User Guide

PAML:

Phylogenetic Analysis
by Maximum Likelihood

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Suggested citations:

Yang, Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood *Computer Applications in BioSciences* **13**:555-556.

Yang, Z. 2007. PAML 4: a program package for phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution* **24**: 1586-1591 (`http://abacus.gene.ucl.ac.uk/software/paml.html`).

Recent changes and bug fixes are documented in the file doc/pamlHistory.txt.

The author can be reached at the following address. I am afraid that I am unable to respond to emails now. Please post your questions and comments at the google discussion site for PAML.

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1 Overview

PAML (for Phylogenetic Analysis by Maximum Likelihood) is a package of programs for phylogenetic analyses of DNA and protein sequences using maximum likelihood.

PAML documentation

Besides this manual, please note the following resources:

- PAML web site: http://abacus.gene.ucl.ac.uk/software/PAML.html has information about downloading and compiling the programs.

- PAML FAQ page: http://abacus.gene.ucl.ac.uk/software/pamlFAQs.pdf

- PAML discussion group at http://www.rannala.org/phpBB2/, where you can post bug reports and questions.

What PAML programs can do

The PAML package currently includes the following programs: baseml, basemlg, codeml, evolver, pamp, yn00, mcmctree, and chi2. A brief overview of the most commonly used models and methods implemented in PAML is provided by Yang (2007). The book (Yang 2006) describes the statistical and computational details. Examples of analyses that can be performed using the package include

- Comparison and tests of phylogenetic trees (baseml and codeml);

- Estimation of parameters in sophisticated substitution models, including models of variable rates among sites and models for combined analysis of multiple genes or site partitions (baseml and codeml);

- Likelihood ratio tests of hypotheses through comparison of implemented models (baseml, codeml, chi2);

- Estimation of divergence times under global and local clock models (baseml and codeml);

- Likelihood (Empirical Bayes) reconstruction of ancestral sequences using nucleotide, amino acid and codon models (baseml and codeml);

- Generation of datasets of nucleotide, codon, and amino acid sequence by Monte Carlo simulation (evolver);

- Estimation of synonymous and nonsynonymous substitution rates and detection of positive selection in protein-coding DNA sequences (yn00 and codeml).

- Bayesian estimation of species divergence times incorporating uncertainties in fossil calibrations (mcmctree).

The strength of PAML is its collection of sophisticated substitution models. Tree search algorithms implemented in baseml and codeml are rather primitive, so except for very small data sets with say, <10 species, you are better off to use another package, such as phylip, paup, or mrBayes, to infer the tree topology. You can get a collection of trees from other programs and evaluate them using baseml or codeml as user trees.

baseml and codeml. The program baseml is for maximum likelihood analysis of nucleotide sequences. The program codeml is formed by merging two old programs: codonml, which implements the codon substitution model of Goldman and Yang (1994) for protein-coding DNA sequences, and aaml, which implements models for amino acid sequences. These two are now distinguished by the variable seqtype in the control file codeml.ctl, with 1 for codon sequences and 2 for amino acid sequences. In this document I use codonml and aaml to mean codeml with seqtype = 1 and 2, respectively. The programs baseml, codonml, and

aaml use similar algorithms to fit models by maximum likelihood, the main difference being that the unit of evolution in the Markov model, referred to as a "site" in the sequence, is a nucleotide, a codon, or an amino acid for the three programs, respectively. Markov process models are used to describe substitutions between nucleotides, codons or amino acids, with substitution rates assumed to be either constant or variable among sites.

evolver. This program can be used to simulate sequences under nucleotide, codon and amino acid substitution models. It also has some other options such as generating random trees, and calculating the partition distances (Robinson and Foulds 1981) between trees.

basemlg. This program implements the (continuous) gamma model of Yang (1993). It is very slow and unfeasible for data of more than 6 or 7 species. Instead the discrete-gamma model in baseml should be used.

mcmctree. This implements the Bayesian MCMC algorithm of Yang and Rannala (2006) and Rannala and Yang (2007) for estimating species divergence times.

pamp. This implements the parsimony-based analysis of Yang and Kumar (1996).

yn00. This implements the method of Yang and Nielsen (2000) for estimating synonymous and nonsynonymous substitution rates (d_S and d_N) in pairwise comparisons of protein-coding DNA sequences.

chi2. This calculates the χ^2 critical value and p value for conducting the likelihood ratio test. Run the program by typing the program name "chi2": it will print out the critical values for different df (for example the 5% critical value with d.f. = 1 is 3.84). If you run the program with one command-line argument, the program enters a loop to ask you to input the d.f. and the test statistic and then calculates the p value. A third way of running the progam is to include the d.f. and test statistic both as command-line argument.

```
chi2
chi2 p
chi2 1 3.84
```

What PAML programs cannot do

There are many things that you might well expect a phylogenetics package should do but PAML cannot. Here is a partial list, provided in the hope that it might help you avoid wasting time.

- Sequence alignment. You should use some other programs such as Clustal or TreeAlign to align the sequences automatically or do a manual alignment, perhaps with assistance from programs such as BioEdit and GeneDoc. Manual adjustment does not seem to have reached the mature stage to be entirely trustable so you should always do manual adjustment if you can. If you are constructing thousands of alignments in genome-wide analysis, you should implement some quality control, and, say, calculate some measure of sequence divergence as an indication of the unreliability of the alignment. For coding sequences, you might align the protein sequences and construct the DNA alignment based on the protein alignment. Note that alignment gaps are treated as missing data in baseml and codeml (if `cleandata` = 1). If `cleandata` = 1, all sites with ambiguity characters and alignment gaps are removed.

- Gene prediction. The codon-based analysis implemented in codonml (codeml for codons with seqtype = 1) assumes that the sequences are pre-aligned exons, the sequence length is an exact multiple of 3, and the first nucleotide in the sequence is codon position 1. Introns, spacers and other noncoding regions must be removed and the coding sequences must be aligned before running the program. The program cannot process sequences downloaded directly from GenBank, even though the CDS information is there. It cannot predict coding regions either.

- Tree search in large data sets. As mentioned earlier, you should use another program to get

a tree or some candidate trees and use them as user trees to fit models that might not be available in other packages.

2 Compiling and punning PAML programs

PAML programs use the old simple command-line interface. You download the archive from the PAML web site, typically named PAML*.*.tgz, and unpack the files onto your hard disk. This is one file for all platforms. Executables for windows are included, while for UNIX or MAC OS X, you need compile the programs before running them.

Windows

The executables for Windows are included in the package.

1. Go to the PAML web site http://abacus.gene.ucl.ac.uk/software/paml.html and download the latest archive and save it on your hard disk. Unpack, say, using WinZip, the archive into a folder, say D:\software\paml\. Remember the name of the folder.

2. Start a "Command Prompt". Go to "Start – Programs – Accessories". Alternatively, choose "Start – Run" and type the command **cmd** and hit OK. You can right click on the title bar to change the font, colour, size etc. of the window.

3. Change directory to the paml folder. For example you type one of the following.

   ```
   d:
   cd \software\paml
   dir
   ```

4. Note that Windows commands and file names are case-insensitive. The folder src\ contains the source files. The examples\ contains various example files, and bin\ contains Windows executables. You can use Windows Explorer to look at the files. To run the program baseml using the default control file baseml.ctl in the current folder, you can a command somewhat like the following.

   ```
   bin\baseml
   ```

   ```
   D:\software\paml4\bin\baseml
   ```

   This causes baseml to read the default control file baseml.ctl in the current folder and do the analysis according to its specifications. Now you can print out a copy of baseml.ctl, and open a text editor to view the relevant sequence and tree files.
   Similarly you can run codeml and look at the control file codeml.ctl.

Next you can prepare your own sequence data files and tree files. Control files and other input files are all plain text files. A common problem occurs due to differences in the way UNIX and Windows deal with carriage return or line breaks. If you use MS Word to prepare the input files, you should save them as "Text with line breaks" or "Text without line breaks". Sometimes only one of those two works. Do not save the file as a Word document. I have collected some notes in the section "Overcoming Windows Annoyances" in Appendix B.

If you insist on double-clicking, you can start Widows Explorer, and copy the executables to the folder that contains the control file, and then double-click on the executables.

UNIX

UNIX executables are not provided in the package, so you will have to compile them using the source files included in the package, in the src/folder. Note that UNIX commands and file or folder names are case-sensitive. The following assumes that you are at the UNIX prompt.

1. Go to the PAML web site http://abacus.gene.ucl.ac.uk/software/paml.html and download the lat

2. est archive and save on your hard disk. Unpack it using gzip, with a command like the following (replace the version numbers and use the correct name for the archive file)

   ```
   tar xf paml4.9g.tgz
   ```

3. You can use ls to look at the files in the folder. Delete the Windows executables (.exe files) in the bin folder. Then cd to the src/ folder to compile using make.

```
ls -lF bin
rm -r bin/*.exe (this removes the .exe files in the bin folder)
cd src
make
ls -lF
rm *.o
mv baseml basemlg codeml pamp evolver yn00 chi2 ../bin
cd ..
bin/codeml
```

4. Those commands compile the programs and generate executables called baseml, basemlg, codeml, pamp, evolver, yn00, and chi2, which you can see with the ls command. Then remove (rm) the intermediate object files *.o, and move (mv) the compiled executables into bin/ folder in the PAML main folder (that is, ../bin from paml/src/). Then cd to the PAML main folder and run codeml, using the default control file codeml.ctl. You can then print out a copy of codeml.ctl and look at it (and the main result file mlc).

If the compilation (the make command) is unsuccessful, you might have to open and edit the file Makefile before issuing the make command. For example, you can change cc to gcc and -fast to -O3 or -O4. If that none of these works, look at the file readme.txt in the src/ folder for compiling instructions. You can copy the compiling commands onto the command line. For example

```
cc -o baseml baseml.c tools.c -lm
cc -o codeml codeml.c tools.c -lm
```

would compile baseml and codeml using the C compiler cc. However, in this case code optimization is not turned on. You should use compiler switches to optimize the code, say,

```
cc -o codeml -O3 codeml.c tools.c -lm
```

Finally, if your current folder is not on your search path, you will have to add ./ in front of the executable file name even if the executable is in your current working folder; that is, use ./codeml instead of codeml to run codeml.

Mac OS X

Since Mac OSX is UNIX, you should follow the instructions for UNIX above. Open a command terminal (Applications-Utilities-Terminal) and then compile and run the programs from the terminal. You cd to the paml/src/ folder and look at the readme.txt or Makefile files. See above. If you type commands gcc or make and get a "Command not found" error, you will have to download the Apple Developer's Toolkit at the Apple web site http://developer.apple.com/tools/. There are some notes about running programs on MAC OS X or UNIX at the FAQ page.

I have stopped distributing executables for old MACs running OS 9 or earlier.

Running a program

As indicated above, you run a program by typing its name from the command line. You should know which folder your sequence file, tree file, and control file are, relative to your working folder. If inexperienced, you may copy the executables to the folder containing your data files. Depending on the model used, codeml may need a data file such as grantham.dat, dayhoff.dat, jones.dat, wag.dat, mtREV24.dat, or mtmam.dat, so you should copy these files as well.

The programs produce result files, with names such as rub, lnf, rst, or rates. You should not use these names for your own files as otherwise they will be overwritten.

Example data sets

The examples/ folder contains many example data sets. They were used in the original papers to test the new methods, and I included them so that you could duplicate our results in the papers.

Sequence alignments, control files, and detailed readme files are included. They are intended to help you get familiar with the input data formats and with interpretation of the results, and also to help you discover bugs in the program. If you are interested in a particular analysis, get a copy of the paper that described the method and analyze the example dataset to duplicate the published results. This is particularly important because the manual, as it is written, describes the meanings of the control variables used by the programs but does not clearly explain how to set up the control file to conduct a particular analysis.

examples/HIVNSsites/: This folder contains example data files for the HIV-1 env V3 region analyzed in Yang *et al.* (2000b). The data set is for demonstrating the NSsites models described in that paper, that is, models of variable ω ratios among amino acid sites. Those models are called the "random-sites" models by Yang & Swanson (2002) since *a priori* we do not know which sites might be highly conserved and which under positive selection. They are also known as "fishing-expedition" models. The included data set is the 10th data set analyzed by Yang *et al.* (2000b) and the results are in table 12 of that paper. Look at the readme file in that folder.

examples/lysin/: This folder contains the sperm lysin genes from 25 abalone species analyzed by Yang, Swanson & Vacquier (2000a) and Yang and Swanson (2002). The data set is for demonstrating both the "random-sites" models (as in Yang, Swanson & Vacquier (2000a)) and the "fixed-sites" models (as in (Yang and Swanson 2002)). In the latter paper, we used structural information to partition amino acid sites in the lysin into the "buried" and "exposed" classes and assigned and estimated different ω ratios for the two partitions. The hypothesis is that the sites exposed on the surface are likely to be under positive selection. Look at the readme file in that folder.

examples/lysozyme/: This folder contains the primate lysozyme *c* genes of Messier and Stewart (1997), re-analyzed by Yang (1998). This is for demonstrating codon models that assign different ω ratios for different branches in the tree, useful for testing positive selection along lineages. Those models are sometimes called branch models or branch-specific models. Both the "large" and the "small" data sets in Yang (1998) are included. Those models require the user to label branches in the tree, and the readme file and included tree file explain the format in great detail. See also the section "Tree file and representations of tree topology" later about specifying branch/node labels.

The lysozyme data set was also used by Yang and Nielsen (2002) to implement the so-callled "branch-site" models, which allow the ω ratio to vary both among lineages and among sites. Look at the readme file to learn how to run those models.

examples/MouseLemurs/: This folder includes the mtDNA alignment that Yang and Yoder (2003) analyzed to estimate divergence dates in mouse lemurs. The data set is for demonstrating maximum likelihood estimation of divergence dates under models of global and local clocks. The most sophisticated model described in that paper uses multiple calibration nodes simultaneously, analyzes multiple genes (or site partitions) while accounting for their differences, and also account for variable rates among branch groups. The readme file explains the input data format as well as model specification in detail. The readme2 file explains the ad hoc rate smoothing procedure of Yang (2004).

examples/mtCDNA/: This folder includes the alignment of 12 protein-coding genes on the same strand of the mitochondrial genome from seven ape species analyzed by Yang, Nielsen, & Hasegawa (1998) under a number of codon and amino acid substitution models. The data set is the "small" data set referred to in that paper, and was used to fit both the "mechanistic" and empirical models of amino acid substitution as well as the "mechanistic" models of codon substitution. The model can be used, for example, to test whether the rates of conserved and radical amino acid substitutions are equal. See the readme file for details.

examples/TipDate/: This folder includes the alignment of 33 SIV/HIV-2 sequences,

compiled and analyzed by Lemey et al. (2003) and re-analyzed by Stadler and Yang (2013). The readme file explains how to duplicate the ML and Bayesian results published in that paper. Note that the sample date is the last field in the sequence name.

Some other data files are included in the package as well. The details follow.

brown.nuc and **brown.trees**: the 895-bp mtDNA data of Brown *et al.* (1982), used in Yang *et al.* (1994) and Yang (1994a) to test models of variable rates among sites.

mtprim9.nuc and **9s.trees**: mitochondrial segment consisting of 888 aligned sites from 9 primate species (Hayasaka *et al.* 1988), used by Yang (1994c) to test the discrete-gamma model and Yang (1995) to test the auto-discrete-gamma models.

abglobin.nuc and **abglobin.trees**: the concatenated α- and β-globin genes, used by Goldman and Yang (1994) in their description of the codon model. abglobin.aa is the alignment of the translated amino acid sequences.

stewart.aa and **stewart.trees**: lysozyme protein sequences of six mammals (Stewart *et al.* 1987), used by Yang *et al.* (1995b) to test methods for reconstructing ancestral amino acid sequences.

3 Data file formats

Sequence data file format

Have a look at some of the example data files in the package (.nuc, .aa, and .nex). As long as you get your data file into one of the formats, PAML programs should be able to read it. The "native" format is the PHYLIP format used in Joe Felsenstein's PHYLIP package (Felsenstein 2005) (but see below). PAML has limited support for the NEXUS file format used by PAUP and MacClade. Only the sequence data or trees are read, and command blocks are ignored. PAML does not deal with comment blocks in the sequence data block, so please avoid them.

Sequential and interleaved formats

Below is an example of the PHYLIP format (Felsenstein 2005). The first line contains the number of species and the sequence length (possibly followed by option characters). *For codon sequences (`codeml` with `seqtype = 1`), the sequence length in the sequence file refers to the number of nucleotides rather than the number of codons.* The only options allowed in the sequence file are I, S, P, C, and G. The sequences may be in either *interleaved* format (option I, example data file `abglobin.nuc`), or *sequential* format (option S, example data file `brown.nuc`). The default option is S, so you don't have to specify it. Option G is used for combined analysis of multiple gene data and is explained below. The following is an example data set in the sequential format. It has 4 sequences each of 60 nucleotides (or 20 codons).

```
     4 60
sequence 1
AAGCTTCACCGGCGCAGTCATTCTCATAAT
CGCCCACGGACTTACATCCTCATTACTATT
sequence 2
AAGCTTCACCGGCGCAATTATCCTCATAAT
CGCCCACGGACTTACATCCTCATTATTATT
sequence 3
AAGCTTCACCGGCGCAGTTGTTCTTATAAT
TGCCCACGGACTTACATCATCATTATTATT
sequence 4
AAGCTTCACCGGCGCAACCACCCTCATGAT
TGCCCATGGACTCACATCCTCCCTACTGTT
```

Species/sequence names. Do not use the following special symbols in a species/sequence name: ", : # ( ) $ =" in a species name as they are used for special purposes and may confuse the programs. The symbol @ can be used as part and end of the sequence name to specify the date of determination of that sequence, for example, virus1@1984. The @ symbol is considered part of the name and the sequence was determined in 1984. The maximum number of characters in a species name (LSPNAME) is specified at the beginning of the main programs `baseml.c` and `codeml.c`. In PHYLIP, exactly 10 characters are used for a species name, which I often found to be too restrictive. So I use a default value of 30. To make this discrepancy less a problem, PAML considers two consecutive spaces as the end of a species name, so that the species name does not have to have exactly 30 (or 10) characters. To make this rule work, you should not have two consecutive spaces *within* a species name. For example the above data set can have the following format too.

```
     4 60
sequence 1   AAGCTTCACCGGCGCAGTCATTCTCATAAT
CGCCCACGGACTTACATCCTCATTACTATT
sequence 2   AAGCTTCACCGGCGCAATTATCCTCATAAT
CGCCCACGGACTTACATCCTCATTATTATT
sequence 3   AAGCTTCACC GGCGCAGTTG TTCTTATAAT
TGCCCACGGACTTACATCATCATTATTATT
sequence 4   AAGCTTCACCGGCGCAACCACCCTCATGAT
TGCCCATGGACTCACATCCTCCCTACTGTT
```

If you want the file to be readable by both PHYLIP and PAML, you should limit the number of characters in the name to 10 and separate the name and the sequence by at least two spaces.

In a sequence, T, C, A, G, U, t, c, a, g, u are recognized as nucleotides (for `baseml`, `basemlg` and `codonml`), while the standard one-letter codes (A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V or their lowercase equivalents) are recognized as amino acids. Ambiguity characters (undetermined nucleotides or amino acids) are allowed as well. Three special characters ".", "-", and "?" are interpreted like this: a dot means the same character as in the first sequence, a dash means an alignment gap, and a question mark means an undetermined nucleotide or amino acid. Non-alphabetic symbols such as ><!'"£$%&^[](){}0123456789 inside a sequence are simply ignored and can be freely used as signposts. Lines do not have to be equally long and you can put the whole sequence on one line.

The way that ambiguity characters and alignment gaps are treated in `baseml` and `codeml` depends on the variable `cleandata` in the control file. In the maximum likelihood analysis, sites at which at least one sequence involves an ambiguity character are removed from all sequences before analysis if `cleandata` = 1, while if `cleandata` = 0, both ambiguity characters and alignment gaps are treated as ambiguity characters. In the pairwise distance calculation (the lower-diagonal distance matrix in the output), `cleandata` = 1 means "complete deletion", with all sites involving ambiguity characters and alignment gaps removed from all sequences, while `cleandata` = 0 means "pairwise deletion", with only sites which have missing characters in the pair removed.

There are no models for insertions and deletions in the PAML programs. So an alignment gap is treated as an ambiguity (that is, a question mark ?). Note also that for codon sequences, removal of any nucleotide means removal of the whole codon.

Notes may be placed at the end of the sequence file and will be ignored by the programs.

Option G: This option is for combined analyses of heterogeneous data sets such as data of multiple genes or data of the three codon positions. The sequences must be concatenated and the option is used to specify which gene or codon position each site is from.

There are three formats with this option. The first format is illustrated by an excerpt of a sequence file listed below. The example data of Brown *et al.* (1982) are an 895-bp segment from the mitochondrial genome, which codes for parts of two proteins (ND4 and ND5) at the two ends and three tRNAs in the middle. Sites in the sequence fall naturally into 4 classes: the three codon positions and the tRNA coding region. The first line of the file contains the option character G. The second line begins with a G at the first column, followed by the number of site classes. The following lines contain the site marks, one for each site in the sequence (or each codon in the case of `codonml`). The site mark specifies which class each site is from. If there are *g* classes, the marks should be 1, 2, ..., *g*, and if *g* > 9, the marks need to be separated by spaces. The total number of marks must be equal to the total number of sites in each sequence.

```
  5 895 G
G 4
3
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
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1231231231231231231231231231231231231231
4444444444444444444444444444444444444444444444444444444444444
4444444444444444444444444444444444444444444444444444444444444
4444444444444444444444444444444444444444444444444444444444444
44444444444444444444
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
1231231231231231231231231231231231231231231231231231123123123
12312312312312312312312312312312312312312312312312312312312
Human
AAGCTTCACCGGCGCAGTCATTCTCATAATCGCCCACGGACTTACATCCTCATTACTATT
CTGCCTAGCAAACTCAAACTACGAACGCACTCACAGTCGCATCATAATC........
Chimpanzee
.........
```

The second format is useful if the data are concatenated sequences of multiple genes, shown below for an example data set. This sequence has 1000 nucleotides from 4 genes, obtained from concatenating four genes with 100, 200, 300, and 400 nucleotides from genes 1, 2, 3, and 4, respectively. The "lengths" for the genes must be on the line that starts with G, *i.e.*, on the second line of the sequence file. (This requirement allows the program to determine which of the two formats is being used.) The sum of the lengths for the genes should be equal to the number of nucleotides, amino acids, or codons in the combined sequence for `baseml` (or `basemlg`), `aaml`, and `codonml`, respectively.

```
5 1000 G
G 4 100 200 300 400
Sequence 1
TCGATAGATAGGTTTTAGGGGGGGGGGGTAAAAAAAAA.......
```

The third format applies to protein-coding DNA sequences only (for `baseml`). You use option characters GC on the first line instead of G alone. The program will then treat the three codon positions differently in the nucleotide-based analysis. It is assumed that the sequence length is an exact multiple of three.

```
5  855  GC
human     GTG CTG TCT CCT ...
```

Option G for codon sequences (codeml with seqtype = 1). The format is similar to the same option for baseml, but note that the sequence length is in number of nucleotides while the gene lengths are in number of codons. This has been a source of confusion. Below is an example:

```
5  300  G
G2   40  60
```

This data set has 5 sequences, each of 300 nucleotides (100 codons), which are partitioned into two genes, with the first gene having 40 codons and the second gene 60 codons.

