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INVITED REVIEW

Revealing the hidden complexities of mtDNA inheritance

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Abstract

Mitochondrial DNA (mtDNA) is a pivotal tool in molecular ecology, evolutionary and population genetics. The power of mtDNA analyses derives from a relatively high mutation rate and the apparent simplicity of mitochondrial inheritance (maternal, without recombination), which has simplified modelling population history compared to the analysis of nuclear DNA. However, in biology things are seldom simple, and advances in DNA sequencing and polymorphism detection technology have documented a growing list of exceptions to the central tenets of mitochondrial inheritance, with paternal leakage, heteroplasmy and recombination now all documented in multiple systems. The presence of paternal leakage, recombination and heteroplasmy can have substantial impact on analyses based on mtDNA, affecting phylogenetic and population genetic analyses, estimates of the coalescent and the myriad of other parameters that are dependent on such estimates. Here, we review our understanding of mtDNA inheritance, discuss how recent findings mean that established ideas may need to be re-evaluated, and we assess the implications of these new-found complications for molecular ecologists who have relied for decades on the assumption of a simpler mode of inheritance. We show how it is possible to account for recombination and heteroplasmy in evolutionary and population analyses, but that accurate estimates of the frequencies of biparental inheritance and recombination are needed. We also suggest how nonclonal inheritance of mtDNA could be exploited, to increase the ways in which mtDNA can be used in analyses.

Keywords: implications, inheritance, mtDNA, nonclonality

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Introduction

A unique mode of inheritance, and a high mutation rate, has made mitochondrial DNA (mtDNA) appealing as a molecular tool in evolutionary biology. In animals, mtDNA is inherited, for the most part, uniparentally through the maternal germline. This, along with the realisation that vegetative segregation efficiently removes heteroplasmy at mitosis, with only rare reported cases of heteroplasmy, led to the early assumption that individuals generally possess only one mtDNA haplotype—a condition known as homoplasmy (Birky 1978, 2001). Homoplasy is also attained through other mechanisms effective at removing genetic diversity, such as selection (Rispe & Moran 2000; Rand 2001), and the mitochondrial bottleneck (Bergstrom & Pritchard 1998). Further, mitochondria are not made *de novo*, but rather self-replicate from existing organelles (Jansen & de Boer 1998).

Homoplasmy precludes recombination between heterogeneous molecules; thus, mtDNA was considered to be effectively nonrecombining. In theory, any base-pair changes that exist between mtDNA molecules should therefore come about by mutation. This leads to a signal of molecular change that is well suited for tracing evolutionary change of species and the dynamics of populations, as it allows for unambiguous definition of matrilineal genetic relationships and, if mutation rates are known, the dating of evolutionary events (Avise *et al*. 1987; Moritz *et al*. 1987). Indeed, mtDNA has been pivotal in answering questions important to many areas of ecology and evolutionary biology including phylogeography (Avise *et al*. 1987), conservation genetics

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(Robertson *et al*. 2007), tracing ancestry (Cann *et al*. 1987), revealing social dynamics (Bass *et al*. 1996), and resolving taxonomic uncertainties (Hebert *et al*. 2003).

However, what if the well-established laws of mtDNA inheritance are over-simplifications of the processes affecting mtDNA, or are in some cases simply untrue? Might the suitability of mtDNA be compromised and the way in which mtDNA data is analysed need reconsideration to ensure that correct conclusions are drawn? Here, we show that such a scenario is not only possible, but likely. Evidence has been accumulating over the last 20 years that challenges many of our core assumptions about mtDNA inheritance (Eyre-Walker 2000; Bromham *et al*. 2003; Slate & Gemmell 2004). Although uniparental inheritance and no recombination are more common than their alternatives, it is now well established that exceptions do occur, to what were once regarded as a strict set of rules for mtDNA inheritance, across the eukaryotes.

Curiously, while deviations are well recognised in plants and fungi (Schnable & Wise 1998; reviewed by Barr *et al*. 2005), the recognition that all may not be as it seems for animal mtDNA has lagged. However, with the advance of technologies with increased sensitivity capable of processing larger sample sizes, e.g. minor SNP alleles can be detected at frequencies as low as 0.05 to 1% (White *et al*. 2005; Wolff & Gemmell 2008a), reported cases of exceptions to the generally held laws of mtDNA inheritance are becoming more abundant. Thus, it is timely to re-address animal mtDNA inheritance. Here, we build from previous reviews of mtDNA (Birky 2001; Ballard & Whitlock 2004) by reassessing the arguments using new evidence, focusing on the implications for molecular ecologists who rely on a simple mode of inheritance.

Biology of mtDNA

Although the vast majority of mitochondrial protein transcription and translation is encoded for and controlled by the nucleus (Ryan & Hoogenraad 2007), mitochondria retain their own DNA — a remnant of an α-proteobacteria ancestry (Gray *et al*. 1999). The roles of mtDNA are well conserved across the eukaryotes, not least the encoding of vital components of the oxidative phosphorylation pathway (Saraste 1999). On the other hand, the size and structure of the mitochondrial genome can vary dramatically across eukaryotes (Burger *et al*. 2003). In animals, they are mostly double-stranded, circular, and lack introns, with highly conserved size and gene content: genomes range between 15 and 20 kb and generally encode 22 transfer RNAs, two ribosomal RNAs and 13 subunits of the oxidative phosphorylation pathway (Wolstenholme & Clary 1985; Kocher *et al*. 1989; Shadel & Clayton 1997). mtDNA may aggregate into nucleoprotein complexes on the inner mitochondrial membrane, referred to as nucleoids, with 2 to 10 mtDNA copies per nucleoid (Satoh & Kuroiwa 1991; Legros *et al*. 2004; Malka *et al*. 2006), the evolution and dynamics of which are now beginning to be understood (Kucej & Butow 2007; Bogenhagen *et al*. 2008). Total cellular mtDNA copy number can vary between cell types (Table 1).

Table 1 mtDNA copy number in mammalian cells. Somatic cells consist of either immortalised cell lines[2], or a combination of immortalised cell lines and primary human fibroblasts[1]

Cell type	Per mitochondrion	Per cell (×10³)
Somatic	1–15 (average 4.6)[2]	1.6–4.1[1]
Mature oocyte	1–2[3,4]	100–200[5,6]
Mature sperm	1[7]	0.1[7]

References: [1](Legros *et al.* 2004), [2](Satoh & Kuroiwa 1991), [3](Shoubridge & Wai 2007), [4](Jansen & de Boer 1998), [5](Piko & Taylor 1987), [6](Cree *et al.* 2008), [7](Hecht *et al.* 1984).

Replication of mtDNA, which occurs semi-autonomously from nuclear control (Larsson *et al*. 1998; Ekstrand *et al*. 2004; Ryan & Hoogenraad 2007), is relaxed so that some of the multiple mtDNA copies in a healthy organelle replicate more than others by chance, or because they possess some intrinsic replicative advantage (Birky 1994, 2001). Segregation of mtDNA between cells in mitosis and meiosis occurs vegetatively due to: (i) relaxed replication of mtDNA, (ii) its stochastic partitioning into daughter organelles, and (iii) the stochastic partitioning of organelles into daughter cells (Birky 2001). Intergenerational transmission of mtDNA is characterised by a genetic bottleneck at early developmental stages, through which mtDNA molecules must pass (Box 1, Fig. 1). This bottleneck, which may result in a reduction from millions to as few as 100 mtDNA copies (Jenuth *et al*. 1996; Wolff *et al*. unpublished), can have a dramatic effect on offspring mtDNA genotype (Koehler *et al*. 1991; Blok *et al*. 1997; Cree *et al*. 2008).

