Complete Mitogenome of Threespot Flounder Grammatobothus polyophthalmus (Pleuronectiformes: Bothidae) and Study on the Mechanism of Gene Rearrangement in 13 Bothids

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Abstract

Background: The mitochondrial genomes (mitogenomes) of 12 bothids (Pleuronectiformes) from eight genera have been obtained. From the data, the genomic-scale and various gene rearrangements revealed the high diversity of variation in these mitogenomes.

Results: A total of 18170 bp of Grammatobothus polyphthalmus mitogenome was determined including 37 genes and two control regions (CRs). Genes encoded by L-strand were grouped to an eight-genes cluster (Q-A-C-Y-S1-ND6-E-P) except for the tRNA-N, other genes encoded by H-strand were grouped together (F-12S ... CytB-T) except for the tRNA-D that was translocated to inside of the eight-genes cluster. The mitogenome of G. polyphthalmus and that of 12 known bothids possessed the similar genomic-scale rearrangements with the only differences in the various combinations of CR, tRNA-D and eight-genes cluster, and the shuffling of tRNA-V. Based on the structure character of all 13 bothid mitogenomes, the Dimer-Mitogenome and Non-Random Loss (DMNR) model was fitted to account for all these rearrangements. And the translocation of tRNA-D occurring after the DMNR process in 10 of 13 bothid mitogenomes was confirmed. The striking finding was that each of degenerated genes existing in the gene rearrangement process in 13 bothids had their counterparts of intergenic spaces.

Conclusions: The result of corresponding relationship between degenerated genes and intergenic spaces provided the significant evidence to support the possibility of the DMNR model, as well as, the existing of dimeric mitogenome in mitochondrion. The findings of this study were rare phenomenon in teleost fish, which not only promoted the understanding of mitogenome structural diversity, but also shed light on studying of mitochondrial rearrangement and replication.

Background
A typical fish mitochondrial genome (mitogenome) contains 37 genes consisting of 13 protein-coding genes, two ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) genes. Among these genes, most are encoded on the heavy strand (H-strand), only ND6 and eight tRNA genes (N, Q, A, C, Y, S1, E and P) are encoded on the light strand (L-strand).

Additionally, two main non-coding regions are also existed, including the origin of replication of L-strand (O_L) as well as the control region (CR) where both of the replication origin of H-strand (O_H) and the transcriptional initiation of two strands are located [1-3].

Three types of gene rearrangement were observed in mitogenome of animals, including shuffling, translocation and inversion [4-7]. Before the gene inversion in tongue fish was firstly discovered [8], only the first two of above gene rearrangement types were reported in fishes [9-11]. Since then, an increasing number of rearranged mitogenomes of flatfishes featuring three rearrangement types have been found [12-16]. Among them, one representative case was mitochondrial rearrangement of blue flounder Crossorhombus azureus [14]. In this mitogenome, genes were grouped with identical transcriptional polarities, including eight genes on L-strand grouped to a cluster (8-cluster, Q-A-C-Y-S1-ND6-E-P) except for the tRNA-N, and other genes (F-12S ... CytB-T) on H-strand were grouped together. Particularly, the order of these genes grouped in each strand was maintained as that in non-rearranged mitogenome of fish, except for the site of tRNA-D. Furthermore, unlike the typical position of CR in fish, CR of this species located between tRNA-D and tRNA-Q, which separated the genes on H-strand and L-strand.

How did this particular order of mitogenome emerge? Four mechanisms had been proposed to account for the gene rearrangements of mitogenome, including duplication-random loss [17], tRNA miss-priming model [18], intramitochondrial recombination [19] and duplication-nonrandom loss [20]. However, none of these four mechanisms could
explain the rearrangement case occurred in C. azureus well. Therefore, a novel mechanism of Dimer-Mitogenome and Non-Random Loss (DMNR) was put forward to specially account for this rearrangement [14]. The inferred DMNR process started from the mitogenome with ancestral gene order in typical fish (Fig. 1A). First, the dimerized event of two-monomers mitogenomes accidently occurred and formed a functionally dimeric molecule linked head-to-tail (Fig. 1I-C). At this stage, the transcription of genes on dimeric mitochondrial DNA (mtDNA) could be normally initiated by the H-strand promoters (HSP and HSP') and the L-strand promoters (LSP and LSP') in two CRs. The transcription on H-strand would terminate at TAS and TAS' in CRs, and that on L-strand particularly at tRNA-L1 and tRNA-L1'. Subsequently, the function of promoters (assumed to be LSP and HSP) in one CR was lost, and the genes controlled by them could not be transcribed, and then these genes degenerated as non-coding sequences or even disappearance. Whereas, the function of promoters (assumed to be LSP' and HSP') in the other CR still worked, the genes controlled by them could be transcribed, and formed the final gene order in the mitogenome of C. azureus (Fig. 1I-D and I-E).

