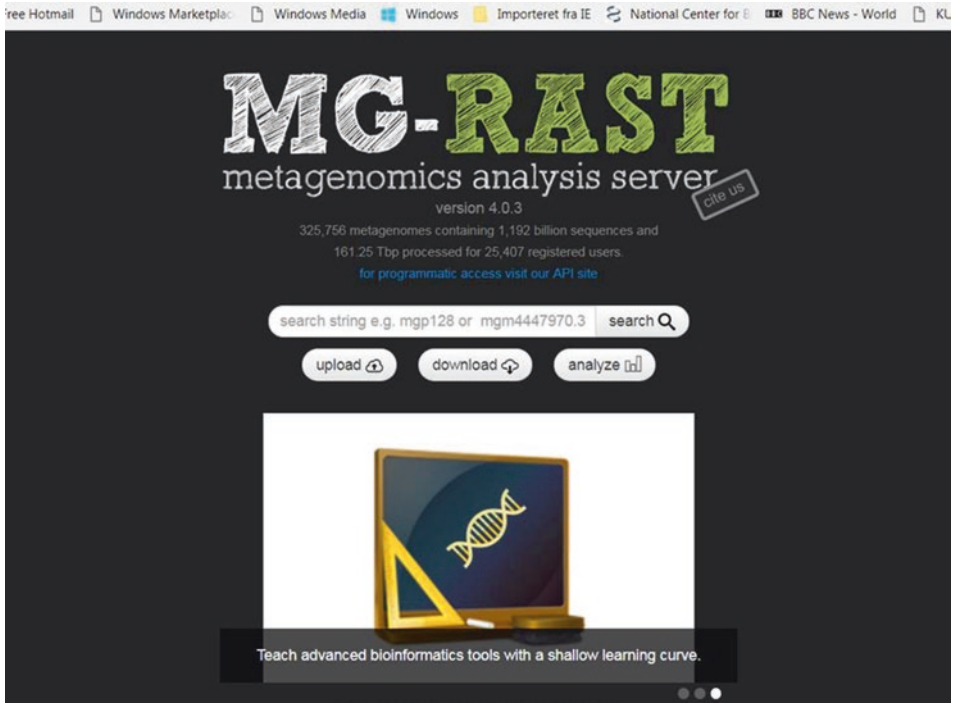

Fig. 9.15 MG-RAST (Meyer et al. 2008) pipeline with the simple interface of download, upload and analyze bottoms (https://www.mg-rast.org/)

Use no. t174 in the column "study" as link. Here, you will get a list of all 20 datasets including mgm acc. numbers. Note that the initial no. mgm4629274.3 is on the list as the first cat. At each line in this table, an overview of number of reads and number of total nucleotides is provided. For the first sample, use number BY170 as link. This brings you to graphics including taxonomic overview and breakdown on functional categories all in nice pie charts. These representations have been used for the figures of this chapter.

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Transcriptomics

10

RNA-seq

Rikke Heidemann Olsen and Henrik Christensen

Contents

R. H. Olsen · H. Christensen (✉)
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: cava@sund.ku.dk; hech@sund.ku.dk

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What This Chapter Will Teach You

A gene is active when it is being transcribed to mRNA. mRNA is acting as a template for protein synthesis. Measuring of how much mRNA is transcribed can be used to estimate how active a gene is under a given circumstance. It is possible to get insight to the regulation status of all genes of a prokaryotic strain at the same time by sequencing the total amount of mRNA (the transcriptome) and annotating the sequences against the fully sequenced genome of the prokaryotic strain. The method is called RNA sequencing (RNA-seq), and this chapter will teach you methods of how to prepare RNA material for sequencing, considerations on experimental design, and how data are analyzed.

10.1 Introduction to Transcriptomics

The ability to measure how genes are regulated under certain developmental stages or physiological conditions has expanded the knowledge of the biology of both human and prokaryotic cells tremendously. A technique called Northern blotting, developed in 1977, was the first method to study gene expression (Alwine et al. 1977). More methods to investigate the regulation of genes, including quantitative PCR (qPCR) and microarrays, have become available. The methods, however, have several limitations, for instance, in qPCR analysis, only few genes can be studied at the same time. For all the methods, the major drawback is hybridization probes (needed, e.g., for Northern blotting and microarrays) and specific primers (for qPCR) need to be manually designed, and consequently, "you only find what you looking for." Despite the bias that the researcher has to decide which contents to put on the hybridization plate, microarrays have been the gold standard to study differential gene expression during the 2000s. As the price of novel high-throughput sequencing has decreased promptly in the same period, RNA sequencing (RNA-seq) is now rapidly replacing hybridization techniques in genome-wide expression studies (Wang et al. 2009).

RNA-seq relies on high-throughput sequencing, and it will allow a genome-wide detection of "active" genes by measuring the level of the genes transcribed. The technique relates to many bioinformatics techniques already described including sequencing, annotation, databases, alignments, and BLAST (Fig. 10.1).

For RNA-seq experiments, often different conditions of the same prokaryotic strain are compared, for instance, the highly oxacillin-resistant strain of *Staphylococcus aureus*, USA300, cultured in normal growth media can be compared to growth media with therapeutic concentrations of oxacillin (the concentration of oxacillin normal used to treat infections with oxacillin-sensitive *S. aureus*). RNA-seq will enable the identification of differently expressed (DE) genes between the two conditions. Such an experiment will allow the identification of DE genes in *S. aureus* under exposure to oxacillin compared to

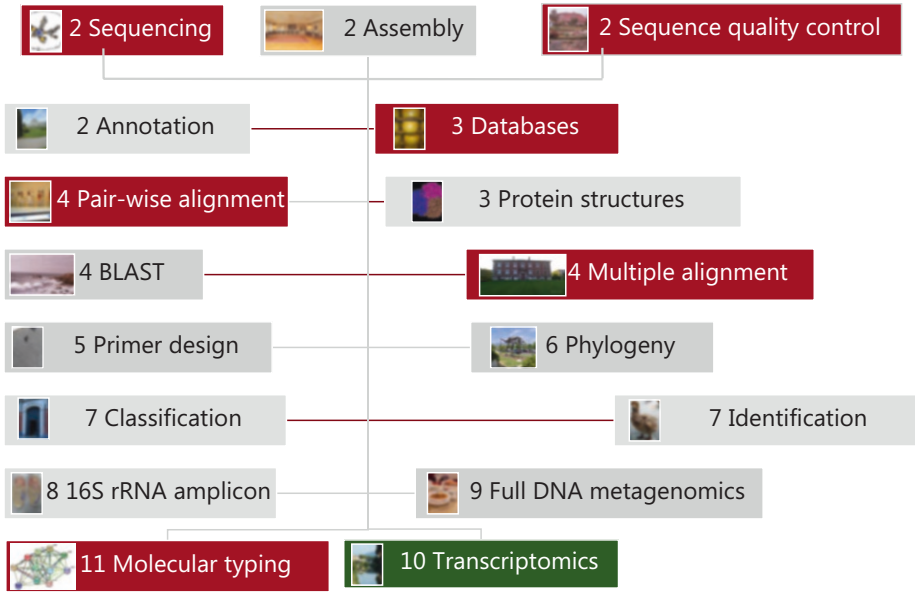

Fig. 10.1 Relationship of the chapter to other chapters of the book. RNA-seq relates to many preceding chapters dealing with sequence assembly, annotation, databases, alignments, and BLAST

control condition and may elucidate the mechanism to why *S. aureus* USA300 is able to withstand the exposure to oxacillin. A part from this example of how RNA sequencing can be used for functional studies, RNA-seq may also been applied for detection of SNPs, finding of novel genes, or a total transcriptome assembly, just to give some examples of the use of transcriptomic data.

In overview, the workflow for RNA-seq is relatively simple: Extracted RNA is converted to cDNA; cDNA is sequenced on a next-generation sequencing platform (NGS) such as either Illumina, Helicos, or SOLiD; and finally, the sequence data are matched to genes by sequence alignment (Chaps. 2 and 4).

The application of NGS has several advantages. In contrast to hybridization-dependent methods, the transcription is studied in an unbiased manner, since no probe sequences are needed to be specified. Secondly, the experimental design does not need to be altered in accordance with differences in genome sequences. Finally, the probeless sequencing allows the discovery of new genetic features (Buermans and den Dunnen 2014). Although the analysis is slightly more user-friendly for microarray data than for RNA sequencing data, RNA-seq is giving a more comprehensive overview of the transcriptome and a better dynamic range and gives the possibility to detect SNPs (Bester-Van Der Merwe et al. 2013). These features taken together are probably the key reasons of why RNA-seq has exceeded microarrays as the method of choice to analyze gene expression (Malone and Oliver 2011).

10.2 Experimental Design

There are several types of RNA that can be sequenced such as total RNA, small RNA, or mRNA. mRNA only constitutes about 2% of the total RNA in the cell; however, the assumption for transcription profiling is that changes in the transcriptional mRNA level correlate with the phenotype (protein expression). This chapter is only focusing on the sequencing of mRNA in a single culture and sequencing by the Illumina short-read technology, although mixed samples (metagenomics) and other sequencing platforms may also be suitable for RNA sequencing (Chu and Corey 2012).

The first thing to do before beginning an experiment is to decide on the experimental design (Fig. 10.2). The prokaryotic growth phase needs to be considered to assess gene expression since gene expression may vary significantly under exponential—compared to stationary growth phase (Rolfe et al. 2012). The number of replicates of each sample needs to be considered. It needs to be considered if rRNA needs to be removed. The sequencing depth required to answer a particular research/biological question needs to be defined. The optimal read length needs to be considered, and it needs to be considered if samples can be pooled. The type of library needs to be selected and the sequencing platform decided including if single-end or paired-end sequencing is required.

