Relaxed selection on male mitochondrial genes in DUI bivalves eases the need for mitonuclear coevolution

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Abstract

Mitonuclear coevolution is an important prerequisite for efficient energy production in eukaryotes. However, many bivalve taxa experience doubly uniparental inheritance (DUI) and have sex-specific mitochondrial (mt) genomes, providing a challenge for mitonuclear coevolution. We examined possible mechanisms to reconcile mitonuclear coevolution with DUI. No nuclear-encoded, sex-specific OXPHOS paralogs were found in the DUI clam *Ruditapes philippinarum*, refuting OXPHOS paralogy as a solution in this species. It is also unlikely that mt changes causing disruption of nuclear interactions are strongly selected against because sex-specific mt-residues or those under positive selection in M mt genes were not depleted for contacting nuclear-encoded residues. However, M genomes showed consistently higher d_N/d_S ratios compared to putatively ancestral F genomes in all mt OXPHOS genes and across all DUI species. Further analyses indicated that this was consistently due to relaxed, not positive selection on M vs. F mt OXPHOS genes. Similarly, selection was relaxed on the F genome of DUI species compared to species with strict maternal inheritance. Coupled with recent physiological and molecular evolution studies, we suggest that relaxed selection on M mt function limits the need to maintain mitonuclear interactions in M genomes compared to F genomes. We discuss our findings with regard to OXPHOS function and the origin of DUI.

Keywords

cytonuclear coevolution; cytonuclear interactions; mitochondrial respiration; mt-miRNAs; nuclear compensation; ORFans; smithRNAs

1 | INTRODUCTION

Almost all eukaryotes rely on mitochondria and mitochondrial (mt)-encoded genes to generate cellular energy via oxidative phosphorylation (OXPHOS) (Karnkowska et al., 2016; Roger et al., 2017). Mt-encoded genes must coordinate with nuclear-encoded mt genes (i.e. N-mt genes) for OXPHOS. Coevolution and coadaptation between mt and N-mt genes are therefore essential and likely a ubiquitous feature of eukaryotes (Havird et al., 2019b; Hill et al., 2019; Rand et al., 2004; Sloan et al., 2018). Not surprisingly, signatures of mitonuclear coevolution have been found in the OXPHOS genes of diverse eukaryotes (Barreto & Burton, 2013; Barreto et al., 2018; Havird & Sloan, 2016; Havird et al., 2017; Yan et al., 2019).

Although unappreciated diversity in mt inheritance exists across eukaryotes (Havird et al., 2019a), strictly maternal inheritance (SMI) of mitochondria is the norm in bilaterian animals (Barr et al., 2005; Birky, 1995; Luo et al., 2018; Wei et al., 2020). One exception is doubly uniparental inheritance (DUI) in many species of bivalves. In DUI taxa, one mt genome is inherited through the female lineage (the F mt genome) and a separate, often divergent, mt genome is inherited through the male lineage (the M mt genome) (Gusman et al., 2016; Zouros, 2013; Zouros et al., 1994). The two mt genomes remain sequestered in separate gametes: egg cells contain only F genomes while sperm exclusively carry M genomes. Somatic tissues in both sexes generally rely on F genomes, although when male somatic tissues were examined closely in one species, appreciable levels of heteroplasmy were found (Ghiselli et al., 2019).

One complication arising from DUI is that both M and F mt genes must interact with the same set of nuclear-encoded genes. Although recent work has supported mitonuclear coevolution in bivalves (Piccinini et al., 2021), extreme divergence between M and F mt genes (e.g. as little as 34% amino acid identity; Capt et al., 2020) raises the possibility that maintaining mitonuclear interactions in all tissues could be a challenge in DUI species. One solution to this problem may be that N-mt paralogs with sex-specific expression have evolved to maintain appropriate interactions with both M and F mt genes (Breton et al., 2007). In this scenario, nuclear-encoded duplicates would undergo sex-specific selection to coevolve with either M or F mt genes, but not both. Sex-specific N-mt paralogs are common in *Drosophila* and mammals, with testis-specific paralogs often showing signs of accelerated evolution, indicating possible positive selection for sex-specific mt function (Eslamieh et al., 2017; Gallach et al., 2010; Havird & McConie, 2019). However, to detect such paralogs, sex- and tissue-specific transcriptomic resources are necessary, which are lacking in DUI taxa.

A second possible solution may be that selection has shaped sex-specific mt evolution to maintain mitonuclear interactions. Mt residues important for maintaining mitonuclear interactions may be under stronger purifying selection than residues distant to nuclear residues. OXPHOS genes are ideally suited to test this prediction, as only a fraction of nuclear-encoded OXPHOS genes directly contact mitochondrial-encoded residues (e.g. Tsukihara et al., 1996). OXPHOS complex II (succinate dehydrogenase) is also entirely nuclear-encoded in many eukaryotes and acts a negative control for examining the influence

of mitonuclear interactions (Ellison & Burton, 2006; Havird et al., 2017). Previous authors have found elevated signatures of mitonuclear coevolution in "contact" vs. "non-contact" OXPHOS genes (Yan et al., 2019), including in bivalves (Piccinini et al., 2021). Similarly, individual amino acid sites in mt genes can be categorized as contact vs. non-contact. Mt contact sites may be under more intense purifying selection to maintain mitonuclear interactions than non-contact mt sites. Under this hypothesis, mt-encoded contact sites should remain relatively conserved between M and F mt genes compared to non-contact residues, which may change more freely.

A third possibility is that maintaining mitonuclear interactions is not as important in M mt genes compared to F mt genes. Studies of individual taxa and genes have reported increased rates of evolution and ratios of non-synonymous to synonymous substitution rates (d_N/d_S) in M mt genes compared to their F counterparts (Hoeh et al., 1996, 1997, 2002; Liu et al., 1996; Ort & Pogson, 2007; Soroka & Burzy ski, 2010; Stewart et al., 1995, 1996; Zbawicka et al., 2010; Zouros, 2013). Increased d_N/d_S ratios could be due to positive or relaxed purifying selection on M mt genes. Adaptive hypotheses for M mt genomes regarding sperm function have been put forward (Bettinazzi et al., 2019; Skibinski et al., 1994), but others have suggested relaxed selection on M mt genes owing to their solitary role in sperm compared to the ubiquitous use of F mt genes in all other tissues (see above citations). If selection is relaxed on mt function in sperm, mitonuclear coevolution with the F mt genome may be favoured over coevolution with the M mt genome (Breton et al., 2007). Some have attempted to differentiate between positive vs. relaxed selection on M mt genes by using population genetic tests of selection (e.g. McDonald-Kreitman or Tajima's *D*). However, results are inconsistent and largely confined to *Mytilus* spp. and single mt genes (Ort & Pogson, 2007; Quesada et al., 1998; mietanka et al., 2009; Smietanka et al., 2013). Recent work has also shown increased d_N/d_S in mt genes of DUI bivalves compared to SMI species (Plazzi & Passamonti, 2019). This was interpreted as a sign of positive selection on at least some mt genes during the transition to DUI.

Here, we examined molecular evolution of mt genes in DUI taxa to evaluate these possibilities. We investigated sex-specific transcriptomes of the model DUI clam *Ruditapes philippinarum* to search for nuclear-encoded, sex-specific OXPHOS paralogs as a possible mechanism to maintain mitonuclear interactions. Using analysis of complete, publicly available mt genomes from DUI bivalves, we also investigated if selection acts differently on mt residues that contact nuclear residues vs. those that lack nuclear contacts. Finally, we used a phylogenetic framework to evaluate the ubiquity of elevated d_N/d_S ratios in M mt genes and if they are caused by relaxed purifying selection or enhanced positive selection.

