Complete mitochondrial genome of *Cultellus attenuatus* and its phylogenetic implications

Haikun Li (✉ 1157474258@qq.com)  
Ocean University of China Fisheries College  
Ruihai Yu  
Ocean University of China Fisheries College  
Peizhen Ma  
Institute of Oceanology Chinese Academy of Sciences  
Chunhua Li  
Ocean University of China Fisheries College

Short Report

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Abstract

The complete mitochondrial genome of *Cultellus attenuatus*, a new aquaculture species, was sequenced and compared with mitogenomes from seven species of Heterodonta bivalve mollusk in GenBank. The mitochondrial genome of *C. attenuatus* is 16888 bp in length and contains 36 genes, including 12 protein-coding genes, 2 ribosomal RNAs and 22 transfer RNAs, and all genes are encoded on the same strand. In comparison with *C. attenuates*, the mitochondrial genes of *Sinonovacula constricta* from the same family were not rearranged, but those of six other species from different families were rearranged to different degrees. The largest noncoding region of *C. attenuatus* is 1173 bp in length and has an A + T content of 68.24%, located between nad2 and trnK. The results of phylogenetic analysis show that *C. attenuates* and *S. constricta* belonging to Cultellidae cluster into one branch while two species of Solenidae (*Solen grandis* and *Solen strictus*) cluster as their sister taxa. In conclusion, we used the mitochondrial genome data to demonstrate the closest relationship between *C. attenuatus* and *S. constricta* in Heterodonta. These data not only contribute to the understanding of the phylogenetic relationship of Heterodonta but also serve as a resource for the development of genetic markers in aquaculture.

Introduction

Most animals’ mitochondrial DNA is a closed circular molecule independent of nuclear DNA, ranging in size from 14 to 17 kb. It contains 37 genes, including 13 proteins, 2 ribosomal RNAs, and 22 transfer RNAs [1]. The mitochondrial genes are quite conserved in replication, featuring matrilineal inheritance, no rearrangement and high substitution rates [2]. Many characteristics make mitochondrial genes valuable and have become a powerful source in genetic evolution, phylogeographic analysis, and species identification [3–5].

In bivalve mollusks, *Mytilus edulis* was the first to obtain complete mitochondrial genetic information [6], and mitochondrial genes were subsequently obtained in other categories, including oysters [7], scallops [8], clams [9] and razor shells [10]. Although mitochondrial genetic resources of bivalves are increasingly being developed, they are limited to only some species with high economic value. For niche classes, molecular information is scarce [11]. In the species of the Cultellidae family, only the mitochondrial genes of *Sinonovacula constricta* have been determined, while those of the other species, including *Cultellus attenuates*, have not been determined, which has been very unfavorable for phylogenetic research in this family [12, 13].

*C. attenuatus* mainly inhabits the shallow sea area of the subtidal zones, and can be found in Japan, South Korea, the Philippines and China (mainly from Liaoning in the north to Taiwan in the south)[14]. *C. attenuatus* has high edible value due to its nutritional composition of low fat and high protein, which makes it increasingly popular among Chinese consumers [15]. In this way, the high market value of *C. attenuatus* gradually increases its artificial catching, while the quantity of wild resources decreases [14].
China, artificial breeding of *C. attenuatus* has been carried out in an attempt to restore diminishing wild resources by introducing hatchery-produced seeds. The study of mitochondrial genes can be applied to produce genetic markers that can monitor the restoration of aquatic animal stocks, which is quite helpful for the restoration and conservation of wild populations\(^3,16-17\). Therefore, it is necessary to obtain the mitochondrial gene sequence of *C. attenuatus*. However, no relevant reports have been reported thus far. In this study, we obtained and analyzed the entire mitochondrial gene of *C. attenuatus* and compared the mitochondrial gene with other family species from Heterodonta. The data not only contribute to our understanding of phylogenetic status but also serve as a source for the development of useful genetic markers in aquaculture.

**Materials And Methods**

Sample collection and DNA extraction

Lived *C. attenuatus* was collected from Bohai Bay (Dongying City, Shandong Province, China). Total genomic DNA was extracted from the adductor muscle of an individual *C. attenuatus* by a DNA extraction kit.

