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ORIGINAL ARTICLE

GWAS analysis using interspecific backcross progenies reveals superior blue catfish alleles responsible for strong resistance against enteric septicemia of catfish

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Abstract

Infectious diseases pose significant threats to the catfish industry. Enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* is the most devastating disease for catfish aquaculture, causing huge economic losses annually. Channel catfish and blue catfish exhibit great contrast in resistance against ESC, with channel catfish being highly susceptible and blue catfish being highly resistant. As such, the interspecific backcross progenies provide an ideal system for the identification of quantitative trait locus (QTL). We previously reported one significant QTL on linkage group (LG) 1 using the third-generation backcrosses, but the number of founders used to make the second- and third-generation backcross progenies was very small. Although the third-generation backcross progenies provided a greater power for fine mapping than the first-generation backcrosses, some major QTL for disease resistance may have been missing due to the small numbers of founders used to produce the higher generation backcrosses. In this study, we performed a genome-wide association study using first-generation backcrosses with the catfish 690 K SNP arrays to identify additional ESC disease resistance QTL, especially those at the species level. Two genomic regions on LG1 and LG23 were determined to be significantly associated with ESC resistance as revealed by a mixed linear model and family-based association test. Examination of the resistance alleles indicated their origin from blue catfish, indicating that at least two major disease resistance loci exist among blue catfish populations. Upon further validation, markers linked with major ESC disease resistance QTL should be useful for marker-assisted introgression, allowing development of highly ESC resistant breeds of catfish.

Keywords Disease resistance · Fish · GWAS · ESC · QTL · SNP

Introduction

Disease resistance describes the overall capacity and efficiency of the host to cope with infections, which is composed of the innate and adaptive immunities. The innate immune system provides physical and chemical barriers, senses pathogens through various receptors to activate microbial defense and stimulate the adaptive immune response (Medzhitov 2007). The adaptive immune system comprises of T and B lymphocytes and their mediated processes. T lymphocytes, including T-helper (Th) cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells), recognize antigenic peptides bound to major histocompatibility complex (MHC) via antigen-specific extracellular receptors. Cytotoxic T cells induce apoptosis of infected cells or kill target cells (Andersen et al. 2006), while Th cells differentiate into effector cells (Th1, Th2, and Th17) upon

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antigen stimulation, producing distinct sets of cytokines for antigen-specific activation of innate host-defense cells such as macrophage and granulocytes as well as for the activation of B cells to generate antigen-specific antibodies (Medzhitov 2007; Annunziato et al. 2009). In addition, B cells can recognize antigens by directly binding to them. The various modules of innate immunity cooperate with cell-mediated and humoral immune responses of adaptive immunity, providing effective protection against infection for vertebrates.

Immunogenetic analysis in fish species has been rare. The practical approaches have focused on the identification of disease resistance-associated DNA markers for marker-assisted selection. The interest for the identification of genetic markers and genomic regions for resistance against diseases in aquaculture species is increasing because rapid progress can be made in this area to reduce disease problems and enhance aquaculture production without deep analysis of the immunological and molecular mechanisms. Among various approaches, quantitative trait locus (QTL) mapping and genome-wide association study (GWAS) have been the most useful approaches for disease resistance studies with aquaculture species.

Disease resistance QTL have been identified in various aquaculture species (For a recent review, see Abdelrahman et al. 2017). For example, QTL have been identified in rainbow trout (*Oncorhynchus mykiss*) for resistance against infectious pancreatic necrosis (Ozaki et al. 2007), infectious hamatopoietic necrosis (Campbell et al. 2014), whirling disease (Baerwald et al. 2011), and bacterial cold water disease (Liu et al. 2015; Palti et al. 2015). In Asian seabass (*Lates calcarifer*), QTL have been detected for viral nervous necrosis disease resistance (Liu et al. 2016a), and for iridovirus resistance (Wang et al. 2017a). For Japanese flounder (*Paralichthys olivaceus*), a single major genetic locus was found to explain 50% of the total variation of disease resistance to lymphocystis disease (Fuji et al. 2006), and the linked DNA marker was successfully used for marker-assisted selection (Fuji et al. 2007). In Atlantic salmon (*Salmo salar*), QTL associated with resistance to Salmon Rickettsial Syndrome were identified (Correa et al. 2015). Moreover, a major QTL in Atlantic salmon that explained most of the genetic variance for resistance to infectious pancreatic necrosis has been commercially utilized by breeding companies (Houston et al. 2008; Moen et al. 2009).

Catfish is the primary aquaculture species in the United States. Channel catfish (*Ictalurus punctatus*) used to be the major cultured species. In recent years, the interspecific hybrid [channel catfish female × blue catfish (*Ictalurus furcatus*) male] is increasingly used by the catfish industry because of its superiority for disease resistance, growth rate, fillet yield, and harvestability (Dunham et al. 2008; Lucas and Southgate 2012). The interspecific hybrids not only promote the aquaculture production but also serve as an excellent model for the analysis of QTL associated with traits such as disease resistance. With enteric septicemia of catfish (ESC), blue catfish is very resistant while channel catfish is highly susceptible (Wolters and Johnson 1994; Wolters et al. 1996). Therefore, the interspecific hybrid provides an ideal system for genetic analysis of ESC disease resistance.