Establishing the laws of mtDNA inheritance

The theory that mitochondrial genes are inherited uniparentally through the maternal germline, in the majority of animals, was initially based on analogy to the inheritance of chloroplasts in plants, and mitochondria in plants and fungi (Birky 1978, 1995). Evidence for a cytoplasmic and uniparental mode of transmission for mtDNA, first came from the observation by yeast geneticists, in the 1950s, that some mitochondrial characters were inherited in a cytoplasmic fashion (reviewed by Williamson 2002). Clones of baker's yeast frequently gave rise to mutants on glucose-limited media, characterised by reduced colony size (so-called 'petite' mutants), resulting from a lack of several enzymes involved in respiration; a phenotype

Box 1 The mitochondrial bottleneck

The paradox of dramatic shifts in frequency of mtDNA genotypes across few generations, despite vast numbers of mtDNA molecules in mature oocytes (Hauswirth & Laipis 1982; Ashley *et al*. 1989), can be explained, in part, by a mitochondrial bottleneck that occurs during early developmental stages (Bergstrom & Pritchard 1998). Although not the only factor likely at work (Birky 2001; Cao *et al*. 2007), this bottleneck may prevent an accumulation of deleterious mutations and 'mutational meltdown', that would otherwise occur in a clonally inherited molecule via Muller's ratchet (Bergstrom & Pritchard 1998; Shoubridge & Wai 2007).

While there is little contention about their existence, what remains debated is whether it is at embryogenesis, or oogenesis, that the strongest effects of mitochondrial bottlenecks are felt (Jenuth *et al*. 1996; Smith *et al*. 2002; Cao *et al*. 2007; Cree *et al*. 2008; Khrapko 2008). During mammalian embryogenesis, total embryonic mtDNA content remains constant during early stages of the cleaving embryo (Cao *et al*. 2007; Cree *et al*. 2008), with mitochondria being equally apportioned to daughter cells. Most of the blastocyst forms extra-embryonic tissues; thus, only a subset of all cells (the inner cell mass, ICM) will contribute to the developing embryo (Hogan *et al*. 1986; Fleming *et al*. 1992). The apportionment of mitochondria to the ICM constitutes a numerical bottleneck, during which rare mtDNA haplotypes are prone to loss (Bergstrom & Pritchard 1998). During mammalian oogenesis, the vast number of germ cells at maturity originates from a limited number of progenitor germ cells (PGCs), each of which contains approximately 10–100 mitochondria (Shoubridge & Wai 2007). There is then an enormous expansion in cell number (to ~25 000 and ~10^7 primary oocytes in mice and human, respectively), and increase in mitochondria number. The number of mtDNA molecules increases dramatically to around 200 000 mtDNA copies in mature oocytes (Jansen & de Boer 1998; Shoubridge & Wai 2007).

This large decrease in mtDNA per cell during embryogenesis, and dramatic increase in oogenesis, means only a subset of maternal mtDNAs will re-populate successive generations. For a heteroplasmic individual this often means a return to homoplasmy, but can lead to strong founder effects (Bergstrom & Pritchard 1998).

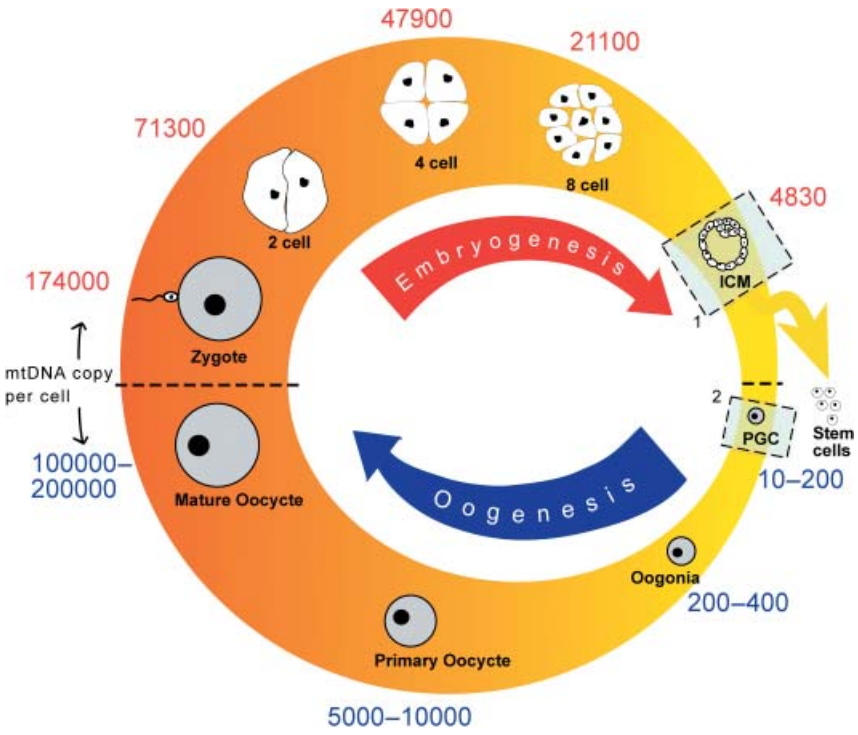

Fig. 1 The mammalian mitochondrial bottleneck. 1 and 2 represent the stages that have the strongest effect on haplotype segregation. ICM, inner cell mass; PGC, progenitor germ cell. Estimates of mtDNA copy per cell come from Cao *et al*. (2007) and Shoubridge & Wai (2007).

associated with cytoplasmic particles precipitable by centrifugation (Ephrussi *et al*. 1955). mtDNA was formally identified and localised in the 1960s (reviewed by Ernster & Schatz 1981; Mounolou & Lacroute 2005), and the first direct evidence for uniparental, and maternal, inheritance of mtDNA in animals was available by 1972 (Dawid & Blackler 1972). Thus, the concept of strict maternal inheritance in animals gained popularity and attained further support from numerous studies which failed to detect paternal mtDNA in a wide range of species (Hutchison *et al*. 1974; Hayashi *et al*. 1978; Kroon *et al*. 1978; Avise *et al*. 1979; Francisco *et al*. 1979; Giles *et al*. 1980; Reilly & Thomas 1980; Gyllensten *et al*. 1985).

More recent observations on the importance of stochastic processes, in preventing paternal mtDNA transmission, support these early findings. For example, the relatively low proportion of paternal mtDNA to maternal mtDNA makes paternal mtDNA prone to loss by drift: mammalian female gametocyte mtDNA copies can outnumber their male counterparts to the order of 10^3 to 10^4 (Table 1). Further, the effects of genetic drift of paternal lineages can be amplified by the mitochondrial bottleneck (Birky 2001; Shoubridge & Wai 2007; Wolff & Gemmell 2008b). Some mechanisms are taxa-specific: in some tunicates, sperm organelles do not enter the egg at all (Ursprung & Schabtach 1965), whereas in other groups, active processes are in place to exclude male mtDNA. For example, elimination of sperm mitochondria within the egg cytoplasm has been shown in mice and hamsters (Hiraoka & Hirao 1988; Kaneda *et al*. 1995). In cattle and primates, this process involves ubiquitination of the sperm mitochondria outer membrane (Thompson *et al*. 2003), followed by subsequent recognition and proteolytic digestion by the female cell (Sutovsky *et al*. 1999, 2000, 2003).

Why mechanisms exist to prevent transmission of paternal mtDNA is not well understood. Bromham *et al*. (2003) have reviewed two possible reasons. The first, more popular explanation, is the prevention of selfish behaviour by competing mtDNAs (Hastings 1992) leading to potentially lethal genome conflict (Hurst & Hamilton 1992). Alternatively, it may be an adaptation to anisogamy which prevents sperm mtDNA, damaged from intense respiration activity, entering the egg and thus being passed on to offspring (Allen 1996).