2 | METHODS

2.1 | Generating sex-specific transcriptomes of *Ruditapes philippinarum*

To investigate sex-specific nuclear OXPHOS paralogs as a possible mechanism to maintain mitonuclear interactions in DUI species, we generated sex- and tissue-specific transcriptomes for the DUI species *Ruditapes philippinarum*. This species has been studied extensively as a model for DUI (Passamonit & Plazzi, 2020) and was also a focus in our additional analyses (see below). Samples were collected in the Northern Adriatic Sea (Italy),

in the river Po delta region (Sacca di Goro, approximate GPS coordinates: 44°50′06″N, 12°17′55″E) during the spawning season (end of July 2015). The collected individuals were kept in the laboratory for 48 h in aerated beakers containing artificial seawater (filtered reverse osmosis water with Red Sea Coral Pro aquariology sea salt; Red Sea Europe, Verneuilsur-Avre, France) that was changed every 12 h. Clams were then opened, sexed by microscopic inspection of gonadal tissue, flash-frozen in liquid nitrogen, and stored at −80°C until RN.

An extraction (usually a few days later). Total RNA was extracted with TRIzol; poly-A transcripts were isolated with magnetic beads and used as template for cDNA synthesis following the protocol in Mortazavi et al. (2008) as modified in Ghiselli et al. (2012). In total, 90 samples were obtained: three different tissues (adductor muscle, mantle, and gonad) of 15 males and 15 females. Gonad samples were highly enriched for sex-specific mt transcripts, although it should be pointed out that only gametes are purely homoplasic. Sequencing was performed on an Illumina HiSeq 2500 platform with a selected insert size of 500 bp to generate 150 bp paired-end reads.

Reads were filtered using trimmomatic v0.36 (Bolger et al., 2014) with the following parameters: PE, -phred33, ILLUMINACLIP:TruSeq3-PE,fa:2:30:10, LEADING:36,TRAILING:36, SLIDINGWINDOW:10:36, MINLEN:45. After trimming, only reads surviving in both left and right ends were maintained. For each sample, low abundance kmers ($C < 5$) were removed using the trim-low-abund.py script, implemented in khmer v2.1.1 (Crusoe et al., 2015). In order to detect sex-specific, or tissue-specific nuclear-encoded OXPHOS transcripts, we assembled reads separately for each combination of tissue and sex. We therefore used Trinity v2.4.0 (Grabherr et al., 2011) with default parameters to obtain 6 transcriptome assemblies: male adductor (m_A), male mantle (m_M), male gonad (m_G), female adductor (f_A), female mantle (f_M), and female gonad (f_G).

We also used a multi-kmer and multi-assembler approach to obtain a single and more accurate reference transcriptome, as suggested in MacManes (2018). For this purpose, reads from all samples were assembled using: (a) Trinity de novo assembly (with 21, 25, and 31 kmer sizes), (b) Velvet 1.2.10-Oases 0.2.8 de novo assembly (with 21, 25, 31, 41, 61, 81, and 101 kmer sizes) (Schulz et al., 2012; Zerbino & Birney, 2008), and (c) Trinity genome-guided assembly using a de novo *R. philippinarum* genome assembly (manuscript in preparation) as reference. Reads were mapped to the reference genome using HISAT2 (Kim et al., 2019), then we used SAMtools (Li et al., 2009) to keep paired reads mapping concordantly one or more times. For the genome-guided assembly, we used default parameters in Trinity with genome_min_coverge of 100. These transcriptome assemblies were merged into a unique, non-redundant reference transcriptome with the tr2aacds.pl script (http://arthropods.eugenes.org/EvidentialGene/trassembly html).

To assess the completeness of the sex/tissue-specific transcriptomes and the reference transcriptome, we used BUSCO (Simao et al., 2015) as implemented in gVolante (Nishimura et al., 2017) using the Metazoa ortholog database.

2.2 | Identifying nuclear-encoded OXPHOS paralogs in *Ruditapes philippinarum*

To annotate nuclear-encoded OXPHOS genes in the sex/tissue-specific transcriptomes and the reference transcriptome, we first downloaded nuclear-encoded OXPHOS protein sequences of the following molluscs and model species from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2016): *Drosophila melanogaster*, *Caenorhabditis elegans*, *Helobdella robusta*, *Octopus bimaculoides*, *Lottia gigantea*, and *Crassostrea gigas*. These sequences were used to create a custom protein database (db). We used findorf (https://github.com/vsbuffalo/findorf; Krasileva et al., 2013) with the custom db to detect nuclear-encoded OXPHOS genes in our transcriptomes and to predict Open Reading Frames (ORFs). The findorf pipeline combined blastx searches against the transcriptomes with HMMER searches against the PFAM db to identify ORFs. To ensure the absence of non-Metazoan contaminants, we performed a blastp search (Altschul et al., 1997) of the putative *R. philippinarum* nuclear OXPHOS proteins against the NCBI nr database.

We used T-COFFEE Version_12.00.7fb08c2 (Notredame et al., 2000) in the psicoffee mode to generate alignments for each identified OXPHOS gene from the sequences identified from the different transcriptomes. For each protein alignment, we used CD-HIT (Fu et al., 2012) with similarity 0.8, to obtain a consensus sequence. To identify putative paralogs, we manually checked for divergent nuclear OXPHOS genes using both protein and nucleotide alignments. In mammals and *Drosophila*, sex-specific OXPHOS paralogs can be up to 50% divergent in amino acid sequence (Eslamieh et al., 2017; Gallach et al., 2010; Havird & McConie, 2019), making them easy to identify in gene alignments. In order to detect the presence of paralogs not identified as OXPHOS genes in the annotation step (and therefore excluded from the alignments), we used Proteinortho6 (Lechner et al., 2011) against the reference transcriptome, with the following parameters: selfblast -p=tblastx+ -sim=0.4 -cov=50.

2.3 | Investigating publicly available DUI mt genomes

Mitochondrial genome sequences for 36 pairs of bivalves in which DUI has previously been confirmed (Gusman et al., 2016) were downloaded from Genbank in Fall 2019. Accession numbers are available in Table S1. Only bivalve species with complete mt genome sequences for both M and F haplotypes were included. While performing analyses, new DUI mt genomes became available for *Geukensia demissa*, *Scrobicularia plana*, and *Limecola balthica* (Capt et al., 2020). These were also included in our analyses when possible. Sequences for *Hyriopsis schlegelii*, *Hyriopsis cumingii*, and *Mytilus coruscus* were included in phylogenetic analyses, but were excluded from most other analyses because their M sequences are likely F haplotypes (Bettinazzi et al., 2016). We also examined partial COX1 sequences obtained via Genbank (Table S1) from M and F sequences of confirmed DUI species representing a wider taxonomic range. We included mt sequences from several SMI bivalves in some analyses, which were gathered from Plazzi and Passamonti, (2019).

2.4 | Phylogenetic analyses

To provide a phylogenetic context for subsequent analyses, the 13 OXPHOS protein-coding genes were extracted from each mt genome sequence and their translated protein sequences were aligned independently via Muscle (Edgar, 2004). Manual adjustments by eye were

performed when necessary in MEGA version 10.0.5 (Kumar et al., 2018). Resulting amino acid sequences were concatenated (except for ATP8, which was unannotated in many genomes) and phylogenetic reconstruction was performed under maximum likelihood using RAxML v8.2.12 and the gamma WAG model of amino acid substitution. Relationships were evaluated with 100 rapid bootstrap replicates (-f a -# 100 -m PROTGAMMAWAG) (Stamatakis, 2014; Stamatakis et al., 2008). Phylogenetic analyses were performed on three datasets: M and F sequences of concatenated gene sets from the DUI species, F sequences of concatenated gene sets from DUI and SMI species, and M and F sequences of COX1 sequences from the wider taxonomic range of DUI and SMI species.

