Mutation and Selection on the Wobble Nucleotide in tRNA Anticodons in Marine Bivalve Mitochondrial Genomes

Hong Yu, Qi Li
Fisheries College, Ocean University of China, Qingdao, Shandong, China

Abstract

Background: Animal mitochondrial genomes typically encode one tRNA for each synonymous codon family, so that each tRNA anticodon essentially has to wobble to recognize two or four synonymous codons. Several factors have been hypothesized to determine the nucleotide at the wobble site of a tRNA anticodon in mitochondrial genomes, such as the codon-anticodon adaptation hypothesis, the wobble versatility hypothesis, the translation initiation and elongation conflict hypothesis, and the wobble cost hypothesis.

Principal Findings: In this study, we analyzed codon usage and tRNA anticodon wobble sites of 29 marine bivalve mitochondrial genomes to evaluate features of the wobble nucleotides in tRNA anticodons. The strand-specific mutation bias favors G and T on the H strand in all the 29 marine bivalve mitochondrial genomes. A bias favoring G and T is also visible in the third codon positions of protein-coding genes and the wobble sites of anticodons, rejecting that codon usage bias drives the wobble sites of tRNA anticodons or tRNA anticodon bias drives the evolution of codon usage. Almost all codon families (98.9%) from marine bivalve mitogenomes support the wobble versatility hypothesis. There are a few interesting exceptions involving tRNATrp with an anticodon CCA fixed in Pectinoida species, tRNAVal with a GCU anticodon fixed in Mytiloida mitogenomes, and the uniform anticodon CAU of tRNAMet translating the AUR codon family.

Conclusions/Significance: These results demonstrate that most of the nucleotides at the wobble sites of tRNA anticodons in marine bivalve mitogenomes are determined by wobble versatility. Other factors such as the translation initiation and elongation conflict, and the cost of wobble translation may contribute to the determination of the wobble nucleotide in tRNA anticodons. The finding presented here provides valuable insights into the previous hypotheses of the wobble determination in tRNA anticodons by adding some new evidence.

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Introduction

Animal mitochondrial DNA has two strands of different mutation pressure which leads to differences in base frequencies between the two strands, usually with H strand being GT-rich and L strand being CA-rich [1]. Strand-specific mutation bias has been supposed to be the main force driving and maintaining the codon usage bias [2]. Animal mitochondrial genomes typically have one tRNA for each synonymous codon families, so that each tRNA anticodon essentially has to wobble to recognize two or four synonymous codons. Several factors have been hypothesized to determine the nucleotide at the wobble site of tRNA anticodons in mitochondrial genomes. One of the traditional hypotheses is the codon-anticodon adaptation hypothesis (GAAH), which states that the codon usage bias is a determining factor, and the tRNA anticodon should correlate with codon usage and match the most abundant codon in a synonymous codon family [3,4]. The correlation between codon usage bias and the anticodon of tRNA has been documented in vertebrate mitochondrial genomes [2]. Another traditional hypothesis is the wobble versatility hypothesis (WVH), which argues that the nucleotide at the wobble site of tRNA anticodon should be occupied by a nucleotide which is the most versatile in wobble-pairing [5-7]. For example, for NNY codon families, tRNA anticodons should have G at wobble sites, because G can pair with both C and U in RNA, whereas for NNR and NNN codon families, the wobble sites should be U because of the high versatility of U in wobble-pairing [8-10]. The wobble versatility hypothesis is generally supported in fungal mitochondrial genomes with a few exceptions [5,9]. Xia [10] integrated the two conventional hypotheses mentioned above and developed a new general hypothesis based on wobble cost as WCH. This hypothesis invokes that the wobble cost may reduce the decoding efficiency and accuracy, and the anticodon wobble site of tRNA should be occupied by a nucleotide with low cost of wobble pairing. WCH was tested by 36 fungal mitochondrial genomes and different costs between two kinds of U:G wobble pairs was concluded [10]. In addition, other factors such as possible suppression of stop codons and historical inertia may also
contribute to the determination of the wobble nucleotides in some tRNA anticodons [9].

