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Comparative analysis of spleen transcriptome detects differences in evolutionary adaptation of immune defense functions in bighead carp and silver carp

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ABSTRACT

The evolutionary divergence of the immune defense functions in bighead carp (*A. nobilis*) and silver carp (*H. molitrix*) is still not understood at the molecular level. Here, we obtained 48,821,754 and 55,054,480 clean reads from spleen tissue libraries prepared for bighead carp and silver carp using Illumina paired-end sequencing technology, respectively, and identified 4976 orthologous genes from the transcriptome data sets by comparative analysis. Adaptive evolutionary analysis showed that 212 orthologous genes and 255 Gene Ontology (GO) terms were subjected to positive selection(Ka/Ks values > 1) only in bighead carp, and 195 orthologous genes and 309 GO terms only in silver carp. Among immune defense functions with significant evolutionary divergence, the positively selected biological processes in bighead carp mainly included B cell-mediated immunity, chemokine-mediated signaling pathway, and immunoglobulin mediated immune response, whereas those in silver carp mainly included the antigen processing and presentation, defense response to fungus, and detection of bacteria. Moreover, we found 2974 genes expressed only in spleen of bighead carp and 3494 genes expressed only in spleen of silver carp, where these genes were mostly enriched in the same biological processes or pathways. These results provide a better understanding of the differences in resistance to some diseases by bighead carp and silver carp, and also facilitate the identification of candidate genes related to disease resistance.

1. Introduction

Bighead carp (*Aristichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) are closely related species in the subfamily Xenocypridinae within Cyprinidae. Recent studies have demonstrated that the split of silver carp and bighead carp only occurred 3 Mya [1]. These species are both filter-feeding fish but they have some significant differences in terms of their physiology and morphology, as follows: (1) both are often considered to be bigheaded carps but there are differences in the size of their skull bones; (2) they prey on different types of plankton, where bighead carp mainly filter zooplankton whereas silver carp mainly filter phytoplankton [2]; (3) they live in different water layers, where bighead carp inhabit the middle water layers and silver carp inhabit the upper–middle water layers; (4) they differ significantly in terms of their sensitivity, where silver carp have a unique and striking tendency to jump up to 3 m when startled, whereas the response of bighead carp is relatively slow; and (5) their growth characteristics are different, where bighead carp grow rapidly to a very large size (80 kg) but silver carp only grow to 20 kg. Thus, due to these differences, bighead carp and silver carp have been investigated widely as ideal model species for studies of toxicology, ecology, physiology, evolutionary genetics, and speciation [3].

Silver carp and bighead carp are two of the four most important pond-cultured fish species in China because of their characteristics such as rapid growth, high tolerance of stress conditions, and low cultivation costs. However, at present, due to the degradation of their germplasm resources and deterioration of aquaculture water environments [4], diseases caused by pathogenic microorganisms occur more frequently in silver carp and bighead carp. Therefore, many breeding studies have aimed to enhance the resistance to diseases in these two species.

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However, although many studies have addressed the breeding, genomics, and genetic resources of silver and bighead carp [5–11], we still have a poor understanding of the resistance to diseases in these two species at the molecular level. The ability to resist some diseases often differs significantly in silver carp and bighead carp during fishery production. For example, in the same natural or artificial aquaculture water body, the silver carp is often affected by the lepidorthosis caused by *Pseudomonas punctata f. ascitae* but the bighead carp does not suffer this disease, while the bighead carp is susceptible to *Branchiomyces spp.* whereas silver carp is not vulnerable to this type of fungus. In addition, among the causes of myxosporidiosis in fish, the parasites comprising *Myxobolus driagini*, *Spirosuturia hypophthalmichthydis*, and *Chloromyxum hypophthalmichthys* are found only in the silver carp [12,13]. Among the causes of dactylogyriasis in fish, the pathogen *Dactylogyrus aristichthys* only parasitizes the gills of the bighead carp whereas the pathogen *D. vaginulatus* only parasitizes the gills of the silver carp. These differences suggest that the immune functions and defenses have diverged significantly in the silver carp and bighead carp during evolution under natural and artificial selection. These evolutionary divergences may explain the significant differences in resistance to some diseases by these two species.

Two main types of culture pattern are employed for the silver carp and bighead carp in China. First, silver carp and bighead carp are cultured as the main fish in lakes and reservoirs, where they breed and produce fish for food. Second, silver carp and bighead carp are cultured together in polyculture ponds at specific proportions with other fish such as the common carp, crucian carp, or grass carp for food production as well as for the biological control of plankton in aquaculture ponds. The silver carp and bighead carp are used as animal models for research into the evolutionary adaptation of immune and defense functions under natural and artificial selection over short periods of time. However, we still have a poor understanding of the evolutionary divergence of the immune functions and defenses in these two species at the molecular level.

In teleosts, the spleen is a primary hematopoietic and peripheral lymphoid organ [14], and it plays an important role in the storage [15] and production of erythrocytes as well as the destruction of aged blood cells. In addition, the teleost spleen contains antibody-producing cells [16] and it also has a role in antigen presentation and initiation of the adaptive immune response [17,18]. The fish spleen acts as a secondary lymphoid organ to filter blood-borne pathogens and it is also an organ that can contribute to protective immunity [19,20]. Therefore, the spleen is a suitable organ for assessing immune responses in fish. Considering the importance of the spleen in fish, we conducted transcriptome sequencing using spleen tissues from silver carp and bighead carp, and performed evolutionary and comparative analyses. The main aim of this study was to determine the differences in the evolutionary adaptation of the immune and defense functions in the bighead carp and silver carp at the molecular level. The results obtained provide a valuable resource to facilitate future research into the molecular mechanisms of disease resistance and differences between the bighead carp and silver carp, as well as supporting breeding to improve disease resistance and disease prevention in these two species.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of the Freshwater Fisheries Research Institute of Henan Province and it did not involve endangered or protected species. All of the animal experiments were conducted in accordance with the Laboratory Animal Management Principles of China.

2.2. Fish sampling

The experimental animals used in this study comprised bighead carp (*A. nobilis*) and silver carp (*H. molitrix*) collected from the Yellow River basin in Xingyang, Henan Province, China. The experimental fish were three years of age and they comprised farmed varieties from the same pond. Samples were taken back to the laboratory where each fish species was stocked in one separate aquarium and an acclimation period was allowed for 48 h. During acclimation, all of the aquaria were maintained under the natural photoperiod, where the dissolved oxygen concentration remained higher than 5 mg/L, the water temperature varied from 20 °C to 25 °C, and the pH ranged from 7.0 to 8.0. Six apparently healthy individuals from each species were anesthetized with MS-222 (100 mg/L, Sigma Chemical Company, St Louis, MO, USA) and their spleen tissues were collected immediately. The average weight of these individuals was 4273.55g for bighead carp and 2750.36g for silver carp, and the average weight of spleen were 4.23g for bighead carp and 3.16g for silver carp. The spleen tissues from the six individuals of each species were mixed equally. The collected samples were then frozen in liquid nitrogen and stored at −80 °C for preservation before use.