2.5 | Mitonuclear interactions and site-specific analyses

If selection acts on M and F genes to maintain mitonuclear interactions, we predicted that contact mt-encoded sites should show signatures of purifying selection compared to non-contact residues. To identify whether mt amino acid residues that tend to change in M vs. F genes are underrepresented for nuclear contacts, we took two approaches. First, we considered all variable sites between M and F OXPHOS gene sequences in the model DUI species *R. philippinarum*. Second, to expand this analysis across all DUI species we examined sites under potential positive selection in M genes, using branch-site tests in PAML v4.9 ("model = 2" and "Nsites = 2"; Yang, 2007). We predicted that mt-encoded contact sites would be underrepresented for positive selection if they mainly evolve under purifying selection. This was performed on the dataset of complete mt genomes from DUI species (i.e. no SMI sequences were included) using the reference topology generated with RAxML. For each gene, analyses were performed where site-specific selection was investigated on all M branches lumped together and when M branches were investigated individually for each origin of M and F genomes (e.g. separate analyses considering just unionid M branches, the *Ruditapes* M branch, the *Permytilus* M branch, etc., see topology in Figure 1). Sites were classified as being under statistically significant positive selection via Bayes Empirical Bayes analyses (Yang et al., 2005).

To determine if mt sites contacted nuclear-encoded residues, we mapped sites of interest onto published structures of OXPHOS complexes I and III–V (complex II is entirely nuclear-encoded) (Fiedorczuk et al., 2016; Iwata et al., 1998; Tsukihara et al., 2003; Zhou et al., 2015). Contact sites between mt- and nuclear-proteins were identified using the "find clashes/contacts" tool in Chimera v1.11.2 (Pettersen et al., 2004) with default parameters, except that minimum overlap was changed to   -1 angstroms to maximize the probability of detecting even distantly interacting residues. The reference mammalian sequences were then aligned to the DUI species to identify homologous residues using the same methods as above.

2.6 | d_N/d_S ratios in M and F mt OXPHOS genes

To compare d_N/d_S ratios between M and F OXPHOS genes, we used branch specific models in PAML. First, we examined d_N/d_S ratios on all branches leading to male sequences (terminal branches and consensus internal M branches) vs. all branches leading to female sequences ("model = 2" in PAML) based on the phylogeny of M and F sequences from the DUI species with complete genomes (i.e. Figure 1). This was evaluated against a model

where all branches had the same d_N/d_S ratio ("model = 0" in PAML). A likelihood ratio test was used to determine if two d_N/d_S ratios was a better fit to the data. This analysis was performed using the concatenated 12 gene dataset and on each gene independently (including ATP8, which had fewer species compared to the other datasets).

To determine if elevated M d_N/d_S ratios were ubiquitous across different origins of M and F genomes, we used a model where different M and F d_N/d_S ratios were applied for each case of independent M and F genome origin. For example, in the COX1 dataset we applied 16 different d_N/d_S ratios, reflecting 8 independent origins of M and F genomes (e.g. separate M and F ratios in unionids, *Ruditapes*, *Permytilus*, etc., according the topology in Figure 1). This was performed using the concatenated 12 gene dataset and on each gene independently.

Finally, we performed a similar analysis examining d_N/d_S ratios in F branches from DUI vs. SMI species. As before, we examined a model with two d_N/d_S ratios (DUI vs. SMI branches) against a model where a single d_N/d_S ratio was applied to all branches using the concatenated 12 gene dataset. M sequences were excluded from this analysis.

2.7 | Positive vs. relaxed selection in M mt OXPHOS genes

To investigate whether elevated estimates of d_N/d_S were the result of positive or relaxed purifying selection, we used the program RELAX in the HyPhy package, both locally and via the online datamonkey server (Pond et al., 2005; Weaver et al., 2018; Wertheim et al., 2014). RELAX first classifies each site in an alignment into one of three d_N/d_S classes: ω_1 corresponds to purifying selection ($d_N/d_S < 1$), ω_2 corresponds to neutral evolution ($d_N/d_S \approx 1$), and ω_3 corresponds to positive selection ($d_N/d_S > 1$). RELAX then estimates the distribution of ω classes for test and reference branches, which is summarized in a "selection intensity parameter" k. $k < 1$ indicates relaxed selection on test branches (d_N/d_S values in test branches tend to converge on 1) and $k \gg 1$ indicates intensified selection on test branches (d_N/d_S values in test branches tend to diverge from 1). The significance of the k parameter is assessed by comparison to a model where a single distribution of d_N/d_S classes is applied to both test and reference branches (see Wertheim et al., 2014 for more detail on RELAX).

The RELAX analyses mirrored the PAML analyses to address two sets of data: (a) M vs. F branches in DUI species and (b) F branches in DUI vs. SMI species. We first compared all M test branches to all F reference branches for DUI species, using the concatenated 12 gene set and for each individual gene. We also limited test and reference branches to each independent origin of M and F genomes (e.g. unionids, *Ruditapes*, *Permytilus*, etc.), while all other branches were left unclassified and not examined (both for the concatenated gene set and for each individual gene). Finally, we used the concatenated 12 gene dataset of F sequences from DUI and SMI species where DUI branches were classified as test and SMI branches were classified as reference.

3 | RESULTS

3.1 | No evidence of sex-specific nuclear OXPHOS paralogs

We sequenced *R. philippinarum* samples to generate sex- and tissue-specific transcriptomes, producing 693,882,864 raw reads and 353,545,680 paired reads after quality trimming for *de novo* transcriptome assemblies (Table S4). Assemblies obtained from adductors (both in males and in females) are characterized by a lower number of transcripts (<23,000) and a higher percentage of missing genes (BUSCO completeness ~50%) compared to other tissues, while in female gonads we found the highest number of transcripts (54,062) and the highest completeness (68%) (Table S5). N50 was comparable in each of the sex- and tissue-specific transcriptomes (~700 bp). Using a multi-kmer and multi-assembler approach to assemble reads from all samples, we obtained a "reference transcriptome" for *R. philippinarum* that had the highest N50 (1005 bp) and the highest BUSCO completeness (81%, Table S6).

We recovered 67 total N-mt OXPHOS genes in the transcriptome assemblies of *R. philippinarum* (Table S7), which is comparable to the number of N-mt OXPHOS genes available in the KEGG database for the model bivalves *Crassostrea gigas* and *Mizuhopecten yessoensis*. In most cases, genes were found in two or more transcriptomes, but NDUC2, NDUV2, QCR10, and COX6B1 were detected in just a single transcriptome. Consensus sequences for each gene were deposited in GenBank (accession numbers MW261528-94) and are available in Table S8.

For N-mt OXPHOS genes found in more than one transcriptome (or found more than once in the same transcriptome), we aligned such sequences to infer the presence of paralogs. By visually inspecting the alignments, we found that genes from different transcriptomes often differ in length, but rarely differ in sequence by more than a few nucleotides, unlike sex-specific N-mt OXPHOS paralogs in mammals and *Drosophila*, which differ up to 50% in amino acid identity. One exception was the SDHC gene of Complex II (succinate dehydrogenase; SDH), for which we found two divergent sequences (nucleotide p-distance 0.98, 33% amino acid identity). The first variant shows a higher sequence similarity with SDHC from other bivalve species (*E*-value = 1e-58, Table S9) and it was found in all the transcriptomes analysed. The second variant shows a higher sequence similarity to SDHC from fish and birds (*E*-value = 1e-32, Table S9) and was detected in assemblies from male gonads, female gonads, and in the reference transcriptome. No other potential paralogs were retrieved.

