

Guidelines for the Choice of Sequences for Molecular Plant Taxonomy

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Abstract

This chapter presents an overview of the major plant DNA sequences and molecular methods available for plant taxonomy. Guidelines are provided for the choice of sequences and methods to be used, based on the DNA compartment (nuclear, chloroplastic, mitochondrial), evolutionary mechanisms, and the level of taxonomic differentiation of the plants under survey.

Key words Nuclear DNA, Chloroplast DNA, Mitochondrial DNA, Repeated DNA, Low-copy DNA, Evolution, Molecular plant taxonomy

1 The Plant Genome and Regions Targeted for Molecular Plant Taxonomy

The nuclear genome in plants is very complex as in many eukaryotes, as illustrated by the “C-value enigma” [1, 2]: although the overall haploid DNA content (C-value) increases with apparent biological complexity, some species have more DNA in their haploid genome than some more complex organisms. Also, for a similar level of biological complexity, some species, such as plants, exhibit a surprisingly wide range of C-values (Fig. 1). This apparent discrepancy can be in part explained by the occurrence of variable amounts of repetitive DNA in the genomes (Fig. 1), most of which is constituted by noncoding sequences [3].

1.1 Repeated Nuclear DNA Sequences

Most nuclear sequences targeted in molecular taxonomy experiments belong to the category of highly repetitive DNA. Nuclear ribosomal RNA genes (nrDNA) are tandemly (side by side) repeated and located at a few loci in plant genomes [4–6] (Fig. 2). These, and particularly the ITS (internal transcribed spacers) [7, 8], have long been widely used for resolving plant taxonomic issues, initially using restriction analysis and then sequencing (Chapter 7). Microsatellite markers, also called STR (simple tandem repeats) or SSR (simple sequence repeats), are tandem repeats of small

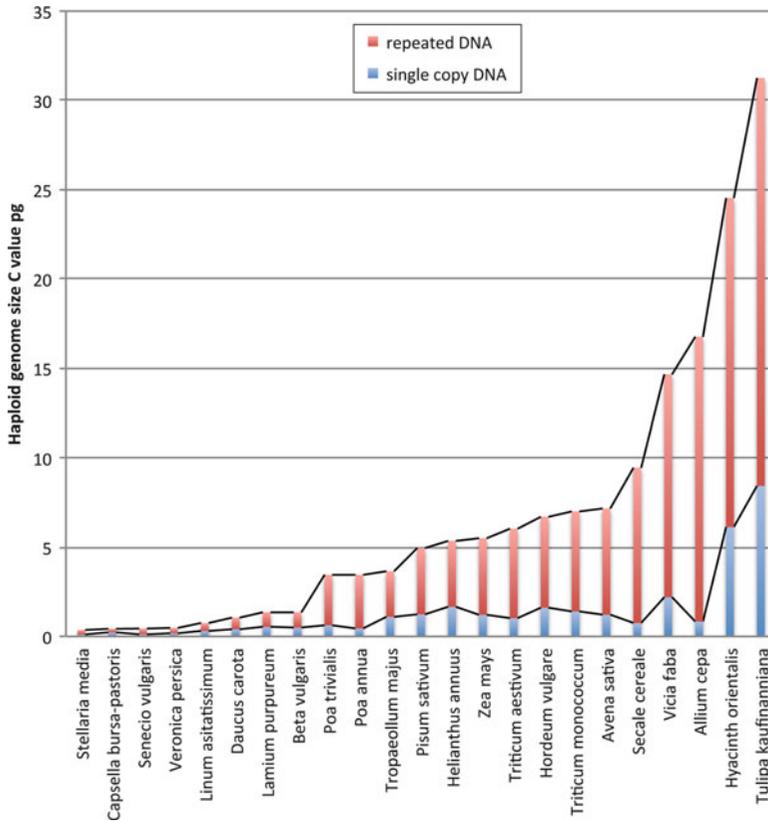


Fig. 1 Haploid genome size and composition for different plant species (graph built from data taken in [50])

stretches of noncoding DNA sequences, discovered in 1989 and named after the discovery of minisatellite and satellite DNA which exhibited a similar tandem structure [9] (Fig. 2). Microsatellites are widely used for diversity studies either as powerful single locus markers easily amplified by PCR (Chapter 9) or in multi-locus profiling methods revealing regions between adjacent SSRs (inter-simple sequence repeats, ISSR) by PCR amplification (Fig. 3) (Chapter 11).

