Molecular markers in plant systematics and population biology

7. DNA sequencing (cpDNA)

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DNA sequencing

- detection the order of nucleotides in a DNA strand

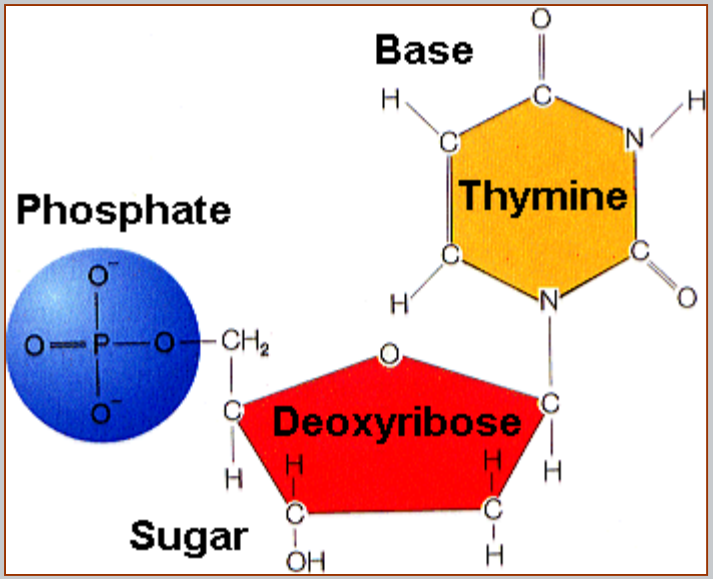
  …ATATATAGGCAAGGAATCTCTATTATTAAATCATT…

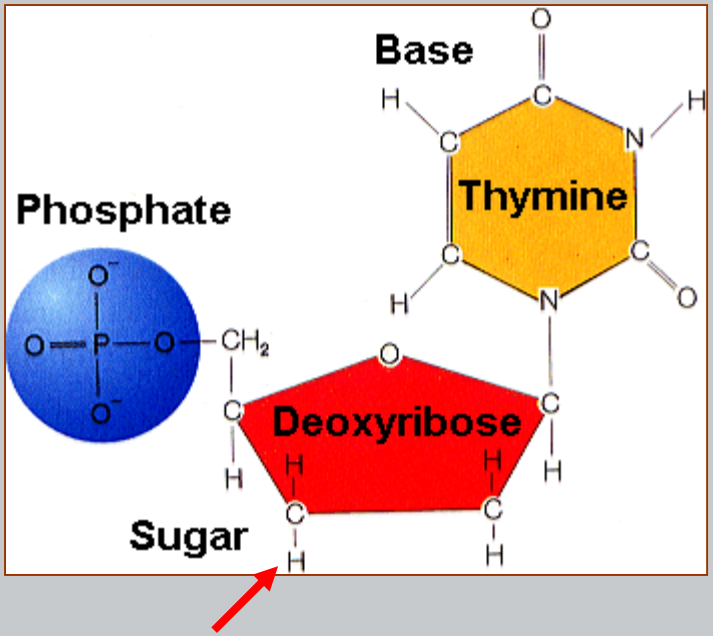
- use the information to model evolutionary and population genetic processes
- make hypothesis about similarity and relationships among taxa

Sequencing principle

- PCR with a primer pair
  - amplification of the target region
- cycle sequencing (dideoxy, Sanger)
  - use of one primer only
  - dNTP as well as ddNTP are present in the mixture
  - produce fragments differing exactly by one base
- electrophoretic separation of fragments in the gel
  - automated sequencer

