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ORIGINAL ARTICLE

Genome-Wide Association Study Reveals Multiple Novel QTL Associated with Low Oxygen Tolerance in Hybrid Catfish

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Abstract Hypoxic condition is common in aquaculture, leading to major economic losses. Genetic analysis of hypoxia tolerance, therefore, is not only scientifically significant, but also economically important. Catfish is generally regarded as being highly tolerant to low dissolved oxygen, but variations exist among various populations, strains, and species. In this study, we conducted a genome-wide association study (GWAS) using the catfish 250 K SNP array to identify quantitative trait locus (QTL) associated with tolerance to low dissolved oxygen in the channel catfish × blue catfish interspecific system. Four linkage groups (LG2, LG4, LG23, and LG29) were found to be associated with low oxygen tolerance in hybrid catfish. Multiple significant SNPs were found to be physically linked in genomic regions containing significant QTL for low oxygen tolerance on LG2 and LG23, and in those regions containing suggestively significant QTL on LG2, LG4, LG23, and LG29, suggesting that the physically linked SNPs were genuinely segregating and related with low oxygen tolerance. Analysis of genes within the associated genomic regions suggested that many of these genes were involved in VEGF, MAPK, mTOR, PI3K-Akt, P53-mediated apoptosis, and DNA damage checkpoint pathways. Comparative analysis indicated that most of the QTL at the species level, as analyzed by using the interspecific system, did not overlap with those identified from six strains of channel catfish, confirming the complexity of the genetic architecture of hypoxia tolerance in catfish.

Keywords Genome · GWAS · Hypoxia · Fish · SNP · Low oxygen tolerance

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Introduction

Low oxygen can pose threats to life, but the levels of tolerance to low oxygen vary greatly among various organisms. It has been long believed that hypoxia-inducible factor (*HIF*) 1 was the master switch of hypoxia responses, and such regulation was believed to be mostly at the level of post-translation (Semenza 2000). However, transcriptional regulation has also been reported in catfish (Geng et al. 2014). *HIF 1α* protein, in spite of being continuously synthesized, is degraded under normal oxygen conditions. Under hypoxia conditions, *HIF 1α* rapidly accumulates, and dimerizes with its partner *HIF 1β*, which then binds to hypoxia-responsive elements of effector genes of various pathways such as those involved in angiogenesis, glucose transport, glycolysis, and erythropoiesis, leading to activation of a wide variety of genes involved in both the cellular and systematic responses (Denko 2008; Kietzmann et al. 2016).

Teleost fish are good models for hypoxia studies because they live in aquatic habitats with various oxygen content, and they have evolved unique capabilities to regulate gene expression patterns in response to hypoxic stress (Nikinmaa and Rees 2005). A number of studies have been conducted to determine gene expression in relation to hypoxia. For example, Ton et al. (2002, 2003) employed zebrafish complementary DNA (cDNA) microarray to determine the changes in gene expression patterns during embryonic development in response to hypoxic stress. They observed downregulation

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of genes encoding both skeletal and cardiac contractile proteins and upregulation of genes encoding enzymes associated with glycolysis pathways such as phosphoglycerate kinase and aldolase. A series of defense processes under hypoxia were identified in zebrafish embryos, including (1) suppression of ATP demand, such as shutting down protein synthesis and cell division processes; (2) reorganizing metabolic pathways by switching from aerobic oxidation to glycolytic pathway; and (3) induction of a number of genes involved in cellular defense and DNA repair, such as HSP70, RAD52, and MSH6. van der Meer et al. (2005) studied long-term adaptive responses to hypoxia in the gill of adult zebrafish and identified the induction of genes for lysosomal lipid trafficking and degradation. Gracey et al. (2001) reported that energy-requiring processes such as cell growth were downregulated, whereas pathways associated with anaerobic metabolism were upregulated in the hypoxic response of *Gillichthys mirabilis*; triglyceride hydrolysis were upregulated, whereas pathways involved in triglyceride synthesis were downregulated (Gracey et al. 2011). In addition, Ju et al. (2007) found that ubiquitin-proteasome and phosphatidylinositol signaling pathways were significantly dysregulated in medaka (*Oryzias latipes*) in response to hypoxic exposure.

Hypoxic condition is common in aquaculture due to high stocking density and nutrient loading under intense aquaculture conditions. Although aerators are wildly used in aquaculture to maintain adequate oxygen levels, extensive use of aerators increases energy cost for production. It is generally acknowledged that fish can employ a complex set of morphological, behavioral, and physiological alterations to cope with short-term hypoxic stress (Nikinmaa and Rees 2005; Kramer 1987; Hochachka 1997; Wu 2002). However, Wu et al. (2003) reported that chronic hypoxia was an endocrine disruptor, which might lead to reduced reproductive performance in carp (*Cyprinus carpio*). Shang and Wu (2004) found that hypoxia might have a teratogenic effect on fish and could delay embryonic development in zebrafish (*Danio rerio*). Padilla and Roth (2001) found that hypoxia caused zebrafish embryos arrested in S and G2 phases of the cell cycle under anoxic conditions. Shang et al. (2006) reported that hypoxia could affect sex differentiation and development in zebrafish, thereby threatening the sustainability of the species. In addition, after being exposed to hypoxic conditions, fish become highly susceptible to diseases which, in turn, lead to mortalities and economic losses (Welker et al. 2007; Kvamme et al. 2013). Therefore, genetic analysis of hypoxia tolerance is important, not only because they provide insight into the mechanisms of hypoxia responses but also because improving hypoxia tolerance is of economic interest.

