Molecular markers in plant systematics and population biology

8. DNA sequencing II. – nrDNA, low-copy markers

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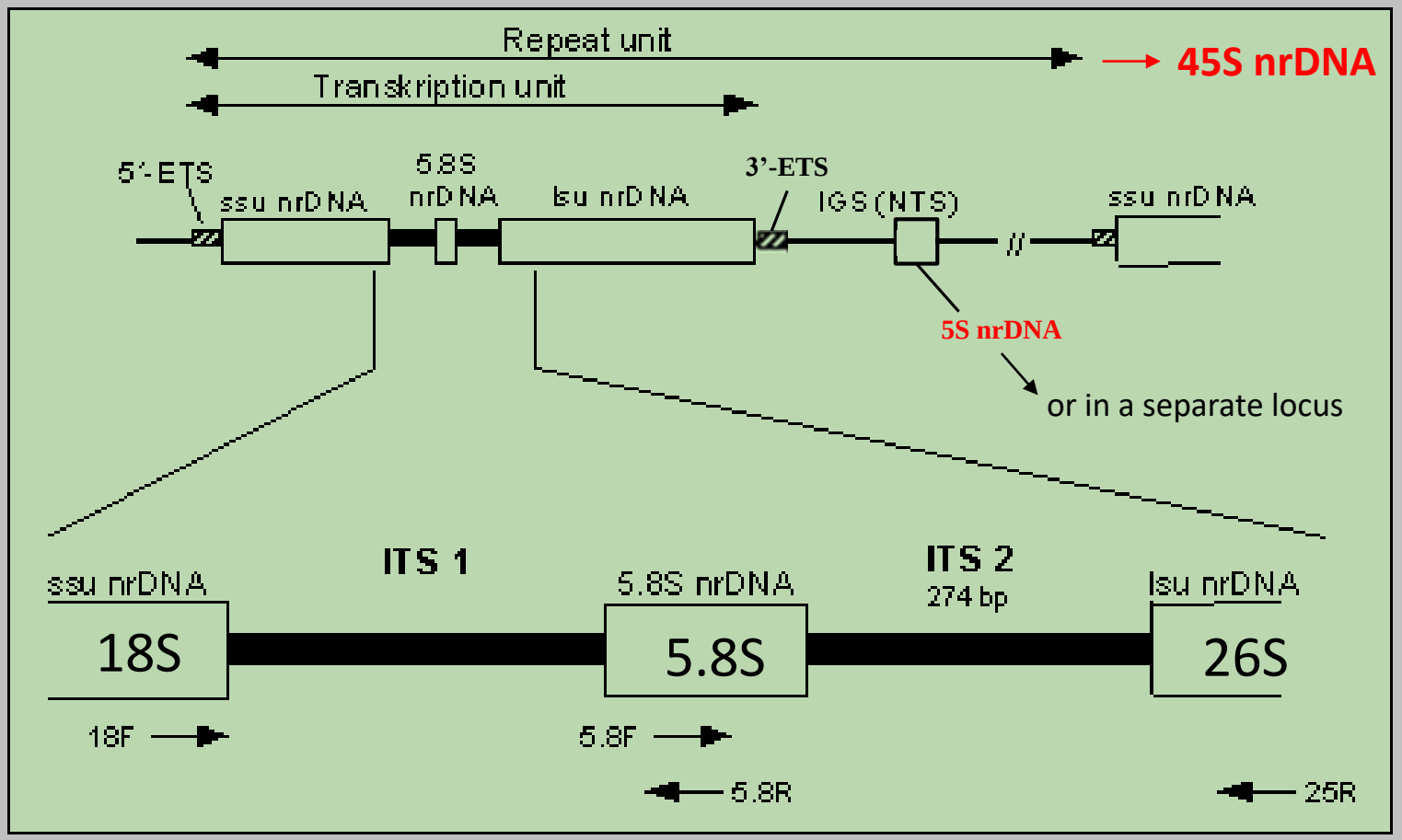
Nuclear genome

- many genes in more copies (*multiple-copy*)
  - homology problem – we do not know what we sequence/observe
  - e.g., genes for rRNA
- *low-copy* or *single-copy* genes
  - problem with primers for the studied group
  - genes for specific proteins – *Adh*, *Tpi*, *Pgi*, phytochrome *c*, *waxy* (GBSSI)…

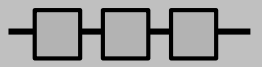
rDNA

- genes for rDNA – commonly used marker in systematics
- hundreds to thousands of tandem repeats (250-2,500 in *Arabidopsis thaliana*, up to 22,000 in *Vicia faba*)
- about 5% of total DNA
- in one or few chromosomal loci
- 45S rDNA locus - transcribed region (ETS-18S-ITS1-5.8S-ITS2-26S) is separated by intergenic spacer (IGS)
- 5S rDNA locus – linked with 45S locus (L-type – streptophyte algae, bryophytes, lycophytes) or separated (S-type arrangement – gymnosperms, angiosperms)
- *concerted evolution* – creates intragenomic uniformity of repeat units
  - if proceeds slowly – several different ITS sequences within genome exists (paralogs) $\rightarrow$ phylogeny inference problematic, take care about hybridization and polyploidization

rDNA structure



5S nrDNA

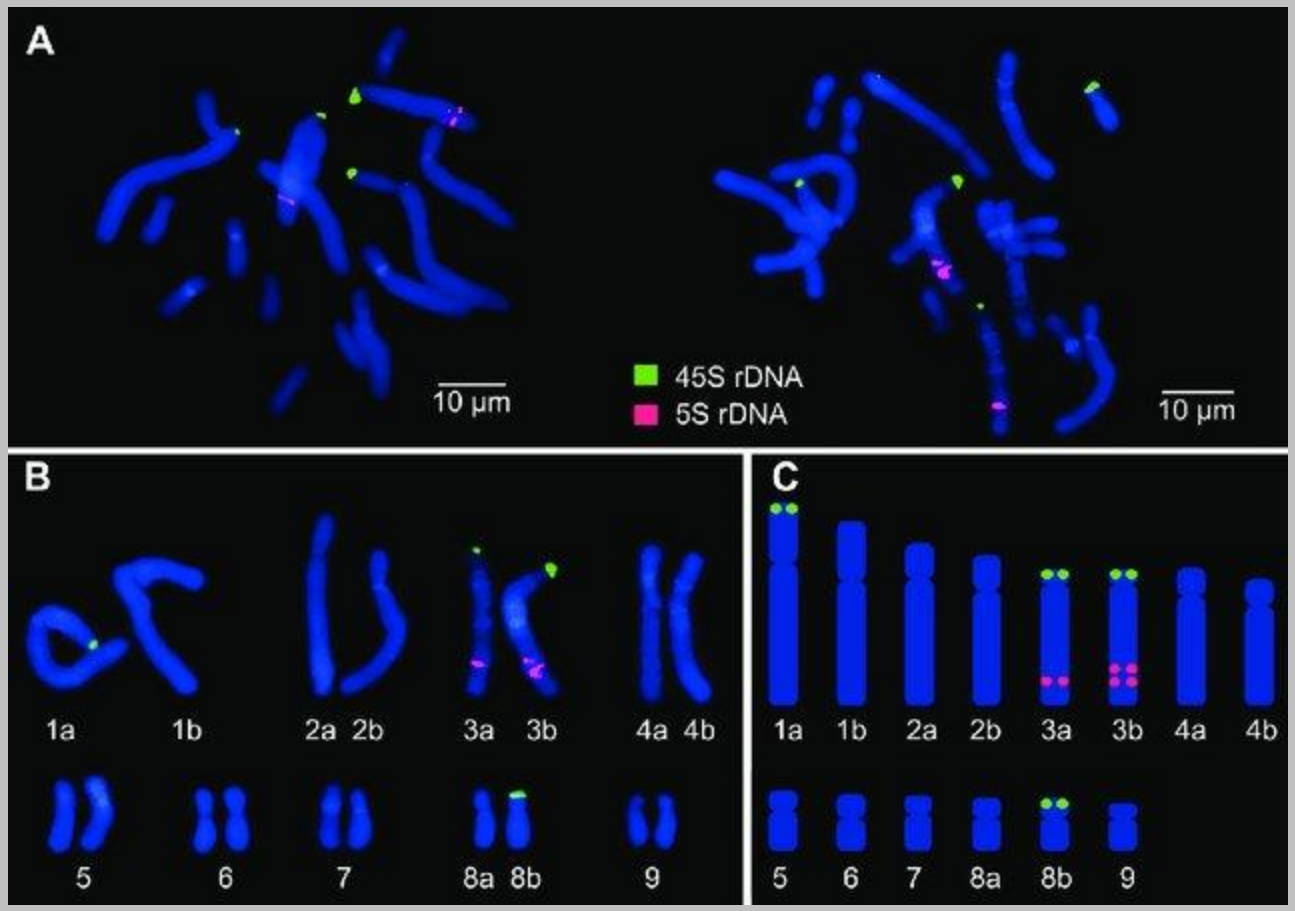


ETS – *external transcribed spacer*

ITS – *internal transcribed spacer*

IGS – *intergenic spacer* (NTS – *non-transcribed spacer*)