Transposable elements (TEs) represent another class of repeated DNA, but the elements are dispersed across the genome instead of being tandemly repeated and these also can represent an important part of the plant nuclear genome. Two main classes of TEs exist in plants: class I retrotransposons (which transpose through a RNA copy which is then reverse transcribed into DNA and inserted at a new site) and class II transposons (which are excised and transpose directly as DNA) (Fig. 4). Class I retrotransposons are more numerous in genomes than class II as the original copy of the transposon is retained after transposition. In maize, for example, LTR (long terminal repeats)-retrotransposons represent up to 70 % of the nuclear genome [10]. Class I transposons (either LTR-retrotransposons or non-LTR-SINEs (short interspersed nuclear

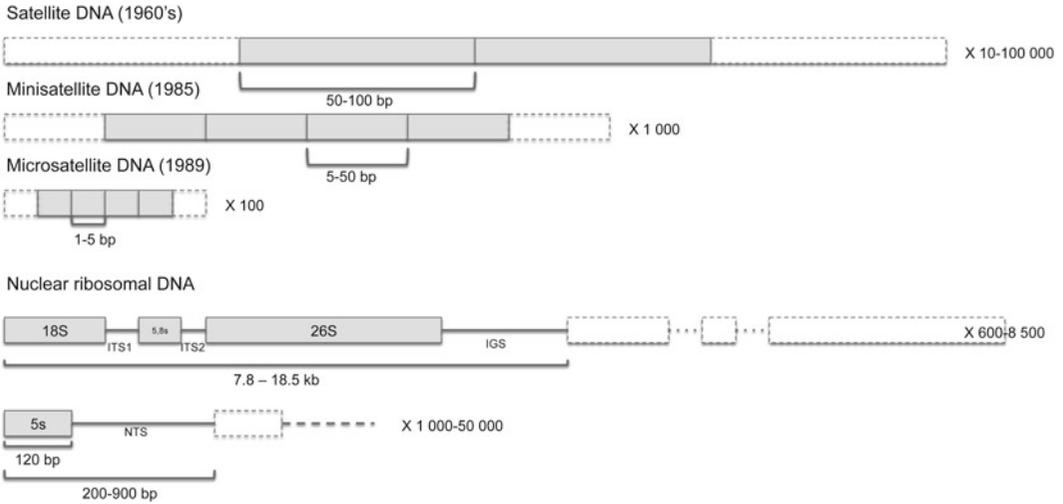


Fig. 2 Tandem repeat sequences used for molecular plant taxonomy: structure and number of tandem repeats

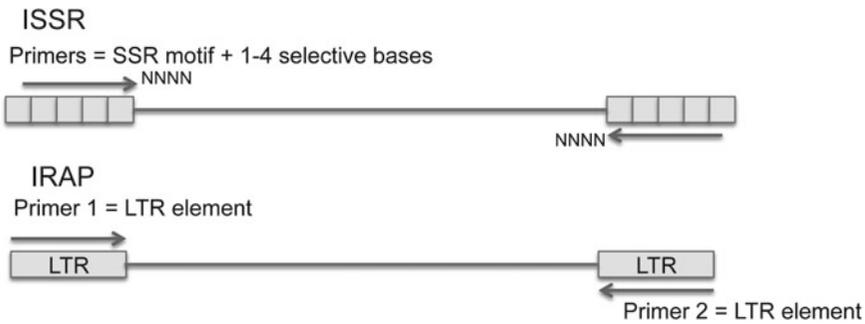


Fig. 3 Multi-locus profiling methods using either SSR or retrotransposons as anchors

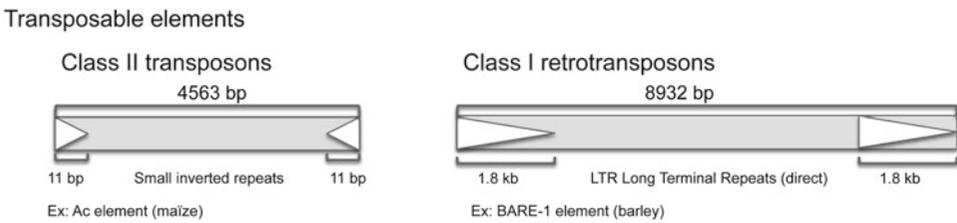


Fig. 4 Transposable elements in plants

elements)) are now commonly used for phylogenetic and taxonomic studies. Many studies use multi-locus PCR-based profiling methods such as inter-retrotransposons amplified polymorphism (IRAP) (Fig. 3) which amplifies regions between adjacent LTR repeats of LTR-retrotransposons (Chapter 12). As described for eukaryotes [11], SINEs are considered as perfect markers and are also being sequenced to build robust plant phylogenies although these studies

SNP

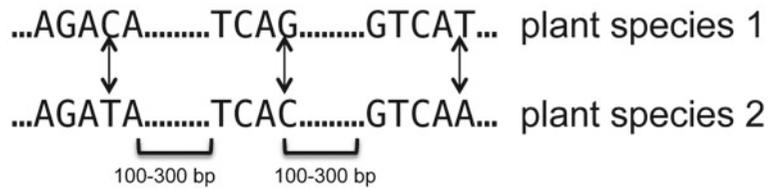


Fig. 5 SNPs in plants

are restricted to a limited number of plant species (mainly cultivated species) for which SINEs have been described and isolated [12, 13].

1.2 Low-Copy Nuclear Genes

Contrary to ribosomal DNA nuclear genes, low-copy nuclear genes (LCNG) do not suffer the possible disadvantages of concerted evolution, paralogy, and homoplasy [7, 8, 14, 15] that can be particularly limiting for taxonomic studies in recent hybrids or polyploids (*see* Chapter 7). However, care must be taken if using low-copy genes belonging to multigenic families for which paralogy and concerted evolution issues might still be problematic [16].