Up to now, the studies on the overall evolution of wobble positions of tRNA anticodons in mitochondria have been reported on vertebrates and fungi [2,5,9,10]. The publications revealed that in vertebrate and fungal mitogenomes the anticodon wobble positions of tRNAs responsible for decoding NNN and NNR codons families were mostly occupied by U, whereas the majority of tRNAs decoding NNY codons possessed G at the anticodon wobble positions. The vertebrate mitochondrial data were unable to ascertain between the selection hypotheses WVH and CAAH, because both hypotheses had the same predictions for the anticodon wobble sites [2], while the fungal mitochondrial data supported WVH in most cases. Many exceptions to the above rules were also found in both vertebrate and fungal mitochondria. The most notable exception was tRNA^{Met} which had a CAU anticodon in all the vertebrate and fungal mitogenomes, violating both CAAH and WVH in most cases [2,9,11]. In spite of studies on the evolution of tRNA anticodons in vertebrate and fungal mitochondria, there are a few documentations of selection and mutation on tRNA anticodons in invertebrate mitochondrial genomes [12,13]. One of the possible reasons is that the asymmetry in the distribution of the protein-coding genes is various, which hinders the generalization of the mitochondrial tRNA anticodon bias to a certain extent [2]. For example, nine protein-coding genes are collinear with the L strand and four are collinear with the H strand for shrimps, whereas 12 protein-coding genes are encoded on the H strand and only one is encoded on the L strand in sea cucumber. Moreover, the extremely variable number of tRNA genes in invertebrate mitogenomes may also limit an overall study on vertebrates. For example, the mtDNA of Chaetognatha encodes only one tRNA and Demospongiae mitogenomes encodes 2 to 27 tRNAs [14].

The class Bivalvia (Mollusca) includes both marine and freshwater species. It is notable that all marine bivalve mitochondrial genomes available in GenBank encode all the genes including protein-coding genes, tRNA and rRNA genes on the H strand, in contrast to four protein-coding genes collinear with the H strand and nine collinear with the L strand in the freshwater bivalve mitogenomes reported by now [15]. Furthermore, marine bivalve mitogenomes generally have a complete set of tRNA genes, with a few exceptions of losses or duplications of some tRNA genes. Thus, marine bivalve species should be ideal materials among animal taxa including flatworms, brachiopods, echinoderms, arachnids and fishes, have been found to show a reverse strand bias [1,16–20].

A bias favoring G and T is also visible in the third codon positions of protein-coding genes (Table 1), which is consistent with the mutation bias of the strand. In particular, NNN and NNY codon families are dominated by the T-ending codons. The result suggests that codon usage bias is maintained by strand-specific mutation bias, which has also been found in vertebrate mitogenomes [2].

tRNA anticodon bias

Except tRNA^{Met}, almost all the tRNAs have G or U at the wobble sites of anticodons. The wobble nucleotides of tRNA anticodons in marine bivalve mitochondrial genomes show a strong bias towards G and U, which is congruent with the mutation bias of the H strand. This observation seems to support the mutation hypothesis of anticodon evolution rather than the selection hypothesis of anticodon adaption described by Xia [2]. The mutation hypothesis of anticodon evolution argues that the strand-specific mutation pressure is the dominant force in shaping anticodon evolution and the anticodon wobble nucleotide bias should be in accord with the strand mutation bias. Conversely, the selection hypothesis of anticodon adaption contends that selection plays a significant role in shaping codon-anticodon adaption and codon usage bias drives the wobble sites of tRNA anticodons [2]. In other words, the tRNA anticodon should evolve to match the most abundant codon in a synonymous codon family. The vertebrate mitogenome data strongly support the selection hypothesis of anticodon adaption, whereas the marine bivalve mitogenome data reject this hypothesis. The selection hypothesis of anticodon adaption is also effectively ruled out in arthropod and fungal mitogenomes, where it is common that the tRNA anticodon does not match the most commonly used codon, especially for 4-fold degenerate codon families [9,21]. However, we still cannot jump to the conclusion that the strand-specific mutation pressure is the main force driving the evolution of tRNA anticodon for marine bivalve mitogenomes. This is because the evolution of the anticodon wobble site also supports the selection on anticodon versatility, given G and U are known to be more versatile in wobble-pairing than C and A.

