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Letter to the Editor

Under the rug: Abandoning persistent misconceptions that obfuscate organelle evolution

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ABSTRACT

The advent and advance of next generation sequencing over the past two decades made it possible to accumulate large quantities of sequence reads that could be used to assemble complete or nearly complete organelle genomes (plastome or mitogenome). The result has been an explosive increase in the availability of organelle genome sequences with over 4000 different species of green plants currently available on GenBank. During the same time period, plant molecular biologists greatly enhanced the understanding of the structure, repair, replication, recombination, transcription and translation, and inheritance of organelle DNA. Unfortunately many plant evolutionary biologists are unaware of or have overlooked this knowledge, resulting in misrepresentation of several phenomena that are critical for phylogenetic and evolutionary studies using organelle genomes. We believe that confronting these misconceptions about organelle genome organization, composition, and inheritance will improve our understanding of the evolutionary processes that underly organelle evolution. Here we discuss four misconceptions that can limit evolutionary biology studies and lead to inaccurate phylogenies and incorrect structure of the organellar DNA used to infer organelle evolution.

1. Recombination, replication, repair mechanisms and genome structure

As bacterial descendants it seemed intuitive that plant organelles would contain circular genomes, and some of the earliest investigations appeared to support this assumption (Kolodner and Tewari, 1975; Sparks and Dale, 1980). Subsequent studies incorporating an array of tools have found that these genomes comprise complex assemblages that include circular molecules but where large linear and branched forms predominate (Bendich, 1993, 2004; Sloan, 2013; Oldenburg and Bendich, 1996, 2015; Kozik et al., 2019). The mechanisms that facilitate replication and repair of organelle genomes are inherent to their dynamic structure. Recombination dependent replication (RDR) can both homogenize and diversify plastomes (Ruhlman and Jansen, 2018; Choi et al., 2019). For example, the identical sequences that make up the inverted repeat (IR), so commonly found among angiosperm plastomes, maintain that identity through gene conversion driven by RDR, not through so called 'flip-flop' recombination occurring within single plastome units (Kolodner and Tewari, 1979; Palmer, 1983; see Zampini et al., 2017 for a review of proposed models of recombination).

The two single copy regions that separate IR sequences in plastomes are typically reported as inversion isomers in equimolar proportion, a phenomenon that simply could not occur in a genome-sized circular molecule (Oldenburg and Bendich, 2004; Maréchal and Brisson, 2010; see Fig. 1 in Bendich, 2004). On the other hand, in plastomes that contain non-IR repeats RDR may initiate through strand invasion at non-allelic sites. Depending on the orientation of the repeats and the specific repair mechanism employed, novel repeats, inversions, deletions and other rearrangements can occur. Where non-allelic repeats employed by replicative repair mechanisms lack complete nucleotide identity, gene conversion can homogenize the repeated sequences, favoring either a 'native' or 'novel' base at any site along the length of the repeat. Furthermore, replication initiation at non-allelic repeats could lead to completely new chromosomal arrangements (Ruhlman and Jansen, 2018). While most plastomes lack large (> 100 bp) repeats, recombination reactions have been demonstrated to initiate at microhomologous regions in wild type plants and in lines where expression of nuclear-encoded DNA maintenance proteins have been impaired (Zampini et al., 2017). Similarly, plant mitogenomes contain repeats that provide substrates for recombination to generate continual structural variation within the cells and tissues of a single individual (Gualberto and Newton, 2017; Kozik et al, 2019). These processes are a source of heteroplasmy (see below) with respect to organelle genome structure and nucleotide substitutions that arise in the context of the nucleoid, the unenveloped aggregation of proteins and nucleic acids that each contain multiple organelle genome-equivalents, and this variation may be heritable. The transmission of organelle genomes to progeny occurs at the level of the organelle, such that several nucleoids, each containing multimers of the genome are included, allowing the possibility that multiple genome types to be transmitted en masse. This heteroplasmy, driven by recombination but separate from biparental inheritance paradigms, is a plausible source of heritable variation that could have implications for evolutionary studies both within and between populations.

