Nuclear integrations: challenges for mitochondrial DNA markers

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The combined use of mitochondrial DNA markers and polymerase chain reaction (PCR) techniques has greatly enhanced evolutionary studies. These techniques have also promoted the discovery of mitochondrial-like sequences in the nuclear genomes of many animals. While the nuclear sequences themselves are interesting, and capable of serving as valuable molecular tools, they can also confound phylogenetic and population genetic studies. Clearly, a better understanding of these phenomena and vigilance towards misleading data are needed.

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In the past two decades, two innovations have had great impact on molecular population genetic studies, particularly as they affect animal evolutionary biology. First was the discovery of mitochondrial DNA (mtDNA) as a useful molecular marker in the late 1970s (for review, see Ref. 1), and more recently came the invention of polymerase chain reaction (PCR) technology[2]. The remarkable characteristics possessed by mtDNA (see Box 1), in combination with the power of PCR, have greatly enhanced evolutionary analysis, and have allowed biologists to study almost any metazoan animal rapidly at molecular and populational levels.

The potential usefulness of mtDNA in evolutionary studies has been thoroughly discussed in a series of review papers (Refs 3–7, and references therein), and several associated problems have been considered, including paternal leakage, heteroplasmy, variable evolutionary rate and non-neutrality[3–8]. However, there is another unexpected problem associated with both mtDNA and PCR that has not been adequately examined: the presence of mitochondrial-like DNA sequences in the nuclear genome of many organisms and their contamination of authentic mtDNA during PCR amplification, and consequently the data obtained. Increasing evidence over the past four years or so[9–16] has shown that nuclear mitochondrial-like DNA sequences could be a substantial problem both in vertebrates and in invertebrates – something that is scientifically very interesting, but that could also lead to erroneous results and much impair the apparent usefulness of mtDNA.

Here, we will first give an overview of nuclear mitochondrial-like sequences observed in various metazoan animals, analyse their different patterns of evolution compared to mtDNA, then examine how they may confound evolutionary studies that use PCR-based techniques. We shall also discuss their potential usefulness, and finally explore how to avoid, in practice, the confusion that can arise.

Nuclear ex-mitochondrial sequences in animals

Until a few years ago, mitochondrial-like sequences were only occasionally observed in the nuclear genomes of animals[17–20]. Since then, they have been found in several vertebrates[9–14,19–21] and invertebrates[15–18], representing many major animal taxa (Table 1). In addition, we have personal communication of a few more studies – on wombats and bandicoots (marsupial mammals) – which suggests the common occurrence of such sequences. A striking example that has been fully characterized comes from the domestic cat[10]: an insertion involving about half of the mitochondrial genome (7.9 kb) was found in the cat's nuclear genome. This insertion contains several mitochondrial genes encoding proteins, ribosomal RNAs (rRNA), transfer RNAs (tRNA) and also a deleted D-loop region, with the mitochondrial gene order having been maintained. It is tandemly repeated 38–76 times on chromosome 2D. Such an insertion seems to be the result of a relatively recent intergenomic transfer that occurred about two million years ago. Many mitochondrial regions have also been found in the human nuclear genome, involving recent and ancient events[11,13,14,20,22,23] (Table 1); some of the insertions were estimated to have a very high copy number in the nuclear genome (i.e. from about a hundred[22] to a thousand[23]).

From data so far available, we can summarize the following features of this phenomenon (Table 1).