It is important to make a distinction between biological and technical replicates. The biological replicates assess variations between samples, whereas the technical replicates can determine variation within sample preparations. RNA extracted from one sample and divided into three samples to be sequenced would be three technical replicates, whereas three samples of RNA harvested from three independently cultured colonies treated under similar conditions would represent three biological replicates. More biological replicates should always be favored over technical replicates but be aware to stratify samples over time. Hence, do not extract RNA for all control samples Monday and all treated samples Wednesday, as factors such a humidity in the weather or even slightly changes of the temperature in the culture water bath may influence the RNA profile. Differences between

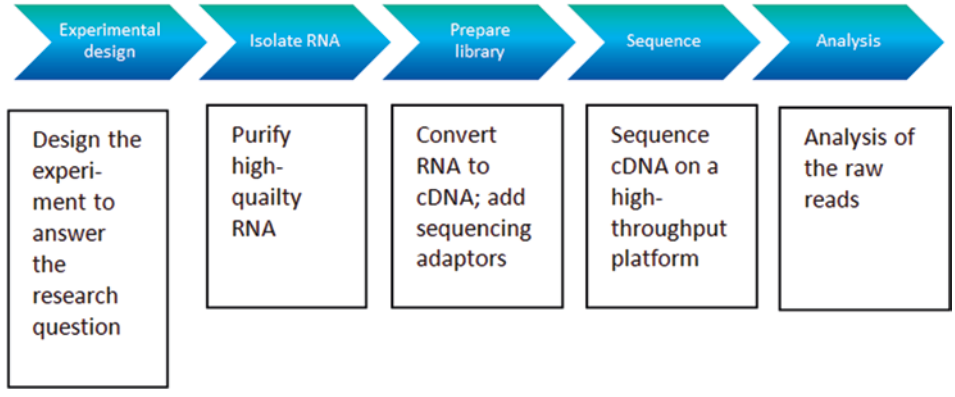

Fig. 10.2 Overview of RNA sequencing workflow

control and treated samples in this setup could then be due to external (environmental) factors rather than due to the treatment. More biological replicates (at least two, three is better) will increase the statistical power in the subsequent analysis.

The adequate read depth needed has to be assessed from experiment to experiment, depending on prokaryotic species and the research question under investigation. If you do not have sufficient read depth, the vast majority of reads will be associated with the highly expressed genes, which may not be the biologically most important genes needed to answer your research question, for example, how does antibiotic x affect genome-wide gene expression (Depardieu et al. 2007). That being said, there is a trade-off between more read depth and replicates, meaning you can add more replicates rather than increase the read depth (Sims et al. 2014).

For highly expressed genes, little effect of an increased sequencing depth is gained on the number of differentially expressed (DE) genes detected. In this case, increasing the number of biological replicates will be more beneficial. However, for low expressed genes, both sequencing depth and biological replicates increases the power to detect DE. According to ENCODE guidelines, 10–20 million reads are sufficient for differential gene expression, but additional unique transcripts are still being found at one billon read (Liu et al. 2014).

The Illumina platform can perform single-end or paired-end sequencing. Paired end is more expensive than single end but improves mapping to reap sequences and improves the accuracy for detection of differential expression for low-expressed genes.

10.3 Preparing a RNA-seq Library

The Illumina Tru-seq RNA protocol is the most commonly used protocol. Using the Illumina Tru-seq RNA protocol, there are six steps in preparing an RNA-seq library:
Step 1: Isolation of RNA
Step 2: Depletion or removal of rRNA
Step 3: Conversion of rRNA into complementary, double-stranded DNA (cDNA)
Step 4: Addition of sequencing adaptors
Step 5: PCR amplification (enrichment)
Step 6: Quality control of the library

10.3.1 Step 1: Isolation of RNA

There are several companies offering sequencing of RNA. It is, however, the responsibility of the researcher to provide a high RNA sample quality, which is essential for successful RNA-seq experiments. Many methods are available for purification of RNA from prokaryotic cells, including different manual protocols (e.g., an acidic phenol-chloroform RNA extraction protocol) and commercial kits (e.g., Qiagen RNA extraction kit). It is important

to use the same RNA extraction protocol for all samples to be compared, since differences between protocols may slightly influence the RNA material (Sultan et al. 2014; Kumar et al. 2017). It is also important to acknowledge that irrespectively of protocol, RNA extraction is much more fragile than DNA extraction due to the ubiquitous and hardly RNases that degrade RNA. Furthermore, RNA extraction is a challenging task due to the short half-life of mRNA (Tan and Yiap 2009). Gloves should always be used when handling/isolating RNA (human skin carries RNases) and all samples be kept on ice. It is acceptable to spin down prokaryotic cells at room temperature, but as soon as the cells have lysed, the temperature should be kept low (by keeping samples on ice), which will inhibit the activity on any luring RNases. Preferably use pipettes that are only handled for work with RNA. If that is not possible, at least make sure always to use RNase-free tips with filter. Be aware that RNases are very stable and will not be eliminated by autoclaving; hence, do only use certified RNase-free water.

The RNA quantity and purity can be evaluated to measuring the UV absorption of the sample using a spectrophotometer, for example, on a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA has a maximum absorption at 260 nm, and the RNA concentration is determined by the OD reading at 260 nm. In addition to the OD260, measurements should also be taken at 280 nm and 230 nm. The A260/A280 ratio provides an indication of the level of protein contamination in the sample. Pure RNA has an A260/A280 ratio of 2.1; however, values between 1.8 and 2.0 are considered acceptable for many protocols. Be aware that OD absorbance measurements can change depending upon the pH of the RNA solution. The best results are obtained when RNA is solubilized in TE buffer. In general, RNA concentrations must be above 20 μg/mL to give reliable readings.

It is needed to control the integrity of the RNA preparation since RNA may be degraded and not perform well in downstream applications. The easiest and cheapest way to assess RNA integrity is by the means of a 1% standard agarose gel and examining the ribosomal RNA (rRNA) bands. The upper ribosomal band (23S in prokaryotic cells) should be about twice the intensity compared to the lower band (16S in prokaryotic cells) and should be crisp and tight. If the rRNA bands are of equal intensity, then it suggests that some degradation has occurred. mRNA runs between the two ribosomal bands and might be seen as a smear. This is acceptable; however, smearing below the rRNA bands suggests that you have poor-quality RNA. Higher-molecular-weight bands that might indicate that the RNA is contaminated with DNA must not be observed.

A second method to control the integrity of your RNA that has become more popular, especially with in microarray analyses, is to use a bioanalyzer, such as the Agilent Bioanalyzer. Bioanalyzers use small amounts of RNA (1–2 μL) and microfluidics to determine the quantity and quality of RNA samples. The analyzer measures the sizes of the rRNA bands and determines an RNA Integrity Number (RIN) to standardize between RNA samples. Bioanalyzers are expensive, but they can often be found in core facilities.

The input needed for the Truseq Stranded mRNA kit (Illumina) is 0.1–4 μg of total RNA. Using the Agilent Bioanalyzer, the RNA Integrity Number (RIN) value should be greater than or equal to 8.

10.3.2 Step 2: Depletion or Removal of rRNA

The majority of the total RNA is rRNA, and rRNA must either be depleted from the sample, or alternatively, mRNA must be captured. For the latter, the selection of mRNA can be done by poly-A-selection, which is done by filtering RNA with 3′ polyadenylated poly(A) tails. The RNA with 3′ poly(A) tails are mature, processed, coding sequences. Poly(A) selection is performed by mixing RNA with poly(T) oligomers covalently attached to a substrate, typically magnetic beads (Cui et al. 2010). If whole RNA is to be sequenced instead, rRNA must be depleted. There are a number of commercially available kits for rRNA depletion, such a RiboZero (TaKaRa).

10.3.3 Step 3: Convertion of RNA into Complementary DNA (cDNA)

The first stage is fragmentation of the RNA to be sequenced. Fragmentation is achieved by using divalent cations, under elevated temperature, which ensures good coverage of the transcriptome. The cleaved RNA fragments are copied into first strand cDNA using reverse-transcriptase and random primers.

One of the advantages of the Illumina Truseq protocol is that it is "stranded" meaning that it will provide information from which of the two DNA strands a given RNA is derived. This provides a large, complete picture of the transcriptome (Wang et al. 2009).

The strand specificity of the samples is achieved by replacing dTTP with dUTP in the second strand synthesis process, which quenches the subsequent amplification of this strand because the polymerase used in the assay will not incorporate past this nucleotide. The final product of this step is blunt-ended, double-stranded cDNA molecules. The addition of an A-base to the ends of the cDNA prevents the blunt-ended fragments from ligating to one another during the adapter ligation reaction.

10.3.4 Step 4: Addition of Sequencing Adaptors

A ligation reaction takes place, which ligates multiple indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

10.3.5 Step 5: PCR Amplification (Enrichment)

The adaptor-added products are purified and enriched with PCR to create the cDNA library. This step is necessary to ensure that the sequencing signal will be strong enough to be detected unambiguously for each base of each fragment.

10.3.6 Step 6: Quality Control of the Library

To assess library quality, 1 µl of the post-enriched library is loaded on one of the following instruments: Advanced Analytical Technologies Standard Sensitivity NGS Fragment Analysis Kit (Advanced analytica, Heidelberg, Germany) or Agilent High Sensitivity DNA Chip (Agilent, Santa Clares, USA). The size of the library is controlled for distribution of the DNA fragments with a size range from approximately 200 bp–1 kb. The manufacturer's instructions should be followed for the respective instruments depending on the kit you are using.