2. Heteroplasmy

Heteroplasmy is the presence of multiple, different copies of an organellar genome within a cell or among cells within an individual. The phenomenon was first discovered in plastids and has been described in nearly all clades of the green plant tree of life (reviewed in Ramsey and Mandel, 2019), as well as in mitochondrial genomes (mitogenomes) of plants and animals (Kmiec et al., 2006; McCauley, 2013; Wu et al., 2020). Heteroplasmy can arise via a number of mechanisms including gene rearrangements or gene chimeras caused by recombination, insertions or deletions, and point mutations. Instances of

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biparental inheritance in which parents differ with respect to their plastomes or mitogenomes would also result in heteroplasmy in the offspring.

A variety of molecular approaches for identifying heteroplasmy have been employed including using fragment length variation, PCR and Q-PCR based approaches, and traditional Sanger and next generation sequencing. In Sanger sequences, heteroplasmy can be detected as an overlay of more than one nucleotide signal (peaks) in an electropherogram (Roy and Schreiber, 2014). While widely noted, these heteroplasmic variants are usually not considered when analyzing data for phylogenetic studies. However, if heteroplasmy is relatively common in studied samples, organellar haplotypes may be scored incorrectly as cautioned by Wolfe and Randle (2004). Ultimately, this situation is similar to the sorts of problems encountered when considering paralogous loci in the nuclear genome since random sampling of organellar haplotypes is likely to affect gene tree topologies.

Rapid advances in high throughput sequencing technologies over recent years have allowed more thorough characterization of organellar genomes including new software aimed at identifying and describing heteroplasmy (e.g., Vellarikkal et al., 2015; Phan et al., 2019). This work has subsequently resulted in the identification of new cases describing heteroplasmy including interest in modeling heteroplasmy in an ontogenetic phylogeny context as it relates to human health (Wilton et al., 2018). In plant species that exhibit biparental transmission of plastids, heteroplasmic progeny tend to retain only one parental haplotype through development (Matsushima et al., 2008) in a stochastic process that occurs rapidly and early (Kirk and Tilney-Bassett, 1978; Birky, 2001). Mutations that arise in the shoot apical meristem, however, may enter the germline and be sexually transmitted, as in *Oenothera* (see Massouh et al., 2016, and references therein). Still, heteroplasmy is not commonly reported in plant evolutionary and phylogenetic studies that utilize organelle genomes despite its apparent ubiquity in nature (Wolfe and Randle, 2004; Kmiec et al., 2006). We urge phylogeneticists to document instances of heteroplasmy in order to provide an avenue for building and describing models that would account for its presence in evolutionary studies.

3. Inheritance

Although research on plastid inheritance in the early 1900s by Bauer, Correns and Renner on *Pelargonium*, *Oenothera* and *Mirabilis* documented biparental inheritance of plastids (Hagemann, 2000), the prevailing view is that both plastomes and mitogenomes are strictly inherited from the maternal parent (Birky, 1995; Reboud and Zeyl, 1994). There is growing evidence that plastome inheritance may not be maternal in at least 20% of angiosperms (Corriveau and Coleman, 1988; Zhang et al., 2003; Hagemann, 2004) and in lineages that do exhibit maternal inheritance there is a low level of paternal leakage (Azhagiri and Maliga, 2007; Thyssen et al., 2012). Inheritance of mitogenomes is considered more strictly maternal but again recent studies have uncovered more cases of paternal leakage (McCauley, 2013; Breton and Stewart, 2015). Thus it is more appropriate to consider organelle inheritance as a quantitative trait that can result in shifts in inheritance patterns between minor and major parental genomes (Ramsey and Mandel, 2019). Such shifts are important for overcoming cytonuclear incompatibility, including plastome genome incompatibility and cytoplasmic male sterility, phenomena that have important implications for speciation (Greiner et al., 2011) and crop breeding (Bohra et al., 2016). There have been a number of hypotheses as to the prevalence of uniparental inheritance of organelle genomes (Birky, 1995; Mogensen, 1996; Kuroiwa, 2010; Bendich, 2013; Sato and Sato, 2013), however a full discussion of these ideas is beyond the scope of this commentary and remains an open question. Regardless of the cause, the misconception that organelle inheritance is strictly uniparental and maternal hinders efforts to uncover the causes and consequences of cytonuclear incompatibility, which in turn inhibits studies of speciation and crop improvement in plants.

