

Novel Methods for Post-Processing Evolutionary Data Using Machine Learning Techniques

Dissertation
by

Lucas Czech

Karlsruhe Institute of Technology (KIT),
Karlsruhe, Germany

Heidelberg Institute for Theoretical Studies (HITS),
Heidelberg, Germany

First Reviewer:
Second Reviewer:

Prof. Dr. Alexandros Stamatakis
Prof. Dr. Emmanuel Müller

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Zusammenfassung

Auf deutsch...

Abstract

In English...

Hiermit erkläre ich, dass ich diese Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe. Ich habe die Satzung des Karlsruher Institutes für Technologie (KIT) zur Sicherung guter wissenschaftlicher Praxis beachtet.

Karlsruhe, September 30, 2018

.....
(Lucas Czech)

Acknowledgment

“Evolution forged the entirety of [...] life on this planet using only one tool – the mistake.”

— Dr. Robert Ford (Anthony Hopkins),
Westworld, Season 1: The Original

falsification, science “irrt sich vorwaerts”... mein Beitrag, aber nicht ohne die Hilfe vieler anderer

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mark, emily, jaime, fred, micah, cedric, colomban, pelin

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phil collins peter gabriel

richard dawkins, ohne den ich nicht auf das Thema aufmerksam geworden waere und daher nicht mit der phd stelle angefangen haette.

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List of Acronyms

RT reference tree

RA reference alignment

QS query sequence

BT backbone tree

CT clade tree

LWR likelihood weight ratio

PhAT Phylogenetic Automatic (Reference) Tree

PCA Principal Components Analysis

MDS Multidimensional scaling

BV Bacterial Vaginosis

NTS Neotropical Soils

TO Tara Oceans

HMP Human Microbiome Project

1. Introduction

TODO: check that the url and access date of all online sources are present in bibliography! TODO: I used a few public domain images from wikipedia as sources, and modified them as needed. make sure that this is okay.

1.1 Motivation

1.2 Objective and Contribution

swarm code contrib [133, 134]

full list of publications is available in D

1.3 Structure and Overview

2. Foundations

This chapter is partially based on the peer-reviewed publications:

- **Lucas Czech**, Pierre Barbera, and Alexandros Stamatakis. "Methods for Automatic Reference Trees and Multilevel Phylogenetic Placement." *Bioinformatics*, 2018.
- **Lucas Czech** and Alexandros Stamatakis. "Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements." *PLOS One*, 2018.

TODO: Contributions: Lucas Czech... Pierre Barbera... Alexandros Stamatakis... and...

TODO: In this chapter, we introduce...

2.1 Evolution and Genetics

Life on Earth is at least 3.77 billion years old [49], and is continuously evolving due to *natural selection* [40]. Driven by *variation*, biological populations diversify through successive generations, leading to the origination of new species. This continuous process is called *evolution* [81]. Heritable characteristics are passed down from parent to offspring, with occasional random mutations leading to variation. Thus, some organisms are better adapted to their environment than others, and have more reproductive success. There is hence a natural selection for advantageous mutations, which can then spread through generations.

The characteristics and traits of an organism are carried by, and inherited via, *deoxyribonucleic acid* (DNA). DNA is the molecule that encodes the genetic information needed for the functioning of all living organisms. It is structured in form of

a double helix [224], and built from two strands of molecules called *nucleotides*. The nucleotides build the backbone of the double helix, and connect the two strands via opposing pairs of *nucleobases*. The redundant structure of pairs of nucleobases gives stability to the DNA molecule, and also serves as a mechanism of error correction when reading the genetic information. In DNA, there are four distinct nucleobases: adenine (A), cytosine (C), guanine (G), and thymine (T), where A pairs with T, and C pairs with G, respectively.

TODO: explain transition vs transversion? TODO: picture of dna and the bases?

The sequence of nucleobases along the strands of DNA is what encodes the genetic instructions used by all known living organisms. Parts of the DNA encode for proteins, which perform a plethora of different functions within organisms. Proteins consist of long chains of amino acid residues, and are assembled in a process called protein (bio)synthesis. This is described by the central dogma of molecular biology [33, 34]: First, DNA is *transcribed* into the intermediary ribonucleic acid (RNA), which is then *translated* into the actual protein.

In each step of this process, the alphabet used to encode information differs. While DNA uses the four nucleobases as described above, in RNA, the nucleobase uracil (U) is used instead of thymine (T). Proteins on the other hand are (mostly) build from a set of 20 standard amino acids. The set of rules used by the molecular machinery for translating nucleobases into amino acids is called the *genetic code*: In a DNA sequence, three consecutive nucleobases are needed to encode one amino acid [198].

The entirety of the genetic material of an organism, that is, its complete DNA sequence, is called its *genome*. A *gene* is a sequence which codes for a molecule that has a particular function, such as a protein [70]. DNA and genes are the basic units of heredity. They are what is varying across generations, and what is selected for in the process of natural selection [41]. The study of genes, variation and heredity is called *genetics* [76].

All life on this planet is related to each other and descends from a common ancestor. Still, it is remarkable that the basic molecular principles and mechanisms of life—DNA, amino acids, and the genetic code—are virtually identical for all living organisms. This implies that by understanding and comparing the genetic information encoded in the DNA of different organisms, one can understand the diversification patterns of evolution.

2.2 Sequence Analysis

TODO: maybe move the paragraph above to here, and make a transition

2.2.1 Genome Sequencing

Prior to analyzing the DNA of an organism, the physical order of nucleobases in the DNA molecule has to be determined. That is, the DNA has to be “read” and stored

in a human-accessible text format, typically a computer file. This technical process is called DNA *sequencing*.

For many decades, the main technique for this purpose was Sanger sequencing [187, 188]. It is labor- and time-intensive, but through improvement and automation, costs were constantly reduced. Eventually, this allowed for large-scale efforts, such as the Human Genome Project [216], which sequenced the whole human genome of more than three billion nucleobases. Sanger sequencing allows to determine long parts of the sequence at once (> 500 nucleobases), which then have to be assembled to build the final sequence.

In the last decades, a variety of novel high-throughput DNA sequencing technologies have been developed [46, 174, 181]. In particular, *Next Generation Sequencing* (NGS) [123, 138] has revolutionized biology by transforming it into a data-driven and compute-intense discipline [60]. The costs of these technologies are decreasing faster than Moore’s law [228]. This leads to a “tsunami” of sequence data, which poses a challenge for computational methods working with these data. Compared to Sanger sequencing, NGS technologies are generally cheaper and faster [152, 221], but come at the price of introducing more errors in the sequence output, or only being able to determine shorter parts of the sequence at once – both of which constitute a challenge for the subsequent assembly of the final sequence.

The result of DNA sequencing is a textual representation of the order of nucleobases. Although this representation ignores the physical and chemical properties of the respective molecules, it is helpful in many applications, and allows to leverage existing algorithms. Each contiguous sequence coming from the sequencing machine is called a *read*. Because of the pairing of nucleobases, both DNA strands can be sequenced, which provides a means of error correction. Such data are typically stored in the **fastq** file format [30]. These so-called paired-end reads are then merged to form a final sequence representation of one strand – that is, a sequence of the characters A, C, G, and T. These data are stored in formats such as the **fasta** file format [172]. Due to the pairing of nucleobases, the length of a DNA sequence is measured in *base pairs* (abbreviated bp): 1 bp represents one character in the file. These files are then the input for computational methods for working with DNA sequences.

2.2.2 Metagenomics

Sanger sequencing requires careful preparation of the genetic material, and is thus best used for sequencing single organisms. There are however many (microbial) organisms that cannot be cultured in a Petri dish, and are hence hard to sequence with this technique. Apart from being cheaper, Next Generation Sequencing machines however “digest” all genetic material presented to them. They thus allow for studying microbial samples directly extracted from their environment [59, 160, 207]. This enables to study environments such as water [71, 75, 98], soil [54, 135], the human body [91, 140, 151, 201], and many others. Each sample from such an environment then represents a geographical location, a body site, a point in time, etc. The DNA of all organisms being present in a sample is sequenced, resulting in a large number

of reads per sample. These reads are anonymous, as it is unclear to which organism they originally belonged.

The study of these data, that is, genetic material from environmental samples, is called *metagenomics* [168]. A first step in such studies is often to characterize the reads obtained from an environment in terms of *reference sequences* of known species. Reads that are similar to (parts of) reference sequences can be assigned to them, while reads with low similarity to known sequences might indicate novel, undescribed species [211]. Key tasks in metagenomic studies are the identification and classification of the anonymous reads (“Who is there?”), and their functional annotation (“What are they doing?”) [47]. Both are introduced in the following.

Functional annotation [204] is the prediction of gene functions of the reads, and the inference of metabolic capacity of microbial communities [24]. As the proteins that are needed in the pathways of such functions can be encoded by genes across the genome, whole-genome sequencing is necessary to capture all genes of interest. For example, in shotgun sequencing [5, 202], the DNA is fragmented into small pieces within the size range that the used sequencing technology can handle, typically a few hundred bp long. This allows to sequence all genetic material contained in a sample. Thus, the resulting reads originate from different parts of the genomes of their organisms, which can then be functionally annotated [73]. This however necessitates to use whole-genome reference sequences in order to be able to assign reads to known species and functions. Typical databases of reference sequences however lack many of the protein sequences from the microbial species present in a sample, mostly because of organisms that cannot be cultured [24].

For the task of identification and classification of reads however, whole genome references are not needed. Instead, specific *marker genes* can be used, which are regions of genes that are particularly suited for delineating between different species [180]. The method of using marker genes to identify species is called *DNA barcoding* [85, 190]. The choice of genes to use as marker is important, and depends on the types of organisms to be studied. The used marker genes should ideally be present in all of the organisms of interest, short enough to be sequenced with current technology, and have enough variation between species to distinguish between them, but have low variation within species [108].

In many metagenomic studies of *bacteria* and *eukaryota*, the 16S [226] and 18S [153] rRNA regions are used as marker genes, respectively [230, 231]. These regions belong to the small subunit (SSU) of the ribosomal ribonucleic acid (rRNA), which is an essential component of the ribosome. The ribosome is a molecular machinery that is responsible for protein synthesis (translation) in all living organisms. Often, prior to sequencing, these regions are amplified by many orders of magnitude, using polymerase chain reaction (PCR) to create copies of these regions [11]. The resulting reads are then de-replicated again, which results in sequences called *amplicons*. While the PCR amplification process is known to introduce bias [24, 124], this inexpensive method is commonly used in practice, particularly for the 16S and 18S rDNA regions. A recent alternative to using PCR for obtaining reads from these regions are _{mi}tags [124]. In this approach, shotgun sequencing is used to get reads

from the whole genomes of the organisms in a sample. These reads are then filtered to only contain reads from the 16S region (for *bacteria*), which capture the diversity of the sample without bias.

Because of the ubiquity of the 16S and 18S rDNA regions in organisms and, consequently, in sequencing studies, many databases provide reference sequences for these marker regions. The reads or amplicons obtained from an environmental sample can then be employed to estimate the microbial diversity of the organisms in the sample by comparison against known species.

2.2.3 Sequence Alignment

Organisms that evolved from a (not too distant) common ancestor share genetic information. Regions of their DNA that have a shared ancestry are called *homologous* regions [105]. This homology is typically inferred from sequence similarity. However, due to mutations, differences in the sequences can occur. There are three main types of sequence mutations that can occur in evolution: a *substitution* is the exchange of a nucleobase for another; a *insertion* adds one or more extra nucleotides into the sequence; a *deletion* removes one or more nucleotides from the sequence. The latter two types of mutations change the length of the sequence; a mutation that is either one of them is called an *indel*.

Because of indels, sequences have to be aligned to each other in order to compare their homologous regions. That is, gap characters (-) have to be added to the sequences such that homologous characters in the sequence get aligned. This results in an $n \times m$ matrix, where n is the number of sequences (rows), and m is the number of homologous characters (columns), called *sites*. This matrix is called a *multiple sequence alignment* (MSA), or simply an *alignment*. Figure 2.1 shows an example of the alignment process and the resulting MSA.

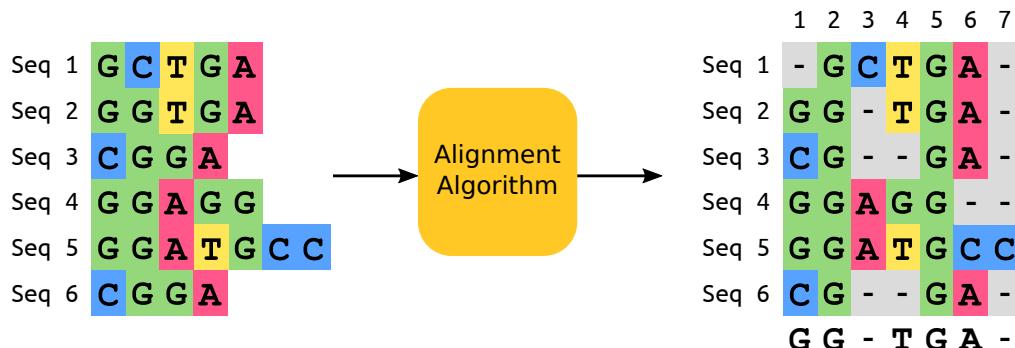


Figure 2.1: Multiple Sequence Alignment. The left hand side shows a set of six sequences. Using an alignment algorithm, gaps are inserted into these sequences at presumed indel positions. The right hand side shows the result of this process, where homologous characters at the sites of the multiple sequence alignment (MSA) are aligned to each other. Below the MSA, the majority rule consensus sequence is shown, see Section 2.2.4.

Sequence alignment can be understood as an optimization problem under a given optimality criterion. On the one hand, *global alignments* attempt to align every character in every sequence, which is most useful for similar sequences of roughly equal size. For example, the Needleman-Wunsch algorithm [161] is a general global alignment technique based on dynamic programming. On the other hand, *local alignments* are better suited for dissimilar sequences which might contain similar regions within a larger sequence context. The Smith-Waterman algorithm [199] is a general local alignment technique using the same dynamic programming scheme, which additionally allows to start and end at any place in the sequence. As both algorithms have their particular use cases [176], hybrid methods have also been developed [25]. Furthermore, heuristic approaches such as BLAST [3] and USEARCH [58] can calculate millions of near-optimal alignments in reasonable time.

These algorithms are efficient for the pairwise alignment of two sequences. Calculating an MSA however has been shown to be NP-hard [95, 223]. Thus, for most empirical data sets, other approaches and heuristics are needed [212]. Tools such as CLUSTAL [87], MUSCLE [57], and MAFFT [99] can calculate near-optimal multiple sequence alignments for many thousands of sequences.

A special use case for aligning sequences arises in metagenomic studies, where environmental sequences are often compared to a set of known reference sequences. In these studies, one often first calculates an MSA of the reference sequences, and then successively aligns the environmental sequences against this MSA. This is because calculating an MSA for millions or billions of sequences from scratch is too expensive even for modern tools. Hence, specialized algorithms for this use case have been developed, such as PAPARA [14, 15] and HMMALIGN, which is a subprogram of the HMMER suite [55, 56].

2.2.4 Consensus Sequences

When working with a number of related but not identical sequences, it is often convenient to “summarize” homologous characters in form of a *consensus sequence*. Such a sequence is typically calculated based on the relative frequencies of the characters per alignment site. It then represents typical features and motifs of the input set of sequences.

The most straight forward method is to construct *majority rule consensus* sequences [43, 147], where each site is represented by the most frequent character at that site. Figure 2.1 shows an example below the MSA on the right hand side. In order to also include information from the less frequent characters at a site in the consensus sequence, *ambiguity characters* can be used [92]. They allow to denote multiple alternative nucleobases as a single character. For example, if the nucleobases A and G are similarly frequent at a site, this site is represented by the ambiguity character R. See Table A.1 for the full list of character representations.

Using ambiguity characters allows for more involved consensus methods. For example, *threshold consensus* sequences [42, 43] include the most frequent characters that are needed to achieve some given frequency threshold (between 50% and 100%)

per site, and represent these characters by their ambiguity character. Furthermore, many methods based on fixed thresholds have been proposed, such as Cavener’s method [27, 28]; see [43] for a critical comparison.

It is theoretically also possible to directly use the relative frequencies of characters per site in the mathematical frameworks of many downstream analysis methods. This would allow to leverage all of the information contained in the input set of sequences. However, to our knowledge, there is no convention or file format to store such information, and consequently, no way of forwarding this information to the respective tools.

2.3 The Tree of Life

The shared evolutionary history of life gives rise to a branching pattern, where new *lineages* split from a common ancestor. This branching pattern forms a tree-like structure, which classifies organisms in a hierarchy based on common descent.

While this *tree of life* is an expedient and, hence, pervasive model [156], it ignores certain biological and evolutionary events. A strict hierarchy does not allow for reticulate events, such as hybridization [131], genetic recombination [86], or horizontal gene transfer [52, 166, 183]. Although approaches such as networks have been proposed to model these events [90], the simplicity of a hierarchy or tree structure still has proven to be useful in classifying and naming organisms, and understanding their evolutionary relationships.

2.3.1 Taxonomy and Nomenclature

Early attempts at classifying organisms date back to the Greek philosopher Aristotle, who used observable attributes to divide living things into groups [113]. This approach as well as the efforts of later centuries were non-uniform and inconsistent. The basis for the modern system of classification was established by Swedish botanist Carl Linnaeus in the mid-18th century [50]. He proposed a *nomenclature*, that is, a naming system for organisms, as well as a *taxonomy*, that is, a rank-based classification of organisms [118, 119].

A taxonomic group of organisms is called a *taxon* (plural: *taxa*). Each taxon is associated with a *taxonomic rank*, which can subsume other ranks, thus forming a hierarchy of higher and lower ranks. A taxonomic rank represents the relative level of a group of organisms in the taxonomy. The principal ranks in modern use are *domain*, *kingdom*, *phylum*, *class*, *order*, *family*, *genus*, and *species*, see Figure 2.2. If needed, further ranks can be included in between (such as *sub-genus*), or more refined lower levels be added (such as *strain*, which is a further distinction within a species).

In order to scientifically name the groups of organisms (taxa) in a taxonomy, the *binomial nomenclature* as introduced by Linnaeus is still prevalent to this day. It uses two terms, often of Latin origin, which respectively specify the taxonomic ranks *genus* and *species* that an organism belongs to, for example *Homo sapiens*.

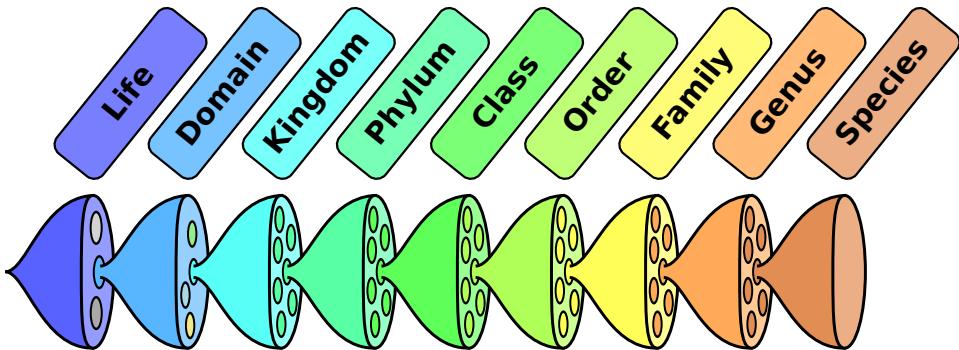


Figure 2.2: Biological classification into taxonomic ranks. The figure depicts a typical set of nested taxonomic ranks [231], which form a hierarchy with increasingly deeper levels towards the right. Source: Image derived from [80].

While early classifications used *phenotypes*, that is, observable characteristics or traits of organisms, modern approaches to taxonomy take genetic information into account [149]. For example, the three-domain system [230, 231] resolves the oldest evolutionary relationships, that is, the highest taxonomic levels, based on 16S rRNA data. Although this classification has been challenged [26, 79, 148], it is widely used. It divides cellular life forms into the three domains *bacteria*, *archaea*, and *eukaryota*; the latter is further separated into kingdoms, which include the kingdoms *fungi*, *plants*, and *animals*.

2.3.2 Phylogenetic Trees

The classification of organisms into a taxonomy is based on (subjective) dissimilarity and thus arbitrary: The number of organisms that are grouped into a taxon at a given rank can vary, and the separation into discrete ranks does not reflect the gradual nature of evolution [72]. A more involved approach that can resolve these issues is *phylogenetics*, which is the study of the evolutionary history and relationships of biological entities (individuals, species, populations).

The evolutionary relationships of such entities are called their *phylogeny*. As the true phylogeny of a set of taxa is unknowable, it has to be inferred from data that is available. A phylogenetic analysis uses inference methods that evaluate observed heritable traits in order to resolve the phylogeny under a given model of evolution of these traits. While phenotypes can be used, modern phylogenetic inference is mostly based on DNA data. The result of a phylogenetic analysis is a *phylogenetic tree*, also called an *evolutionary tree*, or—synonymously—a *phylogeny*. Figure 2.3 shows two examples of phylogenetic trees.

The leaf nodes, or *tips*, of the tree represent living (*extant*) biological entities such as species, strains, individual organisms, or even cells of a multicellular organism. Thus, the tips are often referred to as the *taxa* of the tree, which is meant as a generic term that includes all the above entities. The tips are often named according to the entities they represent (e.g., species names); such a tree is called a *labeled* tree. The inner nodes on the other hand are usually anonymous and represent speciation

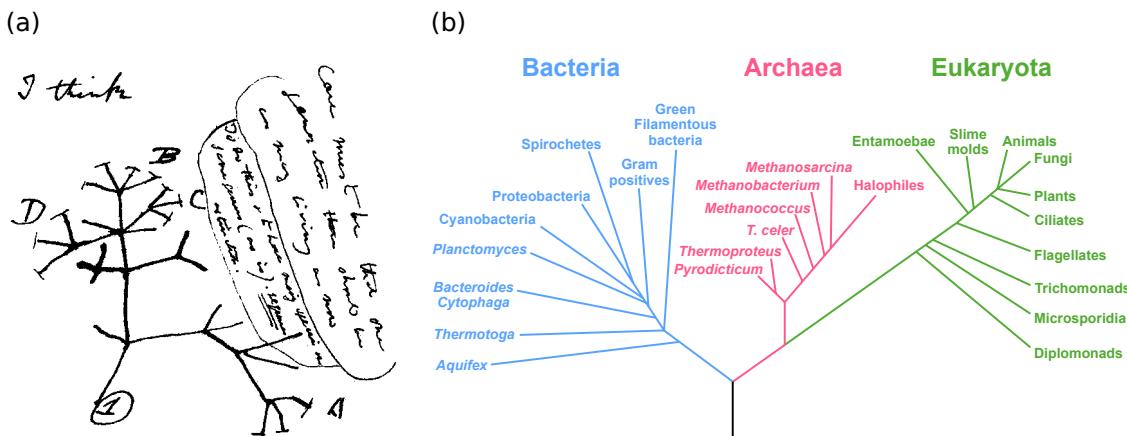


Figure 2.3: Exemplary phylogenetic trees. (a) In 1837, Charles Darwin sketched his first evolutionary tree below the words “I think” in his notebook on “Transmutation of Species”. Source: Image derived from [39]. (b) A modern phylogenetic tree showing the three-domain system [230, 231], which emphasizes the separation of *bacteria*, *archaea*, and *eukaryota* based on 16S rRNA genes. The black branch at the bottom represents the speculative last universal common ancestor of all living organisms. Source: Image derived from [68].

events, where two novel lineages arose from a putative common ancestor. The branching pattern of a phylogenetic tree thus reveals the evolutionary history of its taxa.

The edges of the tree, also called its *branches*, can have associated *branch lengths*, which represent the evolutionary time between the two adjacent nodes, for example, measured as the average change in nucleobases between their respective sequences. The unique path between any two nodes thus can be interpreted as a measure of evolutionary relatedness of the taxa represented by the nodes.

A phylogenetic tree is *rooted* if it is a directed tree that has a unique *root node*, which corresponds to the putative common ancestor of the other nodes in the tree. See Figure 2.3(b) for an example. As evolution is a processes that happens over time, from a biological point of view, every tree has a root. However, most models of DNA evolution are time-reversible, meaning that the direction of change in the sequences cannot be inferred from the data, see Section 2.3.3 for details. Thus, tree inference methods can also yield *unrooted* trees without direction and without a root node. These can be rooted a posteriori, for example by using an *outgroup* of taxa that are closely related to the group of taxa of interest (the *ingroup*), but not part of it. Then, a root node can be placed on the branch that separates the outgroup from the ingroup.

An inner node that has exactly three neighboring nodes is called a *bifurcation* or a *bifurcating* node. In rooted trees, these neighbors are the the parent and the two children of a node, hence the name. An inner node with more neighbors is called a *multiplication* or a *multiplying* node. This naming also applies to the whole tree:

A tree, where each inner node (with the exception of the root node in a rooted tree) is bifurcating, is also called a bifurcating tree. Otherwise (if there is at least one multifurcating node), it is a multifurcating tree. Note that in evolution, an actual multifurcation event is highly unlikely, as it corresponds to the simultaneous formation of more than two new lineages from a single ancestral lineage. Multifurcating trees are for example used when relationships cannot be properly resolved from the existing data, or to summarize a set of otherwise contradicting trees.

Each edge of the tree induces a *bipartition* or *split* of the taxa of the tree into two groups, one on each side of the edge. A set of taxa is *monophyletic*, if there is a bipartition of the tree that splits these taxa from all other taxa of the tree. In a rooted tree, the node at the end of that edge is then the putative common ancestor of these taxa. Furthermore, a monophyletic set of taxa is called a *clade* of the tree; in other words, a clade is a subtree that is separated from the rest of the tree by one edge. For example, the taxa A, B, and C in Figure 2.4 are monophyletic—that is, they form a clade of the tree. Lastly, a non-monophyletic set of taxa is *paraphyletic*.

While the topology of the tree is what models the evolutionary relationships, there are several ways of visualizing or drawing that information. Figure 2.4 shows some examples, which all visualize the same topology (except for the rooting). The figure also summarizes some of the terms and concepts introduced above. Different drawing styles each have their advantages. For example, in a rectangular tree (Figure 2.4(b)), branch lengths are easier to read and compare, while a circular tree (Figure 2.4(c)) can fit more taxa in the same drawing area.

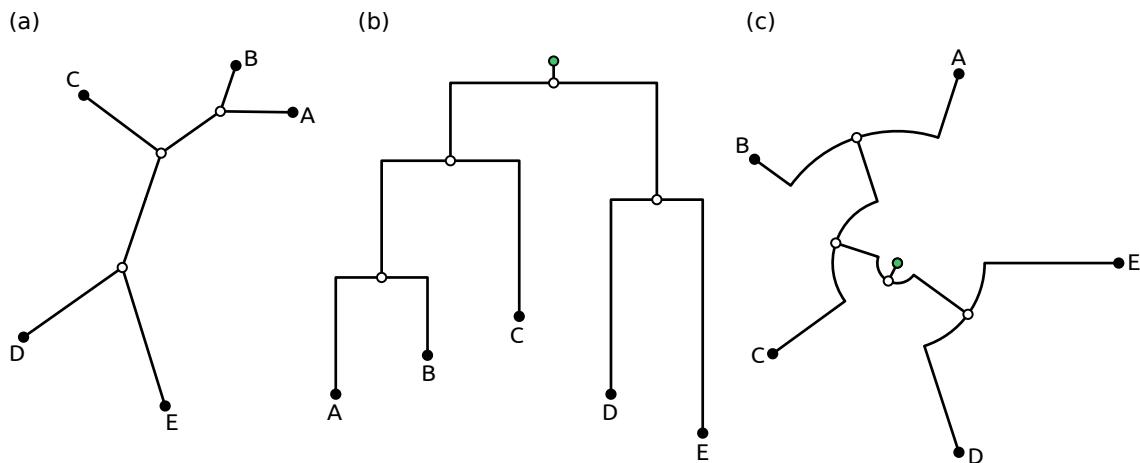


Figure 2.4: Types of phylogenetic trees. Here, we show three different types of labeled, bifurcating trees. Tip nodes are marked with black dots, inner nodes with white dots, and the root node (if present) with a green dot. (a) An unrooted tree with five taxa. (b) The same tree topology, but rooted on the inner branch that splits the taxa D and E from the other taxa. The tree is drawn in rectangular style. (c) The same tree again, but this time drawn in circular style.

Taxonomy and phylogeny serve a related, but different purpose: While the former is a system of classification, the latter reveals evolutionary history. However, there is

a correspondence between a taxonomy and a rooted phylogeny: Inner nodes of the tree constitute older evolutionary relationships, which are represented by the higher ranks of the taxonomy. Figure 2.3(b) shows such a correspondence for the three domains of life. It is however possible that the taxa at one rank of the taxonomy are not monophyletic in the tree. In this case, the two are *incongruent*.

2.3.3 Tree Inference

A phylogeny can be inferred from data that has per-taxon traits which are homologous, that is, which have evolved from the same traits in the common ancestor and are thus comparable [65, 234]. While historically these traits were mostly phenotypes (bone shapes and sizes, metabolism, etc.), the focus has since shifted towards molecular data such as DNA and amino acid sequences, as their *phylogenetic signal* is generally more abundant and less biased [88]. Most often, a multiple sequence alignment is used, whose homologous sites represent the traits of the taxa. To determine the degree of relatedness between taxa, mathematical models of trait evolution are employed.

The general concept of tree inference is then to put closely related taxa close to each other in the phylogeny. Hence, a tree inference can be thought of as an optimization problem, which searches for the best tree given an optimality criterion. However, the space of all possible tree topologies is too large for an exhaustive brute-force search for virtually all empirical datasets. For a given number of taxa n , the number of distinct tree topologies N is given as $N(n) = \prod_{i=3}^n (2i - 5)$, which grows over-exponentially fast [65]. There are thus different approaches and heuristics to conduct tree search.