During this process, the only exception is the transcription of tRNA-N and tRNA-N'. The tRNA-N would not be transcribed but be transcribed and retained, and the tRNA-N' would be transcribed but not be transcribed and then lost. The reason for this exception is the retained tRNA-N related to the structure and function of O_L. The O_L is usually located between tRNA-N and tRNA-C of WANCY region that is form by tRNA cluster of tRNA-W, tRNA-A, tRNA-N, tRNA-C and tRNA-Y [21]. Because tRNA-C and tRNA-Y were rearranged, only a 7-bp intergenic space was left between tRNA-N and COI in which failed to form the second structure of the O_L. But 26-bp middle portion of tRNA-N could form an O_L-like structure (Fig. 2A), therefore, tRNA-N was inferred to act as the function of O_L during L-
strand replication [14, 22]. Additionally, one unsure event occurred in C. azureus mitogenome is when did the tRNA-D translocate from a site between tRNA- \( S_1 \) and COII to between tRNA-T and CR? Based on the feature of mitogenome and process of rearrangement, Shi speculated that the translocation of tRNA-D could occur either before or after the DMNR process as shown in Fig. 1I-B and I-E [14].

So far, mitochondrial genomes of 12 bothid species from eight genera were obtained. Comparing these genomes, the gene orders among all genomes are same, except for the shuffling tRNA-V (Fig. 1IV-B2), the number of CR, and the relative position of CR, tRNA-D and 8-cluster. Further analysis showed that the combination of the tRNA-D, CR and 8-cluster (equal with 5-cluster of Q-A-C-Y-\( S_1 \) plus 3-clusterof ND6-E-P) existed four patterns in 12 bothid mitogenomes (Fig. 1), including pattern A: D-CR-8-cluster (C. azureus, Crossorhombus kobensis and Crossorhombus valderostratus), pattern B: CR-5-cluster-D-3-cluster (Arnoglossus polyspilus, Bothus myriaster and Chascanopsetta lugubris), pattern C: CR-5-cluster-D-3-cluster-CR (Arnoglossus tenuis, Laeops lanceolata, Lophonectes gallus and Psettina iijimae), and pattern D: 5-cluster-D-3-cluster-CR (A. intermedius and Bothus pantherinus). Besides the mentioned rearrangement in C. azureus, the only determined mechanism for gene rearrangement in 12 known bothid mitogenomes was that of B. myriaster (Fig. 1A, II-C, D, F and B). In this mitogenome, Gong (2016) also used the DMNR model to explain the process of gene rearrangement, but different with the tRNA-D of C. azureus translocated to the outside of eight-genes cluster, that of B. myriaster was translocated to the inside of eight-genes cluster (Fig. 1II-B). Whether this translocation occurred before or after the DMNR process was not mentioned [12].

From the data, the genomic-scale and various gene rearrangements revealed the high diversity of variation in 12 bothid mitogenomes. Therefore, to better understand the character of mitochondrial structure of bothids, the mitogenome of threespot flounder
Grammatobothus polyophthalmus was determined in this study. This species is one of few bothids featuring one lateral line on both sides of the body. What are mitogenomic characteristics of this species; and whether did rearrangement occur in this mitogenome, if so, what is the rearrangement type? All these questions were addressed in this study, and the result would reveal more mitochondrial diversity in Bothidae, and provide scientific foundation for further research in mitochondrial rearrangement of fish.

