Molecular evolution and recombination in gender-associated mitochondrial DNAs of the Manila clam *Tapes philippinarum*.

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ABSTRACT

Doubly Uniparental Inheritance (DUI) provides an intriguing system for addressing aspects of molecular evolution and intermolecular recombination of mitochondrial DNA. For this reason, a large sequence analysis has been performed on *Tapes philippinarum* (Bivalvia, Veneridae), which has mitochondrial DNA heteroplasmy that is consistent with a DUI. The sequences of a 9.2 kb region containing 29 genes are analyzed from nine individuals and of a single gene from another 44 individuals. Comparisons suggest that the two sex-related mitochondrial genomes do not experience a neutral pattern of divergence, and that selection may act with varying strength on different genes. This pattern of evolution may be related to the long, separate history of M and F genomes within their tissue-specific “arenas”. Moreover, our data suggest that recombinants, although occurring in soma, may seldom be transmitted to progeny in *T. philippinarum*. Possibly this could be due to a mechanism that somehow selects for robust mitochondrial function in the germ cells, in line with that recently hypothesized in other non-DUI metazoans.

INTRODUCTION

Mitochondrial DNA (mtDNA) has been studied in several animal groups, mainly for phylogenetic purposes. It is physically separate from the nuclear genome and, with few exceptions, it is circular and contains 37 genes (22 for tRNAs, two for rRNAs, 13 for proteins). MtDNA appears to have a strictly maternal inheritance in most organisms and is thought to lack recombination (WOLSTENHOLME 1992), although this is not without controversy (EYRE-WALKER and AWADALLA 2001).

The most serious exception to the matrilineal inheritance of animal mtDNA comes from a few bivalve mollusks, in which two different sex-related mtDNAs have been detected (the so-called M and F mtDNAs), showing unexpected levels of divergence (about 10-30%). Research on the *Mytilus edulis – M. galloprovincialis* species complex demonstrated that the F mtDNA is transmitted from the mother to both daughters and sons, whereas the M mtDNA is transmitted from father to sons only (SKIBINSKI *et al*. 1994a, b; ZOUROS *et al*. 1994a, b). In adult males the F mitochondrial genome prevails in somatic tissues, while the M mitochondrial genome is strongly predominant in gonads, with sperm carrying only M-type mtDNAs. Thus, a double mechanism of transmission is realized, in which both M and F mtDNAs are inherited uniparentally. This peculiar pattern of mitochondrial inheritance was called "doubly uniparental inheritance" (DUI; ZOUROS *et al.* 1994 a,b) or "gender-associated inheritance" (SKIBINSKI *et al.* 1994a,b). This pattern of transmission, detectable through the presence of two mtDNAs showing a tissue-specific distribution in males (SKIBINSKI *et al.* 1994a; DALZIEL AND STEWART 2002), was found in some species of the genus *Mytilus* (*M. edulis, M. galloprovincialis, M. trossulus, M. californianus*), in *Geukensia demissa* (Mytilidae) and in some unionids (fresh water mussels;

Pyganodon fragilis, *P. grandis*, *Fusconaia flava*, *Anodonta grandis*) (see HOEH *et al.* 1997 and references therein). Furthermore, the recent finding of mtDNA heteroplasmy in the venerid Manila clam *Tapes philippinarum* (PASSAMONTI and SCALI 2001) showed that the pattern also occurs in phylogenetically distant families and suggests that it might be widely distributed among bivalves.

Several hypotheses have been suggested for the evolutionary forces involved in sequence divergence of M and F genomes. Analyses of the *Mytilus* species complex showed that mussel mtDNA appears to evolve faster than in other metazoans (HOFFMANN *et al.* 1992); this has been related to relaxed selective constraints acting in DUI systems of inheritance (HOEH *et al.* 1996). Moreover, several studies on *Mytilus* and *Pyganodon* demonstrated that the M lineage evolves faster than the F lineage (LIU *et al.* 1996; QUESADA *et al.* 1998, and references therein). Authors suggested several explanations for this: higher rate of M mtDNA duplication during spermatogenesis and early male embryo development, free-radical damage to sperm, positive selection, or effects of the smaller population size of the M genome (see STEWART *et al.* 1996 for details). Analyses were also performed in order to evaluate the best model of molecular evolution to be applied to the *Mytilus* system. The neutral model has been rejected (STEWART *et al.* 1996), but the nearly neutral theory (OHTA 1992) has been considered to best fit the observed data (QUESADA *et al.* 1998; SKIBINSKI *et al.* 1999). However, SKIBINSKI *et al.* (1999) stated that more information on effective population size for *Mytilus* M and F genomes is considered very important to discriminate a nearly neutral situation (OHTA 1996) from a model of positive selection.

Several attempts have been made to assign a functional role to DUI, but DUI systems are so scarcely known in their molecular aspects that the given

explanations must remain largely speculative. In fact, even in non-DUI systems, the mechanisms of transmission of mitochondria to progeny are scarcely known in detail, but in recent years several studies, mainly on humans, have contributed somewhat to the issue. Despite a high mtDNA copy number (about 100,000) in oocytes, new mtDNA sequence variants segregate rapidly between generations. This paradox can be explained by the occurrence of narrow bottlenecks during oogenesis or early embryo development. Authors speculate that only a few mitochondria are segregated to be passed on to the germline, and this mechanism can be related to the necessity of maintaining the genomic integrity of the organellar genome (JANSEN *et al.* 2000; SHOUBRIDGE 2000). Actually, highly rearranged and/or deleted mtDNAs are commonly observed in several somatic tissues (the so called “sublimons”, see KAJANDER *et al.* 2000), that might be related to the high oxidative stress to which mitochondria are exposed.

