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Thermal tolerance evaluation and related microsatellite marker screening and identification in the large yellow croaker *Larimichthys crocea**

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Abstract Thermal tolerance to high temperature was evaluated in the large yellow croaker *Larimichthys crocea*. The survival thermal maximum for *L. crocea* was 33.0°C, the 50% critical thermal maximum (50% CTMax) was 35.5°C, and the critical thermal maximum (CTMax) was 36.0°C. Three microsatellite markers (LYC0148, LYC0200 and LYC0435), associated with thermal tolerance were screened and identified using a Bulked Segregation Analysis (BSA) method. These markers have six amplified fragments in which four are related to thermal tolerance. These fragments were cloned and sequenced, and the results showed the core motif were all "AC" repeats. For LYC0148 and LYC0200, the lengths of fragments are 181 bp and 197 bp, respectively. For LYC0435, which has two fragments, the fragment lengths are 112 bp and 100 bp. The results provide useful molecular markers for thermal-tolerance breeding of large yellow croaker in the near future.

Keyword: large yellow croaker; thermal tolerance; microsatellite markers

1 INTRODUCTION

The large yellow croaker *Larimichthys crocea* is one of the most important fish species in Chinese mariculture. The annual output of this fish has exceeded any other single net, cage-farmed marine species in China (Li et al., 2013; Shen and Heino, 2014).

Temperature is one of the most important ecological factors that significantly affects the growth, metabolism, development, and other life activities of fish (Brett, 1971; Pankhurst and King, 2010; Quinn et al., 2011). For *L. crocea*, the adapted temperature range is 10–32°C, and the optimum growth temperature is 18–25°C (Xue et al., 2014). The current aquaculture methods for *L. crocea* are still dominated by shallow sea cages at a depth of 4–6 m. In summer, owing to the shallow depth, *L. crocea* are forced to live in high temperature seawater that is near to, or higher than, its adapted endurable temperatures for several days, resulting in a weakened body, susceptibility to disease, and even death. With rising global temperatures (Gleckler et al., 2012), it is necessary to develop thermal-tolerant lines of *L. crocea* using marker-assisted selection breeding. Currently, many markers associated with thermal tolerance have been found in fish, such as *Paralichthys olivaceus* (Lu et al., 2007) and *Scophthalmus maximus* (Ma et al., 2011). However, there are no reports of these in *L. crocea*.

In this study, three microsatellite markers related to thermal tolerance were screened and identified using a Bulked Segregation Analysis (BSA) method (Michelmore et al., 1991). These markers may assist in breeding thermal-tolerant *L. crocea* in the future.

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2 MATERIAL AND METHOD

2.1 High temperature stress experiment

A high temperature stress experiment was conducted from May to June 2014 in Hatcheries of Jinling Fisheries Ltd., the city of Ningde, Fujian Province, China. Before starting the experiment, 700 2-month-old healthy fish were randomly selected and placed into indoor concrete ponds (2 m^3) to acclimate for one week. During that time, the water was maintained at ambient temperature (25.0±0.3°C) and changed once a day. The fish were fed commercially manufactured feed. After holding for 7 d, the water temperature was increased 1°C per day until the experimental fish began to die. We then stopped heating the water and maintained the temperature for 1 d. The temperature was then increased at a rate of 0.5°C daily until all of the fish died. During the experiment, the fish were fed with commercially manufactured feed and the water was changed once a day using preheated water. Although dissolved oxygen measurements were not recorded in the test, water was aerated and vigorously circulated with compressed air released through a submerged air stone to provide sufficient oxygen. The number of deaths, time of death and corresponding temperature for each fish were recorded, and the fins were collected for DNA extraction. In this study, a dynamic heating method was employed to estimate thermal tolerance for the 2-month-old fish. Fish whose opercula had stopped beating for 2 min were defined as dead. The critical thermal maximum (CTMax) was determined by exposing all individuals to water with a constant increasing temperature until all fish were dead (see Bennetti and Judd, 1992; Kita et al., 1996). The temperature at which half the population reached the end-point was reported as the 50% critical thermal maximum (50% CTMax) when the water temperature was increased (Jian et al., 2003; Cheng et al., 2013). The temperature at which the fish started to die was recorded as the survival thermal maximum (STMax) when temperature was increased. This experiment was approved by the Animal Care and Use committee of Fisheries College of Jimei University, Xiamen, China.