ESC is the most prevalent and devastating disease in catfish farming (Hawke 1979; Hawke et al. 1981; Plumb and Hanson 2011), leading to economic loss of $40–60 million in the US catfish industry annually (Shoemaker et al. 2009). In addition to channel catfish, cases have been reported worldwide in white catfish (*Ameiurus catus*), brown bullhead catfish (*Ameiurus nebulosus*), yellow catfish (*Pylodictis olivaris*), striped catfish (*Pangasianodon hypophthalmus*), walking catfish (*Clarias batrachus*), and species of fish other than catfish (Hawke et al. 1981; Kasornchandra et al. 1987; Ye et al. 2009; Dong et al. 2015). ESC occurs most often within a specific temperature range between 20 and 28 °C (Hawke et al. 1998). This disease causes enteritis and septicemia, characterized by ulcerative skin lesions, petechial hemorrhages around the mouth and abdomen, and pimples or holes between eyes (Areechon and Plumb 1983; Hawke et al. 1998). ESC is caused by *Edwardsiella ictaluri*, a Gram-negative, rod-shaped bacterium of the family Enterobacteriacae. *E. ictaluri* can enter catfish through the intestinal tract, the nares, the gills, and the skin (Hawke et al. 1998; Menanteau-Ledouble et al. 2011). *E. ictaluri* is internalized in leukocytes of channel catfish, particularly in macrophages (Miyazaki and Plumb 1985; Shotts et al. 1986; Baldwin and Newton 1993). The initial detection of *E. ictaluri* in the internal organs can occur as early as 15 min post infection by gastric intubation (Baldwin and Newton 1993). Bacteria are released in the blood, and colonize other organs within 48 h (Menanteau-Ledouble 2009). The earliest lesions were observed microscopically at 2 days after an immersion challenge of channel catfish (Newton et al. 1989). Although the disease progression and pathology of ESC are well characterized, little is known about the molecular mechanism underlying the pathogenesis of *E. ictaluri* and associated disease resistance of the host.

Genomic resources of catfish have been made available to facilitate GWAS in catfish, including a large number of SNPs (Liu et al. 2011; Sun et al. 2014), the high-density SNP arrays (Liu et al. 2014; Zeng et al. 2017), and the reference genome sequence (Liu et al. 2016b). Using the 250 K SNP array, several GWAS analyses have been conducted with performance and production traits of catfish, including disease resistance against columnaris (Geng et al. 2015), hypoxia tolerance (Wang et al. 2017b; Zhong et al. 2017), heat tolerance (Jin et al. 2017), albinism (Li et al. 2017a), body weight (Li et al. 2017b), body conformation (Geng et al. 2017a) and head size (Geng et al. 2016). With ESC

disease resistance, GWAS analysis was conducted using the third-generation backcrosses (Zhou et al. 2017). The use of higher generation backcrosses could have provided greater powers for the detection of QTL in a smaller genomic region because of accumulated chromosomal recombinations. However, some significant QTL may have been undetected if such QTL were not included in the few individuals of the first-generation backcrosses used as founders of the higher generation backcrosses.

The QTL by definition must be there in the first generation. However, it would go undetected if the appropriate allelic variation was missing. In this study, we utilized the first-generation backcrosses for determining the genomic regions associated with ESC disease resistance, to ensure detection of species-level QTL, especially those offered from blue catfish.

Materials and methods

Experimental fish, bacterial challenge and sample collection

The bacterial challenge experiment was performed with 1-year-old catfish (average 14.8 cm) produced by crossing male F1 hybrid catfish (channel catfish female × blue catfish male) with female channel catfish (Fig. 1). The female channel catfish were Marion strain (Dunham and Smitherman 1984), including the maternal parent of the F1 hybrid catfish. Four backcross families were used, and the grandparents among families were different. A total of 1200 experiential fish were randomly selected (300 fish per family) at the Auburn University Fish Genetics Facility and were distributed in four tanks (300 L water) by family. A control group containing 200 fish was reared in another tank. The average temperature was maintained at 28 °C, and oxygen level was controlled at optimal level by aeration.

The infestation with *E. ictaluri* was carried out after acclimation. The bacteria challenge procedure was conducted as previously described (Wang et al. 2013). The bacteria were

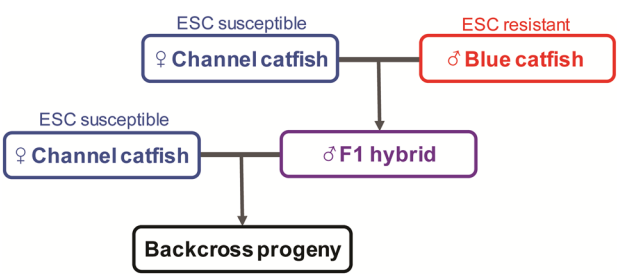

Fig. 1 Backcross strategy for the development of the experimental population

cultured from a single colony, re-isolated from a symptomatic fish, and biochemically confirmed. A single colony was cultured in brain heart infusion (BHI) medium and incubated at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating tenfold serial dilution onto BHI agar plates. The infestation was conducted by immersion exposure for 2 h at a final bacterial concentration of 2×10^7 CFU/mL, whereas control fish were treated with an equal volume of sterile BHI medium. Moribund catfish were continuously sampled by taking blood, and the sampling time was recorded. Fish for further genotyping were selected from the extremes of the disease resistance distribution for each family based on the selective genotyping method (Darvasi and Soller 1992).

DNA isolation and genotyping

DNA was isolated using standard protocols. Briefly, the blood samples in cell lysis solution were incubated at 55 °C overnight. Protease K and protein precipitation solution were used to remove proteins. Extracted DNA was precipitated with isopropanol and collected by brief centrifugation, washed twice with 70% ethanol, air-dried, and rehydrated in TE buffer (pH 8.0). DNA was quantified by spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) and checked by 1% agarose gel electrophoresis stained with ethidium bromide for integrity. DNA was diluted to 50 ng/μL with a final volume of 10 μL. Genotyping with the catfish 690 K SNP array was performed at GeneSeek (Lincoln, Nebraska, USA). Genotype callings were generated using the Axiom Analysis Suite software.

Statistical analysis

To identify association between SNPs and resistance to *E. ictaluri*, the single SNP test was performed using the SVS software package (SNP & Variation Suite, Version 8.3) and PLINK (Version 1.9) (Purcell et al. 2007). Quality control was performed for samples and markers. Samples with genotype missingness larger than 5% were removed. The markers with a minor allele frequency (MAF) lower than 0.05 or a call rate lower than 95% were excluded from the analysis. Linkage disequilibrium (LD) pruning was then conducted to achieve a set of independent SNPs with a window size of 50 SNPs, window increment of 5, and r^2 threshold of 0.5. With the independent SNP markers, identity-by-state (IBS) distances were estimated to reflect relatedness between pairs of samples, and the population structure was analyzed by principal component analysis (PCA).