Results

Organization and gene rearrangement of the G. polyophthalmus mitogenome

A total of 18170 bp in length of G. polyophthalmus mitogenome contained 37 genes, including 13 protein-coding genes, two rRNA genes and 22 tRNA genes. Among these genes, 28 genes were encoded on the H-strand, others of ND6 and eight tRNA genes (N, Q, A, C, Y, S1, E and P) on the L-strand (Additional file 2: Table S2 and Additional file 3: Figure S1). In this mitogenome, after tRNA-C and tRNA-Y were rearranged, a 40-bp intergenic space was left between tRNA-N and COI. The second structure of O_L was formed with 38 bp of this intergenic space and 4 bp of posterior part of tRNA-N (Fig. 2E). This O_L had same sequence motif 5’-TAGA-3’ on 3’ end of arm as that of other 12 bothid species (Fig. 2). But the sequence 5’-GGTGG-3’ of 5’ arm of O_L in G. polyophthalmus was slightly different with both of the motif 5’-GGGGG-3’ in most bothids (Fig. 2F-M) and that of 5’-GCCGG-3’ in most of 17 flatfishes from seven families [23].

Additionally, two large non-coding regions (NC1 and NC2) were found, one of 773 bp located between tRNA-T and tRNA-Q, and the other of 1611 bp between tRNA-P and tRNA-F. Comparing with the conserved structures of the CRs in 12 bothid species and ridged-eye flounder Pleuronichthys cornutus, the similar conserved structures in two NCs of G. polyophthalmus were identified, including the terminal associated sequences (TAS) with
the core sequence ACAT-cTGTA; the conserved sequence blocks (CSB) of central conserved domain, CSB-F, E, D, C and B; the Pyrimidine tract of T; and the other two conserved sequence blocks, CSB-1 and 2 (Fig. 3). Additionally, NC2 had 35 repeating units of 22 bp at the 3' end (Additional file 4: Figure S2). Therefore, based on the conserved structures of two NCs and the repeated unit of NC2, it can be determined that both NC1 and NC2 of *G. polyophthalmus* are control regions named as CR1 and CR2, respectively (Fig. 1III-B and Fig. 3).

Comparing with the mitogenomes of 12 bothid species, the gene order of *G. polyophthalmus* was identical to that of four of these species, *A. tenuis*, *L. gallus*, *L. lanceolata*, and *P. iijimae*. How did genomic-scale rearrangement and duplicated CRs generate in these five species? Although the mitogenomes of above four bothids have been reported for years, the mechanism of their gene rearrangement remains unaddressed. Here, using the *G. polyophthalmus* mitogenome as a representative, the generated process of gene rearrangement was conjectured via DMNR model based on the similar gene orders with that of *C. azureus* and *B. myriaster* mitogenomes.

This process began from the typical mitogenome of fish (Fig. 1A). Firstly, the dimerized event occurred and formed a functionally dimeric mtDNA (Fig. 1III-C). And then the function of the promoters (assumed to be LSP and HSP) in one of CRs was lost, thus the genes controlled by the disabled promoters of LSP and HSP could not be transcribed and then degenerated as non-coding sequences or even disappearance (Fig. 1III-D). Consequently, the genes \((P', E', ND6', S_1', Y', C', A' \text{ and } Q')\) transcribed from LSP' were clustered together forming the eight-genes' cluster\("Q'-A'-C'-Y'-S_1'-ND6'-E'-P'"\), and the other genes \((F, 12S, V \ldots ND5, CytB \text{ and } T)\) transcribed from HSP' were grouped (Fig. 1III-F). Except *tRNA-N*, genes with identical transcriptional polarity were placed together separated by two CRs via the DMNR process. Finally, the gene *tRNA-D* was translocated
from the site between COI and COII to between tRNA-S1 and ND6, and formed the nine-genes cluster “Q-A-C-Y-S1-D-ND6-E-P” (Fig. 1III-B).

Gene-rearrangement mechanism of 13 bothids mitogenome

In summary, the structures of 13 bothids have the same gene order except for the four different combinations of the tRNA-D, CR and 8-cluster, and the shuffling tRNA-V. So far, three types (Type I, II and III) of mitochondrial rearrangement have been accounted for the processes of gene rearrangement in 11 bothid mitogenomes (Fig. 1I, II and III). Based on the similar gene order of all 13 species, the DMNR model was also adopted to speculate the rearrangement process of B. pantherinus and A. intermedius mitogenomes (Fig. 1A, IV-C, D, F, B1 and B2). These two species shared same rearrangement process except for the only difference in the tRNA-V in A. intermedius (Fig. 1IV-B2) shuffling from the location between 12S and 16S (12S-V-16S-L1) to that between 16S and tRNA-L1 (12S-16S-V-L1).

