COMMENTARY

Plant Mitochondria are a Riddle Wrapped in a Mystery Inside an Enigma

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Abstract

A fundamental paradox motivates the study of plant mitochondrial genomics: the mutation rate is very low (lower than in the nucleus) but the rearrangement rate is high. A landmark paper published in *Journal of Molecular Evolution* in 1988 established these facts and revealed the paradox. Jeffrey Palmer and Laura Herbon did a prodigious amount of work in the pre-genome sequencing era to identify both the high frequency of rearrangements between closely related species, and the low frequency of mutations, observations that have now been confirmed many times by sequencing. This paper was also the first to use molecular data on rearrangements as a phylogenetic trait to build a parsimonious tree. The work was a technical tour-de-force, its findings are still at the heart of plant mitochondrial genomics, and the underlying molecular mechanisms that produce this paradox are still not completely understood.

A 1988 paper published in *Journal of Molecular Evolution* presented a fundamental paradox that still dominates the field of plant mitochondrial genome evolution (Palmer and Herbon 1988; https://doi.org/10.1007/BF02143500). These were the early days of molecular biology and genomics; Sanger sequencing was only 11 years old (Sanger et al. 1977), and the first complete flowering plant mitochondrial sequence (*Arabidopsis thaliana*) was still 9 years away (Unseld et al. 1997). In the same year, 1988, the Office of Human Genome Research was established at NIH with the goal of eventually sequencing the human genome. Mammalian mitochondrial genomes had been sequenced, and were known to be small, invariant in gene order, and to have a high mutation rate (Brown et al. 1979), although it was known that the gene order was different in *Drosophila yakuba* than in mammals (Clary et al. 1982; Clary and Wolstenholme 1985). However, Arnold Bendich and co-workers had established that the mitochondrial genomes of flowering plants were very large and variable in size (Ward et al. 1981), the other important conundrum that still vexes the field. It was also known that much of the genome was non-protein-coding, and that gene orders are not well-conserved (Makaroff and Palmer 1987). The time was ripe for a thorough study of plant mitochondrial genome organization to understand the evolution of these two different types of changes: point mutations and rearrangements. To solve the problem, species needed to be compared that were different enough to observe a reasonable number of changes, but not so distantly related as to encounter multi-state problems. Jeffrey Palmer, then at the University of Michigan, chose six species in the Brassicaceae: radish, turnip, cabbage, rutabaga, black mustard, and white mustard, (*Raphanus sativus*, *Brassica campestris* (now known as *Brassica rapa*), *Brassica oleracea*, *Brassica napus*, *Brassica nigra*, and *Brassica hirta* (now known as *Sinapis alba*), respectively).

In the present day, where samples can be sent off for complete genome sequencing, it is hard to appreciate the significant amount of work that had to be done for these comparisons. PCR was not widely used yet, because publication of the method for amplification using thermostable polymerases occurred that same year (Saiki et al. 1988). Instead, Palmer and Herbon purified mitochondrial DNA from green tissues and characterized the structures by restriction mapping. With these genomes being over 200 kb, this required multiple rounds of restriction digestion, gel electrophoresis, Southern blotting, and hybridization with cloned fragments primarily from *B. rapa*. The restriction maps were then compared to find their similarities and differences.

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Three findings emerged from this work, any one of which would have made it an important paper. First, they were able to compare the synteny of the genomes, and build a phylogenetic tree based on the trait of genome rearrangements, rather than synonymous substitutions. Second, they showed that long, non-tandem repeated sequences are not conserved even in close relatives, and are not completely universal. The one result I look most forward to seeing confirmed or refuted by complete genome sequencing is that *Sinapis alba* lacks a long, non-tandem repeat (*Sinapis arvensis* has one). Third, they were able to measure point mutation rates by examining the differences in restriction site cutting, presumably due to single base changes. By comparing the number of point mutations to the number of rearrangements separating related species, they were able to show that rearrangements occur at a higher rate than point mutations, a startling and counter-intuitive result.

