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8 Molecular Phylogenetics: Mathematical Framework and Unsolved Problems

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Abstract. Phylogenetic relationship is essential in dating evolutionary events, reconstructing ancestral genes, predicting sites that are important to natural selection and, ultimately, understanding genomic evolution Three categories of phylogenetic methods are currently used: the distance-based, the maximum parsimony, and the maximum likelihood method. Here I present the mathematical framework of these methods and their rationales, provide computational details for each of them, illustrate analytically and numerically the potential biases inherent in these methods, and outline computational challenges and unresolved problems. This is followed by a brief discussion of the Bayesian approach that has recently been used in molecular phylogenetics.

8.1 Introduction

Biodiversity comes in many colors and shades, and unorganized biodiversity can not only dazzle our eyes but also confuse our minds. Phylogenetics is a special branch of science with the aim to organize biodiversity based on the ancestor-descendent relationship. Molecular phylogenetics uses molecular sequence data to achieve its three main objectives: (1) to reconstruct the branching pattern of different evolutionary lineages such as species and genes, (2) to date evolutionary events such as speciation or gene duplication and subsequent functional divergence, and (3) to understand and summarize the evolutionary processes by substitution models. With the rapid increase of DNA and protein sequence data, and with the realization that DNA is the most reliable indicator of ancestor-descendent relationships, molecular phylogenetics has become one of the most dynamic fields in biology with solid theoretical foundations [1-3] and powerful software tools [4-8]. I will not argue for the importance of molecular phylogenetics other than quoting Aristotle’s statement that “He who sees things from the very beginning has the most advantageous view of them.”

It is not always easy to see things from the very beginning. The evolutionary process depicted in Fig. 1 shows an ancestral population with a single sequence shared among all individuals that have subsequently split into two populations and evolved and accumulated substitutions independently. Twelve substitutions have occurred, but only three differences can be observed between the sequences from the two extant species. The most fundamental difficulty in molecular phylogenetics is to estimate the true number of substitutions (i.e., 12) from the observed number of differences between extant sequences (i.e., 3). In short, the difficulty lies in how to correct for multiple hits.

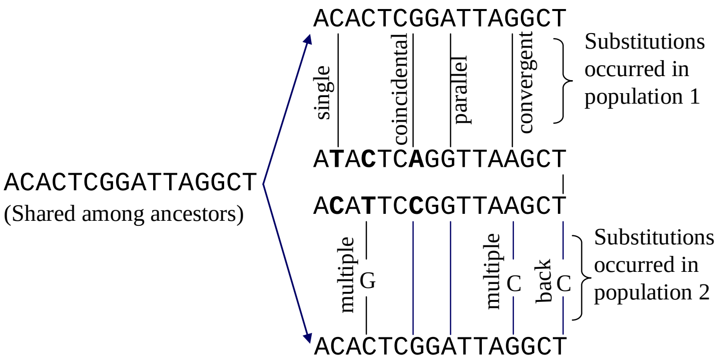


Fig. 1. Illustration of nucleotide substitutions and the difficulty in correcting multiple hits.

The number of substitutions per site is known as a genetic distance. The simplest genetic distance, known as the p-distance (D_p), between two sequences is simply the number of different sites (N) divided by the sequence length (L). For the two sequences in Fig. 1, D_p = 3/16. Because Dp does not correct for multiple hits, it is typically a severe underestimation of the genetic distance and has to be corrected.

In the next few sections, I will first detail commonly used substitution models, derive genetic distances based on the substitution models, and introduce the three categories of molecular phylogenetic methods: the distance-based, the maximum parsimony and the maximum likelihood methods. This is followed by a numerical illustration of the Bayesian inference, together with a brief discussion of the Bayesian approach that has recently been used in molecular phylogenetics [9]. Potential problems with these phylogenetic methods will be highlighted.

8.2 Substitution models

Substitution models reflect our understanding of how molecular sequences change over time. They are the theoretical foundation for computing the genetic distance in the distance-based phylogenetic method and for computing the likelihood value in the maximum likelihood method for phylogenetics. There are three types of molecular sequences, i.e., nucleotide, amino acid and codon sequences. Consequently, there are three types of substitution models, i.e., nucleotide-based, amino acid-based and codon-based. We will focus on nucleotide-based substitution models, with only a brief discussion on amino acid-based and codon-based models to highlight a few potential problems.

8.2.1 Nucleotide-based substitution models and genetic distances

Let $\boldsymbol{p_t}$ be the vector of the four nucleotide frequencies in the order of A, G, C, T at time t. Nucleotide-based substitution models are characterized by a Markov chain of four discrete states as follows:

$$p_{t+1} = p_t \begin{bmatrix} P_{AA} & P_{AG} & P_{AC} & P_{AT} \\ P_{GA} & P_{GG} & P_{GC} & P_{GT} \\ P_{CA} & P_{CG} & P_{CC} & P_{CT} \\ P_{TA} & P_{TG} & P_{TC} & P_{TT} \end{bmatrix} = p_t M \tag{8.1}$$

where $\boldsymbol{M}$ is the transition probability matrix and P_{ij} is the probability of changing from state i to state j in one unit of time. Three frequently used special cases of equation (8.1) will be detailed here: the JC69 model [10], the K80 model [11], and the TN93 model [12].

The simplest nucleotide substitution model is the JC69 one-parameter model, in which all off-diagonal elements in $\boldsymbol{M}$ are identical and designated as α. The four diagonal elements in $\boldsymbol{M}$ are 1-3α constrained by the row sum equal to 1. There is a corresponding rate matrix, designated by $\tilde{Q}$, that differs from $\boldsymbol{M}$ only in that the diagonal elements are -3α, constrained by the row sum equal to 0. It is often more convenient to derive substitution rates by using $\tilde{Q}$ instead of $\boldsymbol{M}$, as will be clear latter. Following equation (8.1), we have

$$\begin{aligned} P_{A.t+1} &= P_{A.t}(1-3\alpha) + P_{G.t}\alpha + P_{C.t}\alpha + P_{T.t}\alpha \\ P_{G.t+1} &= P_{A.t}\alpha + P_{G.t}(1-3\alpha) + P_{C.t}\alpha + P_{T.t}\alpha \\ P_{C.t+1} &= P_{A.t}\alpha + P_{G.t}\alpha + P_{C.t}(1-3\alpha) + P_{T.t}\alpha \\ P_{T.t+1} &= P_{A.t}\alpha + P_{G.t}\alpha + P_{C.t}\alpha + P_{T.t}(1-3\alpha)\,. \end{aligned} \tag{8.2}$$