Different from D process of other three types of rearrangement, besides the loss function of LSP and HSP, the whole of CR1 was also degenerated in Type IV (Fig. 1IV-D). Thus, only one CR was possessed in B. pantherinus and A. intermedius mitogenomes (Fig. 1IV-B1 and B2).

Discussion

Comparison of tRNA-D translocation in four types of rearrangement processes

Comparing the structures of 13 bothid mitogenomes, the position of tRNA-D has two sites: outside (Fig. 1I-E) or inside (Fig. 1II-B, III-B, IV-B1 and IV-B2) of the eight-genes cluster. According to the mechanism of mitochondria rearrangement of C. azureus, the translocation of tRNA-D outside of the cluster could occur before or after the DMNR process (Fig. 1I-B and I-E). As for the case of tRNA-D inside the cluster, if the tRNA-D firstly translocated, it would be between ND5 and ND6 of dimeric mtDNA (Fig. 1II-B′), and
then, \textit{tRNA-D} will be outside the cluster between \textit{ND5} and \textit{CytB} after the DMNR process (Fig. 1II-F’). To the location inside the cluster, this gene needed one more translocation (Fig. 1II-B). Whereas, if the translocation of the \textit{tRNA-D} occurred after the DMNR process (Fig. 1II-B), only this step needed to accomplish the final structure. Therefore, the parsimonious way for the translocation of \textit{tRNA-D} inside the cluster is this translocation occurring after, not before, the DMNR process.

The exception of \textit{tRNA-N} and \textit{tRNA-N’} in DMNR processes

For inferred location of each rearrangement gene during DMNR process in 13 bothid mitogenomes, the only exception is those of \textit{tRNA-N} and \textit{tRNA-N’} that \textit{tRNA-N} should be degenerated but be left and \textit{tRNA-N’} should be left but be degenerated. In mitogenome of \textit{C. azureus} [14], the reason for the retain of the \textit{tRNA-N} was considered that this gene acted as the function of \textit{O}_L due to failing to form the \textit{O}_L structure based on few bases of the intergenic space left between \textit{tRNA-N} and \textit{COI} (Fig. 2A). Comparing this intergenic space in other 12 bothids, two situations of shorter one (12–14 bp) and longer one (47–55 bp) were found. As same as that in \textit{C. azureus} mitogenome, the shorter intergenic space in \textit{A. intermedius}, \textit{C. kobensis} and \textit{C. valderostratus} (Fig. 2B-D) could also not form \textit{O}_L directly, but the middle partial sequence of \textit{tRNA-N} could form an \textit{O}_L-like structure. However, the longer intergenic space in those of other nine species (Fig. 2E-M) adding with only 3–5 bp from 3’ end of \textit{tRNA-N} could form the \textit{O}_L. Therefore, the \textit{tRNA-N} with the longer intergenetic spaces was speculated to assist the functioning of the \textit{O}_L according to the opinion of Seligmann and Krishnan (2006)[22]. Accordingly, the \textit{tRNA-N} plays an important role in forming or assisting of \textit{O}_L functioning. And the finding of the relationship between the \textit{tRNA-N} and the \textit{O}_L in these bothids offers important information for further researching the feature of \textit{O}_L in mitochondrial rearrangement, and it also contributes
foundational works for studying on mitochondria replication.

The evidence for the DMNR model

Several intergenic spaces were found in *G. polyophthalmus* mitogenome, among them, six were unique or longer (length in 2–61 bp) than that of non-rearranged mitogenomes in four flatfishes, *Psettodes erumei, Platichthys stellatus, Peltorhamphus novaezeelandiae* and *Pelotretis flavilatus* (Fig. 4). So many different intergenic spaces raised our interesting to verify do such spaces also occur in other 12 bothid species? The result showed that the total number of the unique or longer spaces in 12 bothids was 121, including most of them with the length from 2 bp to 88 bp, and other six (located at No. 12 intergenic space) from 155 to 511 bp (Fig. 4). Where did the unique or longer spaces come from? And what significance do these mean in rearranged mitogenome? Tracing the DMNR process in each of 13 species, several degenerated genes existed. A striking finding was each of degenerated genes could find their counterparts that were intergenic spaces with shorter length at same location (Fig. 4A and B). This result confirmed that these spaces were traces for remaining of degenerated genes. The corresponding relationship between the degenerated genes and the intergenic spaces provided the direct evidence to support the possibility of inferred DMNR model, as well as, the existing of dimeric mitogenome in mitochondrion.