Efficient Mixed-Model Association eXpedited (EMMAX) analyses using all qualified SNPs were conducted with the first three principal components and body weight as covariates (Kang et al. 2010). The model is listed as follows:

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

where Y is the vector of phenotype; β is the coefficient vector of fixed effects including first three principal components and fish body weight; u is the vector of the random effect, $\mathrm{Var}(u) = G\sigma 2$ g, where $\sigma 2$ g is the additive genetic variance and G is the genomic kinship matrix using the IBS; e is the vector of random residuals; X is the matrix of fixed effects and Z is the matrix of random additive genetic effects.

The Manhattan plot of the $-\log_{10}$ (P value) was generated using the SVS software, with the genetic marker map according to channel catfish genome sequence (Version Coco1.2, Liu et al. 2016a, b). The threshold P value for genome-wide significance was calculated based on Bonferroni correction with estimated number of independent markers and LD blocks.

Family-based association test for quantitative traits (QFAM) was conducted using PLINK (Purcell et al. 2007). QFAM partitions the genotypes into between- and within-family components, and the within-family component is free of sample structure (Fulker et al. 1999; Abecasis et al. 2000). The QFAM-Within procedure in PLINK performed a simple linear regression of phenotype on the within-family component of genotype, and then used permutations to correct for family structure. The model is

$$\hat{y}_{ij} = \mu + \beta_b b_i + \beta_w w_{ij}$$

where y_{ij} denotes the phenotype of individual j in family i; μ is the population mean; β_b is the vector of between-family effect and β_w is the vector of within-family effect; $b_i = (\Sigma g_{ij})/n_i$ if parental genotypes are unknown (g_{ij}: genotype of the jth offspring in the ith family), and $b_i = (g_{i\mathrm{F}} + g_{i\mathrm{M}})/2$ if parental genotypes are available ($g_{i\mathrm{F}}$: genotype of father, $g_{i\mathrm{M}}$: genotype of mother); $w_{ij} = g_{ij} - b_i$.

Sequence analysis

Genes within ± 1 Mb of associated regions were predicted from catfish genome sequence (Liu et al. 2016b) using FGENESH (Solovyev et al. 2006) and annotated by BLAST against the NCBI-nr database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). Synteny analyses and catfish genome information from the NCBI were utilized to provide supporting evidence for the proper annotation.

Results

Experimental fish and sample structure

A total of 1200 catfish from four families were challenged with *E. ictaluri*, and 288 fish with extreme phenotype were chosen for genotyping. The mortality rate after infection is shown in Fig. 2. The information of catfish samples utilized in this study is summarized in Table 1. PCA analysis was conducted using eigenvalues as coordinates to visualize the sample structure. As shown in Fig. 3, each family was grouped into a separate cluster and the four families were distantly related.

Table 1 Catfish samples used in this study

Family ID	Dam	Sire	Sample number	Susceptible sample number	Resistant sample number
1	Channel 1	Hybrid 1	71	36	35
2	Channel 2	Hybrid 2	70	34	36
3	Channel 3	Hybrid 3	70	36	34
4	Channel 4	Hybrid 4	77	36	41

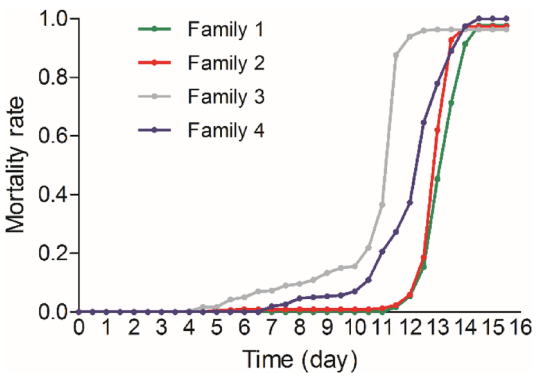

Fig. 2 Mortality rate of the first-generation backcrosses after *Edwardsiella ictaluri* infection

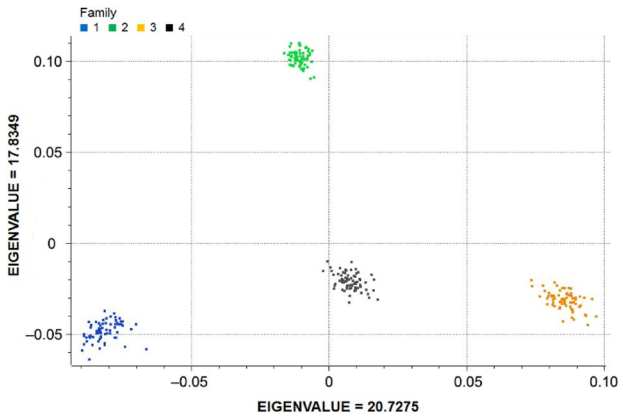

Fig. 3 Sample structure identified by principal component analysis with two major principal components

QTL for ESC resistance

A total of 407,196 SNPs were kept after filtering out SNP markers with genotyping errors, MAF < 5%, or call rate < 95%. The number of independent SNPs and LD blocks was 15,061 after LD pruning, and the genome-wide threshold for statistical significance was calculated to be 3.32e−6 (0.05/15,061) with $-\log_{10}(P \text{ value}) = 5.48$. The threshold of $-\log_{10}(P \text{ value})$ for suggestive association was arbitrarily set as 5 (Geng et al. 2016).

The Manhattan plots generated from EMMAX and QFAM are shown in Fig. 4a, b, respectively. In general, the association results of EMMAX and QFAM were positively correlated. EMMAX showed one genome-wide significant region on linkage group (LG) 23 and two suggestively associated regions on LG1 and LG14, while QFAM revealed two genome-wide significant regions on LG23 and LG1 as well as one suggestive region on LG1. Generally, QFAM is more robust in correcting family structure when using family-based samples than EMMAX (Geng et al. 2017b). In the following sections, we mainly describe the identified regions according to the results generated by QFAM.

