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Maternal inheritance in polyploid fish inferred from mitochondrial ATPase genes analysis

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Abstract

The sequences of the ATPase8/6 genes for the triploid, tetraploid and pentaploid hybrids as well as for their male parent blunt snout bream were determined. In order to examine mitochondrial maternal inheritance, the sequences were subjected to a comparative sequence analysis with the homologous sequences of red crucian carp, their female parent, and zebrafish as the outgroup. Base composition and variation as well as the divergences based on nucleotide sequences and deduced amino acid sequences were calculated. Phylogenetic trees were also constructed with maximum parsimony (MP), minimum evolution (ME), neighbor joining (NJ) and the unweighted pair group method with arithmetic mean (UPGMA) algorithms in MEGA 3.1. The results showed that most nucleotide substitutions occurred at the third codon position of the two genes and thus represented synonymous mutations. The nucleotide sequence divergences of the ATPase8/6 genes ranged from 0.0% to 21.6% among ingroup samples (three types of polyploids and their parents), and 27.0–28.2% between their ingroup and the outgroup samples. All the polyploids were considerably closer in sequence relationship to the female parent red crucian carp (0.0–3.3%) compared to their male parent blunt snout bream (21.0–21.6%). The phylogenetic trees also showed a similar result. In conclusion, the mitochondrial ATPase8/6 genes of artificial polyploid fish stringently indicated maternal inheritance. Our results also suggested that the ATPase8/6 genes are valuable genetic markers to track genealogies and variations in the progenies of the hybrids.

Keywords: Maternal inheritance; ATPase genes; Triploid hybrids; Tetraploid hybrids; Pentaploid hybrids

1. Introduction

Distant hybridization is defined as interspecific, intergeneric or above-generic hybridization, and among all vertebrate species this hybridization process can be achieved easiest in fish [1–3]. Examples of distant hybridization are the F_1 hybrids of both grass carp (*Ctenopharyngodon idella*) × big head carp (*Hypophthalmichthys nobilis*) and grass carp × blunt snout bream (abbreviated as BSB; *Megalobrama amblycephala*), both of which are triploids [4,5]. Additionally, the F_3 hybrids of red crucian carp (abbreviated as RC; *Carassius auratus* red var.) × common carp (*Cyprinus carpio* L.), which are allotetraploids, possessing two chromosome sets of RC and two chromosome sets of common carp [3,6,7] are also an example of distant hybridization. Current publications have reported that the parents used for the formation of polyploid hybrids have the same chromosome numbers, whereas there have been rare reports of the formation of living polyploid hybrids produced by distant parents with different chromosome numbers. Recently, we were successful in obtaining the bisexual fertile tetraploid hybrids (abbreviated as 4*n*RB hybrids) and sterile triploid hybrids (abbreviated as 3*n*RB hybrids) by crossing RC (♀, $2n = 100$) with BSB (♂, $2n = 48$), which

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belong to the Cyprininae and Cultrinae subfamilies, respectively [8]. At the chromosomal level, the 3*n*RB hybrids possess 124 chromosomes with two sets from RC and one set from BSB, and the 4*n*RB hybrids have 148 chromosomes in which two sets are from RC and BSB each. Morphologically, the 4*n*RB hybrids have a pair of barbels, whereas the 3*n*RB hybrids have no barbels. In terms of reproduction, the 3*n*RB hybrids are sterile, yet the 4*n*RB hybrids are bisexually fertile and can produce diploid gametes. As such, the 4*n*RB hybrids provide an opportunity for the formation of a new type of artificial tetraploid species. Interestingly, it was found that the females of 4*n*RB hybrids can produce partly unreduced eggs, which are mated with the haploid sperms produced by BSB. The mating results in the formation of pentaploid hybrids (abbreviated as 5*n*RB hybrids) with 172 chromosomes: two sets from RC and three sets from BSB [8]. In this study, we report the creation of pentaploid hybrids in vertebrates for the first time. Within the evolutionary framework, the fertile tetraploid hybrids are very valuable because their fertility guarantees the formation of a new type of offspring with unique chromosome numbers. In terms of fish genetic breeding, the sterile triploid and pentaploid hybrids have potential value in fish aquaculture owing to features such as high resistance to disease, faster growth rates, sterility and good flesh quality (data not shown). In short, the formation of polyploid hybrids (triploid, tetraploid and pentaploid) from different fish subfamilies will not only further enrich polyploid fish resources but also potentially make a significant contribution to both evolution and fish genetic breeding.