Figure 1 illustrates the complex and detailed data provided. These restriction maps were produced by first purifying the mitochondrial DNA, then digesting it with multiple restriction enzymes, and resolving the different size bands by gel electrophoresis. Because most of the fragments of the *Brassica rapa* genome had been cloned, bands in the other species could be identified by blotting the gel and hybridizing with multiple *B. rapa* probes (Southern 1975). Each of these five enzymes cuts the genome approximately 30 times,

so Palmer and Herbon analyzed 150 bands for each species. They were also able to identify large non-tandem repeats responsible for frequent isomerization of the genomes by recombination (2 kb in *B. rapa*, *napus* and *oleracea*, 9.7 kb in *R. sativa*, and 6.6 kb in *B. nigra*). One can only imagine how difficult it was to deal with the background caused by hybridization from the ubiquitous non-tandem repeats of up to about 500 bp found in these genomes (*B. nigra*, for example, has over 80 such repeats larger than 50 bp (Wynn and Christensen 2019)). Nevertheless, in spite of these technical difficulties, their restriction maps are excellent. Their *Bgl*I map of *B. rapa* compared to accession JF920285 (Chang et al. 2011) shows that the sequence agrees with the restriction map to a remarkable degree, both in sizes and order.

In Fig. 2, they compared the maps of the relatively closely related species *B. rapa*, *B. napus,* and *B. oleracea*. They were able to define multiple rearrangements between these species which defined blocks of synteny and specific inversions that scrambled the genomes. *B. napus* is an allotetraploid of *B. rapa* and *B. oleracea* (Cai et al. 2014; U N 1935). Mitochondrial sequences (Grewe et al. 2014) suggest that *B. napus* allotetraploids have occurred multiple times with different maternal parents, leading to several different cultivars, and agreeing with the Palmer lab's previous findings using chloroplast DNA (Palmer et al. 1983). The map of the *B. napus* cultivar (rutabaga) in Palmer and Herbon (1988)