Channel catfish (*Ictalurus punctatus*) is the major aquaculture species in the USA. It is generally known to be capable of maintaining sufficient oxygen uptake at low levels of dissolved oxygen through a pronounced branchial hyperventilation. However, lactic acidosis usually occurs because of significant anaerobic glycolysis, which may result in decreased growth rate, food intake, and feed conversion ratio (Torrans 2008; Burggren and Cameron 1980; Carlson et al. 1980; Buentello et al. 2000). In addition, hypoxic stress is often associated with increased susceptibility to diseases, leading to outbreaks of infectious diseases, such as enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri*, and columnaris caused by *Flavobacterium columnare* (Welker et al. 2007; Shoemaker et al. 2008). Although channel catfish was most commonly used in the catfish industry, the channel catfish female × blue catfish (*Ictalurus furcatus*) male hybrid are increasingly being used in the catfish industry (Dunham and Masser 2012; Dunham 2011), now accounting for over 60% of the production. The hybrid catfish exhibits superior performance traits such as enhanced growth rates and feed conversion efficiency, and increased resistance and tolerance to diseases and other environmental stresses such as low oxygen (Giudice 1966; Dunham and Smitherman 1987; Dunham and Masser 2012; Ella 1984). Dunham et al. (1983, 2008) reported that the hybrid catfish was more tolerant to hypoxic conditions than channel catfish and blue catfish. Variations in hypoxia tolerance were also reported among different hybrid catfish families and channel catfish strains (Dunham et al. 2014; Wang et al. 2017b).

Genome-wide association study (GWAS) is a powerful strategy for the identification of markers associated with traits of interest with high resolution because it exploits not only linkage disequilibrium of genes with traits, but also historically accumulated recombinations in unrelated populations. Several GWA studies have been conducted in catfish to identify QTL associated with performance and production traits, such as disease resistance (Geng et al. 2015), heat tolerance (Jin et al. 2016), head size (Geng et al. 2016), and low dissolved oxygen tolerance (Wang et al. 2017a). Wang et al. (2017a) reported multiple within- and across-strain QTL for hypoxia tolerance, suggesting a complex genetic architecture of hypoxia tolerance. However, no GWA studies on QTL for low oxygen tolerance were conducted in hybrid catfish. Here, we report a genome-wide association study to identify QTL associated with low oxygen tolerance using the interspecific backcross progenies genotyped with the 250 K SNP arrays (Liu et al. 2014).

Materials and Methods

Experimental Fish and Hypoxia Treatment

The hypoxic stress experiment was conducted with 1-year-old hybrid catfish, which were produced by crossing male F_1 hybrid catfish (female channel catfish × male blue catfish) and female channel catfish. In this study, a total of 347 experimental fish (average body weight 46.23 g) were randomly selected from ten families, including families 1 (51 fish), 2 (25 fish), 3

(17 fish), 4 (39 fish), 5 (62 fish), 6 (36 fish), 7 (22 fish), 8 (51 fish), 9 (22 fish), and 10 (22 fish). Fish were cultured at Fish Genetics Facility of Auburn University. All experimental fish were passive integrated transponder (PIT) tagged before mixed together for low oxygen challenge.

The experimental fish were reared for 1 week in tanks [3.2 m × 0.5 m × 1.0 m (L × W × H)] with flow-through water. The average water temperature was controlled at 25 °C and oxygen level was controlled at a range from 8.0 to 10.0 ppm by using an aeration. During the experiment, dissolved oxygen level was monitored by a dissolved oxygen meter (YSI Model 58, YSI, Ohio). After acclimation, a total of 50 fish were moved to another tank as a control group with 5 fish randomly selected from each family. The remaining 297 hybrid catfish were exposed to hypoxic stress. In the experiment, the aeration and water flow were stopped. Oxygen level was decreased by adding sodium sulfite (Kramer and McClure 1982; Melnychuk and Chapman 2002). The dissolved oxygen (DO) level was 8.50 ppm before hypoxia treatment. The sodium sulfite was used at an approximate rate of 7.9 /1.0 ppm dissolved oxygen and the DO level was gradually decreased to 0.1 ppm in an hour. After that, the DO level was kept constant, and fish were observed for behavior of losing balance. The time of fish that lost balance were recorded. Fish were euthanized using tricaine methanesulfonate (MS-222), and then measured for body weight and draw blood. We considered the first ~35% fish lost balance as hypoxia-intolerant fish and the last ~35% fish as the hypoxia-tolerant fish for each family. In total, 208 fish from ten families were selected for this study (Table 1). Blood samples (500–1000 μL) were collected and mixed with 5 mL of cell lysis buffer (10-mL lysis solution and 20 mg/mL proteinase K) in 15-mL tubes.

DNA Isolation and Genotyping

DNA was extracted using protocol described previously (Kucuktas et al. 2009; Liu et al. 1999). Briefly, blood samples were incubated at 55 °C overnight and the blood cells were broken by cell lysis solution. Protease K and protein precipitation solution were used for removing proteins. DNA was precipitated by isopropanol and collected by brief centrifugation, washed twice with 70% ethanol, air-dried, and resuspended in TE buffer (pH 8.0). The DNA integrity was validated by 1% agarose gel electrophoresis. Nanodrop (Thermo Scientific) was used for DNA quantification and then DNA was diluted to a concentration of 50 ng/μL. Genotyping was conducted using the catfish 250 K SNP array (Liu et al. 2014) at GeneSeek (Lincoln, Nebraska, USA).

Genome-Wide Association Analysis

Genome-wide association analysis was performed using the SVS software package (SNP & Variation Suite, Version 8.0) according to the manufacturer's tutorial. Briefly, SNPs with a minor allele frequency (MAF) <1% or a call rate <95% were removed prior to association analysis. After this SNP quality control, linkage disequilibrium (LD) pruning was analyzed using a window size of 50, window increment of 5, and r^2 threshold of 0.2. After LD pruning, 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 analyzed by using principal component analysis (PCA) with the independent SNP markers. As the first two principal components could explain over 80% of total variance, the plots were constructed with the first two principal components.

To account for the sample structure in this association analysis, Efficient Mixed-Model Association eXpedited (EMMAX) method was conducted with the first two principal components and body weight as covariates (Kang et al. 2010; Wang et al. 2017a). The model is as follows:

$$Y = X\beta + Zu + e$$

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

A Manhattan plot of the $-\log_{10}$ (P value) was also produced by the SVS software. The threshold P value for genome-wide significance was calculated based on Bonferroni correction with the estimated number of independent markers and LD blocks (Geng et al. 2015). The linkage map was constructed based on channel catfish genome sequence (Liu et al. 2016).