The mitochondrial (mt) DNA of fish is a small and circular double-stranded DNA molecule characterized by a simple structure, rapid evolution rate and essentially lacks recombination characteristics [9]. Numerous phylogenetic studies have relied on the mtDNA genes amplification through conserved polymerase chain reaction (PCR) primers [10]. The use of mitochondrial genes can effectively avoid the problems associated with the possible lack of homology in fish compared with nuclear genes. Such a problem can generate noise in the analysis. Due to moderate conservation, the mitochondrial ATPase8/6 genes have been widely applied to the analysis of phylogenetics and genetic relationships of inter- and intra-populations of fish. For instance, Kontula et al. [11] showed that the diversification of the Baikalian cottoids seemed to have started in the Pliocene or early Pleistocene from a total of mtDNA sequences of ATPase8/6 genes. Based on the complete ATPase8/6 and Cyt *b* genes, Perdices et al. [12] speculated that the Danubian *Sabanejewia* (Osteichthyes: Cobitidae) lineages distributed in the European Danube basin waters most likely have a double origin, owing to the influence of Pleistocene glaciations. By using sequences from the mitochondrial ATPase6 genes and a control region, Froufe et al. [13] presented the first insights into four major (internal) clades of the grayling genus *Thymallus* (Salmonidae), which represent two distinct lineages in the Amur basin, one lineage in all remaining Siberian and Mongolian drainages, and one lineage corresponding to European grayling *Thymallus thymallus*. Thai et al. [14] investigated the genetic variation within common carp and developed a global genealogy of common carp strains utilizing the mtDNA ATPase8/6 genes and D-loop analysis. The above studies focused on diploid fish. Currently, few reports exist on the mitochondrial ATPase8/6 genes present in polyploid fish [15]. Thus, we firstly isolated the entire sequences of mtDNA ATPase8/6 genes from the polyploid hybrids of RC♀ × BSB♂, so as to further elucidate the genetic relationship between the polyploid hybrids and their parents, and to explore the variability of mitochondrial genes of polyploidy hybrids.

2. Materials and methods

2.1. Fish and DNA extraction

One individual of BSB, five individuals of 3*n*RB hybrids, six individuals of 4*n*RB hybrids and six individuals of 5*n*RB hybrids were captured from the National Tetraploid Fish Protection Station of China which is located within the Hunan Normal University campus. Total genomic DNA was extracted from whole blood collected from the fish caudal vein using a DNA extraction kit from Sangon (Shanghai, China).

2.2. PCR amplification and sequencing

The complete ATPase8 and ATPase6 genes, which are slightly overlapping mitochondrial genes, were PCR amplified with the following primers: ATPase8/6(+): 5′-AAAG CGTTGGCC TTTTAAGC-3′ and ATPase8/6(−): 5′-GTT AGTGGTCATGGGCTTGGATC-3′. Reactions were tested in total volumes of 50 μl. Each reaction contained 20 ng of template DNA, 1.2 μl of each primer, 4 μl of $MgCl_2$ (25 mmol/L), 10 μl of dNTPs (1 mmol/L), 0.8 μl of *Taq* polymerase (2.5 U/μl; Promega), 5 μl of 10× PCR buffer and 25.6 μl of sterile distilled water. The reactions were conducted in a programmable thermal controller (GeneAmp® PCR System 2700) as follows: an initial denaturation step of 4 min at 94 °C, followed by 45 s at 94 °C, 45 s at 53 °C and 90 s at an extension of 72 °C for 6 cycles; subsequently, 45 s at 94 °C, 45 s at 58 °C and 90 s at an extension of 72 °C for 29 cycles, with a final 10 min extension at 72 °C. Amplified products were separated in 1.5% agarose gels. The separated and specific bands were excised from the gels and extracted using a DNA gel extraction kit (Sangon), and the purified DNA fragments were bidirectionally sequenced using the ABI PRISM 3730 automatic sequencer (ABI, Foster City, CA) in Shanghai Sangon.

