# Utility of the Mitochondrial Genome in Plant Taxonomic Studies

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**SUMMARY**

Size, structure and sequence content lability of plant mitochondrial genome (mtDNA) across species has sharply limited its use in taxonomic studies. Historically, mtDNA variation has been first investigated with RFLPs, while the development of universal primers then allowed studying sequence polymorphisms within short genomic regions (< 3 kb). The recent advent of NGS technologies now offers new opportunities by greatly facilitating the assembly of longer mtDNA regions, and even full mitogenomes. Phylogenetic works aiming at comparing signals from different genomic compartments (i.e. nucleus, chloroplast and mitochondria) have been developed on a few plant lineages, and have been shown especially relevant in groups with contrasted inheritance of organelle genomes. This chapter first addresses the main characteristics of mtDNA and the application offered in taxonomic studies. It then presents a working sequencing protocol based on NGS data to be routinely used in mtDNA-based phylogenetic studies.

## 1. Introduction

**Origin of mitochondrial genomes.** The mitochondrial genome originated from a eubacterial ancestor. More specifically, it is now widely accepted that the mitochondria originated from a single endosymbiotic event which involved a α-proteobacteria-like organism and a common cellular ancestor of eukaryotes (Gray et al., 2001). This symbiotic relationship between a primitive eukaryote nucleus and an aerobic bacteria−the future mitochondria−has enabled the eukaryote to evolve an aerobic lifestyle. In relation with this new endosymbiotic habit, the “resident” mitochondrial genome has undergone a reductive evolution, characterized, for example, by a loss of coding capacities (Andersson and Kurland, 1998). The gene content reduction of mitochondrial genomes has been

primarily attributable to either gene loss or mitochondria-to-nucleus gene transfers (Palmer et al., 2000). This process has been interpreted as a consequence of deleterious accumulation in organelle genomes (Andersson and Kurland, 1998), and as a necessity for multi-cellular organism to keep the function originally coded by organelle genomes. Gene transfer from the mitochondria to the nucleus has been demonstrated to be an ongoing process in plants (Adams et al., 2000), which explains that the mitochondrial gene content varies across distantly related plant lineages (Adams et al., 2002; Adams and Palmer, 2003).

**Mitochondrial structure and genome size**. Land plant mitochondrial DNA (mtDNA or mitogenome) are usually represented as circular maps (e.g. Lonsdale et al., 1984; Palmer and Shields, 1984), yet mtDNA structure is highly variable and should be seen as a complex, dynamic mixture of forms (Palmer and Herbon, 1988; Backert et al., 1997; Nielsen and Nielsen, 2017; Kozik et al., 2019). Indeed, plant mtDNA is composed of multiple alternative subgenomic forms (isoforms) that can recombine due to the presence of one or more pairs of large repeats. This population of isoforms is thus composed of highly complex structures, linear molecules, open circles of variable size and supercoiled molecules. Such a structural lability leads to some difficulties for the definition of universal primers and for the full assembly of mitogenome (but see below).

In sharp contrast to the relative small and homogenous size of animal (usually between 16 and 20 kb; Boore, 1999) and fungal mitogenome (between 19 and 100 kb; Bullerwell and Gray, 2004), land plant mitogenome is large and variable in size (between 104 kb in the moss *Anomodon rugelii* and 11.3 Mb in the angiosperm *Silene conica*; Sloan et al., 2012). This important size variation can be observed between closely-related species (Alverson et al., 2010). Thus, comparative study demonstrated that mtDNA size variation within the *Silene* genus might be related to variable mutation rates, with an accumulation of non-coding sequences in mitogenomes presenting higher mutation rates (Sloan et al., 2012). Angiosperm mtDNA size variation among species is mainly related to differences in the size of non-coding regions, especially large repeats and alien sequences acquired from intercellular gene transfer and/or interspecific horizontal gene transfer (Marienfeld et al., 1999; Choi et al., 2019). Plastid-derived (the so-called *mtpt* regions) and nuclear-derived nucleotide sequences represent respectively from 1 to 12% and from 0.1 to 13.4% of the mitogenome (Kubo and Newton, 2008; Marienfeld et al., 1999; Wang et al., 2007).

Lateral gene transfers (LGT) resulting from mitogenome fusion between distantly related species have been documented, especially in epiphytic and holoparasitic plants (Rice et al., 2013; Gandini and Sanchez-Puerta, 2017; Sanchez-Puerta et al., 2017).