2.3. RNA isolation, cDNA library construction, and sequencing

Total RNA was extracted from the spleen tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol and it was then quantified with a 2100 Bioanalyzer (Agilent Technologies). The cDNA libraries were constructed using the spleen tissues obtained from bighead carp and silver carp with a Truseq™ RNA sample prep Kit (Illumina). Poly(A) mRNA was enriched with 5 μg of the initial total RNA using oligo(dT) magnetic beads (Invitrogen) and the mRNA was then randomly fragmented with fragmentation buffer. The first-strand cDNA chain was synthesized using random hexamer primers and the second-strand cDNA was synthesized with buffer, dNTPs, RNase H, and DNA polymerase I. The double-stranded cDNA was end repaired and a-tailed, and indexed adapters were ligated. Finally, the cDNA library was enriched by PCR amplification and quantified using TBS380 Picogreen (Invitrogen). Transcriptome sequencing was performed using the Illumina HiSeq4000 platform by Majorbio (Shanghai, China). Short paired end sequence reads of 151 bp were generated.

2.4. De novo assembly and gene function annotation

Raw reads were cleaned using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software by removing defective reads, such as reads with adaptors, reads with more than 10% Q < 20 bases (those with a base quality less than 20), and low-quality sequences (reads with ambiguous bases “N”). The clean and high-quality reads were used as the basis of all the subsequent analyses. *De novo* assembly of all the cleaned reads was performed using the short reads assembly program Trinity (version number: trinityrnaseq-r2014-04-13) with the default parameters [21]. Functional annotations of the assembled unigenes were performed based on homology searches against the major public databases, i.e., NCBI non-redundant protein sequences (Nr) [22], Swiss-Prot (a manually annotated and reviewed protein sequence database) [23], String (a database of known and predicted protein-protein interactions) [24], protein family (Pfam) [25], Kyoto Encyclopedia of Genes and Genomes (KEGG) [26], and Gene Ontology (GO; http://www.geneontology.org/). The searches against Nr, String, Swiss-Prot, and KEGG were based on BLASTx with an E-value cutoff of 10^{-5}, while those against Pfam used HMMER3 (http://hmmer.org/) and Blast2GO was used for GO [27]. The putative functions of the transcripts were defined by the first subject hits and the BLAST results with the best hit were extracted for transcript description.

2.5. Determination of orthologs

The combined assembly data were searched to identify protein sequences that matched with a database of eight genomes representing Ostariophysi fishes, i.e., *Takifugu rubripes*, *Oryzias latipes*, *Danio rerio*, *Xiphophorus maculatus*, *Gasterosteus aculeatus*, *Oreochromis niloticus*, *Tetraodon nigroviridis*, and *Gadus morhua*. The matched protein sequences above were used for further orthologous gene analysis. Orthologs among the bighead carp and silver carp were determined using OrthoMCL [28]. Briefly, the all-by-all BLASTP search was first performed based on protein sequence, and then the BLAST results were grouped using MCL software with default settings. Ortholog groups of single copy genes (one-copy orthologs) were chosen according to the following two criteria: (1) each gene family contained the genes of both species, (2) there was one and only one gene from a specific species in each gene family. Meanwhile, these one-copy orthologs were aligned at the protein level using the Prank program [29]. The trimmed alignments with lengths longer than 150 bps were applied for further evolutionary analysis. In addition, to rule out the paralogous genes, the sequence alignment for each homologous gene cluster was performed using the MUSCLE software [30], and the genetic tree of homologous gene clusters was constructed using the RAxML software [31]. Finally, by pruning the genetic tree using the Agalma software package [32], orthologous genes with only one in each species were selected.

2.6. Substitution rate estimation and enrichment analysis for adaptive genes

Evolutionary rates were estimated according to a previously described method [33]. The CodeML program in the PAML package was used to estimate the ratio (Ka/Ks values) of the number of non-synonymous substitutions per non-synonymous site (Ka) relative to the number of synonymous substitutions per synonymous site (Ks), as well as to perform selection analyses for each ortholog [34]. In order to identify genes that are likely to be subject to positive selection, maximum likelihood analyses were performed for each ortholog in the gene set, where runmode = −2 and NSsites = 0 in PAML4.5. In general, a Ka/Ks value > 1 is interpreted as an indicator of positive selection whereas a Ka/Ks value < 1 is interpreted as purifying selection [35,36]. KEGG pathway and GO functional enrichment analyses for rapidly evolving genes and positively selected genes were performed using DAVID [37]. Significantly enriched functional clusters were determined when the *p*-value was less than 0.05.

2.7. Expression level analysis of orthologs

In general, the CDS abundance reflects the expression level for orthologous genes. Therefore, the CDS in each orthologous group was used as the reference sequence in this study. The numbers of reads were calculated for each CDS in the different libraries using RSEM software [38]. The read counts were then normalized using the TPM(transcripts per million), where this value denoted the expression level of an orthologous gene [39]. The TPM value was calculated as follows: (read count × 1,000,000)/total_ read count.

3. Results

3.1. Illumina sequencing, de novo assembly, and gene annotation

After Illumina paired-end sequencing, 49,669,196 and 55,992,800 raw reads were obtained from the spleen tissue libraries prepared for bighead carp and silver carp, respectively. The sequencing reads were deposited in the NCBI Database Sequence Read Archive. The accession numbers for the bighead carp and silver carp sequences are SRP078549 and SRP078474, respectively. After stringent quality assessments, 48,821,754 and 55,054,480 clean reads were obtained for bighead carp and silver carp, respectively, where these reads were used as the basis of all the subsequent analyses. The clean reads obtained from the bighead carp and silver carp spleen tissue libraries were assembled into 48,376 and 49,805 unigenes, respectively, using the Trinity assembly program (Table 1). The unigenes were mainly between 201 and 400 bp in length, which accounted for 21.78% of the total unigenes, followed by sequences measuring 601–800 bp (17.65%), 801–1000 bp (10.34%), 401–600 bp (8.76%), 1001–1200 bp (6.92%), 1201–1400 bp (5.07%), and 2001–2400 bp (4.76%) in length.

Table 1
Statistics for the assembled unigenes.