Arranging the left side to be $P_{A.t+1}$ - $P_{A.t}$ and then applying the continuous approximation, we have

$$\begin{aligned} \frac{\partial P_A}{\partial t} &= -P_{A.t}(3\alpha) + (P_{G.t} + P_{C.t} + P_{T.t})\alpha \\ \frac{\partial P_G}{\partial t} &= -P_{G.t}(3\alpha) + (P_{A.t} + P_{C.t} + P_{T.t})\alpha \\ \frac{\partial P_C}{\partial t} &= -P_{C.t}(3\alpha) + (P_{A.t} + P_{G.t} + P_{T.t})\alpha \\ \frac{\partial P_T}{\partial t} &= -P_{T.t}(3\alpha) + (P_{A.t} + P_{G.t} + P_{C.t})\alpha\,. \end{aligned} \tag{8.3}$$

Equation (8.3) is a special case of a general equation. Designate $\boldsymbol{d}$ as the vector of the four partial derivatives, the general equation is

$$d = P_t\tilde{Q} \qquad (8.4)$$

where $\tilde{Q}$ is the rate matrix mentioned before.

Suppose that we start with nucleotide A, what is the probability that it will stay as A or change to one of the other three nucleotides after time t? Given the initial condition that $P_{A.0} = 1$ and $P_{C.0} = P_{G.0} = P_{T.0} = 0$ and the constrain that that $P_A + P_G + P_C + P_T = 1$, equation (8.3) can be solved to yield

$$\begin{aligned} P_{A.t} &= \frac{1}{4} + \frac{3}{4}e^{-4\alpha t} \\ P_{G.t} &= P_{C.t} = P_{T.t} = \frac{1}{4} - \frac{1}{4}e^{-4\alpha t} \ . \end{aligned} \qquad (8.5)$$

The time t in equation (8.5) is the time from the ancestor to the present. When we compare two extant sequences, the time is $2t$, i.e., from one sequence to the ancestor and then back to the other sequence. So equation (8.5) has its general form as

$$\begin{aligned} P_{ii.t} &= \frac{1}{4} + \frac{3}{4}e^{-8\alpha t} \\ P_{ij.t} &= \frac{1}{4} - \frac{1}{4}e^{-8\alpha t} \ . \end{aligned} \qquad (8.6)$$

The genetic distance (D), which is the number of substitutions per site, is defined as $2t\mu$ where μ is the rate of substitution. For the JC69 mode, $\mu = 3\alpha$, so $\alpha t = D/6$. Now we can readily derive D, now designated D_{JC69}, from the probability of a site being different which is estimated by the p-distance (D_p) defined before. According to equation (8.6),

$$\begin{aligned} D_p &= 1 - P_{ii.t} = \frac{3}{4}(1 - e^{-8\alpha t}) = \frac{3}{4}(1 - e^{-4D_{JC69}/3}) \\ D_{JC69} &= -\frac{3}{4}\ln(1 - \frac{4D_p}{3}) \ . \end{aligned} \qquad (8.7)$$

For the two sequences in Fig. 1, $D_p = 3/16 = 0.1875$ and $D_{JC69} = 0.21576$. The equilibrium frequencies are derived by setting $(p_{i.t+1} - p_{i.t})$ in equation (8.3) to zero. Solving the resulting simultaneous equations with the constraint that the four frequencies sum up to 1, we have $P_{A.t} = P_{G.t} = P_{C.t} = P_{T.t} = 0.25$. In summary, the JC69 model assumes that (1) the four nucleotides can change into each other with equal probability and (2) the equilibrium frequencies are all equal to 0.25.

The variance of D_{JC69} can be obtained by using the "delta" method [13]. When a variable Y is a function of a variable X, i.e., $Y = \mathrm{F}(X)$, the delta method allows us to obtain approximate formulation of the variance of Y if (1) Y is differentiable with respect to X and (2) the variance of X is known. The same can be extended to more variables.

The mathematical concept for the delta method is illustrated below, starting with the simplest case of $Y = \mathrm{F}(X)$. Regardless of the functional relationship between Y and X, we always have

$$\Delta Y \approx \left(\frac{dY}{dX}\right)\Delta X \tag{8.8}$$

$$\left(\Delta Y\right)^2 \approx \left(\frac{dY}{dX}\right)^2 \left(\Delta X\right)^2 \ . \tag{8.9}$$

where ΔY and ΔX are small changes in Y and X, respectively.

Note that the variance of Y is the expectation of the squared deviations of Y, i.e.,

$$\begin{aligned} V(Y) &= E(\Delta Y)^2 \\ V(X) &= E(\Delta X)^2 \ . \end{aligned} \tag{8.10}$$

Replacing $(\Delta Y)^2$ and $(\Delta X)^2$ in equation (8.9) with $V(Y)$ and $V(X)$, we have

$$V(Y) \approx \left(\frac{dY}{dX}\right)^2 V(X) \ . \tag{8.11}$$

This relationship allows us to obtain an approximate formulation of the variance of either Y or X if we know either $V(X)$ or $V(Y)$. For the variance of D_{JC69}, we note that D_{JC69} is a function of D_p, and the variance of D_p is known from the binomial distribution:

$$V(D_p) = \frac{D_p(1-D_p)}{L} \tag{8.12}$$

where L is the length of the two aligned sequences. From the expression of D_{JC69} in equation (8.7), we have

$$\begin{aligned} \frac{\partial D_{JC69}}{\partial D_p} &= \frac{1}{1-\frac{4D_p}{3}} \\ V(D_{JC69}) &= \left(\frac{\partial D_{JC69}}{\partial D_p}\right)^2 V(D_p) = \frac{D_p(1-D_p)}{L\left(1-\frac{4D_p}{3}\right)^2} \ . \end{aligned} \tag{8.13}$$

