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Positive Darwinian selection within *interferon regulatory factor* genes of *Gymnocypris przewalskii* (Cyprinidae) on the Tibetan Plateau

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ABSTRACT

Tibetan Plateau (TP) had experienced phased uplift, resulting in inhospitable environment of low temperature, hypoxia and high ultraviolet radiation for Tibetan wildlife. Many organisms can well adapt to TP, it is of ecological and evolutionary interest to untangle how organisms adapt to extreme environment on TP through evolution. Previous studies mainly focused on hypoxia and metabolism related genes, but we know little about the evolutionary history of immune genes in Tibetan wildlife. In this study, we first identified 10 *interferon regulatory factor* (*IRF*) genes from Tibetan naked carp *Gymnocypris przewalskii*. Within this gene family, *IRF3*, *IRF5*, *IRF7* and *IRF8* contained positive selection sites. Evidences indicated that positive selection may lead to *IRF* genes functional alternations, presumably driving genes towards adaptation to the environmental changes. Taken together, our results suggested 4 candidate genes as interesting targets for further experimental confirmation of their functional variations and contributions to high altitude adaptation in Tibet fish.

1. Introduction

Past evidences indicated that many wildlife species well adapt to life at high altitude on the Tibetan Plateau (TP) [1–6]. TP is one of Earth's most significant continental-scale highlands with extremely inhospitable environment [7], such as low temperature, hypoxia, and high ultraviolet radiation [2,8]. Previous genome-wide studies on multiple Tibetan terrestrial animals had identified that signals of positive selection and expansion were significantly enriched in hypoxia and energy metabolic pathways [2–6]. Noteworthy, recent evidences show that several Tibetan animals' immune genes had undergone significant positive selection [2,4,9,10], but we still know little about the underlying mechanism of immune genes adapt to extreme environment on TP through adaptive evolution.

The naked carp (*Gymnocypris przewalskii*) is one of the best-studied Schizothoracinae fish species on TP, making it a model for evolutionary biology and immunity studies [9–16]. Unlike other widely distributed Schizothoracinae fish species, naked carp narrowly distributed in Lake Qinghai, namely the largest salt lake in China [9,16]. Intriguingly, low pathogen infection rate were found in wild population dwelled in salt water environment [17]. In contrast, naked carp is susceptible to infectious diseases caused by bacteria and parasite in a common laboratory freshwater environment, resulting in high mortality rate [10,13]. To date, this phenomenon has not been well understood. Though naked carp whole genome has not been sequenced currently, its transcriptome data recently become publicly available [9,13], its immune genes have yet to be experimentally investigated.

Interferon regulatory factors (*IRFs*) were originally identified as a family of transcription factors involved in the regulation of IFN expression [18]. *IRFs* also serve as important members in toll like receptor (TLR) signing pathway within innate and adaptive immunity [19], increasing studies suggest that *IRFs* participate in various functions, such as antiviral defense, immune regulation,

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hematopoietic cell development, and maturation of the immune system [20,21]. In vertebrates, *IRFs* have 11 members and classified into 4 subfamilies: *IRF1* (*IRF1*, *2*, and *11*), *IRF3* (*IRF3* and *7*), *IRF4* (*IRF4*, *8*, *9*, and *10*), and *IRF5* (*IRF5* and *6*) [22]. Not all the *IRF* genes exists in one species, for example, the absence of *IRF10* in human and mouse as well as the lack of *IRF3* and *IRF9* in chicken [23,24]. In teleost fish, gene duplication generated more copies of *IRFs* [25], for instance, the duplication of *IRF2*, *IRF4* in half-smooth tongue sole, zebrafish, lamprey and spotted gar [26–28]. Prior study of naked carp immune genes have found that *TLR4* have underwent significant positive selection and tend to be neofunctionalization [10]. *IRFs* as the downstream regulators within TLR signing pathway, it is necessary to understand that whether naked carp *IRFs* underwent positive selection and function had changed.

In this study, for the first time, we obtain the coding regions of 10 *IRFs* through mining naked carp transcriptome and cloning. We assess that whether these sequences display molecular evolutionary patterns consistent with adaptive evolution. In addition, we detect the positively selected sites (PSS) in *gpIRFs* and map them onto predicted three dimensional protein structures. Finally, we examine spatio-expression patterns of *gpIRFs*.

2. Materials and methods

2.1. Samples and ethics statement

Wild adult naked carp samples were collected from Lake Qinghai, China. Fish were dissected after anesthesia with MS-222. Six tissues (blood, spleen, brain, liver, gill and head-kidney) were collected and immediately stored in liquid nitrogen. All animal experiments were approved by Agriculture Department of Qinghai Province, China.

