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ORIGINAL ARTICLE

Multiple across-strain and within-strain QTLs suggest highly complex genetic architecture for hypoxia tolerance in channel catfish

Xiaozhu Wang[1] · Shikai Liu[1] · Chen Jiang[1] · Xin Geng[1] · Tao Zhou[1] · Ning Li[1] · Lisui Bao[1] · Yun Li[1] · Jun Yao[1] · Yujia Yang[1] · Xiaoxiao Zhong[1] · Yulin Jin[1] · Rex Dunham[1] · Zhanjiang Liu[1]

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Abstract The ability to survive hypoxic conditions is important for various organisms, especially for aquatic animals. Teleost fish, representing more than 50 % of vertebrate species, are extremely efficient in utilizing low levels of dissolved oxygen in water. However, huge variations exist among various taxa of fish in their ability to tolerate hypoxia. In aquaculture, hypoxia tolerance is among the most important traits because hypoxia can cause major economic losses. Genetic enhancement for hypoxia tolerance in catfish is of great interest, but little was done with analysis of the genetic architecture of hypoxia tolerance. The objective of this study was to conduct a genome-wide association study to identify QTLs for hypoxia tolerance using the catfish 250K SNP array with channel catfish families from six strains. Multiple significant and suggestive QTLs were identified across and within strains. One significant QTL and four suggestive QTLs were identified across strains. Six significant QTLs and many suggestive QTLs were identified within strains. There were rare overlaps among the QTLs identified within the six strains, suggesting a complex genetic architecture of hypoxia tolerance. Overall, within-strain QTLs explained larger proportion of phenotypic variation than across-strain QTLs. Many of genes within these identified QTLs have known functions for regulation of oxygen metabolism and involvement in hypoxia responses. Pathway analysis indicated that most of these genes were involved in MAPK or PI3K/AKT/mTOR signaling pathways that were known to be important for hypoxia-mediated angiogenesis, cell proliferation, apoptosis and survival.

Keywords Oxygen · Hypoxia tolerance · GWAS · Fish · QTL · Climate change

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✉ Zhanjiang Liu
liuzhan@auburn.edu

[1] The Fish Molecular Genetics and Biotechnology Laboratory, Aquatic Genomics Unit, School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Auburn, AL 36849, USA

Introduction

Oxygen is essential for life of all aerobic organisms. They must adapt to low oxygen environments for survival when being exposed to hypoxic conditions. As a matter of fact, the ability to survive hypoxic conditions determines the distribution of organisms. Land animals are living in high oxygen conditions (~21 %), and even so, animals living in high altitudes may experience frequent exposures to hypoxia. In contrast, aquatic animals are routinely living in low oxygen conditions that have driven the evolutionary changes allowing them to survive in aquatic environments with relatively low dissolved oxygen (in the range of ~10 ppm). Teleost fish are extremely efficient in utilizing low levels of dissolved oxygen in water. Many fish species have evolved various adaptive strategies, allowing them to survive under hypoxic conditions. Behavioral adaptations include shifts from water breathing to surface breathing (Kramer and McClure 1982) and lowering locomotor activities (Nilsson et al. 1993). Physiological adaptations include an enhanced ventilation in gill with extremely large surface areas (Burggren and Cameron 1980), reduction of the metabolic rate and changes of enzymatic activities (Jensen et al. 1993).

Response to hypoxia is a highly complex biological process. Under hypoxic conditions, organisms usually develop alternative strategies of energy metabolism, enzymatic activities and signal transduction pathways to survive. It has been long believed that the control of hypoxia responses is at the posttranslational level. Hypoxia inducible factors (HIFs), especially HIFα, are believed to be a master switch for hypoxia responses (Semenza 2000). HIFα proteins, in spite of being continuously synthesized, are rapidly degraded under normoxic conditions. Under hypoxic conditions, HIFα rapidly accumulates, and dimerizes to its partner HIFβ, which then binds to the hypoxia response element (HRE) of effector genes, leading to activation of a wide variety of genes involved in both the cellular and systematic responses to hypoxia (Kietzmann et al. 2016). Many of these genes, such as vascular endothelial growth factor, glucose transporter 1, and insulin-like growth factors, are involved in erythropoiesis and angiogenesis, apoptosis, vascularization, and metabolism (Semenza et al. 2000). In addition to regulation at post-translational level, *HIF-1α* gene was also found to be transcriptionally induced by hypoxia in catfish (Geng et al. 2014). HIF activation is also regulated by tumor suppressors such as p53 and von Hippel–Lindau, or oncogenes such as *RAS*, *mTOR*, *PI3K*, and *AKT* (Kietzmann et al. 2016). The PI3K/AKT/mTOR pathway or MAPK pathway has been shown to regulate the expression of *HIF-1α* (Xia et al. 2012).

At the genomic level, living in hypoxic conditions can place selection pressure for genotypes that are more tolerant to hypoxia (Bigham et al. 2010). Most of genome level research of hypoxia tolerance has been conducted with plants. For instance, Quantitative trait loci (QTLs) for hypoxia tolerance have been mapped in some plant species, such as in rice (Aggeli et al. 2002) and in maize (Qiu et al. 2007). With animals, much less work has been conducted. However, QTLs associated with tolerance to high altitude hypoxia have been identified in humans. High-altitude human populations such as Sherpas, Tibetans, Ethiopians, and Andeans were found to possess specific SNPs associated with hypoxia tolerance (Beall 2000; Yi et al. 2010; Jha et al. 2016). With fish, especially aquaculture species, genetic analysis of hypoxia tolerance is important, not only because they live in aquatic environments with high adaptability of a range of hypoxic conditions, and thus are best natural models for understanding mechanisms of hypoxia responses, but also because improving hypoxia tolerance is of economic interest. Under aquaculture conditions, acute exposure to hypoxia can lead to high levels of mortalities due to high stocking density. In addition, exposure to hypoxia can also cause depression of the immune system, leading to increased susceptibility to diseases (Welker et al. 2007; Kvamme et al. 2013).

QTL studies have been conducted with various fish species. For instance, some valuable QTLs were identified for traits related to growth in rainbow trout (Wringe et al. 2010), body lipid percentage in Atlantic salmon (Derayat et al. 2007), sexual maturation in Arctic char (Moghadam et al. 2007), and disease resistance in Atlantic salmon (Moen et al. 2009; Houston et al. 2010). A number of studies have been conducted dealing with hypoxia tolerance, but most focused on gene regulation after exposure to hypoxia in various species such as zebrafish (Guan et al. 2011), Rainbow trout (Faust et al. 2004), Atlantic salmon (Anttila et al. 2013), and paddlefish (Aboagye and Allen 2014).