10.4 Sequencing

When preparation of cDNA is done with TruSeq RNA, cDNA sequencing can be done on Illumina MiSeq or HiSeq platform as described elsewhere in the book (Chap. 2). Overall, the sequencing of RNA does not differ from sequencing of genomic DNA. The suggested read length is 50–250 bp.

10.5 Data Management (Sequence Reads)

Data from sequencing will be provided in FASTQ format. Data management includes assessing data for the quality, alignment of the reads to a reference genome, and normalization of the data, before the differential gene expression analysis can be conducted. Some of the most important bioinformatical programs are listed in Table 10.1, and examples on their use will be given in the text.

Table 10.1 Examples of software programs to manage and analysis RNA sequencing data

Design of RNA-seq experiment	Quality control	Alignment	Normalization
RNAtor https://github.com/binaypanda/RNAtor	FastQC https://www.bioinformatics.babraham.ac.uk/projects/fastqc/	Bowtie https://bowtie-bio.sourceforge.net/index.shtml	EdgeR https://bioconductor.org/packages/release/bioc/html/edgeR.html
PROspective Power https://rdrr.io/bioc/PROPER/	dupRadar https://bioconductor.org/packages/release/bioc/html/dupRadar.html	GNUMAP https://dna.cs.byu.edu/gnumap/	DESeq https://bioconductor.org/packages/release/bioc/html/DESeq2.html (Love et al. 2014).
		PerM https://code.google.com/archive/p/perm/	BaySeq https://bioconductor.org/packages/release/bioc/html/baySeq.html

10.5.1 Raw Data

The Illumina platform provides raw sequence reads in FASTQ format, which may be stored directly at the Sequence Read Archive (SRA) (Chap. 3). When assessing the raw data, start by checking whether the FASTQ file is consistent. The format can be validated with software such as FastQValidator (https://github.com/statgen/fastQValidator). Then the base calling quality score, which is part of the sequencing data output, needs to be assessed. Quality scores reflect how confidently the right bases have been called. FastQC (part of the FastQ validator) is an excellent tool for assessing the quality of the sequencing run. The base calling quality score is called a Phred score, Q, which is proportional to the probability p that a base call is incorrect, where $Q = -10 \log_{10}(p)$. For example, a Phred score of 10 corresponds to one error in every ten base calls ($Q = -10 \log_{10}(1/10)$), or 90% accuracy; a Phred score of 20 corresponds to one error in every 100 base calls, or 99% accuracy. A higher Phred score thus reflects higher confidence in the reported base. There is no defined cutoff on how low an acceptable Phred score can be, but one should aim for Phred scores higher than 33. FastQC can also be used to confirm graphically that the GC-content is represented by a nicely bell shaped form.

If the quality score of the run is low, it is may be possible to remove unwanted parts of the raw data. The unwanted parts are either technical contaminations such as low-quality read parts, or technical sequences such as the adaptors or biological contamination like polyA-tails, rRNA, mitochoridal RNA, or tRNA. When these parts are removed, then FastaQC is run again, and it should be decided if you want to proceed with the data or do a re-run.

If you decide that the quality of the reads is sufficient to proceed, the sequences are ready to be aligned to a reference genome.

10.5.2 Alignments of Sequence Reads

Now the reads need to be aligned to the genome(s), which provided the RNA sample(s), and this is called read alignment or mapping. While the term "alignment" describes the process of finding the position of a sequencing read on the reference genome, "mapping" refers to assigning already aligned reads to transcripts, which is also called quantification. The general challenge of short-read alignment is to map millions of reads accurately and in a reasonable time, despite the presence of sequencing errors, genomic variation and repetitive elements. There are several tools to assist in the alignment, depending on whether the reads are aligned to a genome or the reads are assembled de novo, in which the reads need to be assembled first into longer contigs. These contigs can then be considered as the expressed transcriptome to which reads are re-mapped for quantification.

10.5.3 Normalization of Data

Normalization is a process designed for adjustment for sequencing depth and compositional bias. It is done to identify and remove systematic technical differences between samples that occur in the data and to ensure that technical bias has minimal impact on the results. The most common need for normalization is related to differences in the total number of aligned reads.

Library size influences read counts. If one library is sequenced to 20 M reads, and another to 40 M, in the latter case, most genes will be approximately double in their counts. Also, the library composition has importance as highly expressed genes may be overrepresented at cost of lowly expressed genes. One way to normalize data is by the use of Read Per Kilobases per million (RPKM) (for SE-RNA seq) of Fragments per Kilobase Million (FPKM) (For PE-RNA seq). RPKM is calculated by dividing the reads for gene A by the length of gene A times the total number of reads.

$$\text{RPKM} = \text{Reads for gene A} / \left(\text{Length of gene A x Total number of reads}\right)$$

This formula normalizes read counts for: (1) The sequencing depth, since sequencing runs with more depth will have more reads mapping to the gene, and (2) the length of the gene, since longer genes will have more reads mapping to them.

An alternative that might be considered as a better solution is TPM (Transcript Per Kilobase Milo). TPM is very similar to RPKM/FRKM. The only difference is the order of operations:

1. Divide the read counts by the length of each gene in kilobases. This gives you reads per kilobase (RPK).
2. Count up all the RPK values in a sample and divide this number by 1,000,000. This is your "per million" scaling factor.
3. Divide the RPK values by the "per million" scaling factor. This gives you TPM.

Normalization for the library size may also be done using different software, such as DESeq2 and EdgeR (Table 10.1).

Gene length influences the count, as longer transcripts generate more reads. However, the transcript length does not differ between samples. Since it is the relative difference that is of interest, gene length counts do not need to be normalized.

10.6 Differential Gene Expression

RNA-seq is a *relative* abundance measurement technology. The primary goal of the differential gene expression analysis is to quantitatively measure differences in the levels of transcripts between two or more treatments or groups.

Step one in any analysis is always the same: plot the data! You may use a principal component analysis (PCA) or something similar to plot the data. This plot gives a nice

overview of how similar the different replicates are in the RNA composition (the closer, the better) and will give a first impression if you can expect to find interesting difference between you samples (Fig. 10.3).

The actual identification of DEG is typically done using the statistical program R with either edgeR or DEseq and will result in a plot similar to Fig. 10.4. In Fig. 10.4, black dots represent genes that are expressed the same, while each red dot is a gene that is expressed differently between the two sample conditions ("C" and "S" in Fig. 10.4). The X-axis tells you how much each gene is transcribed, while the Y-axis tells you how big the relative difference is between "C" and "S." The red genes are therefore your interesting genes. If

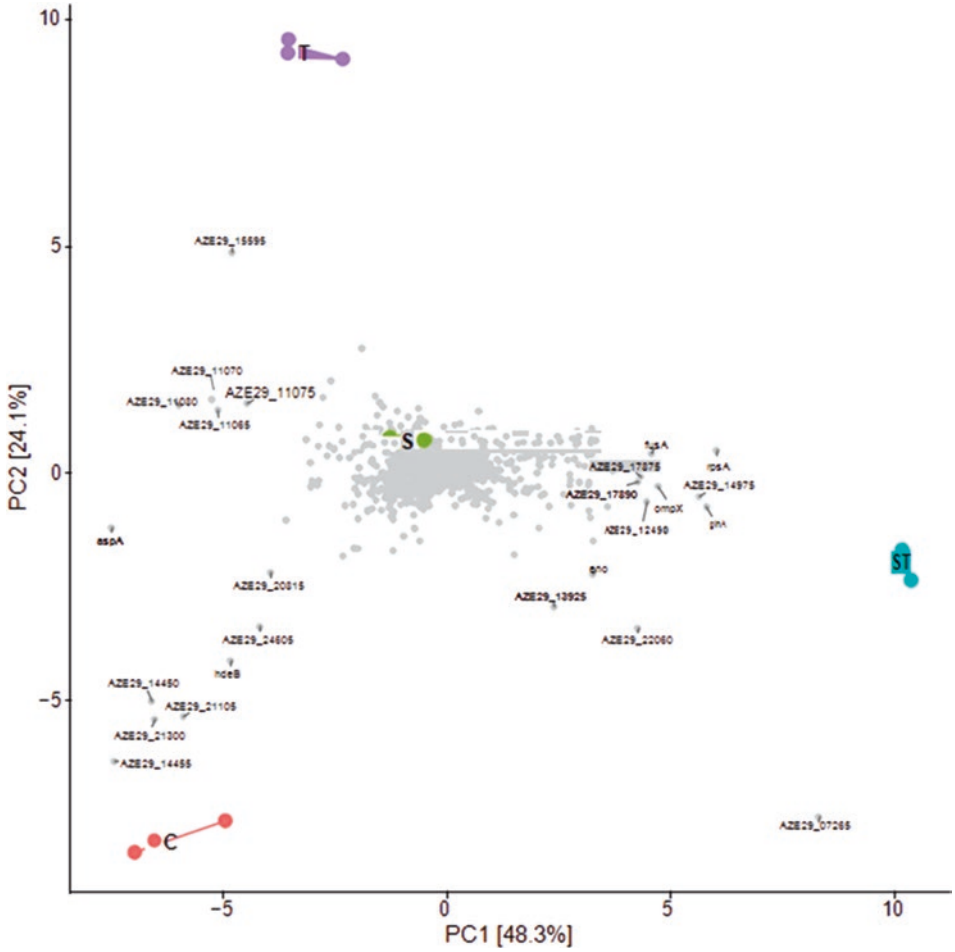

Fig. 10.3 Examples of a principal component analysis (PCA) to visualize the global gene expression of *Escherichia coli* under four different treatment conditions (C, T, S, ST). All conditions were performed in triplicates. Samples located together have similar gene expression. Genes (gray dots) located in the same direction as samples have higher expression in those samples

