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Comprehensive genome-wide identification and functional characterization of *mapk* gene family in northern snakeheads (*Channa argus*)

Chaonan Sun [a], Mingxin Zhu [a], Lingyu Wang [a], Haishen Wen [a], Xin Qi [a], Chao Li [b], Xiaoyan Zhang [b], Donglei Sun [a,**], Yun Li [a,*]

[a] *Key Laboratory of Mariculture (Ocean University of China), Ministry of Education (KLMME), Fisheries College, Ocean University of China, Qingdao, 266003, China*
[b] *School of Marine Science and Engineering, Qingdao Agricultural University, Qingdao, 266109, China*

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ABSTRACT

The mitogen-activated protein kinase (MAPK) signaling cascade, integral to cellular regulation, orchestrates cell growth, differentiation, stress response, and inflammatory reactions to adapt to challenging environments. The northern snakeheads (*Channa argus*), a valuable freshwater species known for its hypoxia tolerance, rapid growth, and high nutritional value, lacks comprehensive research on its *mapk* gene family. In this study, we identified 16 *mapk* genes in northern snakeheads, among which *mapk8*, *mapk12* and *mapk14* have duplicate copies. Phylogenetic analysis confirmed the evolutionary conservation of this gene family. Structural and motif analyses further underscored the conserved nature of these genes. Expression pattern analysis under abiotic and biotic stress conditions showed significant differences expression of *mapks* in the gills and suprabranchial organ (SBO) after air exposure, as well as in the brain following cold stress, highlighting the extensive role of *mapks* in stress regulation. It was worth noting that the significant expression differences of *mapks* were also observed in the spleen after *N. seriolae* infection, implicating that these genes may be involved in the regulation of innate immune responses. Additionally, analysis of protein-protein interaction (PPI) networks suggested that the co-activation of multiple MAPK signaling pathways may play a key role in regulating an organism's response to biotic and abiotic stresses. This study provides a detailed description of the *mapk* gene family in the northern snakeheads and elucidates its biological functions under various stress conditions, offering valuable insights into the regulatory mechanisms of the *mapk* gene family.

1. Introduction

During growth and development, fish may encounter various adverse environmental conditions, such as extreme temperatures, low dissolved oxygen, and pathogenic bacteria. In response to external stressors, physiological, cellular, and molecular adaptations occur within fish to sustain their normal growth and development. The Mitogen-activated protein kinase (MAPK) pathway serves as a crucial conduit for transmitting signals from the extracellular environment to the intracellular milieu [1], playing a pivotal role in regulating the organism's response to these challenges. MAPK is a serine-threonine protein kinase that can be activated by a variety of extracellular stimuli, including hormones, neurotransmitters, cytokines, and stress responses [2]. A typical MAPK cascade consists of three continuously active protein kinases (MAPK kinase kinase/MAPKKK, MAPK kinase/MAPKK and MAPK) [3]. As an important part of this cascade reaction, MAPK can directly act on targets in the cytoplasm or nucleus and regulate the expression of related genes, to regulate cell changes in response to adverse environment. The MAPK family consists of four traditional subfamilies: extracellular signal-regulated kinase (ERK), p38 MAPK (p38), c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase 5 (ERK5), each with distinct roles in specific signaling pathways. ERKs contain a TEY (Thr-Glu-Tyr) activation motif and function within the Ras/Raf/MEK/ERK pathway [4]. Similarly, p38 MAPKs have a TPY (Thr-Pro-Tyr) activation motif and are involved in the p38 pathway. JNKs, also possessing a TPY activation motif, participate in the JNK/SAPK pathway, while ERK5, characterized by a TEY activation motif and a longer amino acid sequence, functions within the ERK5 pathway [5]. Each MAPK subfamily responds to distinct stimuli. The Ras/Raf/MEK/ERK pathway is activated primarily by growth factors [6], whereas the p38 and

* Corresponding author.
** Corresponding author.
E-mail addresses: sdl@stu.ouc.edu.cn (D. Sun), yunli0116@ouc.edu.cn (Y. Li).

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JNK/SAPK pathways are triggered by inflammatory signals, bacterial lipopolysaccharides (LPS), chemokines, ultraviolet radiation, and other stressors [7–9]. The ERK5 pathway is uniquely responsive to both growth factors and environmental stimuli [5]. Collectively, the MAPK subfamilies exhibit diverse roles in regulating downstream signal transduction, enabling fish to adapt to various environmental challenges effectively.

In recent years, genome-wide identification of the *mapk* gene family has become a focal point in ichthyological research. Specifically, *mapk* genes (*mapks*) have been identified in zebrafish (*Danio rerio*) [10], turbot (*Scophthalmus maximus*) [11], atlantic salmon (*Salmo salar*) [12], gilthead seabream (*Sparus aurata*) [13], european seabass (*Dicentrarchus labrax*) [14], spotted seabass (*Lateolabrax maculatus*) [15], japanese flounder (*Paralichthys olivaceus*) [16], largemouth bass (*Micropterus salmoides*) [17], and yellow catfish (*Pelteobagrus fulviadraco*) [18]. In aquatic organism, MAPK cascade responses to abiotic and biological stresses have achieved some results. Studies have shown that zebrafish (*D. rerio*) exposed to triclosan (TCS) an extended period exhibited upregulation at both the transcription level and the protein level to mediate apoptosis of hepatocytes [19]. The phosphorylation levels of p38 and ERK subfamilies in the *mapk* gene family were changed when European bass was re-fed after starvation stimulation [14]. Exposure of Mediterranean fish gilthead seabream (*S. aurata*) to a temperature limit of 24 °C resulted in increased phosphorylation levels of p38 and JNK members, indicating the involvement of the MAPK signaling cascade in high-temperature stress processes [13]. The *mapk7*, *mapk15*, *mapk8a*, *mapk8b*, and *mapk12* in japanese flounder (*P. olivaceus*) were found to be sensitive to the effects of temperature stress [16]. In the spotted seabass (*L. maculatus*), the expressions of *mapk8*, *mapk9*, *mapk11*, *mapk14a* and *mapk14b* were significantly upregulated under salinity stress [15]. The JNK and p38 subfamily members of killifish (*Fundulus heteroclitus*) *mapk* gene family also changed significantly when subjected to salinity stress, suggesting the role of MAPK signaling cascades in osmotic regulation [20,21]. The involvement of MAPK cascade signaling pathway in the process of hypoxia stress has been demonstrated in zebrafish [22], channel catfish (*Ictalurus punctatus*) [23], crucian carp (*Carassius carassius*) [24] and spotted seabass [15]. After infection with *Aeromonas hydrophila*, the expression levels of JNK1a, JNK1b, p38 delta, and p38 alpha b in the *mapk* gene family were significantly up regulated [18]. Japanese flounder *mapk*6, *mapk11* and *mapk14b* were sensitive to the impact of *Edwardsiella tarda* challenge [16]. After rainbow trout is infected with *Vibrio anguillarum*, ERK, p38, JNK signaling pathways are involved in immune regulation [25]. In conclusion, previous studies have shown that MAPK signaling pathways are widely involved in temperature adaptation, osmotic regulation, hypoxic stress, and immune response in fish.

Northern snakeheads (*Channa argus*), is known as an economically important freshwater fish. Due to its hypoxia tolerance, strong growth ability and high nutritive value, it is extremely popular for aquaculture in China. Notably, northern snakeheads possess a suprabranchial organ (SBO), a unique respiratory structure that enables them to breathe air under low oxygen conditions or when exposed to air, an adaptation not commonly found in other fish species. With the intensive development of aquaculture and the increase of aquaculture density, northern snakeheads face numerous adverse factors during the aquaculture process. Among these factors, *Nocardia seriolae* infection leads to many white nodules in the gut (spleen, kidney, liver), which restricts the development of the aquaculture industry [26]. Therefore, in this study, we identified the *mapk* gene family of northern snakeheads for the first time based on existing research results, analyzed the sequence characteristic of different *mapks*, and compared phylogenetic relationships of *mapk* gene family members among different species. With an interest of understanding the involvement of *mapks* of northern snakeheads in response to biotic and abiotic stresses, the mRNA expression profiles of *mapks* in different tissues were analyzed after low temperature, hypoxia, and *N. seriolae* infection challenges. This research aims to enhance our understanding of the role of *mapks* in fish growth, development, stress response, and immune function, potentially guiding future strategies to mitigate the impact of aquaculture stressors.