**Gene arrangement and the importance of homologous recombination**. Due to the presence of numerous repeated regions and to the putative co-existence of more than one type of mitochondrial genome in a cell (heteroplasmy; Kmiec et al., 2006), recombinations are frequent within the mtDNA and gene arrangement (synteny) in higher plants vary enormously (Schuster and Brennicke, 1994). Besides the large size, recombination activity is the most distinctive feature of these genomes (Woloszynska, 2010). Gene arrangement of mtDNA in higher plants varies enormously due to the presence of repeated regions, source of recombination within and between mtDNA genomes (Schuster and Brennicke, 1994). Cole et al. (2018) have demonstrated that rates of mtDNA rearrangements can be very variable between species from the same genus. Importantly, rearrangements lead to the possibility to generate chimerical genes, potentially involved in some traits of interest, such as the cytoplasmic male sterility (Schnable and Wise, 1998). Fortunately, mtDNA coding sequences are highly conserved, facilitating the identification of conserved regions within which universal primers can be defined (Demesure et al., 1995; Duminil et al., 2002) and that can be easily assembled using next generation sequencing data.

**Molecular evolutionary rates of the mtDNA.** In opposition to animals, plant mitochondrial genes evolve very slowly. Comparing coding sequences silent (synonymous) substitution rates among plant genomes [i.e., nuclear DNA (nrDNA), chloroplast DNA (cpDNA), and mtDNA], Wolfe et al. (1987) have demonstrated that mitochondrial genes evolve three times slower ($0.2 - 1.1 \times 10^{-9}$ substitutions per synonymous site per year) than chloroplast genes ($1.1 - 2.9 \times 10^{-9}$ substitutions per synonymous site per year), which in turn evolve two times slower than the nuclear genes (up to $31.5 \times 10^{-9}$ substitutions per synonymous site per year). These results were further confirmed by Gaut et al. (1996) on the comparison of genes from all three genomes between maize and rice. Interestingly, as outlined by Muse (2000), the similarity obtained between Wolfe and Gaut studies, albeit different levels of evolutionary divergence were addressed, might indicate that plant nucleotide substitution features have been constant over higher plant evolution. This is somewhat nuanced by Drouin et al. (2008) that based

on the comparison of 12 genes in 27 seed plant species demonstrated that the overall relative rate of synonymous substitutions of mitochondrial, chloroplast and nuclear genes is 1:3:10 if averaged across studied seed plants, 1:2:4 in gymnosperms, 1:3:16 in angiosperms, and that they go up to 1:3:20 in basal angiosperms. Thought this low molecular evolutionary rate of mitochondrial genes appeared to concern most of plant species, some exceptions were demonstrated (e.g. within *Pelargonium, Plantago*, *Silene*; Cho et al., 2004; Parkinson et al., 2005; Sloan et al., 2012). The generality of slow synonymous sequence evolution in mitogenomes has been investigated across a large and taxonomically widely distributed set of seed plants (Mower et al., 2007). According to this study, earlier finding were confirmed for roughly 80-90% of the studied species, indicating that a surprising number of taxa depart from this common pattern (presenting either an accelerated or a slower synonymous substitution rates). Moreover, Mower et al. (2007) demonstrated that both patterns of faster and slower evolutionary rates can be found within the same species at different genes supporting the idea that all genes evolve independently of one another. Albeit this observation might be related to different artefacts (see the discussion in Mower et al., 2007), independent evidence for mutation rate variation among genes were acquired (Barr et al., 2007; Cole et al., 2018). Therefore, the general idea remains that mitochondrial genes evolve at a slow rate, and that mtDNA polymorphism is very low within one-species and even between closely-related species. This explains the limit use of mtDNA in phylogeography and phylogeny, thought the demonstration of molecular rate heterogeneity within some plant lineages (Barr et al., 2007; Mower et al., 2007) might support the idea that it is worth investigating if this pattern holds true for a given species.

**Mode of inheritance of the mitochondrial genome.** Mitochondrial genomes are generally uniparentally inherited (usually maternally) in seed plants, though some species have been shown to present a paternal (some coniferous species) or a biparental inheritance (Birky, 2001). Uniparental inheritance of organelle genomes is more and more seen as an evolutionarily unstable trait (Greiner et al., 2015). The uniparental inheritance of organellar genomes, together with slow molecular evolutionary rate, explain their success as molecular markers in phylogeography studies (reviewed in Petit and Vendramin, 2007). Mode of inheritance has been shown theoretically and experimentally to have a major effect on the estimation of the among-population genetic differentiation: maternally-inherited genomes generally experience more subdivision than paternally- or

biparentally-inherited ones (Petit et al., 2005). Thus, in conifers, $G_{ST}$ is almost always larger at mtDNA markers than at cpDNA markers, while it is nearly similar at both markers in angiosperms where both are generally maternally-inherited (Petit et al., 2005).