Some inconvenient truths

The absence of both paternal mtDNA and heterologous recombination are hallmarks of mtDNA inheritance (Birky 2001). However, over the last 20 years, evidence of exceptions has accumulated, with multiple cases of heteroplasmy, paternal leakage of mtDNA and recombination now documented. While these exceptions are well accepted, some debate has ensued as to how much consideration should be given to these vs. the more conventional views on mtDNA. A summary of this debate is given in Table 2.

Heteroplasmy

Why heteroplasmy should be common

In the absence of external factors, heteroplasmy should, theoretically, be the default state for mtDNA under a simple mutation-drift scenario. mtDNA has an increased mutation rate when compared to nuclear DNA (Brown *et al*. 1979; Ingman *et al*. 2000; Mishmar *et al*. 2003; Kivisild *et al*. 2006), due to the mutagenic properties of reactive oxygen species (the harmful by-products of oxidative phosphorylation), an error-prone polymerase and limited DNA repair (Bogenhagen 1999). This high mutation rate coupled with the large population size of mtDNA in mature oocytes would make a state of homoplasmy in any cell, tissue or individual surprising under a simple neutral drift model, in the absence of mechanisms that effectively remove genetic diversity from the mtDNA population, e.g. germline bottlenecks, gene transfer and selection against deleterious mutations (Bergstrom & Pritchard 1998; Martin & Herrmann 1998; Rispe & Moran 2000; Rand 2001). Even if selection is actively removing mutant mtDNA types, a return to absolute homoplasmy from a state of significant heteroplasmy can take time, and the resulting transient heteroplasmy is common across many animal taxa (Table 2).

A state of heteroplasmy is also reached if paternal mtDNA enters the egg cytoplasm at fertilisation, referred to as paternal leakage. In this case, a heteroplasmic state has been achieved in the oocyte, not via mutation, but due to the coexistence of mitochondria from two unique ancestral lineages. Until recently, paternal leakage was thought impossible as it was widely held that paternal mtDNA did not reach the egg cytoplasm (Ankel-Simons & Cummins 1996). We now know this to be false, confirmed by examples found in nature (Table 2). These examples show that the mechanisms of preventing paternal mtDNA transmission are not fully understood, and may fail at times (Birky 1995, 2001; Thompson *et al*. 2003). Perhaps the most striking evidence obtained for leakage of paternal mtDNA, to date, is from a 28-year-old-man suffering from a mitochondrial myopathy, caused by a paternally inherited, deleted mtDNA, and present in the patient's muscle cells in a 10-to-1 ratio of paternal to maternal mtDNA (Schwartz & Vissing 2002).

Although less so than homoplasmy, it is now generally accepted that mtDNA heteroplasmy is widespread. However, to influence evolution, heteroplasmy must persist via the germline into future generations, or remain intact in the oocyte cytoplasm long enough for recombination with maternal molecules to occur, and these derivatives then

Table 2 A summary of the debate for and against the strictly clonal evolution of animal mtDNA

Supporting observation	Supporting arguments	Contradictory arguments
Homoplasy	Mechanisms are in place to remove mtDNA genetic variation[1–2] Reported levels of heteroplasmy may be overestimates as may originate from alternate sources such as NUMTs[8] and duplicated mtDNA genes[46] that have not been controlled for Heteroplasmy, where confirmed, is likely to be transient[6-7]	Heteroplasmy is common in fungi, protists and plants, and is increasingly observed in animals[3–5,47,48] Low-level heteroplasmy may be more abundant than first thought, but is generally below the detection limit of standard polymorphism detection technologies[5,52]
Strict maternal inheritance	Early mtDNA studies failed to show any paternal contribution[50,51] Paternal inheritance should be suppressed to avoid potentially lethal genome conflict[9] Virtually no evidence for paternal mtDNA reaching germline; therefore, influence on evolution is not certain	Biparental inheritance has been documented in mammals, birds, reptiles, fish, molluscs, nematodes, and arthropods[11–27], and is the norm in some bivalves[23,25,28] Paternal leakage occurs frequently but its contribution may be below detection limits[10]
No heterologous recombination	Homoplasy precludes heterologous recombination Empirical evidence for recombination is limited to somatic tissues; therefore, it remains unknown if recombination would have any impact on mtDNA evolution[49] In some early cases of reported recombination in humans, through mainly indirect means, quality of polymorphism data was questioned and results were not able to be repeated or were revoked[41–44]	Recombination is common in other eukaryotes including yeast and plants[54,55] Empirical evidence for recombination has been documented in mammals, reptiles, fish, arthropods, molluscs and nematodes[13–14,24,29–34] Indirect evidence for widespread recombination in animal mtDNA is conclusive[56,57] The enzymatic machinery required for recombination has been shown in the mitochondria of several animal species[36, 37, 53] Fusion and fission of mitochondria is common[38-40] Recombination occurs but remains undetected due to: (i) sequence similarity between molecules, (ii) low mtDNA population frequencies, (iii) indirect methods not sufficiently sensitive[45]

References. [1](Birky 2001), [2](Bergstrom & Pritchard 1998), [3](Barr *et al.* 2005), [4](Kann *et al.* 1998), [5](Kmiec *et al.* 2006), [6](Hauswirth & Laipis 1982), [7](Koehler *et al.* 1991), [8](Parr *et al.* 2006), [9](Hurst 1996), [10](Wolff & Gemmell 2008b), [11](Arunkumar *et al.* 2006), [12](Fontaine *et al.* 2007), [13](Gantenbein *et al.* 2005), [14](Guo *et al.* 2006), [15](Gyllensten *et al.* 1991), [16](Kondo *et al.* 1990), [17](Kvist *et al.* 2003), [18](Magoulas & Zouros 1993), [19](Meusel & Moritz 1993), [20](Schwartz & Vissing 2002), [21](Sherengul *et al.* 2006), [22](Shitara *et al.* 1998), [23](Theologidis *et al.* 2007), [24](Ujvari *et al.* 2007), [25](Venetis *et al.* 2006), [26](Zhao *et al.* 2004), [27](Zouros *et al.* 1992), [28](Breton *et al.* 2007), [29](Armstrong *et al.* 2007), [30](Ciborowski *et al.* 2007), [31](Hoarau *et al.* 2002), [32](Lunt & Hyman 1997), [33](Ladoukakis & Zouros 2001a), [34](Kraytsberg *et al.* 2004), [35](Zsurka *et al.* 2005), [36](Lakshmipathy & Campbell 1999a), [37](Thyagarajan *et al.* 1996), [38](Chan 2006), [39](Westermann 2002), [40](Yaffe 1999), [41](Hagelberg *et al.* 2000), [42](Kivisild & Villems 2000), [43](Macaulay *et al.* 1999), [44](Jorde & Bamshad 2000), [45](D. J. White & N. J. Gemmell, unpublished), [46](Abbott *et al.* 2005), [47](Mate *et al.* 2007), [48](Mjelle *et al.* 2008), [49](Slate & Gemmell 2004), [50](Dawid & Blackler 1972), [51](Avise *et al.* 1979), [52](Millar *et al.* 2008), [53](Pontkingdon *et al.* 1995), [54](MacAlpine *et al.* 1998), [55](Bergthorsson *et al.* 2003), [56](Piganeau *et al.* 2004), [57](Tsaousis *et al.* 2005).

reach the germline. The focus of attention should now turn from whether mtDNA heteroplasmy exists, to its frequency in wild populations, how many taxa it affects, its longevity, how stability is maintained where it persists, and under what circumstances paternal leakage may occur (Rokas *et al*. 2003; Fontaine *et al*. 2007).

