Detection of recombinant mitochondrial genomes: Implications for the mechanism of mtDNA inheritance in mussel *Mytilus*

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ABSTRACT

Recombination plays a fundamental role in the creation of biodiversity. It is the mechanism inducing formation of rearrangements within the genomes which, beside mutations, are the major source of genetic variation. In the process of recombination a single or double DNA strand is broken and rejoined with unassociated DNA fragments. There are several types of recombination: homologous recombination, site-specific recombination and transposition. Within mitochondrial genomes, inter- and intra-molecular recombination can occur. Except for intramolecular recombination of mtDNA, the other types of recombination always result in the creation of mosaic genomes. However, in the natural populations mtDNA recombination is detected extremely rarely. It is caused by the clonal inheritance of mitochondrial genomes and consequential lack of sufficient divergence between parental mitochondrial molecules. Mussels of the genus *Mytilus* possess two types of mitochondrial genomes inherited from males and females, respectively, and their mode of mtDNA inheritance is called *doubly uniparental inheritance* (DUI). The presence of two highly diverged parental molecules gives the opportunity for detection of recombinant variants. This feature of *Mytilus* mtDNA can be broadly exploited in the search for and characteristics of recombinant sequences. Apart from the high level of sequence divergence, fusion of mitochondria and appropriate enzymatic toolkit are principal requirements for the occurrence of recombination. The majority of phylogenetic and demographic analysis based on mtDNA assumes the lack of recombination. If this assumption turned out to be erroneous, previous analyses would be weakened. Recombination is associated with DUI abnormalities, e.g. masculinization of mitochondrial genomes. It may even lead to the breakdown of DUI system resulting in the new, unidentified mode of mtDNA inheritance in mussel *Mytilus* that might be regulated by stochastic events.

CHARACTERISTICS OF MITOCHONDRIA AND MITOCHONDRIAL GENOMES

Mitochondria are semiautonomous, self-reproducing organelles responsible for the process of aerobic respiration, found in the cytoplasm of most eukaryotes. They derived from the free living α-proteobacteria. Mitochondria were most likely engulfed by eukaryotic cell in the way of phagocythosis 1.5 billion years ago (Cavalier-Smith 1987). They can move, fuse, divide within a cell and occupy even up to 25% of the cytoplasm volume.

Mitochondria possess their own genetic material (mitochondrial DNA, mtDNA), whose gene products are involved in mitochondrial respiration and oxidative phosphorylation and translation. The metazoan mitochondrial genome consists of a small circular double stranded DNA which within the organelle is organised in mtDNA-protein complexes called nucleoids. The size of mitochondrial genome in the majority of animals is about 16kbp. Each nucleoid comprises of two to eight mitochondrial DNA molecules and there are a few nucleoids in each organelle (Rokas et al. 2003). There can be up to 500-1000 mitochondria per animal cell dependent on the cell type and activity.

Enzymes and structural proteins responsible for the regulation of replication, transcription and translation of mtDNA are encoded by the nuclear genes. In general, mitochondrial genomes do not contain pseudogenes, repetitive DNA, introns and transposons (Avise et al. 1987). Higher rates of evolutionary change in the mitochondrial genome is caused by the relative lack of repair mechanisms, more frequent exposure to reactive oxygen metabolites and the absence of histones (Shoffner and Wallace 1992).

Animal mtDNA normally encodes 37 genes; 24 of them encode components involved in the mitochondrial translation machinery (22 tRNAs and 2 rRNAs). The remaining 13 genes encode protein subunits of respiratory chain complexes

and ATP synthase. Exceptionally, mtDNA of *Mytilus* encodes additional tRNA for methionine, it lacks gene for the ATPase eighth subunit and has longer open reading frame for *coIII* gene (Hoffman et al. 1992).

Mitochondrial DNA has become an important phylogenetic tool because of several significant features, such as its high copy number, high rate of mutation, assumptions of its exclusively maternal transmission and the lack of recombination in animals. However, there are direct evidences for homologous recombination in mtDNA of plants, fungi and protists (Gray et al. 1999).

Recombination seems to be rare or absent in animals because recombinant variants are hardly detectable, but there is no doubt that recombination can occur even if it cannot be detected. Although animal mitochondrial sequences are known to evolve rapidly, their gene arrangements often remain unchanged over a long period of evolutionary time. For example the gene arrangement of human and trout mtDNA are identical (Boore 1999).