Site pattern counts

The sequence alignment can also be input in the form of site patterns and counts of sites having those site patterns. This format is specified by the option "P" on the first line of the input data file, as illustrated by the following example. Here there are 3 sequences, 8 site patterns, with "P" indicating that the data are site patterns and not sites. The "P" option is used in the same way as options "I" for interleaved format and "S" for sequential format (default). The 8 numbers below the alignment are the numbers of sites having the 8 patterns above. For example, at 100 sites, all three species has G, and at 200 sites all three species has T, and so on. In total there are $100 + 200 + 40 +$ … $+ 14 = 440$ sites.

```
3  8  P

human   GTACTGCC
rabbit  GTACTACT
rat     GTACAGAC

100 200 40 50 11 12 13 14
```

This example applies to baseml and basemlg, program for nucleotide-based analysis. To specify multiple genes (site partitions), one may use option G together with option P.

```
3  10  PG
G 2    4 6

human   GTTA CATGTC
rabbit  GTCA CATATT
rat     GTTA CAAGTC

100 200 40 50 120 61 12 13 54 12
```

Here there are 10 site patterns and 2 genes (site partitions). The first 4 patterns are for the first gene while the next 6 patterns are for the second gene, with a total of 10 site patterns. In partition 1 there are 50 sites having the data AAA (nucleotide A in all three species), and while in partition 2 there are 61 such sites.

The same format applies to protein sequences (codeml with seqtype = 2), with amino acids replacing nucleotides in the examples above.

For codon sequences (codeml with seqtype = 1), the format is as follows. There are 3 species, and 8 site patterns, with 6 sites having the first site pattern (which has the codon GTG in all three species). Note that 24 = 8*3. The program requires that you use the number of nucleotide sites on the first line. This is strange but consistent with the sequential or interleaved sequence format, where the sequence length is specified in the number of nucleotides rather than number of codons. (Initially I did this so that the same file can be read by both baseml for nucleotide based analysis and codonml for codon based analysis.)

```
      3   24   P G

human      GTG CTG TCT CCT GCC GAC AAG ACC
rabbit     ... ... ... G.C ... ... ... T..
rat        ... ... ... ..C ..T ... ... ...

   6 1 1 1 1 4 3 1
```

To specify multiple genes for codon site patterns, see the following example.

```
      3   24   P G
G 2      4    4

human      GTG CTG TCT CCT GCC GAC AAG ACC
rabbit     ... ... ... G.C ... ... ... T..
rat        ... ... ... ..C ..T ... ... ...

   6 1 1 1 1 4 3 1
```

Here there are again 8 codon site patterns in total, with the first 4 patterns for gene 1 and the next 4 patterns for gene 2.

Furthermore, option variable P can be used together with option variable I or S. PI means that the site patterns are listed using the interleaved format while PS means that the site patterns are listed using the sequential format. P without I or S uses the default sequential format. Having the whole sequence of all site patterns on one line conforms with both the I and S formats, so there is no need to specify I or S.

If you run baseml and codeml to read the sequential or interleaved formats of sequences, the output will include a print-out in this partitioned format. Look for the line "Printing out site pattern counts". You can move this block into a new file and later on read that file instead, if it takes a long time to pack sites into patterns. Note the restrictions with the P format below.

Here are some restrictions to this option. Some outputs are disabled for this option, including ancestral sequence reconstruction and posterior estimates of rates for sites (or site patterns), that you can get for sequences by using RateAncestor = 1. Second, some of the calculations require the sequence length, which I set to the sum of the site pattern frequencies. If the site pattern frequencies are not counts of sites but are instead site pattern probabilities, calculations involving sequence length will not be correct. Such calculations include the SEs for MLEs, the numbers of sites S and N in codonml, for example.

Possible uses of this option. Sometimes I use evolver to simulate very long sequences (with >1M sites) and it can take minutes or hours to collapse sites into patterns, when the maximum likelihood iteration takes a few seconds. A similar case is analysis of large genomic data of long sequences with >100Mb sites. In this case you can run baseml or codeml once, and then copy the pattern counts from the output file into a data file. Next time, you run the program you can read the new file. This way the program skips the step of counting site patterns. Note that the pattern counts do not have to be integers. You can calculate the site pattern probabilities under a model and then read the probabilities for analysis using a different model to see whether the correct tree is still recovered. The site pattern probabilities under the model amount to a dataset of infinite size (infinitely many sites). This way, you can check whether the tree reconstruction method is still consistent. See Debry (1992) and Yang (1994b) for such analysis. (I need to enable baseml and codeml for printing site pattern probabilities.)

Tree file format and representations of tree topology

A *tree structure file* is used when `runmode` = 0 or 1. The file name is specified in the appropriate control file. The tree topology is typically specified using the parenthesis notation, although it is possible to use a branch representation, as described below.

Parenthesis notation: The first is the familiar parenthesis representation, used in most phylogenetic software. The species can be represented using either their names or their indexes corresponding to the order of their occurrences in the sequence data file. If species names are used, they have to match exactly those in the sequence data file (including spaces or strange characters). Branch lengths are allowed. The following is a possible tree structure file for a data set of four species (human, chimpanzee, gorilla, and orangutan, occurring in this order in the data file). The first tree is a star tree, while the next four trees are the same.

```
      4 5 // 4 species, 5 trees
(1,2,3,4); // the star tree
((1,2),3,4); // species 1 and 2 are clustered together
((1,2),3,4); // Commas are needed with more than 9 species
((human,chimpanzee),gorilla,orangutan);
((human:.1,chimpanzee:.2):.05,gorilla:.3,orangutan:.5);
```

If the tree has branch lengths, baseml and codeml allow you to use the branch lengths in the tree as starting values for maximum likelihood iteration.

Whether you should use rooted or unrooted trees depends on the model, for example, on whether a molecular clock is assumed. Without the clock (clock = 0), unrooted trees should be used, such as ((1,2),3,4) or (1,2,(3,4)). With the clock or local-clock models, the trees should be rooted and these two trees are different and both are different from (((1,2),3),4). In PAML, a rooted tree has a bifurcation at the root, while an unrooted tree has a trifurcation or multifurcation at the root.

Tree files produced by PAUP and MacClade. PAML programs have only limited compatibility with the tree file generated by PAUP or MacClade. First the "[&U]" notation for specifying an unrooted tree is ignored. For the tree to be accepted as an unrooted tree by PAML, you have to manually modify the tree file so that there is a trifurcation at the root, for example, by changing "(((1,2),3),4)" into "((1,2),3,4)". Second, the "Translate" keyword is ignored by PAML as well, and it is assumed that the ordering of the sequences in the tree file is exactly the same as the ordering of the sequences in the sequence data file.

Branch or node labels. Some models implemented in `baseml` and `codeml` allow several groups of branches on the tree, which are assigned different parameters of interest. For example, in the local clock models (clock = 2 or 3) in baseml or codeml, you can have, say, 3 branch rate groups, with low, medium, and high rates respectively. Also the branch-specific codon models (model = 2 or 3 for `codonml`) allow different branch groups to have different ωs, leading to so called "two-ratios" and "three-ratios" models. All those models require branches or nodes in the tree to be labeled. Branch *labels* are specified in the same way as branch *lengths* except that the symbol "#" is used rather than ":". The branch labels are consecutive integers starting from 0, which is the default and does not have to be specified. For example, the following tree

```
((Hsa_Human, Hla_gibbon) #1, ((Cgu/Can_colobus, Pne_langur), Mmu_rhesus), (Ssc_squirrelM, Cja_marmoset));
```

is from the tree file `examples/lysozyme/lysozyme.trees`, with a branch label for fitting models of different ω ratios for branches. The internal branch ancestral to human and gibbon has the ratio ω_1, while all other branches (with the default label #0) have the background ratio ω_0. This fits the model in table 1C for the small data set of lysozyme genes in Yang (1998). See the readme file in the `examples/lysozyme/` folder.

If you want to label all branches within a clade on a big tree, it may be easy to use the clade label $. $ is for Δ, which looks like a good clade symbol but is missing on most keyboards. So (clade) $2 is equivalent to labeling all nodes/branches within the clade with #2. The following two trees are thus equivalent.

```
(((rabbit, rat) $1, human), goat_cow, marsupial);
(((rabbit #1, rat #1) #1, human), goat_cow, marsupial);
```

Here are the rules concerning nested clade labels. The symbol # takes precedence over the symbol $, and clade labels close to the tips take precedence over clade labels for ancestral nodes close to the root. So the following two trees are equivalent. In the first tree below, $1 is first applied to the whole clade of placental mammals (except for the human lineage), and then $2 is applied to the rabbit-rat clade.

```
((((rabbit, rat) $2, human #3), goat_cow) $1, marsupial);
((((rabbit #2, rat #2) #2, human #3) #1, goat_cow #1) #1, marsupial);
```

Note that with this rule, it may make a difference whether or not you include a label $0. For example

```
((a, b) $0, (c, d)) $1;
```

labels the three branches on the left side (to a, to b, and to both a and b) as #0, while the other branches are #1. However the following would label all branches in the tree as #1.

```
((a, b), (c, d)) $1;
```

I have found it convenient to create the tree file with labels and read the tree using Rod page's (1996) TreeView to check that the tree and labels are right. The example trees above should be readable by TreeView. For TreeView X, however, you may have to put the labels inside single quotation marks, like the following.

```
((((rabbit '#2', rat '#2') '#2', human '#3') '#1', goat_cow '#1') '#1', marsupial);
```

This way, the tree is readable by both TreeView and TreeView X (and by baseml/codeml as well). Note that TreeView and TreeView X do not accept labels for tips or tip branches, and may interpret the labels as part of the sequence name. Another program that you can use to create and/or view branch or node labels is Andrew Rambaut's FigTree, available for the MAC. I have no experiencing of using it.

Divergence date symbol @. Fossil calibration information is specified using the symbol @. This is used for the clock and local clock models in baseml and codeml. See the readme file in the examples/MouseLemurs/ folder. So in the following example, the human-chimpanzee divergence is fixed at 5MY.

```
((gorilla, (human, chimpanzee) '@0.05'), orangutan);
```

This kind of calibration information (point calibration) is not used by the Bayesian dating program `mcmctree`. See descriptions later.

Branch representation of tree topology: A second way of representing the tree topology used in PAML is by enumerating its branches, each of which is represented by its starting and ending nodes. This representation is also used in the result files for outputting the estimated branch lengths, but you can also use it in the tree file. For example, the tree ((1,2),3,4) can be specified by enumerating its 5 branches:

```
    5
  5 6   6 1   6 2   5 3   5 4
```

The nodes in the tree are indexed by consecutive natural numbers, with 1, 2, ..., *s* representing the *s* known sequences in the data, in the same order as in the data. A number larger than *s* labels an internal node, at which the sequence is unknown. So in the above tree, node 5 is ancestral to nodes 6, 3, and 4, while node 6 is ancestral to nodes 1 and 2.

This notation is convenient to specify a tree in which some sequences in the data are direct ancestors to some others. For example, the following tree for 5 sequences has 4 branches, with sequence 5 to be the common ancestor of sequences 1, 2, 3, and 4:

```
    4
  5 1   5 2   5 3   5 4
```

☐*Warning.* I did not try to make this tree representation work with all models implemented in baseml and codeml. If you use this representation, you should test the program carefully. If it does not work, you can let me know so that I will try to fix it.

4 baseml

Nucleotide substitution models

For detailed descriptions of Markov models of nucleotide substitution, see Whelan *et al.* (2001), Felsenstein (2004) or Yang (2006: Chapter 1).

Models used in PAML include JC69 (Jukes and Cantor 1969), K80 (Kimura 1980), F81 (Felsenstein 1981), F84 (Felsenstein, DNAML program since 1984, PHYLIP Version 2.6), HKY85 (Hasegawa *et al.* 1984; Hasegawa *et al.* 1985), Tamura (1992), Tamura and Nei (1993), and REV, also known as GTR for general-time-reversible (Yang 1994a; Zharkikh 1994). The rate matrices are parametrized as follows.

$$
\text{JC69}: \qquad Q = \begin{pmatrix} . & 1 & 1 & 1 \\ 1 & . & 1 & 1 \\ 1 & 1 & . & 1 \\ 1 & 1 & 1 & . \end{pmatrix}
$$

$$
\text{K80}: \qquad Q = \begin{pmatrix} . & \kappa & 1 & 1 \\ \kappa & . & 1 & 1 \\ 1 & 1 & . & \kappa \\ 1 & 1 & \kappa & . \end{pmatrix}
$$

$$
\text{F81}: \qquad Q = \begin{pmatrix} . & \pi_C & \pi_A & \pi_G \\ \pi_T & . & \pi_A & \pi_G \\ \pi_T & \pi_C & . & \pi_G \\ \pi_T & \pi_C & \pi_A & . \end{pmatrix}
$$

$$
\text{F84}: \qquad Q = \begin{pmatrix} . & (1+\kappa/\pi_Y)\pi_C & \pi_A & \pi_G \\ (1+\kappa/\pi_Y)\pi_T & . & \pi_A & \pi_G \\ \pi_T & \pi_C & . & (1+\kappa/\pi_R)\pi_G \\ \pi_T & \pi_C & (1+\kappa/\pi_R)\pi_A & . \end{pmatrix}
$$

with $\pi_Y = \pi_T + \pi_C$ and $\pi_R = \pi_A + \pi_G$.

$$
\text{HKY85}: \qquad Q = \begin{pmatrix} . & \kappa\pi_C & \pi_A & \pi_G \\ \kappa\pi_T & . & \pi_A & \pi_G \\ \pi_T & \pi_C & . & \kappa\pi_G \\ \pi_T & \pi_C & \kappa\pi_A & . \end{pmatrix}
$$

$$T92: \qquad Q = \begin{pmatrix} . & \kappa(1-\pi_{GC})/2 & \pi_{GC}/2 & \pi_{GC}/2 \\ \kappa(1-\pi_{GC})/2 & . & \pi_{GC}/2 & \pi_{GC}/2 \\ (1-\pi_{GC})/2 & (1-\pi_{GC})/2 & . & \kappa\pi_{GC}/2 \\ (1-\pi_{GC})/2 & (1-\pi_{GC})/2 & \kappa\pi_{GC}/2 & . \end{pmatrix}$$

$$TN93: \qquad Q = \begin{pmatrix} . & \kappa_1\pi_C & \pi_A & \pi_G \\ \kappa_1\pi_T & . & \pi_A & \pi_G \\ \pi_T & \pi_C & . & \kappa_2\pi_G \\ \pi_T & \pi_C & \kappa_2\pi_A & . \end{pmatrix}$$

$$REV\ (GTR): \qquad Q = \begin{pmatrix} . & a\pi_C & b\pi_A & c\pi_G \\ a\pi_T & . & d\pi_A & e\pi_G \\ b\pi_T & d\pi_C & . & \pi_G \\ c\pi_T & e\pi_C & \pi_A & . \end{pmatrix}$$

$$UNREST \qquad Q = \begin{pmatrix} . & q_{TC} & q_{TA} & q_{TG} \\ q_{CT} & . & q_{CA} & q_{CG} \\ q_{AT} & q_{AC} & . & q_{AG} \\ q_{GT} & q_{GC} & q_{GA} & . \end{pmatrix} = \begin{pmatrix} . & a & b & c \\ d & . & e & f \\ g & h & . & i \\ j & k & l & . \end{pmatrix}.$$

The control file

The default control file for baseml is baseml.ctl, and an example is shown below. Note that spaces are required on both sides of the equal sign, and blank lines or lines beginning with "*" are treated as comments. Options not used can be deleted from the control file. The order of the variables is unimportant.

```
      seqfile = brown.nuc * sequence data file name
      outfile = mlb * main result file
     treefile = brown.trees * tree structure file name

        noisy = 3    * 0,1,2,3: how much rubbish on the screen
      verbose = 0    * 1: detailed output, 0: concise output
      runmode = 0    * 0: user tree;  1: semi-automatic;  2: automatic
                     * 3: StepwiseAddition; (4,5):PerturbationNNI

        model = 5    * 0:JC69, 1:K80, 2:F81, 3:F84, 4:HKY85
                     * 5:T92, 6:TN93, 7:REV, 8:UNREST, 9:REVu; 10:UNRESTu
        Mgene = 0    * 0:rates, 1:separate; 2:diff pi, 3:diff kapa, 4:all diff

*       ndata = 1    * number of data sets
        clock = 0    * 0:no clock, 1:clock; 2:local clock; 3:CombinedAnalysis
      TipDate = 0 100  * TipDate (1) & time unit
    fix_kappa = 0    * 0: estimate kappa; 1: fix kappa at value below; 2: kappa for branches
        kappa = 2.5  * initial or fixed kappa

    fix_alpha = 1    * 0: estimate alpha; 1: fix alpha at value below
        alpha = 0.   * initial or fixed alpha, 0:infinity (constant rate)
       Malpha = 0    * 1: different alpha's for genes, 0: one alpha
        ncatG = 5    * # of categories in the dG, AdG, or nparK models of rates

      fix_rho = 1    * 0: estimate rho; 1: fix rho at value below
          rho = 0.   * initial or fixed rho,   0:no correlation
        nparK = 0    * rate-class models. 1:rK, 2:rK&fK, 3:rK&MK(1/K), 4:rK&MK

        nhomo = 0    * 0 & 1: homogeneous, 2: kappa for branches, 3: N1, 4: N2, 5: user
        getSE = 0    * 0: don't want them, 1: want S.E.s of estimates
  RateAncestor = 0   * (0,1,2): rates (alpha>0) or ancestral states
```

```
    Small_Diff = 1e-6
*    cleandata = 1    * remove sites with ambiguity data (1:yes, 0:no)?
*        icode = 0    * (RateAncestor=1 for coding genes, "GC" in data)
* fix_blength = 0    * 0: ignore, -1: random, 1: initial, 2: fixed, 3: proportional
       method = 0    * 0: simultaneous; 1: one branch at a time
```

The control variables are described below.

seqfile, **outfile**, and **treefile** specifies the names of the sequence data file, main result file, and the tree structure file, respectively. You should not have spaces inside a file name. In general try to avoid special characters in a file name as they might have special meanings under the OS.

noisy controls how much output you want on the screen. If the model being fitted involves much computation, you can choose a large number for noisy to avoid loneliness. verbose controls how much output in the result file.

runmode = 0 means evaluation of the tree topologies specified in the tree structure file, and runmode = 1 or 2 means heuristic tree search by the star-decomposition algorithm. With runmode = 2, the algorithm starts from the star tree, while if runmode = 1, the program will read a multifurcating tree from the tree structure file and try to estimate the best bifurcating tree compatible with it. runmode = 3 means stepwise addition. runmode = 4 means NNI perturbation with the starting tree obtained by a parsimony algorithm, while runmode = 5 means NNI perturbation with the starting tree read from the tree structure file. The tree search options do not work well, and so use runmode = 0 as much as you can. For relatively small data set, the stepwise addition algorithm seems usable.

model specifies the model of nucleotide substitution. Models 0, 1, …, 8 represent models JC69, K80, F81, F84, HKY85, T92, TN93, REV (also known as GTR), and UNREST, respectively. Check Yang (1994a) or Yang (2006: table 1.1) for notation. Two more user-specified models are implemented as special cases of the REV/GTR model (model = 9) or of the UNREST model (model = 10). These models are called REVu and UNRESTu. The format is illustrated in the following examples. The number in the brackets [] is the number of free rate parameters; this is then followed by nucleotide pairs which should have the same rates, grouped in the same pair of parentheses. For example, the line for SYM specifies an UNRESTu model with 5 rates, with one rate for A→C and C→A changes, one rate for A→G and G→A changes, and so on. The model has a symmetrical rate matrix Q, so that the equilibrium frequencies are ¼ for each nucleotide. Note that under UNREST and UNRESTu, the equilibrium nucleotide frequencies are not parameters but are given by the rate parameters: see equation 1.56 in Yang (2006). Similarly model = 9 specifies special cases of the REV/GTR model, or REVu models. In one specifies TC or CT, but not both of them, since those are assumed to have the same rate (exchangeability) parameers.

```
model = 10  [0]  /* JC69 */
model = 10  [1 (TC CT AG GA)]  /* K80 */
model = 10  [11 (TA) (TG) (CT) (CA) (CG) (AT) (AC) (AG) (GT) (GC) (GA) ]  /* UNREST */
model = 10  [5 (AC CA) (AG GA) (AT TA) (CG GC) (CT TC)]  /* SYM */
model = 9   [2 (TA TG CA CG) (AG)]    /* TN93 */
```

Mgene is used in combination with option G in the sequence data file, for combined analysis of data from multiple genes or multiple site partitions (such as the three codon positions) (Yang 1996b; pages 116-8 in Yang 2006). Such models are also called partition models. Choose 0 if option G is not used in the data file.

The description here refers to multiple genes, but apply to any strategy of partitioning sites, such as by codon position. Similar partition models are implemented in codeml for analyzing codon or amino acid sequences.