SNPs associated with ESC resistance are listed in Table S1. Forty-five genome-wide significant SNPs were found on LG1 from 33,233,001 to 33,712,554, spanning approximately 479.6 Kb. The most significant SNP (AX-157732970) on LG1 reached the genome-wide significance level with $-\log_{10}(P \text{ value}) = 6.05$. Similarly, three significantly associated SNPs were located on LG23 in genomic region from 7,793,018 to 8,136,119, spanning approximately 343.1 Kb. The $-\log_{10}(P \text{ value})$ of the most significant SNP (AX-157695826) was 6.25. In addition, 20 SNPs were identified within the suggestive QTL on LG1 from 5,052,936 to 9,817,492. Based on the phenotype and the SNPs placed on the catfish 690 K SNP array (Zeng et al. 2017), the resistance allele of associated SNPs (Table S1) as well as their origins could be determined. Four significant SNPs and four suggestively associated SNPs are all interspecific,

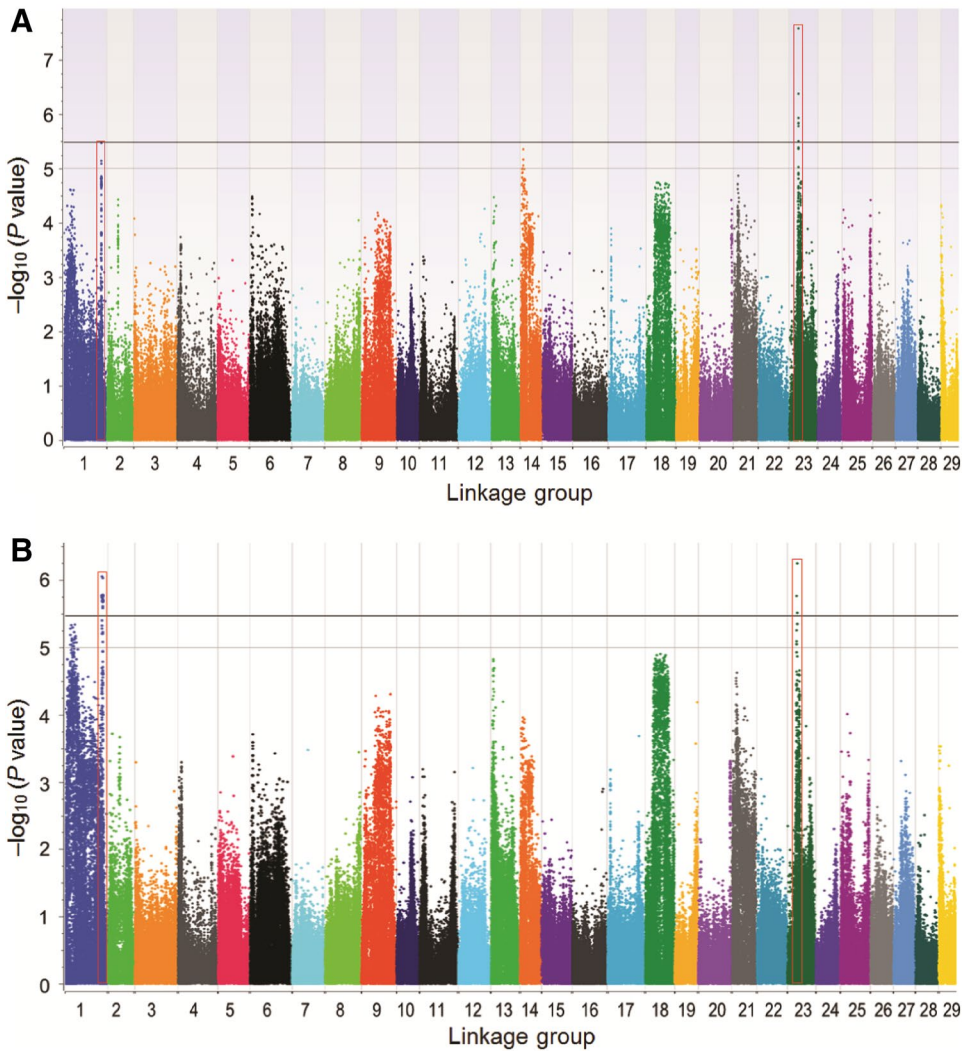

Fig. 4 Manhattan plot of genome-wide association analysis for ESC disease resistance generated from EMMAX (**a**) and QFAM (**b**). The black solid line indicates the threshold *P* value for genome-wide significance. The grey solid line indicates the threshold *P* value for significance of "suggestive association". The red box represents the shared QTL using two methods. (Color figure online)

which means they are SNPs from interspecific origin and not intraspecific variants. For these interspecific SNPs, all resistance alleles originated from blue catfish. EMMAX was utilized to investigate the contribution of significantly associated QTL to the phenotypic variance. The most significant SNP was used to represent the corresponding significant region (Geng et al. 2015), with AX-157732970 on LG1 and AX-157695826 on LG23 accounting for 6.8 and 10.4% of the phenotypic variance, respectively. These two loci alone would indicate a minimum heritability of 0.17 in a first-generation backcross population. According to the phenotypic variance (3827.5) calculated in SVS, fish with the "good" allele of AX-157732970 and AX-157695826 will live 16.1 and 20.0 h longer, respectively.

Genes within the associated QTL for ESC resistance

To explore the potential genes involved in ESC resistance, the ± 1 Mb regions around associated SNPs were examined. Synteny analyses were conducted to compare the extend regions around the significant SNPs. The conserved synteny was observed between catfish and zebrafish, as shown in Figs. 5 and 6. A set of 43 genes were identified within the significantly associated region on LG1 (Table S2). Of these genes, seven genes were found to have known functions in immunity (Table 2), including NCK adaptor protein 1 (*nck1*), angiotensin II receptor type 1 (*agtr1*), transient receptor potential cation channel subfamily C member 1 (*trpc1*), abl interactor 1 (*abi1*), Rap1-GTP-interacting adaptor molecule (*apbb1ip*), ARP3 actin-related protein 3 homolog B (*actr3b*), and vav guanine nucleotide exchange factor 3 (*vav3*). Within the significant QTL on LG23, 45 genes were detected (Table S3) and three genes had immune-related functions (Table 2), including mannose receptor C-type 1 like (*mrc1l*), protein kinase C theta (*prkcq*), and GATA binding protein 3 (*gata3*). Moreover, 49 genes were immune-related, of a total of 271 genes identified in the suggestive region on LG1 (Table S4).

