# The complete mitochondrial DNA of $c$ mitogenomic analyses of three Arcidae species 

# and comparative 

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## A R T I C L E I N F O

## A $C$

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#### Abstract

To better understand the characteristics and the evolutionary dynamics of mt genomes in Arcidae, the complete mitochondrial genome of was firstly determined and compared with other two Arcidae species ( $c \quad c \quad$, and $c \quad c \quad 1 \quad$ ). The complete mitochondrial genome of . was $31,589 \mathrm{bp}$ in length, including 12 protein-coding genes, 2 rRNA genes and 23 tRNA genes, and a major noncoding region. Three tandem repeat fragments were identified in the major non-coding region and the tandem repeat motifs of these fragments can be folded into stem-loop structures. The mitochondrial genome of the three species has several common features such as the AT content, the arrangement of the protein-coding genes, the codon usage of the protein-coding genes and AT/GC skew. However, a high level of variability is presented in the size of the genome, the number of tRNA genes and the length of non-coding sequences in the three mitogenomes. According to the phylogenetic analyses, these mitogenome-level characters are correlated with their phylogenetic relationships. It is the absence of the duplicated tRNAs and large non-coding sequences that are responsible for the length divergence of mitogenomes between . and other two Arcidae species. The phylogenetic analyses were conducted based on 12 partitioned protein genes, which support the relationship at the family level: (((Pectinidae + Ostreidae $)+$ Mytilidae $)+$ Arcidae $)$.


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## 1. Introduction

The typical metazoan mitochondrial ( mt ) genome is a covalently closed circular molecule ranging in size from 14 to 20 kb , encoding 37 genes: 13 protein genes, 22 transfer RNAs and two ribosomal RNAs (Boore, 1999). However, as more and more animal mt genome sequences are determined, an increasing number of animal mtDNAs seem to deviate from this model substantially, and many of them are much larger than 20 kb . At present, many studies have found atypically large mtDNA chains of varying size in the newts (Wallis, 1986), fishes (Gach and Brown, 1997; Richardson and Gold, 1991), lizards (Moritz and Brown, 1986), insects (Boyce et al., 1989) and nematode 1 ' $, ~ c, c$ (Hyman et al., 1988; Hyman and Slater, 1990; Powers et al., 1986). These molecules gained their large size in various ways. Tandem duplications of control region sequences were found to be the main reason of size increases

[^0]in mitogenomes of newts and fishes, whereas the duplications of coding sequences are responsible for the large size of lizard mitogenomes. The large size of mtDNA of the nematode, . c c , is due to many dispersed repeats in the molecular. In bivalves, the mitochondrial genomes of relatively large size (greater than 20 kb ) have been found in the deep sea scallop $c \quad c \quad$ 1 $\quad c$ (up to $40,725 \mathrm{bp}$ ) (Smith and Snyder, 2007) and the Zhikong scallop ।
(21,695 bp) (Xu et al., 2011). The repeated sequences, transposition involving tRNAs or tRNA-like sequences and duplications in the non-coding regions are responsible for the large size of these mitogenomes (Liu et al., 2013; Smith and Snyder, 2007). It was also observed that the mitogenome of Manila clam (AB065375) is larger than 20 kb . A peculiar way of mitochondrial inheritance, doubly uniparental inheritance (DUI), has been found in .

I, which may also influence the genome size in M and F mitochondrial DNA (Passamonti and Scali, 2001; Breton et al., 2007; Ghiselli et al., 2013; Zouros, 2013). The female type is $22,676 \mathrm{bp}$ (GenBank accession number AB065375) and the male type is $21,441 \mathrm{bp}$ (GenBank accession number AB065374).

