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Short communication

Evolution of the tRNA gene family in mitochondrial genomes of five *Meretrix* clams (Bivalvia, Veneridae)

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ABSTRACT

In contrast to the extreme conservation of nuclear-encoded tRNAs, organization of the mitochondrial (mt) tRNA gene family in invertebrates is highly dynamic and rapidly evolving. While gene duplication and loss, gene isomerism, recruitment, and rearrangements have occurred sporadically in several invertebrate lineages, little is known regarding the pattern of their evolution. Comparisons of invertebrate mt genomes at a generic level can be extremely helpful in investigating evolutionary patterns of variation, as intermediate stages of the process may be identified. Variation of mitochondrial tRNA organization among *Meretrix* clams provides good materials to investigate mt tRNA evolution. We characterized the complete mt genome of the lyrate Asiatic hard clam *Meretrix lyrata*, re-annotated tRNAs of four previously sequenced *Meretrix* clams, and undertook an intensive comparison of tRNA gene families in these clams. Our results 1) provide evidence that the commonly observed duplication of *trnM* may have occurred independently in different bivalve lineages and, based on the higher degree of *trnM* gene similarity, may have occurred more recently than expected; 2) suggest that "horizontal" evolution may have played an important role in tRNA gene family evolution based on frequent gene duplications and gene recruitment events; and 3) reveal the first case of isoacceptor "vertical" tRNA gene recruitment (VTGR) and present the first clear evidence that VTGR allows rapid evolution of tRNAs. We identify the $trnS^{-UCR}$ gene in *Meretrix* clams, previously considered missing in this lineage, and speculate that $trnS^{-UCR}$ lacking the D-arm in both *M. lyrata* and *Meretrix lamarckii* may represent the ancestral status. Phylogenetic analysis based on 13 concatenate protein-coding genes provided opportunities to detect rapidly evolved tRNA genes *via* VTGR and gene isomerism processes. This study suggests that evolution of the mt tRNA gene family in bivalves is more complex than previously thought and that comparison of several congeneric species is a useful strategy in investigating evolutionary patterns and dynamics of mt tRNA genes.

1. Introduction

The mitochondrial (mt) genome of Metazoa is a good model system for evolutionary genomic studies (Gissi et al., 2008). Several features, such as genome size, gene arrangement, gene number and structure, can be easily and systematically investigated in the small mt genome. Among these structural genomic features, tRNAs attract more attention because they move within the genome more frequently than do protein-coding genes (PCGs) and ribosomal RNA genes. In contrast to the extreme sequence conservation of the tRNAs themselves, evolution of the tRNA gene family in the mt genome shows complex patterns characterized by extensive variability, such as gene rearrangement, isomerism, duplication, multiplication and loss/deletion (*e.g.*, Gissi et al., 2008; Littlewood et al., 2006; Stamatis et al., 2008; Wang and Lavrov, 2011). Furthermore, in invertebrates, organization of the mt tRNAs is highly dynamic and rapidly evolving in contrast to the extreme conservation of nuclear-encoded tRNAs (Rogers et al., 2010). Among vertebrates, most rearrangements can be explained by a tandem duplication mechanism (Dowton and Austin, 1999; Macey et al., 1997), but little is known of the mechanism(s) by which the distinct rearrangements in various invertebrate lineages (*e.g.*, Kurabayashi et al., 2008; Yang and Yang, 2008) are generated.

The evolutionary patterns of the tRNA gene family in invertebrates appear to be distinct from those of vertebrate species (Dowton et al., 2003). For example, nematode mt genomes possess extremely truncated tRNAs that lack either the T-arm or the D-arm. Out of 22 tRNAs, 20 tRNAs lack the T-arm and two *trnS* genes lack the D-arm (Dirheimer et al., 1995). This feature inspired further investigation that found that the nematode mt translation system employs a unique EF-Tu factor called EF-Tu1. EF-Tu1 has been shown to bind specifically to T-armless

Abbreviations: *atp6* and *atp8*, ATPase subunit 6 and 8 genes; *cob*, cytochrome *b* gene; *cox1–3*, cytochrome *c* oxidase subunit I–III genes; *nad1–6* and *4L*, NADH dehydrogenase subunit 1–6 and 4L genes; rRNA, ribosomal RNA; *rrnL* and *rrnS*, large and small subunits of ribosomal RNA genes; tRNA, transfer RNA.