2.2 DNA extraction and SSR marker analysis

Genomic DNA was extracted from each fish using a standard phenol chloroform protocol (Sambrook et al., 1989). DNA quality and quantity were detected using a UV spectrophotometer. Each DNA concentration was adjusted to 30 ng/μL. Microsatellite primers were synthesized by Shanghai Sangon Biological Engineering Co. Ltd. (Shanghai, China). A total volume of 10 μL of reaction mixture was composed of template DNA, 1 μL (30 ng); 10×PCR buffer, 1.0 μL; 15 mmol/L $MgCl_2$, 1.0 μL; 10 mmol/L dNTPs, 0.2 μL, 10 mmol/L of primers, each 0.2 μL; 5 U/μL Taq enzyme, 0.1 μL; and water, 7.3 μL. The PCR cycle procedure included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, an annealing temperature for 30 s, an extension at 72°C for 30 s, and a 10 min final extension at 72°C. The amplification products were resolved by electrophoresis on 6% polyacrylamide denaturing gels at 1 500 V for 1.5 h, and the marker bands were revealed using a silver-staining protocol and recorded for analysis by photography.

2.3 Marker-phenotype association analysis

Phenotypic extreme bulks (R bulk and S bulk) were made for the marker analysis. The R bulk contained DNA from 15 thermally tolerant fish, and the S bulk contained DNA from 15 thermally sensitive fish. One hundred and sixteen SSR primer pairs (developed by Ye et al.) distributed across the *L. crocea* genome were used for PCR amplification (Ye et al., 2014). The SSR primer pairs, which generated polymorphic markers between the R bulk and S bulk, were surveyed on 60 independent extreme fish to evaluate the association between the microsatellite markers and thermal tolerance using a Chi-squared test.

2.4 Cloning and sequencing of the associated markers

The associated makers detected from 60 independent extreme fish were cloned and sequenced. The steps were as follows: First, the target strips were cut from the polyacrylamide gel, added to ddH_2O and put into water at 95°C for 5 min. The samples were then preserved at room temperature overnight. Second, the mixed solutions were centrifuged (13 800×*g*) for 2 min, and the supernatant fluids were taken as the template for another PCR reaction. Third, the PCR products were gel-purified and cloned into a PMD-19T vector (TaKaRa, Dalian). They were then transformed into competent *Escherichia coli* DH5α cells and sequenced by Shanghai Sangon Biological Engineering Co. Ltd. (Shanghai, China).

Table 1 The number of deaths of *Larimichthys crocea* at different temperatures

Temperature (°C)	Maintained time (h)	Number of deaths	Cumulative number of deaths	Mortality rate (%)	Cumulative mortality rate (%)
25.0–33.0	216	0	0	0.00	0.00
33.0	24	13	13	1.86	1.86
33.5	24	7	20	1.00	2.86
34.0	24	4	24	0.57	3.43
34.5	24	5	29	0.71	4.14
35.0	24	17	46	2.43	6.57
35.5	24	310	356	44.29	50.86
36.0	24	344	700	49.14	100.00

Table 2 The elapsed time from beginning of thermal stress (33°C) to death for thermally sensitive and thermally tolerant *Larimichthys crocea*