Widespread in nature

Cases of heteroplasmy due to point mutations or deletions are now numerous across animals (Table 2). Although rarer, cases of paternal leakage-driven heteroplasmy are also increasing in number. Over the last 2 years, interspecific paternal leakage has been revealed in silkmoths, fruit flies, periodical cicadas and salmonids (Arunkumar *et al*. 2006; Sherengul *et al*. 2006; Ciborowski *et al*. 2007; Fontaine *et al*. 2007). Recent cases of intraspecific paternal leakage include scorpions, fruit flies, and lizards (Gantenbein *et al*. 2005; Sherengul *et al*. 2006; Ujvari *et al*. 2007). To date, the majority of cases come from interspecies or interpopulation hybridisations, most likely as a result of (i) a breakdown of mechanisms to recognise and remove

paternal mtDNA, and (ii) heightened genetic dissimilarity between parental mtDNAs that increases the chance of detection (Kaneda *et al*. 1995; Shitara *et al*. 1998; Sutovsky *et al*. 2000). Overall, the spectrum of taxa in which paternal leakage has been observed is becoming increasingly more diverse, with representation from invertebrates, fish, reptile, bird and mammal groups. It is unlikely that these cases are specific to the taxonomic groups studied but rather represent the amount of research that has focused on these groups, as well as serendipitous opportunities. As research groups interested in other taxa address the issue of paternal leakage, the list will surely grow.

Determining the frequency of paternal leakage is challenging in most settings, due to high genetic similarity in populations between paternal and maternal haplotypes, which makes the detection of nonmaternal haplotypes difficult (hybrid zones being the obvious exception; Kvist *et al*. 2003). The rarity of paternal leakage (Gyllensten *et al*. 1991; Sherengul *et al*. 2006; Wolff & Gemmell 2008b) may also mean that molecular analyses, employing standard technologies, have a limited power of detection (Milligan 1992). Further, adequate screening for paternal mtDNA is seldom part of current mitochondrial studies, unless the authors are explicitly exploring the issue. Such checks add significantly to both the study time and cost, but without them, important cases of paternal leakage may be overlooked.

Persistence in populations

The neutral theory of molecular evolution predicts that persistence of neutral polymorphisms is dependent on the effective population size of mtDNA molecules within an individual (mtDNA N_e) (Kimura & Ohta 1969). Therefore, variation in heteroplasmy longevity is expected between taxa for neutral polymorphisms based on differences in life- history traits. The limited numbers of observations we have for heteroplasmy persistence across taxa reflect this prediction. In mice, apparently neutral, heteroplasmic variants can persist for at least 14 generations (Gyllensten *et al*. 1991). On the other hand, in insects it may take 500 generations to return to homoplasy (Solignac *et al*. 1984; Rand & Harrison 1986), suggesting the mtDNA bottleneck is considerably less strong in insects compared to mammals, either as a result of a considerably larger mtDNA N_e, or fewer cell divisions at oogenesis (Ashley *et al*. 1989). A classic series of studies showed how heteroplasmy can, apparently, return to homoplasy in just a few generations in *Bos taurus* (Hauswirth & Laipis 1982; Ashley *et al*. 1989), and in one instance just one generation was required (Koehler *et al*. 1991). However, although the polymorphism being tracked in this study was located in the noncoding mtDNA control region, it was a guanosine/cytosine transversion close to the phenylalanine tRNA and may have been under selection. Furthermore, as the mtDNA is effectively in complete linkage (in the absence of recombination), there may well have been selection operating at other sites that varied between the mtDNAs examined, affecting the outcome but remaining undetected.

For paternal leakage to influence evolutionary analyses, paternal mtDNA must proliferate considerably during embryogenesis, and to influence the evolution of populations, it will need to reach the germline. Recent studies have demonstrated that both criteria may apply to the dynamics of paternal mtDNA transmission in insects. Fontaine *et al*. (2007) hybridised closely related species of periodical cicadas, and then screened for paternal mtDNA in eggs at various developmental stages. Paternal mtDNA was detected in 4-day old eggs, and up to 16-month old nymphs (but not in 1-day old eggs), in 45 out of 97 instances (Fontaine *et al*. 2007). They were able to detect paternal mtDNA at a ratio of 1:100 paternal to maternal for three cases, and 1:20 in the fourth; however, absolute molecular frequencies were not determined. Their work suggests that, although paternal mtDNA may be too rare for detection early in development, proliferation into later stages occurred. Sherengul *et al*. (2006) examined paternal leakage in both intraspecific and interspecific *Drosophila* hybrids (Sherengul *et al*. 2006), and observed that intraspecific leakage occurred in 18.6 to 48.3% of backcrosses between F_1 females and males of either parental strain (with a sensitivity level of 10^{-3} paternal molecules to maternal), whereas frequencies for interspecific leakage were higher at 30.6 to 63.1% of backcrosses (sensitivity level of 10^{-6}). By screening adult flies, they showed that paternal mtDNA successfully reached later stages of development. These results agree with earlier work (Kondo *et al*. 1990), and confirm that paternal mtDNA can proliferate into the soma and the germline in insects, perhaps support for a wider mitochondrial bottleneck than other animals (Ashley *et al*. 1989). However, what remains unknown across the animal kingdom is the regularity with which paternal mtDNA reaches the gametic tissues and is transmitted.

It may be possible to predict the longevity of neutral heteroplasmy if mtDNA N_e is known. For a population consisting of a constant number of segregating units, where the number of copies of any segregating unit has a Poisson distribution, the expected time for fixation of a mitochondrial mutation is $2N_e$ generations, and $2\ln(2N_e)$ for loss (Kimura & Ohta 1971). For humans, N_e is estimated to be around 100 (Howell *et al*. 1992; Bendall *et al*. 1996; Jenuth *et al*. 1996), and Wolff and colleagues have recently calculated mtDNA N_e for the chinook salmon (*Oncorhynchus tshawytscha*) to be close to 100 (Wolff *et al*. unpublished). This leads to a predicted time to fixation for a neutral, mitochondrial, heteroplasmic variant in humans and chinook salmon of approximately 200 generations.

Influence of selective forces. Intergenerational transmission of heteroplasmy will be strongly affected if variation is non-neutral. Disease-causing mutations in humans have been shown to be extremely skewed in their intergenerational frequencies, often becoming fixed in one generation (Blok *et al*. 1997; White *et al*. 1999; Brandstatter *et al*. 2004). However, persistence has also been observed across multiple generations (Holt *et al*. 1990). Heteroplasmic, pathogenic point mutations may persist if they remain under a certain threshold frequency (generally ≤ 80%, Wallace 1999), as their wild-type counterparts (with whom they coexist in a cell) compensate for functional reductions and prevent negative selection from acting at the cellular level (Chomyn *et al*. 1992). Further, variant mtDNA molecules that harbour deleterious deletions may persist via a replicative advantage (Yoneda *et al*. 1992). For example, mtDNA genomes with large deleterious deletions have been shown to persist for multiple generations in laboratory populations of nematodes, coexisting with wild-type counterparts at a frequency of around 60% (Tsang & Lemire 2002; Liau *et al*. 2007). Likewise, flies with large-scale deletions, including coding sequence, have also shown to be genetically stable (Beziat *et al*. 1997; Petit *et al*. 1998). A plausible hypothesis for these apparently counter-intuitive observations is that deleted mtDNA molecules confer a replication advantage; it takes less resources and time to copy a smaller molecule.