The models are summarized in table 1. The simplest model which assumes complete homogeneity among genes can be fitted by concatenating different genes into one sequence without using the option G (and by specifying Mgene = 0 in the control file). The most general model is equavilent to a separate analysis, and can be done by fitting the same

model to each data set (each gene), but can also be done by specifying `Mgene` = 1 with the option G in the combined data file. The sum of the log-likelihood values over different genes is then the log likelihood of the most general model considered here. Models accounting for some aspects of the heterogeneity of multiple genes are fitted by specifying `Mgene` in combination with the option G in the sequence data file. `Mgene` = 0 means a model that asumes different substitution rates but the same pattern of nucleotide substitution for different genes. `Mgene` = 2 means different frequency parameters for different genes but the same rate ratio parameters (κ in the K80, F84, HKY85 models or the rate parameters in the TN93 and REV models). `Mgene` = 3 means different rate ratio parameters and the same frequency parameters. `Mgene` = 4 means both different rate ratio parameters and different frequency parameters for different genes. Parameters and assumptions made in these models are summarized in the following table, with the HKY85 model used as an example. When substitution rates are assumed to vary from site to site, the control variable `Malpha` specifies whether one gamma distribution will be applied across all sites (`Malpha` = 0) or a different gamma distribution is used for each gene (or codon position).

Table 1. Setups of partition models of nucleotide substitution

Sequence file	Control file	Parameters across genes
No G	`Mgene` = 0	everything equal
Option G	`Mgene` = 0	the same κ and π, but different cs (proportional branch lengths)
Option G	`Mgene` = 2	the same κ, but different πs and cs
Option G	`Mgene` = 3	the same π, but different κs and cs
Option G	`Mgene` = 4	different κ, πs, and cs
Option G	`Mgene` = 1	different κ, πs, and different (unproportional) branch lengths

The different cs for different genes mean that branch lengths estimated for different genes are proportional. Parameters π represent the equilibrium nucleotide frequencies, which are estimated using the observed frequencies (`nhomo` = 0). The transition/transversion rate ratio κ in HKY85 can be replaced by the two or five rate ratio parameters under the TN93 or REV models, respectively. The likelihood ratio test can be used to compare these models, using an approach called the analysis of deviance (McCullagh and Nelder 1989), which is very similar to the more familiar analysis of variance.

ndata: specifies the number of datasets in the sequence file. This variable may be useful for analyzing simulated replicate datasets or gene alignments for the same set of species from genomic sequencing projects. For example, ou can use `evolver` to generate 100 replicate data sets using a tree and a set of parameters, and then set `ndata` = 100 for `baseml` or `codeml` to analyze them. From version 4.10, I have added a maintree option to accommodate the situation where some species are missing for some genes when the sequence data file has alignments for many genes from the same species. The user provides a main tree for all species, and baseml or codeml will prune the main tree to generate the gene trees, for the species present in each gene alignment. There are now four cases with this option variable, as follows. Please see the readme file in the folder examples/ndata/ for details and examples.

(a) ndata = 100 * one tree block used for all datasets/alignments.
    The different alignments in the file must have the same set of sequence names.
(b) ndata = 100 separate_trees # each alignment has its own tree block

(c) `ndata = 100 maintree` # this uses the maintree file to generate the genetrees/subtrees and runs the ML analysis

(c) `ndata = 100 maintree 1` # this uses the maintree file to generate the genetrees/subtrees and runs the ML analysis

(d) `ndata = 100 maintree 0` # this generates the genetrees.trees file but does not run ML

clock specifies models concerning rate constancy or variation among lineages. `clock` = 0 means no clock and rates are entirely free to vary from branch to branch. An unrooted tree should be used under this model. For a binary tree with n species (sequences), this model has ($2n$ – 3) parameters (branch lengths). `clock` = 1 means the global clock, and all branches have the same rate. This model has (n – 1) parameters corresponding to the (n – 1) internal nodes in the binary tree. So a test of the molecular clock assumption, which compares those two models, should have d.f. = n – 2.

`clock` = 2 specifies the local clock models(Yoder and Yang 2000; Yang and Yoder 2003). Most branches in the phylogeny conform with the clock assumption and has the default rate (r_0 = 1), but some pre-defined branches may have different rates. Rates for branches are specified using branch labels (# and $) in the tree file. For example, the tree (((1,2) #1, 3), 4) specifies rate r_1 for the branch ancestral to species 1 and 2 while all other branches have the default rate r_0, which does not have to be specified. The user need to specify which branch has which rate, and the program estimates the unknown rates (such as r_1 in the above example; r_0 = 1 is the default rate). You need to be careful when specifying rates for branches to make sure that all rates can be estimated by the model; if you specify too many rate parameters, especially for branches around the root, it may not be possible to estimate all of them and you will have a problem with identifiability. The number of parameters for a binary tree in the local clock model is (n – 1) plus the number of extra rate parameters for branches. In the above tree of 4 species, you have only one extra rate parameter r_1, and so the local clock model has (n – 1) + 1 = n = 4 parameters. The no-clock model has 5 parameters while the global clock has 3 parameters for that tree.

The option `clock` = 3 is for combined analysis of multiple-gene or multiple-partition data, allowing the branch rates to vary in different ways among the data partitions (Yang and Yoder 2003). To account for differences in the evolutionary process among data partitions, you have to use the option G in the sequence file as well as the control variable Mgene in the control file (baseml.ctl or codeml.ctl). Read Yang and Yoder (2003) and the readme file in the examples/MouseLemurs/ folder to duplicate the analysis of that paper. Also the variable (= 5 or 6) is used to implement the ad hoc rate smoothing procedure of Yang (2004). See the file readme2.txt for instructions and the paper for details of the model.

For clock = 1 or 2, a rooted tree should be used. If fossil calibration information is specified in the tree file using the symbol @ or =, the absolute rate will be calculated. Multiple calibration points can be specified this way, but only point calibrations (where a node age is assumed to be known without error) are accepted and bounds are not accepted. See instructions about the mcmctree program, which accepts bounds or other distributions as calibrations.

TipDate is used to estimate ages of node ages on the rooted tree when the sequences at the tips having sampling dates, as in the case of sequentially sampled viral sequences. The sample dates are the the last field in the sequence name. The time unit is specified by the user on this line. Look at README.txt in examples/TipDate/.

fix_kappa specifies whether κ in K80, F84, or HKY85 is given at a fixed value or is to be estimated by iteration from the data. If `fix_kappa` = 1, the value of another variable, `kappa`, is the given value, and otherwise (`fix_kappa` = 0) the value of `kappa` is used as the initial estimate for iteration. The variables `fix_kappa` and `kappa` have no effect with JC69 or F81 which does not involve such a parameter, or with TN93 and REV which have

two and five rate parameters respectively, when all of them are estimated from the data. The option `fix_kappa` = 2 is used together with `nhomo` = 5 to specify nonstationay models in `baseml` in which the whole rate matrix Q (both parameter κ or the exchangebility parameters and the base frequency parameters) vary among branches. See the descriptions of `nhomo` below.

fix_alpha and **alpha** work in a similar way, where `alpha` refers to the shape parameter α of the gamma distribution for variable substitution rates across sites (Yang 1994c). The model of a single rate for all sites is specified as `fix_alpha` = 1 and `alpha` = 0 (0 means infinity), while the (discrete-) gamma model is specified by a positive value for `alpha`, and `ncatG` is then the number of categories for the discrete-gamma model (`baseml`). Values such as 5, 4, 8, or 10 are reasonable. Note that the discrete gamma model has one parameter (α), like the continuous gamma model, and the number of categories is not a parameter.

The mdel of invariable sites is not implemented in PAML programs. I don't like the model as it generates a strong correlation between the proportion of invariable sites and the gamma shape parameter. It is implemented in PAUP and MrBayes, for example.

To infer rates at individual sites, use RateAncestor = 1. See below. Using a large number of categories (say, ncatG = 40) may be helpful if you are interested in calculating such rates.

For detailed descriptions of those models, see Yang (1996a), Chapter 1 of Yang (2006) and chapters 13 and 16 of Felsenstein (2004).

fix_rho and `rho` work in a similar way and concern independence or correlation of rates at adjacent sites, where ρ (rho) is the correlation parameter of the auto-discrete-gamma model (Yang 1995). The model of independent rates for sites is specified as `fix_rho` = 1 and `rho` = 0; choosing `alpha` = 0 further means a constant rate for all sites. The auto-discrete-gamma model is specified by positive values for both `alpha` and `rho`. The model of a constant rate for sites is a special case of the (discrete) gamma model with $\alpha = \infty$ (`alpha` = 0), and the model of independent rates for sites is a special case of the auto-discrete-gamma model with $\rho = 0$ (`rho` = 0).

nparK specifies nonparametric models for variable and Markov-dependent rates across sites: `nparK` = 1 or 2 means several (`ncatG`) categories of independent rates for sites, while `nparK` = 3 or 4 means the rates are Markov-dependent at adjacent sites; `nparK` = 1 and 3 have the restriction that each rate category has equal probability while `nparK` = 2 and 4 do not have this restriction (Yang 1995). The variable `nparK` takes precedence over `alpha` or `rho`.

nhomo is for `baseml` only, and is used to implement nonstationay models in which the base compositions change along branches on the tree. The basic substitution model (`model`) should be one that has frequency parameters (F81, F84, HKY85, T92, TN93, REV/GTR). The option **nhomo = 1** fits a homogeneous model, but estimates the frequency parameters π_T, π_C and π_A (since π_G is not a free parameter as the frequencies sum to 1) by maximum likelihood iteration. This applies to F81, F84, HKY85, T92 (in which case only π_{GC} is a parameter), TN93, or REV models. With the default option **nhomo = 0**, the frequency parameters are estimated by the averages of the observed frequencies. For both `nhomo` = 0 and 1, you should count 3 (or 1 for T92) free parameters for the base frequencies.

nhomo = 2 uses one transition/transversion rate ratio (κ) for each branch in the tree for the K80, F84, and HKY85 models (Yang 1994a; Yang and Yoder 1999).

Options **nhomo = 3, 4, 5** are used to fit nonhomogeneous models in which base compositions drift on the tree (Yang and Roberts 1995; Galtier and Gouy 1998). The nucleotide substitution is specified by the variable `model` and is one of F84, HKY85, T92, TN93, and REV/GTR. Different frequency parameters are used in the rate matrix for different branches in the tree. Those options may be used together with **fix_kappa = 2** to

allow the exchangebility parameters in the substitution rate matrix (κ in F84, HKY85, and T92; κ_1 and κ_2 in TN93, and *a b c d e* in REV/GTR) to differ among branches of the tree as well. Thus the whole Q matrix can be different for different branches.

The position of the root may make a difference to the likelihood, so that in theory it may be necessary to use rooted trees. Nevertheless parameters around the root including the two branch lengths around the root are expected to be poorly estimated since the model is only weakly identifiable. In short you need to decide whether it is appropriate to use a rooted tree or unrooted tree. Because of the parameter richness, the models may only work if you have very long sequences. Yang and Roberts (1995) used the HKY85 or F84 models, and so three independent frequency parameters are used to describe the substitution pattern, while Galtier and Gouy (1998) used the T92 substitution model and uses the GC content π_{GC} only, with the base frequencies give as $\pi_T = \pi_A = (1 - \pi_{GC})/2$ and $\pi_C = \pi_G = \pi_{GC}/2$. The option **nhomo = 4** assigns one set of frequency parameters for the root, which are the initial base frequencies at the root, and one set for each branch in the tree. This is model N2 in Yang and Roberts (1995) if the substitution model is F84 or HKY85 or the model of Galtier and Gouy (1998) if the substitution model is T92. Option **nhomo = 3** uses one set of base frequencies for each tip branch, one set for all internal branches in the tree, and one set for the root. This is model N1 in Yang and Roberts (1995). The option **nhomo = 5** lets the user specify how many sets of frequency parameters should be used and which branch should use which set. The set for the root specifies the initial base frequencies at the root while the set for any other node is for parameters in the substitution matrix along the branch leading to the node. You use node (branch) labels in the tree file (see the subsection "Tree file and representations of tree topology" above) to tell the program which set each branch should use. There is no need to specify the default set (0). So for example nhomo = 5 and the following tree in the tree file specifies sets 1, 2, 3, and 4 for the four tip branches, set 5 for the root, while all the internal branches (nodes) will have the default set 0. This is equivalent to nhomo = 3.

```
(((1 #1, 2: #2), 3 #3), 4 #4) #5;
```

The output for nhomo = 3, 4, 5 is under the heading "(frequency parameters for branches) [frequencies at nodes] (see Yang & Roberts 1995 fig 1)". Two sets of frequencies are listed for each node. The first set are the parameters (used in the substitution rate matrix for the branch leading to the node), and the second set are the expected base frequencies at the node, calculated from the model ((Yang and Roberts 1995); page 456 column top). If the node is the root, the same set of frequencies are printed twice.

Note that the use of the variable `fix_kappa` with nhomo = 3, 4 or 5 is somewhat unusual. `fix_kappa` = 1 means one common κ is assumed and estimated for all branches, while `fix_kappa` = 0 means one κ is estimated for each branch. `fix_kappa` = 2 means that the same branch/node labels are used to specify the exchangeability parameters (κ in F84, HKY85, and T92; κ_1 and κ_2 in TN93, and *a b c d e* in REV/GTR) for branches.

As an example, suppose we have the following options in the control file

```
model = 7 (REV/GTR)
nhomo = 5
fix_kappa = 2
```

and the tree has the following node/branch labels,

```
(((1 #1, 2: #2), 3 #3), 4 #4) #5;
```

there will be 49 parameters: 6 branch lengths in the rooted tree, 5 sets of exchangeability parameters (*a b c d e*), and 6 sets of base frequency parameters, with $6 + 5 \times 5 + 6 \times 3 = 49$. In other words, the model will assume different GTR rate matrices for the four tip branches (with 8 free parameters in each Q matrix), and one Q matrix for the two internal branches,

plus the initial base frequencies at the root, plus the 6 branch lengths, with $5 \times 8 + 3 + 6 = 49$.

Note that the same labels in the tree are used to label both the nodes (for the frequencies) and the branches (for the exchangeability parameters in the rate matrix). An awkard issue is that we need a set of base frequencies at the root node but we do not need a set of exchangeability parameters for the root branch. Thus with a rooted tree of *s* species, there are $2s – 1$ sets of frequencies and $2s – 2$ sets of exchangeability parameters. If the root has a separate frequency parameters, please use the last (largest) label for the root: in the example above, the root has a new set (#5) of frequency parameters, so that the six sets of frequency parameters are labelled #0, #1, …, #5 while the five sets of exchangeability parameters are labelled #0, #1, …, #4.

As another example, the following two labellings or trees are equivalent

```
(((1 #1, 2: #1), 3), 4);
(((1 , 2) #1, 3 #1) #1, 4 #1)  #1;
```

Either of them will fit the same model, which assumes that the base frequencies have been stationary until the ancestor of species 1 and 2, at which point the two lineages 1 and 2 have been drifting away from the old frequencies. Note that all branches and nodes are labelled, with the default to be #0. In particular, the root node in the first tree has the label #0.

With nhomo = 3, 4, 5, the Expected Markov counting (EMC) method of Matsumoto et al. (2015) generates printouts under the heading "`Expected numbers of nucleotide changes on branches expected`" in the output file. This is available for the model of one rate for all sites only (`fix_alpha` = 1, `alpha` = 0).

getSE = 0, 1, or 2 tells whether we want estimates of the standard errors of estimated parameters. These are crude estimates, calculated by the curvature method, *i.e.*, by inverting the matrix of second derivatives of the log-likelihood with respect to parameters. The second derivatives are calculated by the difference method, and are not always reliable. Even if this approximation is reliable, tests relying on the SE's should be taken with caution, as such tests rely on the normal approximation to the maximum likelihood estimates. The likelihood ratio test should always be preferred. The option is not available and choose `getSE = 0` when tree-search is performed.

RateAncestor = 0 or 1. Usually use 0. The value 1 forces the program to do two additional analyses, which you can ignore if you don't need the results. First under a model of variable rates across sites such as the gamma, `RateAncestor` = 1 forces the program to calculate rates for individual sites along the sequence (output in the file `rates`), using the empirical Bayes procedure (Yang and Wang 1995).

Second `RateAncestor` = 1 forces the program to perform the empirical Bayesian reconstruction of ancestral sequences (Yang *et al.* 1995b; Koshi and Goldstein 1996; Yang 2006 pages 119-124). Ancestral state reconstruction by parsimony (Fitch 1971; Hartigan 1973) is well known (implemented in the program pamp in PAML). It can also be achieved using the likelihood/empirical Bayes approach. Often the two approaches produce similar results, but the likelihood-based reconstruction has two advantages over parsimony: it uses information from the branch lengths and the relative substitution rates between characters (nucleotides), and it provides a measure of uncertainties in the form of posterior probabilities for reconstructed ancestral states.

The outputs are listed, by site, in the file `rst`. You can also use the variable `verbose` to control the amount of output. If `verbose` = 0, only the best nucleotide (the one with the highest posterior proobability) at each node at each site is listed, while with `verbose` = 1 (try 2 if 1 does not work), the full posterior probability distribution from the marginal reconstruction is listed. If the model is homogenous (nhomo = 0, 1) and assumes one rate

for all sites, both the joint and marginal ancestral reconstructions will be calculated. If the model assumes variable rates among sites like the gamma model, only the marginal reconstructions are calculated.

Marginal and *joint* reconstructions. Marginal reconstruction considers character assignments to one single interior node and the character with the highest posterior probability is the best reconstruction (eq. 4 in Yang *et al.* 1995b; or Eq. 4.15 in Yang 2006). The algorithm for marginal reconstruction implemented in PAML works under both the model of a constant rate for all sites and the gamma model of rates at sites. Joint reconstruction considers all ancestral nodes at the same time and the reconstruction (the set of characters at a site assigned to all interior nodes) with the highest posterior probability is the best reconstruction (eq. 2 in Yang *et al.* 1995b; or Eq. 4.16 in Yang 2006). The algorithm for joint reconstruction implemented in PAML is based on that of Pupko *et al.* (2000), which gives the best reconstruction at each site and its posterior probability. This works under the model of one rate for all sites only. (It works under the partition models.) The marginal and joint approaches use slightly different criteria, and expected to produce consistent results; that is, the most probable joint reconstruction for a site should almost always consist of characters that are also the best in the marginal reconstruction. Conflicts may arise in borderline cases where two or more reconstructions have similar posterior probabilities.

A good use of ancestral sequence reconstruction is to synthesize the inferred ancestral proteins and measure their biochemical properties in the lab (Pauling and Zuckerkandl 1963; Chang and Donoghue 2000; Thornton 2004). It is also very popular to use reconstructed ancestral sequences as if they were real observed data to perform further analysis. You should resist this irresistible temptation and use full likelihood methods if they are available (e.g., Yang 2002). See section 4.4.4 in Yang (2006) for a discussion of systematic biases in ancestral reconstruction.

For nucleotide based (`baseml`) analysis of protein coding DNA sequences (option GC in the sequence data file), the program also calculates the posterior probabilities of ancestral amino acids. In this analysis, branch lengths and other parameters are estimated under a nucleotide substitution model, but the reconstructed nucleotide triplets are treated as a codon to infer the most likely amino acid encoded. Posterior probabilities for stop codons are small and reset to zero to scale the posterior probabilities for amino acids. To use this option, you should add the control variable `icode` in the control file `baseml.ctl`. This is not listed in the above. The variable `icode` can take a value out of 0, 1, ..., 11, corresponding to the 12 genetic codes included in PAML (See the control file `codeml.ctl` for the definition of different genetic codes). A nucleotide substitution model that is very close to a codon-substitution model can be specified as follows. You add the option characters GC at the end of the first line in the data file and choose model = 4 (HKY85) and `Mgene` = 4. The model then assumes different substitution rates, different base frequencies, and different transition/transversion rate ratio (kappa) for the three codon positions. Ancestral reconstruction from such a nucleotide substitution should be very similar to codon-based reconstruction.

Ancestral reconstruction under nonhomogeneous models. I have added the option of joint ancestral reconstruction under the nonhomogeneous models. The option variables are nhomo = 3 (N1 in YR1995), 4 (N2 in YR1995), and 5 (user-specified branch types) and model = 3 , 4, 5, 6, 7 (3:F84, 4:HKY85, 5:T92, 6:TN93, 7:REV). This works only for the model of one rate for all sites, and does not work for the model of gamma rates for sites or the partition models (option G). Only joint reconstruction is available as the algorithm I used for marginal reconstruction does not work for nonhomogeneous models.

Small_Diff is a small value used in the difference approximation of derivatives. Use a value between 1e-8 and 1e-5 and check that the results are not sensitive to the value used.

cleandata = 1 means sites involving ambiguity characters (undetermined nucleotides such as N, ?, W, R, Y, etc. anything other than the four nucleotides) or alignment gaps are removed from all sequences. This leads to faster calculation. cleaddata = 0 (default) uses those sites.

method: This variable controls the iteration algorithm for estimating branch lengths under a model of no clock. method = 0 implements the old algorithm in PAML, which updates all parameters including branch lengths simultaneously. method = 1 specifies an algorithm newly implemented in PAML, which updates branch lengths one by one. method = 1 does not work under the clock models (clock = 1, 2, 3).

icode: This specifies the genetic code to be used for ancestral reconstruction of protein-coding DNA sequences. This is implemented to compare results of ancestral reconstruction with codon-based analysis. For example the F3×4 codon model of Goldman and Yang (1994) is very similar to the nucleotide model HKY85 with different substitution rates and base frequencies for the three codon positions. The latter is implemented by using use options GC in the sequence data file and model = 4 and Mgene = 4. To use the option icode, you have to choose RateAncestor = 1.

fix_blength: This tells the program what to do if the tree has branch lengths. The value **0** (ignore) means that branch lengths in the tree file (if they exist) will be ignored and initial branch lengths are generated using pairwise distances and random numbers. The value **–1** (random) means that random initial branch lengths will be used. This option generates very poor branch lengths and might be useful if there are multiple local optima on the likelihood surface. The value **1** (initial) means that branch lengths in the tree file will be used as initial values for ML iteration. Try to avoid using "branch lengths" from a parsimony analysis, as those are numbers of changes for the entire sequence (rather than per site) and are very poor initial values. The value **2** (fixed) means that branch lengths will be fixed at those values given in the tree file and not estimated by ML. In this case, you should make sure that the branch lengths are sensible; for example, if two sequences in the data file are different, but the branch lengths connecting the two sequences in the tree are all zero, the data and tree will be in conflict and the program will abort. The value **3** (proportional) means that branch lengths will be proportional to those given in the tree file, and the proportionality factor is estimated by ML.