Some evidence of mitochondrial recombination has been proposed by LAUDOKAKIS and ZOUROS (2001a,b) in metazoans. As they point out, DUI systems represent a model for studying basic aspects of the evolution and transmission of the mitochondrial genome, mainly due to the presence of two different mtDNAs in the somatic tissues of males. The authors present evidence of recombination between male and female mitotypes in the DUI system of *M. edulis*. They also speculate that mtDNA recombination may occur at a high rate. Based on that, the extent to which the recombination generates new haplotypes may depend only on the frequency of biparental inheritance of mitochondrial genomes.

In order to test the generality of these hypotheses, we conducted a sequence analysis in the Manila clam *Tapes philippinarum*. We analyzed sequences obtained

from somatic tissues of males as well as from mature gonad tissues of both males and females.

MATERIALS AND METHODS

Sampling and DNA extraction: Clams were collected either in the Adriatic Sea (Italy) or in Washington State (U.S.A.). Both stocks have been introduced for commercial purposes, so these populations do not correspond to the original species ranges (FISCHER-PIETTE and METIVIER 1971).

Fully mature, living clams were dissected and gonad liquid was collected with a glass capillary. Separately a sample of somatic tissue (terminal tip of the foot or adductor muscle) was also taken from each specimen. Gonad samples were inspected under a light-microscope to detect mature eggs or sperm. Tissues were utilized both fresh or frozen at –80° for DNA isolation and purification. We analyzed 44 specimens (nine females and 35 males) from the Italian sample and nine specimens (five females and four males) from the U.S. sample.

Total genomic DNA was separately obtained from gonads and somatic tissues either according to the method described in PREISS *et al.* (1988) or utilizing the DNAeasy Tissue Kit (Qiagen). Enriched mtDNA fractions were obtained from tissues using the mtDNA Extractor CT Kit (Wako Chemicals). All those extraction methods were suitable to give good DNA for PCR amplification from clam tissues, but the best results were obtained with the Wako kit, especially for amplifying long fragments.

rRNA partial gene amplification and sequencing: Partial sequence of the mitochondrial large ribosomal subunit RNA gene (*rrnL*) was amplified from various tissues of the 44 Italian specimens as described in PASSAMONTI and SCALI (2001).

Fragments obtained from gonads were directly sequenced, without cloning. In males we were able to obtain good sequences only using gonad liquid from fully mature specimens. At variance with what we did for gonads, we amplified and cloned the *rrnL* partial sequence obtained from somatic tissue of three specimens. Sequencing reactions were performed on both strands with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in a 310 Genetic Analyzer (ABI) automatic sequencer.

Long-PCR and sequencing: Long-PCR amplifications were obtained from the nine U.S. samples with Herculase Enhanced DNA Polymerase (Stratagene) according to the kit protocol. To increase amplification fidelity we used no DMSO in the amplification mixture. The mtDNA region between the mitochondrial large ribosomal subunit RNA gene (*rrnL*) and the Cytochrome Oxidase subunit I gene (*cox1*) (about 9.2 kb) was amplified using 16sbl (PALUMBI *et al.* 1991) and HCO2198 (FOLMER *et al.* 1994) universal mtDNA primers.

The long-PCR products were randomly sheared into fragments of about 1 kb by repeatedly passing the DNA through a narrow aperture under high pressure with a Hydroshear device (GeneMachines), blunt end repaired, and subcloned into a pUC18 vector to make a random insert library. For each sample, 384 recombinant clones were collected and analyzed. Recombinant plasmids were directly amplified from the crude bacterial colony using Rolling Circle Amplification (RCA, DEAN *et al.* 2001) using the TempliPhi kit (Amersham Pharmacia). Sequencing reactions were performed on both strands using Amersham's ET dye terminator kit, reactions were purified using SPRI, and electrophoretic separations and detection were performed with a MegaBACE 1000 automated DNA sequencer. The resulting sequences were processed using Phred (CodonCode Corporation) and assembled using Sequencher

(GeneCodes). All assemblies and the overall sequence quality were verified by eye. Detailed protocols are available at http://www.jgi.doe.gov/Internal/prots_index.html.

Sequence analysis: Sequences were aligned using the Clustal algorithm of the MT Navigator PPC software (Applied Biosystems). Alignments were then edited manually, taking into account gene ends and structure. Proteins and ribosomal RNAs were analyzed by sequence comparison to *Lumbricus terrestris* (BOORE and BROWN 1995), *Katharina tunicata* (BOORE and BROWN 1994) and *Mytilus edulis* (HOFFMANN *et al.* 1992). tRNA genes were identified by using tRNAscan-SE (version 1.1, http://www.genetics.wustl.edu/eddy/tRNAscan-SE; LOWE and EDDY 1997) or, whenever the program failed to predict tRNAs, analyzing the sequence by eye, recognizing potential secondary structures. The 5' end of protein coding genes were inferred to be at the first in-frame, legitimate start codon (ATN, GTG, TTG, GTT; see WOLSTENHOLME 1992) and the 3' ends at the first observed in-frame stop codon (i.e. no "abbreviated" stop codons were inferred). The 5' and 3' ends of rRNA genes were assumed to be adjacent to the flanking genes.

