1 What is Molecular Phylogeny?

Most probably, all life existing today on earth shares a common ancestry billions of years back in the past. A set of indispensable genes necessary for maintenance of basic cell functions were passed on from the unknown common ancestor to its extant descendants by asexual and/or sexual reproduction. During the course of evolution, the genes, the numbers of genes, their functions and the sizes of the genomes (i.e. the total DNA content of a cell) became modified. If genes originate from a common ancestor gene and fulfill the same function in a cell, they are said to be homologous. The degree of divergence between homologous genes is considered a measure for their relatedness (and also for the relatedness of the organisms).

In molecular phylogeny, the relationships among, usually extant, organisms are examined by comparing homologous DNA or protein sequences (i.e. the gene products). The relationships are displayed as trees with branch (or edge) lengths reflecting the degrees of genetic divergence. Each branch tip represents an extant sequence; the internal nodes or vertices represent unknown ancestors to the terminal nodes. The branching pattern and branch lengths describe the evolutionary pathways leading to the sequences at the terminal nodes. Clusters of terminal branches connected to a common ancestor are termed clades.

The construction of phylogenetic trees has been shown to be a NP-hard problem; the number of possible trees increases exponentially with the number of DNA or protein sequences included in the phylogenetic analyses [1]. Due to the large amount of data and the complexity of the task, phylogenetic trees cannot be inferred without help of computers.

Numerous studies addressing the problems of molecular phylogenetic analyses methods in theory or practice have been published. First publications about phylogenetic methods date back into the 60s. The methods and evolutionary models were refined in the course of time, but problems still remain. The cited references in this review represent only few examples from a vast amount of
literature. Also only some of the mostly used methods in molecular phylogeny are presented.

For digging into the mathematics behind the phylogenetic analyses methods introduced below, one may start with Joe Felsenstein’s book \([2]\).

## 2 Phylogenetic Analyses Methods

DNA sequences are based on a four-letter-code representing the four nucleotides (A for adenin, C for cytosin, G for guanin, T for thymin), whereas protein sequences are based on a twenty-letter-code representing the twenty different amino acids. Prior to the phylogenetic analyses, an alignment of the sequences has to be assembled (the single sequence is also termed a “taxon", because it represents a species, genus, individual or strain). If sequences of homologous genes e.g. show differences in lengths due to insertions or deletions, gaps have to be inserted to place functionally corresponding positions in the same vertical column of the alignment (Fig. 1). Non-alignable regions such as insertions of several nucleotides need to be excluded from the phylogenetic analyses. Improperly aligned sequences or inclusions of non-alignable regions in the phylogenetic analyses may result in artefactual phylogenetic trees.

In most standard methods for inferring phylogenetic trees, an optimality criterion and a tree search algorithm have to be chosen. The optimality criterion is used to determine the best among the considered trees by defining a type of “scoring" system. Optimality criteria are e.g. maximum parsimony, distance matrix or maximum likelihood \([2]\).

In unweighted maximum parsimony, each mutation from one nucleotide or amino acid to another, e.g. from a C to a G, costs one “penalty" point. All point mutations are considered equally likely. The mutations along a given tree are summed up and the best tree or maximum parsimony tree is the one with

| Seq 1 | ATCACAGCAATGTGGCAGC--CTCTCT--TTTCTGGGGAGGA----GCG |
| Seq 2 | ACCAAGGCGATGTGGCAGC--CTCTCT--TTAGAGAGGA----GCG |
| Seq 3 | ACCAAGGCGATGTGGCAGC--CTCTCT--TTAGAGAGGA----GCG |
| Seq 4 | ACCAAGGCGATGTGGCAGC--CTCTCT--CTAGAGAGGA----GCG |
| Seq 5 | GCCAATACCGGCTTGGCAGGCTCTCTCTGTGGACATGGAGAGGAAGAACA |
| Seq 6 | GCCTAAACAGTGTGCAAGC--CTCTCT--TTCCAGAGGA----GCG |
| Seq 7 | GCCTAAACAGTGTGCAAGC--CTCTCT--CTTGAGAGGA----GCG |
| Seq 8 | ACCAGACTGTGTGGCAGGCTCTCT--TTCCAGAGGA----GCG |

Fig. 1: Excerpt from an alignment of nuclear ITS2 sequences. The ITS2 or internal transcribed spacer 2 expands between two RNA coding genes of the ribosomal operon. The ribosomal operon is transcribed in one piece. The two internal transcribed spacers between the RNA coding regions fold up in a specific way and are excised. Since the two ITS regions solely function as spacers, they are under low selective pressure and, thus, display high mutation rates. The example alignment shows ITS2 regions of closely related organisms belonging to one genus. The sequences are oriented in horizontal direction, whereas functionally corresponding positions are arranged in columns. Several gaps had to be inserted due to insertions of nucleotides in the sequences 1 and 5.
the lowest sum of penalty points. Unweighted maximum parsimony uses integer values and often several to many equally parsimonious trees are found.