## MITOCHONDRIAL MOLECULAR MARKERS in PHYLOGENETICS and TAXONOMY

**Genomic resources: complete mitochondrial genome**. The first land plant complete mitogenome was obtained for the liverwort *Marchantia polymorpha* (Oda et al., 1992).The number of plant species whom the complete mtDNA sequence is available is now 221 (Figure 1). In comparison, 4020 plant species were completely sequenced for their chloroplast genome (data compiled in October 2019 according to https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/)

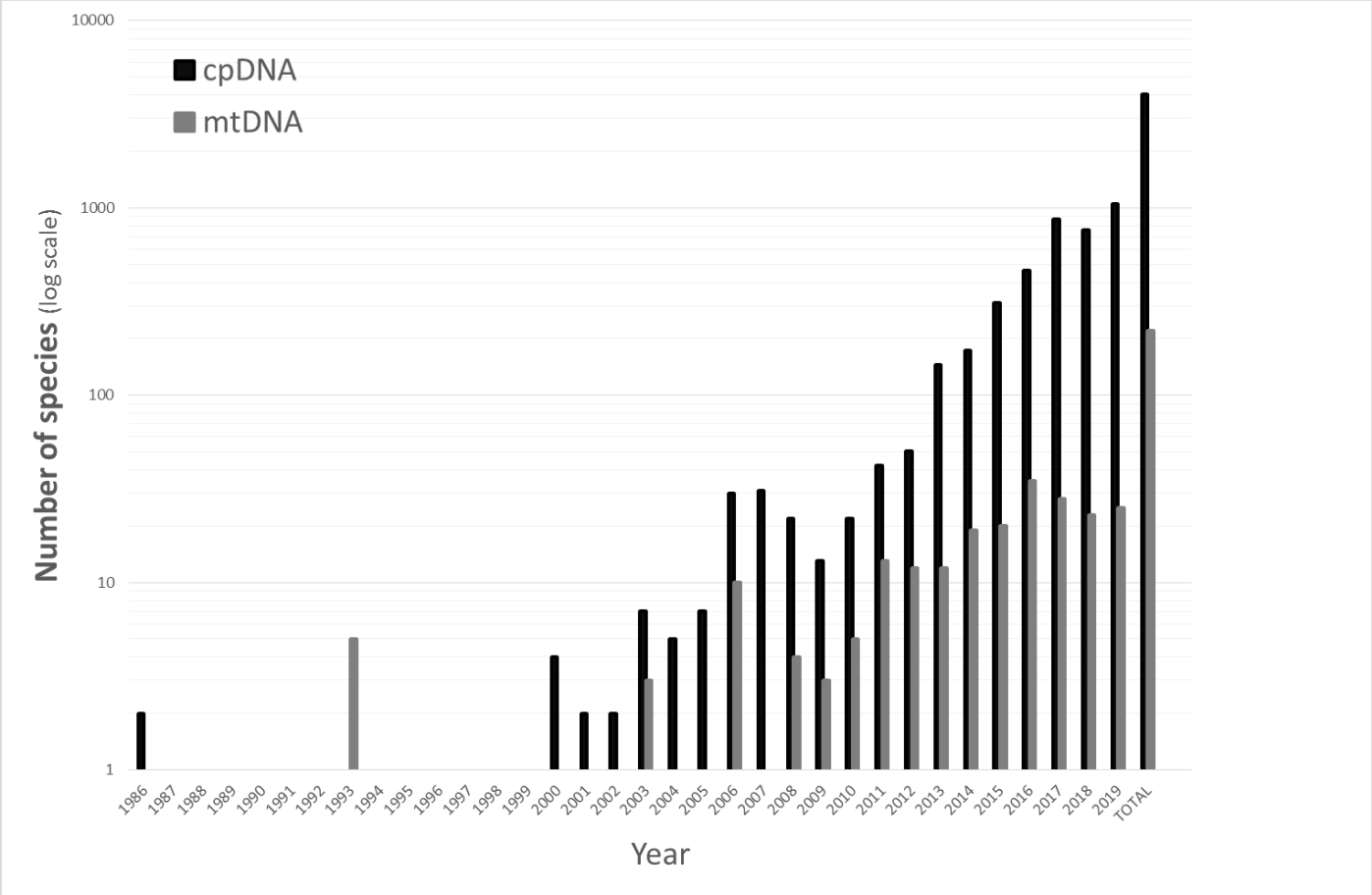


**Figure 1:** Evolution of the number of plant species (log scale) for which mtDNA and cpDNA complete sequences were obtained over time.