Channel catfish is the primary aquaculture species in the United States. Much progress has been made in recent years for integrated genetic and genomic analysis. These included the construction of its physical map (Xu et al. 2007), identification of over eight million genome-wide SNPs (Sun et al. 2014), the development of the 250K SNP array (Liu et al. 2014), the development of high-resolution genetic linkage maps (Li et al. 2015), the production of the reference genome sequence and its annotation (Liu et al. 2016), and analysis of genomic regions associated with performance and production traits (Geng et al. 2015, 2016). For instance, QTLs for columnaris disease resistance (Geng et al. 2015), and for head size (Geng et al. 2016) have been identified using the channel catfish x blue catfish interspecific system. However, no studies have been conducted with hypoxia tolerance in catfish. Here, a GWA study was conducted to identify QTLs associated with hypoxia tolerance using the catfish 250K SNP array.

Materials and methods

Experimental fish, hypoxia challenge and sample collection

Channel catfish used in this experiment were produced at the E. W. Shell Fisheries Research Center, Alabama Agricultural Experiment Station, Auburn University, Alabama. A total of 521 fish were selected from six channel catfish strains, including Marion (54 fish), Marion Select (67 fish), Thompson (82 fish), 103KS (99 fish), Kansas (107 fish), and Kmix (112 fish). Detailed ancestry of the channel catfish strains used in this study is described in Dunham and Smitherman (1984). All experimental fish were PIT (passive integrated transponder) tagged before mixed together for hypoxia treatment. Information of strain, body weight and sex of each fish was recorded with the corresponding PIT tag number.

Fish were acclimated at ambient temperature of 20 °C in the aerated flow-through water for 1 week. The dissolved

Table 1 Information of catfish samples used for this GWA study

Strain name	Total number of fish	Number of sensitive fish	Number of tolerant fish	Mean of body weight (g)
103KS	72	36	36	109.3
Kansas	82	41	41	78.7
Kmix	83	41	42	97.5
Thompson	60	30	30	134.4
Marion Select	48	24	24	100.6
Marion	31	16	15	61.3

oxygen (DO) level was monitored daily using the YSI dissolved oxygen meter. During the experiment, the DO level was reduced gradually from ~9.0 to 0.1 mg/L in an hour using sodium sulfite (Boyd 1982). After that, the DO level was kept constant, and fish were monitored for signs of losing balance to hypoxic stress. The time and sequence of each fish that lost balance were recorded. Blood samples were carefully collected and immediately stored in a 15 mL tube containing 5 mL of cell lysis solution (10 mL lysis solution, proteinase K 20 mg/mL) for DNA isolation. Fish were returned to the well-oxygenated water for recovery after sampling.

Preparation of genomic DNA and genotyping

From each strain, we regarded the first ~35 % fish lost balance as the hypoxia sensitive fish, the last ~35 % fish as the hypoxia tolerant fish. In total, 376 fish from six different strains were selected for this study (Table 1) based on the selective genotyping method, which is economical and efficient using relatively small sample size to achieve high statistical power (Darvasi and Soller 1992; Jin et al. 2004). DNA was isolated using standard phenol/chloroform method. Briefly, the blood cells were broken by cell lysis solution and proteins were removed by protease K and protein precipitation solution after incubated blood samples at 55 °C overnight. DNA was precipitated by isopropanol and collected by brief centrifugation, washed with 70 % ethanol, air-dried, and rehydrated in reduced EDTA TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). DNA was quantified using spectroscopy by Nanodrop (Thermo Scientific). The integrity of DNA samples was checked by 1 % agarose gel electrophoresis stained with ethidium bromide.

Genomic DNA samples were arranged in a 96-well microtiter plate, and normalized to a final concentration of 50 ng/μL with a final volume of 10 μL. We have developed the catfish 250K SNP array with well-distributed markers using Affymetrix Axiom genotyping technology (Liu et al. 2014). Genotyping using the catfish 250K SNP array was performed at GeneSeek (Lincoln, NE, USA). Five samples were excluded due to low quality or low call rate (<95 %). The SNP genotype calling was performed using the Affymetrix Genotyping Console. 176,798 SNPs were kept for subsequent analysis after excluding SNPs failed one or more of the following requirements: a call rate >95 %, a minor allele frequency (MAF) >1 %.

Statistical analysis

To determine potential association between SNPs and phenotypic variants, statistical analysis was carried out using the SNP and Variation Suite (SVS, Version 8.0). To generate a set of independent SNPs, linkage disequilibrium (LD) pruning step was conducted with a window size of 50 SNPs (the number of SNPs at each LD testing), a step of five SNPs (shift the window five SNPs forward), and r^2 threshold of 0.2 using composite haplotype method (CHM) (Weir and Ott 1997; Wang et al. 2009). After LD pruning step, 15,571 independent SNP markers and LD blocks were identified. Identical by state (IBS) between all pairs of samples were estimated using the independent SNPs. The population structure was assessed by principal component analysis (PCA) (Price et al. 2006). Wright's F_{st} was estimated between all pairs of six strains using all the qualified SNPs, which can range from zero (no genetic divergence between the strains) to one (complete isolation of the strains from each other and the overall population) (Nei 1973).

To account for the sample structure in the association test, Efficient Mixed-Model Association eXpedited (EMMAX) model was conducted with the first two principal components (PC1, PC2) and body weight as covariates using all samples from six strains. The model is listed as follows:

$$\mathbf{Y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\boldsymbol{u} + \mathbf{e}$$

where $\mathbf{Y}$ is the vector of time before fish lost balance to hypoxic stress; $\mathbf{X}$ is the matrix of fixed effects including first two PCs and fish body weight; $\boldsymbol{\beta}$ is the coefficient vector; $\mathbf{Z}$ is the matrix of random additive genetic effects, $\boldsymbol{u}$ is the vector representing the coefficients of the random effect, $\mathrm{Var}(\boldsymbol{u}) = \mathrm{G}\sigma_g^2$ where G is the simple IBS allele-sharing matrix and σ_g^2 is the additive genetic variance; $\mathbf{e}$ is the vector of random residuals.

The Manhattan plot of the *P* values was produced according to channel catfish genome sequence (version Coco1.0) (Liu et al. 2016). The threshold *P* value for genome-wide significance was determined based on Bonferroni-correction (Duggal et al. 2008). The threshold *P* value of the 5 % Bonferroni genome-wide significance was $0.05/15571 = 3.21e^{-6}$ ($-\log_{10}$ (*P* value) = 5.49). The threshold *P* value for the significance of "suggestive association", which allowed one false positive effect in a genome-wide test, was $1/15571 = 6.42e^{-5}$ ($-\log_{10}$ (*P* value) = 4.19).

To identify QTLs within strains, the similar statistical analysis was conducted with samples from each of the six strains. For each strain, the Manhattan plot was produced and corresponding threshold *P* value was calculated. In Kansas strain, the threshold $-\log_{10}$ (*P* value) of the 5 % Bonferroni genome-wide significance was 4.51, and the threshold $-\log_{10}$ (*P* value) for the significance of "suggestive association" was 3.21. Similarly, the $-\log_{10}$ (*P* value) thresholds for significant association and suggestive association are 4.77 and 3.47 in Kmix strain, 4.54 and 3.24 in Thompson strain, 4.54 and 3.24 in 103KS strain, 4.39 and 3.09 in Marion strain and finally, 4.68 and 3.38 in Marion Select strain.