Distance based methods such as *Unweighted Pair Group Method with Arithmetic Mean* (UPGMA) [200] and *Neighbor Joining* [186] use a pairwise distance matrix between sequences, and thus do not necessarily need an alignment. *Maximum Parsimony* [189] uses an optimality criterion that is based on Occam’s razor, that is, it yields the tree that explains the observed tip sequences (taxa) with the minimal number of substitutions (mutations). The *Maximum Likelihood* (ML) method [64] employs statistical techniques in order to evaluate the probability of a given phylogenetic tree with respect to a given alignment, and successively search the tree space for the most likely tree. Furthermore, *Bayesian Inference* also relies on the evaluation of tree probability [234], and uses Bayes’ theorem to calculate the posterior distribution of the relevant evolutionary processes; it thus can incorporate prior empirical knowledge into the process.

Maximum Likelihood Tree Inference

In the context of this work, we are mostly interested in Maximum Likelihood (ML) tree inference. It uses a probabilistic framework in which the (phylogenetic) likelihood

$$L(\text{MSA} \mid T, \bar{b}, M, \bar{\theta}) \quad (2.1)$$

is evaluated that an observed MSA is the outcome of an evolutionary history described by a phylogenetic tree with topology T and branch lengths \bar{b} , under a given model of trait evolution M with parameters $\bar{\theta}$. For a fixed model M (see below), the likelihood can be expressed as a function of T , \bar{b} and $\bar{\theta}$, which is known as the *phylogenetic likelihood function* (PLF). By maximizing the PLF using maximum likelihood estimation, the parameter values (including tree topology) are found which best explain the observed data. These estimates are typically obtained by iteratively and independently optimizing the tree topology and the branch lengths and model parameters, which is introduced in the following.

Finding the most likely tree topology is a discrete optimization problem, which has been shown to be NP-hard under the ML criterion [29]. Furthermore, the evaluation of the PLF is computationally expensive, as it involves many floating point operations. A general heuristic for the tree search that avoids an exhaustive evaluation of the tree space is thus as follows. First, a starting tree is generated, either randomly, or using methods such as Neighbor Joining or Maximum Parsimony. Then, the likelihood of the tree is successively improved by applying topological rearrangements (*moves*) of its taxa and clades. For instance, in *greedy hill-climbing* [203], only those moves are applied (*accepted*) that immediately improve the PLF, until a (potentially local) optimum is found.

For a fixed tree topology T , the maximum likelihood estimates of the branch lengths \bar{b} and the model parameters $\bar{\theta}$ are usually obtained with general-purpose numerical optimization methods. Since the derivatives of the PLF can be easily computed, the Newton-Raphson method [236] is often used for optimizing the branch lengths. Model parameters are commonly optimized with Brent's method [23] or with the Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm [67].

Models of Molecular Sequence Evolution

So far, we assumed a fixed model M for describing the evolution of the traits that are used for inferring the tree. For sequence data, such a model yields an estimate of the evolutionary distance between the sequences of different taxa. Most commonly, a continuous-time Markov chain (MC) is used to describe the evolution of a single site within a set of aligned sequences [69]. While the MC model ignores aspects such as natural selection and the molecular mechanisms of evolution, it describes the relative rate of changes in a way that allows multiple substitutions to occur along the same branch ($T \rightarrow A \rightarrow G$).

For DNA data, the MC has four states A, C, G, and T, which correspond to the nucleobases, and transitions between the states correspond to substitutions of the nucleobases, see Figure 2.5(a). **TODO: if transition vs transversion is explained above, we need to clarify here: note that transition is used in two different meanings here...** The process is defined by the instantaneous transition rate matrix Q , where the element q_{ij} is the *rate* of transition from state i to state j , see Figure 2.5(b). The rows of the matrix have the requirement to sum to 0, which defines the diagonal elements q_{ii} .

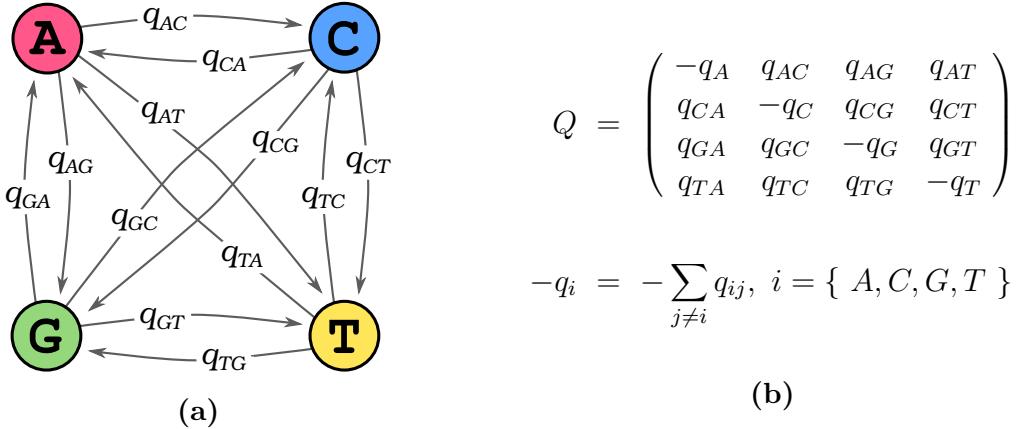


Figure 2.5: Markov chain Model of Nucleotide Substitutions. The evolution of characters at a site in an alignment can be modeled as a Markov chain (MC). (a) The states of the MC for DNA data are the four nucleobases A, C, G, and T. The model allows transitions between all states, which correspond to substitutions between the nucleobases. (b) The instantaneous transition rates between states are given by the substitution rate matrix (Q-matrix), where the rows must sum to 0.

The branch lengths of a phylogeny express the evolutionary time between two nodes, which can be interpreted as the expected number of substitutions between the respective alignment sites. Then, for a given time t , the *transition probabilities* $p_{ij}(t)$ between states in a stationary process are obtained by exponentiating the Q matrix [234]. These probabilities are called the P matrix

$$P(t) = e^{Qt} \quad (2.2)$$

For positive transition rates $q_{ij} > 0, \forall i \neq j$, if the process runs long enough, the Markov chain eventually reaches the unique *stationary* distribution $\Pi = (\pi_A, \pi_C, \pi_G, \pi_T)$, with π_i being the proportion of time spent in state i . If the Markov process reached equilibrium after running long enough, it can be interpreted as generating the sequences of the MSA. In that case, Π is the *equilibrium base composition* of the MSA, and π_i are the *equilibrium* or *stationary base frequencies* of the MSA.

As mentioned before, most models of DNA evolution assume a *time-reversible* Markov process, which means that $\pi_i q_{ij} = \pi_j q_{ji}, \forall i \neq j$. This assumption is biologically not meaningful, as evolution is a process in time, and thus does have a direction. It however allows for simplified calculations: The Q matrix of a time-reversible model can be formulated as the product of a *symmetric* rate matrix $R = \{r_{i \leftrightarrow j}\}$ and a diagonal matrix with the stationary base frequencies:

$$Q = R \cdot \text{diag}(\pi_i) = \begin{pmatrix} -q_A & r_{A \leftrightarrow C} \cdot \pi_C & r_{A \leftrightarrow G} \cdot \pi_G & r_{A \leftrightarrow T} \cdot \pi_T \\ r_{A \leftrightarrow C} \cdot \pi_A & -q_C & r_{C \leftrightarrow G} \cdot \pi_G & r_{C \leftrightarrow T} \cdot \pi_T \\ r_{A \leftrightarrow G} \cdot \pi_A & r_{C \leftrightarrow G} \cdot \pi_C & -q_G & r_{G \leftrightarrow T} \cdot \pi_T \\ r_{A \leftrightarrow T} \cdot \pi_A & r_{C \leftrightarrow T} \cdot \pi_C & r_{G \leftrightarrow T} \cdot \pi_G & -q_T \end{pmatrix} \quad (2.3)$$

The matrix describes the most general model of DNA evolution, where all 6 substitution rates $r_{i \leftrightarrow j}, i \neq j$ and all 4 base frequencies π_i can be different. This model is called the Generalized Time-Reversible (GTR) model [209]. As the sum of the base frequencies must be 1, and as the substitution rates are usually normalized by requiring that $r_{G \leftrightarrow T} = 1.0$, the GTR model has a total of 8 free parameters (that is, 3 base frequencies, and 5 substitution rates).

There are also more restrictive models, which have fewer free parameters, and are thus more robust if data for estimating them is sparse. The Jukes-Cantor model (JC69) [94] has no free parameter and assumes equal substitution rates $r_{i \leftrightarrow j} = 1, i \neq j$ and equal base frequencies $\pi_i = 1/4$. The K80 model [102] adds a free parameter κ , which describes the ratio between two types of substitutions that are not equally likely to occur in evolution: $r_{A \leftrightarrow C} = r_{G \leftrightarrow T} = \kappa \cdot r_{A \leftrightarrow G} = \kappa \cdot r_{A \leftrightarrow T} = \kappa \cdot r_{C \leftrightarrow G} = \kappa \cdot r_{C \leftrightarrow T}$. **TODO: if transition vs transversion was added earlier, add it here, too!** The HKY85 model [84] further adds parameters for the base frequencies, and hence has 4 free parameters. Further models have also been proposed, which offer compromises between the number of free parameters and the expressiveness of the model [234].

The state space of the Markov process becomes significantly larger for protein data, as it needs to comprise 20 standard amino acids instead of 4 nucleobases. Hence, the GTR model for protein data has $(400 - 20)/2 - 1 + 19 = 208$ free parameters. Typical alignments do not contain enough data to reliably estimate these parameters, and thus easily lead to over-fitting. Thus, so-called *empirical* protein models are commonly used, which have substitution rates and equilibrium base frequencies that were pre-estimated on large collections of reference alignments. Among others, some popular models include DAYHOFF [44], WAG [229], and LG [111].

Further Aspects of Tree Inference

constrained trees Models of Rate Heterogeneity among Sites

MC models single site evol ...

wiki by making the simplifying assumption that different sites evolve independently and are identically distributed. . This assumption may be justifiable if the sites can be assumed to be evolving neutrally. If the primary effect of natural selection on the evolution of the sequences is to constrain some sites, then models of among-site rate-heterogeneity can be used. This approach allows one to estimate only one matrix of relative rates of substitution, and another set of parameters describing the variance in the total rate of substitution across sites.

Alignment Partitioning Computation of Phylogenetic Likelihood Function and its Derivatives P-matrix Conditional Likelihood Vectors Likelihood Evaluation at the Root 6.4 Likelihood Derivatives with respect to the Branch Length Parameter

move to phylo trees section:

tree formats newick [6] NEXUS [130] phyloXML [83]

2.4 Phylogenetic Placement

introduce “reference tree”, as a tree inferred from reference sequences.

- . common use case: fixed tree, selected by experts to consist of known, described taxa to represent the diversity expected from an environment.

Since the amount of sequence data produced in metagenomic studies can be quite substantial, computational challenges and bottlenecks arise [193].

2.4.1 Motivation

problem with ngs data: msa is NP-hard [95]. inferring tree for this many sequences is also NP-hard [29] even harder (over exp.), plus short sequences (not phylo signal), so cannot resolve relationships between sequences

A typical task in metagenomic studies is to identify and classify these sequences with respect to known reference sequences, either in a taxonomic or phylogenetic context.

Conventional methods like BLAST [3] are based on sequence similarity. Such methods are fast, but only attain satisfying accuracy levels if the query sequences (e.g., the environmental reads or amplicons) are sufficiently similar to the reference sequences. Furthermore, the best BLAST hit does often *not* represent the most closely related species [106]. This is particularly true for environments where available reference databases do not exhibit sufficient taxon coverage [135]. As insufficient taxon coverage cannot be detected by methods that are based on sequence similarity, they can potentially bias downstream analyses.

Alternatively, so-called phylogenetic (or evolutionary) placement methods [10, 16, 144] identify query sequences based on a phylogenetic tree of reference sequences. Thereby, they incorporate information about the evolutionary history of the species under study and hence provide a more accurate means for read identification. The result of phylogenetic placement is a mapping of the query sequences to the branches of the reference tree. Such a mapping also elucidates the evolutionary distance between the query and the reference sequences.

This information represents useful biological knowledge *per se*. For example, the classification of query sequences can be summarized by means of sequence abundances [89, 169], or to obtain taxonomic annotations [107]. The data can also be utilized to derive further knowledge or hypotheses. Existing methods such as Edge PCA and Squash Clustering [141] are, for instance, able to visualize differences between sets of metagenomic samples, or to cluster samples based on placement similarity. Note that distinct samples from one study are typically mapped to the same underlying reference tree, thus facilitating such comparisons.

phylogenetic placement [10, 16, 144], cited more than 630 times (as of 2018-07-01) considered an established method

There also exist variants of phylogenetic placement that use maximum parsimony [16] and minimum evolution [66] instead of maximum likelihood, variants that calculate Bayesian posterior probabilities [144], and boosting methods to improve the accuracy of the placements [158]. Phylogenetic placement has been used for a variety of applications and derived pipelines, such as species delimitation [97, 238], genome and metagenome analysis [38], taxonomic identification and phylogenetic profiling [164], and identification and correction of taxonomically mislabeled sequences [107].

combine 1,010 more citations (as of 2018-07-01) see also recent art version

there is also a recent approach that yields comparable results (also jplace), but uses an alignment-free, non ML approach [117].

kommt unten noch mal!

, using programs such as PAPARA [14, 15] or HMMALIGN, which is a subprogram of the HMMER suite [55, 56].

2.4.2 Introduction

TODO: pipeline diagram, data flow, etc, reference to chapters?!

In brief, phylogenetic placement calculates the most probable insertion branches for each given query sequence (QS) on a reference tree (RT). The QSs are reads or amplicons from environmental samples; most often barcoding regions or marker genes are used. The RT and the reference sequences it represents are typically assembled by the user so that they capture the expected species diversity in the samples. Nonetheless, we recently presented an automated approach for selecting and constructing appropriate reference sequences from large sequence databases [36]. As phylogenetic placement used a maximum likelihood criterion, the RT has to be strictly bifurcating. Prior to the placement, the QSs need to be aligned against the reference alignment of the RT by programs such as PAPARA [14, 15] or HMMALIGN, which is part of the HMMER suite [55, 56]. The placement is then conducted by initially inserting one QS as a new tip into a branch of the tree, then re-optimizing the branch lengths that are most affected by the insertion, and thereafter evaluating the resulting likelihood score of the tree under a given model of nucleotide evolution, such as the Generalized Time-Reversible (GTR) model [209]. The QS is then removed from the current branch and subsequently placed into all other branches of the RT.

Thus, for each branch of the tree, the process yields a so-called *placement* of the QS, that is, an optimized position on the branch, along with a likelihood score for the whole tree. The likelihood scores for a QS are then transformed into probabilities, which quantify the uncertainty of placing the sequence on the respective branch [206, 222]. Those probabilities are called likelihood weight ratios (LWRs). The accumulated LWR sum over all branches for a single QS is 1.0. Figure 2.6 shows an example depicting the placements of one QS, including the respective LWRs.

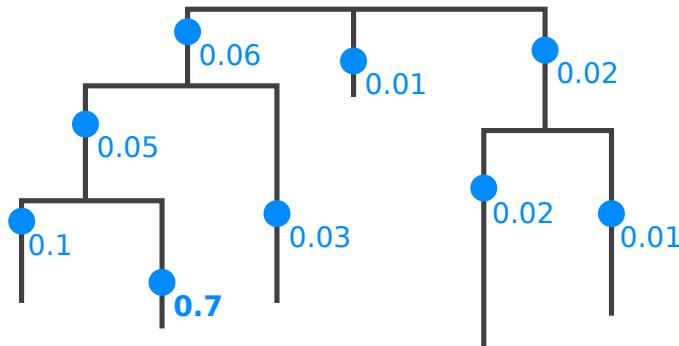


Figure 2.6: Phylogenetic Placement of a Query Sequence. Each branch of the reference tree is tested as a potential insertion position, called a “placement” (blue dots). Note that placements have a specific position on their branch, due to the branch length optimization process. A probability of how likely it is that the sequence belongs to a specific branch is computed (numbers next to dots), which is called the *likelihood weight ratio* (LWR). The bold number (0.7) denotes the most probable placement of the sequence.

This process is repeated for every QS. Note that the placement process is conducted *independently* for each QS. That is, for each QS, the algorithm starts calculating placements from scratch on the original RT.

In summary, the result of a phylogenetic placement analysis is a mapping of the QSSs in a sample to positions on the branches of the RT. Each such position, along with the corresponding LWR, is called a placement of the QS.

TODO: besser unterbringen The data is usually stored in so-called `jplace` files [145].

In the following, we represent a sample by the placement locations of its metagenomic QSSs, including the respective per-branch LWRs. Furthermore, for a specific analysis, we assume the standard use case, that is, all placements were computed on the same fixed reference tree (RT) and reference alignment.

This mapping can be seen as an identification and classification of the QSSs in terms of the RT, similar to a taxonomic assignment. However, phylogenetic placement also allows for more elaborate downstream analyses. Firstly, the reference tree usually offers a higher resolution than simple per-taxon abundance counts, and the amount of mapped QSSs per branch can be directly visualized on the RT [135]. Secondly, established methods such as Edge PCA and Squash Clustering [141] allow for identifying subtle differences between distinct samples, thus enabling comparative studies directly based on phylogenetic placement. Lastly, we recently proposed novel methods for visualizing and clustering phylogenetic placement data [37], which, for example, can reveal correlations of per-sample meta-data features with sequence abundances.

Furthermore, phylogenetic placement is used in a variety of derived tools, such as (M)PTP (species delimitation; [97, 238]), SEPP (boosting; [158]), PHYLOSiFT (metagenome analysis; [38]), SATIVA (identification and correction of taxonomically

mislabeled sequences; [107]) and TIPP (taxonomic identification and phylogenetic profiling; [164]).

A phylogenetic placement can be carried out *if* the QSSs can be aligned to the reference alignment. Often, barcoding regions such as 16S or 18S are used, but there also exist studies that use different, or even a set of, marker genes [207]. Furthermore, other types of sequences such as `mitags` [124] can be used.

2.4.3 Placement Pipeline

aligning against given ref aln, papara, hmmer

ML method, rappas (aln free)

2.5 Phylogenetic Placement Processing

When placing multiple samples, for instance, from different locations, typically, the same RT is used, in order to allow for comparisons of the phylogenetic composition of these samples. In this context, it is important to consider how to properly normalize the samples. Normalization is required as the sample size (often also called library size), that is, the number of sequences per sample, can vary by several orders of magnitude, due to efficiency variations in the sequencing process or biases introduced by the amplification process. Selecting an appropriate normalization strategy constitutes a common problem in many metagenomic studies. The appropriateness depends on data characteristics [227], but also on the biological question asked. For example, estimating indices such as the species richness are often implemented via rarefaction and rarefaction curves [74], which however ignores a potentially large amount of the available valid data [150]. Furthermore, the specific type of input sequence data has to be taken into account for normalization: Biases induced by the amplification process can potentially be avoided if, instead of amplicons, data based on shotgun sequencing are used, such as `mitags` [124]. Moreover, the sequences can be clustered prior to phylogenetic placement analysis, for instance, by constructing operational taxonomic units (OTUs) [58, 133, 134, 184]. Analyses using OTUs focus on species diversity instead of simple abundances. OTU clustering substantially reduces the number of sequences, and hence greatly decreases the computational cost for placement analyses. Lastly, one may completely ignore the abundances (which are called “multiplicities” of placements) of the placed sequences, reads, or OTUs, and only be interested in their presence/absence when comparing samples.

Which of the above analysis strategies is deployed, depends on the specific design of the study and the research question at hand. The common challenge is that the number of sequences per sample differs, which affects most post-analysis methods. Before introducing our methods, we therefore explain how the necessary normalizations of sample sizes can be performed in the following. We also describe general techniques for interpreting and working with phylogenetic placement data. Some of these techniques have been used before as building blocks for methods like Edge PCA and Squash Clustering [61, 141]. **TODO:** see below

2.5.1 Edge Masses

Methods that compare samples directly based on their sequences, such as the UniFrac distance [126, 128], can benefit from rarefaction [227]. However, in the context of phylogenetic placement, rarefaction is not necessary. Thus, more valid data can be kept. To this end, it is convenient to think of the reference tree as a graph (when exploiting graph properties of the tree, we refer to the branches of the tree as edges). Then, the per-branch LWRs for a single QS can be interpreted as mass points distributed over the edges of the RT, including their respective placement positions on the branches, cf. Figure 2.6. This implies that each QS has a total accumulated mass of 1.0 on the RT. We call this the *mass interpretation* of the placed QSs, and henceforth use mass and LWR interchangeably. The *mass of an edge* refers to the sum of the LWRs on that edge for all QSs of a sample, as shown in Figure 2.7(a). The total mass of a sample is then the sum over all edge masses, which is identical to the number of QSs in the sample.

The key idea is to use the distribution of placement mass points over the edges of the RT to characterize a sample. This allows for normalizing samples of different size by scaling the total sample mass to unit mass 1.0. In other words, absolute abundances are converted into relative abundances. This way, rare species, which might have been removed by rarefaction, can be kept, as they only contribute a negligible mass to the branches into which they have been placed. This approach is analogous to using proportional values for methods based on OTU count tables, that is, scaling each sample/column of the table by its sum of OTU counts [227]. Most of the methods presented here use normalized samples, that is, they use relative abundances. As relative abundances are compositional data, certain caveats occur [2, 125], which we discuss where appropriate.

When working with large numbers of QSs, the mass interpretation allows to further simplify and reduce the data: The masses on each edge of the tree can be quantized into b discrete bins, that is, each edge is divided into b intervals (or bins) of the corresponding branch length. All mass points on that edge are then accumulated into their respective nearest bin. The parameter b controls the resolution and accuracy of this approximation. In the extreme case $b := 1$, all masses on an edge are grouped into one single bin. This *branch binning* process drastically reduces the number of mass points that need to be stored and analyzed in several methods we present, while only inducing a negligible decrease in accuracy. As shown in Table 5.1. branch binning can yield a speedup of up to 75% for post-analysis run-times.

Furthermore, using masses allows to summarize a set of samples by annotating the RT with their average per-edge mass distribution. This procedure, also called *squashing* [141], sums over all sample masses per edge and then normalizes them once more to obtain unit mass for this resulting average tree. This normalized tree thereby summarizes the (sub-)set of samples it represents.

2.5.2 Edge Imbalances

So far, we have only considered the per-edge masses. Often, however, it is also of interest to “summarize” the mass of an entire clade. For example, sequences of

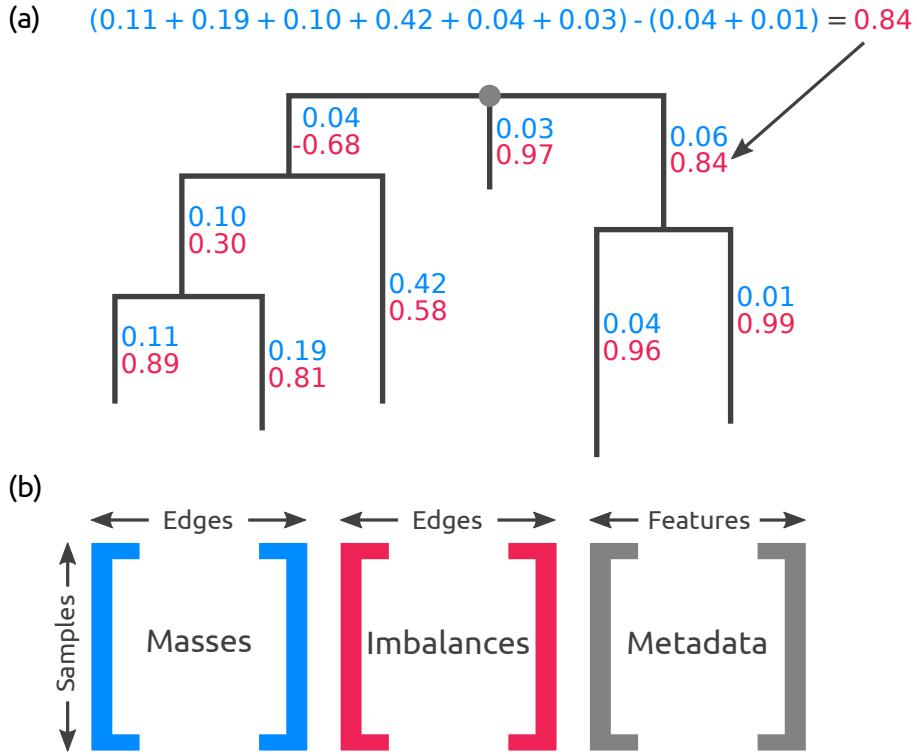


Figure 2.7: Edge Masses and Imbalances. (a) Reference tree where each edge is annotated with the normalized mass (first value, blue) and imbalance (second value, red) of the placements in a sample. The imbalance is the sum of masses on the root side of the edge minus the sum of the masses on the non-root side. The depicted tree is unrooted, hence, its top-level trifurcation (gray dot) is used as “root” node. An exemplary calculation of the imbalance is given at the top. Because terminal edges only have a root side, their imbalance is not informative. (b) The masses and imbalances for the edges of a sample constitute the rows of the first two matrices. The third matrix contains the available meta-data features for each sample. These matrices are used to calculate, for instance, the edge principal components or correlation coefficients.

the RT that represent species or strains might not provide sufficient phylogenetic signal for properly resolving the phylogenetic placement of short sequences [53]. In these cases, the placement mass of a sequence can be spread across different edges representing the same genus or species, thus blurring analyses based on per-edge masses. Instead, a clade-based summary can yield clearer analysis results. It can be computed by using the tree structure to appropriately transform the edge masses. Each edge splits the tree into two parts, of which only one contains the root (or top-level trifurcation) of the tree. For a given edge, its mass difference is then calculated by summing all masses in the root part of the tree and subtracting all masses in the other part, while ignoring the mass of the edge itself [141]. This difference is called the *imbalance* of the edge. It is usually normalized to represent unit total mass, as the absolute (not normalized) imbalance otherwise propagates the effects of differing sample sizes all across the tree. An example of the imbalance calculation is shown

in Figure 2.7(a). The edge imbalance relates the masses on the two sides of an edge to each other. This implicitly captures the RT topology and reveals information about its clades. Furthermore, this transformation can also reveal differences in the placement mass distribution of nearby branches of the tree. This is in contrast to the KR distance, which yields low values for masses that are close to each other on the tree. Examples that illustrate the different use cases for edge mass and edge imbalance metrics are shown in the Results section.

The edge masses and edge imbalances per sample can be summarized by two matrices, which we use for all further downstream edge- and clade-related analyses, respectively. In these matrices, each row corresponds to a sample, and each column to an edge of the RT. Note that these matrices can either store absolute or relative abundances, depending on whether the placement mass was normalized.

Furthermore, many studies provide meta-data for their samples, for instance, the pH value or temperature of the samples' environment. Such meta-data features can also be summarized in a per-sample matrix, where each column corresponds to one feature. The three matrices are shown in Figure 2.7(b). Quantitative meta-data features are the most suitable for our purposes, as they can be used to detect correlations with the placement mass distributions of samples. For example, Edge principal components analysis (Edge PCA) [141] is a method that utilizes the imbalance matrix to detect and visualize edges with a high heterogeneity of mass difference between samples. Edge PCA further allows to annotate its plots with meta-data variables, for instance, by coloring, thus establishing a connection between differences in samples and differences in their meta-data [201]. In the following, we propose several new techniques to analyze placement data and their associated meta-data.

2.5.3 Distance between Samples

kr distance. ref to nh distance?! [61]

2.5.4 Existing Analysis Methods

squash clustering edge pca [61, 141]

we use these methods repeatedly to compare our novel methods to.

3. Phylogenetic Automatic (Reference) Trees

This chapter is based on the peer-reviewed publication:

- **Lucas Czech**, Pierre Barbera, and Alexandros Stamatakis. "Methods for Automatic Reference Trees and Multilevel Phylogenetic Placement." *Bioinformatics*, 2018.

Contributions: Lucas Czech... Pierre Barbera... Alexandros Stamatakis... and...

3.1 Motivation

Phylogenetic placement is particularly helpful for studying new, unexplored environments, for which no closely related sequences exist in reference databases (e.g., 135). However, the selection of suitable reference sequences for inferring the RT constitutes a challenge for studying such environments, as this typically is a manual process. Furthermore, conducting phylogenetic placement requires a higher computational effort with respect to the placement algorithms *per se*, but also the pre- and post-processing, than, for instance, similarity based methods. Due to the continuous advances in molecular sequencing, existing placement methods as well as respective pre- and post-processing tools have already reached their scalability limits.

Here, we introduce methods to overcome the aforementioned limitations, that is, to (1) automatically obtain a high quality reference tree for phylogenetic placement, (2) split up the placement process into two steps using smaller phylogenies, and (3) accelerate the computation of placements via appropriate data pre-processing

approaches. All methods are implemented as part of our GAPPa tool, which is freely available under GPLv3 at <http://github.com/lczech/gappa>.

Molecular environmental sequencing studies, particularly those that aim to conduct phylogenetic placement, often rely on a set of manually selected and aligned reference sequences to infer an RT [45, 135, 210, 213]. Creating and maintaining databases of such reference sequences constitutes a labor-intensive and potentially error-prone process. Moreover, this approach is impractical for samples that contain diverse sequences from many clades of the taxonomy, or samples obtained from unexplored environments. Lastly, even if a large RT is available, the visualization of placements on such an RT might be confusing and thus hard to interpret.

The reference tree (RT) used for phylogenetic placement should ideally (a) cover all major taxonomic groups that occur in the QSSs, (b) use high-quality error-free reference sequences, and (c) not be too large to allow for unambiguous visualization and interpretation. These criteria can be met for small datasets by manually selecting curated sequences from databases. For large and taxonomically diverse samples one key challenge is that sequence databases such as GREENGENES [48], UNITE [1], PR2 [78], EzTAXON [101], SILVA [178], and RDP [31] maintain reference collections of thousands to millions of taxonomically annotated sequences. Therefore, one needs to appropriately sub-sample sequences such that the RT can be inferred in reasonable time *and* sufficiently covers the diversity of the sample.