The Arcidae have a high species richness in the tropical shallow waters and warm temperate seas, comprising about 260 species (Oliver and Holmes, 2006). Two complete mitochondrial genomes were recently made available for this family: $\quad$ c (AB729113) and
c c I (KF750628). Interestingly, the mt genomes of these two Arcidae species deviate the most dramatically from the size of typical metazoan mtDNAs, with a length of 46,985 bp (Liu et al.,

Table 1
Partitioned data for phylogenetic analyses and DNA substitution models applied to each (Bayesian analyses only, RAxML only applies GTR $+G$ for any partition).

| Partition | Alignment length (bp) | DNA substitution models |
| :---: | :---: | :---: |
| c 1 | 1373 | GTR + I + G |
| d 5 | 814 | $\mathrm{GTR}+\mathrm{I}+\mathrm{G}$ |
| d 4 | 674 | GTR $+\mathrm{I}+\mathrm{G}$ |
| d 1 | 646 | $\mathrm{GTR}+\mathrm{I}+\mathrm{G}$ |
|  | 860 | GTR $+\mathrm{I}+\mathrm{G}$ |
| c 2 | 459 | GTR $+\mathrm{I}+\mathrm{G}$ |
| d 6 | 252 | GTR $+\mathrm{I}+\mathrm{G}$ |
| 6 | 375 | HKY + G |
| d 3 | 108 | HKY + G |
| d 4 | 203 | GTR $+\mathrm{I}+\mathrm{G}$ |
| c 3 | 437 | HKY + G |
| d 2 | 281 | GTR $+\mathrm{I}+\mathrm{G}$ |

2013) and $46,713 \mathrm{bp}$ (Sun et al., 2014), respectively. Several special features have been evidenced in the two mitochondrial genomes: (i) . .. and . , have the largest mtDNAs in all metazoan mt genomes sequenced to date; (ii) more than 40 tRNA genes are identified from the two mt genomes, being the largest number of tRNA discovered in metazoan mtDNAs; and (iii) the percentage of non-coding sequences of these two mtDNAs is much larger than that of other metazoan mtDNAs. The sequence duplications in the noncoding regions have been considered one of the factors responsible for the large size of the two mt genomes. Considering the unusual features of the two mt genomes in Arcidae, we attempt to validate whether these characteristics are shared by other species of Arcidae. In the present study we have determined the third complete mt genome of the Arcidae species, c , and compared it with those of . .. and . , . This species is phylogenetically close to . and . . (Oliver and Holmes, 2006; Matsumoto, 2003). The investigation of . mitochondrial genome supplies the gene feature requirements for the comparative analysis of mitochondrial genome of Arcidae. Moreover, this study provided effective information to understand the characteristics and the evolutionary scenarios of mt genomes in Arcidae, explaining the cause of the length differences among Arcidae mt genomes based on the genomic structure. Additionally, in this study, we inferred the phylogenetic topologies with the concatenated nucleotide sequences of 12 protein-coding gene datasets to get insights into the phylogenetic relationship among Arcidae species and the phylogenetic position of Arcidae within Bivalvia.

## 2. Materials and methods

2.1. $C l, A \quad c, \quad 1 \quad f i c \quad d \quad c$

Two live . individuals were collected from the coastal water of Wenzhou, Zhejiang Province, China. Total genomic DNA of . was extracted from adductor muscle by a modification of standard phe-nol-chloroform procedure as described by Li et al. (2002). Short fragments of the genes $c 1$ were amplified by PCR with primers LCO-1490/HCO-2198 (Folmer et al., 1994). Other short fragments, d 2 ,
$d 3, d 4, d 4, d 5, c$ and $c 2$ were obtained from ESTs of (Bao and Lin, 2010). Based on the obtained partial sequences, long-PCR primers were designed to amplify the entire mitochondrial genomes in five long PCR reactions (Supplementary Table 1). PCR were carried out in $50 \mu \mathrm{l}$ reaction mixtures containing $8 \mu \mathrm{l} 2.5 \mathrm{mM}$ dNTPs, $5 \mu \mathrm{l} 10 \times$ reaction buffer $\left(\mathrm{Mg}^{2+}\right.$ plus), $1 \mu \mathrm{l}$ genomic DNA, $1 \mu \mathrm{l}$ $10 \mu \mathrm{M}$ each primer and $0.5 \mu \mathrm{LA}$-Taq polymerase. PCR procedures for all the long fragments were: $94^{\circ} \mathrm{C}$ for 3 min followed by 35 cycles of $94^{\circ} \mathrm{C}$ for $30 \mathrm{~s}, 62{ }^{\circ} \mathrm{C}$ for 30 s and $68{ }^{\circ} \mathrm{C}$ for 10 min . A final extension step of $72{ }^{\circ} \mathrm{C}$ for 10 min was added. PCR products were purified using EZ-10 spin column DNA gel extraction kit (Sangon Biotech), and then directly sequenced with the primer walking method. The sequencing
was conducted on an ABI PRISM 3730 (Applied Biosystems) automatic sequencer.