2´, 3´- dideoxy NTPs





dTTP

ddTTP

3´-CTGGACTGCA-5´
5´-GACCT

Cycle sequencing

3´-TACG-5´ primer

5´-ATGCATGC-3´ template

ddGTP

GTACG
ATGCATGC

ddCTP

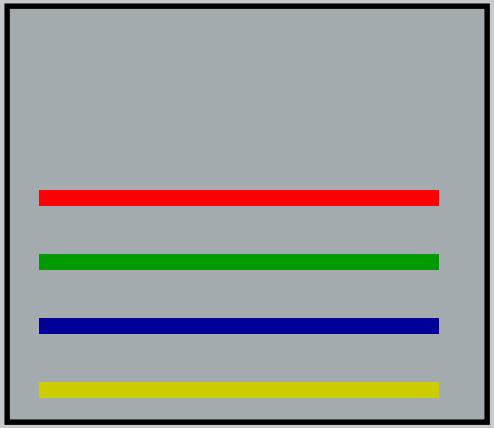
CGTACG
ATGCATGC

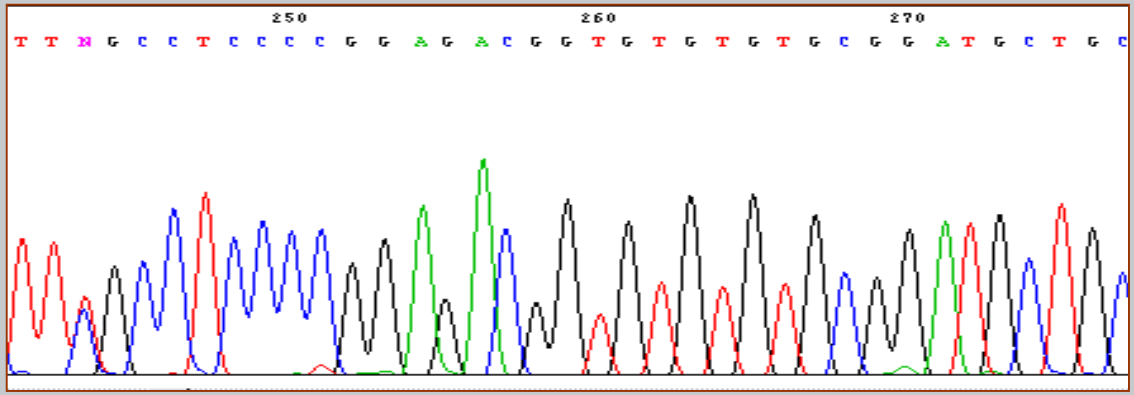
ddATP

ACGTACG
ATGCATGC

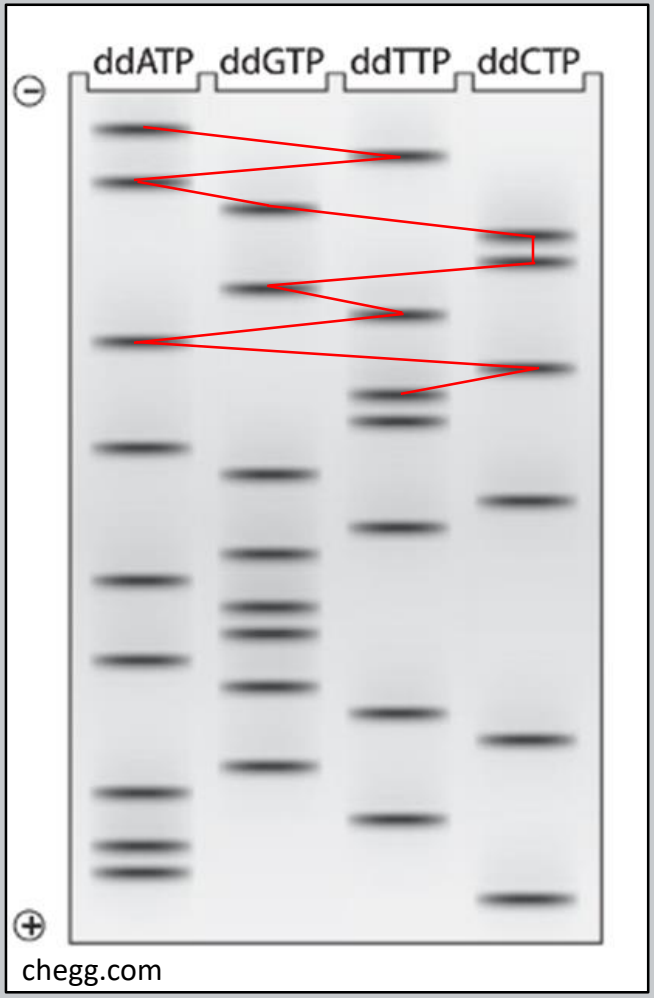
ddTTP

TACGTACG
ATGCATGC

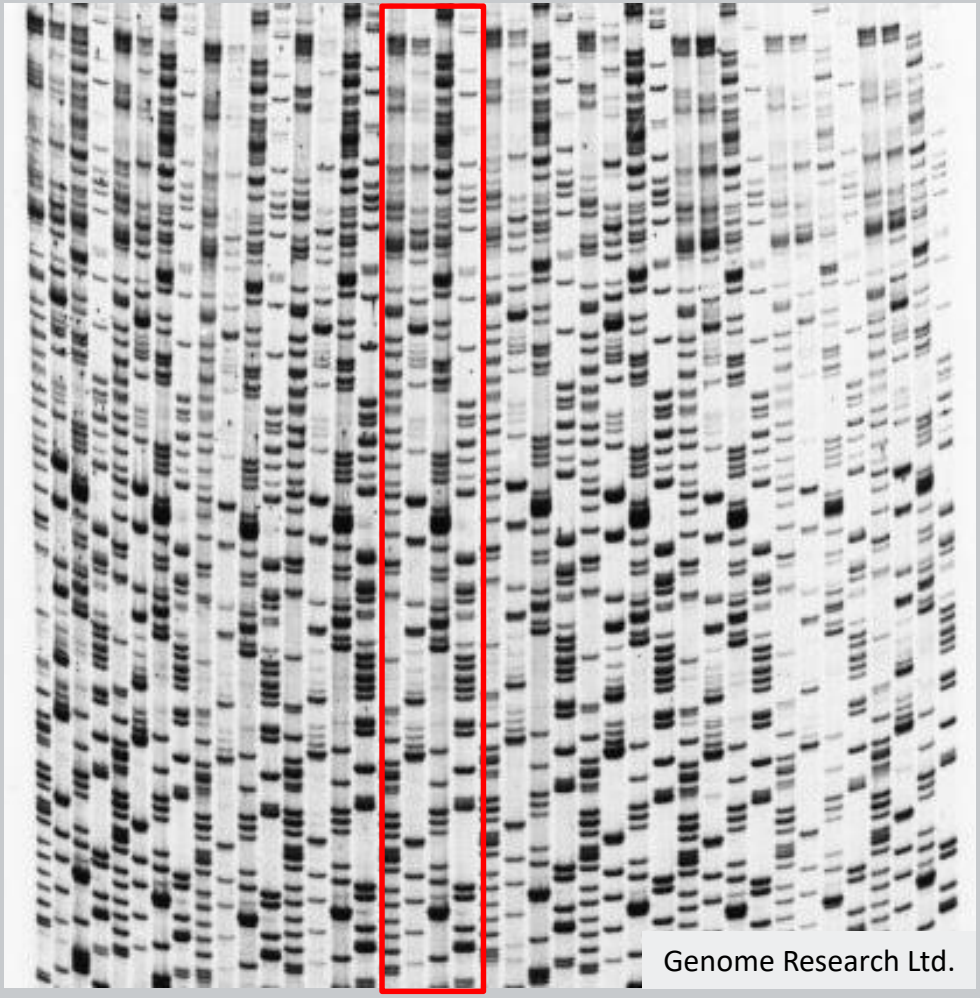




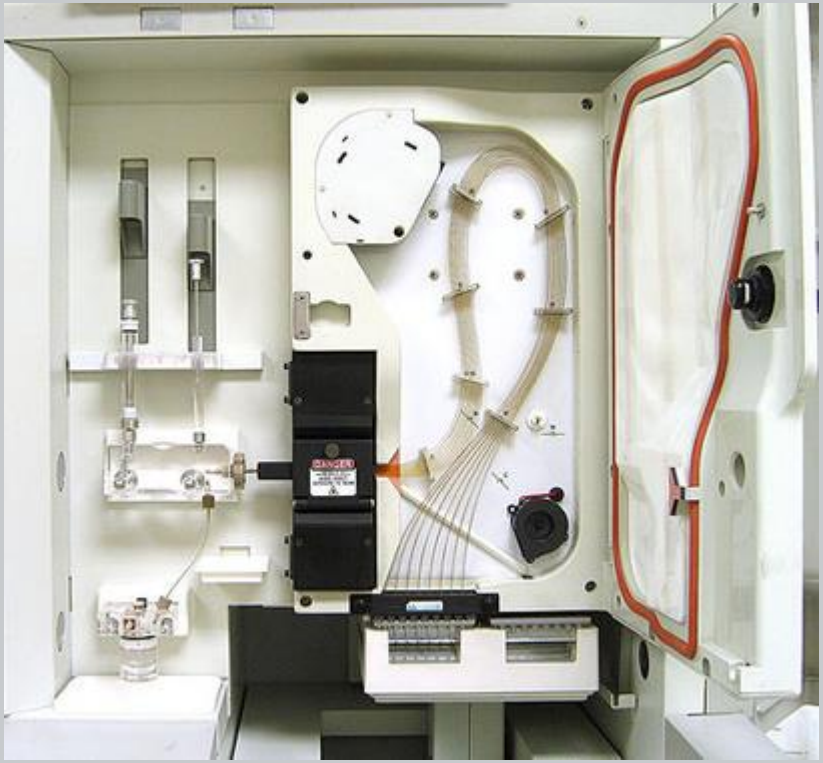
Classical sequencing gel



chegg.com

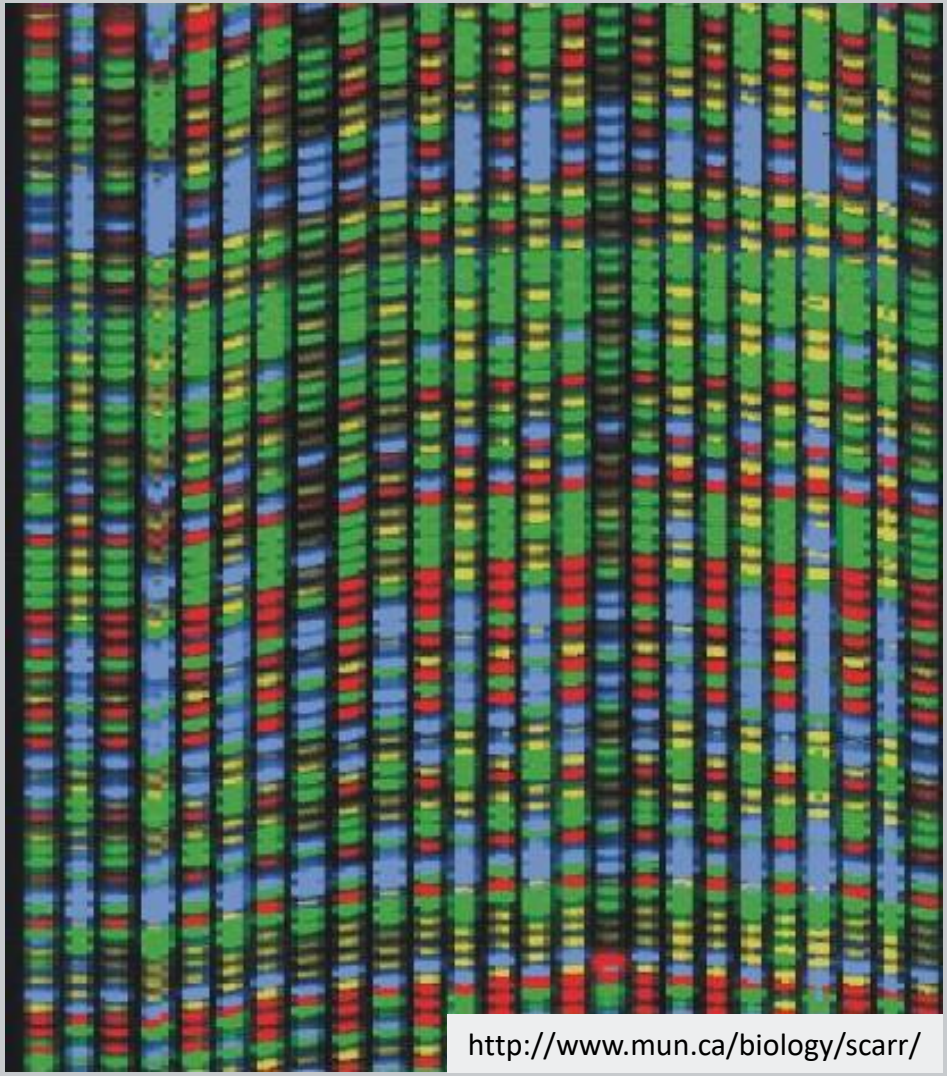

Genome Research Ltd.

Automated sequencer



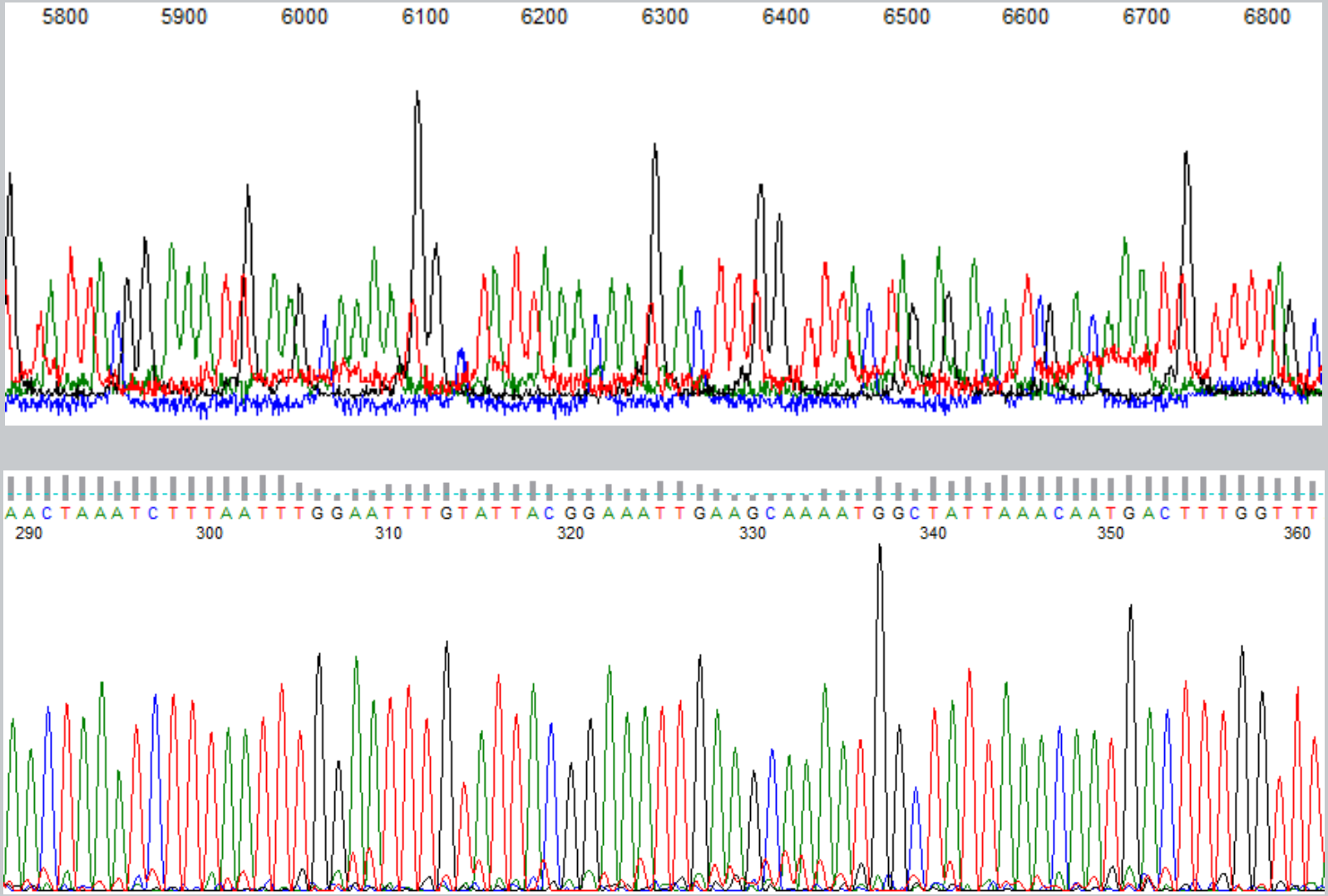
ABI (Applied Biosystems) – slab gel & capillary systems (up to 96)