Candidate Genes and Pathway Analysis

Genes within about ±500 kb of the significant and suggestively significant SNPs were identified from catfish genome sequences (Liu et al. 2016) using FGENESH programs (Solovyev et al. 2006). The predicted amino acid sequences were annotated by BLASTP against NCBI non-redundant protein sequence database. Pathway analysis was conducted using GeneCards (http://www.genecards.org/), DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/home.jsp), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html) and Reactome database (http://www.reactome.org/).

Table 1 The catfish samples used in this study

Family ID	Dam	Sire	Number of samples	Susceptible samples	Resistant samples
1	Channel 1	Hybrid 1	32	16	16
2	Channel 2	Hybrid 2	14	7	7
3	Channel 3	Hybrid 3	8	4	4
4	Channel 4	Hybrid 4	24	12	12
5	Channel 5	Hybrid 5	40	20	20
6	Channel 6	Hybrid 6	22	11	11
7	Channel 7	Hybrid 7	12	6	6
8	Channel 8	Hybrid 8	32	16	16
9	Channel 9	Hybrid 9	12	6	6
10	Channel 10	Hybrid 10	12	6	6

Results

Experimental Fish and Sample Structure

The information of catfish samples used in this study is summarized in Table 1. The experimental fish were communally challenged with low levels of dissolved oxygen. Equal numbers of tolerant and intolerant fish were chosen from each family for genotyping. In total, 208 fish were genotyped for GWAS analysis. Based on the genotype information, the ten families were grouped into five clusters (Fig. 1). Families 1, 3, and 4 was each grouped into a separate cluster, while families 2 and 9 were grouped closely as one cluster, and families 5, 6, 7, 8, and 10 were grouped together as one cluster.

Linkage Groups Associated with Low Oxygen Tolerance

Catfish 250 K SNP array was utilized for SNP genotyping. A total of 208,598 SNPs that passed the quality control criteria were used for further analysis. The number of independent SNP markers was 25,045 after LD pruning. With 25,045 independent haplotype blocks, the genome-wide threshold for statistical significance was calculated to be 1.996×10^{-6} (0.05/25,045), i.e., $-\log_{10}$ (*P* value) = 5.7. Similarly, the threshold for suggestive statistical significance was calculated to be 3.992×10^{-5} (1.0/25,045), i.e., $-\log_{10}$ (*P* value) = 4.4.

The Manhattan plot of the GWAS results is shown in Fig. 2. Two linkage groups, LG2 and LG23, were found to harbor QTL significantly associated with low oxygen tolerance; and two additional linkage groups, LG4 and LG29, were found to contain QTL suggestively associated with low oxygen tolerance in hybrid catfish.

SNP Markers Associated with Low Oxygen Tolerance

The SNP markers significantly and suggestively associated with low oxygen tolerance on linkage groups 2 and 23, and those suggestively associated with low oxygen tolerance on linkage groups 4 and 29 are listed in Table 2. On LG2, six

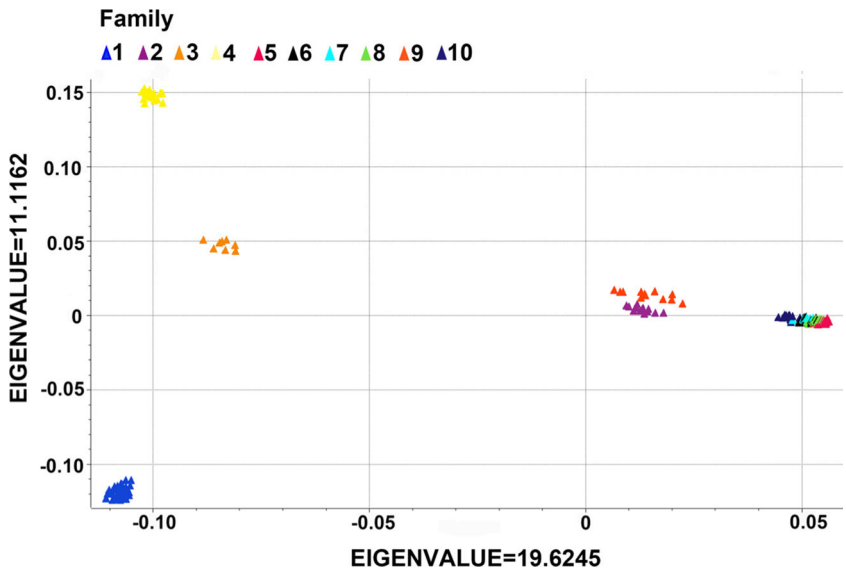

Fig. 1 Sample structure analyzed by using principal component analysis (PCA) with the first two principal components

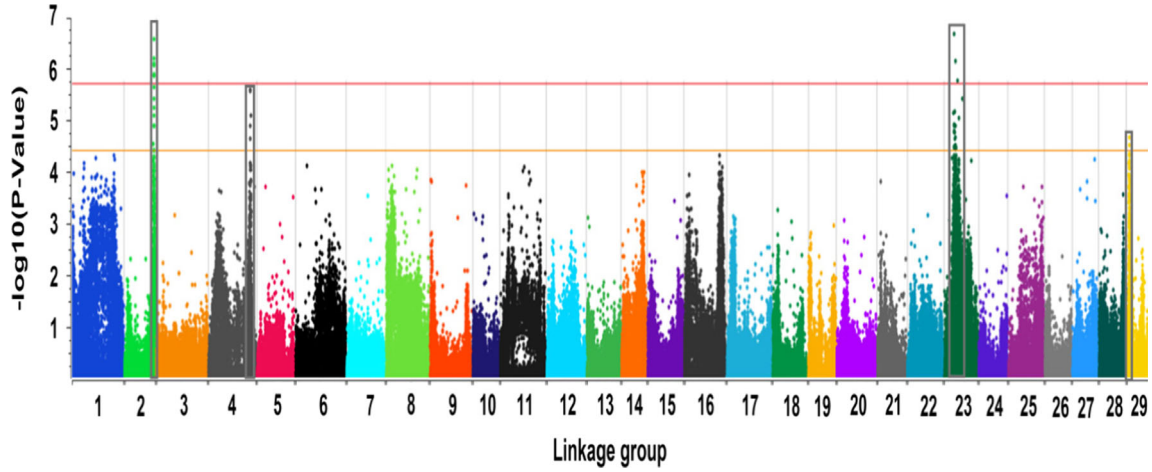

Fig. 2 Manhattan plot of genome-wide association analysis for low oxygen tolerance in hybrid catfish. The *red line* indicates the threshold $-\log_{10}$ (*P* value) for genome-wide significance. The *yellow line* indicates the threshold $-\log_{10}$ (*P* value) for the significance of "suggestive association." The four *boxes* indicate the associated regions

SNPs were significantly associated with low oxygen tolerance, including AX-85209807, AX-85398402, AX-85302510, AX-85203591, AX-85957316, and AX-85279207. These significantly associated SNPs on LG2 spanned a physical distance of approximately 109 kb from 21,356,670 to 21,466,282 bp. In addition, seven SNPs were found to be above the level for suggestive significance in this same genomic region. Altogether, these 13 SNPs were located within a region of 422 kb from 21,112,936 to 21,535,490 bp (Table 2).