ID	Elapsed timeafter 33°C	ID	Elapsed timeafter 33°C
1	0 min	31	200 h 5 min
2	10 min	32	200 h 9 min
3	1 h 20 min	33	200 h 16 min
4	1 h 58 min	34	200 h 20 min
5	2 h 35 min	35	200 h 26 min
6	3 h 1 min	36	200 h 31 min
7	3 h 12 min	37	200 h 38 min
8	3 h 13 min	38	200 h 45 min
9	3 h 14 min	39	200 h 52 min
10	3 h 55 min	40	201 h 8 min
11	4 h 00 min	41	201 h 25 min
12	4 h 35 min	42	201 h 35 min
13	4 h 45 min	43	201 h 42 min
14	25 h 50 min	44	201 h 49 min
15	32 h 10 min	45	201 h 53 min
16	32 h 22 min	46	202 h 5 min
17	32 h 40 min	47	202 h 12 min
18	33 h 10 min	48	202 h 25 min
19	36 h 00 min	49	202 h 45 min
20	36 h 40 min	50	202 h 56 min
21	49 h 35 min	51	203 h 5 min
22	51 h 10 min	52	203 h 35 min
23	52 h 30 min	53	204 h 5 min
24	62 h 10 min	54	204 h 16 min
25	75 h 26 min	55	204 h 20 min
26	83 h 35 min	56	204 h 32 min
27	85 h 54 min	57	204 h 38 min
28	95 h 20 min	58	204 h 50 min
29	96 h 50 min	59	205 h 5 min
30	100 h 13 min	60	205 h 26 min

Table 3 The differential fragments between the R and S bulk by Bulked Segregation Analysis analysis

Locus	Fragment size (bp)	DNA bulk	
		S	R
LYC0137	121	+	-
LYC0154	143	+	-
LYC0148	181	-	+
LYC0200	197	-	+
LYC0161	204	+	-
LYC0211	150	-	+
LYC0435	112	+	-
LYC0435	100	+	-

S represents the DNA bulk of the 15 extreme thermally sensitive fish; R represents the DNA bulk of the 15 extreme thermally tolerant fish. "+" show that the fragments appeared, "-" show that the fragments did not appear.

3 RESULT

3.1 Thermal tolerance evaluation of large yellow croaker

In the high temperature stress experiments, the number of deaths, time and corresponding temperature of *L. crocea* were recorded (Table 1). The survival thermal maximum (STMax) was 33.0°C, 50% critical thermal maximum (50% CTMax) was 35.5°C and critical thermal maximum (CTMax) was 36.0°C.

3.2 Marker-phenotype association analysis

Seven amplification fragments showed frequency differences between the R bulk and S Bulk (Table 2, ID 1-15 DNA were mixed as S Bulk, ID 46-60 DNA were mixed as R bulk).

Four fragments appeared in the S Bulk, and three fragments in the R Bulk (Table 3). These fragments were verified in 60 single extreme fish (ID 1–60 in Table 2), and the results are shown in Table 4. The fragments were amplified at LYC0148 (181 bp) and LYC0200 (197 bp) and showed a frequency difference between the thermally tolerant fish and thermally sensitive fish (Table 4, Figs.1, 2). The frequencies in the thermally tolerant fish were higher than the thermally sensitive fish (P<0.01). By contrast, the fragments amplified at LYC0435 (112 bp and 100 bp) in the thermally sensitive fish were significantly higher than in the thermally tolerant fish (P<0.01) (Table 4, Fig.3).

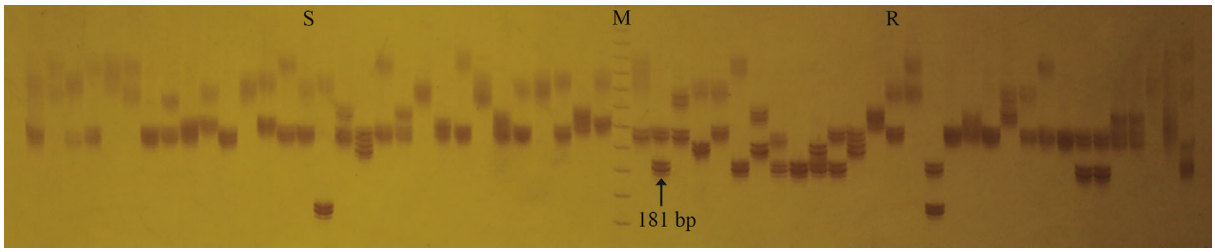

Fig.1 The PCR products amplified at the LYC0148 locus in the 60 extreme fish (*Larimichthys crocea*)

Left of M are the thermally sensitive fish (S). Right of M are the thermally tolerant fish (R). M: 10-bp DNA ladder.

