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Molecular phylogenetics: principles and practice

Ziheng Yang[1,2] *and Bruce Rannala*[1,3]

Abstract | Phylogenies are important for addressing various biological questions such as relationships among species or genes, the origin and spread of viral infection and the demographic changes and migration patterns of species. The advancement of sequencing technologies has taken phylogenetic analysis to a new height. Phylogenies have permeated nearly every branch of biology, and the plethora of phylogenetic methods and software packages that are now available may seem daunting to an experimental biologist. Here, we review the major methods of phylogenetic analysis, including parsimony, distance, likelihood and Bayesian methods. We discuss their strengths and weaknesses and provide guidance for their use.

Systematics
The inference of phylogenetic relationships among species and the use of such information to classify species.

Taxonomy
The description, classification and naming of species.

Coalescent
The process of joining ancestral lineages when the genealogical relationships of a random sample of sequences from a modern population are traced back.

Before the advent of DNA sequencing technologies, phylogenetic trees were used almost exclusively to describe relationships among species in systematics and taxonomy. Today, phylogenies are used in almost every branch of biology. Besides representing the relationships among species on the tree of life, phylogenies are used to describe relationships between paralogues in a gene family[1], histories of populations[2], the evolutionary and epidemiological dynamics of pathogens[3,4], the genealogical relationship of somatic cells during differentiation and cancer development[5] and the evolution of language[6]. More recently, molecular phylogenetics has become an indispensible tool for genome comparisons. In this context, it is used: to classify metagenomic sequences[7]; to identify genes, regulatory elements and non-coding RNAs in newly sequenced genomes[8–10]; to interpret modern and ancient individual genomes[11–13]; and to reconstruct ancestral genomes[14,15].

In other applications, the phylogeny itself may not be of direct interest but must nevertheless be accounted for in the analysis. This ‘tree thinking’ has transformed many branches of biology. In population genetics, the development of the coalescent theory[16,17] and the widespread availability of gene sequences for multiple individuals from the same species have prompted the development of genealogy-based inference methods, which have revolutionized modern computational population genetics. Here, the gene trees that describe the genealogy of sequences in a sample are highly uncertain; they are not of direct interest but nevertheless contain valuable information about parameters in the model. Tree thinking has also forged a deep synthesis of population genetics and phylogenetics, creating the emerging field of statistical phylogeography. In species tree methods[2,18,19], the gene trees at individual loci may not be of direct interest and may be in conflict with the species tree. By averaging over the unobserved gene trees under the multi-species coalescent model[20], those methods infer the species tree despite uncertainty in the gene trees. In comparative analysis, inference of associations between traits (for example, testis size and sexual promiscuity) using the observed traits of modern species should consider the species phylogeny to avoid misinterpreting historical contingencies as causal relationships[21]. In the inference of adaptive protein evolution, the phylogeny is used to trace the synonymous and nonsynonymous substitutions along branches to identify cases of accelerated amino acid change[22], even though the phylogeny is not of direct interest.

Nowadays, every biologist needs to know something about phylogenetic inference. However, to an experimental biologist who is unfamiliar with the field, the existence of many analytical methods and software packages might seem daunting. In this Review, we describe the suite of current methodologies for phylogenetic inference using sequence data. We also discuss various statistical criteria that are useful for choosing the methods that are best suited for a particular question and data type. Next-generation sequencing (NGS) technologies are generating huge data sets. In the analysis of such data sets, reducing systematic errors and increasing robustness to model violations are much more important than reducing random sampling errors. We discuss several issues in the analysis of large data sets, such as the

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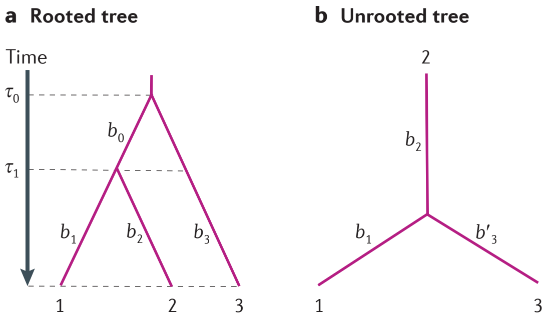
Box 1 | Tree concepts

A phylogeny is a model of genealogical history in which the lengths of the branches are unknown parameters. For example, the phylogeny on the left is generated by two speciation events that occurred at time points τ_0 and τ_1. The branch lengths (b_0, b_1, b_2 and b_3) are typically expressed in units of expected number of substitutions per site and measure the amount of evolution along the branches.

If the substitution rate is constant over time or among lineages, we say that the molecular clock holds[60]. The tree will then have a root and be ultrametric, meaning that the distances from the tips of the tree to the root are all equal (for example, $b_0 + b_1 = b_0 + b_2 = b_3$). A rooted tree for s species can then be represented by the ages of the $s - 1$ ancestral nodes and thus involves $s - 1$ branch-length parameters. The procedure of inferring rooted trees by assuming the molecular clock is called molecular clock rooting. For distantly related species, the clock hypothesis should not be assumed. Most phylogenetic analyses are therefore conducted without the assumption of the clock. If every branch on the tree is allowed to have an independent evolutionary rate, commonly used models and methods are unable to identify the location of the root, so only unrooted trees are inferred. An unrooted tree for s species then has $2s - 3$ branch length parameters. A commonly used strategy to 'root the tree' is to include outgroup species in the analysis, which are known to be more distantly related than the species of interest. Although the inferred tree for all species is unrooted, the root is believed to be located along the branch that leads to the outgroup so that the tree for the ingroup species is rooted. This strategy is called outgroup rooting.



impact of missing data and strategies of data partitioning. The literature of molecular phylogenetics is large and complex[23,24]; the aim of this Review is to provide a starting point for exploring the methods further.

Phylogenetic tree reconstruction: basic concepts

A phylogeny is a tree containing nodes that are connected by branches. Each branch represents the persistence of a genetic lineage through time, and each node represents the birth of a new lineage (BOX 1). If the tree represents the relationship among a group of species, then the nodes represent speciation events. In other contexts, the interpretation might be different. For example, in a gene tree of sequences sampled from a population, the nodes represent birth events of individuals who are ancestral to the sample, whereas in a tree of paralogous gene families, the nodes might represent gene duplication events.