Item	Bighead carp	Silver carp
Total sequence number	48,376	49,805
Total sequence base	63,257,297	63,554,931
Percent GC	44.22%	43.96%
Largest length	28,493 bp	26,593 bp
Smallest length	201 bp	201 bp
Average length	1307.62 bp	1276.08 bp
N50	2055 bp	1989 bp

The assembled unigenes were aligned with sequences in the major databases comprising Nr, Swiss-Prot, String, KEGG, and Pfam using the BLASTx tool. For bighead carp, the numbers of unigenes annotated in the different databases were 17,248 (35.65%) in Pfam, 15,674 (32.40%) in KEGG, 14,603 (30.18%) in String, 21,433 (44.31%) in Swiss-Prot, and 27,367 (56.57%) in Nr. For silver carp, the numbers of unigenes annotated in the Pfam, KEGG, String, Swiss-Prot, and Nr databases were 16,934 (34.00%), 15,558 (31.23%), 14,345 (28.80%), 18,506 (37.16%), and 26,999 (54.20%), respectively. In particular, 8823 and 7550 unigenes were annotated for the bighead carp and silver carp in all five databases, respectively (Fig. 1).

3.2. Identification and adaptive evolution analysis for orthologs

To better understand the evolutionary dynamics of the spleen's immune function in bighead carp and silver carp, we identified 5755 putative orthologs in the two species by comparing the two transcript sets, and 4976 orthologs were retained after alignment and trimming for quality control purposes (**see File 1 in Ref.** [40]). In these orthologs, the overlap region length of the corresponding genes across two species ranged from 150 bp to 15,522 bp, with an average length of 1189.88 bp. For each orthologous gene, we calculated the substitution rates (i.e., Ka, Ks, and Ka/Ks). The number of base substitutions in each orthologous gene ranged from 1 to 559. The average number of base substitutions in each gene was 7.6 for synonymous substitutions and 6.4 for non-synonymous substitutions. This indicated that the detection rate of base substitutions in orthologous genes was still high, although a number of within-species SNP would likely be filtered within the unigene assemblies by using mixed sample sequencing. After examining the Ka/Ks ratios for all the orthologous genes, 439 genes with Ka/Ks > 1 were considered to be divergent genes (i.e., positively selected gene), and the Ka/Ks values for the remaining genes were less than 1 (Fig. 2). The Ka/Ks values for the orthologous genes differed greatly, where they ranged from 0.001 to 50. In total, 1820 orthologous genes with Ka/Ks < 0.1 were considered to be conserved genes during adaptive evolution of the spleen's function in the bighead carp and silver carp. Among the positively selected genes, 90 genes had Ka/Ks = 50, 18 genes had 42 < Ka/Ks < 50, two genes had 10 < Ka/Ks < 38, and 72 genes had 2 < Ka/Ks < 8. This suggests that these orthologous genes exhibited rapid change during adaptive evolution of the spleen's function. Functional enrichment analysis showed that the positively selected genes were closely associated with biological functions, including regulation of cell death, histone modification, and immune response, and they were significantly enriched in pathways such as cytokine–cytokine receptor interaction, hematopoietic cell lineage, complement and coagulation cascades, pertussis, measles, cell

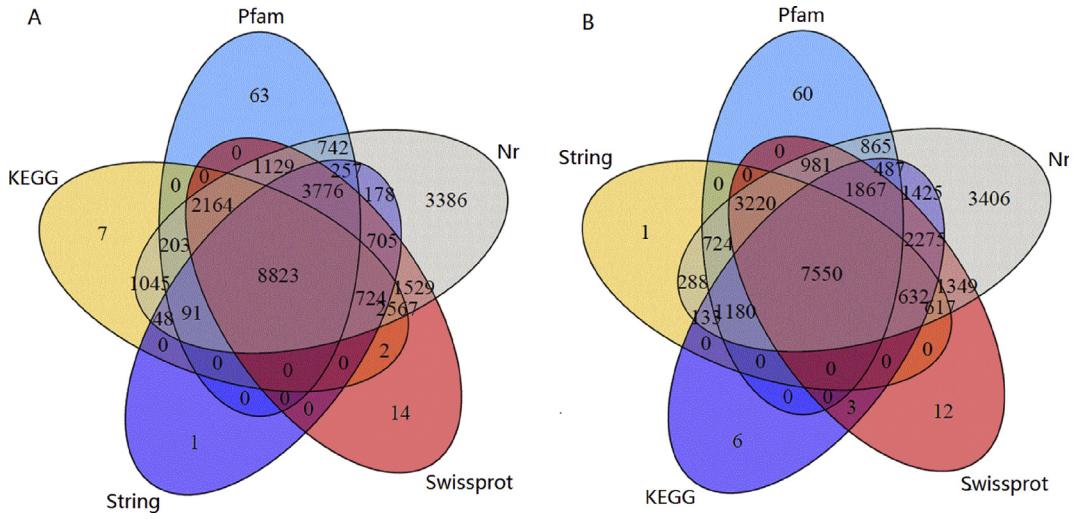

Fig. 1. Venn diagram of the unigenes annotated in five databases. A, bighead carp; B, silver carp.

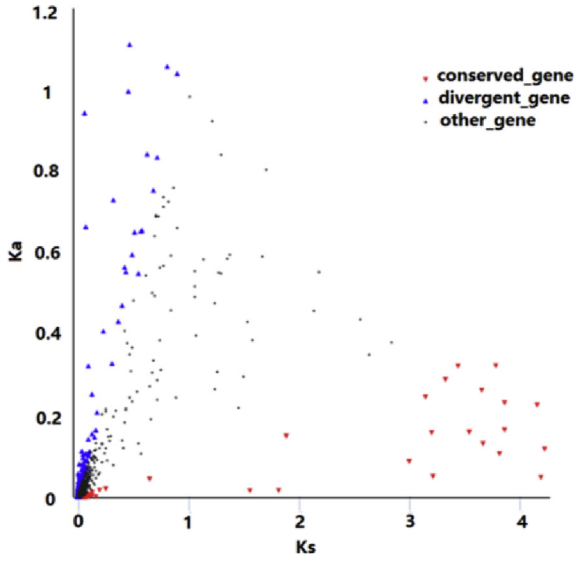

Fig. 2. Distributions of Ka and Ks values for orthologous genes. A blue dot indicates a divergent gene with Ka/Ks > 1 and a red dot indicates a conserved gene with Ka/Ks < 0.1. Ka: non-synonymous substitution; Ks: synonymous substitution. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adhesion molecules, mineral absorption, and legionellosis (FDR < 0.05). The results indicate that these positively selected genes may have played important roles during the evolutionary adaptation of immune and defense functions in the bighead carp and silver carp.

