FACULTAD DE CIENCIAS QUÍMICAS Y FARMACÉUTICAS
UNIVERSIDAD DE CHILE
PROGRAMA DE DIPLOMADO EN BIOINFORMÁTICA Y BIOLOGÍA COMPUTACIONAL

Analysis of Substitution Saturation

The substitutionhttp://www.postgradoquimica.cl/informacion-diplomado-en-bioinformatica-y-biologia-computacional/ saturation estimation is an **essential** analysis to check if the alignment sequences still maintain a phylogenetic signal. Saturation is much more common in nucleotide sequences. There are two methods: one extremely simple and qualitative and the other quantitative, with associated probability (the Xia method, 2009). Both are implemented in the program DAMBE (Xia, 2018).

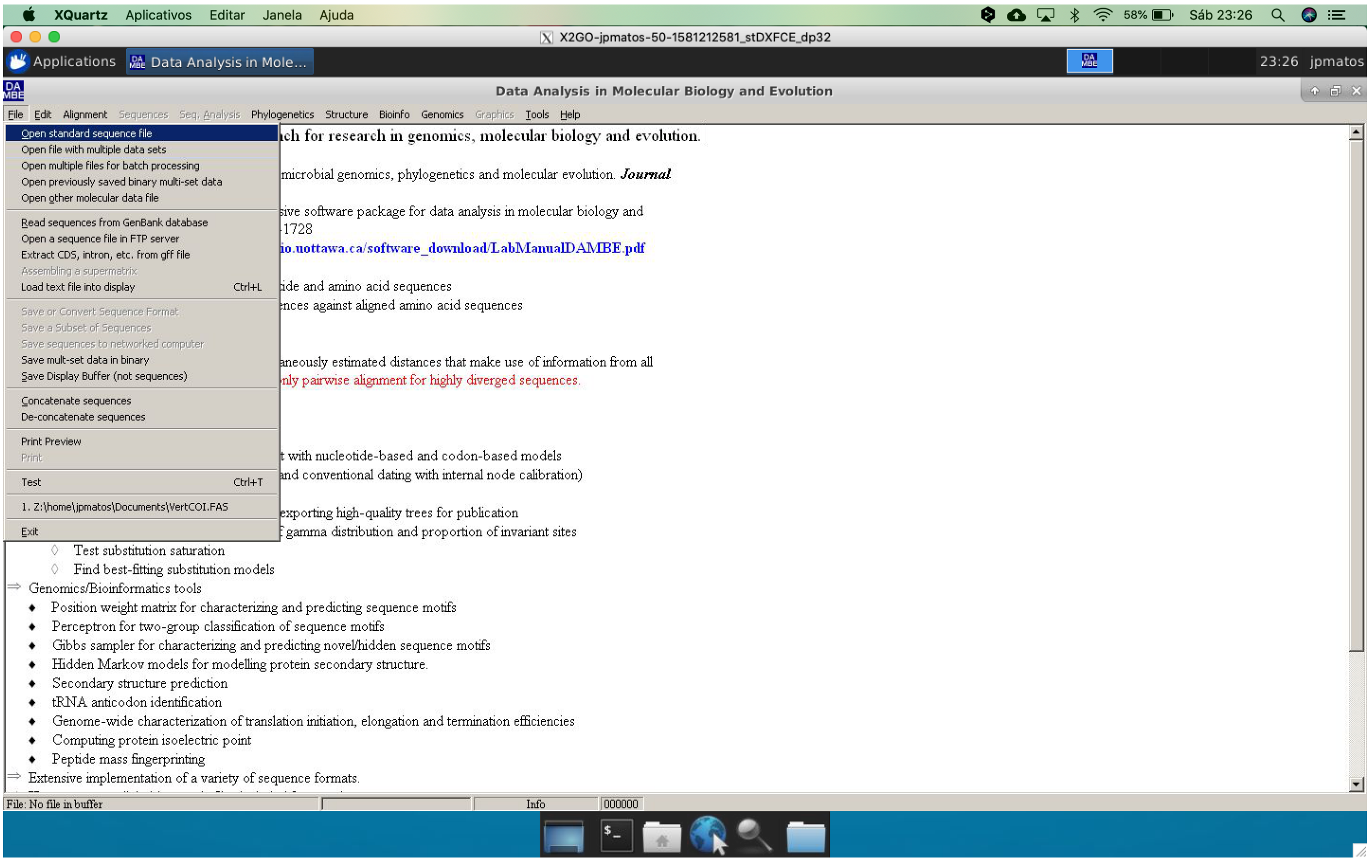
DAMBE (Xia, 2018) is a suite with several phylogenetic and sequence analysis applications. Additionally, it is multi-platform (except if you run MacOS Catalina, as it is not completely 64 bits - Feb 2020) and simple to use. To our knowledge, it is the only phylogenetic analysis program that performs these tests.

The Qualitative Method

The qualitative method of substitution saturation analysis consists of analyzing a plot called "transition and transversals versus divergence" (*transitions and transversals versus divergence*). This method assumes that the distance calculated between the sequences is directly proportional to the divergence time between them, either on any scale.

The steps to obtain this graph are:

- Open the DAMBE program. Click on *File* and then on *Open Standard Sequence File*.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Open standard sequence file
Open file with multiple data sets
Open multiple files for batch processing
Open previously saved binary multi-set data
Open other molecular data file
Read sequences from GenBank database
Open a sequence file in FTP server
Extract CDS, intron, etc. from gff file
Assembling a supermatrix
Load text file into display Ctrl+L
Save or Convert Sequence Format
Save a Subset of Sequences
Save sequences to networked computer
Save mult-set data in binary
Save Display Buffer (not sequences)
Concatenate sequences
De-concatenate sequences
Print Preview
Print
Test Ctrl+T
1. Z:\home\jpmatos\Documents\VertCOI.FAS
Exit

ch for research in genomics, molecular biology and evolution.

microbial genomics, phylogenetics and molecular evolution. *Journal*

sive software package for data analysis in molecular biology and
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io.uottawa.ca/software_download/LabManualDAMBE.pdf

ide and amino acid sequences
nces against aligned amino acid sequences

aneously estimated distances that make use of information from all
nly pairwise alignment for highly diverged sequences.

t with nucleotide-based and codon-based models
and conventional dating with internal node calibration)

exporting high-quality trees for publication
f gamma distribution and proportion of invariant sites
◊ Test substitution saturation
◊ Find best-fitting substitution models