Level of divergence for each analyzed gene and for the whole 9.2 kb sequence were calculated using nucleotide pairwise p-distances, and then the mean value and standard errors (by the Bootstrap procedure) of p-distances within each group (i.e. M vs. M sequences in males, F vs. F in females). The two groups were compared with a basic one way analysis of variance (ANOVA) to test whether variability within M-type and F-type sequences are significantly different.

In order to analyze sequences for neutrality, we performed tests based on protein polymorphism only. We did not use neutrality tests based on DNA polymorphism (i.e. Tajima's test, etc.) because, as stated by several authors (MCDONALD and KREITMAN 1991; BROOKFIELD and SHARP 1994; QUESADA *et al.*

1998), these are strongly influenced by changes in population size. Actually, this is just what happened recently for these samples, since the analyzed populations have been introduced in the Italian and U.S. seas from a small stock of clams obtained from hatcheries.

The number of polymorphic sites within M and F protein-coding sequences (either showing synonymous or non-synonymous mutations) were measured, and a test of positive selection was performed by a one-tailed Fisher's exact test (ZHANG *et al.* 1997), as implemented by MEGA2 (KUMAR *et al.* 2001). If the resulting *P*-value is less than 0.05, then the null hypothesis of neutral evolution (i. e. strictly neutral and purifying selection) is rejected. Moreover, when the number of synonymous differences per synonymous site exceeds the number of non-synonymous differences per non-synonymous site, the test sets *P*=1 to indicate the existence of purifying selection. This test is considered more appropriate than the Z-test when the number of nucleotide substitutions per sequence is small (NEI and KUMAR 2001), which is the case for our comparisons.

Comparisons between M- and F-types were performed by the test of MCDONALD and KRIETMAN (1991), as implemented in DnaSP 3.53 (ROZAS and ROZAS 1999). The test is based on the observation that, under neutrality, the ratio of non-synonymous to synonymous fixed substitutions between M and F types should be the same as the ratio of non-synonymous to synonymous polymorphism within types.

Male and female sequences were also analyzed for gene-conversion and recombination using DnaSP (v. 3.53) (ROZAS and ROZAS 1999) which incorporates the algorithm developed by BETRÁN *et al.* (1997) for detecting gene conversion events.

RESULTS

Sequence features: Sex of each sample was determined using light microscopy of gonads. Both populations showed no apparent sex ratio deviation.

We sequenced 44 Italian samples (nine females and 35 males, all analyzed for the *rrnL* partial sequence only) and nine U.S. samples (five females and four males, analyzed for the 9.2 kb mtDNA fragment).

Sequence data from this article have been deposited with the GenBank Data Libraries under accession nos. AF484288-AF484340 and AF492465.

As expected, sequences belong to two distinct groups: F-like sequences were invariably obtained from ovaries, whereas M-like sequences were obtained from testes. No clam carried haplotypes of the opposite sex, supporting the rarity of "masculinization" events in this species, when compared to *Mytilus* (see HOEH *et al.* 1997 and PASSAMONTI and SCALI 2001 for details).

In the 9.2 kb mtDNA region, we identified 29 genes (7 protein encoding genes, two rRNA genes and 20 tRNA genes). (Animal mitochondrial genomes typically contain 37 genes in total [BOORE 1999].). The region contains no large non-coding regions. All sequenced genes are transcribed from the same strand, as is the case for *Mytilus edulis* (HOFFMANN *et al.*1992).

Comparing male and female gene arrangements, we observed the same gene order with one notable exception: in females the tRNA immediately upstream of *nad6* gene is for valine (tRNA-Val, anticodon TAC, 4927-4989 region), whereas in males, it is for lysine (tRNA-Lys, anticodon TTT, 4862-4921 region). However both sexes also have copies of both of these tRNA genes downstream of *nad6*, so that the difference seems unlikely to be significant for mitochondrial function. The gene for

tRNA-Met also appears in two tandem copies, in both sexes, each having the same anti-codon (CAT) (Fig. 3). The gene arrangement observed here, although incomplete, is novel among sampled metazoans, once more supporting the observed high rearrangement rate in molluscan mtDNAs (BOORE 1999 and references therein). The complete sequencing of the M- and F-like mtDNA genomes is now in progress.

Four out of a possible seven start codons were found to be used in *T. philippinarum*, namely ATA (female *atp6*, *nad6*; male *nad4*, *cox1*), ATG (female *nad4*; male *nad6*), GTG (female *nad3*; male *atp6*, *nad3*) and GTT (female *nad5*, *cox3*, *cox1*; male *nad5*, *cox3*). The stop codon TAA is used in females for *nad4*, *nad3*, *nad5*, *nad6*, *cox3* and in males for *nad4*, *nad3*, *nad6*. The stop codon TAG is used for all other genes (the *cox1* stop codon was not detected since its sequence is incomplete at the 3' end). No abbreviated stop codons, sensu BOORE and BROWN (2000), were inferred for the analyzed portion of the mtDNA. The M sequence is shorter (8986 total nts) compared with the F sequence (9207 nts). This is mainly due to the different length of male genes, which are commonly truncated at both the 5' and 3' ends (see especially *nad4*, *cox3*, and *rrnS* genes). It is unclear, however, whether these deletions may in any way interfere with male mtDNA function.