PCR and DNA sequencing

PCR amplification of the cox1 and rrnL genes was performed with the primer sets LCO1490/HCO2198 and rmLAR-L/rmLAR-H\[^{13}\], respectively. PCR was performed in 50 µl volumes containing 0.5 units Taq polymerase (Takara), 1×PCR buffer, 0.2 mM of each dNTP, 1 µM of each primer, 1.5 mM of MgCl\(_2\) and 50 ng of genomic DNA. The PCR cycle was as follows: 94 ºC for 2 min, then 35 cycles of 94 ºC for 30 s, 40 s at 52 ºC and 1 min at 72 ºC, followed by a final extension at 72 ºC for 5 min.

Based on the above two sequences, two sets of long-PCR primers LB-L/LB-H and LC-L/LC-L, were used for long-PCR amplification\[^{13}\]. Long-PCR was performed in 25 µL volumes containing 1×GC buffer I, 1.25 U of LATaq (Takara), 0.5 mM of each dNTP, 0.4 µM of each primer and 50 ng of genomic DNA. The PCR conditions were as follows: 94 ºC for 2 min, 30 cycles of 94 ºC for 20 s, 1 min/kb at 68 ºC and a final extension at 72 ºC for 10 min. The PCR products were sequenced by Personal Biotechnology Co. Ltd. Qingdao.

Analysis of sequence data

The protein-coding and ribosomal RNA genes were identified by their similarity to published gene sequences through BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/). Transfer RNA was identified by tRNAscan-SE v.1.21 \[^{18}\] and DOGMA \[^{19}\] using the invertebrate mitochondrial genetic code. CGView was used to map the whole mitochondrial genome circle \[^{20}\].

Phylogenetic analysis
To clearly show the phylogenetic relationship of Heterodonta, the mitochondrial gene sequences of 18 species from Heterodonta were obtained from Genbank. *Chlamys farreri* and *Mimachlamys nobilis* were used as distinct outgroups. The amino acid sequences of 12 proteins (except atp8) were adopted for phylogenetic analysis. The amino acid sequences of 12 mitochondrial proteins were aligned by MEAG 7.0. The phylogenetic relationships of heterodont bivalves were reconstructed by neighbor-joining (NJ), and 1000 bootstraps were used for estimation.

**Results And Discussion**

Genome organization and nucleotide composition

The mitochondrial genome of *C. attenuates* is 16,888 bp in length and contains 12 protein-coding genes, 22 transfer RNA genes, and 2 ribosomal RNA genes (Fig. 1). Compared with the genome size of the sequenced mollusk mtDNAs, the mitochondrial genome size was within the normal range. In addition, four overlaps were detected in the mitochondrial genome, with sizes of 1 bp, 6 bp, 1 bp and 3 bp respectively, among which the overlap between trnE and trnS2 was the largest (Table 1). Of the whole mitogenome of *C. attenuates*, its A + T content was 66.46% (Table 2), comparable to that of *S. constricta* (67.00%) and *Solen grandis* (64.84%). In addition, the mitochondrial gene length of *C. attenuates* is 376 bp shorter than that of *S. constricta* in the same family [22] and 104 bp longer than that of *S. grandis* in different families [10].
Table 1
Organization of the mitochondrial genome of *Cultellus attenuates*