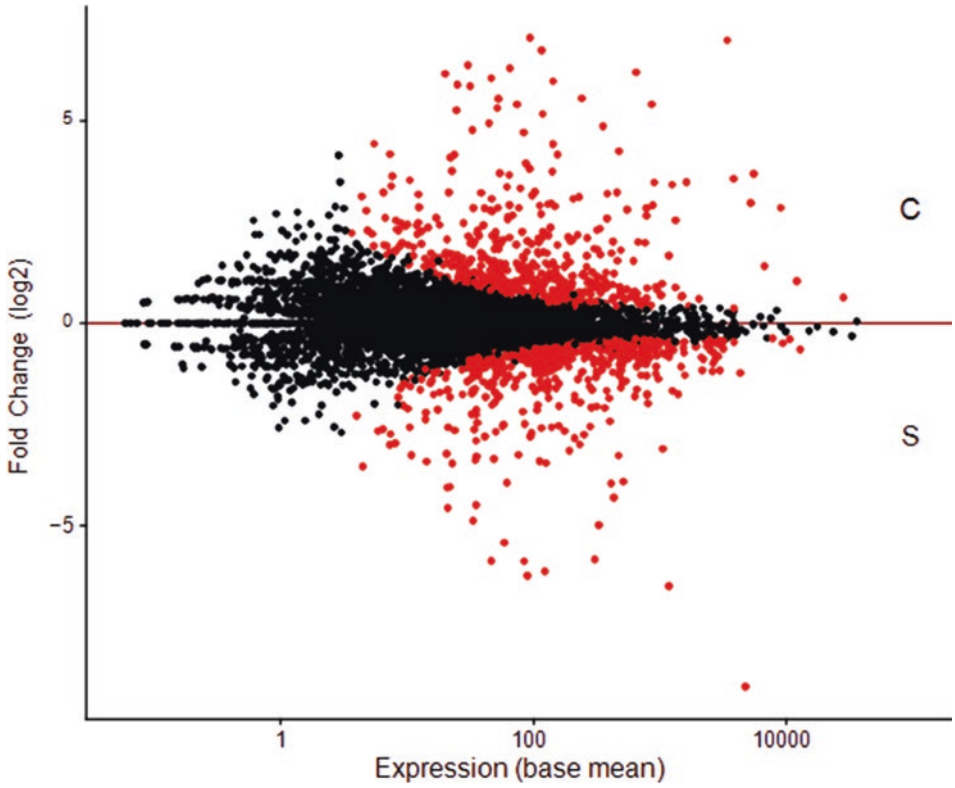

Fig. 10.4 This MA plot shows the fold change between the two treatment conditions of *Escherichia coli* (C and S) compared (log2 scaled) as function of the average expression of each gene. Each point represents a gene; red indicates an adjusted p-value <0.01

you know what you are looking for, you can see if the experiment is validating you hypothesis. If you don't know what you are looking for, you can see if certain pathways are enriched under the different sample conditions using, for example, KeggMapper (https://www.genome.jp/kegg/mapper/) or Cytoscape (Cline et al. 2007).

10.7 Conclusion

Since the first reported studies using RNA seq were published in 2008, our understanding of gene expression has to been taken to a new level. However, there are still some technical issues awaiting resolution such as the PCR amplification stage of the library construction, which may result in redundant sequence read and bias in the final dataset. Nevertheless, RNA-seq holds the promise to continue to replace other genome-wide expression analysis in the future and will likely add in refine our understanding of gene regulation in prokaryotes.

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Sequenced-Based Typing and Prediction of Function

11

Henrik Christensen and John Elmerdahl Olsen

Contents

H. Christensen (✉) · J. E. Olsen
Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
e-mail: hech@sund.ku.dk; jeo@sund.ku.dk

H. Christensen (ed.), *Introduction to Bioinformatics in Microbiology*, Learning Materials in Biosciences, https://doi.org/10.1007/978-3-031-45293-2_11

What You Will Learn

You will learn about sequence-based identification and characterization of populations based on multilocus sequence typing (MLST). This is the starting point for further population level typing such as whole genomic sequence MLST and single nucleotide polymorphism (SNP) analysis based on the whole genomic sequences. You will learn that prediction of functional traits such as serotype, antibiotic resistance gene profile, and virulence is possible from the whole genomic sequence of the best-known pathogens.

11.1 Background of Prokaryotic Populations and Population Genetics

In this chapter, the background of population genetics related to bioinformatics will be presented (Fig. 11.1). Population genetics is the study of the evolutionary change in the genetic composition of populations (Whittam 1995). According to Whittam (1995), population genetics applies both to how the mechanisms (mutation, natural selection, migration, genetic drift) influence the evolutionary rate of change in the populations (Fig. 11.2)

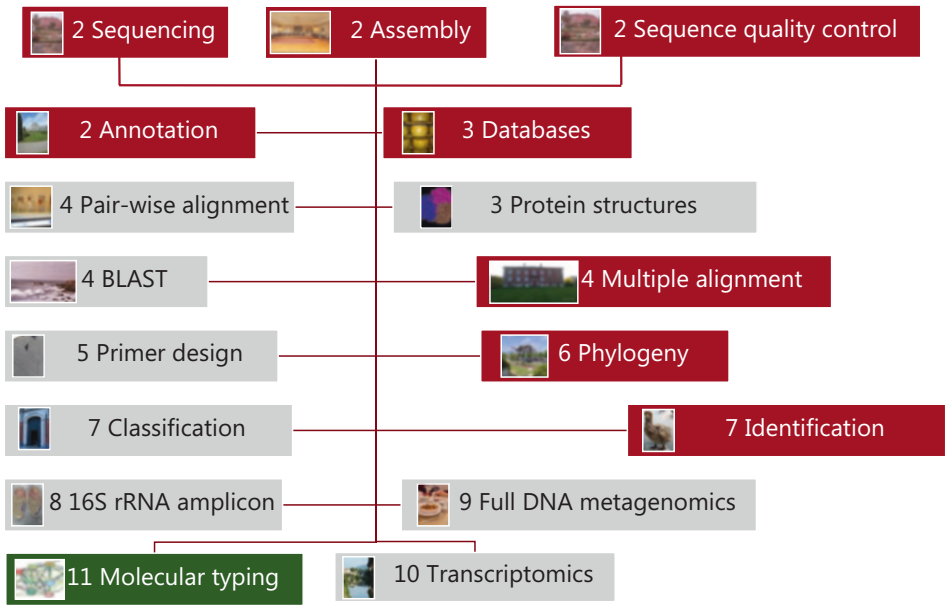


Fig. 11.1 Relationship of the chapter to other chapters of the book. Molecular typing based on sequence comparison relates to many of the preceding chapters dealing with sequence assembly, databases, phylogeny, and identification. For the future, molecular typing could probably also be linked to full DNA metagenomics

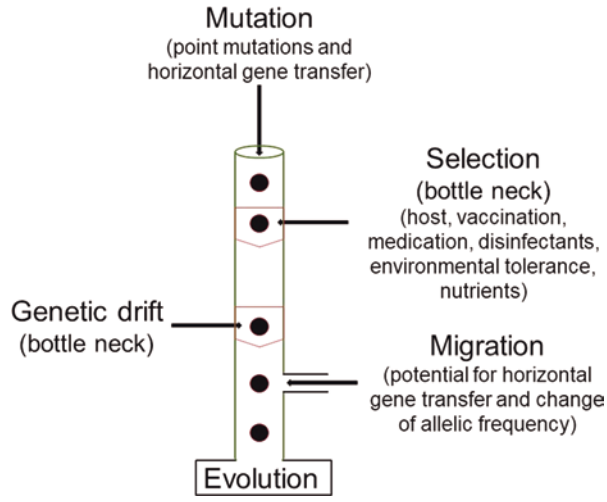
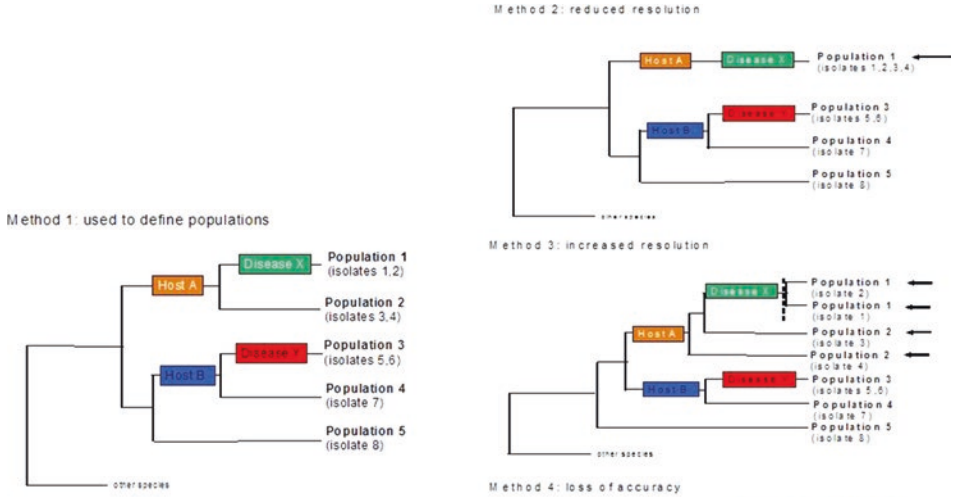


Fig. 11.2 Population genetic mechanisms relevant for the prokaryotes. The mechanisms of selection, mutation and genetic drift contribute to the evolution of populations of by shaping their genetic properties. Migration may also contribute if allelic frequencies are affected

as well as to historical investigations of how and when pathogens have evolved. The outcomes of such investigations will be definition of population structures, knowledge of the nature of allelic variation, and the role of different modes of recombination in generating genotypic variation (Milkman 1973; Selander and Levin 1980; Whittam 1995).