Until recently, direct evidence for the forces that underlie the transmission of pathogenic mtDNA haplotypes to subsequent generations remained elusive. For the past decade, researchers have debated whether selection — positive or negative — or drift has a greater impact (Chinnery *et al*. 2000). Early work was limited to coincidental sampling from human pedigrees diagnosed with mitochondrial disease (Blok *et al*. 1997; White *et al*. 1999; Chinnery *et al*. 2000). However, these models incorporate several factors that make testing for selection inconclusive including (i) small sample sizes introducing potential ascertainment biases, and (ii) temporal changes in the mutation load of somatic tissues (Chinnery *et al*. 2000). Recently, two studies used experimental design that *can* adequately differentiate between drift and selection (Fan *et al*. 2008; Stewart *et al*. 2008). Both generated maternal mice heteroplasmic for pathogenic mutations, and then tracked their progression into future generations. Evidence was found for purifying selection during oogenesis in both: within four generations for one (Fan *et al*. 2008), and after two generations for the other (Stewart *et al*. 2008). Two plausible mechanisms of selection, working at the organelle level, have been put forward by Shoubridge & Wai (2007). One suggests that pathogenic mtDNA mutations may make the mitochondria prone to autophagy, whereas the other suggests that non-silent mutations will simply make these mitochondria less efficient at replicating, leading to them being outnumbered by healthy organelles.

Pseudo mitochondrial heteroplasmy

One final, but important, consideration when screening for heteroplasmy is the detection of a heteroplasmic signal in the absence of true heteroplasmy. Nuclear-encoded, mitochondrial pseudogenes (or NUMTs) are non-functional copies of mitochondrial sequences, transferred and incorporated into the nuclear genome, that can retain close homology to the original mitochondrial genes (Bensasson *et al*. 2001; Tourmen *et al*. 2002; Woischnik & Moraes 2002). In species harbouring NUMTs, polymerase chain reaction (PCR) primers may hybridise to both mitochondrial sequence and nuclear copies (Collura & Stewart 1995; Zhang & Hewitt 1996; Sorenson & Quinn 1998). The resulting co-amplifications can be mistakenly recognised as heteroplasmic sites, or lead to inaccurate estimates of heteroplasmy levels (Hirano *et al*. 1997). It is therefore important to validate the origin of heteroplasmic sites (Parr *et al*. 2006), and various means for the detection of NUMTs and exclusion from analyses have been comprehensively reviewed elsewhere (Bensasson *et al*. 2001). A further, but less common, source of pseudo mtDNA heteroplasmy comes from the presence of duplications within the mitochondrial genome (Kumazawa *et al*. 1996; Eberhard *et al*. 2001; Lee *et al*. 2001; Vali 2002). Single sites may appear heteroplasmic when in fact two separate regions are being typed (Abbott *et al*. 2005). If a primer pair amplifies the target region and its duplication, it is possible variant amplicons may be distinguished by size.

Recombination

Recombination is an important evolutionary mechanism for the removal of deleterious mutations from populations, as well as for generating new, favourable combinations of alleles (Hurst & Peck 1996). Up until the late 1990s, mtDNA was believed not to recombine, relying on alternative mechanisms to maintain fitness of mtDNA populations within individuals (Bergstrom & Pritchard 1998). This viewpoint was reassessed with evidence suggesting that both intra- and intermolecular recombination is possible in animals including: (i) the presence of the required enzymatic machinery in mitochondria (Thyagarajan *et al*. 1996; Lakshmipathy & Campbell 1999a); (ii) widespread mtDNA recombination in plants and fungi (reviewed by Barr *et al*. 2005); (iii) intramolecular recombination in nematodes (Lunt & Hyman 1997) and humans (Holt *et al*. 1997; Tang *et al*. 2000); (iv) and intermolecular recombination in animals with a unique mitochondrial inheritance system (Ladoukakis & Zouros 2001a; Burzynski *et al*. 2006). Further, in 2004 and 2005, two groups analysed the

distribution of polymorphisms in animal mtDNA sequences and both provided statistical support for widespread, low level, mitochondrial recombination across the animal kingdom (Piganeau *et al*. 2004; Tsaousis *et al*. 2005). Tsaousis showed 30 recombination events out of 186 alignments across 29 genera (Tsaousis *et al*. 2005), whereas one of the tests Piganeau and colleagues employed revealed recombination in 14.2% of 279 animal mtDNA data sets (Piganeau *et al*. 2004). Early, indirect studies also revealed recombination in human mtDNA (Awadalla *et al*. 1999; Eyre-Walker *et al*. 1999), but the methodology and quality of sequence data used in these studies have come under scrutiny, and those results, largely, have not been repeated (Macaulay *et al*. 1999; Jorde & Bamshad 2000; Kivisild & Villems 2000). However, empirical evidence for intermolecular recombination across the animal kingdom remains elusive, which may be due to difficulties in its detection (Box 2). Nevertheless, there have been several compelling examples in history, not least the revelation by Kraytsberg and colleagues of heterologous recombination in the skeletal muscle of a muscular myopathy patient that harboured a 10:1 excess of paternal compared to maternal mtDNA. They showed 7% of maternal mtDNA molecules, or 0.7% of total mtDNA, were recombinant (Kraytsberg *et al*. 2004).

Two new, convincing cases of mitochondrial recombination have been documented. Ujvari *et al*. (2007) revealed structure between two populations of Australian frillneck lizard (*Chlamydosaurus kingii*). One anomalous individual was found genetically similar to one population at most of its mtDNA, but not the ND2 gene at which it mirrored the second population. Whole genome sequence revealed a pattern of polymorphism distribution consistent with a recombination event around the ND2 gene, confirmed by four different statistical analyses. As such, it appears the authors have revealed the first case of intraspecific mitochondrial recombination in a wild terrestrial vertebrate (Ujvari *et al*. 2007). However, the possibility that the result may have been influenced by a NUMT was not raised, albeit this would have been a less parsimonious explanation.

Ciborowski *et al*. (2007) examined mtDNA sequence in 717 Atlantic salmon, angled from four rivers in northern Spain between 1948 and 2002. One individual showed an anomalous restriction fragment length polymorphism pattern across the ND1 gene that showed sequence homology at separate regions, to both Atlantic salmon and the closely related brown trout. As neither contamination nor a NUMT were apparently responsible for the result, and since brown trout/Atlantic salmon hybridisations are relatively common in this region, the result was reported as a clear example of interspecific recombination. This was a chance finding as the researchers were not initially using this taxonomic group to search for recombination, and it raises interesting questions. How many other cases of as yet undiscovered recombination exist in nature? And what is the likelihood that recombinant mtDNA molecules are unknowingly incorporated into analyses?

Problems and opportunities

The discovery of high levels of mtDNA heteroplasmy in some populations (Rand & Harrison 1986; Liau *et al*. 2007; Mate *et al*. 2007; Mjelle *et al*. 2008), together with documented cases of paternal leakage (Gyllensten *et al*. 1991; Schwartz & Vissing 2002; Kvist *et al*. 2003; Sherengul *et al*. 2006; Fontaine *et al*. 2007) and recombination (Kraytsberg *et al*. 2004; Ciborowski *et al*. 2007; Ujvari *et al*. 2007), raises a number of intriguing challenges for the molecular ecological community. In particular, we will need to explore whether these 'inconvenient truths' about mtDNA inheritance occur frequently enough to alter our current understanding of organismal evolution derived from this molecule. This issue has to date received little attention due, in part, to the strength of confidence in a strictly clonal model of evolution for this marker.