| Feature | Position   | Length (bp) | Initiation condon | Stop condon | Anticodon | Intergenic nucleotide |
|---------|------------|-------------|-------------------|-------------|-----------|----------------------|
| trnK    | 1–66       | 66          |                   |             | TTT       | 64                   |
| cox2    | 131–968    | 838         | ATG               | T(AA)       |           | 74                   |
| trnY    | 1,043 – 1,106 | 64        |                   |             | GTA       | 9                    |
| nad4l   | 1,116-1,406 | 291         | ATG               | TAA         |           | -1                   |
| trnG    | 1,406-1,472 | 67          |                   |             | TCC       | 8                    |
| trnP    | 1,481-1,546 | 66          |                   |             | TGG       | 103                  |
| nad4    | 1,650-3,020 | 1371        | ATT               | TAA         |           | 13                   |
| trnH    | 3,034 – 3,097 | 64        |                   |             | GTG       | 2                    |
| trnW    | 3,100-3,166 | 67          |                   |             | TCA       | 11                   |
| trnR    | 3,178-3,242 | 65          |                   |             | TCG       | 27                   |
| trnE    | 3,270-3,334 | 65          |                   |             | TTC       | -6                   |
| trnS2   | 3,329-3,391 | 63          |                   |             | TGA       |                      |
| nad3    | 3,392-3,757 | 366         | ATG               | TAG         |           | -1                   |
| trnT    | 3,757-3,823 | 67          |                   |             | TGT       |                      |
| trnI    | 3,824-3,890 | 67          |                   |             | GAT       | 5                    |
| trnD    | 3,896-3,962 | 67          |                   |             | GTC       |                      |
| trnQ    | 3,963-4,029 | 67          |                   |             | TTG       | 25                   |
| trnC    | 4,055 – 4,120 | 66        |                   |             | GCA       | 25                   |
| trnA    | 4,146-4,210 | 65          |                   |             | TGC       | 28                   |
| trnF    | 4,239-4,302 | 64          |                   |             | GAA       | 149                  |
| cox1    | 4,452-6,140 | 1689        | ATT               | TAA         |           | 11                   |
| trnL2   | 6,152-6,218 | 67          |                   |             | TAA       | -3                   |
| nad1    | 6,216-7,145 | 930         | ATA               | TAG         |           | 2                    |
| trnL1   | 7,148-7,214 | 67          |                   |             | TAG       | 10                   |
| trnV    | 7,225-7,289 | 65          |                   |             | TAC       | 12                   |
| trnN    | 7,302-7,367 | 66          |                   |             | GTT       |                      |
| Feature | Position       | Length (bp) | Initiation codon | Stop codon | Anticodon | Intergenic nucleotide |
|---------|----------------|-------------|------------------|------------|-----------|-----------------------|
| nad5    | 7,368-9,128    | 1761        | ATT              | TAG        | 38        |                       |
| cob     | 9,167 – 10,312 | 1146        | ATG              | TAG        | 7         |                       |
| nad6    | 10,320 – 10,850| 531         | ATG              | TAG        |           |                       |
| rrnL    | 10,851 – 12,087| 1237        |                  |            |           |                       |
| atp6    | 12,088 – 12,786| 699         | ATG              | TAA        | 15        |                       |
| trnM    | 12,802 – 12,867| 66          |                  |            | CAT       | 100                   |
| rrnS    | 12,968 – 13,794| 827         |                  |            |           |                       |
| cox3    | 13,795 – 14,583| 789         | ATG              | TAG        | 6         |                       |
| trnS1   | 14,590 – 14,656| 67          |                  |            | TCT       |                       |
| nad2    | 14,657 – 15,715| 1059        | ATG              | TAA        | 1173      |                       |
Table 2
Base composition of the mitochondrial genome, protein-coding genes and ribosomal RNA genes

| Region     | A%  | C%  | G%  | T%  | A + T% | G + C% | AT skew | GC skew |
|------------|-----|-----|-----|-----|--------|--------|---------|---------|
| Whole genome | 23.54 | 10.61 | 22.94 | 42.91 | 66.46 | 33.54 | -0.291 | 0.368   |
| cox2       | 25.89 | 10.26 | 26.13 | 37.71 | 63.60 | 36.40 | -0.186 | 0.436   |
| nad4l      | 21.65 | 7.56  | 24.40 | 46.39 | 68.04 | 31.96 | -0.364 | 0.527   |
| nad4       | 18.75 | 8.17  | 24.07 | 49.02 | 67.76 | 32.24 | -0.447 | 0.493   |
| nad3       | 18.85 | 8.20  | 22.40 | 50.55 | 69.40 | 30.60 | -0.457 | 0.464   |
| cox1       | 20.25 | 12.97 | 23.92 | 42.87 | 63.11 | 36.89 | -0.358 | 0.297   |
| nad1       | 19.14 | 9.57  | 24.73 | 46.56 | 65.70 | 34.30 | -0.417 | 0.442   |
| nad5       | 18.57 | 10.39 | 23.74 | 47.30 | 65.87 | 34.13 | -0.436 | 0.391   |
| cob        | 20.68 | 12.13 | 21.38 | 45.81 | 66.49 | 33.51 | -0.378 | 0.276   |
| nad6       | 20.90 | 8.10  | 23.35 | 47.65 | 68.55 | 31.45 | -0.390 | 0.485   |
| rrnL       | 32.66 | 10.83 | 19.48 | 37.03 | 69.68 | 30.32 | -0.063 | 0.285   |
| atp6       | 20.89 | 10.87 | 21.17 | 47.07 | 67.95 | 32.05 | -0.385 | 0.321   |
| rrnS       | 35.07 | 13.30 | 20.31 | 31.32 | 66.38 | 33.62 | 0.056  | 0.209   |
| cox3       | 21.04 | 12.67 | 23.70 | 42.59 | 63.62 | 36.38 | -0.339 | 0.303   |
| nad2       | 20.68 | 10.48 | 23.70 | 45.14 | 65.82 | 34.18 | -0.372 | 0.387   |