Gel sequencer raw data



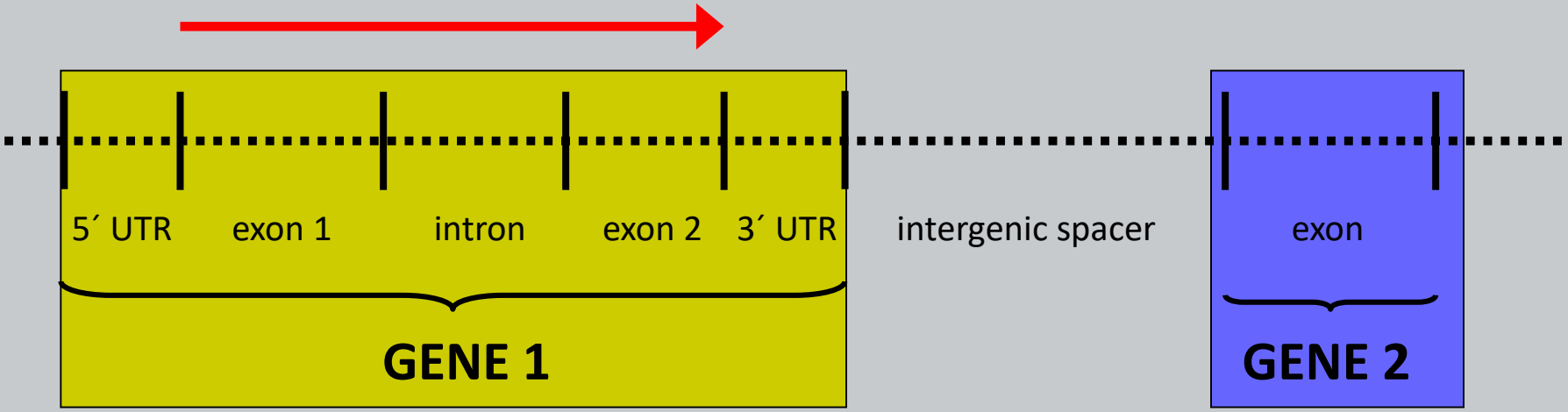
http://www.mun.ca/biology/scarr/

Capillary sequencer raw data



Genome structure

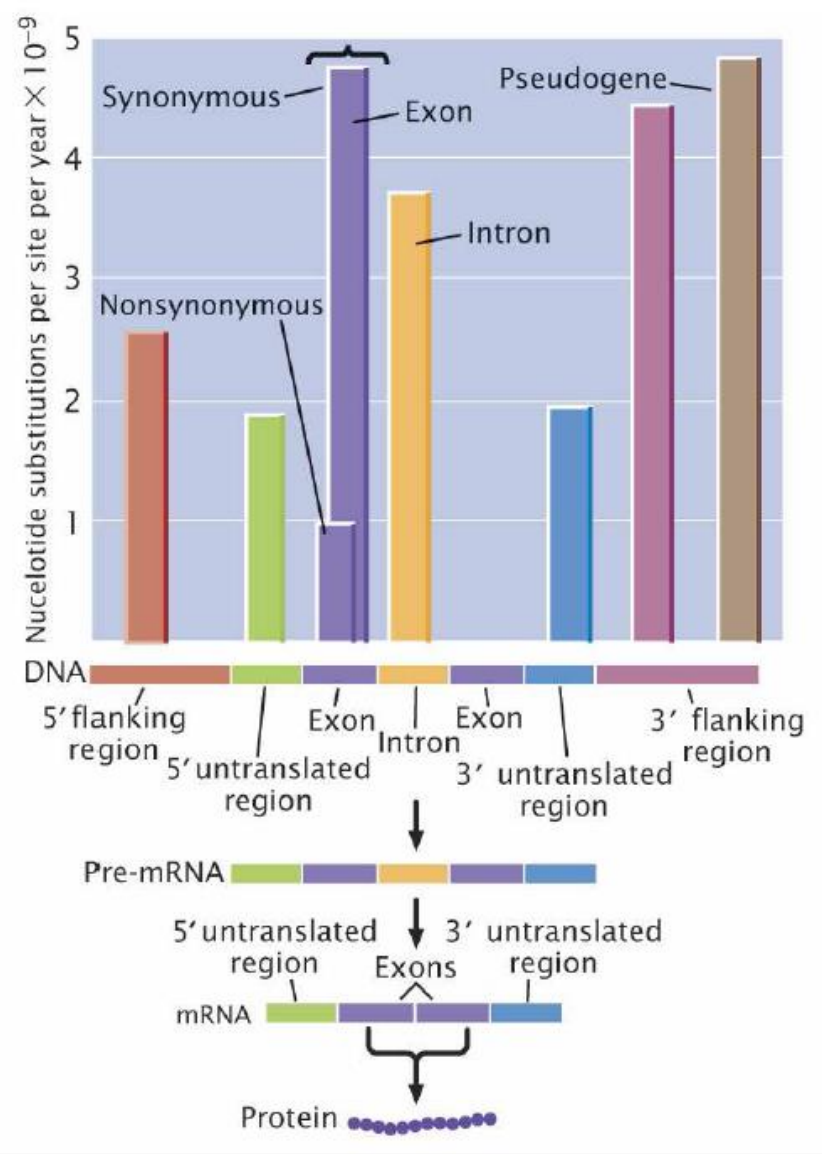
- genetic information – order of nucleotides (ACGT)



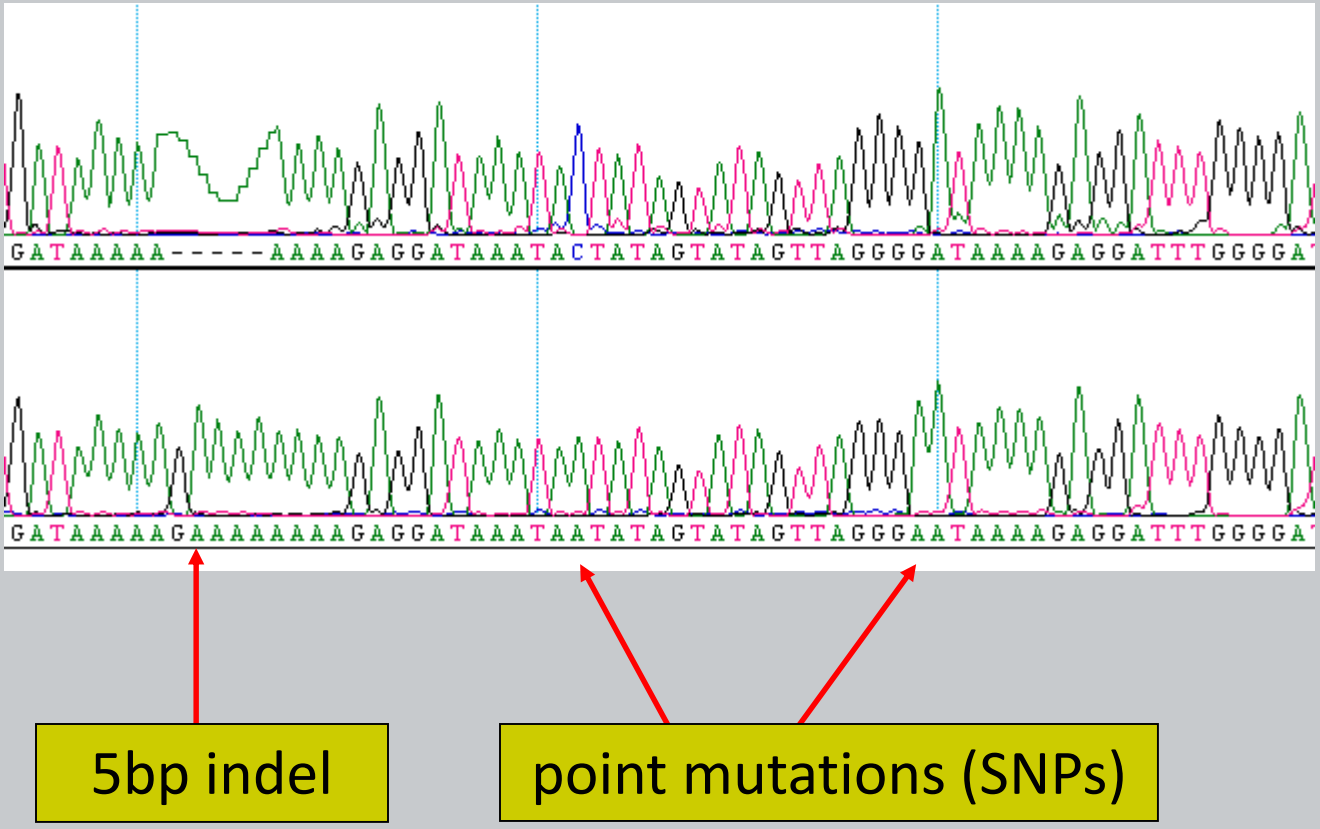
- coding regions – exons – c*onserved*
- non-coding regions – introns, spacers – *variable*
- UTR – untranslated regions