Despite their advantages, single-copy nuclear genes have not so much been used for plant taxonomy as they are much more difficult to isolate and characterize, contrary to cpDNA or ribosomal nuclear DNA which has been extensively used because they are easily amplified using universal primers [16] (Chapters 5 and 7). This situation is however changing rapidly [15, 17]. With the availability and affordability of new sequencing technologies [18], it is now becoming feasible to assess variations at a wide range of single or low-copy genes in nuclear genomes giving access to powerful phylogenomic analyses [19, 20]. Rather than sequencing complete genes for all accessions, single nucleotide polymorphisms (SNPs) can be searched for and analyzed (Fig. 5) (Chapter 8), and various sequence-based SNP assays can then be designed [21].

1.3 Anonymous Sequences

Many molecular technologies also rely on revealing variations at randomly picked anonymous sequences in genomes. In such techniques, the importance is not the nature of the target sequence itself, but rather the high throughput of the technology, which allows revealing numerous markers (loci) covering the genome. The aim is to give an as accurate as possible view of the genome diversity. This is the case for amplified fragment length polymorphism (AFLP) (Chapter 11), randomly amplified polymorphic DNA (RAPD) (Chapter 10), and associated techniques (Fig. 6) which use primers with arbitrary sequence to amplify genomic regions. Some multi-locus profiling techniques use a combination of AFLP associated with the revelation of either SSR loci (selective amplification of microsatellite polymorphic loci, SAMPL) (Chapter 11) or

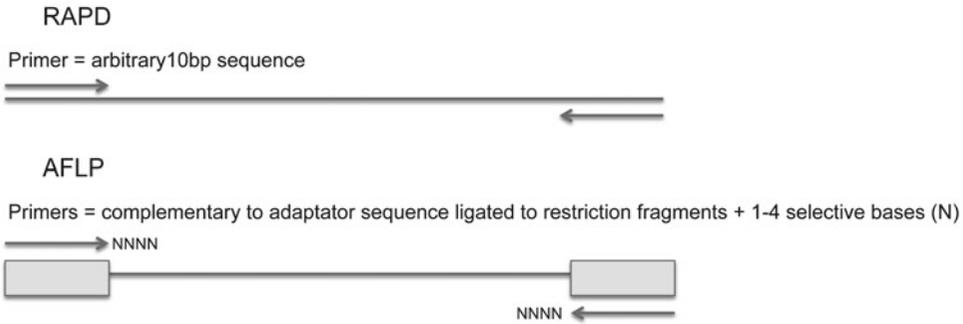


Fig. 6 Markers revealed by RAPD and AFLP

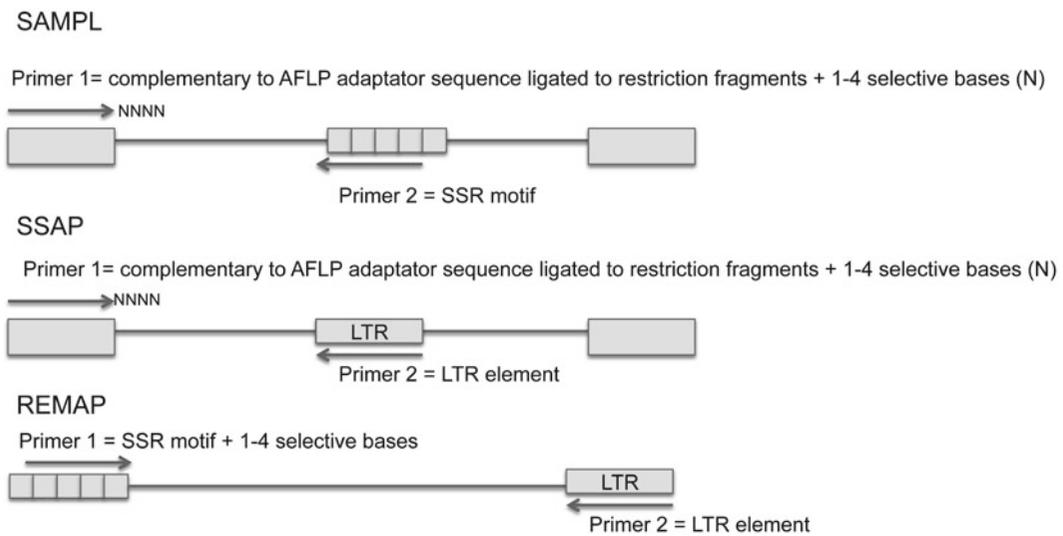


Fig. 7 Multi-locus profiling methods using a combination of anchors based on AFLP, SSR, or LTR

LTR-retrotransposons (sequence-specific amplified polymorphism, SSAP) (Chapter 12); others combine anchor primers in both SSR and LTR-retrotransposon conserved regions (retrotransposon-microsatellite amplification polymorphism, REMAP) (Chapter 12) (Fig. 7). A new technology termed DArT (diversity array technology) [22, 23] was also recently developed. It uses high-throughput DNA-array technology to reveal polymorphisms between individuals without any prior sequence information knowledge and is therefore applicable to non-model species.