Previous approaches mainly relied on phylogenetic diversity [63, 157, 170] and related methods [146]. The major drawback is that they require a comprehensive phylogeny as input. Inferring such large comprehensive phylogenies with hundreds of thousands of taxa, to subsequently reduce the taxon set again, is computationally inefficient and in certain cases infeasible.

To this end, we present a computationally efficient approach for obtaining sequences from large databases to infer an RT. This RT is then used for conducting phylogenetic placement analyses. The input of our method is a database of aligned sequences of known species including their taxonomic labels. Our approach then identifies sets of sequences that are similar to each other based on their entropy. It subsequently reduces the sequences in these sets to a predefined number of consensus sequences. This set of sequences is the output of our method. It represents the taxonomic clades and is then used to infer the RT.

3.2 Method

3.2.1 Sequence Entropy

Conventional methods for sequence similarity are often based on edit distance and other pairwise comparison methods [3, 161, 199]. This however necessitates to transform the pairwise distances to some form of ensemble measure that describes the similarity of all sequences to each other, for which there is no obvious approach [240]. There also exist methods that describe genetic variation and nucleotide diversity of

sets of sequences [20, 162] which could be used for this purpose. We however decided to use entropy for measuring ensemble similarity of a set of sequences.

First, we define a measure to quantify the ensemble similarity of a set s of sequences, based on their entropy [195]. Variants of sequence entropy have been used before in numerous biological and phylogenetic contexts, for example, to asses the information content of sequences [32, 35, 116, 192, 218–220], or to measure substitution saturation [232]. Here, we use entropy for alignment sites, that is, we define the entropy (uncertainty) H at alignment site i as

$$H_i = - \sum_c f_{c,i} \times \log_2 f_{c,i}$$

where $c \in \{A, C, G, T, -\}$ is the set of nucleotide states including gaps, and $f_{c,i}$ is the frequency of character c at site i of the alignment. Including gaps (-) in the summation reduces the contribution of sites that contain a large fraction of gaps. Their contribution is weighed down as all standard phylogenetic inference tools model gaps as undetermined states, that is, they do not contribute anything to the likelihood score. The entropy is 0 for sites that only contain a single character. It increases the more different characters an alignment site contains, and the more similar their frequencies are. Its maximum occurs if all characters appear with the same frequency (each of them 20%). Note that we also treat ambiguous characters as gaps. As only 0.008% of the non-gap characters in our test database (SILVA) are ambiguous, their influence is negligible. Ambiguous characters could however be incorporated by using fractional character counts.

Finally, the total entropy of a set s of aligned sequences is simply the sum over all per-site entropies: $H(s) = \sum_i H_i$. We use this entropy to quantify the ensemble similarity of a set of sequences. This can be regarded as an information content estimate of the sequences.

3.2.2 Sequence Grouping

The goal of this step is to group the sequences of a database into a given target number of groups/sets, such that the groups reflect the diversity of the sequences in the database. We use the taxonomy to identify potential candidate groups of sequences that could be represented by a consensus sequence. We interpret a taxonomy as a sequence labeling, where similar sequences have related labels. Thus, a taxonomy represents a pre-classification of similar sequences that can be exploited to group them.

For a clade t of the taxonomic tree, we denote by $H(t)$ the entropy of all sequences that belong to that clade, including all sequences in its sub-clades, that is, its lower taxonomic ranks. Clades with low entropy imply that they contain highly similar sequences that can in turn be represented by a consensus sequence without sacrificing too much diversity. Inversely, clades with high entropy contain diverse sequences, implying that a consensus sequence is not likely to sufficiently capture the inherent

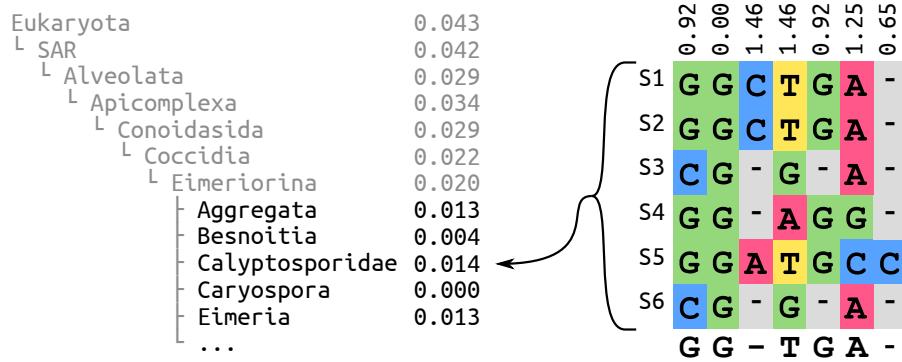


Figure 3.1: Entropy and consensus sequence of a taxonomic clade. The left hand side shows the exemplary clade *Eimeriorina* in its taxonomic context, listing its super- and sub-clades with the normalized entropy of their respective sequences. The right hand side is an excerpt from the alignment of six sequences that belong to the *Calyptosporidae* sub-clade. At its top, the per-site entropies for the alignment columns are shown. At the bottom, the majority rule consensus sequence is shown, which is used to represent the sub-clade.

sequence diversity. It is thus better to expand these clades and construct separate consensus sequences for their respective sub-clades. An example is shown in Figure 3.1. As the clade structure of a taxonomy forms a tree, this criterion can then be applied recursively, as shown in Algorithm 3.1.

Algorithm 3.1 Taxonomy Expansion

```

1: Candidates  $\leftarrow$  list of highest ranking clades
2: TaxaCount  $\leftarrow$  size of Candidates
3: while TaxaCount  $<$  TargetCount do
4:   MostDiverse  $\leftarrow$   $\arg \max_{t \in \text{Candidates}} H(t)$ 
5:   remove MostDiverse from Candidates
6:   add sub-clades of MostDiverse to Candidates
7:   TaxaCount  $\leftarrow$  TaxaCount  $- 1 +$  size of MostDiverse
8: return Candidates

```

The algorithm works as follows: We initialize a list of candidate clades with the highest ranking clades that we want to consider. In the most general case, these can be “Archaea”, “Bacteria”, and “Eukaryota”. We then select the most diverse candidate clade, that is, the clade t whose sequences exhibit the highest entropy $H(t)$. This clade is then expanded, and we do not consider it as a potential candidate for building a consensus sequence. The high entropy clade is then removed from our list and its immediate sub-clades are added as new candidates to the list. Finally, the current count of how many candidates we have already selected is updated accordingly. By expanding clades with high entropy, we descend into the lower ranks of the taxonomy. On average, this decreases the entropy, because low ranking clades generally tend to contain more similar sequences. This process is repeated until our list contains approximately as many candidate clades as the desired target

count of reference sequences, which is provided as input. As the sizes of expanded clades can vary substantially, the target count cannot always be met exactly. In our tests, the average deviation was 0.2%, as shown in Table 3.1.

Given this list of clades from different taxonomic ranks, we can now compute the consensus sequences. For each clade, all sequences in that clade and its sub-clades are used to construct a consensus sequence, which represents the clade diversity, and serves as the reference sequence for that clade. A simple per-site majority rule consensus [43, 147] works well, but we also assessed alternative methods; see Figure 3.5 and Figure 3.6 for details.

Note that it would also be possible to directly use the relative character frequencies at each site to obtain more accurate representations. Maximum likelihood-based phylogenetic inference tools do, in principle, not require discrete input sequences. The likelihood model allows to account for uncertainty in the input data [65], although this is generally not implemented in the mainstream software packages. The above process yields a set of consensus reference sequences which capture the diversity of distinct taxonomic clades.

3.2.3 Inferring a Reference Tree

Once we have identified the consensus sequences, which are already aligned to each other, we can use them to infer a maximum likelihood tree, which we call a *Phylogenetic Automatic (Reference) Tree* (PhAT). As each consensus sequence is associated with a taxonomic clade, the corresponding taxonomic path can be used to label the tips of the tree. Note that since clades with low entropy might not be expanded, the tip labels do not necessarily correspond to species or genera. Also, the PhAT will not necessarily be congruent to the taxonomy.

A PhAT satisfies all criteria we listed: (a) All taxonomic groups occurring in the QSSs can be covered by using a suitable taxonomy as input. (b) By using consensus sequences, potential sequencing errors can be alleviated. (c) The size of the tree can be specified by the user. However, the resolution of the trees is limited by the underlying taxonomy, see Figure 3.8 and Figure 3.11 for details. Thus, one needs to verify that the resulting tree is appropriate for the dataset to be placed on it. This also holds for manually selected reference sequences. Furthermore, using consensus sequences may obscure the degree of sequence diversity in sub-clades, which in turn can affect the accuracy of subsequent phylogenetic placements on that tree. Our algorithm as described here can not fully compensate for this. We present a method to address both issues (tree resolution and obscured diversity) in the next Section.

3.3 Multilevel Placement

When conducting phylogenetic placement, the computationally limiting factors are (i) the number of QSSs to be placed (addressed in the next section) and (ii) the size of the RT (number of taxa) and corresponding alignment length (addressed below). Using RTs with more taxa increases the phylogenetic resolution of the placements, at

the cost of increased computational effort for inferring the RT, aligning the QSSs, and placing the QSSs. Furthermore, longer reference alignments (if appropriate data is available) are required to accurately infer large trees under the maximum likelihood criterion [233], thus further increasing the computational costs. Lastly, placement on large trees that comprise reference sequences with high evolutionary distances can reduce placement accuracy [158]. Thus, using a large number of reference sequences is not always desirable in practice.

One solution is to divide the tree and its alignment into more conquerable subsets, for example as implemented in SATÉ [120, 121]. This approach has also been extended to phylogenetic placement in SEPP [158] and TIPP [164], which divide the tree into disjoint subsets of taxa and conduct placement on each of them. While yielding more accurate placements and taxonomic classifications in less computing time, this method might still result in large reference trees, which are hard to inspect and visualize.

To address this issue, we present an approach called *Multilevel* or *Russian Doll* Placement, which is summarized in Figure 3.2. Instead of working with one large RT comprising *all* taxa of interest, we use a smaller, but taxonomically broad backbone tree (BT) for pre-classifying the QSSs (first level), and a set of refined clade trees (CTs) for the final, more accurate placements (second level). These CTs comprise the reference sequences that are of interest for a particular study. For example, if a study is concerned with *Apicomplexa* and *Cercozoa*, a broad *Eukaryotes* BT can be used for the first level, and two respective CTs for the second level, similar to [135]. Each CT is associated with the set of branches of a specific BT clade.

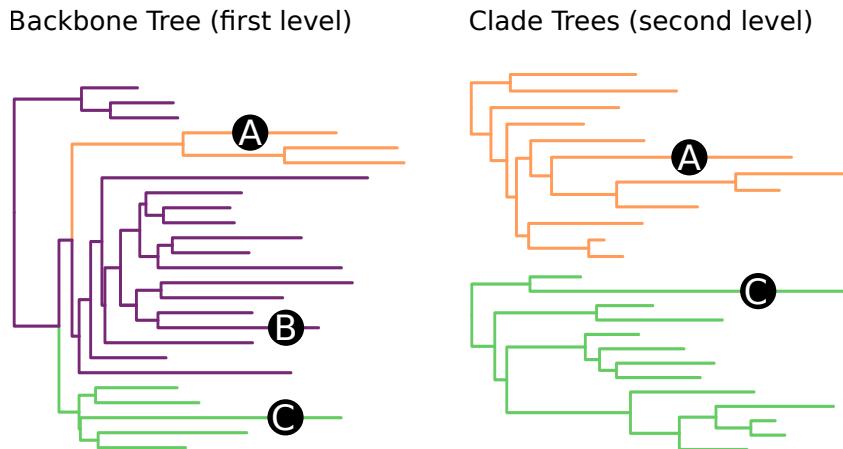


Figure 3.2: Multilevel Placement. The left shows a backbone tree (BT); the right shows two clade trees (CTs) in orange and green. Branches in the BT that are associated with a CT are marked in its color. The trees “overlap” each other, meaning that each CT is represented by multiple branches in the BT. Three sequences A, B and C are placed on the BT, which is the first level. A and C are placed on branches associated with a CT. Hence, their second level placement is conducted on the respective CT. B is placed on a branch that is not associated with any CT, and thus not used in the second level.

The method then works in three steps:

1. Align and place the QSSs using the BT (first level).
2. For each CT, collect the QSSs that are placed on the BT branches associated with the CT.
3. Align and place these QSSs again, using their specific CTs (second level).

While this approach requires some additional bookkeeping, the total computational cost is reduced, because the QSSs do not have to be placed on all branches of all CTs. The gain in speed depends on the sizes of the BT and CTs relative to the size of the (potentially imaginary) large comprehensive tree. For example, by splitting a tree with 10 000 taxa into a BT and 10 CTs with 1000 taxa each, computational cost is reduced to 20% of the original cost (two placement levels with 10% of the cost each). Furthermore, in each level, the amount of computer memory is limited to 10% of what is necessary for the large tree. Lastly, this method allows for fine-grained control over the clades of interest at both placement levels:

Firstly, the BT provides a means for phylogenetically informed sequence filtering – that is, to identify and remove “spurious” QSSs. Sequences with low similarity to known references are often removed in environmental sequencing studies [205]. However, using sequence similarity as a filter criterion can remove too many QSSs, particularly when studying new, unexplored environments [135]. By using phylogenetic placement as a filter instead, substantially more sequences can be retained for downstream analyses. Only the QSSs that are placed onto the inner branches of the BT, that is, branches with no associated CT, are omitted at the second placement level.

Secondly, using specific clade trees for lower level taxonomic clades offers the phylogenetic resolution that is necessary for downstream analyses and for biological reasoning. It is, for example, possible to use manually curated “expert” trees for each clade of interest.

In this setup, the BT is only used for pre-classification, and can, for example, use our PhAT method. The aforementioned issue of obscured diversity in sub-clades can be circumvented by “overlapping” the CTs with the BT. That is, a CT can be associated with several branches of the BT, so that placements on each of these BT branches are collected and placed onto the same CT. See Figure 3.2 and Figure 3.7 for examples. We recommend to ensure that the branches of the BT that are associated with one CT are monophyletic, meaning that there is one split that separates these branches from the rest of the BT. This can be achieved by inferring the BT with a high-level constraint that maintains the monophyly of the CTs. It ensures phylogenetic consistency between the BT and the CTs, and improves the accuracy of the first placement level, as shown in Section 3.6.2. Lastly, it is also possible to use more than two levels, which might become necessary when working with RTs and datasets even larger than what is currently available.

3.4 Data Preprocessing for Phylogenetic Placement

Apart from the RT size, handling the sheer number of QSs also induces computational limitations for conducting phylogenetic placements. Most metagenomic studies publish their data in unprocessed formats, which are sometimes filtered to contain only reads from certain barcoding or marker regions. For instance, they store the raw sequencing output in `fasta` [172] or `fastq` [30] format. Those data often contain duplicates of exactly identical sequences, both *within* and *across* samples. Identical sequences are however treated the same in phylogenetic placement algorithms and therefore induce unnecessary computational overhead. Furthermore, sample sizes, that is, the number of sequences per sample, can vary by several orders of magnitude. If the placement algorithm is parallelized over samples, this leads to an uneven load balance across compute nodes.

In order to solve these issues, that is, reduce computational cost and achieve good load balancing, one can pre-process the sequences with our GAPP tool. First, sequences are de-duplicated across all samples and fused into chunks of equal size. The chunk size should be chosen to allow aligning and placing a chunk within wall time on the intended hardware; we recommend chunk sizes of 50 000 or larger. Our tool assigns an identifier to each unique sequence, and computes a list of abundance counts for each sequence in a sample. Given an RT and its underlying alignment, the QS chunks are then aligned to the reference multiple sequence alignment, using programs such as PAPARA [14, 15] or HMMALIGN [55, 56], and subsequently placed on the RT, for example by PPLACER, RAXML-EPA or EPA-NG [10, 16, 144]. The resulting per-chunk placement result files in combination with the per-sample abundance counts can then be parsed and analyzed by GAPP to generate final per-sample placement files, containing a placement for each sequence in the original sample.

The speedup that can be gained via this preprocessing is proportional to the ratio of total versus unique sequences; the gain in parallel efficiency depends on the ratio of smallest to largest sample (in number of sequences). This approach allows to analyze datasets that are orders of magnitude larger than in previous published studies. For example, in 2012, an analysis of Bacterial Vaginosis (BV) data placed a total of 426 612 sequences, thereof 15 060 unique, on an RT with 796 tips [201]. Using a prototype of GAPP, we were able to analyze a neotropical soils dataset with 50 118 536 total sequences, thereof 10 567 804 unique, with an RT comprising 512 taxa [135]. To demonstrate the scalability of our methods for this paper, we analyzed datasets with up to 116 520 289 total sequences, thereof 63 221 538 unique, from the HMP [91, 151], using RTs with up to 2059 tips. This corresponds to a computational effort that is four orders of magnitude greater than for the BV study.

3.5 Evaluation

To test the Phylogenetic Automatic (Reference) Tree (PhAT) method, we used the “SSU Ref NR 99” sequences of the SILVA database [178] version 123.1 and the

corresponding taxonomic framework [235]. The database contains 598 470 aligned sequences from all three domains of life, classified into 11 860 distinct taxonomic labels.

We constructed four sets of consensus sequences from the SILVA database: a *General* set (“all of life”), as well as separate sets for the domains *Archaea*, *Bacteria*, and *Eukaryota*. For each set except the *Archaea*, the recursive expansion of taxonomic clades was applied to obtain approximately 2000 (*General*) and 1800 (*Bacteria*, *Eukaryota*) consensus reference sequences. This is large enough to cover the diversity well, while still being computationally feasible for the subsequent steps. The *Archaea* taxonomy in SILVA is smaller, containing 248 taxa at *Genus* level, which is the lowest level in their taxonomy. Hence, the *Archaea* tree also comprises 248 taxa. Furthermore, in the three domain-specific trees, we included sequences at the *Phylum* level of the respective two other domains, to ensure that our methods also work with outgroups. The assembly of these four data sets required in total about 30 min and 10 GB of memory on a standard laptop computer. An overview of the tree sizes is shown in Table 3.1. We then inferred constrained and unconstrained maximum likelihood trees for the consensus sequences. The constrained trees comply with the SILVA taxonomy, and are used to assess how taxonomic constraints affect the phylogenetic placement and the subsequent analyses. Details are provided in Supplementary Section 3.6.1, which also discusses differences between the constrained and unconstrained trees. Details of the trees are shown in Table 3.3; Figure 3.7 shows the unconstrained *Bacteria* tree as an example.

In total, our setup yields eight distinct RTs for evaluation: the *General* tree, the three domain trees, and the respective taxonomically constrained variants.

3.5.1 Accuracy

Here, we assess how using our PhAT affects phylogenetic placement accuracy. Each terminal branch of our RTs represents a consensus sequence, which is computed from species level sequences that share the same taxonomic label. We evaluate an RT by placing these species sequences onto the RT: Each species sequence is expected to be placed onto the branch leading to the consensus sequence that represents this particular species sequence. As the consensus sequences are derived from the taxonomy, all terminal branches of the tree have taxonomic labels. These labels thus identify the expected placement position for each species sequence. For example, sequences S1–6 in Figure 3.1 are represented by the consensus sequence for the *Calyptosporidae* clade, which is shown below the 6 sequences in the Figure. They are thus expected to be placed onto the *Calyptosporidae* branch in the RT.

We placed the respective subset of the SILVA database species sequences onto each of the eight RTs. We quantify placement accuracy for a sequence by the distance to its expected placement branch. More precisely, we measured (a) the (discrete) number of branches between the actual placement and the expected branch, and (b) the (continuous) distance in branch lengths units. As a sequence can have multiple placement locations, the distances, are, in fact, weighted averages incorporating the

placement probabilities (likelihood weights). The results for the four unconstrained trees are shown in Figure 3.3; Figure 3.4 depicts the results for the constrained trees. Further details are provided in Table 3.3.

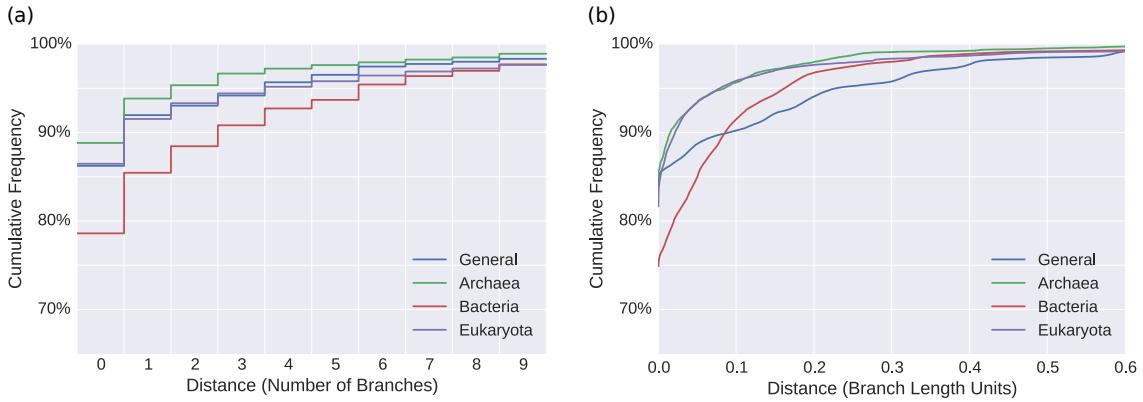


Figure 3.3: Weighted distances to expected edges for unconstrained trees. We evaluated the accuracy of our PhATs by placing sequences and measuring the weighted distances to their respective expected placement branches. The Figure shows the cumulative frequencies of number of sequences versus distances, measured (a) in number of branches and (b) in branch length units. In other words, it shows how many sequences are placed within a certain radius from their expected branches. For example, in (a), more than 85% of the sequences of the *Bacteria* (red) are placed within a radius of at most one branch from their expected branch, and in (b), more than 95% of the Eukaryota (purple) are within a radius of 0.1 branch length units from their expected branches.

Considering the size of the trees, most sequences are placed in close vicinity to their expected branches. This is corroborated by the short average distances reported in Table 3.3. Furthermore, the average expected distance between placement locations [EDPL, 144] is low, indicating that the placements of a specific sequence mostly cluster in a small neighborhood of the tree. We observed that errors occur mostly in parts of the tree with short branches, which might be explained by the inability of 16S SSU sequences to properly resolve certain clades [93]. Also, the placement likelihood differences are small between neighboring, short branches, such that the placement signal is fuzzy.

With 77% of the sequences placed exactly on their expected branch, the accuracy is generally lowest for the *Bacteria* tree. This might be because the *Bacteria* have the most sequences in SILVA, and exhibit a high diversity. In the other three trees, more than 90% of the sequences are placed at most one branch away from their respective expected branch. The constrained trees (Figure 3.4) exhibit similar placement accuracy. Particularly when using Multilevel Placement with overlapping RTs, placement differences of a few branches on the first level tree are acceptable, as they do not change the second level tree on which the sequence is placed. See Section [TODO: sec:Results:sub:MultilevelPlacement](#) for details.

As outlined in the method description, we represent clade diversity via majority rule consensus sequences. To assess the impact of the consensus method, we repeated the above evaluation, using two alternative consensus methods, but found little difference between the methods, see Figure 3.5. Finally, we also tested an automated approach that uses actual sequences (instead of consensus sequences) from the database to represent the taxonomic clades, see Figure 3.6. We found that this approach yields trees that are less accurate for phylogenetic placement.

3.5.2 Empirical Datasets

PhATs are intended for obtaining phylogenetic placements of environmental sequences. As the true evolutionary history of such sequences is unknown, we can not repeat the previous accuracy tests on empirical environmental datasets. Instead, we assess if the PhATs yield meaningful quantitative results for typical post-analysis methods. To this end, we placed two empirical metagenomic amplicon barcoding datasets on our unconstrained *Bacteria* tree. To asses the placement results obtained from the PhAT, we performed Squash Clustering and Edge PCA [141] post-analyses on the placement results (see Section B and Figure 3.8 and Figure 3.9 for details). The results reveal that the PhAT reproduces results of previous studies based on custom RTs with manually selected reference sequences. Furthermore, the PhAT is able to classify samples (e.g., healthy vs sick patients), at least to the extend that is expected from its phylogenetic resolution. That is, samples that only differ in placements at the species level cannot be classified using a broad, high-level tree such as our *Bacteria* tree. In order to obtain finer taxonomic resolution, it is thus necessary to either use a PhAT that contains more taxa, or to use our multilevel approach instead (see next Section).

3.5.3 Taxonomic Assignment and Profiling

Here, we assess how PhATs perform when used for obtaining a taxonomic profile of a set of samples in conjunction with placement. We emphasize though that taxonomic assignment and profiling are neither the focus of PhATs, nor the intended standard applications of phylogenetic placement. To perform the evaluation, we used the *mouse gut* data set of the 2nd CAMI Challenge [22, 194], and phylogenetically placed the reads of the 16S locus ($\approx 0.08\%$ of the total data) on our constrained and unconstrained *Bacteria* trees. We then used this placement data to taxonomically assign the reads based on the underlying SILVA taxonomy of the trees, in analogy to the method used by SATIVA [107]. Unfortunately, the CAMI Challenge uses the NCBI taxonomy for the respective evaluation. We thus had to compute a mapping between the two taxonomies, which introduces some incongruities [9]. The resulting per-read assignment was then used to generate a taxonomic profile of the data.

Despite only using a small fraction of the reads and despite having to use incongruent taxonomies, our PhAT-based taxonomic profiling is in the mid-range of the tools evaluated by CAMI. Therefore, our method yields reasonable accuracy for taxonomic assignment and profiling. Note that the resolution of the assignment is limited by the taxonomy used when running the PhAT method, that is, we could

not assign reads at *Species* level. Details of the process and its results are provided in Section 3.6; Figure 3.11, Figure 3.12, and Table 3.4 show the most important evaluation results.

3.6 Taxonomic Assignment and Profiling

Taxonomic profiling is one potential application of phylogenetic placement. Thus, we also tested how well our PhATs are suited for this purpose. The CAMI Challenge [194] is a community-driven effort to assess taxonomic profiling methods using a common set of benchmark data sets. To assess the feasibility of using trees generated with PhAT to perform taxonomic profiling of microbiome data, we utilized the *mouse gut* data set of the 2nd CAMI Challenge [22]. For this data set, we performed read preprocessing, phylogenetic placement against our constrained and unconstrained *Bacterial* trees, and subsequent taxonomic assignment.

More specifically, we used the unpaired HiSeq reads of the mouse gut data set from CAMI, which comprises 64 samples of simulated reads. The preprocessing involved read de-interleaving following [225], paired-end read merging using PEAR [239], as well as quality filtering and conversion to `fasta` using VSEARCH2 [184]. This yielded a total of 800 341 409 reads. As our trees are based on small ribosomal subunit sequences, we also performed read filtering to obtain reads from the 16S rDNA locus. This filtering was performed using the protocol of [124], which relies on HMMER [55, 56], and respective profiles for the 16S rDNA locus. We performed a global identity based de-replication step on the resulting reads that yielded 616 405 query sequences. We aligned these query sequences to our *Bacteria* reference alignment using PAPARA 2.0 [14, 15]. We then performed phylogenetic placement of the aligned query sequences onto the unconstrained and constrained reference trees, respectively, using EPA-NG [10]. We performed de-de-replication to obtain per-sample data again, resulting in 64 `jplace` files (one per original sample) with placements of the 16S rDNA sequences, for each of the two trees.

Finally, we performed taxonomic assignment and taxonomic profiling of the per-sample results using the `assign` command implemented in GAPPY, which works analogously to the method used in SATIVA [107]. Its basic steps are described in Section C. The CAMI challenge requires taxonomic assignments that conform with the NCBI taxonomy [12, 191]. As our reference tree is however based on the SILVA taxonomy [235], we developed a dedicated mapping procedure to, in a best effort approach, map our results to NCBI taxonomic names and IDs. The mapping is based on the *loose mapping* procedure by [9]. More specifically, we try to map taxonomic paths to their name, rank, and ID in the NCBI taxonomy, if we find a name-based match between the two. When this fails, the phylogenetic placement mass assigned to a taxonomic path by our approach is instead added to the last successful mapping further up in the taxonomic hierarchy. By initiating this procedure for each taxonomic path from its root downwards, we ensure that all placement masses are taken into account.

This mapping is a major disadvantage of our approach when using a SILVA-based reference, as the SILVA and NCBI taxonomies are far from being congruent [9]. Also

note that our reference is limited to 16S rDNA locus. This substantially reduces the volume of data we can evaluate. In this particular test, only $\approx 0.08\%$ of the total data was identified as belonging to 16S. This means that our taxonomic profiling only uses a small fraction of the available data.

Finally, we used the CAMI evaluation tool for taxonomic profilers, OPAL [194], to compare our approach to competing software and the “gold standard” result for the data set. Despite the aforementioned caveats of taxonomic profiling using phylogenetic placements in the CAMI setting, we find that the performance of our approach is “middle of the field”. Note that this is a comparison to tools that are dedicated to taxonomic profiling, which also typically can assign more of the available reads.

We show the most important OPAL results in Figure 3.11, Figure 3.12, as well as in Table 3.4. Furthermore, the full OPAL output can be accessed at <http://github.com/lczech/placement-methods-paper>.

3.6.1 Details about the Phylogenetic Automatic (Reference) Tree Evaluation

To test the Phylogenetic Automatic (Reference) Tree (PhAT) method, we used the “SSU Ref NR 99” sequences of the SILVA database [178] version 123.1 and the corresponding taxonomic framework [235], which are available at <http://www.arb-silva.de>. The database contains 598 470 aligned sequences from all three domains of life, classified into 11 860 distinct taxonomic labels, and mainly contains bacterial sequences. In detail, there are

- 22 913 sequences with 347 taxonomic labels for the *Archaea*,
- 62 436 sequences with 7441 taxonomic labels for the *Eukaryota*, and
- 513 121 sequences with 4072 taxonomic labels for the *Bacteria*.