4. The notion of the single locus and its implications for phylogenetic inference

Early in the use of molecular data to infer phylogenies, Doyle (1992) argued that each of the organellar genomes represents a single locus and this interpretation is still being employed to justify the importance of nuclear datasets for phylogenetic inference (Gitzendanner et al., 2018; Léveillé-Bourret et al., 2017; Wicke et al., 2011). While Doyle's (1992) statement may be true if a "single locus" refers to the plastome as one of the three genomic compartments present in plant cells (i.e., nuclear, plastid and mitochondrion), the statement can also be misleading. When considering the coding-regions of the plastome or mitogenome for phylogenetic inference, it is important to keep in mind that biological and methodological factors can have a significant impact. Biologically, considering genes as completely linked and disregarding that they may have different rates of evolution can produce misleading reconstructions. Methodologically, variation of the phylogenetic signal across the genome and the failure to explore this variation can bias the inference leading to highly supported but spurious phylogenies, a phenomenon documented by Gonçalves et al. (2019) and later confirmed by several studies in both plants and animals (Gruenstaeudl, 2019; Walker et al., 2019; Zhang et al., 2020; Thode et al., 2020; Kim et al., 2020). Another important point is that the variation of phylogenetic signal across different taxonomic levels (Shrestha et al., 2019). It is important to consider any variation of phylogenetic signal, as portions of the genome may be phylogenetically uninformative. Furthermore, the phylogenetically informative regions from different portions of the genome can support incongruent topologies (Leebens-Mack et al., 2005). Therefore, while the complete genome may not be phylogenetically informative, it is still important to explore and define the regions that are informative and assess their congruence. In addition, when inferring phylogenies with concatenated alignments of plastome or mitogenome data, one or a few genes that have high phylogenetic signal may bias the inference (Shen et al., 2017; Gonçalves et al., 2019; Walker et al., 2019). While the concatenation of plastid genes is widely used as a result of "single locus" thinking, the approach has not often been extended to mitogenome data as this genome is less used for phylogenetic inference of plants due to its low rate of nucleotide substitution and high frequency of horizontal gene transfer. However, it is now known that the mutation rates in plant mitogenomes are not as low as previously thought (Christensen, 2018). Systematists should be aware of all these characteristics when inferring phylogenies and should be diligent when proposing new classification systems, especially when their phylogenetic reconstructions are based on one or a few markers from a single genomic compartment.

5. Conclusion

We have outlined four misconceptions that should be abandoned in future phylogenetic analyses of organelle sequences and investigations of structural evolution of these genomes. Major advances in the understanding of organelle genome structure, inheritance and the mechanisms of replication, repair and recombination have been made over the past two decades and this knowledge should not be swept 'under the rug' when using these data for phylogenetic and molecular evolutionary investigations. Table 1 provides recommendations on how the plant biology community can overcome these misconceptions in the future.

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Table 1
Recommendations for overcoming misconceptions regarding plastome and mitogenome evolution and their use in phylogenomics.

Misconceptions	Recommendations
Genome structure	(1) Do not describe, illustrate or publish mitogenomes or plastomes as circular since overwhelming evidence demonstrates that they are mostly linear and branched. (2) Use long-read next-generation sequencing approaches to confirm structure both within and between individuals. (3) Examine multiple cells, individuals and populations to evaluate temporal variation in mitogenome and plastome structure.
Heteroplasmy	(1) Use single cell sequencing with long read platforms to evaluate the frequency of variation of organelle genome variation within and between species at both the sequence and structural levels. (2) Perform more rigorous analyses of sequencing reads to detect variation at particular sites for heteroplasmy. (3) Sample both germline and somatic tissue for heteroplasmy at different developmental stages. (4) Develop models that account for heteroplasmy in evolutionary studies.
Inheritance	(1) Perform more crossing studies, including reciprocal and multiple crosses, and examine multiple progeny to accurately assess the frequency of inheritance from different parents.
Phylogenomic analysis	(1) Examine individual organelle gene phylogenies and compare congruence of gene trees with trees generated from concatenated gene data sets. (2) Consider using Multispecies Coalescence methods to construct phylogenies using organelle genomes. (3) Explore phylogenetic information in different genes and determine which sites and genes are causing significant incongruence.

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Author contributions

All the authors contributed equally to the conception, development and writing of this letter.

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