Sequence analysis

Genes within ±0.5 Mb of the most significant SNPs associated with hypoxia tolerance both across strains and within strains were examined for candidate genes and identified using the catfish reference genome (Liu et al. 2016). Synteny analysis of these genes was conducted between channel catfish and zebrafish to provide evidence for orthology. Similarly, genes most close to the suggestive SNPs identified in this GWAS were also identified as potential candidate genes and analyzed for their functions in hypoxia responses.

Results

Hypoxia challenge

The DO level in water was gradually reduced to 0.1 mg/L and kept for the remaining period of the hypoxia challenge. Under the hypoxic condition, the experimental fish reduced their movement and occasionally swim up to the surface of water for higher level of DO. The Kaplan–Meier analysis was performed to identify the association between probability of fish hypoxia tolerance (time before losing balance) and hypoxia treatment time (Goel et al. 2010). The first fish started to lose balance at 8 min after hypoxia treatment, and the last fish losing balance was observed at 107 min after hypoxia treatment. As expected, the probability of an experimental fish to maintain balance decreased as the hypoxia treatment continued (Fig. 1). For instance, the probability of an experimental fish losing balance exceeds 50 % after 50 min of hypoxia treatment.

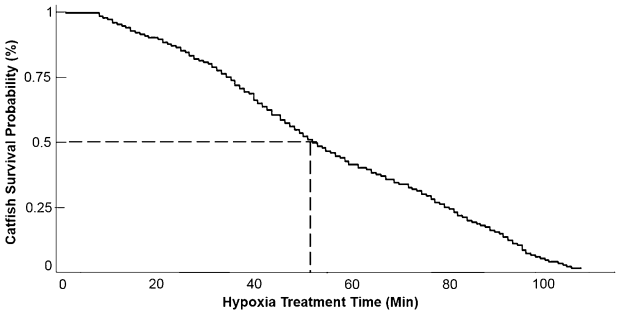

Fig. 1 Overall tolerance of channel catfish under hypoxia treatment

QTLs associated with hypoxia tolerance across strains

The information of fish samples used for this GWA study was summarized in Table 1. After quality control filtering of unqualified samples and SNPs, principal component analysis (PCA) was conducted using eigenvalues as coordinates to visualize the population stratification with samples and SNPs passed quality control (Supplemental Fig. S1). To further determine the relationship of six strains, the values of Wright's F_{st} among six strains were calculated (Supplemental Table S1). The values of F_{st} among strains varied from 0.11 to 0.25, indicating a relatively high level of genetic relatedness of the six strains. EMMAX model was conducted with the first two PCs to estimate the population structure in the association test.

First, QTLs were analyzed using all samples across the six strains. A Manhattan plot of $-\log_{10}$ (*P* value) for all SNPs associated with hypoxia tolerance was shown in Fig. 2. Across the six strains, there was evidence for potential QTLs in five genomic regions on four linkage groups, of which one genomic region on linkage group (LG) 6 was significantly associated with hypoxia tolerance. Four additional genomic regions were found to be suggestively associated with hypoxia tolerance on LG5, LG10, and LG12. As shown in Fig. 2, there appeared to be two suggestive genomic regions on LG5. On LG10 and LG12, there was only one genomic region each suggestively associated with hypoxia tolerance. The most significant SNP (AX-86110011) on LG6 is located at position 28,084,460 bp. Genes surrounding this significant SNP are shown in Fig. 3. Many of these genes are involved in hypoxia response pathways (see below).

The phenotypic variations explained by significantly and suggestively associated SNPs were relatively small. The most significant SNP on LG6 explained approximately

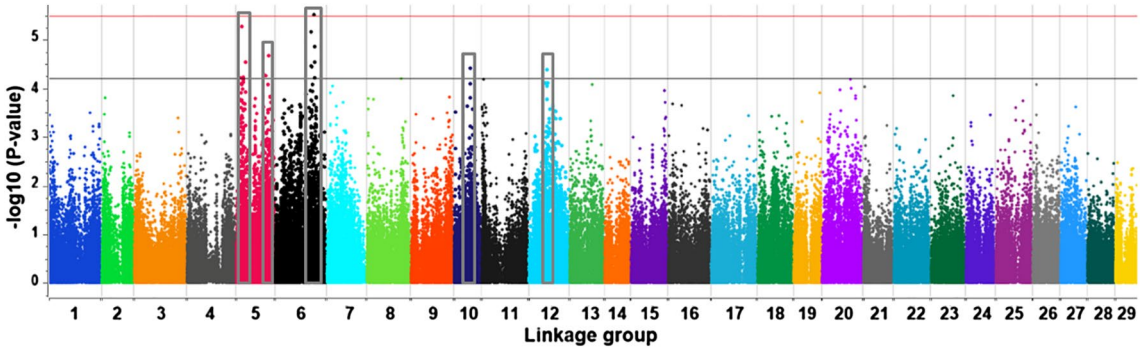

Fig. 2 Manhattan plot reveals across-strain QTLs associated with hypoxia tolerance. The *red solid line* indicates the threshold *P* value for genome-wide significance. The *black solid line* indicates the threshold *P* value for the significance of "suggestive association" (color figure online)