In animals, a population is defined as a group of individuals with a potential for sexual reproduction often limited to a certain geographic region at a certain time point. However, this definition cannot be used for prokaryotes due to the lack of sexual reproduction. Groupings of prokaryotes are better reflected by clonal relationships than physical barriers. A prokaryotic clone was originally defined as a group of prokaryotic isolates "showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin" (Ørskov and Ørskov 1983). Clonal populations are also called genetic lineages.

The most difficult task with prokaryotes is to set up the limits for the population. A practical solution might be to define the population borders according to the role that their members have in causing disease or other properties useful for epidemiology and for the investigation of pathogenesis. However, populations need to be identified by certain genotyping methods and their principles are very much determining for how we observe the populations. These methodological problems with identification of clonal populations are illustrated in Fig. 11.3. Method 1 (e.g., wgsMLST) is optimal in linking genotypic clusters with isolate properties in relation to host and disease association. Method 2 (e.g., MLST) has slightly lower resolution that method 1 and results in the lack of separation of populations 1 and 2. The non-disease-related isolates 3 and 4 are therefore included in this population, which is confusing compared to method 1 that allowed the separation of populations associated disease from the non-pathogenic. Method 3 (e.g., SNP) has higher resolution than method 1, and this will not contribute with any error as long as it is taken care of some clusters that might belong to the same population. Method 4 has the same resolution as



Isolate	Host	Disease
1	A	X
2	A	X
3	A	
4	A	
5	B	Y
6	B	Y
7	B	
8	A and B	

Fig. 11.3 Model illustrating dilemmas between methodological and biological relevance of populations. Clonal population are defined according to method 1 providing optimal biological interpretation (hosts A, B, disease associations X, Y) for the isolates investigated in the table

method 1; however, isolates are clustered differently, and methods 4 will give another interpretation about their role in disease.

The term "strain" is sometimes misused to equal a population or a clone. In the strict sense, a strain is an isolate that has been further characterized, archived, and documented. The term "strain" refers to the culture or subcultures of it. The misuse is related to the fact that two isolates that share phenotype and genotype in principle must be considered as belonging to the same strain.

11.1.1 Mutation

The most likely starting point for an event affecting the genetics of a population is a mutation (Fig. 11.2). The progeny of a prokaryotic cell should in principle be genetically identical to its ancestor. Two genetic mechanisms, point mutations and horizontal gene transfer (HGT), tend to abolish this identity over time. Mutations will increase genetic diversity between individuals. Point mutations accumulate with a nearly constant rate at random positions in the sequence, and we can analyze them by sequence comparison until a certain

level of divergence. HGT, however, is more problematic to analyze, since large fragments of sequences are exchanged between individuals and events of transfer cannot easily be traced since the origin of such fragments is often unknown. ClonalOrigin should be able to model the source of specific recombination events (Didelot et al. 2010).

Recombination in prokaryotes has a completely different meaning compared to eukaryotes. In eukaryotes, recombination refers to the results of crossing over in a symmetrical way between chromosomes during the zygotene stage of meiosis. This happens between members of the same species and even with members of the same population.

Recombination in prokaryotes includes HGT, and it can take place between prokaryotes by transformation, transduction, and conjugation and usually occurs as an asymmetrical exchange events between the partners. Some bacteria like *Haemophilus influenzae* are naturally transformable meaning that they can take up DNA directly from the environment. In bacteria with a double wall structure (gram negative), DNA is taken up as double-stranded across the outer and as a linear single-stranded across the inner membrane, and uptake signal sequences (USS) have been found to favor uptake (Maughan et al. 2008). Transduction is the transfer of genetic material by bacteriophages. There is high variability in the ability of bacteriophages to cross-react with strains within the same species, between different species of the same genus, and between different genera of the same family (Jones and Sneath 1970). Conjugation is when genetic material is transferred between two prokaryotes on a conjugational plasmid. This is the most frequent mechanism for horizontal transmission of antibiotic resistance genes.

Luria and Delbruck (1943) pioneered the investigation of prokaryotic population genetics by investigating how bacteria become resistant to bacteriophages. They found resistance to develop as a random process since resistance to bacteriophages developed independent on the presence of bacteriophages in cultures of bacteria sensitive to bacteriophages. Furthermore, they analyzed mutations in a statistical framework and found that the distribution of mutational events followed a special distribution (Luria and Delbruck distribution). Mutation rates are still measured based on the principles laid down by Luria and Delbruck (1943). The most common procedure is the fluctuation test. In this test, the distribution of mutants in a number of parallel cultures is used to estimate the mutation rate based on knowledge of the expected number of mutation events, the number of cultures, and the size of initial inoculum. A range of assumptions are taken in this calculation of the mutation rate: constant probability of mutations per cell cycle, independent mutation rate of growth phase, no cell death, no revertants formed, only single mutants, wild type and mutants have the same growth rate, a negligible number of mutant cells initially present compared to final numbers in cultures investigated, and that we are able to detect all mutants (Pope et al. 2008). The mutation rate is the probability of a mutation occurring per cell division. Another measure is the mutation frequency being the proportion of mutant prokaryotes present in a culture. The mutation rate is independent on the age of the prokaryotic culture and a more accurate measure of mutations compared to the mutation frequency that is related to the age of the prokaryotic culture. In principle, we should be able to trace mutations directly to the DNA sequence level (Bell 2008). In prac-

tice, this will require an enormous DNA sequencing effort; however, next-generation sequencing technologies might be able to do this job in order to measure mutation rates directly and not via fluctuation tests.

11.1.2 Selection

Periodic selection is the most prominent population genetic mechanism of prokaryotes. If a specific allele is favored in the population and this allele results in higher fitness, then all members of the population with this allele will replace other populations without the allele in the given environment by expansion of this clone. Periodic selection is a form of "bottleneck" (Levin 1981). The higher rate of HGT, the less is the effect of periodic selection (Levin 1981).

The degree of selection acting on a specific coding gene can in theory be predicted by calculation of the so-called dN/dS ratio (Nei 2005). The ratio is the ratio of non-synonymous nucleotide substitutions per non-synonymous site (dN) to that of synonymous nucleotide substitutions per synonymous site (dS). A non-synonymous substitution at DNA level is a change in nucleotide leading to a change in the amino acid translated from the codon where it occurs. It follows that a synonymous substitution is not affecting the amino acid translated from the codon. The theory is based on the "neutral theory": without positive selection, dN/dS = 1. Negative selection (dN/dS < 1) occurs when deleterious alleles (recognized at amino acid level) are eliminated from the population by purifying selection and leaving only the synonymous changes to be observed. In positive selection (dN/dS > 1), polymorphism is assumed to be maintained at the amino acid level, and the changes here will be relatively higher than those observed at synonymous sites. However, high polymorphism at the amino acid level might not necessarily be observed for positive selection to occur (Nei 2005). Textbook examples of positive selection are with peptide-binding sites of MHC genes from humans and mice as well as the antigenic genes of influenza virus (Nei 2005). To carry out the dN/dS calculations, at least two closely related nucleotide sequences need to be compared pair-wise. Computer programs can evaluate the substitutions in regard to all combinations of nucleotides that are legal for each codon. The program DnaSP (Librado and Rozas 2009) (http://www.ub.edu/dnasp/index_v5.html) can be used to calculate selection and many other parameters from DNA sequence data. MEGA11 (Tamura et al. 2021) introduced in Chap. 6 can also calculate the dN/dS parameter and will evaluate if the value is statistically significantly different from neutral: **Data|Open A file/session** and select file saved as fasta format from Bioedit. Click on **Analyse** in **How would you like to open this fasta file** and select **Analyze** and **Nucleotide Sequences** and **ok.**

Say **Yes** to **Protein coding** and then **Change Genetic Code** to **Bacterial Plastid** and **ok.**
Selection|Codon-based Fisher (small number of changes)
Selection|Codon-based Z-test (large number of changes)
and Yes to use current data. **Compute.**

11.1.3 Genetic Drift

"Genetic drift is a random process that can cause gene frequencies in a population to change over time causing evolution without natural selection" (Madigan et al. 2020). The random distribution of certain genotypes within a population may lead to genetic drift if the population is small. The limit has been suggested as 10^8 cells, and with high population size, this effect may not be important. With low population size, then clones developed by periodic selection may be wiped out more or less by chance. The effect is linked to selection in the way that the weaker and more unpredictable periodic selection, the higher the effect of genetic drift.

11.1.4 Migration

Migration is the assimilation of new individuals into a population from another population. For migration to take effect on evolution, the introduction of individuals should have consequences on population genetic processes like allelic frequencies and mutation rates. Investigation of the spread of populations including bacterial spread between animals is not population genetics as long as such spread is not affecting the evolution of the organisms. Investigation of such spread is part of epidemiology.