- Many mitochondrial regions have been found to be integrated into the nuclear genome, including protein-coding, rRNA-coding and noncoding regions – the control regions[10,12,14,15,19], rRNA genes[10,11,15,17,18,20], cytochrome *b* (Cyt *b*) genes[9,12,13,16] and genes for the subunits I and II of the cytochrome oxidase (COI–COII)[10,16,18,22,23] being the most frequently described. This is apparently because these mitochondrial regions have been the most popularly employed.
- The nuclear insertion can be a large mitochondrial fragment[10,13,17,18] and may have a high copy number (being multiple or repetitive[10,22–25]). The nuclear insertions in cat described above provide an extreme example.
- Nuclear insertions show various degrees of homology with their mitochondrial counterparts, depending on the taxa and the regions involved. For example, nucleotide divergence (uncorrected) between the mitochondrial COI–COII sequence and its nuclear homologue in aphids[16] can be <1%; a similar comparison for the Cyt *b* sequences in primates[13] shows a difference of >25%.
- The divergence between different nuclear copies in the same genome varies from organism to organism. For example, divergence can be <1% in some rodents[9] and up to 32% in human[22].
- Nuclear insertions, being under different mutation constraints, show different evolutionary patterns compared to the authentic mitochondrial sequences. Judging from DNA sequence evolution, it appears that nucleotide substitutions in the nuclear copies occur more randomly[12,13,16,22], that is, nuclear copies shows less codon position bias; the ratios of transversion versus transition and replacement versus silent substitutions are usually an order of magnitude higher. Other mitochondrial features, such as mutation pressure, seem to be reduced and the mode of inferred amino acid substitution appears to be different[16]. A number of studies[9,12–14,22] have shown that, in vertebrates, nuclear ex-mitochondrial sequences (1) behave like molecular 'fossils', evolving much more slowly than their mitochondrial homologues, and therefore (2) are closer to the ancestral forms of the present

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Box 1. Mitochondrial DNA as molecular markers

Animal mtDNA possesses several remarkable characteristics, making it a very useful molecular marker in evolutionary studies. Some of these characteristics are listed below (for reviews, see Refs 3–7):

- small genome with simple structure and organization
- ubiquitous presence in almost all animals
- high copy number – thereby easy to isolate
- effective haploidy in DNA sequences
- maternal inheritance (with a few exceptions)
- lack of recombination, introns or other noncoding sequences
- mosaic molecule with faster and slower evolving DNA regions allowing the design of conserved primers and addressing of phylogenetic questions at various taxonomic levels

There are also some particular and general difficulties in the use of mtDNA. These difficulties result from (for reviews, see Refs 3–8):

- biparental inheritance
- length and sequence heteroplasmy
- non-constant molecular clock
- non-neutrality
- rapid lineage sorting
- nuclear mitochondrial-like sequences

mtDNA in their nucleotide sequences. No comparable studies have been carried out in invertebrates yet. The limited data in insects[15,16] indicate that the rate of evolution of some nuclear copies may not differ significantly from that of the authentic mitochondrial sequences.

- Nuclear insertions can often be coamplified with, or amplified instead of, the mitochondrial targets in PCR using conserved primers, with or without size difference (see below).
- Insertions involving the same mitochondrial regions can often be observed in related taxa, such as in different primates[11,13], domestic cats[10], birds[12], sea urchins[26] or aphids[16], and can occur several times in the same species[16,22].
- Such nuclear insertions can be very recent and not yet fixed in natural populations[14], or, no mutations may have accumulated between the nuclear and mitochondrial lineages[22]. On the other hand, some insertions are as ancient as 30 million years or more, as shown in primates[13,22] and sea urchins[26].

The presence of DNA sequences of mitochondrial origin in the nuclear genome *per se*, as a result of intergenomic transfer of genetic materials, is not surprising; it is what one might expect from the endosymbiotic theory[27] of the origin of mitochondria (see Box 2). Indeed, this has been observed in a very diverse group of organisms, including plants, fungi, vertebrate and invertebrate animals (see Refs 28–30, for review). Such a process, however, has been commonly thought to be an infrequent phenomenon that reflects events in the remote past before the diversification of the main animal taxa. What is surprising is that, based on these recent findings, transfer to nuclei appears to be an ongoing process even for the compact mitochondrial genome of metazoan animals. From the frequency and estimated age of the insertion events within and among various animals, and also from the sequence homology observed (Table 1), it can be seen that intergenomic transfer of genetic material from mitochondria to nuclei has occurred frequently and independently in different lineages.

Interference of nuclear ex-mitochondrial sequences in evolutionary studies using mtDNA

Because nuclear insertions have different evolutionary patterns and mode of inheritance compared to the authentic mitochondrial sequences, they will confound phylogenetic and population genetic analyses[9,11–15] when accidentally included in a mitochondrial data set[11–13]. This is particularly relevant for PCR-related studies. Where such nuclear sequences are evolving slowly, such as in vertebrates[9,12–14], nuclear insertions could form a distinct cluster on a phylogenetic tree (Fig. 1), thereby wrongly suggesting an ancient genetic split between mitochondrial lineages. Where the sequence divergence between nuclear insertions and the real mtDNA is very small – owing to a similar rate of evolution or recent nuclear integrations (such as in some insects[15,16]) – it may be difficult to tell them apart using their nucleotide sequences. Because nuclear copies are biparentally inherited and the mitochondrial sequences maternally inherited, conclusions based on a coalescent analysis of sequence data of mixed sources in population studies would certainly be misleading.