On LG23, three SNPs were significantly associated with low oxygen tolerance, including AX-85280487, AX-85232183, and AX-85264562. However, a large genomic region was observed for these three significant SNPs on LG23, spanning approximately 2336 kb from 7,215,927 to 9,552,583 bp. In addition, 17 SNPs in this overall genomic region were also found to be above the suggestively significant level (Table 2), but this entire region involves a large physical distance of 6.29 Mb. It is possible that two or more QTL are involved in this region (see "Discussion").

Two linkage groups, LG4 and LG29, were found to contain suggestively associated SNPs for low oxygen tolerance. On LG4, five SNPs were suggestively significant, and they were located within a genomic region of approximately 430 kb (Table 2). Similarly, on LG29, two suggestively significant SNPs were identified and they are located approximately 121 kb apart (Table 2).

The largest proportion of phenotypic variance explained by the significantly associated SNPs on LG2 was 12.24%, ranging from 10.87 to 12.24%. Conditioned analysis was conducted to examine the correlation of the SNPs associated with low oxygen tolerance (Nishimura et al. 2012). Genotypes of the most significant SNP (AX-85302510) on LG2 were included as a covariate in the mixed linear model. After conditioning, the $-\log_{10}$ (*P* value) of all the other SNPs on LG2 dropped below 3.5, suggesting that these SNPs were strongly correlated because of their linkage. The proportion of phenotypic variance explained by the significantly associated SNPs on LG23 varied from 10.65 to 12.44%. Similar to LG2, conditioned analysis indicated that all the other SNPs on LG23 were not statistically significant when the most significantly associated SNP was included. The proportion of phenotypic variance explained by the suggestively significant SNPs on LG4 accounted for 8.43–10.32% of phenotypic variance and those on LG29 accounted for 8.20–8.49% of phenotypic variance (Table 2).

Genes Included in the Genomic Regions Associated with Low Oxygen Tolerance

To explore the potential genes involved in low oxygen tolerance, genomic sequences with the significant and suggestively significant SNPs were annotated, including a 500-kb window both upstream and downstream of the significantly and suggestively associated SNPs. The information of genes surrounding the SNPs associated with low oxygen tolerance is shown in Figs. 3, 4, and 5. A total of 54 genes were identified in the genomic region containing the significant QTL on LG2 (Fig. 3); Similarly, 126 genes were detected on LG23 (Fig. 4), 34 genes were detected on LG4 (Fig. 5a), and 36 genes were detected on LG29 in the defined region of a little over one million base pairs (Fig. 5b), with the exception of LG23 with which the defined region spanned over 7.3 Mb (Fig. 4).

We further examined the genes within the narrowed genomic regions containing SNPs above the suggestively significant levels. On LG2, this region contained eight genes, and they are diencephalon/mesencephalon homeobox protein 1-A (*dmbx1a*); ATP-dependent DNA helicase PIF1 (*pif1*); prostaglandin E2 receptor EP4 subtype (*ptger4*); artemin (*artn*); ST3 beta-galactoside alpha-2,3-sialyltransferase 3 (*st3gal3a*);

Table 2 The significant (italics) and suggestively significant SNPs associated with low oxygen tolerance, and the proportion of explained variance

Linkage group	SNP marker	SNP position	$-\log_{10}$ (*P* value)	(%) variance explained
2	AX-85206728	21,112,936	4.54	8.27
	AX-85242376	21,342,688	4.41	8.01
	AX-85209807	21,356,670	6.07	11.29
	AX-85365921	21,400,678	5.09	9.36
	AX-86041019	21,418,376	5.41	10.00
	AX-85398402	21,437,246	5.86	10.87
	AX-85302510	21,453,939	6.57	12.24
	AX-85273575	21,461,131	4.87	8.92
	AX-85203591	21,461,796	5.88	10.92
	AX-85957316	21,461,923	6.18	11.5
	AX-85279207	21,466,282	6.06	11.25
	AX-85346358	21,495,820	5.63	10.43
	AX-85286354	21,535,490	5.22	9.62
4	AX-85237561	30,285,674	4.87	8.93
	AX-85196655	30,340,706	4.62	8.43
	AX-85384531	30,403,057	5.58	10.32
	AX-85237732	30,446,851	5.56	10.28
	AX-85261125	30,716,572	5.07	9.33
23	AX-85313535	6,688,447	5.12	9.42
	AX-85314272	6,821,030	4.77	8.72
	AX-85355353	6,842,888	4.90	9.00
	AX-85431600	6,884,169	4.78	8.76
	AX-85947208	6,947,277	4.48	8.16
	AX-86146893	6,977,784	4.89	8.96
	AX-85261919	7,143,103	4.46	8.11
	AX-85280487	7,215,927	6.67	12.44
	AX-85325319	7,433,265	4.64	8.47
	AX-85385627	7,457,563	5.15	9.48
	AX-85431299	7,537,716	4.76	8.71
	AX-85433525	7,877,215	4.88	8.94
	AX-85232183	7,996,431	6.12	11.39
	AX-85996449	8,010,520	4.65	8.49
	AX-85413198	8,071,598	4.50	8.20
	AX-85323279	8,084,454	4.43	8.06
	AX-85279822	8,650,414	4.45	8.10
	AX-85264562	9,552,583	5.75	10.65
	AX-85381605	10,478,135	5.04	9.26
	AX-86076575	12,981,060	5.41	9.99
29	AX-85233788	1,604,095	4.65	8.49
	AX-85391599	1,725,806	4.50	8.20