- nuclear, chloroplast and mitochondrial genome

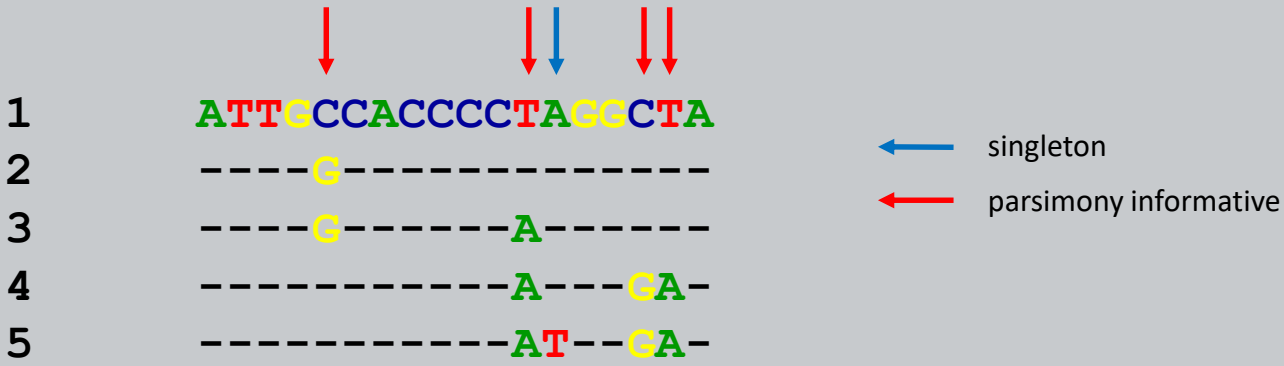
Sequence evolutionary rate



Types of variability in DNA sequences

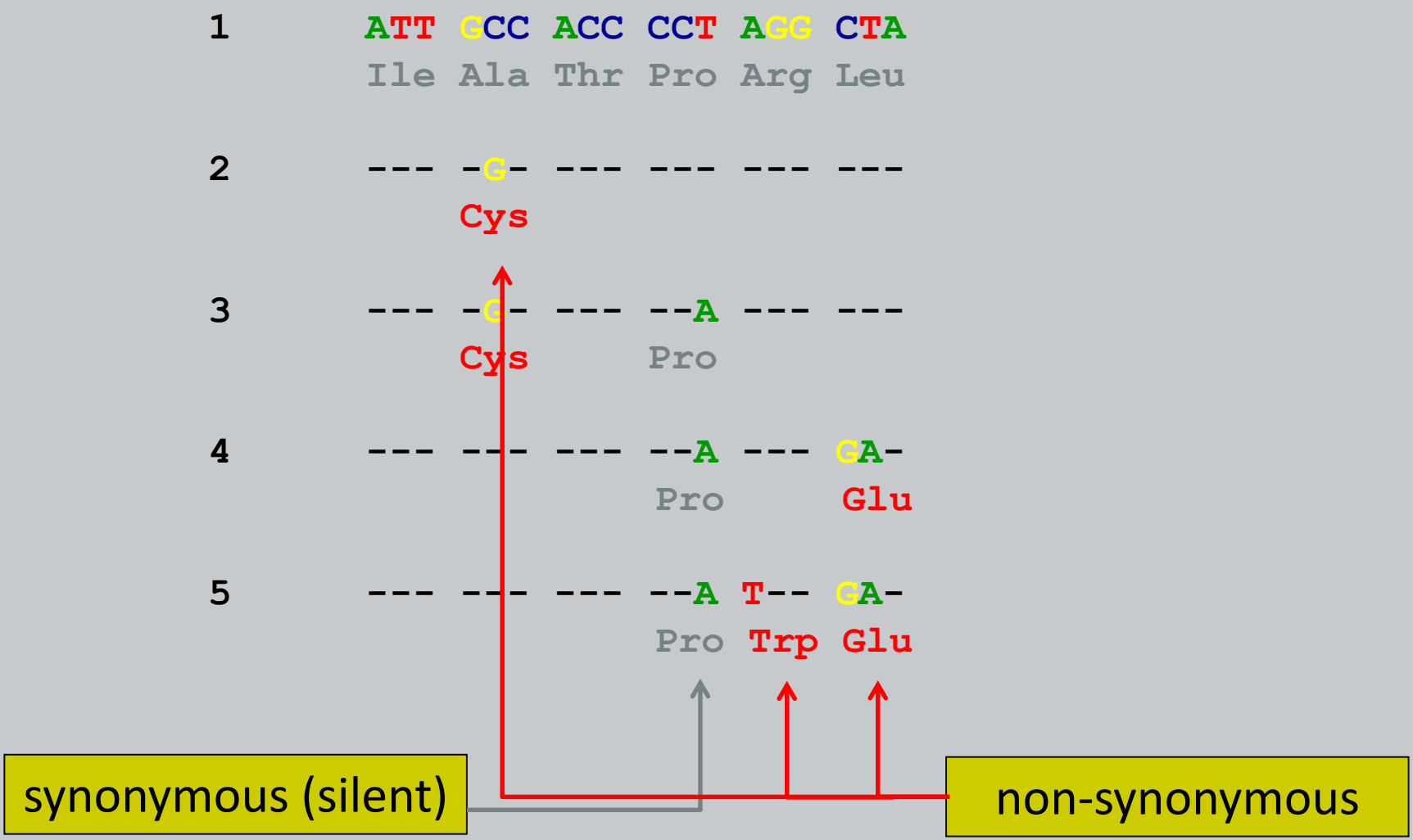


Descriptive statistics for DNA sequences



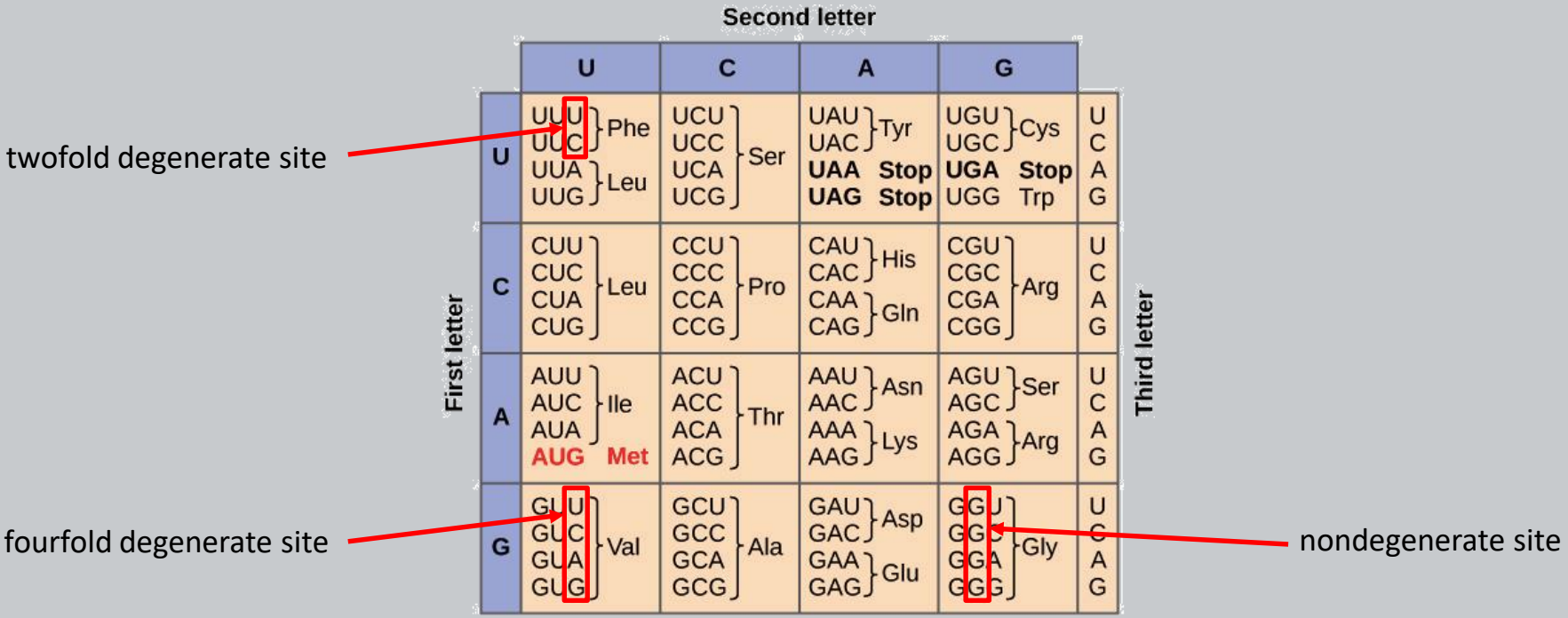
L	sequence length (nr. sites)	18
Π	average number of nucleotide difference	2.8
S	number of segregating (polymorphic) sites	5
π	nucleotide diversity (Π per site)	0.155
s	number of segregating sites per site	0.277

Synonymous vs non-synonymous mutations



Degenaration of the genetic code

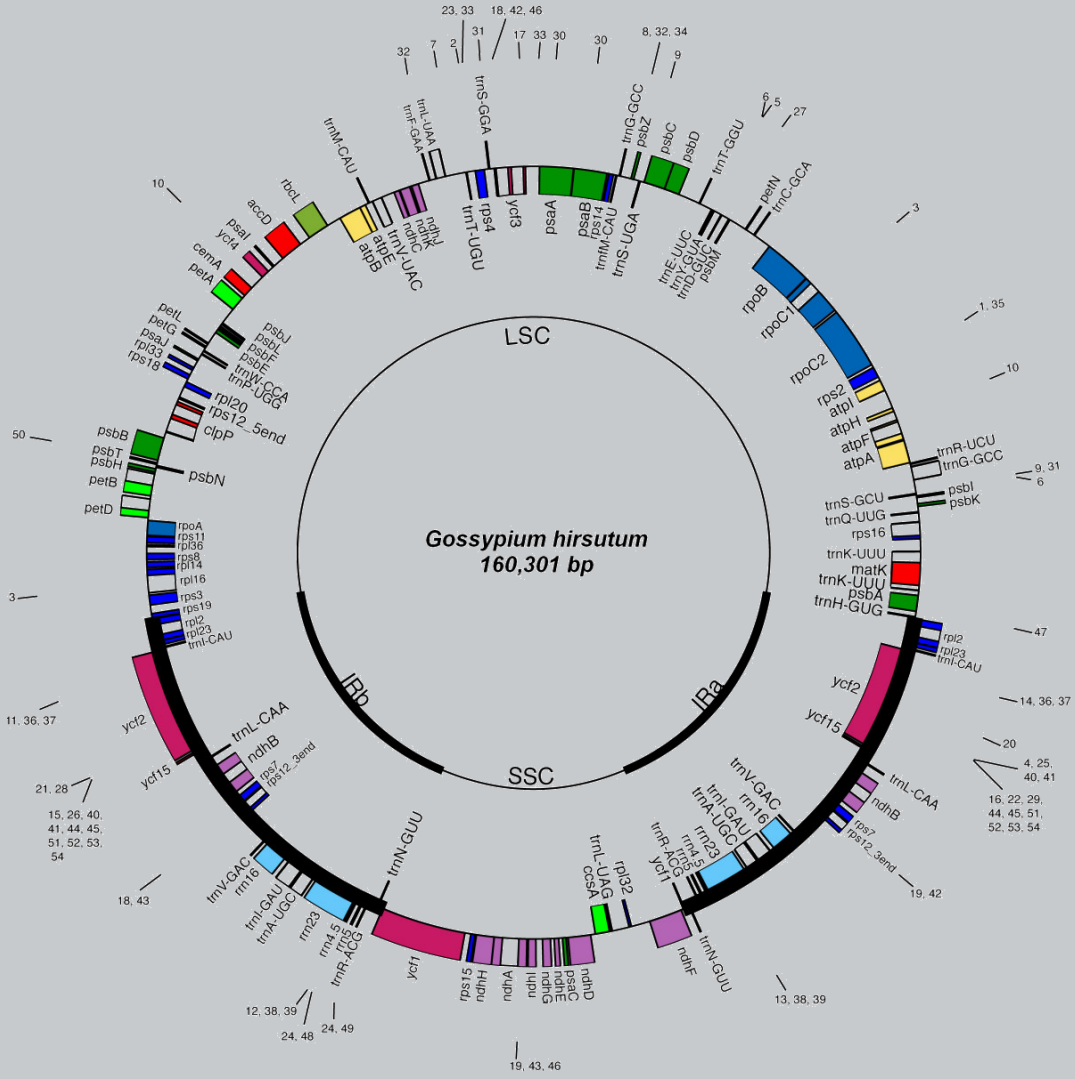
- multiple codons are coding the same aminoacid
  - fourfold degenerate site – any change do not change the aminoacid
  - nondegenerate site – any change also change the aminoacid



Chloroplast genome

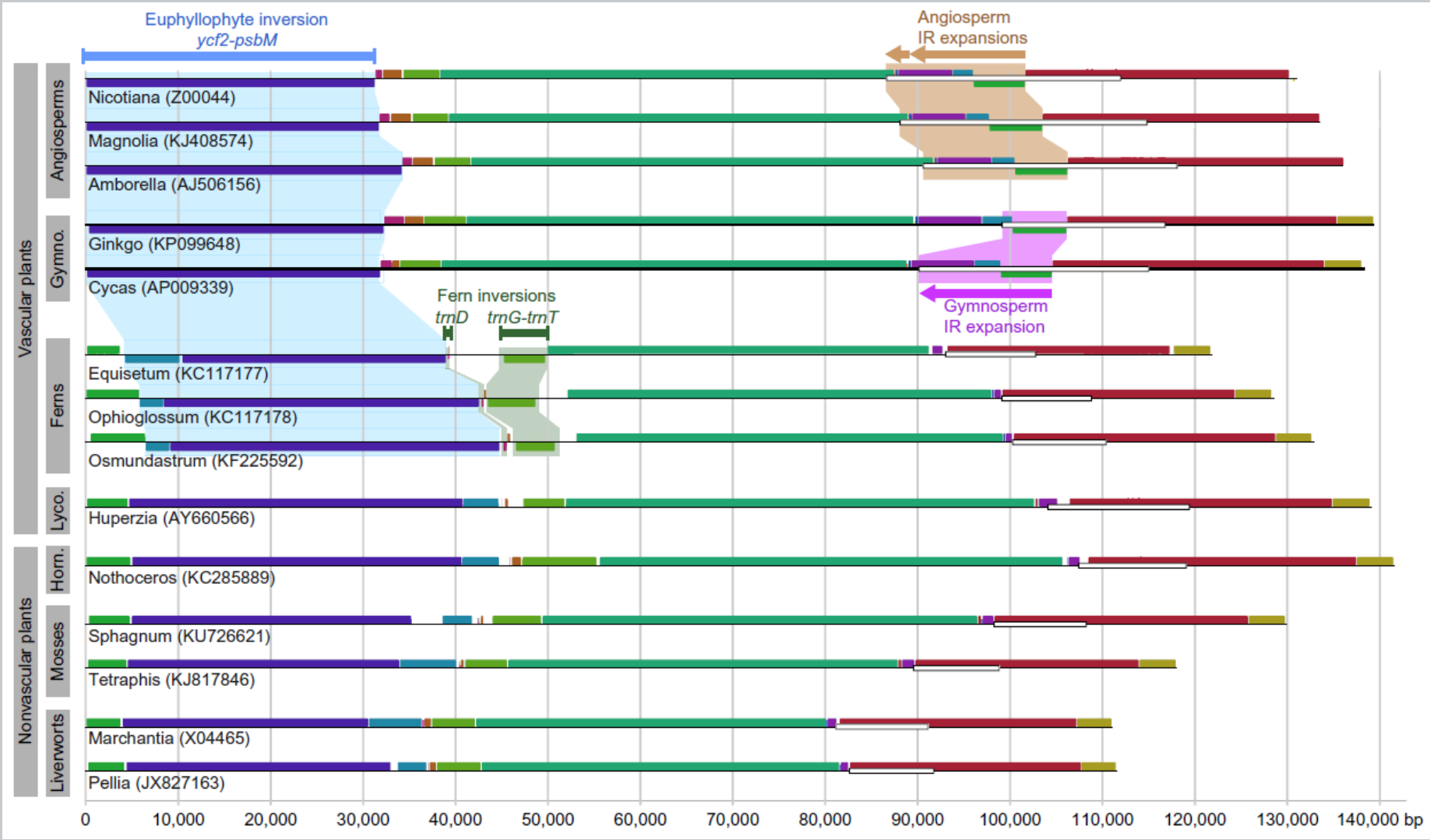
- many genes are *single-copy* (only 1 copy in the whole genome)
- conserved evolution of the chloroplast genome
  - disadvantage when studying intraspecific or population variability
  - many conserved regions can be used as *priming sites*
- structural rearrangements of chloroplast genome
  - mainly on larger evolotionary scale
  - inversion – e.g., 30kb inversion differentiates bryophytes and higher plants
  - extensive deletions
  - loss of specific genes and introns
- *chloroplast capture*
  - chloroplast transfer from one species to another by introgression
  - can influence phylogeny in a wrong way (when not recognized)