Phylogenetic trees are not directly observed and are instead inferred from sequence or other data. Phylogeny reconstruction methods are either distance-based or character-based. In distance matrix methods, the distance between every pair of sequences is calculated, and the resulting distance matrix is used for tree reconstruction. For instance, neighbour joining[25] applies a cluster algorithm to the distance matrix to arrive at a fully resolved phylogeny. Character-based methods include maximum parsimony, maximum likelihood and Bayesian inference methods. These approaches simultaneously compare all sequences in the alignment, considering one character (a site in the alignment) at a time to calculate a score for each tree. The 'tree score' is the minimum number of changes for maximum parsimony, the log-likelihood value for maximum likelihood and the posterior probability for Bayesian inference. In theory, the tree with the best score should be identified by comparing all possible trees. In practice, because of the huge number of possible trees, such an exhaustive search is not computationally feasible except for very small data sets. Instead, heuristic tree search algorithms are used. These approaches often generate a starting tree using a fast algorithm and then perform local rearrangements to attempt to improve the tree score. A heuristic tree search is not guaranteed to find the best tree under the criterion, but it makes it feasible to analyse large data sets. To describe the data, distance matrix, maximum likelihood and Bayesian inference all make use of a substitution model and are therefore model-based, whereas maximum parsimony does not have an explicit model and its assumptions are implicit.

Distance matrix method

Distance calculation. Pairwise sequence distances are calculated assuming a Markov chain model of nucleotide substitution. Several commonly used models are illustrated in FIG. 1. The JC69 model[26] assumes an equal rate of substitution between any two nucleotides, whereas the K80 model[27] assumes different rates for transitions and transversions. Both models predict equal frequencies of the four nucleotides. The assumption of equal base frequencies is relaxed in the HKY85 model[28] and the general time reversible (GTR) model[29,30]. Because of the variation in local mutation rate and in selective constraint, different sites in a DNA or protein sequence often evolve at different rates. In distance calculation, such rate variation is accommodated by assuming a gamma (Γ) distribution of rates for sites[31], leading to models such as JC69 + Γ, HKY85 + Γ or GTR + Γ.

Distance matrix methods. After the distances have been calculated, the sequence alignment is no longer used in distance matrix methods. Here we mention three such methods: least squares, minimum evolution and neighbour joining. The least squares method[32] (see also

Gene trees
The phylogenetic or genealogical tree of sequences at a gene locus or genomic region.

Statistical phylogeography
The statistical analysis of population data from closely related species to infer population parameters and processes such as population sizes, demography, migration patterns and rates.

Species tree
A phylogenetic tree for a set of species that underlies the gene trees at individual loci.

Systematic errors
Errors that are due to an incorrect model assumption. They are exacerbated when the data size increases.

Random sampling errors
Errors or uncertainties in parameter estimates owing to limited data.

Cluster algorithm
An algorithm of assigning a set of individuals to groups (or clusters) so that objects of the same cluster are more similar to each other than those from different clusters. Hierarchical cluster analysis can be agglomerative (starting with single elements and successively joining them into clusters) or divisive (starting with all objects and successively dividing them into partitions).

Markov chain
A stochastic sequence (or chain) of states with the property that, given the current state, the probabilities for the next state do not depend on the past states.

Transitions
Substitutions between the two pyrimidines (T↔C) or between the two purines (A↔G).

Transversions
Substitutions between a pyrimidine and a purine (T or C↔A or G).

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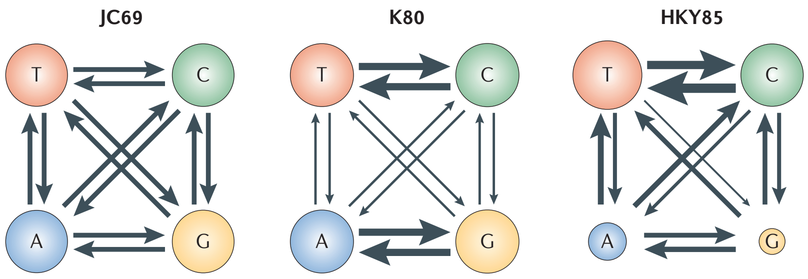

Figure 1 | **Markov models of nucleotide substitution.** The thickness of the arrows indicates the substitution rates of the four nucleotides (T, C, A and G), and the sizes of the circles represent the nucleotide frequencies when the substitution process is in equilibrium. Note that both JC69 and K80 predict equal proportions of the four nucleotides.

REF. 33) minimizes a measure of the differences between the calculated distances (d_{ij}) in the distance matrix and the expected distances ($\hat{d}_{ij}$) on the tree (that is, the sum of branch lengths on the tree linking the two species i and j):

$$Q = \sum_{i=1}^{s}\sum_{i=1}^{s}(\hat{d}_{ij} - d_{ij})^2 \qquad (1)$$

This is the same least squares method used in statistics for fitting a straight line $y = a + bx$ to a scatter plot. Optimizing branch lengths (or $\hat{d}_{ij}$) leads to the score Q for the given tree, and the tree with the smallest score is the least squares estimate of the true tree.

The minimum evolution method[34,35] uses the tree length (which is the sum of branch lengths) instead of Q for tree selection, even though the branch lengths can still be estimated using the least squares criterion. Under the minimum evolution criterion, shorter trees are more likely to be correct than longer trees are.

The most widely used distance method is neighbour joining[25]. This is a cluster algorithm and operates by starting with a star tree and successively choosing a pair of taxa to join together (based on the taxon distances), until a fully resolved tree is obtained. The taxa to be joined are chosen in order to minimize an estimate of tree length[36]. The two joined taxa (for example, species 1 and 2 in FIG. 2) are then represented by their ancestor (for example, node y in FIG. 2), and the number of taxa that are connected to the root (node x in FIG. 2) is reduced by one (FIG. 2). The distance matrix is then updated with the joined taxa replacing the two original taxa. See REF. 36 for a discussion of the neighbour joining updating formula. An efficient implementation of neighbour joining is found in the program MEGA[37] (TABLE 1).

Strengths and weaknesses of distance methods. One advantage of distance methods (especially of neighbour joining) is their computational efficiency. The cluster algorithm is fast because it does not need to compare as many trees under an optimality criterion as maximum parsimony and maximum likelihood do. For this reason, neighbour joining is useful for analysing large data sets that have low levels of sequence divergence. Note that it might be important to use a realistic substitution model to calculate the pairwise distances. Distance methods can perform poorly for very divergent sequences because large distances involve large sampling errors, and most distance methods (such as neighbour joining) do not account for the high variances of large distance estimates. Distance methods are also sensitive to gaps in the sequence alignment[38].

