Advanced methods in DNA sequence and multilocus data analyses
Lesson 1 – DNA sequences I

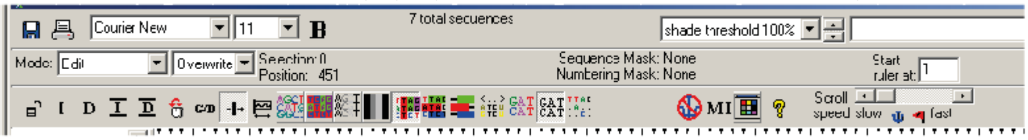
I. Sequence editing [BioEdit, MEGA, FaBox, SeqState, R]
II. Alignment improvement
III. Model selection [jModeltest, PartitionFinder]
IV. Tree reconstruction [RAxML, PAUP, MrBayes]

I. Sequence editing

BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

As an input you can use simply FASTA format (but also some other formats, as you can check in BioEdit).

- *File → Open*
- choose file with suffix ∗.fas (e.g. output from MAFFT) or *.aln (output from CLUSTAL)
- in pop-up menu „*Mode*“ you have to set up the „*Edit*“ mode, that enables you to manipulate with sequences (rewriting, deleting, etc.)
- to highlight differences in sequences you can choose from broad scale of background color styles (e.g. to show only different nucleotides compared to selected reference sequence) using the icons in the main menu bar and/or pop-up menu „*Shade threshold*“ in the top-most bar.



Courier New | 11 | 7 total secuences | shade threshold 100%

Mode: Edit | Overwrite | Selection: 0 | Position: 451 | Sequence Mask: None | Numbering Mask: None | Start ruler at: 1

- alignment editing is intuitive – overwriting bases (only after cross check with original sequence data!), copying particular sequence regions, inserting gaps (using right click), deleting sequence regions, etc.
- you should save your edited alignment under modified name, again in FASTA format (with suffix ∗.fas) or you can choose another format, if needed (check the format offer when saving the file)
- useful shortcuts: Ctrl+A (select all sequences), Ctrl+Del (delete selected sequence(s)), Ctrl+Shift+R (create *reverse complement*)
- using *File → Export → Sequence alignment* you can save the sequence in NEXUS format (with suffix ∗.nex, only *interleave* type), that you can directly use for further analyses e.g. in software PAUP, MrBayes (http://mrbayes.csit.fsu.edu) or SplitsTree. There are some bugs in BioEdit that can cause the problems with saving NEXUS format so it is usually better to save simply FASTA format and use some file convertor to get desired file format for further analyses.
- For the file conversion, you can use e.g. server http://sequenceconversion.bugaco.com/converter/biology/sequences/fasta_to_nexus.php

Can be useful...

- you can differentiate parts of your alignment by using UPPERcase or LOWERcase letters, e.g. in case you want to differentiate **coding** and **non-coding** parts of the sequences. Highlight with mouse desired region of the alignment and select in main menu *Sequences → Manipulations → UPPERCASE/lowercase*

- if you have only **coding sequences** in your alignment (e.g. exons) in correct reading frame, you can translate your nucleotide sequences into **aminoacid sequences** (Ctr+T) and estimate e.g. presence/absence of pseudogenes.

PhyDE software (http://www.phyde.de), for which you need sequences in FASTA or NEXUS sequential format.

- *File → Open*
- select file with suffix ∗.fas
- window with alignment appears
- you can move bases or blocks of sequences using mouse – holding left mouse button highlight desired sequence region, right click, move the sequence block to desired location, right click again. Editing modes are *Align* (enables moving the gaps) or *Edit* (enables editing of sequences).
- *File → Export as… → NEXUS* (or FASTA, etc.)

MEGA software (http://www.megasoftware.net/).

Online manual: http://www.megasoftware.net/web_help_7/helpfile.htm#hc_first_time_user.htm

FaBox software (http://users-birc.au.dk/biopv/php/fabox/)

- using this web tool, you can manipulate fasta format in many different and useful ways. You can e.g. extract (and manipulate) file headers, removing sequences from alignment, joining alignments, identify haplotypes, convert fasta to other formats etc.
- you can also extract only variable positions from the alignment („*Show variable sites only*“)
- to create input file for statistical parsimony network analysis (in TCS) you can use „*Create TCS input file from fasta (fasta2tcs)*“
- to create input file for MrBayes you can use „*Create MrBayes input file from fasta (fasta2mrbayes)*“. The file includes block of commands necessary for MrBayes analysis, but you can also delete these commands and use the input file as pure NEXUS file for other software (e.g. Splitstree, PAUP etc.).