Cases supporting WVH

Almost all codon families (98.9%) from the 29 marine bivalve mitogenomes support WVH (Table 1), which is similar to fungal mitogenomes (94.7%) [9]. For example, for the NNY codon families, the wobble nucleotide of the tRNA anticodon is always G in the 29 mitogenomes, while the CAAH would have always predicted A at the wobble site of the anticodon. This implies that the selection at the wobble site must be very strong. In addition, almost all the tRNAs with the exception of tRNA^{Met} decoding NNR codons possess U at the wobble position of the anticodon. The majority of tRNAs decoding NNN codon families have U at the wobble site. These cases suggest that wobble versatility plays an important role in the evolution of tRNA anticodons in marine bivalve mitogenomes.

A few cases supporting CAAH

A few exceptions in which CAAH is supported occur in Pectinoida mitogenomes (Table 1). Four Pectinoida species have the UGR codon family with their associated tRNA anticodons (CGA) supporting CAAH (Figure 1). The UGR codon family with its tRNA anticodon CCA consistent with CAAH was also found in some fungal mitogenomes [9]. Given UGA is a stop codon in
standard genetic code and might have been captured by \( \text{tRNA}^{\text{Tyr}} \) in mitochondria of an ancestral metazoan [22], the historical inertia may be a possible reason for a CCA anticodon remained for the UGR codon family in fungal mitogenomes [9]. However, in 25 other marine bivalve mitogenomes, the anticodon of \( \text{tRNATrp} \) is UCA but not CCA. Among the 25 mitogenomes with the anticodon \( \text{UCA} \), 15 mitogenomes use more codon-UGA than codon-UGG and support both CAAH and WVH, whereas the other ten mitogenomes use more codon-UGG and support WVH. In this case, the historical inertia may not be a good probable explanation, whereas the hypothesis WVH may interpret the observation well. According to WVH, for NNR codon families, only when \( N_A < N_G \), the cost of wobble pairing for T as the wobble nucleotide (\( M_{wT} \)) is larger than that for C as the wobble nucleotide (\( M_{wC} \)), and WVH predicts a C at the anticodon wobble site. In other cases, \( M_{wT} \) is smaller than \( M_{wC} \), and WVH predicts a T at the anticodon wobble site [10]. The observed \( N_A/N_G \) ratios in the UGR codon family range from 0.098 to 0.226 for the four Pectinoida species (Supporting Information Table S1), so \( M_{wT} \) is estimated to be larger than \( M_{wC} \) and C should be favored at the anticodon wobble site. For the 25 other mitogenomes, the \( N_A/N_G \) ratios in the UGR codon family vary from 0.44 to 2.382 (Supporting Information Table S1), with the mean ratio of 1.244, much larger than those in the four Pectinoida mitogenomes. Thus, the anticodon wobble site should favor the use of T because of lower \( M_{wT} \) compared with \( M_{wC} \). That the four Pectinidae mitogenomes possess a wobble C at the \( \text{tRNATrp} \) anticodon and other 25 mitogenomes possess a wobble T confirms the prediction of WVH.