Chloroplast genome



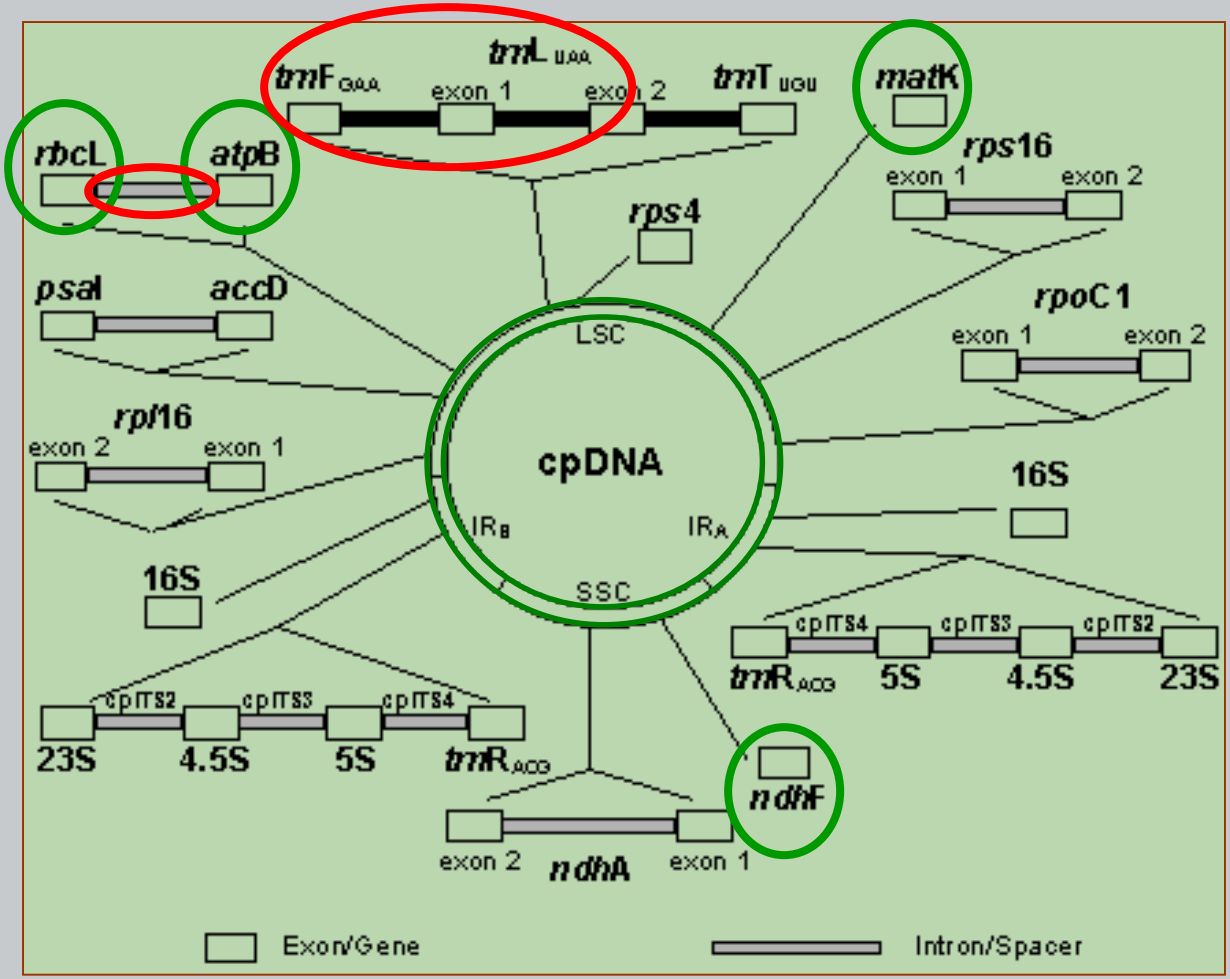
- 4 rRNAs
- 30-11 tRNAs
- 21 ribosomal proteins (*rps*)
- 4 RNA polymerase subunits (*rpo*)
- 28 thylakoid proteins (*ps*)
- *rbc*L (large RuBisCO subunit)
- 11 proteins similar to NADH (*ndh*)

Chloroplast genome



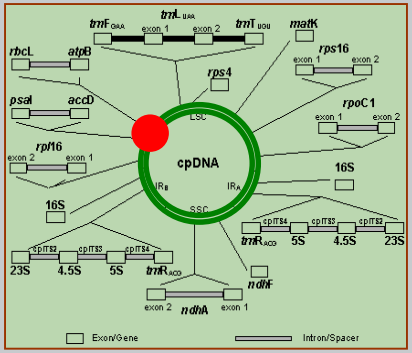
Genome alignment highlighting diagnostic changes among land plant plastomes. Coloured boxes – genome homology segments, horizontal white box – a copy of IR.

Frequently sequenced cpDNA regions



+ many others…

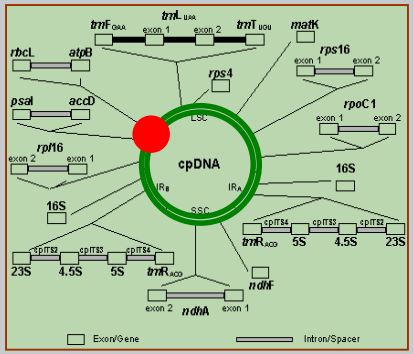
*rbc*L



- gene for large subunit of ribuloso-1,5-bisphosphate-carboxylase/oxygenase (RUBISCO)
- 1,428, 1,431 or 1,434 bp in length – indels are extremely rare
- one of the first sequenced genes
- very conserved, systematics at family or generic level, in some groups at species level

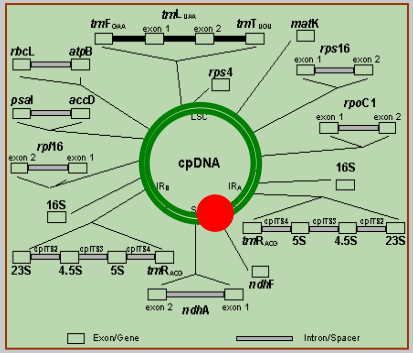
*atp*B

- gene coding beta subunit of ATP synthase
- 1,497 bp in length, indels not found
- similar use as *rbc*L

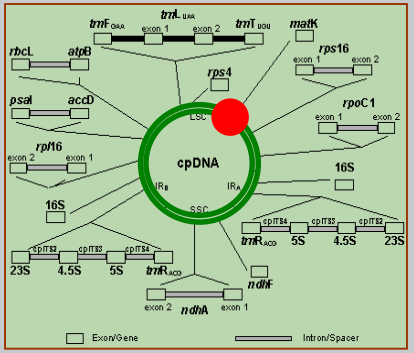


*ndh*F

- codes a subunit of chloroplast NADH-dehydrogenase
- 2,233 bp in length (tobacco)
- about 2x more substitutions then *rbc*L
- for generic level

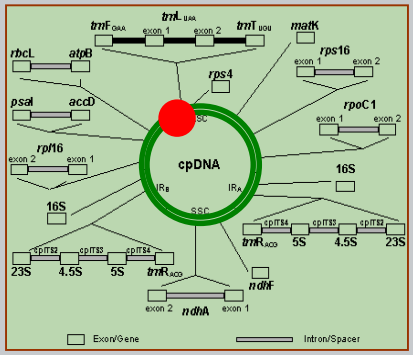


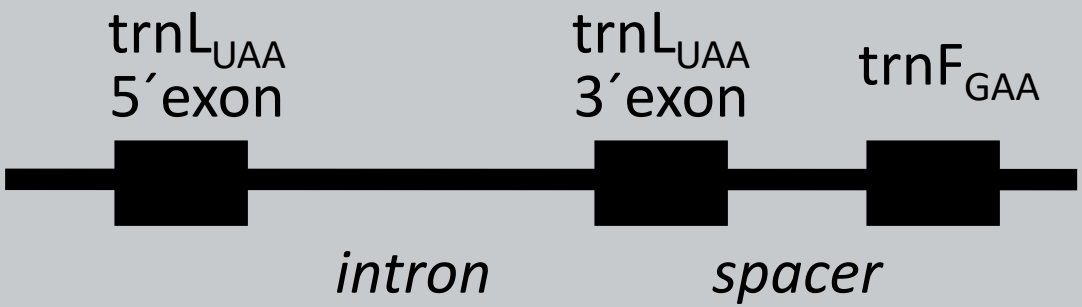
*mat*K



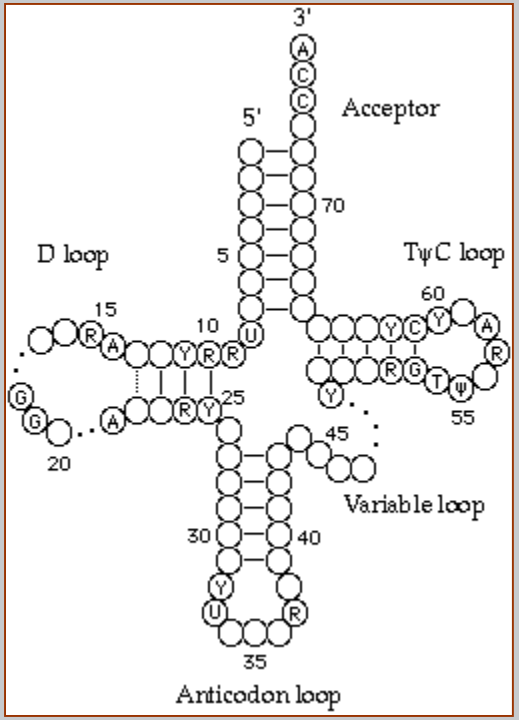
- gene coding maturase (splicing of plastid genes)
- about 1,550 bp in length – low number of indels
- systematics at family and generic level

*trn*L intron and spacer between *trn*L and *trn*F

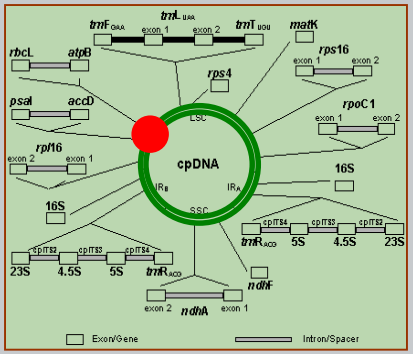




- tRNA genes – secondary structure
- accumulation of insertions/deletions with the same rate as nucleotide substitutions
- alignment problems, especially in distant organisms (sometimes already at family level)
- suitable for systematics of (closely) related species



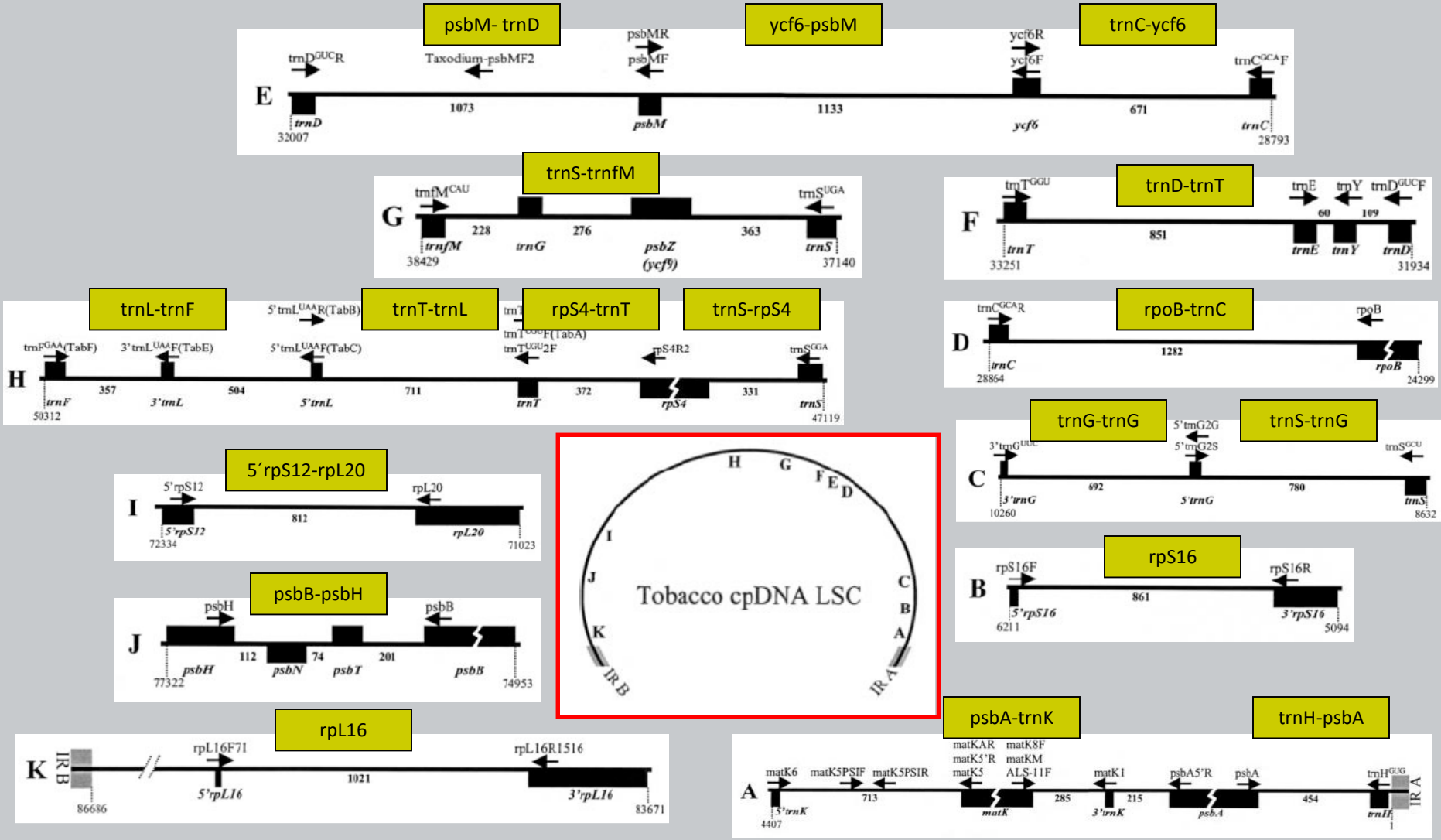
*atp*B-*rbc*L



- spacer of about 900-1,000 bp in length
- systematics at family and generic level