Fig. 1 Originally published as Fig. 1 in Palmer and Herbon (1988). Restriction maps of the master chromosome of three *Brassica* mtDNAs. The circular maps are shown linearized at a *Bgl*I site within the 2-kb repeat. Arrows indicate the two copies of this repeat. Numbers between arrows indicate sizes of subgenomic circles. Restriction fragment summations are given at the right of the maps. Filled triangles indicate eight length mutations, which are arbitrarily shown only as insertions. Variable restrictions sites resulting from length mutation and point mutation are indicated with filled circles and stars, respectively. The *B. campestris* map is modified from (Palmer and Shields 1984)
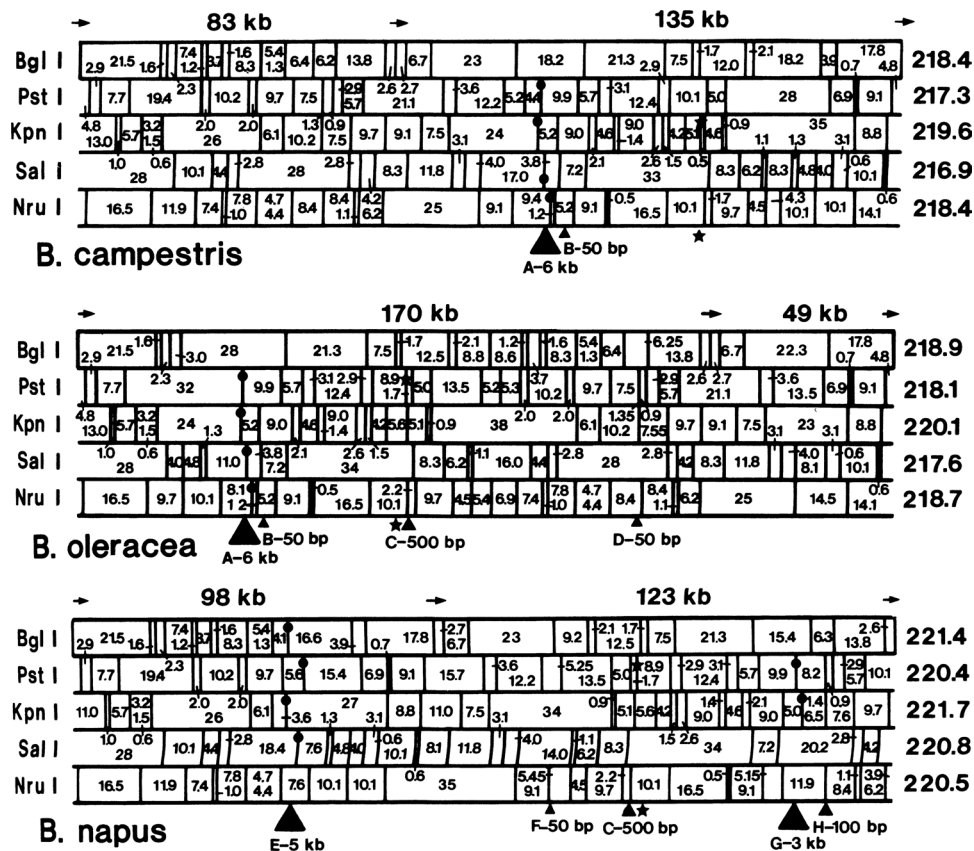