lysine-specific demethylase 4A (*kdm4a*); receptor-type tyrosine-protein phosphatase F (*ptprf*); and cAMP-dependent protein kinase inhibitor beta (*pkib*) (Fig. 3). Similarly, on LG23, this region still contained 106 genes (Fig. 4). On LG4, this region contained nine genes including cytochrome P450 1A1 (*cyp1a1*), collagen and calcium-binding EGF domain-containing protein 1 (*ccbe1*), synaptotagmin-4 (*syt4*), semaphorin-7A (*sema7a*), AT-rich interactive domain-containing protein 3A (*arid3a*), AT-rich interactive domain-containing protein 3B (*arid3b*), protein FAM219B (*fam219b*), P2Y purinoceptor 1 (*p2ry1*) and ras-related protein Rap-2b (*rap2b*) (Fig. 5a). Similarly, this region on LG29 contained three genes, kelch-like protein 5 (*klhl5*), protein Noxp20 (*fam114a1*), and krueppel-like factor 3 (*klf3*) (Fig. 5b).

Potential Gene Pathways

All the genes detected within ±0.5 Mb of the significantly and suggestively associated SNPs were used to perform gene pathway analysis. The most enriched pathways for these genes included vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K)-AKT, P53-mediated apoptosis, and DNA damage checkpoint pathways (Fig. 6). Four genes were involved in VEGF pathway, including metallopeptidase inhibitor 3 (*timp3*), tyrosine-protein kinase CSK (*csk*), protein kinase C theta type (*prkcq*), and mitogen-activated protein kinase 11 (*mapk11*); four genes were found to be involved in MAPK pathway, including caspase-3 (*casp3*), *mapk11*, neurotrophin-3 (*ntf3*), and guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 (*gng12*); three genes were involved in mTOR pathway, including *prkcq*, *mapk11*, and eukaryotic translation initiation factor 4 gamma 1 (*eif4g1*); five genes were involved in PI3K-AKT pathway, including collagen alpha-2(IV) chain (*col4a2*), collagen alpha-4(IV) chain (*col4a4*), *gng12*, *prolactin* (*prl*), and interleukin-4 receptor subunit alpha (*il4ra*); four genes were involved in the P53-mediated apoptosis pathway, including *ntf3*, growth arrest-specific protein 1 (*gas1*), *timp3*, and *casp3*; and two genes were involved in DNA damage checkpoint pathway, including cyclin-dependent kinase inhibitor 1 (*cdkn1a*) and growth arrest and DNA damage-inducible protein GADD45 alpha (*gadd45a*).

Discussion

In this study, a GWA study was conducted with the channel catfish × blue catfish backcross progenies genotyped by using the catfish 250 K SNP array. We successfully identified significant QTL on LG2 and LG23, and suggestively significant SNPs on LG4 and LG29. Apparently, the ability to achieve significance is not only related to the nature of the genetic architecture of the QTL and their contribution toward the phenotypic variances but also the sample sizes used for association test. In our case, the sample sizes were relatively small

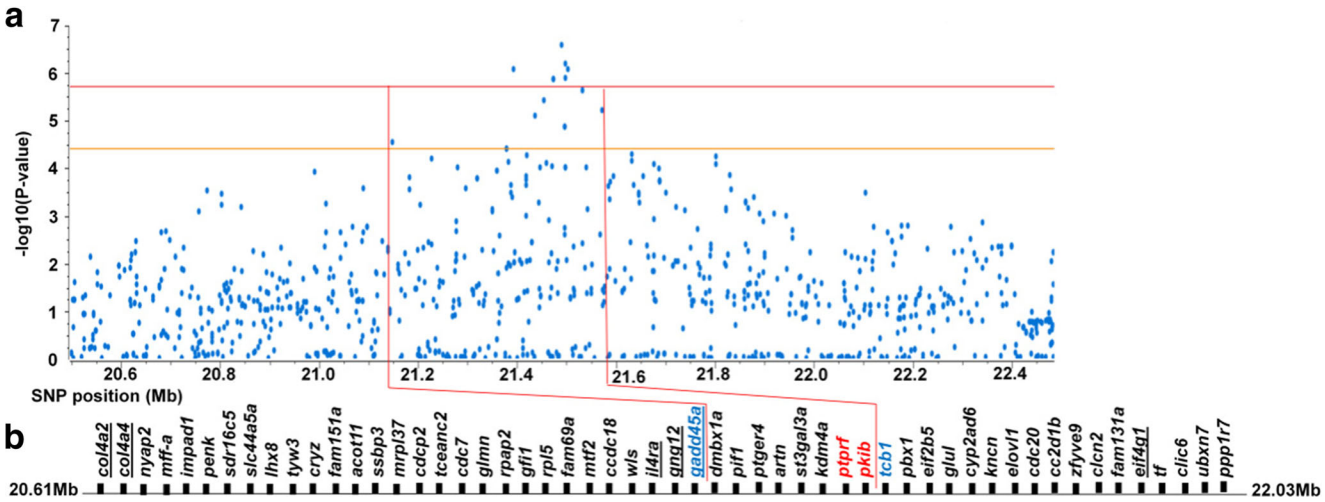

Fig. 3 Genes surrounding the significant and suggestively significant SNPs associated with hypoxia tolerance on LG2. **a** Regional $-\log_{10}$ (*P* value) plot for the QTL. The *horizontal red line* indicates the threshold $-\log_{10}$ (*P* value) for genome-wide significance. The *horizontal yellow line* indicates the threshold $-\log_{10}$ (*P* value) for the significance of "suggestive association." **b** Genes within ±0.5 Mb genomic region of the significant and suggestively significant SNPs. Genes with *red colors* are the nearest genes of the significant SNPs. Genes with *blue colors* are the nearest genes of the suggestively significant SNPs. Genes detected on the gene pathways (Fig. 6) are *underlined*

due to financial limitations, but the use of a large number of families was helpful for the detection of the QTL.