Furthermore, the evolutionary rate of each biological function was analyzed at the GO level based on the Ka, Ks, and Ka/Ks values determined for the orthologous genes. In total, 70 biological functions with Ka/Ks values greater than 1 for their GO term were under greater selective pressure, and thus they exhibited accelerated evolution. In particular, the Ka/Ks values were up to 48–50 for GO terms corresponding to mitochondrial translational elongation, UTP metabolic process, UTP biosynthetic process, GTP biosynthetic process, mitochondrial electron transport, response to exogenous dsRNA, snRNA transport, snRNA import into nucleus, RNA import into nucleus, and others. In addition, accelerated evolution was detected for the immune defense biological functions involved with regulation of production of molecular mediator of immune response, regulation of immunoglobulin production, T cell-mediated cytotoxicity, T cell-mediated immunity, regulation of adaptive immune response, and others.

3.3. Divergence of adaptive evolution genes in A. nobilis and H. molitrix

The Ka/Ks ratios for each ortholog were evaluated in bighead carp and silver carp. To increase the accuracy of subsequent analysis, genes were discarded if they had any one of the following values in bighead carp or silver carp: Ka = 0, Ks = 0, and Ka/Ks = 999 or 0.0001. Finally, 1981 orthologous genes were retained for adaptive evolutionary analysis**(see File 2 in Ref.** [40]), and the distributions of the Ka/Ks values for these orthologous genes were shown in Fig. 3. In these two species, we found that the Ka/Ks values of 68 orthologous genes were greater than 1 whereas those of 1506 orthologous genes were less than 1. The evolutionary direction of these orthologous genes was the same but their selective pressures were different because their Ka/Ks values differed significantly in the two species. In addition, the Ka/Ks values of 212 orthologous genes were greater than 1 in the bighead carp and less than 1 in the silver carp, whereas the Ka/Ks values of 195 orthologous genes were less than 1 in the bighead carp and greater than 1 in the silver carp. This indicates that these orthologous genes were selected in the different direction (positive selection or purifying selection) in the

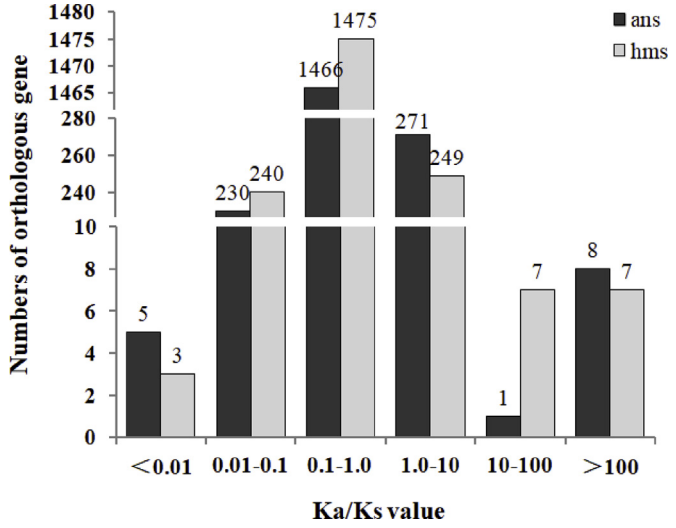

Fig. 3. The distributions of the Ka/Ks values for the orthologous genes in bighead carp and silver carp. The abscissa indicates the interval of Ka/Ks value and the ordinate denotes the number of orthologous genes in the corresponding interval. ans: *Aristichthys nobilis*; hms: *Hypophthalmichthys molitrix*.

spleens of bighead carp and silver carp. For the adaptive genes mentioned above, we evaluated the evolutionary adaptation of their biological functions in the bighead carp and silver carp based on the GO annotations. In terms of biological processes, 622 GO terms with positive selection were identified, where 58 GO terms were positively selected in both bighead carp and silver carp, 255 GO terms only in bighead carp, and 309 GO terms only in silver carp. Moreover, 3187 GO terms with purifying selection were identified in both bighead carp and silver carp. These results indicate that the evolution of adaptive genes differed significantly between bighead carp and silver carp, and the corresponding biological functions also appeared to differ with the evolution of these genes.

To further evaluate the adaptive evolutionary divergence of immune functions in bighead carp and silver carp, we analyzed the GO terms related to immune system process and response to stimulus. We found that some of immune-related biological processes including T cell activation, T cell differentiation, T cell lineage commitment, dendritic cell differentiation, lymphoid progenitor cell differentiation, type 2 immune response, defense response to protozoan, and response to exogenous dsRNA were positively selected in both bighead carp and silver carp. The positively selected genes related to the immune response in both species mainly comprised *cfh, casp9, itgam, il2rb,* and *pkp4* (Table 2). In bighead carp, the positively selected biological processes mainly comprised B cell-mediated immunity, chemokine-mediated signaling pathway, immunoglobulin mediated immune response, leukocyte migration, and defense response, where the positively selected genes mainly included *cf1, c1r, bfb,* and *c9* in the complement pathway, and *ccr6a, ccr12b.2,* and *cxcr3.3* as chemokine receptors (Table 2). In silver carp, the positively selected biological processes included many aspects of immune defense, such as adaptive immune response, antigen processing and presentation, cytokine biosynthetic process, innate immune response, leukocyte-mediated immunity, natural killer cell-mediated immunity, regulation of immune effector process, cellular response to stimulus, defense response to fungus, detection of bacteria, and regulation of defense response, where the positively selected genes comprised *il13ra2, crfb1, tnfsf14, tlr1, tlr3*, and others (Table 2). However, most of the biological processes and their corresponding genes in immune system process and response to stimulus were under purifying selection in both bighead carp and silver carp. The main evolutionarily divergence in bighead carp and silver carp are shown in Fig. 4 for the biological processes related to the immune system and in Fig. 5 for the biological processes involved with response to stimulus. Therefore, in terms of the evolutionary adaptation of immune and defense functions in the bighead carp and silver carp, most of the functions maintained a consistent evolutionary direction and only some specific function exhibited large differences.

3.4. Functions of genes expressed in spleen

We estimated the expression levels of orthologous genes in spleen tissues obtained from bighead carp and silver carp based on the TPM values (**see File 3 in Ref.** [40]). Comparative analysis showed that some orthologous genes were differentially expressed in the spleen tissues from the two species. However, most of these genes were expressed at lower abundances in the spleens of the two species, where these genes were enriched in pathways such as protein digestion and absorption, and pancreatic secretion (FDR < 0.05), which are not associated with functions of the spleen. By contrast, a number of orthologous genes were expressed at higher abundances but their expression levels were not significantly different in the two species. In particular, 633 orthologous genes with TPM ≥ 100 were found in the bighead carp or silver carp, where they accounted for 12.7% of the total orthologous genes. High abundance orthologous genes were significantly enriched in biological processes such as translation, mRNA splicing, protein folding, proteolysis, mitochondrial electron transport, ribosome biogenesis, and response to lipopolysaccharide (FDR < 0.05).