Correlation of the SNPs with ESC resistance

Conditioned analyses were performed to determine whether the associations detected on a linkage group were independent (Nishimura et al. 2012). The lead SNP on each associated QTL was included as a covariate in the mix linear model. After conditioning, associations of the surrounding SNP on the same linkage group disappeared, while SNPs on other linkage groups generally remained the same, indicating there was no independently associated SNP marker within the same linkage group.

Discussion

Identification of polymorphisms affecting ESC disease resistance could facilitate effective breeding through marker-assisted selection and introgression, and thereby reduce losses for the catfish aquaculture industry. Here, GWAS was conducted using the channel catfish × blue catfish backcross progenies genotyped with the newly designed catfish 690 K

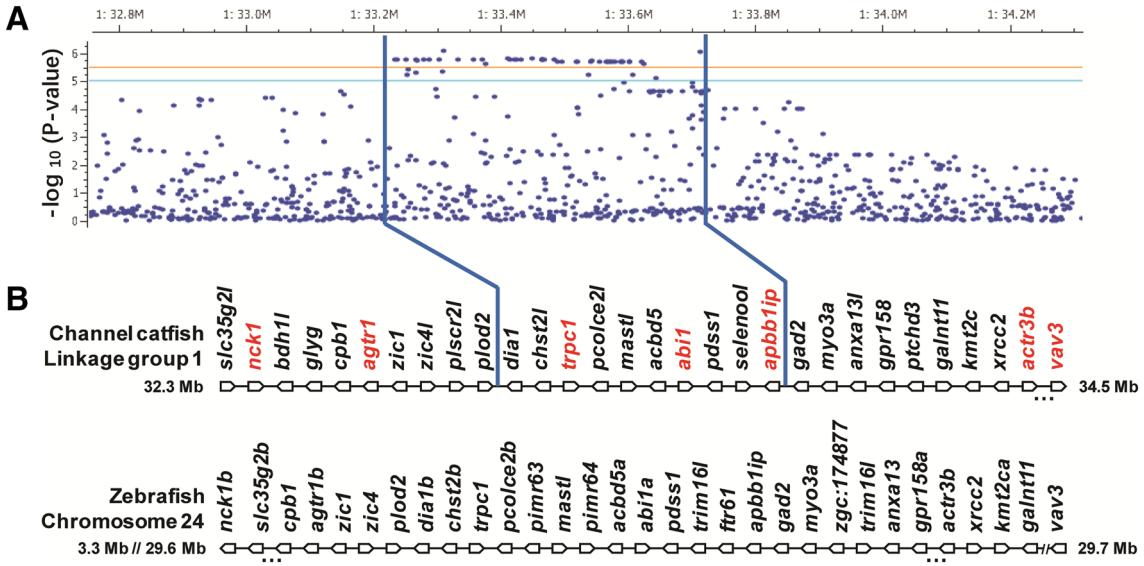

Fig. 5 Genes within the significant region associated with ESC resistance on LG1. **a** Regional Manhattan plot for the QTL on LG1. The yellow horizontal line indicates the threshold *P* value for genome-wide significance. The blue horizontal line indicates the threshold *P* value for significance of "suggestive association". **b** Synteny analysis between catfish and zebrafish. Immune-related genes in channel catfish are highlighted in red. (Color figure online)

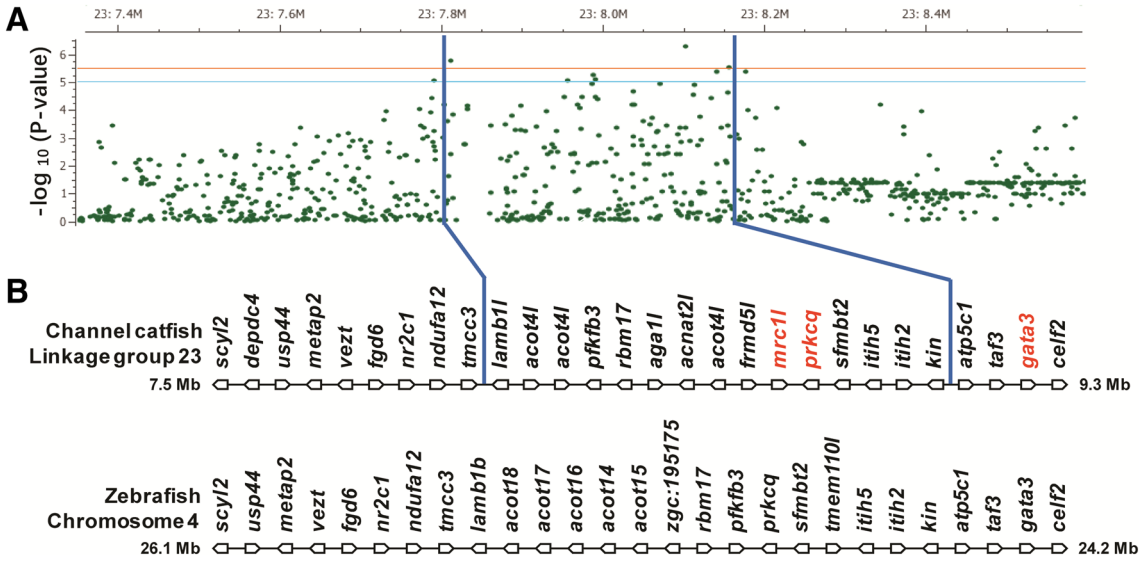

Fig. 6 Genes within the significant region associated with ESC resistance on LG23. **a** Regional Manhattan plot for the QTL on LG23. The yellow horizontal line indicates the threshold *P* value for genome-wide significance. The blue horizontal line indicates the threshold *P* value for significance of "suggestive association". **b** Synteny analysis between catfish and zebrafish. Immune-related genes in channel catfish are highlighted in red. (Color figure online)

Table 2 Immune-related genes within the genome-wide significant QTL on linkage group (LG) 1 and 23