Maximum parsimony

Parsimony tree score. The maximum parsimony method minimizes the number of changes on a phylogenetic tree by assigning character states to interior nodes on the tree. The character (or site) length is the minimum number of changes required for that site, whereas the tree score is the sum of character lengths over all sites. The maximum parsimony tree is the tree that minimizes the tree score.

Some sites are not useful for tree comparison by parsimony. For example, constant sites, for which the same nucleotide occurs in all species, have a character length of zero on any tree. Singleton sites, at which only one of the species has a distinct nucleotide, whereas all others are the same, can also be ignored, as the character length is always one. The parsimony-informative sites are those at which at least two distinct characters are observed, each at least twice. For four species, only three site patterns are informative: xxyy, xyxy and xyyx, where x and y are any two distinct nucleotides. There are three possible unrooted trees for four species, and which of them is the maximum parsimony tree depends on which of the three site patterns occurs most often in the alignment.

An algorithm for finding the minimum number of changes on a binary tree (and for reconstructing the ancestral states to achieve the minimum) was developed by Fitch[39] and Hartigan[40]. PAUP[41], MEGA[37] and TNT[42] are commonly used parsimony programs.

Parsimony was originally developed for use in analysing discrete morphological characters. During the late 1970s, it began to be applied to molecular data. A controversy arose concerning whether parsimony (without explicit assumptions) or likelihood (with an explicit evolutionary model) was a better method for phylogenetic analysis[23]. The controversy has subsided, and the importance of model-based inference methods is broadly recognized. The use of parsimony is still common: not because it is believed to be assumption-free, but because it often produces reasonable results and is computationally efficient.

Strengths and weaknesses of parsimony. A strength of parsimony is its simplicity; it is easy to describe and to understand, and it is amenable to rigorous mathematical analysis. The simplicity also helps in the development of efficient computer algorithms.

A major weakness of parsimony is its lack of explicit assumptions, which makes it nearly impossible to incorporate any knowledge of the process of sequence evolution in tree reconstruction. The failure of parsimony to correct for multiple substitutions at the same site

Unrooted trees
Phylogenetic trees for which the location of the root is unspecified.

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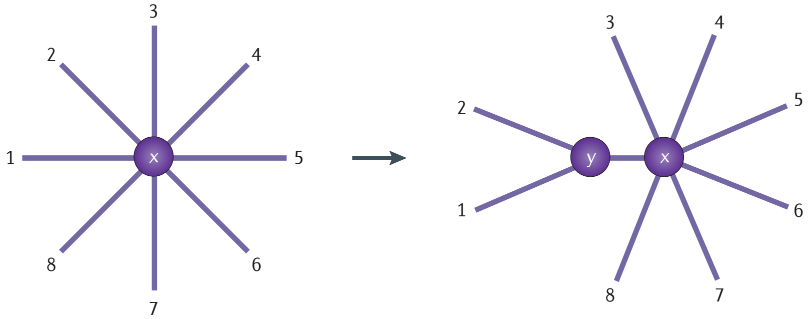

Figure 2 | **The neighbour joining algorithm.** The neighbour joining algorithm is a divisive cluster algorithm. It starts from a star tree: two nodes are then joined together on this tree (in this example, nodes 1 and 2), reducing the number of nodes at the root (node *x*) by one. The process is repeated until a fully resolved tree is generated.

makes it suffer from a problem known as long-branch attraction[43]. If the correct tree (T_1 in FIG. 3a) has two long external branches separated by a short internal branch, parsimony tends to infer the incorrect tree (T_2 in FIG. 3b), and the long branches are grouped together. When the branch lengths in T_1 are extreme enough, the probability for site pattern xxyy, which supports the correct tree T_1, may be smaller than that for xyxy, which supports the incorrect tree T_2. Thus, the more sites there are in the sequence, the more probable it is for the pattern xxyy to be observed at fewer sites than xyxy, and the more certain that the incorrect tree T_2 will be chosen to be the maximum parsimony tree. Parsimony thus converges to a wrong tree and is statistically inconsistent. Long-branch attraction has been demonstrated in many real and simulated data sets[44] and is due to the failure of parsimony to correct for multiple changes at the same site or to accommodate parallel changes on the two long branches. See REFS 24,45 for more discussions of the issue.

Note that model-based methods (namely, distance, likelihood and Bayesian methods) also suffer from long-branch attraction if the assumed model is too simplistic and ignores among-site rate variation[46]. In the reconstruction of deep phylogenies, long-branch attraction (as well as unequal nucleotide or amino acid frequencies among species) is an important source of systematic error[47,48] (FIG. 3c,d). In such analyses, it is advisable to use realistic substitution models and likelihood or Bayesian methodologies. Dense taxon sampling to break long branches and removing fast-evolving proteins or sites can also be helpful.

Long-branch attraction
The phenomenon of inferring an incorrect tree with long branches grouped together by parsimony or by model-based methods under simplistic models.

Maximum likelihood

Basis of maximum likelihood. Maximum likelihood was developed by R. A. Fisher in the 1920s as a statistical methodology for estimating unknown parameters in a model. The likelihood function is defined as the probability of the data given the parameters but is viewed as a function of the parameters with the data observed and fixed. It represents all information in the data about the parameters. The maximum likelihood estimates (MLEs) of parameters are the parameter values that maximize the likelihood. Most often, the MLEs are found numerically using iterative optimization algorithms. The MLEs have desirable asymptotic (large-sample) properties: they are unbiased, consistent (they approach the true values) and efficient (they have the smallest variance among unbiased estimates).

Maximum likelihood tree reconstruction. The first algorithm for maximum likelihood analysis of DNA sequence data was developed by Felsenstein[49]. The method is now widely used owing to the increased computing power and software implementations and to the development of increasingly realistic models of sequence evolution. Note that two optimization steps are involved in maximum likelihood tree estimation: optimization of branch lengths to calculate the tree score for each candidate tree and a search in the tree space for the maximum likelihood tree. From a statistical point of view, the tree (topology) is a model instead of a parameter, whereas branch lengths on the given tree and substitution parameters are parameters in the model. Maximum likelihood tree inference is thus equivalent to comparing many statistical models, each with the same number of parameters. The attractive asymptotic properties of MLEs mentioned above apply to parameter estimation when the true tree is given but not to the maximum likelihood tree[24,50].