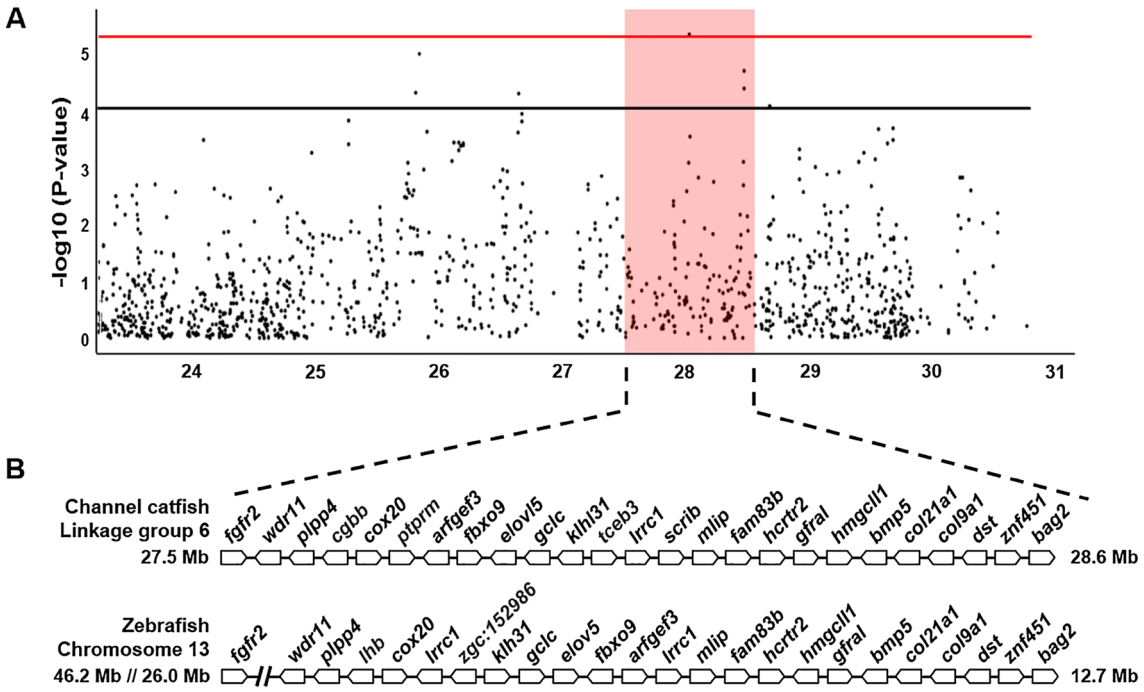

Fig. 3 Genes surrounding the most significant SNP associated with hypoxia tolerance across strains on LG6. **a** Regional −log10 (*P* value) plot for the QTL. The *red shade* indicates ±0.5 Mb genomic region of the most significant SNP. The *red solid line* indicates the threshold *P* value for genome-wide significance. The *black solid line* indicates the threshold *P* value for the significance of "suggestive association". **b** Genes within ±0.5 Mb genomic region of the most significant SNP. Synteny analysis was conducted between catfish and zebrafish. Genes with *gray* names are not conserved between channel catfish and zebrafish (color figure online)

6 % of phenotypic variation, while other associated SNPs on LG6 explained 4–5 % of phenotypic variation (Table 2). On LG5, two separate genomic regions were identified to be suggestively associated with hypoxia tolerance, including a set of SNPs with $-\log_{10}(P \text{ value})$ ranging from 4.19 to 5.27 (Table 2). One region spans from 3,073,377 to 6,575,020 bp, and the other region spans from 20,194,928 to 23,640,298 bp. They can explain 4–5 % of phenotypic variation. One suggestively associated QTL was identified on LG10 and LG12, respectively (Table 2). On LG10, one SNP at position 11,248,369 bp was suggestively associated with hypoxia tolerance and explained ~4 % of phenotypic variation. On LG12, one SNP at position 12,924,457 bp was suggestively associated with hypoxia tolerance, explaining ~4 % of phenotypic variation (Table 2).

QTLs associated with hypoxia tolerance within strains

To investigate QTLs within strains, EMMAX model was conducted with samples from each of the six strains. Many genomic

Table 2 SNPs associated with hypoxia tolerance across strains

Linkage group	SNP ID	SNP position	$-\log_{10}$ (*P* value)	% Variance
LG6	AX-85193644	25,756,477	4.45	4.51
	AX-85410024	25,789,234	5.15	5.30
	AX-85313934	26,633,015	4.43	4.49
	AX-86110011	28,084,460	5.52	5.71
	AX-85214060	28,549,254	4.53	4.60
	AX-85254568	28,584,932	4.86	4.97
	AX-85252972	28,764,133	4.21	4.24
LG5	**AX-85353272**	**3,073,377**	**4.21**	**4.23**
	AX-85216281	**3,976,205**	**5.27**	**5.43**
	AX-85324750	**4,869,369**	**4.22**	**4.25**
	AX-85271686	**5,189,256**	**4.19**	**4.22**
	AX-85224364	**6,575,020**	**4.53**	**4.60**
	AX-85367587	*20,194,928*	*4.26*	*4.30*
	AX-85338386	*21,541,353*	*4.25*	*4.28*
	AX-85961782	*23,640,298*	*4.66*	*4.75*
LG10	AX-85217471	11,248,369	4.41	4.47
LG12	AX-85197805	12,924,457	4.38	4.43

Bold and italics indicate two separate associated genomic regions on LG5

regions were found to be associated with hypoxia tolerance within these strains. Significant SNPs were detected within three strains (Fig. 4). With Kansas strain, significant SNPs were found on LG4, LG22, and LG25. In addition to these significant genomic regions, suggestive SNPs were found on many other linkage groups, including LG2, LG5, LG6, LG8, LG9, LG11, LG13, LG17, and LG20 (Fig. 4a). Apparently, many of these could be pseudo-positive (see "Discussion"), but some of these may potentially contain real QTLs for hypoxia tolerance. With Kmix strain, two genomic regions were detected to contain significant SNPs on LG7 and LG12. Additional suggestively associated SNPs were detected on LG2, LG3, LG5, LG10, LG17, LG18, and LG19 (Fig. 4b). With Thompson strain, one SNP on LG7 was found to be significantly associated with hypoxia tolerance, and suggestively associated SNPs were detected on LG9, LG13, LG20, and LG21 (Fig. 4c). With 103KS, Marion, and Marion Select strains, no significant SNPs were detected, but suggestive SNPs were found on LG10 for 103KS strain (Fig. 5a), on LG4, LG13, and LG15 for Marion strain (Fig. 5b), and on LG3, LG9, LG13, LG22, and on LG23 for Marion Select strain (Fig. 5c).

The effects on phenotypic variation appeared to be relatively large for QTLs significantly associated with hypoxia tolerance within strains (Table 3). With Kansas strain, the most significant SNPs on LG25, LG22 and LG4 explained approximately 25, 21 and 20 % of phenotypic variation, respectively. With Kmix strain, the most significant SNPs on LG12 and LG7 can explain approximately 23 and 22 % of phenotypic variation, respectively. With Thompson strain, the most significant SNP on LG7 itself explained 32 % of phenotypic variation.

Genes within the QTL regions significantly associated with hypoxia tolerance

As the reference genome sequence is available and the assembly was validated to be highly accurate, examination of the genes in genomic regions surrounding the significant SNPs may provide insights into potential candidate genes and potential mechanisms of hypoxia responses. We determined genes on LG6 within the ±0.5 Mb genomic region of the most significant SNP. A total of 25 genes exist in this region, and these genes and their locations relative to the most significant SNP are shown in Fig. 3. Many of these genes are involved in hypoxia response pathways (see "Discussion"), such as *lrrc1* (leucine rich repeat containing 1), *tceb3* (transcription elongation factor B polypeptide 3), *mlip* (muscular LMNA-interacting protein), *fam83b* (family with sequence similarity 83 member b), *gclc* (glutamate-cysteine ligase catalytic subunit), *fgfr2* (fibroblast growth factor receptor 2), *plpp4* (phospholipid phosphatase 4), *fbxo9* (F-box protein 9), *bmp5* (bone morphogenetic protein 5), and *bag2* (BAG family molecular chaperone regulator 2).