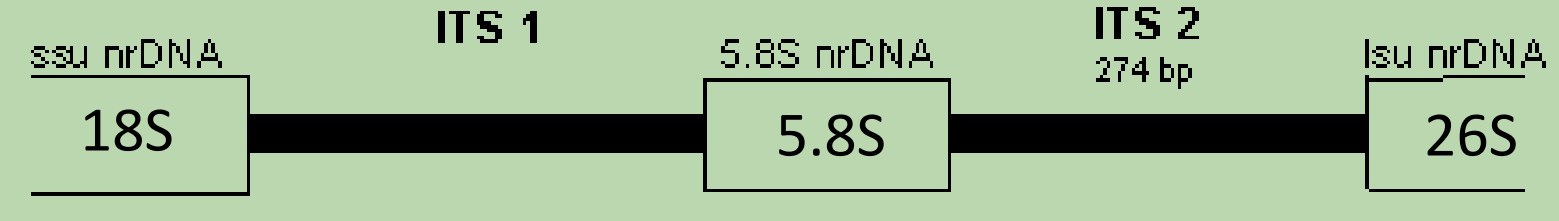
rDNA on chromosomes



Organizations of 45S rDNA and 5S rDNA loci on metaphase chromosomes

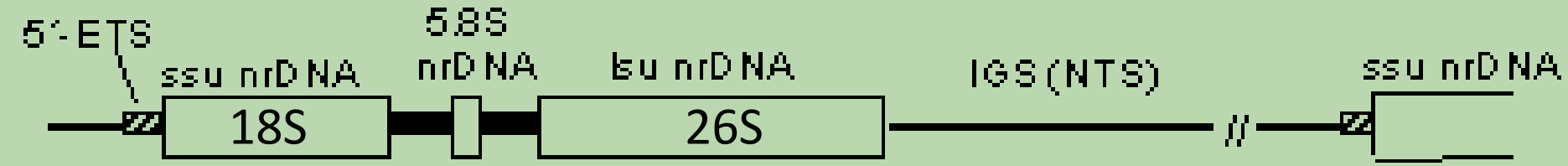
Monkheang et al. 2016

ITS – internal transcribed spacer



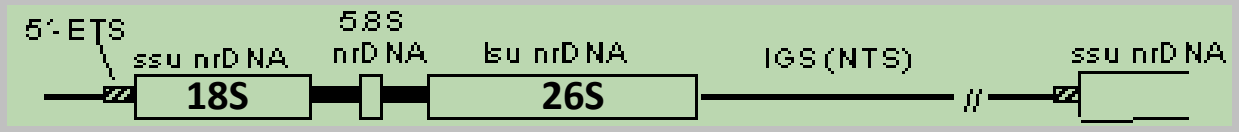
- ITS1 (200-300 bp) – greater length variation than ITS2
- ITS2 (180-240 bp)
- frequently used to detect relationships among closely related genera and at the species level (but sometimes low variability)
- have certain function when forming ribosomal units
- i.e., some evolutionary constrain of structure and sequence does exits
  - 40% of ITS2 conserved among all angiosperms
  - 50% of ITS2 is possible to align across family and higher level
- much longer in gymnosperms, high length variability (1,550-3,125 bp in *Pinaceae*)

ETS – external transcribed spacer



- at least same evolutionary rate as ITS
- 258-635 bp
- problem with sequencing – missing conserved region at 5´-end of the spacer
- *long-distance* PCR for amplification of the whole IGS (using universal primers from 18S and 26S DNA)
- after sequencing of the product from 3´-end it is possible to design internal primers

18S rDNA



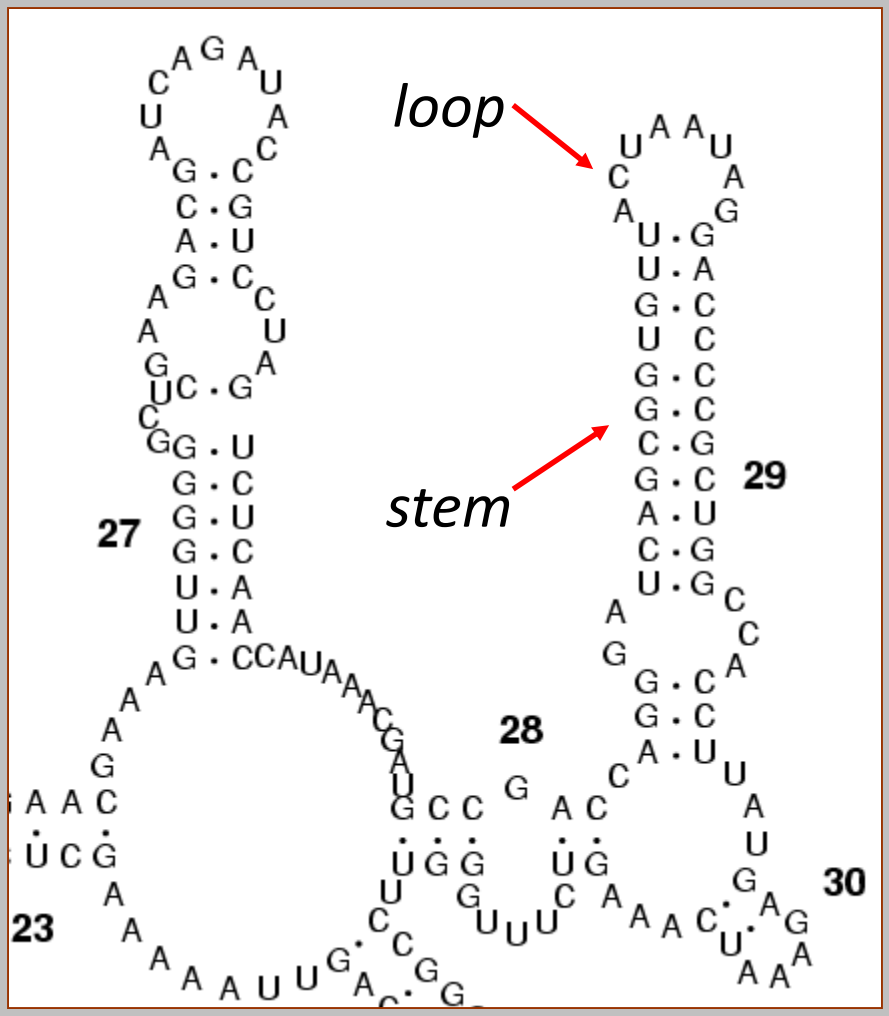
- about 1,800 bp
- length mutations often just 1 bp, at particular sites
- i.e., simple alignment