After sequencing the regions between $d 4$ and $c 3$, we used the genome-walking based method to obtain the whole mitogenome sequence. The primary PCR procedures were performed following the methods described in the BD GenomeWalker Universal Kit User Manual (BD Biosciences). The primer sequences used for genome-walking are presented in Supplementary Table 2. All the PCR amplicons were sequenced using the methods described above.

$$
\text { 2.2. } A \quad c d \quad d
$$

All sequence data were analyzed and arranged to create the full genome using the Seqman program from DNASTAR (http://www. DNASTAR.com). Manual examinations were applied to ensure correct assembly. To define protein coding genes, ORF Finder (http://www. ncbi.nlm.nih.gov/gorf/gorf.html), BLASTx (http://blast.st-va.ncbi.nlm. nih.gov/Blast.cgi?PROGRAM=blastx\&PAGE_TYPE=BlastSearch\&LINK_ LOC=blasthome) and HMMER web server (Finn et al., 2011; Eddy, 2011) were used. Signal peptides (SPs) were identified using InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/, Zdobnov and Apweiler, 2001) and Phobius (http://phobius.sbc.su.se/, Käll et al., 2004). TMpred (http://www.ch.embnet.org/software/TMPRED_form. html, Hofmann and Stoffel, 1993) and Phobius (http://phobius.sbc.su. se/, Käll et al., 2004) were used to localize putative transmembrane helices (TM-helices). @TOME 2 (http://atome.cbs.cnrs.fr/AT2B/meta.html, Pons and Labesse, 2009) was used to find similarities with known proteins. The tandem repeat sequences were searched by Tandem Repeats Finder 4.0 (Benson, 1999). Prediction of potential secondary structure was performed by the online version of the mfold software, version 3.2 (Zuker, 2003). The tRNA genes were identified by tRNA-scan SE Search Server (Lowe and Eddy, 1997) and ARWEN (Laslett and Canback, 2008), using the mito/chloroplast genetic code and the default search mode. Codon usage analysis was performed by MEGA 5 (Tamura et al., 2011). The $\mathrm{A}+\mathrm{T}$ content values and nucleotide frequencies were computed using Editseq program from DNASTAR. The GC and AT skews


Fig. 1. Gene map of the mitochondrial genome of transcribed from the plus strand. The largest non-coding region is designated as

## 2.3.

A total of 16 taxa were selected for phylogenetic analysis, including the complete mt sequences of . (this study). The representative species we chose for each taxonomic group were: (AB729113) and , (KF750628) (Arcida)

| $729113)$ and . <br> c (AY905542), | (KF750628) (Arcidae), <br> d (FJ841967) and cc |
| :---: | :---: |
| (FJ841968) (Ostreidae), | c (AB271769), |
| (EU715252), A | d (EU023915), l c |

(FJ415225) and . I . c (DQ088274) (Pectinidae),
d (AY484747), $\quad$ (AY497292),
(GU936625) and , c. (GQ527172) (Mytilidae). Ic 1... (HM347668) from the subclass Palaeoheterodonta was used as outgroup.

The twelve-partitioned nucleotide sequences of protein coding genes were aligned with MAFFT (Katoh et al., 2005). Areas of dubious
were calculated according to the formulae by Perna and Kocher (1995): AT skew $=(\mathrm{A}-\mathrm{T}) /(\mathrm{A}+\mathrm{T})$; GC skew $=(\mathrm{G}-\mathrm{C}) /(\mathrm{G}+\mathrm{C})$. The mitochondrial genome was initially generated with the program CGView (Stothard and Wishart, 2005). The complete mtDNA sequence of was deposited in the GenBank database under accession number (KJ607173).