Calculation of the likelihood on a given tree under various substitution models is explained in REFS 23,24. All substitution models used in distance calculation can be used here. Indeed, joint comparison of many sequences by likelihood makes it feasible to accommodate much more sophisticated models of sequence evolution. Most models used in molecular phylogenetics assume independent evolution of sites in the sequence so that the likelihood is a product of the probabilities for different sites. The probability at any particular site is an average over the unobserved character states at the ancestral nodes. Likelihood and parsimony analyses are similar in this respect, although parsimony only uses the optimal ancestral states, whereas likelihood averages over all possible states.

Early maximum likelihood implementations include PHYLIP[51], MOLPHY[52] and PAUP* 4.0 (REF. 41). Modern implementations, such as PhyML[53], RAxML[54] and GARLI[55], are not only computationally much faster but are also more effective in finding trees with high likelihood scores. The recent inclusion of maximum likelihood in MEGA 5 (REF. 37) has made the method more accessible to biologists who are not experienced computer users (TABLE 1).

Strengths and weaknesses of the maximum likelihood method. One advantage of the maximum likelihood method is that all of its model assumptions are explicit, so that they can be evaluated and improved. The availability of a rich repertoire of sophisticated evolutionary models in the likelihood (and Bayesian) method is one of its major advantages over maximum parsimony. Modern inferences of deep phylogenies using conserved proteins almost exclusively rely on likelihood and Bayesian methods. For such inference, it is important

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Table 1 | **Functionalities of a few commonly used phylogenetic programs**

Name	Brief description	Link	Refs
Bayesian evolutionary analysis sampling trees (BEAST)	A Bayesian MCMC program for inferring rooted trees under the clock or relaxed-clock models. It can be used to analyse nucleotide and amino acid sequences, as well as morphological data. A suite of programs, such as Tracer and FigTree, are also provided to diagnose, summarize and visualize results	http://beast.bio.ed.ac.uk	135
Genetic algorithm for rapid likelihood inference (GARLI)	A program that uses genetic algorithms to search for maximum likelihood trees. It includes the GTR + Γ model and special cases and can analyse nucleotide, amino acid and codon sequences. A parallel version is also available	http://code.google.com/p/garli	55
Hypothesis testing using phylogenies (HYPHY)	A maximum likelihood program for fitting models of molecular evolution. It implements a high-level language that the user can use to specify models and to set up likelihood ratio tests	http://www.hyphy.org	136
Molecular evolutionary genetic analysis (MEGA)	A Windows-based program with a full graphical user interface that can be run under Mac OSX or Linux using Windows emulators. It includes distance, parsimony and likelihood methods of phylogeny reconstruction, although its strength lies in the distance methods. It incorporates the alignment program ClustalW and can retrieve data from GenBank	http://www.megasoftware.net	37
MrBayes	A Bayesian MCMC program for phylogenetic inference. It includes all of the models of nucleotide, amino acid and codon substitution developed for likelihood analysis	http://mrbayes.net	71
Phylogenetic analysis by maximum likelihood (PAML)	A collection of programs for estimating parameters and testing hypotheses using likelihood. It is mostly used for tests of positive selection, ancestral reconstruction and molecular clock dating. It is not appropriate for tree searches	http://abacus.gene.ucl.ac.uk/software	137
Phylogenetic analysis using parsimony* and other methods (PAUP* 4.0)	PAUP* 4.0 is still a beta version (at the time of writing). It implements parsimony, distance and likelihood methods of phylogeny reconstruction	http://www.sinauer.com/detail.php?id=8060	
PHYLIP	A package of programs for phylogenetic inference by distance, parsimony and likelihood methods	http://evolution.gs.washington.edu/phylip.html	
PhyML	A fast program for searching for the maximum likelihood trees using nucleotide or protein sequence data	http://www.atgc-montpellier.fr/phyml/binaries.php	53
RAxML	A fast program for searching for the maximum likelihood trees under the GTR model using nucleotide or amino acid sequences. The parallel versions are particularly powerful	http://scoh-its.org/exelixis/software.html	54
Tree analysis using new technology (TNT)	A fast parsimony program intended for very large data sets	http://www.zmuc.dk/public/phylogeny/TNT	42

Note: all programs can run on Windows, Mac OSX and Unix or Linux platforms. Except for PAUP*, which charges a nominal fee, all packages are free for download. See Felsenstein's comprehensive list of programs at http://evolution.genetics.washington.edu/phylip/software.html. GTR, general time reversible; MCMC, Markov chain Monte Carlo.

for the model to accommodate variable amino acid substitution rates among sites[56] or even different amino acid frequencies among sites[57,58].

Maximum likelihood has a clear advantage over distance or parsimony methods if the aim is to understand the process of sequence evolution. The likelihood ratio test can be used to examine the fit of evolutionary models[59] and to test interesting biological hypotheses, such as the molecular clock[60,49] and Darwinian selection affecting protein evolution[61–63]. See REFS 22,24,64,65 for summaries of such tests in phylogenetics.

The main drawback of maximum likelihood is that the likelihood calculation and, in particular, tree search under the likelihood criterion is computationally demanding. Another drawback is that the method has potentially poor statistical properties if the model is misspecified. This is also true for Bayesian analysis (TABLE 2).

Bayesian methods

Basis of Bayesian inference. Bayesian inference is a general methodology of statistical inference. It differs from maximum likelihood in that parameters in the model are considered to be random variables with statistical distributions, whereas in maximum likelihood they are unknown fixed constants. Before the analysis of the data, parameters are assigned a prior distribution, which is combined with the data (or likelihood) to generate the posterior distribution. All inferences concerning the parameters are then based on the posterior distribution. In the past two decades, Bayesian inference has gained popularity thanks to advances in computational methods, especially Markov chain Monte Carlo algorithms (MCMC algorithms).