11.1.5 The Biological Consequences of Population Genetics of Prokaryotes

In theory, the population structure of a prokaryote species is determined by the "ratio of genetic changes caused by recombination relative to de novo mutation" (Spratt and Maiden 1999) meaning that if HGT is relatively higher than point mutation rates, the population structure will be very complex and diverse, whereas low degree of recombination relative to point mutation will result in well-defined populations at the sequence level. Only in the last case are we able to recognize clonal populations (Fig. 11.4). The ratio between "recombination and point mutations" can be estimated by comparing recombination between the alleles of the genes used in the MLST (Sect. 11.2) analysis to actual point mutations observed with the same genes.

The distribution of alleles investigated by MLST (Sect. 11.2) can be used to predict if the population structure is clonal or non-clonal (panmictic). With equal assortment of alleles, there should be an equal random distribution of alleles between populations of a species. The expected variance V_E should equal the observed variance V_O. If the populations have evolved like clones, the alleles will be identical or highly related within clones and very different between clones, and V_O will be higher than V_E. To compare V_E and V_O, the index of association is calculated ($I_A = ((V_O/V_E) - 1)$, and it follows that I_A is not

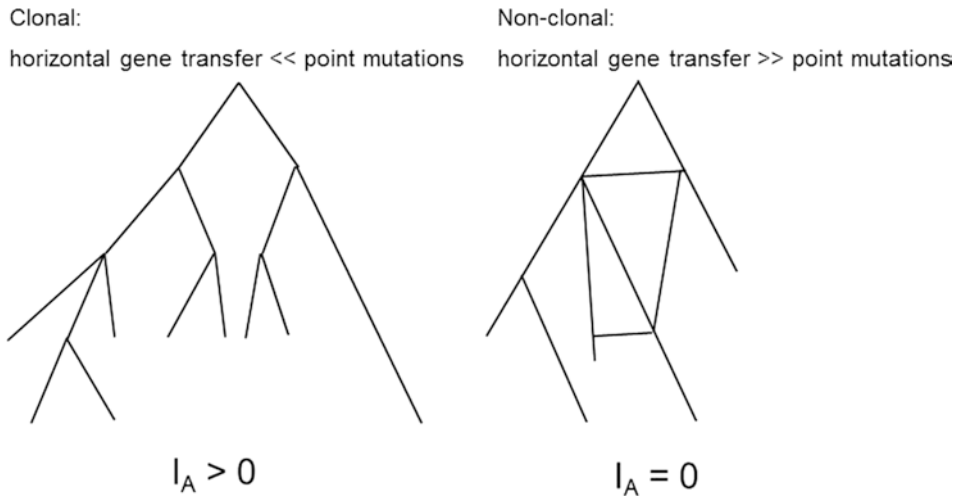


Fig. 11.4 Population structures are related to the ratio between recombination and point mutations: For most eukaryotes, the expected variance of allele frequency, V_E, should equal the observed variance V_O. For most prokaryotes where populations have evolved like clones, the alleles will be identical or highly related within clones and very different between clones, and V_O will be higher than V_E. The index of association is calculated as $I_A = ((V_O/V_E) - 1)$

significantly different from 0 with a non-clonal (panmictic) population structure but significantly different with a clonal population structure (Fig. 11.4).

Some clonal lineages of a species seem to have adapted to specific hosts. In addition, these lineages often cause disease to a higher extent than other lineages, and for epidemiological investigations, it is therefore of great importance to identify and understand prokaryotes at the population level.

One outcome of population genetics investigations is more realistic diagnostic methods for prokaryotic populations involved in disease. If population structures reflect disease patterns and hosts, we will be able to determine the populations that are really responsible for disease and not their commensal sister groups (Fig. 11.2).

Ecotypes are groups of prokaryotes playing ecological distinct roles defined based on DNA sequences. Ecotypes are analyzed by construction of phylogenetic trees based on housekeeping genes of isolates representing populations of a species. At a certain level of depth of a cluster in the tree, a group of populations that equal an ecotype is defined. Simulations are used to select this level of cutoff for the ecotype as well as to estimate periodic selection and genetic drift. Ecotypes are as a consequence one or more clonal populations if we refer to the current species concept of prokaryotes. Ecotypes can be regarded as species if we redefined the prokaryotic species concept; however, multiple ecotypes are usually recovered within the traditional species (Koeppel et al. 2008).

11.2 Multilocus Sequence Typing (MLST)

11.2.1 MLST

Multilocus sequence typing (MLST) is based on the comparison of DNA sequences of conserved genes in strains of a species (Maiden et al. 1998). For each gene (locus), all different versions of sequences are scored as alleles and designated a random number. This number is not reflecting the quantitative difference between the sequences but just stating that the sequences are different. An allele profile, for instance, 1, 3, 4, 5, 2, 3, 1, is defined as a sequence type (ST). If there is only one difference in the allelic profiles between two STs, these STs are single locus variants (SLV). This will happen if, for instance, the one ST is 1, 1, 1, 1, 1, 1, 1 and the other 1, 1, 1, 1, 1, 1, 2. Double locus variants (DLV) share five alleles and have two variants, and as the name indicates, then triple locus variants (TLV) have three different alleles. If a group of STs are linked as SLVs or DLVs, they form a clonal complex (CC). The clonal complexes are named after the ST estimated to be the ancestor of the complex—the founder.

MLST typing has mainly been performed on the server https://pubmlst.org/general.shtml dedicated to a series of species. The databases on these servers have been maintained by curators who have uploaded new sequence types and information of isolates. Users are able to upload sequences to the servers and to obtain a sequence type.

11.2.2 Multilocus Sequence Analysis

The information in the DNA sequences compared for MLST can also be directly analyzed by phylogeny (Chap. 6) and is then called multilocus sequence analysis (MLSA). MLSA has also been considered in Chap. 7 with respect to classification. For MLSA, the DNA sequences of the genes (loci) used for each ST are concatenated meaning that they are joined end by end. For instance, if a 7 locus scheme includes partial regions of 500 nt, the concatenated sequence will be 3500 nt. For most of the MLST servers, concatenated datasets can be downloaded for this type of phylogenetic analysis. The information gained from such an analysis differs from the MLST analysis since the phylogeny will show the actual evolutionary diversity of the STs. The MLSA analysis works best for prokaryotes with a clonal population structure (Fig. 11.4). For a true clonal population structure, the phylogeny of all seven genes should be the same (congruent). More frequent horizontal gene transfer will lead to more linkage equilibrium and will result in a different phylogeny for each gene. In this case, a concatenation of all genes will make little sense.

11.3 Whole Genome-Based Typing

The relative low cost of generating whole genomic draft sequences has enabled the use of the most common typing methods based on the direct comparison of the whole genomic sequences. Even for MLST typing, which only relies on a few kilo bases of sequence, it is

now cheaper to sequence the whole genome and used that to determine the allelic profile than to sequence the seven genes by the traditional Sanger method. For the more common species of clinical importance, servers have become available that can predict serotypes, antibiotic resistance gene profiles, and virulence gene profiles.

11.3.1 Whole Genomic Multilocus Sequence Typing (wgMLST)

Whole genomic MLST (wgMLST) is an extension of the MLST concept to more than seven conserved genes of the species. In principle, all conserved gene sequences can be included in such wgMLST scheme. We are aware that the term wgMLST in some other texts is used for the pangenome and cgMLST reserved only for the conserved part of the pangenome (Table 11.1). For simplicity, we will use wgMLST here and only consider to include the conserved genes of a species in such a scheme. However, if all conserved genes of the species are included, the number of variations in alleles and sequence types will be very high, and there will be a risk of too high resolution of the typing system (Fig. 11.2 method 3). To focus on clones with certain properties, a set of genes can be selected, which includes genes predicted to encode for virulence or other important functional factors. A

Table 11.1 Definition of pan and accessory parts of genomes

Comparative genomic component	Definition	Importance	Reference
Pan-genome (supragenome)	Sum of core and dispensable genome	All genes	Medini et al. (2005), Lapierre and Gogarten (2009)
Core genome	Genes present in all strains of a species	Genes responsible for basic metabolic functions. Genes included in MLST	Medini et al. (2005)
Extended core	Genes present in at least 99% of the sampled genomes		Lapierre and Gogarten (2009)
Dispensable genome	Genes present in two or more strains and unique genes in some but not all strains of a species	Genes encoding supplementary biochemical pathways responsible for selection including host colonization and antibiotic resistance. Genes present on genomic islands. Genes recently acquired by horizontal gene transfer. Genes for prediction of serotypes	Medini et al. (2005)
Unique genes (ORFans)	Genes only present in one strain		Medini et al. (2005), Lapierre and Gogarten (2009)

wgMLST typing system can be set up on the BIGSdb platform https://pubmlst.org/software/database/bigsdb/ (Jolley and Maiden 2010). This platform can be used both for MLST and for wgMLST. To set up the platform, you will need assistance from a system administrator. The platform allows users to upload whole genomic sequences and extract the sequence type. Curators of the database associated with BIGSdb are able to add new sequence types and information of isolates to the database. The platform has, for instance, been set up to predict the serotype of members of Pasteurellaceae: https:\\ivsmlst.sund.ku.dk.

11.3.2 Single Nucleotide Polymorphisms (SNP)

Single nucleotide polymorphisms (SNP) are point mutations that only occur as single nucleotide changes in sequences. The SNP concept will be introduced here in relation to the comparison of raw reads of isolates obtained by high-throughput sequencing to a reference sequence. The reference can be a fully assembled genome of a well-characterized strain of the bacterial species investigated. It is important that this reference is closely related to the isolates that are being typed. The isolates for SNP typing are whole genome sequenced, and the reads are mapped to the reference. All positions where SNPs are identified between the reads and reference are then scored. The positions can be scored as a multiple alignment, and it can be used to construct a phylogeny as described in Chap. 6. To construct such a phylogeny, many isolates are compared at one time to the same reference.