Fig. 2 Originally published as Fig. 3 in Palmer and Herbon (1988). Relative arrangement of similar sequences in three *Brassica* mtDNAs. Numbered horizontal arrows above and below the complete *Sal*I maps indicate blocks of sequences whose arrangement has been conserved between genomes. The crossing lines between *Sal*I fragments connect regions of cross-hybridization. Double-headed vertical arrows indicate inversion endpoints. Horizontal arrowheads indicate recombination repeats
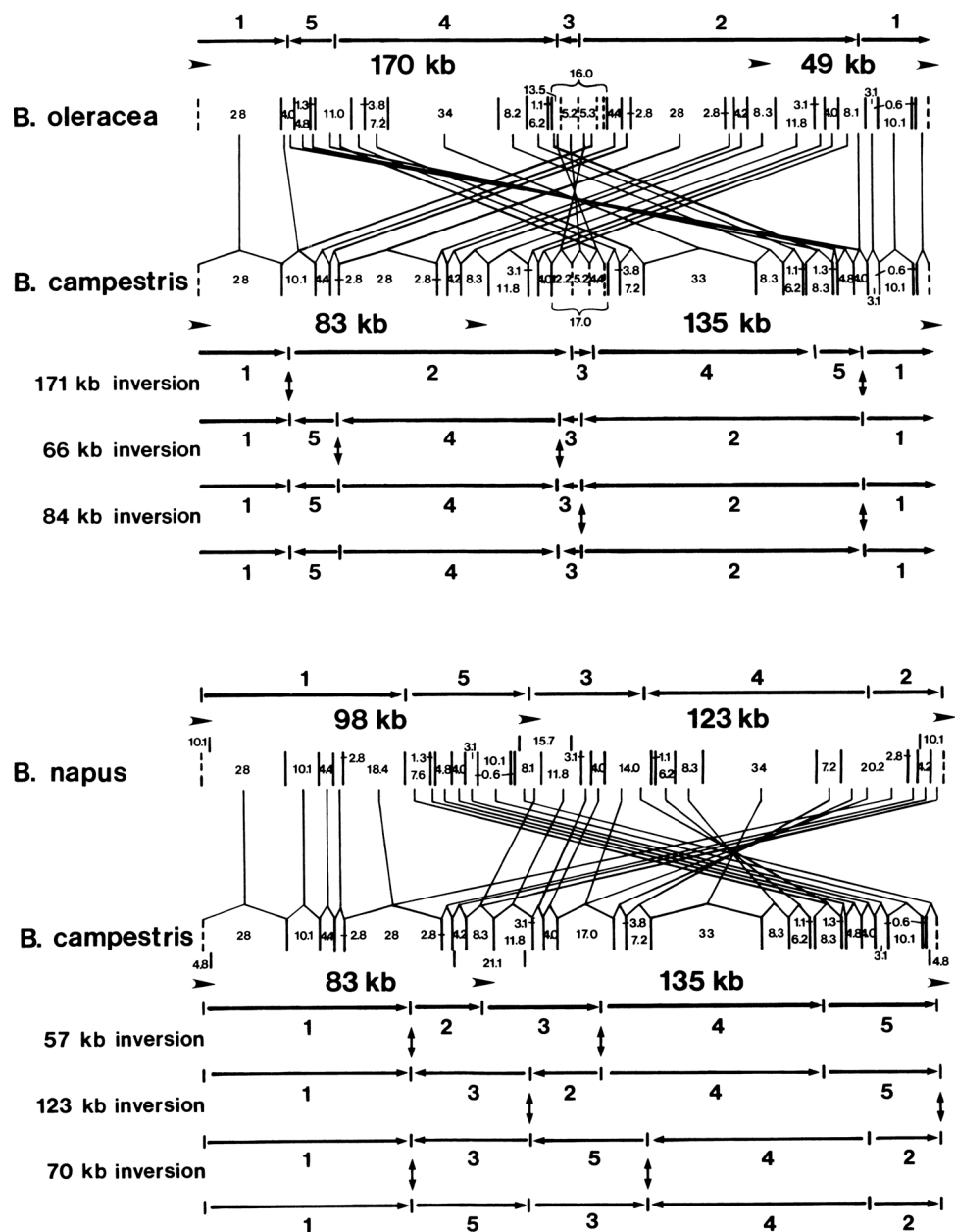
appears to be most similar to the sequenced cultivar *B. napus* napus, accession number AP006444.1 (Handa 2003) which is very similar to the *B. oleracea* sequenced genome (Grewe et al. 2014).