2. Materials and methods

2.1. Identification and characterization of mapk genes in C. argus

To identify *mapks* of *C. argus*, *mapks* of human (*Homo sapiens*) and zebrafish (*D. rerio*) sequence as a query to retrieve from NCBI database (http://www.ncbi.nlm.nih.gov/) and used TBLASTN (cutoff E-value: $1e^{-5}$) to search the reference genome (GenBank: GCA_027943205.1) and transcriptome database (PRJNA834927, PRJNA599026, PRJNA615958, PRJNA832507, PRJNA877371, PRJNA693677, PRJNA772548) of *C. argus*. To eliminate duplicate and obtain a unique set of sequences, using the ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) to compare initial sequence. Open reading frame (ORF) were predicted, and the retrieved corresponding sequence were translated by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Predicted ORFs were verified by comparing the NCBI non-redundant (NR) protein database with BLASTP. The conserved domains of *mapks* were identified and predicted by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/). Using ExPASyProt-Param (https://web.expasy.org/protparam/) to calculate each MAPK protein molecular weight (MW) and isoelectric point (pI). The *mapks* copy numbers of different species were statistically compared according to genome databases of several other vertebrates. According to the location of *mapks* on the reference genome, the MapGene2chromosome web v2 (MG2C) software tool (http://mg2c.iask.in/mg2c_v2.0/) was used to locate the genes on the chromosome.

2.2. Phylogenetic analysis of mapk genes

In order to evaluate the evolutionary relationship of the *mapk* gene family, the amino acid sequences of *mapks* from *C. argus* and several representative vertebrates were downloads from the NCBI and Ensemble databases (http://www.ensembl.org/), including human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), zebrafish (*D. rerio*), climbing perch (*A. testudineus*), gobie (*P. magnuspinnatus*), barramundi perch (*L. calcarifer*), zig-zag eel (*M. armatus*), siamese fighting fish (*B. splendens*), spotted seabass (*L. maculatus*), and spotted gar (*L. oculatus*). Multiple amino acid sequences were aligned by ClustalW2 program (https://www.ebi.ac.uk/Tools/msa/clustalw2/), with the default parameters (Edgar, 2004). The phylogenetic tree was further prettified by Interactive Tree of Life (iTOL, https://itol.embl.de/).

2.3. Syntenic analysis of mapk genes

To provide additional evidence for the identification and annotation of *mapks*, we identified the adjacent genes of *mapk8*, *mapk12*, and *mapk14* from the reference genome of *C. argus*. Briefly, the amino acid sequences of neighboring genes of *mapk8*, *mapk12*, and *mapk14* were predicted from the *C. argus* chromosome sequences by FGENESH program of MolQuest software (Softberry Int.). Subsequently, the identified amino acid sequences were annotated against NCBI non-redundant (NR) database and UniProt Knowledgebase (UniProtKB) by BLASTP. Meanwhile, conserved syntenic regions and their flanking genes of other species (*D. rerio*, *A. testudineus*, *P. magnuspinnatus* and *B. splendens*) were identified from the NCBI and Ensemble databases. Subsequently, syntenic analysis was performed on the duplicate copies of *mapk8a* and *mapk8b*, *mapk12a* and *mapk12b, mapk14a* and *mapk14b*. For interchromosome syntenic analysis, genomic sequences of *C. argus*, *D. rerio* and *B. splendens* were used for genome scale analysis of syntenic relationship with lotus in MCScanX, then visualized with TBtools software (v 2.012).

2.4. Conserved motifs and gene structure analysis of mapk genes

Conserved motifs of the *mapks* were determined using the MEME software (http://meme.nbcr.net/MEME/). The untranslated regions (UTR), coding sequence (CDS) of each member were obtained from the reference genome of the *C. argus*, and the gene structure can be obtained from the Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/) to build.

2.5. Analysis of MAPK protein interaction patterns

To detect protein-protein interaction (PPI) networks, the predicted MAPK protein sequences were aligned using the String online software (https://cn.string-db.org/) with the default setting. The composite score of protein interactions was obtained based on STRING information such as homology, co-expression, experimentally determined interactions, database annotation and automatic textmining. The resulting PPI networks between MAPK proteins were then visualized using Cytoscape software (v 3.9.1).

2.6. Transcriptomic data analysis

To observed the *mapks* expression in different tissues and under response to biotic (*N. seriolae* infection) and abiotic (low temperature) stresses, the mRNA expression profiles, the online available transcriptomic data were downloaded from NCBI (PRJNA834927, PRJNA599026, PRJNA615958, PRJNA832507, PRJNA877371, PRJNA693677, PRJNA772548) as depicted in previous studies [26–29]. The transcriptomic data were mapped to the reference genome of *C. argus* (GenBank: GCA_027943205.2) using Hisat2 (v 2.2.1). Then, the expression level of each gene was calculated and normalized to the transcripts per million (TPM) value used for measuring gene expression levels using StringTie.

2.7. Sample collection of air breathing experiment

To investigate the expression patterns of *C. argus* MAPKs under air exposure stress conditions, gill and suprabranchial organ (SBO) tissues were collected at four time points. The adult *C. argus* used in the experiment were sourced from Daqiang Fishery Co., Ltd., in Linyi, Shandong Province, China. Healthy fish with an average body length of 23.69 ± 2.68 cm and an average body weight of 101.72 ± 12.23 g were selected for the study. The fish were acclimated in freshwater at 22–24 °C for one week before exposure experiments and sample collection. In the control group (0 h), the fish were maintained under standard rearing conditions. In the treatment group, fish were placed on a moist sponge and exposed to air, with efforts made to ensure the water temperature matched the ambient air temperature as closely as possible. At 3 h, 6 h, and 24 h of air exposure, the fish were anesthetized with MS-222 at a concentration of 200 mg/L. Gill and SBO tissues were collected from three biological replicates per group, flash-frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction.

2.8. The analysis of the expression patterns of mapk genes in the air-breathing status

For the transcriptome analysis of air-breathing experiment, gills and SBOs were obtained from *C. argus* after breathing in air for 0 h, 3 h, 6 h and 24 h. Equal amounts of RNA from 3 individuals for each sample were pooled, and a total of 24 sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's instructions. Then the libraries were sequenced on the Illumina HiSeq X Ten platform with the PE150 strategy at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The raw sequencing data was first processed by removing adapters and low-quality reads using Trimmomatic (v 0.39). Transcriptomic data was evaluated by Hisat2 and StringTie and bioinformatics (https://www.bioinformatics.com.cn) software were used to generate the expression heatmap.

2.9. Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± SEM. Statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software Inc., USA). Univariate analysis of variance (ANOVA) was carried out with SPSS 25.0 (IBM, USA), with $P < 0.05$ considered statistically significant.

3. Results

3.1. Identification of mapk genes in C. argus

To identify the *mapks* of *C. argus*, we used the protein sequences of *H. sapiens* and *D. rerio* as query sequences for BLAST search. Further, a total of 16 *mapks* sequences were identified in *C. argus* (Table .1). The length of the *mapks* transcripts ranged from 1080 bp (*mapk1*) to 3372 bp (*mapk7*), and the predicted amino acid ranged from 359 aa (MAPK1) to 1123 aa (MAPK7). The molecular weights of MAPKs ranged from 41.48 kDa (MAPK11) to 123.81 kDa (MAPK7), and pI ranged from 4.83 (MAPK6) to 9.63 (MAPK15).According to the variety of double phosphorylation sites, these *mapks* were divided into three subfamilies: *mapk1*, *mapk3*, *mapk4*, *mapk6*, *mapk7*, and *mapk15* belong to extracellular signal regulating kinase (ERK) subfamily, *mapk8a*, *mapk8b*, *mapk9*, and *mapk10* belong to c-Jun N-terminal kinase (JNK) subfamily, *mapk11*, *mapk12a*, *mapk12b*, *mapk13*, *mapk14a*, and *mapk14b* belong to p38 subfamily.

3.2. Phylogenetic and syntenic analysis

The amino acid sequences of MAPKs of 12 vertebrate species were used to construct the phylogenetic tree, determing the evolutionary relationships among these representative species and identifying the annotation of the *mapks* in *C. argus* (Fig. 1). To further confirm the annotation of duplicate *mapks*, syntenic analysis was performed on *mapk8a*, *mapk12a*, and *mapk14a*. As shown in the results of Fig. 2, syntenies were clearly conserved for these 6 *mapks* in the studied species (*C. argus*, *D. rerio*, *A. testudineus*, *P. magnuspinnatus*, *B. splendens*), Moreover, through the interchromosomal syntenic analysis of *C. argus*, *D. rerio,* and *B. splendens* (Fig. 3), revealed that the three species still retained a large number of similar sequences from the same ancestral genome after a long evolutionary process. This suggests that MAPKs were relatively conserved throughout evolutionary history.

3.3. Analysis of gene copy number and chromosome localization

The copy numbers of *mapks* of *C. argus* and other representative vertebrates were summarized in Table .2. From mammals to fish, the copy numbers of the *mapks* were relatively conserved. The total gene numbers of *mapk* were varied slightly from 10 to 16. Among them, *mapk2* and *mapk5* were absent from all the species listed in (Table .2). Furthermore, we identified the presence of duplicate copies of *mapk8*, *mapk12*, and *mapk14* in *C. argus*, *D. rerio*, and *P. magnuspinnatus*, whereas only a single copy was observed in higher vertebrates.