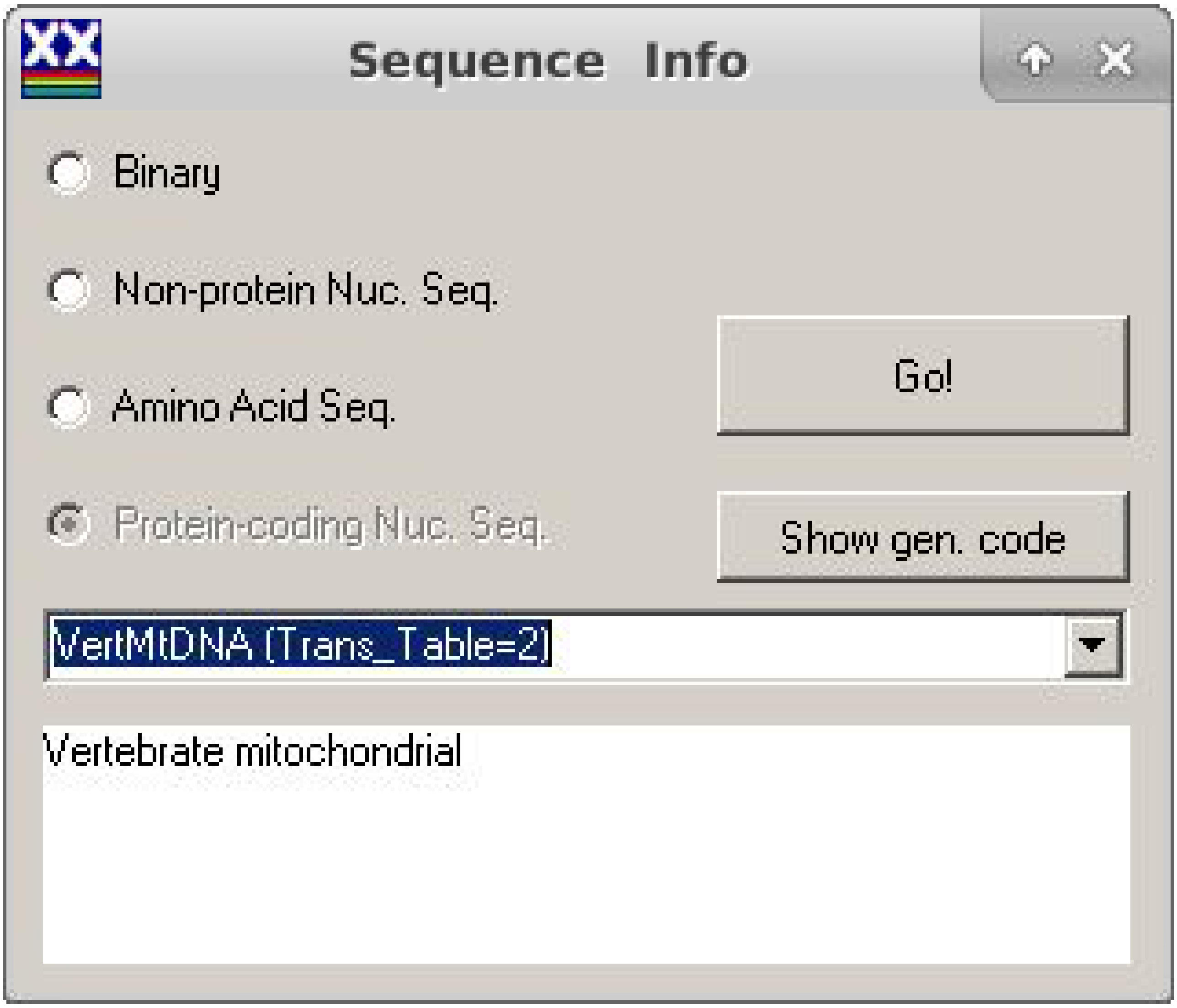
⇒ Genomics/Bioinformatics tools

- Position weight matrix for characterizing and predicting sequence motifs
- Perceptron for two-group classification of sequence motifs
- Gibbs sampler for characterizing and predicting novel/hidden sequence motifs
- Hidden Markov models for modelling protein secondary structure.
- Secondary structure prediction
- tRNA anticodon identification
- Genome-wide characterization of translation initiation, elongation and termination efficiencies
- Computing protein isoelectric point
- Peptide mass fingerprinting

⇒ Extensive implementation of a variety of sequence formats.

File: No file in buffer | Info | 000000

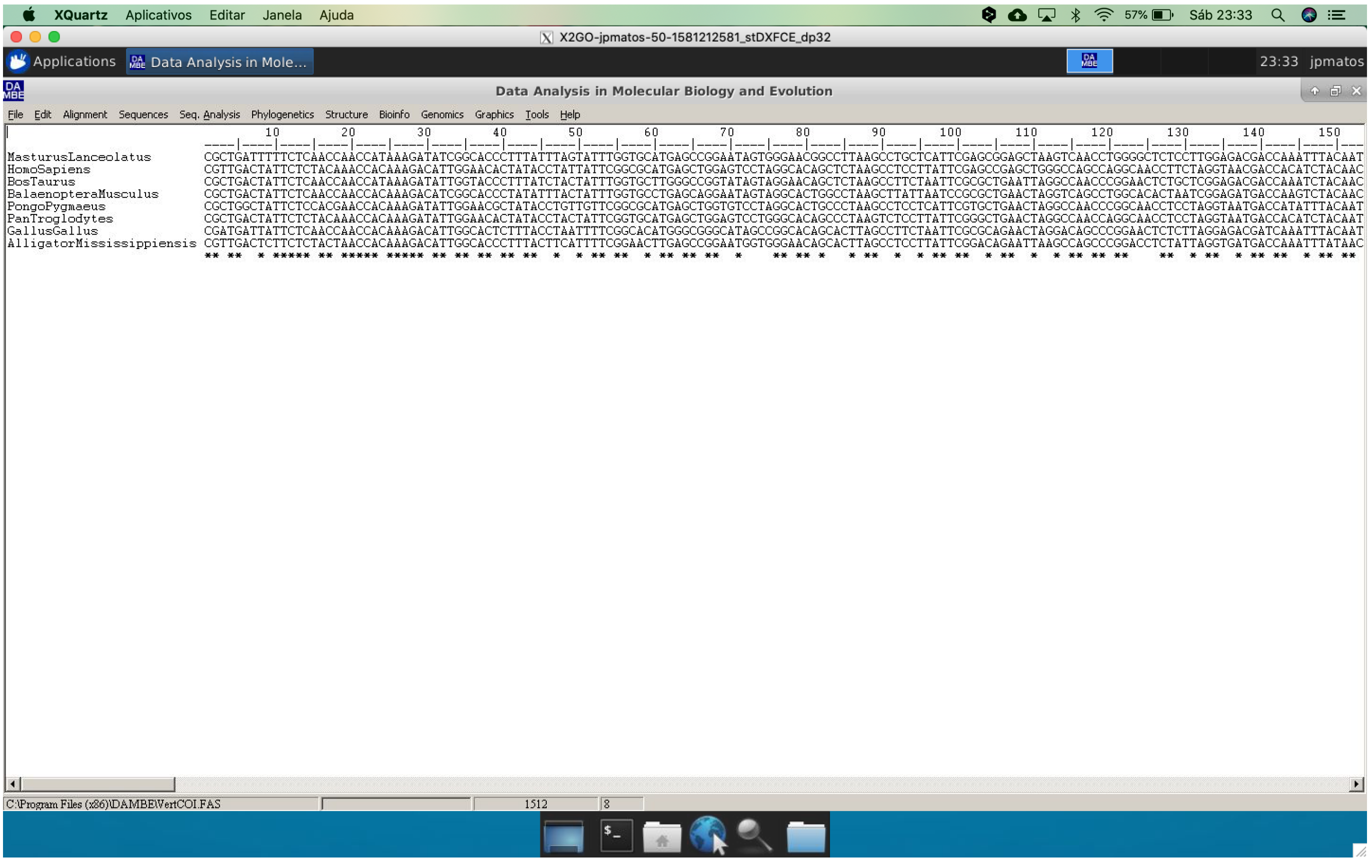
- Select the *dataset* VertCOI.fas. Click `Open`.
- In the dialog box that appears, called *SequenceInfo*, check the option *Protein-coding Nuc-Seq*. In this same window, in the Genetic Code options, choose the number 2 (from the Vertebrate mitochondria).