3.2 | Divergent M and F genomes have evolved independently multiple times

As shown previously based on more limited datasets, a maximum likelihood phylogenetic analysis of concatenated M and F mt OXPHOS genes from 38 DUI species (Table S1) shows a clear sister relationship between M and F genes within the Unionoida, confirming a single origin of sex-specific mt genomes in the last common ancestor of unionids (Figure 1). In non-unionids, M and F genes generally show a sister relationship within a species, supporting independent origins of separate M and F genomes within the Mytilidae, Veneridae, and Scrobiculariidae. Within *Mytiluls* spp., it is unclear if there was a single

origin in the last common ancestor of the genus, or two origins (once for *M. trossulus* + *M. edulis* + *M. galloprovincialis* and once for *M. californianus*). In another phylogenetic analysis, we included only F genomes from DUI species along with 62 F genomes from SMI species (Figure S1) and found DUI species did not cluster together, again confirming multiple gains or losses of DUI in bivalves. The analyses based on complete mt genomes support at least nine independent origins of M and F mt genomes in bivalves. A dataset based on partial COX1 sequences, which are available from a wider range of taxa, revealed additional possible, independent origins of M and F mt genomes in Solemyidae (*Modiolus modiolus*), Mytilidae (*Mytella charruana*, *Geukensia demissa*, and three *Brachidontes* spp.), Yoldiidae (*Yoldia hyperborea*), Tellinoidea (*Limecola balthica* + *Scrobicularia plana*), and Mactridae (*Pseudocardium sachalinensis*) (Figure S2). All told, the COX1 analysis supports at least 15 independent origins of M and F genomes across bivalves.

Amino acid identity (uncorrected p-distance after pairwise alignment) between concatenated protein coding M and F genes from DUI species with complete mt genomes ranged from as little as 34% (in *L. balthica*; Capt et al., 2020) to 84% (in *Mytilus edulis*), with an average of 55% (Table S10).

3.3 | Residues that are altered in M genes are enriched, not depleted for nuclear contacts

We found no evidence that mt residues that contacted nuclear-encoded residues were more likely to be conserved between M and F sequences. Variable sites between M and F OXPHOS genes in *R. philippinarum* (which shows 66% M vs. F amino acid identity) were *more* likely to be contact residues compared with conserved sites (Figure 2a). This pattern was consistent across many genes and statistically significant for four individual genes and the summed dataset, where 30% of variable sites contacted nuclear-encoded residues vs 24% of conserved sites ($p < 0.001$, Fisher's exact).

More broadly, we examined all sites under potential positive selection in M genes of DUI species. When all genes were combined, there was a slight, but statistically non-significant trend for positively selected sites to be enriched for mitonuclear contacts ($p = 0.580$; Figure 2b). This trend was only statistically significant when considering COX1 by itself (43% of positively-selected sites were contact vs. 27% in sites not under positive selection, $p = 0.044$).

When only considering sites that were identified as statistically significant for positive selection in M genes via a Bayes empirical Bayes analysis, the same statistically non-significant trend was observed (Figure 3): positively selected sites were more likely to contact nuclear-encoded residues ($p = 0.090$, Figure 2c). This comparison was only statistically significant for a single gene, ND5 (34% vs. 24%, $p = 0.042$).

3.4 | M genes and DUI species show consistently elevated d_N/d_S ratios

As suggested based on previous studies, we observed consistently higher d_N/d_S for M mt OXPHOS genes compared to F genes (Figure 4). When all M branches were compared against all F branches, d_N/d_S ratios were significantly elevated in M branches in all datasets ($p < 0.05$, likelihood ratio test), except when the small subset of ATP8 sequences were analysed by themselves (Figure 4a). d_N/d_S ratios were 1.65-fold higher for M genes in

the concatenated gene set and up to ~5-fold higher when examining single gene datasets (COX1). When M vs. F genes were compared for each independent origin of separate M and F genomes, (e.g. M unionid branches compared to the F unionid branches), there was a clear trend for elevated d_N/d_S ratios in M genes across datasets (Figure 4b). When the concatenated gene set was used, d_N/d_S ratios were always elevated in M branches when examining all origins of DUI.

In a separate analysis, we examined F mt OXPHOS genes of DUI species compared to those from SMI species (following the topology in Figure S1). When all DUI branches were compared against all SMI branches for the 12 gene concatenated dataset, d_N/d_S ratios were slightly, but statistically significantly higher in DUI branches (0.065 vs. 0.051, $p < 0.001$ likelihood ratio test).

3.5 | Elevated d_N/d_S ratios in M mt genes are caused by relaxed purifying selection

RELAX analyses consistently suggested relaxed purifying selection in M mt OXPHOS genes compared to F genes. When comparing all M branches to all F branches in DUI species, the selection intensity parameter k was estimated at 0.44 for the concatenated 12 gene dataset (Figure 5a; $p < 0.001$). This indicates that F mt OXPHOS genes are under about 2.3 times more intense selection compared to M mt OXPHOS genes. Similar to the d_N/d_S analyses, each individual gene was consistent with this pattern with the exception of the smaller ATP8 dataset (Figure 5b). When the same analyses were performed using clade-specific M branches (e.g. M vs. F genes in unionids), the 12 gene concatenated data set consistently showed signatures of relaxed selection on M branches compared to F branches in all clades, which was generally supported when examining individual genes (Figure 5c). However, the power to detect relaxed selection in gene- and clade-specific datasets was greatly reduced due to the smaller number of branches and sites being compared.

In a separate analysis, we compared F mt OXPHOS genes of DUI species to those from SMI species (following the topology in Figure S1). When all DUI branches were compared against all SMI branches for the 12 gene concatenated dataset, there was a slight, but significant signature of relaxed selection in DUI species ($k = 0.94$, $p = 0.045$).

4 | DISCUSSION

4.1 | Relaxed selection may limit the need for nuclear coevolution with M mt genomes

The hypothesis of mitonuclear coevolution has been supported across eukaryotes (Barreto & Burton, 2013; Barreto et al., 2018; Havird & Sloan, 2016; Havird et al., 2017; Osada & Akashi, 2012; Yan et al., 2019). Recently, a strong correlation in evolutionary rates among nuclear- and mt-encoded OXPHOS genes was found in bivalves, providing support for mitonuclear coevolution in this taxa as well (Piccinini et al., 2021). However, DUI bivalves face a challenge when it comes to mitonuclear coevolution: the same nuclear genome must coevolve with two mt genomes, which are often highly divergent. The main findings of our study are that sex-specific nuclear paralogs and selection against mt changes that alter nuclear interactions likely do not facilitate mitonuclear coevolution in DUI bivalves, at least in *R. philippinarum*. Rather, relaxed selection on M mt OXPHOS genes may indicate that

nuclear coevolution with the M mt genome is secondary to maintaining proper nuclear interactions with the F mt genome. In other words, nuclear genes may coevolve with F mt genes, while coevolution with M mt genes may be under less stringent selection.

In DUI species, male-specific nuclear OXPHOS paralogs could coevolve with M mt genes, while paralogs with ubiquitous expression could coevolve with F mt genes. However, when we examined sex- and tissue-specific transcriptomes of *R. philippinarum*, one of the most heavily studied DUI species (Passamonti & Plazzi 2020); the only possible paralog was for SDHC, which lacks mitonuclear interactions because SDH (OXPHOS Complex II) is entirely nuclear-encoded in bivalves. This paralog also lacked sex-specific expression. Therefore, it is unlikely that sex-specific, nuclear-encoded OXPHOS paralogs facilitate M mitonuclear coevolution in *R. philippinarum*. A recent study also failed to find nuclear-encoded OXPHOS paralogs across diverse bivalves (Piccinini et al., 2021), although sex- and tissue-specific transcriptomes were not used in that study and DUI species were not well represented, likely preventing detection of sex-specific paralogs. This is in line with recent work concluding that elevated rates of evolution in N-mt OXPHOS paralogs of mammals and *Drosophila* are likely due to relaxed, not positive selection, bringing into question the idea that these paralogs are shaped by mitonuclear coevolution (Havird & McConie, 2019). However, this possibility should be explored further in other DUI lineages, especially unionids with extreme M vs. F divergence.