In addition, further analyses showed that both degenerated genes and its corresponding intergenic spaces are evolutionarily diverse. The number of unique or longer intergenic spaces in each of 13 bothid species ranged from 5 to 11, which indicated that the evolution states of degenerated genes were the progressive degeneration and complete disappearance. As well as, the number of intergenic spaces corresponding to each of degenerated genes varied widely from 1 to 13, which meant these un-transcribed genes were degenerated at different rate in different species. Such as the degenerated genes
corresponding to No. 3, 4, and 5 intergenic spaces were in progressive degeneration stage in all species, whereas that of No. 10 intergenic space was complete disappearance in all species, except \textit{B. myriaster}.

As for seven locations of intergenic spaces that occurred with no relationship of the DMNR process, among them, three spaces of the 12S-16S-V-\textit{L}_2 region were generated by the shuffling of \textit{tRNA-V} only in \textit{A. intermedius}. The double-A space between \textit{tRNA-H} and \textit{tRNA-S}_2 had the same base as the last one of \textit{tRNA-H}. And the other three spaces were also found in non-rearrangement mitogenomes of flatfishes and other teleosts.

Conclusions

In summary, the new determined complete mitogenome of \textit{G. polyophthalmus} and that of 12 known bothids possessed the genomic-scale and various rearrangements. The only differences among these mitogenomes are the combination of CR, \textit{tRNA-D} and 8-cluster and the shuffling of \textit{tRNA-V}. Based on the structure character of mitogenomes, this study found the DMNR model was fitted to account for the gene rearrangement in all 13 bothid mitogenome. And the translocation of \textit{tRNA-D} occurring after, not before, the DMNR process in 10 of these mitogenomes was also confirmed in this study. One striking finding was that each of degenerated genes existing in the gene rearrangement processes of 13 species had their counterparts of intergenic spaces. This result provided the significant evidence to support the possibility of inferred DMNR model, as well as, the existing of dimeric mitogenome in mitochondrion. What's more, the findings of this study were rare phenomena in teleost fish, which not only promoted the understanding of mitogenome structural diversity, but also shed light on studying of mitochondrial rearrangement and replication.

Methods
Sampling, DNA extraction, PCR and sequencing

The specimen of *G. polyophthalmus* was collected from landing of fishing activity in Fangcheng Port, China, conducted by artisanal bottom-trawling, therefore no specific information is available on depth of capture, substrata, or longitude and latitude for it. Fresh specimen were stored in crushed ice immediately after collection, and then frozen at −20°C in the laboratory until ready for further processing. Total genomic DNA was extracted from epaxial musculature of the specimen using the SQ Tissue DNA Kit (OMEGA) following the manufacturer’s protocol. Nine primer pairs were designed for amplification of the mtDNA genome (Additional file 1: Table S1), based on the known complete mitochondrial sequences of bothids. More than 70 bp of overlaps between sequenced fragments ensured correct assembly of the complete mtDNA genome.

PCR was performed in a 25 μl reaction volume containing approximate 50 ng DNA template, 0.5 μM of each primer, 1.0 U Taq polymerase (Takara, China), 2.0 mM MgCl₂, 0.4 mM of each dNTP, and 2.5 μl of 10× Taq buffer. The PCR cycling protocol included one cycle at 95°C for 3 min (initial denaturation), followed by 35 cycles at 95°C for 30s (denaturation), 45–55°C for 50 s (annealing) and 68–72°C for 1.5–5 min (extension), followed by a final extension at 68–72°C for 10 min. The PCR products were purified by the Takara Agarose Gel DNA Purification Kit (Takara, China) and then were used directly as templates for cycle sequencing reactions. Sequence specific primers were designed for walking primers of each fragment with an ABI 3730 DNA sequencer (Applied Biosystems, USA). Sequenced fragments were assembled to a complete mitochondrial genome by using CodonCode Aligner v3 and BioEdit v7 [24]. For large fragments and walking sequences, regular manual examinations were made to ensure reliable assembly of the mitogenome. The complete sequence of *G. polyophthalmus* mitogenome was submitted to GenBank under the accession number MK770643.
Sequence analysis

