Footprints of unconventional mitochondrial inheritance in bivalve phylogeny: Signatures of positive selection on clades with doubly uniparental inheritance.

Footprints of unconventional mitochondrial inheritance in bivalve phylogeny: Signatures of positive selection on clades with doubly uniparental inheritance

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Abstract

The doubly uniparental inheritance (DUI) of some bivalve mollusks is the major exception to the common maternal inheritance of mitochondria in animals. DUI involves two mitochondrial lineages with paternal and maternal transmission routes, and it appears as a complex phenomenon requiring both nuclear and mitochondrial adaptations. DUI distribution seems to be scattered among the Bivalvia, and there are several clues for its multiple origins. In this paper, we investigate whether the incipient DUI systems had left possible selective signatures on mitochondrial genomes. Alongside the outstanding divergence of amino acid sequences, we confirmed strong purifying selection to act on mitochondrial genes. However, we found evidence that distinct episodes of intense directional pressure are associated with the origins of different DUI systems: We interpret these signals as footprints of the coevolution with the nuclear genome that ought to take place at the base of a DUI clade. Six genes (*atp6*, *cox1*, *cox2*, *cox3*, *nad4L*, and *nad6*) seem to be more commonly linked to the appearance of DUI. We also identified few putative DUI specific mutations, thus extending support to the hypothesis of multiple independent origins of this complex phenomenon.

Sommario

Impronte di un'eredità mitocondriale non convenzionale nella filogenesi dei bivalvi: gli episodi di selezione direzionale nei cladi a Eredità Uniparentale Doppia

L'Eredità Uniparentale Doppia (DUI) di alcuni molluschi bivalvi è la principale eccezione alla comune eredità matrilineare dei mitocondri negli animali. La DUI prevede due linee mitocondriali, una con una via di trasmissione paterna e una con una via di trasmissione materna, e costituisce un fenomeno complesso che richiede una coevoluzione tra nucleo e mitocondrio. La distribuzione della DUI è molto irregolare tra i bivalvi e ci sono diverse evidenze di un'origine multipla. In questo lavoro ci siamo interessati dei possibili marchi di selezione positiva sui genomi mitocondriali che hanno cominciato ad adattarsi a un sistema DUI. Oltre la notevole divergenza delle sequenze proteiche, abbiamo confermato la forte pressione selettiva purificatrice sui geni mitocondriali; abbiamo anche evidenziato, tuttavia, episodi distinti di intensa selezione direzionale associati con l'origine dei diversi sistemi DUI: interpretiamo questi segnali come tracce della coevoluzione tra genoma nucleare e mitocondriale che deve aver avuto luogo all'origine di ogni clade DUI. Sei geni in particolare (*atp6*,

cox1, *cox2*, *cox3*, *nad4L* e *nad6*) sembrano collegati più spesso degli altri all'adattamento alla DUI incipiente. Abbiamo identificato solo alcune possibili mutazioni diagnostiche del sistema DUI, il che corroborerebbe ulteriormente l'ipotesi di un'origine multipla di questo complesso fenomeno.

KEYWORDS
Bivalvia, doubly uniparental inheritance, mitochondrial genomics, mito-nuclear coevolution, selective pressure

1 | INTRODUCTION

The generally accepted view of mitochondria as mere "powerhouses" of eukaryotic cells has been repeatedly challenged by several studies from different fields: Far from being just the site of massive ATP production through oxidative phosphorylation, mitochondria are now known to be involved in cell signaling and differentiation, fertilization, embryonic development, aging, and apoptosis (Babayev et al., 2016; Chandel, 2014; López Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Prieto & Torres, 2017; Scheffler, 2008; Spikings, Alderson, & St. John, 2007; Van Blerkom, 2011).

This growing body of evidence about the central role of mitochondria in eukaryotic cells, in turn, increased the interest in the inheritance mechanisms of these organelles. The model of strict maternal inheritance (SMI) of mitochondria is currently considered the most widespread rule in animals (Birky, 2001); however, a major exception to SMI is posed by the system of doubly uniparental inheritance (DUI) of mitochondrial DNA, found in many species of bivalve mollusks (reviewed in Breton, Doucet Beaupré, Stewart, Hoeh, & Blier, 2007; Passamonti & Ghiselli, 2009; Zouros, 2013; Gusman, Lecomte, Stewart, Passamonti, & Breton, 2016).

After fertilization, two mitochondrial lineages are passed to the zygote under DUI: the male (M) type, which was found in sperm, and the female type (F), which was found in oocytes. However, their fate depends on the sex of the developing embryo: While in female embryos M type, mitochondria are dispersed and/or disrupted, and in male embryos, they are aggregated in the primordial germ cells (Cao, Kenchington, & Zouros, 2004). As a consequence, while females are essentially homoplasmic for F type mitochondria, males are heteroplasmic, with M type mitochondria dominating the germ line and F type counterparts often dominating the soma (Garrido Ramos, Stewart, Sutherland, & Zouros, 1998). Notably, exceptions are known to this broad figure: M type mitochondrial genomes may dominate the soma as well, and heteroplasmic females may be found (Batista, Lallias, Taris, Guerdes Pinto, & Beaumont, 2011; Brannock, Roberts, & Hilbish, 2013; Chakrabarti et al., 2007; Ghiselli, Milani, & Passamonti, 2011; Kyriakou, Zouros, & Rodakis, 2010; Obata, Sano, & Komaru, 2011).

To date, DUI has been found in more than one hundred bivalve species (reviewed in Gusman et al., 2016), in most cases using sex linked heteroplasmy as a proxy (Boyle & Etter, 2013; Dégletagne, Abele, & Held, 2016; Gusman et al., 2016; Passamonti & Scali, 2001; Plazzi, 2015; Plazzi, Cassano, & Passamonti, 2015; Theologidis, Fodelianakis, Gaspar, & Zouros, 2008; Vargas, Pérez, Toro, & Astorga, 2015). However, it is possible that many other bivalve, if not molluscan, DUI species are still to be discovered (Gusman et al., 2016).

Given the status of DUI as the major exception to SMI in animals, the question of its origin is of great interest in the field of mitochondrial biology. DUI is a complex phenomenon, and a single origin may seem the most parsimonious hypothesis (Boyle & Etter, 2013; Hoeh, Stewart, Saavedra, Sutherland, & Zouros, 1997). However, information on DUI distribution among bivalves is increasing, and it still shows a scattered pattern (Gusman et al., 2016; Plazzi, 2015). It must always be remembered that DUI detection is particularly prone to false negatives, and many DUI species may have been overlooked (Theologidis et al., 2008; Zouros, 2013).

Nonetheless, the hypothesis of multiple DUI origins is becoming more than a speculative alternative (Milani, Ghiselli, Guerra, Breton, & Passamonti, 2013; Milani, Ghiselli, & Passamonti, 2016; Zouros, 2013). A complex cell machinery is needed to maintain DUI and obvious similarities are shared, yet DUI species display many differences as well. For instance, as extensively reviewed, for example, in Zouros (2013) and Plazzi (2015), different DUI systems may show different (and somewhat opposite) heteroplasmy levels in somatic and germ cells, and different genome architectures are known with respect to gene content (see also Section 4 below).