The 16 *mapks* of *C. argus* were located on 11 chromosomes. As shown in the results (Fig. 4), each of the individual chromosomes (2, 7, 8, 9, 17, and 19) contains 1 *mapk* gene, whereas chromosomes (1, 10, 12, 20, and 21) contain 2 *mapks*. Notably, *mapks* that belonging to the same subfamily were not observed to be located on the same chromosome.

3.4. Gene structure analysis

The results of gene structure analysis of 16 *mapks* (Fig. 5A) showed

Table 1
Characteristics of *mapks* in *C. argus*.

Gene Name	Chromosome ID	Activation Sites	Subfamily Classification	mRNA(bp)	Size of Amino Acid (aa)	MW(kDa)	pI	Accession Number
mapk1	Chr12	TEY	ERK	1080	359	41.87	7.87	PQ240837
mapk3	Chr17	TEY	ERK	1458	485	54.30	7.53	PQ240838
mapk4	Chr12	SEG	ERK	2979	992	111.39	6.42	PQ240839
mapk6	Chr2	SEG	ERK	2277	758	86.13	4.83	PQ240840
mapk7	Chr20	TEY	ERK	3372	1123	123.81	7.99	PQ240841
mapk8a	Chr1	TPY	JNK	1785	594	66.99	7.03	PQ240842
mapk8b	Chr20	TPY	JNK	1317	438	49.49	6.75	PQ240843
mapk9	Chr9	TPY	JNK	1269	422	47.86	4.94	PQ240844
mapk10	Chr19	TPY	JNK	1707	568	64.20	8.54	PQ240845
mapk11	Chr21	TGY	p38	1083	360	41.48	6.02	PQ240846
mapk12a	Chr8	TGY	p38	1086	361	42.03	7.85	PQ240847
mapk12b	Chr21	TGY	p38	1641	546	61.79	7.97	PQ240848
mapk13	Chr10	TGY	p38	1400	467	52.79	7.44	PQ240849
mapk14a	Chr10	TGY	p38	1095	364	42.05	7.94	PQ240850
mapk14b	Chr7	TGY	p38	1230	409	47,15	6.02	PQ240851
mapk15	Chr1	TEY	ERK	1827	608	67.79	9.63	PQ240852

that the number of introns in *mapks* ranged from 7 (*mapk3*, *mapk7*) to 14 (*mapk4*, *mapk12b* and *mapk15*), indicating that introns loss occurred during evolution [30]. In addition, in the analysis of the *mapks*, except for *mapk3*, *mapk4*, *mapk8a*, *mapk10*, *mapk12b*, and *mapk14a*, the remaining *mapks* all contain 5′ UTR and 3′ UTR.

The MEME software was used to determine the conserved motifs of *mapks* in *C. argus*. A total of 8 conserved motifs (motif 1–8) were identified in this study (Fig. 5B). The numbers and types of conserved motifs were relatively conservative. Except motif 6 and motif 8, the other motifs were conserved in the protein sequences of MAPKs. We found that motif 8 exists only in *mapk8a*, *mapk8b*, *mapk9* and *mapk10*, all of which belong to the JNK subfamily, suggesting that motif8 may be involved in the function of JNK subfamily proteins.

3.5. Protein-protein interaction network analysis of mapk gene family members

The protein-protein interaction (PPI) network analysis was performed with the aim of revealing potential interacting proteins and associated signaling pathway transduction pathways. The results showed that the *mapks* from three different subfamilies could interact with each other (Fig. 6). For example, *mapk3* and *mapk7* from the ERK subfamily, as well as *mapk9* and *mapk10* from the JNK subfamily, exhibited interactions with two copies of *mapk14* (*mapk14a*, *mapk14b*) from the p38 subfamily. Additionally, *mapk11* was observed to interact with two copies of *mapk14* from the same subfamily. Moreover, *mapk4* from the ERK subfamily and *mapk9* from the JNK subfamily interacted with two copies of *mapk8* from the JNK subfamily. Similar PPI patterns were observed in other genes, including *mapk3* and *mapk7* from the ERK subfamily. Moreover, ERK subfamily *mapks* interacted with those of the JNK and p38 subfamilies, while JNK subfamily *mapks* interacted with those of the ERK and p38 subfamilies.

3.6. Expression patterns of the mapk genes in the different tissues of C. argus

The tissue expression profiles of *mapks* were determined using the published transcriptome sequencing data of *C. argus* in fifteen different tissues under normal conditions, including the jaw, skin, gill, suprabranchial organ, eye, intestine, spleen, swim bladder, heart, liver, brain, spinal cord, testis, ovary, and muscle (Fig. 7). The log10(TPM+1) are shown in Supplementary Table 1. All identified *mapks* were ubiquitously and differentially expressed in all tissues. *mapk4*, *mapk14b*, *mapk3*, *mapk1*, *mapk6* and *mapk9* showed relatively high overall expression levels, while the rest of the *mapks* overall expression is relatively low. Most *mapks* exhibited higher expression level in the brain and spinal cord, while all *mapks* were showed lower expression level in muscle. We found higher expression level of *mapk12a* in intestine, *mapk15* in testis, and *mapk13*, *mapk12a*, and *mapk14a* of the p38 subfamily in skin, gill, and suprabranchial organ. It is worth noting that some *mapks* have tissue-specific expression patterns, such as *mapk11* only being expressed in the spleen, brain, and spinal cord, while *mapk10* only being expressed in the eye, brain, and spinal cord. Moreover, *mapk12b* was hardly expressed in all tissues.

3.7. Expression profiles of mapk genes after air-breathing in C. argus

To further understand the changes of *mapks* expression in response to air-breathing, the relative expression levels of the major respiratory organs gill and SBO after 0 h (control group), 3 h, 6 h, and 24 h of air exposure were analyzed based on RNA-seq data (Fig. 8). The value of log10(TPM+1) are shown in Supplementary Table 2. The results showed that all *mapks*, except *mapk10* and *mapk12b,* showed different expression patterns after air exposure, and most *mapks* showed an up-regulation trend. In the gills, the expression levels of *mapk15* and *mapk9* were continuously upregulated and reached a peak after 24 h. It was worth noting that the relationship between the expression profiles of some genes and the time after air-breathing was not a simple linear increase or decrease. Specifically, the expression levels of *mapk4*, *mapk12a* and *mapk13* showed a downward trend after 3 h of air exposure compared with the control group (0h), and then an upward trend similarly reached the highest expression level at 24 h. The highest expression levels of *mapk14b*, *mapk3*, *mapk7*, *mapk8a*, *mapk1*, *mapk6*, *mapk8b* and *mapk11* all appeared at 6 h after hypoxic treatment. In the SBOs, the expression levels of *mapk15*, *mapk7*, *mapk12a*, *mapk14a*, *mapk6*, *mapk9* and *mapk8b* exhibited a continuous rise during air exposure, peaking at 24 h. After 3 h of air exposure, the expression levels of *mapk8a*, *mapk1* and *mapk11* exhibited a decline compared to control group. Subsequently, a gradual increase was observed, reaching a peak after 24 h. The highest expression levels of *mapk3* and *mapk4* occurred 6 h after air exposure. It is worth noting that *mapk6, mapk12a*, *mapk13*, *mapk14a*, and *mapk15* showed a significant upregulation trend in both tissues, indicating that they play a critical role in the process of responding to air-breathing stress in *C. argus*.

3.8. Cold stress induced the differential expression of the mapk genes in C. argus

To investigate the expression profiles of *mapks* after cold stress in *C. argus,* the relative expression levels of the liver and brain following low temperature treatment for different times (4, 6, 8, 10, and 16 h) were analyzed based on published transcriptome sequencing data (Fig. 9) [31]. The value of log10(TPM+1) are shown in Supplementary Table 3. In the liver, all *mapks*, except *mapk4*, *mapk10*, *mapk11*, *mapk12b*