2.3. Sequence analysis

The homologous nucleotide sequences of RC (AY714387) and zebrafish (*Danio rerio*, AC024175) were

retrieved from the GenBank database. The nucleotide sequences, together with amino acid sequences deduced from the ATPase8/6 genes, were aligned using the CLUSTAL W package (http://www.ebi.ac.uk/clustalw) [16] and confirmed by eye. The base composition bias for all used samples was calculated by MEGA3.1 software [17]. Pairwise sequence comparisons to determine the distribution and amount of variation as well as the degree of saturation by the codon position were performed using the MEGA3.1 software [17]. The percentage divergences of nucleotide and amino acid sequences among all samples were estimated with Kimurap's two-parameter model [18]. Phylogenetic relationships were valuated by different algorithms, including maximum parsimony (MP), minimum evolution (ME), neighbor joining (NJ) and the unweighted pair group method with an arithmetic mean (UPGMA) from the MEGA3.1 software package, with the zebrafish as the outgroup [17]. The reliability of tree topology was assessed by 1000 bootstrap replications [19].

3. Results

3.1. Amplification of the ATPase8/6 genes and base compositional bias

The size of the amplification products among all fish samples was about 951 bp, including the complete ATPase8/6 genes, partial $tRNA^{Lys}$ and partial COIII gene sequences. By comparison with other fish mtDNA sequences, the entire sequences of the ATPase8 and ATPase6 genes of all samples used were obtained. The lengths of the ATPase8 and ATPase6 genes were 165 and 684 bp, respectively, and they have been deposited in the GenBank database (Table 1). There is a 7 bp overlap between the ATPase8 and ATPase6 genes, and this is consistent with the previous results [15]. Five 3*n*RB hybrids, one BSB and six 5*n*RB hybrids shared one haplotype. A total of six 4*n*RB hybrids as well as one RC hybrid shared one haplotype.

The average compositions of T, C, A and G nucleotides from the ATPase8/6 genes accounted for 29.5%, 26.4%, 31.9% and 12.3%, respectively (Table 1). Similar to cases reported in the mitochondrial control region of the three types of polyploid hybrids [20] and other fish mitochondrial DNA [15,21], the base compositions of the ATPase8/6 genes were skewed, with higher content of A+T than G+C, with the lowest content being G. The pattern of nucleotide composition at the different codon positions of the ATPase8/6 genes from all samples differed. At the first position, the frequencies were A>T>C>G in the ATPase8 gene, and C>A>G>T in the ATPase6 gene. At the second position, the frequencies were T≈C>A>G in the ATPase8 gene, and T>C>A>G in the ATPase6 gene. However, at the third position, the frequencies were similar in the ATPase8 and ATPase6 genes, i.e. A>T>C>G. The highest bias against G content occurred at the third codon position of the ATPase8/6 genes and it has been suggested that the selection might restrict the nucleotide frequencies at the third positions [22].

3.2. Sequence variation analysis

Base insertions and deletions were not found in the ATPase8/6 genes of all fish samples. Of the 842 bp obtained from the ATPase8/6 genes isolated from the three types of polyploid hybrids and their parents, 164 (19.48%) and 10 (1.19%) were variable sites and parsim-informative sites, respectively. Furthermore, a total of 678 conserved sites (80.52%) were detected in the three types of polyploid hybrids and their parents. For the 3*n*RB hybrids and their parents, there existed 153 variable sites in the ATPase8/6 genes, of which, only two sites were variable between the 3*n*RB hybrids and their maternal parent RC. Here, the base at position 425 was A in the 3*n*RB hybrids, whereas this base was G in the female parent RC. In the second case, the base at position 680 was T in the 3*n*RB hybrids, whereas the base was C in the female parent RC. A total of 152 variable sites were found for the ATPase8/6 genes between the 4*n*RB hybrids and their parents. The variability existed only between the 4*n*RB hybrids and their male parent BSB. The total number of variable sites detected was 164 between the 5*n*RB hybrids and their parents, in which the variable sites (27) between the 5*n*RB hybrids and their female parent RC were far fewer than those (155) between the 5*n*RB hybrids and their male parent BSB. The percentage divergences of nucleotide sequences were calculated, and are presented in Table 2. From Table 2, it can be noticed that the percentage divergences (0.2% and 0.0%, respectively) among the 3*n*RB hybrids, 4*n*RB hybrids and their female parent RC were far lower than

Table 1
The average base composition (%) of ATPase8/6 genes in the six fish species.

Fish species	T	C	A	G	GenBank Accession No.
BSB	27.0	29.8	30.5	12.7	EU434747
RC	29.5	25.4	32.8	12.4	AY714387
3*n*RB	29.6	25.3	32.9	12.2	EU350363
4*n*RB	29.5	25.4	32.8	12.4	EU350364
5*n*RB	29.9	25.3	32.3	12.5	EU350365
Zebrafish	31.4	27.0	30.2	11.5	AC024175
Average	29.5	26.4	31.9	12.3	

Table 2
The percentage divergence of nucleotide sequences (above diagonal) and amino acid sequences (below diagonal) deduced from the ATPase8/6 genes in the six fish species.