1.4 Organellar DNA

In plants, the genetic information is also carried on the mitochondrial as well as chloroplast genomes (organellar DNA). Although mitochondrial genome (mtDNA) has received little attention in plant taxonomic studies (but *see* Chapter 6) because of numerous rearrangements and low levels of sequence variation, chloroplast DNA (cpDNA) has been widely used in molecular plant phylogeny (Chapter 5) through sequencing, restriction, or chromatography.

2 Evolutionary Considerations

The molecular clock hypothesis suggests that nucleotide substitutions occur at a roughly constant rate between and within evolutionary lineages across time [24] and has given rise to different models to estimate this evolutionary rate and its constancy [25]. According to the neutral theory of evolution, the speed of this rate (the amount of molecular variation accumulated over time) depends on the structural and functional constraints of the molecule [26]. This can be illustrated by noncoding DNA molecules (such as introns or intergenic sequences) evolving much faster than coding DNA as they accumulate more variations over time. Also it is now well admitted that third position bases in codons evolve much faster than other positions due to the redundancy of the genetic code [26] (less functional constraint on the third position allows for more variations to accumulate over time). Most markers generated using RAPD or AFLP technology have been shown by genome-mapping experiments to cluster around the centromeres of chromosomes [27–30], a heterochromatin region with mainly noncoding sequences. Consequently, these markers often reveal an important amount of variation.

The evolutionary rate of a molecule is also driven by its evolutionary mechanisms. Microsatellite markers are the most variable molecules known to date. They are mostly noncoding molecules and vary in length (due to the variation in the number of tandem repetitions or VNTR) due to replication slippage (SMM model [31]), which occurs at a high frequency (10^{-6} to 10^{-2}) in plants [32]. Microsatellites with shorter motifs and greater number of repeats are more prone to replication slippage and are thus the most variable [33]. ISSR, SAMPL, and REMAP markers, which use a microsatellite locus as an anchor, also benefit to a certain extent from the microsatellite length hyper-variability. Minisatellite sequences that tend to evolve through unequal crossing-over (IAM model [31]), which is a phenomenon with greater frequency than simple base mutations, also vary in length (i.e., number of tandem repeats) with great frequency. Both types of sequences have been for this reason used for generating powerful DNA fingerprints in human [34, 35] and subsequently in numerous species including plants.

Most tandemly repeated sequences in the genome evolve through what is known as “concerted evolution” or molecular drive [36, 37], which involves mechanisms such as unequal crossing-over or biased gene conversion. Over time, the sequences that compose a family of tandem repeats within an individual genome are maintained similar, thanks to this concerted evolution [6, 38–40]. Such sequences also tend to be maintained identical through close lineages within a species and will therefore display a slower evolutionary rate than molecules without concerted evolution.

In the cpDNA, like in the nDNA, intergenic noncoding sequences evolve faster than coding sequences. For example, by testing seven different sequences on a range of land plants, [41] classified these sequences by order of variation as follows: *psbK-psbI* > *trnH-psbA* > *atpF-atpH* > *matK* > *rpoB* > *rpoC1* > *rbcL*, illustrating that cpDNA intergenic regions are more variable than coding regions. Globally, in plants, organellar sequences evolve more slowly than nuclear sequences: mtDNA evolves three times slower than cpDNA, which in turn evolves two times slower than nDNA (average synonymous substitution rates per site per year for mtDNA and cpDNA are $0.2\text{--}1.0 \times 10^{-9}$ and $1.0\text{--}3.0 \times 10^{-9}$, respectively [42]) (Chapter 6). Even the most variable of intergenic regions in cpDNA is less variable than nuclear ITS: ITS reveals 2.81 % sequence divergence in a range of plant families compared to 1.24 % divergence for *trnH-psbA*, one of the most variable intergenic cpDNA regions [43].

Finally, class I TEs are good classification criteria to evaluate species phylogenetic relationships; their mode of transposition (“copy–paste” mode) makes them numerous and implies no ambiguity in the ancestral state definition, which is, for a given locus, the absence of TE [11, 12]. Class II TEs are less appropriate for phylogenetic issues mainly because of their direct mode of transposition (“cut–paste” mode) which, associated with possibilities of horizontal transfer, can lead to erroneous classifications (TE phylogenetic trees not concordant with species phylogenetic history) [44, 45].

3 Choice of Sequences for Molecular Taxonomy

These evolutionary considerations are of primary importance when one wants to use a DNA sequence to infer phylogenetic relationships between a set of accessions. Two questions have to be considered when starting a molecular taxonomy project:

1. What is the degree of time divergence between the accessions under study? Do we want to address variations at the intra-specific level (population level) or are we comparing species from the same genus or different genera from the same family or above?
2. What is the evolutionary rate of the molecule that will be used to infer relationships between accessions?

The rule to keep in mind is that the further we need to go in evolutionary times, the slower the molecule must evolve. Going too far with too much diverging sequences will lead to homoplasy (characters identical by state, not by descent) through convergence or reversion. On the opposite, slow evolving sequences will not be enough in discriminating for groups that have evolved recently (Fig. 8).