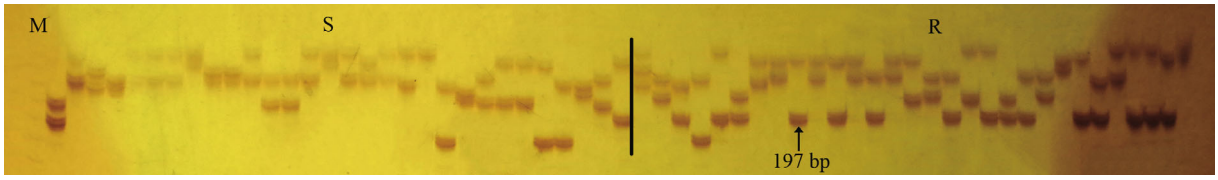

Fig.2 The PCR products amplified at the LYC0200 locus in the 60 extreme fish (*Larimichthys crocea*)

Left of vertical line are the thermally sensitive fish (S). Right of vertical line are the thermally tolerant fish (R). M: 10-bp DNA ladder.

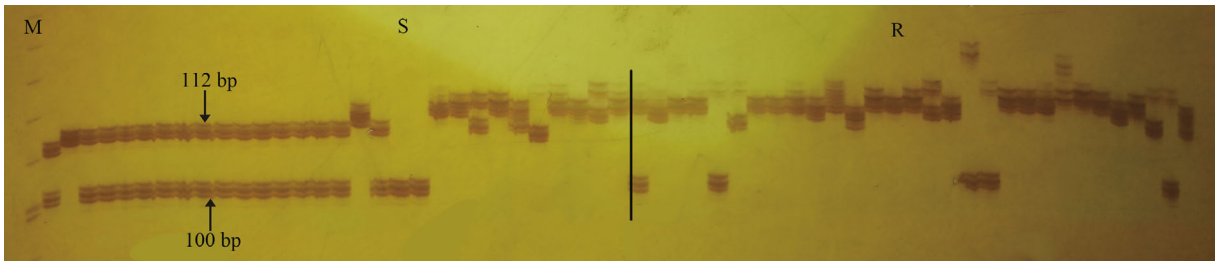

Fig.3 The PCR products amplified at the LYC0435 locus in the 60 extreme fish (*Larimichthys crocea*)

Left of vertical line are the thermally sensitive fish (S). Right of vertical line are the thermally tolerant fish (R). M: 10-bp DNA ladder.

Table 4 The number of differential fragments amplified in the 30 thermally tolerant and 30 thermally sensitive *Larimichthys crocea*

Locus	LYC0137	LYC0154	LYC0148	LYC0200	LYC0161	LYC0211	LYC0435	LYC0435
Fragment size (bp)	121	143	181	197	204	150	112	100
S	1	1	0	2	5	0	18	17
R	0	0	10	15	0	5	2	4
P value	1	1	0.002	0.001	0.062	0.062	0.000	0.001

Table 5 Sequencing results of the differential fragments in this study

Locus	Sequence
LYC0148 (181 bp)	GTGACAAACGCACAAGAAGCCCAGATGAGTCTGTGCAGTGAACACACACACACACACACACA-CACACACACACACAGAACTGTAGCCTACAGTGATAAGATAACTTCTAACTTTGTTTCTGGAAA-GAGAAAGGAACACCTCTGCTTTTCCCAGGACCCAACACATTTGTTAGTTCAGCCCC
LYC0200 (197 bp)	GAGATGAGGGATAAGTGCCTTTTTAACACACACACACACACACACACCTACGGAGTATTCCCACG-CAGAACACTGAGAGGTGTGTAAAAAAAAAAAACCCCAAAAGAAACCTCCACACGGTCCACCGAAAAAG-CCGATCATTAGCAGTTAAACAGAAGCATTTCAACTGGAGACAGAGTGTATCCTAATGGGAACTAT
LYC0435 (100 bp)	TGACACAGAACAGAGCAGGGGAATCATTGCACAGCGGGAAGAAAACAAAAACA-CAAACATCTCATTTTGACACACACACACCGTTTCTTCATATCCATGA
LYC0435 (112 bp)	TGACACAGAACAGAGCAGGGGAATCATTGCACAGCGGGAAGAAAACAGAAAAACACAAA-CATCTCATTTTGACACACACACACACACACACCGTTTCTTCATATCCATGA