Our results using structural information on OXPHOS complexes (Figures 2 and 3) suggest that mitonuclear interactions may play a role in DUI mt evolution, but not as we predicted. If selection on M mt genes was acting to preserve mitonuclear interactions, we reasoned residues that diverge in M vs. F genomes would be underrepresented for nuclear contacts. However, the results suggest the opposite: residues that were variable between M vs. F genes in *R. philippinarum* or under positive selection in M mt genes were enriched for contacting nuclear-encoded residues (although not always at statistically significant levels, Figure 2). These results should be interpreted cautiously, given that bivalve evolution was mapped onto mammalian structures. However, these are some of the most highly conserved proteins in eukaryotes and similar techniques have been used in more divergent taxa (Havird et al., 2015).

Nuclear interactions may therefore be actively manipulated or disrupted, rather than preserved, during M mt genome evolution. This fits with a growing body of evidence that M mt genomes in DUI species may influence nuclear processes in order to preserve parental inheritance or drive sex differentiation (Stewart et al., 2020; Zouros, 2020). M mt genomes encode M-specific ORFan genes and often have expansions in OXPHOS genes (especially COX2; Bettinazzi et al., 2016; Capt et al., 2020). The purpose of sex-specific mt ORFans and insertions remains largely unknown, but they may play roles in OXPHOS. Transcribed small RNAs from the M mt genome may also affect expression of nuclear-encoded genes as part of sex determination in DUI species (Pozzi et al., 2017). Recent work suggests that such mt-encoded small RNAs (termed "smithRNAs" or "mt-miRNAs") may be ubiquitous across animals (Pozzi & Dowling, 2019, 2020). Our results complement these findings and suggest that evolution of M mt OXPHOS genes may also result in altered mitonuclear interactions, possibly through altering OXPHOS function (see below).

Another possibility is that mt-encoded contact sites are evolving under relaxed selection. The branch-site tests we used to detect positive selection essentially test for $d_N/d_S > 1$ in a subset of codons on a branch (Yang & dos Reis, 2010). While positive selection most readily explains this pattern, relaxed selection on individual codons could also cause elevated d_N/d_S. It is therefore possible that mitonuclear interactions may be under relaxed selection in M mt genes, which is supported by the RELAX results. However, the RELAX results are based on analyses of entire genes (or concatenated gene sets), which still allows for individual sites within those genes to evolve via positive selection. In any case, mt sites that contact nuclear residues do not appear to be conserved during M mt genome evolution in DUI bivalves.

Although elevated d_N/d_S ratios have been documented in M mt genes previously, we find that elevated d_N/d_S ratios in M vs. F mt OXPHOS genes is a nearly ubiquitous feature across genes and species (Figure 4). This is consistently due to relaxed, not positive selection on M vs. F mt OXPHOS genes (Figure 5). Previous studies using population tests of selection in *Mytilus* species and reduced datasets support this result (Hoeh et al., 1996; Ort & Pogson, 2007; Riginos et al., 2004; Skibinski et al., 1999; Smietanka et al., 2013). Relaxed selection on M mt genomes is likely due to their limited physiological roles compared with F mt genes (Obata et al., 2011; Ort & Pogson, 2007; Stewart et al., 1996). Although individual sites may still be under positive selection in M mt genes and purifying selection likely still dominates M mt genome evolution, our analyses demonstrate conclusively that elevated d_N/d_S ratios are caused by relaxed selection on M mt OXPHOS genes.

Given that selection is relaxed on M compared to F mt OXPHOS genes, it is likely that maintaining proper mitonuclear interactions with M mt genes is also less important. This was supported in the evolutionary rate correlations of Piccinini et al. (2021): although only four DUI taxa were examined, correlations between mt and N-mt OXPHOS genes were stronger when using F mt sequences than M mt sequences. Mitonuclear coevolution is still likely important in M mt genomes, but our results suggest that relaxed selection on M mt genes may have shifted the balance to preferentially preserving nuclear interactions with F over M mt genes.

4.2 | Physiological consequences of relaxed M mt selection

Examining the physiology of tissues with M vs. F genomes may reveal the consequences of relaxed selection on M mt OXPHOS genes. Although M mt genomes are found in a heteroplasmic state in somatic tissues of males in some DUI species (Ghiselli et al., 2019), sperm rely exclusively on M mt genomes, suggesting sperm physiology and function may underlie differences in M vs. F mt selection pressures. Some hypotheses suggest positive selection for enhanced sperm function in male DUI species (Breton et al., 2007; Burt & Trivers, 2006; Skibinski et al., 1994). DUI species can also theoretically escape the "mother's curse" inherent to SMI (Bettinazzi et al., 2019,2020; Cosmides & Tooby, 1981; Frank & Hurst, 1996; Gemmell et al., 2004; Stewart et al., 2020), allowing mt variants with adaptive male-specific functions to be selected for. However, experiments using "masculinized" mt genomes in *Mytilus edulis* do not support this (Everett et al., 2004; Jha et al., 2008). Masculinization occurs when a F mt genome invades the male lineage and becomes paternally inherited, which has only been documented in *Mytilus* spp. (Zouros,

2013). Masculinized F genomes result in slightly *better* sperm performance than the native M genomes via increased OXPHOS enzyme activities (Breton et al., 2009; Everett et al., 2004; Jha et al., 2008), contrary to the idea that M genomes are under positive selection for enhanced sperm performance. A recent comparison of SMI vs. DUI species supports these results, as SMI sperm were faster than DUI sperm (Bettinazzi et al., 2020).

Because the central role of mt genes is to perform OXPHOS, examining OXPHOS in DUI species may uncover how selection has acted on M mt genes. Recent work has addressed this in two marine DUI species: *Arctica islandica* and *M. edulis*. In both species, OXPHOS coupling efficiency and excess OXPHOS complex IV capacity are lower in sperm compared to eggs, while in SMI species sperm and eggs show comparable phenotypes (Bettinazzi et al., 2019). The apparent reduction in OXPHOS functionality of tissues with M mt genomes may be caused by relaxed selection on M mt genes in DUI bivalves, although fitness remains to be directly linked to these phenotypes.

Relaxed selection on OXPHOS may suggest DUI bivalve sperm rely mostly on glycolysis, not OXPHOS, for ATP production. Sperm from mammals appear to rely primarily on glycolysis (du Plessis et al., 2015), but the SMI bivalve *Crassostrea gigas* requires OXPHOS to sustain the later "long motility" phase of spermatozoa (Boulais et al., 2015). Recent work in *M. edulis* and *R. philippinarum* supports this in DUI species as well: OXPHOS inhibitors strongly impeded sperm velocity, while glycolysis only appeared to play a role in sperm performance when in the presence of egg attractants (Bettinazzi et al., 2020). Because OXPHOS is necessary for sperm function in DUI species, M mt OXPHOS genes must remain functional. Given this, it is not surprising that M mt genes show no obvious signs of pseudogenization or frame shifts. Again, purifying selection likely dominates M mt evolution and mitonuclear interactions are likely still under strong selection to be maintained. Previous work has also shown that the most highly conserved sites across invertebrates in mt genes remain largely unaltered in M mt genes, while less conserved sites are altered at a higher rate in M vs. F mt genes (Stewart et al., 1996). This may also explain why nuclear-contact sites may be more likely to change in M mt genes: nuclear contact sites on often on the periphery of OXPHOS structures and may be less functionally important for ion transport.