An even worse situation occurs when the real mitochondrial data are contaminated by nuclear mitochondrial-like sequences from other organisms. In several independent studies[11,13,31], human nuclear mitochondrial-like sequences constituted a dangerous source of contamination for those working with primates, ancient DNA and forensic analyses. Indeed, human nuclear contamination has been shown[13,31] to be responsible for the surprising phylogenetic placement of the amplified 'dinosaur' bone mtDNA[32]. Because human beings are always involved and the real targets are present at very low concentrations in these analyses, false results owing to such contaminations unfortunately can be reproduced independently in different laboratories[13], thus providing erroneous positive support.

In practice, the presence of mitochondrial-like sequences in the nuclear genome can negate the advantages of mtDNA as a molecular marker in population studies. One important advantage is effective haploidy (i.e. genetic homogeneity of the mtDNA molecules), despite high copy number in the cell and in the individual. This advantage greatly facilitates experimental work, and promises the rapid analysis of a large number of individuals from many populations followed by the application of coalescent analysis. However, from a purely practical viewpoint, the existence of nuclear copies has an effect similar to sequence heteroplasmy, and demands much complicated data collection and analysis. Another advantage of mtDNA is that PCR primers can be designed that are highly conserved between distant organisms. This promises the isolation of mitochondrial regions of interest by PCR from many organisms without much prior knowledge of the structure and evolution of their mitochondrial genomes. Sadly, such primers can also amplify the nuclear insertions[9–16].

In applications where nonspecific PCR primers (versatile primers and degenerate primers) are used, nuclear copies may be preferentially amplified because of better matches between primers and the nuclear targets. This is because nuclear sequences in some organisms evolve more slowly than their mitochondrial counterparts (see above) and so are likely to be more conserved. This has been exemplified in studies carried out in akodontine rodents[9], various primates[11,13] and snow geese[21], where mitochondrial Cyt *b*, 12S rRNA and D-loop sequences were employed as diagnostic markers. Also, where rearrangements of gene order have occurred in the mitochondrial genome, but with little change in the nuclear copies, only nuclear copies may be amplified by PCR[9]. As PCR amplified bands from the two different targets may be indistinguishable in size, such errors may not be easily noticed by investigators. This is particularly so when indirect mutation-detecting methods are used for screening polymorphism, such as SSCP (single-strand conformation polymorphism), HA (heteroduplex

analysis) or D/TGGE (denaturing/temperature gradient gel electrophoresis).

In several cases[16,22,23,25], it has been demonstrated that nuclear insertions are present as multiple copies or else are repetitive[10,24]. This further increases their chance of being amplified along with the authentic mitochondrial sequences in PCR.

Nuclear integrations: new opportunities for evolutionary studies

As discussed above, nuclear ex-mitochondrial sequences can embarrass phylogenetic and population genetic studies employing mtDNA. However, they do not exist just to complicate a biologist's life, they also can be used as a novel and powerful tool in various aspects of evolutionary biology.

Nuclear integrations provide a real opportunity to study the relative rate of evolution of nuclear and mitochondrial sequences. The relative rates of evolution of mtDNA and single-copy nuclear DNA (scnDNA) have major significance for their use as molecular markers in evolutionary studies. In mammals, mtDNA appears to evolve several times faster than scnDNA[33]. Whether this is applicable to other metazoan groups is a continuing debate, particularly for insects where considerable controversy exists[4,7,34]. One of the difficulties in dealing with this problem in insects is that mtDNA and scnDNA are not directly related, and that their rate of evolution may be influenced by a number of factors, such as population history, functional constraints on a given sequence, and so on. Knowing that mtDNA and its nuclear insertions share a common ancestor and that most nuclear ex-mitochondrial sequences so far identified are nonfunctional pseudogenes, it should be feasible to study their relative patterns and rates of evolution using a proper outgroup.