The overall number of taxonomic labels is counted here, that is, it includes higher level labels.

As explained in the main text, we constructed four sets of consensus sequences from these data using our PhAT method: a *General* set (“all of life”), as well as separate sets for the domains *Archaea*, *Bacteria*, and *Eukaryota*. The assembly of the four data sets with our method required in total about 30 min and 10 GB of memory on a standard laptop computer. This includes counting alignment characters, calculating entropies and constructing consensus sequences. The resulting data set sizes and the fraction of sequences from each domain the PhATs contain are shown in Table 3.1.

Our implementation of the method furthermore contains some details that are worth mentioning for reproducibility: It is possible to constrain the maximal size of clades in order to not build a consensus sequence for an overly large clade, which might not be a good representative of that clade. For the same reason, it is possible to first expand the highest ranks of the taxonomy into separate candidates. We used

conservative values for these two constraints (a maximal clade size of 2000 and an expansion of only the first two taxonomic ranks), in order to give more weight to the sequence entropy. Lastly, some clades contain only one sub-clade. Those were immediately expanded, as they do not change the length of the candidate list during the algorithm.

We then inferred unconstrained and constrained maximum likelihood trees on the sequences, running 50 independent tree searches for each tree and selecting the best-scoring tree. Unconstrained trees were inferred using RAxML 8.2.8 [203]. Constrained trees were inferred with SATIVA 0.9-55 [107], which internally again relies on RAxML, and offers a convenient way to transform a taxonomy into a constraint tree.

The relative Robinson-Foulds distances [182] between the four pairs of trees (constrained versus unconstrained) are between 45.8% and 49.7%. The differences between the trees however mostly concern inner branches. As QSSs generally tend to be placed more towards the terminal branches of the tree, the differences in the inner branches thus are acceptable for our evaluation purposes. Furthermore, we performed significance tests comparing the constrained trees to the unconstrained ones, as shown in Table 3.2. The tests show that in all cases, the unconstrained trees fit the data significantly better.

Given these eight PhATs, the evaluation was conducted as explained in the main text. As the sequences in SILVA are already aligned to each other, no alignment step was necessary. As they contain no phylogenetic signal, we removed sites consisting entirely of gaps from the alignment, in order to reduce the memory footprint of downstream steps. Phylogenetic placement was conducted using EPA-NG [10], which is substantially faster and more scalable than RAxML-EPA [16] and PPLACER [144].

We evaluated the accuracy of the placements using the taxonomic labels of the sequences in SILVA as an indicator of the expected branch of each sequence. Thus, we have to assume the taxonomic label of each sequence to be correct. However, errors are expected due to incongruity between the taxonomy and the phylogeny [159], as well as due to taxonomically mislabeled sequences [107]. For example, SATIVA [107], found 9934 mislabeled sequences in the SILVA database. Furthermore, 17 452 sequences contain one of “incertae”, “unclassified” or “unknown” in their name, indicating that those sequences might not be reliable. In total, there are 25 910 (or 4.3%) such dubious sequences in version 123.1 of the SILVA database. Not all sequences are hence expected to be placed on their expected branches. We also evaluated how these dubious sequences affect the accuracy of the trees. To this end, we used the same four trees as before (that is, they were constructed with all sequences, including the dubious sequences), but for the evaluation step excluded the dubious sequences. That is, those sequences were not placed on the trees, and their distance to the expected branch was not used for the evaluation. In most cases, this improved the results slightly (data not shown). Therefore, we decided to only report the unfiltered results.

3.6.2 Sub-clades and Multilevel Placement

We selected five bacterial clades to evaluate PhAT accuracy on smaller clades, as well as to assess some properties of the Multilevel Placement approach. The same clades were already scrutinized in SATIVA [107]. Figure 3.7 shows the *Bacteria* tree with the five test clades highlighted.

First, using the sequences and taxonomies of these five clades, we built unconstrained and constrained PhATs. We then conducted the same accuracy analysis as explained before on these ten trees. That is, we placed the SILVA sequences of the five clades onto their respective PhAT and evaluated distances to expected branches. Thereby, we evaluated the accuracy of these PhATs when used as second level clade trees. The results are shown in Figure 3.10. The placement accuracy is slightly worse for the clade trees than for the eight comprehensive PhATs evaluated before. This is again likely due to 16S SSU sequences being unable to properly resolve lower taxonomic levels [93].

Next, using the five clades, we evaluated the accuracy of the first placement level when conducting Multilevel Placement. So far, our evaluation focused on the distance from a sequence placement to its expected placement branch. For the first placement level on a backbone tree (BT), it is however more important that a sequence is placed into the correct clade. Thus, we used the unconstrained *Bacteria* BT again, and assessed how many sequences were placed in the clades shown in Figure 3.7. Of the 450 313 sequences in SILVA in these clades, 98.0% were placed (most likely placement) into a branch of their corresponding clade. Thus, for multilevel placement, they will be assigned to the correct second level clade tree (CT). More specifically, the *Firmicutes* perform worst, as only 94.7% of the *Firmicute* sequences are placed into the corresponding clade. This can be explained by the high amount of paraphyletic branches of this clade, cf. Figure 3.10, which is a known issue [171]. The sequences of the other four clades we tested achieve a clade identification accuracy exceeding 99%.

As mentioned before, a high-level taxonomic constraint can improve the accuracy of placing a sequence into the correct BT clade. To show this, we inferred the *Bacteria* RT again, but used a *Phylum* level constraint that separates the five clades from each other and from the rest of the tree. All branches within the clades were resolved using maximum likelihood. The tree (not shown) is similar to the tree in Figure 3.7, but all five clades are now monophyletic. Using this tree, 99.3% of the sequences were placed into the correct clade. Particularly the accuracy for *Firmicutes* improved, yielding an accuracy of 99.5%.

Overall, our experiments show that the first level placement is highly accurate, even if an extremely diverse “all bacteria” backbone tree is used. The accuracy on the second level is slightly worse when using PhATs as CTs.

3.7 Conclusion and Outlook

We presented algorithms and software tools to facilitate and accelerate phylogenetic placement of large environmental sequencing studies.

The Phylogenetic Automatic (Reference) Tree (PhAT) method provides a means for automatically obtaining suitable reference trees by using the taxonomy of large sequence databases. Using the Silva database as a test case, we showed that it can be applied for accurately (pre-)placing environmental sequences into taxonomic clades. The method can also be used for rapid data exploration in environmental sequencing studies: An PhAT might be useful to obtain an overview of the taxa that are necessary to capture the diversity of a sequence dataset, without the substantial human effort and potential bias of manually selecting reference sequences. As we showed, PhATs can also be used to obtain taxonomic assignments and profiles for a set of samples, in conjunction with phylogenetic placement. To capture clade diversity with finer resolution, for example for a second placement level, clade-specific PhATs can be inferred. If species-level resolution is required, we recommend that the sequences are inspected by an expert, in order to make sure that the tree is suitable for the dataset to be placed on it. Furthermore, as our automated approach inevitably suffers from errors in the database it is based on, we recommend using SATIVA [107] to identify potentially mislabeled sequences in the database. One should also keep in mind that phylogenetic placement does not necessarily provide resolution at the *species* level [53].

As we show, our multilevel placement method as well as the preprocessing pipeline accelerate the placement process without sacrificing accuracy. By first placing the query sequences on a broad backbone tree (BT), as described in the method, novel environments with sequences of unknown evolutionary origin can be classified without having to process a large tree comprising all taxa of interest. A second placement on a set of clade trees (CTs) provides sufficient resolution for biological interpretation. Placement accuracy can be further improved by inferring the BT with a high-level constraint that separates the clades of the CTs from each other and thus ensures monophyly of these clades.

The methods presented here are implemented as part of our GAPP^A tool, which is freely available under GPLv3 at <http://github.com/lczech/gappa> (see Section C for an overview of the corresponding commands). All scripts and data used for this paper are available at <http://github.com/lczech/placement-methods-paper>.

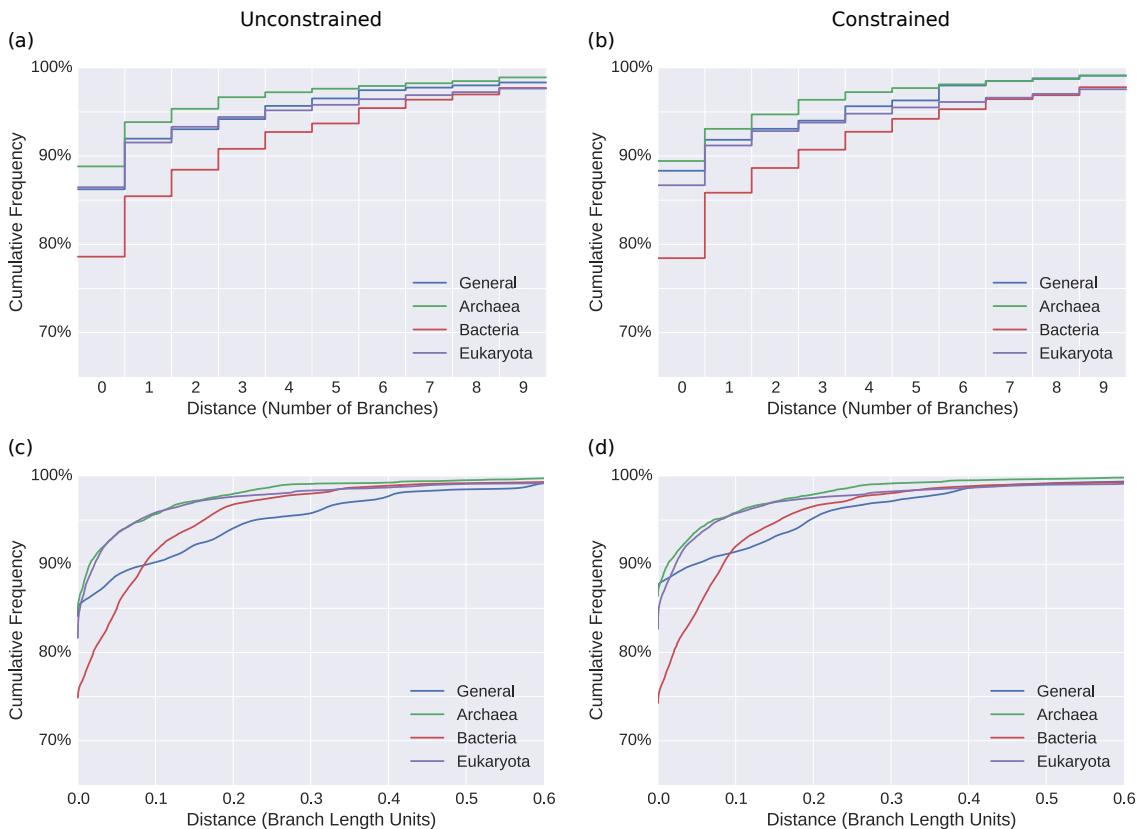


Figure 3.4: Accuracy comparison between unconstrained and constrained trees. Here, we compare how a taxonomic constraint changes the weighted distances to correct edges when placing our evaluation sequences on PhATs. The evaluation method is explained in the main text. Subfigures (a) and (c) are identical to Figure 3.3 of the main text and included here for ease of comparison. Subfigures (b) and (d) show the results when using the SILVA taxonomy as constraint for the tree inference. The relative Robinson-Foulds distances [182] between the four pairs of trees range between 45.8% and 49.7%. The differences probably occur because our trees span diverse clades, whose ancient branches are hard to resolve. Also, single gene data might not be sufficient to resolve these clades. See Section 3.6.1 and Table 3.2 for a more detailed comparison of the constrained and unconstrained trees. However, the differences mostly concern inner branches. Most of the placements are however expected to be near the terminal branches, which are more stable across the trees.

This is confirmed by the fact that, overall, the results are similar between the unconstrained and constrained trees. A slight improvement can be observed for the constrained General tree (blue), which performs better according to both distance measures. However, when considering only the distance of the most likely placement (highest LWR) to its correct edge instead of using average distances weighted by the LWR per QS, the constrained trees consistently yield better results (data not shown). For example, the most significant change is observed for the Eukaryota tree, with 84% correct placements for the unconstrained tree, but 89% for the constrained one. We suspect that this is an artifact of our evaluation process, as we consider a sequence to be correctly placed if the placement branch belongs to the consensus sequence to which the sequence contributed. As the selection of sequences for each consensus sequence is guided by the taxonomy, using the same taxonomy as constraint for the tree thus might also improve the placement accuracy.

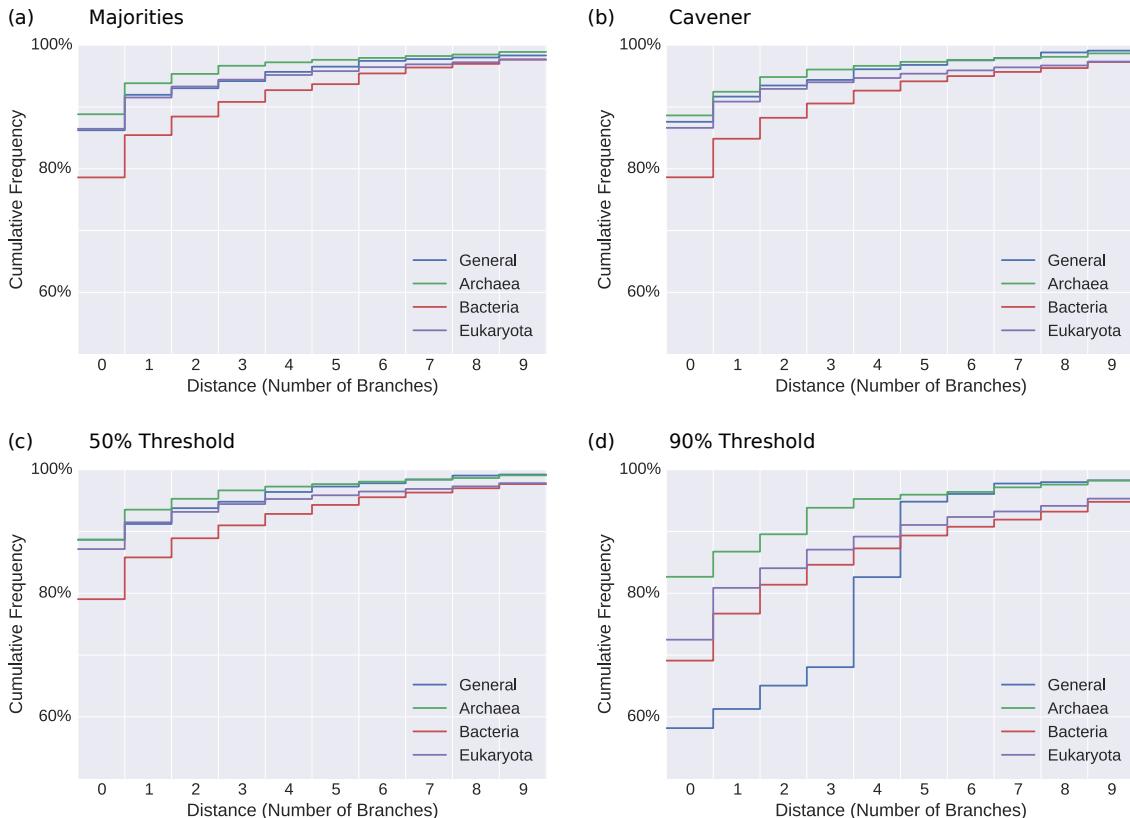


Figure 3.5: Effect of different consensus sequence methods on placement accuracy. In the main evaluation of our PhAT method, we used a reference tree and alignment based on majority rule consensus sequences [43, 147] of the SILVA database sequences. Here, we evaluate the effect of using other consensus sequence methods on phylogenetic placement accuracy. The evaluation method is described in the main text. In addition to (a) majority rule consensus, we tested (b) Cavener’s method [27, 28], as well as threshold consensus sequences [42, 43] using thresholds of 50%, 60%, 70%, 80%, and 90%, of which two are shown in (c) and (d). The three remaining threshold methods exhibit accuracies almost exactly in between the shown plots, that is, accuracy decreases with increasing thresholds. For comparison, we also included Figure 3.3(a) of the main text again, here as Subfigure (a), using the same y-axis scaling as the other plots. We only show distances measured in number of branches here, because this is more relevant in the context of our methods (e.g., Multilevel Placement).

By using alternative consensus methods, the consensus sequences and thus the sites in the alignments change. Hence, the obtained reference trees (not shown) differ substantially from each other. Across the corresponding trees of the consensus methods tested here, we observed an average relative RF distance of 49.5%. This is similar to our findings depicted in Figure 3.4. Here too, the accuracy of the constrained variants of these trees (data not shown) does not change much compared to the accuracy obtained for the unconstrained trees shown here. Thus, the differences in accuracy seen here are most likely due to the interplay of alignment and placed sequences (which is what we are interested in), and not due to differences in the trees (which are not of interest here).

The first three plots (a)-(c) exhibit similar accuracies. On average, majority rule, Cavener’s, and low threshold ($\leq 70\%$) consensus methods place 82-83% of the sequences on the expected branch. As a general trend, the *Archaea*, being the smallest tree, tend to have the highest accuracy. On the other hand, the *Bacteria*, having

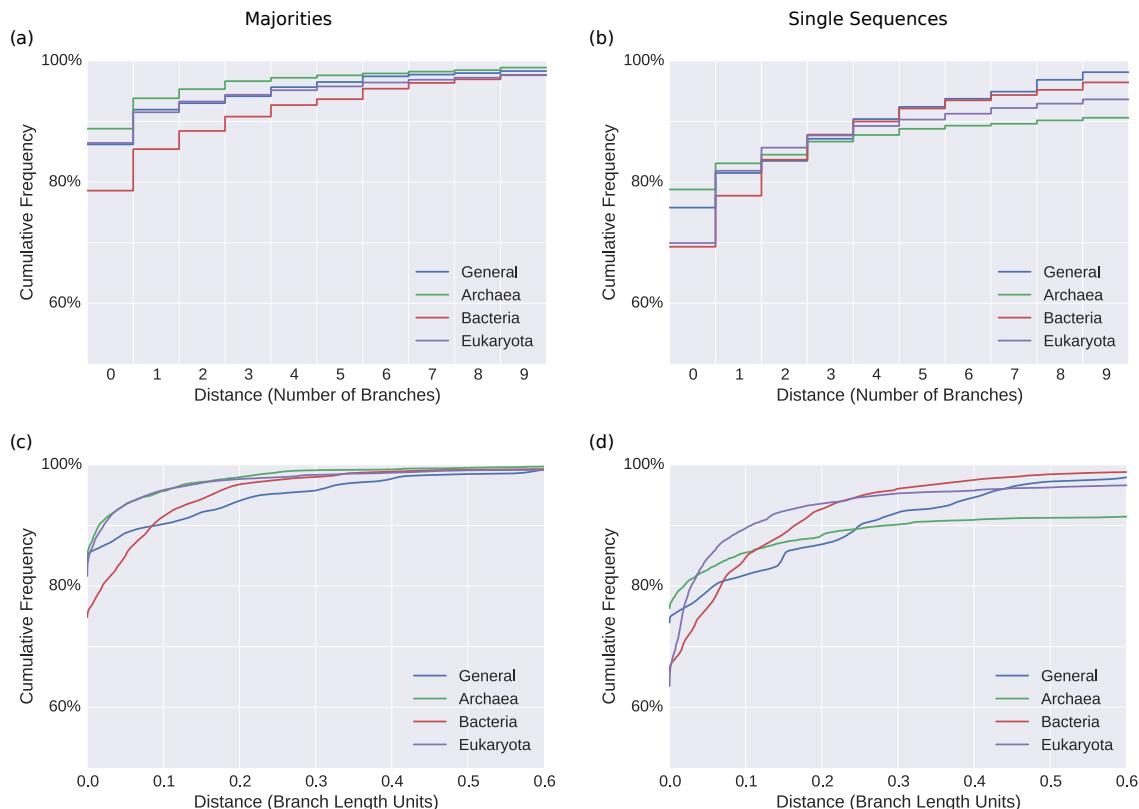


Figure 3.6: Effect of using actual sequences (instead of consensus sequences) on placement accuracy. For most of our evaluation of the PhAT method, we used some form of consensus sequence representation for the clades of the taxonomy, see e.g., Figure 3.4 and Figure 3.5. However, we also tested how the method behaves when using actual sequences instead, thus avoiding to unnecessarily blur the phylogenetic signal, and other potential drawback of consensus sequences. As manually selecting representative sequences from the database was not practical, we used the following automated approach. First, we took the 90% threshold consensus sequences of the PhAT method that were already evaluated in Figure 3.5(d). By using a high threshold, most of the diversity of each clade is included. Then, for each such consensus sequence, we calculated a score for all sequences from the database that were used to construct this consensus sequence. This score is the number of different nucleotides between the consensus sequence and the database sequence. The sequence with the lowest score (that is, with most matching nucleotides) was then used as representative of the clade. Thereby, the taxonomic clades are represented by actual sequences from the database. However, as these sequences are close to the respective consensus sequence, they are still good representatives of the diversity of the clade. Using this set of sequences, we then again inferred a tree and conducted the evaluation procedure by placing all sequences of the database on that tree, as described in the main text.

Subfigures (a) and (c) are identical to Figure 3.3 of the main text and included here for ease of comparison, however with the y-axis scaled to fit the remaining subfigures. That is, they show the evaluation of the majority rule consensus sequences. Subfigures (b) and (d) show the evaluation of the approach as described here. The resulting accuracy is worse in all cases. That is, on average, the sequences were placed further from their respective expected branch. We suspect that this is because single sequences do not capture the diversity of their clade as well as consensus sequences, and because they do not incorporate as much biological information (e.g., insertion/deletion information).

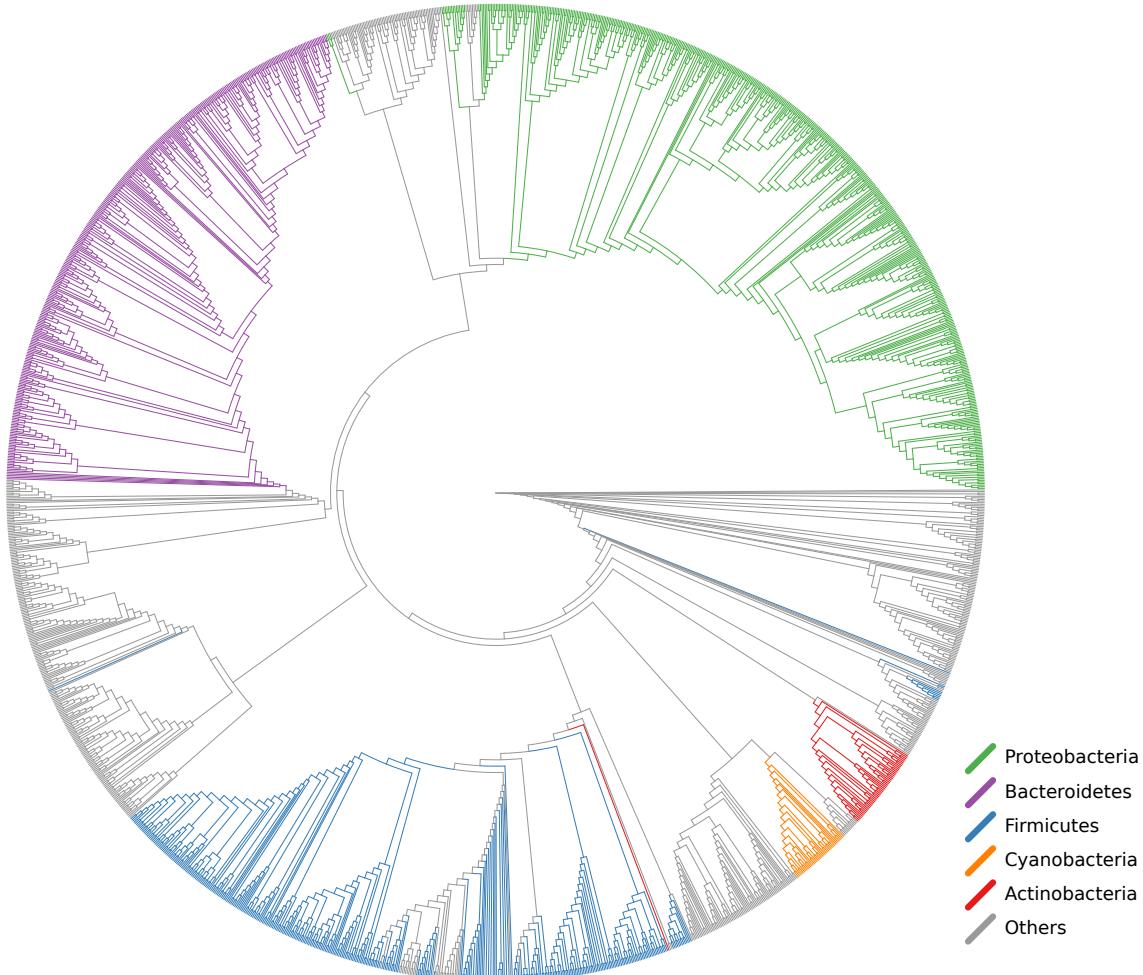


Figure 3.7: Unconstrained *Bacteria* tree with five bacterial sub-clades. This tree is the result of our PhAT method applied to the *Bacteria* sequences in SILVA. The tree contains a total of 1914 taxa. Colorized are the five *Phylum* level sub-clades that we used for testing multilevel placement: *Proteobacteria* (505 taxa), *Bacteroidetes* (362 taxa), *Firmicutes* (360 taxa), *Cyanobacteria* (39 taxa) and *Actinobacteria* (53 taxa). The incongruence between taxonomy and phylogeny is visible here as non-monophyletic colored branches. We thus here define a clade to consist of all branches that are part of a monophyletic split of the tree with respect to the taxa in the clade. In other words, all branches on one side of a split are considered to belong to a clade, if that side of the split only contains taxa from that clade. These branches then receive the same color here. Then, for multilevel placement, a sequence is considered to be part of a clade if its most probable placement falls into that clade. For example, a sequence that is placed onto one of the orange branches on this tree is subsequently placed in the *Cyanobacteria* tree for the second level placement. Each of the five sub-clades is represented by multiple branches here, which we call the “overlap” with the *Bacteria* tree.

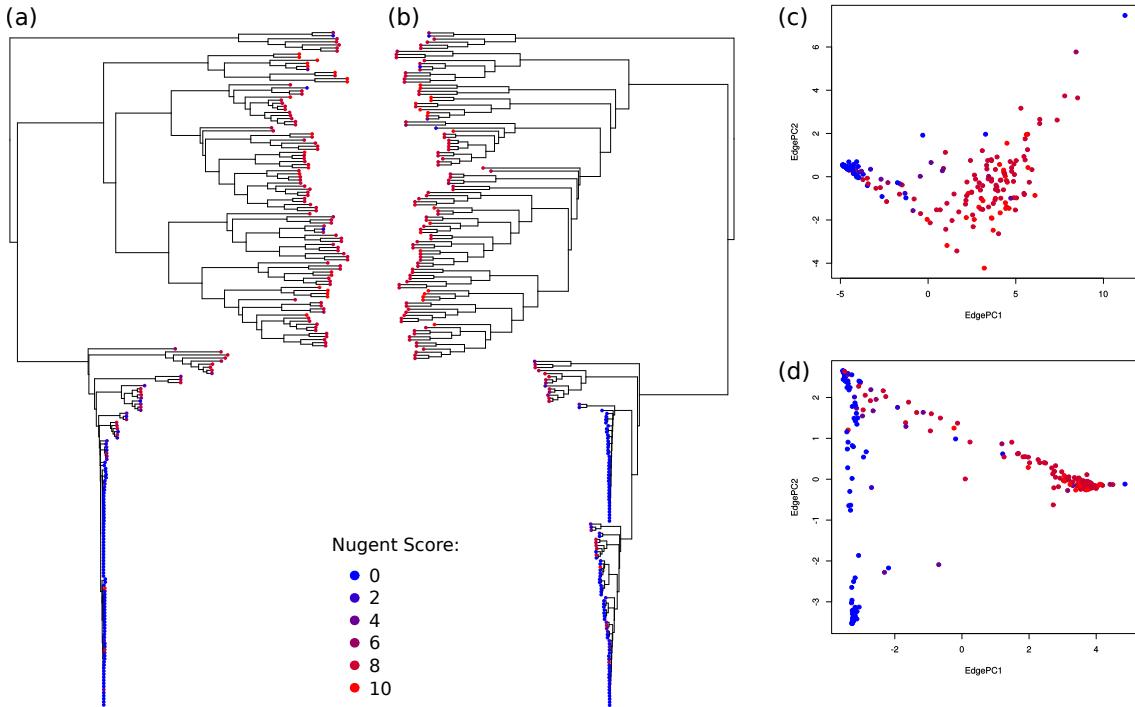


Figure 3.8: TODO: this is small. lets see if this needs to stay small! **Assessment of a PhAT for conducting Squash Clustering and Edge PCA.** Here, we test the behavior of the unconstrained *Bacteria* tree obtained via our PhAT method when used for standard post-analyses of phylogenetic placement results. For this, we used a sequence dataset of the vaginal microbiome of 220 women [201]. For details on the processing, see Section B. The original study showed associations between the presence of certain bacterial species and the diagnosis of Bacterial Vaginosis (BV), a condition caused by changes in the vaginal microbiome. In the study, the Nugent score [165] was used as a clinical diagnostic criterion for BV, which ranges from 0 (healthy) to 10 (severe illness). We placed the sequences of the dataset on their original tree and on our PhAT, and reproduce some of the results from the original study to assess differences induced by using distinct references trees.