26S rDNA

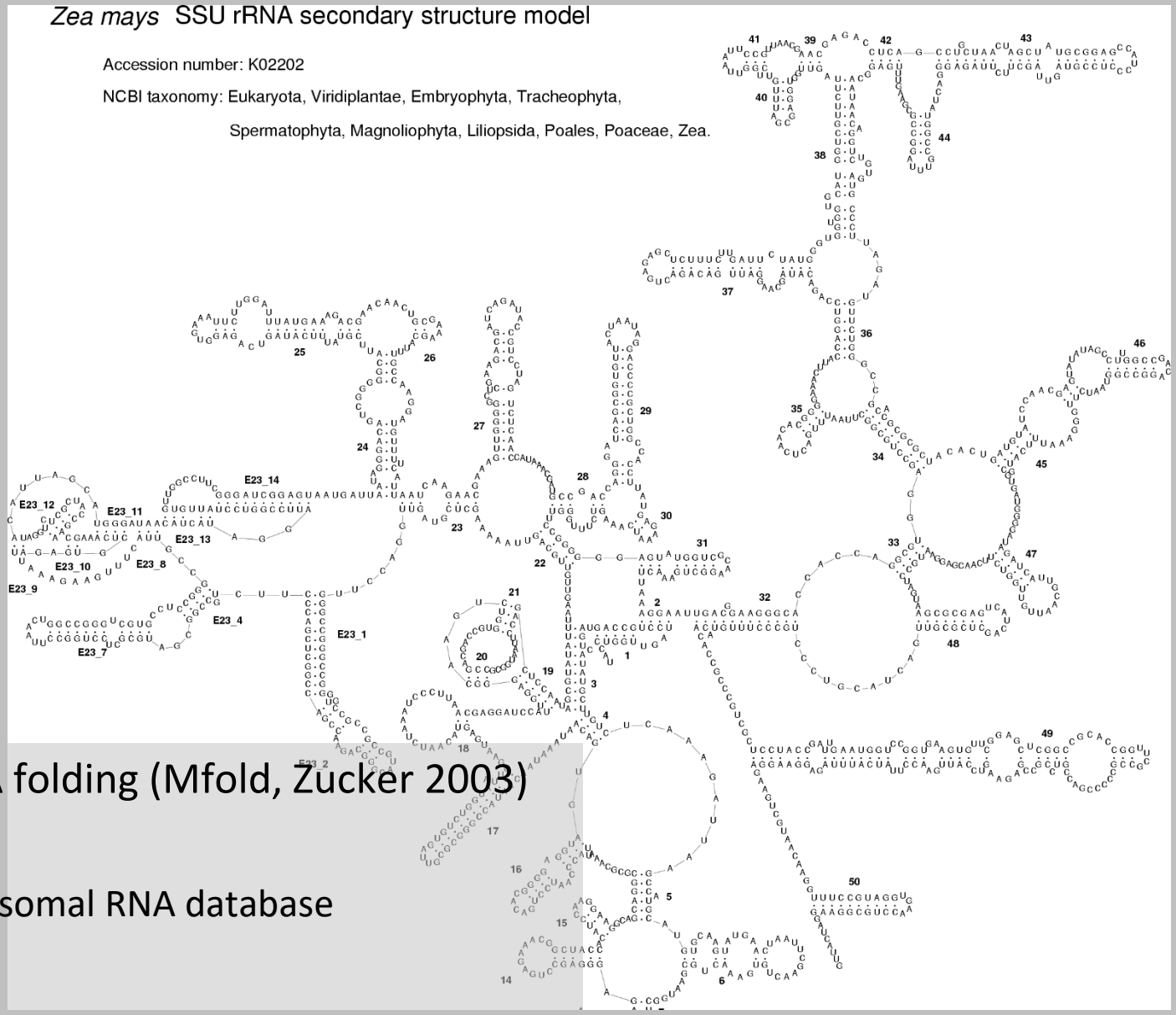
- between 3,375 and 3,393 bp
- very conserved
- evolves 1.6-2.2x faster than 18S rDNA
- contains conserved regions and expansion segments
- *conserved core regions* – systematics at higher taxonomic level
- *expansion segments* – evolves up to 10× faster

rRNA – model of secondary structure

- *loops* and *stems*
- Watson-Crick pairing (A-U and G-C, but often G-U as well)
- *CBC - compensatory base changes* (substitutions to hold *stem* structure)
- i.e., bases are not independent – different weight than *loop*

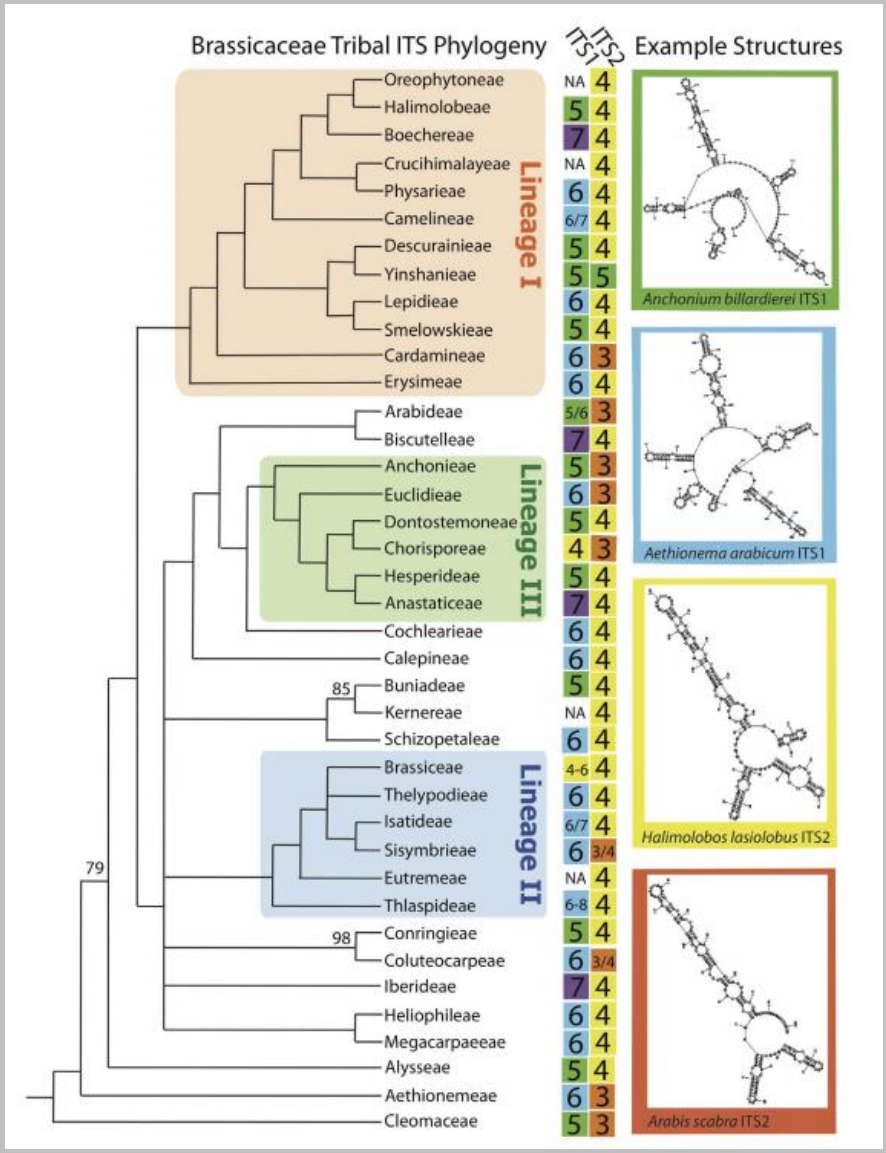


Secondary structure of RNA



- prediction – RNA folding (Mfold, Zucker 2003)
- databases
  - European ribosomal RNA database
  - ITS2

ITS secondary structure



Edger P.P. et al. (2014): *Secondary structure analyses of the nuclear rRNA internal transcribed spacers and assessment of its phylogenetic utility across the* Brassicaceae *(mustards)*. PLoS ONE 9(7): e101341.

rDNA markers, ITS

pros

- many copies – easily sequenced
- universal primers
- favorite marker – many sequences in databases
- variable (ITS)
- biparentally inherited (useful for parent identification of a hybrid)