Although multiple linkage groups were detected to contain significant SNPs for hypoxia tolerance within strains, only one linkage group, LG25, within Kansas strain was found to contain multiple significant SNPs associated with hypoxia tolerance. Therefore, we determined the genes surrounding this specific genomic region (±0.5 Mb of the most significant SNP on LG25). A total of 34 genes exist in this genomic region (Fig. 6). Similar to the situation for QTLs across strains, many genes included in this QTL region are involved in hypoxia response pathways, such as *nf1* (neurofibromin 1), *lgals9* (Lectin, galactoside binding soluble 9), *ucp2* (uncoupling protein 2), *slc1a3* (solute carrier family 1 member 3), *gdnf* (glial cell derived neurotrophic factor), and *dhrs13* (dehydrogenase/reductase 13).

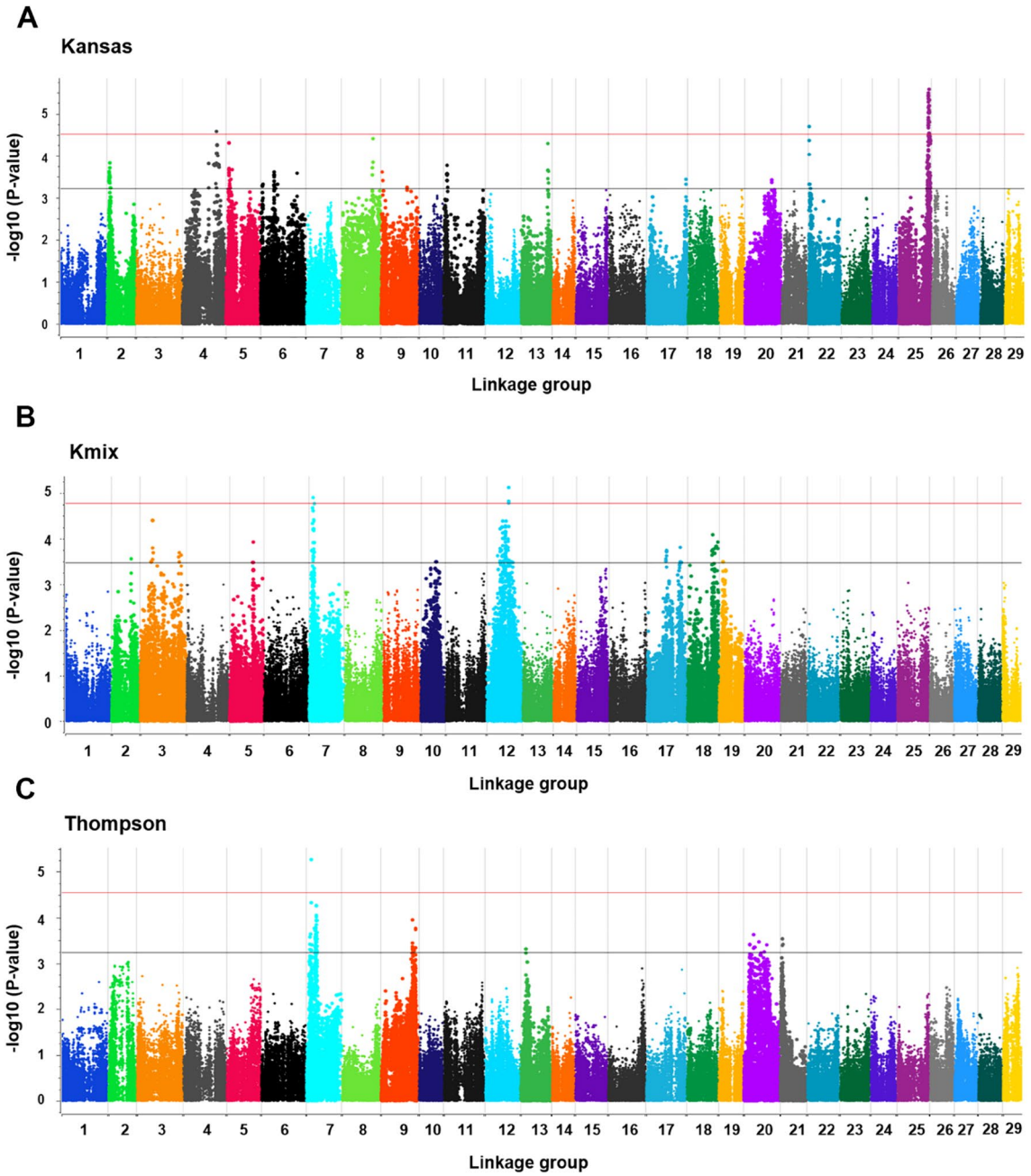

Fig. 4 Manhattan plots reveal QTLs for hypoxia tolerance within Kansas, Kmix and Thompson strains. **a** QTLs within Kansas strain. **b** QTLs within Kmix strain. **c** QTLs within Thompson strain. The *red solid line* indicates the threshold *P* value for genome-wide significance. The *black solid line* indicates the threshold *P* value for the significance of "suggestive association" (color figure online)

Correlation of the SNPs associated with hypoxia tolerance

Conditioned analyses were conducted to evaluate whether the SNPs associated with hypoxia tolerance were independent (Geng et al. 2015). The significant associated SNP on LG6 was included as a covariate in EMMAX model. After conditioning, the $-\log_{10}$ (*P* value) of the remaining associated SNPs on LG6 drastically dropped below 2.0, indicating strong correlations among SNPs within the same linkage group. While the $-\log_{10}$ (*P* value) of SNPs remain the same on different linkage groups. Similarly, when the conditioned analysis was conducted with associated SNPs on the same linkage group for QTLs detected within strains, after conditioning, the $-\log_{10}$ (*P* value) of the remaining associated SNPs on the same linkage group