Palmer and Herbon further compared the arrangements of these genomes with the additional species mentioned above, and were able to characterize the rearrangements that separated the genomes. A parsimony-based analysis using rearrangements as a trait allowed them to construct the phylogenetic tree in Fig. 3. An analysis of this type would be done today using sequence data, with software tools such as GRAPPA (Bader et al. 2001) or GRIMM (Tesler 2002) that only became available in the decade

following the Palmer and Herbon (1988) paper. Chromosomal rearrangements had been used as a taxonomic trait previously in Drosophila, using the banding patterns of polytene chromosomes (Dobzhansky and Sturtevant 1938; Sturtevant and Dobzhansky 1936). However, I am not aware of any earlier attempt to use molecular biology data to determine rearrangements for phylogenetic purposes than the work of Palmer and Herbon (1988). It is also not clear to me which method is more tedious. Palmer and Herbon's findings showed that it could be done, opening the door to subsequent work in metazoans and fungi (Blanchette et al. 1999; Boore and Brown 1998; Sankoff

Fig. 3 Originally published as Fig. 7 in Palmer and Herbon ([1988](#)). Phylogenetic history of mtDNA rearrangements in *Brassica*. *Top* Cytoplasmic phylogeny for *Brassica* based on cp-DNA restriction site mutations (Palmer et al. [1983](#)). This phylogeny is cladistically derived and is not intended to convey divergence times. Numbers of rearrangements are given relative to the reference genome, *B. campestris*, except for the ten rearrangements that have been shown (Makaroff and Palmer [1988](#)) to distinguish the CMS and fertile mtDNAs of *R. sativa*. *Bottom* Sizes of master chromosomes and (where present) subgenomic circles resulting from high frequency recombination at the indicated recombination repeats
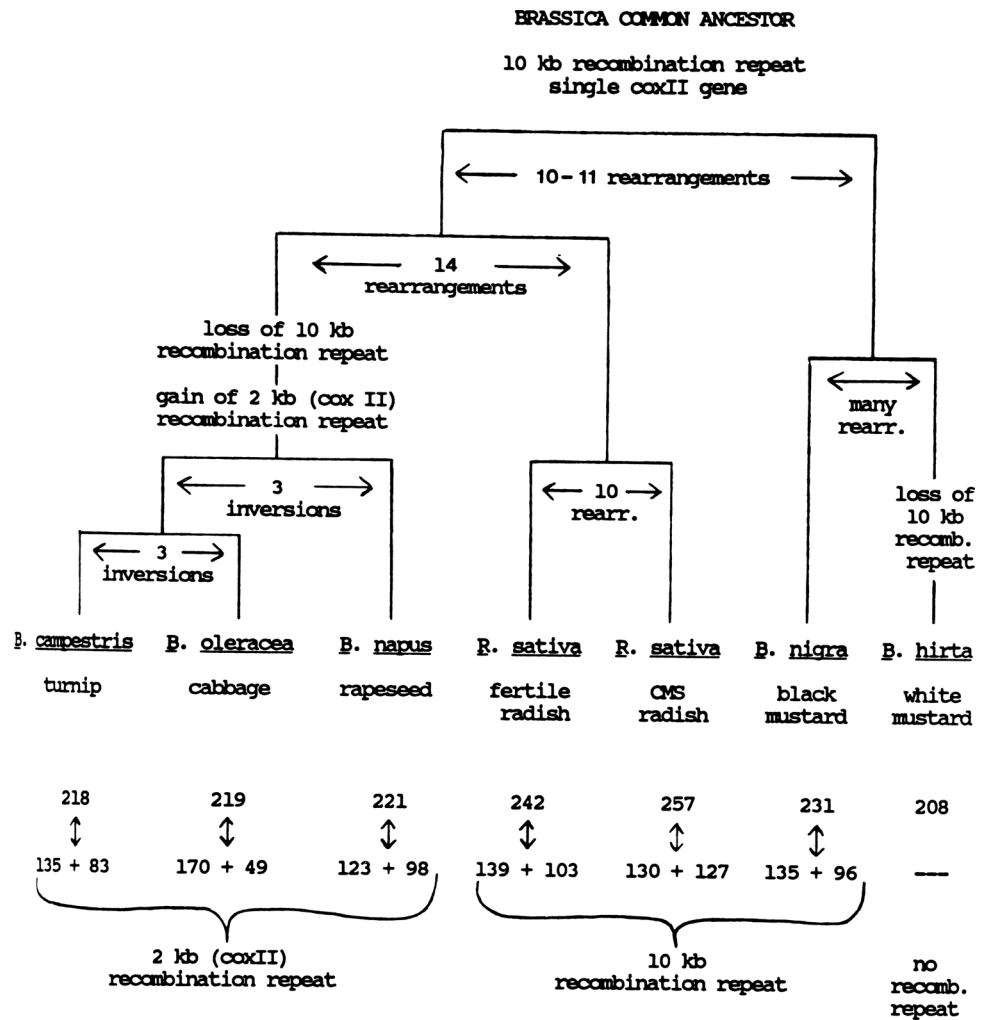
et al. [1992](#); Smith et al. [1993](#)) and ultimately in flowering plants (Cole et al. [2018](#); Darracq et al. [2010](#)).

The final results that make this a landmark paper are mapping Restriction Fragment Length Polymorphisms (RFLPs) in order to measure mutation rates. For this, Palmer and Herbon added an additional 15 restriction enzymes to their analysis, and used Southern blotting against double-digested DNA to find RFLPs. What they found was remarkable. Point mutation rates are very low in plant mitochondria, lower even than in the nucleus, while the rearrangement rates are quite high, essentially scrambling the genomes of even close relatives.