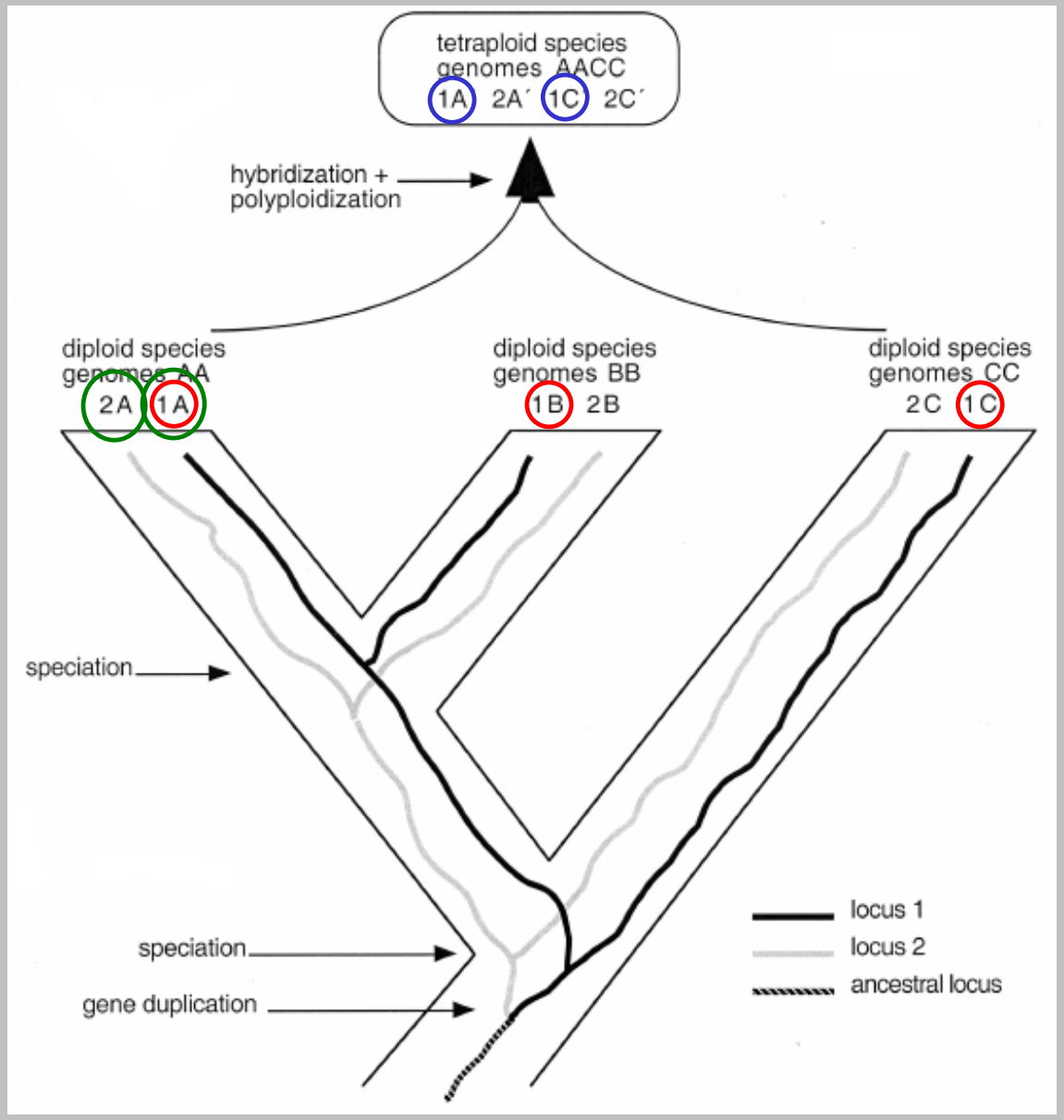
cons

- multiple-copy marker
- more copies after hybridization/polyploidization (if concerted evolution is slow) – cloning necessary
- sometimes not enough variable (even ITS) in closely related species

Low-copy nuclear markers

- genes that are only in several copies in the genome
- × multiple copy – hundreds to ten thousands copies (nrDNA…)
- higher variability than ITS and non-coding cpDNA (at least some of them)
- homology problem
  paralogy × orthology × homeology

Paralogue, orthologue and homeologue genes



orthologue genes

- originated by speciation

paralogue genes

- originated by gene duplication

homeologue genes

- originated by polyploidization

orthologue genes

1A-1B-1C

paralogue genes

1A-2A, 1B-2B, 1C-2C

1A-2B, 1A-2C atd…

homeologue genes

1A´-1C´, 2A´-2C´

Low-copy markers

pros

- higher evolutionary rate than organelar sequences
- possibility to use many independent (unrelated) loci
- biparental inheritance

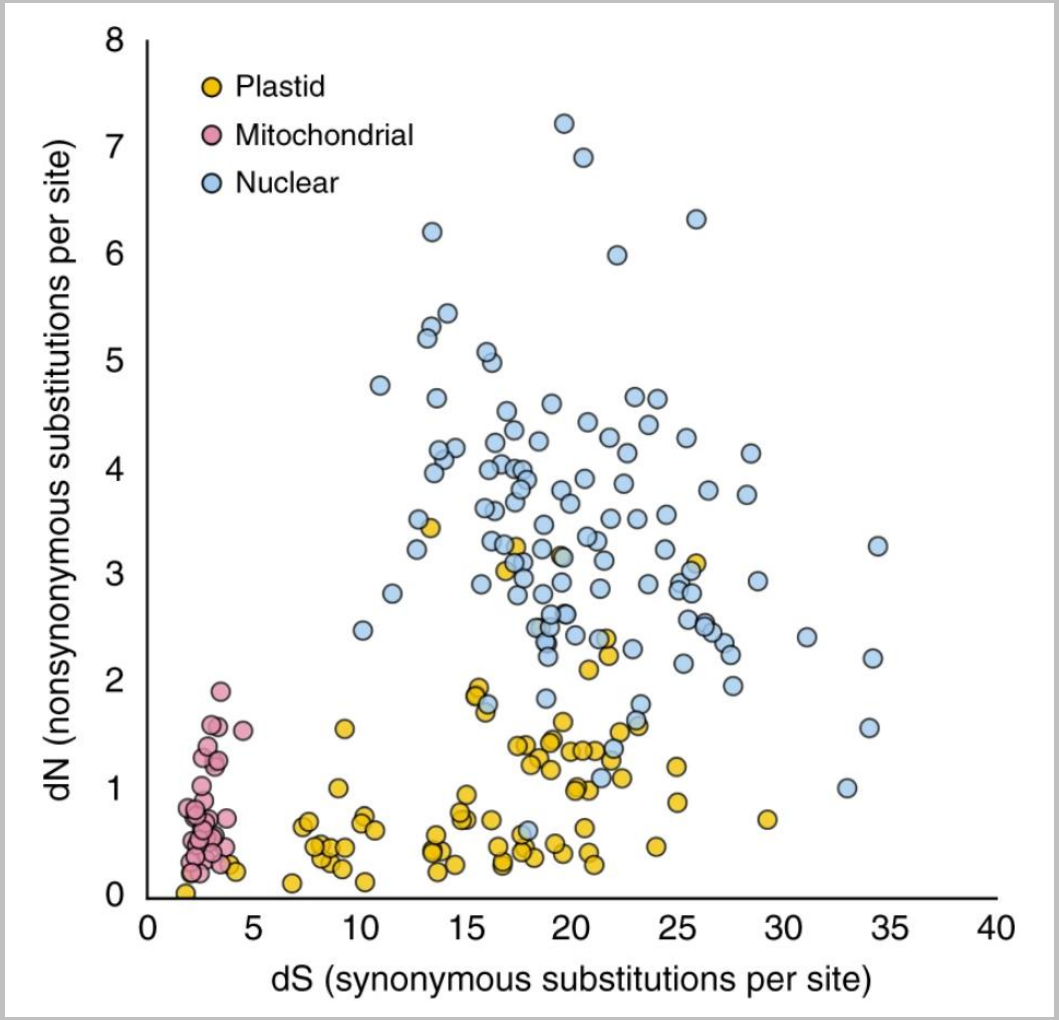
cons

- complex genetic structure (gene duplication)
- difficuilt identification of orthologous loci
- within-species, within-population and within-individual variability (heterozygosity)

Evolutionary rate variation

- synonymous substitutions – 5× faster than cpDNA genes and 20× faster than mtDNA
- e.g., relationships within *Gossypium* – cotton (Small et al. 1998)
  - 7,000 bases of non-coding cpDNA provided incomplete and poorly supported resolution
  - 1,650 bases of *Adh*C – complete and robust resolution
  - great differences in variability (mutation rate) among different genes – up to 7× differences
- i.e., it is necessary to test more markers for each group and select the variable loci