Moreover, after analyzing the transcriptome sequencing data, we also found a large number of genes expressed only in spleen of bighead carp or silver carp (referred to as specifically expressed genes, SEG). There were 2974 and 3494 SEGs in bighead carp (**File 4 in Ref.** [40])

Table 2
Some positively selected genes involved with immune system processes and response to stimulus in *A. nobilis* and *H. molitrix*.

Gene_name	Orthology_id	ans_Ka/Ks	hms_Ka/Ks	Description
cfh	ORTHOMCL7889	1.5182	5.6323	complement factor H
itgam	ORTHOMCL8005	1.4352	3.4693	integrin, alpha M (complement component 3 receptor 3 subunit)
il2rb	ORTHOMCL7701	1.2928	1.8601	interleukin 2 receptor, beta
casp9	ORTHOMCL6142	1.6208	1.5763	caspase 9, apoptosis-related cysteine peptidase
pkp4	ORTHOMCL5055	2.1665	1.0770	plakophilin 4
CFI	ORTHOMCL7534	2.4149	0.8044	complement factor I
tlr20.4	ORTHOMCL7944	1.9263	0.7461	toll-like receptor 20, tandem duplicate 4
ifi35	ORTHOMCL5173	1.7246	0.2099	interferon-induced protein 35
itfg1	ORTHOMCL4942	1.7158	0.1694	integrin alpha FG-GAP repeat containing 1
tnfrsf9a	ORTHOMCL6539	1.4940	0.5288	tumor necrosis factor receptor superfamily, member 9a
ccr12b.2	ORTHOMCL4386	1.4020	0.1744	chemokine (C-C motif) receptor 12b, tandem duplicate 2
c9	ORTHOMCL5201	1.3618	0.8481	complement component 9
bfb	ORTHOMCL5552	1.3237	0.3283	complement component bfb
il2rga	ORTHOMCL7468	1.2113	0.4378	interleukin 2 receptor, gamma a
ccr6a	ORTHOMCL2468	1.1213	0.5241	chemokine (C-C motif) receptor 6a
c1r	ORTHOMCL2311	1.1032	0.0820	complement component 1, r subcomponent
cxcr3.3	ORTHOMCL4908	1.0893	0.4116	chemokine (C-X-C motif) receptor 3, tandem duplicate 3
rap2b	ORTHOMCL4924	1.0545	0.9168	RAP2B, member of RAS oncogene family
gig2i	ORTHOMCL3452	1.0527	0.8584	grass carp reovirus (GCRV)-induced gene 2i
aatf	ORTHOMCL6255	1.0023	0.3365	apoptosis antagonizing transcription factor
il13ra2	ORTHOMCL5140	0.8320	89.2285	interleukin 13 receptor, alpha 2
il2rb	ORTHOMCL6174	0.7352	3.4797	interleukin 2 receptor, beta
tnfsf14	ORTHOMCL6979	0.9471	2.9393	tumor necrosis factor (ligand) superfamily, member 14
tlr1	ORTHOMCL5282	0.2745	1.7954	toll-like receptor 1
nfil3-6	ORTHOMCL6400	0.2340	1.6076	nuclear factor, interleukin 3 regulated, member 6
crfb1	ORTHOMCL7619	0.2619	1.3883	cytokine receptor family member b1
itln2	ORTHOMCL2695	0.5127	1.3184	intelectin 2
tlr3	ORTHOMCL4813	0.2124	1.1392	toll-like receptor 3
ifi30	ORTHOMCL3062	0.7861	1.0254	interferon, gamma-inducible protein 30

ans: *Aristichthys nobilis*; hms: *Hypophthalmichthys molitrix*.

