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The dynamics of mito-nuclear coevolution: A perspective from bivalve species with two different mechanisms of mitochondrial inheritance

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Abstract

The proteins involved in the main process of energy production of most eukaryotes (oxidative phosphorylation, OXPHOS) are encoded by either nuclear or mitochondrial genomes. The importance of mito-nuclear interactions suggests that the two genomes might be under coevolution. While most eukaryotes are characterized by a strictly maternal inheritance (SMI) of mitochondria, some bivalves show doubly uniparental inheritance (DUI), where two distinct mitochondrial lineages coexist in the same individual, and the nuclear OXPHOS genes have to cofunction with genes produced by two different mitochondrial genomes. We took advantage of the natural heteroplasmy of a DUI species to get insights into the dynamics of mito-nuclear coevolution. We used RNA-seq to investigate transcription and rate of protein evolution of OXPHOS genes in two related bivalves: *Ruditapes decussatus* (Bivalvia, Veneridae), a species with SMI, and *Ruditapes philippinarum* (Bivalvia, Veneridae), a species with DUI. Surprisingly, our results are not consistent with most of the proposed hyphotheses about the mechanisms of mito-nuclear coevolution. In particular, despite observing a rate of protein evolution of mitochondrial subunits an order of magnitude higher compared to that of most animal taxa investigated so far, we found no evidence for nuclear compensation. Moreover, we found no correlation between rate of protein evolution and transcription level in both nuclear and mitochondrial OXPHOS subunits. In this work, we report clear deviations from the expected results predicted by widely accepted hypotheses built around data obtained from a restricted representation of biodiversity. This should encourage further investigations based on broad comparative analyses encompassing usually overlooked non-model species.

KEYWORDS
dN/dS, oxidative phosphorylation, protein evolution, RNA-seq

1 | INTRODUCTION

Mitochondria are a primary component of the eukaryotic cell derived from a free-living α-proteobacterium (Martin, Garg, & Zimorski, 2015, for a thorough review). Since their endosymbiotic origin, a massive gene transfer from the ancestral mitochondrial genome to the nucleus occurred, and most of the genes involved in mitochondrial function and biogenesis are now encoded by the nucleus (Timmis, Ayliffe, Huang, & Martin, 2004). Nevertheless, mitochondria retained part of their original genome, and the proteins

involved in the main process of energy production of most eukaryotes—that is, oxidative phosphorylation (OXPHOS)—are encoded by either the nuclear or the mitochondrial genome (mtDNA), so that they have to function together. Accordingly, it was proposed that tight coevolution and co-regulation of these two genomes is essential to maintain efficient mitochondrial activity (see, for example, Allen, 2015; Bar-Yaacov, Blumberg, & Mishmar, 2012; Rand, Haney, & Fry, 2004).

Still, mitochondrial OXPHOS subunits seem to be subject to different evolutionary forces compared to nuclear subunits. For instance, the rate of amino acid sequence evolution of mitochondrial subunits is remarkably lower than that of nuclear subunits in most previously investigated taxa (see, for example, Nabholz, Ellegren, & Wolf, 2012; Popadin, Nikolaev, Junier, Baranova, & Antonarakis, 2013). Accordingly, there is evidence for strong purifying selection acting on mitochondrially encoded OXPHOS subunits (Piganeau & Eyre-Walker, 2009; Popadin et al., 2013), even if signs of positive selection were also reported (Castellana, Vicario, & Saccone, 2011; Gibson, Niehuis, Verrelli, & Gadau, 2010; James, Piganeau, & Eyre-Walker, 2016; Pavlova et al., 2017).

Three hypotheses were proposed to explain such differences: first, mitochondrial OXPHOS subunits could be subject to tighter functional constraints, since they constitute the core of OXPHOS complexes, while nuclear subunits—that are instead more peripheral—could be under more relaxed selection (Popadin et al., 2013; Zhang & Broughton, 2013). Second, because mitochondrial genomes in animals tend to accumulate mutations from nine to 25 times faster than the nuclear genome (Lynch, Koskella, & Schaack, 2006), positive selection would act on nuclear OXPHOS subunits to compensate the insurgence of mutations in mitochondrial genes, ensuring a proper structural/functional match among the subunits of OXPHOS complexes. This theory, called the "nuclear compensation hypothesis", was adopted by several authors (Aanen, Spelbrink, & Beekman, 2014; Burton & Barreto, 2012; Burton, Ellison, & Harrison, 2006; Dowling, Friberg, & Lindell, 2008; Havird & Sloan, 2016; Osada & Akashi, 2012). Third, Nabholz et al. (2012) proposed that transcription level could be the main factor responsible in affecting the rate of protein evolution in OXPHOS genes, supporting the assumption that a negative correlation—called "E-R correlation"—exists between transcript abundance and rate of protein evolution (Zhang & Yang, 2015).

How important is coevolution between mitochondrial and nuclear genomes? So far, many works attempted to answer this question by producing cytoplasmic hybrids to break up coadapted mitochondrial and nuclear genomes and measure the resulting changes in function/fitness. Some of these experiments included hybrids carrying mitochondrial and nuclear genome of different species (McKenzie, Chiotis, Pinkert, & Trounce, 2003; Niehuis, Judson, & Gadau, 2008; Sackton, Haney, & Rand, 2003), showing good evidence for detrimental effects, such as reduction of OXPHOS activity, oxidative damage, and disruption of mitochondrial functions (see, for example, Kazuno et al., 2006; Moreno-Loshuertos et al., 2006). Similar results were obtained when interpopulation hybrids carried mitochondrial variants different from those coadapted with their respective nuclear background (Barreto & Burton, 2013; Ellison & Burton, 2008; Sharpley et al., 2012).

The emergence of genomic conflicts and/or mito-nuclear incompatibilities can be fostered also by different mitochondrial haplotypes coexisting in the same individual (Sharpley et al., 2012). The non-Mendelian mechanism of mitochondrial inheritance typical of Metazoa avoids—or at least greatly reduces—the presence of several mtDNA haplotypes within organism (Birky, 1995; Lane, 2012). Indeed, animals are almost invariably characterized by strictly maternal inheritance (SMI) of mitochondria, namely only females transmit mitochondria to the offspring, while paternal mitochondrial contribution is prevented in different ways across eukaryotes (Birky, 1995; Sato & Sato, 2013). The only known evolutionarily stable exception to SMI in Metazoa is doubly uniparental inheritance (reviewed in: Breton, Beaupré, Stewart, Hoeh, & Blier, 2007; Passamonti & Ghiselli, 2009; Zouros, 2013; Milani, Ghiselli, & Passamonti, 2016), a peculiar mechanism of mitochondrial heredity observed, so far, in ~100 species of bivalve mollusks (Gusman, Lecomte, Stewart, Passamonti, & Breton, 2016). In DUI species, two mitochondrial lineages are present: the F-type, inherited through eggs, and the M-type, inherited through sperm; as a result, female somatic tissues are generally homoplasmic for the F-type, male somatic tissues are heteroplasmic, and gametes carry only the sex-specific mitochondrial lineage. Therefore, in contrast to all other Metazoa, in DUI bivalves, the same nuclear background has to coevolve with two distinct mtDNA lineages which present a high nucleotide divergence (up to 40%; Zouros, 2013) and different replication and transcription dynamics (Ghiselli, Milani, & Passamonti, 2011; Obata, Sano, & Komaru, 2011; Milani & Ghiselli, 2015; Ghiselli et al., 2013; Milani, Ghiselli, Iannello, & Passamonti, 2014; Milani & Ghiselli, 2015; Guerra, Ghiselli, Milani, Breton, & Passamonti, 2016). Therefore, DUI species offer a unique opportunity to investigate the coevolution between nuclear and mitochondrial genomes and the effects of heteroplasmy.