Output: The output should be self-explanatory. Descriptive statistics are always listed. The observed site patterns and their frequencies are listed, together with the proportions of constant patterns. Nucleotide frequencies for each species (and for each gene in case of multiple gene data) are counted and listed. lmax = ln(Lmax) is the upper limit of the log likelihood and may be compared with the likelihood for the best (or true) tree under the substitution model to test the model's goodness of fit to data (Goldman 1993; Yang *et al.* 1995a). You can ignore it if you don't know what it means. The pairwise sequence distances are included in the output as well, and also in a separate file called 2base.t. This is a lower-diagonal distance matrice, readable by the NEIGHBOR program in Felesenstein's PHYLIP package (Felsenstein 2005). For models JC69, K80, F81, F84, the appropriate distance formulas are used, while for more complex models, the TN93 formula is used. baseml is mainly a maximum likelihood program, and the distance matrix is printed out for convenience and really has nothing to do with the later likelihood calculation.

With getSE = 1, the S.E.s are calculated as the square roots of the large sample variances and listed exactly below the parameter estimates. Zeros on this line mean errors, either caused by divergence of the algorithm or zero branch lengths. The S.Es of the common parameters measure the reliability of the estimates. For example, $(\kappa - 1)/SE(\kappa)$, when κ is estimated under K80, can be compared with a normal distribution to see whether there is real difference between K80 and JC69. The test can be more reliably performed by comparing the log-likelihood values under the two models, using the likelihood ratio test. It has to be stressed that the S.E.'s of the estimated branch lengths should not be misinterpreted as an evaluation of the reliability of the estimated tree topology (Yang 1994b).

If the tree file has more than one tree, the programs `baseml` and `codeml` will calculate the bootstrap proportions using the RELL method (Kishino and Hasegawa 1989), as well as the method of Shimodaira and Hasegawa (1999) with a correction for multiple comparison. The bootstrap resampling accounts for possible data partitions (option G in the sequence data file).

5 basemlg

basemlg uses the same control file `baseml.ctl`, as `baseml`. Tree-search or the assumption of a molecular clock are not allowed and so choose `runmode` = 0 and `clock` = 0. Substitution models available for `basemlg` are JC69, F81, K80, F84 and HKY85, and a continuous gamma is always assumed for rates at sites. The variables `ncatG, given_rho, rho, nhomo` have no effect. The S.E.'s of parameter estimates are always printed out because they are calculated during the iteration, and so getSE has no effect.

Because of the intensive computation required by `basemlg`, the discrete-gamma model implemented in `baseml` is recommended for data analysis. If you choose to use `basemlg`, you should run `baseml` first, and then run `basemlg`. This allows `baseml` to collect initial values into a file named `in.basemlg`, for use by `basemlg`. Note that `basemlg` implements only a subset of models in `baseml`.

6 codeml (codonml and aaml)

Codon substitution models

There is now a large collection of codon substitution models. See Yang and Bielawski (2000), Yang (2002) and Yang (2006: Chapter 8) for detailed discussions.

The basic model is a simplified version of the model of Goldman and Yang (1994) and specifies the substitution rate from codon *i* to codon *j* as

$$q_{ij} = \begin{cases} 0, & \text{if the two codons differ at more than one position,} \\ \pi_j, & \text{for synonymous transversion,} \\ \kappa\pi_j, & \text{for synonymous transition,} \\ \omega\pi_j, & \text{for nonsynonymous transversion,} \\ \omega\kappa\pi_j, & \text{for nonsynonymous transition,} \end{cases}$$

(Yang *et al.* 1998). The equilibrium frequency of codon *j* (π_j) can be considered a free parameter, but can also be calculated from the nucleotide frequencies at the three codon positions (control variable CodonFreq). Under this model, the relationship holds that $\omega = d_N/d_S$, the ratio of nonsynonymous/synonymous substitution rates. This basic model is fitted by specifying model = 0 NSsites = 0, in the control file codeml.ctl.

The ω ratio is a measure of natural selection acting on the protein. Simplistically, values of $\omega < 1$, = 1, and > 1 means negative purifying selection, neutral evolution, and positive selection. However, the ratio averaged over all sites and all lineages is almost never > 1, since positive selection is unlikely to affect all sites over prolonged time. Thus interest has been focused on detecting positive selection that affects only some lineages or some sites.

The *branch model*s allow the ω ratio to vary among branches in the phylogeny and are useful for detecting positive selection acting on particular lineages (Yang 1998; Yang and Nielsen 1998). They are specified using the variable model. model = 1 fits the so-called *free-ratios* model, which assumes an independent ω ratio for each branch. This model is very parameter-rich and its use is discouraged. model = 2 allows you to have several ω ratios. You have to specify how many ratios and which branches should have which rates in the tree file by using branch labels. See "Branch or node labels" in the section "Tree file format" in Chapter 4. The lysozyme example data files are included in the examples/lysozyme/ folder; check the readme file.

The *site model*s allow the ω ratio to vary among sites (among codons or amino acids in the protein) (Nielsen and Yang 1998; Yang *et al.* 2000b). A number of such models are implemented in codeml using the variable Nssites (and model = 0). You can run several Nssites models in one go, by specifying several values for NSsites. For example, NSsites = 0 1 2 7 8 will fit 5 models to the same data in one go. The site models have been used in real data analyses and evaluated in computer simulation studies (Anisimova *et al.* 2001, 2002; Anisimova *et al.* 2003; Wong *et al.* 2004). Two pairs of models appear to be particularly useful, forming two likelihood ratio tests of positive selection. The first compares M1a (NearlyNeutral) and M2a (PositiveSelection), while the second compares M7 (beta) and M8 (beta&ω). M1a (NearlyNeutral) and M2a (PositiveSelection) are slight modifications of models M1 (neutral) and M2 (selection) in (Nielsen and Yang 1998). The old models M1 and M2 fix $\omega_0 = 1$ and $\omega_1 = 1$, and are unrealistic as they do not account for sites with $0 < \omega < 1$. In the new models M1a and M2a, described in Wong *et al.* (2004) and Yang *et al.* (2005) and implemented since version 3.14 (2004), $0 < \omega_0 < 1$ is estimated from the data while $\omega_1 = 1$ is fixed. Also since version 3.14, the BEB procedure for identifying positively selected sites is included besides the NEB procedure (Yang *et al.* 2005). In both tests comparing M2a against M1a or M8 against M7, df = 2 may be used. The M1a-M2a comparison appears to be more stringent than

the M7-M8 comparison. See the table below.

Table 2. Parameters in the site models

Model	NSsites	#p	Parameters	Note
M0 (one ratio)	0	1	ω	(Goldman and Yang 1994; Yang and Nielsen 1998)
M1a (neutral)	1	2	p_0 ($p_1 = 1 - p_0$), $\omega_0 < 1$, $\omega_1 = 1$	(Nielsen and Yang 1998; Yang *et al.* 2005)
M2a (selection)	2	4	p_0, p_1 ($p_2 = 1 - p_0 - p_1$), $\omega_0 < 1$, $\omega_1 = 1$, $\omega_2 > 1$	(Nielsen and Yang 1998; Yang *et al.* 2005)
M2a_rel	22	4	p_0, p_1 ($p_2 = 1 - p_0 - p_1$), $\omega_0 < 1$, $\omega_1 = 1$, $\omega_2 > 0$	$\omega_2 > 0$, for use as null for testing the clade model (Weadick and Chang 2012)
M3 (discrete)	3	5	p_0, p_1 ($p_2 = 1 - p_0 - p_1$) $\omega_0, \omega_1, \omega_2$	(Yang *et al.* 2000b)
M7 (beta)	7	2	p, q	(Yang *et al.* 2000b)
M8 (beta&ω)	8	4	p_0 ($p_1 = 1 - p_0$), $p, q, \omega_s > 1$	(Yang *et al.* 2000b)

NOTE.— #p is the number of free parameters in the ω distribution. Parameters in parentheses are not free and should not be counted: for example, in M1a, p_1 is not a free parameter as $p_1 = 1 - p_0$. In both likelihood ratio tests comparing M1a against M2a and M7 against M8, df = 2. The site models are specified using `NSsites`.

A third test compares the null hypothesis M8a (beta&ω_s =1) and M8 (Swanson *et al.* 2003; Wong *et al.* 2004). M8a is specified using `NSsites = 8, fix_omega = 1, omega = 1`. The null distribution is the 50:50 mixture of point mass 0 and χ_1^2 (Self and Liang 1987). The critical values are 2.71 at 5% and 5.41 at 1% (as opposed to 3.84 for 5% and 6.63 for 1% for χ_1^2). Note that the *p* value for a 50:50 mixture of χ_j^2 and χ_k^2 is just the average of the two *p* values from the two distributions, in the case of M8a-M8 comparison, you get the *p* value from χ_1^2 and then half it to get the *p* value for the mixture distribution. You can also use χ_1^2 (Wong *et al.* 2004).

We suggest that The M0-M3 comparison should be used as a test of variable ω among sites rather than a test of positive selection. However, the model of a single ω for all sites is probably wrong in every functional protein, so there is little point of testing.

The naïve empirical Bayes (NEB) (Nielsen and Yang 1998; Yang *et al.* 2000b) and the Bayes empirical Bayes (BEB) (Yang *et al.* 2005) are available for calculating the posterior probabilities for site classes, and can be used to identify sites under positive selection if the likelihood ratio test is significant. NEB uses the MLEs of parameters (such as the proportions and ω ratios) but do not account for their sampling errors, while BEB deals with the sampling errors by applying a Bayesian prior. BEB is implemented under models M2a and M8 only. We suggest that you ignore the NEB output and use the BEB results only.

> The BEB output has the following format:
>
> Prob(w>1) mean w
>
> 135 K 0.983* 4.615 +- 1.329
>
> Interpretation: 4.615 is the approximate mean of the posterior distribution for w, and 1.329 is the square root of the variance in the posterior distribution for w.   The program prints out an * if the posterior probability is >95%, and ** if the probability is > 99%.

Suzuki and Gojobori's (1999) method for detecting sites under positive selection. In the terminology used here, The SG99 method tests whether the ω ratio for each site is >1 or <1. It uses parsimony to reconstruct ancestral sequences, and then for each site, counts the numbers of synonymous and nonsynonymous differences and the numbers of synonymous and nonsynonymous sits. It then test whether the ω ratio at the site is significantly different from 1. Errors in the ancestral sequence reconstruction are ignored. Suzuki has a program called AdaptSite that implements the test. This is an intuitive test, and is known to lack power.

In codeml, a test of this kind is implemented as a by-product of ancestral sequence reconstruction in codeml and baseml, which use ML to reconstruct ancestral sequences. Use RateAncestor = 1. The choice of baseml versus codeml and also the choice of substitution model for each program affects ancestral sequence reconstruction only. The later steps are the same, and follow Suzuki & Gojobori (1999). For codeml, you can use M0 (NSsites = 0 and model = 0). If you want, you can try some other models, such as NSsites = 2 or 8. The models for ancestral reconstruction typically make little difference. For baseml, you should have "GC" on the first line of the sequence data file to indicate that the sequences are protein coding. Use icode (= 0 for the universal code and 1 for vertebrate mitochondrial code) in the control file to specify the genetic code, as in codeml. The following "multiple-gene" model is close to M0: model = 4 Mgene = 4 (see (Yang 1996b) and the section titled "Models for combined analysis of partitioned data").

NSsites = 22 now specifies the site model M2a_rel of Weadick & Chang (2012). This is the same as M2a except that $\omega_2 > 0$, while model M2a (NSsites = 2) has $\omega_2 > 1$. M2a_rel is the null model for the likelihood ratio test based on clade model C (Weadick and Chang 2012).

The *branch-site* models allow ω to vary both among sites in the protein and across branches on the tree and aim to detect positive selection affecting a few sites along particular lineages (called foreground branches). Initially Yang and Nielsen (2002) implemented model A (model = 2 NSsites = 2) and model B (model = 2 NSsites = 3). The tests did not work well in simulations (Zhang 2004), so a change was introduced to model A (table 3) (Yang *et al.* 2005; Zhang *et al.* 2005), with two tests constructed. Test 2, also known the branch-site test of positive selection, is the recommended test. This compares the modified model A with the corresponding null model with ω_2 = 1 fixed (fix_omega = 1 and omega = 1). The null distribution is the 50:50 mixture of point mass 0 and χ_1^2, with critical values 2.71 at 5% and 5.41 at 1%. To calculate the *p* value based on this mixture distribution, you calculate the *p* value using χ_1^2, and then divide it by 2. Suppose your $2\Delta\ell$ = 2.71, you will get 0.10 from χ_1^2, the the *p* value for the mixture is 0.10/2 = 5%. We recommend that you use χ_1^2 (with critical values 3.84 and 5.99) instead of the mixture to guide against violations of model assumptions.

Similarly both the NEB and BEB methods for calculating posterior probabilities for site classes are implemented for the modified branch-site model A (not for model B). You should use model A in combination with the BEB procedure and ignore the NEB output.

Table 3. Parameters in branch-site model A (np = 4)

Site class	Proportion	Background	Foreground
0	p_0	$0 < \omega_0 < 1$	$0 < \omega_0 < 1$
1	p_1	$\omega_1 = 1$	$\omega_1 = 1$
2a	$(1 - p_0 - p_1)\, p_0/(p_0 + p_1)$	$0 < \omega_0 < 1$	$\omega_2 \geq 1$
2b	$(1 - p_0 - p_1)\, p_1/(p_0 + p_1)$	$\omega_1 = 1$	$\omega_2 \geq 1$

NOTE.— Branch-site model A is specified using model = 2 NSsites = 2. This is the alternative model in the branch-site test of positive selection. The null model fixes $\omega_2 = 1$. The likelihood ratio test has df = 1 (see text).

Clade model C is specified by model = 3 Nssites = 2 while clade model D is specified by model = 3 NSsites = 3 (Bielawski and Yang 2004; see also Forsberg and Christiansen 2003). In both models, the number of site classes (ncatG) is fixed at 3. Clade model C went through a modification, in a similar way to branch-site model A. The new model C replaces $\omega_0 = 0$ by $0 < \omega_0 < 1$ and has 5 parameters in the ω distribution (if there are two clades or branch types): p_0, p_1, ω_0, ω_2, and ω_3. The new model C can be compared with the new M1a (NearlyNeutral), which has 2 parameters, with d.f. ≈ 3. A more powerful test is to use the site model M2a_rel of Weadick & Chang (2012) as the null model, with df = 1.

Table 4. Parameters in clade models C or D, with more than two clades (branch types)

Site class	Proportion	Clade 1	Clade 2	Clade 3	Clade 4
0	p_0	ω_0	ω_0	ω_0	ω_0
1	p_1	ω_1	ω_1	ω_1	ω_1
2	$p_2 = 1 - p_0 - p_1$	ω_2	ω_3	ω_4	ω_5

In clade model C, $\omega_1 = 1$ is fixed, while in clade model D, ω_1 ($0 < \omega_1 < \infty$) is estimated as a free parameter. In both models, ω_0 is estimated under the constraint $0 < \omega_0 < 1$.

The BEB procedure is implemented for clade models C and D. Note that BEB is preferred over NEB.

An extension has been made to clade models C and D to allow for more than two clades or branch types. The branch types are specified using labels in the tree file. If you have four branch types (labelled using #0, #1, #2, #3), the clade model will look like the following. Here ω_2, ω_3, ω_4, ω_5 are independent parameters, optimized in the range $(0, \infty)$. The BEB calculation under clade model C is very expensive (with each additional branch type increasing the computation by 10 folds), so that the model should be used with just a few branch types. The maximum number of clades is set by the variable NBTYPE near the beginning of the source file codeml.c. I think this is right now set to 10. However, the BEB calculation may be too expensive if you have 5 clades. In the case of 4 clades (as in table 4), the calculation uses about 4.4GB of memory. There are 8 dimensions in the grid: two dimensions for p_0-p_1 (see figure 1 in Yang et al. 2005), ω_0 for site class 0, ω_1 for site class 1, and ω_2, ω_3, ω_4, ω_4 for site class 2, so that the grid has 10^8 points.

The mutation-selection models of Yang and Nielsen (2008) are implemented using the control variables

```
CodonFreq = 7  * 0:1/61 each, 1:F1X4, 2:F3X4, 3:codon table
               * 4:F1x4MG, 5:F3x4MG, 6:FMutSel0, 7:FMutSel
  estFreq = 0  * 0: use observed freqs; 1: estimate freqs by ML
```

where CodonFreq = 6 specifies FMutSel0 and 7 specifies FmutSel (Yang and Nielsen 2008). The FmutSel model assigns a fitness to every codon with 60 (= 61 – 1) codon fitness parameters for the universal genetic code. The FmutSel0 model is a special case of FmutSel and assigns the same fitness value for all synonymous codons, so that only 19 (= 20 – 1) amino acid fitness parameters are used. This model assumes that the amino acid frequencies are determined by the functional requirements of the protein, and given the amino acid frequencies, the relative frequencies of synonymous codons are determined solely by the mutational-bias parameters.

If estFreq = 1, the frequency/fitness parameters are estimated by ML from the data, while if estFreq = 0, they are calculated using the observed frequencies in the data. See Yang and Nielsen (2008) for details of the model. Look at the README file in the examples/mtCDNAape/ folder, to see how to duplicate results of table 1 in that paper.

Amino acid substitution models

I made a distinction between empirical and mechanistic models of amino acid substitution (Yang *et al.* 1998; 2006: Chapter 2). Empirical models implemented in codeml include Dayhoff (Dayhoff *et al.* 1978), JTT (Jones *et al.* 1992), WAG (Whelan and Goldman 2001), mtMam (Yang *et al.* 1998), mtREV (Adachi and Hasegawa 1996b), etc. These are estimates of substitution rate parameters under the general time reversible model from real datasets.

Mechanistic models are formulated by considering the mutational distance between the amino acids as determined by the locations of their encoding codons in the genetic code, and the filtering of mutations by natural seletion operating on the protein level (Yang *et al.* 1998). The program `aaml` implements a few such models, specified by the variable `aaDist`.

seqtype = 2 model = 6 (`FromCodon1`) specifies the mechanistic amino acid substitution model of Yang et al. (1998; table 3), which uses the Markov chain for codons and aggregates the synonymous codons into one state (the coded amino acid) to construct a model of amino acid substitution. This is an approximate formulation since the process after state aggregation is not Markovian. The model involves the parameter κ, but ω is not estimable. Branch lengths are in the expected number of amino acid substitutions per amino acid.

seqtype = 2 model = 5 (`FromCodon0`) specifies another codon-based amino acid substitution model, which treats amino acids as ambiguities codons. This is an exact formulation. This model involves both parameters κ and ω, but because ω is weakly identifiable, it cannot be reliably estimated, and as a result, the branch lengths are not reliably estimated either. Branch lengths are in the expected number of nucleotide substitutions per codon. This model is described in (Weber *et al.* 2021).

The control file

Since the codon based analysis and the amino acid based analysis use different models, and some of the control variables have different meanings, it may be a good idea to use different control files for codon and amino acid sequences. The default control file for `codeml` is `codeml.ctl`, as shown below.

```
      seqfile = stewart.aa * sequence data file name
      outfile = mlc * main result file name
     treefile = stewart.trees * tree structure file name

        noisy = 9  * 0,1,2,3,9: how much rubbish on the screen
      verbose = 0  * 1: detailed output, 0: concise output
      runmode = 0  * 0: user tree;  1: semi-automatic;  2: automatic
                   * 3: StepwiseAddition; (4,5):PerturbationNNI; -2: pairwise

      seqtype = 2  * 1:codons; 2:AAs; 3:codons-->AAs
    CodonFreq = 2  * 0:1/61 each, 1:F1X4, 2:F3X4, 3:codon table
 *        ndata = 10
        clock = 0   * 0:no clock, 1:clock; 2:local clock
```

```
       aaDist = 0  * 0:equal, +:geometric; -:linear, 1-6:G1974,Miyata,c,p,v,a
                   * 7:AAClasses
    aaRatefile = wag.dat * only used for aa seqs with model=empirical(_F)
                  * dayhoff.dat, jones.dat, wag.dat, mtmam.dat, or your own

        model = 2
                 * models for codons:
                     * 0:one, 1:b, 2:2 or more dN/dS ratios for branches
                 * models for AAs or codon-translated AAs:
                     * 0:poisson, 1:proportional,2:Empirical,3:Empirical+F
                     * 5:FromCodon0, 6:FromCodon1, 8:REVaa_0, 9:REVaa(nr=189)

       NSsites = 0  * 0:one w;1:neutral;2:selection; 3:discrete;4:freqs;
                    * 5:gamma;6:2gamma;7:beta;8:beta&w;9:beta&gamma;
                    * 10:beta&gamma+1; 11:beta&normal>1; 12:0&2normal>1;
                    * 13:3normal>0

         icode = 0  * 0:universal code; 1:mammalian mt; 2-11:see below
         Mgene = 0  * 0:rates, 1:separate;

     fix_kappa = 0  * 1: kappa fixed, 0: kappa to be estimated
         kappa = 2  * initial or fixed kappa
     fix_omega = 0  * 1: omega or omega_1 fixed, 0: estimate
         omega = .4 * initial or fixed omega, for codons or codon-based AAs

     fix_alpha = 1  * 0: estimate gamma shape parameter; 1: fix it at alpha
         alpha = 0. * initial or fixed alpha, 0:infinity (constant rate)
        Malpha = 0  * different alphas for genes
         ncatG = 3  * # of categories in dG of NSsites models

       fix_rho = 1  * 0: estimate rho; 1: fix it at rho
           rho = 0. * initial or fixed rho,    0:no correlation

         getSE = 0  * 0: don't want them, 1: want S.E.s of estimates
   RateAncestor = 0  * (0,1,2): rates (alpha>0) or ancestral states (1 or 2)

     Small_Diff = .5e-6
   *   cleandata = 0  * remove sites with ambiguity data (1:yes, 0:no)?
   * fix_blength = 0  * 0: ignore, -1: random, 1: initial, 2: fixed, 3: proportional
        method = 0   * 0: simultaneous; 1: one branch at a time
```

The variables seqfile, outfile, treefile, noisy, Mgene, fix_alpha, alpha, Malpha, fix_rho, rho, clock, getSE, RateAncestor, Small_Diff, cleandata, ndata, fix_blength, and method are used in the same way as in baseml.ctl and are described in the previous section. The variable seqtype specifies the type of sequences in the data; seqtype = 1 means codon sequences (the program is then codonml); 2 means amino acid sequences (the program is then aaml); and 3 means codon sequences which are to be translated into proteins for analysis.