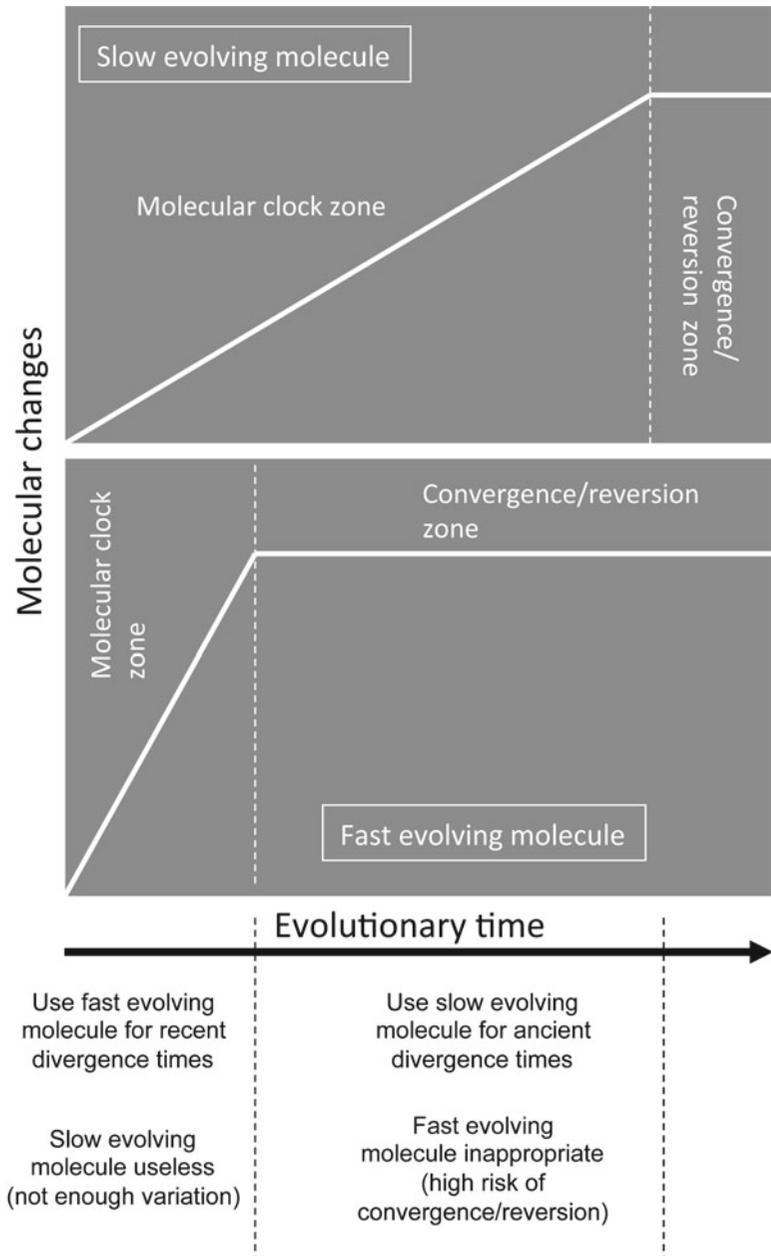


Fig. 8 Illustration of the usefulness of rapidly evolving versus slow evolving sequences in molecular taxonomy assessment of recently or anciently diverged groups. The curvilinear relationship between molecular changes and time is represented theoretically starting with a constant accumulation rate (molecular clock hypothesis) which plateaus as a consequence of the saturation of the sequence over time. The faster the sequence evolves, the faster the plateau is reached

Figure 9 illustrates this rule: if a very slow evolving sequence is used, it might be unable to differentiate the two hypothetical species under study (Fig. 9a). A sequence with an intermediate rate of evolution and concerted evolution would allow the identification