**Use of mtDNA in phylogeography and phylogenetics.** Due to the absence of recombination within the cpDNA molecule and its slightly faster evolutionary rate, cpDNA-derived molecular markers were more popular in phylogeography and phylogenetic studies than mtDNA-derived ones. However, the acquisition of mtDNA data can also be very interesting to characterize species evolutionary history, notably in addition to and comparison with cpDNA data (Dumolin-Lapegue et al., 1999; Govindarajulu et al., 2015; Hosaka and Sanetomo, 2009; Jaramillo-Correa et al., 2006; Liepelt et al., 2002; Rydin et al., 2017; Van de Paer et al., 2018).

Historically, mtDNA variation was first evaluated with the Restriction Fragments Length Polymorphisms technique (RFLPs; Levings and Pring, 1976; Quetier and Vedel, 1977). This approach allowed revealing large rearrangements that were particularly useful to investigate linkage disequilibrium between chloroplast and mitochondrial polymorphisms within some species due to the common maternal inheritance of cytoplasmic organelles (Desplanque et al., 2000; Besnard et al., 2002). With the advent of PCR methods, the amplification of mtDNA loci with universal primers become then more popular (Demesure et al., 1995; Duminil et al., 2002; Dumolin-Lapegue et al., 1997; Froelicher et al., 2011; Jaramillo-Correa et al., 2003; Jeandroz et al., 2002). Universal primers were defined in conserved regions (exons) and were used to amplify mtDNA regions with conserved micro-synteny. Polymorphism in amplified fragment has been revealed with various methods: PCR-RFLP (Boonruangrod et al., 2008; Godbout et al., 2005; San Jose-Maldia et al., 2009; Moriguchi et al., 2009; Naydenov et al., 2007), RFLP-SSR (Burban and Petit, 2003; Godbout et al., 2005; Jaramillo-Correa et al., 2003), mtDNA-SSR (Hosaka and Sanetomo, 2009), the variable number of tandem repeats (VNTR) in minisatelite regions (Bastien et al., 2003; Fievet et al., 2007; Honma et al., 2011; Yoshida et al., 2012), and finally in DNA sequences (Avtzis and Aravanopoulos, 2011; Eckert et al., 2008; Edwards et al., 2005; Goodall-Copestake et al., 2010; Gugger et al., 2011). The choice of the candidate loci was limited and was depending on the taxonomic level addressed by the phylogenetic study. At the lowest taxonomic level (intra-specific or among closely-related species), intergenic or intronic sequences were particularly interesting. Instead, at higher taxonomic level (polymorphism among a set of species in a phylogenetic framework), generally focus on coding sequences. Based on such approaches, only a few studies have combined cpDNA and mtDNA polymorphisms to reconstruct the phylogeography of species (whereas cpDNA has remained the most frequently used marker). Yet, some contrasted phylogeographic patterns have been

revealed in some species. This was particularly true in conifers, in which cpDNA and mtDNA can be transmitted by different parents, providing complementary information on species pollen- and seed-mediated gene flow (Jaramillo-Correa et al., 2006; Liepelt et al., 2002).

We have now entered the high-throughput sequencing area. This offers new opportunities for the use of mtDNA in phylogeography and phylogenetics. Given the low polymorphism nature of mtDNA, acquiring long mtDNA fragments, or even full mtDNA genomes allows capturing useful genomic variations. However, these new technologies also brings along new challenges, notably in terms of mtDNA assembly and comparison between species. As mentioned above, the mtDNA assembly is complicated by the presence of numerous short and long repeated fragments (some reaching more than 10 kb), as well as exogenous fragments (intercellular gene transfers and/or LGTs; Marienfeld et al., 1999). Assembly of non-repeated sequences is feasible on relatively long contigs (> 10 kb; Donnelly et al., 2017; Malé et al., 2014), but the integration of all fragments in a master chromosome can be challenging (Van de Paer et al., 2016). The combined use of long reads (Oxford Nanopore Technologies) with short reads (Illumina technologies) can help mtDNA assembly (Wang, Song, et al., 2018; Kozik et al., 2019), with the possibility to observe recombination in long repeated regions (alternative conformation of mitogenomes). The parallel reconstruction of plastid and mitochondrial genomes is also necessary to resolve the assembly of *mtpt* regions; by applying a step-by-step approach, it was possible to reconstruct the master chromosomes in Oleaceae even on very fragmented DNA from old herbarium specimens (Van de Paer et al., 2016, 2018). All parts of mitogenome are, however, not informative for phylogeographic or taxonomical studies, since some regions are not shared between species, even at the genus level, whereas some homologous regions are not necessarily orthologous (because could be recurrently transferred, in particular from the plastome). As a consequence, mtDNA phylogenies should be reconstructed with the pan-mitogenome or the core fragments (shared by all mitogenomes; e.g. Wang et al., 2018), and thus focus on regions with functional genes (i.e. exons and introns). Using this approach, the comparison of phylogenetic topologies obtained with cpDNA and mtDNA have shown subtle differences in several groups (Cole et al., 2018; Fonseca and Lohmann, 2019; Van de Paer et al., 2018; Wang, Song, et al., 2018), but it can also demonstrate strong informative incongruences (Govindarajulu et al., 2015; Rydin et al., 2017). At the species level, the use of complete mitogenomes could be possible, but beforehand, orthology of *mtpt* regions has to be verified (by testing, for each *mtpt*, phylogenetic clustering of accessions of the same species compared to other genera). Such a strategy has been applied on the olive tree, and allowed resolving the phylogeny of maternally