*Codon sequences (*seqtype *= 1)*

CodonFreq specifies the equilibrium codon frequencies in codon substitution model. These frequencies can be assumed to be equal (1/61 each for the standard genetic code, CodonFreq = 0), calculated from the average nucleotide frequencies (CodonFreq = 1), from the average nucleotide frequencies at the three codon positions (CodonFreq = 2), or used as free parameters (CodonFreq = 3). The number of parameters involved in those models of codon frequencies is 0, 3, 9, and 60 (for the universal code), for CodonFreq = 0, 1, 2, and 3 respectively.

clock See the notes for the baseml control file.

aaDist specifies whether equal amino acid distances are assumed (= 0) or Grantham's matrix is used (= 1) (Yang *et al.* 1998). The example mitochondrial data set analyzed in that paper is included in the example/mtdna folder in the package. aaDist = 7 (AAClasses), which is implemented for both codon and amino acid sequences, allow you to have several types of amino acid substitutions and let the program estimate their different rates. The model was implemented in Yang *et al.* (1998). The number of substitution types and which pair of amino acid changes belong which type is specified in a file called OmegaAA.dat. You can use the model to fit different *ω* ratios for "conserved" and "charged" amino acid substitutions. The folder examples/mtCDNA contain example files for this model; check

the readme file in that folder.

runmode = -2 performs ML estimation of d_S and d_N in pairwise comparisons of protein-coding sequences (seqtype = 1). The program will collect estimates of d_S and d_N into the files `2ML.dS` and `2ML.dN`. Since many users seem interested in looking at d_N/d_S ratios among lineages, examination of the tree shapes indicated by branch lengths calculated from the two rates may be interesting although the analysis is *ad hoc*. If your species names have no more than 10 characters, you can use the output distance matrices as input to Phylip programs such as `neighbor` without any change. Otherwise you need to edit the files to cut the names short. For amino acid sequences (seqtype = 2), option runmode = -2 lets the program calculate ML distances under the substitution model by numerical iteration, either under the model of one rate for all sites (alpha = 0) or under the gamma model of rates for sites (alpha ≠ 0). In the latter case, the continuous gamma is used and the variable ncatG is ignored. (With runmode = 0, the discrete gamma is used.)

`runmode = -3` implements a Bayesian method for estimating distance *t* and dN/dS ratio *ω* in pairwise comparisons (Angelis *et al.* 2014). The default gamma priors are $t \sim G(1.1, 1.1)$ and $\omega \sim G(1.1, 2.2)$. To change the gamma parameters use

        `runmode = -3 1.1 1.1 1.1 2.2   * -3: pairwise Bayesian`

The four numbers are α_t, β_t, α_ω, β_ω.

model specifies the *branch* models (Yang 1998; Yang and Nielsen 1998). `model = 0` means one *ω* ratio for all branches; 1 means one ratio for each branch (the free-ratio model); and 2 means an arbitrary number of ratios (such as the 2-ratios or 3-ratios models). When `model = 2`, you have to group branches on the tree into branch groups using the symbols # or $ in the tree. See the section above about specifying branch/node labels.

With `model = 2`, the variable `fix_omega` fixes the last *ω* ratio (ω_{k-1} if you have *k* ratios in total) at the value of `omega` specified in the file. This option is used to test whether the ratio for the foreground branch is significantly greater than one. See the `examples/lysozyme/` folder to duplicate the results of Yang (1998).

NSsites specifies the site models, with `NSsites` = *m* corresponds to model M*m* in Yang *et al.* (2000b). The variable `ncatG` is used to specify the number of categories in the *ω* distribution under some models. In Yang *et al.* (2000b), this is 3 for M3 (discrete), 5 for M4 (freq), 10 for the continuous distributions (M5: gamma, M6: 2gamma, M7: beta, M8:beta&w, M9:beta&gamma, M10: beta&gamma+1, M11:beta&normal>1, and M12:0&2normal>1, M13:3normal>0). For example, M8 has 11 site classes (10 from the beta distribution plus 1 additional class for positive selection with $\omega_s \geq 1$). See the section Codon substittion models above about the changes to M1 and M2 introduced in PAML version 3.14.

You can run several `Nssites` models in one batch, as follows, in which case the default values of `ncatG`, as in Yang *et al.* (2000b), are used.

        `NSsites = 0 1 2 3 7 8`

The posterior probabilities for site classes as well as the expected *ω* values for sites are listed in the file `rst`, which may be useful to identify sites under positive selection.

Look at the examples/hivNSsites/ and examples/lysine/ folders for example of analysis under the site models.

The *branch-site* model A (see the section Codon substitution models above) is specified by using both variables `model` and `NSsites`.

        `Model A: model = 2, NSsites = 2,  fix_omega = 0`

This is the alternative model for the branch-site test of positive selection. The null model is

also the branch-site model A but with $\omega_2 = 1$ fixed, specified by

```
Model A1: model = 2, NSsites = 2,  fix_omega = 1, omega = 1
```

Here are some notes about two old tests that we do not recommend. The old branch-site model B (Yang and Nielsen 2002) is specified by

```
Model B: model = 2, NSsites = 3
```

The null model for the old test B is the NSsites model 3 (discrete) with 2 site classes:

```
Site model 3: model = 0, NSsites = 3,  ncatG = 2
```

Use d.f. = 2. The large lysozyme data set analyzed by Yang and Nielesn (2002) is in the examples/lysozyme folder.

Also branch-site test 1 described by Yang *et al.* (2005) and Zhang *et al.* (2005) uses the modified branch-site model A as the alternative hypothesis, while the null hypothesis is the new site model M1a (NearlyNeutral), with d.f. = 2. This test can be significant when the foreground branches are either under relaxed selective constraint or under positive selection. The advice is that you use test 2 instead, which is also known as the branch-site test of positive selection.

The *clade* models C and D of Bielawski and Yang (2004) are specified by

```
Model C: model = 3, NSsites = 2

Model D: model = 3, NSsites = 3  ncatG = 2
```

See that paper for details. Similarly model C is modified and the BEB procedure is implemented for model C only; see above.

icode specifies the genetic code. Eleven genetic code tables are implemented using `icode` = 0 to 10 corresponding to transl_table 1 to 11 in GenBank. These are 0 for the universal code; 1 for the mammalian mitochondrial code; 3 for mold mt., 4 for invertebrate mt.; 5 for ciliate nuclear code; 6 for echinoderm mt.; 7 for euplotid mt.; 8 for alternative yeast nuclear; 9 for ascidian mt.; and 10 for blepharisma nuclear. There is also an additional code, called Yang's *regularized code*, specified by `icode` = 11. In this code, there are 16 amino acids, all differences at the first and second codon positions are nonsynonymous and all differences at the third codon positions are synonymous; that is, all codons are 4-fold degenerate. There is yet no report of any organisms using this code.

Mgene, in combination with option G in the sequence data file, specifies partition models (Yang and Swanson 2002), as summarized in table 6. The lysin data set used in that paper is included in the examples/ folder of the package. The analysis separates the buried and exposed amino acids in the lysin into two partitions ("genes"), and heterogeneity between the partitions are accounted for in the analysis. You can read the readme file and try to duplicate the results in table 6 of Yang & Swanson (2002).

Table 5. Setups of partition models of codon substitution

Sequence file	Control file	Parameters across genes
No G	Mgene = 0	everything equal
Option G	Mgene = 0	the same (κ, ω) and π, but different cs (proportional branch lengths)
Option G	Mgene = 2	the same (κ, ω), but different πs and cs
Option G	Mgene = 3	the same π, but different (κ, ω) and cs

Option G	Mgene = 4	different (κ, ω), πs, and cs
Option G	Mgene = 1	separate analysis

fix_alpha, alpha: For codon models, the pair fix_alpha and alpha specify the model of gamma rates for sites, in which the relative rate for a site varies among codons according to the gamma distribution, but the ω ratio stays the same over all sites. This is a lazy extension of the gamma model of rates for nucleotides and amino acids. I don't like this model and suggest that you use the NSsites models instead (which is specified using the NSsites variable, with fix_alpha = 1, alpha = 0). The program should abort if you specify both NSsites and alpha.

RateAncestor: See descriptions for the baseml control file.

Output for codon sequences (seqtype = 1): The codon frequencies in each sequence are counted and listed in a genetic code table, together with their sums across species. Each table contains six or fewer species. For data of multiple genes (option G in the sequence file), codon frequencies in each gene (summed over species) are also listed. The nucleotide distributions at the three codon positions are also listed. The method of Nei and Gojobori (1986) is used to calculate the number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N) and their ratio (d_N/d_S). These are used to construct initial estimates of branch lengths for the likelihood analysis but are not MLEs themselves.

Results of ancestral reconstructions (RateAncestor = 1) are collected in the file rst. Under models of variable d_N/d_S ratios among sites (NSsites models), the posterior probabilities for site classes as well as positively selected sites are listed in rst.

Amino acid sequences (seqtype = 2)

model specifies the model of amino acid substitution: 0 for the Poisson model assuming equal rates for any amino acid substitutions (Bishop and Friday 1987); 1 for the proportional model in which the rate of change to an amino acid is proportional to the frequency of that amino acid. Model = 2 specifies a class of empirical models, and the empirical amino acid substitution rate matrix is given in the file specified by aaRatefile. Files included in the package are for the empirical models of Dayhoff (dayhoff.dat), JTT (jtt.dat), WAG (wag.dat), mtMAM (mtmam.dat), mtREV24 (mtREV24.dat), etc.

Those files have the same structure, consisting of two parts: a lower-triangular matrix (s_{ij}) followed by 20 amino acid frequencies (π_j). The triangular matrix specifies the symmetrical part of the matrix, s_{ij} with $s_{ij} = s_{ji}$; these are called exchangeabilities in Whelan & Goldman 2001. The amino acid substitution rate is then given by the product of the two: $q_{ij} = s_{ij}\pi_j$ (see Yang 2014, eq. 2.2). Please see notes in dayhoff.dat, say. Note that both the exchangeabilities (s_{ij}) and substitution rates (q_{ij}) are relative, so that multiplying the whole matrix by a constant has no effect. The whole matrix is scaled in codeml so that the average rate is 1.

If you want to specify your own substitution rate matrix, you should take a look at one of those files. Also when you choose to estimate the GTR rate matrix from the amino acid alignment (model = 9), the rate matrix will also be printed in this format.

model = 3 is a slight variation to model = 2, with the amino acid frequencies in the aaRatefile replaced by the amino acid frequencies observed in the alignment. The model may be considered an extension to the empirical model (model = 2) with amino acid frequencies treated as free parameters (which are estimated by using the observed frequencies in the sequence alignment). If the user estimated the GTR rate matrix from the alignment, model = 2 should be used.

model = 5 and 6 are mentioned above. Modl = 6 (FromCodon1) specifies the mechanistic

amino acid substitution model of Yang et al. (1998; table 3), while model = 5 (`FromCodon0`) specifies another codon-based amino acid substitution model, which treats amino acids as ambiguities codons ().

model = 9 (REVaa) specifies the GTR model for amino acids. This forces codeml to estimate the 189 exhangeability rates while the amino acid frequencies are estimated using the average observed frequencies. This model is described in Yang et al. (1998). You can use a reasonable tree to estimate the GTR rate matrix from your alignment and then use the empirical matrix to conduct tree search in IQ-tree, RAxML, or PhyloBayes. See (Kapli *et al.* 2023) for such an example.

model = 8 (REVaa0) specifies a special case of the GTR model for amino acids in which amino acid pairs that are separated by two or three differences have the rate 0; in other words, only amino acids that are one-step apart can change instantaneously. This model is described in Yang et al. (1998).

Finally note that some option variables are for codon models only and should be ignored in analysis of amino acid sequenecs. Specifically, the variables `model`, `aaDist`, `CodonFreq`, `NSsites`, and `icode` are used for codon sequences (`seqtype` = 1), while `model`, `alpha`, and `aaRatefile` are used for amino acid sequences.

Mgene, in combination with option G in the sequence data file, specifies partition models (Yang and Swanson 2002), as summarized in table 6. The lysin data set used in that paper is included in the examples/ folder of the package. The analysis separates the buried and exposed amino acids in the lysin into two partitions ("genes"), and heterogeneity between the partitions are accounted for in the analysis. You can read the readme file and try to duplicate the results in table 6 of Yang & Swanson (2002).

Table 6. Setups of partition models of amino acid substitution

Sequence file	Control file	Parameters across genes
No G	Mgene = 0	everything equal
Option G	Mgene = 0	the same π, but different cs (proportional branch lengths)
Option G	Mgene = 2	different πs and cs
Option G	Mgene = 1	separate analysis

runmode also works in the same way as in `baseml.ctl`. Specifying runmode = −2 will forces the program to calculate the ML distances in pairwise comparisons. You can change the following variables in the control file `codeml.ctl`: `aaRatefile`, `model`, and `alpha`.

If you do pairwise ML comparison (`runmode` = -2) and the data contain ambiguity characters or alignment gaps, the program will remove all sites which have such characters from all sequences before the pairwise comparison if `cleandata` = 1. This is known as "complete deletion". It will remove alignment gaps and ambiguity characters in each pairwise comparsion ("pairwise" deletion) if `cleandata` = 0. [[This does not seem to be true. The program currently removes all sites with any ambiguities if `runmode` = -2. Need checking. Note by Ziheng 31/08/04.]] Note that in a likelihood analysis of multiple sequences on a phylogeny, alignment gaps are treated as ambiguity characters if `cleandata` = 0, and both alignment gaps and ambiguity characters are deleted if `cleandata` = 1. Note that removing alignment gaps and treating them as ambiguity

characters both underestimate sequence divergences. Ambiguity characters in the data (`cleandata = 0`) make the likelihood calculation slower.

Output for amino acid sequences (`seqtype = 2`): The output file is self-explanatory and very similar to the result files for the nucleotide- and codon-based analyses. The empirical models of amino acid substitution (specified by dayhoff.dat, jones.dat, wag.dat, mtmam.dat, or mtREV24.dat) do not involve any parameters in the substitution rate matrix. When `RateAncestor = 1`, results for ancestral reconstruction are in the file `rst`. Calculated substitution rates for sites under models of variable rates for sites are in `rates`.

7 evolver

This program generates a naïve menu, like the following.

```
(1) Get random UNROOTED trees?
(2) Get random ROOTED trees?
(3) List all UNROOTED trees into file trees?
(4) List all ROOTED trees into file trees?
(5) Simulate nucleotide data sets (use MCbase.dat)?
(6) Simulate codon data sets      (use MCcodon.dat)?
(7) Simulate amino acid data sets (use MCaa.dat)?
(8) Calculate identical bi-partitions between trees?
(9) Calculate clade support values (evolver 9 treefile maintreefile <pick1tree>)?
(0) Quit?
```

Options 1, 2, 3, 4. The program can be used to generate a random tree, either unrooted or rooted, either with or without branch lengths. It can also list all the trees for a fixed number of species. Of course, you should do this for a small number of species only as otherwise your hard drive will be filled by useless trees. Option 8 is for reading many trees from a tree file and then calculating bi-partition distances either between the first and all the remaining trees or between every pair.

Option 9 (Clade support values) can be used to summarize bootstrap or Bayesian analyses. This reads two tree files. The first file should include one tree, say, the maximum likelihood tree. You should have the number of species and the number of tree (should be 1) at the beginning of this file. The second tree file should include a collection of trees, such as 1000 maximum likelihood trees estimated from 1000 bootstrap pseudo-samples. This option will then calculate the bootstrap support value for each clade on the ML tree in the first tree file, that is, the proportion of trees in the second file that contain the node or clade in the tree in the first file. The second tree file does not have to have the numbers of species and trees on the first line. If you run MrBayes, you can move the maximum likelihood tree or maximum *a posteriori* tree into the first file, and the second tree file can be the .t file generated by MrBayes, with no change necessary. Right now species are represented by numbers only in the tree files, I think. You can choose this option by running evolver, then option 9. The program will then ask you to input two file names. An alternative way, which bypasses the naïve menu, is to put the option and two file names at the command line:
    evolver 9 <MaintreeFile> <TreesFile>

Options 5, 6, 7 (Simulations). The program evolver simulates nucleotide, codon, and amino acid sequences with user-specified tree topology and branch lengths. The user specifies the substitution model and parameters in a control file; see below. The program generates multiple data sets in one file in either PAML (output mc.paml) or PAUP* (output mc.paup) format. If you choose the PAUP* format, the program will look for files with the following names: paupstart (which the program copies to the start of the data file), paupblock (which the program copies to the end of each simulated data set), and paupend (which the program incorporates at the end of the file). This makes it possible to use PAUP* to analyze all data sets in one run. Parameters for simulation are specified in three files: MCbase.txt, MCcodon.txt, and MCaa.txt for simulating nucleotide, codon, and amino acid sequences, respectively. Run the default options while watching out for screen output. Then have a look at the appropriate .dat files. As an example, the MCbase.dat file is reproduced below. Note that the first block of the file has the inputs for evolver, while the rest are notes. The tree length is the expected number of substitutions per site along all branches in the phylogeny, calculated as the sum of the branch lengths. This variable was introduced when I was doing simulations to evaluate the effect of sequence divergence while keeping the shape of the tree fixed. evolver will scale the tree so that the branch lengths sum up to the specified tree length. If you use –1 for the tree length, the program will use the branch lengths given in the tree without the re-scaling. Also note that the base frequencies have to be in a fixed order; this is the same for the amino acid and codon frequencies in MCaa.dat and MCcodon.dat.

```
   seed = -1
  noisy = 3
seqtype = 0 * 0 (nucleotides), 1 (codons), 2 (amino acids)
seqfile = myseqfile.txt 1  * comment out this line if you don't want seqs
            * 0,1:seqs or patterns in paml format (mc.paml); 2:paup format (mc.nex); 3: paup JC69 format
```

```
nseq-nsites-nrepl = 3 10 2
        fix_tree = 1:
              (A: 0.1, B: 0.2, C: 0.3);
      treelength = -1    * use -1 if tree has absolute branch lengths.
           model = 7          * model: 0:JC69, 1:K80, 2:F81, 3:F84, 4:HKY85, 5:T92, 6:TN93, 7:REV
          Qrates = 0  10 5 5 5 5 10   * 1: fixed; 0: dirichlet, for TC TA TG CA CG AG
           freqs = 0  10  10  10  10  * 0: random, Dirichlet(aT, aC, aA, aG), for base frequencies
    alpha_siterate = 1  0.5  5     * discrete-gamma with alpha = 0.5 and K = 5
    alpha_siterate = 0  20 10  0.5 5  * G(a, b) for alpha for sites & K for discrete gamma (0 for continuous)
```

The simulation options (5, 6, 7) of evolver can be run using a command line format, bypassing the naïve menu. So here are all the possible ways of running evolver:

```
evolver
evolver 5 MyMCbaseFile
evolver 6 MyMCcodonFile
evolver 7 MyMCaaFile
```

Simulation under codon models (option 6). You specify the frequencies for all 64 codons. Unlike codeml, there is no variable for genetic code here: you simply specify 0 for the stop codons. Also unlike codeml, there is no CodonFreq variable. You can specify the same frequency for all (sense) codons to specify the Fequal model. For models such as F1x4 and F3x4, you can calculate the resulting frequencies for the sense codons and specify them in MCcodon.dat. If you choose verbose = 1 when you run codeml under F1x4 or F3x4 models, the program will print out the codon frequencies expected under those models, calculated using the observed nucleotide frequencies in the dataset. Look in the result file for "Codon frequencies under model, for use in evolver".

Also the model of codon substitution used here assumes the same ω ratio for all branches in the phylogeny and for all sites in the gene. This is known as model M0 (one-ratio). To simulate under the site models with variable ω's among sites, or the branch models with different ωs among branches, or the branch-site models with ω varying both among sites and among branches, please read the file CodonSimulation.txt in the paml/Technical/Simulation/Codon/ folder.

The first variable controls the sequence data format to be used: with 0 meaning the paml/phylip format, 1 site pattern counts and 2 the nexus format. The site pattern counts may be read by baseml or codeml later, and is useful if you have large data sets with many ($>10^6$) sites. (See the section on sequence data file format above.)

evolver also can simulate data using a random tree with random branch lengths for each simulated data set. You will have to change the source code and recompile. Open evolver.c and find fixtree=1 in the routine Simulate() and change the value 1 into 0. Then recompile and name the program as evolverRandomTree, say.

```
cc -o evolverRandomTree -O2 evolver.c tools.c -lm
evolver 5 MCbaseRandomTree.dat
```

The control data file such as MCbase.dat has to be changed as well. An example file named MCbaseRandomTree.dat is included in the package. This has the lines for "tree length" and tree topology replaced by a line for the birth rate λ, death rate μ, sampling fraction ρ, and the tree height (the expected number of substitutions per site from the root to the tips). The trees are generated using the birth-death process with species sampling (Yang and Rannala 1997). The clock is assumed. You will have to change the source code to relax the clock. If you choose 0 (paml) for the file format, the random trees are printed out in the file ancestral.txt (?); this you can read from within Rod Page's TreeView. If you choose 2 (nexus) for file format, the program prints out the tree in a tree block in the sequence data file.

The evolver program also has a few options for listing all trees for a specified small number of species, and for generating random trees from a model of cladogenesis, the birth-death process with species sampling (Yang and Rannala 1997). It also has an option for calculating the partition distance between tree topologies.

Monte Carlo simulation algorithm used in evolver. You can read about more details in the section "Models and Analyses". See also Chapter 9 in Yang (2006). Here are some brief notes. Evolver simulates data sets by "evolving" sequences along the tree. First, a sequence is generated for the

root using the equilibrium nucleotide, amino acid, or codon frequencies specified by the model and/or the data file (`MCbase.dat`, `MCcodon.dat`, and `MCaa.dat`, respectively). Then each site evolves along the branches of the tree according to the branch lengths, parameters in the substitution model etc. When the sites in the sequence evolve according to the same process, the transition probability matrix is calculated only once for all sites for each branch. For so called site-class models (such as the gamma, and the NSsites codon models), different sites might have different transition probability matrices. Given the character at the start of the branch, the character at the end of the branch is sampled from a multinomial distribution specified by the transition probabilities from the source character. Sequences at the ancestral nodes are generated during the simulation and printed out in the file ancestral.txt.