LG	Gene	Location (bp)	Function
1	*nck1*	32,352,283–32,413,183	Actin filament organization Phagocytosis T-cell activation B-cell receptor signaling
	agtr1	32,480,471–32,494,698	regulation of inflammatory response
	trpc1	33,472,942–33,504,891	calcium ion transport B-cell receptor signaling
	abi1	33,560,300–33,609,297	Actin polymerization or depolymerization Phagocytosis
	apbb1ip	33,632,904–33,677,852	T-cell activation
	actr3b	34,277,661–34,297,129	Actin nucleation Phagocytosis
	vav3	34,542,282–34,629,025	Phagocytosis B-cell receptor signaling
23	*mrc1l*	7,943,812–7,946,175	Cellular response to lipopolysaccharide Endocytosis T-cell activation
	prkcq	7,954,997–7,964,953	Inflammatory response T-cell activation
	gata3	8,239,408–8,259,340	Inflammatory response T-cell differentiation humoral immune response

Gene function was collected form Uniprot (http://www.uniprot.org) and REACTOME (http://www.reactome.org)

SNP arrays. We successfully identified QTL associated with ESC resistance on LG1 and LG23. Channel catfish and blue catfish exhibit great contrast in the resistance to ESC, with blue catfish being highly resistant, almost never becoming infected naturally and having low mortality when challenged artificially (Bilodeau-Bourgeois et al. 2008; Hanson 2006; Wolters and Johnson 1994; Wolters et al. 1996). In the backcross progenies, both genes and the disease resistance traits are segregating, allowing mapping of disease resistance to chromosomal locations. Although the F2 intercrosses of

hybrids between the two species with phenotypic divergence are appropriate populations for mapping QTL (Cnaani et al. 2003; Poompuang and Hallerman 1997), it is extremely difficult to produce the interspecific F2 hybrids due to the very low hatching rate (Dunham and Argue 2000). Therefore, the backcross progenies provide a best available alternative for the study of interspecific ESC resistance/susceptibility-associated QTL.

Two methods, EMMAX and QFAM, were utilized for family-based samples in the present study. Both are effective for correcting population stratification which can lead to biased or spurious results. The statistical approaches of EMMAX and QFAM to control the population stratification are different. EMMAX uses a pairwise relatedness matrix as random effect to correct for sample structures including hidden relatedness and population stratification (Kang et al. 2010). QFAM partitions the genotypes into between- and within- family components (Fulker et al. 1999; Abecasis et al. 2000). The within-family analysis is robust to population stratification, which assesses transmission of alleles within a family, but without making use of allelic association observed across families. When using family-based samples, QFAM was found to be more robust in correcting family structure than EMMAX (Geng et al. 2017b). Family-based samples were utilized in the present study and the suggestive QTL on LG14 found by EMMAX but not by QFAM was suggested to be false positive due to family structure. Furthermore, the suggestive region on LG1 was not found by EMMAX but identified by QFAM in this study. Geng et al. (2016) reported that QFAM possessed more power compared with EMMAX in a family-based study. Recent work has shown that inclusion of candidate markers in the relatedness matrix could lead to decreased power due to double-fitting of candidate markers in the mix linear model (Yang et al. 2014). Therefore, QFAM results were chosen for further analysis.

Examination of the associated SNPs revealed that the superior disease resistance alleles were from blue catfish. Four suggestively associated SNPs and four significant SNPs including the most significant SNP (AX-157732970) on LG1 were all interspecific. All of the resistance alleles were found to be derived from blue catfish, explaining the very strong resistance of blue catfish against the ESC disease. For LG23, all associated SNPs were channel catfish-specific, which means the single nucleotide variation on these loci occurred only in channel catfish. Further investigation showed that all the SNPs that could be mapped to blue catfish genome sequence are homologous with the two resistance alleles. The results implied that certain blue catfish alleles were associated with increased ESC resistance.

Within the two significantly associated regions on LG1 and LG23 (indicated by red boxes in Fig. 4), an examination of genes and related pathways provided insights into the molecular mechanisms underlying ESC resistance. The most studied, closest relative of catfish, zebrafish, has 696 genes involved in the immune system process in PANTHER database (http://www.pantherdb.org). The channel catfish has 27,143 genes based on NCBI annotation. The approximate proportion of immune genes in the genome is 0.026. The proportion of immune genes within our QTL region for LG1 and LG23 is 0.163 (7/43) and 0.067 (3/45), which in the case of LG1 is more than expected (Chi-square test, $P < 0.001$) compared to the rest of the genome. The immune-related genes observed in the significant QTL were found to be mainly involved in phagocytosis (Fig. 7) and T-cell activation (Fig. 8). Phagocytosis is a principal component of the innate immunity in which phagocytes, including macrophages, neutrophils and dendritic cells, internalize targets in an actin-dependent manner (Botelho and Grinstein 2011). *E. ictaluri* is internalized in catfish phagocytes after infection, especially in macrophages (Miyazaki and Plumb 1985; Shotts et al. 1986; Baldwin and Newton 1993), suggesting that phagocytic activities play crucial roles in immunity for combating ESC. T cells have a central role in adaptive immunity by regulating immune responses of various phagocytes and B cells through cytokine secretion or by directly

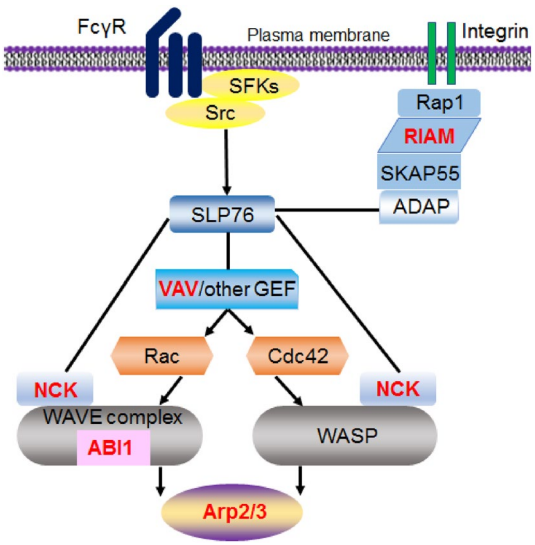

Fig. 7 Signal transduction pathway involved in Fcγ receptor-mediated phagocytosis. Corresponding proteins of immune-related genes identified in this study are highlighted in red. SFKs, Src family tyrosine kinase; Src, proto-oncogene tyrosine-protein kinase Src; SLP76 Src homology 2 domain-containing leukocyte protein of 76 kilodaltons, VAV, VAV guanine nucleotide exchange factor, Rac, small GTPase Rac; Cdc42, small GTPase Cdc42; NCK, NCK adaptor protein; WASP, Wiskott–Aldrich syndrome protein; WAVE, WASP family Verprolin-homologous protein; ABI1, abl interactor 1; Arp2/3, the actin nucleation complex Arp2/3; ADAP, Adhesion and degranulation-promoting adapter protein; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; RIAM, Rap1-GTP-interacting adaptor molecule; Rap1, small GTPase Rap1. (Color figure online)