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tRNAs, revealing the co-evolution of tRNA and tRNA-binding proteins in nematode mitochondria (Sakurai et al., 2006). tRNA secondary structure changes (*e.g.*, loss and acquisition of the DHU and/or TψC arm of *trnC*, *trnF*, and *trnR*) have also been observed occasionally in several flatworm lineages (Littlewood et al., 2006). In most other cases, however, due to the effort required to sequence an entire mt genome, taxonomic sampling of invertebrates remains relatively poor, such that conclusions are based on relatively few samples. The paucity of data makes inference about the evolutionary mechanisms difficult; the mechanistic relics of tRNAs evolution are likely to have been erased by long intervening periods. For example, tRNA gene duplication or multiplication is sporadically occurring in different invertebrate lineages, yet the general mechanisms controlling tRNA gene multiplication remain unclear. There are several groups of species in which the duplicated tRNA genes occur next (or very close) to the original gene, suggesting that tandem duplication has occurred (*e.g.*, Lavrov and Lang, 2005; Ren et al., 2009). In many cases, however, different isoacceptor tRNAs are scattered within the genome. Several previous studies reported an unexpectedly high similarity between a duplicated tRNA and the other alloacceptor tRNA (Burger et al., 1995; Cedergren et al., 1980; Lavrov and Lang, 2005; Wu et al., 2012b), suggesting that at least some tRNAs could have evolved independently of the genetic code from duplicated genes for alloacceptor tRNAs, a process named "tRNA gene recruitment (TGR)." The importance of TGR in tRNA evolution remains uncertain.

Comparisons of invertebrate mt genomes at low taxonomic levels can be extremely helpful in investigating patterns of variation and evolutionary dynamics, as intermediate stages of the process may be identified. Bivalve mt genomes are highly variable in size, and gene organization is known to vary extensively even among species from the same genus (*e.g.*, Gissi et al., 2008; Wu et al., 2012a; Xu et al., 2012). We recently compared the mt genomes from four *Paphia* clams and found that genome reorganization among the four congeneric species is not random but follows phylogenetic trends (Xu et al., 2012). A novel model of alloacceptor tRNA gene recruitment, named "vertical" tRNA gene recruitment, was used to explain the origin of the extra *trnK* and *trnQ* genes in the mt genomes of five congeneric Asian oysters. We speculated that this recruitment process may be a common phenomenon in the evolution of the tRNA multigene family, but the importance of this process, and whether it is a common phenomenon in tRNA evolution, awaits further investigation (Wu et al., 2012a).

The genus *Meretrix* (Bivalvia, Veneridae) contains five species, namely *M. meretrix*, *M. lusoria*, *M. lamarckii*, *M. petechialis* and *M. lyrata* (Habe, 1977; Zhuang, 2001). These species are widely distributed along the coastal and estuarine areas of the Indian Ocean and Western Pacific Ocean and they have important commercial value (Wang et al., 2010). Complete mtDNA sequences of four *Meretrix* clams were recently reported (Ren et al., 2009; Wang et al., 2010); *M. lyrata* was not sequenced. The mt genome of *M. lyrata* consists of the same 13 PCGs and two rRNAs as other species, but it contains 26 tRNAs, with extra copies of *trnI*, *trnT*, *trnN* and *trnM*. This discrepancy from the canonical tRNA composition is not unique in the *Meretrix* genus. Ren et al. (2009) found that *M. petechialis* has one copy of *trnS* ($trnS^{-UCR}$) but a duplicated *trnQ* gene. We found an un-annotated $trnS^{-AGN}$ gene in *M. meretrix* (GenBank accession number: NC_013188) as well as two extra *trnQ* genes. In *M. lyrata* and *M. lamarckii*, the putative secondary structure of $trnS^{-UCR}$ lacks the D-arm. Furthermore, several tRNA genes are rearranged in comparisons of *M. lyrata* mtDNA to that of the other four clams. These discrepancies, including tRNA gene content, and rearrangements led us to look more closely at *Meretrix* mt tRNA evolution. Therefore, the main objective of this study is to describe the evolutionary pattern of mt tRNA genes among congeneric clams and to explore its possible evolutionary mechanism.