The majority of phylogenetic reconstruction methods assume a single evolutionary history for all mtDNA sequences. However in result of recombination, different parts of examined sequences have different evolutionary histories and they cannot be analysed as a whole (in one alignment). Undetected recombination can lead to overestimation of the length of terminal branches and the total branch length, underestimation of the time to the most common ancestor of the sequences and the rejection of the molecular clock hypothesis (Schierup and Hein 2000). It becomes obvious that data sets should be tested for the presence of recombination prior to phylogenetic analysis.

INSTANCES OF RECOMBINATION IN THE ANIMAL KINGDOM

The first evidence of intramolecular recombination in animal mtDNA was found in the nematode *Meloidogyne javanica* (Lunt and Hyman 1997). The authors demonstrated that the mtDNA control region containing a variable number of tandem repeats (VNTR) can self-recombine creating a figure of eight-like structure resulting in two circular molecules: a large one containing all the genes and the part of the control region and the small one encompassing the fragment of D-loop. The more parsimonious explanation of this result implicates multiplication and deletion of the part of the control region rather than the occurrence of intramolecular recombination.

Signatures of intermolecular recombination have been found within the mitochondrial control region of the flatfish *Platichthys flesus* (Hoarau et al. 2002), three halibut species: *Hippoglossus hippoglossus, Hippoglossus stenolepsis, Reinharditius hippoglossoides* (Mjelle et al. 2008), in mitochondrial sequences of scorpion family Buthidae encoding *rnl* (16S ribosomal RNA) and coxI (Gantenbein et al. 2004) and for the first time in mtDNA of terrestrial vertebrate: Australian frillneck lizard *Chlamydosaurus kingii* (Ujvari et al. 2007).

Ladoukakis and Zouros (2001) detected recombinant mitochondrial molecules coexisting within the same individual together with non-recombinant parental genomes providing the evidence of homologous recombination. However, none of the recombinant molecules have been proven to be transmitted to the offspring. Furthermore, the recombinant haplotypes have been found in a series of research on mussels *Mytilus* from the interspecies hybridization zones: *M. edulis-M. trossulus* and *M. edulis-M. galloprovincialis* (Breton et al. 2005; Burzyński et al. 2003, 2006; Filipowicz et al. 2008; Rawson 2005) extended in the Baltic Sea, along Atlantic coasts of Europe and north America and from the homogenous zones like the Azov Sea and the Black Sea inhabited only by *M. galloprovincialis* (Filipowicz et al. 2008).

The existence of recombinant variants in the Baltic Sea is confined only to males. On the contrary, recombinants from the Azov Sea, Black Sea and the Bay of Biskay have been reported in both male and female individuals. The high frequency of their occurrence implies the transmission of recombinant haplotypes into the next generations. The strong evidence of recombination comes from a human individual who has been shown to have both paternal and maternal mtDNA in his muscle tissue (Schwartz and Vissing 2002).

To investigate the question how prevalent recombination is the compilation of protein-coding sequence data sets were made and subjected to statistical tests of recombination (Piganeau et al. 2004; Tsaousis et al. 2005). It was shown that recombination is a fairly pervasive event in mtDNA of animals. Pigneau et al. (2004) have found that 14.2% of analyzed alignments provide the evidence for recombination supported by one to four statistical tests which they applied. Their results suggest that scarcity of the recombination examples in animal kingdom results from difficulties in the detection of recombinant sequences rather than from the lack of its of occurrence.

HETEROPLASMY AS A KEY CONDITION IN DETECTION OF RECOMBINANTS

Detection of recombinant haplotypes requires two highly diverged parental mitochondrial genomes. In the majority of animal species mtDNA is exclusively maternally inherited. Besides rare mutants all copies of mtDNA within the organism have identical DNA sequence, a condition called homoplasy. On the other hand heteroplasmy is defined as a state in which more than one mitochondrial genotype occurs within individual. The ratio of different types of mtDNA in a heteroplasmy may be variable, but usually one mitotype prevails against the others, which may occur in a very low proportion (Kmiec et al. 2006). The phenotype of the organism is determined by the predominant mtDNA variant. In animals heteroplasmy can be related to mitochondrial diseases (Wallace 1994; Zeviani and Antozzi 1997) and aging (Szibor and Holtz 2003). It is also supposed to play a role in

cancer (Chinnery et al. 2002). Successful detection of heteroplasmy depends on the type of change in the mitochondrial genome and the ratio of the heteroplasmic variants.