Converting sequence formats using various web tools:

- Sequence conversion (http://sequenceconversion.bugaco.com/converter/biology/sequences)
- FormatConverter (http://hcv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html)

SeaView software (http://doua.prabi.fr/software/seaview)

- Using SeaView you can simply open one of the sequence format you have (FASTA, NEXUS, PHYLIP, etc.)
- *File → Save as*
- change the type of the output file in the bottom right of the pop-uped window and save

SeqState software (http://bioinfweb.info/Software/SeqState).

If the alignment includes **gaps,** i.e. insertion/deletion (shortly 'indels') that might be phylogenetically informative, it might be necessary to code such indels as additional characters. You can do this automatically using this software. For theoretical background, you should read e.g. Müller (2006): Incorporating information from length-mutational events into phylogenetic analysis. *Molecular Phylogenetics and Evolution*, 38, 667-676.

Practically:

- *File* → *Load NEXUS file* (or *Load FASTA file*)
- choose file with suffix *.fas or ∗.nex (it have to be sequential format, created e.g. by PhyDE or reformated using some web tool, see above)
- *IndelCoder* → *simple indel coding* (or another way of coding, see the theoretical paper mentioned above)
- it creates NEXUS file (in interleave format, and it adds suffix '_mcic.nex' to original file name). In addition to original data matrix the matrix including coded gaps is included. This file can be directly used e.g. in PAUP.

R software (https://www.r-project.org/)

A few simple R scripts to manage molecular data:

# save sequence names

```
library(phytools)
align = read.FASTA(file = "ITS_1.fas")
write.csv(names(align),file="ITS_names.csv")
# taky pomocí savelist (seqinr)
```

# replace sequence names

```
library(seqinr)
al <- read.fasta(file = "ITS_1.fas")
jmena=read.csv(file="ITS_names_new.csv")
write.fasta(sequences = al, names = as.vector(jmena$x),
        nbchar = 80, file.out = "ITS_1N.fas")
```

# minimize alignment to a mask

```
library(seqinr)
align = read.fasta(file = "Actin_1_MAFFT.fas")
vyber=which(align$mask %in% "1")
masked=lapply(align, "[", vyber)
write.fasta(sequences = masked, names = names(masked),
        nbchar = 80, file.out = "Actin_2_MAFFT.fas")
```

# minimize alignment to selected sequences

```
library(seqinr)
#ITS
align_ITS  <- read.alignment(file = "ITS_2.fas", format = "fasta")
data=read.csv2(file="its-vyber.csv")
vyber_I=match(as.vector(data$ITS),align_ITS$nam)
vyber_ITS=vyber_I[!is.na(vyber_I)]
write.fasta(sequences = align_ITS$seq[vyber_ITS], names = align_ITS$nam[vyber_ITS],
        file.out = "ITS_vyber.fas")
```

```
# concatenate
library(ape)
A <- read.dna("actin_concat.fas", format="fasta")
B <- read.dna("ITS_concat.fas", format="fasta")
C <- cbind(A,B)
write.dna(C, "concatenated.fas", format="fasta", colsep="")
```

