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Molecular characterization of salt regulatory genes in *Oreochromis niloticus* and *Oreochromis mossambicus*

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Abstract. Polymorphisms in microsatellite DNA loci located in the salt regulatory genes have been found to show variations in salinity tolerance and growth in fish. Five microsatellite loci located in the salt regulatory genes prolactin 1 (Prl1), transferrin-A (TFA) and transferrin-B (TFB)have been characterized in *Oreochromis niloticus* and *O. mossambicus*. The microsatellite markers were amplified from a total of 78 fish by polymerase chain reaction and genotyped by polyacrylamide and agarose gel electrophoresis. The Prl1 (L-K), PrlI-MS01, TFA and TFB loci in *O. niloticus* and Prl I-MS01, TFA lociin*O. mossambicus* were found to be polymorphic. The average observed (H_o) and expected heterozygosity (H_e) values in *O. niloticus* were higher than the values in *O. mossambicus*. Except locus Prl1(L-K) in *O. niloticus*, the F_{IS} values were negative in all other cases which indicates that both the two populations (*O. niloticus* and *O. mossambicus*) had excess of heterozygosity. The loci Prl I-MS01 and TFA were both in *O. mossambicus* and *O. niloticus*and Prl1(L-K) in *O. niloticus* were found to be significantlydeviated from Hardy-Weinberg expectations. Polymorphisms detected in four loci in *O. niloticus* can be used for studying association of polymorphisms with salt tolerance and selection of salt tolerant individuals of this species through marker assisted selection.
Keywords: Salt tolerance, polymorphisms, tilapia, molecular breeding

Introduction

Tilapia is the second most important farmed fish species globally next to carps with a production of more than 5.6 million tons and described as the world's leading aquaculture species of the 21st century with the Nile tilapia, *Oreochromis niloticus* at the forefront (FAO 2017, Yue *et al.* 2016). However, this contribution is apprehended to be affected by sea-level rise in the coastal regions due to the increasing scarcity of freshwater available for aquaculture. Tilapias show a great variation in salinity tolerance, ranging from 0 to 120 ppt (Trewavas 1982, Morgan *et al.* 2004). Due to high salinity tolerance tilapias are cultured around the world in fresh, brackish and sea water bodies. *O. mossambicus* and its hybrids, for example, are commonly cultured in saline waters, while *O. niloticus* are mainly cultured in freshwatersbecause of its low salinity tolerance (Cnaani and Hulata 2011).

Prolactin (Prl) belongs to a group of peptide hormones which also includes growth hormone (GH), mammalian placental lactogen (PL) and teleosteansomatolactin (SL) (Wallis 1992). It plays a freshwater adapting role which increases plasma osmolality by reducing gill Na^+-K^+-ATPase activity (Sakamoto *et al.* 1997). Nicoll (1974) described 85 different actions of Prl into 5 categories: (a) reproduction, (b) osmoregulation, (c) growth, (d) integument, and (e) synergism with steroids. In teleost, the main function ofprolactin is regulation of water and electrolyte homeostasis (Loretz and Bern 1982). Streelman and Kocher (2002) reported that microsatellite polymorphism in the tilapia Prl1 promoter is associated with differences in Prl1gene expression and growth response of salt-challenged fishes. They crossed females of the

salt-tolerant *O. mossambicus* homozygous for long alleles(LL:CA31/CA31) with a freshwater-adapted *O. niloticus* male heterozygous (SL) for microsatellite alleles that differed by 17 repeat units (CA31/CA14). Fish homozygous for the long allele grew more slowly at 16 ppt and their weight was only half of those of the other two genotypes (SL and SS:CA14/CA14), while in freshwater growth rate did not differ significantly among the three genotypes. Velan *et al.* (2015) detected association between polymorphism in a repetitive element within the promoter of the Prl1 gene and growth rate of tilapia in saline water in three F2 families of *O. mossambicus* × *O. niloticus* hybrids challenged in the first year. The same pattern of improved growth was observed in genotypes with shorter alleles originating from the *O. niloticus* grand-parental fish, although *O. mossambicus* was considered to be a more salt tolerant species.

Transferrin is a single monomeric iron binding glycoprotein comprising approximately of 700 amino acids in length with molecular weight of 80 kDa where iron exists as a nonheme protein compounds to be transported as a redox-inactive form (Aisen *et al.* 1978). Rengmark and Lingaas (2007) investigated the role of transferrin, known to have an important role in the immune system and salinity tolerance. They cloned and sequenced the entire transferrin gene of tilapia, and identified two microsatellites closely linked to the gene as well as many single nucleotide polymorphisms (SNPs) within it. Studies of the segregation of alleles in these two closely-linked microsatellite loci showed that they had defined two haplotypes;the less salt-tolerant individuals possess haplotype 1 (287/184) whereas the salt-tolerant individuals showed a strong tendency to possess haplotype 2 (289/188). The objective of the present study was to characterize the microsatellite loci located in the salt regulatory genes Prl1, TFA and TFB of *O. niloticus* and *O. mossambicus*.