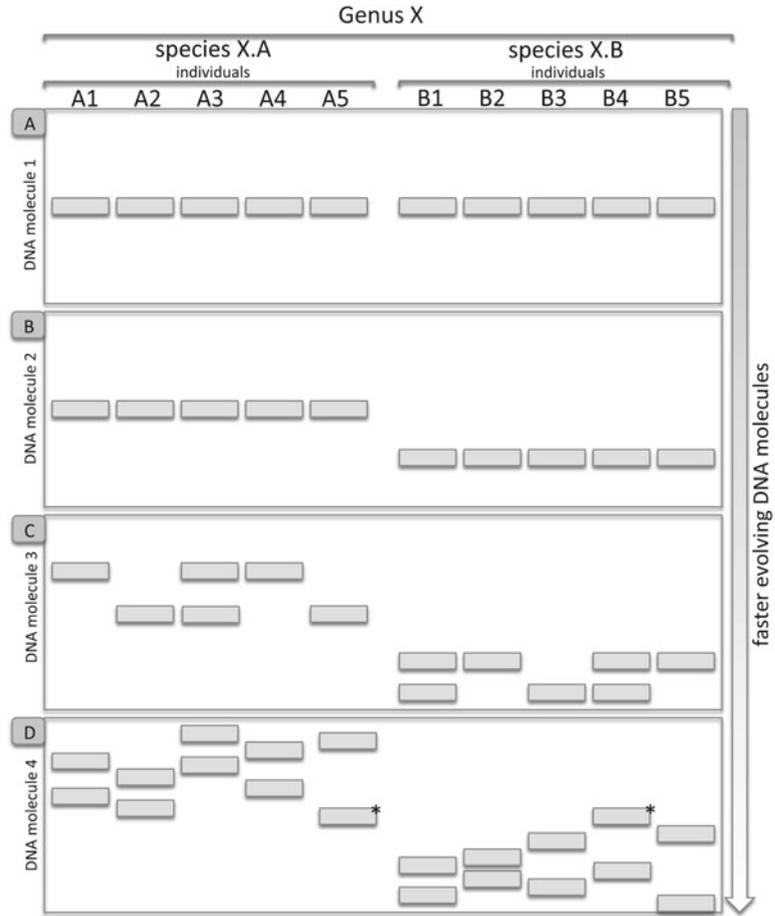


Fig. 9 Illustration of the differentiation power of DNA molecules depending on their evolutionary rates

of each species, but would be unable to reveal any intraspecific variability (Fig. 9b). To reach such level of informativeness, one would need to use a single-copy gene (Fig. 9c) or a microsatellite marker (Fig. 9d), but the latter, due to high evolutionary rate, may generate homoplasy (*) which could lead to erroneous interpretations if comparing species A and B, as individual B4 would appear more related to species A than to individuals from species B. Such rapidly evolving sequences are therefore not appropriate for studying relationships at too high taxonomic levels.

Guidelines for the choice of sequences to be used depending on the level of taxonomic divergence are illustrated (Fig. 10). It must be kept in mind that the level of taxonomic differentiation can vary considerably depending on the species group; therefore one always needs to perform preliminary tests of various sequences on a representative subset of accessions to assess their power in differentiating our own individuals, species, or genera of interest.

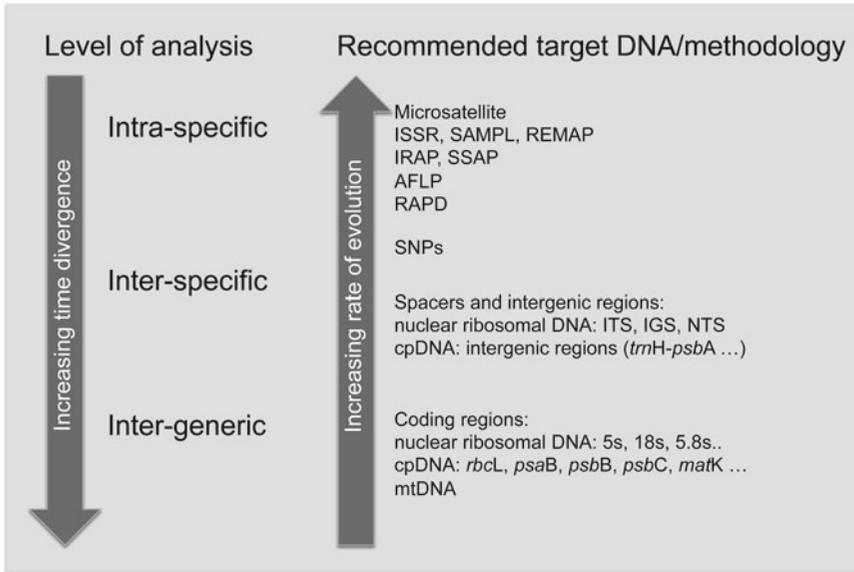


Fig. 10 General guidelines for the choice of markers to be used for plant taxonomy

4 Genetic Considerations

Knowledge of the mode of inheritance of the molecules under study is also of great importance. Nuclear sequences are inherited in a Mendelian fashion, with contribution from both parents. Organellar (chloroplastic and mitochondrial) sequences are almost always uniparentally inherited (generally maternally, but see [46]). This can have important consequences when building a molecular phylogeny, as individuals or species of interspecific origin will appear inconsistently on the trees generated with each type of markers (Fig. 11): a species B of hybrid origin will be grouped with its mother species A using cytoplasmic sequences, although it will appear different from it on the nuclear tree.

AFLP, RAPD, ISSR, and other multi-locus profiling methods generate >90 % dominant markers [47]. The polymorphism revealed is mainly due to mutations in the hybridization region of one of the primers, leading to either amplification of the locus (presence) or null allele (absence of amplification), i.e., a dominant system (Fig. 12). Consequently, such methods provide only biallelic markers.

On the other hand, microsatellites are very powerful monolocus markers as they are multiallelic and codominant (Fig. 12). They are indeed widely used in molecular ecology and population genetic studies as heterozygous loci can be clearly identified and allelic frequencies can be calculated to test for deviations from Hardy-Weinberg equilibrium. One microsatellite multiallelic marker provides as much genetic information as four to ten biallelic AFLP markers [48].