II. Alignment improvement

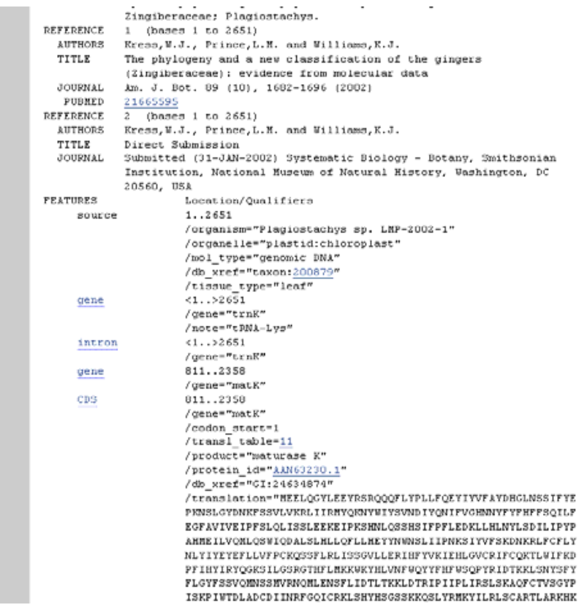
R script to show the saturation plot

```
library(ape)
data <- read.dna("Chlorella_vulgaris.fas", format="fasta")
#Convert to genetic distances
dist<-dist.dna(data, model="raw")
dist.corrected<-dist.dna(data, model="TN93")
#Make plot
plot(dist~dist.corrected, pch=20, col="red", xlab="TrN model distance", ylab="Uncorrected genetic distance", main="Saturation Plot")
abline(0,1, lty=2)
```

III. Model selection

Testing the models of DNA evolution. Properly chosen model of DNA evolution is the key for estimation of likelihoods of gene tree topologies using Maximum likelihood and/or Bayesian methods.

Before testing models of DNA evolution, it is important to know the **structure of analyzed DNA sequence**, i.e. what regions represent coding sequences, what region represent non-coding sequences, etc. The important step is to realize, that some positions in alignment can evolve in different manner than the others, i.e. under different evolutionary model. Information about studied sequence (and potential borders between coding and non-coding regions) you can found e.g. using on-line tool **BLAST**), where the annotations and characteristics of some closely related sequence might be available. The exemplar annotation is shown on the figure on the right.



```
                Zingiberaceae; Plagiostachys.
REFERENCE   1  (bases 1 to 2651)
  AUTHORS   Kress,W.J., Prince,L.M. and Williams,K.J.
  TITLE     The phylogeny and a new classification of the gingers
            (Zingiberaceae): evidence from molecular data
  JOURNAL   Am. J. Bot. 89 (10), 1682-1696 (2002)
   PUBMED   21665595
REFERENCE   2  (bases 1 to 2651)
  AUTHORS   Kress,W.J., Prince,L.M. and Williams,K.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-JAN-2002) Systematic Biology - Botany, Smithsonian
            Institution, National Museum of Natural History, Washington, DC
            20560, USA
FEATURES             Location/Qualifiers
     source          1..2651
                     /organism="Plagiostachys sp. LMP-2002-1"
                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:200879"
                     /tissue_type="leaf"
     gene            <1..>2651
                     /gene="trnK"
                     /note="tRNA-Lys"
     intron          <1..>2651
                     /gene="trnK"
     gene            811..2358
                     /gene="matK"
     CDS             811..2358
                     /gene="matK"
                     /codon_start=1
                     /transl_table=11
                     /product="maturase K"
                     /protein_id="AAN63230.1"
                     /db_xref="GI:24634874"
                     /translation="MEELQGYLEEYRSRQQQFLYPLLFQEYIYVFAYDHGLNSSIFYE
                     PKNSLGYDNKFSSVLVKRLIIRMYQKNYWIYSVNDIYQNIFVGHNNVFYFHFFSQILF
                     EGFAVIVEIPFSLQLISSLEEKEIPKSHNLQSSHSIFPFLEDKLLHLNYLSDILIPYP
                     AHMEILVQMLQSWIQDALSLHLLQFLLHEYYNWNSLIIPNKSIYVFSKDNKRLFCFLY
                     NLYIYEYEFLLVFPCKQSSFLRLISSGVLLERIHFYVKIEHLGVCRIFCQKTLWIFKD
                     PFIHYIRYQGKSILGSRGTHFLMKKWKYHLVNFWQYYFHFWSQPYRIDTKKLSNYSFY
                     FLGYFSSVQMNSSMVRNQMLENSFLIDTLTKKLDTRIPIIPLIRSLSKAQFCTVSGYP
                     ISKPIWTDLADCDIINRFGQICRKLSHYHSGSSKKQSLYRMKYILRLSCARTLARKHK
```

Since you know the structure of studied region you can partition the dataset into the coding and non-coding parts, i.e. create from single dataset two (or more) datasets for which you can estimate proper DNA evolution model separately. You can divide the original dataset 'physically' in BioEdit (or another alignment editor) by preparing different datasets from the original one and saving them under different names (e.g. '..._exon.fas', '..._intron.fas').