Squash Clustering is a hierarchical clustering method for phylogenetic placement data [141] that uses the phylogenetic Kantorovich-Rubinstein (KR) distance [61] to measure cluster similarity. The result of Squash Clustering is a cluster tree of samples, where samples that are similar according to the KR distance are closer to each other, with branch lengths according to that distance. The left side of the figure compares the cluster trees resulting from using (a) our tree and (b) the original reference tree. Subfigure (b) is a recalculation of Figure 1(A) of [201]. The tips, which correspond to samples, are colored by the Nugent score of each sample, and thus indicate which women are affected by BV. The general features of the two cluster trees are comparable, indicating that our tree is able to distinguish between healthy and sick patients. However, there is a major difference in the lower half of the trees: While (b) shows some small branch lengths and even a separated sub-clade of samples with low Nugent score, these branches have a length of virtually zero in (a). As shown in [201], the healthy patients are divided into two classes, based on the presence of two species of *Lactobacillus*. The original reference tree contains sequences of those species, and can thus distinguish between them. Our broad *Bacteria* tree however does not have this degree of species-level resolution and thus treats them the same, yielding a negligible KR distance between the samples. Although this finding is expected, it serves as an example for the limits of our method. TODO: missing text!!!!!!!!!!!!!!!!!!!!!!!!!!!!!! dimensions too large!

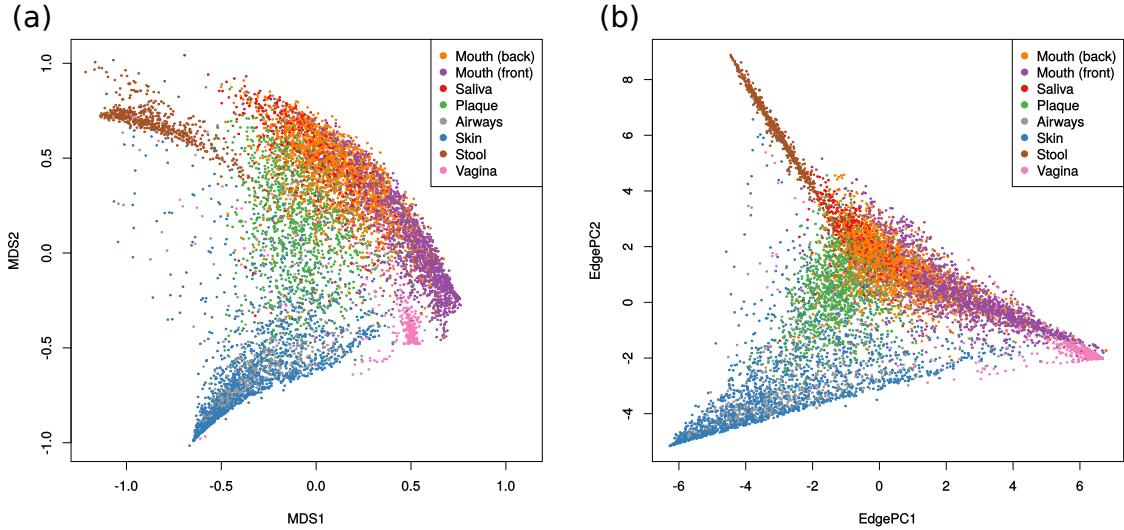


Figure 3.9: Assessment of a PhAT for large dataset analyses. Here, we test the unconstrained *Bacteria* tree generated by our PhAT method for placing and analyzing a large sequence dataset. For this, we use the Human Microbiome Project (HMP) [91, 151] data, and selected 9192 samples from different body sites with a total of 117 million sequences. For details on the processing, see Section B. We categorized the 19 original body site labels into 8 regions, in order to make the plot more readable. See Table B.2 for the mapping between the original labels and the ones used here. The sequences were placed on the tree, and subsequently analyzed with two different methods. Both subfigures show that the tree, despite only representing higher taxonomic levels, suffices to separate different body site regions from each other.

In subfigure (a), we visualized the pairwise KR distances between all samples. The phylogenetic Kantorovich-Rubinstein (KR) distance [61, 141] between two samples, also known as the Earth Mover’s distance, measures how much placement “mass” has to be moved by how much along the branches of the tree in order to transform one sample into the other. It is a generalization of the UniFrac distance [126, 128]. The high-dimensional pairwise KR distance matrix was then embedded into the plot by performing Multidimensional scaling (MDS). MDS [62, 110, 137] is a dimensionality reduction technique that finds an embedding of a distance matrix into lower dimensions (in this case, 2 dimensions) preserving higher dimensional distances as well as possible.

In subfigure (b), we also perform Edge PCA [141] on the samples. The grouping of body sites is again clearly visible with this method. Similar to Figure 3.8(d), the plot forms a triangle of samples, roughly separated by body site regions.

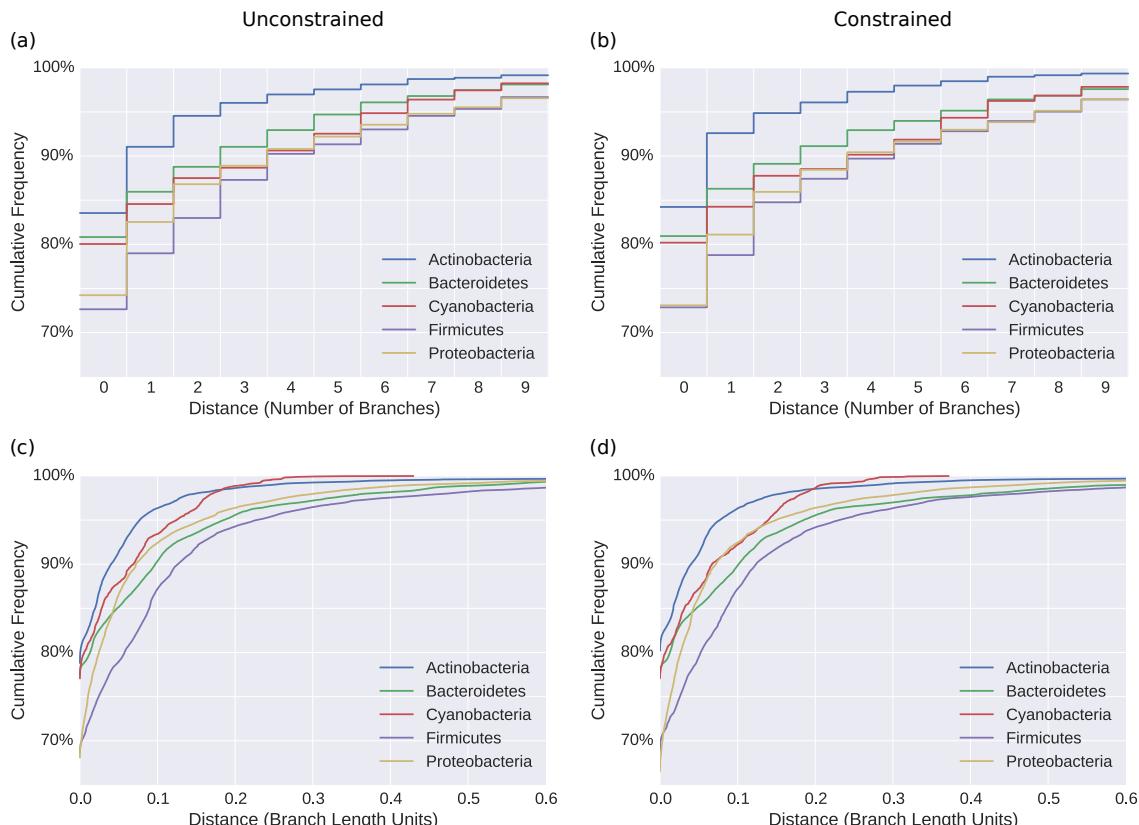


Figure 3.10: Accuracy of the PhATs of five bacterial sub-clades. We used five sub-clades of the *Bacteria* in SILVA, which were already scrutinized in [107], to test how our PhAT method works for less diverse sets of sequences. These five clades are also highlighted in Figure 3.7; see there for a description of the clades. The evaluation was conducted as explained in the main text; in short, we placed the SILVA sequences of the clades on their respective tree, and measured how far each of them is away from the branch of the consensus sequence it is represented by.

The placement accuracy on these sub-clades is slightly worse compared to the broad *Bacteria* tree, which can be seen by comparison to Figure 3.4. On average, 73.4% of the sequences were placed exactly on their expected branch, dominated by *Proteobacteria* and *Firmicutes*, which combined make up 75% of the sequences in the five clades, and have an accuracy of 71%. The *Actinobacteria* have the highest accuracy, with 82% of their sequences placed on the expected branch.

The two smallest clades, *Actinobacteria* and *Cyanobacteria*, exhibit the shortest distances in branch length units. In fact, the longest distance of any sequence from its expected branch in the *Cyanobacteria* clade is around 0.4, which is indicated by the end of the red line in the lower two plots. On the other hand, the *Firmicutes* generally have the lowest accuracy here. In Figure 3.7, which shows the unconstrained *Bacteria* tree, the *Firmicutes* clade exhibits many paraphyletic branches, which is a known issue [171]. This indicates that there is a high incongruence between the *Firmicutes* taxonomy and phylogeny in SILVA, which might explain why the *Firmicutes* score worst here.

These results are likely due to the inability of 16S SSU sequences to properly resolve lower taxonomic levels [93, 155, 175]. For example, Table 2 of [93] lists 10 bacterial genera that are known to be hard to identify using 16S sequences. These genera account for 7.9% of the 2846 taxa that are represented by the five bacterial trees tested here. Furthermore, 95 553 of the 450 313 sequences that were placed on those trees (21.2%) belong to one of these genera. This might explain the worse scores of

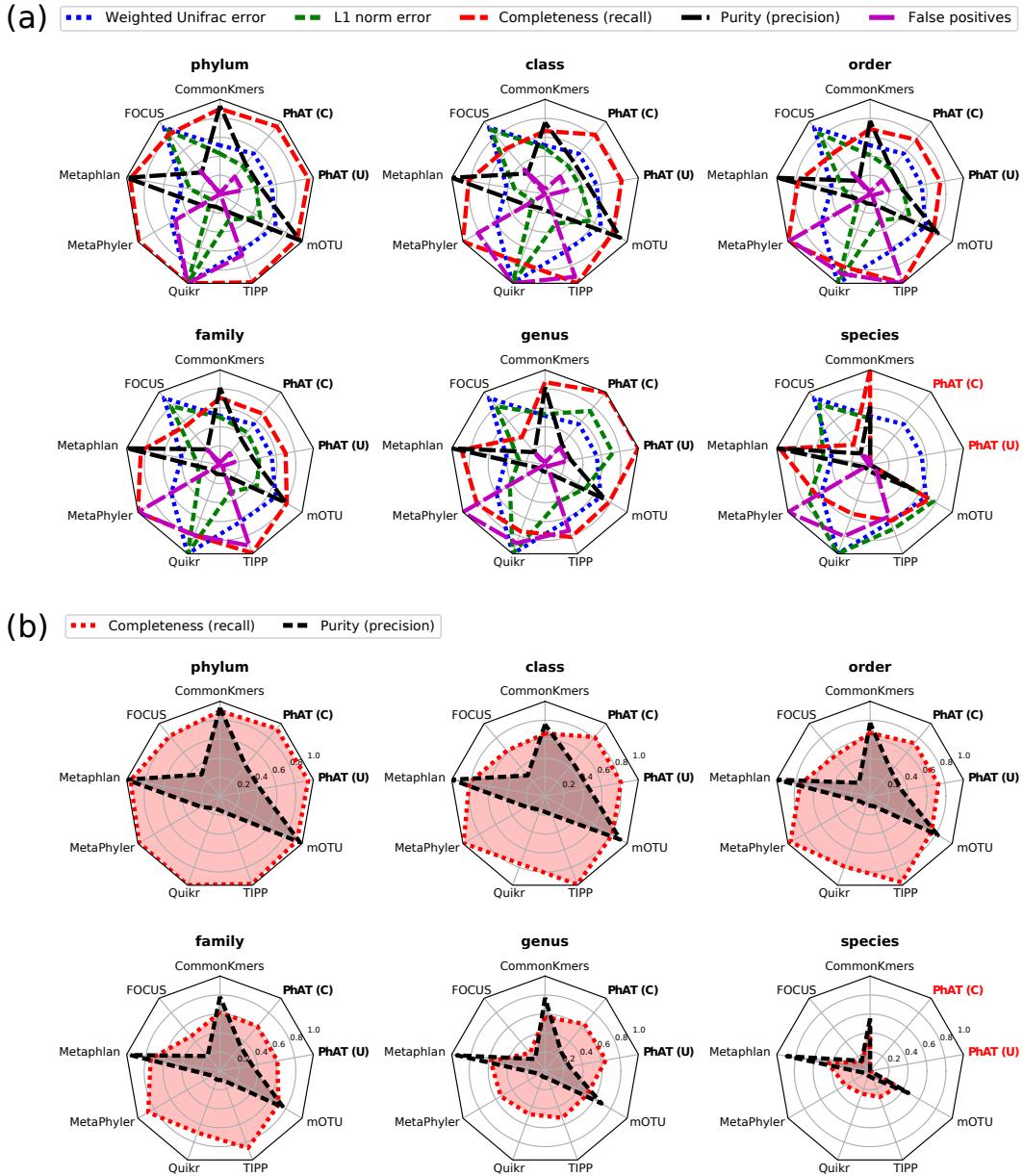


Figure 3.11: CAMI Profiling Results. The figure shows the profiling results of different tools evaluated with data from, and following the protocol of, the 2nd CAMI challenge [22, 194]. Here, we compare the taxonomic assignment and profiling conducted with our `assign` command to the tools that took part in the 2nd CAMI challenge. For this, we used the unconstrained and constrained *Bacterial* tree, which are abbreviated here as “PhAT (U)” and “PhAT (C)”, respectively. See Section 3.6 for details on our evaluation, and see [194] for details on CAMI and their metrics.

Subfigure (a) shows the relative performance of the tools for different taxonomic ranks by displaying the error metrics used by CAMI: Weighted Unifrac error, L1 norm error, recall (completeness), precision (purity) and false positives. The error metrics were divided by their respective maximal value to allow viewing them on the same scale and to allow relative performance comparisons. Subfigure (b) shows the absolute recall (completeness) and precision (purity) for each tool across the taxonomic ranks. In both subfigures, the red text for our PhAT evaluations indicates that no predictions at the corresponding taxonomic rank were returned. This is because our SILVA-based tree does not have *species* resolution and does hence not allow for taxonomic profiling at this level.

Table 3.1: Taxonomic composition of the four PhATs. The table lists the four trees and their sizes (in number of tips), as well as how many of these tips originate from each of the three domains of life. The target size of the *General* tree was 2000 taxa, while the *Bacteria* and *Eukaryota* tree were targeting 1800 domain-specific taxa, which is approximately reached, but not exactly (underlined values). This is because the sizes of sub-clades in the taxonomy vary. Because each tip of the tree is a consensus sequence that represents the respective lowest taxonomic level, the number of available taxa is smaller than the total number of taxonomic labels in the SILVA database. For example, the *Archaea* have a total of 347 taxonomic labels across all ranks, but only 248 labels at *Genus* level. Thus, the *Archaea* tree shown here represents the *Archaea* taxonomy resolved at the *Genus* level. In the three domain specific trees, we furthermore included consensus sequences at the *Phylum* level of the respective two remaining domains, in order to make sure that the evaluation also works well if such “outgroups” are included.

Tree	Size	Thereof number of		
		Archaea	Bacteria	Eukaryota
<i>General</i>	<u>1998</u>	210	508	1280
<i>Archaea</i>	511	248	205	58
<i>Bacteria</i>	1914	59	<u>1797</u>	58
<i>Eukaryota</i>	2059	59	205	<u>1795</u>

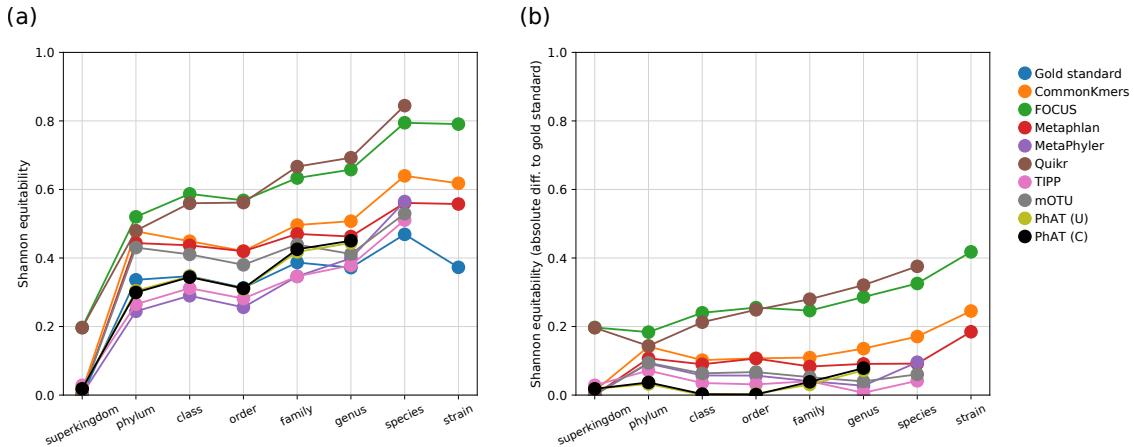


Figure 3.12: CAMI: Alpha Diversity. The figure shows the alpha diversity as estimated by different tools evaluated with data from, and following the protocol of, the 2nd CAMI challenge [22, 194]. Here, we compare the taxonomic assignment and profiling conducted with our `assign` command to the tools that took part in the 2nd CAMI challenge. For this, we used the unconstrained and constrained *Bacterial* tree, which are abbreviated here as “PhAT (U)” and “PhAT (C)”, respectively. **TODO:** add some more text here

Table 3.2: Tree Topology Significance Tests. Here, we report significance tests comparing the four pairs of unconstrained (U) and constrained (C) trees used in our evaluation. The tests were performed with IQ-TREE v1.5.6 [163] under the GTR+G model and 10 000 resamplings using the RELL method [104]. The table shows that the unconstrained trees fit the data significantly better in all four cases and in all tests.

Columns are as follows. logL and deltaL: log likelihood and difference between constrained and unconstrained tree. bp: bootstrap proportion using REL method [104]. p-(W)KH: p-value of the one sided and the weighted Kishino-Hasegawa test [103]. p-(W)SH: p-value of the (weighted) Shimodaira-Hasegawa test [197]. c-ELW: Expected Likelihood Weight [206]. p-AU: p-value of approximately unbiased (AU) test [196].

TODO: table size! set to small now, but maybe make the page landscape instead??

Table 3.3: Overview of the PhATs and their evaluation statistics. Details of four unconstrained (U) and four constrained (C) trees are shown. “Size” is the number of leaves of the tree, that is, the number of consensus sequences that the tree was inferred from. “% Seqs.” the percentage of sequences from SILVA placed on it. The *General* tree does not cover all sequences, because there are some sequences labels in the database that could not be mapped to the taxonomy. “ \emptyset Br. Len.” is the average branch length in the tree. The evaluation results are reported in the remaining columns: Average distances of the sequences to their respective expected branch are listed in numbers of branches (Discrete) and in branch length units (Continuous), as explained in the text. Furthermore, “Exp. Br. Hits” shows how often the most probable placement was placed exactly on the expected branch. Lastly, the average expected distance between placement locations (EDPL) is shown. The EDPL is the sum of the distances between the placements of a sequences weighted by their probability [144].

Reference Tree	Size	% Seqs.	\emptyset Br. Len.	Average Distance			Exp. Br. Hits	\emptyset EDPL
				Discrete	Continuous			
<i>General</i> (U)	1998	98.7%	0.084	0.63	0.034	85.9%	0.00058	
<i>General</i> (C)	1998	98.7%	0.086	0.57	0.027	88.2%	0.00046	
<i>Archaea</i> (U)	511	3.4%	0.070	0.46	0.013	86.4%	0.00038	
<i>Archaea</i> (C)	511	3.4%	0.071	0.45	0.013	88.2%	0.00041	
<i>Bacteria</i> (U)	1914	84.6%	0.067	1.13	0.031	77.0%	0.00095	
<i>Bacteria</i> (C)	1914	84.6%	0.071	1.11	0.031	76.6%	0.00091	
<i>Eukaryota</i> (U)	2059	10.0%	0.080	0.79	0.022	84.9%	0.00032	
<i>Eukaryota</i> (C)	2059	10.0%	0.083	0.81	0.024	85.7%	0.00031	

Table 3.4: CAMI: Scores and Ranks. The table shows the scores and ranks of different tools evaluated with data from, and following the protocol of, the 2nd CAMI challenge [22, 194]. Here, we compare the taxonomic assignment and profiling conducted with our `assign` command to the tools that took part in the 2nd CAMI challenge. For this, we used the unconstrained and constrained *Bacterial* tree, which are abbreviated in the table as “PhAT (U)” and “PhAT (C)”, respectively. See Section 3.6 for details.

Four metrics are used in CAMI for evaluating tools: Recall (completeness), precision (purity), L1 norm error (abbreviated here as L1 NE), and Weighted Unifrac Error (abbreviated here as WUE). For each metric, the comparative scores of the tools are shown, as well as their rankings, relative to each other. Also, the total sum of scores and the total rank are shown, which add up the values of the four metrics. The procedure of the scoring and ranking is explained in detail in the Online Supplement of [194].

Despite the caveats and limitations that are explained in Section 3.6, using our PhATs trees to obtain a taxonomic profile yields rankings in the middle of the field for all metrics.

Tool	Total		Recall		Precision		L1 NE		WUE	
	Sum	Rank	Score	Rank	Score	Rank	Score	Rank	Score	Rank
Metaphlan	1814	1	958	3	75	1	731	2	50	1
MetaPhyler	2995	2	314	1	2119	7	322	1	240	5
CommonKmers	3333	3	1448	6	409	2	1318	7	158	3
mOTU	3751	4	1703	8	488	3	1260	5	300	6
PhAT (C)	3784	5	1202	4	1116	4	1280	6	186	4
PhAT (U)	3933	6	1436	5	1153	5	1208	4	136	2
TIPP	4263	7	892	2	2126	8	930	3	315	7
FOCUS	6153	8	2079	9	1636	6	2018	8	420	8
Quikr	6838	9	1488	7	2398	9	2453	9	499	9

4. Visualization

This chapter is based on the peer-reviewed publication:

- **Lucas Czech** and Alexandros Stamatakis. "Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements." *PLOS One*, 2018.

Contributions: Lucas Czech... Alexandros Stamatakis...

4.1 Motivation

A first step in analyzing phylogenetic placement data is often to visualize them. For small samples, it is possible to mark individual placement locations on the RT, as offered for example by iTOL [114], or even to create a tree where the most probable placement per QS is attached as a new branch, as implemented in the GUPPY tool from the PPLACER suite [144], RAxML-EPA [16, 203], and our tool GAPPA. For larger samples, one can alternatively display the per-edge placement mass, either by adjusting the line widths of the edges according to their mass, or by using a color scale, as offered in GGTREE [237], GUPPY, and GAPPA. Using per-edge colors corresponds to binning all placement of an edge into one bin. For large datasets, the per-edge masses can vary by several orders of magnitude. In these cases, it is often preferable to use a logarithmic scaling, as shown in [135].

TODO: refine more advanced version for example when doing tax assignment can be done with hierarchical pie charts, as for instance offered by the Krona tool [167].

pie charts are typical [91]

These simple visualizations directly depict the placement masses on the tree. When visualizing the accumulated masses of multiple samples at once, it is important to

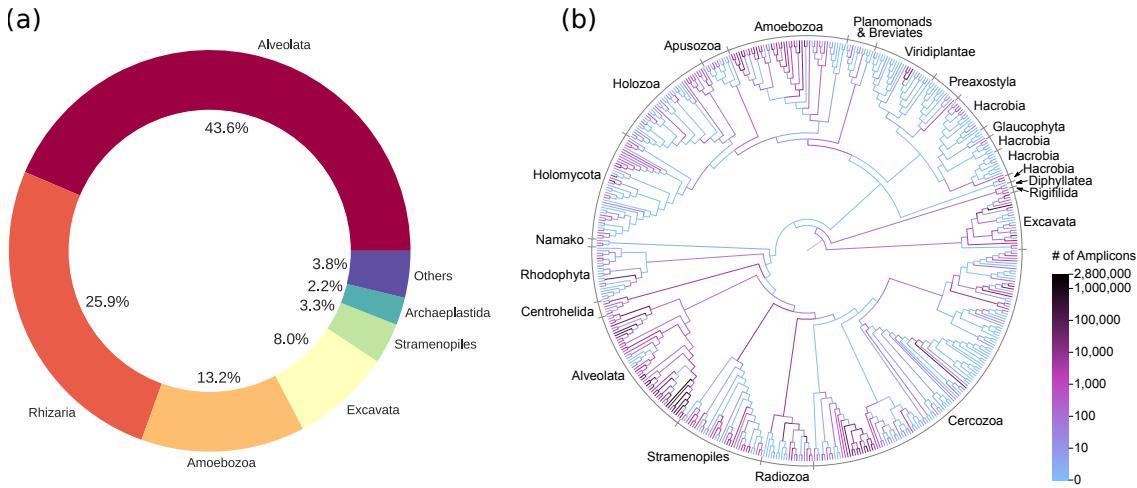


Figure 4.1: Visualization of read abundances. Using neotrop data, we show a typical pie chart of taxonomic abundances in (a) and the much more informative per-branch visualization in (b), similar to [135]. note that pie charts are usually not considered good visualization practice, and thus better use bar plots. but, it seems to be a default in many papers... **TODO: cite them!**

chose the appropriate normalization strategy for the task at hand. For example, if samples represent different locations, one might prefer to use normalized masses, as comparing relative abundances is common for this type of data. On the other hand, if samples from the same location are combined (e.g., from different points in time, or different size fractions), it might be preferable to use absolute abundances instead, so that the total number of sequences per sample can be visualized.

The visualizations provide an overview of the species abundances over the tree. They can be regarded as a more detailed version of classic abundance pie charts. When placing OTUs, or ignoring sequence abundances, the resulting visualizations depict species diversity. Moreover, these visualizations can be used to assess the quality of the RT. For example, placements into inner branches of the RT may indicate that appropriate reference sequences (i) have not been included or (ii) are simply not yet available.

Here, we introduce visualization methods that highlight (i) regions of the tree with a high variance in their placement distribution (called *Edge Dispersion*), and (ii) regions with a high correlation to meta-data features (called *Edge Correlation*).

4.2 Edge Dispersion

The Edge Dispersion is derived from the edge masses or edge imbalances matrix by calculating a measure of dispersion for each of the matrix columns, for example the standard deviation σ . Because each column corresponds to an edge, this information can be mapped back to the tree, and visualized, for instance, via color coding. This allows to examine which edges exhibit a high heterogeneity of placement masses

across samples, and indicates which edges discriminate samples. As edge mass values can span many orders of magnitude, it might be necessary to scale the variance logarithmically. Often, one is more interested in the branches with high placement mass. In these cases, using the standard deviation or variance is appropriate, as they also indicate the mean mass per edge. On the other hand, by calculating the per-edge Index of Dispersion [62], that is, the variance-mean-ratio σ^2/μ , differences on edges with little mass also become visible. As Edge Dispersion relates placement masses from different samples to each other, the choice of the normalization strategy is important. When using normalized masses, the magnitude of dispersion values needs to be cautiously interpreted [125]. The Edge Dispersion can also be calculated for edge imbalances. As edge imbalances are usually normalized to $[-1.0, 1.0]$, their dispersion can be visualized directly without any further normalization steps. An example for an Edge Dispersion visualization is shown in Figure 4.2(a), and discussed in Section Results.

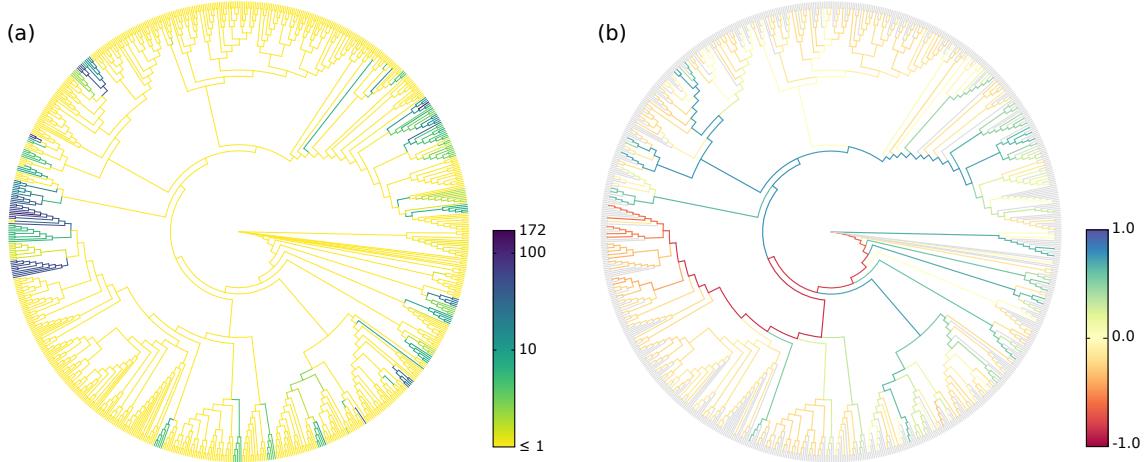


Figure 4.2: Examples of Edge Dispersion and Edge Correlation. We applied our novel visualization methods to the BV dataset to compare them to the existing examinations of the data. (a) Edge Dispersion, measured as the standard deviation of the edge masses across samples, logarithmically scaled. (b) Edge Correlation, in form of Spearman’s Rank Correlation Coefficient between the edge imbalances and the Nugent score. Tip edges are gray, because they do not have a meaningful imbalance. This example also shows the characteristics of edge masses and edge imbalances: The former highlights individual edges, the latter paths to clades.