It is intriguing to find that the anticodon CCA of \( \text{tRNATrp} \) only occurs in Pectinoida species among the marine bivalves. According to the phylogenetic analysis, Ostreoida and Pectinoida are reciprocally monophyletic with Mytiloida being sister to Ostreoida+Pectinoida (Figure 1). Although Ostreoida and Pectinoida form

### Table 1. Nucleotide biases of the total genome and protein-coding genes, and number of codon families unambiguously supporting the codon-anticodon adaptation hypothesis (\( N_{\text{CAAH}} \)) and the wobble versatility hypothesis (\( N_{\text{WVH}} \)) in each marine bivalve mitochondrial genome.

| Species | GenBank Accession No. | Total GenBank Accession No. | Total Protein-coding genes | N CAAH | NWVH |
|---------|-----------------------|----------------------------|---------------------------|--------|------|
| Crassostrea angulata | EU672832 | 57.69 | 2 | 0.13 | 0.20 | 61.29 | 0 | 16 |
| Crassostrea ariakensis | EU672835 | 57.79 | 2 | 0.13 | 0.20 | 60.30 | 0 | 15 |
| Crassostrea gigas | AF177226 | 57.70 | 2 | 0.13 | 0.20 | 61.18 | 0 | 16 |
| Crassostrea hongkongensis | EU672834 | 57.28 | 2 | 0.10 | 0.19 | 58.99 | 0 | 12 |
| Crassostrea virginica | HM015198 | 56.61 | 2 | 0.10 | 0.21 | 59.78 | 0 | 14 |
| Crassostrea viridis | FJ841967 | 56.82 | 2 | 0.10 | 0.20 | 59.78 | 0 | 14 |
| Saccostrea mordax | EU672833 | 57.85 | 2 | 0.13 | 0.21 | 61.77 | 0 | 15 |
| Ostrea denselamellosa | EU672835 | 57.79 | 2 | 0.13 | 0.20 | 60.30 | 0 | 15 |
| Sinonovacula constricta | EU880278 | 63.59 | 2 | 0.23 | 0.36 | 70.48 | 0 | 17 |
| Argopecten irradians | EU151999 | 58.34 | 2 | 0.15 | 0.19 | 60.93 | 0 | 16 |
| Mimachlamys nobilis | EU880278 | 63.59 | 2 | 0.23 | 0.36 | 70.48 | 0 | 17 |
| Chlamys farrei | EU715252 | 62.34 | 2 | 0.18 | 0.34 | 67.42 | 1 | 18 |
| Placopecten magellanicus | DQ088274 | 66.57 | 2 | 0.27 | 0.40 | 79.54 | 2 | 18 |
| Meretrix petechialis | EU145977 | 65.02 | 2 | 0.26 | 0.39 | 72.73 | 0 | 17 |
| Meretrix meretrix | NC_013188 | 64.89 | 2 | 0.25 | 0.39 | 72.45 | 0 | 17 |
| Acanthocardia tuberculata | DQ632743 | 58.85 | 2 | 0.18 | 0.17 | 58.51 | 0 | 16 |
| Hiattella arctica | DQ632742 | 59.80 | 2 | 0.15 | 0.29 | 61.12 | 0 | 14 |
| Lucinella discairata | EF043342 | 63.44 | 2 | 0.24 | 0.33 | 64.98 | 0 | 17 |
| Loripes lacteus | EF043341 | 63.29 | 2 | 0.23 | 0.32 | 66.09 | 0 | 19 |
| Venerupis philippinarum F | AB065373 | 60.08 | 2 | 0.13 | 0.36 | 60.31 | 0 | 16 |
| Venerupis philippinarum M | AB065374 | 59.99 | 2 | 0.15 | 0.31 | 62.96 | 0 | 16 |
| Mytilus trossulus M | DQ198225 | 56.47 | 2 | 0.09 | 0.20 | 54.77 | 0 | 14 |
| Mytilus trossulus F | DQ198231 | 58.09 | 2 | 0.11 | 0.25 | 57.98 | 0 | 15 |
| Mytilus galloprovincialis F | NC_006161 | 58.06 | 2 | 0.11 | 0.25 | 57.98 | 0 | 15 |
| Mytilus galloprovincialis F | DQ198231 | 58.09 | 2 | 0.11 | 0.25 | 57.98 | 0 | 15 |
| Mytilus edulis M | NC_006886 | 58.06 | 2 | 0.11 | 0.25 | 57.98 | 0 | 15 |
| Mytilus edulis F | DQ198231 | 58.09 | 2 | 0.11 | 0.25 | 57.98 | 0 | 15 |
| Total | | | 54 | 5 | 454 |