The methods commonly applied are based on PCR, DNA sequencing and Southern hybridization. Heteroplasmy sometimes results from rearrangements mediated by recombination. However, heteroplasmy is rare in the animal kingdom with the exception of some families of mussels, where it is prevalent and is a key condition enabling more frequent detection of recombinants.

Heteroplasmy can be gained in a few ways: through mutations, paternal inheritance often resulting from paternal leakage, caused by interspecies hybridizations and *doubly uniparental inheritance* (DUI). The rate of recombination and the frequency of recombinant variants in the population depends on the number of heteroplasmic mitochondrial genomes within the cell and the level of their sequence divergence. Genetic divergence between the mtDNA genomes of the two hybridizing species could play a pivotal role in the production of recombinant molecules. Mitochondrial DNA will not recombine if the degree of genetic divergence is too high. When the level of sequence divergence exceeds 5% and more than three recombination events occurred then the opportunity for detection of recombinants will be considerably increased (Wiuf et al. 2001). By contrast, increase in a degree of genetic divergence between species prone to hybridize decreases the probability that sperm mitochondria are recognized and eliminated.

In the majority of animal species paternal mitochondrial inheritance is inhibited. Sperm mitochondria are tagged with ubiquitin and finally subjected to proteolysis within the developing zygote. Elimination of the male mitochondria from the early embryo can be disrupted in interspecific crosses. Leakage of paternal mitochondrial genomes observed in animal interspecies hybrids indicates that elimination of sperm mitochondria from the zygote could be a species-specific process (Kaneda et al. 1995). Paternal leakage has been observed in the crosses of *Drosphila melanogaster* (Kondo et al. 1990), mice (Gyllensten et al. 1991), anchovies (Magoulas and Zouros 1993), honeybee (Meusel and Moritz 1993), in tit (Kvist et al. 2003) and also in human individual (Schwartz and Vissing 2002).

Another way of heteroplasmy acquisition is *doubly uniparental inheritance* of mtDNA. It is a special type of mitochondrial inheritance, characteristic for several bivalve families: sea mussels Mytilidae, e.g. „*Mytilus edulis* complex" (Skibinski et al. 1994), *Mytilus californianus* (Ort and Pogson 2007), *Geukensia demissa, Musculista senhousia* (Passamonti 2007); Veneridae (Passamoti and Scali 2001), Donacidae (Theologidis et al. 2008) and freshwater mussels: Unionidae (Hoeh et al. 1996; Liu et al. 1996), Margaritiferidae (Hoeh et al. 2002) and Hyriidae (Curole and Kocher 2005).

According to DUI, maternal and paternal genome is transmitted to the next generation respectively. The fate of the male mtDNA in the zygote depends on the mussel sex. In the female offspring sperm mitochondria containing genome M are eliminated within 24 hours (Sutherland et al. 1998). It results in the presence of only one type of mtDNA called genome F. In the male zygote sperm mitochondria are retained and translocated to the blastomere that gives birth to the gonad tissue. In the result males are heteroplasmic. Their reproductive tissue contains exclusively genome M and somatic tissue is dominated by the genome F (Venetis et al. 2006).

Apart from considerable sequence divergence between mitochondrial genomes existing inside heteroplasmic cell, two other requirements must be fulfilled for the recombination to occur. Firstly mitochondria must show ability for fusion to exchange mtDNA from different organelles present within the cell. Secondly they must possess necessary enzymes catalysing the process of recombination.

Fusion of mitochondria has been documented in *Drosophila* (Yaffe 1999). Homologs of one of the highly conserved genes (*fuzzy onions*) involved in the fusion have been found in humans (mitofusins *Mfn1* and *Mfn2*) and yeast (*Fzo1p*) (Santel and Fuller 2001). Despite of the molecular machinery necessary for fusion there is no spectacular evidence for efficient mitochondrial fusion in animals. Recombination plays a crucial role in the mtDNA replication and repair (Rokas et al. 2003). DNA ligazeIII, a key enzyme involved in the replication, recombination and DNA repair is localised in mitochondria (Thyagarajan et al. 1996). Apart from this the homologous and non-homologous recombination activities have been documented in human mitochondria (Thyagarajan et al. 1996).