Bayesian phylogenetics. Bayesian inference was introduced to molecular phylogenetics in the late 1990s[66–69]. The early methods assumed a molecular clock[60]. Development of more efficient MCMC algorithms[70] that eliminate the clock assumption (allowing independent branch lengths on unrooted trees) and the release of the program MrBayes[71] made the method popular among molecular systematists. A more recent Bayesian implementation in the program BEAST[72] uses the so-called relaxed-clock models to infer rooted trees even though the model allows substitution rates to vary across lineages (TABLE 1).

Likelihood ratio test
A general hypothesis-testing method that uses the likelihood to compare two nested hypotheses, often using the χ^2 distribution to assess significance.

Molecular clock
The hypothesis or observation that the evolutionary rate is constant over time or across lineages.

Prior distribution
The distribution assigned to parameters before the analysis of the data.

Posterior distribution
The distribution of the parameters (or models) conditional on the data. It combines the information in the prior and in the data (likelihood).

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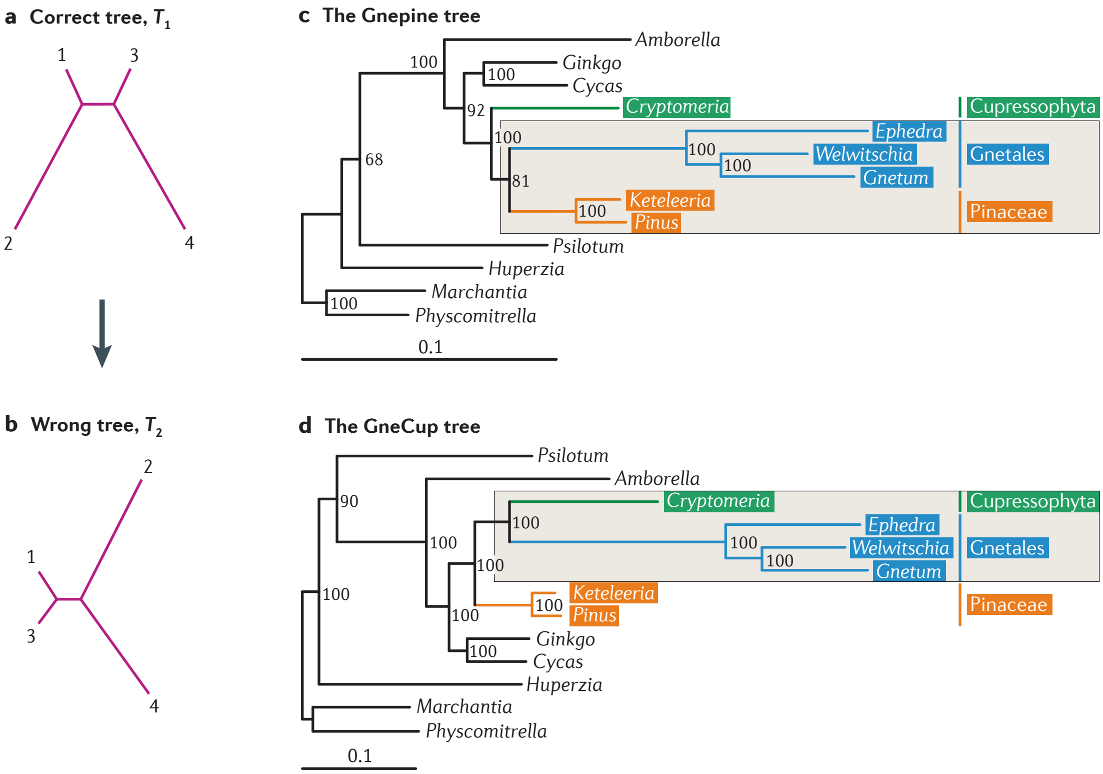

Figure 3 | **Long-branch attraction in theory and in practice.** Panels **a** and **b** show the four-species case analysed by Felsenstein[43]. If the correct tree (T_1 in **a**) has two long branches separated by a short internal branch, parsimony (as well as model-based methods such as likelihood and Bayesian methods under simplistic models) tends to recover a wrong tree (T_2 in **b**), in which the two long branches are grouped together. Panels **c** and **d** show a similar phenomenon in a real data set, concerning the phylogeny of seed plants[134]. The Gnetales is a morphologically and ecologically diverse group of Gymnosperms including three genera (*Ephedra*, *Gnetum* and *Welwitschia*), but its phylogenetic position has been controversial. Maximum likelihood analysis of 56 chloroplast proteins produced the GneCup tree (**d**), in which the Gnetales are grouped with Cupressophyta, apparently owing to a long-branch attraction artefact. However, the Gnepine tree (**c**), in which the Gnetales joins the Pinaceae, was inferred by excluding the fastest-evolving 18 proteins as well as three proteins (namely, psbC, rpl2 and rps7) that had experienced many parallel substitutions between the Cryptomeria branch and the branch ancestral to the Gnetales. The Gnepine tree (**c**) is also supported by two proteins from the nuclear genome and appears to be the correct tree. Branch lengths and bootstrap proportions are all calculated using RAxML. See REF. 134 for details.

Bayesian inference relies on Bayes's theorem, which states that

$$P(T,\theta|D) = \frac{P(T,\theta)P(D|T,\theta)}{P(D)} \quad (2)$$

where $P(T,\theta)$ is the prior probability for tree T and parameter θ, $P(D|T,\theta)$ is the likelihood or probability of the data given the tree and parameter, and $P(T,\theta|D)$ is the posterior probability. The denominator $P(D)$ is a normalizing constant, as its role is to ensure that $P(T,\theta|D)$ sums over the trees and integrates over the parameters to one. The theorem states that the posterior is proportional to the prior times the likelihood, or the posterior information is the prior information plus the data information.

In general, the posterior probabilities of trees cannot be directly calculated. In particular, the normalizing constant $P(D)$ involves high-dimensional integrals (over all possible parameter θ values) and summation over all possible trees. Instead, Bayesian phylogenetic inference relies on MCMC algorithms to generate a sample from the posterior distribution. This is illustrated in BOX 2. See Chapter 5 of REF. 24 for an introduction to MCMC.

Strengths and weaknesses of the Bayesian inference method. Both likelihood and Bayesian methods use the likelihood function and thus share many statistical properties, such as consistency and efficiency. However, maximum likelihood and Bayesian inference represent opposing philosophies of statistical inference. The same feature of Bayesian inference may thus be viewed as either a strength or a weakness, depending on one's philosophy. See REF. 24 for a brief description of the controversy. Here we comment on two issues relating to the interpretability of the results and to the practicalities of incorporating prior information in the model.