In the present paper, we decided to investigate the evolutionary transitions which lead to clusters of DUI species, which are scattered across the bivalve evolutionary tree, looking for genomic and selective signatures that might be related to the shift from SMI to DUI.

2 | MATERIALS AND METHODS

We retrieved all 98 complete mitochondrial genomes (mtDNAs) that were already collected and characterized in Plazzi, Puccio, and Passamonti (2016), which is presently the most comprehensive appraisal to bivalve mitogenomics, and we re examined this dataset with special reference to DUI species. Given the issues raised by Bettinazzi, Plazzi, and Passamonti (2016), *Hyriopsis* spp. mtDNAs were not considered as DUI genomes throughout the whole work. The 98 bivalve species included in this paper are listed in Supporting Information Table S1 along with their family, abbreviation, and GenBank Accession Number.

The original publication presented data from the following genes: the *ATP synthase membrane subunit 6* (*atp6*), *cytochrome c oxidases* I III (*cox1 3*), *cytochrome b* (*cytb*), *NADH:ubiquinone oxidoreductase* core subunits 1 6 (*nad1 6*), and the *NADH:ubiquinone oxidoreductase* core subunit 4L (*nad4L*). For the present study, we added the *ATP synthase membrane subunit 8* (*atp8*) gene alignment, which was missing; we followed the same procedure for *atp8* that was originally followed for the aforementioned 12 protein coding genes (PCGs). Namely, we used the software masking package (detailed in Plazzi et al., 2016), written for bash and R (R Development Core Team, 2008) environments and loading the package seqinr (Charif & Lobry, 2007) to (a) perform a structural alignment using T Coffee (Notredame, Higgins, & Heringa, 2000); (b) clean alignment from possible phylogenetic noise using the four tools Aliscore 2.0 (Misof & Misof, 2009), BMGE 1.1 (Criscuolo & Gribaldo, 2010), Gblocks 0.91b (Castresana, 2000), and Noisy (Dress et al., 2008); (c) compare all outputs and keep only sites selected by at least 3 tools out of 4. All options were set as in Plazzi et al. (2016).

The 13 final amino acid alignments (*atp6*, *atp8*, *cox1 3*, *cytb*, *nad1 4*, *nad4L*, and *nad5 6*) are available as Supporting Information Dataset S1. Cleaned amino acids alignments were back translated into codons using a simple custom R script, retaining the original nucleotide sequences. The 13 back translated alignments, plus the complete concatenated matrix, are available as Supporting Information Dataset S2.

The pairwise uncorrected (p) amino acid distance was computed using the distmat binary of the EMBOSS 6.6.0 (Rice, Longden, & Bleasby, 2000) suite, with the exclusion of *atp8* due to its high variability. The pairwise number of nonsynonymous changes for nonsynonymous sites (dN), the number of synonymous changes for synonymous sites (dS), and the dN/dS ratio were computed with KaKs Calculator 2.0 (Wang, Zhang, Zhang, Zhu, & Yu, 2010) for each gene (again, with the exclusion of *atp8* due to its high variability), using the default model averaging method. The non linear correlation between pairwise p distances and dN/dS ratios was explored by fitting an exponential model of the form $y = a + e^{b+cx}$ via the minpack.lm R package (Elzhov, Mullen, Spiess, & Bolker, 2016).

Aiming to characterize variability and dN/dS ratios of DUI species, we selected six subsets from all pairwise comparisons: (a) Unionidae, comparisons between the two DUI mtDNAs from the same species of the family Unionidae; (b) Mytilidae, comparisons between the two DUI mtDNAs from a single species of the family Mytilidae; (c) other DUI species, comparisons between the two DUI mtDNAs from either *Venerupis philippinarum* or *Meretrix lamarckii*; (d) within genus, comparisons between two non DUI mtDNAs from two different species from the same genus; (e) within family, comparisons between two non DUI mtDNAs from two different genera from the same family; and (f) within subclass, comparisons between two non DUI mtDNAs from two different families from the same subclass.

Furthermore, dN rate, dS rate, and dN/dS ratio were computed with PAML 4.8a (Yang, 1997, 2007) for each gene (including *atp8*) along the best known likelihood (BKL) tree by Plazzi et al. (2016) which is shown in Figure 1. Briefly, this tree was the result of the exploration of several maximum likelihood parameter combinations and is therefore the best single tree estimate of the whole mitochondrial phylogeny of bivalves. To explore the possibility of different selective pressures on different branches, we used the free ratios model, allowing an independent dN/dS ratio for each branch: It was already demonstrated that the free ratios model always outperforms the single ratio model (Plazzi, Puccio, & Passamonti, 2017). Equilibrium codon frequencies were used as free parameters.

Branches were then assigned to two different categories (Figure 1): (a) DUI origin, branches leading to splits between published F and M mtDNAs in known DUI systems and (b) DUI unlinked, all remaining branches; the phylogenetic tree was graphically edited using Dendroscope 3.5.9 (Huson & Scornavacca, 2012). The two tailed nonparametric Mann Whitney test (Hollander & Wolfe, 1999) was carried out to test differences in dN, dS, and dN/dS between DUI origin and DUI unlinked branches.

Given the limited number (5) of available DUI origin branches, we devised a method to exclude the possibility that such a limited number of samples, when compared to a larger set, yield a significant result because of a sample size issue. Namely, five random branches were selected from the same tree (as if they had been DUI origin branches), and differences in dN, dS, and dN/dS were again explored with a two tailed Mann Whitney test. This procedure was repeated 1,000 times; the original *p* value was considered significantly low when smaller than the 5^{th} (one tailed test) or 2.5^{th} (two - tailed test) percentile of replicated values. Percentiles were computed setting type = 8 in the quantile function of the R environment (Hyndman & Fan, 1996).