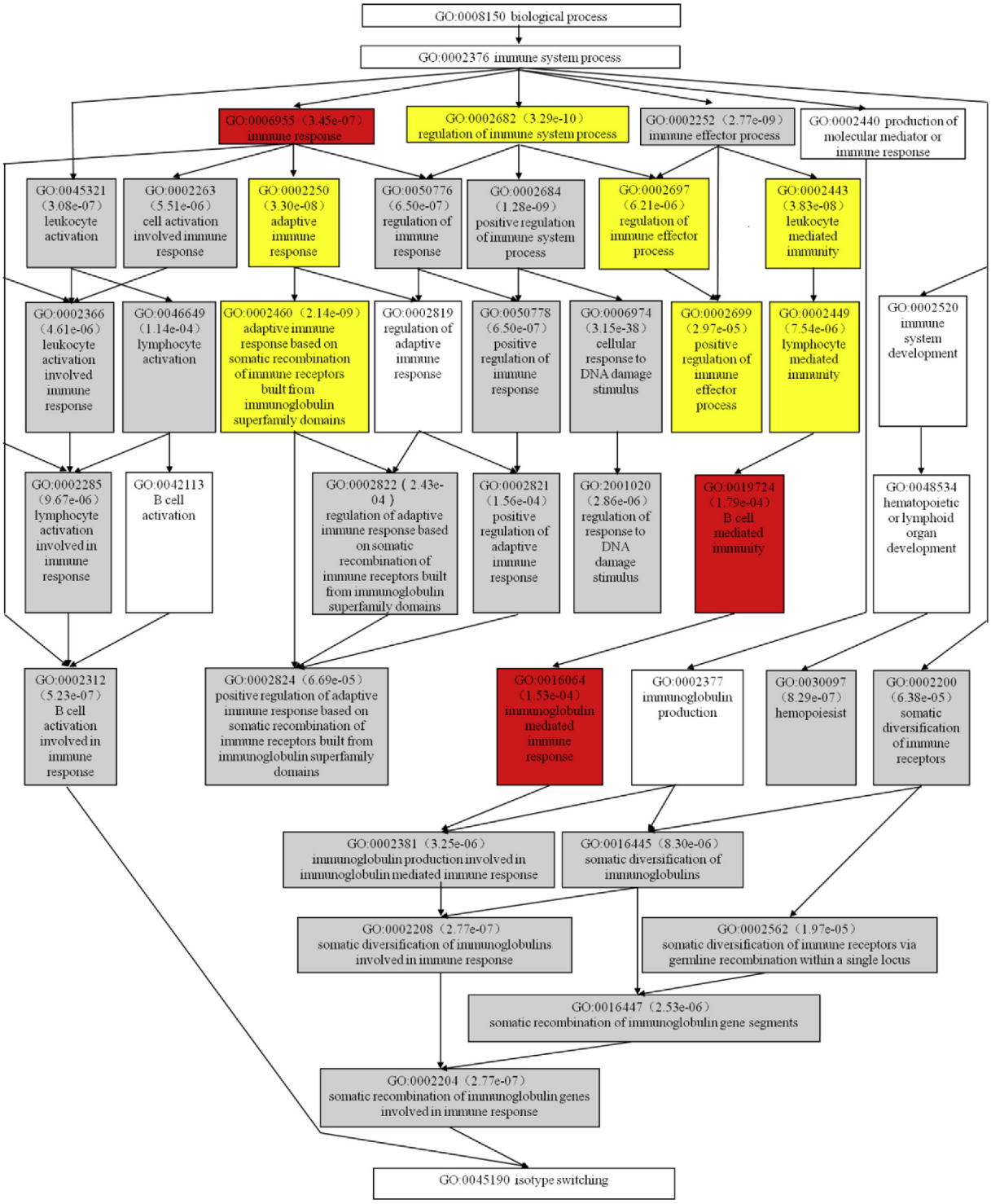

Fig. 4. Hierarchy enrichment of evolutionarily divergent GO terms related to immune system processes in *A. nobilis* and *H. molitrix*. The results were calculated using the hypergeometric distribution in the GO-Term Finder module. The Bonferroni correction was handled by the AmiGO module. The *p*-value cutoff was 0.0005. The minimum number of gene products was 12. Each of the terms in the figure was significantly enriched. Connections between broad high-hierarchy terms and more specific low-hierarchy terms are indicated by arrows. Gray boxes represent terms where the Ka/Ks ratio was less than 1 in both *A. nobilis* and *H. molitrix*. Red boxes represent terms where the Ka/Ks ratio was greater than 1 in *A. nobilis*, but less than 1 in *H. molitrix*. Yellow boxes represent terms where the Ka/Ks ratio was greater than 1 in *H. molitrix*, but less than 1 in *A. nobilis*. White boxes denote associated terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and silver carp (**File 5 in Ref.** [40]), respectively. In the bighead carp spleen, the TPM values of these SEGs ranged from 0.1 to 7366.17, where the number of genes with TPM ≥50 was 276, which accounted for 9.3% of the total SEGs. In the silver carp spleen, the TPM values of SEGs ranged from 0.12 to 6881.1, and 335 genes with TPM ≥50 accounted for 9.6% of the total SEGs. Thus, most of the SEGs that emerged in the evolutionary process were expressed at lower abundances. In addition, functional annotations were made for these SEGs. Surprisingly, functional annotations of these SEGs were strikingly similar between two species, and their functions were nearly

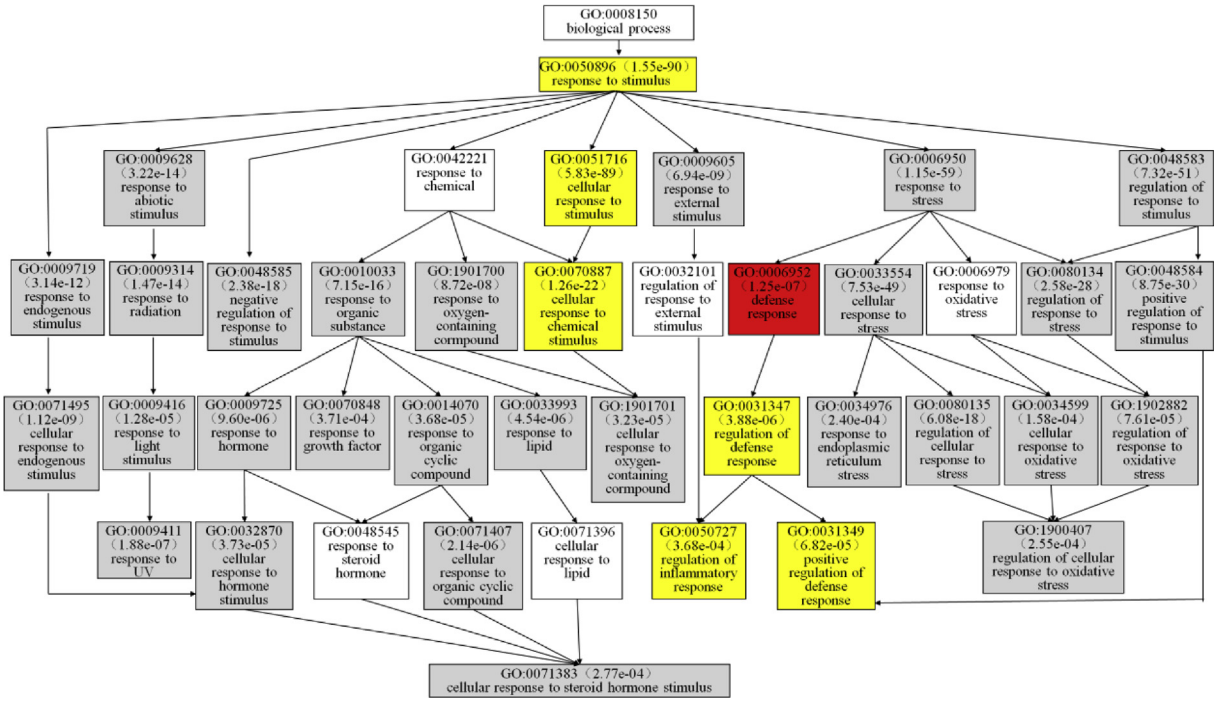

Fig. 5. Hierarchy enrichment of evolutionarily divergent GO term related to response to stimulus in *A. nobilis* and *H. molitrix*. The results were calculated using the hypergeometric distribution in the GO-Term Finder module. The Bonferroni correction was handled by the AmiGO module. The *p*-value cutoff was 0.0005. The minimum number of gene products was 12. Each of the terms in the figure was significantly enriched. Connections between broad high-hierarchy terms and more specific low-hierarchy terms are indicated by arrows. Gray boxes represent terms where the Ka/Ks ratio was less than 1 in both *A. nobilis* and *H. molitrix*. Red boxes represent terms where the Ka/Ks ratio was greater than 1 in *A. nobilis*, but less than 1 in *H. molitrix*. Yellow boxes represent terms where the Ka/Ks ratio was greater than 1 in *H. molitrix*, but less than 1 in *A. nobilis*. White boxes denote associated terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