Table 3
List of total size, AT content, AT and GC skew for , with length of genes and NCR.

| Gene | . .. | . 1 , | . |
| :---: | :---: | :---: | :---: |
| Total size | 46,985 | 46,713 | 31,589 |
| \%AT | 67.89 | 62.75 | 60.18 |
| AT skew | -0.1657 | -0.0990 | -0.1353 |
| GC skew | 0.3574 | 0.1066 | 0.4127 |
| NCR | 31,658 | 32,982 | 16,394 |
|  | 1324 | 1332 | 1343 |
|  | 673 | 675 | 641 |
| c 1 | 1584(ATG/TAG) | 1575(ATA/TAA) | 1578(ATG/TAG) |
| d 5 | 1707(ATA/TAA) | 1002(ATG/TAA) | 1716(ATA/TAA) |
| d 1 | 924(ATA/TAG) | 747(ATA/TAG) | 795(ATA/TAG) |
| d 4 | 1296(ATA/TAA) | 804(ATA/TAA) | 1227(ATG/TAA) |
|  | 1278(GTG/TAA) | 1080(ATA/TAA) | 1221(ATA/TAG) |
| c 2 | 666(ATG/TAA) | 666(ATG/TAG) | 1227(ATG/TAA) |
| d 6 | 468(ATA/TAA) | 408(ATG/TAG) | 492(ATG/TAA) |
| 6 | 525(ATA/TAA) | 675(ATG/TAA) | 648(ATG/TAG) |
| d 3 | 360(ATA/TAA) | 246(ATG/TAG) | 402(ATG/TAG) |
| d 4 | 285(ATG/TAA) | 252(ATA/TAA) | 252(ATA/TAG) |
| c 3 | 759(GTG/TAA) | 537(ATG/TAA) | 903(ATA/TAA) |
| d 2 | 567(ATA/TAG) | 801(ATA/TAA) | 1170(ATG/TAG) |

remaining 10,000 sampled trees were used to estimate the $50 \%$ majority rule consensus trees and the Bayesian posterior probabilities.

## 3. Result and discussion

3.1. $\quad d \quad 1 \quad A c d$

The mt genome of .
contains 12 protein-coding genes, two rRNAs, 23 tRNAs and non-coding regions, with all the 37 genes transcribed from the plus strand (Fig. 1, Table 2). The arrangement of the 12 protein-coding genes in . is identical to that of and . $\quad 1 \quad$, in the order $c$ 1- d5- d 1$d 4-c-c 2-d 6-6-d 3-d 4-c 3-d 2$, corresponding evidently to their closer genetic relationships. However, the location and the number of the tRNA genes of . are different from those of the other two Arcidae genomes (Fig. 2). Mt-gene order appears to be dramatically variable in the major groups of bivalves. Pectinidae species seem to be a good example to prove this. Comparing gene orders of . , A. d , . and . I $c$, even after excluding the tRNA genes from the comparison, the four mt genomes still show no identical gene arrangement (Xu et al., 2011). In bivalves, it is also common that species belonging to the same genus have different gene orders. For example, in congeners . . c and the six Asian , only protein-coding gene is arranged in an identical order, but tRNAs are extensively rearranged (Wu et al., 2010). The relatively conserved gene order in the three Arcidaes suggests that the divergence of the species may have occurred recently. The complete mitochondrial genome of .
is $31,589 \mathrm{bp}$ in length, and is far
less than those of the other two Arcidae species,
( $46,985 \mathrm{bp}$ ) and . , ( $46,713 \mathrm{bp}$ ) (Table 3). However, it is still an uncharacteristically large mitochondrial genome. Such differences may be mainly resulted from the variable number and size of non-coding regions (NCRs). The length of the non-coding regions found in the mt genome of .
( $16,394 \mathrm{bp}$ ) is markedly shorter than that of . . ( $31,658 \mathrm{bp}$ ) and . . ( $32,982 \mathrm{bp}$ ). Furthermore, in the mt genomes of . ... and . , , most of the non-coding DNAs were observed within two distinct zones. One lies between $c 2$ and $d 6$, and the other lies between $d 2$ and c 1. Unlike . and . , . . has only one concentrated non-coding region, located between $d 2$ and $c \quad 1$, the same position of one of the two non-coding segments in .
and . $\quad 1 \quad$ (Fig. 2). Even so, some protein-coding genes in . , such as $c \quad 2, \quad d 3, c \quad 3$ and $d 2$, are much longer than in c c species (Table 3). There are 3877 codons for the 12 proteincoding genes of . with the stop codons excluded, compared with 3473 and 2931 for . .. and . . . We attempt to explore whether the divergence of the mitogenomes among Arcidae species is phylogenetically based or owing to speciesspecific mitogenome maintenance mechanisms (Xu et al., 2012). According to the phylogenetic framework, we argue that genome reorganization among these congeneric species is not random, but correlated with their phylogenetic relationships (Fig. 2).