In distance analyses, the sequences are pair-wise compared. Their genetic divergences are transformed into distance values and listed in a triangular distance matrix. Whereas maximum parsimony treats all mutations as equally likely, the computation of distance matrices allows for different mutation rates and other variations of parameters (i.e. evolutionary models, see chapter below). To infer trees from a distance matrix, usually the neighbor-joining algorithm is used (see below).

Maximum likelihood is a probabilistic and the computationally most costly method (Fig. 2). It searches for the tree that optimizes the probability of observing the data. The likelihood of a tree is expressed as negative natural logarithm. The maximum likelihood method also allows for different evolutionary models, but differs from distance matrix methods in that it uses discrete characters and may result in more than one optimal tree (however, rarely more than two).

The numbers of sequences used to infer phylogenetic trees in biological research projects almost always prohibited exhaustive searches of the complete tree space due to limitations of computation time. Thus, maximum parsimony or maximum likelihood were usually combined with heuristic tree search algorithms. For a heuristic search a first tree is generated e.g. by adding the sequences step-by-step to the growing tree. This first tree is then subjected to local and/or global rearrangements by swapping internal branches or cutting the tree into pieces and rejoining the parts in different places. This procedure is supposed to overcome potential local optima and to find the global optimum. The construction of a tree by neighbor-joining, the preferred method used with distance matrices, starts with a star-like tree. The pair of sequences with the lowest genetic divergence is joined (i.e. they are said to be neighbors) and the distance matrix recalculated. These steps are repeated with the next closest related sequences or clusters of sequences until the tree is completely resolved.

In Bayesian analyses, posterior probabilities for trees and evolutionary parameters are calculated using the Bayes theorem. With the Bayes formula the posterior probability of a tree given the data is calculated using prior probabilities of the data and the tree, and the likelihood of a tree. Since it is impossible to calculate all trees and evolutionary parameters from the space of the joint posterior probability distribution, samples are drawn using Metropolis-coupled Markov chain Monte Carlo simulations. This means, at start of a Bayesian analysis, several chains are initialized to search for the global optimum in the space of the joint posterior probability distribution. Once initialized, the chains cross the space for several hundredthousands to millions of generations by slightly modifying the parameters (tree topology, branch lengths, evolutionary model parameters). Trees and evolutionary model parameters are sampled only from the cold chain; the other so-called heated chains traverse the space more easily and exchange their status data from time to time with the cold chain. By doing so, the heated chains help the cold chain to reach the global optimum, which comprises a set of the best trees and evolutionary parameters. The presumed global optimum is found when the likelihoods of the trees sampled from the cold chain reach stationarity.

The phylogenetic trees inferred by the above mentioned methods are usually bifurcating trees. They may be rooted or unrooted. In rooted trees, the closest related sistergroup is used to define the direction of evolution in the
Fig. 2: Computation of the likelihood of a tree. To obtain the overall likelihood value of a tree, for each position of the alignment the probabilities of all possible combinations of ancestral character states are computed. The site-wise likelihood comprises the sum of all probabilities. The site-wise log likelihoods are then multiplied and result in the log likelihood of a given tree.
sequences. To e.g. examine the relationships among chimpanzee, gorilla and man, the orangutan would be the appropriate outgroup. Unrooted trees are like looking onto the treetop from above without knowing where the stem is. In unrooted trees it is not possible to tell, where evolution started and in which direction the sequences evolve.

3 Models of Molecular Evolution

In addition to exponentially growing numbers of possible trees, phylogenetic analyses are further complicated by the fact that substitution rates of nucleotides or amino acids may vary. Evolutionary models are an attempt to approximate the complexity of molecular evolution as close as possible.