Fish species	BSB	RC	3*n*RB	4*n*RB	5*n*RB	Zebrafish
BSB		21.0	21.2	21.0	21.6	27.0
RC	7.8		0.2	0.0	3.3	27.9
3*n*RB	7.8	0.0		0.2	3.3	27.7
4*n*RB	7.8	0.0	0.0		3.3	27.9
5*n*RB	7.8	0.0	0.0	0.0		28.2
Zebrafish	16.6	18.3	18.3	18.3	18.3	

those (21.2% and 21.0%, respectively) among the 3*n*RB hybrids, 4*n*RB hybrids and their male parent BSB. Similarly, the percentage divergence (3.3%) between the 5*n*RB hybrids and their female parent 4*n*RB hybrids was even lower than that (21.6%) between the 5*n*RB hybrids and their male parent BSB.

The start codon of the ATPase8 and ATPase6 genes was ATG; however, the stop codons differed, namely, TAG and TAA, respectively. The deduced residue lengths of the ATPase8 and ATPase6 genes were 52 and 227, respectively. The amino acid sequences were absolutely identical between the three types of polyploid hybrids and their respective female parent; however, there were definite divergences (7.8%) for the amino acid sequences between the three types of polyploid hybrids and their male parent BSB.

3.3. Phylogenetic relationship analysis

The MP, ME, NJ and UPGMA phylogenetic trees based on parsimonious sites and pairwise distances were constructed using the entire mitochondrial ATPase8/6 genes. The phylogenetic trees were constructed such as to take into account the particular parameters (transitions, transversions and transitions/transversions) and the empirical frequencies of nucleotides. The phylogenetic trees with the support of rather higher bootstrap values recovered in the equal weighting analysis for all characters had the same topology. Thus, the NJ tree can represent the other three phylogenetic trees (Fig. 1). The phylogenetic tree showed that the outgroup zebrafish was clearly separated from the ingroup samples. Among the ingroup samples, the 3*n*RB, 4*n*RB and 5*n*RB hybrids formed a subcluster with their respective female parent, whereas they split significantly earlier from their male parent.

4. Discussion

In this study, the lengths of the ATPase genes obtained from fish were similar in length to those previously reported for other vertebrates. The two differences found were that the ATPase8 gene was one codon shorter (165 bp instead of 168 bp) and the overlap sequence with the ATPase6 gene was thus shorter (7 bp instead of the usual 10 bp). Following the retrieval of the sequences from the GenBank, we found this feature present in only two Cyprinidae (i.e. *Cyprinus carpio* and *Carassius auratus*), whereas other species of Cypriniformes, such as *Crossostoma lacustre*, showed the general vertebrate sequence pattern. This change in sequence may have occurred in a common ancestor of the family Cyprinidae [15].

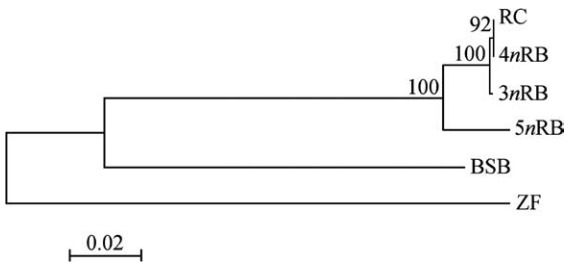

Fig. 1. Phylogenetic tree constructed by the neighbor joining algorithm. One thousand bootstrap replication values were shown in the nodes of the tree.