Another, non-mutually exclusive possibility for relaxed selection on M mt OXPHOS genes is that lower effective population sizes in M mt genes could result in changes in selection on M mt genes (and for maintaining mitonuclear interactions; Stewart et al., 1996, Stewart et al., 1995), causing changes in OXPHOS efficiency. Comparing mtDNA copy numbers and the mt transmission "bottleneck" in sperm vs. eggs of DUI species may clarify mtDNA effective population sizes for these genomes. However, these metrics are generally not available outside of mammals.

4.3 | Unanswered questions in the origin of DUI

As in previous studies, our phylogenetic analyses suggest multiple, independent origins of separate M and F genomes in bivalves (Figure 1, Figures S1 and S2) (Gusman et al., 2016; Milani et al., 2013, 2014; Plazzi & Passamonti, 2019; Plazzi et al., 2016; Zouros, 2013). Although some have suggested that a single origin of DUI with multiple losses may be more

parsimonious based on the distribution of DUI species in bivalve phylogenomic analyses (Bettinazzi et al., 2020), our analyses using both M and F genes clearly refute this in its simplest form because sister relationships between M and F mt genomes of the same species are often recovered. If DUI was lost, we would expect sequences to still cluster based on sex, but M genomes would be missing in some lineages (e.g. as in the basal unionid clade Etherioidea; Guerra et al., 2017). Sequences do cluster based on sex rather than taxonomy in unionids, some *Mytilus* species, and *Scrobicularia/Limecola*, suggesting a single origin of independent M and F genomes in these lineages (Figure 1, Figures S1 and S2).

There is a subtle difference between the origin of DUI and the origin of independent M and F mt genomes. Our results do not refute the possibility that DUI evolved once (or a few times), but was then subsequently "reset" to SMI many times, followed by the establishment of new M and F mt genomes. Our most inclusive analyses of COX1 sequences suggest at least 15 independent origins or resets of DUI (Figure S2) spaced across both relatively quick time scales (e.g. within genera or families) and longer ones (e.g. in distant orders). The "masculinization" phenomenon, which has been observed in natural populations of mytilids (Ladoukakis et al., 2002), may explain such losses and could be a route to resetting DUI through recombination of M and F mt genomes (Zouros, 2020): divergent M genomes may be replaced through recombination with F genomes during speciation but then immediately begin to diverge again. The parasitic life cycle of unionids may make this scenario intractable, while it could be more feasible in mytilids. Clearly, more work is needed to test whether DUI has evolved many times independently or is reset frequently, possibly concordantly with speciation in some lineages.

Given the phylogenetic placement of M and F mt sequences, it is clear that the origins and maintenance of DUI systems are complex. Several hypotheses have been put forward to explain how DUI might arise in bivalves (reviewed in Ghiselli et al., 2019; Milani et al., 2016; Zouros, 2020). Many models assume there is positive selection on the mtDNA itself to become paternally transmitted, possibly through gaining novel genes via viral gene endogenization in the mt genome (Milani et al., 2013, 2014, 2016). Even though we found relaxed selection on M mt OXPHOS genes, our results do not necessarily contradict such models because we did not examine mt ORFans, smithRNAs, tNRAs, or rRNAs. Positive selection on ORFans or other M mt elements for paternal transmission may have also resulted in the fixation of non-optimal OXPHOS genes given that mtDNA is inherited as a single linked molecule (Hill & Robertson, 1966). Hypotheses in line with this reasoning suggest that M mtDNA may act as a selfish element in DUI species (Hurst & Hoekstra, 1994), as has been shown in other systems (Havird et al., 2019a). Although M mt genes could act selfishly, they have remained entirely functional in DUI species, somewhat contradicting this idea in its purest form.

Another set of models suggests high membrane potential in M mitochondria allow entry into primordial germ cells to establish paternal transmission (Bettinazzi et al., 2019; Milani, 2015; Milani & Ghiselli, 2015). This "metabolic remodelling" of M mitochondria would putatively involve mt-encoded OXPHOS genes and positive selection, because those mitochondria that had high membrane potential would be most likely to be passed on. Our results do not support this, as we found consistent signatures of relaxed selection

on M mt OXPHOS genes during every independent origin of M mt genomes. Similarly, we also found slight, but Significantly elevated d_N/d_S ratios in DUI species compared to SMI species (Figure S2). While this pattern has been reported previously (although using a slightly different approach where M and F genes of DUI species were compared to SMI genes), it was thought to reflect positive selection on DUI compared to SMI species (Plazzi & Passamonti, 2019). Our results contradict this. Therefore, if membrane potential or remodelling of OXPHOS in M mitochondria lead to their transmission, it is likely not due to positive selection on M mt OXPHOS genes, but may be a case of constructive neutral evolution (Stoltzfus, 1999) or the result of sex-specific mt ORFans and genome expansions. The latter hypothesis deserves attention as mt chimeric ORFans have been suggested to originate via ATP8 duplications in unionids (Guerra et al., 2017). This is very similar to mt chimeric ORFans that disrupt OXPHOS and cause male sterility in many plants (Chen & Liu, 2014; Havird et al., 2019a; Schnable & Wise, 1998) and a number of interesting parallels exist between DUI and cytoplasmic male sterility systems (Breton et al., 2010; Mitchell et al., 2016). While ATP8 annotations are lacking in many DUI mt genomes, the ATP8 sequences we were able to examine showed a different pattern in d_N/d_S ratios (Figure 4) and RELAX analyses (Figure 5), which should prompt further study.

Given the number of independent origins of M and F genomes, it is remarkable that the pattern of elevated d_N/d_S and relaxed selection in M mt OXPHOS genes is so consistent. While early reports from partial gene sequences in *Arcuatula (Musculista) senhousia* suggested this species may show the opposite pattern (Passamonti, 2007), here we used complete mt genomes to show it follows the typical pattern. Indeed, while performing our analyses, new M and F mt genomes became available for *Geukensia demissa*, *Scrobicularia plana*, and *Limecola balthica* (Capt et al., 2020). Although the timing of these releases precluded including them in all of our analyses, they also show signs of elevated d_N/d_S and relaxed selection in M mt genes (e.g. $d_N/d_S = 0.025$ vs. 0.037, $k = 0.75$ in *L. balthica* for 12 mt genes). Our results suggest that while the origins of DUI may be complex and numerous, the resulting patterns of selection on M vs. F mt OXPHOS genes are extraordinarily predictable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

Sequence data from DUI and SMI species analysed here are publicly available via Genbank (see Table S1 for accession numbers). Files from the analyses described here are available via FigShare (dataset # 13179983). Raw sequencing reads from *R. philippinarum* were

uploaded to NCBI's sequence read archive (SRA) (see Table S2). Transcriptome assemblies were uploaded to NCBI's transcriptome shotgun assembly (TSA) database (see Table S3).