Kimura [11] noted that transitional substitutions typically occur much more frequently than transversions, and consequently proposed the two-parameter K80 model in which the rate of transitional substitutions (A↔G and T↔C) is designated as α and the rate of transversion substitutions (A↔T, A↔C, G↔T and G↔C) as β. Substituting this new $\tilde{Q}$ into equation (8.4) and solve the equations with the initial condition that $P_{A.0} = 1$ and $P_{C.0} = P_{G.0} = P_{T.0} = 0$ and the constrain that that $P_A + P_G + P_C + P_T = 1$as before, we have

$$
\begin{aligned}
P_{A.t} &= \frac{1}{4} + \frac{1}{4}e^{-4\beta t} + \frac{1}{2}e^{-2(\alpha+\beta)t} \\
P_{G.t} &= \frac{1}{4} + \frac{1}{4}e^{-4\beta t} - \frac{1}{2}e^{-2(\alpha+\beta)t} \\
P_{C.t} &= P_{T.t} = \frac{1}{4} - \frac{1}{4}e^{-4\beta t}\ .
\end{aligned}
\tag{8.14}
$$

Note again that time t in equation (8.14) should be 2*t* when used between two extant sequences. So equation (8.14) has its general form as

$$
\begin{aligned}
P_{s.t} &= \frac{1}{4} + \frac{1}{4}e^{-8\beta t} - \frac{1}{2}e^{-4(\alpha+\beta)t} \\
P_{v.t} &= \frac{1}{2} - \frac{1}{2}e^{-8\beta t}
\end{aligned}
\tag{8.15}
$$

where $P_{s.t}$ and $P_{v.t}$ are the probabilities that a site differs by a transition and a transversion, respectively, between two sequences that have diverged for time *t*, and can be estimated by the proportion of sites differ by a transition (*P*) and a transversion (*Q*), respectively. This leads to

$$
\begin{aligned}
\beta t &= -\frac{\ln(1-2Q)}{8} \\
\alpha t &= -\frac{\ln(1-2P-Q)}{4} + \frac{\ln(1-2Q)}{8}\ .
\end{aligned}
\tag{8.16}
$$

Recall that the genetic distance is defined as $2t\mu$ where $\mu = \alpha + 2\beta$ for the K80 model. Therefore,

$$
\begin{aligned}
&D_{K80} = 2\alpha t + 4\beta t = \frac{1}{2}\ln(a) + \frac{1}{4}\ln(b), \text{ where} \\
&a = \frac{1}{1\text{-}2P\text{-}Q} \text{ and } b = \frac{1}{1\text{-}2Q}\ .
\end{aligned}
\tag{8.17}
$$

For the two sequences in Fig. 1, $P = 2/16$, $Q = 1/16$, $D_{K80} = 0.22073$. The equilibrium frequencies are derived by setting ***d*** in equation (8.4) to the ***0*** vector. Solving the resulting simultaneous equations with the constraint that the four frequencies sum up to 1, we have $P_{A.t} = P_{G.t} = P_{C.t} = P_{T.t} = 0.25$. Thus, the K80 model shares with the JC69 model the assumption that the equilibrium frequencies are all equal to 0.25. You might have noticed this because nucleotide frequencies are not featured in the expression of D_{JC69} or D_{K80}.

The variance of D_{K80} can be derived by the delta method as before:

$$
dD_{K80} = \left(\frac{\partial D_{K80}}{\partial P}\right)dP + \left(\frac{\partial D_{K80}}{\partial Q}\right)dQ = d_1(dP) + d_2(dQ)
\tag{8.18}
$$

$$\begin{aligned}(dD_{K80})^2 &= \left[d_1(dP) + d_2(dQ)\right]^2 \\ &= d_1^2(dP)^2 + 2d_1d_2(dPdQ) + d_2^2(dQ)^2 \\ &= d_1^2V(P) + 2d_1d_2Cov(P,Q) + d_2^2V(Q) \\ &= \begin{bmatrix} d_1 & d_2 \end{bmatrix} \begin{bmatrix} V(P) & Cov(P,Q) \\ Cov(P,Q) & V(Q) \end{bmatrix} \begin{bmatrix} d_1 \\ d_2 \end{bmatrix} .\end{aligned} \tag{8.19}$$

Recall that P and Q stand for the proportion of sites that differ by a transitional change and differ by a transversional change, respectively. Designate R as the proportion of identical sites ($R = 1 – P – Q$). From the trinomial distribution of $(R + P + Q)^L$, we have:

$$\begin{aligned}V(P) &= \frac{P(1-P)}{L} \\ V(Q) &= \frac{Q(1-Q)}{L} \\ Cov(P,Q) &= -\frac{PQ}{L} \ .\end{aligned} \tag{8.20}$$

Substituting these into equation (8.19), we have the variance of D_{K80}:

$$V(D_{K80}) = (dD_{K80})^2 = \frac{a^2P + c^2Q - (aP + cQ)^2}{L} \tag{8.21}$$

where $c = (a + b)/2$, with a and b defined in equation (8.17).

Note that equation (8.19) is a general equation for computing the variance by the delta method. For any function $Y = \mathrm{F}(X_1, X_2, ..., X_\mathrm{n})$, the variance of Y is obtained by the variance-covariance matrix of X_i multiplied left and right by the vector of partial derivatives of Y with respect to X_i.

Tamura and Nei [12] noticed the rate difference between C↔T and A↔G transitions and proposed the TN93 model with the following rate matrix:

$$\tilde{Q} = \begin{bmatrix} T & \bullet & \alpha_1\pi_C & \beta\pi_A & \beta\pi_G \\ C & \alpha_1\pi_T & \bullet & \beta\pi_A & \beta\pi_G \\ A & \beta\pi_T & \beta\pi_C & \bullet & \alpha_2\pi_G \\ G & \beta\pi_T & \beta\pi_C & \alpha_2\pi_A & \bullet \end{bmatrix} . \tag{8.22}$$

where π_i designates equilibrium nucleotide frequencies, and the diagonal is constrained by the row sum equal to 0.