The base compositions and the AT contents of the mitogenomes of the three Arcidae species have been shown in Table 3. The overall AT content of . mt genome is $60.18 \%$, the lowest among the three Arcidae species (. , 67.89\% and . $62.75 \%$ ). The nucleotide compositions are all strongly skewed away from C in favor of G (the GC-skews are from 0.107 to 0.413 ) and from A in favor of T (the AT-skews are from -0.166 to -0.100 ).

## 3.2. $-c d$

In total, 12 protein-coding genes (PCGs) were identified in the mitogenome of . . The 8 gene was not identified, as apparently is the case of most marine bivalve species studied so far (Yu et al., 2008). However, several exceptions have been found. For example, 1 c 1 (Wu et al., 2013), (Wang et al., 2010), and c. c (Dreyer and Steiner, 2006) show an

8 gene in their mtDNA. All these 12 PCGs start with conventional invertebrate start codons, with 7 PCGs using ATG and the other 5 PCGs employing ATA. Compared the initiation codon usage of the three Arcidaes, we found that almost all PCGs of the Arcidae mitogenomes initiated with ATG and ATA, and only one alternate initiation codon, GTG, which was detected in $c 3$ gene of . . The alternate initiation codon GTG was also discovered in mitochondrial genomes of other bivalves, such as ( $d 4$ and $c$ 3: GTG) (Xu et al., 2012) and $c \quad d, c \quad(d 4$ and : GTG) (Yuan et al., 2012). Two PCGs contain the same initiation codons in all three


Fig. 3. Pairwise genetic divergence among the three Arcidaes based on separate and concatenated protein coding genes.


Fig. 4. Putative secondary structure of the 23 tRNA genes predicted based on the

Arcidae species ( $d$ 1: ATA; $c \quad 2$ : ATG) and four genes used the same termination codon ( d 5: TAA; d 1: TAG; d 4: TAA; c 3: TAA). Comparing the usage of start and stop codon, we found that they were random cases unrelated to the phylogenetic relationships of Arcidae. Similar to the other two Arcidae species, UUU is the most frequently used codon in . (7.9\%).

Pairwise divergence among the three Arcidae mitogenomes was calculated based on the separate 12 protein-coding genes (Fig. 3). The c 1 gene showed the smallest divergence among the 12 proteincoding genes (average divergence: 0.212 , Kimura two-parameter distance; K2P), while d 2 gene revealed the largest divergence (average divergence: $0.553, \mathrm{~K} 2 \mathrm{P}$ ), which supported the existence of different mutation constraints among genes. In most metazoans, the genes coding for the three subunits of the cytochrome $c$ oxidase and cytochrome had a higher degree of conservation ( $>0.750$ ) than the NADH dehydrogenase genes (Saccone et al., 1999). 0TD(Fan54uanC-173)-9.3e15(h)042.Fan54uanotens(v)14(e3.3(n)0(c)2yan)-25w.8(v)14.6(s.4(e)97.9(a)0(1)2)25(s)102


