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Re-Interpreting Mitogenomes: Are Nuclear/Mitochondrial Sequence Duplications Correctly Characterised in Published Sequence Databases?

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From the late 1970s when Fred Sanger and Alan Coulson developed their chain termination method for rapid determination of DNA sequence [1], Sanger sequencing dominated nucleic acid and genome research until about a decade ago when technical developments led to the "next big thing" in sequencing genomes. In 2005, the first workable sequencing strategies entailing arrays of millions of DNA templates sequenced in parallel became publicly available [2,3]. It was not long before such high-throughput, massively-parallel approaches transformed biological research, generating vast amount of genome data. However, for the successful interpretation of sequence data, the quality of primary sequence reads and correct assembly of contigs is paramount. While no sequencing technique is error-free, and biases have been accounted for with both first generation and next generation sequencing (NGS) technologies, when it comes to "difficult genomic regions", it can be anticipated that results relying solely on just one sequencing approach may produce fundamental errors within published sequences, which are difficult if not impossible to identify without closer examination.

Mitochondria are the major energy providers in eukaryotes. They are cytoplasmic, semi-autonomous organelles with the majority of their ~16.5kbp (in a standard vertebrate) original precursor genome now integrated into the nuclear genome of the cell but with a small portion consisting of 37 genes and ~1kb of non-coding sequence referred to as the control region (CR) remaining within the organelle itself [4]. Due to their high abundance, small genome size relative to nuclear DNA (nuDNA), and non-recombinant inheritance, mitochondria have been extensively employed in genetic studies. For example, mitochondrial DNA (mtDNA) has been widely used in forensic investigations [5], archaeogenomic research [6,7], population genetics to establish genetic relationships, as well as molecular systematics and reconstruction of species history [8,9]. Equally, the quality and quantity of mtDNA is often employed as a marker of mitochondrial activity and, considering its bioenergetics role within cells, mtDNA variants and defects have been implicated in a plethora of pathologies, metabolic syndromes (such as diabetes), aging, aging-associated degenerative diseases and cancer [10,11]. Consequently, if it is the mtDNA sequence on which a whole populations' history is based, aetiology of a disease explained, or on which a judicial verdict depends – the sequence assembly must be 100% accurate.

However, even with the current power of NGS platforms and decades of experience with Sanger sequencing, erroneous mitogenome sequences are published, have been deposited, and remain available within the databases. There are three main biological issues that have to be accounted for in order to obtain a true and accurate mitochondrial sequence. 1) mitochondrial heteroplasmy: the high copy number that makes mtDNA an easily accessible and attractive tool for population genetics and ancient DNA studies may prove a tripping stone as sequence genotype can vary from organelle to organelle between different cell/tissue types and even over time [12]. 2) the fragments of mtDNA that are also integrated within germline nuclear sequences – referred to as "numts" (nuclear mitochondrial sequences, [13-16]): while these "molecular fossils" provide exciting opportunities to study mtDNA and species evolution [15,17-18], it is not uncommon that numt sequences are mistaken for authentic mtDNA and included within mitosequences [19,20]. 3) mitochondrial gene duplications/deletions: a phenomenon associated with abnormality in mammals [21], but which have been found in normally functioning mitochondria in other organisms [22-24]. Birds are a particularly good example, as mitochondrial gene duplications and/or non-coding control region duplications (YCR), and different arrangements of the gene order have been observed arisen independently multiple times across the avian family [25-28].

When sequencing avian mitochondrial genomes, all of these three impediments will have to be taken into account: even though numts and heteroplasmy have been shown to exist in a wide number of taxa [16,29-30] and tandem repeat sequences within the CR have been observed in many mammalian species such as horse, deer, shrew, bat and various carnivores [31-35], birds are the only endotherms with reported genic duplications within the actual mitochondrial DNA [26,28,36,37]. To complicate matters further, some birds have been recorded to possess portions of the CR duplications integrated within the nuclear genome and with heteroplasmy in terms of the repeat number within the variable domain of the YCR in mtDNA and/or nuDNA. For instance, a set of consistent underlying peaks at 5-20% of maximum peak intensity can be clearly determined when analysing Sanger sequencing chromatogram of the YCR-specific PCR amplicons of the mitochondria of the Red kite (*Milvus milvus*) [38]. The double signal, which is present only in female birds, originates from the presence of mitochondrial DNA sequences translocated to the W chromosome [39,40]. Illumina shotgun sequencing failed to characterise the mitochondrial and W-chromosome duplications, as most of the reads from these regions remained unmapped because of the highly repetitive and relatively long repeat structure (~1.5kb), thus they were discarded by the assembly package in the first place, during early contig assembly. A recent study by Nacer and do Amaral reported a striking pseudogenization in avian phylogenetics and concluded that avian numts may be much more frequent and longer than previously thought [41]. For instance, a nuclear copy of mtDNA covering 93.6% of the mitogenome was found in the Peregrine falcon and numt sequences in falcons totalled ~49kb or ~0.004% of the whole nuclear genome. In another instance, a phylogenetic study of cranes based on standard PCR amplification of the mtDNA sequences by Krajewski et al. [42] did not report evidence for a duplication within crane mtDNA. Most of the mtDNA sequences used were obtained from ~500-1000bp overlapping amplicons providing ≥2× coverage for ~25% of the mtDNA molecule. ND6 and CR sequences of all cranes were obtained from Krajewski et al. [43] and Fain [44], respectively, and incorporated within the sequence assembly. For seven years the deposited sequences

were believed to be a complete and accurate mitochondrial picture of the Crane (Gruidae) family members and these sequences are still accessible via NCBI [45]. However in 2017, Akiyama et al. [46] using a mix of initial long-range PCR (LR-PCR) and then nested PCRs to study the structure of mtDNA, showed that all the 13 analysed *Gruidae* species previously sequenced by Krajewsi et al. [42] possessed a duplication block consisting of Cytb, tRNAThr, tRNAPro, ND6, tRNAGlu and CR. The duplication was conserved across all the species and was similar to those detected in other unrelated avian species such as albatrosses (36,47], spoonbills [48], and boobies [49], in a stroke increasing the average size of Gruidae mitochondrial genome from 16.5kb to ~22kb. This error not only changed the perception of the linage divergence of the cranes, but also radically altered our understanding of the evolution of the YCR within the avian phylogeny [37,46,49,50].

To conclude, living in an era when obtaining large quantities of sequencing data is no longer an obstacle, the effort should be focused on ensuring its correct validation. Whilst no sequencing technique is perfect and warning signs have been issued on numerous occasions [51,52], strategic approaches to dealing with "difficult sequence" such as mtDNA duplications, deletions and inerchromosomal transfers should be developed, whether these be mixtures of laboratory-based techniques such as LR-PCR, sequence capture and use of more than one sequencing approach (i.e. combining Sanger with NGS or use of the new long-read sequencers such as MinION) or the generation of novel bioinformatics pipelines for resolving these issues in existing datasets.

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