The proportions of the four nucleotides in a DNA sequence may differ from gene to gene and, thus, need to be considered in phylogenetic analyses (base frequencies). To account for differing substitution rates for the six types of point mutations, a substitution rate matrix is used (Fig. 3A). However, depending in the positions in the alignment, these rates may be higher or lower. Some positions are highly conserved and do not change at all. Others evolve at differing rates (Fig. 3B). Both parameters, the proportion of invariable sites and site-specific rate variation, modelled as a gamma-distribution (Fig. 3C), belong to the among-site substitution rate variation and can be explained by functional constraints on the gene products.

For most data sets used in biological studies, it is impossible to infer phylogenetic trees in a reasonable time by optimizing all likelihood parameters at once during a maximum likelihood analysis, i.e. tree topology, branch lengths of the trees, base frequencies, substitution rate matrix, proportion of invariable sites and continuously gamma-distributed among-site rate variation. An often practised approach consisted of determining first the parameters of the evolutionary model fitting best the data \[4\]. To find the appropriate evolutionary model, a tree is inferred with a fast method (usually distance matrix with neighbor-joining) and the likelihood values for this tree are calculated for each available evolutionary model. The model fitting best the data is then chosen by e.g. hierarchical likelihood ratio tests (hLRT) or by the Akaike information criterion (AIC). Also, a discrete instead of a continuous gamma-distributed among-site rate variation is used to reduce computation times (Fig. 3C). Thus, during heuristic tree search only tree topology and branch lengths need to be optimized, whereas the evolutionary model parameters have been already estimated from the data set using an approximate tree topology prior to the heuristic tree search.

An additional evolutionary parameter, the covarion/covariotide model takes lineage-specific evolutionary rates into consideration, i.e. complete sequences may evolve faster than others. The covarion/covariotide model, however, until today was only implemented in Bayesian phylogenetic analyses programmes.

Protein coding DNA sequences are in vivo first transcribed into messenger RNA, then translated into a protein consisting of a string of amino acids (Fig. 3B). The function of the protein is determined by folding up into tertiary and quaternary structures and by amino acids with specific chemical properties in specific positions. Maximum likelihood analyses of DNA sequences are quite time intensive. Maximum likelihood analyses with 20 character states for the
Fig. 3: Substitution rate matrices and among-site rate variation. Fig. 3A. Examples for substitution rate matrices. To the left, the most complex type implemented in phylogeny software programmes, the general time reversible model (GTR) with six different substitution rates. To the right, a modified GTR model, the Tamura-Nei model with three different mutation rates. Fig. 3A. Among-site rate variation in RNA and protein coding DNA. Sites with high mutation rates are usually found in loop regions of RNA secondary structure, whereas helices are more conserved (left). In protein coding DNA, the third position of the codons is usually the most variable. The degenerate code allows for several codons to represent the same amino acid. In this example, codons for the amino acids serine, arginine and valine are shown. Between DNA and protein, a transcription step to messenger RNA is necessary. Bold face, positions with higher mutation rates. Fig. 3C. Modelling the among-site rate variation using a gamma distribution. Examples for continuous gamma distribution with different shape parameters to the left and a discrete gamma distribution with seven rate categories to the right. The discrete gamma distribution approximates a continuous gamma distribution with a shape parameter $\alpha$ of 1.
amino acids are even more time-consuming. Thus, in protein phylogenies, substitution rate matrices were usually not computed from the data sets, instead pre-defined substitution rate matrices empirically derived from large alignments of other proteins were used.

Phylogenetic trees can also be inferred from the DNA sequences of protein coding genes, which however offers some pitfalls. In protein coding genes, three nucleotides code for one amino acid, but the genetic code is degenerate. This means that several three-nucleotide combinations may code for the same amino acid (e.g. six codons are known to code for arginine, leucine or serine; see Fig. 3B). As a consequence, a nucleotide change in one codon position may be either without effect on the amino acid (= silent or synonymous substitution), or cause a change of one amino acid to another (= nonsynonymous substitution). Only nonsynonymous substitutions can result in a loss or decrease of function, and, thus are subject to functional constraints. However, the sophisticated evolutionary model parameters mentioned above were in first place developed to cope with RNA coding genes. The three-nucleotide codon structure is ignored and synonymous and nonsynonymous mutations are treated equally. Also, often several evolutionary pathways are possible to evolve from one codon to another, which further complicates the evolutionary model parameters. Often the third positions of codons show nucleotide biases towards higher GC or AT contents.