Mitochondrial heteroplasmy

Mitochondrial heteroplasmy can create some ambiguity in phylogenetic and network interpretations of population data (see the case study below). If unaccounted for, paternal mtDNA could potentially lead to inaccurate estimates of divergence times if molecular clocks are employed, and confuse haplogroup designation (Eyre-Walker 2000; Slate & Gemmell 2004). However, if the heteroplasmy is stable, it may provide useful additional information for defining haplotypes, and resolving further the relationships among individuals at a population level. The most famous instance in which heteroplasmy has helped clarify the relationships among individuals is the identification of the remains of Russia's last Tsar, Nicholas II, and his family (Gill *et al*. 1994; Ivanov *et al*. 1996). In July 1991, nine skeletons were exhumed in Yekaterinburg, Russia, which were believed to include the Russian Tsar Nicholas II, the Tsarina Alexandra, and three of their daughters. To identify the remains, mtDNA sequences were obtained and analysed against Prince Philip, a living maternal relative of Tsarina Alexandra. The mtDNA from the four female skeletons exactly matched those of Prince Philip, providing strong evidence that the remains were those of the Tsarina and her daughters. However, the analysis of the mtDNA from the presumed skeleton of the Tsar was more complicated, because the sample was heteroplasmic, possessing both a C and a T at a single position. Sequencing of mtDNA from the Tsar's brother, Georgij Romanov, showed that he too was heteroplasmic for the same nucleotides at the same site, providing powerful evidence supporting the identification of Tsar Nicholas II (Ivanov *et al*. 1996).

Box 2 Detecting recombination

It is now widely accepted that intermolecular recombination can occur in animal mtDNA (Rokas *et al*. 2003; Piganeau *et al*. 2004; Tsaousis *et al*. 2005), although whether it occurs frequently enough to cause concern is contentious (Elson & Lightowlers 2006). Empirical evidence is required to determine the frequency of intermolecular recombination in animals. However, many factors exist which may not only prevent recombination, but also its detection when it does occur.

Several events are required to occur simultaneously for mtDNA recombination to be detectable. First, there is the necessity for two mtDNA molecules to be present within a cell that differ at two or more nucleotide sites. If individuals are homoplasmic, or heteroplasmic but differ at only one site, recombination will be undetectable (Fig. 2). Second, molecules need to be in close enough physical proximity to allow a crossover event which, unlike nuclear chromosomes, is not part of the life cycle of mtDNA. This may require aggregation of nucleoids, or fusion of organelles (reviewed by Yaffe 1999). Third, essential nuclear-encoded enzymes, such as those for fusion and genome exchange and repair need to be available (Thyagarajan *et al*. 1996; Lakshmipathy & Campbell 1999b; Yaffe 1999; Santel & Fuller 2001). To complicate matters, alignment of homologous regions may be hindered by its circular nature and, for a recombinant molecule to remain intact, two crossovers events are needed.

Even if recombination has occurred between heterologous mtDNA molecules, detection is not guaranteed. The low frequency of recombinant mtDNA molecules, compared to the vast majority of intact molecules, means that they are particularly prone to loss by drift during the stochastic processes of vegetative segregation and the genetic bottleneck. Without proliferation, frequencies will likely be too low for the detection limits of standard technologies (Loeb 2001; Kmiec *et al*. 2006), and the likelihood of proliferation is reduced with low initial frequencies.

One way to circumvent the cost and time of direct analyses is to employ indirect tests, which, through statistical frameworks, estimate the likelihood that patterns of polymorphism distribution can be explained by recombination (Posada & Crandall 2001; Bruen *et al*. 2006), and many tests are currently available. A useful collection of some methods can be accessed via the RDP3 (http://darwin.uvigo.es/rdp/rdp.html, Martin *et al*. 2005), and RecombiTEST (http://www.lifesci.sussex.ac.uk/CSE/test/index.php, Piganeau *et al*. 2004) websites. It should be noted that, as well as levels of recombination, the effectiveness of indirect tests is associated with other parameters, including recombinant frequency and sequence diversity (Posada & Crandall 2001; Wiuf *et al*. 2001; Bruen *et al*. 2006). This means that detection of a recombination event is not guaranteed by any one indirect test, particularly at low levels of recombination (Bruen *et al*. 2006). Further, the power of indirect tests is often determined using sequences simulated under a set of general parameters (Posada & Crandall 2001; Bruen *et al*. 2006). When they are tested using sequence simulated under mitochondrial parameters, overall they do less well (D. J. White & N. J. Gemmell, unpublished). A consensus view is that wherever indirect tests are required, multiple tests should be used.

A final consideration is that, even if statistical support is given for recombination, true recombination may not be detected. Instead, the signal may derive from a molecular artefact that resembles a recombinant molecule, resulting from polymerase infidelity during the amplification reaction (termed 'jump PCR'). In the study of Kraytsberg and colleagues, where definitive evidence for intermolecular recombination in humans was revealed, they incorporated single-molecule PCR to control for this phenomenon (Kraytsberg *et al*. 2004).

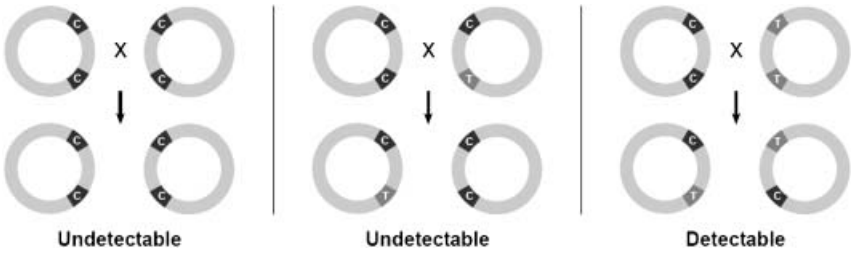

Fig. 2 Detectable recombination in mtDNA.

A common approach to dealing with heteroplasmic bases in mtDNA sequence data is to ignore, or eliminate, those sites from the data. There is some justification in eliminating this data if it is ambiguous, particularly where enough other data are available that the elimination of one or two bases leads to little change in resolution. However, are we losing important information that may tell us more about the relationships among, and within, populations at

a contemporary level, by eliminating heteroplasmic bases from our analyses? Our own work on the relationships among humans in the Pacific suggests this may well be the case (see below). Thus, given that there are ways to incorporate heteroplasmic data into our analyses, incorporation of these data on a more regular basis is warranted.

At present, several phylogenetic analysis programmes that implement parsimony methods are able to incorporate a fifth character state to account for indels in the data (Felsenstein 1989; Swofford 1996; Tamura *et al*. 2007). Using these packages, the alternate states of heteroplasmic sites can be coded as indels to provide additional resolution in some circumstances. Unfortunately, most of the population genetic analysis programmes used routinely by molecular ecologists are incapable of handling heteroplasmic data, but there are statistical approaches to identifying alleles from heterozygous genotypes (Clark 1990; Stephens *et al*. 2001; Adkins 2004; Niu 2004). Haplotypes present in a heteroplasmic individual could thus be determined, permitting the independent examination of the relationship of each mtDNA haplotype, strengthening further our analyses.

The effect of mtDNA recombination on evolutionary inference

Simulations show that a low level of recombination may not have any great impact on the ability of phylogenetic methods to reconstruct trees above the species level and, despite some reticulation, may also appear as expected below the species level (Schierup & Hein 2000; Posada & Crandall 2002). However, while the tree topology recovered when there is recombination may agree with the general features of demographic history, not accounting for recombination may result in an erroneous picture of coalescent events (Schierup & Hein 2000) and, thus, the estimates derived from the coalescent, including divergence times and patterns of population expansion (Kuhner *et al*. 1995; Kuhner *et al*. 1998; Beerli & Felsenstein 1999).

Some attempts have been made to estimate the effect of undetected recombination on estimates of the age of the most recent common ancestors (MRCAs) for humans (Cann *et al*. 1987; Ingman *et al*. 2000). 'Mitochondrial Eve', the female mitochondrial lineage that carried the last common ancestor of all human mitochondria, is estimated to be ~171 000 years old using mitochondrial phylogenies, based explicitly on the assumption of strict maternal inheritance (Ingman *et al*. 2000). However, if recombination occurs, some suggest that the age of the mitochondrial Eve could be underestimated by a factor of 40–50% (Eyre-Walker 2000; Schierup & Hein 2000). This places the migration of modern humans from Africa closer to (although still slightly more recent than) estimates derived from nuclear genes (Templeton 2002). However, with paternal inheritance, the last common ancestor of human mitochondria could be up to four times older than current estimates based on the maternal inheritance model (Gyllensten *et al*. 1991; Bromham *et al*. 2003; Rokas *et al*. 2003; Slate & Gemmell 2004).