Alternatively, you can add the block of commands into original NEXUS format (or in the separate file), where the borders of particular regions are specified. The final parts of the alignments are called '**partitions**'.

The particular way of partition definition is always better to consult with documentation for particular downstream analysis because the formats might differ (e.g. PAUP, MrBayes, PartitionFInder etc..). Exemplar nexus block for differentiation of coding sequences according the position of nucleotides in coding triplets for the purpose of Bayesian analysis is shown below.

```
begin mrbayes; # začátek nového nexus bloku (může být např. i “Begin sets;”)
outgroup 22;
charset CHS_pos1 = 1-957\3; # definice úseků - kódující sekvence, první nukleotidy v tripletu
charset CHS_pos2 = 2-957\3; # kódující sekvence, druhé nukleotidy v tripletu
charset CHS_pos3 = 3-957\3; # kódující sekvence, třetí nukleotidy v tripletu
partition Position = 3: CHS_pos1, CHS_pos2, CHS_pos3; # rozdělení datasetu
end;
```

The DNA models for particular partitions you can test using software **jModeltest** (http://code.google.com/p/jmodeltest2/). The resulting models can be than used e.g. for ML analysis. Software jModeltest do not accept input datasets that are divided into partitions, therefore, in order to analyze different partitions you have to analyze different parts of the dataset one by one (e.g. separate data file for exons and separate data file for introns).

- *File → Load DNA alignment*   # choose file in Nexus or Phylip format
- *Analysis → Compute likelihood scores* → settings of analysis complexity (i.e. how many different evolutionary models will be tested, from 3 to 88 models).
- If you want to analyze your sequences with ML, set up maximum number of models (I.e. „Number of substitution rates“ = 11 and tick all the boxes for „Base frequencies“ and „Rate variation“). Other settings we can leave as default.
- If you want to analyze your sequence data using MrBayes, it is enough to set up only few selected models, as MrBayes also only allows you to set up only few selected models. For testing the models for MrBayes analysis, you can also use more specific software MrModeltest, that is in default tests only those models that are further useful for MrBayes analysis.
- *Analysis → Do AIC calculation* → use default settings and tick the box „write *PAUP block”
- in an output you will find the comparisons of particular models tested and their likelihood scores (the measure of likelihood of particular models given your data). In section „AKAIKE INFORMATION CRITERION (AIC)“ you will find the optimal model for your data selected based on Akaike criterion (AIC). Below you copy set of commands that codes the selected model and its usage in ML analysis. This commands you can add to the Nexus format with your sequence data.

```
--
PAUP* Commands Block:
 If you want to load the selected model and associated estimates in PAUP*,
 attach the next block of commands after the data in your PAUP file:
[!
Likelihood settings from best-fit model (HKY+G) selected by AIC
with jModeltest 0.1.1 on Thu Oct 17 13:26:26 CEST 2013]

BEGIN PAUP;
Lset  base=(0.4010  0.2062  0.1059  0.2870)  nst=2  tratio=3.3506  rates=gamma  shape=0.4330  ncat=4
pinvar=0;
END;
--
```

Alternatively, you can test DNA models and simultaneously find the best partition for you data using **PartitionFinder** (http://www.robertlanfear.com/partitionfinder/). We will describe its usage for further ML analysis of your data. The advantage of this approach is the possibility to load single dataset that can be divided into the partitions (as described above). Software then test all DNA models for all partitions and further decide, which partitions should use different evolutionary models and potentially also which partitions can be described by the evolutionary model and therefore can be joined together. This is especially useful when we want to know, if it's necessary to differentiate in our DNA region only exons and introns or if we want to differentiate also different positions within the coding regions. In the latter case you usually end up with more than three partitions so it is more practical to use PartitionFinder for model testing in such a cases. PartitionFinder is the python script so in order to run it, you have to have the Python installed on your computer (see http://www.python.org/).