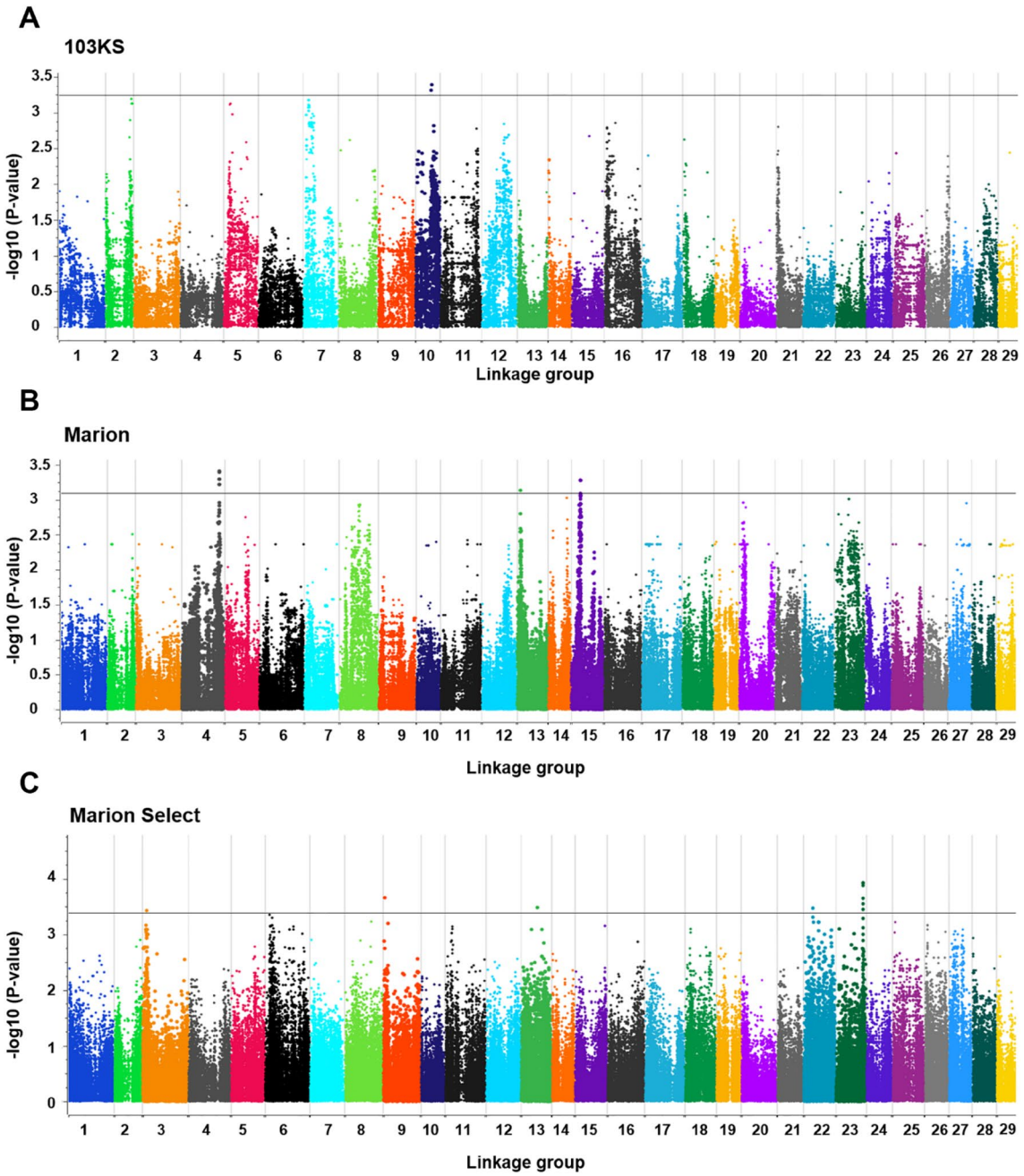

Fig. 5 Manhattan plots reveal QTLs for hypoxia tolerance within 103KS, Marion and Marion Select strains. **a** QTLs within 103KS strain. **b** QTLs within Marion strain. **c** QTLs within Marion Select strain. The *black solid line* indicates the threshold *P* value for the significance of "suggestive association"

drastically dropped, while SNPs on different linkage group remain the same.

Discussion

In this study, we conducted a GWA study with six channel catfish strains using the catfish 250K SNP array to identify QTLs associated with hypoxia tolerance. A large number of QTLs were identified for hypoxia tolerance both across strains and within strains. Across the six strains, one significant genomic region and four additional suggestively associated genomic regions were identified to contain QTLs for hypoxia tolerance. Analyses of QTLs associated with hypoxia tolerance within the six strains allowed detection of multiple significant QTLs in three strains,

Table 3 SNPs significantly associated with hypoxia tolerance within strains

Linkage group	SNP ID	Position (bp)	$-\text{Log}_{10}$ (*P* value)	% Variance
Kansas				
LG25	AX-85273690	23,848,240	4.83	22.02
	AX-85382689	24,066,260	4.73	21.53
	AX-85278184	24,083,110	4.72	21.51
	AX-85401604	24,116,919	4.73	21.53
	AX-85366674	24,133,568	4.77	21.75
	AX-85208027	24,147,963	5.41	24.60
	AX-85404384	24,149,673	5.02	22.84
	AX-85240400	24,161,130	5.14	23.39
	AX-86065291	24,169,339	5.48	24.91
	AX-85272411	24,171,584	4.90	22.32
	AX-85265728	24,189,546	5.26	23.95
	AX-85248269	24,210,520	5.36	24.38
	AX-85382884	24,246,000	4.86	22.14
	AX-86065374	24,349,412	4.81	21.90
	AX-85368724	24,368,217	5.32	24.21
	AX-85317869	24,463,914	5.58	25.32
	AX-85333027	24,525,559	5.03	22.91
	AX-85305506	24,528,322	5.18	23.59
	AX-85234612	24,530,224	5.31	24.14
	AX-85208143	24,530,446	4.53	20.63
	AX-85242477	24,571,757	5.04	22.93
	AX-85297757	24,576,963	5.00	22.78
	AX-85953964	24,582,978	5.08	23.12
	AX-85280870	24,609,404	4.79	21.80
LG22	AX-85325454	383,931	4.69	21.37
LG4	AX-85294968	27,711,266	4.57	20.83
Kmix				
LG12	AX-85394840	17,561,280	4.78	21.51
	AX-85426612	17,792,919	5.12	23.04
	AX-85357080	17,800,330	4.81	21.68
LG7	AX-85370491	3,225,533	4.90	22.06
Thompson				
LG7	AX-85302554	3,746,317	5.26	32.04

and suggestive QTLs in the other strains. In Kansas strain, three genomic regions were found to contain SNPs significantly associated with hypoxia tolerance, while additional nine genomic regions were found to contain SNPs suggestively associated with hypoxia tolerance. In Kmix strain, two significantly associated genomic regions and seven suggestively associated genomic regions were identified for hypoxia tolerance. In Thompson strain, one significant genomic region and four additional suggestively associated genomic regions were identified to contain QTLs for hypoxia tolerance. With the other three strains, 103KS, Marion, and Marion Select, no significant SNPs were identified to be associated with hypoxia tolerance, but a large number of suggestive SNPs were identified.

The identification of a large number of QTLs both across strains and within strains suggested a very complex genetic architecture of hypoxia tolerance in teleost fish. It is widely believed that hypoxia tolerance is a polygenic trait, involving both HIF-dependent and HIF-independent pathways (Li et al. 2013). With HIF-dependent pathway, HIF-1α is believed to be the master switch. Instead of degradation when under normoxic conditions, HIF-1α is no longer hydrolyzed, and it then coupled to HIF-1β, and the heterodimer bind to HRE to activate transcription of the effector genes under hypoxic conditions (Kietzmann et al. 2016). A large number of genes were reported to contain HRE, and therefore, any genomic sequence variations in the form of SNPs within HRE may potentially be involved in QTLs controlling hypoxia tolerance. With HIF-independent pathways, multiple genes, such as *PI3K*, *AKT*, *RAF*, *JNK*, and *COX2*, were involved in response to hypoxia (Mizukami et al. 2007). Therefore, any SNPs within these genes may also contribute to hypoxia tolerance. In addition, our preliminary results for analysis of hypoxia tolerance using various strains of catfish indicated strong strain difference in hypoxia tolerance (Wang et al., unpublished), suggesting