Following the same protocol as before, and designate P_1, P_2 and Q as the probabilities of C↔T transitions, A↔G transitions and R↔Y transversions (R means either A or G and Y means either C or T), respectively, we can obtain,

$$\begin{aligned} P_1 &= \pi_T P_{TC}(2t) + \pi_C P_{CT}(2t) \\ &= \frac{2\pi_T \pi_C (\pi_Y + \pi_R e^{-2\beta t} - e^{-2(\alpha_1 \pi_Y + \beta \pi_R)t})}{\pi_Y} \end{aligned} \tag{8.23}$$

$$\begin{aligned} P_2 &= \pi_A P_{AG}(2t) + \pi_G P_{GA}(2t) \\ &= \frac{2\pi_A \pi_G (\pi_R + \pi_Y e^{-2\beta t} - e^{-2(\alpha_2 \pi_R + \beta \pi_Y)t})}{\pi_R} \end{aligned} \tag{8.24}$$

$$Q = 2\pi_R \pi_Y (1 - e^{-2\beta t}) \ . \tag{8.25}$$

Solving for $\alpha_1 t$, $\alpha_2 t$ and βt from equations (8.23)-(8.25), we have

$$\alpha_1 t = \frac{-\ln(1 - \frac{Q}{2\pi_Y} - \frac{P_1 \pi_Y}{2\pi_T \pi_C}) + \pi_R \ln(1 - \frac{Q}{2\pi_R \pi_Y})}{2\pi_Y} \tag{8.26}$$

$$\alpha_2 t = \frac{-\ln(1 - \frac{Q}{2\pi_R} - \frac{P_2 \pi_R}{2\pi_A \pi_G}) + \pi_Y \ln(1 - \frac{Q}{2\pi_R \pi_Y})}{2\pi_R} \tag{8.27}$$

$$\beta t = -\frac{\ln(1 - \frac{Q}{2\pi_R \pi_Y})}{2} \ . \tag{8.28}$$

$$\begin{aligned} D_{TN93} &= 2t[\pi_A(\beta\pi_T + \beta\pi_C + \alpha_2\pi_G) + \pi_C(\beta\pi_A + \beta\pi_G + \alpha_1\pi_T) \\ &\quad + \pi_T(\beta\pi_A + \beta\pi_G + \alpha_1\pi_C) + \pi_G(\beta\pi_T + \beta\pi_C + \alpha_2\pi_A)] \\ &= 4[\pi_R\pi_Y\beta t + \pi_A\pi_G\alpha_2 t + \pi_C\pi_T\alpha_1 t] \ . \end{aligned} \tag{8.29}$$

Because we can estimate P_1, P_2 and Q by the proportion of sites with C↔T transitions, A↔G transitions and R↔Y transversions, respectively, D_{TN93} can be readily computed. For the two sequences in Fig. 1, D_{TN93} is 0.2525. The variance of $\mathrm{D}_{\mathrm{TN93}}$ can be easily obtained by left- and right-multiplying the variance-covariance matrix of P_1, P_2 and Q with the vector of the three derivatives of D_{TN93} with respect to P_1, P_2 and Q in the same way shown in the last term of equation (8.19). The variance and covariance of P_1, P_2 and Q can be obtained in the same way as in equation (8.20).

Many more substitution models and genetic distances have been proposed [14], with the number of all possible time reversible models of nucleotide substitution being 203 [15]. In addition, there are more complicated models underlying the LogDet and the paralinear distances [16, 17] that can presumably accommodate the nonstationarity of the substitution process. Different substitution models often lead to different trees produced and constitute a major source of controversy in molecular phylogenetics [18-20].

8.2.2 Amino acid-based and codon-based substitution models

Amino acid-based models [21, 22] are similar in form to those nucleotide-based models in the previous section, except that the discrete states of the Markov chain will be 20 instead of only 4. Because of the large size of the transition matrix, the transition probabilities are typically derived from empirical transition matrices [23, 24].

There are three inherent difficulties with amino acid-based models. First, protein-coding genes often differ much in substitution patterns, and one can never be sure if any of the empirical transition matrices is appropriate for the protein sequences one is studying. Second, note that an amino acid replacement is effected by a nonsynonymous codon replacement. Two codons can differ by 1, 2, or 3 sites, and an amino acid replacement involving two codons differing by one site is expected to be more likely than that involving two codons differing by 3 sites. Only a codon-based model can incorporate this information. Third, two similar amino acids are expected to, and do, replace each other more frequently than two different amino acids [25]. However, the similarity between amino acids is difficult to define. For example, polarity may be highly conserved at some sites but not at others. Two very different amino acids rarely replace each other in functionally important domains but can replace each other frequently at unimportant segment. Moreover, the likelihood of two amino acids replacing each other also depends on neighboring amino acids [26]. For example, whether a stretch of amino acids will form a α-helix may depend on whether the stretch contains a high proportion of amino acids with high helix-forming propensity, and not necessarily on whether a particular site is occupied by a particular amino acid.

The codon-based substitution models [27, 28] were proposed to overcome some of the difficulties in amino acid-based models. These models share the third difficulty above with the amino acid-based models, and have additional problems of their own. For example, one cannot get good estimate of codon frequencies because protein-coding genes are typically very short. An alternative is to use the F3x4 codon frequency model [8, 29]. However, codon usage is affected by many factors, including differential ribonucleotide and tRNA abundance as well as biased mutation [30-32]. For example, the site-specific nucleotide frequencies are poor predictors of codon usage (Table 1) of protein-coding genes in *Escherichia coli* K12. The A-ending codon is used frequently for coding lysine, but the G-ending codon used frequently for coding glutamine (Table 1). The reason for this is simple. Six Lys-tRNA genes in *E. coli* K12 all have anticodons being UUU which can translate the AAA lysine codon better than the AAG lysine codon. For glutamine codons, there are two copies of Glu-tRNA genes (glnX and glnV) with a CUG anticodons matching the CAG codon and another two copies (glnW and glnU) with the UUG anticodon matching the CAA codon. However, the former is more abundant than the latter in the *E. coli* cell [33], which would favor the use of CAG against the CAA codon for glutamine. One should expect the F3x4 codon frequency model to perform poorly in such a situation which unfortunately is frequently encountered.

Table 1. Site-specific nucleotide frequencies and codon usage in two codon families. AA – amino acid; N_{cod} – number of codon; CS - codon site. Results based on eight highly expressed genes (*gapC, gapA, fbaB, ompC, fbaA, tufA, groS, groL*) from the *Escherichia coli* K12 genome (GenBank Accession: NC_000913)

Nuc. Freq. by codon sites (CS)	Codon freq.