Typical mutations of DUI genomes were searched for in each gene alignment: A first screening was carried with a custom R script that computed a site wise dichotomy informativeness (SDI) score, which is defined as follows. At first, we define a set of sequences (which are screened for diagnostic mutations) as the ingroup; remaining sequences are defined as the outgroup. In our case, the ingroup was initially set to DUI mtDNAs. Then, for each site *i*, the most common residue (MCR) within the ingroup is found: We define its number of occurrences as MCR_{in}. Subsequently, the occurrences of the same residue at the same site are computed in the outgroup: We define this number as MCR_{out}. In case of ties, we conservatively select the MCR with the highest MCR_{out}; in case of ties, the MCR is randomly chosen between those with the highest MCR_{out}. If $MCR_{out} > MCR_{in}$, MCR_{out} is set to MCR_{in}. Finally, the SDI score of the *i* th site is computed as

$$SDI_i \quad \frac{MCR_{in} \quad MCR_{out}}{N}$$

where *N* is the number of operational taxonomic units (OTUs) within the ingroup. Following this definition, $0 < SDI_i < 1$; an SDI score equal to 1 means that all the OTUs in the ingroup share the same residue at a given site *i* and that no OTU in the outgroup shows the same residue; an SDI score equal to 0 means that the number of OTUs sharing the most common residue in the ingroup at the site *i* is equal to, if not smaller than, the number of OTUs in the outgroup

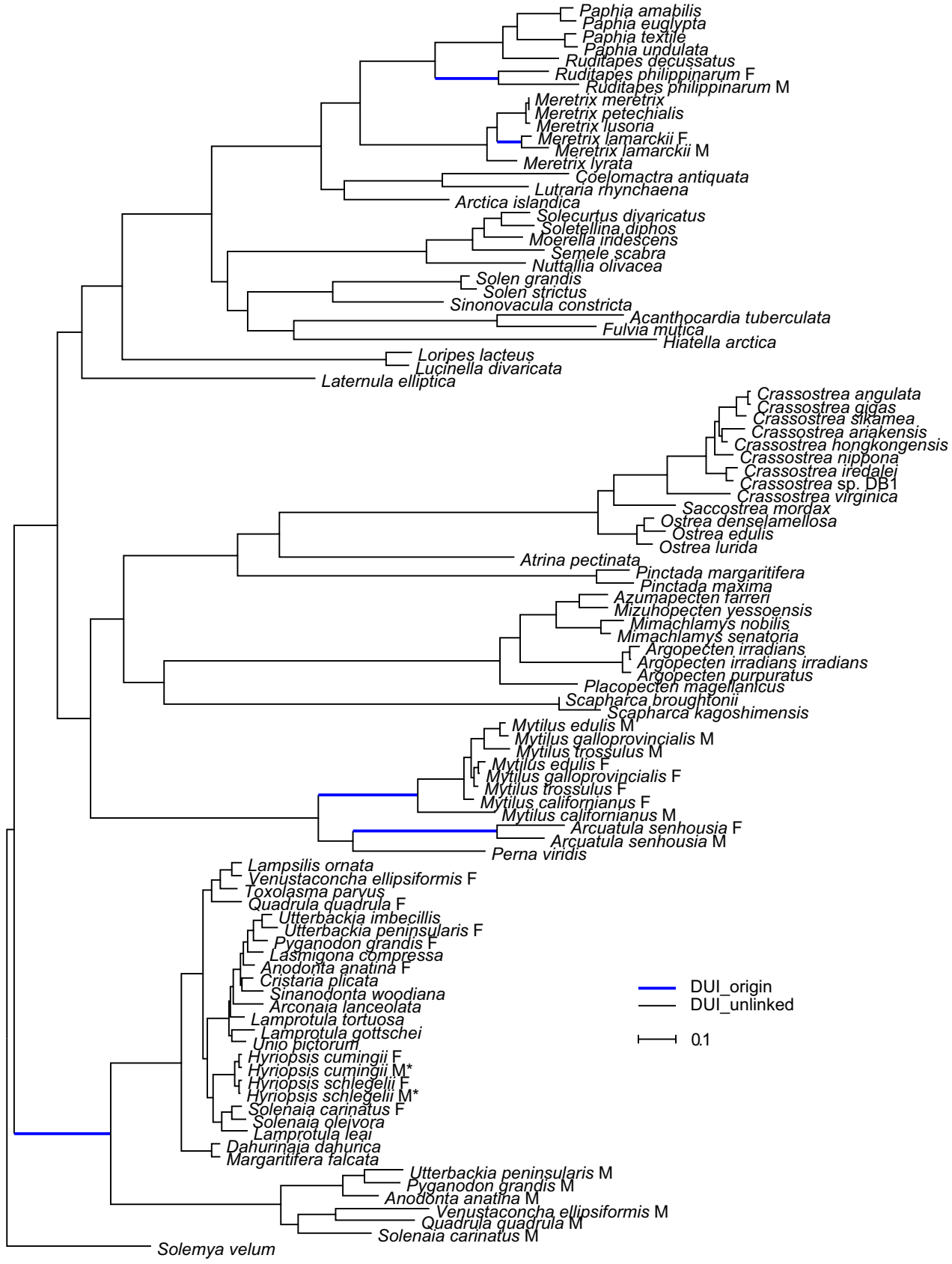

FIGURE 1 The BKL tree by Plazzi et al. (2016) showing branches leading to splits between F and M mtDNAs in blue ("DUI origin" branches); all remaining branches were classified as "DUI unlinked." Asterisks refer to the fact that, notwithstanding the current GenBank annotation, the *Hyriopsis* spp. mtDNAs are probably all female type ones, as detailed in Bettinazzi et al. (2016). Thus, a single DUI origin branch was used for Unionidae