While GWAS provides a higher power for the detection of associated markers, it is more prone to produce type I errors. GWAS often encounters an issue of population structure, which may produce spurious associations if not properly corrected (Campbell et al. 2005; Tian et al. 2008). Spurious associations were primarily detected at markers with allele frequency differences among subpopulations. In our case, relatively small samples from all 10 families were used (Table 1), which, to a certain extent, prevented strong structural stratification problems. In addition, we used EMMAX model that has been shown to be applicable for correcting population structure, as well as family structure and hidden relatedness (Price et al. 2010). This approach can use high-density markers to calculate a pairwise relatedness matrix representing the sample structure and correct for the structure during the mapping (Kang et al. 2010). To exclude false positive results produced by sample structure observed in our study, EMMAX method was utilized and adjusted for the first two principal components after calculation of kinship matrix-pairwise IBS distance.

It is evident that these identified QTL regions are genuinely associated with low oxygen tolerance. The strongest evidence

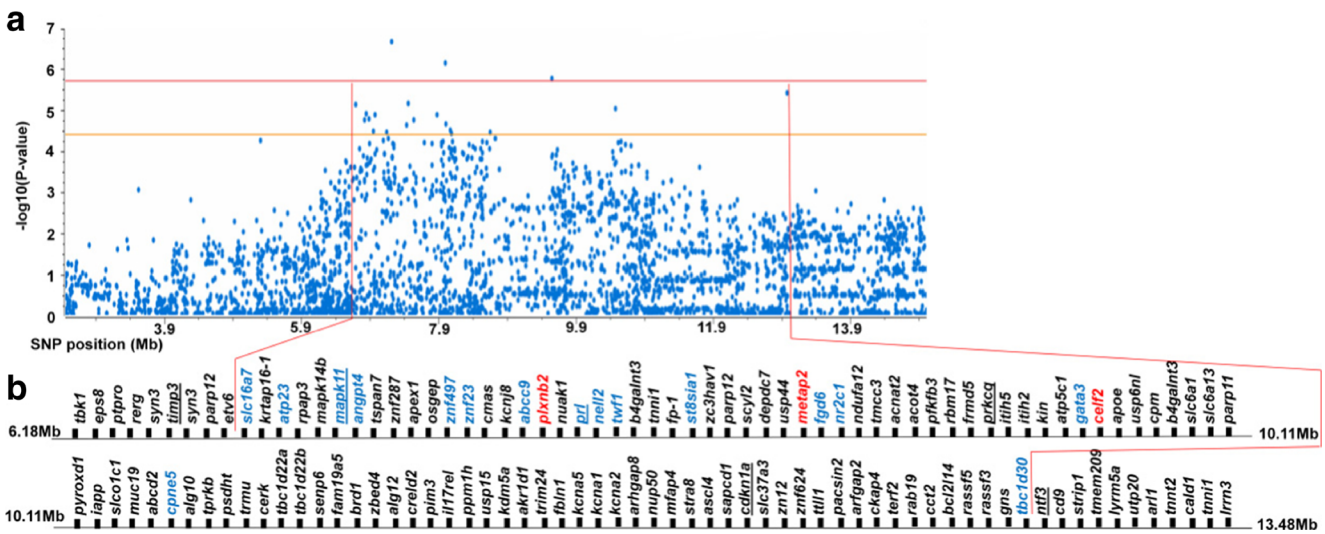

Fig. 4 Genes surrounding the significant and suggestively significant SNPs associated with hypoxia tolerance on LG23. **a** Regional $-\log_{10}$ (*P* value) plot for the QTL. The *horizontal red line* indicates the threshold $-\log_{10}$ (*P* value) for genome-wide significance. The *horizontal yellow line* indicates the threshold $-\log_{10}$ (*P* value) for the significance of "suggestive association." **b** Genes within ±0.5 Mb genomic region of the significant and suggestively significant SNPs. Genes with *red colors* are the nearest genes of the significant SNPs. Genes with *blue colors* are the nearest genes of the suggestively significant SNPs. Genes detected on the gene pathways (Fig. 6) are *underlined*