Protein-coding genes and codon usage

The mitochondria of *C. attenuates* contain 12 protein-coding genes, which, range in size from 291 bp to 1761 bp and, have a total length of 11470 bp, accounting for 67.92% of the total length of the genes (Table 1). There were two starting codons in these 12 protein-coding genes, ATG for 7 protein-coding genes (cox2, cox3, cob, atp6, nad2, nad3 and nad6), ATT for 3 protein-coding genes (cox1, nad4 and nad5) and ATA for 2 protein-coding genes (nad41 and nad1), which differed from the initial codon composition of the protein-coding genes in the mtDNA of *S. grandis*. Except for cox2, all 11 protein-coding genes had complete termination codons, including TAA (N = 5) and TAG (N = 6). The A + T content of all the protein-coding genes exceeds 60%, indicating that the protein-coding genes prefer AT.

Of the entire mitochondrial genome, the values of AT skew and GT skew of the entire mitochondrial genome are -0.291 and 0.368, respectively, suggesting that the entire mitochondrial gene is biased toward T and G, a phenomenon also found in some other bivalves. All 12 protein-coding genes were similar to the whole genome, with a negative AT skew and a positive GT skew, showing a bias toward T and G (Table 2).
Ribosomal RNAs and transfer RNAs

Similar to most bivalves, the mitochondrial genomes of *C. attenuates* contain 22 transfer RNA genes and 2 ribosomal RNA genes. The size of 22 tRNA genes varies from 63 bp to 67 bp, and all of them can be folded into typical secondary structures. The two ribosomal RNA genes include rrnL and rrnS — the former is 1237 bp, located between nad6 and atp6, and the latter is 827 bp located between trnM and cox3 (Table 1). The content of A + T in rrnL was 69.68%, which was the highest of all protein-coding genes and rRNA genes. The A + T content of rrnS was 66.38%, which was slightly lower than the average level of the whole mitochondrial genome. The contents of AT skew and GC skew of rrnL are −0.063 and 0.285, respectively, which is similar to the protein coding gene and has a bias toward T and G. The contents of AT skew and GC skew of rrnS are 0.056 and 0.209, respectively, showing a bias toward A and G, which is different from other genes in the whole mitochondrial genes (Table 2).

Noncoding regions

The mitochondrial genome of the whole *C. attenuates* contains 24 noncoding regions, ranging in size from 2 bp -1173 bp, with a total length of totaling 1917 bp, accounting for 11.35% of the entire mitochondrial genome. Four of the 24 noncoding regions were larger, with sizes greater than or equal to 100 bp, which were 103 bp (trnP-nad4), 149 bp (trnF-cox1), 100 bp (trnM-rrnS) and 1173 bp (nad2-trnK). The largest noncoding region (NCR) is 1173 bp in length and has an A + T content of 68.24%, located between nad2 and trnK. In addition, in the largest NCR, a sequence (15884 bp -15974 bp) with low A + T content (53.85%) was found, which is considered to be the origin of L-strand replication. The remaining sequence of the largest NCR is 914 bp in length, with an AT content of 69.80%, and is identified as a putative control region. Compared with the largest NCR of the *C. attenuates*, the largest NCR of *S. constricta* in the same family is at the same position (nad2 – trnK) with a slightly larger size (1492 bp) and lower A + T content (66.89%)[22]. The largest NCR positions and sizes of *C. attenuates* varied considerably compared with the other 6 species of different families (Table 3), indicating the highly rearranged gene order in bivalves.
Table 3
The comparison of noncoding regions (NCRs) within the eight mitochondrial genomes of Heterodonta

| Species                  | No. of NCR | Total length (bp) | Proportion of the mt genome (%) | Largest NCR | Lenth (bp) | A + T % | Location       |
|--------------------------|------------|-------------------|--------------------------------|-------------|-----------|---------|----------------|
| *Paphia euglypta*        | 28         | 2431              | 13.04                          |             | 1774      | 65.80   | nad4 l-trnI    |
| *Acanthocardia tuberculata* | 24         | 1751              | 9.60                           |             | 1103      | 59.11   | trnaM-trnaH    |
| *Hiatella arctica*       | 30         | 2558              | 14.05                          |             | 614       | 66.10   | trnaA-atp8     |
| *Solen grandis*          | 27         | 1699              | 10.12                          |             | 435       | 66.44   | trnN-trnA      |
| *Sinonovacula constricta*| 25         | 2134              | 12.39                          |             | 1492      | 66.89   | nad2-trnK     |
| *Culpellus attenuates*   | 24         | 1917              | 11.35                          |             | 1173      | 68.24   | nad2-trnK     |
| *Lucinella divaricata*   | 31         | 3825              | 20.20                          |             | 1050      | 69.24   | trnC-trnL2     |
| *Solecurtus divaricatus* | 22         | 1160              | 6.93                           |             | 775       | 65.81   | rmS-trnM       |