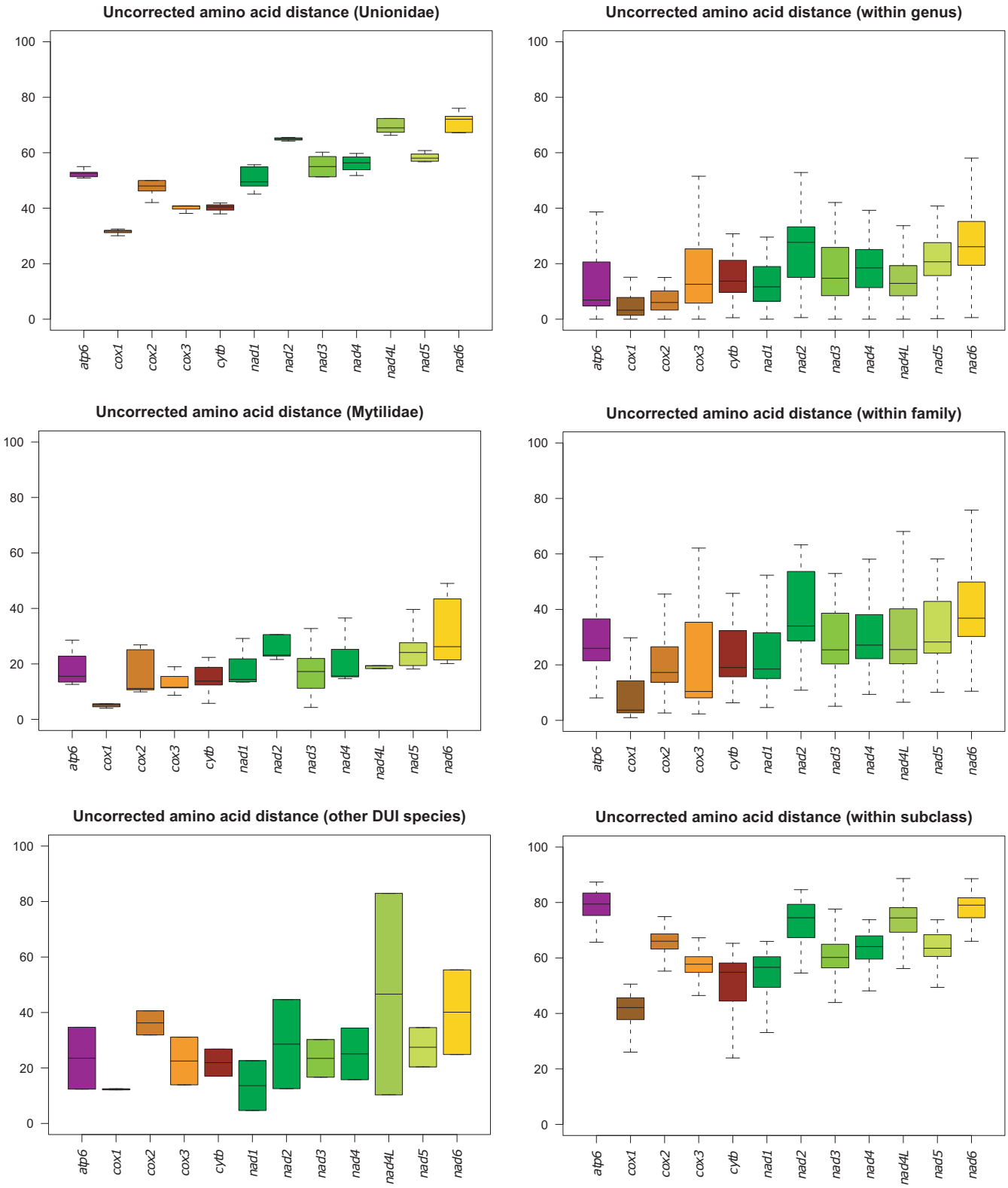

FIGURE 2 Distributions of uncorrected (p) amino acid distances by gene. The black line is the median; the two hinges of the box approximate the first and the third quartile; whiskers, when present, extend to a roughly 95% confidence interval. (a) Unionidae, comparisons between the two DUI mtDNAs from a same species of the family Unionidae; (b) Mytilidae, the same for Mytilidae; (c) other DUI species, the same for *Venerupis philippinarum* and *Meretrix lamarckii*; (d) within genus, comparisons between two non DUI mtDNAs from two different species from the same genus; (e) within family, comparisons between two non DUI mtDNAs from two different genera from the same family; and (f) within subclass, comparisons between two non DUI mtDNAs from two different families from the same subclass

showing the same residue and that the site is henceforth not informative regarding the selected ingroup/outgroup dichotomy. The same procedure as above was also applied to amino acid categories instead of single residues: acidic/basic/polar uncharged/hydrophobic nonpolar; external/ambivalent/internal; and chemical properties of the functional groups.

Before considering potential diagnostic sites for our dataset, we tested whether our procedure tends to retrieve such sites irrespective of the biological significance of the proposed dichotomy. For this purpose, we excluded DUI mtDNAs from the dataset and extracted random subsets from the remaining taxa. Since DUI OTUs are 26 out of 98 (26.53%) in the complete dataset, we followed the same proportion and 1,000 random subsets of size 19 were drawn from the 72 non DUI OTUs (26.38%). The SDI score was computed as above for each random subset: The consideration threshold for a given site was arbitrarily set to 0.5, meaning that a site was taken into consideration if and only if its SDI score was higher than 0.5.

After the automatic screening based on the SDI score, different DUI mtDNA subsets were explored and, eventually, selected sites were individually checked. The final alignment of relevant sites was graphically edited with the TeXshade package (Beitz, 2000).

3 | RESULTS

Overall pairwise p distances and dN/dS ratios are shown for each gene in Supporting Information Figure S1, and the exponential correlation between variability and selective pressure patterns depicts the agreement between the two variables (Supporting Information Figure S2). In agreement with Plazzi et al. (2016), higher dN/dS ratios (and amino acid divergence as well) are associated with *atp6*, *nad2*, *nad4L*, and *nad6*. The order of magnitude of either variable is similar for within genus and within family comparisons, while, as expected, values for within subclass comparisons are higher (Figures 2 and 3). With respect to DUI comparisons (i.e., comparisons between the two sex specific mtDNAs from a given DUI species), while the pattern of Unionidae is similar to that of within subclass comparisons, the patterns of Mytilidae and remaining DUI species (i.e., the two venerid species) show the same order of magnitude of the within genus and within family groups.

When considering single branches of the bivalve phylogeny, median dN rates, dS rates, and dN/dS ratios are always higher for DUI origin branches than for DUI unlinked branches (Figures 4 and 5). This difference was significant only in some cases, but in all (and only in) these cases, the *p* value is significantly lower than *p* values from random replicates (Table 1). In particular, the dN/dS ratio of