Fig. 6. Stem-loop structures of the tandem repeat motif in the MNR of .
mt genome. A) The first stem-loop structure of the tandem repeat motif with 132 bp . B) The second stemloop structure of the tandem repeat motif with 129 bp . C) The last stem-loop structure of the tandem repeat motif with 132 bp .
$53.64 \%, 63.74 \%$ and $52.74 \%$, respectively. These results showed that the and are stable among Arcidae groups.
In . $3^{\prime}$ end boundary of identification, a 19 bp-long sequence was used, which can be folded into a small stem-loop structure (5-bp stem, 9-bp loop). Furthermore, this structure was similar to the $3^{\prime}$ terminus stem-loops (4-bp stem, 7-bp loop) found in .
and
(Fig. 5). As for the , its boundaries were tentatively defined immediately adjacent to the genes, $A-$ and $A$ $A$. Boundaries were identified by sequence similarity to . and . , . In a wide range of organisms, a heptamer TGGCAGA( N$)_{5} \mathrm{G}$ box is well-conserved near the $3^{\prime}$ end of , which is regarded as the signal of the mitochondrial rRNA transcription termination (Valverde et al., 1994). In bivalves, this motif is present in the $5^{\prime}$ end of the 8 in $\left.\right|_{\quad,}$, which is adjacent to the
(Wang et al., 2011). The same situation also occurs in . (Wang et al., 2010) and . . ( AB065375). But this motif was not found in the mt genomes of Arcidae species. Although the putative boundaries of . and have been found, these cannot be precisely determined until transcript mapping is carried out.

## 3.4. $-c d$

As mentioned above, the variable lengths of the non-coding regions (NCRs) in the three mt genomes are the source of their different mt genome sizes. In total, $16,394 \mathrm{bp}$ of the . mt genome was predicted to be non-coding sequence, accounting for $51.9 \%$ of the entire mtDNA. This percentage of non-coding mtDNA is much lower than those of
(67.4\%) and .
(70.6\%). Within these non-coding sequences, an $11,848 \mathrm{bp}$ nucleotide segment was putatively identified as the major non-coding region (MNR), which contained three distinct tandem repeat units (24,693-25,037, 26,23526,953 and $30,292-30,877$ ). They were $344 \mathrm{bp}, 718 \mathrm{bp}$ and 585 bp in length, respectively. The first repeat family contained two identical copies and a third copy with a $60 \%$ length of a 132 -base sequence. The second one had a $129-\mathrm{bp}$ repeat motif and five identical copies and a sixth copy with a $57 \%$ length of the repeat motif were found. The last one comprised four identical copies and a fifth copy with a $50 \%$ length of a 130 bp sequence. Furthermore, each tandem repeat motif of the MNR formed five secondary structures with stem-loop when the sequence is folded to minimize the free energy of the structure (Fig. 6), supporting the view that the stem-loop structures play an important part in sequence duplications in animal mtDNA (Stanton et al., 1994; Wilkinson and Chapman, 1991). A large number of reports have described tandem repeats in the non-coding regions of mitogenomes of metazoan (Lunt
et al., 1998; Rand, 1993; Wilkinson et al., 1997; Yokobori et al., 2004). Tandem repeat units have also been regarded as a common feature for mitogenomes of bivalves. For example, they have been described in the mt genome of . (Xu et al., 2011) .d. c (Yuan et al., 2012) and $\quad$. (Meng et al., 2012). Smith and Snyder (2007) have also found repeated sequences in the mt genome of .I c and the repeat units of the non-coding regions are associated with tRNA or pseudo-tRNA structures. This feature was also

Table 4
Signal peptide and transmembrane helix prediction in the ORFs.