More generally, the failure to account for undetected recombination in phylogenetic inference can result in not only an underestimation of MRCAs, especially when divergence is recent, but also in an overestimation of the number of mutations, the apparent exponential growth of populations and the incorrect rejection of a constant molecular clock (Schierup & Hein 2000; Posada *et al*. 2002).

If inheritable recombination in mtDNA occurs, and affects our evolutionary interpretations, it is important to ask how we can build recombination into models of mtDNA evolution. Models have been developed that do not require gene tree reconstruction, and therefore, can allow for recombination, selection and demographic processes (Rannala & Bertorelle 2001). So far, these have only been applied to nuclear DNA, mainly to date relatively recent mutations, but they could presumably be also adapted for mtDNA.

A case study. In the presence of heteroplasmy, mutation events that define splits within a phylogenetic tree may persist over a much longer time frame than the currently assumed single generation (Hauswirth & Laipis 1982). If new mutations were to occur on a heteroplasmic background, recombination between the three or more different mtDNA molecules could result in a set of outcomes that mimic recurrent mutations in phylogenetic reconstructions. A putative illustration of this is seen in a recent survey of mtDNA control region variation within a human Polynesian population, in which two instances of apparent heteroplasmy (at position 16247) in two samples were revealed (Pierson 2007). A transition to guanine at this position defines the distinctive control region haplotype known as the 'Polynesian motif' (PM), which reaches high frequencies in Polynesian populations. Its current distribution in Oceania suggests it arose before the colonisation of Remote Oceania began, about 3000 years ago (Kirch 2000).

Could the heteroplasmy observed in these two individuals have persisted from the time of mutation to the present day? A simple calculation based on the archaeological estimate for the beginning of Remote Oceanic settlement, allowing 20 years per generation, requires the persistence of the heteroplasmic state for at least 150 generations. This figure is consistent with predictions from neutral population genetic theory (~$2N_e$ generations, Kimura & Ohta 1971), if we assume mtDNA N_e in humans is 100 (Jenuth *et al*. 1996).

Whole mtDNA sequences provide a finer resolution to the relationships inferred from the control region (Pierson *et al*. 2006). The 18 complete mtDNA sequences of the N'R'B4a1a1 haplogroup, to which the PM haplotypes belong, include a subgroup of three linked by a transition at nt6905 in the COI gene. Two of these three samples have

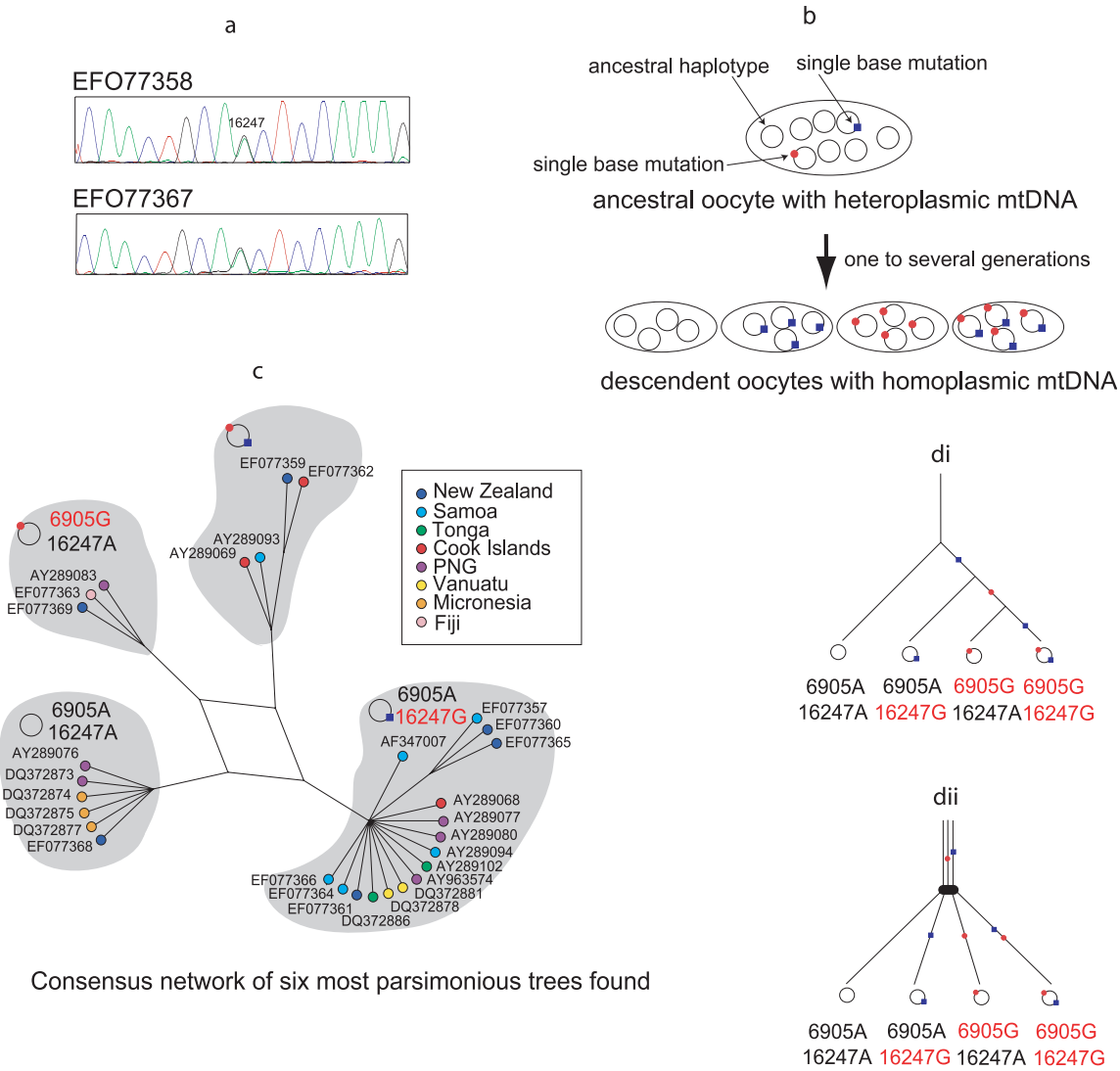

Fig. 3 The effect of unaccounted for heteroplasmy on phylogenetic inference. (a) Chromatogram showing a heteroplasmic, mitochondrial, variant at nucleotide 16247. If heteroplasmy persists within a lineage for several generations, it is possible that a new mutation could arise on a heteroplasmic background, effectively creating a third type (b). If these three haplotypes recombine freely a fourth distinct mtDNA haplotype may be observed (c), carrying the two new variants relative to the ancestral type. Phylogenetic relationships could then become ambiguous (di and dii).

the PM nt16247G transition, while the third retains the ancestral nt16247A variant. This, and the samples heteroplasmic at nt16247 in the control region, can be explained by recurrent mutation at nt16247. However, unlike several other nucleotide positions in the control region, this base position is not regarded as 'hypervariable' (Stoneking 2000), and the time frame for repeat mutation is very short. Might there be another mechanism that better explains these observations, involving intralineage recombination? Heterologous recombination in the somatic tissues of humans has been documented previously (Kraytsberg *et al*. 2004).