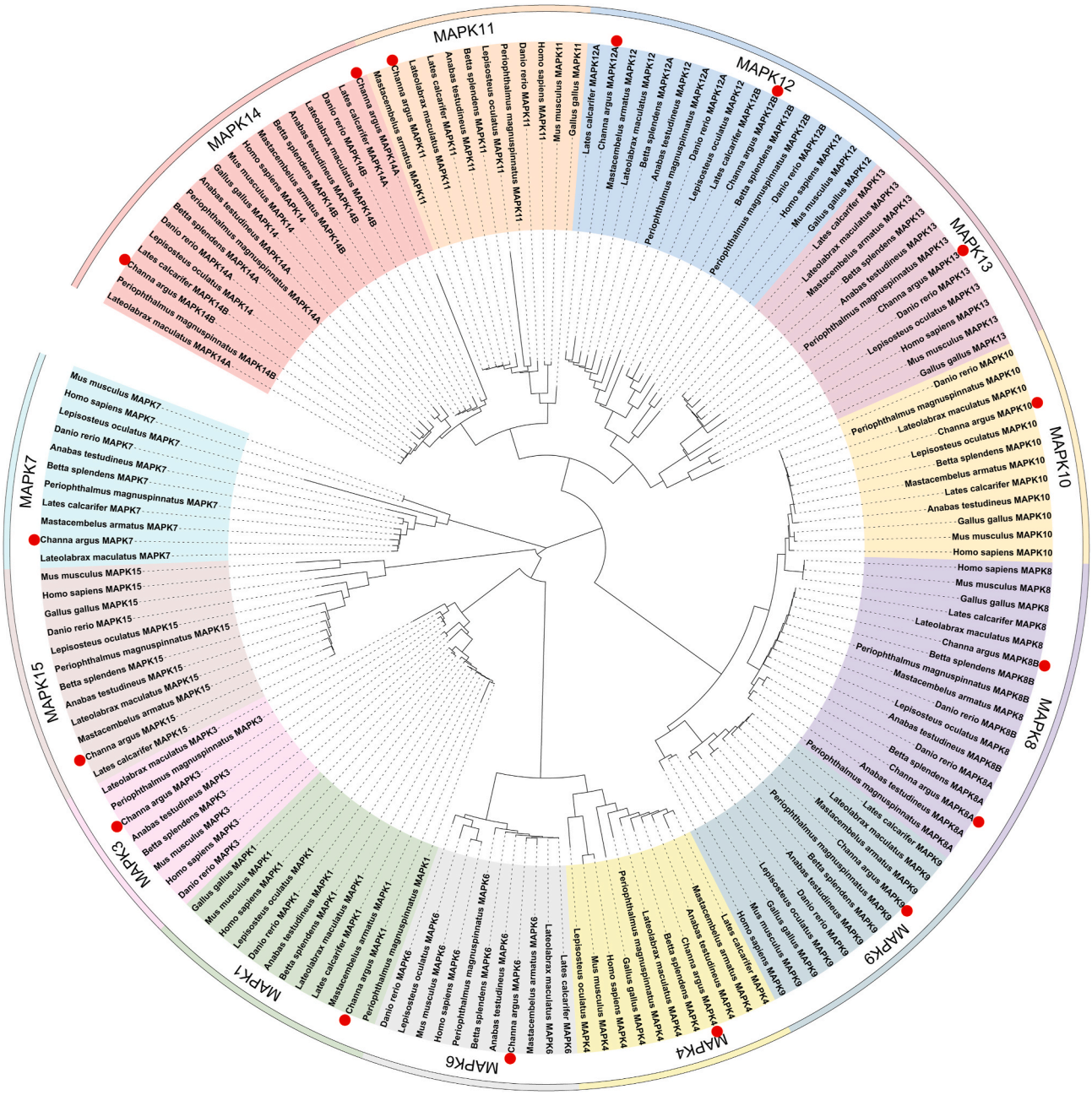

Fig. 1. Phylogenetic relationships of *C. argus* MAPKs. The phylogenetic tree was constructed by the amino acid sequences from several representative mammals and teleosts with 1000 bootstrap relationships in MEGA7. MAPKs of *C. argus* was labeled with red dot. The phylogentic tree was divided into 13 groups with different colored background blocks.

and *mapk14a,* were generally differentially expressed. The expression levels of *mapk6* and *mapk14b* showed an upregulated trend at 4 h of cold stress, while *mapk3*, *mapk7*, *mapk8b*, *mapk12a* and *mapk15* showed a downregulated trend. In the brain, all *mapks* except *mapk1*, *mapk8a*, *mapk9*, *mapk14b*, *mapk11*, and *mapk14a* were ubiquitously differentially expressed. After 4 h of cold stress *mapk6*, *mapk8b*, *mapk10*, *mapk13* and *mapk14b* showed an up-regulated trend, whereas *mapk12a* and *mapk12b* showed a down-regulated trend. Interestingly, the expression of *mapk6* and *mapk14b* showed an up-regulated trend at most time points, while *mapk15* showed a down-regulated trend in both tissues. In addition, the expression trend of *mapk3* and *mapk13* were opposite in the two tissues. In detail, *mapk3* and *mapk13* were down-regulated in liver, but up-regulated in brain. In summary, *mapk10*, *mapk8b*, *mapk13*, *mapk14b* and *mapk12b* in brain, as well as *mapk6*, *mapk9* and *mapk14b* in liver, may be involved in modulating the process of low-temperature stress.

3.9. Identification of mapk genes involved in immune response of C. argus

Previous studies showed that bacterial infection could cause the activation of the MAPK signaling pathway [16,18,25]. To further understand the expression profiles of *mapks* in response to *N. seriolae* infection, the relative expression levels of the spleen 48 h, 96 h, and 144 h after bacterial infection were analyzed based on the RNA-seq data, and heatmaps were obtained. The value of log10(TPM+1) are shown in Supplementary Table 4. The expression profile of the spleen after bacterial infection was shown in (Fig. 10). With the exception of *mapk15*, *mapk12b*, and *mapk14b*, which did not respond to the bacterial infection, the expression of the remaining *mapks* was up-regulated or

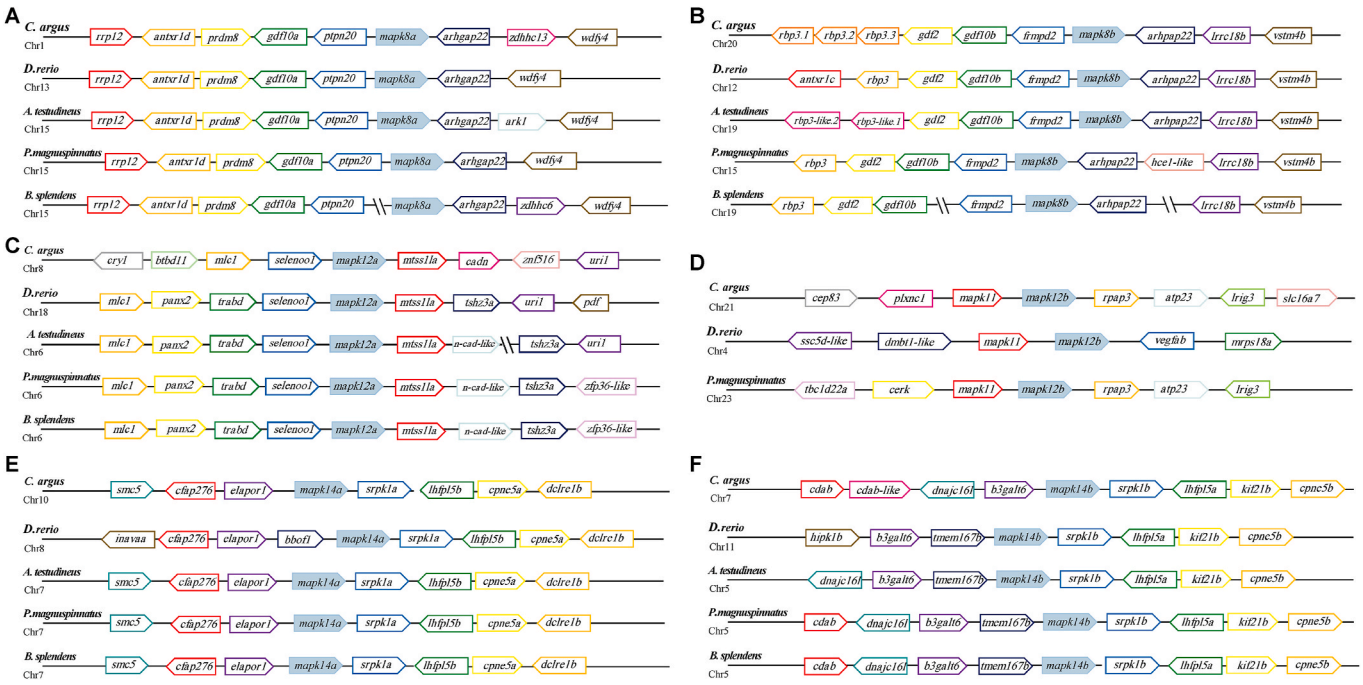

Fig. 2. Syntenic analysis of *C. argus mapk8, mapk12,* and *mapk14* with *D. rerio, A. testudineus, P.magnuspinnatus,* and *B. splendens.* A. *mapk8a*; B. *mapk8b*; C. *mapk12a*; D. *mapk12b*; E. *mapk14a*; F. *mapk14b*; These syntenies were generated with the information obtained from the NCBI. Full gene names were provided in Appendix: Supplementary Table 5.