Some people wanted to specify the sequence at the root rather than letting the program generate a random sequence. This can be achieved by putting a sequence in the file RootSeq.txt. The sequence cannot have ambiguities or gaps or stop codons. In almost all simulations, it is simply wrong to fix the root sequence, so you should resist the temptation of making the mistake. If you want the simulation to reflect your particular gene, you may estimate parameters under a model from that gene and then simulate data sets using the parameter estimates.

8 yn00

The program yn00 implements the method of Yang and Nielsen (2000) for estimating synonymous and nonsynonymous substitution rates between two sequences (d_S and d_N). The method of Nei and Gojobori (1986) is also included. The ad hoc method implemented in the program accounts for the transition/transversion rate bias and codon usage bias, and is an approximation to the ML method accounting for the transition/transversion rate ratio and assuming the F3x4 codon frequency model. We recommend that you use the ML method (runmode= -2, CodonFreq = 2 in codeml.ctl) as much as possible even for pairwise sequence comparison.

```
        seqfile = abglobin.nuc * sequence data file name
        outfile = yn          * main result file
        verbose = 0      * 1: detailed output (list sequences), 0: concise output

          icode = 0  * 0:universal code; 1:mammalian mt; 2-10:see below
       weighting = 0  * weighting pathways between codons (0/1)?
      commonf3x4 = 0  * use one set of codon freqs for all pairs (0/1)?
```

The control file yn00.ctl, an example of which is shown above, specifies the sequence data file name (seqfile), output file name (outfile), and the genetic code (icode). Sites (codons) involving alignment gaps or ambiguity nucleotides in any sequence are removed from all sequences. The variable weighting decides whether equal weighting or unequal weighting will be used when counting differences between codons. The two approaches will be different for divergent sequences, and unequal weighting is much slower computationally. The transition/transversion rate ratio κ is estimated for all sequences in the data file and used in subsequent pairwise comparisons. commonf3x4 specifies whether codon frequencies (based on the F3x4 model of codonml) should be estimated for each pair or for all sequences in the data. Besides the main result file, the program also generates three distance matrices: 2YN.dS for synonymous rates, 2YN.dN for nonsynonymous rates, 2YN.t for the combined codon rate (t is measured as the number of nucleotide substitutions per codon). Those are lower-diagonal distance matrices and are directly readable by some distance programs such as NEIGHBOR in Felesenstein's PHYLIP package.

9 mcmctree

Overview

The program `mcmctree` may be the first Bayesian phylogenetic program (Yang and Rannala 1997; Rannala and Yang 1996), but was very slow and decommissioned since the release of MrBayes (Huelsenbeck and Ronquist 2001).

Since PAML version 3.15 (2005), mcmctree implements the MCMC algorithms of Yang and Rannala (2006) and then of Rannala and Yang (2007) for estimating species divergence times on a given rooted tree using multiple fossil calibrations. This is similar to the multidivtime program of Jeff Thorne and Hiro Kishino. The differences between the two programs are discussed by Yang and Rannala (2006) and Yang (2006, Section 7.4); see also below.

Please refer to any book on Bayesian computation, for example, Chapter 5 in Yang (2006) for the basics of MCMC algorithms.

Here are some notes about the program.

- Before starting the program, resize the window to have 100 columns instead of 80. (On Windows XP/Vista, right-click the command prompt window title bar and change Properties - Layout - Window Size - Width.)

- The tree, supplied in the tree file, must be a rooted binary tree: every internal node should have exactly two daughter nodes. You should not use a consensus tree with polytomies for divergence time estimation using MCMCTREE. Instead you should use a bifurcating ML tree or NJ tree or traditional morphology tree. Note that a binary tree has a chance of being correct, while a polytomy tree has none.

- The tree must not have branch lengths. For example, ((a:0.1, b:0.2):0.12, c:0.3) '>0.8<1.0'; does not work, while ((a, b), c) '>0.8<1.0'; is fine.

- Under the relaxed-clock models (clock = 2 or 3) and if there is no calibration on the root, a loose upper bound (maximal age constraint) must be specified in the control file (RootAge). (There should be no need to use RootAge if clock = 1, but the program insists that you have it. I will try to fix this.)

- *Choice of time unit.* The time unit should be chosen such that the node ages are roughly in the range 0.01-10. If the divergence times are around 100-1000MY, then 100MY may be one time unit. The priors on times and on rates and the fossil calibrations should all be specified based on your choice of the time scale. For example, if one time unit is 100MY, the following

    ```
    rgene_gamma = 100 1000 2 0  * gamma dirichlet prior for locus rates
    sigma2_gamma = 10 100 2     * gamma dirichlet prior for sigma^2  (for clock=2 or 3)
    ```

  means an overall average rate of $100/1000 = 0.1$ substitutions per site per 100MY or 10^{-9} substitutions per site per year. If you change the time unit, you should keep the shape parameter fixed and change the scale parameter β_μ to have the correct mean. In other words, to use one time unit to represent 10MY, the prior should become

    ```
    rgene_gamma = 100 100 2 0   * gamma dirichlet prior for locus rates
    sigma2_gamma = 10 100 2     * gamma dirichlet prior for sigma^2  (for clock=2 or 3)
    ```

  Note that under the independent-rates model (clock=2), the change of the time unit should not lead to a change to the prior for σ^2, because σ^2 is the variance of the log rate: the variance of the logarithm of the rate does not change when you rescale the rate by a constant. However, for the correlated-rates model (clock=3), the change of the time unit should also lead to a change to σ^2: under that model, the variance of the log-normal distribution is $t\sigma^2$, where t is the time gap separating the midpoints of the branches.

When you change the time unit, the fossil calibrations in the tree file should be changed accordingly. While ideally one would want the biological results to be unchanged when one changes the time unit, we know that two components of the model are not invariant to the time scale: the log normal distribution for rates and the birth-death model for times. Nevertheless, Groussin et al. (2011) suggested that the choice of time scale had minimal effects on the posterior time and rate estimates.

- *Specifying the prior on rates.* Choose α_μ for `rgene_gamma` (μ) based on how confident you are about the overall rate. For example, $\alpha_\mu = 1$, 1.5, or 2 mean quite diffuse (uninformative) priors. Then adjust β_μ so that the mean α_μ/β_μ is reasonable. To get a rough mean for the overall rate, you can use a few point calibrations to run the ML program baseml or codeml under a strict clock (clock = 1). For example, if a node has the calibration B(0.06, 0.08), you can fix the node age at 0.07 when you run baseml/codeml. If you are analyzing multiple loci/partitions, which have quite different rates, you can use an intermediate value, or the mean or median among the locus rates. The program uses the same prior for μ for all loci.

- It is important that you run the same analysis at least twice to confirm that the different runs produced very similar (although not identical) results. It is critical that you ensure that the acceptance proportions are neither too high nor too low. See below about the variable finetune.

- It is important that you run the program without sequence data (usedata = 0) first to examine the means and CIs of divergence times specified in the prior. In theory, the joint prior distribution of all times should be specified by the user. Nevertheless it is nearly impossible to specify such a complex high-dimensional distribution. Instead the program generates the joint prior by using the calibration distributions and the constaint on the root as well as the birth-death process model to generate the joint prior. This the prior used by the program in the dating analysis. You have to examine it to make sure it is sensible, judged by your knowledge of the species and the relevant fossil record. If necessary, you may have to change the fossil calibrations so that the prior look reasonable.

- The program right now does a simple summary of the MCMC samples, calculating the mean, median and the 95% CIs for the parameters. If you want more sophisticated summaries such as 1-D and 2-D density estimates, you can run a small program ds at the end of the mcmctree run, by typing   ds mcmc.out.

- To use hard bounds, you can specify the tail probabilities as 10^{-300} instead of the default 0.025. See table 8 below.

The control file

You can use the files in the folder examples/SoftBound/ to duplicate the results of Yang and Rannala (2006: table 3) and Rannala and Yang (2007: table 2). Below is a copy of the control file mcmctree.ctl.

```
        seed = -1234567
     seqfile = mtCDNApri123.txt
    treefile = mtCDNApri.trees
    mcmcfile = mcmc.txt

     outfile = out
*   checkpoint = 1 0.01 mcmctree.ckpt1 * flag (0: nothing; 1: save;  2: resume) prob filename
*   checkpoint = 2 0.01 mcmctree.ckpt1 * flag (0: nothing; 1: save;  2: resume) prob filename
*  duplication = 1 (this force some nodes in the tree to have the same age)
       ndata = 3
      usedata = 1    * 0: no data; 1:seq like; 2:use in.BV; 3: out.BV
       clock = 1    * 1: global clock; 2: independent rates; 3: correlated rates
*     TipDate = 1 100  * TipDate (1) & time unit

     RootAge = '>0.8<1.2'
       model = 0    * 0:JC69, 1:K80, 2:F81, 3:F84, 4:HKY85
       alpha = 0    * alpha for gamma rates at sites
       ncatG = 5    * No. categories in discrete gamma
```

```
      cleandata = 0     * remove sites with ambiguity data (1:yes, 0:no)?
   BlengthMethod = 0     * 0: arithmetic; 1: geometric; 2: Brownian

          BDparas = 1 1 0    * birth, death, sampling
     kappa_gamma = 6 2       * gamma prior for kappa
     alpha_gamma = 1 1       * gamma prior for alpha

      rgene_gamma = 2 2 1 0   * prior for locus rates (rates for genes)
    sigma2_gamma = 1 1 1     * prior for sigma^2     (for clock=2 or 3)

         finetune = 1: .01 .2 .1 .1 .2 .1 * auto (0 or 1): times, musigma2, rates, mixing, paras,
   FossilErr

            print = 1 (print = 2 to print rates for branches with clock=2 or 3)
           burnin = 4000
          sampfreq = 2
           nsample = 10000
```

seed should be assigned a negative or positive integer. A negative integer (such as –1) means that the random number seed is determined from the current clock time. Different runs will start from different places and generate different results due to the stochastic nature of the MCMC algorithm. You should use this option and run the program at least twice, to confirm that the results are very similar between runs (identical to 1MY or 0.1MY, depending on the desired precision). If you obtain intolerably different results from different runs, you obviously won't have any confidence in the results. This lack of consistency between runs can be due to many reasons: including slow convergence, poor mixing, insufficient samples taken, or errors in the program. Thus you can check to make sure (i) that the chain is at reasonably good place when it reached 0% (the end of burn-in), indicating that the chain may have converged; (ii) that the acceptance proportion of all proposals used by the algorithm are neither too high nor too low (see below about **finetune**) indicating that the chain is mixing well; (iii) that you have taken enough samples (see **nsample** and **burnin** below). If you give **seed** a positive number, that number will be used as the real seed. Then running the program multiple times will produce exactly the same results. This is useful for debugging the program and should not be the default option for real data analysis.

checkpoint is a switch (1 for save and 2 for resume) to turn on checkpointing. This does not save the memory image etc. and is not checkpointing in the usual sense of the word. Instead it saves the current state of the Markov chain (such as the divergence times, rates for loci, and the step lengths) in a file (**mcmctree.ckpt**, say). It does not save the conditional probability vectors, which are recalculated when the run is resumed. The prob variable specifies how often it saves into the file so that prob = 0.01 means saving 100 times if the run is uninterrupted. When the file already exists, it will be overwritten.

With the resume option, the program will read the control file and sequence alignments, allocate memory, and then fix the state of the Markov chain by reading from the checkpoint file (**mcmctree.ckpt**, say), which will have the last saved state of the chain, and then restart the MCMC (by setting **burnin = 0**). In effect it is using the last saved parameter values as initial values, without running the burnin. It will then take **nsample** samples, with a sampling frequency **sampfreq**, where **nsample** and **sampfreq** are read from the control file. The old mcmc sample file is destroyed. To use this option, you will need to rename the sample file and then change to **checkpoint** = 2, and then merge the new samples with the old samples to summarize.

It should also be fine if you use the same saved state (or the same checkpoint file) to restart multiple MCMC runs in different folders using different random number seeds (**seed = -1**, say). Even though the initial state is the same, the different chains will generate different samples because of the different random numbers used. You can then merge or combine the mcmc samples from the multiple runs.

This may be rather tedious. Sandra has written some scripts for combining the mcmc sample files.

I should think of some other options. For example, one can save the random number so that the save-resume option will produce exactly identical results as running the whole chain without using checkpointing. This will be closer to checkpointing in computer science, but is clearly not so useful.

duplication is a switch (0 for no and 1 for yes) to turn on the option for duplicated genes/proteins so that some nodes in the tree corresponding to the same speciation events in different paralogues share the same age. The driver/mirror nodes which share the same age are identified using node labels in the tree file, such as #1, #2, ...

```
(((A1, A2) [#1], A3) #2, ((B1, B2) [#1 B{0.2, 0.4}], B3) #2 ) >0.9<1.1;

(((A1, A2) #1, A3) #2, ((B1, B2) [#1 B{0.2, 0.4}], B3) [#2] ) >0.9<1.1;

(((A1, A2) [#1 >0.2 <0.4], A3) #2, ((B1, B2) [#1 >0.2 <0.4], B3) #2 ) >0.9<1.1;
```

The above three trees are equivalent ways of specifying *equality constraints* on the tree of figure 2a. They are all acceptable by the program. In this example, A and B are paralogs, while 1, 2, 3 are different species. Node *r* represents a duplication event while other nodes are speciation events. Node *a* (the A1-A2 ancestor) and node *b* (the B1-B2 ancestor) are assigned the same label (#1), so they share the same age: $t_a = t_b$. Similarly node u and node v have the same age: $t_u = t_v$. Also there is a calibration on nodes *a* or *b*: $0.2 < t_a = t_b < 0.4$. There is another calibration on the root: $< 0.9 < t_r < 1.1$.

Among the nodes on the tree with the same label, one is chosen as the driver node while the others are mirror nodes. If calibration information is provided on one of the shared nodes, the same information obviously applies to all shared nodes. If calibration information is provided on multiple shared nodes, that information has to be the same. The time prior (or the prior on all node ages on the tree) is constructed by using a density at the root of the tree, specified by the user, while the ages of all non-calibration nodes are given by the uniform density. This time prior is similar to that used by Thorne *et al.* (1998). The parameters in the birth-death-sampling process (λ, μ, ρ specified using the variable BDparas) are ignored.

Note that more than two nodes can have the same label, but one node cannot have two or more labels. Also the prior on rates does not distinguish between speciation and duplication events.

The `duplication` option (i.e., the equality constraint) may be used to specify *inequality constraints* (figure 2b). To constrain node <u>u</u> to be older than node *v* ($t_u > t_v$), we include a ghost species in the tree, creating a new node *g* that is ancestral to node *v*. Then by constraining node *g* to have the same age as node *u* (they are both labelled #2), we enforce the inequality constraint $t_u > t_v$. Note that the ghost species does not appear in the sequence data file as there is no data avialable for it; it is used in the tree only to create a new node (*g*) that has the same age as *u* and is older than *v*. The tree of figure 2b can be specified as follows:

```
(((A1, A2) [#1], A3) #2,
(((B1, B2) [#1 B{0.2, 0.4}],
B3), ghost) #2 ) >0.9<1.1;
```

One possible scenario for this is that there is a lateral gene transfer (LGT) event, in which branch *u* is the donor and branch *v* is the recipient so that node *u* is older than node *v*. There will be no information about the precise time of the LGT event. The *inequality constraints* here merely specify the relative order of the ages of two nodes on the tree, with the donor species being older than the recipient.
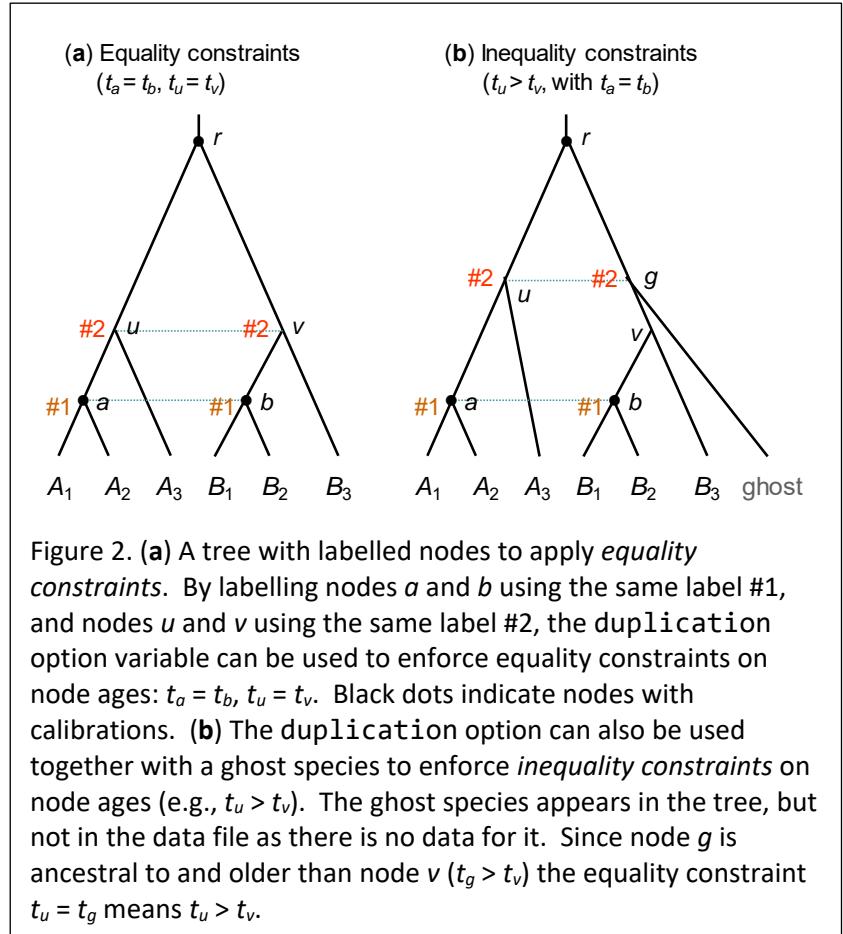

Figure 2. (**a**) A tree with labelled nodes to apply *equality constraints*. By labelling nodes *a* and *b* using the same label #1, and nodes *u* and *v* using the same label #2, the `duplication` option variable can be used to enforce equality constraints on node ages: $t_a = t_b$, $t_u = t_v$. Black dots indicate nodes with calibrations. (**b**) The `duplication` option can also be used together with a ghost species to enforce *inequality constraints* on node ages (e.g., $t_u > t_v$). The ghost species appears in the tree, but not in the data file as there is no data for it. Since node *g* is ancestral to and older than node *v* ($t_g > t_v$) the equality constraint $t_u = t_g$ means $t_u > t_v$.

ndata is the number of loci (or partitions) in a combined analysis. The program allows some species to be missing at some loci. The mt primate data included protein-coding genes, and the three codon positions are treated as three different partitions. In the combined analysis of multiple gene loci, the same substitution model is used, but different parameters are assigned and estimated for each partition.

usedata = 0

usedata = 1

usedata = 2 inBVfilename

usedata. 0 means that the sequence data will not be used in the MCMC, with the likelihood set to 1, so that the MCMC approximates the prior distribution. This option is useful for testing and debugging the program, and is also useful for generating the prior distribution of the divergence times. The fossil calibrations and the constraints on the root you specify are not the real prior that is implemented in the program; for example, they may not even satisfy the requirement that ancestors should be older than descendents. The prior that is used by the program can be generated by running the chain without data. usedata = 1 means that the sequence data will be used in the MCMC, with the likelihood calculated using the pruning algorithm of Felsenstein (1981), which is exact but very slow except for very small species trees. This option is available for nucleotide sequences only, and the most complex model available is HKY85+Γ. usedata = 2 and 3 implement a method of approximate likelihood calculation (dos Reis and Yang 2011). They can be used to analyze nucleotide, amino acid, and codon sequences, using nucleotide, amino acid, and codon substitution models, respectively. specifies the approximate likelihood calculation, with the input

(gradient & Hessian matrix etc.) in the file.

clock. The clock variable is used to implement three models concerning the molecular clock: 1 means global molecular clock, so that the rate is constant across all lineages on the tree (even though the rate may vary among multiple genes); 2 means the independent-rates model, and 3 the auto-correlated rates model. See Rannala and Yang (2007) and Section §7.4 in Yang (2006) for details.

TipDate. This option is used to estimate ages of internal nodes on the given rooted tree when the sequences at the tips having sampling dates, as in the case of sequentially sampled viral sequences. The sample dates are the the last field in the sequence name. The time unit is specified by the user on this line. Look at the section Dating viral divergnces and README.txt in examples/TipDate/.

RootAge. The RootAge variable is used to specify a loose constant on the age of the root, to constrain the root age from above. It is used if no such constraint is available through a fossil calibration on the root. Note that fossil calibrations are specified in the tree file. Two formats are accepted, specifying either a maximum bound (e.g., RootAge = '<1.2') or a pair of minimum and maximum bounds (e.g., RootAge = '>0.8<1.2'). The RootAge variable is ignored if a fossil calibration on the root is specified in the tree file in the form of a maximum bound, a pair of minimum and maximum bounds, or a gamma distribution. If the fossil calibration in the tree file is a minimum bound on the root (e.g. '>0.9'), and you specify RootAge = '<1.2', then the program implements the pair of bounds, equivalent to specifying the calibration '>0.9<1.2' on the root.

model, alpha, ncatG are used to specify the nucleotide substitution model. These are the same variables as used in baseml.ctl. If alpha $\neq$ 0, the program will assume a gamma-rates model, while alpha = 0 means that the model of one rate for all sites will be used. Those variables have no effect when usedata = 2.

cleandata = 0 means that alignment gaps and ambiguity characters will be treated as missing data in the likelihood calculation (see pages 107-108 in Yang 2006). = 1 means that any sites at which at least one sequence has an alignment gap or ambiguity character will be deleted before analysis. This variable is used for usedata = 1 and 3 and has no effect if usedata = 2.