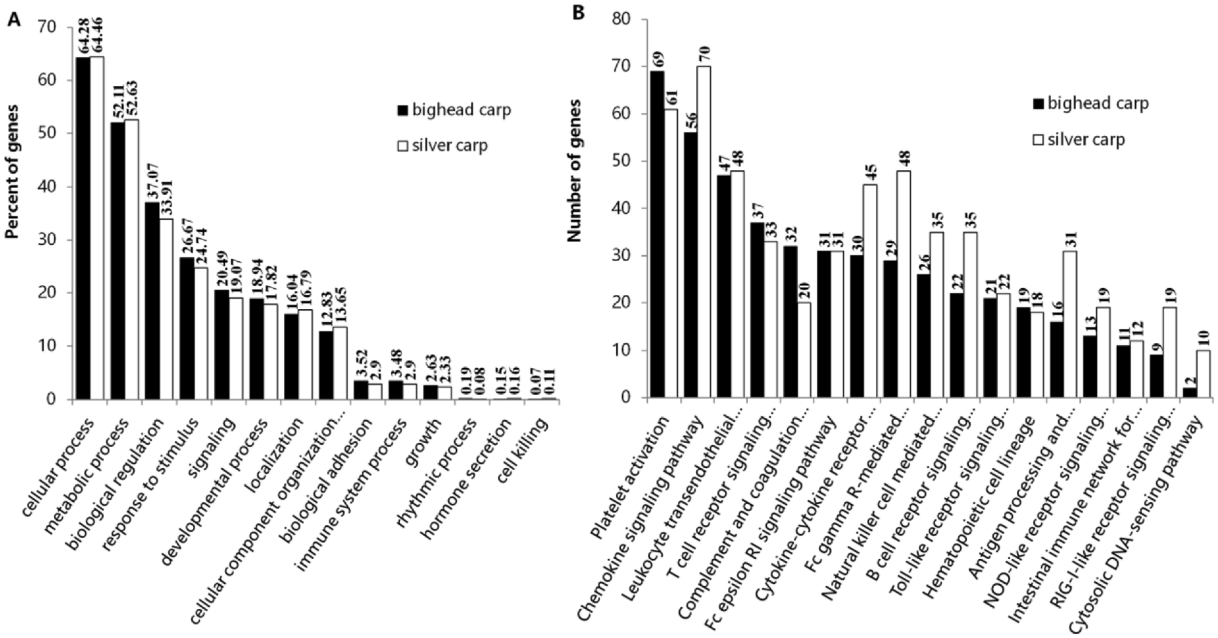

Fig. 6. Enriched GO terms (A) and KEGG pathways (B) for genes expressed only in spleen of bighead carp or silver carp. The bars represent the numbers of gene enriched for each GO term or KEGG pathway. The total numbers of sequences in bighead carp and silver carp were 2587 and 3,686, respectively.

concentrated in the same biological processes and pathways(Fig. 6). The GO annotations showed that the functions of SEGs were mostly involved with basic biological functions, such as cellular process, metabolic process, and biological regulation, as well as immune defense functions, including response to stimulus, immune system process, and cell killing (Fig. 6A). According to the KEGG pathway information, the SEGs were associated with 320 pathways in the bighead carp and silver carp, where metabolic pathways were the most common with 284 and 440 genes assigned in bighead carp and silver carp, respectively. In addition, 17 pathways related to immune system process were also identified (Fig. 6B), which suggests that these SEGs may have played important roles during the evolution of immune functions in the

bighead carp or silver carp. In particular, we found that SEGs were assigned to many disease-related pathways according to the KEGG annotations, where these pathways included pathways in cancer and various types of cancer, infectious diseases caused by bacteria, viruses, and protozoa, diseases related to autoimmune deficiency, and other types of diseases**(see File 6 in Ref.** [40]).

4. Discussion

Bighead carp (A. *nobilis*) and silver carp (*H. molitrix*) are two closely related species from the subfamily Xenocypridinae within Cyprinidae, which are found in the rivers of China. They are also the most important aquaculture fish species in ponds, lakes, and reservoirs in China. In the unique polyculture pattern employed in China, these two species provide an ideal model system for studying rapid evolutionary adaptation in response to natural and artificial selection over short periods of time. In recent decades, bighead carp and silver carp have exhibited accelerated evolutionary adaptation due to various factors, including habitat fragmentation or loss caused by ongoing human activities such as water pollution, dam construction, and over-fishing, the mixing of wild populations and cultured individuals due to frequent flooding and unscientific artificial release, as well as the deleterious effects of inbreeding, hybridization, and invasion [41]. For example, a previous study confirmed that the morphological characteristics of silver and bighead carp in the Yangtze River have changed dramatically over the past 50 years, where they have developed larger heads but smaller trunks and tails [8]. This suggests that the morphological changes in these two species are due to rapid adaptation to the effects of artificial selection. Positive selection is an important source of evolutionary innovation and adaptation, so the main aim of this study was to identify orthologous genes under positive selection. We found a total of 439 orthologous genes with Ka/Ks $>$ 1, which might have been subject to positive selective pressure in bighead carp and silver carp. These orthologs were significantly enriched in biological functions or pathways such as cell death, immune response, hematopoietic cell lineage, and complement and coagulation cascades. In addition, we found four genes that served as markers of spleen development in the sequencing data, i.e., *Tlx1*, *Tcf21*, *Nkx2.5*, and *Ikaros* [42–46]. These genes were expressed at higher abundances in the spleens of bighead carp and silver carp, but their expression levels were not significantly different in the two species **(see File 7 in Ref.** [40]). The orthologous gene “ORTHOMCL7164” encoding “T-cell leukemia, homeobox 1” was found to be subject to strong positive selection. It is known that Tlx1 is a marker of early spleen mesenchymal cells and it acts downstream of the genetic cascade that governs spleen development by promoting cell fate specification and organ expansion [42,43]. During spleen development, the loss of Tlx1 reduces the proliferation of the splenic mesenchyme and arrest growths [46]. In fish, the spleen is a primary hematopoietic and peripheral lymphoid organ, and it plays important roles in cellular responses and host defense. Our results indicate that spleen development and function have also been evolving rapidly in the bighead carp and silver carp under natural and artificial selection, but also because of increasingly severe environmental stress.