The Polynesian control region sample of 13 individuals was typed for status at nt6905. Figure 3c shows the consensus network of the most parsimonious trees found when the control region sequences and nt6905 status were combined from the Polynesian sample (excluding the two sequences with nt16247A/G heteroplasmy) and existing complete mtDNA sequences. The ancestral states for nt6905 and nt16247 are labelled in black, with new states in red. The four groupings fit the four haplotypes predicted by a mechanism of intralineage recombination between inherited heteroplasmic mtDNA molecules, and result in the expected network of most parsimonious reconstructions,

rather than a tree. Resolving this network to a tree requires inferring a recurrent mutation (shown at nt16247 in Fig. 3dii). But if indeed the current sequences reflect intralineage recombination, Fig. 3dii may be an example of a more 'correct' history — meaning that relationships and time estimates inferred from a reconstruction, such as that in Fig. 3dii, are misleading.

Current state of affairs

It is apparent that mtDNA heteroplasmy caused by mutation is widespread across the animal kingdom, leading to variation in length or sequence composition. Depending on where in the mitochondrial genome mutations arise, heteroplasmy may either persist, become fixed, or be lost due to either the forces of drift or selection, or both. To predict its longevity, we will first need to know the position in the genome, and the implied function of the mutation. In animals, persistence of heteroplasmy caused by paternally leaked mtDNA will likely be determined by the stochastic processes of vegetative segregation, underlying mtDNA inheritance. As such, although unlikely due to a low initial relative frequency, sufficiently high frequencies of paternal mtDNA could be reached in cells that lead to the unknowing incorporation of paternal mtDNA into evolutionary studies. A question of interest is should molecular ecologists be concerned? If leakage occurs between closely related populations, paternal mtDNA may not be detectable due to high similarity between sequences, and even if it was detected, it may not influence interpretations. If leakage occurs between distantly related groups, then differences in mtDNA haplotypes should be obvious, and detection of paternal mtDNA straightforward. However, it is in a scenario that combines these two situations that may be of most concern, i.e. where paternal mtDNA is sufficiently different to alter evolutionary and ecological interpretations, but where its detection is not straightforward because, for example, it occurred in a population with high background genetic diversity.

If stochastic processes alone were responsible for paternal leakage, the probability of it occurring could be estimated from ratios of paternal to maternal mtDNA in zygotes, and the number of segregating units between generations. Such frequencies are likely to vary significantly across the animal kingdom, due to differences in gamete investment and gamete morphology. For example, Wolff & Gemmell (2008b) recently determined the ratio of paternal to maternal mtDNA, in zygotes of a teleost fish, to be around $1{:}10^9$. The relative contribution of paternal mtDNA in teleost zygotes is therefore around four orders of magnitude smaller than that revealed for the mammalian system (Ankel-Simons & Cummins 1996; Shoubridge & Wai 2007). Similarly, the effective number of mtDNAs that pass through the bottleneck has been demonstrated to vary moderately among taxa, ranging from 1 to 349 in humans (Howell *et al*. 1992; Bendall *et al*. 1996; Jenuth *et al*. 1996), 200 in mice (Cree *et al*. 2008) and 370 to 740 in *Drosophila* (Solignac *et al*. 1984).

In addition, failure of mechanisms that prevent biparental inheritance, such as the ubiquitination or exclusion of sperm mitochondria, must be considered. Unlike stochastic processes, such as dilution of paternal mtDNA in the egg, and drift at the genetic bottleneck, species-specific mechanisms may actively exclude paternal mtDNA. However, if we extend the observation of ubiquitination in three mammal species (Sutovsky *et al*. 1999, 2000, 2003) to all mammals, evidence for paternal leakage in humans clearly demonstrates that such mechanisms can fail (Schwartz & Vissing 2002).

There is adequate evidence now to suggest that if heteroplasmy does persist, recombination between heterologous mtDNA molecules can occur. It seems clear, however, that the chance of recombination is rare. Further, the impact on evolution will depend on the inheritability of recombinant molecules, and as yet, there is no direct evidence for this in mammals, although indirect evidence does exist for humans (Zsurka *et al*. 2007). The lack of direct evidence for the persistence of recombinant mtDNA into the germline is likely due to low initial frequencies, exposing recombinant molecules to the forces of drift that all mtDNA molecules face during replication and segregation (Birky 2001). It seems likely that with increased, more sensitive surveillance, and time, recombinant molecules will be discovered in the germline, and the range of taxa in which recombinant mtDNA is detected will increase.

What remains to be clarified are the levels of recombination in nature, if enough occurs to permanently alter this tenet of mitochondrial inheritance from no heterologous recombination to low heterologous recombination, and whether levels (if it occurs at all) are sufficient to influence mtDNA evolution and mtDNA-focused evolutionary analyses. Whether recombination occurs frequently enough in nature to be of concern to evolutionary biologists is the subject of much debate (Ladoukakis & Zouros 2001b; Innan & Nordborg 2002; Piganeau *et al*. 2004; Tsaousis *et al*. 2005; Elson & Lightowlers 2006). Models of mtDNA evolution have been developed that incorporate both recombination and biparental inheritance, and both phenomena can strongly influence evolution of the molecule (Birky *et al*. 1989; Kaplan *et al*. 1989). Further, only a small amount of recombination may be required to prevent mutation accumulation (Charlesworth *et al*. 1993). Similarly, the use of mtDNA as a molecular tool in evolutionary analyses, particularly when concerned with the coalescent, may be compounded by modest amounts of recombination (Schierup & Hein 2000; Posada & Crandall 2002; Zsurka *et al*. 2007). Barr *et al*. (2005) raise the point that recombination may be occurring frequently enough to help purge mutations from populations, but too infrequently to be detected by standard detection techniques.

The future

Reports of paternal leakage of mtDNA and heterologous recombination have increased in recent years. We feel efforts need to be focused on obtaining reliable estimates of the frequency of both phenomena, so that they can be appropriately accounted for in models of mitochondrial evolution and, of central focus to this review, in analyses performed by molecular ecologists. By using parameters of mtDNA life history including mutation rates, mtDNA N_e, and egg and sperm mtDNA content, estimates could be made of the expected level of heteroplasmy, induced through paternal leakage and mutation, and recombination. For this, further research is required to determine: (i) the size of the mtDNA bottleneck (N_e) in different species, (ii) the ratio of paternal to maternal mtDNA in zygotes, (iii) whether the ubiquitination of sperm mitochondria applies to groups other than mammals, and identify any additional species- or taxa-specific mechanisms that prevent paternal leakage. Further, simulation studies could be developed that incorporate different levels of these phenomena, to determine if threshold limits exist under which the vast majority of analyses performed by molecular ecologists will remain unaffected.

It may be logistically challenging to obtain true frequencies in nature for paternal leakage and recombination due to the large sample sizes that are needed. In this regard, new technologies that increase throughput and sensitivity of polymorphism detection will prove valuable. In addition, the collective of indirect tests of recombination grows, and their interpretation becomes increasingly more reliable. Overall, our ability to detect rare events is improving and will lead to more reliable frequency estimates of the more incommodious events of mtDNA transmission.

Although rare, we feel that exceptions to the traditionally held laws of mtDNA inheritance exist in nature, and probably at higher frequencies than available evidence suggests. In this review, we have summarised recent studies that reveal paternal leakage and heterologous recombination, not only due to their biological relevance, but also to demonstrate approaches that have resulted in their successful detection. However, mtDNA has been pivotal in answering questions pertaining to evolutionary and conservation biology and will continue to be so. We do not advocate the removal of mtDNA from evolutionary research, but rather to account for, where necessary, the less common properties of mtDNA inheritance. This will not only prevent misleading inferences from being made, but potentially increase the amount of information this molecule provides for evolutionary and ecological analyses.

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