Paradoxes are important in science. Molecular biology was founded by scientists who were inspired by the works of Erwin Schrödinger and Niels Bohr, who had seen physics move forward by analyzing paradoxes (Stent [1968](#)). Nature, they thought, is not paradoxical; it operates according to natural laws. A paradox reveals where our understanding is flawed, and offers an opportunity for important research. Finding paradoxes is important and exciting, because by studying and analyzing the phenomena that appear paradoxical we have the opportunity to learn important new things about nature.

Palmer and Herbon's work, along with Arnold Bendich's papers on the large and variable sizes and structures of plant mitochondrial genomes (Bendich [1993](#), [1996](#); Ward et al. [1981](#)), established the fundamental paradoxes that continue to drive plant mitochondrial genomics. How can a genome be so full of non-conserved, seemingly useless, junk DNA (Graur et al. [2015](#))? How can a genome that is so good at repairing nucleotide changes be simultaneously so susceptible to rearrangements? How is it possible that the remarkable variation in junk DNA content and synteny leave mitochondrial function intact? Why is the plant mitochondrial genome so different from the chloroplast and nucleus?

The importance of the Palmer and Herbon ([1988](#)) paper is underscored by the citation record. It has been cited every year since its publication except for 1999, emphasizing the

central role of the paradox they revealed in plant mitochondrial research. One reason for this is that plant mitochondrial genomes are very different in structure, organization and mutation rates than animal mitochondria or the nucleus, and any paper on plant mitochondrial DNA needs to cite the works of Bendich, Palmer, and others (Wolfe et al. 1987) to remind the reader of these traits. In addition, mitochondrial genome replication, recombination, and maintenance are still not fully understood.

How is it possible that three decades later we do not completely understand why plant mitochondrial genomes behave this way? Although I am convinced that the expansions and variation in non-coding DNA, the low mutation rate in genes, and the high rearrangement rate in non-genes all emerge from the same fundamental process of DNA repair, that process is not understood fully. My preferred hypothesis is that damage, even to a single base in a single strand, is converted to a break and repaired by double-strand break repair (DSBR) (Christensen 2013, 2014). DSBR mechanisms fall into two categories: non-template-directed and template-directed. Non-template-directed repair can be very accurate, but can also lead to rearrangements. Duplications can be produced this way, and probably explain the expansions. Inaccurate repair in genes would be eliminated by purifying selection, and even synonymous substitutions would be reduced in frequency by template-directed repair of even a small region of DNA (Sloan and Taylor 2010; Wynn and Christensen 2015). Rearrangements in noncoding DNA must be neutral, or nearly so, because they accumulate more quickly, as first observed by Palmer and Herbon. Evidence for this hypothesis has been difficult to come by, in part because we are still unable to transform plant mitochondria, and in part because we still do not know all of the proteins involved in repair (Gualberto and Newton 2017). An additional problem is that the repair mechanism is so effective that even an introduced cytidine deaminase in a Uracil-*N*-glycosylase mutant led to a small number of mutations, none of them fixed, after 10 generations (Wynn et al. 2020). The MSH1 protein, which has novel features in angiosperms possibly allowing it to make double-strand breaks at sites of damage or mismatch has recently been shown to play an important role in DNA repair in plant organelles, providing support for the hypothesis (Wu et al. 2020).

In the years since 1988, plant mitochondrial genomics has not gotten simpler, only more complex. The Palmer lab has shown surprising levels of horizontal gene transfer of mitochondrial DNA between plant species, further complicating the phylogenetics of plants (Bergthorsson et al. 2003; Mower et al. 2010; Rice et al. 2013; Richardson and Palmer 2007). Bendich and co-workers have revealed that the mitochondrial genomes do not exist as circular molecules, even though they can be mapped to circles (Bendich 1993, 1996, 2007), and the DNA in mature tissues is different from in

meristematic tissue (Bendich 2013; Kumar et al. 2015; Oldenburg et al. 2013).