Identification and annotation of protein coding genes and rRNA genes were performed by using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The tRNA genes and their secondary structures were determined by using tRNAscan-SE 1.21 [25], setting the cut-off values to 1 when necessary. The secondary structure of $O_L$ was identified by using the mfold web server (http://unafold.rna.albany.edu/?q=mfold). The gene map of *G. polyophthalmus* mitogenome was generated by using CGView [26]. Mitogenomes of eight out of 12 bothid fishes used in this study were determined in our laboratory, including *A. intermedius*, *A. tenuis*, *B. myriaster*, *C. azureus*, *C. lugubris*, *C. valerostratus*, *L. gallus*, and *P. iiijimae*, which were under the accession numbers MK256952, KP134337, KJ433563, JQ639068, KJ433561, KJ433566, KJ433567 and KP134336, respectively. And those of other four out of 12 bothids retrieved from GenBank, including *A. polypilus*, *B. pantherinus*, *C. kobensis*, and *L. lanceolata*, which were under the accession numbers NC_024946, NC_024947, NC_024949, and AP014591, respectively.

Abbreviations

Mitogenome: Mitochondrial genome, rRNA: Ribosomal ribonucleic acid, tRNA: Transfer ribonucleic acid, H-strand: Heavy strand, L-strand: Light strand, $O_L$: Origin for L-strand replication, CR: Control region; $O_H$: Origin for H-strand replication, 8-cluster: Eight genes ($Q$-$A$-$C$-$Y$-$S_1$-$ND6$-$E$-$P$) on L-strand grouped to a cluster, DMNR: Dimer-Mitogenome and Non-Random Loss mechanism of mitochondrial rearrangement, mtDNA: Mitochondrial DNA, HSP: H-strand promoters, LSP: L-strand promoters, WANCY: tRNA cluster of *tRNA-W*, *tRNA-A*, *tRNA-N*, *tRNA-C* and *tRNA-Y*, 5-cluster: Five genes ($Q$-$A$-$C$-$Y$-$S_1$) on L-strand grouped to a cluster, 3-cluster: Three genes (*ND6$-$E$-$P*) on L-strand grouped to a cluster, PCR: Polymerase chain reaction, NC: Non-coding region, TAS: Terminal associated sequences,
Declarations

Ethics approval and consent to participate

The specimen of *G. polyophthalmus* was collected from the commercial fishery. This species was not involved in the International Union for Conservation of Nature Red List of Threatened Species. Specimen collection and maintenance were performed in strict accordance with both of the recommendations of Animal Care Quality Assurance in Chinese Mainland and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Consent for publication

Not applicable.

Availability of data and materials

The sequence of complete mtDNA of *G. polyophthalmus* in this study was submitted to GenBank under the accession number MK770643. Mitogenomes of other 12 bothids fishes (*A. intermedius*, *A. polyspilus*, *A. tenuis*, *B. myriaster*, *B. pantherinus*, *C. azureus*, *C. kobensis*, *C. lugubris*, *C. valderostratus*, *L. gallus*, *L. lanceolata* and *P. iijimae*) were under the GenBank accession numbers MK256952, NC_024946, KP134337, KJ433563, NC_024947, JQ639068, NC_024949, KJ433561, KJ433566, KJ433567, AP014591 and KP134336, respectively.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

HRL sampled specimen, collected the datasets and drafted the manuscript. XYK directed the entire research project and revised the manuscript. SXC carried out the experiments. WS revised the analysis of datasets. All authors read and approved the final manuscript.