BDparas = 2 2 .1 specifies the three parameters (birth rate λ, death rate μ and sampling fraction ρ) in the birth-death process with species sampling (Yang and Rannala 1997), which is used to specify the prior of divergence times (Yang and Rannala 2006). The node times are order statistics from a kernel density, which is specified by those parameters. A few kernel densities are shown in figure 2 of Yang and Rannala (1997) or figure 7.12 in Yang (2006). The Mathematica code for plotting the density for given parameters λ, μ and ρ is posted at the web site http://abacus.gene.ucl.ac.uk/ziheng/data.html. By adjusting parameters λ, μ and ρ to generate different tree shapes, one can assess the impact of the prior on posterior divergence time estimation. Intuitively, the node ages and thus the shape of the tree are determined by the parameters as follows. There are $s - 1$ internal nodes and thus $s - 1$ node ages in the rooted tree of s species. The age of the root is fixed, so the $s - 2$ node ages are relative to the root age (they are all between 0 and 1). We draw $s - 2$ independent random variables from the kernel density and order them. Those ordered variables will then be the node ages. Thus if the kernel density has the L shape, all internal nodes tend to be young (relative to the root), and the tree will have long internal branches and short tip branches. In contrast, if the kernel density has the reverse L shape, the node ages are large and the nodes close to the root, then the tree will be bush-like. See pages 250-251 in Yang (2006). (Strictly speaking the above description is accurate if fossil calibration is available for the root only but not for any other nodes. Otherwise the kernel density specifies the distribution of the ages of non-calibration nodes only, and the impact of the kernel on the joint distribution of all node ages may be complex, depending on the locations of the calibration nodes.)

kappa_gamma = 6 2 specifies the shape and scale parameters (α and β) in the gamma prior for parameter κ (the transition/transversion rate ratio) in models such as K80 and HKY85. This has no effect in models such as JC69, which does not have the parameter. Note that the gamma distribution with parameters α and β has the mean α/β and variance α/β^2. Those variables are used only when

usedata = 1 and have no effect when usedata = 2 or 3.

alpha_gamma = 1 1 specifies the shape and scale parameters (α and β) in the gamma prior for the shape parameter for gamma rates among sites in models such as JC69+Γ, K80+Γ etc. The gamma model of rate variation is assumed only if the variable **alpha** is assigned a positive value. This prior is used only when usedata = 1 and has no effect when usedata = 2 or 3.

rgene_gamma = 100 1000 1 0 specifies the parameters in the prior for the locus rates. Two priors are implemented for locus rates (μ_i), as summarized in Zhu et al. (2015), specified in the form

```
rgene_gamma = au bu a prior
```

where au is α_μ, bu is β_μ, a is α, and prior = 0 (default) is the gamma-Dirichlet prior (dos Reis et al. 2014 Sys Biol, equations 3-5) while prior = 1 is the conditional i.i.d. prior (Zhu et al. 2015 Sys Biol, p.279 equation 8). Thus the above specifies the gamma-Dirichlet prior with $\alpha_\mu = 100$, $\beta_\mu = 1000$, $\alpha = 0.5$ for locus rates μ_i.

The gamma-Dirichlet and conditional i.i.d. priors are expected to be similar especially if the number of genes or loci or partitions is large. In the implementation, $\overline{\mu}$ is a parameter in the conditional i.i.d. prior but not in the gamma-Dirichlet prior, so you will see one more parameter if you use the gamma-Dirichlet prior.

The σ_i^2 prior (**sigma2_gamma**) has parameters au, bu, a, specified in the same way. The same form of prior (gamma-dirichlet or conditional iid) is used for both μ_i and σ_i^2.

```
  rgene_gamma = 100 1000 1.0 0  * gamma dirichlet prior for locus rates
sigma2_gamma = 4 100 1.0       * gamma dirichlet for sigma^2 (for clock=2 or 3)
```

The default value of α is 1 and the default prior is 0 (gamma-Dirichlet), so that the above is equivalent to the following.

```
  rgene_gamma = 100 1000  * gamma-dirichlet prior for locus rates
sigma2_gamma = 4 100      * gamma-dirichlet for sigma^2 (for clock=2 or 3)
```

The conditional i.i.d. prior is specified as follows.

```
  rgene_gamma = 100 1000 1 1  * conditional iid prior for locus rates
sigma2_gamma = 4 100 1        * conditional iid for sigma^2 (for clock=2 or 3)
```

Here are some notes about the prior models. See dos Reis et al. (2014) and Zhu et al. (2015) for details. Under the global-clock model (clock=1), the independent-rates model (clock = 2), and the correlated-rates model (clock = 3), μ_i is the overall rate for locus i. In the example, μ_i has the prior mean $100/1000 = 0.1$, that is, one change per site per time unit. If one time unit is 100MY, this means an overall average rate of 10^{-9} substitutions per site per year. The variance ($100/1000^2 = 0.01$ in the example) of this gamma prior specifies how confident you are about the overall rate. Parameter α (= 1 in the example) specifies how variable the overall rates are among loci. This has more or less the same interpretation as the shape parameter α for the gamma model of variable rates among sites (Yang 1993), with a large α (100, say) meaning nearly identical rates among loci and small values (such as 1 or 0.5) highly variable rates among loci.

You need to adjust this prior to suit your data and the chosen time scale. Don't use the default. If ou do not have information about the overall rate, one way of deriving a rough rate estimate (for use as the prior mean) may be to run mcmctree with the clock (clock = 1).

sigma2_gamma = 4 100 specifies the shape and scale parameters (α and β) in the conditional i.i.d. or gamma-Dirichlet prior for parameter σ_i^2. See notes above about **rgene_gamma**. Note that σ_i^2 specifies how variable the rates are across branches or how seriously the clock is violated at the locus. This prior is used for the two variable-rates models (clock = 2 or 3), with a larger σ^2 indicating more variable rates (Rannala and Yang 2007). If clock = 1, this prior has no effect.

In the independent-rates model (clock = 2), rates for branches are independent variables from a log-normal distribution (Rannala and Yang 2007: equation 9).

$$f(r \mid \mu,\sigma^2) = \frac{1}{r\sqrt{2\pi\sigma^2}} \exp\left\{-\frac{1}{2\sigma^2}\left[\log\left(r/\mu\right)+\tfrac{1}{2}\sigma^2\right]^2\right\}, \quad 0 < r < \infty. \tag{1}$$

Here σ^2 is the variance in the logarithm of the rates. The rate r has mean μ and variance $(e^{\sigma^2}-1)\mu^2$.

The correlated-rates model (clock = 3) specifies the density of the current rate r, given that the ancestral rate time t ago is r_A, as

$$f(r \mid r_A, t\sigma^2) = \frac{1}{r\sqrt{2\pi t\sigma^2}} \exp\left\{-\frac{1}{2t\sigma^2}(\log\left(r/r_A\right)+\tfrac{1}{2}t\sigma^2)^2\right\}, \quad 0 < r < \infty \tag{2}$$

(Rannala and Yang 2007: equation 2). Parameter σ^2 here is equivalent to ν in Kishino *et al.* (2001). Thus r has mean r_A and variance $(e^{t\sigma^2}-1)r_A^2$.

Note that σ^2 (clock = 2) or $t\sigma^2$ (clock = 3) is not the variance of the rate; it is the variance of the logarithm of the rate.

finetune. The following line in the control file

```
finetune = 0: 0.04 0.2 0.3 0.1 0.3     * auto (0 or 1) : times, musigma2, rates, mixing, paras,
finetune = 1: .05 .05 .05 .05 .05 .05  * auto (0 or 1) : times, musigma2, rates, mixing, paras,
```

is about the step lengths used in the proposals in the MCMC algorithm. The first value, before the colon, is a switch, with 0 meaning no automatic adjustments by the program and 1 meaning automatic adjustments by the program. Following the colon are the step lengths for the proposals used in the program. The proposals are as follows: (a) to change the divergence times, (b) to change μ (and σ^2 in the relaxed rates models), (c) to change the rate for loci for the relaxed clock models, (d) to perform the mixing step (page 225 in Yang and Rannala 2006), and (e) to change parameters in the substitution model (such as κ and α in HKY+Γ). If you choose to let the program adjust the step lengths, burnin has to be >200, and then the step lengths specified here will be the initial step lengths, and the program will try to adjust them using the information collected during the burnin step. It does this twice, once at half of the burnin and another time at the end of the burnin. The option of automatic adjustment is not well tested.

The following notes are for manually adjusting the step lengths. You can use them to generate good initial step lengths as well for the option of automatic step length adjustment.

```
-20% 0.33 0.01 0.25 0.00 0.00  1.022 0.752 0.252 0.458 0.133 0.843 - 0.074 0.787 -95294.7
-15% 0.33 0.01 0.25 0.00 0.00  1.021 0.751 0.253 0.457 0.130 0.841 - 0.067 0.783 -95295.4
-10% 0.33 0.00 0.26 0.00 0.00  1.022 0.752 0.254 0.458 0.129 0.842 - 0.065 0.781 -95294.6
 -5% 0.33 0.00 0.25 0.00 0.00  1.022 0.751 0.254 0.457 0.128 0.841 - 0.063 0.780 -95292.4
  0% 0.32 0.00 0.25 0.00 0.00  1.022 0.751 0.254 0.457 0.128 0.841 - 0.063 0.780 -95290.2
  2% 0.32 0.00 0.27 0.00 0.00  1.014 0.746 0.253 0.453 0.126 0.833 - 0.059 0.784 -95290.4
```

A few seconds or minutes (hopefully not hours) after you start the program, the screen output will look like the above. The output here is generated from a run under the JC model and global clock (clock = 1). The percentage % indicates the progress of the run, with negative values for the burn-in. Then the five proportions (e.g., 0.33 0.01 0.25 0.00 0.00 on the first line) are the acceptance proportions (P_{jump}) for the corresponding proposals. The optimal acceptance proportions are around 0.3, and you should try to make them fall in the interval (0.2, 0.4) or at least (0.15, 0.7). If the acceptance proportion is too small (say, <0.10), you decrease the corresponding finetune parameter. If the acceptance proportion is too large (say, >0.80), you increase the corresponding finetune parameter. In the example here, the second acceptance proportion, at 0.01 or 0.00, is too small, so you should stop the program (Ctrl-C) and modify the control file to decrease the corresponding finetune parameter (change 0.2 into 0.02, for example). Then run the program again (use the up $\downarrow$ and down $\uparrow$ arrow keys to retrieve past commands), observe it for a few seconds or minutes and then kill it again if the proportions are still not good. Repeat this process a few times until every acceptance proportion is reasonable. This is not quite so tedious as it may sound.

The finetune parameters in the control file are in a fixed order and always read by the program even if the concerned proposal is not used (in which case the corresponding finetune parameter has no

effect). In the above example, JC does not involve any substitution parameters, so that the 4th finetune parameter has no effect, and the corresponding acceptance proportion is always 0. This proportion is always 0 also when the approximate likelihood calculation is used (usedata = 2) because in that case the likelihood is calculated by fitting the branch lengths to a normal density, ignoring all substitution parameters like κ, α etc. If clock = 1, there are no parameters in the rate-drift model, so that the 5th acceptance proportion is always 0.

Note that the impact of the finetune parameters is on the efficiency of the algorithm, or on how precise the results are when the chain is run for a fixed length. Even if the acceptance proportions are too high or too low, reliable results will be obtained in theory if the chain is run sufficiently long. This effect is different from the effect of the prior, which affects the posterior estimates.

print = 1 means that samples will be taken in the MCMC and written to disk and the posterior results will be summarized. 0 means that the posterior means will be printed on the monitor but nothing else: this is mainly useful for testing the program. The relaxed-clock models (clock=2 or 3) generates a lot of output with rates for branches for each locus (partition), so those rates are printed out only if you choose print = 2.

burnin = 2000, **sampfreq** = 5, **nsample** = 10000. In the example here, the program will discard the first 2000 iterations as burn-in, and then run the MCMC for 5 × 10000 iterations, sampling (writing to disk) every 5 iterations. The 10000 samples will then be read in and summarized. I think you should take at least 2000 samples.

Fossil calibration

Fossil calibration information, in the form of statistical distributions of divergence times (or ages of nodes in the species tree), is specified in the tree file. See table 8 for a summary. Here "fossil" means any kind of external calibration data, including geological events. For a sensible analysis, one should have at least one lower bound and at least one upper bound on the tree, even though they may not be on the same node. The gamma, skew normal, and skew *t* distributions can act as both bounds, so one such calibration is enough to anchor the tree to enable a sensible analysis.

(1) Lower bound (minimal age) is specified as '>0.06' or 'L(0.06)', meaning that the node age is at least 6MY. Here we assume that one time unit is 100 million years. In PAML version 4.2, the implementation of the minimum bound has changed. Instead of the improper soft flat density of Figure 2a in Yang and Rannala (2006) or figure 7.11a in Yang (2006), a heavy-tailed density based on a truncated Cauchy distribution is now used (Inoue *et al.* 2010). The Cauchy distribution with location parameter $t_L(1 + p)$ and scale parameter ct_L is truncated at t_L, and then made soft by adding $\alpha_L = 2.5\%$ of density mass left of t_L. The resulting distribution has mode at $t_L(1 + p)$. The $\alpha_L = 2.5\%$ limit is of course at t_L and the 97.5% limit is at

$$t_{97.5\%} = t_L[1 + p + c\cot(\tfrac{\pi A \alpha_R}{1-\alpha_L})],$$

where $\alpha_R = 1 - 0.975$ and $A = \tfrac{1}{2} + \tfrac{1}{\pi}\tan^{-1}\left(\tfrac{p}{c}\right)$. This is slightly more general than the formula in the paragraph below equation (26) in (Inoue *et al.* 2010), in that α_L and α_R are arbitrary: to get the 99% limit when t_L is a hard minimum bound, use $\alpha_L = 0$ and $\alpha_R = 0.01$ so that $t_{99\%} = t_L[1 + p + c\cot(0.01\pi A)]$.

If the minimum bound t_L is based on good fossil data, the true time of divergence may be close to the minimum bound, so that a small p and small c should be used. It is noted that c has a greater impact thatn p on posterior time estimation. The program uses the default values $p = 0.1$ and $c = 1$. However, you are advised to use different values of p and c for each minimum bound, based on a careful assessment of the fossil data on which the bound is based. Below are a few plots of this density. The minimum bound is fixed at $t_L = 1$, but one time unit can mean anything like 100Myr or 1000Myr. For each value of p (0.1 and 0.5), the four curves correspond to $c = 0.2, 0.5, 1, 2$ (from

top to bottom nearthe peak). The 2.5% limit is at 1, while the 97.5% limits for those values of c are 4.93, 12.12, 24.43, 49.20, respectively, when $p = 0.1$, and are 4.32, 9.77, 20.65, 44.43 when $p = 0.5$. (Note that those values were incorrectly calculated in Inoue *et al.* 2010)
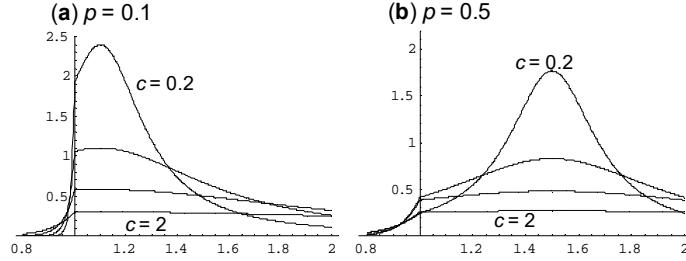

Table 7. Calibration distributions

Calibration	#p	Specification	Density
L (lower or minimum bound)	4	'>0.06' or 'L(0.06)' or 'L(0.06, 0.2)' or 'L(0.06, 0.1, 0.5)' or 'L(0.06, 0.1, 0.5, 0.025)'	$L(t_L, p, c, p_L)$ specifies the minimum-age bound t_L, with offset p, and scale parameter c, and left tail probability p_L. The default values are $p = 0.1$, $c = 1$, and $p_L = 0.025$, so >0.06 or L(0.06) means L(0.06, 0.1, 1, 0.025), and L(0.06, 0.2) means L(0.06, 0.2, 1, 0.025). If you would like the minimum bound to be hard, use $p_L = $ 1e-300, but do not use $p_L = $ 0. In other words, use L(0.06, 0.2, 1, 1e-300), not L(0.06, 0.2, 1, 0).
U (upper or maximum bound)	2	'<0.08' or 'U(0.08)' or 'U(0.08, 0.025)'	Eq. 16 & fig. 2b in YR06. $U(t_U, p_R)$ specifies the maximum-age bound t_U, with right tail probability p_R. The default value is $p_U = 0.025$, so <0.08 or U(0.08) means U(0.08, 0.025). For example U(0.08, 0.1) means that there is 10% probability that the maximum bound 8MY is violated (i.e., the true age is older than 8MY).
B (lower & upper bounds or minimum & maximum bounds)	4	'>0.06<0.08' or 'B(0.06, 0.08)' or 'B(0.06, 0.08, 0.025, 0.025)'	Eq. 17 & fig. 2c in YR06 $B(t_L, t_U, p_L, p_U)$ specifies a pair bound, so that the true age is between t_L and t_U, with the left and right tail probabilities to be p_L and p_U, respectively. The default values are $p_L = p_U = 0.025$.
G (Gamma)	2	'G(alpha, beta)'	Eq. 18 & fig. 2d in YR06
SN (skew normal)	3	'SN(location, scale, shape)'	Eq. 2 & plots below
ST (skew *t*)	4	'ST(location, scale, shape, df)'	Eq. 4 & plots below
S2N (skew 2 normals)	7	'SN2(p_1, loc1, scale1, shape1, loc2, scale2, shape2)'	p_1: $1 - p_1$ mixture of two skew normals.

Note .— #p is the number of parameters in the distribution, to be supplied by the user. Figure 2 in

YR06 (Yang and Rannala 2006) is figure 7.11 in Yang (2006).

(2) Upper bound (maximal age) is specified as '<0.08' or 'U(0.08)', meaning that the node age is at most 8MY.

(3) Both lower and upper bounds on the same node are specified as '>0.06<0.08' or 'B(0.06, 0.08)', meaning that the node age is between 6MY and 8MY.
Note that in all the above three calibrations (L, U, B), the bounds are soft, in that there is a 2.5% probability that the age is beyond the bound (see figure 2 in Yang and Rannala 2006; or figure 7.11 in Yang 2006).

(4) The gamma distribution. 'G(188, 2690)' specifies the gamma distribution with shape parameter $\alpha = 188$ and rate parameter $\beta = 2690$. This has the mean $\alpha/\beta = 0.07$ and the 2.5 and 97.5 percentiles at ### and ###. In earlier versions (3.15, 4a & 4b), the gamma was specified as '`>.06=0.0693<.08`', but this format is not used anymore.

```
(((((human, (chimpanzee, bonobo)) 'G(188, 2690)', gorilla), (orangutan, sumatran))
'>.12<.16', gibbon);
```

In the tree above, the human-chimp divergence time has a gamma distribution G(188, 2690), while the orang-utan divergence time has soft bounds between 12MY and 16MY.

The above tree can be read in TreeView, with the calibration information in quotation marks treated as node labels.

You can use the MS Excel function GAMMADIST(X, alpha, beta, 0) to calculate and plot the density function (pdf) of the gamma distribution, and the function GAMMAINV(0.025, alpha, beta) to calculate the 2.5% percentile. However, note that beta in Excel is $1/\beta$ in MCMCTREE (and other PAML programs). In other words, the mean is α/β in MCMCTREE and $\alpha\beta$ in Excel.

(5) Skew normal distribution SN(location, scale, shape) or SN(ξ, ω, α) (Azzalini and Genton 2008). The basic form of the skew normal distribution has density

$$f(z;\ \alpha) = 2\phi(z)\Phi(\alpha z), \tag{1}$$

where $\phi()$ and $\Phi()$ are the PDF and CDF of the standard normal distribution respectively. Then $x = \xi + \omega z$, has the skew normal distribution SN(ξ, ω, α) with location parameter ξ, scale parameter ω, and shape parameter α. The density is

$$f_{SN}(x;\ \xi,\ \omega,\ \alpha) = \frac{2}{\omega} \times \frac{1}{\sqrt{2\pi}} e^{-\frac{(x-\xi)^2}{2\omega^2}} \int_{-\infty}^{\alpha\left(\frac{x-\xi}{\omega}\right)} \frac{1}{\sqrt{2\pi}} e^{-\frac{u^2}{2}} du. \tag{2}$$

for $-\infty < \xi < \infty$, $0 < \omega < \infty$, $-\infty < \alpha < \infty$. Let $\delta = \alpha/\sqrt{1+\alpha^2}$. The mean and variance are

$$E(x) = \xi + \omega\delta\sqrt{2/\pi},$$
$$\mathrm{Var}(x) = \omega^2\left(1 - \frac{2\delta^2}{\pi}\right). \tag{3}$$

(6) **Skew t distribution, ST(location, scale, shape, df) or ST(ξ, ω, α, ν)** (Azzalini and Genton 2008), with location parameter ξ, scale parameter ω, shape parameter α, and degree of freedom ν, has density

$$f_{ST}(x;\xi,\omega,\alpha,\nu) = \frac{2}{\omega}t(z;\nu)T\left(\alpha z\sqrt{(\nu+1)/(\nu+z^2)};\nu+1\right), \tag{4}$$

where $z = (x - \xi)/\omega$, t and T are the PDF and CDF of the standard t distribution, respectively. These are defined as follows.

$$t(z;\nu) = \frac{\Gamma\left(\frac{1}{2}(\nu+1)\right)}{\sqrt{\pi\nu}\,\Gamma\left(\frac{1}{2}\nu\right)}\left[1+\frac{z^2}{\nu}\right]^{-(\nu+1)/2},$$

$$T(z;\nu) = \begin{cases} \frac{1}{2}I_{\nu/(\nu+z^2)}\left(\frac{1}{2}\nu,\frac{1}{2}\right), & \text{if } z < 0, \\ 1-T(-z;\nu), & \text{if } z \geq 0. \end{cases}$$

(5)

where $\Gamma()$ is the gamma function, and

$$I_p(a,b) = \frac{1}{B(a,b)}\int_0^p u^{a-1}(1-u)^{b-1}\,\mathrm{d}u$$

(6)

is the incomplete beta function ratio, or the CDF of the beta(a, b) distribution, while

$$B(a,b) = \int_0^1 u^{a-1}(1-u)^{b-1}\,\mathrm{d}u = \frac{\Gamma(a)\Gamma(b)}{\Gamma(a+b)}$$

(7)

is the beta function.