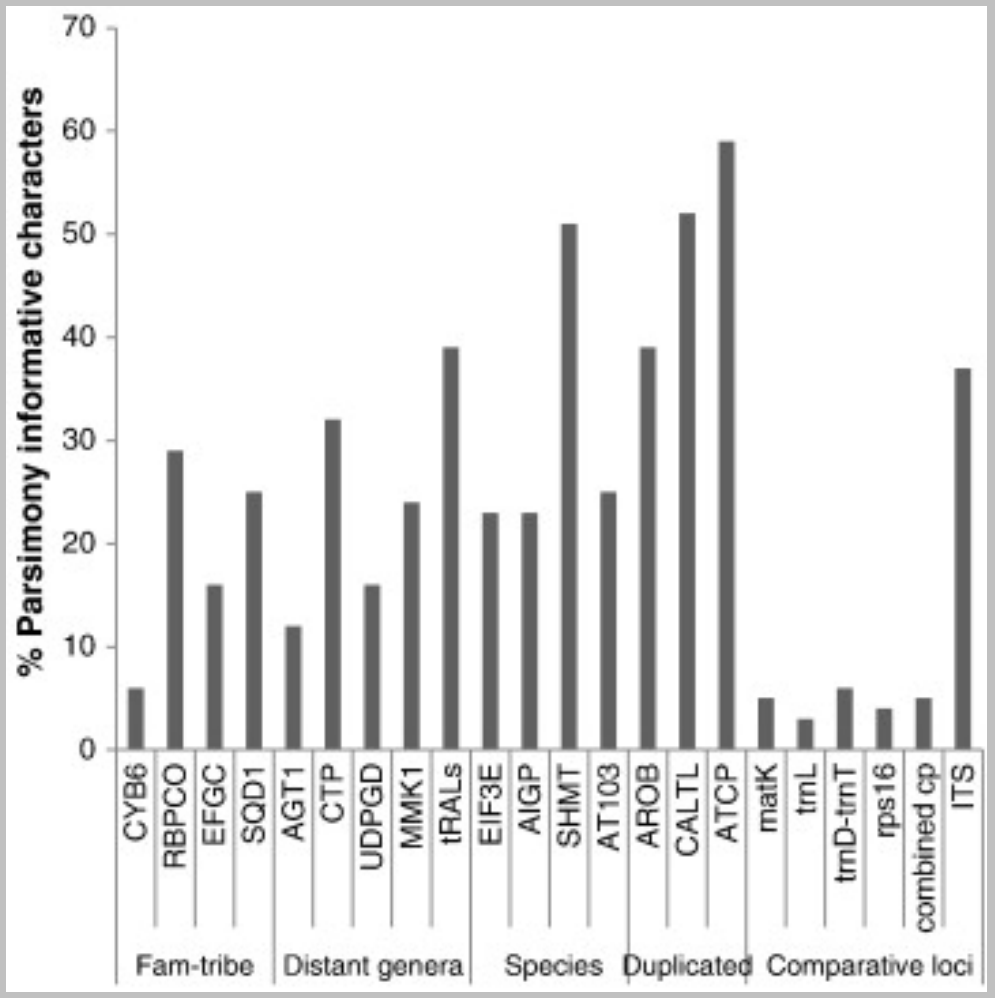
Evolutionary rate variation



Total gene tree depth in synonymous (dS) and non-synonymous (dN) substitutions per site for protein-coding genes in three genomic compartments across Bryophyta.

Liu et al. (2019): *Resolution of the ordinal phylogeny of mosses using targeted exons from organellar and nuclear genomes*. Nat. Comm. 10: 1485

Parsimony informative sites



Babineau et al. (2013): *Phylogenetic utility of 19 low copy nuclear genes in closely related genera and species of caesalpinioid legumes*. S. Afr. J. Bot. 89: 94-105

Structure of eucaryote genes

- *5´ UTR (untranslated region)* – promotors for gene regulation (conserved), sometimes includes highly variable introns
- *exons* – more conserved at non-synonymous positions (first and second codon position), at synonymous (third codon) positions similar to non-coding regions
- *introns* – fewer functional constrains at the sequence level, often limited length
- *3´ UTR (untranslated region)* – controls maturation of mRNA and addition of poly-A signal, but often highly variable as well
- different functions, various evolutionary constrains

Multiple unlinked loci

- independent reconstructions of evolution
- markers at different chromosomes (or distant enough from each other at a single chromosome) – evolutionary independent
- incongruence among different markers – possibility to detect, i.e., hybridization, introgression, polyploidization, ILS (*incomplete lineage sorting*)

Biparental inheritance

- low-copy markers are less often a subject of *concerted evolution*

- i.e., they are ideal candidates for identification of parental genomes in putative ***hybrids*** or ***polyploids***

Gene families

- multiple copies of homologous genes originating by duplication
- gene families differs in copy number
  - single copy – GBSSI in diploid *Poaceae*
  - hundreds of copies – actine, small heat-shock proteins
- gene and whole-genome duplication (and consequent loss of genes) – dynamic and ongoing process
- characteristics of a gene family – taxon (group) specific
- gene family characteristics in one group need not to be applied in another group
  - *Adh* generally – 1 to 3 loci
  - in *Gossypium* or *Pinus* – up to 7 loci
- wrong characterisation of gene family leads to erroneous phylogeny reconstruction (it is always necessary to compare orthologous copies!)

Study of ortholog sequences

1. design of *universal primers* – amplification of more PCR products (of different length) $\rightarrow$ characterisation of the gene family (identification the number of loci?)

2. design of *locus-specific primers* – only orthologous sequences are amplified

- evidence of orthology
  - overall sequence similarity (orthologs are mutually more similar than paralogs)
  - *expression pattern* – orthologous sequences share the same pattern
- great differences in the variability among genes and loci – preliminary study for detection of sufficient variability is necessary

Intraspecific variability

- allelic variability within and among populations
- ***coalescence within species*** – alleles evolved within a single species – it does not violate correct phylogeny inference, i.e., this is a useful variability for within-species studies – population, phylogeographic etc.
- ***deep coalescence*** – allelic variability exceeds species boundary, i.e., some alleles are more related to alleles from a different species rather than to other alleles from the same species – more probable in species with high population sizes
- loci under balanced selection (maintain high allelic variability) – unsuitable for phylogeny reconstruction
  - e.g., self-incompatibility genes in *Solanaceae* – allelic variability exceeds species and even generic boundaries
- also due to hybridization and introgression

Recombination

1. *allelic recombination*
   - recombination at the individual locus level
   - generates allelic variability
     - do not violate the assumption of bifurcate relationships among alleles
     - introduce a reticulate evolution
     - do not violate correct phylogeny reconstruction as far as alleles are monophyletic within a species
2. *non-homologous recombination*
   - recombination among paralogous loci
   - can be rare or specific for particular gene

Concerted evolution

- common in highly repetitive loci (nrDNA)
- exists in low-copy markers as well – *non-homologous recombination*

- *do not occur* → sequencing of all genes from the gene family produces orthology-paralogy tree (OP-tree)
- *completed* (assumed in nrDNA) → sequencing of whichever gene from the gene family produces the correct phylogenetic tree
- *incomplete* → mixture of orthologous and incompletely homogenised paralagous sequences, i.e., correct phylogenetic reconstruction is practically impossible

Concerted evolution

- common in highly repetiti
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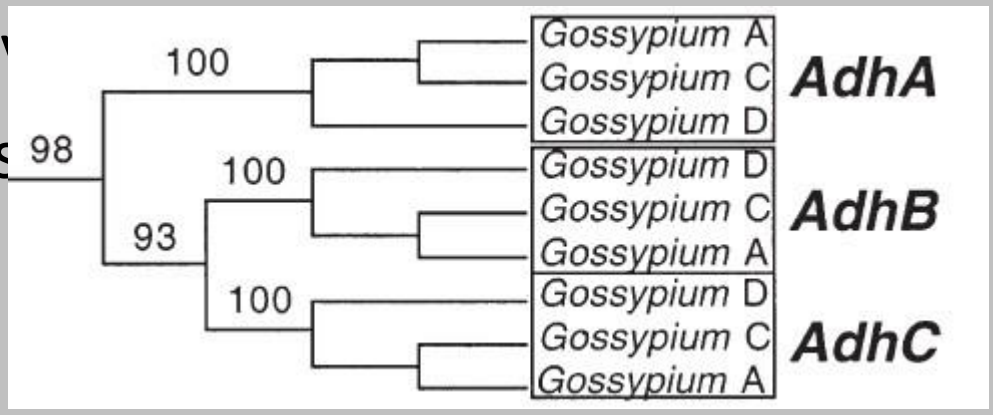

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PCR-mediated recombination

- *in vitro* non-homologous recombination
- sources
  - 1. template interchange during PCR
  - 2. incompletely amplified copies of one locus serve as primers for amplification of paralogous locus
- degree depends on
  - degree of sequence similarity among paralogous loci
  - universality/specificity of primers
  - PCR conditions (optimization of annealing temperature, product length and extension time)

How to determine suitable nuclear markers for phylogenetic analyses

1. candidate gene selection (and representative taxa) for preliminary study
2. candidate gene islation from representative taxa
3. assessment of orthology among isolated sequences
4. determination of relative rate of sequence evolution – selection of suitable locus
5. generating sequences from all studied taxa for the particular locus