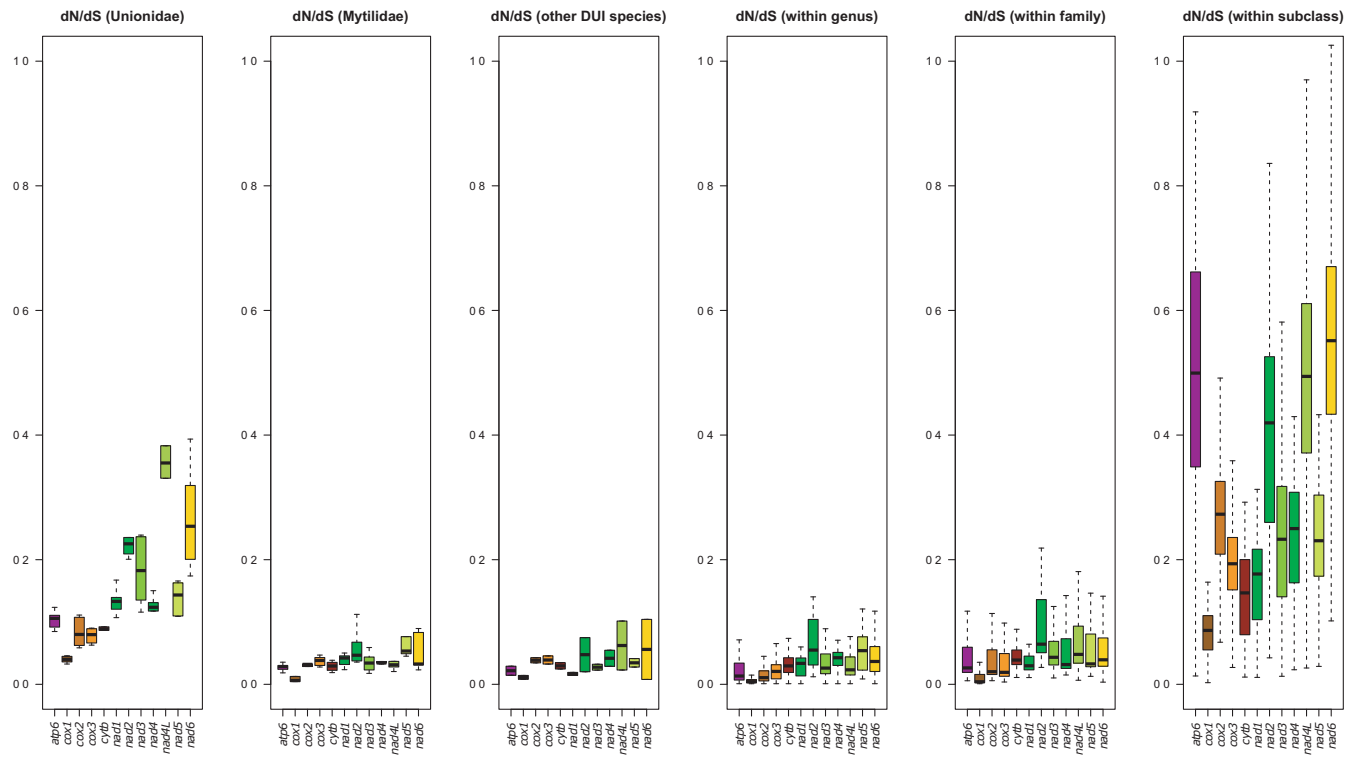

FIGURE 3 Distributions of dN/dS ratios by gene. The black line is the median; the two hinges of the box approximate the first and the third quartile; whiskers, when present, extend to a roughly 95% confidence interval. (a) Unionidae, comparisons between the two DUI mtDNAs from a same species of the family Unionidae; (b) Mytilidae, the same for Mytilidae; (c) other DUI species, the same for *Venerupis philippinarum* and *Meretrix lamarckii*; (d) within genus, comparisons between two non DUI mtDNAs from two different species from the same genus; (e) within family, comparisons between two non DUI mtDNAs from two different genera from the same family; and (f) within subclass, comparisons between two non DUI mtDNAs from two different families from the same subclass

atp6, *cox1*, *cox2*, *cox3*, *nad4L*, and *nad6* is significantly higher in DUI origin branches than in other branches.

The preliminary screening on single residues yielded 4 diagnostic sites discriminating between DUI and non DUI OTUs with SDI > 0.5; conversely, among 1,000 random subsets of 19 out of 72 non DUI OTUs, putative diagnostic sites were never retrieved.

Different ingroup settings led to an increase in the number of putative diagnostic sites. When mytilid and male unionid mtDNAs were used as the ingroup, 137 sites with a promising SDI score (i.e., $SDI_i > 0.5$) were obtained, using either single residues or functional properties.

Notably, 33 sites out of 137 are *cox1* sites (Supporting Information Table S2). However, only 18 sites showed a nearly complete pattern: mytilid and male unionid DUI mtDNAs, on one side, and other mtDNAs, on the other side (Figure 6 and Supporting Information Dataset S3). Out of these 18 sites, 2 are *atp6* sites, while 6 are *cox1* sites. For example, the most complete pattern is that of site 15 of Figure 6 (site 140 of *nad1* alignment): All mytilid and male unionid mtDNAs show a proline, and only *Perna viridis* shows a proline at the same site among the outgroups. Conversely, most sites show exceptions in both ingroup and outgroup: For example, site 3 (site 19 of *cox1* alignment) is a serine with the exception of male *Venustaconcha ellipsiformis*, and only *P. viridis* and *Scapharca broughtonii* were found to have a serine at the same site.

4 | DISCUSSION

The question of the first origin and evolution of DUI is of outstanding interest, because DUI is a complex phenomenon, with many links to other key biological issues, and most importantly to sex determination (Breton et al., 2011, 2014; Passamonti & Ghiselli, 2009; Zouros, 2013) and genomic conflicts (Milani et al., 2016; Passamonti & Ghiselli, 2009). Recently, several lines of evidence point toward a multiple origin of DUI. Milani et al. (2013, 2016) suggested that viral gene endogenizations in the mitochondrial genomes could be related to DUI origin, a causal mechanism that might account for the scattered distribution of DUI across bivalves.

Mitochondrial ORFans (i.e., ORFs with no known homology to typical mitochondrial genes; Fischer & Eisenberg, 1999) are often found in bivalve mitochondrial genomes (Breton et al., 2014; Plazzi et al., 2016) and may be connected with the DUI phenomenon (Breton et al., 2009, 2011). These may also originate from gene duplication events (Mitchell, Guerra, Stewart, & Breton, 2016) rather than from viral horizontal gene transfers; however, sequence or structure similarities are rarely retrieved between ORFans of different, even if related, bivalve species (Milani et al., 2013; Mitchell et al., 2016; Plazzi et al., 2016) and these supernumerary genes may be not homologous at all (Plazzi et al., 2016).

On the other hand, the alternative hypothesis involves a single origin of DUI with the radiation of Eulamellibranchiata (or, possibly,