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one clade, the usage of codon UGG is different between the two orders. The usage of UGG is more frequent in the codon UGR family in Pectinoida, whereas it decreases in Ostreoida (Supporting Information Table S1). We refer that a mutation C at the wobble site of tRNATrp anticodon may occur in the common ancestor of Ostreoida and Pectinoida and was selected in Pectinoida which consequently drove the evolution of synonymous codons toward to the maximum of the codon UGG pairing with the anticodon.

The UCN codon family with the tRNAser anticodon AGA in P. magellanicus mitogenome also supports CAAH, whereas all the
other mitogenomes have the UGA anticodon supporting WVH. It is seldom to find an anticodon AGA of tRNA^ser in other animal mitogenomes. It is therefore possible that this predicted anticodon is the result of a sequencing error, and therefore may not represent a true case supporting CAAH.

Exceptional cases in AGN codon family

Most of the marine bivalve mitogenomes have tRNA^ser with a UCU anticodon for AGN codon family supporting WVH. However, in Mytiloida mitogenomes (Figure 1), the anticodon of tRNA^ser becomes GCCU which does not support any available hypothesis. These cases are unlikely sequencing errors. For one thing, six mitogenomes including female-transmitted (F) genomes and male-transmitted (M) genomes of three Mytiloida species all have GCCU as the anticodon. For another thing, tRNA^ser with a GCCU anticodon for the AGN codon family has also been found in different vertebrate mitogenomes, such as Asterias, Loligo, and some arthropod mitogenomes [12,23,24].

For the AGN codons, there have been a number of genetic code changes. The AGN codons, which correspond to Arg in the standard code, have been reassigned to Ser, Gly, and stop codons in different metazoan lineages [22]. Previous work suggested that the reassignment of AGN codons from Arg to Ser could have occurred in a common ancestor of all triploblastic metazoa, and the subsequent changes occurred within deuterostomes [22]. For instance, AGN codons have been reassigned to Gly in urochordates [25], and to stop codon in vertebrate mtDNA [26]. Recently, some special realignments of AGN have also been observed. Abascal et al. [12] found parallel evolution of the genetic code in Arthropod mitogenomes, in which the AGG codon was reassigned between Lys and Ser. In addition, Temperley et al. [27] reported that human mitochondria could avoid AGA and AGG “hungry” codons by frameshifting -1 to result in a UAG stop codon, casting doubts as to whether AGN is a bona fide stop codon. Overall, the AGN codon is labile.

The molecular basis of the AGN codon family with UCU-tRNA^ser can be interpreted by the high versatility of U in wobble-doubts as to whether AGR is a bona fide stop codon. Overall, the codons by frameshifting -1 to result in a UAG stop codon, casting doubts as to whether AGR is a bona fide stop codon. Overall, the AGN codon is labile.

The peculiar phylogenetic distribution suggests that m^G may have been lost early in triploblastic metazoan diversification, and re-acquired independently in several different lineages of echinoderm and mollusk.

Mytiloida species possess an unusual system termed doubly uniparental inheritance of mtDNA (DUI) [15]. Whether the unusual anticodon GCC of tRNA^ser in Mytiloida mitogenomes is related to the unusual inheritance of mtDNA? Besides Mytiloida, the marine bivalve Venusuphis philippinarum was also found to be DUI-possessing organism [15]. However, tRNA^ser-AGN is absent in V. philippinarum mitogenomes, so that it can not confirm the supposition. Six freshwater bivalve species have also been reported to possess DUI [15], but all the six species have the anticodon UCU for tRNA^ser-AGN in both F and M mitogenomes. Hence, the anticodon GCC of tRNA^ser in Mytiloida mitogenomes is not correlated to DUI.