Is the high variability of mtDNA in DUI species matched by a high rate of nucleotide substitution in nuclear subunits? Is transcription level associated with the rate of protein evolution in OXPHOS genes? To address such questions, we investigated the rate of protein evolution and the transcription level of nuclear and mtDNA-encoded OXPHOS subunits in two related bivalve species: *Ruditapes decussatus*, characterized by SMI of mitochondria (Ghiselli et al., 2017), and *Ruditapes philippinarum*, with DUI of mitochondria. We tested the above-mentioned hypotheses about mito-nuclear coevolution, focusing on nuclear compensation and E-R correlation.

2 | MATERIALS AND METHODS

2.1 | Dataset

Transcriptome data of mature gonads from 12 individuals (six females and six males) of *R. decussatus* and 12 individuals (six females and six males) of *R. philippinarum* were retrieved from Ghiselli et al. (2018). Raw reads and transcriptome assembly from both experiments are available on NCBI, under BioProjects PRJNA68513 (*R. philippinarum*)

and PRJNA170478 (*R. decussatus*); further data are available on figshare: https://doi.org/10.6084/m9.figshare.5398618.v1.

The clams were collected from the Northern Adriatic Sea, in the river Po delta region (Sacca di Goro, approximate GPS coordinates: 44°50′06″N, 12°17′55″E). Same tissue and developmental stage (ripe gonads) were used for both the species. In more detail, samples were collected during the spawning season (end of July). The consistency of gonad maturation stage across the individuals was confirmed by microscope inspection of gonadal liquid—necessary also for sexing. Samples were frozen in liquid nitrogen and stored at −80°C. For both the species, library preparation and sequencing were performed following the same procedure. Briefly, total RNA was extracted with TRIzol, poly-A transcripts were selected using magnetic beads and used as template for cDNA synthesis, following the protocol as in Mortazavi, Williams, McCue, Schaeffer, and Wold (2008) and the modifications in Ghiselli et al. (2012). Samples were sequenced with two technical replicates, using an Illumina Genome Analyzer IIx machine. Filtered reads were assembled with Trinity-v2.4.0. For technical details about transcriptome de novo assembly refer to Ghiselli et al. (2018).

2.2 | OXPHOS subunit detection

Open reading frames (ORF) of nuclear-encoded OXPHOS genes were retrieved using findorf (Krasileva et al., 2013), that uses a BLASTX (Altschul et al., 1997) search against a user-defined database and a HMMER (Mistry, Finn, Eddy, Bateman, & Punta, 2013) search against Pfam database 30.0 (Finn et al., 2016). Concerning the user-defined database, we chose to include OXPHOS genes—obtained from the KEGG database (Kanehisa & Goto, 2000)—from seven model species (Supporting Information Table S1). Genes that were found in only one of the two species were added to the database, and the improved database used in one additional round of search.

Due to the polycistronic transcription of mtDNA, a fully automated annotation was not possible for mitochondrial-encoded OXPHOS genes, since the largest ORF retrieved from each transcript can compromise the identification of other mitochondrial genes in the same transcript. For this reason, mitochondrial transcripts were identified with a local BLASTX against all molluskan mitochondrial protein coding genes (PGCs) downloaded from NCBI, and filtered for contaminants following a RNA-seq annotation pipeline for non-model organisms developed in-house (manuscript in preparation). Briefly, contaminants were identified blasting our transcripts against the nr database with BLASTX (default parameters). Then, since most of the expected contaminants of bivalves are represented by fungi, plantae, bacteria and some marine invertebrates (e.g., Platyhelminthes and Nematoda), we used BLAST "taxid" to select only transcripts belonging to "Molluska." Open reading frames were extracted using ORFfinder (www.ncbi.nlm.nih.gov/orffinder) and validated with BLASTP against NCBI nr database.

OrthoVenn (Wang, Coleman-Derr, Chen, & Gu, 2015) with default parameters was used to validate the orthology of OXPHOS subunits between the two species.

2.3 | Transcription of OXPHOS genes

In order to obtain the transcription level of OXPHOS subunits, we used Bowtie 2 (Langmead & Salzberg, 2012) to map reads of each sample to nuclear and mitochondrial OXPHOS subunits in both the species. Since males and females of *R. philippinarum* are characterized by two different mitochondrial genomes, we retrieved mitochondrial reads by mapping female reads against the F-type mitochondrial OXPHOS subunits and male reads against M-type mitochondrial subunits. SAMtoolts (Li et al., 2009) were used to keep reads mapping exactly one time, with mapping quality ≥10 and concordant pairs match. We used the NOISeq R package (Tarazona et al., 2015), to obtain transcription level from raw counts, by using the trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack, 2010), and fragments per kilobase of transcript per million mapped reads (FPKM; Mortazavi et al., 2008).

Differences in the transcription level between nuclear and mitochondrial subunits were assessed by plotting the distribution of $\log_2$(TMM) separately for males and females in both the species, and the Wilcoxon rank-sum test was performed for statistical support. A hierarchical clustering analysis (see below for details) was applied to generate transcription-level (TMM) heatmaps of both nuclear and mitochondrial subunits in each sample. Spearman's rank correlation coefficient was calculated across transcription levels of males and females for each species and across males and females between species. In order to detect sex- and/or species-specific co-transcriptional patterns of OXPHOS subunits, we calculated correlation matrices separately for the following conditions: *R. decussatus* males and *R. decussatus* females (which are characterized by the same mitochondrial genome), *R. philippinarum* males and *R. philippinarum* females—characterized by different mitochondrial genomes. Specifically, for each pair of OXPHOS subunits, we calculated the Spearman correlation coefficient to estimate the transcriptional correlation among all samples belonging to a given condition.

All the heatmaps were produced using the heatmap.2 function of the gplots v3.0.1 R package (https://CRAN.R-project.org/package=gplots). The hierarchical clustering was computed using Ward's method, and the distance matrix was calculated using euclidean distances. Positive correlation between subunit transcription levels is reported in red, negative correlation in blue, and no correlation in white.