VertMtDNA (Trans_Table=2)

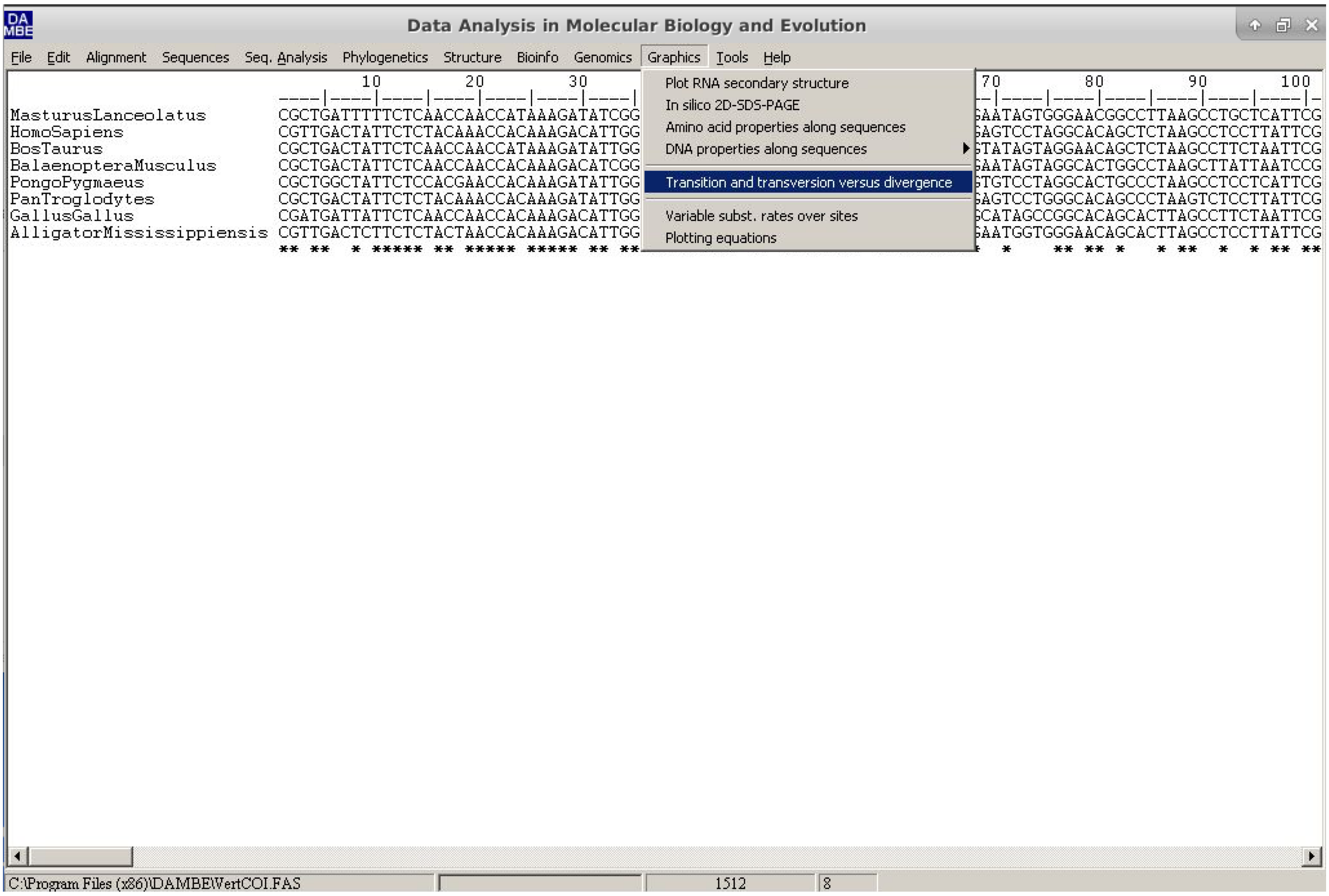
Vertebrate mitochondrial

- In the main window, the alignment will appear in Clustal format.



```
                                  10        20        30        40        50        60        70        80        90       100       110       120       130       140       150
                          ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---
MasturusLanceolatus       CGCTGATTTTTCTCAACCAACCATAAAGATATCGGCACCCTTTATTTAGTATTTGGTGCATGAGCCGGAATAGTGGGAACGGCCTTAAGCCTGCTCATTCGAGCGGAGCTAAGTCAACCTGGGGCTCTCCTTGGAGACGACCAAATTTACAAT
HomoSapiens               CGTTGACTATTCTCTACAAACCACAAAGACATTGGAACACTATACCTATTATTCGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGGGCCAGCCAGGCAACCTTCTAGGTAACGACCACATCTACAAC
BosTaurus                 CGCTGACTATTCTCAACCAACCATAAAGATATTGGTACCCTTTATCTACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAGCTCTAAGCCTTCTAATTCGCGCTGAATTAGGCCAACCCGGAACTCTGCTCGGAGACGACCAAATCTACAAC
BalaenopteraMusculus      CGCTGACTATTCTCAACCAACCACAAAGACATCGGCACCCTATATTTACTATTTGGTGCCTGAGCAGGAATAGTAGGCACTGGCCTAAGCTTATTAATCCGCGCTGAACTAGGTCAGCCTGGCACACTAATCGGAGATGACCAAGTCTACAAC
PongoPygmaeus             CGCTGGCTATTCTCCACGAACCACAAAGATATTGGAACGCTATACCTGTTGTTCGGCGCATGAGCTGGTGTCCTAGGCACTGCCCTAAGCCTCCTCATTCGTGCTGAACTAGGCCAACCCGGCAACCTCCTAGGTAATGACCATATTTACAAT
PanTroglodytes            CGCTGACTATTCTCTACAAACCACAAAGATATTGGAACACTATACCTACTATTCGGTGCATGAGCTGGAGTCCTGGGCACAGCCCTAAGTCTCCTTATTCGGGCTGAACTAGGCCAACCAGGCAACCTCCTAGGTAATGACCACATCTACAAT
GallusGallus              CGATGATTATTCTCAACCAACCACAAAGACATTGGCACTCTTTACCTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAGCACTTAGCCTTCTAATTCGCGCAGAACTAGGACAGCCCGGAACTCTCTTAGGAGACGATCAAATTTACAAT
AlligatorMississippiensis CGTTGACTCTTCTCTACTAACCACAAAGACATTGGCACCCTTTACTTCATTTTCGGAACTTGAGCCGGAATGGTGGGAACAGCACTTAGCCTCCTTATTCGGACAGAATTAAGCCAGCCCGGACCTCTATTAGGTGATGACCAAATTTATAAC
                          ** **  * ***** ** ***** ***** ** ** ** ** **  *  * ** **  * ** ** **  *    ** ** *   * **  *  * ** **  * **  *  * ** ** **    **  * **  * ** **  * ** **
```

On DAMBE's main menu, go to *Graphics* and click on ***transition and transversion versus divergence**.


