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High altitude adaptation of the schizothoracine fishes (Cyprinidae) revealed by the mitochondrial genome analyses

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ABSTRACT

The schizothoracine fishes, also known as "mountain carps" are widely distributed in the Qinghai-Tibetan Plateau and its peripheral regions. Although they provide a prime example of high altitude adaptation, the phylogenetic relationships and the divergence times among these carp lineages are still controversial. Moreover, the genetic basis for high altitude adaptation is also poorly understood. In this study, we determined the mitochondrial genomes from two species of the schizothoracine fishes, representing a "morphologically primitive" clade and "morphologically specialized" clade, respectively. The phylogenetic tree and the divergence times were estimated within the evolutionary framework of the entire order Cypriniformes. Our results indicate a polyphylyetic relationship of the schizothoracine fishes and suggest two independent migration events into the Qinghai-Tibetan Plateau: one by the "morphologically primitive" clade in the Late Miocene and another by the "morphologically specialized" clade in the Eocene. Rapid speciation events of each clade from the Late Miocene to the Pliocene correspond to the timing of the geologic acceleration of the Qinghai-Tibetan Plateau. Interestingly, we found evidence for positive selection acting on the protein coding genes in the mitochondrial genomes of the "morphologically specialized" clade, implying a possible genetic basis for high altitude adaptation in this derived lineage of cypriniform fishes.

1. Introduction

The Qinghai-Tibetan Plateau is the largest plateau in the world, occupying 2.5 million km^2 with an average altitude of 4000 m above sea level, and has been designated as a global hotspot of biodiversity (Qi et al., 2012). Its environment is characterized by hypoxia and low temperature (Wang et al., 2011). Accordingly, we expect to observe a high efficiency in the metabolisms of organisms evolving in such environments. Recent molecular studies have detected a number of signals of adaptive evolution for high altitude. Mitochondria, in particular, play an essential role in ATP synthesis and heat generation, and it is possible that intense selection pressures may be operating on them in high altitude environments. Previous studies have detected signals of positive section in the mitochondrial genomes of organisms living at high altitude, including goats (Hassanin et al., 2009), chirus (Xu et al., 2005), alpacas (da Fonseca et al., 2008), Tibetan asses (Luo et al., 2012), Tibetan horses (Xu et al., 2007), pikas (Luo et al., 2008), Chinese snub-nosed monkeys (Yu et al., 2011), and bar-headed goose (Scott et al., 2010),. However, most of these studies are focused on mammals or aves, and the high altitude adaption of fishes is little known.

The schizothoracine fishes (Teleostei: Cyprinidae), which are also called "mountain carps" are distributed throughout the Qinghai-Tibetan Plateau and its peripheral regions. Therefore, this taxon provides an excellent opportunity for investigating high altitude adaptation of teleost fishes. Cao et al. (1981) indicated that the schizothoracine fishes can be divided into three groups from the morphological characters such as reductions of scales, numbers of pharyngeal teeth, and numbers of barbels. From an evolutionary perspective, these groups are called morphologically "primitive", "specialized", and "highly specialized" schizothoracine fishes. Recent molecular studies (He et al., 2004; Qi et al., 2012) indicated the "primitive" schizothoracine fishes form a monophyletic clade, while the "specialized" and the "highly specialized" schizothoracine fishes form another clade. However, the "specialized" and "highly specialized"

Abbreviations: GTR, general time reversible; ML, maximum likelihood; mt, mitochondrial; OXPHOS, oxidative phosphorylation; ω (dN/dS), The nonsynonymous over synonymous substitution rates.

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schizothoracine fishes are intermingled with each other. Hereafter, we call the former "morphologically primitive clade" and the latter "morphologically specialized clade". A reliable phylogenetic tree and divergence times among lineages are an essential prerequisite for understanding the history of biological events that have occurred during the evolutionary process. However, these issues for schizothoracine fishes are still controversial. For example, although the previous studies showed the monophyly of schizothoracine fishes (He et al., 2004; Qi et al., 2012), this was likely an artifact of sparse taxon sampling (e.g., exclusion of the "barbine" group *sensu stricto*), or mis-specification of an outgroup (assuming barbine *sensu stricto* as an outgroup). Ruber et al. (2007) also indicated that barbine *sensu stricto* is closely reated to schizothoracine, but they treated barbine *sensu stricto* and two lineages of schizothoracine as an unresolved trifurcation. Divergence times among these groups are also little known. According to the estimation of He et al. (2004) based on *cytochrome b*, the common ancestor of the schizothoracine was in the Late Miocene (about 10 Ma). On the contrary, according to the estimation of Ruber et al. (2007) based on *cytochrome b*, it was in the Oligocene-Miocene boundary (around 23 Ma) or older. Although the mitochondrial genome analysis of Saitoh et al. (2011) included only one species of the schizothoracine fishes (*Gymnocypris przewalskii*), the estimated divergence time between *G. prezwalskii* and *B. barbus* was in the Paleocene (61 Ma: 95% credible interval was 47.9 Ma–73.5 Ma).

To better resolve phylogenetic relationships and divergence time estimations, we newly determined the mitochondrial genomes of two schizothoracine species, and inferred the phylogenetic tree together with the published mitochondrial genome data of Cypriniformes. We also added the *cytochrome b* data of the schizothoracine fishes covering all genera. Subsequently, we estimated the divergence times among them within the evolutionary framework of the entire order Cypriniformes, and also reconstructed the ancestral geographic distributions. Finally, we tested for positive selection on codon sites and inferred both the timing and the geographic regions associated with positive selection and the adaptive evolution of schizothoracine fishes.