2.4 | Rates of protein evolution of OXPHOS subunits

For each OXPHOS subunit in *R. decussatus* and *R. philippinarum*, we used TranslatorX (Abascal, Zardoya, & Telford, 2010) to translate nucleotide sequences, compute protein alignment with MUSCLE (Edgar, 2004) between orthologs in the two species, and to back-translate amino acid alignments into nucleotide alignments. KaKs_Calculator 2.0 (Wang, Zhang, Zhang, Zhu, & Yu, 2010) with default settings was used to obtain the ratio of non-synonymous to synonymous nucleotide substitution (dN/dS) between *R. decussatus* and
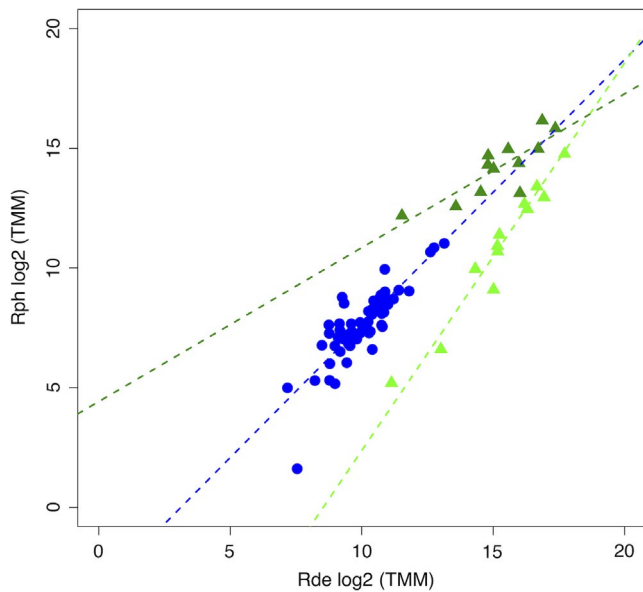
FIGURE 1 Correlation among OXPHOS subunits transcription levels, measured as $\log_2$(TMM), *in Ruditapes decussatus* (Rde) and *Ruditapes philippinarum* (Rph). Blue dots = transcription level of nuclear subunits; light green triangles = transcription level considering the F-type mitochondrial DNA of *R. philippinarum*; dark green triangles = transcription level considering the M-type mitochondrial DNA of *R. philippinarum*. Dashed lines represent linear regression between nuclear subunits (blue line) and between mitochondrial subunits, considering separately F-type (light green line) and M-type (dark green line)

R. philippinarum. Since males and females of *R. philippinarum* have different mitochondrial genomes, we reported two distinct dN/dS for mitochondrial subunits: one referred to female mitochondrial subunits (dN/dS between *R. decussatus* and *R. philippinarum* F-type) and one referred to male mitochondrial subunits (dN/dS between *R. decussatus* and *R. philippinarum* M-type). We plotted the distribution of dN/dS of nuclear and mitochondrial complexes, and the Wilcoxon test was performed to evaluate significant differences. To investigate the presence of E-R correlation between dN/dS and transcription levels, we plotted $\log_2$(TMM) of nuclear subunits and their dN/dS for both *R. decussatus* and *R. philippinarum*. Concerning mitochondrial subunits, we plotted $\log_2$(TMM) of *R. philippinarum* males against dN/dS between *R. decussatus* and *R. philippinarum* M-type; likewise, we plotted $\log_2$(TMM) of *R. philippinarum* females against dN/dS between *R. decussatus* and *R. philippinarum* F-type. Finally, we chose to plot $\log_2$(TMM) of *R. decussatus* mitochondrial subunits against dN/dS between *R. decussatus* and *R. philippinarum* F-type, since F-type is considered the ancestral mitochondrial genome (Zouros, 2013). The Spearman rank correlation coefficient was calculated for each comparison. Since previous analyses used FPKM instead of TMM to normalize transcription level, we also reported the relationship between dN/dS and FPKM to investigate the E-R correlation; in any case, we prefer to use TMM because it is considered more robust for between-sample comparisons (Conesa et al., 2016).

2.5 | Rates of protein evolution of nuclear genes involved in mitochondrial processes

Both transcriptomes of *R. decussatus* and *R. philippinarum* were previously annotated in Ghiselli et al. (2018). In order to identify genes involved in mitochondrial processes, we used AmiGO2 (Balsa-Canto, Henriques, Gábor, & Banga, 2016) to select loci annotated with the GO term "Mitochondrion" and every associated child term in both transcriptomes. When genes were recognized as orthologs between the two species, we obtained dN/dS following the same pipeline we used for OXPHOS subunits. When orthologous genes with mitochondrial GO annotation had a dN/dS higher than 0.2, we used BLASTX for identification.

3 | RESULTS

3.1 | Differential transcription of OXPHOS genes

Following the annotation steps described in Materials and Methods, we retrieved 59 OXPHOS nuclear-encoded subunits in both *R. decussatus* and *R. philippinarum* (FASTA sequences provided as Supporting Information Data S1). All mtDNA-encoded subunits were retrieved except *atp8*, likely because of known annotation/alignment issues of this gene in bivalves (Breton, Stewart, & Hoeh, 2010).

The transcription level of OXPHOS subunits in the two species is reported in Supporting Information Tables S2 and S3. The transcription level between the two species is highly correlated (Figure 1) for both nuclear-encoded subunits (in blue; Spearman's rank correlation = 0.80, *p*-value < 2.2E-16) and mtDNA-encoded subunits (in light green between *R. decussatus* and *R. philippinarum* F-type: Spearman's rank correlation = 0.95 and *p*-value < 2.2E-16; in dark green between *R. decussatus* and *R. philippinarum* M-type: Spearman's rank correlation = 0.79, *p*-value = 0.003).

Considering sexes separately, we found the highest correlation between males and females of *R. philippinarum* (Supporting Information Figure S1: Spearman's rank correlation = 0.96 for both nuclear and mitochondrial subunits, blue dots and triangles, respectively), and in mitochondrial subunits between females of the two species (Spearman's rank correlation = 0.97, red triangles); all other comparisons show a slightly lower correlation (Supporting Information Table S4 and Figure S1).