However, at least three factors could complicate such analysis. One involves the possible presence of multiple copies of nuclear insertions, which could have arisen separately during different stages of evolution. Inclusion in the analysis of an outgroup taxon that lineaged out before any nuclear transfer took place may help to overcome this. The second point is that when a high level of mtDNA heteroplasmy existed in the lineage that recently produced nuclear integrations, it will be difficult to separate the divergence contributed by heteroplasmic states from divergence accumulated between mitochondrial and nuclear copies (see also Ref. 16). The third point involves rapid mtDNA lineage sorting. Owing to maternal inheritance, the mtDNA type that gave rise to the nuclear integrations in question could become extinct after such events. Therefore, mtDNA and its nuclear copies observed today in an organism may not always share a direct common ancestor (only their ultimate

Table 1. Nuclear mitochondrial-like sequences in metazoan animals

Organisms	Nuclear insertions	Size (kb)[a]	Copy number	Age of transfer (My)[b]	Nuclear-mt divergence[c]	Identification methods	Refs[d]
Invertebrates							
Locusts							
Locusta migratoria	12S and 16S rRNAs, tRNA[leu]	3.5	hundreds	–	<10%	blotting[e]/cloning	17,25
Schistocerca gregaria	12S rRNA, control region, tRNA[ile]	>1	multiple	–	~1.5	PCR	15
Aphids							
Sitobion	COI, COII, tRNA[leu], Cyt *b*	0.8	multiple	–	0.6–2.9	PCR/blotting	16
Sea urchins							
Strongylocentrotus purpuratus	16S rRNA, COI	2.8	low	30	9–17	blotting/cloning	18,26
Vertebrates							
Primates							
human	12S and 16S rRNAs, ND2, ND4, ND4L, Cyt *b*, ND5, COI, COII, tRNAs, D-loop	0.2 to >3	from a few to 1000	recent–43	0–33	blotting/ cloning/PCR	11,13,14, 20,22,23
others[f]	12S rRNA, Cyt *b*	0.4 >3	1–5+	recent–30	25–30	PCR	11,13
Rodents							
rats	D-loop	0.5	repetitive	13.6	20	cloning	19,24
akodontines	Cyt *b*	0.35	–	–	25–26	PCR	9
Bovids	Cyt *b*, D-loop	–	–	–	–	–	12
Whales	Cyt *b*, D-loop	–	–	–	–	–	12
Cats	12S and 16S rRNAs, ND1, ND2, COI, COII, D-loop, tRNAs	7.9	38–76 (tandem repetition)	1.8 – 2.0	3.1–8.0	blotting/cloning	10
Birds							
snow geese	D-loop	0.18	–	–	9–11.8	blotting/PCR	21
Scytalopus	Cyt *b*	0.32	–	>5	~16–19	PCR	12

[a]Refers to sizes of the sequenced or characterized parts of nuclear mitochondrial insertions (normally representing their minimum sizes).
[b]My, million years.
[c]Refers to percentage of nucleotide differences (uncorrected, in most cases) between a nuclear copy and its mitochondrial counterpart. Therefore, such values depend on the regions compared and their sizes. Where sequence data for mtDNA and its nuclear copies from the same individual are available, values given refer to within individual comparisons. Some of the values shown were calculated by us from the published sequences.
[d]In some cases, only the most recent publications of a given author are given, but data from their previous publications have been included here.
[e]Blotting, genomic Southern blotting analysis.
[f]Other primates, in which nuclear mitochondrial-like sequences have been identified, include chimpanzees, orang-utans, gibbons, baboons and various monkeys (see Refs 11 and 13).