Data Analysis in Molecular Biology and Evolution
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Structure
Bioinfo
Genomics
Graphics
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Help
Plot RNA secondary structure
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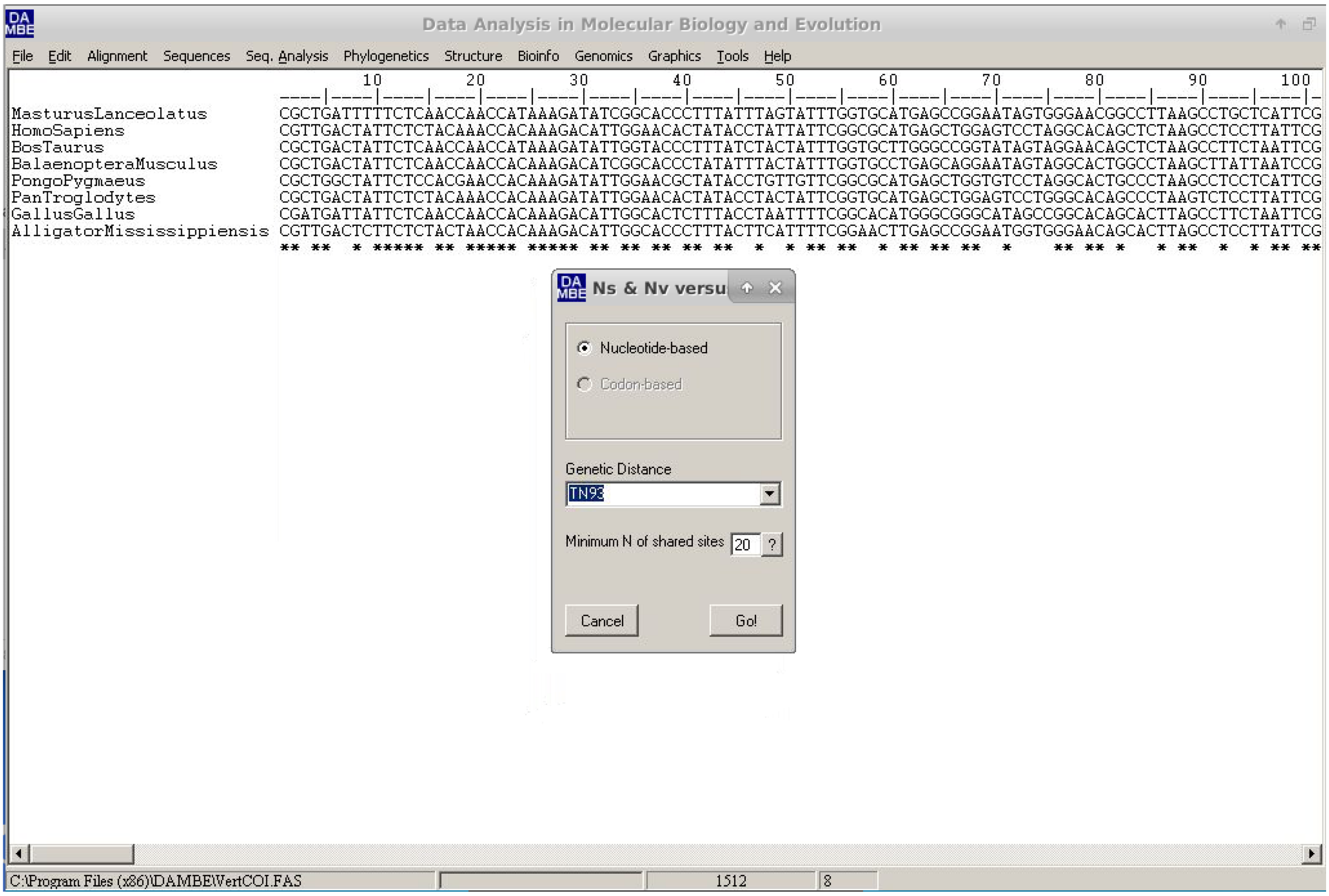
Amino acid properties along sequences
DNA properties along sequences
Transition and transversion versus divergence
Variable subst. rates over sites
Plotting equations

```
                                         10        20        30
                               ----|----|----|----|----|----|----|
MasturusLanceolatus            CGCTGATTTTTCTCAACCAACCATAAAGATATCGG
HomoSapiens                    CGTTGACTATTCTCTACAAACCACAAAGACATTGG
BosTaurus                      CGCTGACTATTCTCAACCAACCATAAAGATATTGG
BalaenopteraMusculus           CGCTGACTATTCTCAACCAACCACAAAGACATCGG
PongoPygmaeus                  CGCTGGCTATTCTCCACGAACCACAAAGATATTGG
PanTroglodytes                 CGCTGACTATTCTCTACAAACCACAAAGATATTGG
GallusGallus                   CGATGATTATTCTCAACCAACCACAAAGACATTGG
AlligatorMississippiensis      CGTTGACTCTTCTCTACTAACCACAAAGACATTGG
                               ** **  * ***** ** ***** ***** ** **
```

```
 70        80        90        100
--|----|----|----|----|----|----|-
GAATAGTGGGAACGGCCTTAAGCCTGCTCATTCG
GAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCG
GTATAGTAGGAACAGCTCTAAGCCTTCTAATTCG
GAATAGTAGGCACTGGCCTAAGCTTATTAATCCG
GTGTCCTAGGCACTGCCCTAAGCCTCCTCATTCG
GAGTCCTGGGCACAGCCCTAAGTCTCCTTATTCG
GCATAGCCGGCACAGCACTTAGCCTTCTAATTCG
GAATGGTGGGAACAGCACTTAGCCTCCTTATTCG
*  *     ** ** *    * **  *   * ** **
```

C:\Program Files (x86)\DAMBE\VertCOI.FAS | 1512 | 8

A new dialog box will appear. In it, the default values can be used. However, we will change the genetic distance (*Genetic distance* field) to TN93, as shown in the figure below:



```
                                      10        20        30        40        50        60        70        80        90       100
                              ----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-
MasturusLanceolatus           CGCTGATTTTTCTCAACCAACCATAAAGATATCGGCACCCTTTATTTAGTATTTGGTGCATGAGCCGGAATAGTGGGAACGGCCTTAAGCCTGCTCATTCG
HomoSapiens                   CGTTGACTATTCTCTACAAACCACAAAGACATTGGAACACTATACCTATTATTCGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCG
BosTaurus                     CGCTGACTATTCTCAACCAACCATAAAGATATTGGTACCCTTTATCTACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAGCTCTAAGCCTTCTAATTCG
BalaenopteraMusculus          CGCTGACTATTCTCAACCAACCACAAAGACATCGGCACCCTATATTTACTATTTGGTGCCTGAGCAGGAATAGTAGGCACTGGCCTAAGCTTATTAATCCG
PongoPygmaeus                 CGCTGGCTATTCTCCACGAACCACAAAGATATTGGAACGCTATACCTGTTGTTCGGCGCATGAGCTGGTGTCCTAGGCACTGCCCTAAGCCTCCTCATTCG
PanTroglodytes                CGCTGACTATTCTCTACAAACCACAAAGATATTGGAACACTATACCTACTATTCGGTGCATGAGCTGGAGTCCTGGGCACAGCCCTAAGTCTCCTTATTCG
GallusGallus                  CGATGATTATTCTCAACCAACCACAAAGACATTGGCACTCTTTACCTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAGCACTTAGCCTTCTAATTCG
AlligatorMississippiensis     CGTTGACTCTTCTCTACTAACCACAAAGACATTGGCACCCTTTACTTCATTTTCGGAACTTGAGCCGGAATGGTGGGAACAGCACTTAGCCTCCTTATTCG
                              ** **  * ***** ** ***** ***** ** ** ** ** **  *  * ** **  * ** ** **  *    ** ** *   * **  *  * ** **
```