Sequence variability: In *T. philippinarum* the substitution rate in M sequences is higher than in F for all but one analyzed gene. Values of sequence divergence, evaluated by p-distance, are between 0.0020 (*rrnL*, *nad3*) and 0.0081 (*nad6*) in F-type, and between 0.0028 (*rrnL*) and 0.0094 (*cox3*) in M-type sequences. However, ANOVA, performed on each sequenced gene, showed a quite complex situation, since differences in substitution ratios are statistically significant in only some genes (*rrnL*, *nad3*, *nad5*, *cox1*, *cox3* and [collectively] tRNAs) but not in others (*rrnS*, *atp6*,

nad4, *nad6*). Nonetheless, when the complete 9.2 kb sequences were compared, a highly significant value is obtained (p=0.0022), thus indicating that a higher M-type substitution ratio is a general feature of the mtDNA of *T. philippinarum*, even if some gene-specific differences do exist (Table 1).

Values of divergence are considerably higher for pairwise M- versus F-type comparisons, ranging from 0.1549 (*rrnL*) to 0.3284 (*nad6*). Moreover, different genes show different levels of divergence, but it is worth mentioning that rRNA and tRNA genes are less variable than protein-coding genes (Table 1). The whole set of data on sequence variability appears to be in line to what is observed in other DUI species, but the level of divergence within M- and F-type sequences still remains much lower in *T. philippinarum* than in *M. edulis* (see RAWSON and HILBISH 1995).

Plotting the percentage of transitions as a function of sequence divergence, we observed a strong transition bias for all analyzed genes (single-gene comparisons not shown), either within or between M- and F- types, with the only exception of the partial sequence of *rrnL*, in which M- and F-type sequences appear to mutate without any bias toward transitions (Fig. 1). It is unclear whether this deviation is somehow related to structural constraints on the LrRNA product, or whether it is influenced by the Italian sample history (i.e. variation present in the founding populations).

Comparisons within groups (i.e. M- and F-type sequences) by the Fisher's exact test (Table 2) reveal that a selection against non-synonymous mutations is working for all analyzed protein-coding genes (i.e. purifying selection). McDonald and Krietman's test of neutrality indicates that for 6 out of 7 analyzed genes, M- and F-type sequences evolve, over a long time of divergence, in a non-neutral way (Table 3).

Recombination: Analysis failed to show any trace of recombination in the 9.2 kb partial mtDNA sequences obtained from the U.S. samples. Although this could reflect a sampling bias, the whole sequencing effort (a total of about 132 kb analyzed) might reduce such a shortcoming. It should be noted that we analyzed samples obtained from gonads so that our sequencing procedures were targeted toward detecting the haplotype that is commonly passed to progeny. This approach was taken mainly because we wanted to know whether recombinant mtDNAs, as detected by LAUDOKAKIS and ZOUROS (2001a), are commonly inherited or not. However, if the PCR sample is cloned and different clones analyzed, it is virtually impossible to determine whether the analyzed sequences are really from germline cells or not, due to the inevitable contamination by somatic cells.

Out of 30 analyzed clones of *rrnL* partial sequences obtained from 3 samples of somatic tissue, we obtained a single sequence that may be interpreted as recombinant in origin and appears fully similar to that observed by LAUDOKAKIS and ZOUROS (2001a). This sequence, although clearly male-like, presents a series of contiguous female-like diagnostic sites in the 429-459 region (Fig. 2, GenBank accession number AF492465).

This may indicate that in *T. philippinarum* recombination of somatic mitochondria is more common than in the germline. However, it has to be mentioned that for this clone a PCR artifact cannot be excluded (see PÄÄBO *et al.* 1990).

DISCUSSION

Molecular evolution of sex-related mtDNA genomes: These data confirm the presence of a sex-related heteroplasmy in the Manila clam, *Tapes philippinarum*.

The observed tissue-specific distribution of M and F mitochondrial sequences fully parallels that predicted by a doubly uniparental mechanism of mitochondrial inheritance (SKIBINSKI *et al.* 1994a,b; ZOUROS *et al.* 1994a,b). Moreover, the analysis over a large part of the mitochondrial genome shows an overall higher substitution rate in M than in F mtDNA. However, not all genes behave the same way, because some do not show a significantly higher level of mutation in the M sequence (see Table 1).

As already mentioned, three main mechanisms have been proposed to explain a higher substitution rate of M mtDNA: enhanced duplication rate during spermatogenesis, free-radical damage to sperm, or the effects of the smaller population size of the M genome. However, all of them would produce an evenly distributed higher level of mutation of the M genome, because they invariably act on the entire mtDNA molecule. Although the previously mentioned mechanisms may act on the M genome, the observed gene-specific variation in effect seems to indicate that different selective forces are acting on individual M genes.