Gene arrangement

One species was selected from six families and one superfamily of Heterodonta, and its mitochondrial gene was compared with the gene arrangement of *C. attenuates* (Fig. 2). The mitochondrial genes of *S. constricta* and *C. attenuates* were exactly in the same order. For mitochondrial genes, species in different families have a large degree of rearrangement, while species in the same family tend to have a small degree of rearrangement, and species in the same genus even have almost no rearrangement [8, 24]. Therefore, the mitochondrial genes of *S. constricta* and *C. attenuates* were not rearranged, further confirming the closer relationship between the two species [12]. The same phenomenon was also found in this comparison of mitochondrial gene arrangement. There were only three gene blocks, nad5-cob, rmL-atp6-rrnS-cox3 and nad4 l-nad4, shared between *S. grandis* and *C. attenuates*. However, the remaining five species from different families shared fewer gene blocks, indicating a greater rearrangement. This enormous gene rearrangement in bivalves is associated with single-stranded coding, because double-stranded coding tends to inhibit gene rearrangement compared to single-stranded coding [25].

Phylogenetic analysis
With the development of mitochondrial genome research, an increasingly number of researchers have conducted phylogenetic research on species through mitochondrial genes, but to date, there has been relatively little information about the mitochondrial genome of heterodonts\(^{10,11}\). In this study, the phylogenetic tree was reconstructed through the amino acid sequence of the mitochondrial protein gene of heterodont bivalves (Fig. 3). Two species of Lucinidae clustered into a single clade, and other species clustered into a large clade containing three small clades, similar to previous studies\(^{11,26}\). \textit{Paphia euglypta, Venerupis philippinarum, Meritrix meretrix} and \textit{Meritrix petechialis} clustered together, supporting their genetic relationship within Veneridae\(^{26}\). \textit{Moerella iridescens, Sanguinolaria diphos, Sanguinolaria olivacea, Semele scaba} and \textit{Solecurtus divaricatus} belonging to the superfamily Tellinoidea clustered together, consistent with previous studies\(^{22}\). The new finding of this study is that \textit{C. attenuates} had the closest relationship with \textit{S. constricta}, which further determines that \textit{C. attenuates} and \textit{S. constricta} belong to the same family (Cultellidae)\(^{12}\). \textit{S. grandis} and \textit{Solen strictus} had a close relationship, with \textit{C. attenuates} and \textit{S. constricta} being the sister taxa.

**Declarations**

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**Consent for publication**

All authors read and approved the final manuscript.

**Availability of data and material**

The mitochondrial genome sequence is available from Gene Banks\(\text{MW653805}\).

**Code availability**

There is no relevant code.

**Authors' contributions**
Ruihai Yu designed and supervised the project, and reviewed drafts of the paper. Peizhen Ma provided technical assistance and collected the samples. Chunhua Li provided technical assistance. Haikun Li wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript before they read and approved the final manuscript.

**Compliance with Ethical Standards**

**Conflicts of interest/Competing interests**

The authors declare that they have no conflicts of interest.

**Ethics approval**

The present study was performed according to the standard operation procedures (SOPs) of the Guide for the Use of Experimental Animals of the Ocean University of China. All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

**Consent to participate**

This article does not contain any studies with humans

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**Figures**
Figure 1

The complete genome circle of mitochondria in Cultellus attenuates. From inside to outside, the first circle represents the scale; the second circle represents the GC skew; the third circle represents GC content; and the fourth and fifth circles represent the arrangement of protein-coding genes, tRNA genes and rRNA genes on the genome.
Figure 2

Linear representation of the mitochondrial gene arrangement in eight species of heterodonta. The bars indicate identical gene blocks.
Figure 3

Phylogenetic trees derived from neighbour-joining (NJ) of concatenated amino acid sequences of 12 protein-coding genes