Base	CS_1	CS_2	CS_3	Codon	AA	N_{cod}
A	0.273	0.32	0.18	AAG	Lys	24
C	0.189	0.24	0.326	AAA	Lys	149
G	0.409	0.16	0.219	CAG	Gln	73
U	0.129	0.28	0.275	CAA	Gln	7

8.3 Tree-building methods

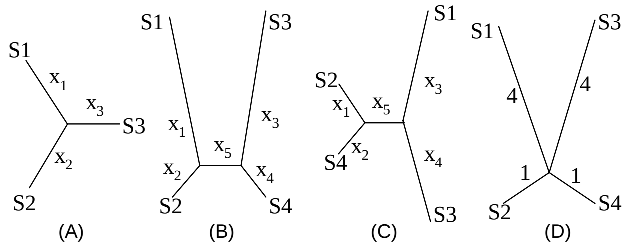
Three categories of tree-building methods are in common use: the distance-based, the maximum parsimony and the maximum likelihood methods. These methods have their respective advantages and disadvantages and I will provide mathematical details for the reader to understand their problems.

8.3.1 Distance-based methods

The distance-based methods build trees from a distance matrix, and are represented by the neighbor-joining (NJ) method [34], the Fitch-Margoliash (FM) method [35] and the FastME method [36]. The calculation of genetic distances has already been covered in previous sections. Other than the simplest UPGMA method, each tree-building method consists of two steps: (1) the evaluation of branch lengths for a given topology by the least-squares (LS) method, the NJ method or the FM method, and (2) the selection of the best tree based on either the minimum evolution (ME) criterion or the least-squares or the weighted least-squares criterion referred to hereafter as the Fitch-Margoliash (FM) criterion. One should not confuse, e.g., the FM way of evaluating branch lengths with the FM criterion for choosing the best tree.

There are many ways of evaluating branch lengths for a given tree, and I will only present the LS method here. For the three-OTU (operational taxonomic unit) tree in Fig. 2A, the branch lengths (x_i) can be solved uniquely by the following equations:

$$\begin{aligned} d_{12} &= x_1 + x_2 \\ d_{13} &= x_1 + x_3 \\ d_{23} &= x_2 + x_3 \; . \end{aligned} \tag{8.30}$$



	S2	S3	S4
S1	5	8	5
S2		5	2
S3			5
S1	4	5	4
S2		4	2
S3			4
S1	6	12	6
S2		6	2
S3			6

Fig. 2. Topologies for illustrating the distance-based methods in phylogenetic reconstruction.

For the four-OTU tree in Fig. 2B, we can write down the equations in the same way as in equation (8.30), but there will be six equations for five unknowns. The LS method finds the x_i values that minimize the sum of squared deviations (*SS*),

$$\begin{aligned} SS &= \sum (d_{ij} - d'_{ij})^2 \\ &= [d_{12} - (x_1 + x_2)]^2 + \ldots + [d_{34} - (x_3 + x_4)]^2 \ . \end{aligned} \tag{8.31}$$

By taking the partial derivatives with respect to x_i, setting the derivatives to zero and solving the resulting simultaneous equations, we get

$$\begin{aligned} x_1 &= d_{13}/4 + d_{12}/2 - d_{23}/4 + d_{14}/4 - d_{24}/4 \\ x_2 &= d_{12}/2 - d_{13}/4 + d_{23}/4 - d_{14}/4 + d_{24}/4 \\ x_3 &= d_{13}/4 + d_{23}/4 + d_{34}/2 - d_{14}/4 - d_{24}/4 \\ x_4 &= d_{14}/4 - d_{13}/4 - d_{23}/4 + d_{34}/2 + d_{24}/4 \\ x_5 &= -d_{12}/2 + d_{23}/4 - d_{34}/2 + d_{14}/4 + d_{24}/4 + d_{13}/4 \ . \end{aligned} \tag{8.32}$$

With four OTUs, there are three unrooted trees. There are two commonly used criteria for choosing the best tree. The first is the ME criterion based on the tree length (*TL*) which is the summation of all x_i values. The tree with the smallest *TL* is chosen as the best tree. In contrast, the FM criterion chooses the tree with the smallest *SS*

$$SS = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \frac{\left(d_{ij} - d'_{ij}\right)^2}{d_{ij}^P} \tag{8.33}$$

where n is the number of OTUs and P often takes the value of 0 or 2.

Whether a distance-based method will recover the true tree depends critically on the accuracy of the distance estimates. We will briefly examine this problem with both the ME criterion and the FM criterion. Let TL_B and TL_C be the tree length for Trees B and C in Fig. 2. Suppose that OTUs 1 and 3 have diverged from each other so much as to have experienced substitution saturation [37] to cause difficulty in estimating the true D_{13}. Let pD_{13} be the estimated D_{13}, where p measures the degree of underestimation ($p < 1$) or overestimation ($p > 1$). Designate D_{TL} as the difference in *TL* between the two trees,

$$D_{TL} = TL_B - TL_C = \frac{d_{12} + d_{34} - (pd_{13} + d_{24})}{4} \ . \tag{8.34}$$

According to the LS method of branch evaluation, Tree B is better than Tree C if $D_{TL} < 0$, and worse than Tree C if $D_{TL} > 0$. Simple distances such as the p-distance or JC69 distance tend to have $p < 1$ and consequently increase the chance of having $D_{TL} > 0$, i.e., favoring the incorrect Tree C. This is the long-branch attraction problem, first recognized in the maximum parsimony method. Genetic distances corrected with the gamma-distributed rates over sites [12, 38-40] tend to have $p > 1$ when there is in fact no rate heterogeneity over sites, and consequently would favor Tree B over Tree C, leading to long-branch repulsion [41].

The long-branch attraction and repulsion problem is also present with the FM criterion. Let SS_B and SS_C be *SS* in equation (8.33) for Trees B and C, respectively. With P = 0 in equation (8.33) and letting $D_{SS} = SS_B - SS_C$, we have

$$\begin{aligned} 4D_{SS} &= (d_{13}+d_{24})^2 - (d_{12}+d_{34})^2 + 2(d_{14}+d_{23})[(d_{12}+d_{34})-(d_{13}+d_{24})] \\ &= x^2 - y^2 + 2z(y-x) \end{aligned} \tag{8.35}$$

where $x = d_{13}+d_{24}$, $y = d_{12}+d_{34}$ and $z = d_{14}+d_{23}$.