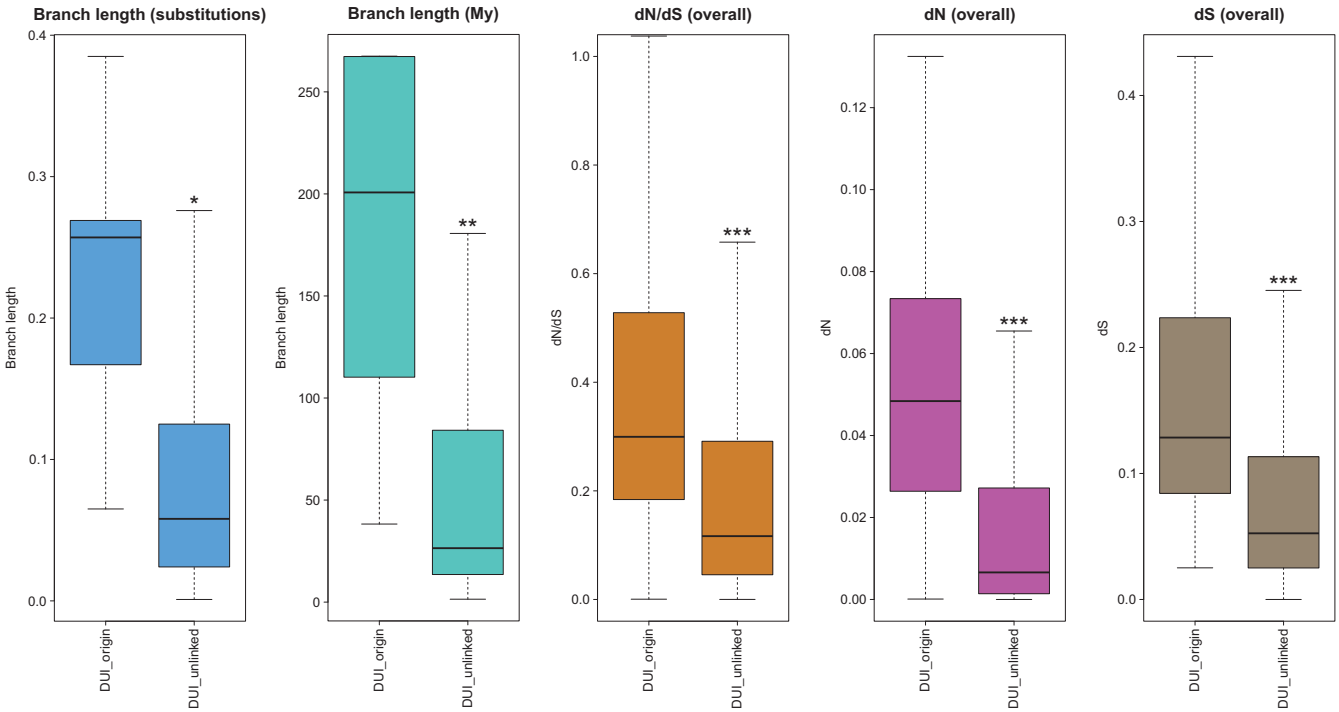

FIGURE 4 Overall comparison between branches leading to a DUI driven split and other branches. The black line is the median; the two hinges of the box approximate the first and the third quartile; whiskers, when present, extend to a roughly 95% confidence interval. Branch lengths, in terms of substitutions/site, were taken from the BKL tree of Plazzi et al. (2016) which is depicted in Figure 1; in terms of million years, they were taken from the ultrametric tree of Plazzi et al. (2016). "dN/dS," "dN," and "dS" refer to estimates from all genes and the concatenated dataset joined together. Branches leading to a DUI driven split ("DUI origin") are shown in Figure 1; other branches are called "DUI unlinked." All comparisons are significant (asterisks above the DUI unlinked box; see Table 1 for details)

at or near the origin of the modern class Bivalvia; Boyle & Etter, 2013), followed by its loss in some lineages. Zouros (2013) argued that, assuming that such a loss implies the loss of the M mtDNA lineage, in the single origin scenario either F mtDNAs ought to cluster together with non DUI mtDNAs or a masculinization event is required at the base of each DUI clade. The former is not the case; the latter may not be the most parsimonious explanation.

In fact, there are clear differences between different DUI systems and sex linked mtDNAs show different features in different DUI species (Plazzi, 2015; Zouros, 2013): We recall here three major differences. (a) The mitochondrial gene *cox2* has a male specific extension in freshwater mussels of the order Unionoida (Breton et al., 2011; Curole & Kocher, 2002), while it is duplicated in the female mitochondrial genome (mtDNA) of the venerid clam *Ruditapes philippinarum* (Breton et al., 2014). Among mussels, the *cox2* gene is duplicated in the M mtDNA of *Arcuatula senhousia* (Breton et al., 2014). (b) The occurrence of masculinization of F mtDNA was broadly demonstrated in the genus *Mytilus* (Zouros, 2013), but it is not found in Unionidae (Curole & Kocher, 2002, 2005; Stewart, Breton, Blier, & Hoeh, 2009; Walker et al., 2006), and never directly observed in Veneridae (Passamonti, 2007; Passamonti & Scali, 2001; Stewart et al., 2009). (c) DUI is highly correlated with gonochorism (Breton et al., 2011; Guerra et al., 2017), but still this is not the only key to DUI distribution: In some groups, such as the family Unionidae, DUI is common and widespread and strictly absent in hermaphroditic taxa; in other cases, only one or two species were found to have DUI in a given family (Gusman et al., 2016), with many gonochoric species showing no evidence of DUI (Plazzi et al., 2015).

Bivalve lineages experienced hundreds of million years of evolutionary divergence, the first appearance of the class being dated to 520 million years ago (Mya) by fossil records (Brasier & Hewitt, 1978). During this time span, it is conceivable that considerable novelties arose from a single ancestral machinery. Different DUI systems can be treated as biological replicates of this multi faceted phenomenon, and it is possible to search for repeated features: The present work aims to characterize the evolutionary signature of DUI on mitochondrial genomes, with special reference to the evolutionary transition from SMI to DUI. In the case of a single origin of DUI followed by divergence and neutral/directional evolution in various lineages, we do not expect to see particular selective signatures on branches leading to sex specific, DUI linked mtDNA splits: These branches should behave like the general figure of the inferred phylogenetic tree. Conversely, in case of multiple DUI origin, we expect to observe selective signatures on these branches, which are connected to each DUI ongoing evolution.

The detected strong purifying selection (Figure 3 and Supporting Information Figure S1) is in agreement with previous findings on bivalves (Gaitán Espitia, Quintero Galvis, Mesas, & D'Elía, 2016; Plazzi et al., 2016; Zbawicka, Wenne, & Burzyński, 2014) and other