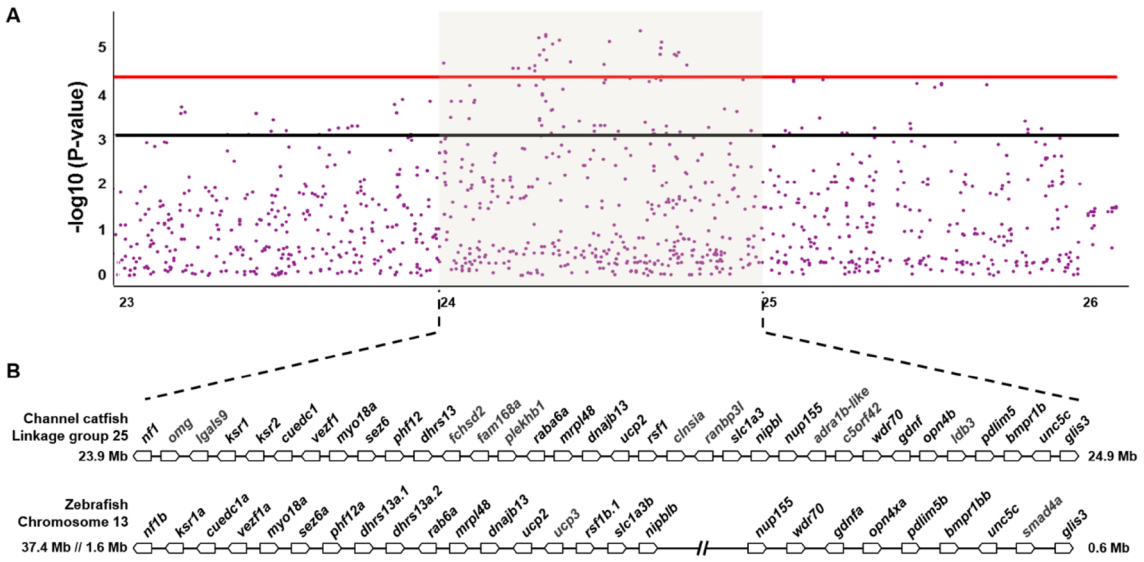

Fig. 6 Genes surrounding the most significant SNP associated with hypoxia tolerance within Kansas strain on LG25. **a** Regional −log10 (*P* value) plot for the QTL. The *gray shade* indicates ±0.5 Mb genomic region of the most significant SNP. The *red solid line* indicates the threshold *P* value for genome-wide significance. The *black solid line* indicates the threshold *P* value for the significance of "suggestive association". **b** Genes within ±0.5 Mb genomic region of the most significant SNP. Synteny analysis was conducted between catfish and zebrafish. Genes with *gray* names are not conserved between channel catfish and zebrafish (color figure online)

various QTLs may be at work within various strains. However, this study is the very first QTL analysis for hypoxia tolerance in fish, there is no existing results for us to compare. The future analyses using even more strains, more families within each strain and large sample sizes are required to validate the genetic architecture for hypoxia tolerance.

QTLs identified across strains barely overlapped with those identified within strains. This is expected because within-strain analyses may have more power to identify rare QTLs within each strain, while across-strain analyses may be more efficient to detect common QTLs for multiple strains (Ogut et al. 2015). In addition, the detected QTLs across strains reflect the superiority of one allele over the other among strains, and they may explain variations of hypoxia tolerance among strains; while the allele differences within strains may reflect variations of hypoxia tolerance among families within each strain. As such, perhaps the QTLs detected within strains are even more important for genetic enhancement programs. However, we must exercise caution because the sample sizes were quite small for both across-strain analyses and for within-strain analyses, especially for within-strain analyses. With small samples, it is prone to both type I and type II errors. One guiding principle we can use to provide some levels of confidence is the observation of many physically linked significant SNPs within the genomic region containing the most significant SNPs. For instance, for the QTL on LG25 of Kansas strain, twenty-four significant SNPs were detected within approximately 760 kb genomic region containing the QTL associated with hypoxia tolerance (Fig. 4a). The linked SNPs were all included in one haplotype block, suggesting that the association to hypoxia tolerance is related to the inclusion of the genomic segments due to co-segregation rather than genotyping errors or other related causes.

Analysis of genes included in the QTL regions associated with hypoxia tolerance suggested co-location in the genome and coordinated functions. Although it is not possible to delineate the candidate genes because of the relatively large numbers of genes existing in the identified QTL regions, analysis of the genes and their known functions are still important. First, such analysis can provide a pool of genes for the determination of potential candidate genes for hypoxia tolerance. In order not to miss any potentially important genes, we included all genes within the ±0.5 Mb genomic regions of the most significant SNPs (Figs. 3, 6), and the closest genes surrounding the suggestive SNPs (Supplemental Table S2) in both across-strain analyses and within-strain analyses. With channel catfish, 0.5 Mb physical distance correspond to, on average, almost 3 cM of genetic distance (Liu et al. 2016). Second, by examination of genes within these genomic regions and their known functions, insights can be gained as to if these genes have known functions related to hypoxia responses, and/or what hypoxia related pathways they are involved.

Genes surrounding the most significant SNP on LG6 for hypoxia tolerance across strains were identified using the catfish reference genome (Fig. 3). Most of these candidate

genes were involved in MAPK or PI3K/AKT/mTOR signaling pathways; and these pathways are known to be essential for hypoxia-mediated angiogenesis, cell proliferation, apoptosis and survival (Emerling et al. 2005). For instance, the most significant SNP (AX-86110011) was located in *lrrc1* gene on LG6. Gene *LRRC1*, encoding the cell polarity regulator, shares a high level of sequence and function similarity with *C/EBP* (Descombes et al. 1990). *C/EBP* plays a critical role in regulating cellular response to stress and is regulated by p38 MAPK (Huggins et al. 2015). Gene *tceb3*/*elongin-a* was located immediately upstream of *lrrc1* in the catfish genome. Knockout of *TCEB3* was reported to induce apoptosis and cellular senescence through activation of the p38 MAPK pathway and the hypoxia response genes (Miyata et al. 2007). *GCLC* was also reported to downregulate in hypoxic wild-type mice when compared with normoxic wild-type mice (Hoshikawa et al. 2001; Eba et al. 2013). *MLIP* is required for precocious cardiac adaptation to stress in mice by impacting cardiac activity of Akt/mTOR pathways, which may play a similar role for cardiac adaption to hypoxic stress in catfish (Cattin et al. 2015). Inhibition of *FAM83B*, a key regulator of RAF/MAPK signaling pathway, can decrease AKT phosphorylation by altering the subcellular location of multiple PI3K signaling components (Cipriano et al. 2014). *FGFR2* transduces *FGF* signals to PI3K-Akt signaling cascade, which is involved in cell survival and polarity control (Katoh 2009). *PLPP4*, preferentially expressed in endothelial cells, may play a role in angiogenesis (Takeuchi et al. 2007). *COX20* acts as the last enzyme in the respiratory electron transport chain of mitochondria (Bourens et al. 2014). *FBXO9* can minimize energy-consuming and procure survival to avoid early cell death by directly regulating mTOR signaling pathway (Fernandez-Saiz et al. 2013). *BMP5* can induce apoptosis through activation of p38 MAPK pathway (Zuzarte-Luıs et al. 2004). *BAG2* is involved in the regulation of oxidative phosphorylation and energy metabolism; and is necessary for the assembly of mitochondrial respiratory supercomplexes (Ueda et al. 2004). Some genes neighboring the most significant SNP on LG25 for Kansas strain were also found to be contributed to hypoxia tolerance (Fig. 6). For instance, *NF1*, as a tumor suppressor gene, may involve in hypoxia responses mediated by HIF-1α (Opocher et al. 2005). *LGALS9* and *VEZF1* were reported to involve in the regulation of angiogenesis (Miyashita et al. 2004; Thijssen and Griffioen 2014). *SLC1A3*, *GDNF* and *DHRS13* were found to be upregulated in response to hypoxia (Yamagata et al. 2002; Wang et al. 2010; Hu et al. 2014). *UCP2* can inhibit ROS-mediated apoptosis under hypoxic conditions (Deng et al. 2012). The colocalization of these functional related genes for hypoxia tolerance also supported our hypothesis of "functional hubs" within the genomes (Geng et al. 2015).