Special cases of tRNA^met anticodon

Among the 29 marine bivalve mitogenomes, six have one tRNA^met gene with the anticodon CAU, 22 have two tRNA^met genes, and P. magellanicus has nine tRNA^met genes (Table 2). Among the 22 mitogenomes with two tRNA^met genes, six Mytiloida mitogenomes have a CAU-tRNA^met gene and a UAU-tRNA^met gene, whereas the other 16 mitogenomes have two CAU-tRNA^met genes. P. magellanicus possesses four CAU-tRNA^met genes and five UAU-tRNA^met genes. It is intriguing to find variable numbers of tRNA^met genes in marine bivalve mitochondrial genomes. It is commonly reported that the number of tRNA^met gene in one group of species is stable. For example, all the vertebrate mitogenomes have only one tRNA^met gene, while all the tunicate mitogenomes have two tRNA^met genes. Two distinct genes for an initiator and an elongator tRNA^met, both with CAU anticodon, have been identified in the phylum Placozoa, the basal metazoan lineage [28,29], and it has been postulated that the elongator and initiator tRNA^met have been lost early in metazoan diversification, and re-acquired independently in the two distant lineages of mollusc bivalves and tunicates [14]. All the available mtDNAs of tunicates have one CAU-tRNA^met gene and one UAU-tRNA^met gene. However, in marine bivalve mtDNAs, single CAU-tRNA^met, duplicated CAU-tRNA^met, one CAU-tRNA^met plus one UAU-tRNA^met, and even nine tRNA^met genes are observed (Table 2). There is no distinct phylogenetic distribution of different numbers of tRNA^met.

The CAU-tRNA^met corresponding to both AUG and AUA codons was found to have 5-formylcytidine (F) at the anticodon wobble position in bovine, nematode and squid mitochondria [30–32], and thus the AUG codon could be recognized. In Drosophila mitochondria, two kinds of CAU-tRNA^met were found, one having N^6-threonylcarbamoyladenosine (t6A) at position 37 and the other having A at the position 37 is expected to play an important role in the decoding of the AUG codon as Met [13]. Among the 29 marine bivalve mitogenomes, 22 mitogenomes have only CAU-tRNA^met genes to decode both AUG and AUA codons. It is likely that tRNA^met (F/C34/A37) or/and tRNA^met (C34/F/A37) may exist in the 22 marine bivalve mitogenomes to stabilize the interaction between anticodon (CAU) and codon (AUA).
CAU-tRNA\textsuperscript{Met} genes. However, whether the mitochondrial translation systems of these seven marine bivalves acquire distinct elongator and initiator tRNA\textsuperscript{Met} genes needs further investigations.

Methionine is coded by both AUA and AUG codons in the 29 marine bivalve mitogenomes, with AUG more frequent than AUA in most cases, so do vertebrate and fungal mitogenomes [2,9]. This raises the question of why the wobble C does not simply mutate to U, making the base modification unnecessary. Xia et al. [11] put forward the translation initiation and elongation conflict hypothesis to explain the unusual usage of the CAU anticodon in tRNA\textsuperscript{Met}. This hypothesis argues that the anticodon CAU would increase the translation initiation rate but decrease the translation elongation rate, because AUG is the most efficient initiation codon, while AUA is usually more frequently used in mitogenomes. There is a conflict between translation initiation and translation elongation. According to this translation conflict hypothesis, AUA should be used relatively less frequently compared to UUA in the UUR codon family, as the anticodon CAU would impose selection against the use of AUA codon. This prediction has been confirmed in fungal mitogenomes by estimating P\textsubscript{AUA} and P\textsubscript{UUA} [11]. In the 29 marine bivalve mitogenomes, the mean P\textsubscript{AUA} value is smaller than the mean P\textsubscript{UUA} value (Table 2), in accordance with the prediction from the translation conflict hypothesis, suggesting a selection force against AUA codon. Seven mitogenomes with both CAU and UAU anticodons are predicted to favor an increased usage of AUA codon. The result confirmed this prediction. The mean (P\textsubscript{UUA}-P\textsubscript{AUA}) value is only -2.91 in the seven mitogenomes, in contrast to the other 22 mitogenomes with only CAU anticodon, where the mean (P\textsubscript{UUA}-P\textsubscript{AUA}) value is 9.53.