However, from theoretical and simulation studies, but also empirically, it became obvious that using wrong assumptions about the underlying evolutionary processes may result in biased phylogenetic trees.

4 Simulation Studies

The accuracy of a method comprises consistency, efficiency and robustness. A method is consistent, if it infers the correct phylogenetic tree with an infinite amount of data. Efficiency describes the sensitivity of a method concerning the lengths of sequences. The shorter the sequences can be for a method to converge to the correct tree topology, the more efficient is the method. Robustness considers using wrong assumptions about the underlying evolutionary model. A method is robust, if it infers the correct phylogenetic tree although a wrong evolutionary model was used. Since biologists use DNA or protein sequences of finite lengths, in practice only consistency and robustness of a method are of interest.

In a simulation study by Huelsenbeck, e.g. four-taxon data sets of differing sequence lengths were generated in silico from a random starting sequence according to pre-specified evolutionary models and phylogenetic trees (see parameter space in Fig. 4A). Different phylogenetic analyses methods were then used to infer trees from the data sets and the conditions determined that caused the methods to infer wrong tree topologies. The so-called long branch attraction artefact (LBA) is the most well-known phenomenon causing biased tree topologies. Usually, LBAs were found in phylogenetic trees with extremely long terminal (i.e. branches with high evolutionary rates) but short internal branches (Fig. 4B). In most test situations, maximum likelihood outperformed other methods, but it also failed in finding the correct tree, if the assumed evolutionary models were too different from the evolutionary processes under which the simulated data sets had evolved.
Fig. 4: The long branch attraction artefact (LBA). Fig. 4A. The parameter space with different tree topologies usually used in simulation studies with four-taxon trees. Fig. 4B. An example for a LBA of a four-taxon tree. The tree to the left corresponds to the tree in the top left corner of the parameter space in Fig. 4A. The tree to the right shows the typical LBA bias. The high evolutionary rates displayed by the long branches of the taxa A and B cause reversals in the nucleotides, e.g. a C mutates to a G, a T and back to a C. In combination with a high background noise, which blurs phylogenetic signals, these reversals are presumably interpreted erroneously as positives for genetic relatedness. The region in the parameter space resulting in biased trees is also sometimes called the “Felsenstein” zone of a method. This region is predominantly located in the top left, sometimes extended to the top right of the parameter space shown in Fig. 4A. The larger this “Felsenstein” zone is, the less robust the phylogenetic method.
5 Phylogenetic Analyses and Real Life Data

Since divergent branch lengths were almost always found in phylogenetic analyses of \textit{in vivo} evolved sequences, the effects of potential LBAs were a frequent matter of concern \cite{6}. Especially in large scale phylogenies comprising sequences of very different organisms, long-branch taxa were often gathered ladder-like close to the root of the trees, which may indicate a potential bias caused by LBAs. The farther back in time the examined relationships of organisms reach, the worse the resolution at the internal branches of a tree. It was found that an addition of sequences to the data set and a complex evolutionary model with a gamma-distributed among-site rate variation were the best options to reduce artefacts in a phylogenetic tree \cite{7}, \cite{8}. Especially, adding more sequences of the problematic type could break up long branches, increase the resolution in this part of a tree and thereby neutralise the LBA.

An example of how taxon sampling and choice of evolutionary model may affect the results of a molecular phylogeny can be found in the cryptophytes, a group consisting of microscopic flagellated unicells. Most of the genera in this group are algae, i.e. they contain a pigmented plastid which is used to turn the energy of light into chemical energy by photosynthesis. Two genera are, however, colourless. \textit{Goniomonas} is phagotrophic; it feeds from ingesting bacteria. The other genus, formerly classified as \textit{Chilomonas} feeds from organic molecules, but still harbours a leukoplast, i.e. a colourless plastid. In a phylogenetic analysis with a low number of nuclear 18S ribosomal DNA sequences, \textit{Goniomonas} and “\textit{Chilomonas}” clustered together indicating a relationship of both genera \cite{9}. In a later analysis, sequences of the photosynthetic genus \textit{Cryptomonas} were added \cite{10}. It turned out that \textit{Goniomonas} was the most basally diverging taxon, whereas “\textit{Chilomonas}” was a colourless \textit{Cryptomonas}. The clade with the genera \textit{Cryptomonas} and “\textit{Chilomonas}” seemed to be the most basal group of the plastid-bearing cryptophytes. Thus, the sisterhood of \textit{Goniomonas} and “\textit{Chilomonas}” were caused by a LBA due to inappropriate taxon sampling. The analysis in \cite{10}, however, was done using maximum likelihood under a simple evolutionary model, i.e. without considering an among-site rate variation. In a study using a complex evolutionary model with among-site rate variation, the basal position of the \textit{Cryptomonas}/“\textit{Chilomonas}” clade was also shown to be an artefact caused by long branch attraction \cite{11}.