Figure 2 shows the transcription levels of nuclear- and mtDNA-encoded orthologous genes involved in OXPHOS, subdivided by cell compartment (nuclear, mitochondrial), species, and sex. In both *R. decussatus* and *R. philippinarum*, the transcription of mitochondrial subunits is markedly higher than that of nuclear subunits (Figure 2, Supporting Information Table S5). In *R. decussatus*, the median transcription level of mitochondrial subunits is 43-fold higher than that of nuclear subunits in males (40,872 TMM as opposed to 949 TMM, respectively; Wilcoxon test *p*-value = 8.942E-08) and 34-fold higher in females (37,745 TMM as opposed to 1,101 TMM, respectively; Wilcoxon test *p*-value = 1.253E-07). In *R. philippinarum*, the median transcription level of M-type mitochondrial subunits in males
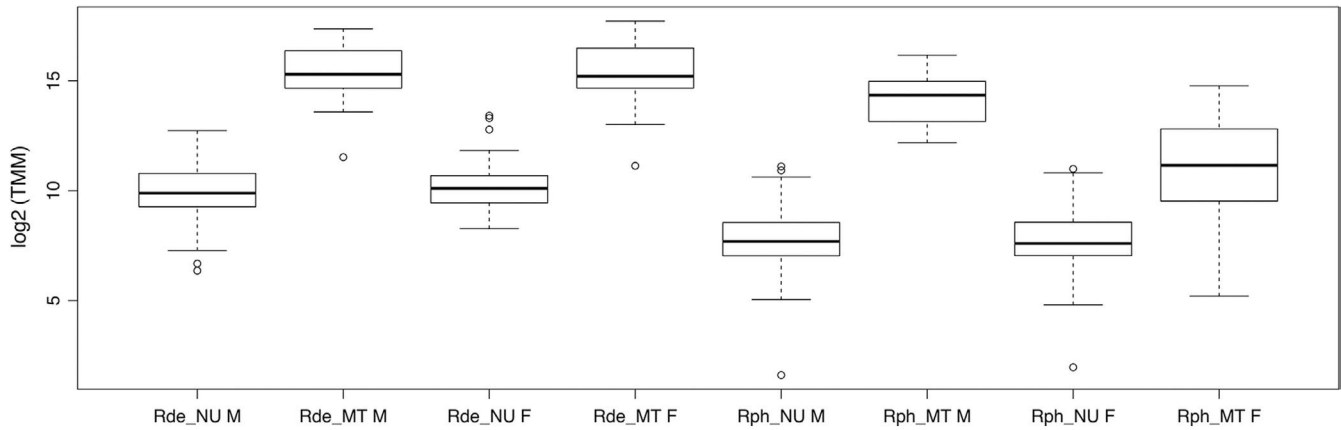
FIGURE 2 Transcritpion level, measured as $\log_2$(TMM), of nuclear (NU) and mitochondrial (MT) OXPHOS subunits in males (M) and females (F) of *Ruditapes decussatus* (Rde) and *Ruditapes philippinarum* (Rph)
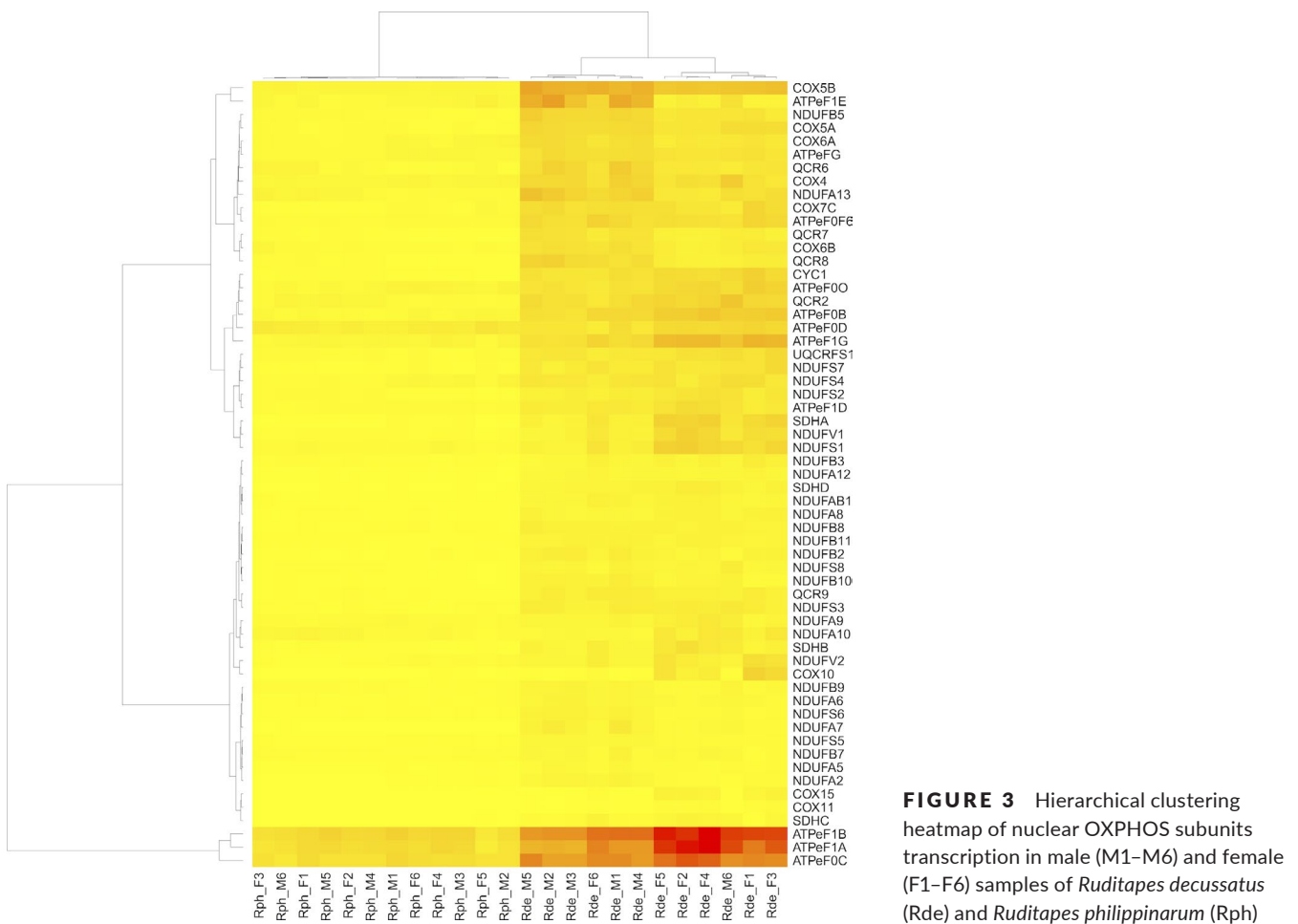

FIGURE 3 Hierarchical clustering heatmap of nuclear OXPHOS subunits transcription in male (M1–M6) and female (F1–F6) samples of *Ruditapes decussatus* (Rde) and *Ruditapes philippinarum* (Rph)

is 100-fold higher than that of nuclear subunits (20,767 TMM vs. 206 TMM, respectively; Wilcoxon test *p*-value = 5.837E-08), while in females, F-type mitochondrial subunits are 12 times more transcribed than nuclear subunits (2,310 TMM vs. 193 TMM, respectively; Wilcoxon test *p*-value = 0.0002). In nuclear complexes, the transcription level seems to be more similar between males and females within species—particularly for Complex II, IV, and ATPase (Supporting Information Figure S2A and Table S5). On the contrary,

the transcription level of mitochondrial complexes is similar among males of *R. philippinarum* and males and females of *R. decussatus*, while females of *R. philippinarum* are characterized by lower and more variable values (Supporting Information Figure S2B and Table S5).