In conclusion, most of the nucleotides at the wobble sites of tRNA anticodons in marine bivalve mitogenomes are determined by wobble versatility. There is no evidence that the codon usage bias drives the evolution of tRNA anticodons or the tRNA anticodon bias drives the evolution of codon usage in marine bivalve mitogenomes. There are some unusual tRNA anticodons in marine bivalve mitogenomes, which may be explained by other factors such as the translation initiation and elongation conflict, and the cost of wobble translation.

**Methods**

To date complete mitochondrial genomes of 23 marine bivalve species are publicly available in GenBank, of which four species (Mytilus galloprovincialis, M. edulis, M. trossulus and V. philippinarum) possess doubly uniparental inheritance of mtDNA and have F and M mitochondrial genomes. Twenty seven raw mitogenomes of marine bivalve species mentioned above were downloaded from GenBank. Mitochondrial genomes for two additional marine bivalve species (Crassostrea nippona and Ostrea denselamellosa) were sequenced and added into the analyses. The information of the 29 mitochondrial genomes is shown in Table 1. All the bivalve mitochondrial genomes use genetic code 5.

The protein-coding sequences from each mitochondrial genome were extracted and codon usage quantified by using DAMBE [33]. The tRNA genes were identified by tRNAscan-SE v.1.21 [34] and DOGMA [35] using invertebrate mitochondrial genetic code and compared with the original annotations from GenBank in order to exclude incorrect annotations. Some mitochondrial genomes in GenBank were annotated incorrectly. For example, the tRNA\textsuperscript{Ser} gene for AGN codon family in the mitochondrial genome of Saccostrea mordax locates at the position 6314-6383 with an anticodon UCA, and the tRNA\textsuperscript{Ser} gene for UCN codon family is assigned to the position 6043-6112 with an anticodon of UGA. However, the GenBank file [FJ841968] annotated the tRNA\textsuperscript{Ser} gene for UCN codon family at the position 6314-6383 with an anticodon CGA and omitted tRNA\textsuperscript{Ser} for AGN codon family.

To investigate the nucleotide bias, skew was calculated as (A-T)/(A+T) or (G-C)/(G+C) [36]. The statistical analyses of codon usage bias were conducted according to Carullo and Xia [9]. The values of PN\textsubscript{AUA} were calculated according to equation as described by Xia et al. [11], in order to test whether there is tRNA\textsuperscript{Met}-mediated selection.

Phylogenetic relationships among the marine bivalves were analyzed using concatenated nucleotide sequences from 12 protein-coding genes based on Bayesian inference (BI). Gene ATP8 was excluded from the analysis as most marine bivalve species lack this gene. Two freshwater bivalves *Hyriopsis cumingii* [FJ529186] and *Lampsilis ornate* [AY365193] were used as outgroups. The nucleotide sequences of 12 protein-coding genes were concatenated and aligned using ClustalW2 [http://www.ebi.ac.uk/Tools/clustalw2/index.html] with default parameters. Areas of dubious alignment