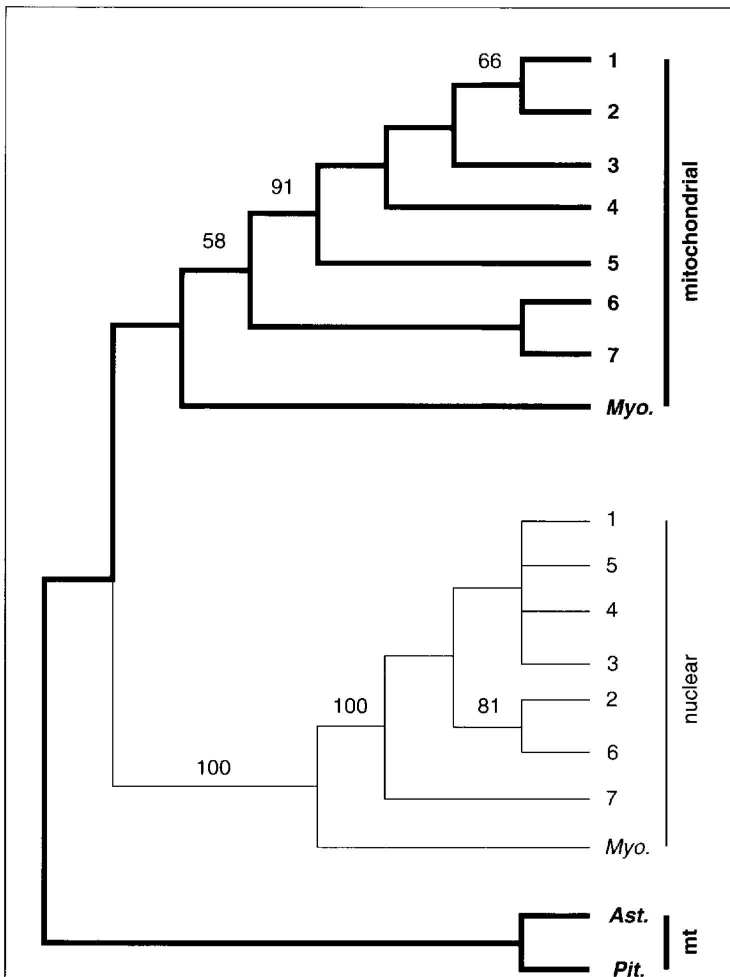

Fig. 1. Phylogenetic placement of mitochondrial Cyt *b* sequence and its nuclear homologue in birds revealed by parsimony analysis. Taxa 1 to 7 represent seven species of the South American bird genus *Scytalopus*. *Myornis* (*Myo.*) is used as the outgroup to the *Scytalopus* sequences. Mitochondrial sequences of *Asthenes dorbigney* and *Pitta sordida* (*Ast.* and *Pit.*, respectively) are included to root the entire data set. Numbers on the branches are bootstrap proportions; only values >50% are given. Mitochondrial sequence and its nuclear counterpart have been isolated from the same individual by PCR/cloning. Mitochondrial (mt) lineages are shown with heavy lines and the nuclear insertions in thin lines. Because of different evolutionary patterns and rate, nuclear ex-mitochondrial sequences involved here form a distinct cluster with strong bootstrap support. The two types of sequences in *Scytalopus* differ at 16–19% of the nucleotide positions. *Modified, with permission, from Ref. 12.*

one). Similarly, as pointed out by Sunnucks and Hales[16], sexual reproduction may introduce into an individual mtDNA and nuclear copies that are not directly related.

Nuclear integrations in vertebrates can be used as effective outgroups to mtDNA in phylogenetic studies[14,21,22], because such sequences evolve more slowly than the authentic mitochondrial sequences and thus look like molecular 'fossils'[12–14,22] with their nucleotide substitution patterns resembling more the ancestral state of mtDNA[22]. Indeed, this has proven very helpful in the study of both snow goose[21] and human[14] evolution, using the mitochondrial control region as the DNA marker, where the corresponding nuclear copy has been successfully employed as the outgroup (and, otherwise, no suitable outgroup organism can be found[14]).

Nuclear integrations, which appear to occur frequently and be taxonomically widespread, allow us to study the molecular mechanisms of endosymbiotic transfer and intergenomic interaction. Data available so far suggest that, in many organisms, DNA transfer from mitochondria to nucleus is not only an historical event but also an ongoing process. Recent endosymbiotic transfer resulting in function has not been reported in animals, but recent functional transfers from mitochondria to nucleus have been observed in plants and fungi (see Ref. 30 for review). Although animal mtDNA genomes are already very compact, there does not seem to be any real reason to think that they have reached their ultimate state of endosymbiotic evolution.