Variable non-coding cpDNA regions

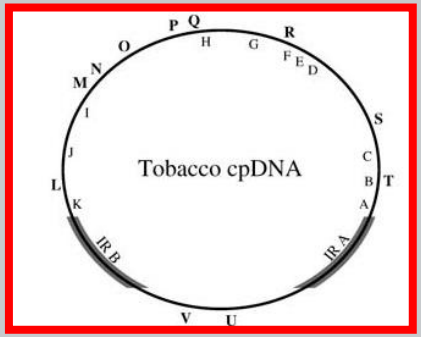
Shaw et al. (2005): The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *Am. J. Bot.* **92**: 142-166



Variable non-coding cpDNA regions

Shaw et al. (2007): Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *Am. J. Bot.* **94**: 275–288.

- another 13 regions



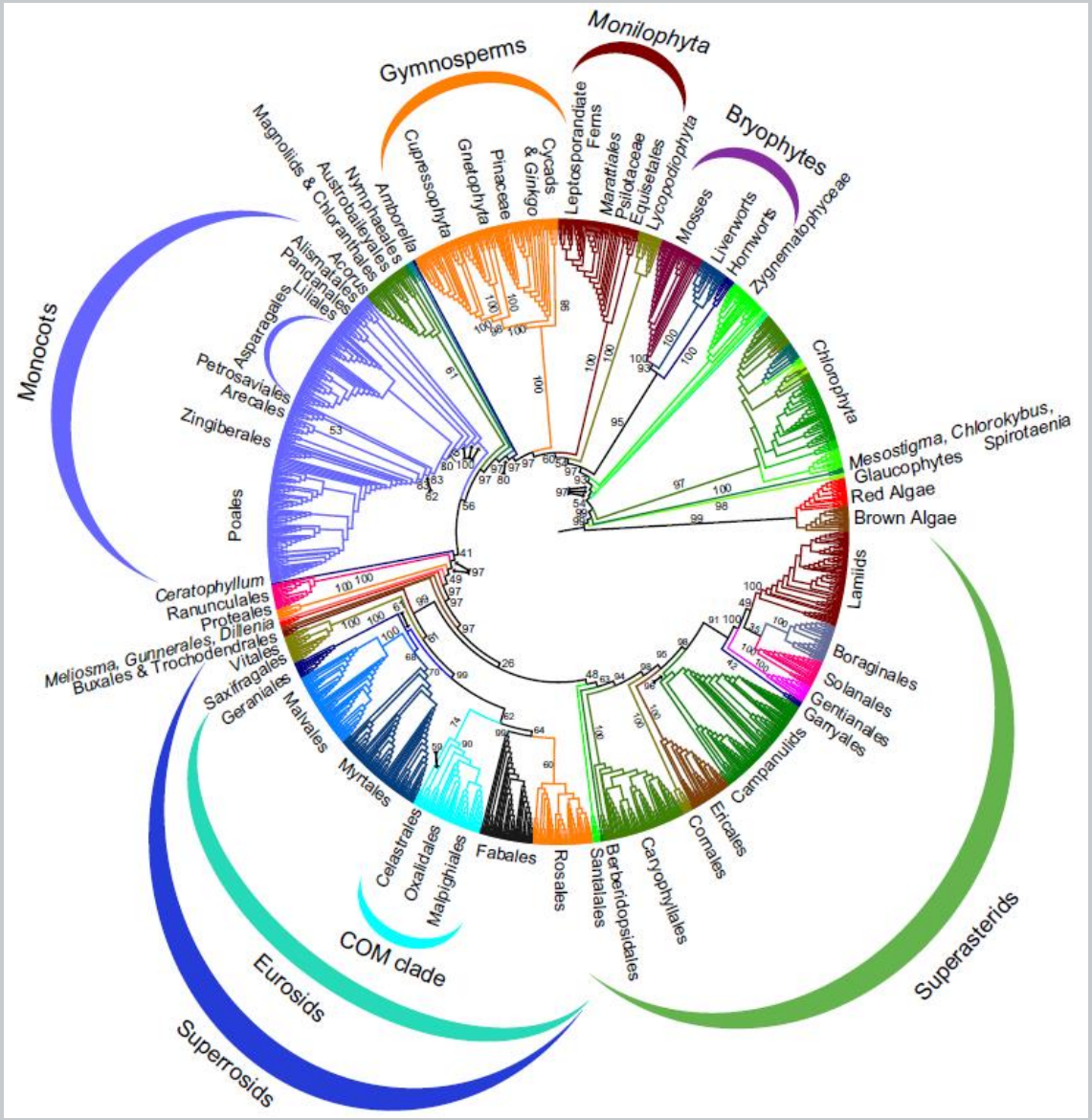
Shaw et al. (2014): Chloroplast DNA sequence utility for the lowest phylogenetic and phylogeographic inferences in angiosperms: The tortoise and the hare IV. *Am. J. Bot.* **101**: 1987–2004.

- top 13 regions within each major evolutionary lineage

Use of chloroplast sequences

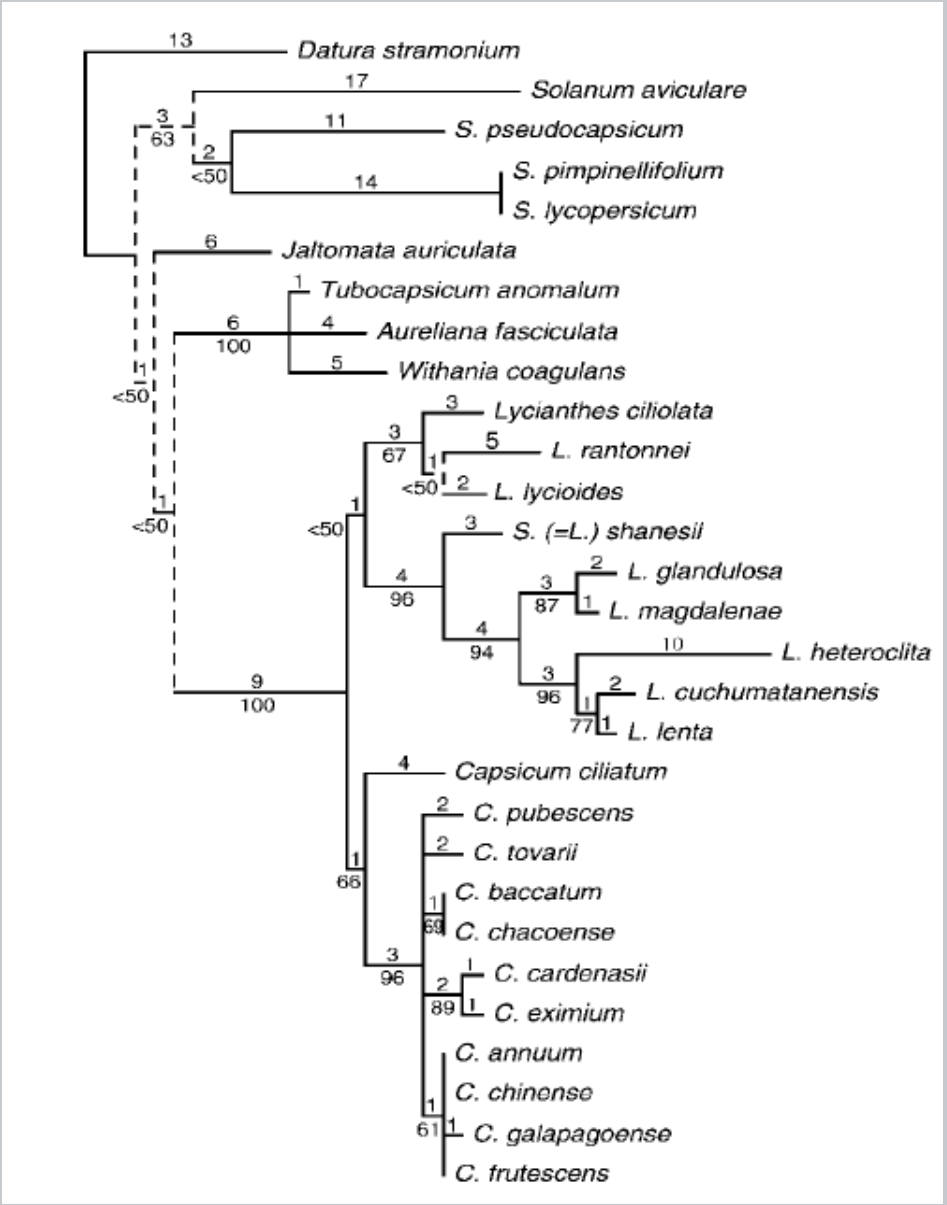
- phylogeny of large groups
- among-species relationships within a genus
- within-species phylogeography (haplotype definition)
- hybridization – inference of the maternal taxon (individual) – cpDNA maternaly inherited in angiosperms

Viridiplantae plastid phylogeny



Gitzendanner et al. (2018)
78 coding plastid genes
1827 taxa + 52 outgroups

Relationships among species



Capsicum
*atp*B-*rbc*L spacer
Walsh & Hoot (2001)