If so, we might also speculate that the hypothesis of STEWART *et al.* (1996) fits our data. This differentiates three selective “arenas” for mitochondria, namely the somatic cell line, the female germline, and the male germline, and assumes that there might be some tradeoffs in terms of optimal functioning. In DUI species, the M genome has to function in only one of the three arenas (i.e. the male germline), whereas the F genome has to function in two (i.e. somatic tissue and the female germline); in non-DUI species, the sole mtDNA type must function in all three. Therefore, perhaps those functioning in fewer arenas can evolve more quickly, adapting to that specific environment, whereas the need to function in more arenas would result in greater purifying selection, accounting for the varying rates of

evolution. It is possible that the clam mtDNAs are adapted with greater specialization to the arenas in which they function, accounting for the M and F mtDNAs not having switched roles in *T. philippinarum* (i.e. low “masculinization” frequency), in contrast to the observation in *Mytilus* (HOEH *et al*. 1997).

This is also in line with the observation that between M and F genomes, divergence is very high, both for synonymous and non-synonymous positions. In fact, the splitting of the M and F lineages seems to have occurred near to the origin of the species itself, as suggested by the relative-rate test performed on *rrnL* (PASSAMONTI and SCALI 2001). The McDonald and Kreitman test also showed that the two variants largely diverged in a non-neutral way, even if some exception occurs (*nad3*). The ratio of fixed synonymous to non-synonymous substitutions is significantly different from the ratio of polymorphic synonymous to non-synonymous substitutions, which does not meet the prediction of a neutral model of evolution. This should be taken as evidence that some sort of selective pressure might act during the divergence of M and F genomes, likely to better adapt them to their specialized tasks. It may be that gene-specific selection has produced in *T. philippinarum* two stable (i.e. purifying selection detected within mtDNAs) and divergent (i.e. positive selection detected between mtDNAs) mitochondrial genomes.

This scenario largely parallels to what has been observed by STEWART (1996) on American *Mytilus edulis* and *M. trossulus*, which rejects neutral evolution on mussels’ mitochondrial genes, but seems to contrasts with QUESADA (1998), which found an excess of non-synonymous substitutions in European mussels.

It has to be noted however, that we cannot totally exclude a nearly neutral pattern of evolution for *T. philippinarum* (OHTA 1992), according to the QUESADA’s rationale (QUESADA et al., 1998). Actually, in a nearly-neutral scenario, repeated

fluctuations of effective population size can result in a relaxed selection on slightly deleterious mutations (OHTA, 1972; 1992); while the major part of deleterious mutations would be eliminated over a long time, some would be permitted by the more relaxed selection on the M genome (still in a gene-specific pathway), thus resulting in replacement substitutions between the M and F genomes.

Mitochondrial recombination in the Manila clam DUI system: Absence of recombination in metazoan mtDNA has been a “tenet” for several years and this is still considered one of the most important features of mitochondrial DNA as a phylogenetic tool.

However, the subject of mitochondrial recombination has again become controversial in recent years (ARCTANDER 1999; AWADALLA *et al.* 1999; EYRE-WALKER *et al.* 1999; ELSON *et al.* 2001; HAGELBERG *et al.* 1999; INGMAN *et al.* 2000; KIVISILD and VILLEMS 2000; MERRIWEATHER and KAESTLE 1999). Although it is quite clear that there is no molecular reason to consider recombination structurally absent from metazoan mitochondria (THYAGARAJAN *et al.* 1996; LAUDOKAKIS and ZOUROS 2001a,b), it has to be noted that within an all-maternal, homoplasmic mtDNA population, recombinants are hardly detectable by direct observation of gene-converted tracts, since recombinants will generally be identical in sequence to the typical mtDNAs. For this reason, doubly uniparental inheritance represents a unique system to test recombination events, because these species show a high level of mtDNA heteroplasmy. Following this, LAUDOKAKIS and ZOUROS (2001a) pointed out the possibility that the ratio of recombinants, which are commonly produced in metazoan cells, is directly proportional to the level of heteroplasmy.

Further, even if we accept that mtDNA recombination is possible, then we still must ask whether recombinants are commonly inherited by progeny. This issue is

limited by lack of understanding of mechanisms for germline mitochondrial segregation.

Data on *T. philippinarum* may give some insights on these issues. As reported above, recombinant sequences were not detected in mtDNAs obtained from gonads, but a recombinant sequence is obtained from the soma. This may be an indication that clams do not transmit recombinants to their progeny at a detectable level, even if recombination may occur in their cells with characteristics similar to that of *M. edulis* (LAUDOKAKIS and ZOUROS 2001a). This may interface with studies that show a narrow "bottleneck" during early embryonic development (not limited to DUI organisms) which may enable "screening" of mitochondria for proper functioning and thereby elimination of deleterious mitochondrial mutations.

In conclusion, from present analysis a quite different picture is emerging for mitochondrial inheritance and selection, and further analyses of DUI systems will probably play a pivotal role on building up a new model for metazoans. A better understanding of the distribution of mtDNAs in both soma and germline may illuminate these processes, and this can be addressed by exploiting the different, co-occurring sex-related haplotypes of mtDNA in DUI species.