We performed a hierarchical clustering of transcription level in nuclear subunits of males and females of *R. decussatus* and *R. philippinarum* (Figure 3). While many of these subunits seem to have similar
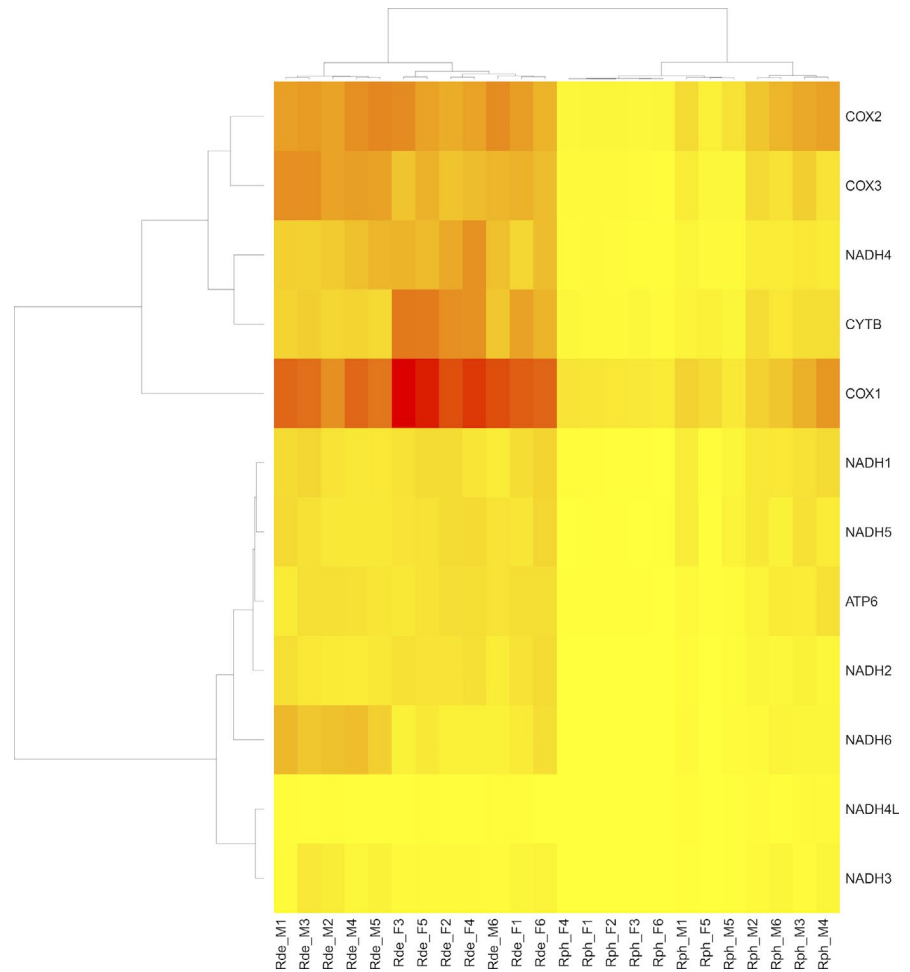
FIGURE 4 Hierarchical clustering heatmap of mitochondrial OXPHOS subunits transcription in male (M1–M6) and female (F1–F6) samples of *Ruditapes decussatus* (Rde) and *Ruditapes philippinarum* (Rph)

levels of transcription in both the species, some subunits of ATPase are more transcribed in both the species. The cluster analysis was able to separate species based on their transcription level, but males and females are mixed in the same cluster. Also, the heatmap did not cluster together subunits belonging to the same complex.

The same analysis was performed on mitochondrial subunits (Figure 4): also, in this case, species are clustered apart, but differently from nuclear subunits, males and females within species cluster separately as well. Finally, subunits were not clustered together based on complex. For instance, *nad4* clusters together with *cytb* and with subunits of Complex IV (*cox1, cox2, cox3*), due to its higher transcription.

When we considered all OXPHOS subunits in the cluster analysis, we found that mitochondrial subunits tend to cluster separately from almost all nuclear subunits, because of their higher transcription level (Supporting Information Figure S3). Among samples, the two species group separately and males and females belong to different clusters in most cases.

We performed correlation heatmaps of OXPHOS subunits transcription (Supporting Information Figures S4-S11), separately for males and females of both species. We report absence of correlation among subunits belonging to the same complex. Some signal of positive correlation was observed among mitochondrial subunits

of males and females of *R. philippinarum* (Supporting Information Figures S9 and S11) and among subunits belonging to ATPase in males of *R. philippinarum* (Supporting Information Figure S11).

3.2 | Evolutionary rates of OXPHOS genes

The ratio of non-synonymous to synonymous nucleotide substitution (dN/dS) was calculated between nuclear subunits of *R. decussatus* and *R. philippinarum*, between mitochondrial subunits of *R. decussatus* and *R. philippinarum* F-type, and between mitochondrial subunits of *R. decussatus* and *R. philippinarum* M-type (Supporting Information Table S6). As shown in Figure 5, dN/dS of nuclear and mitochondrial subunits is comparable (median nuclear = 0.12; median *R. decussatus* vs. *R. philippinarum* F-type = 0.11; median *R. decussatus* vs. *R. philippinarum* M-type = 0.15), and the differences in their distribution are not statistically significant. Among nuclear subunits (Figure 6, blue boxplots), dN/dS is variable, with highest values in Complex III (median = 0.44) and lowest values in Complex II (median = 0.04) (Supporting Information Table S7). Among mitochondrial subunits (Figure 6, green boxplots), Complexes IV and ATPase have the highest dN/dS in the *R. decussatus* vs. *R. philippinarum* M-type comparison (dN/dS ≥ 0.16), and *R. decussatus* vs. *R. philippinarum*
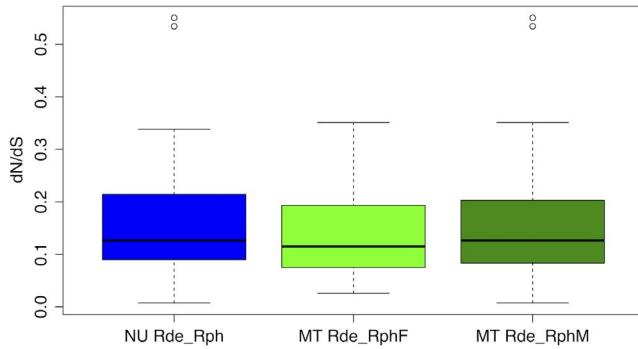
FIGURE 5 Rate of protein evolution between nuclear OXPHOS subunits (NU) of *Ruditapes decussatus* and *Ruditapes philippinarum* (Rde_Rph; blue boxplot) and between mitochondrial OXPHOS subunits (MT) of *R. decussatus* and *R. philippinarum* F-type (Rde_RphF; light green boxplot) and *R. decussatus* and *R. philippinarum* M-type (Rde_RphM; dark green boxplot)

F-type comparison (dN/dS ≥ 0.19), while Complex III is characterized by the lowest value in both F-type and M-type comparisons (dN/dS = 0.089 when F-type is considered, dN/dS = 0.094 for M-type), as well as Complex I in the F-type comparison (dN/dS = 0.082) (Supporting Information Table S7).

3.3 | Correlation between transcription level and evolutionary rate in OXPHOS genes

Figure 7 shows the relationship between log$_2$(TMM) and dN/dS in nuclear and mitochondrial subunits. We did not find correlation between transcription level and dN/dS of nuclear subunits neither in *R. decussatus* (Spearman's correlation = 0.06, *p*-value = 0.6; Figure 7 on the top, blue dots) nor in *R. philippinarum* (Spearman's correlation = 0.17, *p*-value = 0.19; Figure 7 on the bottom, blue circles). In the same way, we did not find correlation between transcription level and rate of protein evolution in mitochondrial subunits, neither in *R. decussatus* (Spearman's correlation = −0.03, *p*-value = 0.92; Figure 7 on the top, light green triangles) nor in *R. philippinarum* M-type (Spearman's correlation = −0.32, *p*-value = 0.3; Figure 7 on the bottom, dark

green triangles) nor in *R. philippinarum* F-type (Spearman's correlation = −0. 29, *p*-value = 0.35; Figure 7 on the bottom, light green triangles). The same lack of negative correlation was obtained when we considered FPKM instead of TMM in the relationship with dN/dS, as reported in other studies (Supporting Information Figure S12, FPKM values reported in Supporting Information Tables S8 and S9).

3.4 | Annotation of genes involved in mitochondrial biology

We found 1,224 orthologous genes in *R. decussatus* and *R. philippinarum* annotated either with the GO term "Mitochondrion" or any of its child terms. Among such genes, we retrieved 12 loci with a rate of protein evolution >0.2 and annotated them with BLASTX (Supporting Information Table S10). We found that these genes are mainly involved in mitochondrial translation, regulation of transcription, and respiratory chain assembly.