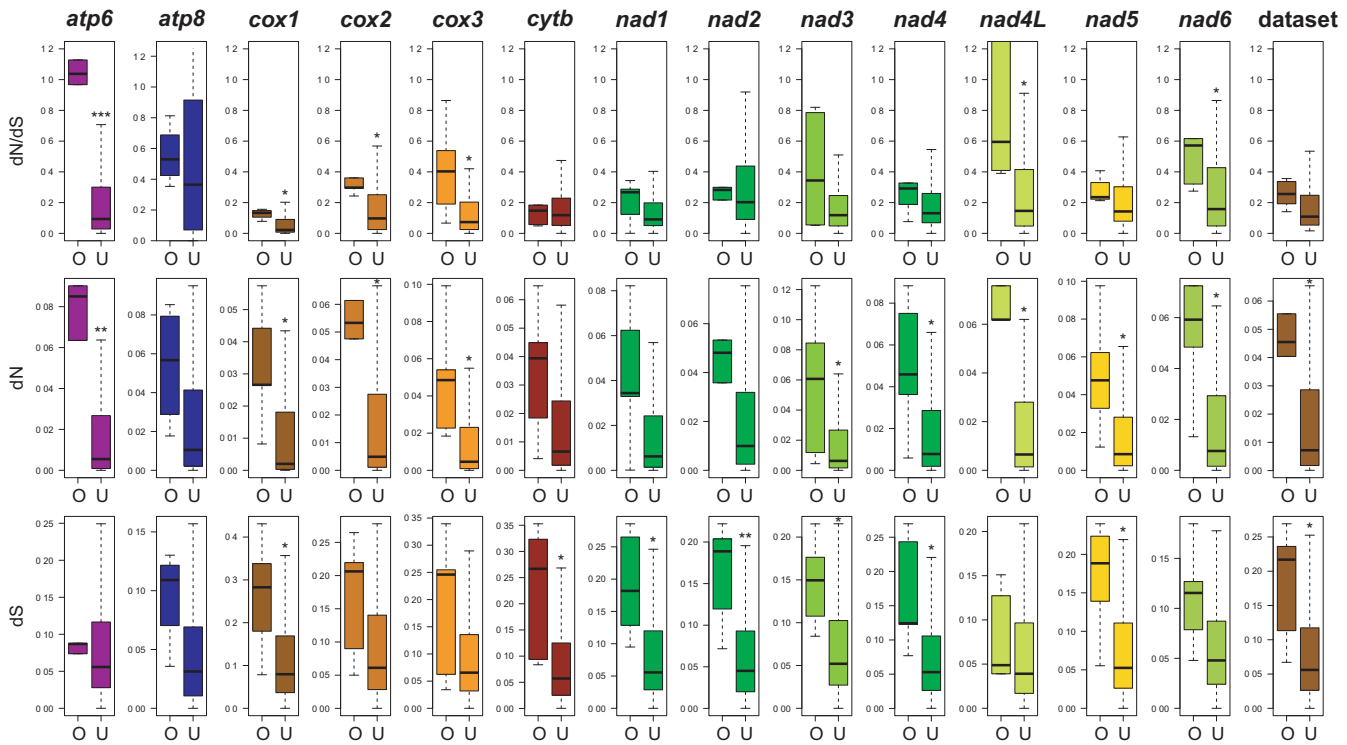

FIGURE 5 Gene by gene comparison of dN/dS ratios (top), dN values (middle), and dS values (bottom) between branches leading to a DUI driven split ("O," i.e., "DUI origin") and other branches ("U," i.e., "DUI unlinked"). The black line is the median; the two hinges of the box approximate the first and the third quartile; whiskers, when present, extend to a roughly 95% confidence interval. Branches leading to a DUI driven split ("O") are shown in Figure 1; other branches were labeled as "U." Significant comparisons are shown with asterisks above the U box (see Table 1 for details)

eukaryotes (Havird & Sloan, 2016; Nabholz, Ellegren, & Wolf, 2013; Popadin, Nikolaev, Junier, Baranova, & Antonarakis, 2013). High dN/dS levels were detected only between species from different families of the same subclass, and divergence patterns seem to be associated more to genes and phylogeny than to DUI presence/absence, recall that Unionidae show higher divergence values than Mytilidae and other DUI species (Figure 2), as reported elsewhere (Bettinazzi et al., 2016; Zouros, 2013).

Six genes show significantly higher dN/dS ratios along branches leading to a DUI driven split (Figure 5; Table 1), in two cases with particularly large median values (~0.4 for *cox3*, ~0.6 for *nad4L* and *nad6*, but >1 for *atp6*). It is known that *atp6* experienced several periods of noteworthy selective constraints (Plazzi et al., 2017) and that *nad4L* and *nad6* have highly variable sequence (Plazzi et al., 2016). However, two facts lead us to discard the possible interpretation of these results as incidental features of these genes: (a) The same significant result obtained for *cox1*, *cox2*, and *cox3*, which have a long, generally highly conserved sequence (Figure 5); (b) the finding that, if not dN/dS ratio, at least either dN or dS rate is significantly higher along DUI splitting branches for all genes and the concatenated dataset, with the exception of *atp8* (Table 1). Observed dN/dS ratios, as well as dN and dS rates, are also highly significantly different when considering all data together (Figure 4).

This increase in mutational (dN and dS rates) and selective (dN/dS ratios) pressure seems to be associated with the emergence of DUI in all the available replicates: When DUI switches on, it increases the mutational pressure and requires some sort of directional selection on at least some mitochondrial genes. Furthermore, larger branch lengths (either in terms of substitutions/site or in terms of million years) are also associated with these lineages (Figure 4; Table 1); however, it is difficult to understand whether this is a specific effect of the taxon sampling of the tree by Plazzi et al. (2016) or not.

Despite the common claim that M mtDNA evolves faster than F mtDNA (Zouros, 2013; and reference therein), at least one opposite situation has been found (Passamonti, 2007). Moreover, SNP calling in *Venerupis philippinarum* demonstrated that F type variability may be largely underestimated (Ghiselli et al., 2013), and similar analyses are still to be performed in other DUI species. Therefore, the significantly higher dN/dS levels of DUI origin branches should not be universally explained by the relaxation of the selective pressure in the diverging M lineage, and this phenomenon should not be regarded as the only driver of our results.

It is common to observe higher rates of nonsynonymous amino acid changes in non recombining sex chromosomes (Crowson, Barrett, & Wright, 2017; Papadopulos, Chester, Ridout, & Filatov, 2015; White, Kitano, & Peichel, 2015). Actually, mtDNA or, at least, mtDNA encoded factors may act as sex determination drivers (Breton et al., 2007; Capt et al., 2018; Passamonti & Ghiselli, 2009; Pozzi, Plazzi, Milani, Ghiselli, & Passamonti, 2017; Yusa, Breton, & Hoeh, 2013), and a role for DUI in the maintenance of gonochorism itself is emerging (Breton et al., 2011; Guerra et al., 2017; Milani et al., 2016). Recall that mitochondrial recombination is currently understood to be a widespread phenomenon in animals (Zouros, 2013), sex specific mtDNAs may share some features with canonical sex chromosomes, including the tendency toward recombination

TABLE 1 dN rates, dS rates, and dN/dS ratios that are significantly higher in DUI linked branches (two tailed Mann Whitney test)

		Significance over 1,000 random replicates[b]	
	p-Value[a]	One-tailed test[c]	Two-tailed test[d]
dN/dS (*atp6*)	3.5206E-03***	***	***
dN (*atp6*)	9.6263E-03**	**	*
dN/dS (*cox1*)	2.2720E-02*	*	N/S
dN (*cox1*)	1.2395E-02*	*	*
dS (*cox1*)	3.3809E-02*	*	N/S
dN/dS (*cox2*)	2.2780E-02*	*	*
dN (*cox2*)	1.1889E-02*	*	*
dN/dS (*cox3*)	2.4383E-02*	*	*
dN (*cox3*)	1.4733E-02*	**	*
dS (*cytb*)	1.6182E-02*	*	*
dS (*nad1*)	1.7258E-02*	*	*
dS (*nad2*)	6.8233E-03**	***	**
dN (*nad3*)	4.9898E-02*	*	N/S
dS (*nad3*)	3.4776E-02*	*	N/S
dN (*nad4*)	2.9939E-02*	*	N/S
dS (*nad4*)	2.1002E-02*	*	*
dN/dS (*nad4L*)	1.4624E-02*	*	*
dN (*nad4L*)	1.0874E-02*	**	*
dN (*nad5*)	2.1921E-02*	*	N/S
dS (*nad5*)	2.1926E-02*	*	*
dN/dS (*nad6*)	3.4300E-02*	*	N/S
dN (*nad6*)	1.7164E-02*	*	*
dN (dataset)	2.1218E-02*	*	*
dS (dataset)	2.3851E-02*	*	N/S
Branch length (substitutions; overall)	2.1678E-02*	*	N/S
Branch length (time; overall)	1.1810E-02*	*	*
dN/dS (overall)	8.8413E-10***	***	***
dN (overall)	9.4594E-17***	***	***
dS (overall)	1.0727E-12***	***	***

[a]Two-tailed Mann Whitney test; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.005. [b]For each replicate, 5 random branches were set as DUI origin branches. The one-tailed test was considered significant whenever the original Mann Whitney p-value was lower than the 5th percentile of the resulting 1,000 p-values; the two-tailed test was considered significant whenever it was lower than the 2.5th percentile. [c]*original p-value < 5th percentile; **original p-value < 1st percentile; ***original p-value < 0.5th percentile; N/S, not significant. [d]*original p-value < 2.5th percentile; **original p-value < 0.5th percentile; ***original p-value < 0.25th percentile; N/S, not significant.