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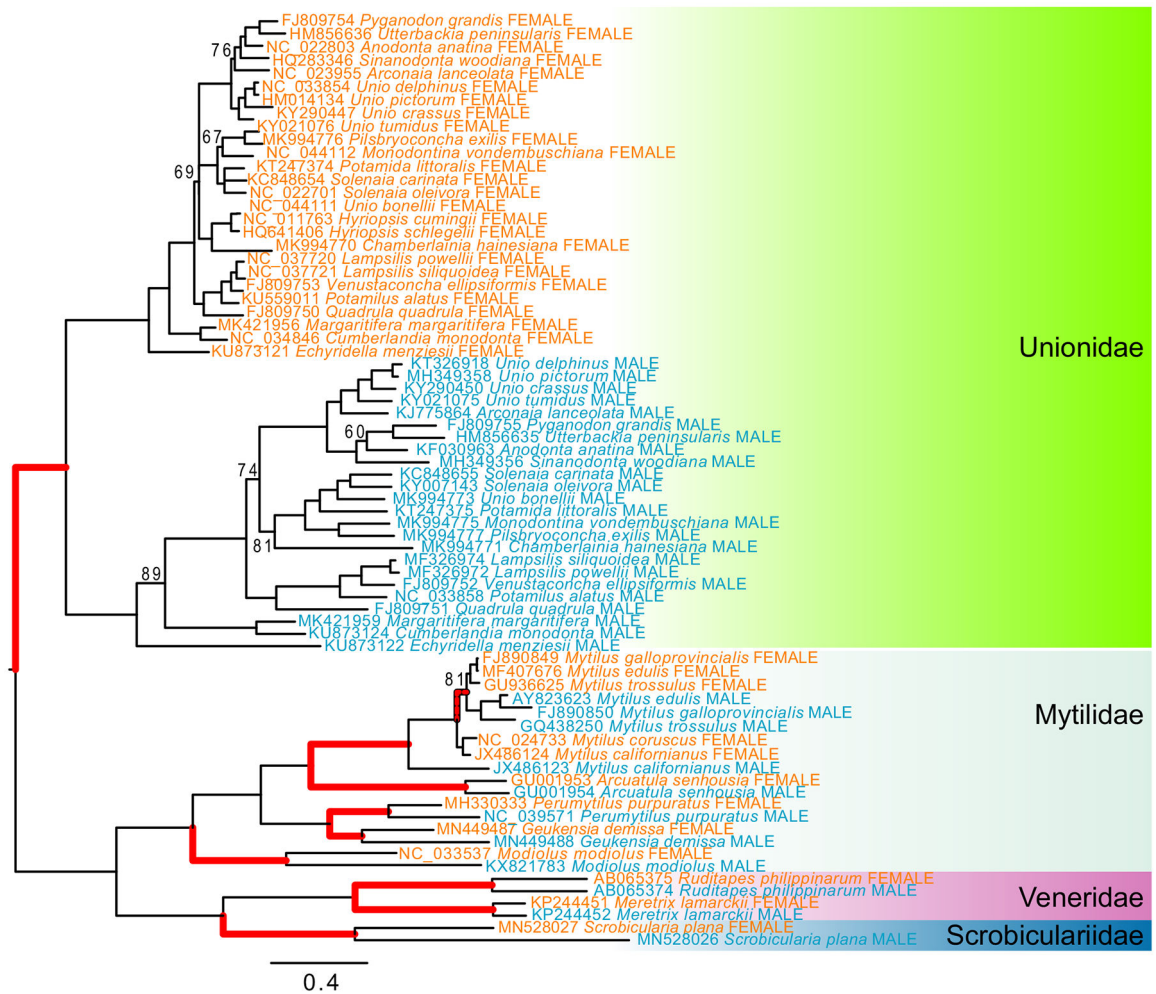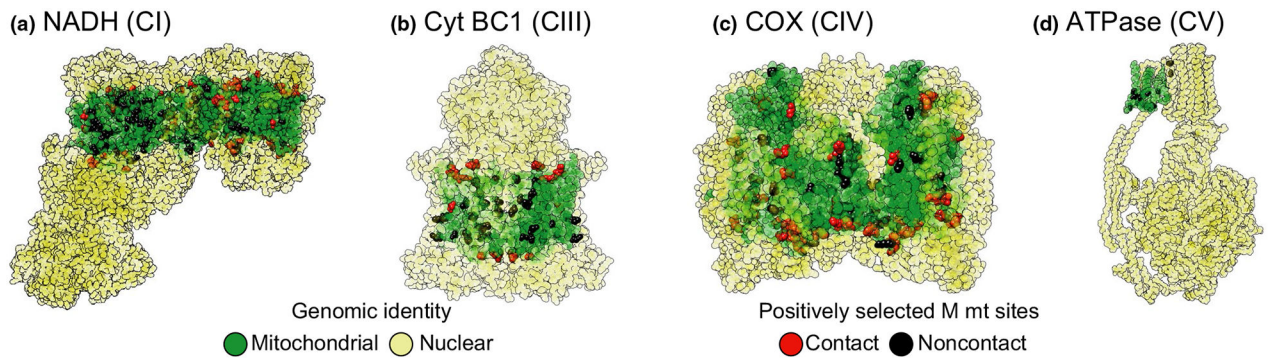
FIGURE 1.

Maximum likelihood phylogeny of male and female sequences from DUI bivalves (in blue and orange, respectively) based on amino acid sequences from 12 concatenated mt genes (all except ATP8). Red branches indicate independent origins of separate M and F genomes. Nodal support values are based on 100 rapid bootstraps calculated via RAxML and are not presented for nodes with support values of 95 or higher. One relationship with less than 50% support was collapsed into a polytomy. Scale bar indicates amino acid replacements per site
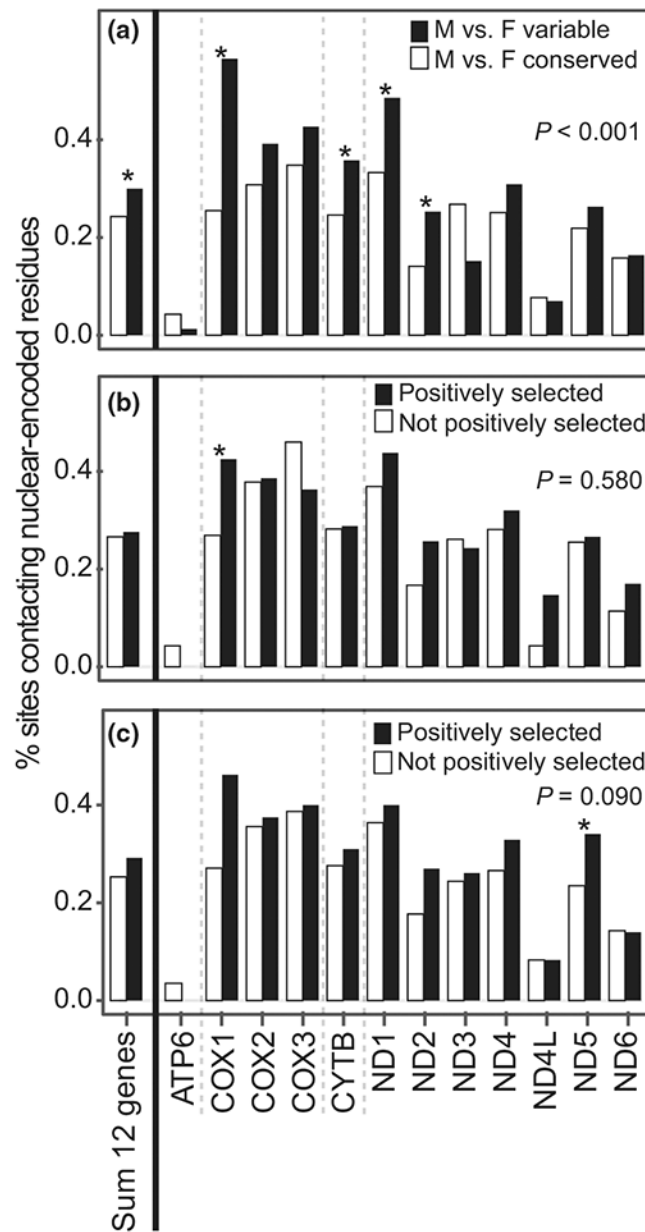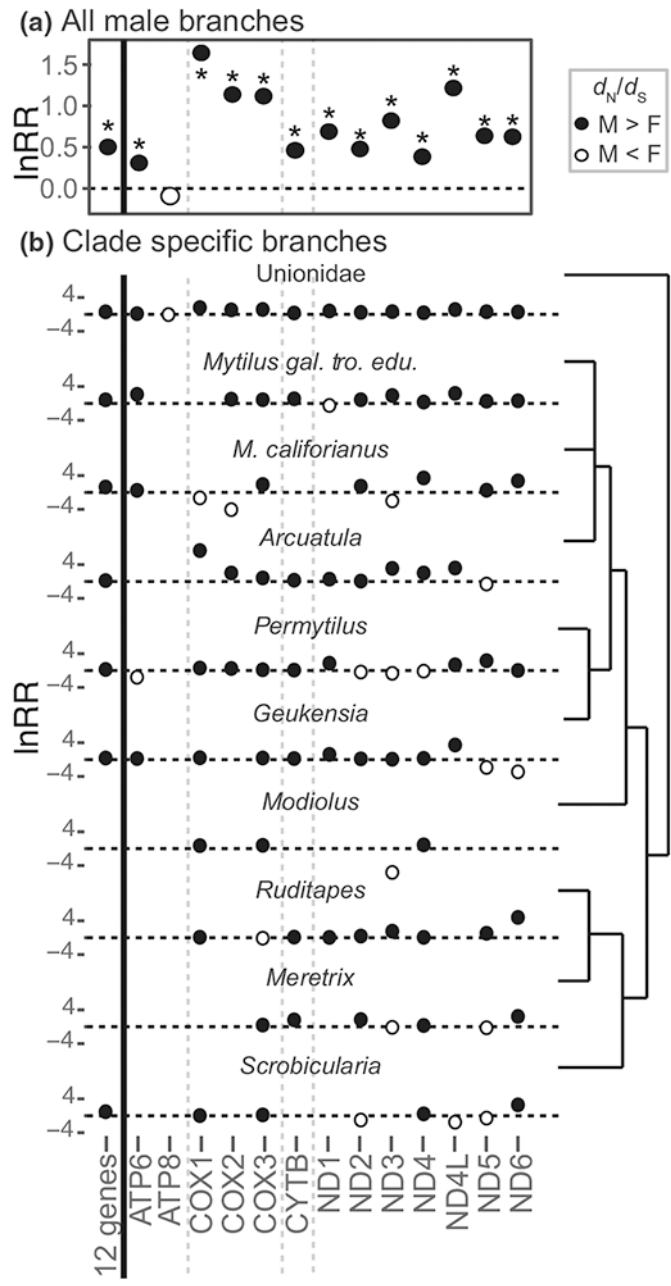
FIGURE 2.