4 | DISCUSSION

4.1 | Rate of protein evolution of OXPHOS subunits

The rate of protein evolution (dN/dS) of mtDNA-encoded OXPHOS subunits has been previously investigated in some metazoan taxa (see, for example, Nabholz et al., 2012; Piganeau & Eyre-Walker, 2009; Popadin et al., 2013), commonly revealing values very close to zero. To explain such strong purifying selection, it was hypothesized that mitochondrial subunits are subject to strict functional constraints because they represent the core of OXPHOS complexes, and the accumulation of non-synonymous substitution may compromise their function and/or the proper protein-protein interaction. Yet, evidence of positive selection on mitochondrial OXPHOS subunits was reported as well, for example, James et al. (2016)—who analyzed over 500 animal species from a wide range of taxonomic groups—estimating that 26% of non-synonymous substitutions are fixed by adaptive evolution. Other studies suggested that species with high energy needs are characterized by a lower dN/dS of
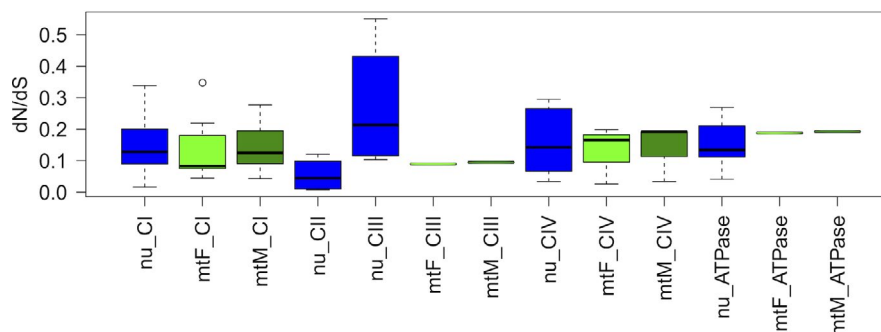

FIGURE 6 Rate of protein evolution of nuclear (NU; blue boxplots) and mitochondrial (MT) subunits separately for each OXPHOS complex. Light green boxplots = rate of protein evolution between mitochondrial subunits of *Ruditapes decussatus* and *Ruditapes philippinarum* F-type; dark green boxplots = rate of protein evolution between mitochondrial subunits of *R. decussatus* and *R. philippinarum* M-type

FIGURE 7 Relationship between transcription level [log$_2$(TMM)] and rate of protein evolution (dN/dS) of OXPHOS subunits in *Ruditapes decussatus* (top) and *Ruditapes philippinarum* (bottom). Blue dots = nuclear subunits; light green triangles = dN/dS between mitochondrial subunits of *R. decussatus* and *R. philippinarum* F-type; dark green triangles= dN/dS between mitochondrial subunits of *R. decussatus* and *R. philippinarum* M-type. Dashed lines represent linear regression between nuclear subunits (blue line) and between mitochondrial subunits (light green line considering F-type; dark green line considering M-type)
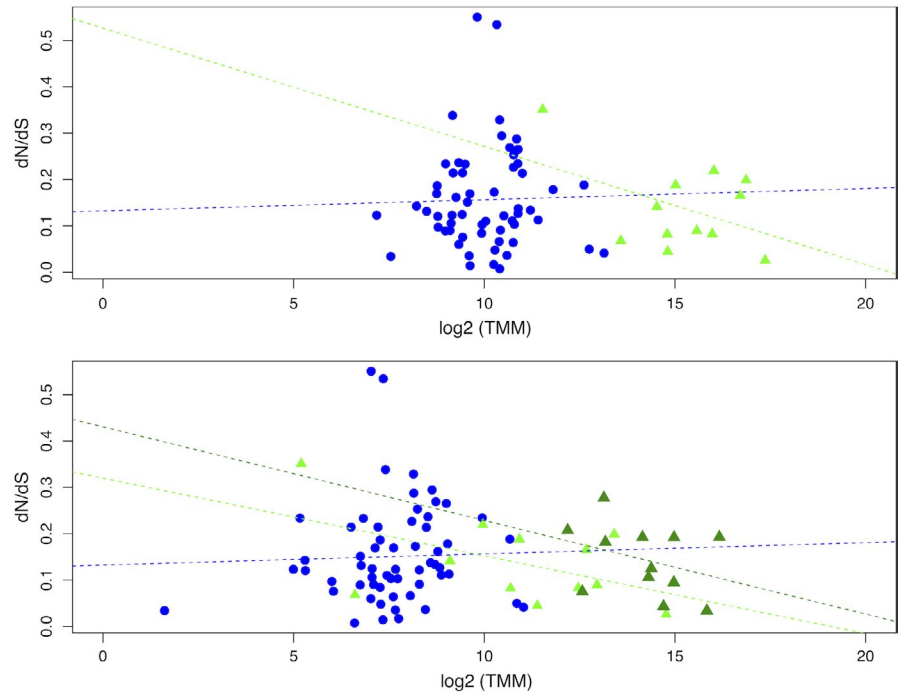
mitochondrial OXPHOS subunits, compared to species with lower energy needs; accordingly, mitochondrial OXPHOS subunits of species with a more sedentary life showed a higher dN/dS (Chong & Mueller, 2013; Mitterboeck & Adamowicz, 2013; Shen, Shi, Sun, & Zhang, 2009; Strohm, Gwiazdowski, & Hanner, 2015). Considering the lower energy needs, the cited authors hypothesized a relaxed purifying selection acting on mitochondrial OXPHOS subunits, rather than adaptive evolution.

Here, we show that dN/dS of mitochondrial OXPHOS subunits between *R. decussatus* and *R. philippinarum* is an order of magnitude higher compared to that of most animal taxa investigated so far (dN/dS ~ 0.13 vs. 0.02; Nabholz et al., 2012; Popadin et al., 2013), both considering *R. philippinarum* F-type and *R. philippinarum* M-type in the comparison with *R. decussatus* (Figure 5). Apparently, the selective constraints on mitochondrial subunits of these species are weaker, and non-synonymous substitutions accumulate easier compared to most taxa investigated so far. As mentioned before, higher dN/dS could reflect either relaxed or positive selection. On the one hand, in this work, we considered two bivalve species leading a very sedentary life, spending most of the time buried in the sand; therefore, the higher dN/dS might be due to a relaxation of functional constraints. Still, the sedentary life of these animals is restricted to the adult stage, and the highly motile larval stages (i.e., presettlement and metamorphosis) might be characterized by a more intense metabolic activity, so this hypothesis needs further investigation. On the other hand, the two species analyzed here are characterized by different mechanisms of mitochondrial inheritance, with *R. philippinarum* having two mtDNA lineages showing high sequence divergence (average amino acid p-distance of PCGs ~34%). Investigations on mtDNA of DUI species showed a faster PCG sequence evolution compared

with mtDNAs of species with SMI (Zouros, 2013). However, it is not clear whether such faster evolution is the result of a relaxation of constraints on mitochondrially encoded OXPHOS proteins in bivalves—hypothesis that might explain why DUI is possible in this taxon (Milani et al., 2016)—or whether it is driven by positive selection due to, for example, adaptation of each lineage with specific functions in the respective sex (F-type with female functions, M-type with male functions), and/or gamete competition (Ghiselli et al., 2013; Milani & Ghiselli, 2015; Skibinski, Ghiselli, Diz, Milani, & Mullins, 2017). Interestingly, as discussed more in detail in the following section, such faster evolution seems to be limited to mitochondrially encoded OXPHOS subunits.