3.3 Cloning and sequencing of the associated makers

To verify the associated markers were the existing microsatellites, and obtain the microsatellite sequences, the associated makers were gel-purified, cloned and sequenced. The sequencing results are shown in Table 5. For these loci, the core motif was "AC" repeats. Online BLAST analyses with the sequences was undertaken (Table 5) to align to the genome of the large yellow croaker (GenBank assembly accession: GCA_000972845.1) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_SPEC=Assembly&ASSEMBLY_NAME=GCA_000972845.1). The locus LYC0148 had significant alignments with "the *Larimichthys crocea* isolate SSNF unplaced genomic scaffold scaffold761, whole genome shotgun sequence" (accession No.: KQ041183.1). The locus LYC0200 had significant alignments with "the *Larimichthys crocea* isolate SSNF unplaced genomic scaffold scaffold45, whole genome shotgun sequence" (accession No.: KQ041981.1). The locus LYC0435 had significant alignments with "the *Larimichthys crocea* isolate SSNF unplaced genomic scaffold scaffold33, whole genome shotgun sequence" (accession No.: KQ041581.1).

4 DISCUSSION

Within a certain temperature range, fish can acclimate to a change of ambient temperature by adjusting their physiological activity and metabolism. However, if the temperature exceeds a fish's tolerance limits, it will cause internal environmental disorders and even death (Feng and Wang, 1984). Currently, there are two main methods for evaluating the temperature tolerance of fish, acute heating and slow heating (Bevelhimer and Bennett, 2000; Rajaguru and Ramachandran, 2001; Mora and Maya, 2006; Eme and Bennett, 2009). Fish are ectotherms. Therefore, slow heating allows the fish to have a sufficient time to adapt to a wide range of temperature shifts (Carveth et al., 2007; Ndong et al., 2007). Based on this, the present study used the slow heating method. We found that the STMax for *L. crocea* was 33.0°C, 50% CTMax was 35.5°C, and CTMax was 36.0°C. The STMax in this study is slightly higher than that of our former study (Li et al., 2015), and this may be related to experimental subjects. In the present study, 2-month-old fish were used, whereas in the previous study, 12-month-old fish were used. The thermal-tolerance of fry is higher than adult fish which is also observed in other fish such as redband trout *Oncorhynchus mykiss gairdneri* (Rodnick et al., 2004) and rohu *Labeo rohita* (Das et al., 2005).

In this study, three markers (four fragments) associated with high temperature were found. However, these markers are only associated markers and not specific markers (i.e., only appeared in thermally tolerant fish or thermally sensitive fish), suggesting that these markers may be closely linked with the thermal tolerance gene. In addition, thermal tolerance is a quantitative trait that may be determined by many genes. According to Ye et al. (2014), the locus LYC0148 was assigned to linkage group LG20, the loci LYC0200 and LYC0435 were assigned to the same linkage group LG12 and the genetic map distance between the two markers is 9.1 cm. This gives an indication that there is one gene in this region of the genome that is associated with thermal tolerance. The associated markers found in this study can only partly explain the thermal tolerance of *L. crocea*. Therefore, it will be necessary to undertake a more thorough study and screen additional markers or genes associated with high temperature.

5 CONCLUSION

In this study, thermal tolerance of *L. crocea* was evaluated and three associated microsatellite markers were screened and identified using a Bulked Segregation Analysis (BSA) method. Our results provide reference data for breeding and domestication of thermally tolerant *L. crocea*.

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