We now focus on Tree D, for which y is expected to equal z. Now equation (8.35) is reduced to

$$4D_{SS} = (x-y)^2 \tag{8.36}$$

If branch lengths are accurately estimated, then $x = y = 10$, and $D_{SS} = 0$, i.e., neither Tree B nor Tree C is favored. However, if d_{13} (i.e., the summation of the two long branches) is under- or overestimated, then $D_{SS} > 0$ favoring Tree C. This means that both under- and overestimation of the distance between divergence taxa will lead to long-branch attraction. This can be better illustrated with a numerical example with Tree D in Fig. 2 which also displays three distance matrices. The first one is accurate, the second one has genetic distances more underestimated for more divergent taxa, and the third has genetic distances more overestimated for more divergent taxa (e.g., when gamma-distributed rates are assumed when the rate is in fact constant over sites). Note that Tree B and Tree C converge to Tree D when $x_5 = 0$. Table 2 shows the results by applying the ME and LS criterion in analyzing the three distance matrices.

When the distances are accurate, both the ME criterion and the FM criterion recovers Tree D (the true tree) with $x_5 = 0$, *TL* = 10, and *SS* = 0. However, ME criterion favors Tree C when long branches are underestimated, and Tree B when long branches are overestimated. In contrast, the FM criterion would favor Tree C with both under- and overestimated distances (Table 2) when negative branches are allowed.

Table 2. Effect of under- and over-estimation of genetic distances

	Distance matrix					
	Correct		Under-		Over-	
	TreeB	TreeC	TreeB	TreeC	TreeB	TreeC
TL	10	10	7.75	7.5	12.5	13
SS	0	0	0.25	0	1	0
x_5	0	0	-0.25	0.5	0.5	-1

8.3.2 Maximum parsimony methods

In contrast to the distance-based methods, maximum parsimony (MP) and maximum likelihood methods are character-based methods. The six aligned sequences in Fig. 3 have nine sites, with sites 2, 4, 9 being monomorphic, and the rest of sites being polymorphic. A polymorphic site with at least two different states each represented by at least two OTUs is defined as an informative site. The MP method operates on informative sites only.

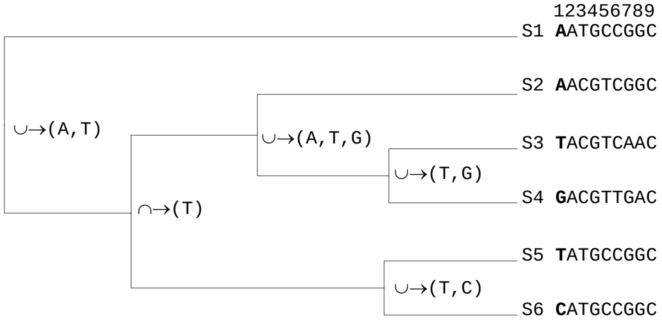


Fig. 3. Computing the minimum number of changes for the first site of the six alignment sequences in phylogenetic reconstruction using the maximum parsimony method.

Given a topology, the minimum number of changes for each sequence site is computed, with the computation of the first site illustrated in Fig. 3. Each node is represented by a set of characters, with the terminal nodes (leaves) each represented by a set containing a single character. The method traverses through each internal node, starting from the node closest to the leaves. If two sets of the two daughter nodes have an empty intersection, then the node will be represented by the union of the two daughter sets, otherwise the node will be represented by the intersection. Once the operation reaches the root, then the number of union operations is the minimum number of changes needed to map the site to the tree. Site 1 in Fig. 3 requires four union operations (Fig. 3), whereas sites 3, 5, and 8 each require only one union operation. Sites 6 and 7, which are polymorphic with two nucleotide states but not informative, will require one change for any topology. So the minimum number of changes, also referred to as the tree length, given the topology and the sequences in Fig. 3, is nine. The same computation is done for other possible topologies and the tree with the smallest tree length is taken as the MP tree.

The MP method is known to be inconsistent [42, 43] and I will provide a simple demonstration here by using trees in Fig. 4. With four species, we have three possible unrooted topologies, designated T_i (i = 1, 2, 3), with T_1 being the correct topology.

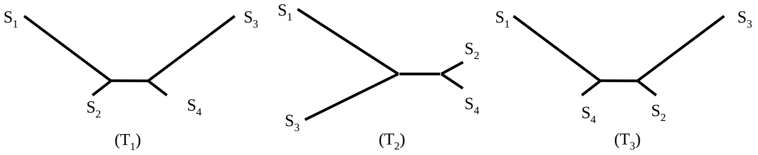


Fig. 4. The long-branch attraction problem in the maximum parsimony methods.

Let X_{ij} be nucleotide at site j for species X_i, and L be the sequence length. For simplicity, assume that nucleotide frequencies are all equal to 0.25. Suppose that the lineages leading to X_1 and X_3 have experienced full substitution saturation, so that

$$\Pr(X_{1j} = X_{ij,i\neq 1}) = \Pr(X_{3j} = X_{ij,i\neq 3}) = 0.25 \qquad (8.37)$$

where *Pr* stands for probability. The lineages leading to X_2 and X_4 have not experienced substitution saturation and have

$$\Pr(X_{2j} = X_{4j}) = P \tag{8.38}$$

where $P > 0.25$. For simplicity, let us set $P = 0.8$, and $L = 1000$.

We now consider the expected number of informative sites, designated by n_i (i = 1, 2, 3), favoring T_i. By definition, site j is informative and favoring T_1 if it meets the following three conditions: $X_{1j} = X_{2j}$, $X_{3j} = X_{4j}$, $X_{1j} \neq X_{3j}$. Similarly, site j favors T_2 if $X_{1j} = X_{3j}$, $X_{2j} = X_{4j}$, $X_{1j} \neq X_{2j}$. Thus, the expected numbers of informative sites favoring T_1, T_2 and T_3, respectively, are

$$\begin{aligned} E(n_1) &= \Pr(X_{1j} = X_{2j}, X_{3j} = X_{4j}, X_{1j} \neq X_{3j})L \\ &= 0.25 \times 0.25 \times 0.75 \times 1000 \approx 47 \\ E(n_2) &= \Pr(X_{1j} = X_{3j}, X_{2j} = X_{4j}, X_{1j} \neq X_{2j})L \\ &= 0.25 \times 0.8 \times 0.75 \times 1000 = 150 \\ E(n_3) &= E(n_1) \approx 47\ . \end{aligned} \tag{8.39}$$

The equations mean that, in spite of T_1 being the true topology, we should have, on average, only about 47 informative sites favoring T_1 and T_3, but 150 sites supporting the wrong tree T_2. This is one of the several causes for the familiar problem of long-branch attraction [44] or short-branch attraction [45]. Because it is the two short branches that contribute a large number of informative sites supporting the wrong tree, “short-branch attraction” seems a more appropriate term for the problem than “long-branch attraction”.