- There are two input files that should be located in the single folder - the file including your alignment in Phylip format and the configuration file „partition_finder.cfg“ in which you specify the name of the input sequence file (*.phy), define the partitions of your dataset and select the way of model testing. When editing the *.cfg file it is better to check the original documentation that comes with PartitionFinder. Default/exemplar setting can be like this:

```
## ALIGNMENT FILE ##
alignment = test.phy;

## BRANCHLENGTHS: linked | unlinked ##
branchlengths = linked;

## MODELS OF EVOLUTION for PartitionFinder: all | raxml | mrbayes | beast | <list> ##
##              for PartitionFinderProtein: all_protein | <list> ##
models = all;

# MODEL SELECCTION: AIC | AICc | BIC #
model_selection = BIC;

## DATA BLOCKS: see manual for how to define ##
[data_blocks]
Gene1_pos1 = 1-1062\3;
Gene1_pos2 = 2-1062\3;
Gene1_pos3 = 3-1062\3;

## SCHEMES, search: all | greedy | rcluster | hcluster | user ##
[schemes]
search = greedy;

#user schemes go here if search=user. See manual for how to define.#
```

- You run analysis from the command line. In Windows you can call the command line using Start → Run → cmd
- In the command line, you write command of following structure:
  python ”<PartitionFinder.py>” “<inputFolderName>”
  where ”<PartitionFinder.py>” is the PATH to the python script PartitionFinder.py (that can be wherever in the computer) and “<inputFolderName>” that represents PATH to the folder with input files (it also can be locate wherever). Both PATH you can import to the command line after command “python” by mouse drag and drop.

- After the end of analysis the new folder with name „analysis“ appears in your working directory. In this folder you will be mainly interested in file “best_schemes.txt”, where you will find the information about the best option for grouping of particular partitions into the groups with the same model of evolution.

```
Subset | Best Model | Subset Partitions| Subset Sites | Alignment
```

```
1 | F81+G | Gene1_pos1, Gene2_pos1, Gene3_pos1 | 1-789\3, 790-1449\3, 1450-2208\3 | .....
2 | F81+G | Gene1_pos2, Gene2_pos2, Gene3_pos2 | 2-789\3, 791-1449\3, 1451-2208\3 | .....
3 | K80+G | Gene1_pos3 | 3-789\3 | .....
4 | TrN+G | Gene2_pos3, Gene3_pos3 | 792-1449\3, 1452-2208\3 | ....

Scheme Description in PartitionFinder format
Scheme_step_5  =  (Gene1_pos1,  Gene2_pos1,  Gene3_pos1)  (Gene1_pos2,  Gene2_pos2,  Gene3_pos2)
(Gene1_pos3) (Gene2_pos3, Gene3_pos3);
```

- The software, however, do not generate Nexus block of commands that you could directly paste into your alignment file. You have to create this block by yourself based on the information from the file „best_schemes.txt".

IV. Tree reconstruction

Fast construction of ML tree and other useful analyses in MEGA

Software MEGA can be useful from several points of view. You can view your alignment and convert FASTA file into other sequence formats (e.g. NEXUS, PHYLIP), test your sequence data if they evolve according molecular clocks or construct ML tree in rather fast way. We recommend to use other software for serious ML reconstruction for your final datasets (e.g. RaxML, Garli), however, you can use MEGA for the first insight into your data.

- Fast construction of ML tree
    - open Fasta alignment in MEGA
    - Phylogeny – Construct/Test Maximum LikelihoodTree:
        - Test ofPhylogeny – none (or you can choose here if you want to use Bootstrap analysis to estimate reliability of your gene topology)
        - Substitution Model &RatesamongSites – choose suitable DNA evolution model according previous jModeltest (or another) analysis
        - Compute
    - After the end of analysis final tree appears. You can save it using: File – Export CurrentTree (Newick)
- Format conversion
    - open Fasta alignment in MEGA
    - Data → Explore active data. Your alignment appears in new window. In this active window, it is better to click and deactivate the icon 'Use identical symbol' to see particular nucleotides in the alignment.
    - Data → Export data
    - in section 'Format' choose the desired format for export and click 'OK'. You can also check some other options for the export, if needed.