FIGURE 6 The 18 most structure informative sites. Male unionid and all mytilid DUI species (16 sequences) comprise the ingroup, while all other taxa comprise the outgroup: All features were computed on the complete outgroup (82 sequences), but only 16 random sequences are shown for clarity. Residues, as well as the logo at the top, are shaded following the rasmol amino acid clustering, which is explained in the legend at the bottom; conservation is shown in the bar chart below the alignment. Sites not from *atp6/cox1* alignments (right portion) are tinted; numbering refers to Supporting Information Table S2. See Supporting Information Table S1 for species abbreviations; see Figure 1 for the complete names

suppression, which would facilitate the separation of sex determining factors (Charlesworth & Charlesworth, 2000; Charlesworth, Charlesworth, & Marais, 2005). The observation of significantly higher dN/dS ratios along branches leading to an emerging DUI system may thus be compatible with a pair of sex linked mitochondrial chromosomes caught while evolving recombination suppression.

Conversely, the evolution of a DUI system does not involve well defined mitochondrial apomorphies. A total of 3,113 amino acid sites comprise the complete, concatenated dataset, yet only 137 showed an SDI score > 0.5 (Supporting Information Table S2), and only 18 out of 137 turned out to really show some informative pattern (Figure 6). Moreover, this pattern does not involve, as initially expected, a dichotomy between DUI and non DUI species; the only suitable split is between mytilid and male unionid DUI species, on one side, and other mtDNAs, on the other side (Supporting Information Dataset S3). It is conceivable that this pattern is partly due to ancestral unresolved polymorphism: Indeed, Unionidae is the sister group to other eulamellibranchiates in mitochondrial gene trees, while Mytilidae is the sister group to other pteriomorphians (Doucet Beaupré et al., 2010; Plazzi, Ceregato, Taviani, & Passamonti, 2011; Plazzi et al., 2016; Stöger & Schrödl, 2013). However, there is a large difference in the geological first appearance of either family in the geological record, mytilids being dated to ~420 Mya (Upper Silurian; Kříž, 2008) and unionids to ~245 Mya (Lower Triassic; Tillyard & Dunstan, 1916; Cromptok & Parrington, 1955; Drysdall & Kitching, 1963; Nesbitt et al., 2010). It is known that M unionid genomes typically experience higher mutational rates and are therefore expected to be more derived than F counterparts (Guerra et al., 2017; Gusman et al., 2016; Plazzi et al., 2016), and this may account for the fact that only M mtDNAs of unionids cluster with mytilids for these sites.

All this considered, it is conceivable that, in a few cases, the origin of a DUI system triggered convergent mutations: Many of these sites (6 out of 18) belong to the *cox1* alignment, which is indeed included in those gene that were demonstrated to experience directional selection when DUI turns on along a branch. However, the examination of possible structural effects of these mutations is well beyond the scopes of the present paper, and the pattern in diagnostic sites is never completely dichotomic, albeit for subsets of DUI mtDNAs. An increased taxon sampling is mandatory to unravel this

issue; yet, those species that frequently share mutations with mytilid and male unionid DUI species, such as *Perna viridis* and *Laternula elliptica*, may be regarded as good DUI species candidates. In any case, diagnostic mutations, if any, are really few: This, along with the fact that independent events of directional selection were detected at the base of each DUI system, is consistent with the hypothesis of multiple DUI origins.

Because of (a) the repeated significant increase in dN/dS at the base of DUI systems and (b) the substantial lack of shared mutations, the present study lends support to the scenario proposed by Milani et al. (2016) and to the multiple origin hypothesis. If the endogenization of a viral element triggers a series of reproductive transitions (from hermaphroditism to androdioecy to gonochorism) that end up in a DUI system, it is conceivable that either mitochondrial genome ought to coevolve with the nuclear genome to undergo these complex biological modifications.

It is often a single element, or portion, of the mtDNA that is involved in DUI origin and maintenance, and not the whole molecule. Such circumscribed mitochondrial regions are different in different DUI systems, which may be connected to different viral triggers. In mytilids, specific sequences of the control region are known to drive masculinization events and therefore to be involved in determining the persistence or the loss of a mitochondrion (Zouros, 2000; Burzyński, Zbawicka, Skibinski, & Wenne, 2003; Venetis, Theologidis, Zouros, & Rodakis, 2007; Zouros, 2013; and reference therein). The male extension of *cox2* and sex specific ORFans has been suggested to be correlated with DUI maintaining in Unionidae (Breton et al., 2009, 2011); in venerids, the putative viral elements described by Milani et al. (2013, 2016) are also sex specific mitochondrial ORFans. Finally, at least in *Venerupis philippinarum*, nuclear genome is regulated through mitochondrially encoded sncRNAs, called smithRNAs, which may be connected to sex determination and DUI maintenance (Pozzi et al., 2017).

Interestingly, 5 out of 6 genes that showed DUI associated increased directional pressure (*atp6*, *cox1*, *cox3*, *nad4L*, *nad6*) were never cited among these putative DUI drivers, and we detected a general increase in mitochondrial evolutionary rates (Figure 4). In other words, this means that the mitochondrial genome as a whole may be affected by the evolution of the different way of inheritance which is entered as DUI is switched on. The coevolution of the mtDNA with the nuclear DUI machinery left phylogenetic signatures that are evident in Figure 5 and Table 1, with special reference to the clues of directional selection we detected at least for six genes (mainly from mitochondrial complex IV).

With the current knowledge about DUI distribution, it is not advisable to draw conclusions about why these genes are specially affected by these selective constraints. A better understanding of DUI distribution will allow a test of our results, avoiding all drawbacks connected to the current sample of DUI systems. The present findings may also prove useful in identifying further DUI species: The detection of an unusually high dN/dS score along a terminal branch makes the relative OTU a good candidate to look for DUI.

With an improved knowledge of DUI distribution, it will be possible to describe the process of the origin of the DUI more properly, a process which can be divided, in our view, into three evolutionary phases. (a) The first step is a point, unpredictable event that triggers the DUI possibly a viral infection by an element able to distort segregation and avoid degradation of sperm mitochondria in embryos (Milani et al., 2016). (b) DUI itself switches on, with all the connected molecular machinery, mostly nuclear encoded and only partly driven by mtDNAs; (c) meanwhile, the corresponding coevolution of mitochondrial genome takes place, which entails a general increase in mutational events (Figures 4 and 5; Table 1) and selective pressures on at least some specific genes.

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