4.3 Edge Correlation

In addition to the per-edge masses, the Edge Correlation further takes a specific meta-data feature into account, that is, a column of the meta-data matrix. The Edge Correlation is calculated as the correlation between each edge column and the feature column, for example by using the Pearson Correlation Coefficient or

Spearman’s Rank Correlation Coefficient [62]. This yields a per-edge correlation of the placement masses or imbalances with the meta-data feature, and can again be visualized via color coding of the edges. It is inexpensive to calculate and hence scales well to large datasets. As typical correlation coefficients are within $[-1.0, 1.0]$, there is again no need for further normalization. This yields a tree where edges or clades with either a high linear or monotonic correlation with the selected meta-data feature are highlighted. Figure 4.2(b) shows an example of this method. In contrast to Edge PCA [141] that can use meta-data features to annotate samples in its scatter plots, our Edge Correlation method directly represents the influence of a feature on the branches or clades of the tree. It can thus, for example, help to identify and visualize dependencies between species abundances and environmental factors such as temperature or nutrient levels. Again, the choice of normalization strategy is important to draw meaningful conclusions. However, the correlation is *not* calculated between samples or sequence abundances. Hence, even when using normalized samples, the pitfalls regarding correlations of compositional data [125] do not apply here.

4.4 Results

4.4.1 BV Dataset

We re-analyzed the BV dataset by inferring a tree from the original reference sequence set and conducting phylogenetic placement of the 220 samples. The characteristics of this dataset were already explored in [201] and [141]. We use it here to give exemplary interpretations of our Edge Dispersion and Edge Correlation methods, and to evaluate them in comparison to existing methods.

Figure 4.2 shows our novel visualizations of the BV dataset. Edge Dispersion is shown in Figure 4.2(a), while Figure 4.2(b) shows Edge Correlation with the so-called Nugent score [165] is a clinical standard for the diagnosis of Bacterial Vaginosis, ranging from 0 (healthy) to 10 (severe illness). The connection between the Nugent score and the abundance of placements on particular edges was already explored in [141], but only visualized indirectly (i.e., not on the RT itself). For example, Figure 6 of the original study plots the first two Edge PCA components colorized by the Nugent score. We recalculated this figure for comparison in Figure 5.3(i). In contrast, our Edge Correlation measure directly reveals the connection between Nugent score and placements on the reference tree: The clade on the left hand side of the tree, to which the red and orange branches lead to, are *Lactobacillus iners* and *Lactobacillus crispatus*, respectively, which were identified in [201] to be associated with a healthy vaginal microbiome. Thus, their presence in a sample is anti-correlated with the Nugent score, which is lower for healthy subjects. The branches leading to this clade are hence colored in red. On the other hand, there are several other clades that exhibit a positive correlation with the Nugent score, that is, were green and blue paths lead to in the figure, again a finding already reported in [201].

Both trees in Figure 4.2 highlight the same parts of the tree: The dark branches with high deviation in Figure 4.2(a) represent clades attached to either highly correlated (blue) or anti-correlated (red) paths Figure 4.2(b). This indicates that edges that have a high dispersion also vary between samples of different Nugent score.

We further compared our methods to the visualization of Edge PCA components on the reference tree. To this end, we recalculated Figures 4 and 5 of [141], and visualized them with our color scheme in Figure 4.6 for ease of comparison. They show the first two components of Edge PCA, mapped back to the RT. The first component, Figure 4.6(a), reveals that the *Lactobacillus* clade represents the axis with the highest heterogeneity across samples, while the second component, Figure 4.6(b), further distinguishes between the two aforementioned clades within *Lactobacillus*. Edge Correlation also highlights the *Lactobacillus* clade as shown in Figure 4.2(b), but does not distinguish further between its sub-clades. This is because a high Nugent score is associated with a high abundance of placements in either of the two relevant *Lactobacillus* clades.

Further examples of variants of Edge Dispersion and Edge Correlation on this dataset are shown in Figure 4.3 and Figure 4.4. We also conducted Edge Correlation using Amsel’s criteria [4] and the vaginal pH value as shown in Figure 4.5, both of which were used in [201] as additional indicators of Bacterial Vaginosis. We again found similar correlations compared to the Nugent score.

4.4.2 Tara Oceans Dataset

We analyzed the Tara Oceans (TO) dataset to provide further exemplary use cases for our visualization methods. To this end, we used the unconstrained *Eukaryota* RT with 2059 taxa as provided by our Automatic Reference Tree method [36]. The meta-data features of this dataset that best lend themselves to our methods are the sensor values for chlorophyll, nitrate, and oxygen concentration, as well as the salinity and temperature of the water samples. Other available meta-data features such as longitude and latitude are available, but would require more involved methods. This is because geographical coordinates yield pairwise distances between samples, whose integration into our correlation analysis methods is challenging. The Edge Correlation of the 370 samples with the nitrate concentration, the salinity, the chlorophyll concentration, and the water temperature are shown in Figure 4.7.

We selected the diatoms and the animals as two exemplary clades for closer examination of the results. In particular, the diatoms show a high correlation with the nitrate concentration, as well as an anti-correlation with salinity, which represent well-known relationships [127, 177]. See Figure 4.7 for details. These findings indicate that the method is able to identify known relationships. It will therefore also be useful to investigate or discover insights of novel relationships between sequence abundances and environmental parameters.

4.4.3 Performance

Both methods (Edge Dispersion and Edge Correlation) are computationally inexpensive, and thus applicable to large datasets. The calculation of the above visual-

izations took about 30 s each, which were mainly required for reading in the data. Furthermore, in order to scale to large datasets, we reimplemented Edge PCA, which was originally implemented as a command in the GUPPY program [144]. For the BV dataset with 220 samples, GUPPY required 9 min and used 2.2 GB of memory, while our implementation only required 33 s on a single core, using less than 600 MB of main memory. For the HMP dataset, as it is only single-threaded, GUPPY took 11 days and 75.1 GB memory, while our implementation needed 7.5 min on 16 cores and used 43.5 GB memory.

4.5 Conclusion and Outlook

Edge Dispersion highlights branches of the phylogenetic tree that exhibit variations in the number of placements, and thus allows to identify regions of the tree with a high placement heterogeneity. Edge Correlation additionally takes meta-data features into account, and identifies branches of the tree that correlate with quantitative features, such as the temperature or the pH value of the environmental samples. These methods complement existing methods such as Edge PCA, and are data exploration tools that can help unravel new patterns in phylogenetic placement data. The variants of the methods presented here are hence best used in combination with each other.

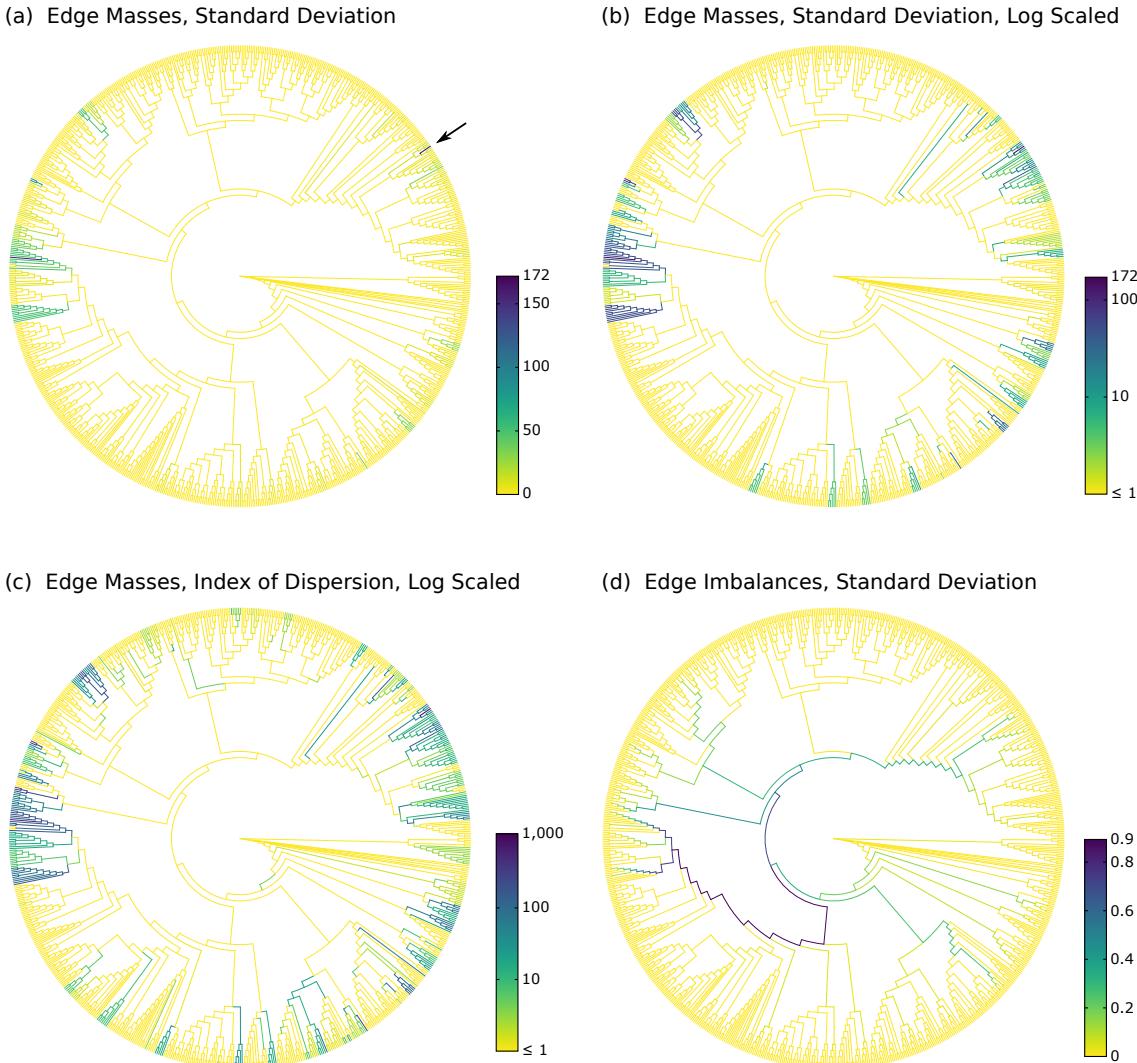


Figure 4.3: Examples of variants of Edge Dispersion. We re-analyzed the BV dataset to show variants of our Edge Dispersion method. All subfigures highlight the same branches and clades as found by other methods such as Edge PCA. The method is useful as a first exploratory tool to detect placement heterogeneity across samples. In contrast to Edge Correlation, it can however not explain the reasons of heterogeneity. Subfigure (a) shows the standard deviation of the absolute edge masses, without any further processing. It is striking that one outlier, marked with an arrow, is dominating, thus hiding the values on less variable edges. This outlier occurs at the species *Prevotella bivia* in one of the 220 samples, where 2781 out of 2782 sequences in the sample have placement mass on that branch. Upon close examination, this outlier can also be seen in Figure 1D of [201], but is less apparent there. Subfigure (b) is identical to Figure 4.2(a) of the main text and shows the standard deviation again, but this time using logarithmic scaling, thus revealing more details on the edges with lower placement mass variance. Furthermore, when comparing it to Figure 4.4(c), we see that the same clades that exhibit a high correlation or anti-correlation with meta-data there are also highlighted here. There are only few medium values, which indicates that there are two classes of edges: Those which distinguish patients and those who have almost no placement on them at all. Subfigure (c) shows the Index of Dispersion of the edge masses, that is, the variance normalized by the mean. Hence, edges with a higher number of placements are also allowed to have a higher variance. We again use a logarithmic scale because of the outlier. The figure reveals more details on the edges with lower variance.

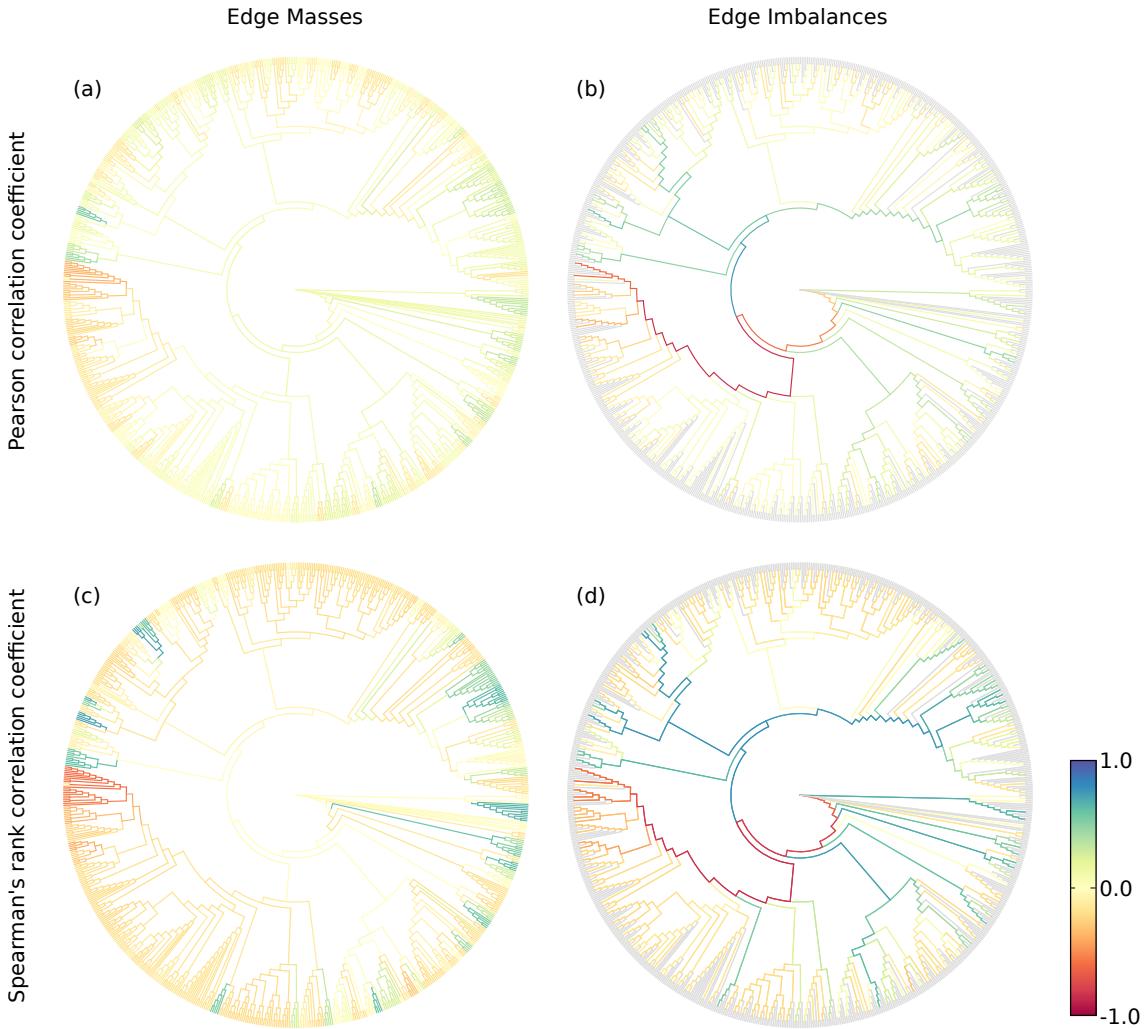


Figure 4.4: Examples of variants of Edge Correlation. We again use the BV dataset, and show the correlation of edge masses and imbalances with the Nugent score. The Nugent score measures the severeness of Bacterial Vaginosis, and ranges from 0 for healthy subjects to 10 for heavily affected patients. Subfigures (a) and (b) use the Pearson Correlation Coefficient, that is, they show the linear correlation with the meta-data feature, while subfigures (c) and (d) use Spearman's Rank Correlation Coefficient and thus show monotonic correlations. Subfigure (d) is identical to Figure 4.2(b) of the main text. All subfigures show red edges or red paths at the *Lactobacillus* clade. This indicates that presence of placements in this clade is anti-correlated with the Nugent score, which is consistent with the findings of [201] and [141]. In other words, presence of *Lactobacillus* correlates with a healthy vaginal microbiome. On the other hand, blue and green edges, which indicate positive correlation, are indicative of edges that correlate to Bacterial Vaginosis. The extent of correlation is larger for Spearman's Coefficient, indicating that the correlation is monotonic, but not strictly linear.

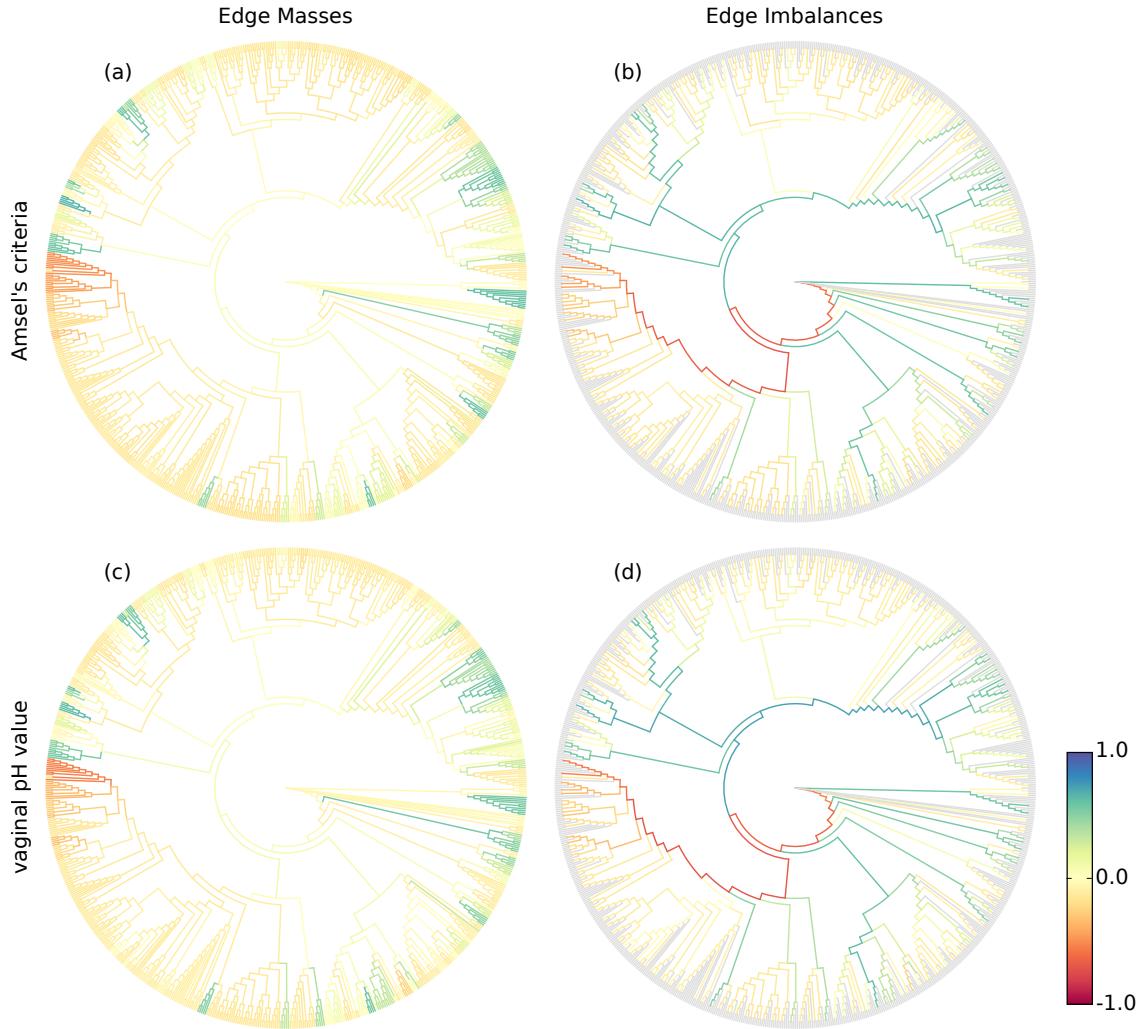


Figure 4.5: Edge Correlation with more meta-data features. Here, we use additional meta-data features of the BV dataset to show that Edge Correlation yields consistent results with existing methods. In particular, we calculated Spearman’s Coefficient with Amsel’s criteria [4] in subfigures (a) and (b), as well as with the vaginal pH value in subfigures (c) and (d). Both features were also used in [201] as indicators of Bacterial Vaginosis. The figures are almost identical to the ones shown in Figure 4.4; that is, they yield results that are consistent with the previously used Nugent score, as well as consistent with existing methods.

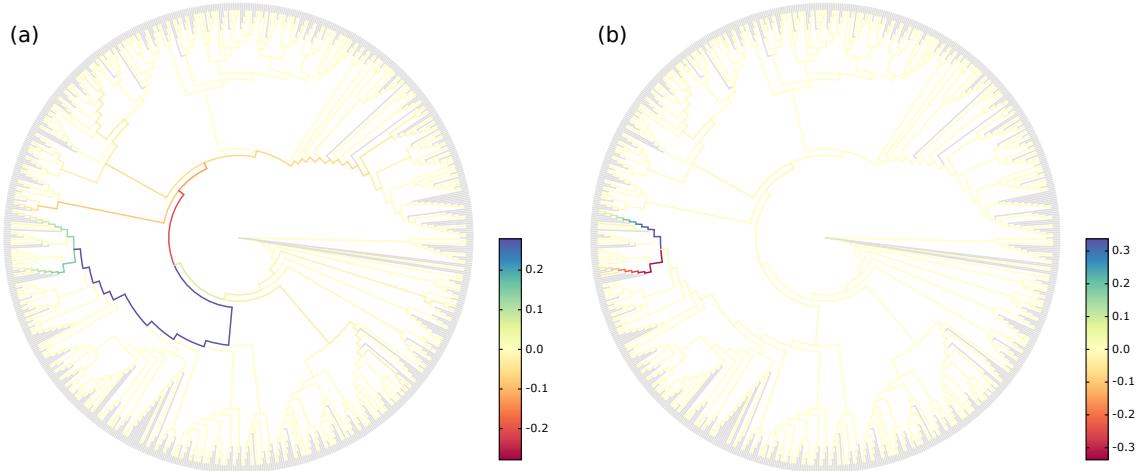


Figure 4.6: Recalculation of the Edge PCA tree visualization. Subfigures (a) and (b) are recalculations of Figures 4 and 5 of [141], respectively. However, we show them here in our coloring scheme in order to facilitate comparison with other figures. The original publication instead uses two colors for a positive and a negative sign of the principal components, and branch width to show their magnitude. Note that the actual sign is arbitrary, as it is derived from principal components.

The figure shows the first two Edge PCA components, visualized on the reference tree. This form of visualization is useful to interpret results such as the Edge PCA projection plot as shown in Figure 5.1(e) of the main text. It reveals which edges are mainly responsible for separating the samples into the PCA dimensions. Here, the first principal component in (a) indicates that the main PCA axis separates samples based on the presence of placements in the *Lactobacillus* clade, which is what the blue and green path leads to. The second component in (b) then further distinguishes between two species in this clade, namely *Lactobacillus iners* and *Lactobacillus crispatus*.

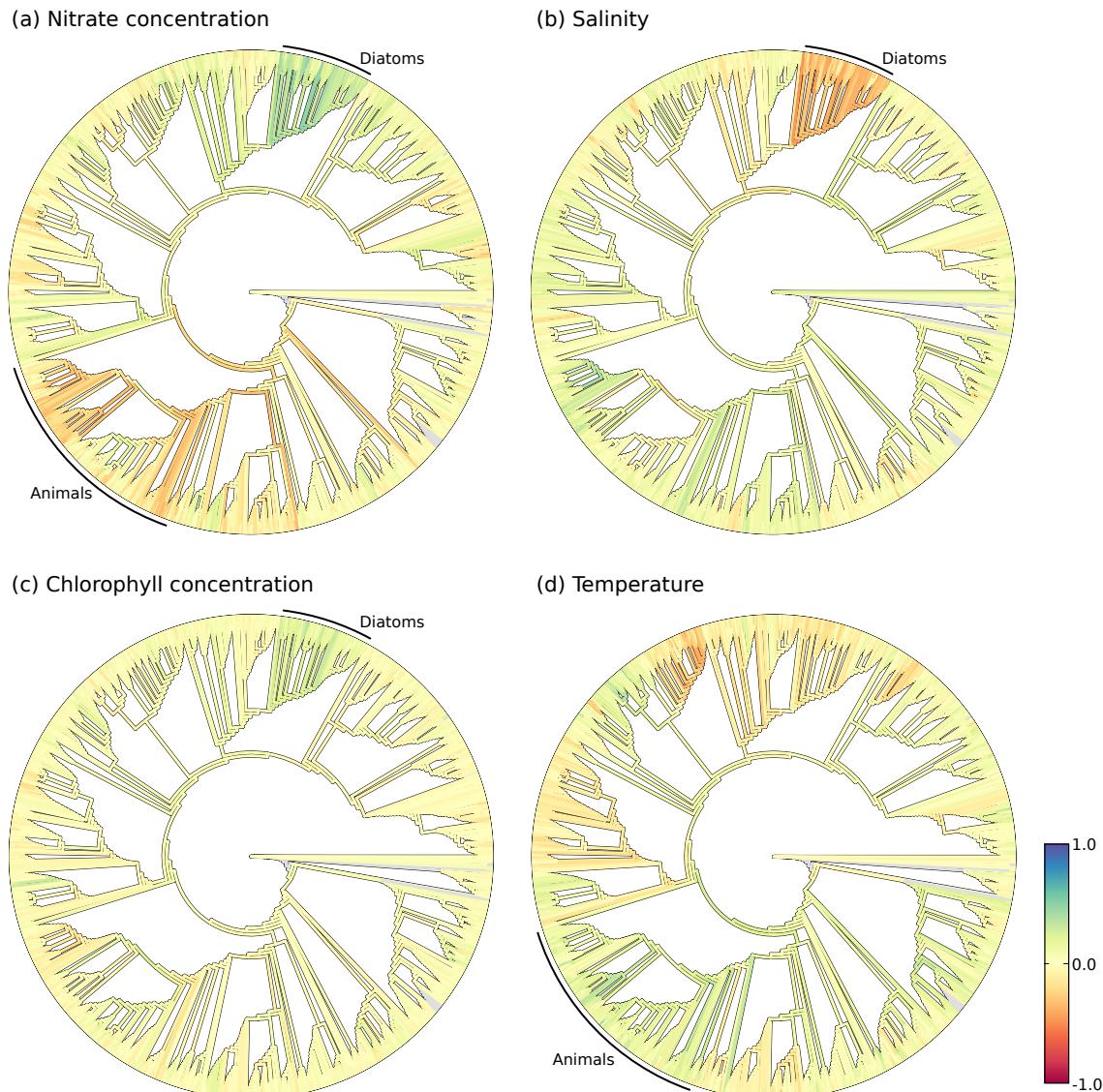


Figure 4.7: Examples of Edge Correlation using Tara Oceans samples. The figure shows the correlation of Tara Oceans sequence placements with (a) the nitrate, (b) the salinity, (c) the chlorophyll, and (d) the temperature sensor data of each sample. The sensor values range from -2.2 to $33.1 \mu\text{mol/l}$ (nitrate), from 33.2 to 40.2 psu (salt), from -0.02 to 1.55 mg/m^3 (chlorophyll), and from -0.8 to 30.5°C (temperature), respectively. The negative nitrate and chlorophyll concentrations are values below the detection limit of the measurement method (pers. comm. with L. Guidi), and hence simply denote low concentrations. We used Spearman's Rank Correlation Coefficient, and examine two exemplary clades, namely the *Animals* and the *Diatoms*.

Diatoms are mainly photosynthetic, and thus depend on nitrates as key nutrients, which is clearly visible by the high correlation of the clade in (a). Furthermore, the diatoms exhibit positive correlation with the chlorophyll concentration (c), which again is indicative of their photosynthetic behavior. On the other hand, they show a high anti-correlation with the salt content (b). Salinity is a strong environmental factor which heavily affects community structures and species abundances [127], particularly diatoms [177].

The correlations of the animal clade are less pronounced. They exhibit a negative correlation with nitrate (a), as well as an increase in absolute abundance with higher temperatures (d). While these findings are not surprising, they show that

5. Clustering

This chapter is based on the peer-reviewed publication:

- **Lucas Czech** and Alexandros Stamatakis. "Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements." *PLOS One*, 2018.

Contributions: Lucas Czech... Alexandros Stamatakis...

TODO: distance measures, nhd, simulations, mantel test

5.1 Motivation

Given a set of metagenomic samples, one key question is how much they differ from each other. A common distance metric between microbial communities is the (weighted) UniFrac distance [126, 128]. It uses the fraction of unique and shared branch lengths between phylogenetic trees to determine their difference. UniFrac has been generalized and adapted to phylogenetic placements in form of the phylogenetic Kantorovich-Rubinstein (KR) distance [61, 141]. In other contexts, the KR distance is also called Wasserstein distance, Mallows distance, or Earth Mover's distance [115, 136, 179, 217]. The KR distance between two metagenomic samples is a metric that describes by at least how much the normalized mass distribution of one sample has to be moved across the RT to obtain the distribution of the other sample. The distance is symmetrical, and becomes larger the more mass needs to be moved, and the larger the respective displacement (moving distance) is. As the two samples being compared need to have equal masses, the KR distance operates on normalized samples; that is, it compares relative abundances.

Given such a distance measure between samples, a fundamental task consists in clustering samples that are similar to each other. Standard linkage-based clustering methods like UPGMA [112, 154, 200] are solely based on the distances between samples, that is, they calculate the distances of clusters as a function of pairwise sample distances. Squash Clustering [141, 201] is a method that also takes into account the intrinsic structure of phylogenetic placement data. It uses the KR distance to perform agglomerative hierarchical clustering of samples. Instead of using pairwise sample distances, however, it merges (*squashes*) clusters of samples by calculating their average per-edge placement mass. Thus, in each step, it operates on the same type of data, that is, on mass distributions on the RT. This results in a hierarchical clustering tree that has meaningful, and hence interpretable, branch lengths.