In any case, the present work cannot provide evidence in support of either relaxed or positive selection; further analyses including more species—both DUI and SMI—and more refined methods—like detection of positive selection at single codon sites or on single branches of a phylogenetic tree—will help to clarify this point.

4.2 | Mito-nuclear coevolution

Few works have studied the rate of protein evolution of both nuclear- and mtDNA-encoded OXPHOS subunits and the coevolution between these two genomes (Aanen et al., 2014; Burton & Barreto, 2012; Burton et al., 2006; Dowling et al., 2008; Havird & Sloan, 2016; Nabholz et al., 2012; Osada & Akashi, 2012; Popadin et al., 2013). In particular, most of these studies focused on model organisms, and some investigated the effects of mito-nuclear mismatches by producing cytoplasmic hybrids. One of the main hypotheses about the dynamics of mito-nuclear coevolution is the "nuclear compensation hypothesis," which posits that positive selection for compensatory amino acid substitutions counteracts the rapidly accumulating

mtDNA mutations, allowing the proper functioning of OXPHOS complexes. We assessed the nuclear response to the high dN/dS of mitochondrial subunits observed in the present study. The finding that Complex II shows the lowest dN/dS values (median = 0.04; Figure 6 and Supporting Information Table S7) is consistent with what predicted by the nuclear compensation hypothesis: Complex II is all nuclear-encoded, so it should be unaffected by mito-nuclear coevolution. For all the other OXPHOS complexes, we expected to see an increase of dN/dS in nuclear subunits as a consequence of the higher rate of protein evolution in mitochondrial genes. Surprisingly, while dN/dS of the mitochondrial subunits is an order of magnitude higher compared to the species where mito-nuclear coevolution was investigated so far, dN/dS of nuclear subunits is comparable to that of other species (dN/dS = 0.12); furthermore, there are no significant differences in the distribution of dN/dS between nuclear- and mtDNA-encoded subunits (Figure 5). Therefore, no evidence for nuclear compensation was found in these bivalves in response to the higher rate of evolution of mitochondrial genome. In addition, this lack of compensation is remarkably evident when we investigate the evolution of sequences for each OXPHOS complex separately: among nuclear subunits, Complex III has the highest dN/dS (median dN/dS = 0.21); on the contrary, Complex III has the lowest rate of protein evolution among mitochondrial complex (median dN/dS ~ 0.09); furthermore, in Complex IV and ATPase, the median dN/dS is higher in mitochondrial subunits compared to nuclear subunits (Figure 6, Supporting Information Table S7). Similar results were obtained by analyzing synonymous substitution rates (dS) and non-synonymous substitution rates (dN) of OXPHOS and non-OXPHOS genes in vertebrates (Zhang & Broughton, 2013). The authors found that dN of mitochondrial subunits is not always higher than that of nuclear subunits in each complex and suggested a minor role for compensatory mechanism in the evolution of OXPHOS genes.

Given the high dN/dS of the species investigated here and the strong heteroplasmy of *R. philippinarum*, one might expect to observe an even higher rate of compensatory amino acid substitutions in the nuclear counterparts. Our results seem to indicate that sequence evolution of nuclear-encoded OXPHOS subunits is not affected by such an extreme mitochondrial variability. One explanation might be that it is not possible to fully evaluate mito-nuclear interactions by simply looking at sequence divergence. The interaction among OXPHOS subunits—and most of all the underlying molecular changes—is extremely complex and need to be studied beyond the nucleotide/amino acid sequence level. Most works on this subject report dN/dS in OXPHOS subunits, but only few have examined the effects of mutations on protein structure and/or function (Azevedo et al., 2009; da Fonseca, Johnson, O'Brien, Ramos, & Antunes, 2008; Osada & Akashi, 2012; Schmidt, Wu, Goodman, & Grossman, 2001; Skibinski et al., 2017). Therefore, it is possible that OXPHOS proteins can tolerate mutations while maintaining their function intact (i.e., robustness, Kitano, 2004), particularly if such mutations affect domains not directly involved in the interactions among subunits, or if they affect disordered regions, or if such mutations are compensated by other non-synonymous substitutions in the same

subunit, or in a functionally linked subunit. In addition, even a non-synonymous substitution can be functionally neutral, having no effects on the protein structure and yielding a perfectly working complex, particularly in species with low metabolic requirements. As a general note, it is worth mentioning that dN/dS is not a particularly powerful method for estimating the type, extent, and strength of natural selection, and that might not be appropriate for broad comparisons. First, dN/dS becomes ineffective when dS reaches saturation—which is a likely event when comparing evolutionarily distant genes (Anisimova & Liberles, 2012). Second, as shown by Havird and Sloan (2016), the relationship between mitochondrial and nuclear dN/dS varies dramatically across eukaryotes mainly because of differences in dS. Unfortunately, more sensitive and robust methods (e.g., McDonald–Kreitman test, methods based on linkage disequilibrium) require data not yet available for most organisms, so dN/dS is often the only possible choice.

An alternative hypothesis to explain our results might be that because the products of a single nuclear genotype have to cofunction with the products of two different mtDNAs, compensatory coevolution is precluded, or slowed down. If mutations in nuclear subunits compensate the changes in one mitochondrial genotype, they might make such nuclear subunits incompatible (or less compatible) with the other mitochondrial genotype.

Other nuclear genes could be involved in mito-nuclear incompatibilities as proposed by Ellison and Burton (2008). It is known that there are ~1,500 nuclear genes involved in mitochondrial biology (Wallace, 2005), that have to constantly interact—either directly or indirectly (Baris et al., 2017)—with mitochondrial DNA, RNAs, and proteins, and some of these genes could be responsible for mito-nuclear incompatibilities. Since the nuclear genome of *R. decussatus* has to interact with only one mitochondrial variant, while the nuclear genome of *R. philippinarum* evolved interacting with two different mitochondrial variants, these species provide a unique chance to investigate which genes, if any, evolved faster in response to mitochondrial variability. For this purpose, we retrieved orthologous nuclear genes with putative mitochondrial function and dN/dS higher than 0.2. We found that such genes are involved in translation, regulation of transcription, and respiratory chain assembly (Supporting Information Table S10). Ellison and Burton (2008) proposed that nuclear genes involved in mitochondrial transcription could be responsible for mito-nuclear incompatibilities and thus mainly involved in mito-nuclear coevolution. Here, we report that genes involved particularly in translation, but also in regulation of transcription, as well as respiratory chain assembly factors, have a higher rate of protein evolution in these species and could be subject to a faster evolution in response to the exceptional mtDNA variability.