SNP markers are monolocus, codominant, but are biallelic. Indeed, they evolve through the infinite sites (IAM) model: given

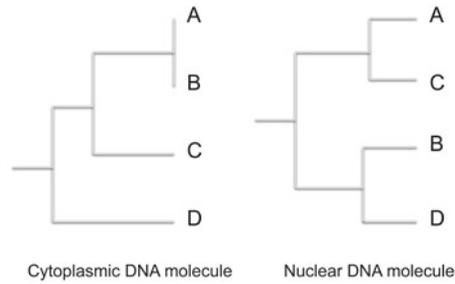


Fig. 11 A hypothetical phylogeny involving a hybrid species B whose maternal parent is species A

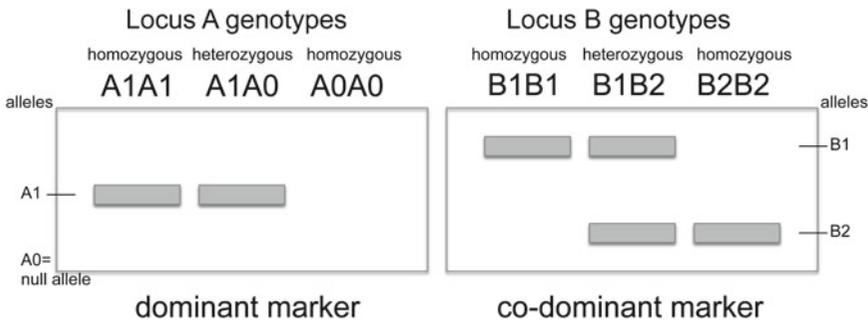


Fig. 12 Different genetic profiles: dominant versus codominant markers

the low rate of substitutions in genomes (the average synonymous substitution rate in plant nuclear genome is about $5.0\text{--}30.0 \times 10^{-9}$ per site per year [42]), the probability of more than one mutation at a given site is negligible; therefore each SNP is almost exclusively found only with two different states among the four possible (A, G, C, or T). For population genetic studies, it will be necessary to compensate the low allelic diversity of SNP markers by increasing the number of studied loci (2–6 times more SNP locus are needed as compared to microsatellites [49] to reach the same level of informativeness).

5 Analyzing Results

Fragment length data (different band sizes visualized and coded after electrophoretic separation) will only be analyzed using distance-based methods (e.g., UPGMA or neighbor joining), whereas sequence data will be analyzed either using distance-based methods or more powerfully using character-based methods (e.g., using maximum parsimony or maximum likelihood), allowing true phylogenetic trees to be constructed rather than phenetic trees (Chapter 13). Always remember that the tree built is a sequence tree, not a species trees. For all the reasons discussed above, using different sequences can lead to different trees reflecting the different evolutionary patterns of the sequences under study.

6 Further Exploration: Chromosomal Organization

In plants, genome organization is very complex and polyploidy can be an important speciation mode. It will be almost impossible to differentiate, for example, a diploid species from a related autopolyploid species in a phylogenetic tree. Molecular taxonomy can be greatly enhanced in some taxonomic complex plant groups by assessing not only phylogenetic relationships but also genome organization to determine introgression, hybridization, or polyploidization (by analyzing either chromosomes or simply genome size) (Chapters 14–16).

References

- Gregory T (2001) Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biol Rev* 76:65–101
- Thomas C (1971) The genetic organization of chromosomes. *Annu Rev Genet* 5:237–256
- Schmidt T, Heslop-Harrison JS (1998) Genomes, genes and junk: the large-scale organization of plant chromosomes. *Trends Plant Sci* 3:195–199
- Hamby RK, Zimmer EA (1992) Ribosomal RNA as a phylogenetic tool in plant systematics. In: Soltis PS, Soltis DE, Doyle JJ (eds) *Molecular systematics of plants*. Chapman & Hall, New York
- Schaal BA, Learn GH (1988) Ribosomal DNA variations between and among plant populations. *Ann Mo Bot Gard* 75:1207–1216
- Hillis DM, Dixon MT (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453
- Alvarez IA, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phylogenet Evol* 29:417–434
- Poczai P, Hyvönen J (2010) Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. *Mol Biol Rep* 37:1897–1912
- Ellegren H (2004) Microsatellites: simple sequences with complex evolution. *Nat Rev Genet* 5:435–445
- SanMiguel P, Bennetzen JL (1998) Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. *Ann Bot* 82:37–44
- Ray DA (2007) SINEs of progress: mobile element applications to molecular ecology. *Mol Ecol* 16:19–33
- Deragon JM, Zhang X (2006) Short interspersed elements (SINEs) in plants: origin, classification, and use as phylogenetic markers. *Syst Biol* 55:949–956
- Schmidt T (1999) LINEs, SINEs and repetitive DNA: non-LTR retrotransposons in plant genomes. *Plant Mol Biol* 40:903–910
- Feliner GN, Rosselló JA (2007) Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Mol Phylogenet Evol* 44:911–919
- Zimmer EA, Wen J (2013) Using nuclear gene data for plant phylogenetics: progress and prospects. *Mol Phylogenet Evol* 66:539–550
- Small RL, Cronn RC, Wendel JF (2004) Use of nuclear genes for phylogeny reconstruction in plants. *Aust Syst Bot* 17:145–170
- Schlötterer C (2004) The evolution of molecular markers – just a matter of fashion? *Nat Rev Genet* 5:63–69
- Hudson ME (2008) Sequencing breakthroughs for genomic ecology and evolutionary biology. *Mol Ecol Resour* 8:3–17
- Grover CE, Salmon A, Wendel JF (2012) Targeted sequence capture as a powerful tool for evolutionary analysis. *Am J Bot* 99:312–319
- Timme RE, Bachvaroff TR, Delwiche CF (2012) Broad phylogenomic sampling and the sister lineage of land plants. *PLoS One* 7:1–8
- Syvänen AC (2001) Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet* 2:930–942
- Jaccoud D, Peng K, Feinstein D, Killian A (2001) Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Res* 29:1–7
- James KE, Schneider H, Ansell SW, Evers M, Robba L, Uszynski G, Pedersen N, Newton AE, Russell SJ, Vogel JC, Kilian A (2008) Diversity arrays technology (DART) for pan-genomic evolutionary studies of non-model organisms. *PLoS One* 3:1–11
- Zuckermandl E, Pauling L (1965) Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel H (eds) *Evolving genes and proteins*. Academic, New York, pp 97–166
- Arbogast BS, Edwards SV, Wakeley J, Beerli P, Slowinski JB (2002) Estimating divergence