It is also interesting that pre-genomics methods are very accurate. Bendich's lab established the sizes of plant mitochondrial genomes using reassociation kinetics (C_0t curves) and the sizes are remarkably close to those determined decades later by sequencing. Palmer's lab used restriction mapping and Southern blotting, and their results are also remarkably close to current data from sequencing.

Palmer and Herbon (1988) deserves recognition as an important paper in the history of the *Journal of Molecular Evolution*. The paper showed that plant mitochondrial genomes undergo fundamentally different DNA maintenance processes than the nucleus or animal mitochondria. This 32-year old paper was, and still is, important and relevant, underpinning and informing the field of plant mitochondrial biology.

References

Bader DA, Moret BME, Yan M (2001) A linear-time algorithm for computing inversion distance between signed permutations with an experimental study. J ComputBiol 8:483

Bendich AJ (1993) Reaching for the ring: the study of mitochondrial genome structure. Curr Genet 24:279

Bendich A (1996) Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed field gel electrophoresis. J MolBiol 255:564

Bendich AJ (2007) The size and form of chromosomes are constant in the nucleus, but highly variable in bacteria, mitochondria and chloroplasts. BioEssays 29:474

Bendich AJ (2013) DNA abandonment and the mechanisms of uniparental inheritance of mitochondria and chloroplasts. Chromosome Res 21:287

Bergthorsson U, Adams KL, Thomason B, Palmer JD (2003) Widespread horizontal transfer of mitochondrial genes in flowering plants. Nature 424:197

Blanchette M, Kunisawa T, Sankoff D (1999) Gene order breakpoint evidence in animal mitochondrial phylogeny. J MolEvol 49:193

Boore JL, Brown WM (1998) Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. CurrOpin Genet Dev 8:668

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. ProcNatlAcadSci USA 76:1967

Cai G, Yang Q, Yi B, Fan C, Edwards D, Batley J, Zhou Y (2014) A complex recombination pattern in the genome of allotetraploid-*Brassica napus* as revealed by a high-density genetic map. PLoS ONE 9:e109910

Chang S, Yang T, Du T, Huang Y, Chen J, Yan J, He J, Guan R (2011) Mitochondrial genome sequencing helps show the evolutionary

mechanism of mitochondrial genome formation in Brassica. BMC Genomics 12:497

Christensen AC (2013) Plant mitochondrial genome evolution can be explained by DNA repair mechanisms. Genome BiolEvol 5:1079

Christensen AC (2014) Genes and junk in plant mitochondria—repair mechanisms and selection. Genome BiolEvol 6:1448

Clary DO, Wolstenholme DR (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J MolEvol 22:252

Clary DO, Goddard JM, Martin SC, Fauron CM, Wolstenholme DR (1982) Drosophila mitochondrial DNA: a novel gene order. Nucleic Acids Res 10:6619

Cole LW, Guo W, Mower JP, Palmer JD (2018) High and variable rates of repeat-mediated mitochondrial genome rearrangement in a genus of plants. MolBiolEvol 35:2773

Darracq A, Varre JS, Touzet P (2010) A scenario of mitochondrial genome evolution in maize based on rearrangement events. BMC Genomics 11:233

Dobzhansky T, Sturtevant AH (1938) Inversions in the chromosomes of *Drosophila pseudoobscura*. Genetics 23:28

Graur D, Zheng Y, Azevedo RBR (2015) An evolutionary classification of genomic function. Genome BiolEvol 7(3):642–645

Grewe F, Edger PP, Keren I, Sultan L, Pires JC, Ostersetzer-Biran O, Mower JP (2014) Comparative analysis of 11 Brassicales mitochondrial genomes and the mitochondrial transcriptome of *Brassica oleracea*. Mitochondrion 19(Part B):135

Gualberto JM, Newton KJ (2017) Plant mitochondrial genomes: dynamics and mechanisms of mutation. Annu Rev Plant Biol 68:225–252