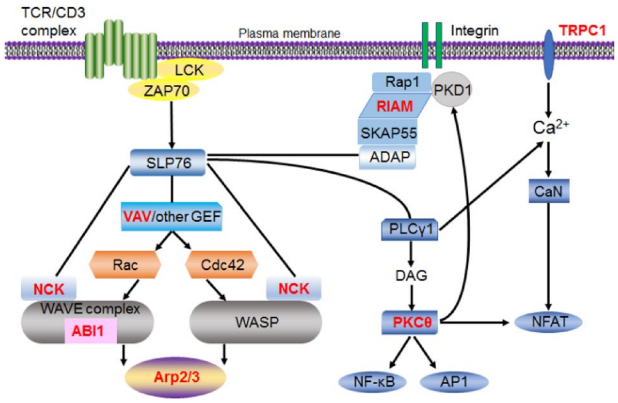

Fig. 8 Signal transduction pathway leading to T-cell activation. Corresponding proteins of immune-related genes identified in this study are highlighted in red. LCK, LCK proto-oncogene; Src family tyrosine kinase, ZAP70, zeta chain of T-cell receptor associated protein kinase 70; SLP76, Src homology 2 domain-containing leukocyte protein of 76 kilodaltons; VAV, VAV guanine nucleotide exchange factor; Rac, small GTPase Rac; Cdc42, small GTPase Cdc42; NCK, NCK adaptor protein; WASP, Wiskott–Aldrich syndrome protein; WAVE, WASP family Verprolin-homologous protein; ABI1, abl interactor 1; Arp2/3, the actin nucleation complex Arp2/3; ADAP, Adhesion and degranulation-promoting adapter protein; SKAP55, Src kinase-associated phosphoprotein of 55 kDa; RIAM, Rap1-GTP-interacting adaptor molecule; Rap1, small GTPase Rap1; PLCγ1 ,phospholipase C-γ1; DAG, diacylglycerol; PKCθ, protein kinase C theta; PKD1, polycystin 1; transient receptor potential channel interacting, TRPC1, transient receptor potential cation channel subfamily C member 1; CaN, calcineurin; NF-κB, nuclear factor-kappa B; AP-1, activator protein-1; NFAT, nuclear factor of activated T cells. (Color figure online)

destroying antigen-bearing cells (Medzhitov 2007). Russo et al. (2009) demonstrated that macrophages from vaccinated fish were more efficient in rapid clearance of infection upon re-exposure to virulent *E. ictaluri*, reflecting that memory lymphocytes were involved and lymphocytes in adaptive immunity were important in enhancing macrophage activities during immune responses.

One of the similar process of both phagocytosis and T-cell activation is cytoskeleton rearrangement. In phagocytes, local polymerization of actin filaments supports the protrusion of pseudopodia that facilitates the engulfment of pathogens or particles. In T cells, cytoskeleton reorganization is crucial for the formation of immunological synapse, which is crucial for cell adhesion and T-cell activation. Four genes within the significantly associated region on LG1, including *nck1, vav3, abi1*, and *actr3b*, were involved in actin cytoskeleton reorganization. Nck proteins are adapter proteins which play pivotal role in the phagocytic process (Coppolino et al. 2001) and T-cell receptor (TCR) induced actin cytoskeleton reorganization during T-cell activation (Lettau et al. 2009). Upon the phosphorylation of tyrosine kinases, SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) recruits Nck. Nck then promotes the recruitment of the multidomain adapter protein Wiskott–Aldrich syndrome protein (WASP) and WASP family Verprolin-homologous protein (WAVE), whose activations facilitate the actin-related protein 2/3 (Arp2/3) complex to drive actin filament formation. SLP-76 also recruit the Vav proteins, promoting the GTP-loading of Rho GTPases to facilitate the activation of WASP family proteins. The Vav family proteins (Vav1, Vav2, and Vav3) are guanine nucleotide exchange factors (GEFs) for Rho family GTPases (Hornstein et al. 2004; Tybulewicz 2005), playing crucial roles in the regulation of actin dynamics during phagocytosis (Patel et al. 2002; Hall et al. 2006) and T-cell activation (Villalba et al. 2000, 2002). Abl interactor 1 (Abi1) is crucial in actin cytoskeleton dynamics due to its participation in the WAVE complex, which stimulates Arp2/3 complex-dependent actin assembly (Innocenti et al. 2004; Kheir et al. 2005). Arp2/3 complex plays a major role in the regulation of the actin cytoskeleton (Machesky and Gould 1999), essential for actin polymerization in the phagocytic cup and subsequent particle engulfment in both FcγR- and CR3-mediated phagocytosis (May et al. 2000). Moreover, the Arp2/3 complex was involved in actin cytoskeleton remodeling during T-cell activation (Krause et al. 2000). Actin-Related Proteins ARP3 (*actr3*) is one subunit of the Arp2/3 complex. In the present study, the homology of *actr3, actr3b*, was in the significantly associated region on LG1 that associated with ESC disease resistance.