STRUCTURE OF THE CONTROL REGION OF *MYTILUS* MITOCHONDRIAL GENOME

Both male and female *Mytilus* mitochondrial genomes encompass a noncoding sequence between the *l-rRNA* and *tRNA^{Tyr}* which is the most diverged part of mtDNA. It consists of three domains. Two of them laterally situated are variable domains (VD) and the third one is the central most conservative domain (CD). CD domain has diverged by only 1.5% between F and M genomes, while the average divergence over the whole molecule is about 20%. The presence of differentially diverged domains suggests that different parts of the major noncoding region are under various selection constraints.

The major noncoding region of mtDNA (D-loop) is also the main control region of the mussel mtDNA. It was stated on the basis of the following facts. AT-rich parts of the noncoding region are capable of producing secondary structures characteristic for regions that perform control functions. Besides, the motif of the sea urchin mtDNA molecule for which there is an evidence that it plays a pivotal role in replication and transcription was found in the D-loop (Cao et al. 2004). There are several similarities between major noncoding region of mussels and the control region of

mammals. The average female control region is approximately 1kb in length and that of the male is about 100bp shorter. There are some reports on longer (Zbawicka et al. 2003) and rearranged control regions in *Mytilus* (Breton et al. 2005; Mizi et al. 2005; Rawson 2005). Increase in length is mainly caused by the presence of multiplicated fragments within D-loop, containing M-F specific, mosaic parts obtained in the result of recombination.

THE IMPACT OF RECOMBINATION ON DOUBLY UNIPARENTAL INHERITANCE SYSTEM

The system of *doubly uniparental inheritance* can be broken in mussels *Mytilus* inhabiting the interspecies hybridization zones (Fisher and Skibinski 1990) or within hybrid individuals generated in the result of experimental interspecies crosses (Rawson et al. 1996). The breakdown of DUI is the disruption of the mechanism regulating the process of male mtDNA elimination from the developing embryo. It can lead to the creation of heteroplasmic females and homoplasmic males. Failure of DUI associated with hybridization events can invoke the disruption of nuclear-cytoplasmic interactions which prompt mitochondrial genomes instabilities. MtDNA instabilities found in natural populations of hybrid animals can be caused by several mechanisms such as duplications, deletions, inversions and recombinations (Abott et al. 2005; Campbell and Barker 1999; Gach and Brown 1997; Kumazawa et al. 1996; Lavrov et al. 2002; Shao et al. 2004). Control region length variation was observed within mtDNA of mussel individuals from interspecies hybridization zones (Filipowicz et al. 2008; Zbawicka et al. 2003).

Two categories of mechanisms have been proposed to account for mtDNA length increase. The first category invokes strand slippage. This involves the mispairing of a slipped strand in an array of repeated motifs followed by polymerase repair, which results in the gain or loss of repeat (Boore 2000). The second model based on recombination assumes that intra or inter-recombination could account for the generation of repeat (Mueller and Boore 2005). Among mussels *Mytilus* some males seem to lack a typical M genome and instead of it they have two types of F genomes. One of them probably invaded the male route of inheritance and since then it has been transmitted to next generations with the sperm. This type of the F genome is referred to as recently masculinised M genome (Hoeh et al. 1997; Ladoukakis et al. 2002; Śmietanka et al. 2004) and the process is called masculinization.

Some authors assume that recombination plays crucial role in the process of masculinization (Burzyński et al. 2003, 2006; Venetis et al. 2007). This assumption was based on the fact that masculinized genomes found in distant *Mytilus* populations contained M-specific insertion within the F-specific control region of their mtDNA (Breton et al. 2005; Rawson 2005). Burzyński et al. (2006) speculated that occasional invasions of the male transmission route by the F genome could be possible through the addition of M control region sequences to the control region of F genomes in the process of recombination. However, the recombinant mitochondrial genomes containing mosaic M-F specific control regions are not present exclusively in sperm (Filipowicz et al. 2008; Rawson 2005). They were also found in the male somatic tissue together with typical M or F genome, in eggs and in the female somatic tissue. Comparable frequencies of recombinant mtDNA in male and female individuals from the Black Sea (Filipowicz et al. 2008) allow to presume that mosaic mitochondrial molecules might be transmitted from generation to generation in the peculiar way, most likely not operated by *doubly uniparental inheritance system*.

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