In addition to the analysis of genes within the significant QTL regions, examination of the genes surrounding the suggestive SNPs may provide some insights as to if such genomic regions would include real QTLs, upon increases of sample sizes. As listed in Table S2, some genes close to the suggestively associated SNPs on different linkage groups appeared to have known functions related to hypoxia tolerance. With across-strain QTLs, for instance, *cox7a2l* gene is located 17 kb upstream of the suggestive SNP marker AX-85410024 on LG6. *COX7A2L* is involved in the regulation of energy metabolism and responsible for the assembly of mitochondrial respiratory supercomplexes (Ikeda et al. 2013). This gene was reported to be significantly differentially expressed under hypoxia as a cardiac gene in *Fundulus grandis* (Everett et al. 2012). On LG5, *btbd17* gene is located 1 kb upstream, and *map2k6*/*mkk6*of is located 21 kb downstream of the suggestive SNP marker AX-85216281. While the function of *BTBD17* remains unknown, *MAP2K6*/*MKK6* is an essential component of the MAPK pathway that mediates apoptotic cell death in thymocytes (Raingeaud et al. 1996). Cells deficient in *MKK6* failed to activate p38 MAPK and stabilize *HIF-1* during hypoxia, indicating that the hypoxic activation of p38 MAPK and *HIF-1* is in a *MKK6*-dependent manner (Emerling et al. 2005). On LG10, the suggestively associated SNP marker AX-85217471 is located within the *itsn1* gene. *ITSN1* codes a long (*ITSN1-L*) and a short (*ITSN1-S*) protein isoform, and hypoxia specifically upregulates the long isoform *ITSN1-L* (Weigand et al. 2012). *ITSN* also regulates PI3K-C2beta and AKT signaling pathway necessary for cell survival (Das et al. 2007). Additionally, *ITSN1* was reported to negatively regulate the mitochondrial apoptotic pathway in endothelial cells (Predescu et al. 2007). Similarly, on LG12, the suggestively associated marker AX-85197805 is within the *cic* gene. *CIC* acts as an important regulator of the homeostatic control of mitochondria in tumor tissues, and high expression level of *CIC* in tumors allows adaptation for metabolic and respiration stress (Catalina-Rodriguez et al. 2012). With within-strain QTLs, for instance, *rap2b*, located in SNP AX-85237540 on LG4 for Marion strain, can be induced by various stress and regulate pro-survival function in a p53-dependent manner (Zhang et al. 2013). Gene *bad*, located in SNP AX-85325943 on LG13 for Marion Select strain, was reported to induce apoptosis in cells by EGFR/MAPK and PI3K/Akt kinase pathways (She et al. 2005). Gene *aplp1*, located in SNP AX-85339135 on LG22 for Marion Select strain, is required for the proliferation in epithelial and fibroblastic cell types (Tang et al. 2007). Taken together, there are reasons to believe that the suggestive QTLs deserve to be further explored. It is apparent that use of larger sample sizes may make these suggestive QTLs statistically significant.

A number of gene pathways are known to be involved in hypoxia responses including HIF, NOTCH, MAPK and PI3K pathways. Here, the vast majority of the genes within QTLs identified in this GWAS are involved in MAPK pathway or/and PI3K/AKT/mTOR pathway. In particular, p38 MAPK signaling, known as stress-activated protein kinase pathway, is essential for *HIF1* activation under hypoxic conditions (Emerling et al. 2005). Similar to MAPK pathway, constitutively activated PI3K/AKT involved in hypoxic activation of *HIF-1α*, while the activity of *mTOR1* is suppressed under hypoxic conditions in cancer cells (Cam et al. 2010). In our study, nine genes were involved in the MAPK pathway, including *tceb3*, *klhl31*, *fam83b*, *hcrtr2*, *bmp5*, *bag2*, *map2k6*, *ucp2* and *bad*; seven genes were involved in PI3K/AKT/mTOR pathway, including *elovl5*, *mlip*, *fam83b*, *fgfr2*, *fbxo9*, *itsn* and *bad*. All these genes are known to be involved in MAPK or PI3K/AKT/mTOR pathway through different mediators to regulate cell response to extracellular stress and maintain cell homeostasis for organisms' survival. Taken together, the presence of these genes within the detected QTL regions may suggest that the allelic variations within these genes could be potentially the causes of the observed phenotypic variations of hypoxia tolerance. Future studies of the sequence variations in contrasted haplotypes of these QTL regions should elucidate the molecular basis of the detected QTLs for hypoxia tolerance.

In summary, a large number of significant and suggestive QTLs were identified for hypoxia tolerance in channel catfish. Within-strain QTLs explained relatively large proportions (>20 %) of phenotypic variations, while across-strain QTLs had a relatively small effect on phenotypic variations (4–6 %) for hypoxia tolerance. Many genes surrounding the identified QTLs are known to be functionally related to cell adaption and response to hypoxic stress, and they are mostly involved in MAPK or/and PI3K/AKT/mTOR pathways. The fact that many QTLs were detected both across strains and within strains, and that very few overlaps of the QTLs among strains suggested a highly complex genetic architecture for hypoxia tolerance. If validated, these findings would suggest that marker-assisted selection for hypoxia tolerance would be essentially very difficult, while traditional and/or genome-based selection may have to be adopted for genetic enhancement programs in catfish.

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Compliance with ethical standards

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Conflict of interest All authors declare that no conflict of interest exist in this work.

Ethical approval All procedures involving the handling and treatment of fish used during this study followed the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. Blood samples were collected after euthanasia. All animal procedures were carried out according to the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the United States. This article did not use any personal data.

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