The analysis can be performed at Center for Genomic Epidemiology (http://www.genomicepidemiology.org). The files with all reads of a genome can be uploaded to https://cge.cbs.dtu.dk//services/CSIPhylogeny/ (Leekitcharoenphon et al. 2012). A reference genome needs to be defined, and then files with reads each representing isolates for typing can be uploaded. The programs on the server will extract all SNPs identified between the isolates compared to the reference. The SNPs can be downloaded in the form of a multiple alignment, and a phylogeny can be visualized by MEGA11 (Tamura et al. 2021), a procedure referred to as phylotyping. The benefit with this typing method is that is relatively easy to perform and has high resolution (Schürch et al. 2018). A database is not needed for this type of SNP like for wgMLST.

Unfortunately, there are at least two drawbacks with the SNP concept. One is that rates of SNPs differ in different species. When *Listeria monocytogenes* was subcultured from a frozen stock every 3 months during a 3 year period, only one SNP detected (Kwong et al. 2016) and almost at the same level of SNPs was found in *Mycobacterium tuberculosis* with four SNPs over 4 years, whereas *Helicobacter pylori* accumulated 30 SNPs per year (Schürch et al. 2018). In this review, the range is further extended from 2 SNPs per year for *Salmonella enterica* to 37 for *Pseudomonas aeruginosa*. Further thresholds are described in Pightling et al. (2018). The other problem is that SNP results only are valid in comparison with a specific reference. If another reference is selected, another result may

be obtained. For species of *Salmonella*, wgMLST was preferred compared to SNP in a large comparative study (Alikhan et al. 2018).

REALPHY (Bertels et al. 2014) can use multiple references, which in some way is solving the problem of biases related to different references selected.

SNP analysis can be performed at Center for Genomic Evolution (CGE): https://cge.cbs.dtu.dk/services/CSIPhylogeny/ (Kaas et al. 2014). First, the reference WGS is uploaded in FASTA format. Then all isolates (three or more) to be compared to the reference are uploaded. For each strains sequenced by Illumina short-read paired-end technology, there will be two files (R1 and R2) in fastq format. Read files on "zipped" form are preferred for upload related to smaller file size. All sequences need to be uploaded at one time by holding down the ctrl bottom and selecting all the files to be uploaded with the mouse. For many isolates, assembled contigs on FASTA format can be uploaded as an alternative to read files to speed up upload of data and further analysis. The results will be available after a few hours and a link sent by email. The important file "Snp-tree-aln.fasta" (alignment file in fasta format) is downloaded for analysis of number of SNPs between strains. The SNPs can be enumerated from this multiple alignment. The phylogeny shown online is difficult to interpret. If a phylogeny is needed, it is recommended to use the aligned SNPs downloaded (Snp-tree-aln.fasta) and use ClustalX or similar multiple alignment program to construct the phylogeny and FigTree or (http://tree.bio.ed.ac.uk/software/figtree/) or other similar tool like MEGA11 to illustrate the phylogeny graphically. The phylogeny is used to confirm the clonal relationships between SNPs.

11.3.3 Typing of Virulence, Antibiotic Resistance, and Serotype Based on the Whole Genomic Sequence

This approach is only possible for certain well-characterized species where all or most virulence genes and antibiotic resistance genes have been identified and characterized and where the genetic background for the antigenic profile is well characterized. With this information, a typing system can be established on a server with internet access. Such a tool is available Center for Genomic Epidemiology (DTU) (http://www.genomicepidemiology.org/) (Larsen et al. 2012; Joensen et al. 2014, 2015). The whole genomic sequences can be uploaded in different formats including assembled genomes or raw reads.

11.3.3.1 Prediction of Virulence Genes

VFDB 2022 (http://www.mgc.ac.cn/VFs/main.htm) (Liu et al. 2022).

Dedicated prediction for *Escherichia coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* is available from CGE (https://cge.food.dtu.dk/services/VirulenceFinder/) (Joensen et al. 2014; Malberg Tetzschner et al. 2020).

11.3.3.2 Prediction of Antibiotic Resistance

CARD, ResFinder, and AMRFinderPlus linked to NCBI are the most common for prokaryotes (Table 11.2). For fungi, the Mycology Antifungal Resistance database allows prediction of antimycotic resistance.

11.3.3.3 Secondary Metabolites

AntiSMASH (antibiotics and secondary metabolites analysis shell) database and prediction tool (Medema et al. 2011) aim to identify secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. A special facility is the analysis of non-ribosomal peptide synthetases and polyketide synthetases and other biosynthesis pathways (NRPS/PKS).

The server can be accessed from https://antismash.secondarymetabolites.org/#!/start via interactive graphical user interface. The search can be done with three levels of detection strictness. The tool has been described in several follow-up papers (Blin et al. 2021). Prediction of secondary metabolites is based on profile Hidden Markov Models (pHMM). The database can also be used for search with full DNA metagenome data (Chap. 9).

Bacteriocin production can be predicted by BAGEL4 (http://bagel4.molgenrug.nl/) (van Heel et al. 2018). Bacteriocin production is important to test in bacteria with a potential to be used as probiotics.

11.3.3.4 Prophages

Genomes can be uploaded to PHASTER (http://phaster.ca/) (Arndt et al. 2019) and PHAST (http://phast.wishartlab.com/) prophages predicted. There are probably no databases to predict lytic phages.

11.3.3.5 Plasmids Mobile Genetic Elements

PlasmidFinder (Carattoli et al. 2014) (https://cge.food.dtu.dk/services/PlasmidFinder/) based on whole genomic sequences as query and replicon prediction based on selected plasmid genes mainly associated with replication types. ICEberg 2.0 (https://bioinfo-mml.sjtu.edu.cn/ICEberg2/index.php) is used for the prediction of integrative and conjugative

Table 11.2 Prediction of antimicrobial resistance databases and servers

Database/server	URL	Reference
CARD	https://card.mcmaster.ca/	Alcock et al. (2023)
ResFinder	https://cge.food.dtu.dk/services/ResFinder/	Zankari et al. (2012)
AMRFinderPlus	https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/	Feldgarden et al. (2021)
MARDy	Mycology Antifungal Resistance database http://mardy.dide.ic.ac.uk/	

elements from whole genomic sequences (Liu et al. 2019). The insertion sequence (IS) database https://www-is.biotoul.fr/ allows search and submissions of new IS elements (Siguier et al. 2015).

11.3.3.6 Restriction and Modification of DNA

REBASE http://rebase.neb.com/rebase/rebase.html (Roberts et al. 2023). DNA methyl transferases have recently extensively been predicted by real-time single-molecule real-time (SMRT) sequencing as determined on the PacBio sequencing platform (Chap. 2). PRODORIC allows position weight matrices for regulons and transcription factor binding sites to be identified (Dudek and Jahn 2022) (https://www.prodoric.de/).

11.3.3.7 CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) may have a role in prokaryotes defense against bacteriophages and other foreign DNA. Their structure with spacers and repeats can be predicted in the whole genomic sequences. CRISPRFCasFinder identifies the regions and extracts the repeated elements and the unique spacers (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index).

11.4 Organisms Specific Platforms for Whole Genome Sequence-Based Typing

Enterobase is dedicated work with *Escherichia*, *Salmonella*, *Clostridioides* (*Clostridium difficile*), and *Yersinia* (https://enterobase.warwick.ac.uk/) (Alikhan et al. 2018). At this server, the raw Illumina reads can be uploaded and compared to traditional MLST (legacy MLST) and different variants of wgMLST databases. It is also possible only to perform MLST based on genes encoding for the ribosomal proteins (rMLST) (Jolley et al. 2012).

Pathogenwatch (https://pathogen.watch/) includes prediction tools for the most common bacterial pathogens.

11.5 Association of Genetic Trains with Metadata and Phylogeny

Analysis of the genome can be performed by ROARY (Page et al. 2015). Such analysis will define the accessory and core genome (Table 11.1). ROARY analysis is based on the annotated genomes such as obtained by Prokka (Chap. 2). The core genome identified can then be used for genomic phylogenetic analysis (phylogenome). Such a phylogeny can be linked to metagenomics traits and single level genome distribution and traits by Scoary including statistical evaluation (Brynildsrud et al. 2016). ROARY and Scoary both require installation in a Linux (Ubuntu) environment and some bindings, which are beyond the scope of this book. A system administrator will be able to install the programs in the right environment. Sitto and Battistuzzi (2020) provide rather detailed instructions for installations as well as further hints to the use of ROARY.

11.6 Activities

11.6.1 MLST Typing of *Pasteurella multocida*

This species is mainly an important animal pathogen; however, it can also affect humans if bitten by animals. A well-curated MLST scheme has been set up at:

https://pubmlst.org/bigsdb?db=pubmlst_pmultocida_seqdef. (Davies et al. 2004; Jolley and Maiden 2010; Subaaharan et al. 2010). Select **Sequence query all loci** and upload a genome of *P. multocida*. To get a genome, follow the instruction in 3.8.2 and use acc. no. LT906458. The result should be ST13 in the **RIRDC MLST** scheme and ST3 in the **Multiple host scheme**. The reason for two sequence types is that there exist two MLST typing systems for this species. For most species, only one MLST scheme is available.