So far, the majority of reports on fish mtDNA have only provided documentation for natural polyploid fish species. For example, Murakami et al. [23] found that the triploid ginbuna has been derived from two different maternal lineages, the diploid ginbuna and the goldfish, on the basis of the hyper-variable segments (323327 bp) of the mitochondrial D-loop. Machordom and Doadrio [24] split the current *Barbus* genus, including diploids and tetraploids as well as hexaploids, into five main mitochondrial lineages by analyzing the complete sequences of three mitochondrial genes: ATPase8/6 and Cyt *b*. Based on the complete mtDNA control region sequences of triploid gibel carp (*Carassius auratus gibelio*) in four hatcheries, Li and Gui [25] confirmed that the differences in sequences varied remarkably among hatcheries, with the Fangzheng and Qihe lines demonstrating the highest diversity and Wuhan and Pengze lines showing no variation. The results suggest that the Fangzheng and Qihe lines represent two distinct matrilineal sources. The above reports mainly focused on the analysis of natural polyploid fish, so the maternal inheritance of these species was not accurately and clearly determined. In contrast, the 3*n*RB, 4*n*RB and 5*n*RB hybrids are crosses from artificial polyploid hybrids, so their original parents are clearly known and thus provide a convenient approach to study fish mtDNA genetic relationships. Until now, with the exception of the study provided by Guo et al. [15], there existed little work on the mtDNA genes in the artificial polyploid hybrids. In this study, the divergences of the sequences and the variable nucleotide sites detected between three types of polyploid hybrids and their respective female parent were lower than those between the three types of polyploid hybrids and their male parent. The results suggest that these artificial polyploid hybrids possess the mtDNA of the maternal parent regardless of their ploidy level. The phylogenetic trees also reflected clearly their maternal inheritance.

The 5*n*RB hybrids were produced by backcrossing of 4*n*RB♀ × BSB♂, whereas the RC is the female parent of the 4*n*RB hybrids. Due to the mtDNA maternal inheritance, the nucleotide sequence divergence between the 5*n*RB hybrids and the original female parent RC should be lower than that between the 5*n*RB hybrids and the paternal BSB. In the present study, the nucleotide sequence divergence was 3.3% between the 5*n*RB hybrids and RC, and 21.6% between the 5*n*RB hybrids and BSB. This result was consistent with the maternal inheritance characteristic. Theoretically, the nucleotide sequence divergence between 3*n*RB and RC should be identical between the 4*n*RB hybrids and RC. In fact, the nucleotide sequence divergence was 0.2% between the 3*n*RB hybrids and RC,

whereas no differences were observed in the nucleotide sequences between the 4*n*RB hybrids and RC. We speculate that the presence of different haplotypes in the 3*n*RB and 4*n*RB hybrids is possibly attributed to different female parent individuals.

In the present study, 678 conserved nucleotide sites among all fish species were detected, which were possibly generated during the continuous evolution of different fish subfamilies. Two variable nucleotide sites were present between the 3*n*RB hybrids and their female parent, and 27 variable nucleotide sites were detected between the 5*n*RB hybrids and their female parent. However, the amino acid sequences were unambiguously similar between the two types of polyploid hybrids and their female parent, which suggested that the variation appeared mainly at the third codon position in the ATPase8/6 genes. This observation is consistent with the regular rule of rapid evolution rates at the third codon position.

The maternal inheritance of mtDNA is a common feature in all eukaryotes. However, some studies have previously demonstrated the presence of leaked paternal mtDNA and biparental transmission of mtDNA [26,27]. Awadalla et al. [28] found that the linkage disequilibrium in human and chimpanzee mtDNAs declined as a function of the distance between sites, which could be attributed to one mechanism only: recombination. In comparison with the complete mtDNA of triploid crucian carp and its parents, Guo et al. [29] confirmed that triploid crucian carp possessed the recombination mtDNA fragment $tRNA^{Val} \rightarrow$ NADH5 (12759 bp) derived from the paternal fish allotetraploids. This demonstrated that mtDNA recombination was generated from the fusion of the maternal and paternal mtDNAs. In this paper, five nucleotide sites detected in the 5*n*RB hybrids were similar to their male parent, yet different from their female parent. Are these sites caused by recombination of the maternal and paternal mtDNAs? It is well known that the mtDNAs are usually in the cytoplasm, and recombination does not easily occur in mtDNAs. Furthermore, the nucleotide sequence divergence between the 5*n*RB hybrids and their female parent was far lower than that observed between the 5*n*RB hybrids and their male parent. Therefore, another explanation could be that the appearance of variable sites in the 5*n*RB hybrids was caused by their origination from different female parents, rather than the recombination of maternal and paternal mtDNAs. At the same time, 21 nucleotide sites (3*n*RB hybrids: 2 and 5*n*RB hybrids: 19) that differed from their parents were detected. Similar results were indicated in the analysis of mtDNA 12S rRNA genes of allotetraploid fish, triploid crucian carp and their parents [30]. This study suggested that these variable nucleotide sites were possibly generated by point mutations during artificial genetic breeding or without adjustment and repair of mismatched bases produced at the time of mtDNA replication. The mechanism of appearance of those variable sites is worthy of further study and represents future work.

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