8.3.3 Maximum likelihood methods

The maximum likelihood (ML) method is based on explicit substitution models. Many different types of computer simulation have demonstrated the superiority of the ML method in recovering the true tree. I now use the four aligned sequences in Fig. 5 to illustrate numerically the computation involved in the ML method based on the JC69 model. With four sequences, we have three possible unrooted topologies of which one is shown in Fig. 5.

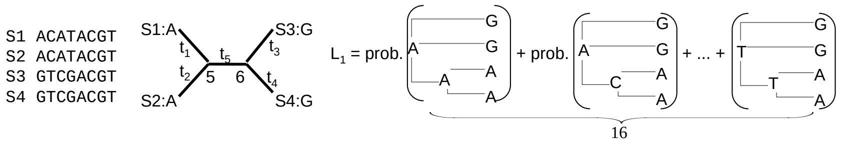


Fig. 5. Likelihood calculation for the first site of the four aligned sequences.

The sequences have 8 sites, with the first four sites sharing one site pattern and the last four sites sharing another site pattern. So we need only two site-specific likelihood functions. The likelihood function of the first site, given the topology in Fig. 5, is the summation of the 16 probabilities corresponding to the 16 nucleotide combinations of the two internal nodes with unknown nucleotides (Fig. 5). Thus, the likelihood of the first site is,

$$\begin{aligned} L_1 = \pi_A & P_{AA.t_1} P_{AA.t_2} P_{AA.t_5} P_{AG.t_3} P_{AG.t_4} \\ & + \pi_C P_{CA.t_1} P_{CA.t_2} P_{CA.t_5} P_{AG.t_3} P_{AG.t_4} \\ & + \ldots \\ & + \pi_T P_{TA.t_1} P_{TA.t_2} P_{TT.t_5} P_{TG.t_3} P_{TG.t_4} \end{aligned} \tag{8.40}$$

where $P_{ij.t}$ for the JC69 model has already been given in equation (8.6) except that "8αt" should be replaced by "4αt". Note that $L_2 = L_3 = L_4 = L_1$. We can write L_5 (= $L_6 = L_7 = L_8$) in a similar fashion.

The sequences in Fig. 5 allow us to simplify equation (8.40) greatly. Note that S1 = S2 and S3 = S4 (Fig. 5) so that αt_1, αt_2, αt_3, and αt_4 are all zero. Now we have

$$\begin{aligned} L_1 &= 0.0625 - 0.0625e^{-4\alpha t_5} \\ L_5 &= 0.0625 + 0.1875e^{-4\alpha t_5} \ . \end{aligned} \tag{8.41}$$

With the assumption that all sites evolve independently, the likelihood function for all eight sites is simply

$$\begin{aligned} L &= L_1^4 L_5^4 \\ \ln L &= 4\ln(L_1) + 4\ln(L_5) \\ &= 4\ln(0.0625 - 0.0625e^{-4\alpha t_5}) + 4\ln(0.0625 + 0.1875e^{-4\alpha t_5}) \ . \end{aligned} \tag{8.42}$$

The αt_5 value that maximizes ln*L* is 0.27465, which leads to ln*L* = -21.02998. The branch length between nodes 5 and 6 is $3\alpha t_5 = 0.82396$. We can do the same calculation for the other two possible topologies, and then choose the tree with the largest lnL value as the ML tree. In this particular example, the tree in Fig. 5 is the ML tree because it has the lnL value greater than that of the other two trees. One may also find that the ML tree, including its estimated branch lengths, is identical to the tree from a distance-based method such as the neighbor-joining [34], the FastME [36] or the Fitch-Margoliash method [35] as long as the JC69 distance is used.

There are two major criticisms on the ML method in phylogenetics. The first is that the application of the likelihood in phylogenetics is not really a ML method in its conventional sense because the topology is not in the likelihood function [3, 46]. To see this point, we can illustrate the conventional ML method with a simple example.

Suppose we wish to estimate the proportion of males (*p*) of a fish population in a large lake. A random sample of *N* fish contains *M* males. With the binomial distribution, the likelihood function is

$$L = \frac{N!}{M!(N-M)!} p^M (1-p)^{N-M} \ . \tag{8.43}$$

The maximum likelihood method finds the value of *p* that maximizes the likelihood value. This maximization process is simplified by maximizing the natural logarithm of L instead:

$$\ln L = A + M\ln(p) + (N - M)\ln(1 - p)$$

$$\frac{\partial \ln L}{\partial p} = \frac{M}{p} - \frac{N - M}{1 - p} = 0 \tag{8.44}$$

$$p = \frac{M}{N}\ .$$

The likelihood estimate of the variance of p is the negative reciprocal of the second derivative,

$$Var(p) = -\frac{1}{\frac{\partial^2 \ln(L)}{\partial p^2}} = -\frac{1}{-\frac{M}{p^2} - \frac{N - M}{(1 - p)^2}} = \frac{p(1 - p)}{N}\ . \tag{8.45}$$

Note that, in contrast to the likelihood in equation (8.44) which is a function of p (the parameter to be estimated), the likelihood in equation (8.42) does not have the topology as a parameter. Without the convenient "∂lnL/∂θ = 0" formulation, we have to do either exhaustive or branch-and-bound search in order to find the topology that maximizes that likelihood. In practice, exhaustive or branch-and-bound search is rarely done, which implies that few of the published ML trees are authentic ML trees. Thus, Nei's criticism highlights more of a practical difficulty than a theoretical one because the likelihood principle does not require the parameter to be continuous and differentiable [47]. The criticism can also be applied to other phylogenetic methods. However, other methods are generally faster and can search the tree space more thoroughly than the ML method. Therefore, while it is not particularly controversial to claim that an authentic ML tree is generally better than a tree satisfying the MP, ME or FM criterion, it is not unreasonable for one to expect the latter to be as good as or better than a "ML" tree that is derived from searching a small subset of all possible topologies. This is particularly pertinent with reconstructing very large phylogenies [48].