Handa H (2003) The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus L*): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. Nucleic Acids Res 31:5907

Kumar RA, Oldenburg DJ, Bendich AJ (2015) Molecular integrity of chloroplast DNA and mitochondrial DNA in mesophyll and bundle sheath cells of maize. Planta 241:1221

Makaroff CA, Palmer JD (1987) Extensive mitochondrial specific transcription of the *Brassica campestris* mitochondrial genome. Nucleic Acids Res 15:5141

Makaroff CA, Palmer JD (1988) Mitochondrial DNA rearrangements and transcriptional alterations in the male-sterile cytoplasm of Ogura radish. Mol Cell Biol 8:1474

Mower JP, Stefanović S, Hao W, Gummow JS, Jain K, Ahmed D, Palmer JD (2010) Horizontal acquisition of multiple mitochondrial genes from a parasitic plant followed by gene conversion with host mitochondrial genes. BMC Biol 8:150

Oldenburg DJ, Kumar RA, Bendich AJ (2013) The amount and integrity of mtDNA in maize decline with development. Planta 237:603

Palmer JD, Herbon LA (1988) Plant mitochondrial DNA evolves rapidly in structure, but slowly in sequence. J MolEvol 28:87

Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307:437

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid Brassica species. TheorAppl Genet 65:181

Rice DW, Alverson AJ, Richardson AO, Young GJ, Sanchez-Puerta MV, Munzinger J, Barry K, Boore JL, Zhang Y, dePamphilis CW, Knox EB, Palmer JD (2013) Horizontal transfer of entire genomes via mitochondrial fusion in the angiosperm Amborella. Science 342:1468

Richardson AO, Palmer JD (2007) Horizontal gene transfer in plants. J Exp Bot 58:1

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. ProcNatlAcadSci USA 74:5463

Sankoff D, Leduc G, Antoine N, Paquin B, Lang BF, Cedergren R (1992) Gene order comparisons for phylogenetic inference: evolution of the mitochondrial genome. ProcNatlAcadSci USA 89:6575

Sloan DB, Taylor DR (2010) Testing for selection on synonymous sites in plant mitochondrial DNA: the role of codon bias and RNA editing. J MolEvol 70:479

Smith MJ, Arndt A, Gorski S, Fajber E (1993) The phylogeny of echinoderm classes based on mitochondrial gene arrangements. J MolEvol 36:545

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J MolBiol 98:503

Stent GS (1968) That was the molecular biology that was. Science 160:390

Sturtevant AH, Dobzhansky T (1936) Inversions in the third chromosome of wild races of *Drosophila pseudoobscura*, and their use in the study of the history of the species. ProcNatlAcadSci USA 22:448

Tesler G (2002) GRIMM: genome rearrangements web server. Bioinformatics 18:492

U N (1935) Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7:389

Unseld M, Marienfeld JR, Brandt P, Brennicke A (1997) The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nat Genet 15:57

Ward BL, Anderson RS, Bendich AJ (1981) The mitochondrial genome is large and variable in a family of plants (cucurbitaceae). Cell 25:793

Wolfe K, Li W, Sharp P (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. ProcNatlAcadSci USA 84:9054

Wu Z, Waneka G, Broz AK, King CR, Sloan DB (2020) MSH1 is required for maintenance of the low mutation rates in plant mitochondrial and plastid genomes. ProcNatlAcadSci USA 117:16448

Wynn EL, Christensen AC (2015) Are synonymous substitutions in flowering plant mitochondria neutral? J MolEvol 81:131

Wynn EL, Christensen AC (2019) Repeats of unusual size in plant mitochondrial genomes: identification, incidence and evolution. G3 9:549

Wynn E, Purfeerst E, Christensen A (2020) Mitochondrial DNA repair in an *Arabidopsis thaliana* uracil N-glycosylase mutant. Plants (Basel) 9:261