2. Materials and methods

2.1. Specimens, DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from adductor muscles of *M. lyrata* using the TIANamp Marine Animal DNA kit (Tiangen, Beijing). Short fragments from the genes *cox1*, *cob*, *nad5*, *rrnS*, and *rrnL* were amplified by PCR with universal primer pairs designed based on the alignment of the published *Meretrix* mt genome sequences. Based on the sequences of these fragments, long-PCR primers were designed and employed to amplify overlapping segments of the entire mt genome (Supplementary file 1). PCR reactions were performed in a 25 μL volume with 0.5 μL template DNA (approximately 30 ng), 0.5 μL 10 mM dNTP mix, 2.5 μL 10× buffer (Mg^{2+} plus), 1 μL of each primer (10 μM), and 0.25 μL (1 U) of LA Taq polymerase (Takara, Dalian, China). The PCR reactions were performed on an ABI Veriti Thermal Cycler (Applied Biosystems, California, USA) with the following parameters: pre-denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 20 s, 45–55 °C annealing temperature (listed in Supplementary file 1) for 20 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR products were separated by electrophoresis in a 1.0% agarose gel, purified with a QIAquick PCR Purification kit (QIAGEN, California, USA) and bi-directionally sequenced on an ABI 3730xl DNA Sequencer (Applied Biosystems, California, USA). Part of *cob* gene was amplified using another broad-spectrum primer pair (CobF: GGWTAYGTWYTWCCW TGRGGWCARAT; CobR: GCRTAWGCRAAWARRAARTAYCAYTCWGG) which can successfully amplify the *cob* gene fragment in bivalves from different subclasses (Plazzi and Passamonti, 2010) as well as in species employing doubly uniparental inheritance (DUI) heredity system (*e.g.*, Breton et al., 2007). PCR products were TA-cloned and 50 positive clones were randomly selected for sequencing to detect the possible presence of male-transmitted mtDNA in adductor muscles.

2.2. Sequence assembly, annotation and analysis

Sequences were assembled using SeqMan program (DNASTAR, Madison, Wisconsin). Manual examinations ensured correct assembly. PCGs, *rrnL* and *rrnS* genes were identified by comparison with orthologous mt DNA sequences of previously published *Meretrix* clams. tRNA genes were identified by the programs tRNAscan-SE1.21 (Lowe and Eddy, 1997) and ARWEN (Laslett and Canbäck, 2008). Pairwise analysis of genome rearrangements was made with SPRING (Sorting Permutation by Reversals and block-INterchanGes: http://algorithm.cs.nthu.edu.tw/tools/SPRING). Mt genome map was generated by CGView (Stothard and Wishart, 2005). A complete mt genome has been deposited in GenBank under accession number KC832317.

2.3. Phylogenetic analyses

MEGA 5 (Tamura et al., 2011) was used for sequence alignments. Neighbor-joining (NJ) trees based on uncorrected *p* distances among mt tRNA genes from *M. meretrix* (NC_013188), *M. petechialis* (NC_012767), *M. lusoria* (GQ903339), *M. lamarckii* (NC_016174), *M. lyrata* and *Paphia textile* (JF969277) were constructed using MEGA

Fig. 1. (A) Gene content and organization of *Meretrix lyrata* mitochondrial genome. All genes are encoded by the H-strand. Protein coding and rRNA genes are abbreviated as in the text, and transfer RNA genes are depicted by their corresponding one-letter amino acid code. (B) Linear mitochondrial genomic organizations of *M. meretrix* (Mmer), *M. petechialis* (Mpet), *M. lusoria* (Mlus), *M. lamarckii* (Mlam) and *M. lyrata* (Mlyr). Putative evolutionary pathway of tandem duplication and random loss and the inferred intermediate gene order are shown between *M. lyrata* and *M. lamarckii*. tRNA gene duplication, isomerism, and recruitment are marked as letters D, I, and R, respectively.