Ns & Nv versu

● Nucleotide-based

○ Codon-based

Genetic Distance: TN93

Minimum N of shared sites: 20

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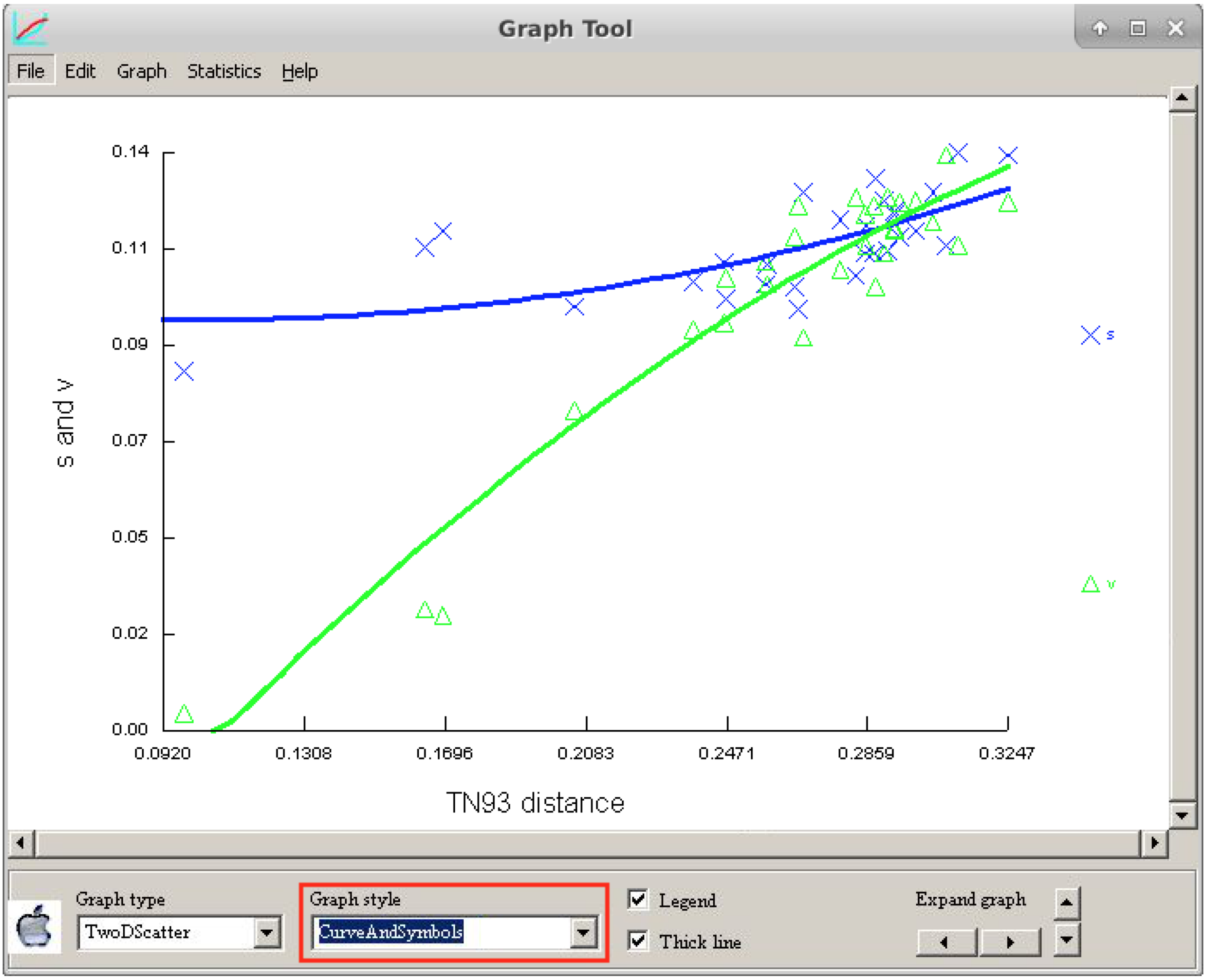
- Click on `Go` and watch the resulting graph. To make the visualization easier, leave the options as below:



Graph type: TwoDScatter

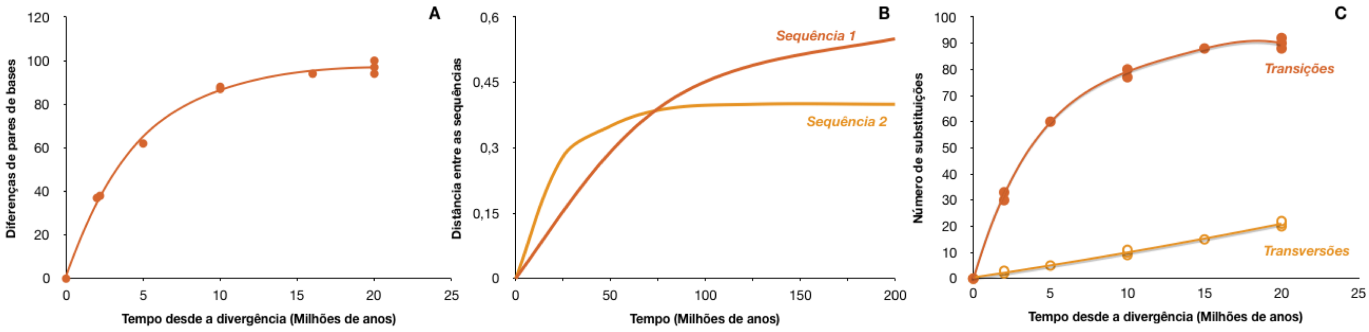
Graph style: CurveAndSymbols

☑ Legend

☑ Thick line

> The letter "s" refers to the transitions and the letter "v" to the transversions*.