First, Bayesian statistics is known to answer the biological questions directly and yields results that are easy to interpret: the posterior probability of a tree is simply the probability that the tree is correct, given the

Markov chain Monte Carlo algorithms
(MCMC algorithms). A Monte Carlo simulation is a computer simulation of a biological process using random numbers. An MCMC algorithm is a Monte Carlo simulation algorithm that generates a sample from a target distribution (often a Bayesian posterior distribution).

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Table 2 | **A summary of strengths and weaknesses of different tree reconstruction methods**

Strengths	Weaknesses
Parsimony methods	
• Simplicity and intuitive appeal • The only framework appropriate for some data (such as SINES and LINES)	• Assumptions are implicit and poorly understood • Lack of a model makes it nearly impossible to incorporate our knowledge of sequence evolution • Branch lengths are substantially underestimated when substitution rates are high • Maximum parsimony may suffer from long-branch attraction
Distance methods	
• Fast computational speed • Can be applied to any type of data as long as a genetic distance can be defined • Models for distance calculation can be chosen to fit data	• Most distance methods, such as neighbour joining, do not consider variances of distance estimates • Distance calculation is problematic when sequences are divergent and involve many alignment gaps • Negative branch lengths are not meaningful
Likelihood methods	
• Can use complex substitution models to approach biological reality • Powerful framework for estimating parameters and testing hypotheses	• Maximum likelihood iteration involves heavy computation • The topology is not a parameter so that it is difficult to apply maximum likelihood theory for its estimation. Bootstrap proportions are hard to interpret
Bayesian methods	
• Can use realistic substitution models, as in maximum likelihood • Prior probability allows the incorporation of information or expert knowledge • Posterior probabilities for trees and clades have easy interpretations	• Markov chain Monte Carlo (MCMC) involves heavy computation • In large data sets, MCMC convergence and mixing problems can be hard to identify or rectify • Uninformative prior probabilities may be difficult to specify. Multidimensional priors may have undue influence on the posterior without the investigator's knowledge • Posterior probabilities often appear too high • Model selection involves challenging computation[138,139]

data and model. By contrast, concepts such as the confidence interval in a likelihood analysis have a contrived interpretation that eludes many users of statistics. In phylogenetics, it has not been possible to define a confidence interval for the tree. The widely used bootstrap method[73] (BOX 3) has been difficult to interpret despite numerous efforts[74–77]. However, the odds are not entirely against maximum likelihood. Posterior probabilities for trees and clades that have been calculated from real data sets often appear to be too high[66,78–80]. In many analyses, nearly all nodes had posterior probabilities of ~100%. Posterior tree probabilities are also sensitive to model violations, and use of simplistic models may lead to inflated posterior probabilities[81].

Second, the prior probability allows incorporation of *a priori* information about the trees or parameters. However, such information is rarely available, and specification of the prior is most often a burden on the user; almost all data analyses are conducted using the 'default' priors in the computer program. High-dimensional priors are notoriously hard to specify, and an innocent-looking prior can have an undue and unexpected influence on the posterior. For example, it has recently been pointed out that the independent exponential prior on branch lengths used by MrBayes can induce a strongly informative and unreasonable prior on the tree length, producing unreasonably long trees in some data sets[82–84]. It is therefore important to conduct Bayesian robustness analysis to assess the impact of the prior on the posterior estimates.

Clades
Groups of species that have descended from a common ancestor.

Statistical assessments of phylogenetic methods

The aim of phylogenetic inference is to estimate the tree topology and possibly also the branch lengths. Four criteria have been used to judge tree reconstruction methods.

Consistency. An estimation method is said to be consistent if the estimate converges to the true parameter value when the amount of data approaches infinity. A tree reconstruction method is consistent if the estimated tree converges to the true tree when the number of sites in the sequence grows. Model-based methods (that is, distance matrix, maximum likelihood and Bayesian inference) are consistent if the assumed model is correct. Parsimony may be inconsistent under some model tree combinations; Felsenstein's[43] demonstration of this has spurred much heated discussion.

Efficiency. In the statistical estimation of a parameter, an unbiased estimate with a smaller variance is more efficient than one with a larger variance. In phylogenetics, efficiency may be measured by the probability of recovering the correct tree or subtree given the number of sites. This can be estimated by computer simulation. The complexity of tree reconstruction means that the asymptotic theory of MLEs does not apply. Nevertheless, computer simulations have generally found a higher efficiency of maximum likelihood than maximum parsimony or neighbour joining in recovering the correct tree[23].

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Box 2 | Markov chain Monte Carlo

Markov chain Monte Carlo (MCMC) is a simulation algorithm in which one moves from one tree (or parameter value) to another and, in the long run, visits the trees (or parameters) in proportion to their posterior probabilities. The tree parameter set (T,θ) constitutes the state of the algorithm. Here, parameters θ may include the branch lengths of the tree and parameters in the evolutionary model, such as the transition/transversion rate ratio. The following scheme demonstrates the main features of MCMC algorithms.

Step 1. Initialization. Choose a starting tree and starting parameters at random (T,θ).

Step 2. Main loop.

- Step 2a. Proposal to change the tree T. Propose a new tree, T*, by changing the current tree, T. If T* has higher posterior probability than the current tree, $P(T^*,\theta|D) > P(T,\theta|D)$, accept the new tree T*. Otherwise, accept T* with probability:

$$\frac{P(T^*,\theta|D)}{P(T,\theta|D)} = \frac{P(T^*,\theta)P(D|T^*,\theta)}{P(T,\theta)P(D|T,\theta)} \tag{3}$$

  If T* is accepted, set $T = T^*$.

- Step 2b. Proposal to change parameters θ. Propose new parameter value, θ*, by changing the current θ. Here, for simplicity, we assume that the proposals are symmetrical so that the probability of proposing θ* from θ equals the probability of proposing θ from θ*. If $P(T,\theta^*|D) > P(T,\theta|D)$, accept new θ*. Otherwise, accept θ* with probability:

$$\frac{P(T,\theta^*|D)}{P(T,\theta|D)} = \frac{P(T,\theta^*)P(D|T,\theta^*)}{P(T,\theta)P(D|T,\theta)} \tag{4}$$

  If the new θ* is accepted, set $\theta = \theta^*$.