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TABLE 1

Sequence variability and analysis of variance (ANOVA)

Comparisons		mean pD±s.e.	d.f.	F	p
Italian samples					
rrnL	F-type vs F-type	0.0020±0.0007			
	M-type vs M-type	0.0028±0.0010			
	M-type vs F-type	0.1549±0.0152	(1;1079)	10.2115	0.0014**
U.S. samples					
9.2 kbs	F-type vs F-type	0.0034±0.0004			
	M-type vs M-type	0.0077±0.0007			
	M-type vs F-type	0.2697±0.0045	(1;14)	13.9329	0.0022**
rrnS	F-type vs F-type	0.0038±0.0011			
	M-type vs M-type	0.0049±0.0015			
	M-type vs F-type	0.1963±0.0117	(1;14)	0.9425	0.3481
atp6	F-type vs F-type	0.0042±0.0016			
	M-type vs M-type	0.0065±0.0021			
	M-type vs F-type	0.2931±0.0159	(1;14)	3.3247	0.0897
nad3	F-type vs F-type	0.0020±0.0013			
	M-type vs M-type	0.0062±0.0013			
	M-type vs F-type	0.2832±0.0210	(1;14)	6.0608	0.0274*

TABLE 1 - continued

nad4	F-type vs F-type	0.0044±0.0012			
	M-type vs M-type	0.0066±0.0017			
	M-type vs F-type	0.3006±0.0130	(1;14)	1.6748	0.2166
nad5	F-type vs F-type	0.0028±0.0009			
	M-type vs M-type	0.0093±0.0018			
	M-type vs F-type	0.3156±0.0126	(1;14)	13.5779	0.0024**
nad6	F-type vs F-type	0.0081±0.0027			
	M-type vs M-type	0.0065±0.0025			
	M-type vs F-type	0.3284±0.0205	(1;14)	0.9354	0.3499
cox1	F-type vs F-type	0.0042±0.0015			
	M-type vs M-type	0.0106±0.0027			
	M-type vs F-type	0.2238±0.0147	(1;14)	17.0207	0.0010**
cox3	F-type vs F-type	0.0039±0.0014			
	M-type vs M-type	0.0094±0.0022			
	M-type vs F-type	0.2742±0.0151	(1;14)	18.8915	0.0001**
tRNAs	F-type vs F-type	0.0011±0.0005			
	M-type vs M-type	0.0059±0.0015			
	M-type vs F-type	0.1925±0.0103	(1;14)	39.6078	0.0000**

pD = nucleotide p-distance; s.e.= standard error; d.f. = degrees of freedom of the ANOVA test; F = F statistic; p = significance levels (* = $p<0.05$; ** = $p<0.01$).

Mean and standard error values of nucleotide p-distance are reported for each mitochondrial gene. tRNA genes have been pooled. One way analysis of variance

(ANOVA) have been performed comparing intra-female and intra-male p-distance distributions, in order to test whether M and F mutation ratios are significantly different or not.

TABLE 2

Number of polymorphic sites (synonymous and non-synonymous mutations) within M- and F-type sequences and test of positive selection within sex-related sequence groups.

Genes [a]		synonymous (S)	non-synonymous (NS)	Fisher's exact test [b]
atp6	F-type	8	0	1.000
	M-type	7	2	1.000
nad3	F-type	2	0	1.000
	M-type	1	4	0.880
nad4	F-type	8	5	1.000
	M-type	15	1	1.000
nad5	F-type	9	2	1.000
	M-type	26	4	1.000
nad6	F-type	7	2	1.000
	M-type	4	1	1.000
cox1	F-type	7	0	1.000
	M-type	13	0	1.000
cox3	F-type	8	0	1.000
	M-type	15	1	1.000

[a] = protein-coding genes only; [b] = average p value obtained from one-tailed Fisher's exact test.

Test of positive selection performed by the one tailed Fisher's exact test. No comparison gives significant values. Significant values would indicate positive selection.

TABLE 3

McDonald and Krietman's test of neutrality performed between M- and F-type protein sequences.

genes[a]		substitutions		
		fixed (F)	polymorphic (P)	probability[b]
atp6	Non-synonymous	105	2	
	Synonymous	106	15	0.0012**
nad3	Non-synonymous	62	3	
	Synonymous	52	4	0.7028
nad4	Non-synonymous	180	6	
	Synonymous	197	23	0.0060**
nad5	Non-synonymous	248	6	
	Synonymous	273	34	0.0001**
nad6	Non-synonymous	92	3	
	Synonymous	64	11	0.0010**
cox1	Non-synonymous	35	0	
	Synonymous	119	18	0.0258*
cox3	Non-synonymous	105	1	
	Synonymous	126	23	0.0001**

[a] = protein-coding genes only; [b] = probability (from Fisher's exact test)

probability levels as in Table 1

Numbers of fixed and polymorphic sites (synonymous and non-synonymous substitutions) between F- and M-type sequences of *T. philippinarum* and probabilities from contingency test reported.

FIGURE LEGENDS

FIGURE 1. – Plotting of the transition rate as a function of percentage sequence divergence on *rrnL* partial sequence (Italian sample set) and on the 9.2 Kb mtDNA fragment (U.S.A. sample set). The following comparisons were carried out in *T. philippinarum*: M-type versus M-type (triangles), F-type versus F-type (squares) and M-type versus F-type (rhombs).

FIGURE 2. – Putative recombinant clone (*rrnL* partial sequence) obtained from the somatic tissue of an analyzed male (Italian sample set). M-type sequence regions marked in pale gray, F-type sequence regions marked in deep gray.