Another shared process during T-cell activation and phagocytosis is the integrin activation, where Rap1-GTP-interacting adaptor molecule (RIAM, *apbb1ip*) is implicated. Integrins are transmembrane heterodimers responsible for mediating cell/cell or cell/matrix adhesions (Hynes 2002), which are crucial for development, tissue maintenance and repair, immune response and hemostasis (Hynes 2002; Harburger and Calderwood 2009). Binding affinity and avidity of integrins for their ligands facilitate T-cell activation by providing stable contact with APCs and extracellular proteins (Burbach et al. 2007). A key integrin regulator is the small GTPase Ras-proximity-1 (Rap1), which enhances T-cell activation by mediating TCR-induced adhesion to intercellular adhesion molecule (ICAM) (Katagiri et al. 2000; Sebzda et al. 2002). In addition, FcγR-mediated responses have been shown to require the presence of β2 integrins, include adhesion to immune complexes and enhanced Fc-dependent phagocytosis (Ortiz-Stern and Rosales 2003).

The calcium ion (Ca^{2+}) is the main common second messenger involved in signaling transduction following immunoreceptor activation. For the T-cell activation, TCR-induced increase in intracellular Ca^{2+} level is crucial for many cellular functions, including the translocation of NFAT (nuclear factor of activated T cells) to the nucleus through calcineurin (CaN) activation (Macian 2005). TRPC1, a

membrane-spanning subunit of cation channel, was involved in store-operated Ca^{2+} entry (SOCE) by interacting with calcium release-activated calcium channel protein 1 (Orai1) and stromal interaction molecule 1 (STIM1) (Ong et al. 2007), both of which were up-regulated during T-cell activation (Lioudyno et al. 2008). Furthermore, TRPC1 was shown to be expressed in a human T-cell line (HPB-ALL) and contributed to cannabinoid-induced Ca^{2+} influx in these cells independently of intracellular Ca^{2+} store depletion (Rao and Kaminski 2006).

Pattern recognition receptors (PRRs) are well known for their significant roles in immunity. Mannose receptor C-type 1 (MRC1) is a C-type lectin primarily present on macrophages and dendritic cells. MRC1 played significant roles in both innate and adaptive immune responses against various microorganisms (Taylor et al. 2005), and it was implicated in pathogen recognition, phagocytosis, cytokine production, antigen processing and presentation, cell adhesion and migration (Apostolopoulos and McKenzie 2001; East and Isacke 2002; Taylor et al. 2005; Gazi and Martinez-Pomares 2009). Due to the crucial roles in immune responses, much work on MRC1 has been done in human and mice, with a few studies in fish as well. In grass carp (*Ctenopharyngodon idella*), MRC1 was significantly up-regulated after *Aeromonas hydrophila* infection in liver, spleen, head kidney and intestine (Wang et al. 2014). Similarly, the expression of MRC1 was dramatically induced in the spleen of tilapia (*Oreochromis niloticus*) at all three studied timepoints (5 h, 50 h, and 7 days) following *Streptococcus iniae* infection (Zhu et al. 2015). In this study, the gene *mrc1 like* was within the QTL on LG23, and its expression in catfish was significantly up-regulated at 3 and 72 h after *E. ictaluri* infection in a previous study (Li et al. 2012).

The gene *prkcq* was located within the associated region on LG23, coding for protein kinase C theta (PKCθ). Predominantly expressed in T lymphocytes, PKCθ plays essential roles for T-cell activation, proliferation, differentiation, survival, and cytokine production (Anderson et al. 2006; Isakov and Altman 2002; Barouch-Bentov et al. 2005; Hayashi and Altman 2007). PKCθ is the only serine/threonine protein kinase C (PKC) recruited selectively to the center of the immunological synapse following stimulation with APCs, required for T-cell activation and downstream signaling (Monks et al. 1997; Bi et al. 2001). T lymphocytes in PKCθ-deficient mice displayed impaired activation of transcription factors including NF-κB (nuclear factor-kappa B), AP-1 (activator protein-1) and NFAT (Pfeifhofer et al. 2003; Sun et al. 2000). PKCθ was found to be significantly up-regulated in intestine of *Pelodiscus sinensis* after intragastric challenge with lipopolysaccharide (LPS) (Xu et al. 2016).

One interesting finding of our study was that the functionally related genes in immunity were in relatively close proximity on the chromosome. Of the seven immune-related genes within the significantly associated region on LG1, six genes were implicated in phagocytosis (*nck1, vav3, abi1, actr3b, apbb1ip*, and *agtr1*), and six genes were involved in T-cell activation (*nck1, vav3, abi1, actr3b, apbb1ip*, and *trpc1*). In plants, some resistance genes often cluster together in certain chromosome, sometimes so tightly that they can be considered as complex loci (Vale et al. 2001). The structural organization may facilitate coordinated expression and function (Michalak 2008). Further studies need to be conducted to confirm the involvement of these genes and their specific function in ESC disease resistance.

Our long-term goal is to enhance catfish stocks with a broad spectrum of disease resistance as well as other favorable traits, and support a sustainable and profitable aquaculture industry. To achieve this goal, the genetic basis underlying desirable traits should be understood, especially the accurate location of QTL affecting the traits. In this study, we identified two genomic regions associated with ESC resistance on LG1 and LG23. We previously reported one QTL in LG1 using the third-generation backcrosses (Zhou et al. 2017), which was produced by mating with channel catfish female in each generation. The QTL was validated here in the first-generation backcrosses, suggesting that this QTL was operating in various populations of a broad genetic background. SNPs which were associated with ESC resistance were identified in the present study. Individuals must be generated that are homozygous for these key SNPs, identified and selected as these SNPs are likely associated with the blue catfish resistance alleles that are incompletely dominant since blue catfish are almost totally resistant to ESC, but the F1 channel catfish female × blue catfish male (heterozygous) is not totally resistant though improved. Research should continue to identify the exact location and identity of the ESC resistance alleles using more families or populations. This would allow additional options to interspecific introgression to establish the ESC resistance alleles in a homozygous state in cultured catfish. These include gene transfer or even CRISPR/Cas9 technology to mutate the channel catfish alleles into their blue catfish ESC resistance counterparts (Dunham et al. 2014).

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Compliance with ethical standards

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

Ethical approval All the procedures involving the handling and treatment of fish were approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. Blood samples were col-

lected 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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