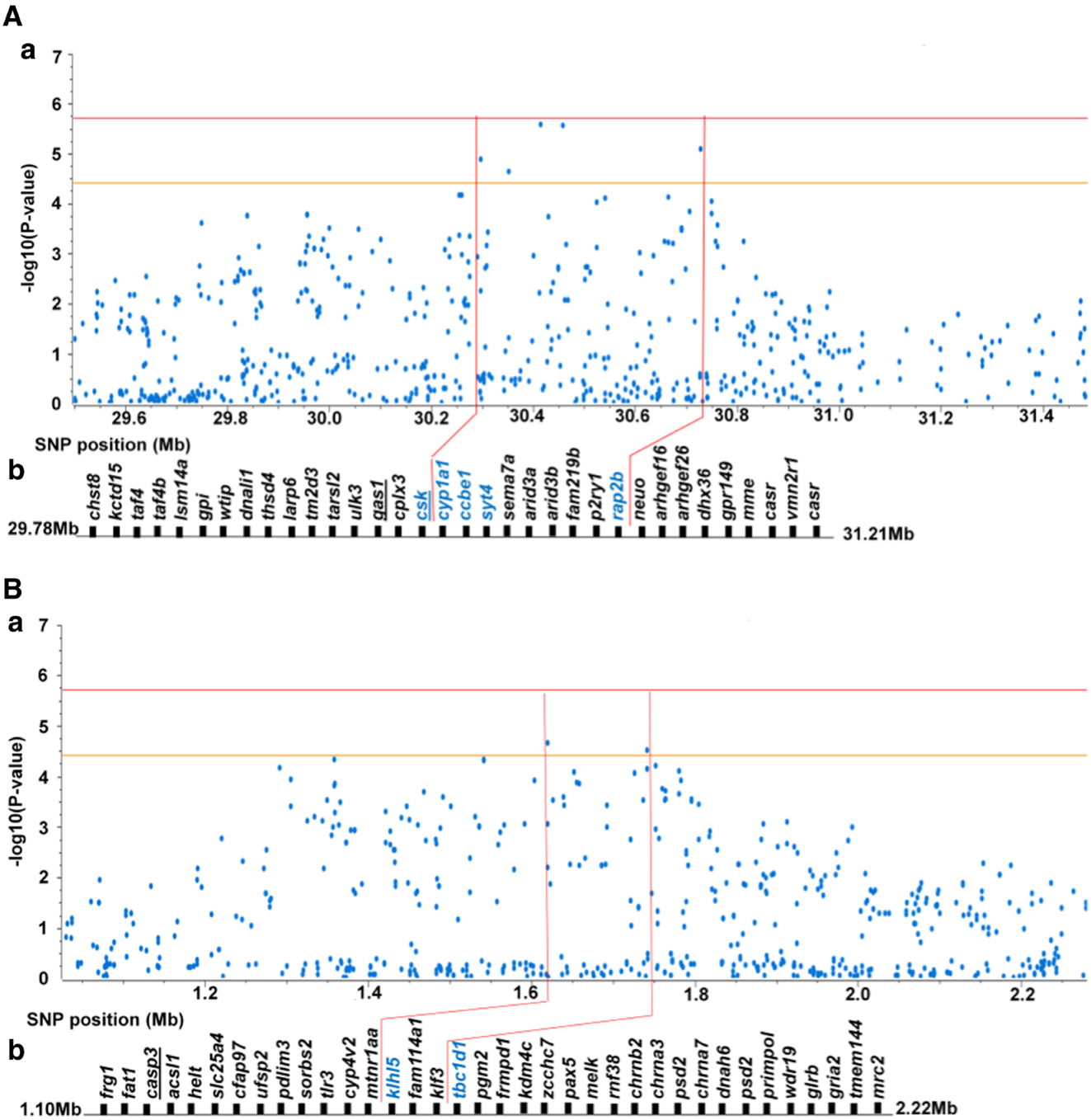

Fig. 5 Genes surrounding the suggestively significant SNP associated with hypoxia tolerance on LG4 (**a**) and LG29 (**b**). *a* Regional $-\log_{10}$ (*P* value) plot for the QTL. The *horizontal red line* indicates the threshold $-\log_{10}$ (*P* value) for genome-wide significance. The *horizontal yellow line* indicates the threshold $-\log_{10}$ (*P* value) for the significance of "suggestive association." **b** Genes within ±0.5 Mb genomic region of the suggestively significant SNPs. Genes with *blue color* are the nearest genes of the suggestively significant SNPs. Genes detected on the gene pathways (Fig. 6) are *underlined*

came from the identification of a large number of significant or suggestively significant SNPs within a small genomic region where they are linked one another within a physical distance of several hundred kilobases, with the exception of the genomic region identified on LG23 (see below). When the SNPs are closely linked, they are physically located together in the genome, and they are expected to segregate together, with rare cases of recombination. This is exactly what was observed, suggesting that the QTL regions contained SNPs whose co-segregation was the major reason of the observed association with the low oxygen tolerance. The use of a large number of families (10) allowed us to detect the QTL at a stronger power by taking advantage of historically accumulated recombination.

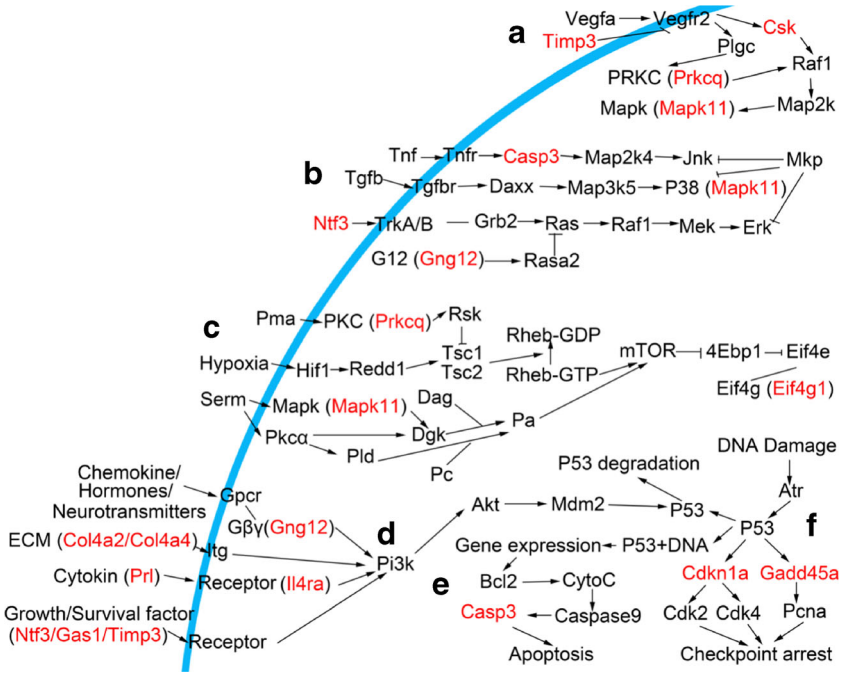

Fig. 6 The gene pathways associated with low oxygen tolerance in hybrid catfish. *A* VEGF pathway, *B* MAPK pathway, *C* mTOR pathway, *D* PI3K-AKT pathway, *E* P53-mediated apoptosis, *F* DNA damage checkpoint pathway. The genes with *red colors* are the candidate genes associated with low oxygen tolerance identified in this study

For the most part, the SNPs above the suggestively significant levels were located in a narrow genomic region. For instance, on LG29, the region spanned a distance of only 121 kb. However, the genomic region identified on LG23 spanned a relatively large distance (Fig. 4), suggesting the potential that more than one QTL are involved on LG23. The expanded look of the genomic region suggested that a group of SNPs were centered around 6.6–8.2 Mb; and a second group of SNPs were centered around 9.5–10.5 Mb, although only a few SNPs in this region were above the suggestively significant region. Only one SNP around 12-Mb region was above the suggestively significant level, significantly reduced the level of confidence of the SNP being genuinely associated with low oxygen tolerance. In this regard, perhaps the genomic region between 6.6 and 8.2 Mb on LG23 are more likely to contain one real QTL because multiple SNPs in this region are above the suggestively significant level (Table 2 and Fig. 4). It is possible that a second QTL exists in the region of 9.5–10.5 Mb. Additional mapping is required to confirm this.