FIGURE 3. – Partial gene arrangement of *T. philippinarum* M- and F-like sequences for the 9.2 Kb analyzed sample (U.S.A. sample set). Differences between sexes are marked in gray.

Figure 1

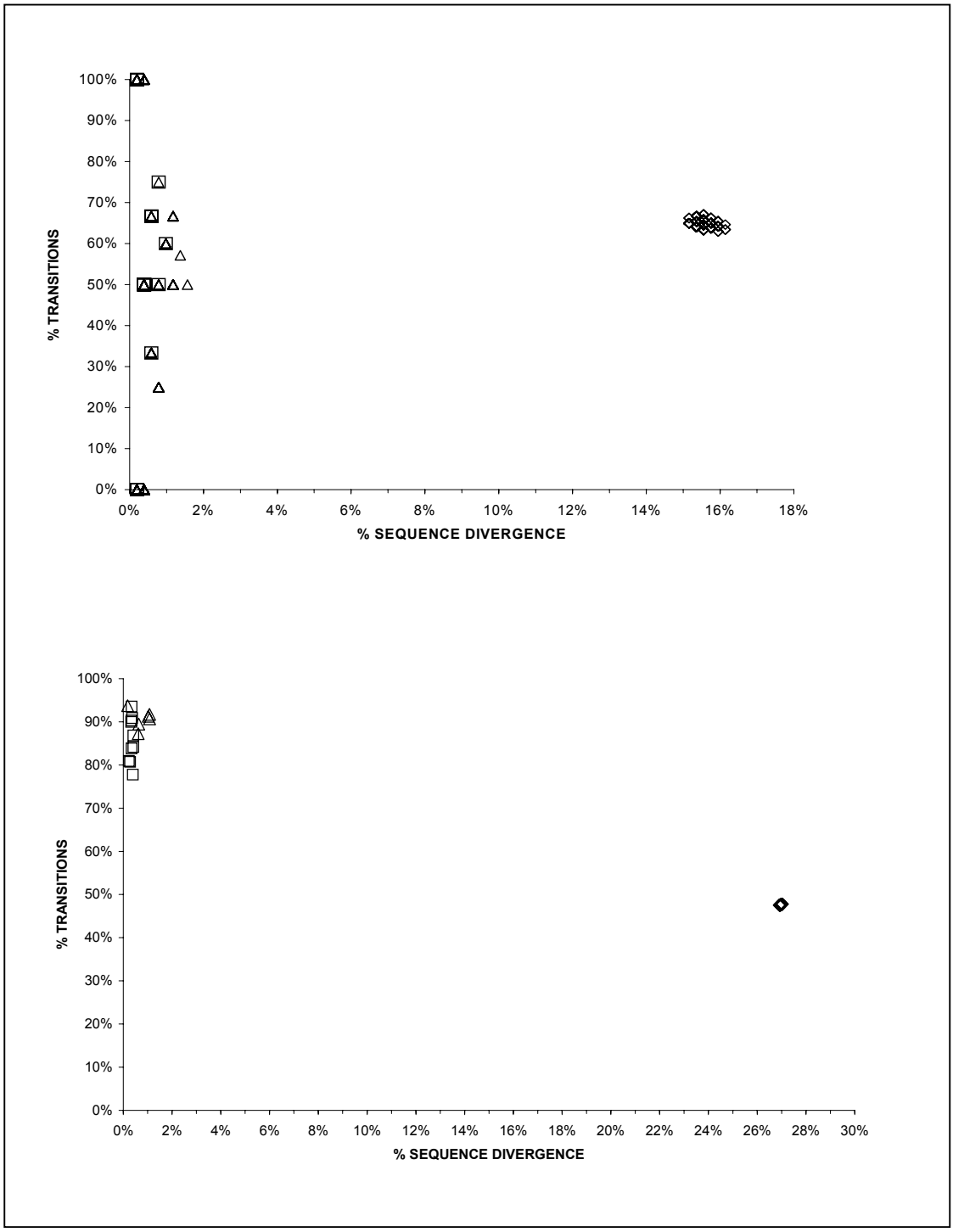
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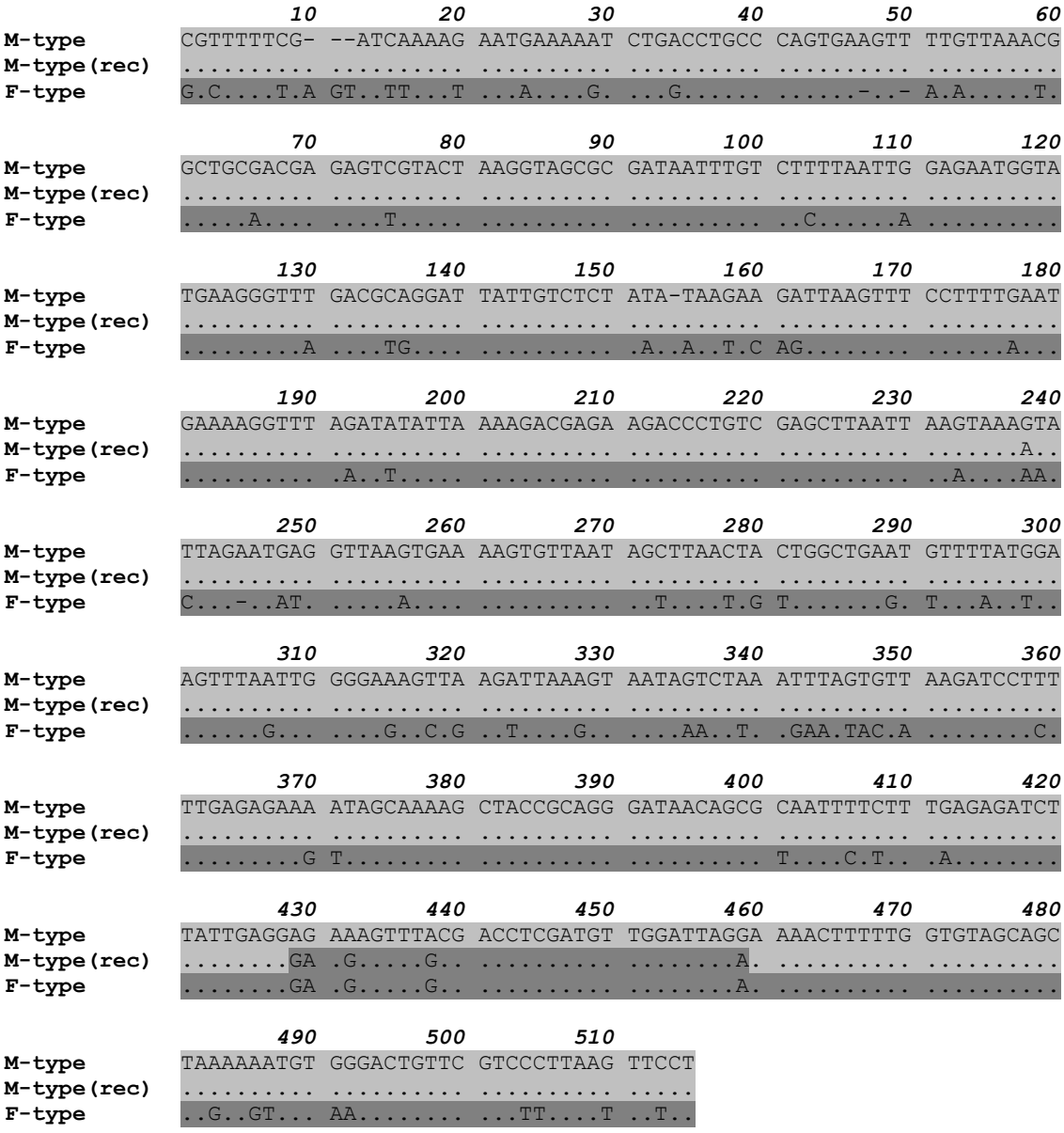
Figure 2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M-type(rec) ....
F-type G.C....T.A GT..TT...T ...A....G. ...G...... ......-..- A.A.....T.
70 80 90 100 110 120
M-type GCTGCGACGA GAGTCGTACT AAGGTAGCGC GATAATTTGT CTTTTAATTG GAGAATGGTA
M-type(rec) ....
F-type .....A.... ....T..... ....C......A ....
130 140 150 160 170 180
M-type TGAAGGGTTT GACGCAGGAT TATTGTCTCT ATA-TAAGAA GATTAAGTTT CCTTTTGAAT
M-type(rec) ....
F-type ....A ....TG.... .... .A..A..T.C AG........ ......A...
190 200 210 220 230 240
M-type GAAAAGGTTT AGATATATTA AAAGACGAGA AGACCCTGTC GAGCTTAATT AAGTAAAGTA
M-type(rec) ....A..