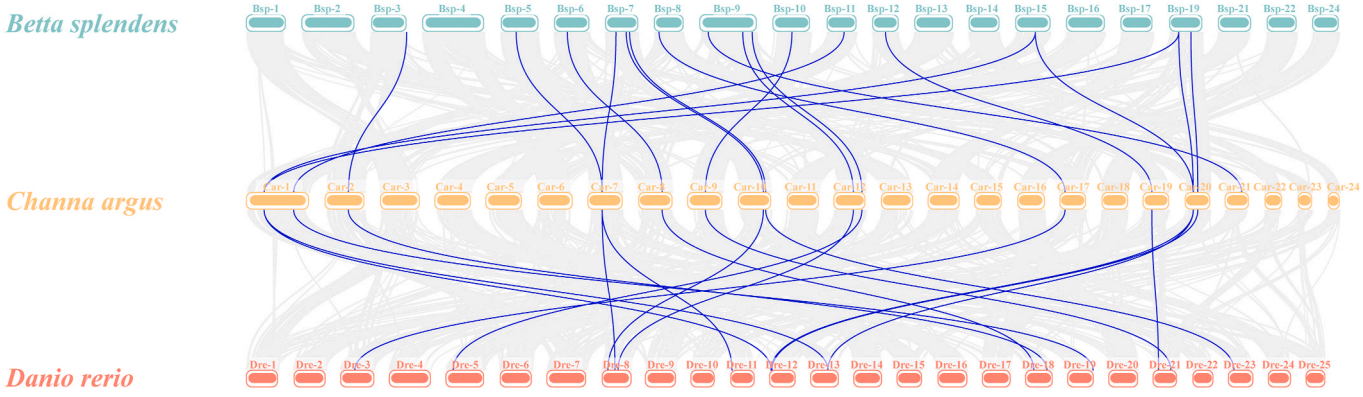

Fig. 3. Syntenic analysis of chromosomes between *C. argus, D.rerio,* and *B. splendens.* Chromosomes of different species are represented by different color blocks, where green represents *B. splendens*, orange represents *C. argus* and red represents *D.rerio*, and the blue connecting line shows the syntenic relationship of *mapks* between different chromosomes in the three species.

down-regulated. In detail, the *mapk9*, *mapk1*, *mapk3*, *mapk11*, *mapk6* and *mapk14b* expression were continuously upregulated after bacterial infection and reaching a peak at 144 h. However, the expression of *mapk10* showed a trend of lower after a bacterial infection and reaching a minimum value at 144 h of *N. seriolae* infection. In addition, the lowest expression of *mapk7*, *mapk12a*, and *mapk8a* appeared 96 h after infection.

4. Discussion

MAPK is an evolutionarily conserved serine-threonine protein kinase that can be activated by various stimuli outside the cell and transduced into the cell, where it participates in various MAPK signaling cascades [2]. Previous studies on the MAPK signaling pathway in fish has demonstrated its pivotal role in temperature adaptation, osmotic pressure regulation, oxygen adaptation and immune regulation [13–15, 17–19]. The northern snakehead (*C. argus*) are valuable freshwater species known for its hypoxia tolerance, rapid growth, and high nutritional value. However, the characteristics and functions of its *mapk* gene family have not yet been systematically studied. Accordingly, this study represents the inaugural identification of the *mapk* gene family in *C. argus*, with the discovery of 16 *mapks*. Furthermore, phylogenetic analysis of the *mapk gene family* among different species was also performed. To ascertain the accuracy of gene annotation and chromosome localization, a syntenic analysis was employed. Additionally, the gene structure and conserved motifs of the genes were analyzed. Transcriptome sequencing data were used to analyze the expression patterns of *C. argus* under the biological (*N. seriolae* infection) and abiotic (low temperature, air exposure) stresses.

Based on the double-phosphorylation sites of MAPKs amino acid sequence and phylogenetic analysis, the *mapk* gene family of *C. argus* is divided into three subfamilies (ERK, JNK and p38) and *mapks* are highly conserved in every subfamily, which is similar to the results of previous studies [15,17]. The analysis of gene copy number in 12 representative

Table 2
Copy number of *mapks* in different species.

Name	*Channa argus*	*Danio rerio*	*Anabas testudineus*	*Periophthalmus magnuspinnatus*	*Lates calcarifer*	*Mastacembelus armatus*	*Betta splendens*	*Lateolabrax maculatus*	*Lepisosteus oculatus*	*Gallus gallus*	*Mus musculus*	*Homo sapiens*
mapk1	1	1	1	1	1	1	1	1	1	1	1	1
mapk2	0	0	0	0	0	0	0	0	0	0	0	0
mapk3	1	1	1	1	0	0	1	1	0	0	1	1
mapk4	1	1	1	1	1	1	1	1	1	0	1	1
mapk5	0	0	0	0	0	0	0	0	0	0	0	0
mapk6	1	1	1	1	1	1	1	1	1	1	1	1
mapk7	1	1	1	1	1	1	1	1	1	0	1	1
mapk8	2	2	2	2	1	1	2	1	1	1	1	1
mapk9	1	1	1	1	1	1	1	1	1	1	1	1
mapk10	1	1	1	1	1	1	1	1	1	1	1	1
mapk11	1	1	1	1	1	1	1	1	1	1	1	1
mapk12	2	2	1	2	2	1	1	1	1	1	1	1
mapk13	1	1	1	1	1	1	1	1	1	1	1	1
mapk14	2	2	2	2	2	2	2	2	1	1	1	1
mapk15	1	1	1	1	1	1	1	1	1	1	1	1
Total	16	16	15	16	14	13	15	14	12	10	13	13

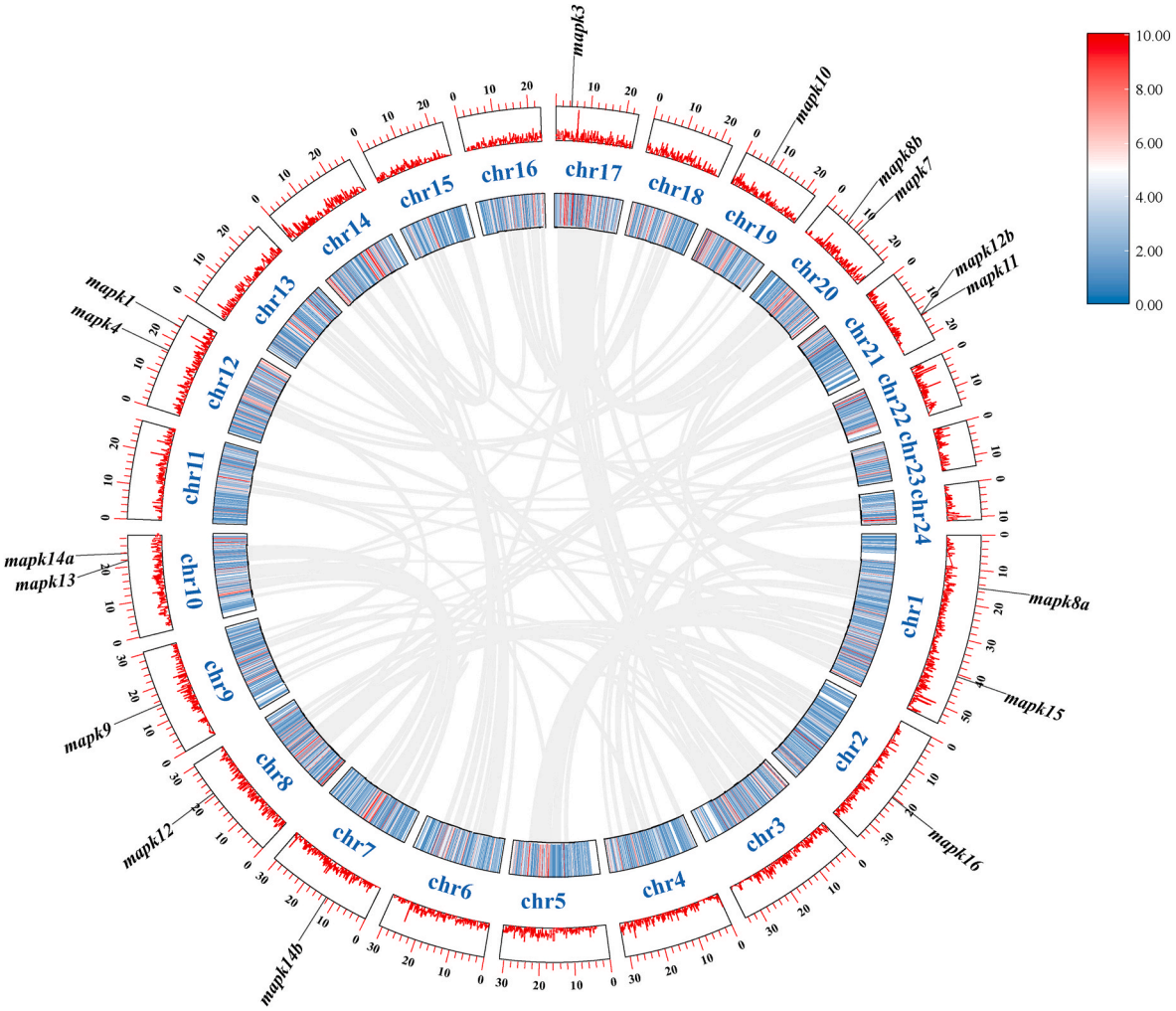

Fig. 4. Chromosomal locations of *mapks* of *C. argus*. The black lines represent the location of each *mapk* gene on the chromosome, and the collinearity of all genes in the genome is represented by the gray lines. The red outer ring represents gene density on each chromosome.

vertebrates provides valuable insights into the evolution of *mapk* gene family and enhances our understanding of the biological mechanism of *mapks* regulation. The analysis, which included *C. argus*, *D. rerio*, *L. calcarifer*, *M. armatus*, *B. splendens*, *L. maculatus*, *L. oculatus*, *A. testudineus* and *P. magnuspinnatus*, identified 12 to 16 *mapk genes*. Additionally, previous studies on the identification of plant *mapks* demonstrated the existence of 20, 28 and 43 *mapks* in arabidopsis (*Arabidopsis thaliana* (L.) Heynh), sunflower (*Helianthus annuus* L.) and strawberry (*Fragaria* × *ananassa* Duch.) respectively [32–34]. It is hypothesized that the difference in gene copy number between plants and animals may be attributed to the strategy of gene replication and genome polyploidy that plants usually employ during evolution.