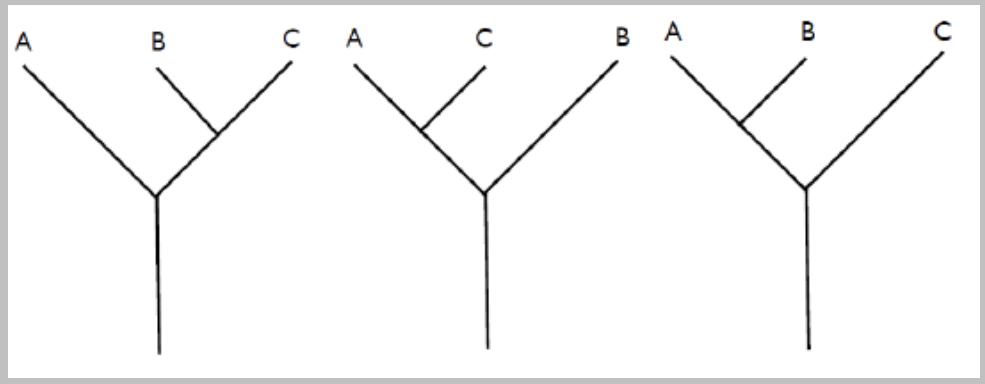
Candidate gene selection

- no general assumptions of universality for any particular gene
- not necessary to use frequently sequenced genes (*Adh*…)
- little known or even anonymous nuclear loci is possible to use
- where to start?
  - previous studies within the group
  - literature search for the utility of a gene at given taxonomical level in different groups
  - GenBank, EMBL… – taxon × gene name combination search
  - BLAST search – search for similar sequences → primer design, gene structure identification (exons, introns)…
  - NGS sources – transcriptome, genome skimming

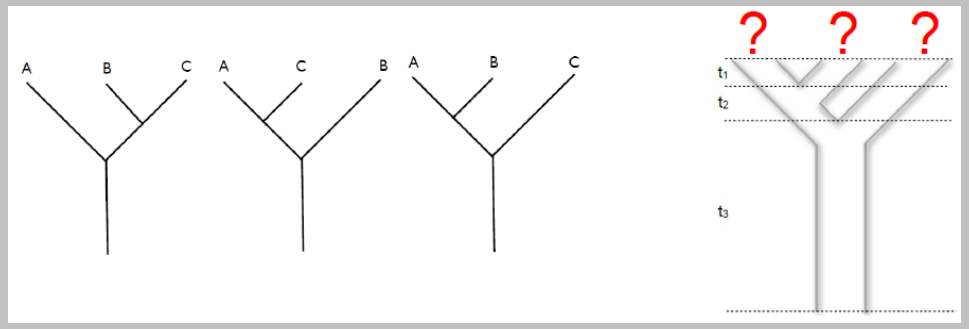
Li M. et al. (2008): *Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species*. Cladistics 24: 1-19.

Incongruencies among markers: gene trees vs species tree

- gene duplications and losses (orthology problem)
- incomplete lineage sorting/deep coalescence
- hybridization
- polyploidization
- recombination



Species tree estimation



- concatenation

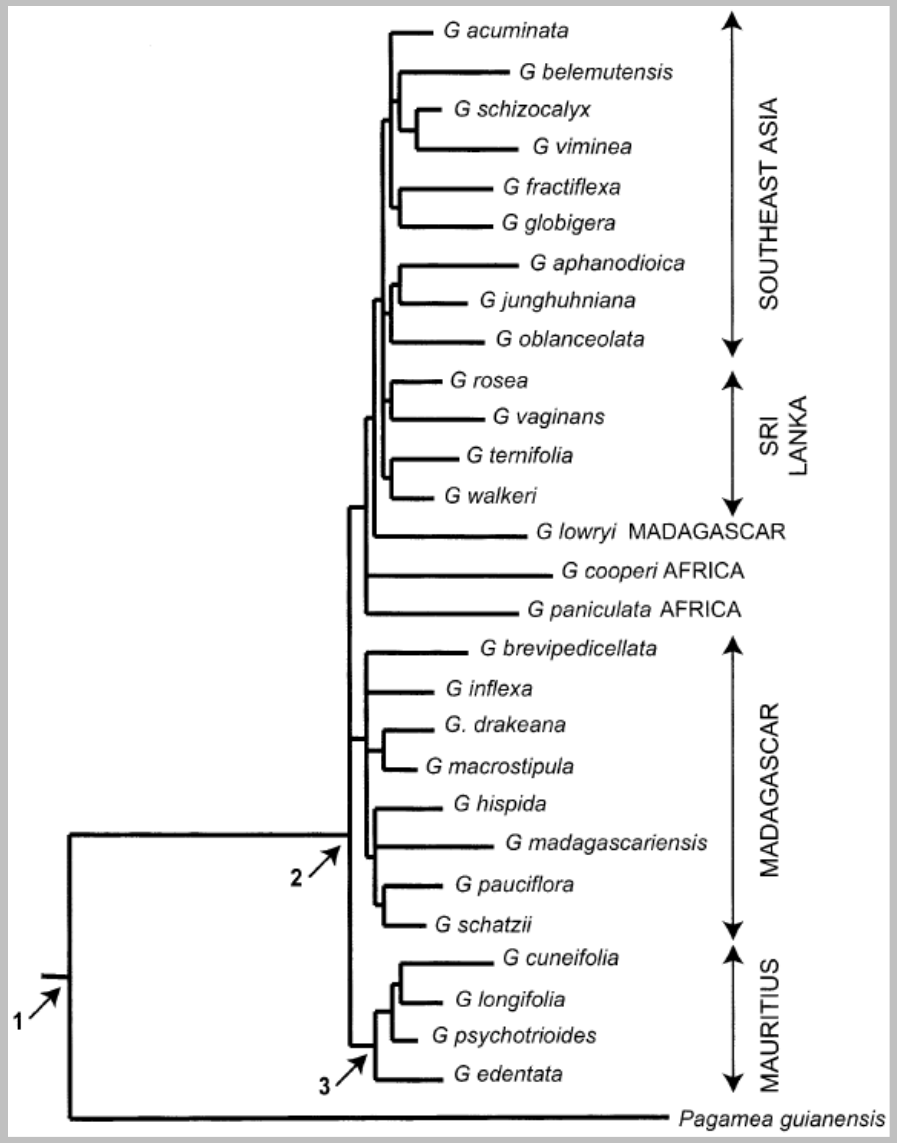
- multispecies coalescence
  - coestimation of gene trees and species tree
  - summary methods

(more in the Hyb-Seq lesson…)

Application of single-copy genes

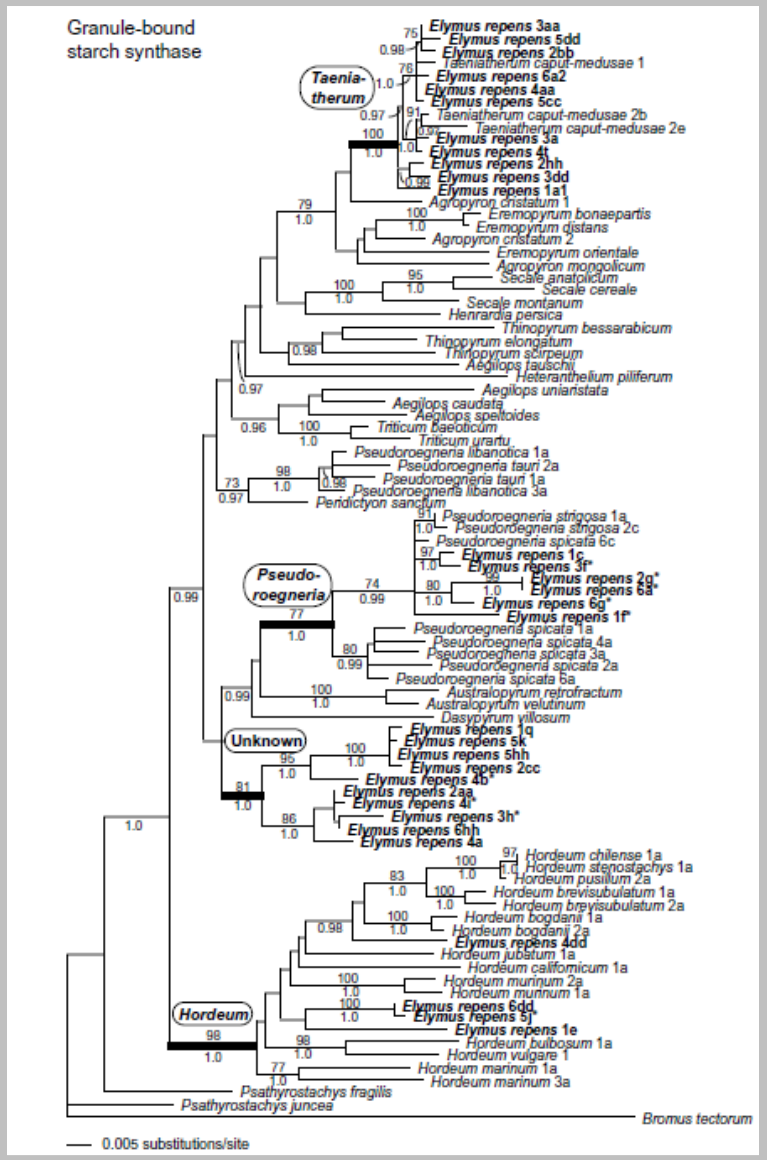
- phylogenetic studies – can provide enough variability for the full resolution at lower taxonomical level (e.g., relationships among closely related species)
- study of polyploids – ‘picking’ individual parental sequences from the polyploid genome and identification of complex allopolyploid pattern
- phylogeography – variability within species