SN(2, 1.5, 6)
ST(2, 1.5, 6, 2.2)

SN(2, 1.5, –6)
ST(2, 1.5, –6, 2.2)

SN(2, 1.5, 10)
ST(2, 1.5, 10, 3)

SN(2, 1.5, 50)
ST(2, 1.5, 50, 1)

Figure 1. Density functions for skew normal (blue) and skew t (red) distributions. Skew t has heavier tails than skew normal.

Here are a few notes about the skew normal and skew t distributions.

- When the shape parameter $\alpha = 0$, the distributions become the standard (symmetrical) normal and t distributions.

- Changing α to $-\alpha$ flips the density at $x = \xi$ (the location parameter). Fossil calibrations should have long right tails, which means $\alpha > 0$.

- A larger $|\alpha|$ means more skewed distributions. When $|\alpha| = \infty$, the distribution is called folded normal or folded t distribution, that is, the normal or t distribution truncated at ξ from the left ($\alpha = \infty$) or from the right ($\alpha = -\infty$).

- When the degree of freedom $\nu = \infty$, the t or skew t distribution becomes the normal or skew normal distribution. The smaller ν is, the heavier the tails are. A small ν (1-4 , say) with a large shape parameter α in the skew t distribution represents virtually hard minimal bound and very uncertain maximal bound. When $\nu = 1$, the t distribution is known as Cauchy distribution, which does not have mean or variance.

- Both skew normal and skew t distributions go from $-\infty$ to ∞. In MCMCTREE, negative values are automatically truncated, so only the positive part is used. If feasible, try to construct the distribution so that the probability for negative values is small (<0.1%, say).

- Please visit the web site http://azzalini.stat.unipd.it/SN/ to plot skew normal and skew t distributions. R routines are also available for such plots. The equations above are for my testing and debugging. I think I should remove them later on.

Dating viral divergences

This option is specified by the following line in the control file:

```
TipDate = 1 100  * TipDate (1) & time unit
```

The example data file is in the examples/TipDate/. When you run the default analysis, you will see the following printout on the monitor.

```
TipDate model

Date range: (1994.00, 1956.00) => (0, 0.38). TimeUnit = 100.00.
```

The end of each sequence name has the sampling year, which goes from 1994 to 1956. The program then sets the most recent sequence date (1994) to time 0, and then the oldest sequence has age 0.38, as 1956 is 38 years earlier than 1994 and one time unit is specified to be 100 years.

Other control variables work in the same way as in the case of dating species divergence using fossil calibrations. The prior on the age of the root is believed to be important. Please use the sample dates and time unit to specify the bounds on the rate age. For the example dataset mentioned above, the following specifies a soft uniform for the root age in the interval (1914, 1874), with tail probability 10^{-10} on both the left and right tails. This uniform prior is soft but quite sharp.

```
RootAge = B(0.8, 1.2, 1e-10, 1e-10)  * root age constraints, used if no fossil for root
```

Ideally you should use whatever biological information available to specify the prior. Also you should change this prior to assess its impact on the posterior time estimates.

Similarly, the prior on mutation rate (rgene_gamma) may be important as well. The relaxed clock models are species using clock = 2 or 3, while clock = 1 is the strict clock.

The newly implemented prior of times is based on Tanja Stadler's birth-death-sampling model. You should use rho = 0, and psi > 0, for dating viral divergences. You can change the parameters lambda, mu, and psi to assess the impact of the prior.

```
BDparas = 2 1 0 0.8  * lambda, mu, rho, psi for birth-death-sampling model
```

Approximate likelihood calculation

Thorne *et al.* (1998) suggested the use of the multivariate normal distribution of MLEs of branch lengths to approximate the likelihood function. To implement this approximation, one has to obtain the MLEs of the branch lengths and calculate their variance-covariance matrix or equivalently the matrix of second derivatives of the log likelihood with respect to the branch lengths (this matrix is

also called the Hessian matrix). In Thorne's multidivtime package, this is achieved using the program estbranches.

I have implemented this approximation using the option usedata = 3. With this option, mcmctree will prepare three temporary files for each locus and then invoke baseml or codeml to calculate the MLEs of branch lengths and the Hessian matrix. These results are generated in the file rst1 and copied into the file out.BV by mcmctree. The three temporary files for each locus are the control file tmp#.ctl, the sequence alignment tmp#.txt, and the tree file tmp#.trees, where # means the index for the locus. The tree for the locus is generated by mcmctree by pruning the master tree of all species so that only those species present at the locus remain, and by de-rooting the resulting tree. You should not edit this tree file. You can edit the control file tmp#.ctl to use another model implemented in baseml or codeml, and this option should allow you to use amino acid or codon substitution models. The calculation of the Hessian matrix may be sensitive to the step length used in the difference approximation, and it is advisable that you change the variable Small_Diff in the control file tmp#.ctl to see whether the results are stable.

The output file out.BV from usedata = 3 should then be renamed in.BV. This file has one block of results for each locus. If you manually edit the control file tmp#.ctl and then invoke baseml or codeml from the command line (for example, by typing codeml tmp2.ctl), you will have to manually copy the content of rst1 into in.BV.

With usedata = 2, mcmctree will read the MLEs and Hessian matrix from in.BV and apply the approximate method for calculating the likelihood in the MCMC.

In effect, mcmctree/usedata = 3 performs the function of estbranches and you can manually perform this step by running baseml or codeml externally after the tree file tmp#.ctl is generated. Similarly mcmctree/usedata = 2 performs the function of mlutidivtime.

Models of amino acid or codon substitution are not implemented in the mcmctree program for the exact likelihood calculation. The only way to use those models is through the approximate method (usedata = 3 and 2). If is advisable that you edit the intermediate control file tmp#.ctl to choose the appropriate model of amino acid or codon substitution in the codeml analysis, and then copy the results into the in.BV file. Also have a look at the estimated branch lengths in the tree. If many of them are near 0, you should be concerned as perhaps you have too little data or the tree is wrong for the locus. Finally run mcmctree/usedata = 2.

In the description here, a gene or locus means a site partition. For example, since the three codon positions typically have very different rates, different base compositions, etc., you may treat them as separate partitions.

The theory is described in detail in dos Reis and Yang (2011). The default transformation used in the program is the JC transformation.

Infinitesites program

You can compile infinitesites as follows.

```
cc -o infinitesites -DINFINITESITES -O3 mcmctree.c tools.c -lm
```

This generates the limiting posterior distribution when the number of sites in the sequence alignment approaches infinity (Yang and Rannala 2006; Rannala and Yang 2007). Instead of reading and analyzing sequence alignments, the program use the estimated branch lengths as the data, considering them to be without errors. For the clock model (clock = 1), the input file is called FixedDsClock1.txt, while for clock = 2 or 3, the file is called FixedDsClock23.txt. There is an example in the examples/DatingSoftBound/ folder, and the mcmctree tutorial explains how to run this program.

With clock=1, the file FixedDsClock1.txt should have the following format.

```
9
1.0  0.7  0.2  0.4  0.1  0.8  0.3  0.5
1.5
0.8
1.8
```

The first number is the number of species, $s = 9$ in the example. The next line has $s - 1$ node ages (the distances from the $s - 1$ internal nodes in the tree to the present time). Here the implied tree topology must be the same as that in the tree file referred to in the control file mcmctree.ctl, so that the node numbers stay the same. If you used baseml or codeml (with clock=1) to estimate the branch lengths under the clock using the same rooted tree, the output should be in the correct order.

If thre are more than one locus, the next lines will have the ages of the root for those loci, again measured by distance. The example above shows 4 loci. The distance from the root to the tips are 1, 1.5, 0.8 and 1.8 at the four loci respectively. Note that if the clock holds, the node ages should be proportional between loci, so that additional loci provide no extra information about the relative node ages.

With clock=2 or 3, the input file FixedDsClock23.txt should have the branch lengths for the unrooted trees at the multiple loci. The following example is for 3 loci, and there are 7 species in the tree.

```
7

((((human: 0.029043, (chimpanzee: 0.014557, bonobo: 0.010908): 0.016729):
0.015344, gorilla: 0.033888): 0.033816, (orangutan: 0.026872, sumatran:
0.022437): 0.069648): 0.073309, gibbon: 0.024637);

((((human: 0.012463, (chimpanzee: 0.002782, bonobo: 0.003835): 0.003331):
0.004490, gorilla: 0.014278): 0.006308, (orangutan: 0.010818, sumatran:
0.008845): 0.030551): 0.004363, gibbon: 0.029246);

((((human: 0.270862, (chimpanzee: 0.066698, bonobo: 0.056883): 0.124104):
0.139082, gorilla: 0.310797): 0.391342, (orangutan: 0.152555, sumatran:
0.114176): 0.696518): 0.017607, gibbon: 1.394718);
```

The tree topology is rooted, with a bifurcation at the root. The program then collaspses the two branches around the root into one branch before doing any analysis. I think the rooted tree should be the same tree as in the tree file referred to by mcmctree.ctl. Every species has to be present at every locus.

10 Miscelaneous notes

Also see the FAQ page, but please note that document is not up to date.

Analysing large data sets and iteration algorithms

The maximum likelihood method estimates parameters by maximizing the likelihood function. This is multi-dimensional optimisation problem that has to be solved numerically (see Yang 2000a for an exception). PAML implements two iteration algorithms. The first one (`method` = 0) is a general-purpose minimization algorithm that deals with upper and lower bounds for parameters but not general equality or inequality constraints. The algorithm requires first derivatives, which are calculated using the difference approximation, and accumulates information about the curvature (second derivatives) during the iteration using the BFGS updating scheme. At each iteration step, it calculates a search direction, and does a one-dimensional search along that direction to determine how far to go. At the new point, the process is repeated, until there is no improvement in the log-likelihood value, and changes to the parameters are very small. The algorithm updates all parameters including branch lengths simultaneously.

Another algorithm (method = 1) works if an independent rate is assumed for each branch (clock = 0) (Yang 2000b). This algorithm cycles through two phases. Phase I estimates branch lengths with substitution parameters (such as the transition/transversion rate ratio κ and the gamma shape parameter α) fixed. Phase II estimates substitution parameters using the BFGS algorithm, mentioned above, with branch lengths fixed. The procedure is repeated until the algorithm converges. In phase I of the algorithm, branch lengths are optimized one at a time. The advantage of the algorithm is that when the likelihood is calculated for different values of one single branch length, as is required when that branch length only is optimised, much of likelihood calculations on the phylogeny is the same and can be avoided by storing intermediate results in the computer memory. A cycle is completed after all branch lengths are optimized. As estimates of branch lengths are correlated, several cycles are needed to achieve convergence of all branch lengths in the tree, that is, to complete phase I of the algorithm.

If branch lengths are the only parameters to be estimated, that is, if substitution parameters are fixed, the second algorithm (method = 1) is much more efficient. Thus to perform heuristic tree search using stepwise addition, for example, you are advised to fix substitution parameters (such as κ and α). The second algorithm is also more efficient if the data contain many sequences so that the tree has many branch lengths.

☐ **Tip**: To get good initial values for large data sets of protein coding DNA sequences, you can use baseml. Add the options characters "GC" at the end of the first line in the sequence data file. Then run the data with baseml. In the result file generated by baseml (say mlb), look for "branch lengths for codon models" and copy the tree with branch lengths into the tree file. Then run codeml and choose "1: initial values" when asked about what to do with the branch lengths in the tree.

Tree search algorithms

One heuristic tree search algorithm implemented in `baseml`, codonml and `aaml` is a divisive algorithm, called "star-decomposition" by Adachi and Hasegawa (1996a). The algorithm starts from either the star tree (`runmode` = 2) or a multifurcating tree read from the tree structure file (`runmode` = 1). The algorithm joins two taxa to achieve the greatest increase in log-likelihood over the star-like tree. This will reduce the number of OTUs by one. The process is repeated to reduce the number of OTUs by one at each stage, until no multifurcation exists in the tree. This algorithm works either with or without the clock assumption. The stepwise addition algorithm is implemented with the option `runmode` = 3. Options `runmode` = 4 or 5 are used for nearest neighbor interchanges , with the intial tree determined with stepwise addition under the parsimony criterion (`runmode` = 4) or read from the tree structure file (`runmode` = 5). The results are self-explanatory.

Besides the fact that ML calculations are slow, my implementations of these algorithms are crude. If the data set is small (say, with <20 or 30 species), the stepwise addition algorithm (`runmode` = 3) appears usable. Choose `clock` = 0, and `method` = 1 to use the algorithm that updates one branch at a time, and fix substitution parameters in the model (such as κ and α) so that only branch lengths are optimized. Parameters κ and α can be fixed in the tree search using `fix_kappa` and `fix_alpha` in the control files. Other parameters (such as substitution rates for genes or codon positions or site partitions) cannot be fixed this way; they can instead be specified in the file of initial values (`in.baseml` or `in.codeml`). Suppose you use a candidate tree to estimate branch lengths and substitution parameters with `runmode` = 0. You can then move the substitution parameters (but not the branch lengths) into the file of initial values. You then change the following variables for tree search: `runmode` = 3, `method` = 1. The program will use the substitution parameters as fixed in the tree search, and optimizes branch lengths only. It is important that the substitution parameters are in the right order in the file; so copy-and-paste from PAML output is probably the safest. It is also important that you do not change the parameter specifications in the control file; the control file should indicate that you want to estimate the substitution parameters, but when the program detects the file of initial values, fixed parameter values are used instead.

Generating bootstrap data sets

To generate bootstrap pseudo-samples from your original data, use the control variable `bootstrap` in the control files `baseml.ctl` or `codeml.ctl`, as follows

```
bootstrap = 1000    * generate 1000 bootstrap datasets
```

The resulting file is named `boot.txt`. You may want to rename it. Use `baseml` to generate samples for nucleotide-based analysis and `codeml` for amino acid and codon-based analysis. If the data are partitioned (using option G), the programs use stratified sampling to generate bootstrap samples, preserving the number of sites in each partition.

To analyze the bootstrap samples using `baseml` or `codeml`, you can set `ndata = 1000`, say.

The rub file recording the progress of iteration

If you use a large value for the variable noisy (say >2), the programs `baseml` and `codeml` will log output to the screen, indicating the progress of the iteration process, i.e., the minimization of the negative log-likelihood. They will also print in the `rub` file, the size (norm) of the gradient or search direction (*h*), the negative log likelihood, and the current values of parameters for each round of iteration. A healthy iteration is indicated by the decrease of both *h* and the negative log likelihood, and *h* is particularly sensitive. If you run a complicated model hard to converge or analyzing a large data set with hundreds or thousands of sequences, you may switch on the output. You can check this file to see whether the algorithm has converged. A typical symptom of failure of the algorithm is that estimates of parameters are at the preset boundaries, with values like 2.00000, 5.00000. When `method` = 1, the output in the `rub` file lists the log likelihood and parameter estimates only.

Specifying initial values

You may change values of parameters in the control file such as kappa, alpha, omega, etc. to start the iteration from different initial values. Initial values for the second and later trees are determined by the program, and so you do not have much control in this way.

You can collect initial values into a file called `in.baseml` if you are running `baseml` or `in.codeml` if you are running `codeml`. This file should contain as many numbers, separated by white spaces, as the number of parameters that are being optimized by the program. So if the program is estimating 56 parameters (say 51 branch lengths, 1 kappa, and 5 other parameters from the ω distribution), you should put 56 numbers in the file. The parameters are ordered internally in

the program and you have no control of the ordering. Nevertheless, the order is the same as in the main output (below the lnL line for each tree). One way of generating the in.codeml or in.baseml files is to run a data set, and then copy initial values from the rub file or from the main output file. The `rub` file records the iteration process and has one line for each round of iteration. Each line lists the current parameter values after the symbol x; you can copy those numbers (not the symbol x) into the file of initial values, and if you like, change one or a few of the parameter values too. When you run the program, look at lnL0 printed out on the screen and check that it is the same as recorded in rub.

When the program runs, it checks to see whether a file of initial values exists, and it does, the program will read initial values from it. This may be useful if the iteration is somehow aborted, and then you can collect current values of parameters from the file `rub` into this file of initial values, so that the new iteration can have a better start and may converge faster. The file of initial values may also be useful if you experience problems with convergence. If you have already obtained parameter estimates before and do not want the program to re-estimate them and only want to do some analysis based on those estimates such as reconstructing ancestral sequences, insert -1 before the initial values.

Warning: A complication is that in some models a transformation is applied during the iteration while the printout uses the original variables. Examples of this are the frequency/proportion parameters for base frequencies (nhomo = 1 in baseml), proportions of site classes in the NSsites models (except for models always having only two classes in which case no transformation is applied), and times or node ages in clock models (clock = 1, 2, 3, 5, 6, but not 0). For those models, you can see that the line of output in the main output file looks different from the last line of rub after the iteration finishes. In the file of initial values, if you use -1 at the start, the program assumes the original variables, while if you don't, the program assumes transformed variables.

Fine-tuning the iteration algorithm

The iteration algorithm uses the difference approximation to calculate derivatives. This method changes the variable (x) slightly, say by a small number e, and see how the function value changes. One such formula is $df/dx = [f(x + e) - f(x)]/e$. The small number e should be small to allow accurate approximation but should not be too small to avoid rounding errors. You can change this value by adding a line in the control files `baseml.ctl` or `codeml.ctl`

```
Small_Diff = 1e-6
```

The iteration is rather sensitive to the value of this variable. This variable also affects the calculation of the SE's for parameters, which are much more difficult to approximate than the first derivatives. If the calculated SE's are sensitive to slight change in this variable, they are not reliable.

If you compile the source codes, you can also change the lower and upper bounds for parameters. I have not put these variables into the control files (See below).

Adjustable variables in the source codes

This section is relevant only if you compile the source codes yourself. The maximum values of certain variables are listed as constants in uppercase at the beginning of the main programs (`baseml.c`, `basemlg.c`, `codeml.c`). These values can be raised without increasing the memory requirement by too much.

NS: maximum number of sequences (species)

LSPNAME: maximum number of characters in a species name

NGENE: maximum number of "genes" in data of multiple genes (option G)

NCATG: maximum number of rate categories in the (auto-) discrete-gamma model (baseml.c, codeml.c)

You can change the value of `LSPNAME`. Other variables that may be changed include the bounds for parameters, specified at the beginning of the function `testx` or `SetxBound` in the main programs (`baseml.c` and `codeml.c`). For example, these variables are defined in the function `SetxBound` in `codeml.c`:

```
double tb[]={.0001,9}, rgeneb[]={0.1,99}, rateb[]={1e-4,999};

double alphab[]={0.005,99}, rhob[]={0.01,0.99}, omegab[]={.001,99};
```

The pairs of variables specify lower and upper bounds for variables (`tb` for branch lengths, `rgeneb` for relative rates of genes used in multiple gene analysis, `alphab` for the gamma shape parameter, `rhob` for the correlation parameter in the auto-discrete-gamma model, and `omegab` for the d_N/d_S ratio in codon based analysis.

Windows notes

Turn on file extensions in Windows Explorer. Windows Explorer by default hides file extensions for known file types. You should go to "Windows Explorer - Tools - Folder options - View" and un-tick "Hide extensions for known file types", so that you can see the full file names from Windows Explorer.

Using Task Manger to change job priority. Start Task Manger (for example, right click on task bar and choose Task Manager). Click on Processes button. Locate the big job, say, codeml. Right click and Set Priority to Low. Note that the process running the Command Prompt is cmd. If you change the priority of cmd to low, all jobs started from that window will run at low priority. You can change View – Update Speed to Low and change View – Select Columns. Change Options – Minimize on Use. Then you can minimize rather than close Task Manger.

All input and output files are plain text files. In the Command Prompt box (Start - Programs – Accessaries – Command Prompt), you can use **type** or **more** to view a text file. If you see strange characters on the screen and perhaps also hear beeps, the file is not a plain text file. You can also use a text editor to view and edit a plain text file. If you use Microsoft Word or Wordpad to save a file, make sure that the files are saved as a plain text file. Use File – Save As and change the file type. When you do, Word or other programs might automatically add the file extension .txt, and you will have to rename the file if you don't want the .txt.

Changing search path to include paml programs. You can create a folder called bin under your account. The folder name should be shown when you move (cd) to that folder. Let's say this is D:\bin. Copy or move the executable files from the paml/bin/ folder into this folder.

Now change the environment variable path. On Windows Vista or XP, open Control Panel – System – Advanced System Settings – Environment variables. Under "User variables for Ziheng", find path, and Edit. Add the folder name (D:\bin in my example) to the end or beginning of the string. The fields are separated by semi-colons. Note that Windows/Dos is case-insensitive, so D:\bin is the same as D:\BIN or d:\bin.

UNIX/Linux/Mac OSX notes

How to change your search path to include paml programs. You can create a folder bin in your root directory and copy paml executables into that folder, and then include the folder in your search path. This way you can run the program no matter where you are. If you have several versions of the paml, you may even name the executables baseml3.15, baseml4, etc., to distinguish them. Similarly you can copy other programs such as mb, dnadist, etc. into that folder.

Type cd   to move to your root directory. Create a bin/ folder in your root directory.

mkdir bin

Copy executables such as codeml, baseml, and mcmctree into the bin/ folder.

Then modify your path environment variable to include the bin/ folder in the initialization file for the shell. You can use more /etc/passwd to see which shell you run. Below are notes for the C shell and bash shell. There are other shells, but these two are commonly used.

1) If you see /bin/csh for your account in the /etc/passwd file, you are running the C shell, and the intialization file is .cshrc in your root folder. You can use more .cshrc to see its content if it is present. Use a text editor (such as emacs, vi, SimpleText, etc.) to edit the file, and insert the following line

set path = ($path . ~/bin)

The different fields are separated by spaces. Here . means the current folder, and ~/ means your root folder, and ~/bin means the bin folder you created, and $path is whatever folders are already in the path.
If the file .cshrc does not exist, you create one, and insert the line above.

2) If you see /bin/bash in the file /etc/passwd for your account, you are running the bash shell, and the initialization file is .bashrc. Use a text editor to open .bashrc and insert the following line

PATH=$PATH:./:~/bin/

This changes the environment variable PATH. The different fields are separated by colon : and not space. If the file does not exist, create one.

After you have changed and saved the initialization file, every time you start a shell, the path is automatically set for you.

Note that UNIX is case-sensitive.

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