F-type .... .A..T..... ....A....AA.
250 260 270 280 290 300
M-type TTAGAATGAG GTTAAGTGAA AAGTGTTAAT AGCTTAACTA CTGGCTGAAT GTTTTATGGA
M-type(rec) ....
F-type C...-..AT. .....A.... ....T....T.G T.......G. T...A..T..
310 320 330 340 350 360
M-type AGTTTAATTG GGGAAAGTTA AGATTAAAGT AATAGTCTAA ATTTAGTGTT AAGATCCTTT
M-type(rec) ....
F-type ......G... ....G..C.G ..T....G.. ....AA..T. .GAA.TAC.A ....C.
370 380 390 400 410 420
M-type TTGAGAGAAA ATAGCAAAAG CTACCGCAGG GATAACAGCG CAATTTTCTT TGAGAGATCT
M-type(rec) ....
F-type ....G T......... .... T....C.T.. .A........
430 440 450 460 470 480
M-type TATTGAGGAG AAAGTTTACG ACCTCGATGT TGGATTAGGA AAACTTTTTG GTGTAGCAGC
M-type(rec) ....GA .G.....G.. ....A. ....
F-type ....GA .G.....G.. ....A. ....
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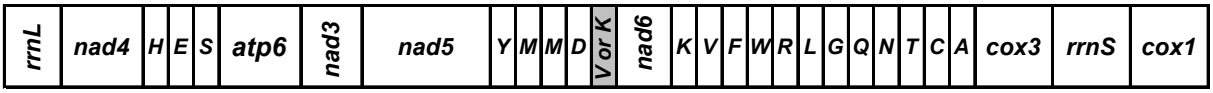
F-type ..G..GT... AA......... ...TT....T ..T..

Figure 3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