| ORF | Signal peptides |  | Transmembrane helices |  |
| :---: | :---: | :---: | :---: | :---: |
|  | InterProScan | Phobius | TMpred | Phobius |
| 01 | - | - | - | - |
| 02 | 1-31 | - | 27-44/55-72 | 27-48/60-79 |
| 03 | - | - | 26-45/55-73 | 20-44/51-73 |
| 04 | 1-31 | - | 19-41/55-74 | 24-46/58-76 |
| 05 | - | 1-34 | 4-21/80-97 | 81-99 |
| 06 | - | - | 35-51 | 33-51 |
| 07 | - | - | 25-40 | 25-42 |
| 08 | - | - | 37-61 | 41-65 |
| 09 | - | - | 7-22 | - |
| 10 | - | - | 22-37 | 21-40 |
| 11 | - | - | 18-35 | 16-35/42-59 |
| 12 | 1-21 | 1-21 | 1-17 | - |
| 13 | 1-50 | 1-50 | - | - |
| 14 | - | - | 23-52 | 23-53 |
| 15 | - | - | - | - |
| 16 | - | - | 13-37/32-52 | 12-30 |
| 17 | - | - | 1-18/11-41 | 12-33 |
| 18 | - | - | - | - |
| 19 | - | - | 26-41 | 24-41 |
| 20 | 1-22 | 1-22 | 3-23 | - |
| 21 | - | - | - | - |
| 22 | 1-21 | 1-21 | 1-17 | - |
| 23 | - | - | 15-30 | 12-33 |
| 24 | - | - | 13-29 | 6-28 |
| 25 | - | - | - | - |
| 26 | - | - | - | - |
| 27 | 1-23 | 1-23 | - | - |
| 28 | - | - | - | - |
| 29 | - | - | - | - |
| 30 | - | - | 6-22 | 6-32 |
| 31 | - | - | - | - |
| 32 | - | - | 7-32 | 6-32 |

Note-Signal peptides: Only signal peptides statistically supported (Phobius posterior label probability $>0.5$, significance test not provided by InterProScan) are shown; Transmembrane helices: Only transmembrane helices considered significant (TMpred score $>500$; Phobius posterior label probability $>0.5$ ) are shown.
reported in the mt genome of brachiopod
(Endo et al.,
2005). However, this characteristic was not found in .

Among the large non-coding sequences of . , a total of 32 open reading frames for polypeptides were found, ranging in size from 34 to 1641 amino acids. No significant amino acid sequence similarity was detected between the . ORFs and known proteins or any of the ORFs found in other mtDNAs. Of the 32 ORFs, 6 had both signal peptide (SP) and transmembrane helix (TM-helix), 17 had either of them, and the remaining nine contained neither SP nor TM (Table 4). In the 6 ORFs, SPs and TM-helices shared the same region. The signal peptides (SPs) are peptide chains of hydrophobic amino acids, so it's difficult for software to discern them from TM-helices (Käll et al., 2004).

Dramatic change was shown when comparing the non-coding regions of . to those of the other two Arcidae species, which was discussed above. Such structural divergence is reasonable and consistent with the previous finding that the non-coding sequence was the most changeable segment, followed by tRNA genes and PCGs (Cunha et al., 2009; Shao et al., 2006). We favor the hypothesis that this structural divergence may be generated by sequence duplications. The emergence of two large non-coding segments in . ... and 1. could be explained reasonably if we suppose that the ancestor of the two $c \quad c$ species had the segment as . cur-

Pectinidae appears as sister to this group. In our study, according to the phylogenetic tree, we can clearly see that Ostreidae and Pectinidae have the closest relationship, with Mytilidae being the sister taxon. Arcidae is well supported to be the sister group to the ((Pectinidae + Ostreidae) + Mytilidae) clade. This result suggests that the family Arcidae diverges early and is positioned at the base of the ((Pectinidae + Ostreidae) + Mytilidae) clade, which is incompatible with the previous viewpoints based on short fragments of nuclear gene or mtDNA.

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## Appendix A. Supplementary data

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[^0]:    A : 6, ATPase subunit 6 genes; , cytochrome b gene; $c$ 1-3, cytochrome coxidase subunits I-III genes; NCR, non-codingregion; d 1-6and d4,NADHdehydrogenase subunits 1-6 and 4L genes; ML, maximum likelihood; rRNA, ribosomal RNA;
    and , large and small subunits of ribosomal RNA genes; tRNA, transfer RNA; PCG, protein coding gene; K2P, Kimura two-parameter distance; TDRL, tandem duplication and random loss; mtDNA, mitochondrial DNA; mt genome, mitochondrial genome; MNR, major non-coding region; $\mathrm{Sb}, \mathrm{c}$ c .; Sk, c c $\quad$, Tg, ${ }^{c}$

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