- times from molecular data on phylogenetic and population genetic timescales. *Annu Rev Ecol Syst* 33:707–740
26. Kimura M, Ohta T (1974) On some principles governing molecular evolution*(population genetics/mutational pressure/negative selection/random drift). *Proc Natl Acad Sci USA* 71:2848–2852
 27. Saliba-Colombani V, Causse M, Gervais L, Philouze J (2000) Efficiency of RFLP, RAPD, and AFLP markers for the construction of an intraspecific map of the tomato genome. *Genome* 43:29–40
 28. Qi X, Stam P, Lindhout P (1998) Use of locus-specific AFLP markers to construct a high-density molecular map in barley. *Theor Appl Genet* 96:376–384
 29. Saal B, Wricke G (2002) Clustering of amplified fragment length polymorphism markers in a linkage map of rye. *Plant Breed* 121:117–123
 30. Young WP, Schuppert JM, Keim P (1999) DNA methylation and AFLP marker distribution in the soybean genome. *Theor Appl Genet* 99:785–792
 31. Shriver MO, Jin L, Chakraborty R, Boerwinkle E (1993) VNTR allele frequency distributions under the stepwise mutation model: a computer simulation approach. *Genetics* 134:983–993
 32. Bhargava A, Fuentes FF (2010) Mutational dynamics of microsatellites. *Mol Biotechnol* 44:250–266
 33. Buschiazzo E, Gemmell NJ (2006) The rise, fall and renaissance of microsatellites in eukaryotic genomes. *BioEssays* 28:1040–1050
 34. Jeffreys AJ, Wilson V, Thein SL (1985) Individual-specific fingerprints of human DNA. *Nature* 316:76–79
 35. Jobling MA, Gill P (2004) Encoded evidence: DNA in forensic analysis. *Nat Rev Genet* 5:739–752
 36. Dover G (1982) Molecular drive: a cohesive mode of species evolution. *Nature* 299:111–117
 37. Dover G (1994) Concerted evolution, molecular drive and natural selection. *Curr Biol* 4:1165–1166
 38. Pohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. *Gene* 409:72–82
 39. Nei M, Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. *Annu Rev Genet* 39:121–152
 40. Ganley ARD, Kobayashi T (2007) Highly efficient concerted evolution in the ribosomal DNA repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data. *Genome Res* 17:184–191
 41. Hollingsworth ML, Clark A, Forrest LL, Richardson J, Pennington RT, Long DG, Cowan RO, Chase MW, Gaudeul M, Hollingsworth PM (2009) Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. *Mol Ecol Resour* 9:439–457
 42. Wolfe KH, Li W-H, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. *PNAS* 84:9054–9058
 43. Kress WJ, Wurdack KJ, Zimmer EA, Weig LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. *PNAS* 102:8369–8374
 44. Capy P, Anxolabehere D, Langin T (1994) The strange phylogenies of transposable elements: are horizontal transfers the only explanation. *Trends Genet* 7–12
 45. Syvanen M (1994) Horizontal gene transfer: evidence and possible consequences. *Annu Rev Genet* 28:237–261
 46. McCauley DE, Sunby AK, Bailey MF, Welch ME (2007) Inheritance of chloroplast DNA is not strictly maternal in *Silene vulgaris* (Caryophyllaceae): evidence from experimental crosses and natural populations. *Am J Bot* 94:1333–1337
 47. Bensch S, Akesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Mol Ecol* 14
 48. Mariette S, Corre VL, Austerlitz F, Kremer A (2002) Sampling within the genome for measuring within population diversity: trade-offs between markers. *Mol Ecol* 11:1145–1156
 49. Morin PA, Luikart G, Wayne RK, The SNP workshop group (2004) SNPs in ecology, evolution and conservation. *Trends Ecol Evol* 19:208–216
 50. Flavell R, Bennett M, Smith J, Smith D (1974) Genome size and the proportion of repeated nucleotide sequence DNA in plants. *Biochem Genet* 12:257–259



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