Phylogeography

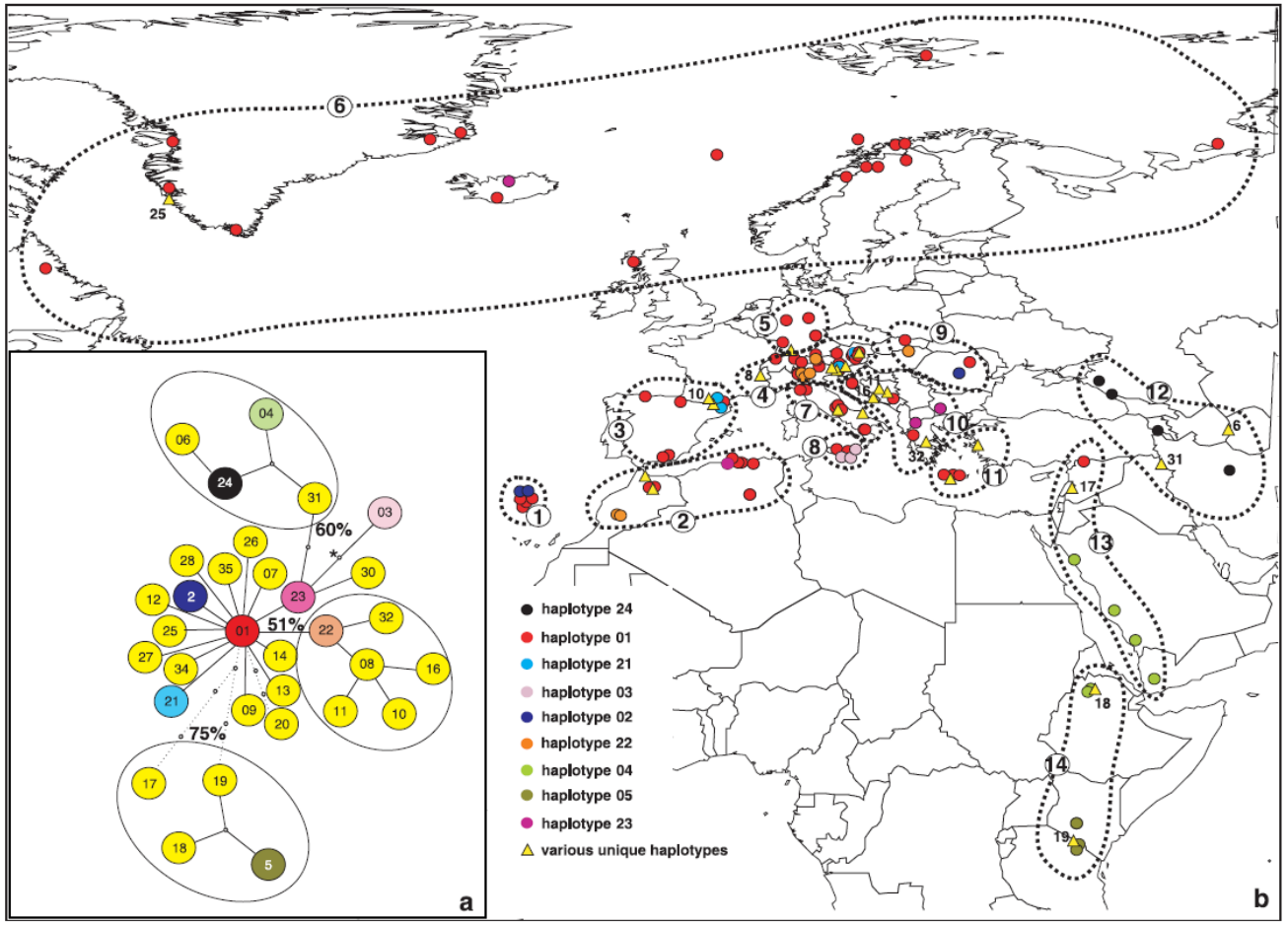
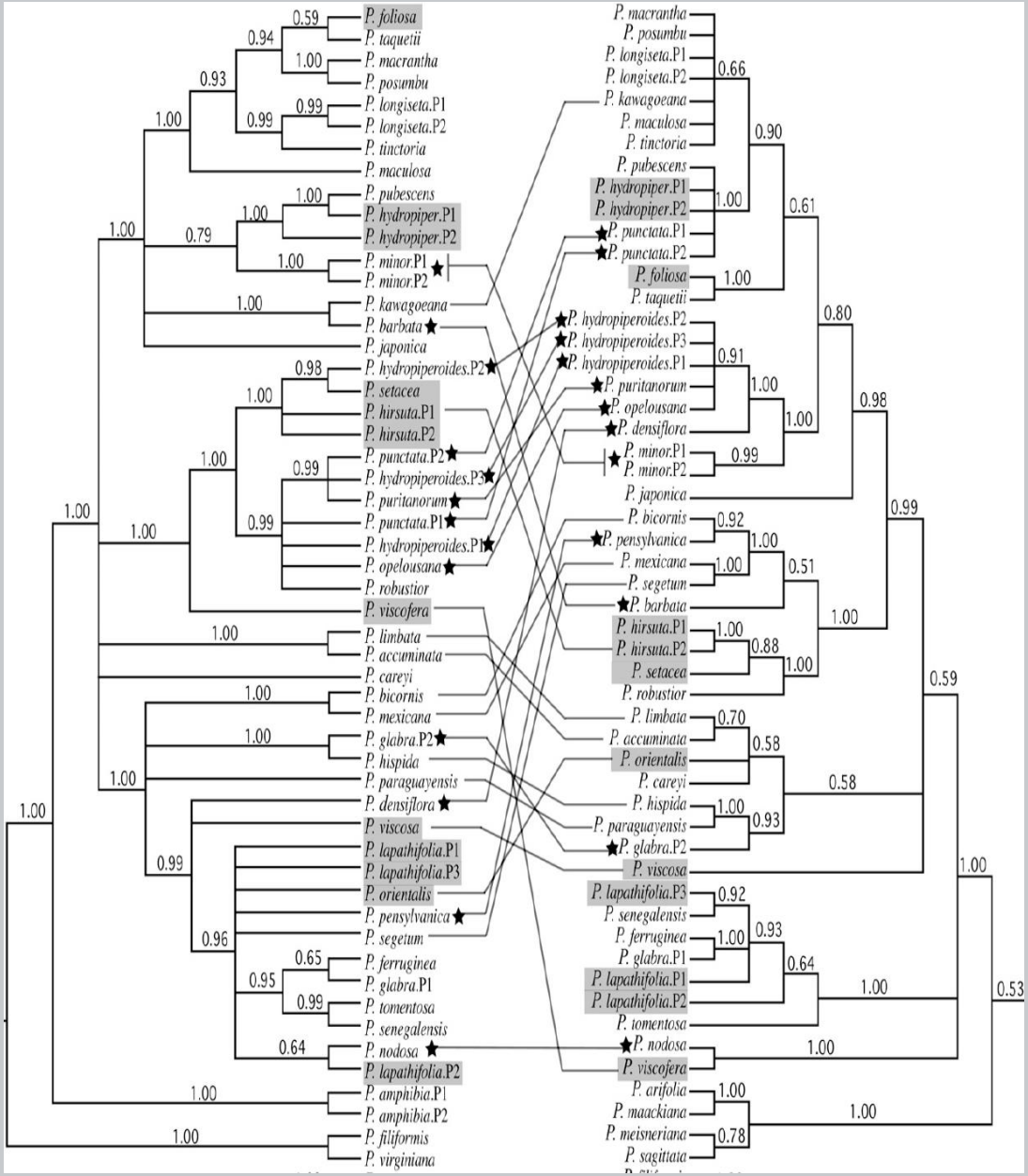


Fig. 1 (a) *Arabis alpina* cpDNA haplotype network as inferred from TCS (see Material and methods section for details). The position of the root as revealed from parsimony and ML analyses is indicated (*). Groups of haplotypes circled correspond to clades that have been also recognized by parsimony and ML analyses (bootstrap values redrawn from parsimony analysis). Colour code and haplotype designation follow Fig. 1 (b), with yellow symbols showing unique haplotypes. (b) Distribution of *A. alpina* cpDNA haplotypes. The distribution range of *A. alpina* is represented by the accessions. Regional subdivision of the accessions into 14 areas is indicated (refer to Table 1 and Table S1, Supplementary materials).

Arabis alpina
*trn*L-*trn*F
Koch et al. 2006

Inter-specific hybridization



incongruence between cpDNA and nDNA

Persicaria
*mat*K, *psb*A-*trn*H, *trn*L-*trn*F
versus ITS
Kim & Donoghue 2008

Data analysis

- *multiple alignment*

```
S206          ATATATATATAGGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
S207          ATATATATA--GGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
S208          ATATATATA--GGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
S209          ATATATATA--GGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
S210          ATATATATA--GGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
S0G3          ATATATATA--GGCAAGGAATCTCTATTATTAAATCATTTAGAATCCATA
TL            ATATATATATAGGCAAGGAATCTCTATTATTAAATCATTCATAATTCATA
```

- construction of phylogenetic tree
  - distance methods
  - maximum parsimony (MP)
  - maximum likelihood (ML)
  - Bayesian inference (BI)

Maximum parsimony (MP)

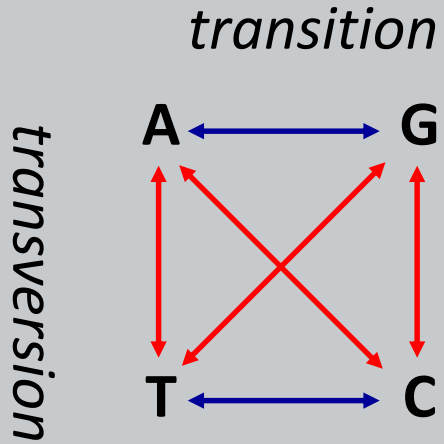
- cladistic method
- search for the simplest tree (*most parsimonious tree*)
- i.e., tree in which the evolution is explained by minimum number of substitutions
- software
  - PAUP *
    Phylogenetic **A**nalysis **U**sing **P**arsimony
    (* and other methods)
  - TNT
    Tree Analysis Using **N**ew **T**echnology

Maximum likelihood (ML)

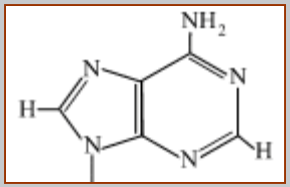
- search for tree with the highest probability (likelihood – L)
- probability that observed sequences evolved under given tree topology (and under given evolutionary model)
- software GARLI, PhyML, RAxML, RAxML-ng, PAML…

Evolutionary models for DNA sequences

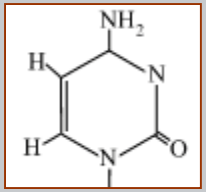
- models for sequence changes



purines



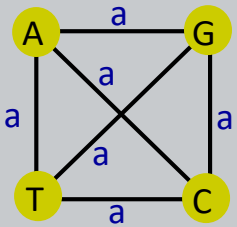
pyrimidines



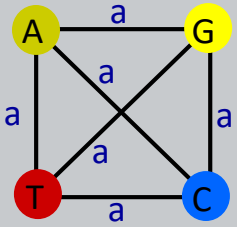
- parameters
  - base frequences
  - substitution types (transitions, transversions)
  - heterogeneity in substitution rates (G)
  - proportion of invariant sites (I)

Substitution models

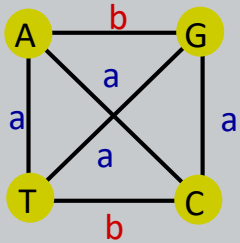
Increasing number of parameters



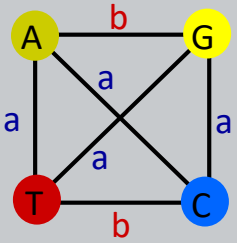
JC (Jukes-Cantor 1969)
– same substitution rates
– same base frequencies



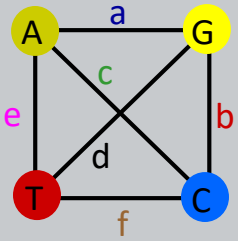
F81 (Felsenstein 1981)
– same substitution rates
– different base frequencies



K2P (Kimura 2 parameter 1980)
– two different substitution rates
– same base frequencies



HKY (Hasegawa, Kishino & Yano 1985)
– two different substitution rates
– different base frequencies



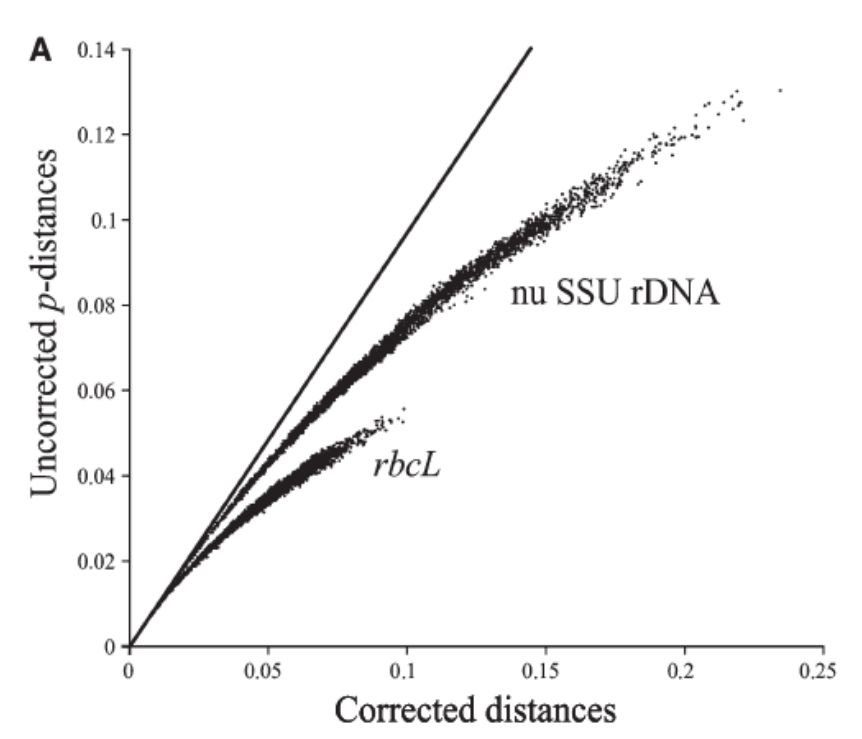
GTR (General time-reversible model)
(Tavaré et al. 1986)
– six different substitution rates
– different base frequencies

Which model to select?