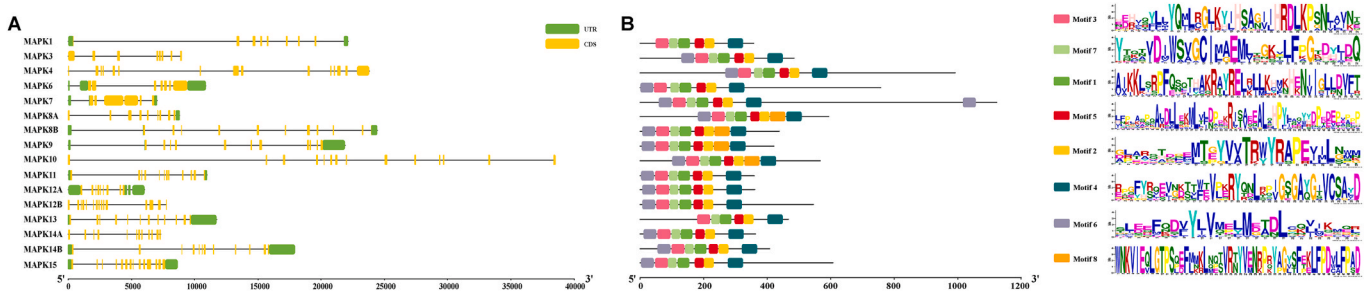

Fig. 5. Gene structure (A) and motif (B) content of MAPK predicted by using the MEME suite tool. A. Gene structure analysis of *C. argus* MAPKs, the untranslated regions (UTR) are marked with green boxes, the coding sequence (CDS) are marked with yellow boxes and black lines indicate introns. B. Motif analysis of *C. argus* MAPKs, different motif is marked with different colored boxes.

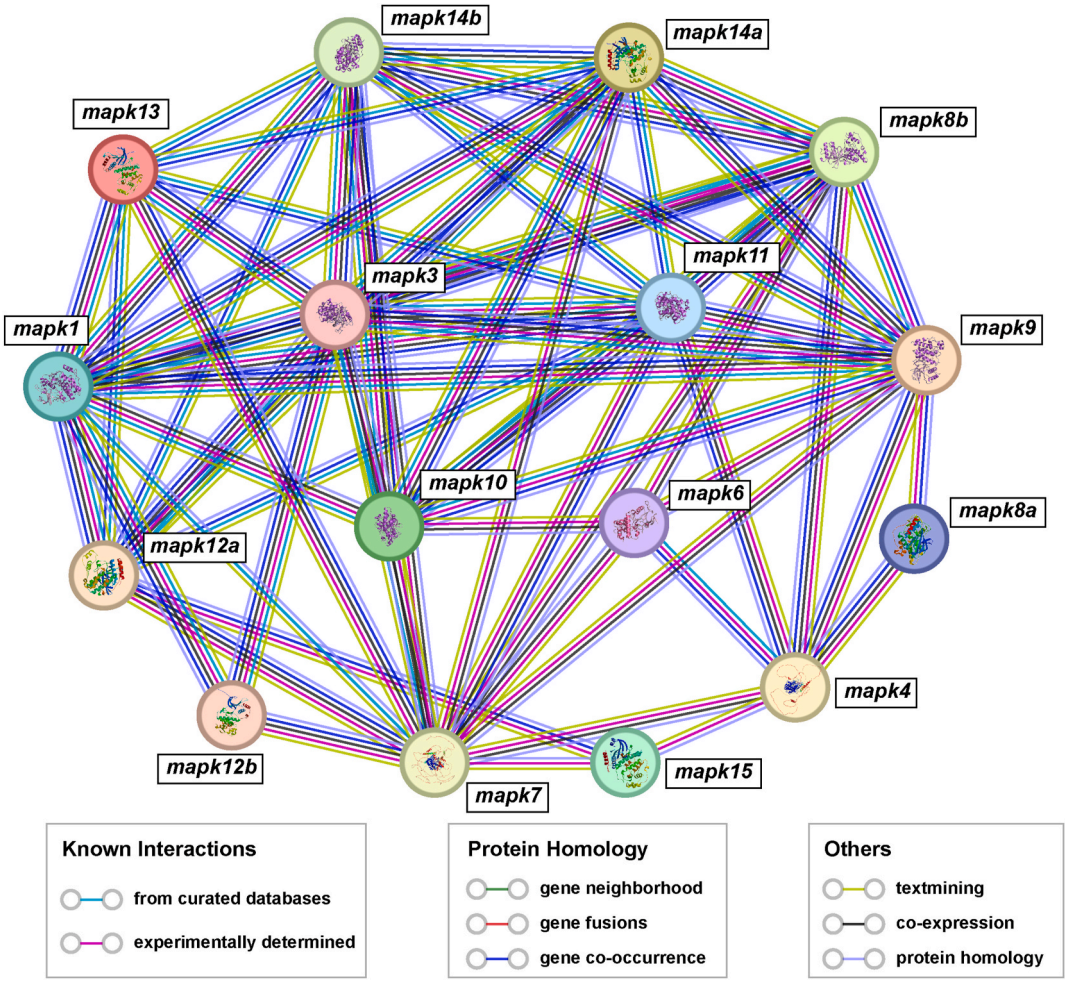

Fig. 6. Protein-protein interactions which show predicted functional partners of *C. argus*. Different colored nodes represent different genes. Different connection lines have different colors, representing the interaction type. Different connection lines have different widths, which represent the interaction strength.

Notably, duplicate copies of *mapk8*, *mapk12*, and *mapk14* have been found in the teleost (*D. rerio*, *P. magnuspinnatus* and *C. argus*), whereas only one copy exists in higher vertebrates. Prior research has demonstrated that the majority of vertebrate undergo two rounds of whole genome duplication, whereas fish undergo three or more rounds of whole genome duplication [35]. Chromosomal localization of 16 *mapks* identified in *C. argus* shows that they exist on 11 different chromosomes, suggesting that the probability of the replicated copies produced may not be the result of tandem replication, but rather a whole-genome duplication.

The possible function of the *mapk gene family* in *C. argus* can also be predicted by structural analysis. The number of introns contained in the *mapks* ranged from 7 (*mapk3*, *mapk7*) to 14 (*mapk4*, *mapk12b* and *mapk15*), suggesting that introns may have been lost to adapt to evolutionary processes. Intron loss is one of the types of genomic variation and may influence gene regulation and expression to promote the evolution of eukaryotic genes [36].

The *mapks* were ubiquitously and differentially expressed in all 15 examined tissues. Among them, those belonging to the ERK subfamily (*mapk1*, *mapk3*, *mapk4* and *mapk6*), the JNK subfamily (*mapk9*) and the p38 subfamily (*mapk14b*) exhibited the higher overall expression level. This suggests that different members of the *mapk* gene family may

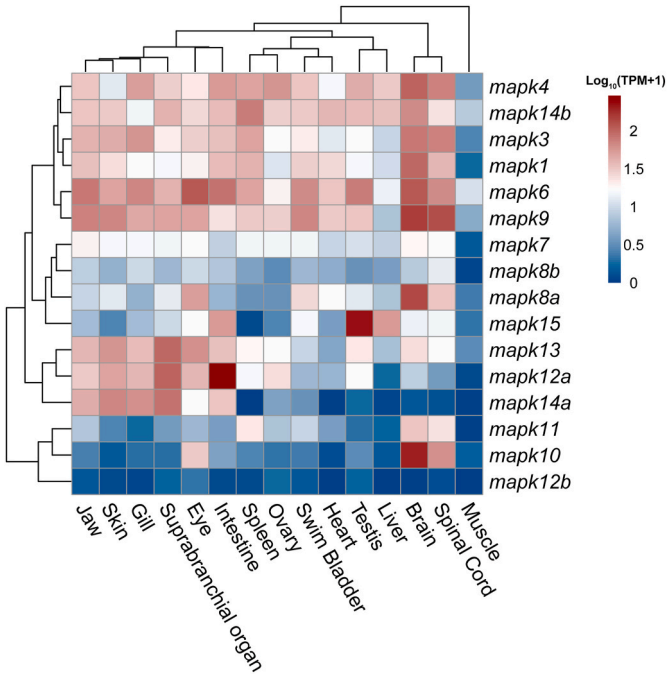

Fig. 7. Heatmap of *C. argus mapks* expression in different tissues. The examined tissues comprise the jaw, skin, gill, suprabranchial organ, eye, intestine, spleen, ovary, swim bladder, heart, testis, liver, brain, spinal cord, and muscle. The color depth indicates the level of expression, with red representing relatively high expression and blue representing relatively low expression. Each cell with different color has a concrete value of $\log_{10}$(TPM+1) to represent the expression level.