In a recent study, Wang et al. (2017a) also conducted a GWAS analysis to identify QTL for hypoxia tolerance using channel catfish from six strains. One significant QTL and four suggestive QTL were identified across strains, and six significant QTL and many suggestive QTL were identified within strains (Wang et al. 2017a). These QTL do not overlap with those identified in our study. The only exception is the suggestive QTL detected around 30.7 Mb on LG4, which appeared to be the same QTL identified by Wang et al. (2017a) within Marion strain. These results, once again, suggest extremely complex genetic architecture of tolerance for low dissolved oxygen.

It has been shown in various studies that GWAS findings from one population may not be always easy to be generalized to another population (Bustamante et al. 2011; Holmes et al. 2008; Kumar et al. 2011; Wu et al. 2009). In catfish, this is also expected because multiple QTL identified across strains and within strains of channel catfish were rarely overlapped (Wang et al. 2017a), and here, the identified QTL would represent across-species QTL. These interspecific QTL should be useful for elucidation of additional genes involved in hypoxia tolerance in catfish, and for introgression breeding programs. However, the effects of these across-species QTL were larger than those of across-strain QTL, where each QTL accounted for only 4–6% of the phenotypic variance (Wang et al. 2017a).

In this study, five genes were nearest to the nine significant SNPs identified on LG2 and LG23, including *ptprf* and *pkib* on LG2, and plexin-B2 (*plxnb2*), methionine aminopeptidase 2 (*metap2*) and CUGBP Elav-like family member 2 (*celf2*) on LG23. Those two genes on LG2 were mainly involved in mTOR and PI3K-AKT pathway, which were known to play an important role in hypoxic stress (de Lorenzo et al. 2013; Dou et al. 2016; Laplante and Sabatini 2009; Karar and Maity 2011). In addition, those three genes on LG23 were reported to involve in angiogenesis under hypoxic stress (Miao et al. 1999; Zielonka et al. 2010; Towner et al. 2013; Wang et al. 1998; Zhang et al. 2000; Subramaniam et al. 2011; Ohno et al. 2012).

The results of gene pathway analysis suggested that genes identified in the associated genomic regions for low oxygen tolerance were mostly enriched in six pathways, including VEGF, mTOR, PI3K-AKT, DNA damage checkpoint, P53-mediated apoptosis, and MAPK pathways. Among these six

pathways, three pathways (MAPK, mTOR, and PI3K-AKT signaling pathway) were also found to be involved in low oxygen tolerance in channel catfish (Wang et al. 2017a). VEGF signaling pathway was known to play an important role in maintaining the vascular density and oxygen supply in tissue hypoxia by regulating angiogenesis and vascular permeability (Ohno et al. 2012; Maitland et al. 2010). The vascular endothelial growth factor (*VEGF*), one of target genes of *HIF*, is master regulator of angiogenesis (Ohno et al. 2012; Conway et al. 2001). The *timp3* gene, identified within the associated region on LG23 in our study, was reported to block the ability of *VEGF* to bind to *VEGF* receptor-2, thereby inhibiting both downstream signaling and angiogenesis (Qi et al. 2003).

In addition to activating angiogenesis, hypoxic stress also suppresses protein synthesis to conserve energy by the mTOR pathway (Laplante and Sabatini 2009). Brugarolas et al. (2004) reported that the regulated in development and DNA damage responses 1 (*REDD1*) protein was a transcriptional target of the hypoxic response and acted as a negative regulator of mTOR, thus reduced expression level of eukaryotic translation initiation factor 4E-binding protein 1 (*4EBP1*) phosphorylation and increased its binding with eukaryotic translation initiation factor 4E (*EIF4E*). This prevents cap-dependent translation initiation by preventing binding of *EIF4E* with eukaryotic translation initiation factor 4 gamma (*EIF4G*) (Richter and Sonenberg 2005; Petroulakis et al. 2006). The *eif4g1*, identified within the associated region on LG2 in our study, was known to act as a scaffold protein for the assembly of *EIF4E* and eukaryotic initiation factor 4A (*EIF4A*) to form the translation initiation machinery—*EIF4F* complex (Marcotrigiano et al. 1999). Karar and Maity (2011) found that PI3K/AKT/mTOR pathway played a key role in angiogenesis under hypoxia. It was reported that *AKT* regulated mTOR pathway to control protein synthesis and cell growth by directly phosphorylating *TSC2* of the *TSC1–TSC2* complex, thus upregulating *HIF 1α* and increasing *VEGF* expression and other angiogenic factors such as nitric oxide and angiopoietins (Potter et al. 2003).

Low oxygen conditions have been shown to induce DNA damage in fish (Liepelt et al. 1995; Mustafa et al. 2011). The classical DNA damage responses include activation of a DNA damage checkpoint, which arrests cell cycle to allow repair of damaged DNA, and apoptosis, which eliminates heavily damaged cells (Ciccia and Elledge 2010). The *cdkn1a* gene, identified within the associated region on LG23, was reported to be required in the G1/S and G2/M checkpoints in DNA damage checkpoint pathway. The *gadd45a* gene, identified within the associated region on LG2, was required in the G2/M checkpoint (Dotto 2000; Wang et al. 1999). P53 activation was reported to occur in response to hypoxia and DNA damage, which will lead to cell cycle arrest, DNA repair, or apoptosis (Lakin and Jackson 1999). Caspases are crucial mediators of apoptosis in P53-mediated apoptosis pathway, and *casp3* was included within the associated region on LG29 in this study. It was reported that P53 could functionally interact with MAPK pathways in response to stressful stimuli (Wu 2004). The *mapk11* gene was included within the associated region on LG23 in this study. *MAPK11* is known to encode *P38β* MAPK, which is a member of the P38 MAP kinase family (She et al. 2001). In principle, many of these genes are downstream effector genes of the HIF pathway, suggesting that genomic variations of any effector genes of HIF pathway can have impact on tolerance to low dissolved oxygen. It is possible that performance traits that involve gene pathways with multiple effector genes, such as those of the HIF-1 alpha, may have very complex genetic architecture for the control of the traits, thereby having multiple QTL resulting from mutations accumulated in different strains, populations, and families.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The experiment was undertaken with the approval of 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.

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