- Test dataset for **molecular clocks**
    - open Fasta alignment in MEGA
    - Clocks → Test Molecular Clocks (ML)
    - You have to specify the tree topology for your data in the section 'Tree to Use' using 'Use tree from file' and then navigate the software to the directory where you store your tree (e.g. in newick format)

- You should specify the evolutionary model for your data in section 'Model/Method'
- other settings you can leave as it is by defaults and run analysis using 'Compute'
- After the end of analysis the new window will appear with all the information

Construction of a ML tree using RAxML (http://sco.h-its.org/exelixis/web/software/raxml/index.html)
Python version: http://sourceforge.net/projects/raxmlgui/
Windows version: https://github.com/stamatak/standard-RAxML/tree/master/WindowsExecutables_v8.1.20

Basic parameters

-m	specification of substitution model
-p	random seed
-t	starting tree (if not specified, a parsimony tree is automatically created)
-s	name of input file (in phylip format)
-#	number of replications
-n	name appendix of resulting files

1. ML on binary data

raxmlHPC -m BINGAMMA -p 12345 -s binary.phy -# 20 -n resultBIN

- using GAMMA model
- 20 replications

2. ML on DNA data

raxmlHPC -m GTRGAMMA -p 12345 -s dna.phy -# 20 -n resultDNA

3. bootstrapping

a) first a best-scoring ML tree is found

raxmlHPC -m GTRGAMMA -p 12345 -# 20 -s dna.phy -n bestML

b) a bootstrap analysis is followed

raxmlHPC -m GTRGAMMA -p 12345 -b 12345 -# 100 -s dna.phy -n boot

- bootstrap trees are generated to RAxML_bootstrap.boot

c) bootstrap values are mapped on the best-scoring ML tree

raxmlHPC -m GTRGAMMA -p 12345 -f b -t RAxML_bestTree.bestML -z RAxML_bootstrap.boot -n finalboot

- RAxML_bipartitions.finalboot file (bootstrap values as nodes) and RAxML_bipartitionsBranchLabels.finalboot file (bootstrap values on branches)

4. rapid bootstrapping

- complete analysis in one step(ML search+ bootstrapping):

raxmlHPC -f a -m GTRGAMMA -p 12345 -x 12345 -# 100 -s dna.phy -n rbs

- RAxML_bipartitions.rbs file is created – the best ML tree with bootstrap values mapped

Construct a MP tree using PAUP

Open a file:

1. File -> Open… (select a NEXUS file)
or File -> Import Data … (can open FASTA, PHYLIP, etc.)

2. File -> Execute (or CTRL + R)
3. File -> Log Output To Disk

Perform a ML analysis:
1. Analysis -> Parsimony
2. Analysis -> Parsimony Settings…
Optimization – ACCTRAN or DELTRAN
Gaps – Missing Data
3. Analysis -> Heuristic Search
General: Keep – optimal trees only, Set MaxTrees – 1000, Leave unchanged
Starting trees – Get by stepwise addition
Stepwise addition: Addition sequence – random, # reps – 10
Branch swapping: swapping algorithm – TBR
4. Trees - > Root Trees…
Rooting Options… - Define Outgroup… - click to outgroup species
5. Trees -> Print/View Trees…
6. Trees -> Compute Consensus… - Strict, Majority-rule (50%), Include values in treefile… as node labels, Output to treefile
7. Trees -> Print/View Consensus Tree(s)…

Bootstrapping:
1. Analysis -> Bootstrap/Jackknife Analysis
Resampling method – Bootstrap
Number of replicates
2. Trees -> Print/View Bootstrap Consensus…

Construct a Bayesian tree using MrBayes (http://mrbayes.sourceforge.net/)

- test alignments:
    - test.nex – concatenated dataset
- Bayesian inference
    - a MrBayes block is added at the end of Nexus file:

```
begin mrbayes;                              -
charset 18S = 1-1782;                       1st partition definition
charset psa = 1783-2702;                    2nd partition definition
charset cox = 2703-3285;                    3rd partition definition
partition marker = 3:18S,psa,cox;           partitioning definition
set partition = marker;                     set partitions

prset applyto=(all) ratepr=variable;        priors definition (partitions are given in brackets);
                                               ratepr=variable: partitions may have different
                                               evolutionary rates
lset applyto=(1)   nst=6  rates=invgamma;   substitution models definition (partitions in brackets,
lset applyto=(2)   nst=6  rates=propinv;       again); nst=1 (JC, F81), nst=2 (K80, HKY), nst=6
lset applyto=(3)   nst=2  rates=equal;         (GTR); rates=equal / gamma (Γ) / propinv (I)
```