Relationships among closely related species



Gaertnera
PepC, Tpi
Malcomber 2002

Allohexaploid origin



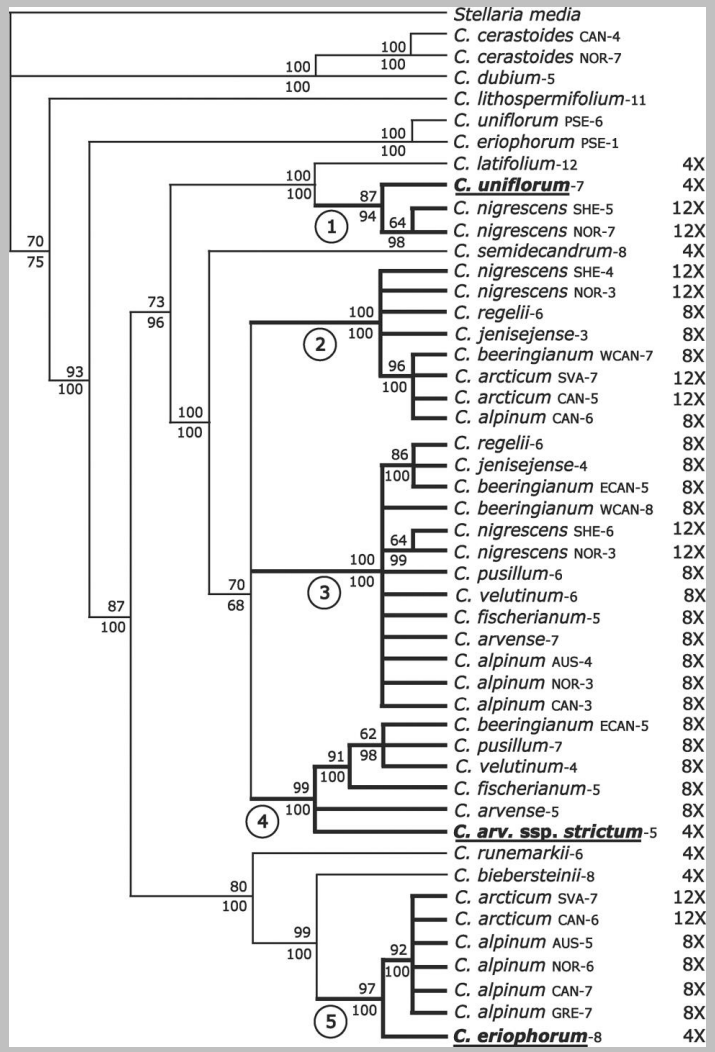
Taeniatherum

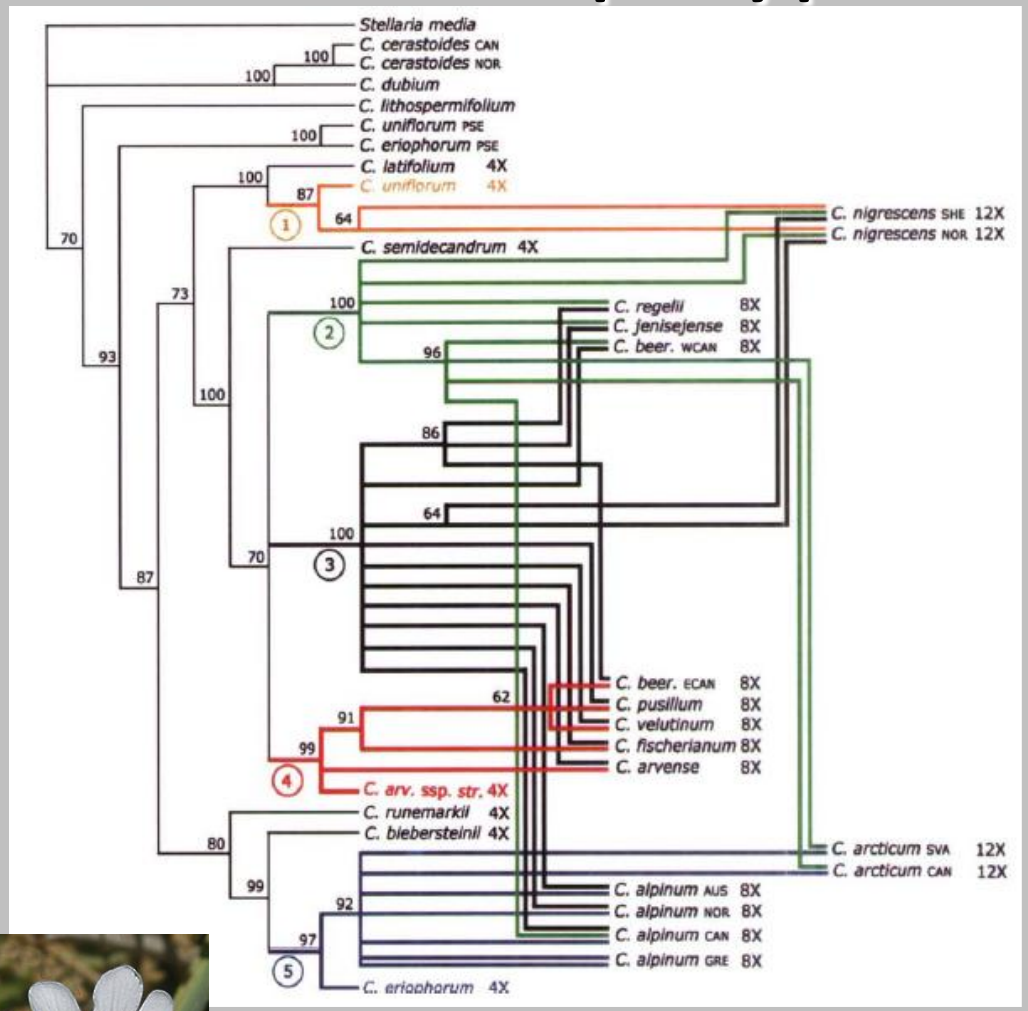
Pseudoroegneria

Hordeum

Elymus repens
GBSSI (single-copy)
Mason-Gamer 2008

Parental genomes within allopolyploids





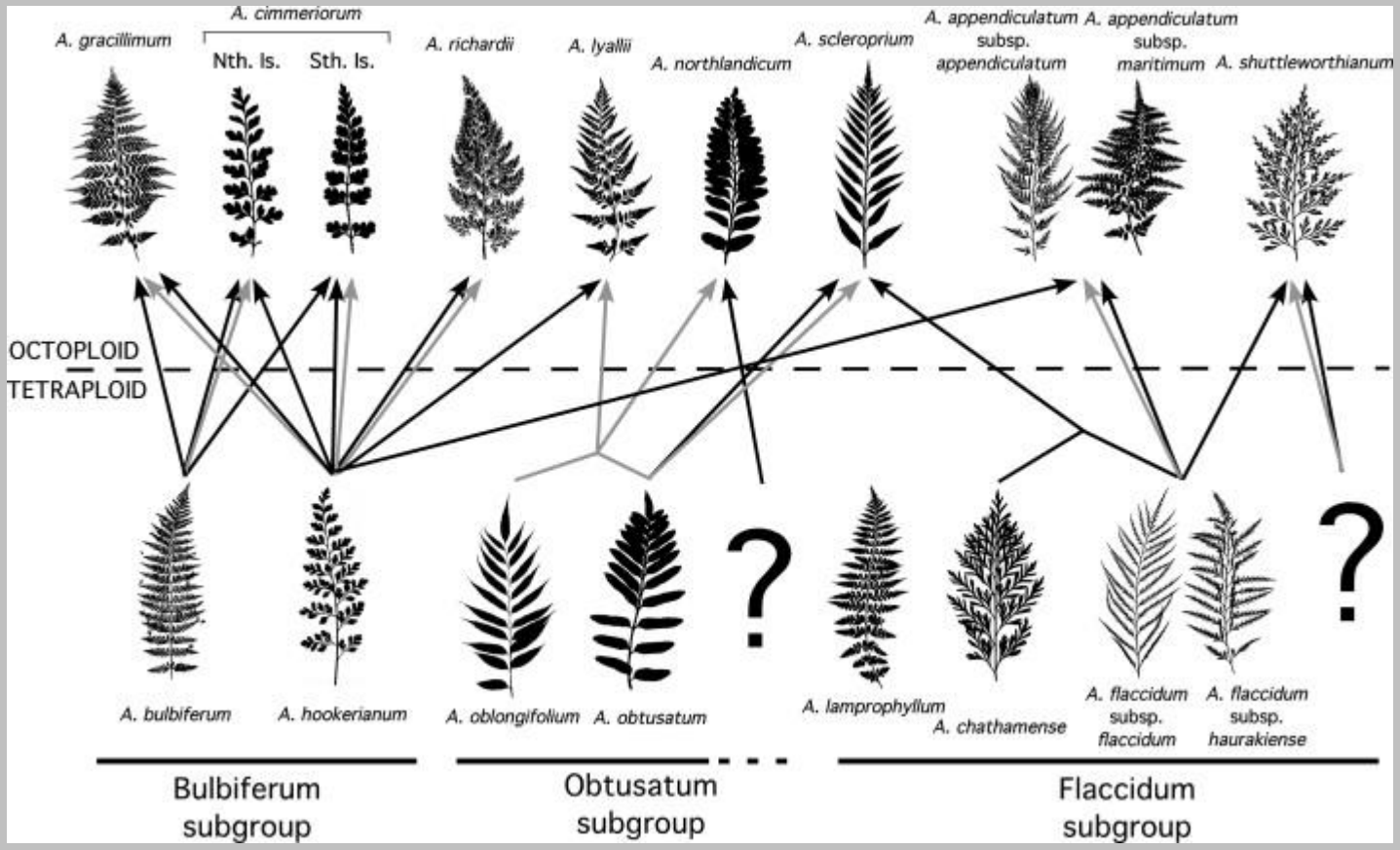
Cerastium

4x, 8x, 12x

RPB2 tree, network

Brysting et al. 2007

Parental genomes within allopolyploids



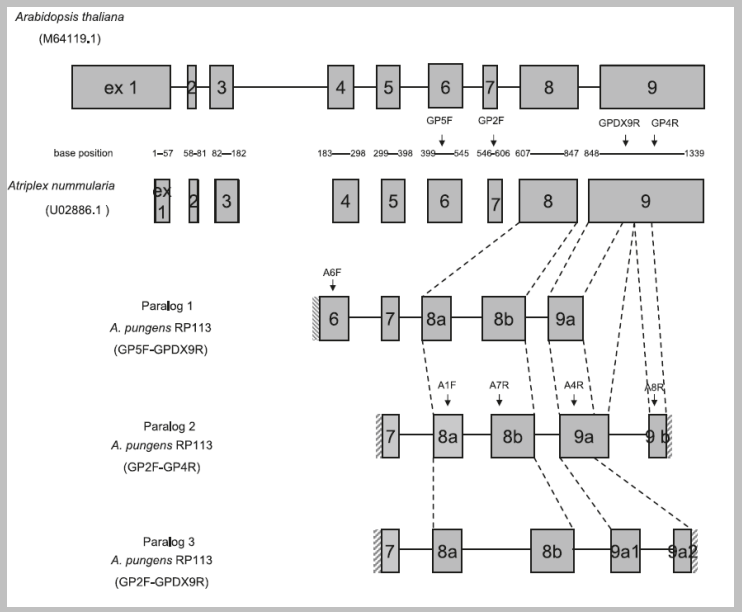
Asplenium