Remark: Squash clustering uses the KR distance, which is based on mass distributions on the edges of the RT. It thus “suffers” from the same shortcomings that Edge PCA is solving by using mass imbalance instead. For instance, Figure 7 of [141] shows the result of a normal principal component analysis on a dataset of Bacterial Vaginosis, a disease of the vagina. On the left hand side of the figure, the blue data points, representing healthy women, cluster close together, while the red data points, which belong to sick patients, spread over the rest of the graph. The result of Squash Clustering on this dataset is presented in Figure 1 of [201]. The bottom half of the clustering tree, containing mainly healthy samples, has short branches, while the top half, with mostly samples from sick patients, has many long branches. Thus, Squash Clustering and normal PCA represent almost equivalent information in this case.

5.2 Phylogenetic k -means

The number of tips in the resulting clustering tree obtained through Squash Clustering is equal to the number n of samples that are being clustered. Thus, for datasets with more than a few hundred samples, the clustering result becomes hard to inspect and interpret visually. We propose a variant of k -means clustering [129] to address this problem, which we call *Phylogenetic k -means*. It uses a similar approach as Squash Clustering, but yields a predefined number of k clusters. It is hence able to work with arbitrarily large datasets. Note that we are clustering samples here, instead of sequences [100]. We discuss choosing a reasonable value for k later.

The underlying idea is to assign each of the n samples to one of k cluster centroids, where each centroid represents the average mass distribution of all samples assigned to it. Note that all samples and centroids are of the same data type, namely, they are mass distributions on a fixed RT. It is thus possible to calculate distances between samples and centroids, and to calculate their average mass distributions, as described earlier. Our implementation follows Lloyd’s algorithm [122], as shown in Algorithm 5.1.

By default, we use the k -means++ initialization algorithm [8] to obtain a set of k initial centroids. It works by subsequent random selection of samples to be used as

Algorithm 5.1 Phylogenetic k -means

```

1: initialize  $k$  Centroids
2: while not converged do
3:   assign each Sample to nearest Centroid
4:   update Centroids as mass averages of their Samples
5: return Assignments and Centroids

```

initial centroids, until k centroids have been selected. In each step, the probability of selecting a sample is proportional to its squared distance to the nearest already selected sample. An alternative initialization is to select samples as initial clusters entirely at random. This is however more likely to yield sub-optimal clusterings [96].

Then, each sample is assigned to its nearest centroid, using the KR distance. Lastly, the centroids are updated to represent the average mass distribution of all samples that are currently assigned to them. This iterative process alternates between improving the assignments and the centroids. Thus, the main difference to normal k -means is the use of phylogenetic information: Instead of euclidean distances on vectors, we use the KR distance, and instead of averaging vectors to obtain centroids, we use the average mass distribution.

The process is repeated until it converges, that is, the cluster assignments do not change any more, or until a maximum number of iterations have been executed. The second stopping criterion is added to avoid the super-polynomial worst case running time of k -means, which however almost never occurs in practice [7, 21].

The result of the algorithm is an assignment of each sample to one of the k clusters. As the algorithm relies on the KR distance, it clusters samples with similar relative abundances. The cluster centroids can be visualized as trees with a mass distribution, analogous to how Squash Clustering visualizes inner nodes of the clustering tree. That is, each centroid can be represented as the average mass distribution of the samples that were assigned to it. This allows to inspect the centroids and thus to interpret how the samples were clustered. Examples of this are shown in Figure 5.4.

The key question is how to select an appropriate k that reflects the number of “natural” clusters in the data. There exist various suggestions in the literature [19, 82, 173, 185, 214, 215]; we assessed the Elbow method [214] as explained in Figure 5.6, which is a straight forward method that yielded reasonable results for our test datasets. Additionally, for a quantitative evaluation of the clusterings, we used the k that arose from the number of distinct labels based on the available meta-data for the data. For example, the HMP samples are labeled with 18 distinct body sites, describing where each sample was taken from, c.f. Figure 5.2.

5.2.1 Algorithmic Improvements

In each assignment step of the algorithm, distances from all samples to all centroids are calculated, which has a time complexity of $\mathcal{O}(n \cdot k)$. In order to accelerate this step, we can apply branch binning as introduced in Section 2.5.1. For the BV

dataset, we found that even using just 2 bins per edge does not alter the cluster assignments. Branch binning reduces the number of mass points that have to be accessed in memory during KR distance calculations; however, the costs for tree traversals remain. Thus, we observed a maximal speedup of 75% when using one bin per branch, see Table 5.1 for details.

Furthermore, during the execution of the algorithm, empty clusters can occur, for example, if k is greater than the number of natural clusters in the data. Although this problem did not occur in our tests, we implemented the following solution: First, find the cluster with the highest variance. Then, choose the sample of that cluster that is furthest from its centroid, and assign it to the empty cluster instead. This process is repeated if multiple empty clusters occur at once.

5.3 Imbalance k -means

We further propose *Imbalance k -means*, which is a variant of k -means that makes use of the edge imbalance transformation, and thus also takes the clades of the tree into account. In order to quantify the difference in imbalances between two samples, we use the euclidean distance between their imbalance vectors (that is, rows of the imbalance matrix). This is a suitable distance measure, as the imbalances implicitly capture the tree topology as well as the placement mass distributions. As a consequence, the expensive tree traversals required for Phylogenetic k -means are not necessary here. The algorithm takes the edge imbalance matrix of normalized samples as input, as shown in Figure 2.7(b), and performs a standard euclidean k -means clustering following Lloyd’s algorithm.

This variant of k -means tends to find clusters that are consistent with the results of Edge PCA, as both use the same input data as well as the same distance measure. Furthermore, as the method does not need to calculate KR distances, and thus does not involve tree traversals, it is several orders of magnitude faster than the Phylogenetic k -means. For example, on the HMP dataset, it runs in mere seconds, instead of several hours needed for Phylogenetic k -means; see Section Performance for details.

5.4 Results

We now evaluate our Phylogenetic k -means clustering (which uses edge masses and KR distances) and Imbalance k -means clustering (which uses edge imbalances and euclidean distances) methods in terms of their clustering accuracy. We used the BV as an example of a small dataset to which methods such as Squash Clustering [141] are still applicable, and the HMP dataset to showcase that our methods scale to datasets that are too large for existing methods.

5.4.1 BV Dataset

We again use the re-analyzed BV dataset to test whether our methods work as expected, by comparing them to the existing analysis of the data in [201] and [141].

To this end, we ran both Phylogenetic k -means and Imbalance k -means on the BV dataset. We chose $k := 3$, inspired by the findings of [201]. They distinguish between subjects affected by Bacterial Vaginosis and healthy subjects, and further separate the healthy ones into two categories depending on the dominating clade in the vaginal microbiome, which is either *Lactobacillus iners* or *Lactobacillus crispatus*. Any choice of $k > 3$ would simply result in smaller, more fine-grained clusters, but not change the general findings of these experiments. An evaluation of the number of clusters using the Elbow method is shown in Figure 5.6. We furthermore conducted Squash Clustering and Edge PCA on the dataset, thereby reproducing previous results, in order to allow for a direct comparison between the methods, see Figure 5.1. The figure shows the results of Squash Clustering, Edge PCA, and two alternative dimensionality reduction methods, colorized by the cluster assignments *PKM* of Phylogenetic k -means (in red, green, and blue) and *IKM* of the Imbalance k -means (in purple, orange, and gray), respectively. We use two different color sets for the two methods, in order to make them distinguishable at first glance. Note that the mapping of colors to clusters is arbitrary and depends on the random initialization of the algorithm.

As can be seen in Figure 5.1(a), Squash Clustering as well as Phylogenetic k -means can distinguish healthy subjects from those affected by Bacterial Vaginosis. Healthy subjects constitute the lower part of the cluster tree. They have shorter branches between each other, indicating the smaller KR distance between them, which is a result of the dominance of *Lactobacillus* in healthy subjects. The same clusters are found by Phylogenetic k -means: As it uses the KR distance, it assigns all healthy subjects with short cluster tree branches to one cluster (shown in red). The green and blue clusters are mostly the subjects affected by the disease.

The distinguishing features between the green and the blue cluster are not apparent in the Squash cluster tree. This can however be seen in Figure 5.1(c), which shows a Multidimensional scaling (MDS) plot of the pairwise KR distances between the samples. MDS [62, 110, 137] is a dimensionality reduction method that can be used for visualizing levels of similarity between data points. Given a pairwise distance matrix, it finds an embedding into lower dimensions (in this case, 2 dimensions) that preserves higher dimensional distances as well as possible. Here, the red cluster forms a dense region, which is in agreement with its short branch lengths in the cluster tree. At the same time, the green and blue cluster are separated in the MDS plot, but form a coherent region of low density, indicating that $k := 3$ might be too large with Phylogenetic k -means on this dataset. That is, the actual clustering just distinguishes healthy from sick patients (Figure 5.6).

A similar visualization of the pairwise KR distances is shown in Figure 5.1(d). It is a recalculation of Figure 4 in the preprint [142], which did not appear in the final published version [141]. It is a recalculation of Figure 4 of [142], but can also be found at [143]. The figure shows a standard Principal Components Analysis (PCA) [62, 110] applied to the distance matrix by interpreting it as a data matrix, and was previously used to motivate Edge PCA. However, although it is mathematically sound, the direct application of PCA to a distance matrix lacks a simple interpre-

tation. Again, the red cluster is clearly separated from the rest, but this time, the distinction between the green and the blue cluster is not as apparent.

In Figure 5.1(b), we compare Squash Clustering to Imbalance k -means. Here, the distinction between the two *Lactobacillus* clades can be seen by the purple and orange cluster assignments. The cluster tree also separates those clusters into clades. The separate small group of orange samples above the purple clade is an artifact of the tree ladderization. The diseased subjects are all assigned to the gray cluster, represented by the upper half of the cluster tree. It is apparent that both methods separate the same samples from each other.

Lastly, Figure 5.1(e) compares Imbalance k -means to Edge PCA. The plot is a recalculation of Figure 3 of [142], which also appeared in Figure 6 in [141] and Figure 3 in [201], but colored using our cluster assignments. Because both methods work on edge imbalances, they group the data in the same way, that is, they clearly separate the two healthy groups and the diseased one from each other. Edge PCA forms a plot with three corners, which are colored by the three Imbalance k -means cluster assignments.

In Figure 5.3, we report more details of the comparison of our k -means variants to the dimensionality reduction methods used here. Furthermore, examples visualizations of the cluster centroids are shown in Figure 5.4, which further supports that our methods yield results that are in agreement with existing methods.

5.4.2 HMP Dataset

The HMP dataset is used here as an example to show that our method scales to large datasets. To this end, we used the unconstrained *Bacteria* RT with 1914 taxa as provided by our Automatic Reference Tree method [36]. The tree represents a broad taxonomic range of *Bacteria*, that is, the sequences were not explicitly selected for the HMP dataset, in order to test the robustness of our clustering methods. We then placed the 9192 samples of the HMP dataset with a total of 118 701 818 sequences on that tree, and calculated Phylogenetic and Imbalance k -means on the samples. The freely available meta-data for the HMP dataset labels each sample by the body site where it was taken from. As there are 18 different body site labels, we used $k := 18$. The result is shown in Figure 5.2. Furthermore, in Figure 5.5, we show a clustering of this dataset into $k := 8$ broader body site regions to exemplify the effects of using different values of k . This is further explored by using the Elbow method as shown in Figure 5.6.

Ideally, all samples from one body site would be assigned to the same cluster, hence forming a diagonal on the plot. However, as there are several nearby body sites, which share a large fraction of their microbiome [91], we do not expect a perfect clustering. Furthermore, we used a broad reference tree that might not be able to resolve details in some clades. Nonetheless, the clustering is reasonable, which indicates a robustness against the exact choice of reference taxa, and can thus be used for distinguishing among samples. For example, stool and vaginal samples are clearly clustered. Furthermore, the sites that are on the surface of the body (ear, nose, and arm) also mostly form two blocks of cluster columns.

5.4.3 Performance

The complexity of Phylogenetic k -means is in $\mathcal{O}(k \cdot i \cdot n \cdot e)$, with k clusters, i iterations, and n samples, and e being the number of tree edges, which corresponds to the number of dimensions in standard euclidean k -means. As the centroids are randomly initialized, the number of iterations can vary; in our tests, it was mostly below 100. For the BV dataset with 220 samples and a reference tree with 1590 edges, using $k := 3$, our implementation ran 9 iterations, needing 35 s and 730 MB of main memory on a single core. For the HMP dataset with 9192 samples and 3824 edges, we used $k := 18$, which took 46 iterations and ran in 2.7 h on 16 cores, using 48 GB memory.

In contrast to this, Imbalance k -means does not need to conduct any expensive tree traversals, and instead operates on compact vectors, using euclidean distances. It is hence several orders of magnitude faster than Phylogenetic k -means. For example, using again $k := 18$ for the HMP dataset, the algorithm executed 75 iterations in 2 s. It is thus also applicable to extremely large datasets.

Furthermore, as the KR distance is used in Phylogenetic k -means as well as other methods such as Squash Clustering, our implementation is highly optimized and outperforms the existing implementation in GUPPY [144] by orders of magnitude (see below for details). The KR distance between two samples has a linear computational complexity in both the number of QSSs and the tree size. As a test case, we computed the pairwise distance matrix between sets of samples. Calculating this matrix is quadratic in the number of samples, and is thus expensive for large datasets. For example, in order to calculate the matrix for the BV dataset with 220 samples, GUPPY can only use a single core and required 86 min. Our KR distance implementation in GENESIS is faster and also supports multiple cores. It only needed 90 s on a single core; almost half of this time is used for reading input files. When using 32 cores, the matrix calculation itself only took 8 s. This allows to process larger datasets: The distance matrix of the HMP dataset with 9192 samples placed on a tree with 3824 branches for instance took less than 10 h to calculate using 16 cores in GENESIS. In contrast, GUPPY needed 43 days for this dataset. Lastly, branch binning can be used to achieve additional speedups, as shown in Table 5.1.

5.5 Conclusion and Outlook

Furthermore, we presented adapted variants of the k -means method, which exploit the structure of phylogenetic placement data to identify clusters of environmental samples. The method builds upon ideas such as Squash Clustering and can be applied to substantially larger datasets, as it uses a pre-defined number of clusters. For future exploration, other forms of cluster analyses could be extended to work on phylogenetic placement data, for example, soft k -means clustering [18, 51] or density-based methods [109]. The main challenge when adopting such methods consists in making them phylogeny-aware, that is, to use mass distributions on trees instead of the typical \mathbb{R}^n vectors.

Table 5.1: Effect of Branch Binning on the KR Distance of the HMP Dataset. Here we show the effect of per-branch placement binning on the run-time and on the resulting relative error when calculating the pairwise KR distance matrix between samples, by example of the Human Microbiome Project (HMP) [91, 151] dataset. Because of the size of the dataset (9192 samples) and reference tree (1914 taxa), we executed this evaluation in parallel on 16 cores. The first row shows the baseline performance, that is, without binning. When using fewer bins per branch, the run-time decreases, at the cost of slightly increasing the average relative error. Still, even when compressing the placement masses into only one bin per branch (that is, just using per-branch masses), the average relative error of the KR distances is around 1%, which is acceptable for most applications. However, considering that the run-time savings are not substantially better for a low number of bins, we recommend using a relatively large number of bins, e.g., 32 or more. This is because run-times of KR distance calculations also depend on other effects such as the necessary repeated tree traversals. We also conducted these tests on the BV dataset, were the relative error is even smaller.

Bins	Time (h:mm)	Speedup	Relative Δ
-	9:46	1.00	0.000000
256	6:58	1.40	0.000008
128	6:39	1.47	0.000015
64	6:30	1.50	0.000035
32	6:25	1.52	0.000124
16	6:13	1.57	0.000272
8	6:08	1.59	0.000669
4	6:07	1.60	0.002747
2	6:04	1.61	0.004284
1	5:35	1.75	0.011585

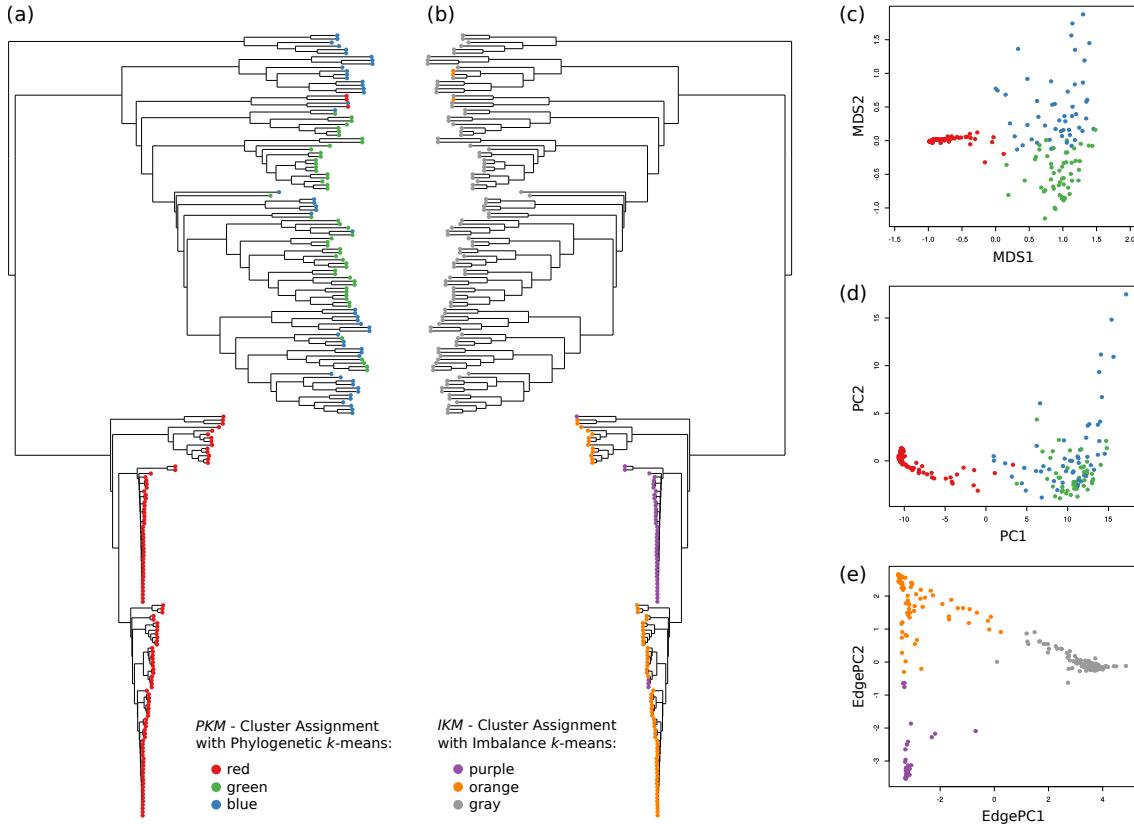


Figure 5.1: Comparison of k -means clustering to Squash Clustering and Edge PCA. We applied our variants of the k -means clustering method to the BV dataset in order to compare them to existing methods. See [201] for details of the dataset and its interpretation. We chose $k := 3$, as this best fits the features of the dataset. For each sample, we obtained two cluster assignments: First, by using Phylogenetic k -means, we obtained the cluster assignment PKM . Second, by using Imbalance k -means, we obtained assignment IKM . In each subfigure, the 220 samples are represented by colored circles: red, green, and blue show the cluster assignments PKM , while purple, orange, and gray show the cluster assignments IKM .

(a) Hierarchical cluster tree of the samples, using Squash Clustering. The tree is a recalculation of Figure 1(A) of [201]. Each leaf represents a sample; branch lengths are KR distances. We added color coding for the samples, using PKM . The lower half of red samples are mostly healthy subjects, while the green and blue upper half are patients affected by Bacterial Vaginosis. (b) The same tree, but annotated by IKM . The tree is flipped horizontally for ease of comparison. The healthy subjects are split into two sub-classes, discriminated by the dominating species in their vaginal microbiome: orange and purple represent samples where *Lactobacillus iners* and *Lactobacillus crispatus* dominate the microbiome, respectively. The patients mostly affected by BV are clustered in gray. (c) Multidimensional scaling using the pairwise KR distance matrix of the samples, and colored by PKM . (d) Principal component analysis applied to the distance matrix by interpreting it as a data matrix. This is a recalculation of Figure 4 of [142], but colored by PKM . (e) Edge PCA applied to the samples, which is a recalculation of Figure 3 of [142], but colored by IKM .

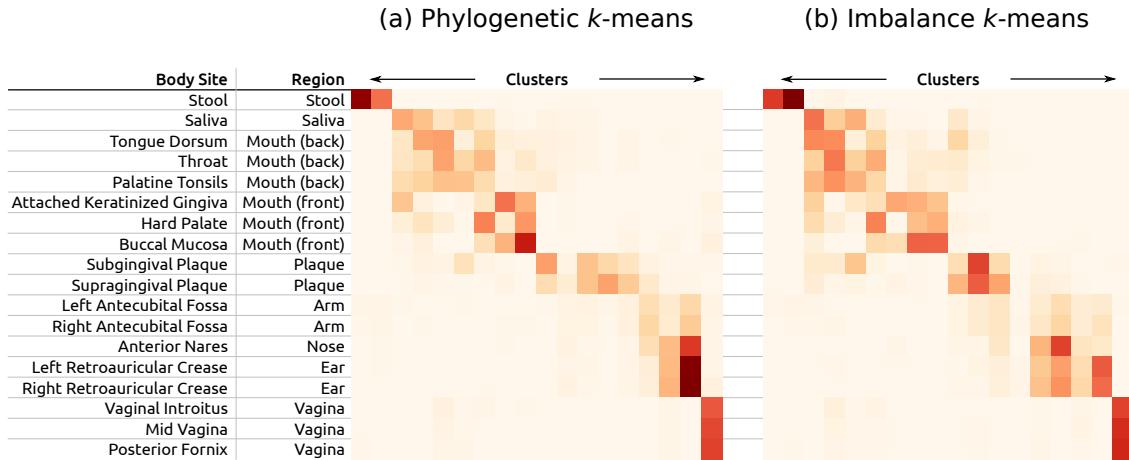


Figure 5.2: k -means cluster assignments of the HMP dataset with $k := 18$. Here, we show the cluster assignments as yielded by Phylogenetic k -means (a) and Imbalance k -means (b) of the HMP dataset. We used $k := 18$, which is the number of body site labels in the dataset, in order to compare the clusterings to this “ground truth”. Each row represents a body site; each column one of the 18 clusters found by the algorithm. The color values indicate how many samples of a body site were assigned to each cluster. Similar body sites are clearly grouped together in coherent blocks, indicated by darker colors. For example, the stool samples were split into two clusters (topmost row), while the three vaginal sites were all put into one cluster (rightmost column). However, the algorithm cannot always distinguish between nearby sites, as can be seen from the fuzziness of the clusters of oral samples. This might be caused by our broad reference tree, and could potentially be resolved by using a tree more specialized for the data/region (not tested). Lastly, the figure also lists how the body site labels were aggregated into regions as used in Figure 5.5. Although the plots of the two k -means variants generally exhibit similar characteristics, there are some differences. For example, the samples from the body surface (ear, nose, arm) form two relatively dense clusters (columns) in (a), whereas those sites are spread across four of five clusters in (b). On the other hand, the mouth samples are more densely clustered in (b).

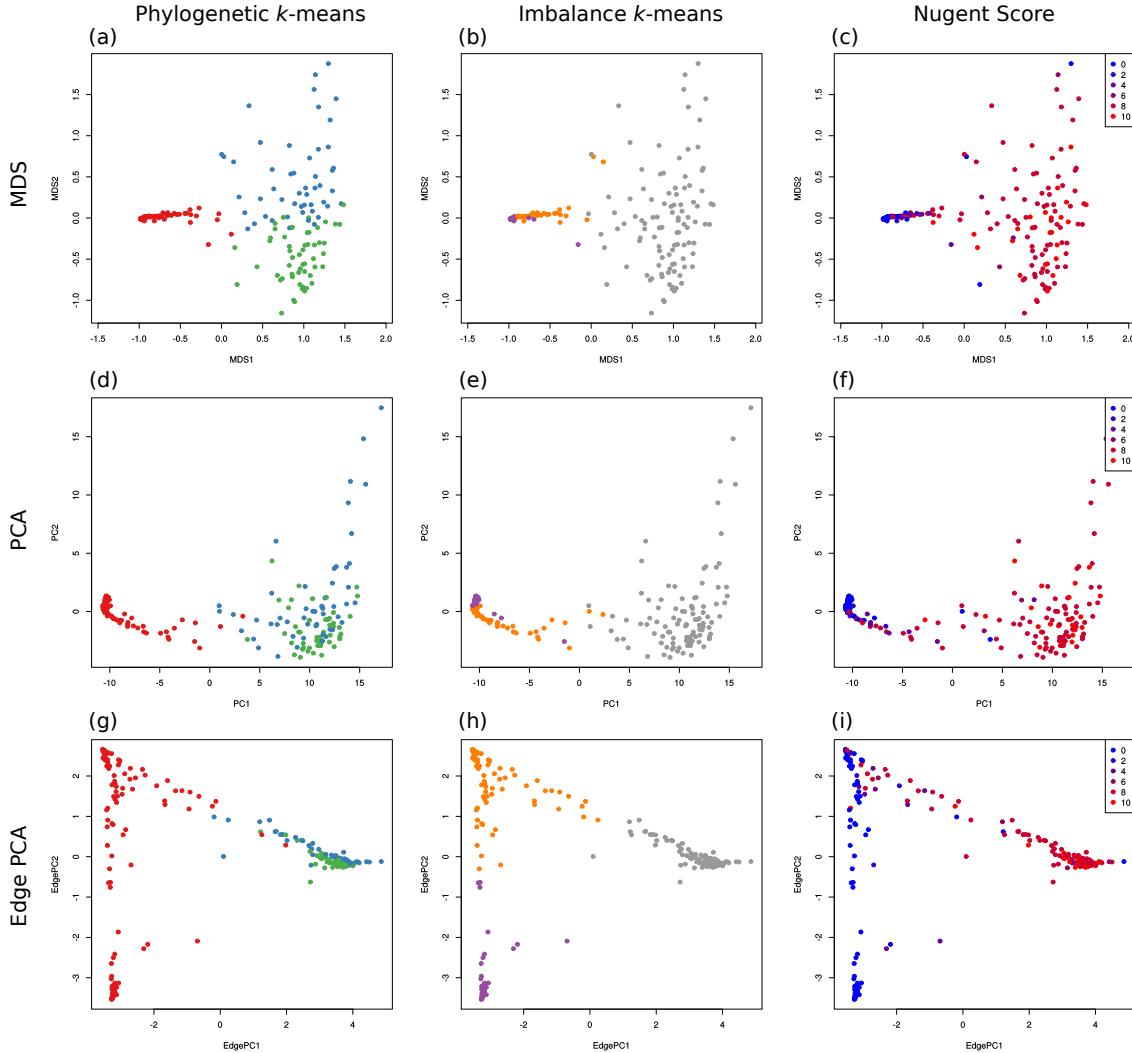


Figure 5.3: Comparison of k -means clustering to MDS, PCA, and Edge PCA. Here, we show and compare the dimensionality reduction methods MDS, PCA, and Edge PCA (one per row). MDS and PCA were calculated on the pairwise KR distance matrix of the BV dataset, Edge PCA was calculated using the placements on the re-inferred RT of the original publication [201]. The plots are colored by the cluster assignments as found by our k -means variants (first two columns), and by the Nugent score of the samples (last column). The Nugent score is included to allow comparison of the health status of patients with the clustering results. (a), (d) and (h) are identical to Figure 5.1(c), (d) and (e) of the main text, respectively. (f) and (i) are recalculations of Figures 4 and 3 of [142], respectively. This figure reveals additional details about how the k -means method works, that is, which samples are assigned to the same cluster. For example, the purple cluster found by Imbalance k -means forms a dense cluster of close-by samples on the left in (b) and (e), which is in accordance with the short branch lengths of this cluster as shown in Figure 5.1(b) of the main text.

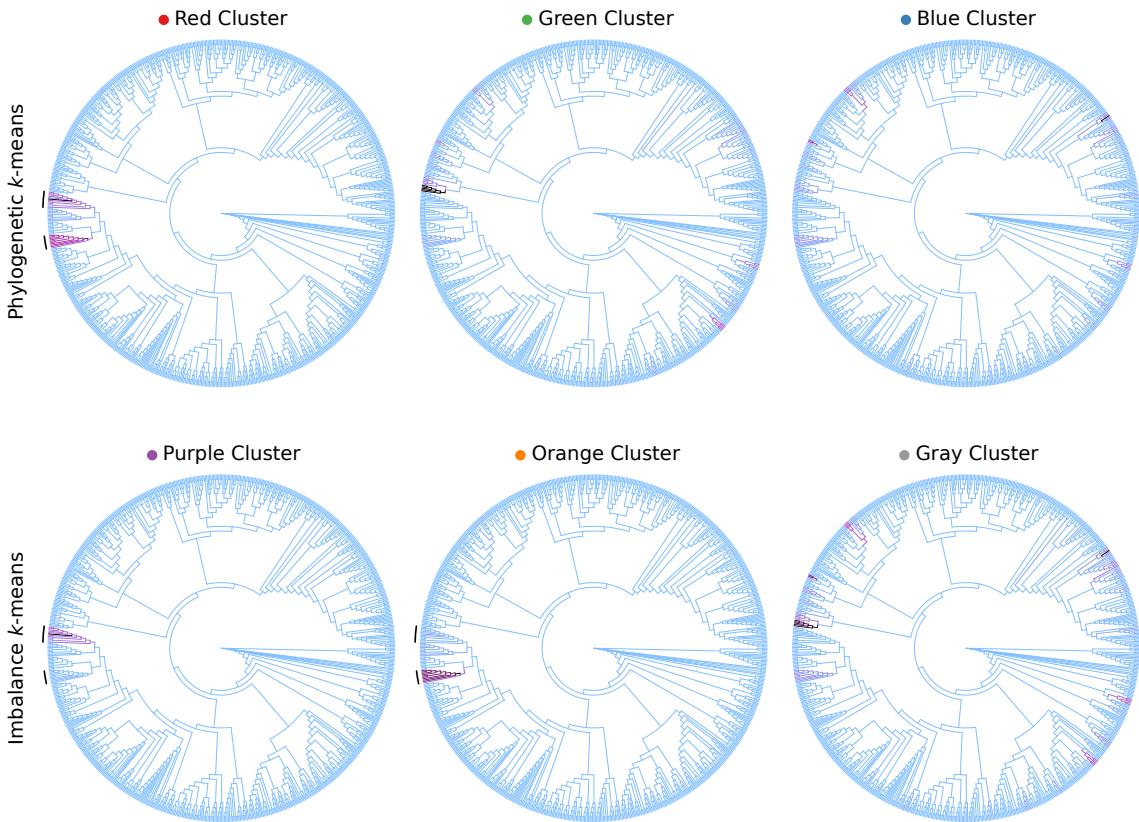


Figure 5.4: Example of k -means cluster centroids visualization. Here we show the cluster centroids as found by our k -means variants using the BV dataset, visualized on the reference tree via color coding. The cluster assignments are the same as in Figure 5.1 of the main text; the first row show the three clusters found by Phylogenetic k -means, the second row the clusters found by Imbalance k -means. Each tree represents one centroid around which the samples were clustered, that is, it shows the combined masses of the samples that were assigned to that cluster. The edges are colored relative to each other, using a linear scaling of light blue (no mass), purple (half of the maximal mass) and black (maximal mass).