/invgamma (Γ + I)

```
unlink statefreq=(all) revmat=(all) tratio=(all) shape=(all)
pinvar=(all);

mcmc ngen=5000000 samplefreq=100 relburnin=no;

end;
```

each partition may have unique parameters of substitution models

set a mcmc analysis: ngen=number of generations; samplefreq=rate of writing parameters into files

- in addition to the above-mentioned parameters, the analysis is based on several default parameters. Their values can be listed by typing „help“:
  - help prset = list of priors
  - help lset = list of model parameters
  - help mcmc = list of mcmc parameters
- Bayesian analysis is run by typing „mrbayes“ and „exe FILENAME.nex“

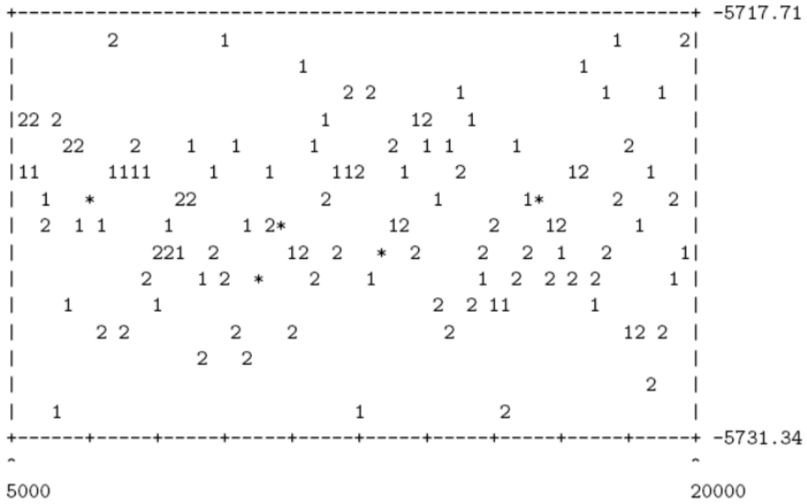
- analysis evaluation
  - at the end of analysis, MrBayes asks asks whether or not you want to continue with the analysis. Before answering that question, examine the average standard deviation of split frequencies. If the value is higher than 0.05, the analysis should be prolonged. Values less than 0.01 indicate a good convergence.
- drawing a consensus tree:

```
sump

-or-

sump relburnin=no burninfrac=0.1

sumt contype=allcompat

-or-

sumt relburnin=no burninfrac=0.1
contype=allcompat
```

summary of model parameters.

*Burnin = number of discarded generations. Should be set to obtain a stationary phase (a graph includes randomly placed letters 1, 2 and *) – see the picture below. If only “sump” is typed, a 25% of generations are discarded. To set the burnin value manually, “relburnin=no“ should be typed.*

creating a consensus tree, setting a burnin parameter as indicated above. “contype=allcompat“ returns a fully bifurcated tree.



```
+------------------------------------------------------------+ -5717.71
|        2         1                                  1     2|
|                         1                        1         |
|                             2 2       1            1    1  |
|22 2                       1       12   1                   |
|    22    2    1   1      1      2  1 1     1         2     |
|11      1111     1    1     112   1    2         12     1   |
|  1   *       22           2         1       1*      2    2 |
|  2  1 1     1      1 2*         12       2    12      1    |
|            221  2      12  2   *  2     2   2  1   2      1|
|           2    1 2  *    2    1         1  2  2 2 2      1 |
|    1       1                        2  2 11       1        |
|       2 2         2    2             2               12 2  |
|                2   2                                       |
|                                                        2   |
|   1                          1            2                |
+------+-----+-----+-----+-----+-----+-----+-----+-----+-----+ -5731.34
^                                                            ^
5000                                                         20000
```

Ideal sumt result

If the numbers are not intermixed, or If you see an obvious trend in the plot, either increasing or decreasing, the analysis should be run longer. Finally, the values of PSRF+ parameters should be checked. The values should be close to 1.00.

results of you analysis.