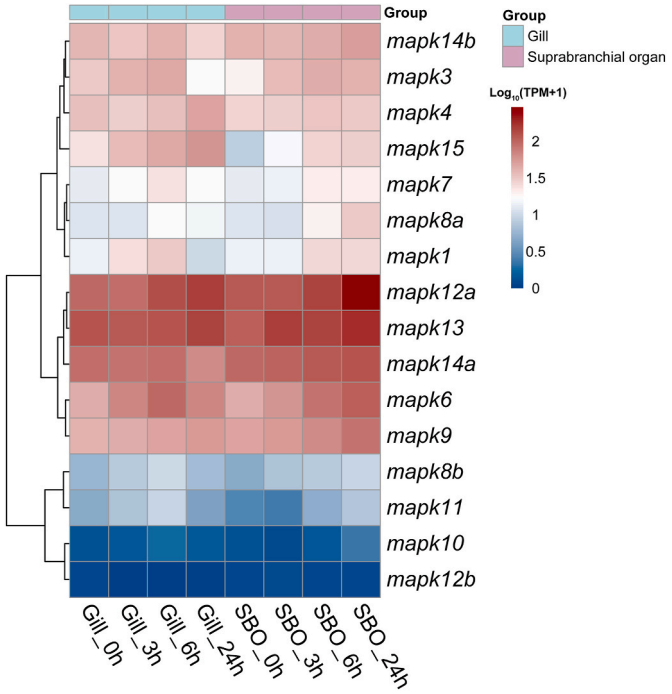

Fig. 8. Heatmap of *C. argus mapks* expression in gill and suprabranchial organ after air-breathing. SBO represents suprabranchial organ, and the labels 0 h, 3 h, 6 h and 24 h indicate the time points 0 h (control), 3 h, 6 h and 24 h after hypoxia, respectively. Each cell with different color has a concrete value of $\log_{10}$(TPM+1) to represent the expression level. Red representing relatively high expression and blue representing relatively low expression.

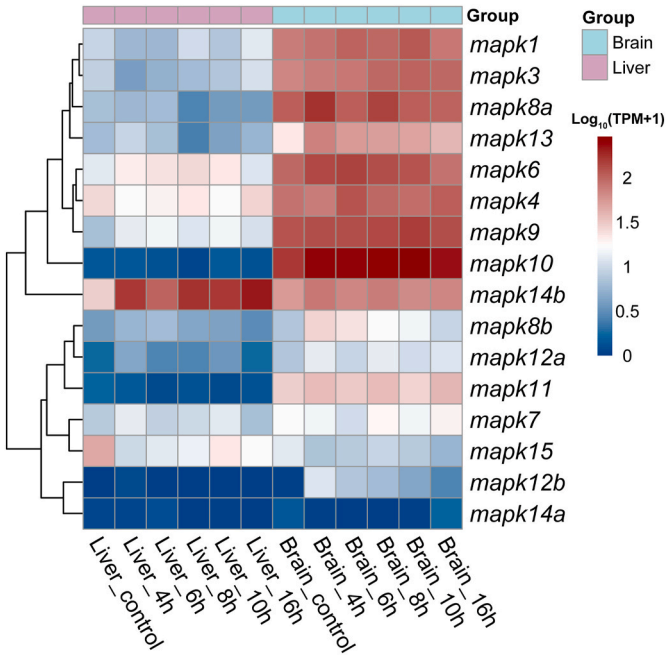

Fig. 9. Heatmap of *C. argus mapks* expression profiles of liver and brain after cold stress. The labels control, 4 h, 6 h, 8 h, 10 h and 16 h indicate the time points 0 h, 4 h, 6 h, 8 h, 10 h and 16 h after cold stress, respectively. Each cell with different color has a concrete value of $\log_{10}$(TPM+1) to represent the expression level. Red representing relatively high expression and blue representing relatively low expression.

participate in various biological processes of the organism respectively. For example, mapk1 is involved in a variety of cellular processes, including cell proliferation, differentiation, and transcriptional regulation [37]. It is noteworthy that the majority of *mapks* exhibit relatively high expression levels in both the brain and spinal cord. Specifically, the following *mapks* were identified in the brain: *mapk4*, *mapk14b*, *mapk3*, *mapk1*, *mapk6*, *mapk9*, *mapk8a*, *mapk11*, *mapk10*. Similarly, the following *mapks* were identified in the spinal cord: *mapk4*, *mapk3*, *mapk1*, *mapk6*, *mapk9*, *mapk10*. Furthermore, *mapk11*, *mapk10* showed tissue-specific expression in the brain and spinal cord. However, the expression of all *mapks* was detected at lower levels in muscle, suggesting that these members may play an important role in the formation and functioning of the nervous system of *C. argus*. For example, *mapk1* plays an important role in synaptic plasticity, neural activity, and connectivity [38]. Different *mapk* members can also activate the MAPK cascade, which plays a role in apoptosis of intact brain cells [39]. This result is similar to the result of high expression of *mapks* in brain tissues of largemouth bass [17]. In addition, we found that *mapk13*, *mapk12a* and *mapk14a* of the p38 subfamily present higher expression level in the gill and SBO. It is well known that gill and SBO are the main respiratory organs and auxiliary respiratory organs of *C. argus*, which enable the fish to obtain the necessary oxygen by air exchange in the water environment or air exposure, and the gills also play the role of nitrogen metabolic waste and osmotic regulation within the fish [40]. This indicates that certain p38 subfamily genes may be involved in air exchange and osmotic regulation in *C. argus*. The highest expression level of *mapk12a* and *mapk15* were observed in the intestine and testis respectively. The intestine, as a classic mucosal immune system, plays a role in mediating both humoral and cellular immune processes in fish. This potentially suggests that *mapk12a* may be involved in immune activity or sperm cell function in fish. For example, some studies have shown that MAPK signaling cascade is related to spermatogenesis and apoptosis [41,42].

Previous studies have shown that ERK1/2, p38 and JNK phosphorylation levels in the heart and p38 in the brain of *C. carassius* increased after 7 days of hypoxia [24]. This implies the regulatory role of MAPK

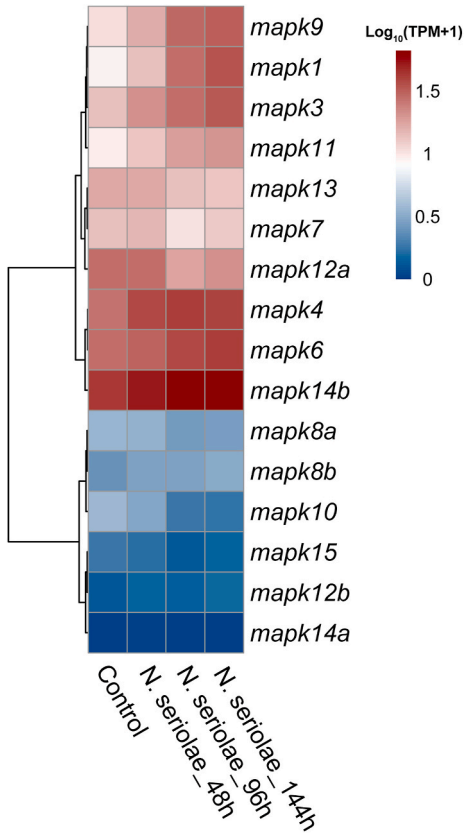

Fig. 10. Heatmap of *C. argus mapks* expression profiles of spleen after *N. seriolae* infection. The labels control, 48 h, 96 h, and 144 h indicate the time points 48 h under normal culture conditions, 96 h, and 144 h after cold stress, respectively. Each cell with different color has a concrete value of $\log_{10}$(TPM+1) to represent the expression level. Red representing relatively high expression and blue representing relatively low expression.