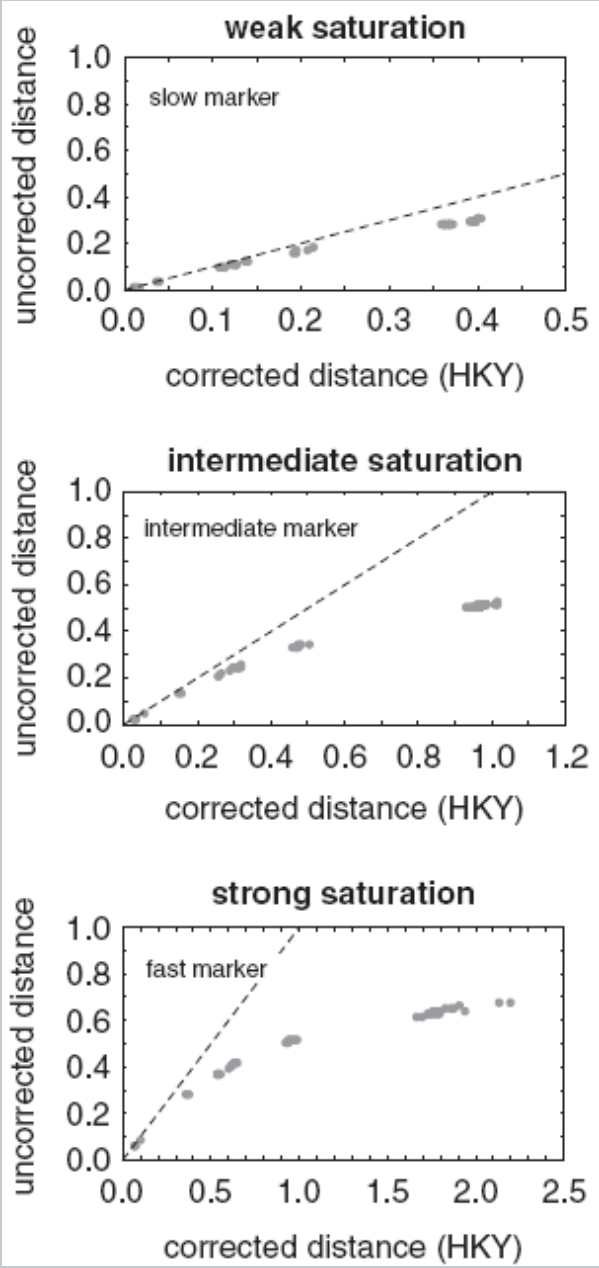
- MODELTEST: A tool to select the best-fit model of nucleotide substitution (Posada et al.)
- testing different models – selecting the simpliest that sufficiently explain the data using
  - hierarchical likelihood ratio tests (hLRTs)
  - Akaike information criterion (AIC)
- jModelTest2 (https://code.google.com/p/jmodeltest2/)
- ModelTest-NG (https://github.com/ddarriba/modeltest/)

Saturation

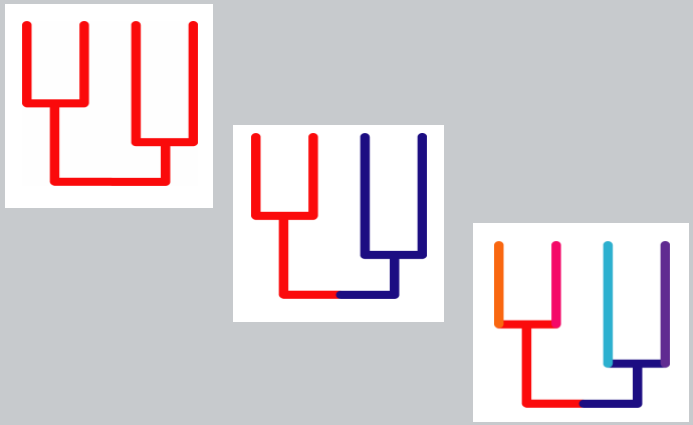
- signal and noise in the data
- corrected versus uncorrected distance
- skewness (g_1-statistics), I_{SS}



Gontcharov & Melkonian 2008



Molecular clock

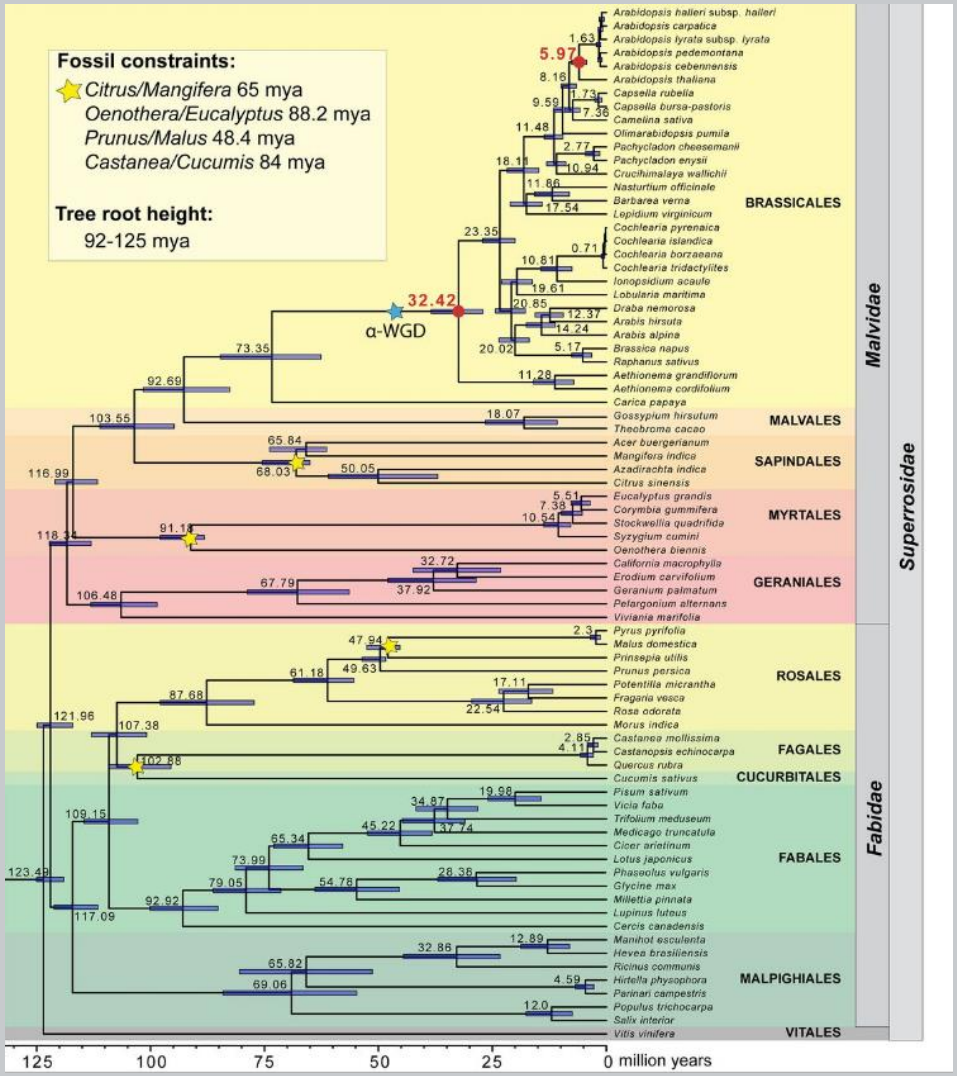


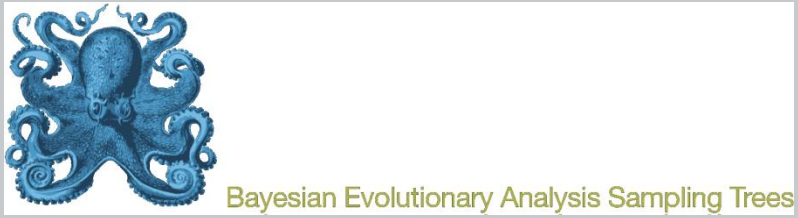
- strict (global)
  - *clocklike evolution*
- local
- *relaxed clocks*
  - autocorrelated (closely related taxa have similar mutation rates)
  - uncorrelated (lognormal, exponential)
- calibration
  - substitution rates from another study or generally assumed rate (e.g., for cpDNA)
  - fossils
  - biogeography
- software
  - BEAST (Bayesian), r8s (non-parametric rate smoothing, penalized likelihood), …

http://sydney.edu.au/science/biology/meep/documents/Workshop_Lecture4.pdf

Estimates of divergence times

(BEAST – Bayesian Evolutionary Analysis Sampling Trees)





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Gene banks – databases of sequences

- **GenBank**
  National Centre for Biotechnology Information (NCBI)
  http://www.ncbi.nlm.nih.gov/
- **EMBL**
  European Bioinformatics Institute (EBI)
  http://www.ebi.ac.uk/embl/

GenBank example

```
LOCUS       JQ409881                562 bp    DNA     linear   PLN 31-DEC-2012
DEFINITION  Curcuma ecomata voucher JLS 73353 18S ribosomal RNA gene, partial
            sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA
            gene, complete sequence; and internal transcribed spacer 2, partial
            sequence.
ACCESSION   JQ409881
VERSION     JQ409881.1
KEYWORDS    .
SOURCE      Curcuma ecomata
  ORGANISM  Curcuma ecomata
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Zingiberales;
            Zingiberaceae; Curcuma.
REFERENCE   1  (bases 1 to 562)
  AUTHORS   Zaveska,E., Fer,T., Sida,O., Krak,K., Ma
            Leong-Skornickova,J.
  TITLE     Conquest of ginger paradise: first insig
            based on plastid and nuclear sequences
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 562)
  AUTHORS   Zaveska,E., Fer,T., Sida,O., Krak,K., Ma
            Leong-Skornickova,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JAN-2012) Department of Bo
            Prague, Faculty of Science, Benatska 2,
            Czech Republic
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     misc_RNA        9..179
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ORIGIN
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      121 gaagcakagg gccccttgct gtgagcgggg agcccaatgc atcgaagatt cctcggaatc
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      481 tgaacgtcgt cctcgtcgtt tcgggatgag tcctcaagag accctgtgtg attgcggagt
      541 cggttgaaag tgccgtgtca at
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Population study

Sanz M. et al. (2014): Southern isolation and northern long-distance dispersal shaped the phylogeography of the widespread, but highly disjunct, European high mountain plant *Artemisia eriantha* (Asteraceae). *Botanical Journal of the Linnean Society* 174: 214–226.

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Renner S.S. (2004): A chloroplast phylogeny of *Arisaema* (Araceae) illustrates Tertiary floristic links between Asia, North America, and East Africa. *American Journal of Botany* 91(6): 881–888

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