The second criticism is on the assumptions shared by nearly all the substitution models currently implemented in the likelihood framework: (1) the substitutions occur independently in different lineages, (2) substitutions occur independently among sites, and (3) the process of substitution is described by a time-homogeneous (stationary) Markov process . The first assumption is false in taxa with a history of horizontal gene transfer [49-54]. The problem of the second assumption can be illustrated with the following example involving the GAT and GGT codons. Both codons end with a T. Whether a T→A substitution would occur depends much on whether the second position is an A or a G. The T→A substitution is rare when the second codon position is A because a T→A mutation in the GAT codon is nonsynonymous, but relatively frequent when the second codon position is G because such a T→A mutation in a GGT codon is synonymous. So nucleotide substitutions do not occur independently among sites. This is one of the reasons for using codon-based models but these models have their own problems as mentioned before. The third assumption is also problematic. Suppose we wish to reconstruct a tree from a group of orthologous sequences from both invertebrate and vertebrate species. There is little DNA methylation in invertebrate genomes, but heavy DNA methylation in some vertebrate genomes. DNA methylation greatly enhanced the C→T transition (and consequently the G→A transition on the opposite strand [55]. The net result is a much elevated transition/transversion bias and increased AT% in the lineages with DNA methylation, violating the third assumption.

More complicated models have been proposed in response to our increased knowledge of the substitution process. However, such parameter-rich models require more data for reliable parameter estimation. The dilemma is that increasing the sequence length also increases the heterogeneity of substitution processes [56] including heterotachy [57] operating on different sequence segments and consequently increase the number of parameters to be estimated. Such heterogeneity over sites implies that the consistency of the ML method [47, 58] is not of much value because we cannot get long sequences for a fixed and small number of parameters. Take for example the estimation of the proportion of male fish in the lake. If we get only six male fish in a sample with no female, then the likelihood estimation of *p* is 1 which is worse than our wildest guess without any data.

8.3.4 Bayesian inference

The Bayesian approach has only recently been used in phylogenetic inference [9]. Here I illustrate the basic principle of the Bayesian approach by using the problem of estimating the proportion (*p*) of males when the sample of six fish being all males. For a continuous variable such as *p*, the Bayes' theorem is

$$f(\theta \mid y) = \frac{f(y \mid \theta) f(\theta)}{\int f(y \mid \theta) f(\theta) d\theta} \qquad (8.46)$$

where θ is the parameter of interest, *y* is the observed sample data, $f(\theta)$ is the prior probability for incorporating our prior knowledge on θ, $f(y|\theta)$ is the likelihood, and $f(\theta|y)$ is the posterior probability. In practice, equation (8.46) is rarely used because the integration in the denominator is difficult unless $f(\theta)$ and $f(y|\theta)$ are very simple, although the MCMC (Markov chain Monte Carlo) approach [59, 60] can alleviate the problem. Two alternative approaches have been devised to ease the computation burden, one being to use discrete approximations to continuous probability models, and the other being to use the conjugate prior distributions. For our example involving a stationary and independent Bernoulli process in estimating *p*, the conjugate prior distribution is the beta distribution with the following $f(p)$:

$$f(p) = \frac{(n-1)!}{(r-1)!(n-r-1)!} p^{r-1} (1-p)^{n-r-1} \, . \qquad (8.47)$$

Let's designate n and r as as n' and r' in the prior distribution, n" and r" in the posterior distribution, and just as n and r in the sample. If we expect the fish species to have equal number of males and females, then for a sample of six fish (n' = 6), we expect r' to be 3. The prior probability can be calculated from equation (8.47) and shown in Fig. 6.

It can be proven that, if the prior distribution of *p* is a beta distribution, then the posterior distribution will also be a beta distribution with the two parameters computed according to Eq. (8.48) below. In our actual sample with six males and 0 female (n = 6 and r = 6),

$$\begin{aligned} r'' &= r' + r = 3 + 6 = 9 \\ n'' &= n' + n = 6 + 6 = 12 \end{aligned} \tag{8.48}$$

Now the posterior probability can be calculated by using equation (8.47) and shown in Fig. 6 in comparison with the prior probability. Thus, our prior expectation of p = 0.5 has been revised by the actual sample to p = 0.8. One may note that if the population of fish is indeed made of all males, e.g., when there is a high concentration of androgen masculinizing all individuals to males [61], then the likelihood estimate of p = 1 is correct and the Bayesian estimate of p = 0.8 is wrong, and the wrong estimate may lead to our failure to identify an environmental crisis.

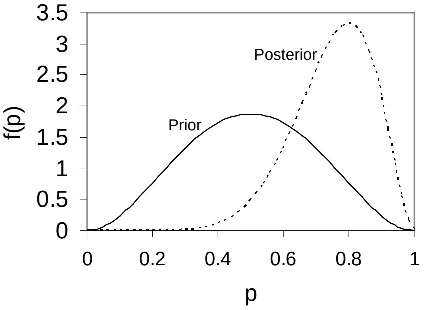


Fig. 6. Comparison between prior and posterior probabilities.

In Bayesian phylogenetics, θ is a collection of the tree topology, the rate matrix and the branch lengths, and the likelihood function is formulated as in the maximum likelihood method. The main difficulty is the justification of the prior probability [1, 62, 63] which is problematic even for the simple example of estimating p.

8.4 Final words

What I have presented is only the tip of the iceberg. One need to go down and get wet to see what is truly big (and truly messy). The models and equations are presented more for convenience than for mathematical rigor, but should work well for pedagogical purposes. I used to tell my son that his toys were alive with their own minds so he should be nice to them otherwise they would be upset and refuse to play with him. Such a lousy worldview nevertheless seemed to work perfectly well for him. I am sure that my son will grow out of this worldview, just as I am sure that the reader will grow out of the conceptual framework of molecular phylogenetics that is presented in this paper.

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