Changes in gene expression are considered to underlie many of the phenotypic differences between species [47]. But, in this study, we found that some differentially expressed orthologous genes with lower abundances were significantly enriched in pathways such as protein digestion and absorption, which are not associated with functions of the spleen. By contrast, many orthologous genes with higher abundances such as ribosomal protein and beta-2 microglobulin precursor were not significantly differentially expressed in the bighead carp and silver carp spleen tissues. However, these genes play fundamental and important roles in the functions of spleen. Furthermore, it has frequently been shown that natural variation involve alterations in tissue-specific gene expression [48]. Thus, we identified many genes expressed only in spleen of bighead carp or silver carp (SEGs). It should be noted that these SEGs were mostly enriched in the same biological processes and pathways, where their functions mainly had basic physiological roles such as cellular process, metabolic process, response to stimulus, immune system process, and metabolic pathways in the spleen(Fig. 6). These results suggest that the evolution of the basic physiological function of the spleen in the bighead carp and silver carp is consistent with the evolutionary direction, and it has been relatively well conserved during evolution. This evolutionary pattern may be closely related to the unique polyculture pattern in China as well as the similar ecology of the bighead carp and silver carp because unrelated species often evolve predictably similar features when present separately in the same environment [49]. Under polyculture, the two species comprising bighead carp and silver carp have lived in the same water environment for a long period of time under the same environmental stresses.

The spleen has important immune and defense functions in fishes. Our comparative analysis of orthologous genes under selective pressure demonstrated found significant differences in the evolutionary adaptation of immune and defense functions in the spleens of bighead carp and silver carp. These differences mainly belonged to the following two types. First, orthologous genes and functions under purifying selection were dominant in both bighead carp and silver carp spleens, but the selective pressure differed between the two species. In general, purifying selection is expected to increase the Ka/Ks value due to random substitution throughout the genome. We found that 1056 orthologous genes and 3187 GO terms were under purifying selection in both the bighead carp and silver carp, which is similar to a previous report that the evolution of gene expression in mammals is strongly shaped by purifying selection [47]. The functions under purifying selection were involved mainly with leukocyte activation, lymphocyte activation, regulation of immune response, and somatic diversification of immunoglobulin (Fig. 4). Second, the orthologous genes and functions under positive selection in the spleen differed significantly in the bighead carp and silver carp. In general, positive selection is expected to increase the Ka/Ks value in specific loci and this is an important source of evolutionary adaptation, where the signatures of positive selection indicate that these genes have important roles in the adaptation of organisms to environmental change [50]. We found that the functions with significant differences under positive selection conditions were related mainly to the adaptive immune response in the spleen of bighead carp and silver carp (Fig. 4). In silver carp, the immune response and regulation of immune system processes were under strong positive selection, and thus the adaptive immune response based on the somatic recombination of immune receptors as well as the positive regulation of immune effector processes associated with them were mainly under selection during evolution. In contrast to the silver carp, B cell-mediated immunity and immunoglobulin-mediated immune responses were mainly under selection during evolution because of strong positive selection for leukocyte-mediated immunity in the bighead carp (Fig. 5). Among the immune functions that differed significantly, B cells can differentiate into follicular B cells and marginal zone B cells in the spleen [51], where they are responsible for the production of specific antibodies [52] and they participate in the immune response to pathogen infections such as bacteria, viruses, and parasites [53]. Immunoglobulin is an important functional molecule in the acquired immune system in fishes and other vertebrates, and it is widespread in the humoral circulation system and local mucosal immune system in fishes [54]. It is known that the main types of immunoglobulin in teleost fish are IgM [55,56], IgD [57], IgT [58,59], IgZ [60], and IgH [61], which play important roles in antibacterial and antiviral responses. Moreover, some genes that encode complement factors were under strong positive selection in bighead carp, whereas some genes encoding interferon were under strong positive selection in silver carp (Table 2). It is known that complement and interferon are important molecules related to non-specific immunity in the blood or mucus in fishes. Complement molecules can eliminate pathogenic microorganisms such as bacteria, fungi, and viruses via the complement pathway [62,63]. Interferon can

induce cells to produce antiviral proteins in vertebrates and it has a broad range of antiviral activities [64,65]. In addition, the GO terms associated with defense response to fungus and detection of bacteria were found to be under strong positive selection only in silver carp but not in bighead carp. These results indicate that adaptive immunity have clearly diverged in the bighead carp and silver carp during their evolutionary adaptation to the environment. These changes may explain the differences in resistance to infectious diseases by these two species.

In recent years, diseases induced by viruses and bacteria have emerged as the main problems that affect bighead carp and silver carp aquaculture in China. Coincidentally, we found that the SEGs in bighead carp and silver carp were enriched in many disease-related pathways **(see File 6 in Ref**. [40]). Meanwhile, we also found that the defense response, regulation of defense response, defense response to protozoans, and other processes were under strong positive selection in both the silver carp and bighead carp. These results are consistent with the current situation in terms of the disease outbreaks that are more frequent in silver carp and bighead carp. In general, the occurrence of diseases is closely related to deterioration of the ecological environment in aquaculture. We found that many forms of stress caused by abiotic factors were detected according to the adaptive evolutionary analysis of orthologous genes(Fig. 5). Detection of these factors was related to water pollution caused by a high breeding density, excessive food, the unreasonable use of drugs, and other problems. Among the different types of stress responses, endoplasmic reticulum (ER) stress is an adaptive response to the accumulation of misfolded proteins within the ER [66–68], and plays important roles in some diseases [69]. Oxidative stress leads to an environment where pro-oxidant species overwhelm antioxidant species in the body, which is considered to be associated with many diseases [70]. Therefore, the presence of these factors may be a fundamental cause of the frequent occurrence of diseases in bighead carp and silver carp during aquaculture in China. Moreover, we found that the cellular response to chemical stimulus and regulation of inflammatory response were only under strong positive selection in silver carp but not in bighead carp (Fig. 5), which may explain why the sensitivity to some stress factors during production differs between the bighead carp and silver carp. However, because of budget reasons, the pooled spleen samples were used for the transcriptome sequencing in this study. This strategy could not reveal the differences in gene expression levels between bighead carp and silver carp, and it also couldn't rule out statistically a few outliers. In particular, the disease-related pathways enriched above were caused by the genetic differences or environmental influence, which was also impossible to be separated out. Thus, we will further verify these results through independent sampling and sequencing in future studies.

In this study, we sequenced spleen transcriptomes for the silver carp and bighead carp using Illumina paired-end sequencing for the first time, and identified many genes and functions that are under positive selection in the spleens of these two species. The results showed that the evolutionary adaptation of the spleen in bighead carp and silver carp in the unique poly-culture pattern employed in China has led to convergence in the basic physiological functions but significant divergence in terms of adaptive immunity. The transcriptome resources produced by this study may provide an important basis for comparative genomic studies of adaptation in silver carp, bighead carp, and other closely related species, as well as for understanding the genetic basis of differences in resistance to some diseases in silver carp and bighead carp.

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