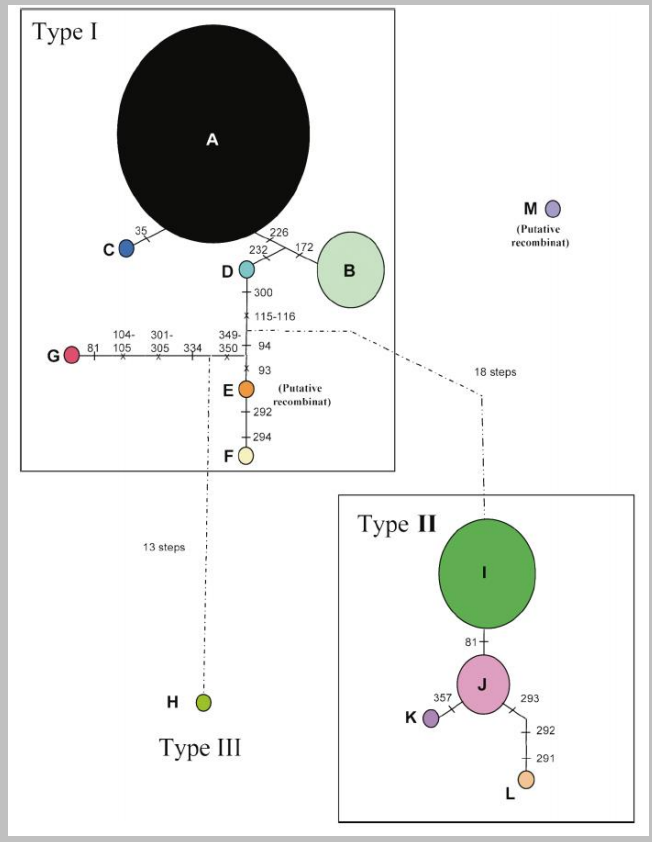
4x, 8x

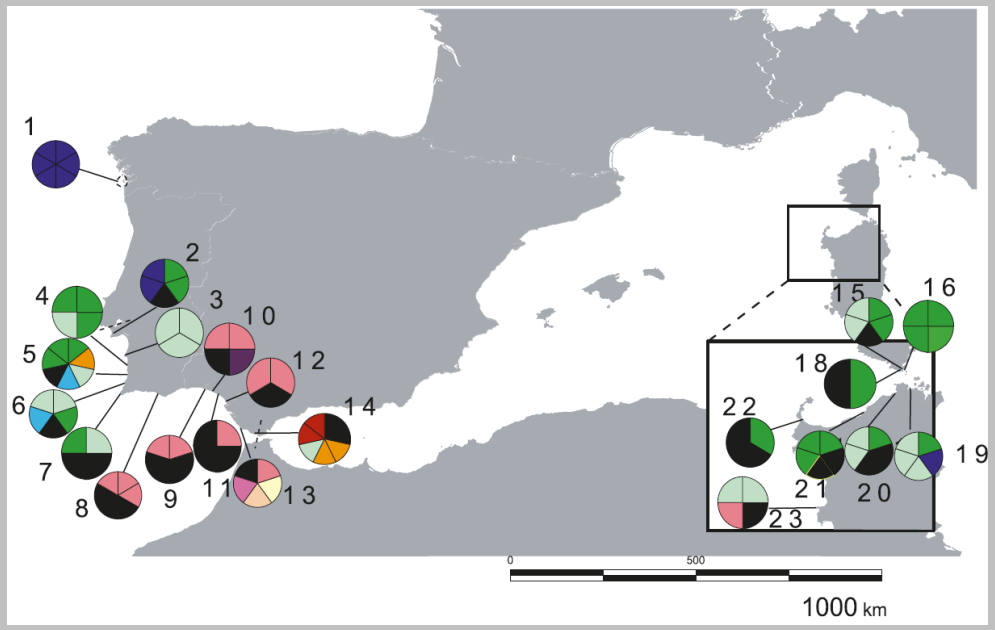
LFY, 2nd intron, cpDNA

Shepherd et al. 2008

Phylogeography with low-copy gene







Armeria pungens
*Gap*C
Pineiro et al. 2008

Systematic study

Salas-Leiva D.E.S. et al. (2013): Phylogeny of the cycads based on multiple single-copy nuclear genes: congruence of concatenated parsimony, likelihood and species tree inference methods. *Annals of Botany* 112(7): 1263–1278

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