- Step 2c. Sample from the chain. Print out (T,θ).

Note that first the algorithm does not need calculation of the normalizing constant P(D), as it cancels in the posterior ratios in proposal steps 2a and 2b. Second, in the long run, a tree parameter set (T_1,θ_1) will be visited more often by the algorithm than another set (T_2,θ_2) if its posterior probability is higher: $P(T_1,\theta_1|D) > P(T_2,\theta_2|D)$. Indeed, the expected proportion of time that the algorithm spends in any tree T is exactly its posterior probability: $P(T|D)$. Thus, by counting the frequencies at which each tree is visited in the algorithm, we get an MCMC estimate of the posterior probabilities for the trees.

The sequence (or chain) of values for (T,θ) generated by the algorithm has the property that, given the current state (T,θ), the probabilities by which it moves to new states do not depend on past states. This memory-less property is known as the Markovian property, which states that given the present, the future does not depend on the past. The generated sequence is called a Markov chain. The algorithm is called Markov chain Monte Carlo because the Markov chain is generated by Monte Carlo simulation.

Robustness. A method is robust if it gives correct answers even when its assumptions are violated. Some assumptions matter more than others. With the rapid accumulation of sequence data, sampling errors in tree reconstruction are considerably reduced, so systematic errors or robustness of the method become more important.

Computational speed. This property is easy to assess. Neighbour joining uses a cluster algorithm to arrive at a tree and is very fast. Methods that search for the best tree under a criterion, such as maximum evolution, maximum parsimony and maximum likelihood, are slower. The computational speed of the Bayesian method depends on the length of the chain (generated by MCMC algorithms), which is highly data-dependent. As calculation of the phylogenetic likelihood is expensive, maximum likelihood and Bayesian inference are typically slower than maximum parsimony. Nevertheless, considerable advancements in computational algorithms[53–55] have made likelihood-based methods feasible for the analysis of large data sets. Algorithms that take advantage of new computers with multicore processors and graphical processing units (GPUs)[85,86] are pushing the boundary even further.

Graphical processing units (GPU). Specialized units that are traditionally used to manipulate output on a video display and have recently been explored for use in parallel computation.

Phylogenomic analysis of large data sets

With the advent of new sequencing technologies and the completion of various genome projects, phylogenetics has entered the era of genome-scale data sets. Here we discuss a few issues relating to the analysis of such large data sets.

Supertree and supermatrix approaches. Two approaches have been advocated for the phylogenetic analysis of hundreds or thousands of genes or proteins, especially when some loci are missing in some species. The supertree approach separately analyses each gene and then uses heuristic algorithms to assemble the subtrees for individual genes into a supertree for all species[87]. The separate analysis is useful for studying the differences in the reconstructed subtrees or the prevalence of horizontal gene transfer. However, it is inefficient for estimating a common phylogeny that underlies all genes.

In the supermatrix approach, sequences for multiple genes are concatenated to generate a data supermatrix, in which missing data are replaced by question marks, and the supermatrix is then used for tree reconstruction[88]. Most supermatrix analyses ignore differences in evolutionary dynamics among the genes. Note that a supermatrix analysis that assumes different evolutionary models and different trees and branch lengths for the genes is equivalent to a separate or supertree analysis. When a common tree underlies all genes, the ideal approach should be a combined (supermatrix) analysis of all genes, using the likelihood to accommodate the among-gene heterogeneity in the evolutionary process[89–91]. Our comments below relate to this combined approach.

Impact of missing data. Many genomic data sets are highly incomplete, and so most cells in the species by gene matrix will be empty. Although, in theory, the likelihood function (in the maximum likelihood and Bayesian methods) can properly accommodate missing data[23,24], the impact of such large-scale missing data and alignment gaps is not well-understood. Simulations suggest that maximum likelihood and Bayesian inference generally perform better than neighbour joining or maximum parsimony in dealing with missing data, and Bayesian inference was found to perform the best[92–94]. The poor performance of neighbour joining may be understood if one considers extreme cases in which, after removal of alignment gaps, the pairwise distances are calculated from different sets of genes or sites, some of which are fast-evolving, whereas others are slowly evolving.

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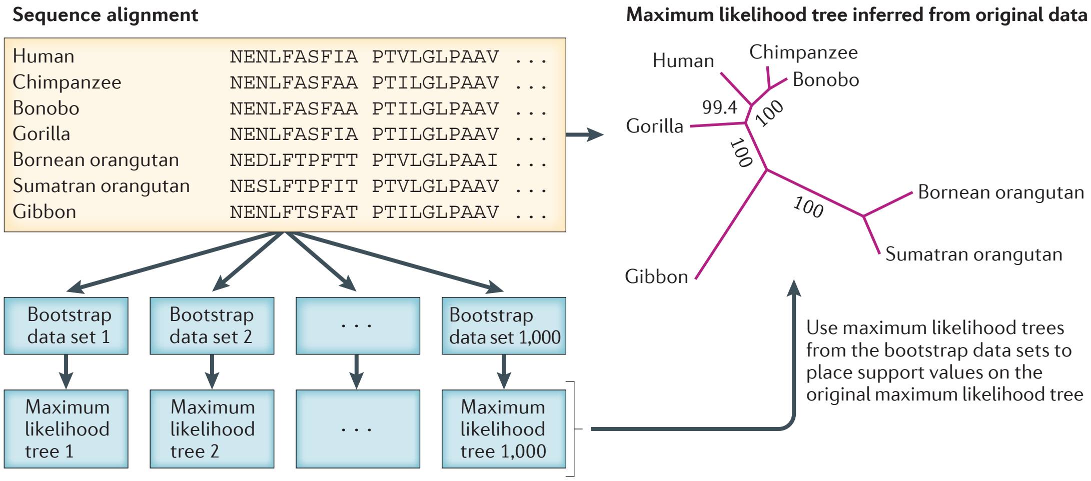
Box 3 | Sampling error in the estimated tree and bootstrap analysis