2. Materials and methods

2.1. Muscle samples and DNA Extraction

The muscle samples of the two mountain carps *Schizothorax waltoni* (morphologically primitive clade) and *Schizopygopsis younghusbandi* (morphologically specialized clade) were collected in the Yarlung Zangbo River (28°00′-31°16′ N, 82°00′-97°07′) from Tibet (2057 km long), China in 2010, and then immediately preserved in 95% ethanol at −20 °C until DNA isolation. Voucher specimens were deposited at the School of Life Sciences of Fudan University, China. Total genomic DNA was extracted from muscle of a single specimen using the TIANGEN genomic DNA extraction Kit.

2.2. PCR Amplification and Sequencing

The complete mt genomes were amplified from the genomic DNA of the two schizothoracine fishes using 21 overlapping amplification primers by PCR methods. PCR primers were partially derived from the literature (Chang et al., 1992; Xiao et al., 2001) and from original DNA sequences of cyprinid fish mitochondrial genomes (Table S1).

PCR reactions were prepared at 50 uL total volume as follows: 10×PCR buffer, 2.5-3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μg/μL BSA, 0.4 mM of each primer, 3.0-5.0 U Taq DNA polymerase, and 20–50 ng of genomic DNA. The PCR conditions included an initial denaturation step at 95 °C for 2 min followed by 30–35 cycles at 94 °C for 50 s, 54-60 °C for 45 s-1 min, and 72 °C for 50 s-2 min; with a final extension of 10 min at 72 °C. Annealing temperatures and extension times were varied within the above-listed ranges in order to optimize the efficiency of different primers.

2.3. Sequence editing and analysis

The assembled complete mt genomes were annotated and analyzed by Geneious 4.8.3 (http://www.geneiouscom). Thirteen protein-coding genes, 2 ribosomal RNAs and the D-loop region for the two species were confirmed through BLAST comparisons of GenBank sequences from *Gymnocypris przewalskii* (Saitoh et al., 2006). The tRNA genes were identified with the software tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE) and modified on the basis of secondary structure (Lowe and Eddy, 1997). Pairwise comparisons and statistical information from the two mitochondrial genomes specially sequenced for this study were obtained using the DNAstar 5.0 software package (DNAstar Inc).

2.4. Phylogenetic analyses based on mitochondrial genomes

Original mitochondrial genome sequences of *St. waltoni* and *Sp. younghusbandi* were determined in this study, and the published mitochondrial genome sequences of 60 teleost species from GenBank were used to conduct phylogenetic analyses. The "loaches" were selected as outgroups (Saitoh et al., 2011). Accession numbers for all sequences used in this study are summarized in Table S2.

Twelve protein-coding genes encoded in the heavy-strand of DNA and 2 rRNA genes were used for the analyses. We excluded the *ND6* gene, because this gene is encoded on the light-strand, and nucleotide compositions are very different from other genes. Each gene sequence was automatically aligned by using the Clustal W program (Thompson et al., 1997), and carefully checked by eye and all ambiguous parts were excluded. After removing the start and stop codons, as well as the overlapping regions between the genes for *ATP8*, *ATP6*, *ND4L*, *ND4*, *ND5*, and *ND6*, 12 protein coding genes and 2 rRNA genes were concatenated.

We inferred the phylogenetic relations with the maximum likelihood (ML) method (Felsenstein, 1981). For the ML analyses, we used the RAxML program version 7.2.6 (Stamatakis et al., 2008) with the GTR + I + Γ model. Taking into account the different tempo and mode of the nucleotide substitutions, the parameters of the nucleotide substitution model and the branch lengths of 1st, 2nd, 3rd codon positions and ribosomal RNAs were separately estimated. To evaluate the confidence of the internal nodes, the rapid bootstrap method (Stamatakis et al., 2008) was applied with 1000 replications.

Sasaki et al. (2005) demonstrated that the codon substitution (CS) model (Yang et al., 1998) is superior to normal nucleotide substitution models such as GTR model. Therefore we also applied the CS + Γ model for the concatenated 12 protein coding genes. Miyata et al. (1979)'s physicocemichal distances among 20 amino acid were used as amino acid distance with geometric formula (Yang et al., 1998). This analysis was carried out by using the CODEML program of PAML (version 4.4, Yang, 2007). Since the program is too slow to apply for the heuristic search, the CS + Γ model was only applied for the exhaustive search among limited numbers of the candidate trees.

2.5. Phylogenetic analyses based on cytochrome b

Currently complete mitochondrial genome sequence data are available only from three species of schizothoracine fishes (*G. przewalskii, St. waltoni* and *Sp. younghusbandi*), and it is not sufficient to draw a complete evolutionary picture for schizothoracine fishes. On the other hand, *cytochrome b* has been the most frequently used DNA marker for cyprinid fishes (Ruber et al., 2007), and is available for all genera of the subfamily Schizothoracinae (Qi et al., 2012). Thus we collected the complete *cytochrome b* sequences of schizothoracine fishes and related species from GenBank (accession numbers are listed in Table S3).

The procedures of the alignment and phylogenetic inference were basically same with the Section 2.4. The parameters of the nucleotide