2.2. Data retrieval and sequence analysis

Recent publication of transcriptome of naked carp has revealed abundant immune genes [9,13], and allow build local database for mining genetic resource of interest. Published IRF protein sequences (Fig. 1) were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and used as queries, *IRF* genes of *G. przewalskii* were obtained by TBLASTN search against local nucleotide database. To increase the stringency, only hits returning E-values of ≤0.001 were considered for further analysis. After resulting in putative *gpIRFs*, we confirmed and corrected each of *IRFs* by RT-PCR (primers, Table S1), and sequenced using ABI 3730 (Applied Biosystems, USA). Open reading frames (ORFs) of each *IRFs* were predicted and translated using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The functional domains and motifs of *IRFs* protein were identified using MEME suite (http://meme.nbcr.net/meme/).

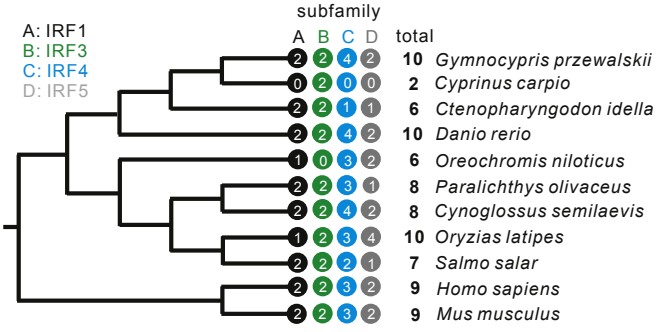

Fig. 1. The naked carp *IRF* genes in comparison with other teleosts and mammals *IRFs* which published (Data are available on NCBI, Table S2). The *IRF* gene family contains four subfamilies: *IRF1* (black), *IRF3* (green), *IRF4* (blue) and *IRF5* (gray). Numbers of *IRFs* within these 11 species are mapped on the species phylogenetic tree, four subfamilies are labeled by A, B, C and D with colors, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Phylogeny and molecular evolutionary analyses

Deduced amino acid sequences of *IRFs* were aligned using ClustalW program implemented in MEGA5 [29]. Phylogenetic analysis was conducted by neighbor-joining (NJ) and maximum-likelihood (ML) method using MEGA5 with bootstrap values following 1000 replicates. Splitstree (version 4.6) [30] was also used to confirm the systematics of *IRFs*.

A sliding window analysis were delivered to test selection pressures on each member within *IRF* family, and full-length of coding sequences of each species *IRFs* (Table S2) were aligned using ClustalW. The generated codon alignments were subjected to computation of dS and divergence levels (dN/dS ratios) using DnaSP software (version 5.10) (http://www.ub.edu/dnasp/). The ω value (ω = dN/dS) is used to measure direction and magnitude of selection pressure on amino acid replacement. The ω values greater than 1 demonstrate high rate variation and possible signs of positive selection, ω = 1 representing neutral evolution, and ω < 1 representing purifying selection. Given sliding window analyses define the range of positive selection regions and may result in false positive results [10,31], we use PAML 4.7a [32] to detect positively selected sites within *gpIRFs*. In PAML analysis, the branch-site models allow ω to vary both among sites in the sequence and across branches of the phylogenetic tree. The null hypothesis (Model A) assumes a fixed ω_2 (≤1) whereas the alternative hypothesis (Model B) estimates ω_2 with its initial value > 1. By comparing these two models through likelihood ratio test, whether positive selection affected a few sites along particular lineages could be detected [33].

2.4. Protein three dimensional (3D) structure modeling

Protein 3D structures of gpIRFs under positive selection were predicted and built by I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). PyMOL [22] was used to analyze protein structure. At last, PSS were mapped onto the protein 3D structure of gpIRF.

2.5. Spatio-specific expression analysis

Total RNA was isolated from above six tissues and following the method as previously described [10]. Quantitative Real-time PCR (RT-qPCR) was used to detect spatio-specific expression of *gpIRFs* (primers, Table S1). *β-actin*, a previously validated housekeeping gene in naked carp was used as an internal control [12]. RT-qPCR was performed using an ABI ViiA™7 in 20 μL reactions following the protocol as previously described [10]. No-template reaction was included as a negative control for all experiments. All samples were analyzed in triplicate as technical replicates and fold changes were calculated using $2^{-\Delta\Delta CT}$ method [34]. Statistical analysis was performed by SAS v9.1.3 (SAS Institute Inc., USA). Significant differences between samples were analyzed by one-way ANOVA using Duncan's test. Multiple comparisons analysis was conducted using Tukey's posthoc test. Differences between means were considered significant at $p < 0.05$. All data are shown as mean ± standard error of the mean (SEM).