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Figures

Figure 1

Inferred four types of the DMNR process for rearrangement mitogenome in 13 bothids. The letters A to F following the type number I-IV represent the steps of
four types of DMNR process. A: Ancestral gene order in typical fish. The rRNA, protein-coding genes and CRs are indicated by boxes, and the tRNA genes are indicated by columns. Genes labeled above the columns are encoded by the H-strand, those below the columns by the L-strand. TAS is an acronym for the terminal associated sequences. B: The translocation of tRNA-D shown by arrow. C: The dimeric molecule with two monomers linked head-to-tail. The LSP, LSP', HSP and HSP' indicate the L- and H-strand promoters of transcription. The tRNA-L1, tRNA-L1', TAS and TAS' indicate the L- and H-strand terminations of transcription. The direction of transcription is shown by arrow. D: Functional loss of LSP and HSP. The broken lines indicate untranslatable regions. Black boxes indicate the degeneration of LSP, HSP and related genes. The triangle marks the abnormal transcriptions of the tRNA-N and tRNA-N'. E: The mitogenomic structure generated after the degeneration of dimeric molecule or the translocation of tRNA-D. F: The mitogenomic structure generated after the degeneration of dimeric molecule. The diagrams of I-E, II-B, III-B, IV-B1, and IV-B2 represent the complete mitogenome in 13 bothids. The path from A, II-C, II-D, II-F to I-E in Type I indicates the translocation of tRNA-D occurred after the DMNR process. That from A, II-B', II-C', II-D', II-F' to II-B in Type II indicates the translocation of tRNA-D occurred before DMNR. Comparing the processes of B', C', D' and F' with that of B, C, D and F, the only difference is the location of tRNA-D. The broken line in IV-B2 indicates the omitted genes as same as those shown in IV-B1. Type I includes C. azureus, C. valderostratus and C. kobensis; Type II includes B. myriaster, C. lugubris and A. polyspilu; Type III includes G. polyophthalmus, L. lanceolata, L. gallus, P. iijimae and A. tenuis; Type IV includes B. pantherinus (IV-B1) and A. intermedius (IV-B2).
Figure 2

Second structures of the OL in 13 bothid mitogenomes. The underline indicates conserved bases, and the bases in red color come from the tRNA-N.
Figure 3

Aligned sequences of control regions of mitogenomes in 13 bothids and Pleuronichthys cornutus. The shaded blocks represent the conservative sequences. TAS is an acronym for the terminal associated sequence. CSB is an acronym for the conserved sequence block. Abbreviations of species names are given as follows and the number 1 and 2 after it mean CR1 and CR2. P.co: Pleuronichthys cornutus; G.p1 and G.p2: Grammatobothus polyophthalmus; A.t1 and A.t2: Arnoglossus tenuis; L.g1 and L.g2: Lophonectes gallus; L.i1 and L.i2: Laeops lanceolate; P.i1 and P.i2: Psettina iijimae; C.az: Crossorhombus azureus; C.ko: Crossorhombus kobensis; C.va: Crossorhombus valderostratus; A.po: Arnoglossus polyspilus; B.my: Bothus myriaster; C.lu: Chascanopsetta lugubris; A.in: Asterorhombus intermedius; B.pa: Bothus pantherinus.

Figure 4

The unique and longer intergenic spaces in mitogenomes of 13 bothids. A: The diagram of the degenerated genes located in dimeric molecule. The grey boxes indicate the degenerated genes. B: The diagram of mitogenome combining genes and intergenic spaces of the 13 bothid mitogenomes. The dark grey boxes
indicate the intergenic spaces. The lines between A and B indicate the corresponding relationship between the degenerated genes in A and intergenic spaces in B. The black horizontal lines indicate the range of degenerated genes.

The solid and broken lines in blue and brown indicate two corresponding relationships between underlined degenerated genes and the intergenic spaces among different species, respectively. The boxes of OL in green and purple indicate this structure overlapped and inside of the tRNA-N, respectively. The dotted boxes of tRNA-V and tRNA-D represent the different location of these two tRNA in four species. The numbers in the dark grey boxes indicate the order of intergenic spaces. C: The length of unique and longer intergenic spaces in 13 bothid mitogenomes. Abbreviations of species names are given as same as the abbreviations in the figure legend of Fig. 3. The numbers below the No. 3 intergenic space representing the length of spaces between OL and COI are black, and that of spaces between tRNA-N and COI are purple. The italic numbers indicate the length of intergenic spaces with no relationship related to the DMNR process.

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