| Species                        | tRNA\textsuperscript{Met} anticodon | P\textsubscript{UUA} | P\textsubscript{AUA} | P\textsubscript{UUA-P\textsubscript{AUA}} |
|-------------------------------|-------------------------------------|----------------------|----------------------|----------------------------------------|
| Crassostrea angulata          | CAU/CAU                             | 63.20                | 49.74                | 13.46                                  |
| Crassostrea ariviakensis      | CAU/CAU                             | 62.93                | 56.67                | 6.27                                   |
| Crassostrea gigas             | CAU/CAU                             | 65.27                | 48.98                | 16.29                                  |
| Crassostrea hongkongensis     | CAU/CAU                             | 71.94                | 64.52                | 7.42                                   |
| Crassostrea sikamea           | CAU/CAU                             | 66.67                | 46.07                | 20.59                                  |
| Crassostrea virginica         | CAU/CAU                             | 63.01                | 49.49                | 13.52                                  |
| Crassostrea nippona           | CAU/CAU                             | 70.98                | 64.32                | 6.66                                   |
| Crassostrea iredalei          | CAU/CAU                             | 73.26                | 55.43                | 17.82                                  |
| Saccostrea mordax             | CAU/CAU                             | 69.04                | 59.46                | 9.58                                   |
| Ostrea denselamellosa         | CAU/CAU                             | 66.42                | 49.46                | 16.96                                  |
| Sinonovacula constricta       | CAU/CAU                             | 64.69                | 56.41                | 8.28                                   |
| Argopecten iradians           | CAU                                 | 50.39                | 37.76                | 12.63                                  |
| Mtmachlamys nobilis           | CAU/CAU                             | 57.62                | 50.61                | 7.01                                   |
| Chlamys farrei                | CAU/CAU                             | 60.90                | 58.08                | 2.82                                   |
| Placopeoten magellanicus      | 4CAU/5UA                            | 37.60                | 33.33                | 4.27                                   |
| Meretrix petechialis          | CAU                                 | 56.97                | 54.55                | 2.42                                   |
| Meretrix meretrix             | CAU                                 | 58.65                | 54.55                | 4.11                                   |
| Acanthocardia tuberculata     | CAU/CAU                             | 65.90                | 56.42                | 9.48                                   |
| Hiattella arctica             | CAU/CAU                             | 73.96                | 60.73                | 13.23                                  |
| Lucinella divaricata          | CAU                                 | 56.60                | 55.62                | 0.99                                   |
| Lariopes lacteus             | CAU/CAU                             | 55.71                | 49.71                | 5.99                                   |
| Venerupis philippinarum F     | CAU/CAU                             | 80.05                | 75.40                | 4.65                                   |
| Venerupis philippinarum M     | CAU/CAU                             | 75.24                | 65.73                | 9.52                                   |
| Mytilus trossulus M           | CAU/UAU                             | 68.24                | 72.44                | 4.20                                   |
| Mytilus galloprovincialis M   | CAU/UAU                             | 66.32                | 73.68                | -7.36                                  |
| Mytilus edulis M              | CAU/UAU                             | 69.75                | 69.47                | 0.28                                   |
| Mytilus trossulus F           | CAU/UAU                             | 63.12                | 67.44                | -4.32                                  |
| Mytilus galloprovincialis F   | CAU/UAU                             | 63.70                | 67.62                | -3.92                                  |
| Mytilus edulis F              | CAU/UAU                             | 63.12                | 68.27                | -5.15                                  |
| Average                       |                                    | 64.18                | 57.65                |                                  |

**Table 2.** The effect of anticodons of tRNA\textsuperscript{Met} in 29 marine bivalve mitogenomes on P\textsubscript{UUA} and P\textsubscript{AUA}.
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Supporting Information

Table SI Usage of UGA and UGG in 29 marine bivalve mitogenomes.

Author Contributions

Conceived and designed the experiments: QL, HY. Performed the experiments: HY. Analyzed the data: HY. Contributed reagents/materials/analysis tools: QL. Wrote the paper: HY.