Thus, long branch attraction artefacts are a real problem in phylogenies inferred from \textit{in vivo} evolved sequences. The best options to cope with LBAs, i.e. adding more taxa, and using complex evolutionary models and robust methods, however, collide with another problem biologists were and are still confronted with computation times. The larger the amount of sequences, the more reliable the phylogenetic analyses methods do work, but exponentially more time is also needed to obtain results.

Bayesian analysis was introduced as a potential faster alternative to maximum likelihood analysis \cite{3}. However, for large data sets Markov chains often need to be run for more generations to reach a plateau of likelihood values, which also increases computation times. In addition, the posterior probabilities given for the different branches of the consensus tree, in which the sampled trees are summarised, are more optimistic than support values obtained from nonparametric bootstrapping using the maximum likelihood criterion (i.e. a subsampling method with at least 100, often more than 100 subsample data sets, to
test the stability of the branches of a tree). Bayesian analysis may be speeded up by running the different Markov chains on separate CPUs of a computing server or a cluster.

In heuristic tree searches using the maximum likelihood criterion, some parallelised versions of programmes have been introduced e.g. [12]. The tasks of tree generation and tree evaluation were distributed among a master (tree generation and comparison) and worker programmes (calculation of branch lengths and likelihoods).

Another attempt to decrease computation times was quartet-puzzling [13]. In quartet-puzzling, trees are computed from quartets of sequences of a larger data set using the maximum likelihood criterion and weighted accordingly. The best of the three possible 4-trees for each quartet are used to first assemble a large number of n-trees (quartet-puzzling) and finally to obtain a consensus n-tree. This method is much faster than a heuristic trees search, but more vulnerable to LBA. Among hundreds to thousands computed four-taxon trees, only a low number of biased 4-trees suffices to pass on a topological error to the final n-tree. In simulation studies, global character maximum likelihood almost always outperformed quartet-puzzling or related methods [14].

Other studies tried to overcome LBA and exponentially growing computing times with longer sequences, e.g. by using complete genomes to infer phylogenetic trees. Phylogenetic analyses of longer sequences increase the computing times only linearly. Since sequencing of complete genomes need much more time and resources than that of single genes or smaller sets of genes, the taxon sampling in these studies generally was lower. It has been shown, however, that long sequences cannot compensate for an extended taxon sampling. The low number of taxa included in a genome-scale analysis resulted in high bootstrap support even for biased tree topologies [15]. Also genome-scale alignments cannot be refined by eye anymore. They depend in automatic alignment algorithms, which may perform badly by producing more or less biologically meaningless alignments [16]. A better option than using complete genomes presumably is to sequence a set of genes, to refine the alignment of each gene by eye, and to concatenate the genes [17].

Additional problems occur, if the evolution of a gene and/or a group of organisms cannot be described by bifurcating trees. In sexually reproducing populations, the examined gene may be present in differing alleles. Each individual of a population inherits two alleles, one from its mother, the other from its father. In addition, parts of the alleles can be exchanged by genetic recombination. Genetic material may also be transferred between unrelated organisms, e.g. by infection with viruses, by endosymbiosis or in bacteria by exchange of plasmids. Whereas the inheritance of genes from parents to child is called vertical gene transfer, the exchange of genetic material between unrelated organisms is called lateral gene transfer. The results of sexual reproduction or lateral gene transfers are genetic chimaeras and reticulate evolutionary trees.

6 Conclusions

Until yet, there seems to be no easy way out of the treadmill of extremely increasing computing times for phylogeneticists. New algorithms to reduce time consumption in phylogenetic analysis have been proposed until recently, e.g. [18].
However, only if the algorithms are offered in software programmes suitable for the tasks of phylogenetic analysis, if they are presented in an understandable way to biologists and if they prove to be robust, they will accepted and used.

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