11.6.2 Graphics

Phylogenetic inference and data visualization (PHYLOViZ) is used for visualization of results generated by sequence-based typing (http://www.phyloviz.net/) (Francisco et al. 2012). It is a freeware program that can visualize population genetics structures as a system of lines and circles with sizes of circles proportional to number of isolates in each ST.

The program can both be used online from the URL and as a downloaded program. Two files are needed, one with the MLST profiles and one with the iso late information (auxillary data). At least one of the columns in the isolates file needs to be labeled the same way as in the profiles. This can, for example, be "ST" (Fig. 11.5).

The program can be used online, but to obtain graphics for publications, you need to download and install the program from the URL. For Windows, save the zip file to your computer, unzip to a folder on c: for instance, **c:/phyloviz**. Open the folder and the "**bin**" folder, press the **phyloviz64.exe** icon, which will activate the program.

Prepare the two input files (Fig. 11.5) with a text editor like **word pad**. Separate the columns with one tab stroke and save each file in txt format. Start the program and select **File | Load dataset | Dataset name** where you can select any name. Select dataset type: **MLST**, select **Next** and upload the profiles file, press Open and **Finish** and then the isolates file and again press **Open** and **Finish**. At **Datasets** in the top left corner, you will now see the name of the dataset just uploaded. Double click on it, and you will see the name of the two files with the isolate and profile (MLST) information. Right click on the MLST set and select **Compute | geoBURST** and select **Next**. Select the level of links between STs, SLV, DLV, or TRV, for instance, TRV. Click on the key to left to the MLST name, which will show the file **geoBURST**. Click on it, and you will see the graphics. Now go to the upper left corner of the window, double click on Isolates, which will show the table with isolates information press **Select** and **View**. Format the information from the **Options** panel below left to the graphics by unselecting **Group** and select all other options. Use **control** to adjust the line lengths and node sizes. You can arrange the nodes and lines by

Profiles file

ST	gene1	gene2	gene3	gene4	gene5	gene6	gene7
1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	2
3	3	1	1	1	1	1	2
4	3	1	1	2	1	1	2
5	3	1	2	1	2	1	1
6	1	2	1	3	2	2	1
7	3	3	2	3	2	2	1
8	3	3	1	2	1	2	3
9	3	3	2	3	2	2	3
10	3	2	2	1	3	2	1

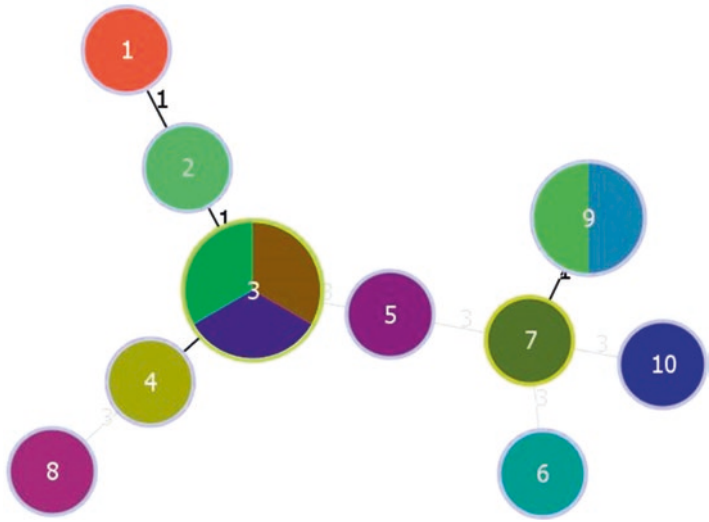
Isolates file (note that the ST column links this file to the Profiles file)

ST	isolate	country	host
1	A1	USA	Mouse
2	B2	Germany	Mouse
3	C4	Australia	Mouse
3	D5	Germany	Mouse
3	E6	Germany	Mouse
4	G2	Australia	Rat
5	H3	Netherlands	Mouse
6	I1	Netherlands	Rabbit
7	J6	Netherlands	Mouse
8	K9	Denmark	Mouse
9	L3	Germany	Mouse
9	M7	Netherlands	Mouse
10	N4	Australia	Rat

Fig. 11.5 Input files for PHYLOViZ. This example is showing 10 MLST types in the "profiles file" and 13 isolates linked to the STs in the "isolates file"

dragging with the mouse. When done pressing the pause (‖) sign, it should result in the output like A11.1. If there are problems, then control the input files with respect to profiles and isolates information and make sure that columns have been separated by exactly one tab field. If the nodes are not nice pie shaped, try to open the isolates table from the program and press **Select** and **View** again. When the graphics is satisfactory, pause the viewer, save the result by clicking on the camera, and select appropriate format for instance jpg. Insert the jpg in power point as a picture (A11.1).

You can now use this setup with the fictive dataset as a learning tool by changing the allelic profiles in the profile file and see the effect on the graphics. If there too few common alleles between the STs (less than 4), they will no longer be linked in the graphic structure.



Picture A11.1 Output from PHYLOViZ based on the data in Fig. 11.5. The nodes are labeled with the STs, and the numbers at the branches are the number of alleles differences between STs. The size of nodes reflects the number of isolates in each ST, and the color can be used for a legend indicating the origin of the isolates

Further Reading

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Appendix

Abbreviation of Amino Acids

A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic acid	Asp
E	Glutamic acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
R	Arginine	Arg
Q	Glutamine	Gln
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Ambiguity Table Symbols

G or A	R	puRine
T or C	Y	pYrimidine
A or C	M	aMino
G or T	K	Keto

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G or C	S	Strong interaction (3 H bonds)
A or T	W	Weak interaction (2 H bonds)
A or C or T	H	not-G, H follows G in the alphabet
G or T or C	B	not-A, B follows A
G or C or A	V	not-T (not-U), V follows U
G or A or T	D	not-C, D follows C
G or A or T or C	N	aNy

Codon Tables

The standard codon table (1) is used for most animals and plants. Codons labelled with green are start codons and those labelled red are the stop codons.

All codon tables are listed at
https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi

1. The Standard Code (transl_table=1)
By default all transl_table in GenBank flatfiles are equal to id 1, and this is **not** shown. When transl_table is not equal to id 1, it is shown as a qualifier on the CDS feature.
All code tables
http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=t#SG1

	pos. 2				
pos. 1	A	C	G	T	pos. 3
A	AAA K Lys	ACA T Thr	AGA R Arg	ATA I Ile	A
A	AAC N Asn	ACC T Thr	AGC S Ser	ATC I Ile	C
A	AAG K Lys	ACG T Thr	AGG R Arg	ATG M Met i	G
A	AAT N Asn	ACT T Thr	AGT S Ser	ATT I Ile	T
C	CAA Q Gln	CCA P Pro	CGA R Arg	CTA L Leu	A
C	CAC H His	CCC P Pro	CGC R Arg	CTC L Leu	C
C	CAG Q Gln	CCG P Pro	CGG R Arg	CTG L Leu i	G
C	CAT H His	CCT P Pro	CGT R Arg	CTT L Leu	T
G	GAA E Glu	GCA A Ala	GGA G Gly	GTA V Val	A
G	GAC D Asp	GCC A Ala	GGC G Gly	GTC V Val	C
G	GAG E Glu	GCG A Ala	GGG G Gly	GTG V Val	G
G	GAT D Asp	GCT A Ala	GGT G Gly	GTT V Val	T
T	TAA * Ter	TCA S Ser	TGA * Ter	TTA L Leu	A
T	TAC Y Tyr	TCC S Ser	TGC C Cys	TTC F Phe	C
T	TAG * Ter	TCG S Ser	TGG W Trp	TTG L Leu i	G
T	TAT Y Tyr	TCT S Ser	TGT C Cys	TTT F Phe	T

Codon table 11 is used for prokaryotes. Codons labelled with green are start codons and those labelled red are the stop codons. Compared to codon table 1, table 11 has more alternative start codons; however, ATG is most frequently used as start codon.

All codon tables are listed at

https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi

11. The Bacterial and Plant Plastid Code (transl_table=11)					
	pos. 2				
pos. 1	A	C	G	T	pos. 3
A	AAA K Lys	ACA T Thr	AGA R Arg	ATA I Ile	A
A	AAC N Asn	ACC T Thr	AGC S Ser	ATC I Ile	C
A	AAG K Lys	ACG T Thr	AGG R Arg	ATG M Met i	G
A	AAT N Asn	ACT T Thr	AGT S Ser	ATT I Ile	T
C	CAA Q Gln	CCA P Pro	CGA R Arg	CTA L Leu	A
C	CAC H His	CCC P Pro	CGC R Arg	CTC L Leu	C
C	CAG Q Gln	CCG P Pro	CGG R Arg	CTG L Leu i	G
C	CAT H His	CCT P Pro	CGT R Arg	CTT L Leu	T
G	GAA E Glu	GCA A Ala	GGA G Gly	GTA V Val	A
G	GAC D Asp	GCC A Ala	GGC G Gly	GTC V Val	C
G	GAG E Glu	GCG A Ala	GGG G Gly	GTG V Val	G
G	GAT D Asp	GCT A Ala	GGT G Gly	GTT V Val	T
T	TAA * Ter	TCA S Ser	TGA * Ter	TTA L Leu	A
T	TAC Y Tyr	TCC S Ser	TGC C Cys	TTC F Phe	C
T	TAG * Ter	TCG S Ser	TGG W Trp	TTG L Leu i	G
T	TAT Y Tyr	TCT S Ser	TGT C Cys	TTT F Phe	T

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