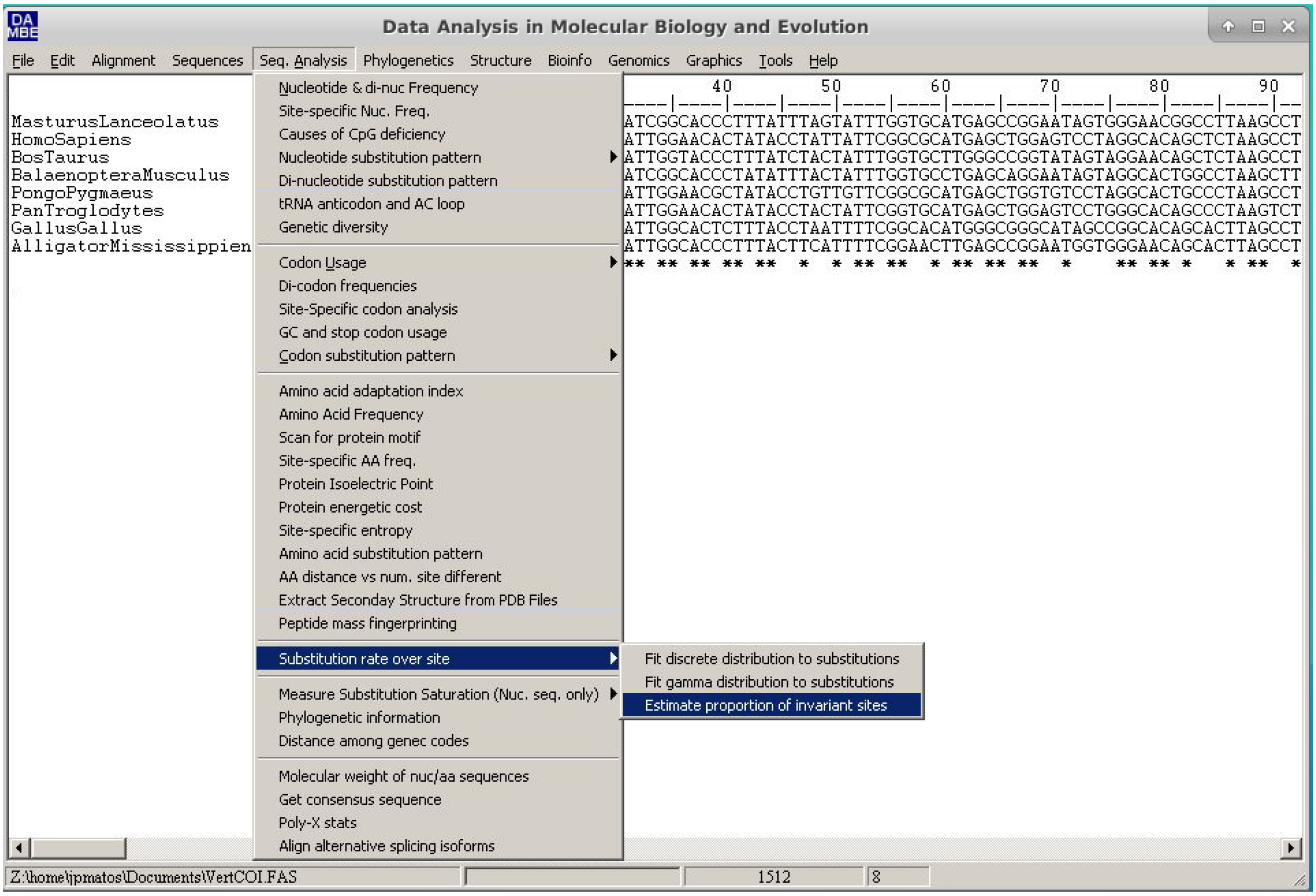
Compare the graph above with the figure below and draw your conclusions about the saturation of these sequences.



The quantitative method

The Xia method is a quantitative method, based on entropy, that assigns a significant value to the saturation of substitutions. It returns two criteria: Iss and Iss.c. The first is the substitution saturation index. The second is the critical substitution saturation index, which is the value of Iss at which sequences can no longer return the correct tree. When an observed Iss value is significantly lower than Iss.c (Iss < Iss.c), substitution saturation is not a problem for the *dataset* in question. The steps to perform this method are:

- Open the DAMBE program. Click on *File* and then on *Open Standard Sequence File*.
- Select VertCOI.fas dataset. Click on `Open`.
- In the dialog box that appears, called *SequenceInfo*, check the option *Protein-coding Nuc-Seq*. In this same window, in the Genetic Code options, choose the number 2 (from the Vertebrate mitochondria).
- In the main window, the alignment will appear in Clustal format. Before continuing, the proportion of invariable sites (Pinvar) should be calculated for this *dataset*. This parameter is crucial for sequences with very different substitution rates along with the sites.
- To do this, follow the path: *Seq.Analysis* > *Substitution rates over site* > *Estimate proportion of invariant sites*.