Sequence alignment
Human NENLFASFIA PTVLGLPAAV ...
Chimpanzee NENLFASFAA PTILGLPAAV ...
Bonobo NENLFASFAA PTILGLPAAV ...
Gorilla NENLFASFIA PTILGLPAAV ...
Bornean orangutan NEDLFTPFTT PTVLGLPAAI ...
Sumatran orangutan NESLFTPFIT PTVLGLPAAV ...
Gibbon NENLFTSFAT PTILGLPAAV ...
Bootstrap data set 1
Bootstrap data set 2
. . .
Bootstrap data set 1,000
Maximum likelihood tree 1
Maximum likelihood tree 2
. . .
Maximum likelihood tree 1,000
Maximum likelihood tree inferred from original data
Human
Chimpanzee
Bonobo
Gorilla
99.4
100
100
100
Bornean orangutan
Sumatran orangutan
Gibbon
Use maximum likelihood trees from the bootstrap data sets to place support values on the original maximum likelihood tree

In traditional parameter estimation, we attach a confidence interval to indicate the uncertainty involved in the point estimate of the parameter. This has not been possible in molecular phylogenetics, as concepts such as the variance and confidence interval are not meaningful when applied to trees. For distance, parsimony and likelihood methods, the most commonly used procedure to assess the confidence in a tree topology estimate is the bootstrap analysis[73]. In this approach, the sites in the sequence alignment are resampled with replacement as many times as the sequence length, generating a bootstrap pseudo-sample that is of the same size as the original data set. Typically, 100 or 1,000 bootstrap samples are generated in this way, and each one is analysed in the same way as the original sequence alignment. An example that uses the maximum likelihood method is illustrated in the figure. The inferred trees from those bootstrap samples are then tabulated to calculate the bootstrap support values. For every clade in the estimated tree, its bootstrap support value is simply the proportion of bootstrap trees that include that clade[24,65,133]. The commonly used but less satisfactory approach is to use the bootstrap trees to generate a majority-rule consensus tree, which shows a clade if — and only if — it occurs in more than half of the bootstrap trees.

Importance of systematic errors. In the analysis of very large data sets, almost all bootstrap support values or Bayesian posterior probabilities are calculated to be 100%, even though the inferred phylogenies might be conflicting across genes or might depend on the method and model used[47]. Systematic biases are thus much more important than random sampling errors in such analyses, and methods that are robust to violations of model assumptions, even if they are less efficient, should be preferable.

Data-partitioning strategies. The rationale for data partitioning is to group genes or sites with similar evolutionary characteristics into the same partition so that all sites in the same partition are described using the same model, and different partitions use different models[89,90]. Partitioning too finely increases computation time and can cause over-fitting, but partitioning too coarsely may lead to under-fitting or model violation. However, the situation is complicated, as some models instead allow random variation among sites in substitution rate[31,56,61], in amino acid frequencies[57,58,95] or in the pattern of substitution[96]. Such mixture models use a statistical distribution to accommodate the among-site heterogeneity without data partitioning. Often, the choice between using partition or mixture models is a philosophical one: it corresponds, respectively, to the preference for fixed-effects models or random-effects models in statistics.

Current strategies for data partitioning include partitioning genes according to their relative substitution rates[97] and separating the three codon positions in coding genes into different partitions[89]. The likelihood ratio test has also been used to decide whether two genes should be in the same or different partitions[98]. In summary, data partitioning is more of an art than a science, and it should rely on our knowledge of the biological system: for example, on whether it is reasonable to assume that the same phylogeny underlies all genes.

Perspectives

We focus here on three research areas that are currently the focus of much methodological development. The first is multiple sequence alignment. Many heuristic methods and programs for aligning sequences exist[99,100], and improved algorithms continue to appear[101,102]. Efforts have also been taken to infer alignment statistically under an explicit model of insertions and deletions[103,104] and to infer alignment and phylogeny jointly in a Bayesian framework[105,106]. An advantage of those model-based alignment methods is that they produce estimates of insertion and deletion rates. For now, those algorithms are based on simplistic insertion–deletion models and involve heavy computation, and so they do not compare favourably against good heuristic algorithms either in computational efficiency or alignment quality. Nevertheless, they are biologically appealing, and improvements are very likely.

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The second area of development is molecular clock estimation of divergence dates. Under the clock assumption, the distance between sequences increases linearly with the time of divergence, and if a particular divergence can be assigned an absolute geological age based on the fossil record, the substitution rate can be calculated, and all divergences on the tree can be dated. Similar ideas can be used to estimate divergence times of viral strains when sample dates for viral sequences are available and act as calibrations. However, in practice, the molecular clock may be violated, especially for distantly related species, and the fossil record can never provide unambiguous times of lineage divergence. In the past several years, advancements have been made using the Bayesian framework to deal with those issues. Since the pioneering work of Thorne and colleagues[107,108], models of evolutionary rate drift over time have been developed to relax the molecular clock[72,109]. Soft age bounds and flexible probability distributions have been implemented to accommodate uncertainties in fossil calibrations[72,110,111]. The fossil record (that is, the presence and absence of fossils in the rock layers) has also been statistically analysed to generate calibration densities for molecular dating analysis[112,113].

The third area of exciting development, which was mentioned at the beginning of this Review, is statistical phylogeography[20,114–116]. The availability of genomic data at species and population levels offers unprecedented opportunities for addressing interesting questions in evolutionary biology. Multi-locus sequence data can be used to estimate divergence times between closely related species and the sizes of both extant and extinct populations[117,118] to infer population demographic changes and to estimate migration patterns and rates[119,120]. Such data can also be used to delimit species (that is, to determine whether a population consists of one or two species, for example)[121,122]. The past few years have seen the appearance of many individual genome sequences and the rise of population genomics. Currently, the data are mostly from humans and their close relatives, but genomes from other species are being sequenced as well, such as mastodons and mammoths[123] and the bacterium *Yersinia pestis* from Black Death victims[124]. Genomic sequence data from humans and apes are used to infer the species divergence times and to test for possible hybridization during the human–chimpanzee separation[125–131]. Comparison of a few human individual genomes provides insights into the recent demographic history of our species[12,13], whereas sequencing of the Neanderthal genome allows estimation of the Neanderthal contribution to the genome of modern humans[11,132]. The size of the data and the complexity of the model pose great statistical and computational challenges. Again, Bayesian MCMC algorithms, under the multi-species coalescent model[118], provide the natural framework for inference.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

Ziheng Yang's homepage: http://abacus.gene.ucl.ac.uk
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A comprehensive list of phylogenetic programs maintained by Joe Felsenstein: http://evolution.genetics.washington.edu/phylip/software.html
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