Mt amino acid residues that are altered in M genes tend to contact nuclear-encoded residues in DUI bivalves. (a) Sites were classified as being either conserved or variable in M vs. F mt genes in *Ruditapes philippinarum*. (b) Sites were classified as being under positive selection or not in M mt genes in any analysis on any M phylogenetic branch (statistical significance was not considered). (c) Only sites classified as being under significant positive selection via Bayes Empirical Bayes analysis ($p < 0.05$) were considered (Yang et al., 2005). Asterisks indicate datasets where positively selected or variable sites were significantly over-represented for nuclear contacts (Fisher's exact test). *P* values are for the "12 genes" concatenated dataset of all mt genes except ATP8

(a) NADH (CI) **(b)** Cyt BC1 (CIII) **(c)** COX (CIV) **(d)** ATPase (CV)

Genomic identity

● Mitochondrial ○ Nuclear

Positively selected M mt sites

● Contact ● Noncontact

FIGURE 3.

Structural representations of OXPHOS complexes and sites under positive selection in M mt genes of DUI bivalves. Nuclear- and mt-encoded residues not under positive selection are shown in yellow and green, respectively. For mt sites identified as being under significant positive selection in M branches ($p < 0.05$ Bayes Empirical Bayes test), those that contact nuclear-encoded sites are shown in red and those that do not are shown in black (i.e. data summarized in Figure 4C). (a–d) show OXPHOS complexes I–V; CII is excluded because it is entirely nuclear-encoded in bivalves

FIGURE 4.

d_N/d_S ratios are elevated in M genomes of DUI bivalves. The y-axis is the natural log of the response ratio metric: $\ln\left(\frac{M\,dN\,/\,dS}{F\,dN\,/\,dS}\right)$, where lnRR > 0 (shown with a dashed line) indicates higher M vs. F d_N/d_S (shown as filled points). (a) All M branches were compared to all F branches based on the phylogeny in Figure 1; asterisks indicate a significantly better fit when using separate M and F d_N/d_S ratios compared to a single ratio (likelihood ratio test p < 0.05). (b) M branches were compared to F branches for independent origins of M and F genomes. "12 genes" represents a concatenated dataset of all mt genes except ATP8
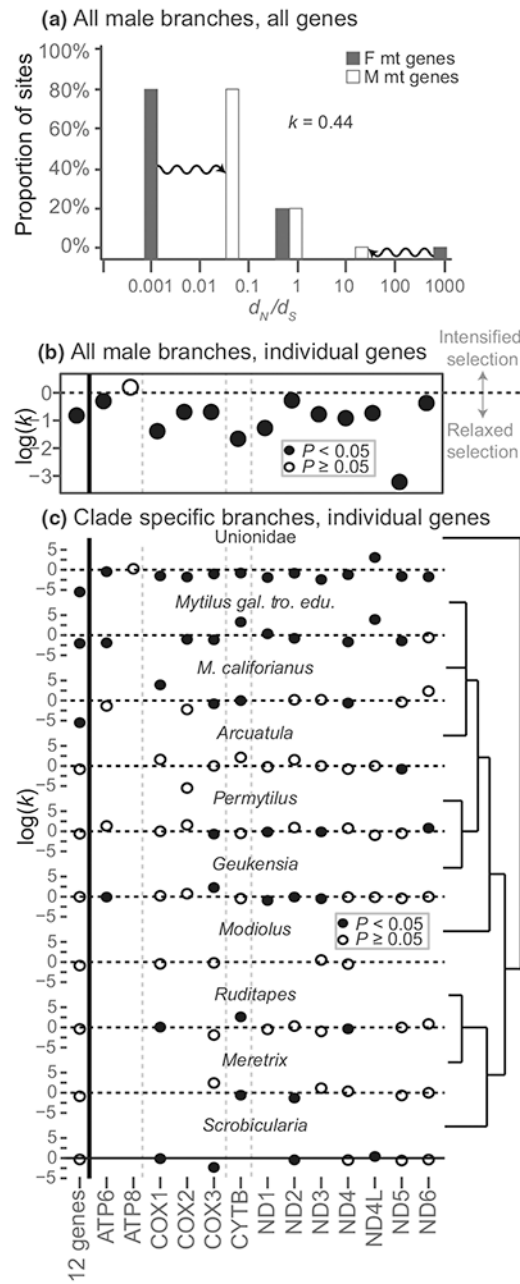
FIGURE 5.

Male mt genes are under relaxed selection compared to F genes in DUI bivalves. The k metric from the RELAX program (Wertheim et al., 2014) indicates relaxed selection on M branches when $k < 1$ or $\log(k) < 0$. Values that are significantly different from $k = 1$ are indicated as filled points. (a) Distribution of site classes for all M and F branches from the 12 gene concatenated set based on the phylogeny in Figure 1. (b) All M branches were compared to all F branches for each individual gene. (c) M branches were compared to F branches for each independent origin of M and F genomes. "12 genes" represents a concatenated dataset of all mt genes except ATP8