Being this work based on two species, our results do not allow to draw general conclusions about the ubiquity of mito-nuclear coevolution, or about the mechanisms behind it. However, we report clear deviations from the expected results predicted by the nuclear compensation hypothesis. We do not know whether such deviations are exceptions due to specific features of bivalve mollusks or DUI species, but since such hypothesis was built around data obtained from

a restricted representation of animal biodiversity, we need broad comparative analyses—encompassing usually overlooked taxa—to address these issues. It is entirely possible that the importance of mito-nuclear coevolution is not the same across different taxa, especially if mito-nuclear mismatches are less maladaptive in organisms with low energetic requirements.

4.3 | Transcription level of mitochondrial and nuclear OXPHOS subunits

The transcription level of mtDNA-encoded OXPHOS subunits is much higher than that of nuclear-encoded subunits in both the species (Figure 2, Supporting Information Table S5). This pattern was already reported in a wide range of eukaryotes, where the transcript abundance of mitochondrial subunits is, on average, 20-fold higher than that of nuclear subunits in animals, 18-fold higher in plants, and 6-fold higher in fungi (Havird & Sloan, 2016; Nabholz et al., 2012). Here, we report a high abundance of mitochondrial transcripts in both males and females of *R. decussatus*, in females of *R. philippinarum*, and a particularly high abundance in males of *R. philippinarum*—respectively, 43-fold, 34-fold, 12-fold, and 100-fold higher than the transcription of nuclear subunits. The reason behind this remarkable difference in transcript abundance between mitochondrial and nuclear subunits is not clear. This pattern was proposed to be ascribed to peculiar property of mitochondrial transcription machinery, and different hypotheses were taken in consideration, such as inefficient mitochondrial translation (Havird & Sloan, 2016; Woodson & Chory, 2008).

It is not clear whether the transcription level is correlated among OXPHOS subunits; previous works reported patterns of co-transcription among subunits belonging to the same OXPHOS complex (Garbian, Ovadia, Dadon, & Mishmar, 2010; van Waveren & Moraes, 2008). We found that the cluster analysis can usually separate samples based on species and, in the case of mitochondrial subunits, also based on sex. Interestingly, the cluster analysis is not able to separate subunits based on the complex they belong to, in any case (Figures 3 and 4). This observation suggests that transcript abundance is considerably variable among subunits, and no pattern of complex-specific co-transcription is found in these species. In addition, we performed correlation matrices among subunits, separately for males and females in each species. Even in this case, a lack of complex-specific correlation pattern is evident in almost every condition (Supporting Information Figures S4–S11). This is interesting, because most of the OXPHOS subunits exist at same ratio in all complexes, except for some subunits of the ATPase (Hüttemann, Lee, Samavati, Yu, & Doan, 2007): accordingly, F0-ATPase c—which is present as a dodecamer in Complex V—and F1-ATPase α and β—both trimers in Complex V—(Kochegarov, 2001), show the highest TMM values among nuclear subunits. Concerning mitochondrial subunits, it should be highlighted that the knowledge about mitochondrial transcription, as well as post-transcriptional regulatory mechanisms and protein turnover, is very restricted (Dennerlein, Wang, & Rehling, 2017; Sirey & Ponting, 2016; Small, Rackham,

& Filipovska, 2013). Most of the studies about these topics focus on mammals (see, for example, Asin-Cayuela & Gustafsson, 2007; Rackham, Mercer, & Filipovska, 2012) and, in any case, many genes and mechanisms remain uncharacterized. Even more surprisingly, the role of polyadenylation in mitochondrial transcripts is still not clear. It is commonly known that poly(A) tail confers stability to cytosol transcripts, ensures their exit from the nucleus, and allows the initiation of translation (Rorbach & Minczuk, 2012). On the contrary, a poly(A) tail mediates transcript degradation in bacteria and plant mitochondria (Gagliardi, Stepien, Temperley, Lightowlers, & Chrzanowska-Lightowlers, 2004). So, what is the role of poly(A) tail in animal mitochondrial transcripts? Does it confer stability, or is it rather required to degradation, similarly to prokaryotes? Slomovic, Portnoy, and Schuster (2008) proposed that these two mechanisms could coexist in mitochondria. Thus, the use of poly(A) selection in RNA-seq library preparation introduces some complications in the analysis of mitochondrial transcription. On the one hand, if the length of the poly(A) tail in mitochondrial transcripts is not consistent, they would be selected with less efficiency than nuclear transcripts leading to an underestimation of mitochondrial transcription level. On the other hand, if poly(A) tail is required for both stabilization and degradation, a quantification based on polyadenylated transcripts would represent an overestimation of the transcripts that will be eventually expressed. Therefore, even if poly-A selection has been largely used in studies that quantified transcription of both nuclear and mitochondrial genes (including this one), it is important to take into account this potential confounding factor that could explain the absence of the expected transcription correlation within mitochondrial OXPHOS subunits reported here. Other studies have solved this problem by using approaches such as Ribo-Zero which depletes rRNA instead of selecting mRNA based on polyadenylation (see, for example, Havird & Sloan, 2016), but, unfortunately, such an approach is not available yet for most animal species. That said, interestingly, we observed a lack of co-transcription also among nuclear subunits; in such case, polyadenylation yields stable transcripts, so the transcription level should be more indicative of the amount of the final product. However, in general, a low correlation between transcription level and protein abundance has been reported, and it was estimated that ~60% of the variation in protein concentration is due to post-transcriptional regulation (Vogel & Marcotte, 2012).

4.4 | The relationship between transcription level and rate of protein evolution in OXPHOS subunits

According to a widely accepted hypothesis, transcription level is the main factor determining the rate of protein evolution, and highly transcribed genes are characterized by lower dN/dS (Zhang & Yang, 2015, and references therein). Therefore, there is a negative correlation (defined E-R correlation) between transcript abundance and dN/dS. Recently, the E-R correlation was taken in consideration to explain the low rate of protein evolution affecting mitochondrial OXPHOS subunits. Nabholz et al. (2012) found a negative correlation between

transcription level of OXPHOS subunits and dN/dS in different organisms and proposed that the high transcription level of mitochondrial genes is the main factor responsible for the low rate of protein evolution. In the present work, we investigated the relationship between transcripts abundance and dN/dS in OXPHOS genes of two bivalve species. This is an interesting analysis, since *R. philippinarum* has a particularly high transcription level of mitochondrial subunits compared to nuclear subunits: up to 100-fold higher for M-type, a difference about fivefold higher than in other animals (Havird & Sloan, 2016; Nabholz et al., 2012). Therefore, according to the E-R correlation hypothesis, a much lower dN/dS should characterize mitochondrial subunits of *R. philippinarum*. Still, dN/dS of such subunits is high as well, while the same value is associated with a considerably lower transcription level in *R. decussatus*. In any case, we did not find negative correlation—neither in mitochondrial nor in nuclear subunits—in both the species (Figure 7). Similar results were obtained by Havird and Sloan (2016) who analyzed 84 eukaryotic taxa and concluded that transcript abundance cannot be responsible for dN/dS. Moreover, Ghiselli et al. (2018)—who analyzed sequence evolution and transcription level in sex-biased nuclear genes—already discussed the need to be careful in searching for a causal correlation between these two variables, and here, we confirm no evidence for E-R correlation. Also, our results show that transcription level of both nuclear and mitochondrial subunits is highly variable, and it is not uniform even across samples within species—despite using the same tissue at the same stage of life cycle. Our opinion is that dN/dS is not affected by transcript abundance and we suggest caution in investigating the relationship between transcription level and rate of protein evolution.

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