cascade reaction under different oxygen regimes. *C. argus* is one of the representative vertebrate animals that can breathe with gills and SBO in conditions of low oxygen or air exposure. Accordingly, we studied the expression pattern of *mapks* in response to air stress. The RNA-seq analysis of the expression level of gill and SBO *mapks* after air stress showed that multiple members of *mapks* exhibited different patterns of response. This suggests that many members of the *mapk gene family* are either directly or indirectly involved in the regulation of hypoxic stress. It has been suggested that the activation of multiple parallel MAPK pathways may play an important role in the cardiac response to cellular stress [43], which is in accordance with our speculation. It is worth noting that *mapk6* and *mapk15 of ERK, mapk12a*, *mapk13*, *mapk14a of p38* showed a significant upregulation trend in both tissues, suggesting their importance in regulating air exposure. In the study of catfish under hypoxic conditions, a significant single-nucleotide polymorphism (SNP) site was identified, located on the cell polarity regulator LRRC1 gene. Thia SNP had a high sequence similarity to the C/EBP. C/EBP is a gene that regulates the cellular stress response and is regulated by p38 *mapks* [44]. In particular, the p38 MAPK signaling pathway is a necessary condition for the activation of HIF (hypoxia-inducing factor) dependent pathway under hypoxia conditions [45]. In the hypoxia-inducible factor (HIF)-dependent signaling pathway, HIF-1α is a master switch involved in biological processes under hypoxia conditions. This is similar to hypoxia studies in mice, which found that hypoxia and hypoxia/reoxidation lead to activation of the p38 MAPK signaling pathway [46]. Moreover, the activation of p38 and JNK subfamilies is thought to be related to cellular stress. Published data suggesting that Gq/11PCR agonists can activate multiple MAPK signaling pathways in the heart, which may contribute to the development of cardiomegaly phenotypes [47]. Other studies have shown that cardiomegaly, narrowing of ventricular outflow tract or cardiac lacunae in zebrafish shorten the diffusion distance of oxygen in the heart, thereby contributing to the development of cardiomyocytes during hypoxia [22]. In our research, we also observed a significant upregulation of *mapk6* and *mapk15* of ERK in both tissues. This may be due to relatively small experimental sample sizes or individual differences, limiting the ability to statistically significant and generalize the results. It is also possible that some ERK genes which are not directly related to HIF may also be involved in hypoxia tolerance.

In addition to hypoxia stress, fish growth is also affected by temperature stress. The fish body can sense the change of water temperature through the skin epidermal microvessels and gill vessels. Once the temperature fluctuation occurs, the signal will be transmitted to the hypothalamus, which controls body temperature, and the brain will respond immediately [48]. Furthermore, previous studies have reported that multiple genes of *mapk* gene family can respond to temperature changes to different degrees and show significant differences in liver [16,17]. Therefore, we used published transcriptome sequencing data [31] to analyze the expression patterns of *mapks* in the liver and brain of *C. argus* under low temperature stress. We found that *mapk10*, *mapk8b* of JNK subfamily, *mapk13*, *mapk14b* and *mapk12b* of p38 subfamily in brain, as well as *mapk6* of ERK subfamily, *mapk9* of JNK subfamily and *mapk14b* of p38 subfamily in liver were sensitive to the effects of low temperature stimulation. The findings with significant changes after cold stress in large yellow croaker is contrary to our research, the expression of *mapk6 and* LcERK2 (also known as *mapk1*) is down-regulated at most time points after cold stress [49]. However, low temperature can activate ERK in silkworm [50], which may be caused by different species. The positive feedback regulation formed by ERK can mediate the activation of JNK [51], so we speculated that the increased expression levels of *mapk9*, *mapk10* and *mapk8b* might be related to the increased expression levels of *mapk6*. However, the up regulation of certain genes within the p38 family was observed to parallel findings in largemouth bass and Japanese flounder, where both species exhibited an increase in *mapk12* expression [16,17]. In addition, we found that the expression profiles of some genes in the liver and brain were opposite. For example, the expression of *mapk3* and *mapk13* was decreased in the liver tissue, whereas it was increased in the brain. We considered that this might be related to the temporal and spatial expression of genes.

In recent years, *N. seriolae* has become the main pathogen of *C. argus* [26], which can cause skin ulceration and obvious white nodules in the internal organs (spleen, liver and kidney) [52], thus restricting the development of *C. argus* culture. We thus sought to investigate the changes in the expression pattern of the spleen, a pivotal immune-related organ, following *N. seriolae* infection. The reported transcriptome data information was subjected to analysis, which revealed that *mapk1*, *mapk3* and *mapk6* of the ERK subfamily, *mapk9* of the JNK subfamily, and *mapk11* and *mapk14b* of the p38 subfamily were significantly upregulated after *N. seriolae* infection. This observation suggests that these genes may be more sensitive to the effects of *N. seriolae* infection. The expression of *Pomapk6*, *Pomapk11* and *Pomapk14b* in the kidneys of Japanese flounder after infection with *E. tarda* showed significant changes [16], which is similar to the results of our study. It is well known that effector T cells and memory T cells are key to the immune response against infection, and the survival and functional role of these two types of cells is associated with the NF-κB-induced kinase (NIK)-Tpl2-ERK signaling pathway [53]. In addition to the ERK signaling pathway, the p38 and JNK signaling pathways are also involved in immunoregulatory processes. Moreover, a study has also reported that the expression levels of JNK1a, JNK1b, p38 delta, and p38 alpha b in the *mapk gene family* were significantly up-regulated after infection with *A. hydrophila*, indicating that *mapks* were involved in the inflammation and innate immune response of fish [34]. After orange-spotted grouper (*Epinephelus coioides*) was infected

with SGIV, p38 played a crucial role in regulating apoptosis [54]. The notable differential expression of *mapk1*, *mapk3*, and *mapk6* of the ERK subfamily, *mapk9* of the JNK subfamily, and *mapk11* and *mapk14b* of the p38 subfamily following *N. seriolae* infection was observed in our study, indicating that the spleen plays a pivotal role in the immune response in *C. argus*. It is possible that a splenic infection may result in an increased inflammatory response and apoptosis in the spleen of *C. argus*.

We constructed a PPI network for each member of the *mapk* gene family to analyze their interactions in response to biotic and abiotic stresses. As shown in Fig. 6, *mapks* from three different subfamilies can interact, which corresponds to the results of *mapks* responses to biotic and abiotic stressors. For example, *mapk14a* from the p38 subfamily is able to interact with members of the JNK family, such as *mapk9*, as can mapk12a, mapk13, and mapk14a from the same subfamily. This also explains the involvement of different members from the p38 subfamily in the hypoxic stress response process, as evidenced by previous studies [46,55]. Our analyses demonstrated that the JNK subfamily members *mapk10*, *mapk8b*, the p38 subfamily members *mapk13*, *mapk14b* and *mapk12b*, and the ERK subfamily member *mapk6* were particularly susceptible to the effects of low-temperature stimuli. The ERK subfamily (*mapk1*, *mapk3* and *mapk6*), the JNK subfamily (*mapk9*), and the p38 subfamily (*mapk11* and *mapk14b*) exhibited a notable increase in expression following infection with *N. seriolae*. The PPI prediction seems to demonstrate that the interaction of *mapk14b* with other *mapks* (*mapk3, mapk6, mapk8b, mapk9, mapk10, mapk11, and mapk13*) allows these genes to be jointly involved in the process of responding to low-temperature stress and immune responses. To conclude, our research results seem to corroborate the hypothesis that p38 members mediate environmental stress and inflammatory signaling responses [56]. Additionally, the predicted interaction between *mapk6* and *mapk9* is noteworthy in the involvement in stress responses and immune regulation processes. Therefore, we believe that the interactions between *mapks* play a significant role in the response to stress pressure in the *mapk* cascade signaling pathway and should not be overlooked. It is evident that further bioinformatics and molecular biology experiments are required to elucidate the interactions and potential connections between *mapks* and their signaling pathways that are involved in a specific stress process.

5. Conclusion

In this study, the *mapk* gene family of *C. argus* was analyzed comprehensively and systematically. Sixteen *mapks* were identified, including duplicate copies of *mapk8*, *mapk12*, and *mapk14*. Phylogenetic analysis and syntenic analysis provided evidence for gene annotation and homology. The analysis of gene structure and motif composition provided evidence for the conservation of *mapks* in the evolution of *C. argus*. Expression patterns analysis showed that *mapks* were universally and differentially expressed in 15 tested tissues. Furthermore, we observed that different *mapks* showed diverse immune response patterns to air stress, cold stress, and *N. seriolae* infection, suggesting their potential involvement in regulating biotic stress and abiotic stress processes. Moreover, the co-activation of multiple parallel MAPK signaling pathways may play a critical role in regulating the response of organisms to abiotic and biological stresses. In conclusion, this study provides valuable insights into the regulatory mechanisms of *mapks* in aquatic vertebrates, particularly their involvement in biotic and abiotic stress responses.

CRediT authorship contribution statement

Chaonan Sun: Writing – original draft, Data curation, Formal analysis, Software, Validation, Visualization. **Mingxin Zhu:** Validation, Investigation. **Lingyu Wang:** Writing – review & editing. **Haishen Wen:** Conceptualization. **Xin Qi:** Supervision. **Chao Li:** Funding acquisition, Investigation. **Xiaoyan Zhang:** Visualization. **Donglei Sun:** Software, Methodology, Resources, Writing – review & editing. **Yun Li:** Conceptualization, Methodology, Project administration, Writing – review & editing.

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Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.110076.

Data availability

Data will be made available on request.

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