inherited genome, which was not possible with the plastome only (Van de Paer et al., 2018). Overall, at this taxonomical level, more information was recovered from the whole mtDNA (> 0.6 Mb) than from the plastome (ca. 0.15 Mb).

This chapter finally provides a working sequencing protocol based on NGS data to assemble mtDNA sequences for phylogenetic studies [see Duminil (2014) for methods based on a PCR approach]. The protocol is defined to sequence conserved mtDNA regions (i.e. parts of the pan-mitogenome) among distantly related species. The approach is relatively simple and is based on shot-gun sequencing of total genomic DNA (the so called "genome skimming" approach; Straub et al., 2012). Considering the high number of cytoplasmic organites in a cell, organelle DNA is expected to be highly represented in such data (ca. 5-10% of total genomic reads). These data can thus be used for the assembly of different genomic regions from both the nuclear genome (especially the ribosomal DNA cluster that is highly repeated) and the organellar genomes (e.g. Straub et al., 2012; Malé et al., 2014; Van de Paer et al., 2018; Fonseca and Lohmann, 2019; Olofsson et al., 2019).

## 2. Materials

Micropipette and TIPs for dispensing from 1 to 1000 µL
Microtubes (1.5 and 2 mL)
Material for grinding leaves or seeds
Kit for DNA extraction
Heating block for 2-mL tubes
Centrifuge
dsDNA quantitation kit, fluorescence assay
Kit for library preparation
Sequencer

## 3. Methods

For studied samples, total genomic DNA has to be extracted with an appropriate protocol, that allows recovering a relatively clean extract with enough double-stranded DNA (at least 50 ng). For instance, the BioSprint 15 DNA Plant Kit (Qiagen Inc.) has been successfully used for distinct plant groups, including relatively old museum specimens (e.g. Zedane et al., 2016). With this method, each leaf sample needs to be ground in a 2-mL tube containing three tungsten beads with a TissueLyser (Qiagen Inc., Texas) before to be extracted. Double-stranded DNA concentration of final extracts is then quantified (e.g. PicoGreen or Qubit, ThermoFisher). Between 50 and 500 ng of double stranded DNA are usually used to construct sequencing libraries with a kit (e.g. TruSeq DNA Sample, Illumina). For herbarium specimens, DNA libraries can be generated without prior DNA sonication because the DNA is supposedly moderately to highly degraded (e.g. Zedane et al., 2016; Olofsson et al., 2019). Purified fragments are then A-tailed and ligated to sequencing indexed adapters. Fragments are enriched with 8-12 cycles of PCR before library quantification and validation. Each sample is then paired-end sequenced (usually reads of 150 bp) on a sequencer lane (e.g. HiSeq or NovaSeq, Illumina). A pool of 24 to 48 libraries can be hybridized to the flow cell. Bridge amplification is performed to generate clusters, and paired-end reads are collected on the sequencer.

Before starting the assembly, duplicated reads have to be removed and overlapping paired-end reads can be merged. Because no automated approach of full plant mitogenome assembly based on short-read data is currently available, we recommend to focus on the conserved mtDNA regions and map reads on a reference that has been previously defined on

complete mitogenome. For instance, in the Oleaceae family, 36 protein-coding genes and 16 introns, for a total of ca. 55 kb) have been targeted (Van de Paer et al., 2018). Mapping merged and unmerged reads against the reference can be done with various approach, for instance with the Geneious software (Kearse et al., 2012). We recommend to check the quality of the mapping and the homogeneity of the sequencing depth to detect any chimeric genes or duplication/deletion among the targeted regions.

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