Data Analysis in Molecular Biology and Evolution
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Nucleotide & di-nuc Frequency
Site-specific Nuc. Freq.
Causes of CpG deficiency
Nucleotide substitution pattern
Di-nucleotide substitution pattern
tRNA anticodon and AC loop
Genetic diversity
Codon Usage
Di-codon frequencies
Site-Specific codon analysis
GC and stop codon usage
Codon substitution pattern
Amino acid adaptation index
Amino Acid Frequency
Scan for protein motif
Site-specific AA freq.
Protein Isoelectric Point
Protein energetic cost
Site-specific entropy
Amino acid substitution pattern
AA distance vs num. site different
Extract Seconday Structure from PDB Files
Peptide mass fingerprinting
Substitution rate over site
Measure Substitution Saturation (Nuc. seq. only)
Phylogenetic information
Distance among genec codes
Molecular weight of nuc/aa sequences
Get consensus sequence
Poly-X stats
Align alternative splicing isoforms
Fit discrete distribution to substitutions
Fit gamma distribution to substitutions
Estimate proportion of invariant sites
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```
                                  40        50        60        70        80        90
                            ----|----|----|----|----|----|----|----|----|----|----|----|--
MasturusLanceolatus         ATCGGCACCCTTTATTTAGTATTTGGTGCATGAGCCGGAATAGTGGGAACGGCCTTAAGCCT
HomoSapiens                 ATTGGAACACTATACCTATTATTCGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCT
BosTaurus                   ATTGGTACCCTTTATCTACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAGCTCTAAGCCT
BalaenopteraMusculus        ATCGGCACCCTATATTTACTATTTGGTGCCTGAGCAGGAATAGTAGGCACTGGCCTAAGCTT
PongoPygmaeus               ATTGGAACGCTATACCTGTTGTTCGGCGCATGAGCTGGTGTCCTAGGCACTGCCCTAAGCCT
PanTroglodytes              ATTGGAACACTATACCTACTATTCGGTGCATGAGCTGGAGTCCTGGGCACAGCCCTAAGTCT
GallusGallus                ATTGGCACTCTTTACCTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAGCACTTAGCCT
AlligatorMississippien      ATTGGCACCCTTTACTTCATTTTCGGAACTTGAGCCGGAATGGTGGGAACAGCACTTAGCCT
                            ** ** ** ** **  *  * ** **  * ** ** **  *    ** ** *   * **  *
```

- In the dialog box that follows, check the "*Use a new tree*" option. In the new window, choose the *Neighbor-Joining* method, choose the appropriate *outgroup* (for this example, we will use *Alligator mississippis*) and keep the rest of the options the way it is. Click on `Run` and then click on `Go`. At the end of the text output, Pinvar will be described, as below:

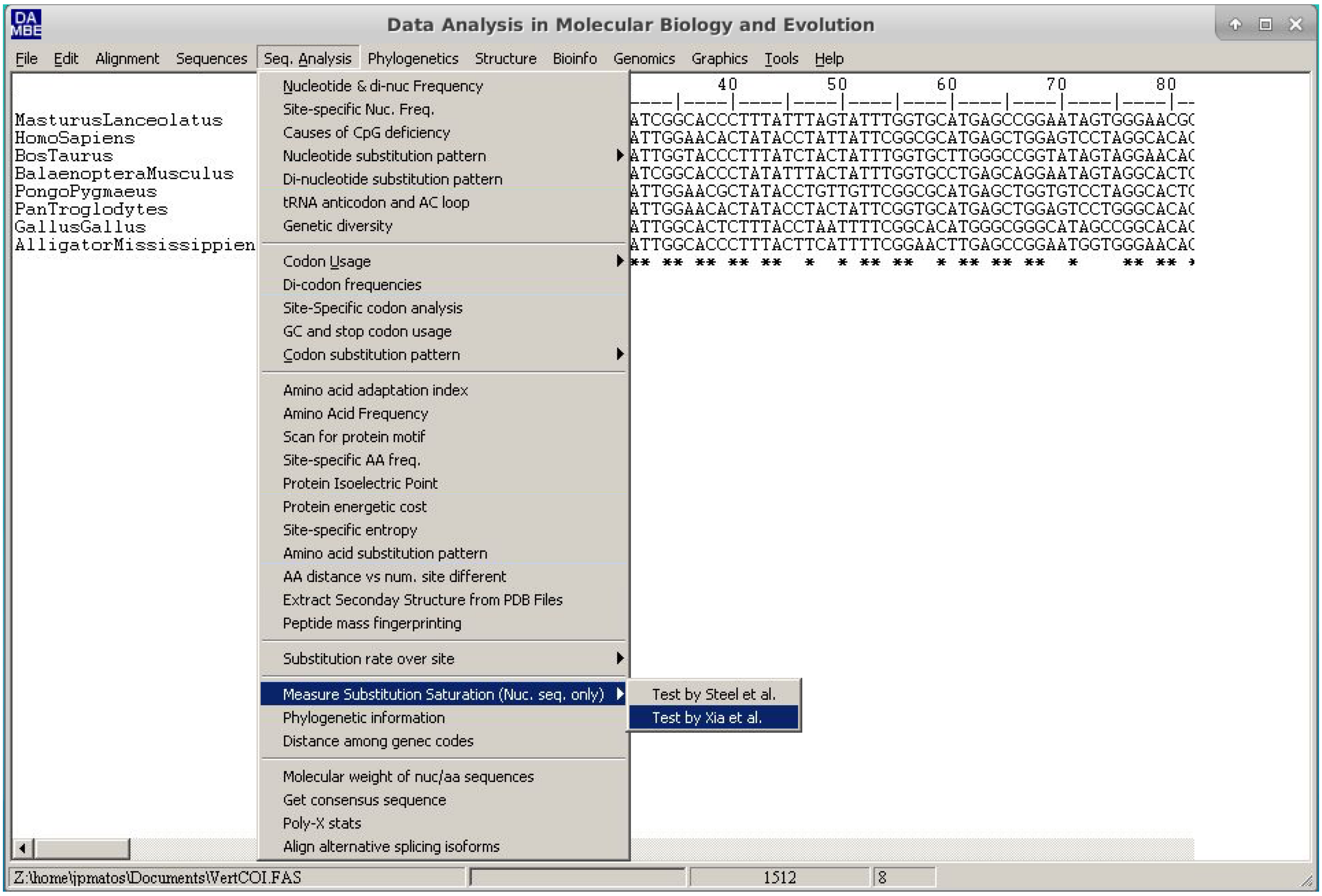
```
Estimation based on tree:
(AlligatorMississippiensis:0.13730,(MasturusLanceolatus:0.15730,
(((HomoSapiens:0.04522,PanTroglodytes:0.04795):0.02113,PongoPygmaeus:0.08982):0
.05970,
(BosTaurus:0.09378,BalaenopteraMusculus:0.10744):0.01982):0.01484):0.01673,Gall
usGallus:0.12297);