Reported cases in animals have revealed different patterns of genomic organization of transferred sequences in the nuclear genome: apparent random location for low-copy sequences, dispersed distribution[22,23] and tandem repetition[10]. This possibly means that different molecular mechanisms were involved in different events. Study of these nuclear ex-mitochondrial sequences in animals is just at its beginning, and most reports are not extensive even at the nucleotide sequence level. Comprehensive characterization of the whole transferred unit, its flanking regions, copy number, chromosome location and involvement of other nuclear elements (such as repetitive elements[17,18,20]) will throw light on the molecular mechanisms involved and the nature of intergenomic interaction.

Avoiding the nuclear pitfalls: practical considerations

Intergenomic transfer from mitochondria to nucleus is probably not an uncommon phenomenon in animals. In light of this and the potential problems discussed earlier, investigators using mtDNA markers and PCR-based techniques should check for the presence of nuclear copies of mitochondrial sequences in their organisms, carefully avoiding contamination of the data by such sequences. The following consensus approach was reached by researchers experienced in such problems[9,11–13,15].

One should seriously consider the possibility of nuclear copies of mitochondrial sequences if (1) PCR amplification constantly produces more than one band or different bands, (2) sequence ambiguities or background bands persist, (3) unexpected deletions/insertions, frameshifts or stop codons occur, (4) nucleotide sequences obtained are radically different from those expected, or (5) phylogenetic analysis yields an unusual or contradictory tree topology.

To selectively amplify the mitochondrial targets, the use of target-specific primers is recommended (this necessitates a fuller characterization of both the mitochondrial and nuclear sequences), or a combination of several primers, or enriched or purified mtDNA. Alternatively, a different mitochondrial region can be used. However, there may well be some further difficulties. First, it may not always be possible to design target-specific primers because of, for example, recent integrations or low divergence between nuclear and mitochondrial sequences, particularly when the DNA regions involved are biased in nucleotide composition[15]. Second, when nonspecific primers are used, a better match between primers and the nuclear targets can frustrate attempts to purify mtDNA, as noticed by Collura and Stewart[13]. In such a context, when nuclear ex-mitochondrial sequences are likely to be a problem, traditional non-PCR-based methods using purified mtDNA seem to be more robust and much less sensitive to the nuclear contamination. Data produced by these assays are fairly free from errors resulting from nuclear pseudogenes.

Concluding remarks

Studies of animal mtDNA have been very fruitful, but there are pitfalls as we have seen above. Nuclear mitochondrial-like sequences, while capable of serving as valuable

Box 2. Endosymbiotic theory of the origin of mitochondria

This theory[27] hypothesizes a xenogenous origin of mitochondria, that is, mitochondria are evolutionary remnants of bacterial-like ancestors that were harboured in prototype eukaryotic cells possessing primitive forms of the present nuclear genome. Endosymbiotic relationships maintained their coexistence. As many mtDNA genomes observed today are substantially smaller in size and simpler in complexity than bacterial genomes, it has been postulated that many genes or DNA sequences in the genomes of the progenitors of mitochondria have been lost or transferred into the host nuclear genome during evolution. Most functionally successful transfers appear to have occurred early in evolution, because the genes that have been retained in the mitochondrial genomes of animals, plants, fungi, algae and protists are fundamentally similar. Between deeply diverged animal lineages, such as vertebrates and invertebrates, a nearly identical set of functional genes is maintained in the mtDNA genome (see Refs 6,27,29 and 30 for reviews).

Different terms are used in the scientific literature to describe nuclear sequences of mitochondrial origin, including: nuclear copies of mtDNA, nuclear mitochondrial-like sequences, nuclear ex-mitochondrial sequences, nuclear insertions, nuclear integrations, transposed mitochondrial sequences, and so on. These terms are employed interchangeably in this paper.

molecular tools, are particularly challenging for the employment of PCR-based technologies in evolutionary studies using mtDNA markers. We must be aware of this and check for erroneous results. Some assays have been suggested here to help to overcome this problem, but we also acknowledge the need to study the nuclear sequences themselves. In fact, only with a comprehensive analysis of the presence and evolution of these nuclear homologues, in vertebrates and in invertebrates, can we safely guard against confusion. Such approaches, in return, will provide novel opportunities for a more effective use of mtDNA markers in evolutionary studies.

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