As explained in the main text, the samples can be split into three groups: The diseased subjects, which have placement mass in various parts of the tree, as well as two groups of healthy subjects, with placement mass in one of two *Lactobacillus* clades (marked with black arcs on the left of the trees). This grouping is also clearly visible in these trees. The red cluster for example represents all healthy subjects, and thus most of its mass is located in the two *Lactobacillus* clades. The purple and orange clusters on the other hand show a difference in placement mass between those clades. Furthermore, the placement mass of the gray cluster is mostly a combination of the masses of the green and blue cluster, all of which represent diseased subjects. These observations are in accordance with previous findings as explained in the main text.

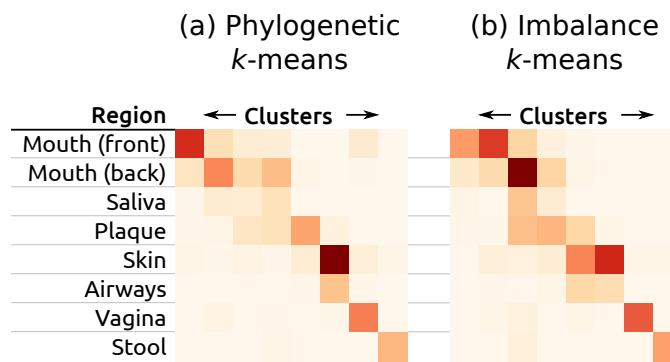


Figure 5.5: Clustering using Phylogenetic k -means on the HMP dataset. k is set to 8, instead of $k := 18$ as in the main text, based on a coarse aggregation of the original body site labels. See Figure 5.1 for the cluster assignment where k is set to the original number of labels; there, we also list how the labels were aggregated. Each row represents a body site; each column one of the 8 clusters. The color values indicate how many samples of a body site were assigned to each cluster. Some of the body sites can be clearly separated, while particularly the samples from the oral region are distributed over different clusters. This might be due to homogeneity of the oral samples.

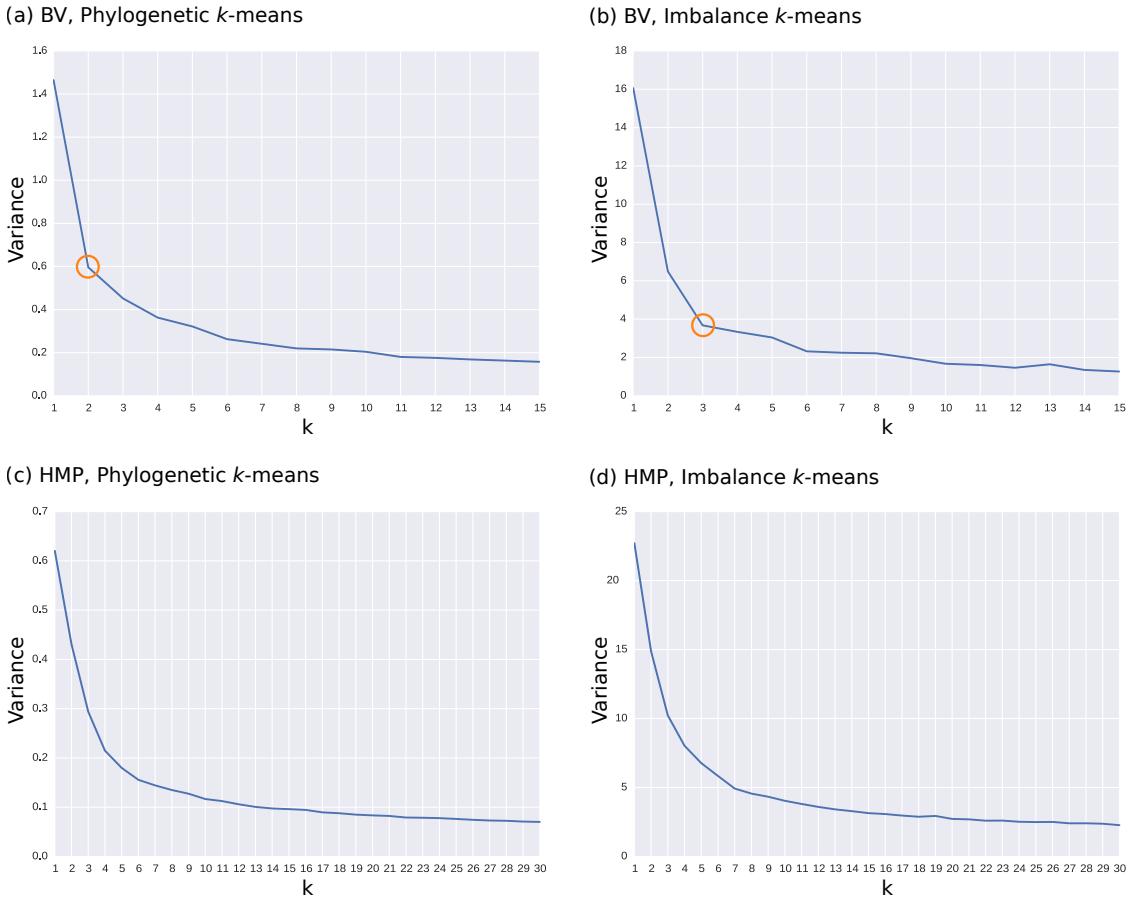


Figure 5.6: Variances of k -means clusters in our test datasets. The figures show the cluster variance, that is, the average squared distance of the samples to their assigned cluster centroids, for different values of k . The first row are clusterings of the BV dataset, the second row of the HMP dataset. They were clustered using Phylogenetic k -means (first column), and Imbalance k -means (second column), respectively. Accordingly, (a) and (c) use the KR distance, while (b) and (d) use the euclidean distance to measure the variance. These plots can be used for the Elbow method in order to find the appropriate number of clusters in a dataset [214]. Low values of k induce a high variance, because many samples exhibit a large distance from their assigned centroid. On the other hand, at a given point, higher values of k only yield a marginal gain by further splitting clusters. Thus, if the data has a natural number of clusters, the corresponding k produces an angle in the plot, called the “elbow”.

For example, (a) and (b) exhibit the elbow at $k := 2$ and 3 , respectively, which are marked with orange circles. These values are consistent with previous findings, for instance, Figure 5.1: There, Phylogenetic k -means splits the samples into a distinct red cluster and the nearby green and blue clusters, while Imbalance k -means yields three separate clusters in purple, orange, and gray.

For the HMP dataset, the elbow is less pronounced. We suspect that this is due to the broad reference tree not being able to adequately resolve fine-grained differences between samples, see Section B for details. Likely candidates for k are $4 - 6$ for (c) and around 7 for (d). These values are consistent with the number of coherent “blocks” of clusters, which can be observed in Figure 5.2. Clearer results for this dataset might be obtained with other methods for finding “good” values for k , although we did not test them here.

6. Conclusion and Outlook

we showed...

in the future... future work

A. Supporting Information

Table A.1: IUPAC notation of nucleobases and ambiguity characters. The table lists the character representations of nucleobases and their combinations (which are used to denote ambiguity) as suggested by the IUPAC Commission [92]. The names and symbols for ambiguity characters are chosen based on bio-chemical properties of the nucleobases. See Section 2.2.4 for more information.

Symbol	Description	Represented Bases			Complement
A	Adenine	A			T
C	Cytosine		C		G
G	Guanine			G	C
T	Thymine			T	A
U	Uracil			U	A
W	Weak	A		T	2
S	Strong		C	G	2
M	aMino	A	C		2
K	Keto			G T	2
R	puRine	A		G	2
Y	pYrimidine		C	T	2
B	not A (B comes after A)		C G	T	3
D	not C (D comes after C)	A		G T	3
H	not G (H comes after G)	A	C		3
V	not T (V comes after T and U)	A	C G		3
N	any Nucleotide (not a gap)	A	C G	T	4
Z	Zero				0

B. Empirical Datasets

This chapter is based on the peer-reviewed publications:

- **Lucas Czech**, Pierre Barbera, and Alexandros Stamatakis. "Methods for Automatic Reference Trees and Multilevel Phylogenetic Placement." *Bioinformatics*, 2018.
- **Lucas Czech** and Alexandros Stamatakis. "Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements." *PLOS One*, 2018.

Contributions: Lucas Czech... Alexandros Stamatakis...

We used three real world datasets to evaluate our methods:

- Bacterial Vaginosis (BV) [201]. This small dataset was already analyzed with phylogenetic placement in the original publication. We used it as an example of an established study to compare our results to. It has 220 samples with a total of 15 060 unique sequences.
- Tara Oceans (TO) [77, 98, 208]. This world-wide sequencing effort of the open oceans provides a rich set of meta-data, such as geographic location, temperature, and salinity. Unfortunately, the sample analysis for creating the official data repository is still ongoing. We thus were only able to use 370 samples with 27 697 007 unique sequences.
- Human Microbiome Project (HMP) [91, 151]. This large data repository intends to characterize the human microbiota. It contains 9192 samples from

different body sites with a total of 63 221 538 unique sequences. There is additional meta-data such as age and medical history, which is available upon special request. We only used the publicly available meta-data.

Details of the datasets (download links, data statistics, data preprocessing, etc.) are provided in **TODO: S1 Text**. At the time of writing, about one year after we initially downloaded the data, the TO repository has grown to 1170 samples, while the HMP even published a second phase and now comprises 23 666 samples of the 16S region. This further emphasizes the need for scalable methods to analyze such data.

These datasets represent a wide range of environments, number of samples, and sequence lengths. We use them to evaluate our methods and to exemplify which method is applicable to what kind of data. To this end, the sequences of the datasets were placed on appropriate phylogenetic RTs as explained in **TODO: S1 Text**, in order to obtain phylogenetic placements that our methods can be applied to. In the following, we present the respective results, and also compare our methods to other methods where applicable. As the amount and type of available meta-data differs for each dataset, we could not apply all methods to all datasets. Lastly, we also report the run-time performance of our methods on these data.

The analyses and figures presented here were conducted on distinct reference alignments and trees. Firstly, for the BV dataset, we used the original set of reference sequences, and re-inferred a tree on them. Secondly, for the TO and HMP datasets, we used our Phylogenetic Automatic (Reference) Tree PhAT method [36] to construct sets of suitable reference sequences from the SILVA database [178, 235]. We used the 90% threshold consensus sequences; see [36] for details.

For all analyses, we used the following software setup: Unconstrained maximum likelihood trees were inferred using RAxML v8.2.8 [203]. For aligning reads against reference alignments and reference trees, we used a custom MPI wrapper for PA-PARA 2.0 [14, 15], which is available at [13]. We then applied the `chunkify` procedure as explained in [36] to split the sequences into chunks of unique sequences prior to conducting the phylogenetic placement, in order to minimize processing time. Phylogenetic placement was conducted using EPA-NG [10], which is a faster and more scalable phylogenetic placement implementation than RAxML-EPA [16] and PPLACER [144]. Lastly, given the per-chunk placement files produced by EPA-NG, we executed the `unchunkify` procedure of [36] to obtain per-sample placement files. These subsequently served as the input data for the methods presented here.

B.1 Bacterial Vaginosis

We used the Bacterial Vaginosis dataset [201] in order to compare our novel methods to existing ones such as Edge PCA and Squash Clustering [61, 141]. The dataset contains metabarcoding sequences of the vaginal microbiome of 220 women, and was kindly provided by Sujatha Srinivasan. This small dataset with a total of 426 612 query sequences, thereof 15 060 unique, was already analyzed with phylogenetic placement methods in the original publication. We re-inferred the reference

Table B.1: Overview of the dataset dimensions. The “Samples” columns show how many metagenomic samples there were in the originally downloaded data and how many of those we actually used for our experiments after filtering out spurious ones. The columns “Filtered Sample Sizes” show how many sequences each of the filtered samples has. The “Sequence Count” columns show the total number of sequences in the filtered samples, and how many of them are unique. The columns “Sequence Length” show statistics of the length of the sequences. Lastly, the “Chunks” column shows into how many chunks of size 50 000 the samples were distributed.

Dataset	Samples		Filtered Sample Sizes			Filtered Sequence Count		Filtered Min
	Source	Filtered	Min	Max	Avg	Total	Unique	
Bacterial Vaginosis		220				426,612	15,060	
Neotropical Soils		154				50,118,536	10,567,804	
Tara Oceans		370				49,023,231	27,697,007	
Human Microbiome	9,815	9,194				118,701,818	63,221,538	

tree of the original publication using the original alignment, which contains 797 reference sequences specifically selected to represent the vaginal microbiome. As the query sequences were already prepared, no further preprocessing was applied prior to phylogenetic placement. The available per-sample quantitative meta-data for this dataset comprises the Nugent score [165], the value of Amsel’s criteria [4], and the vaginal pH value. We used all three meta-data types in our analyses.

TODO: from art:

For testing the accuracy of our unconstrained *Bacteria* tree on real data, we used a vaginal microbiome dataset of 220 women [201], which was provided by Sujatha Srinivasan. See Figure 3.8 for the respective results. This small dataset with a total of 426 612 query sequences, thereof 15 060 unique, was already analyzed with phylogenetic placement methods in the original publication. We used it as an example of a well-designed study to asses our results using an PhAT as reference tree. In addition to the *Bacteria* PhAT, we re-inferred the reference tree of the original publication using their alignment, again using RAxML 8.2.8 [203]. The query sequences of the dataset were then aligned to our reference tree and alignment, as well as to the reference alignment of the original publication and our re-inferred tree. For aligning, we used a custom MPI wrapper of PAPARA 2.0 [14, 15], which is available at [13]. Finally, the query sequences were placed on these trees using EPA-NG [10], and the analyses were subsequently performed as explained in Figure 3.8.

B.2 Tara Oceans

The Tara Oceans (TO) dataset [77, 98, 208] that we used here contains amplicon sequences of protists, and is available at <https://www.ebi.ac.uk/ena/data/view/>

PRJEB6610. At the time of download, there were 370 samples available with a total of 49 023 231 sequences. As the available data are raw **fastq** files, we followed [132] to generate cleaned per-sample **fasta** files. For this, we used our tool PEAR [239] to merge the paired-end reads; CUTADAPT [139] for trimming tags as well as forward and reverse primers; and VSEARCH [184] for filtering erroneous sequences and generating per-sample **fasta** files. We filtered out sequences below 95 bps and above 150 bps, to remove potentially erroneous sequences. No further preprocessing (such as chimera detection) was applied. This resulted in a total of 48 036 019 sequences, thereof 27 697 007 unique. The sequences were then used for phylogenetic placement as explained above. We placed the sequences on the unconstrained *Eukaryota* reference tree obtained via our PhAT method [36], which comprises 2059 taxa, thereof 1795 eukaryotic sequences. The remaining 264 taxa are *Archaea* and *Bacteria*, and were included as a broad outgroup. The TO dataset has a rich variety of per-sample meta-data features; in the context of this paper, we mainly focus on quantitative features such as temperature, salinity, as well as oxygen, nitrate and chlorophyll content of the water. Furthermore, each sample has meta-data features indicating the date, longitude and latitude, depth, etc. of the sampling location. This data might be interesting for further correlation analyses based on geographical information. We did not use them here, as for example longitude and latitude would require a more involved method that also accounts for, e.g., ocean currents. Furthermore, geographical coordinates yield pairwise distances between samples, which are not readily usable with our correlation analysis. Lastly, in order to use features such as the date, that is, in order to analyze samples over time, the same sampling locations would need to be visited at different times during the year, which is not the case for the Tara Oceans expedition.

B.3 Human Microbiome Project

We used the Human Microbiome Project (HMP) dataset [91, 151] for testing the scalability of our methods. In particular, we used the “HM16STR” data of the initial phase “HMP1”, which are available from <http://www.hmpdacc.org/hmp/>. The dataset consists of trimmed 16S rRNA sequences of the V1V3, V3V5, and V6V9 regions. The data are further divided into a “by_sample” set and a “healthy” set, which we merged in order to obtain one large dataset, with a total of 9811 samples. We then removed 10 samples that were larger than 70 MB as well as 605 samples that had fewer than 1500 sequences, because we considered them as defective or unreliable outliers. Finally, we also removed 2 samples that did not have a valid body site label assigned to them. This resulted in a set of 9192 samples containing a total of 118 702 967 sequences with an average length of 413 bps. From these samples, sequences with a length of less than 150 bps as well as sequences longer than 540 bps were removed, as we considered them potentially erroneous. No further preprocessing (such as chimera detection) was applied. This resulted in a total of 116 520 289 sequences, of which 63 221 538 were unique. We then used the unconstrained *Bacteria* tree of our PhAT method [36] for phylogenetic placement. The tree comprises 1914 taxa, thereof 1797 bacterial sequences. The remaining 117 taxa are *Archaea*.

and *Eukaryota*, and were included as a broad outgroup. Each sample is labeled with one of 18 human body site locations where it was sampled. This is the only publicly available meta-data feature.

TODO: from art:

We used the Human Microbiome Project (HMP) dataset [91, 151] for further testing our methods (see Figure 3.9). In particular, we used the “HM16STR” data of their initial phase “HMP1”, which are available from <http://www.hmpdacc.org/hmp/>. The dataset consists of trimmed 16S rRNA sequences of the V1V3, V3V5, and V6V9 regions. Each sample of the dataset is labeled with the human body site where it was obtained. See Table B.2 for an overview of those labels. The data are further divided into a “by_sample” set and a “healthy” set, which we merged in order to obtain one large dataset, with a total of 9811 samples. We then removed 10 samples that were larger than 70 MB as well as 605 samples that had fewer than 1500 sequences, because we considered them as outliers, and finally 2 samples that did not have a valid body site label assigned to them. This resulted in a set of 9192 samples containing a total of 118 702 967 sequences with an average length of 413 bps. From these samples, sequences with a length less than 150 bps as well as sequences longer than 540 bps were removed. No further preprocessing (e.g., chimera detection) was applied. This resulted in a total of 116 520 289 sequences, of which 63 221 538 were unique. These were split into 1265 chunks of size 50 000 each, which were subsequently aligned to and placed on the unconstrained *Bacteria* tree with 2059 tips using the steps explained above. The chunk placements were then transformed again into per-sample placement files, before finally running the steps explained in Figure 3.9.

Table B.2: HMP Dataset Overview. The table lists the 19 body site labels used by the Human Microbiome Project (HMP) [91, 151]. We used this dataset to evaluate the applicability of typical analysis methods for phylogenetic placement using our PhATs, see Section B and Figure 3.9 for details. In order to simplify the visualization in Figure 3.9, we summarized some of the labels into eight location regions, as shown in the second column. The last column lists how many samples from each body site were used in our evaluation.

Body Site	Region	Samples
Tongue Dorsum	Mouth (back)	610
Palatine Tonsils	Mouth (back)	599
Throat	Mouth (back)	638
Attached Keratinized Gingiva	Mouth (front)	600
Hard Palate	Mouth (front)	566
Buccal Mucosa	Mouth (front)	597
Saliva	Saliva	529
Supragingival Plaque	Plaque	608
Subgingival Plaque	Plaque	595
Anterior Nares	Airways	541
Left Retroauricular Crease	Skin	596
Right Retroauricular Crease	Skin	604
Left Antecubital Fossa	Skin	290
Right Antecubital Fossa	Skin	328
Stool	Stool	600
Vaginal Introitus	Vagina	292
Mid Vagina	Vagina	298
Posterior Fornix	Vagina	301
Sum		9192

C. Pipeline and Implementation

This chapter is based on the peer-reviewed publications:

- **Lucas Czech**, Pierre Barbera, and Alexandros Stamatakis. "Methods for Automatic Reference Trees and Multilevel Phylogenetic Placement." *Bioinformatics*, 2018.
- **Lucas Czech** and Alexandros Stamatakis. "Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements." *PLOS One*, 2018.

Contributions: Lucas Czech... Alexandros Stamatakis...

TODO: add genesis and gappa logos. because we can.

The methods described here are implemented in our tool GAPPA, which is freely available under GPLv3 at <http://github.com/lczech/gappa>. GAPPA internally uses our C++ library GENESIS, which offers functionality for working with phylogenies and phylogenetic placement data, and also contains methods to work with taxonomies, sequences and many other data types. GENESIS is also freely available under GPLv3 at <http://github.com/lczech/genesis>.

GAPPA offers a command line interface for conducting typical tasks when working with phylogenetic placements. The methods that we described here are implemented via the following sub-commands:

- **dispersion:** The command takes a set of jplace files (called samples), and calculates and visualizes the Edge Dispersion per edge of the reference tree.

- **correlation**: The command takes a set of `jplace` samples, as well as a table containing metadata features for each sample. It then calculates and visualizes the Edge Correlation with the metadata features per edge of the reference tree.
- **phylogenetic-kmeans** and **imbalance-kmeans**: Performs k -means clustering of a set of `jplace` files according to our methods.
- **squash** and **edgepca**: Reimplementations of the two existing methods [61, 141].

These are the GAPPA commands that are relevant for this paper. The tool also offers additional commands that are useful for phylogenetic placement data, such as visualization or filtering. At the time of writing this manuscript, GAPPA is under active development, with more functions planned in the near future. Lastly, we provide prototype implementations, scripts, data, and other tools used for the tests and figures in this paper at <http://github.com/lczech/placement-methods-paper>.

TODO: ART:

An implementation of the methods described here is freely available in our tool GAPPA, which is published under GPLv3 at <http://github.com/lczech/gappa>. GAPPA is based on our C++ library GENESIS, which offers functionality concerning phylogenies and phylogenetic placement data, but also has functions to work with sequences, taxonomies and many other data types. GENESIS is also published under GPLv3 and is available at <http://github.com/lczech/genesis>.

GAPPA offers a command line interface for typical tasks of working with phylogenetic placements. The methods described in this paper are implemented via four sub-commands:

- **phat**: Phylogenetic Automatic (Reference) Tree method. The command expects a taxonomy file and a sequence file of a sequence database, e.g., SILVA [178, 235], as well as the target number of consensus sequences to be generated for the intended phylogeny. The result is a `fasta` file with consensus sequences representing taxonomic clades. The command can be further customized, e.g., by changing the consensus sequence method, using only a specified subclade of the taxonomy for running the algorithm, as well as several detail settings for the method. It can also output additional info files that allow to inspect details of the calculations, like the number of sequences and their entropy per clade.
- **extract**: Extract/collect placements in specific sub-clades of the tree. The command performs the main step of the multilevel placement approach. Its input is a set of `jplace` files containing placements on the backbone tree, as well as a file listing the clade name that each taxon of the backbone tree belongs to. For each clade, it then writes a new `jplace` file, containing all queries that were placed in that clade with more than a customizable threshold of their

placement mass.

Furthermore, if provided with the sequence files that were used to make the input `jplace` files, the corresponding sequence of each query are also written to `fasta` files per clade. Thus, a per-clade collection of sequences is created, where each result file contains the sequences that were placed in this clade of the backbone tree. These can then be used for the second level placement on separate clade-specific trees.

- **chunkify:** Split a set of `fasta` files into chunks of equal size, and write abundance maps. The command re-names the sequences using a configurable hash function (MD5, SHA1 or SHA256), and de-duplicates across all input sequences. Its output are chunk files of sequences, as well as an abundance map file for each input sequences file. The sequence chunk files can then be used to perform phylogenetic placement to obtain per-chunk `jplace` files.
- **unchunkify:** Take the per-chunk `jplace` files as well as the abundance map files, and generate a `jplace` for each original sequence file, including the correct abundances. This command is the second step of the `chunkify` command, and reverts its effect, so that the resulting `jplace` files are as if they were created using the original sequence files.
- **assign:** Perform taxonomic assignment using phylogenetic placements. While this is not the main focus of this work, we briefly introduce this method here. The command uses a taxonomic labeling of the tips of the reference tree to annotate all inner branches of the tree with the longest common taxonomic label for the induced subtree of the inner branch, in analogy to SATIVA [107]. Then, each query sequence in the provided `jplace` files is taxonomically assigned according to the labels of the branches where it does have placement mass. This can subsequently either be used for taxonomic assignment of the query sequences themselves, or to obtain a taxonomic profile of one or more samples.

These are the commands of GAPPA relevant for this paper, but it also offers more commands that are useful when working with phylogenetic placements. For details on the commands, and additional potentially useful commands, see the GAPPA documentation at <https://github.com/lczech/gappa/wiki>. At the time of writing, it is under active development, and more functions are planned for the near future. Furthermore, we provide prototype implementations, scripts, data and other tools used for the tests and figures in this paper at <http://github.com/lczech/placement-methods-paper>.

D. List of Publications

unieuk: [17] art: [36] pppp: [37]

1. **L. Czech**, S. Berger, D. Krompaß, J. Zhang, P. Kapli, P. Pavlidis and A. Stamatakis. Evolutionary Placement of Short Reads - Methods, Applications, and Visualization. Poster at EMBO/EMBL Symposium: A New Age of Discovery for Aquatic Microeukaryotes, Heidelberg, Germany, January 2016, and at Hellenic Bioinformatics Conference (HBio) 2016, Thessaloniki, Greece, November 2016.
2. F. Mahé, C. de Vargas, D. Bass, **L. Czech**, A. Stamatakis, E. Lara, D. Singer, J. Mayor, J. Bunge, S. Sernaker, T. Siemensmeyer, I. Trautmann, S. Romac, C. Berney, A. Kozlov, E. A. D. Mitchell, C. V. W. Seppey, E. Egge, G. Lentendu, R. Wirth, G. Trueba, and M. Dunthorn. Parasites dominate hyperdiverse soil protist communities in Neotropical rainforests. *Nature Ecology & Evolution*, vol. 1, no. 4, p. 0091, 2017.
3. **L. Czech**, J. Huerta-Cepas, and A. Stamatakis. A Critical Review on the Use of Support Values in Tree Viewers and Bioinformatics Toolkits. *Molecular Biology and Evolution*, vol. 17, no. 4, pp. 383–384, 2017.
4. T. Flouri, J. Zhang, **L. Czech**, K. Kobert, A. Stamatakis. An efficient approach to merging paired-end reads and the incorporation of uncertainties. Chapter in Algorithms for Next-Generation Sequencing Data: Techniques, Approaches and Applications. 1st ed., M. Elloumi, Ed. Springer International Publishing AG, 2017, pp. 299–326.
5. P. Barbera, A. Kozlov, T. Flouri, D. Darriba, **L. Czech** and A. Stamatakis. Massively Parallel Evolutionary Placement of Genetic Sequences. Poster at ISC 2017 PhD Symposium, Frankfurt am Main, Germany, June 2017.

6. C. Berney, A. Ciuprina, S. Bender, J. Brodie, V. Edgcomb, E. Kim, J. Rajan, L. W. Parfrey, S. Adl, S. Audic, D. Bass, D. A. Caron, G. Cochrane, **L. Czech**, M. Dunthorn, S. Geisen, F. O. Glöckner, F. Mahé, C. Quast, J. Z. Kaye, A. G. B. Simpson, A. Stamatakis, J. del Campo, P. Yilmaz, and C. de Vargas. UniEuk : Time to Speak a Common Language in Protistology! *Journal of Eukaryotic Microbiology*, vol. 38, no. 1, pp. 42–49, 2017.
7. X. Zhou, S. Lutteropp, **L. Czech**, A. Stamatakis, M. von Looz, A. Rokas. Quartet-based computations of internode certainty provide accurate and robust measures of phylogenetic incongruence. *bioRxiv*, 168526, 2017.
8. P. Barbera, A. Kozlov, **L. Czech**, B. Morel, A. Stamatakis. EPA-ng: Massively Parallel Evolutionary Placement of Genetic Sequences. *bioRxiv*, 291658, 2018.
9. D. Bass, **L.Czech**, B. Williams, C. Berney, M. Dunthorn, F. Mahe, G. Torruella, G. Stentiford and T. Williams. Clarifying the Relationships between Microsporidia and Cryptomycota. *Journal of Eukaryotic Microbiology*, 2018.
10. **L. Czech**, A. Stamatakis. Methods for Automatic Reference Trees and Multilevel Phylogenetic Placement. *bioRxiv*, 299792, 2018.
11. **L. Czech**, A. Stamatakis. Scalable Methods for Post-Processing, Visualizing, and Analyzing Phylogenetic Placements. *bioRxiv*, 346353, 2018.
12. TODO: 1KITE
13. TODO: long reads
14. TODO: swarm 3?
15. TODO: genesis and gappa

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