Estimating the proportion of invariant sites by iteration.
(Poisson+I)
 Iter       Phi      Pinv
-------------------------------------
    0   0.85377   0.28746
    1   0.85377   0.25871
    2   0.85377   0.31960
    3   0.73189   0.42154
    4   0.62860   0.47955
    5   0.57154   0.51389
    6   0.56361   0.52394
    7   0.56361   0.53330
    8   0.56361   0.52737
    9   0.56361   0.52462
   10   0.56361   0.52224
   11   0.56361   0.52258
   12   0.56361   0.52122
   13   0.56361   0.52089
   14   0.56361   0.51988
   15   0.56361   0.51954
   16   0.56361   0.51887
   17   0.56361   0.51821
   18   0.56361   0.51754
   19   0.56361   0.51688
   20   0.56361   0.51621
   21   0.56361   0.51555
-------------------------------------
P(invariant) = 0.51555
```

- Save or write down the Pinvar value (0.51555).

> Depending on your operating system's language settings, you will have to use decimal notation as a period and not as a comma. Pay close attention to this.*

Now let us estimate the saturation of substitutions (the program may present some problems, and so, in some cases, the sequence file should be opened again).

- Click on "*Seq.Analysis > Measure substitution saturation > Test by Xia et al.*".


Data Analysis in Molecular Biology and Evolution
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Site-specific Nuc. Freq.
Causes of CpG deficiency
Nucleotide substitution pattern
Di-nucleotide substitution pattern
tRNA anticodon and AC loop
Genetic diversity
Codon Usage
Di-codon frequencies
Site-Specific codon analysis
GC and stop codon usage
Codon substitution pattern
Amino acid adaptation index
Amino Acid Frequency
Scan for protein motif
Site-specific AA freq.
Protein Isoelectric Point
Protein energetic cost
Site-specific entropy
Amino acid substitution pattern
AA distance vs num. site different
Extract Seconday Structure from PDB Files
Peptide mass fingerprinting
Substitution rate over site
Measure Substitution Saturation (Nuc. seq. only)
Phylogenetic information
Distance among genec codes
Molecular weight of nuc/aa sequences
Get consensus sequence
Poly-X stats
Align alternative splicing isoforms
Test by Steel et al.
Test by Xia et al.
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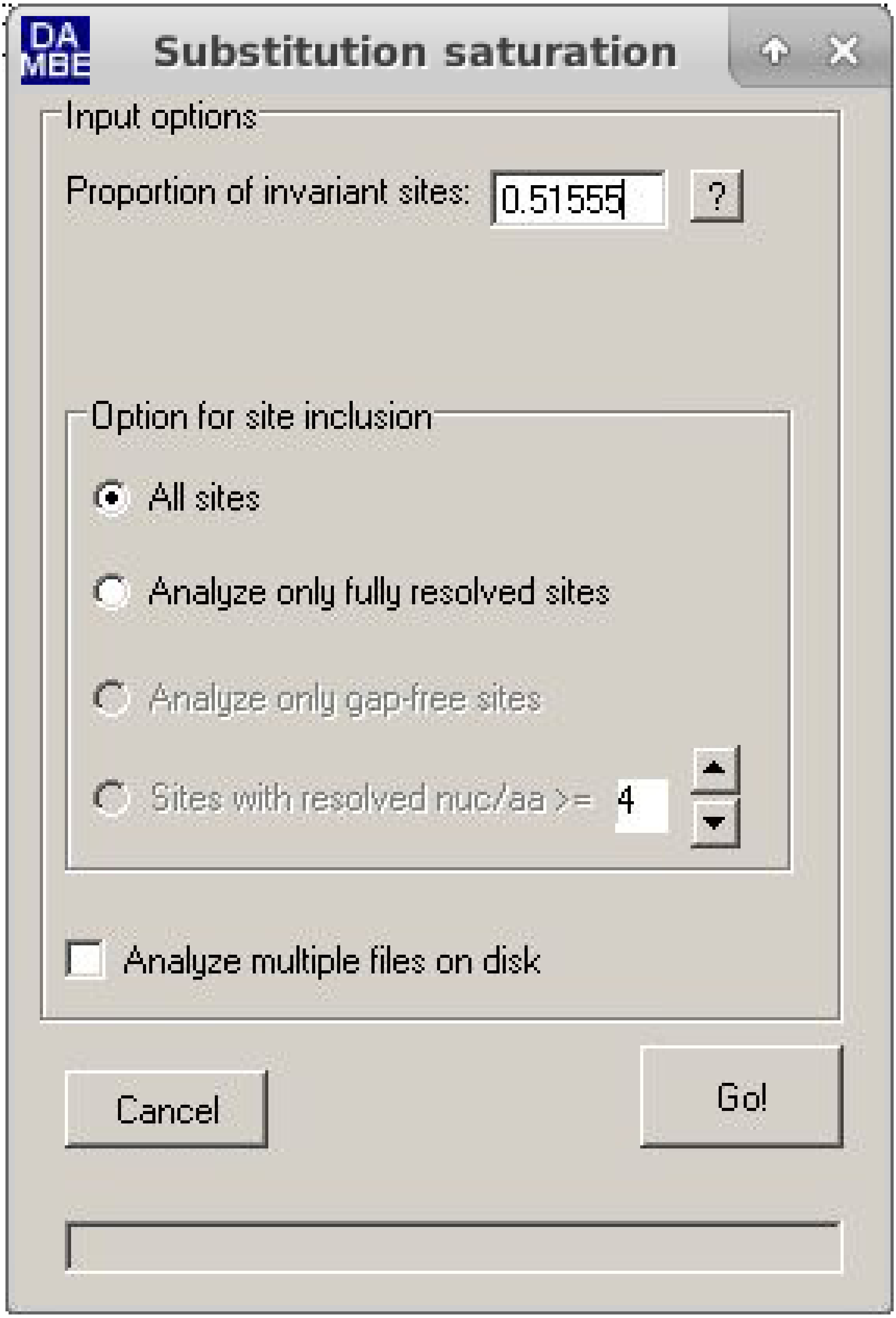
```
                                 40        50        60        70        80
                           ----|----|----|----|----|----|----|----|----|--
MasturusLanceolatus        ATCGGCACCCTTTATTTAGTATTTGGTGCATGAGCCGGAATAGTGGGAACGC
HomoSapiens                ATTGGAACACTATACCTATTATTCGGCGCATGAGCTGGAGTCCTAGGCACAC
BosTaurus                  ATTGGTACCCTTTATCTACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAC
BalaenopteraMusculus       ATCGGCACCCTATATTTACTATTTGGTGCCTGAGCAGGAATAGTAGGCACTC
PongoPygmaeus              ATTGGAACGCTATACCTGTTGTTCGGCGCATGAGCTGGTGTCCTAGGCACTC
PanTroglodytes             ATTGGAACACTATACCTACTATTCGGTGCATGAGCTGGAGTCCTGGGCACAC
GallusGallus               ATTGGCACTCTTTACCTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAC
AlligatorMississippien     ATTGGCACCCTTTACTTCATTTTCGGAACTTGAGCCGGAATGGTGGGAACAC
                           ** ** ** ** **  *  * ** **  * ** ** **  *    ** ** *
```

- In the window, just put the Pinvar value calculated and then click Go.



Substitution saturation

Input options

Proportion of invariant sites: 0.51555

Option for site inclusion

● All sites

○ Analyze only fully resolved sites

○ Analyze only gap-free sites

○ Sites with resolved nuc/aa >= 4

□ Analyze multiple files on disk

- Analyze the results (further comments will be made during the practice).

```
Test of substitution saturation (Xia et al. 2003; Xia and Lemey 2009)

Testing whether the observed Iss is significantly
lower than Iss.c.

Part I. For a symmetrical tree.
========================================================
Prop. invar. sites                0.5156
Mean H                            0.9205
Standard Error                    0.0183
Hmax                              1.6517
Iss                               0.5573
Iss.c                             0.8093
T                                13.7506
DF                              731
Prob (Two-tailed)                 0.0000
95% Lower Limit                   0.5214
95% Upper Limit                   0.5933

========================================================
Part II. For an extreme asymmetrical (and generally very
unlikely) tree.
========================================================
Iss.c                             0.7095
T                                 8.3050
DF                              731
Prob (Two-tailed)                 0.0000

95% Lower Limit                   0.5214
95% Upper Limit                   0.5933

========================================================

Interpretation of results:
               Significant Difference
               ----------------------
               Yes                 No
-------------------------------------------------------
Iss < Iss.c    Little             Substantial
               saturation         saturation
-------------------------------------------------------
Iss > Iss.c    Useless            Very poor
               sequences          for phylogenetics
-------------------------------------------------------

Please cite:

Xia, X., Z. Xie, M. Salemi, L. Chen, Y. Wang. 2003. An index of substitution
saturation and its application. Molecular Phylogenetics and Evolution 26:1-7.
```

```
Xia, X. and Lemey, P. 2009. Assessing substitution saturation with DAMBE. Pp.
615-630 in Philippe Lemey, Marco Salemi and Anne-Mieke Vandamme, eds. The
Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny. 2nd
edition Cambridge University Press.
```

It can be verified that both scenarios have Iss<Iss.c. Therefore these sequences do not present substitution saturation and can be used for phylogenetic analysis.

Influence of the position on the codon

Due to the genetic code characteristics, nucleotide sequences coding for proteins tends to show a higher number of changes in the 3rd position of the codon than in the 1st and 2nd position. Therefore, in these cases, the ideal is to perform both methods for all codon positions (this option will be shown during practice). The table below shows all Pinvar, Iss, and Iss.c data for all codon positions.

Positions	Pinvar	*Iss*	*Iss.c*	P
All	0.51555	0.5573	0.8093	0.0000
1st position	0.64014	0.3814	0.7519	0.0000
2nd position	0.42518	0.0655	0.7519	0.0000
3rd position	0.02485	0.7340	0.7519	0.3540
1st e 2nd positions	0.37664	0.1391	0.7886	0.0000

Final Remarks

You should always perform the analysis of substitution saturation whenever working with a new or not widely known marker. If you are using genes or proteins already known and commonly used in the literature studies, these estimates, although still recommended, are not indispensable. However, you can still perform these tests to confirm if the marker is suitable to the taxonomic level/rank that you are working on. Sometimes qualitative analysis is sufficient, but you can (and should) use the quantitative method if the graph presents a problematic interpretation.sudo apt install ttf-mscorefonts-installer -y