Materials and Methods

***Experimental fish*:** A total of 78 tilapia including 45 Mozambique tilapia, *O. mossambicus* and 33 Nile tilapia, *O. niloticus* were collected from Khulna and Mymensingh districts of Bangladesh, respectively, tagged with Passive Integrative Tags (PIT) and stocked in cemented cisterns. Tissue samples were collected from caudal fin and immediately preserved in separate microfuge tubes containing 95% ethanol and stored at -20°C.

***DNA extraction and PCR amplification*:** Genomic DNA was extracted from the collected fin tissues according to the method described by Islam and Alam (2004). The DNA samples were quantitated using a spectrophotometer (Biophotometer plus Eppendorf, Germany)and adjusted to a final concentration 50 ng/μl.The 20 μl PCR reactions contained 2 μl 10X PCR buffer, 1.6 μldNTPs (2.5mM each), 2 μl(1μM) of forward and reverse primers (Table I), and 1 unit of*Taq*DNA polymerase (Takara, Japan), and 2 μl (100 ng) template DNA. The thermal profile of the PCR wasas follows: For Prl1(S-K), TFA and TFB- initial denaturation at 95°C for 3 min, 35 cycles comprising denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by a by a final extension step at 72° C for 7 min; for Prl1(L-K), Prl I-MS01: initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension at 72 ° C for 7 min. The PCR products of Prl1(S-K), TFA and TFB were separated by polyacrylamide gel electrophoresis while those of Prl1(L-K) and Prl I-MS01 were separated by 3% agarose gel electrophoresis. The primer sequences along with the annealing temperature are shown in Table I.

***Microsatellite data analysis*:** The bands representing particular alleles at the microsatellite loci were scored by using AlphaEaseFC version 4.0 software from the gel image and the sizes of the bands/alleles were determined with respect to the 100bp DNA marker. The software GenAlEx version 6.503 (Peakall and Smouse 2012) was used to analyze the allelic variation and conformity to Hardy-Weinberg expectationof microsatellite markers in the samples of two tilapia species.

Table I. Primer sequences of prl1, tfa and tfb loci used for polymerase chain reaction with the annealing temperature

Locus name	Primer Sequence		Annealing Temp. (°C)	References
Prl1(S-K)	F	5′ - GCATGATCACCCTGCCTATAGG-3′	60	Streelman and Kocher (2002)
	R	5′ -CCCATACAACGAACTGTTCGA-3′		
Prl1(L-K)	F	5′ - GTTAACATTTTCCACCTTCACG-3′	53	Lee and Kocher (1998)
	R	5′-CTTGCCTCCATTTTATAGTTCCTT-3′		
Prl I-MS01	F	5′- GTTAGCCCCCTCCTCACTCT-3′	53	Chi *et al*. (2014)
	R	5′- ACCTTGCTCGTCACACCTG-3′		
TFA	F	5′ -TTTTACTGCATCGCGTTTGA-3′	60	Rengmark and Lingaas (2007)
	R	5′ -ATCGGCTTCATTTTCAACCA -3′		
TFB	F	5`-GCGAGTTAATTAGAAGCGCAGT-3′	60	
	R	5′ -TGGAGCAGTTAATTACGTGAGG-3′		

Results

***Genetic variation in salt regulatory genes ofO. niloticus and O. mossambicus*:** Three microsatellite loci located in the Prl1 gene [(Prl1(S-K), Prl1(L-K) and Prl1-MS01] and two microsatellite loci (TFA and TFB)located in the transferrin gene of *O. niloticus* and *O. mossambicus* were analyzed in this study. The locus Prl1(S-K)was found to be monomorphic bothin *O. niloticus* and *O. mossambicus* with an allele size of 487bp and 500bp, respectively. The loci TFA, TFB, Prl1 (L-K) and PrlI-MS01 were found to be polymorphic in *O. niloticus*. On the other hand, twoloci TFA and PrlI-MS01 were found to be polymorphic and loci TFB and Prl1(L-K) was found to be monomorphic in *O. mossambicus* (Table II).Microsatellite profile of the locus TFB is shown in Fig. 1.

A total of four alleles were found in locus TFA while three alleles were found in TFB. Two alleles were found in Prl1 (L-K) locus and three alleles were found in PrlI-MS01 locus. The 254bp allele of Prl1 (L-K) was absent in *O. niloticus* samples, while the 253bp and 281bp alleles of Prl1 (L-K) were absent in *O. mossambicus* samples (Table II). The 296bp and 320bp alleles of TFA were present in *O. niloticus* but absent in *O. mossambicus* samples, while the 310bp and 340bp alleles of TFA were present in *O. mossambicus* but absent in *O. niloticus* samples. The 166bp and 254bp alleles of TFB were present in *O. niloticus* but absent in *O. mossambicus* populations while the 188bp allele was present in *O. mossambicus* and absent in *O. niloticus* samples (Table II).

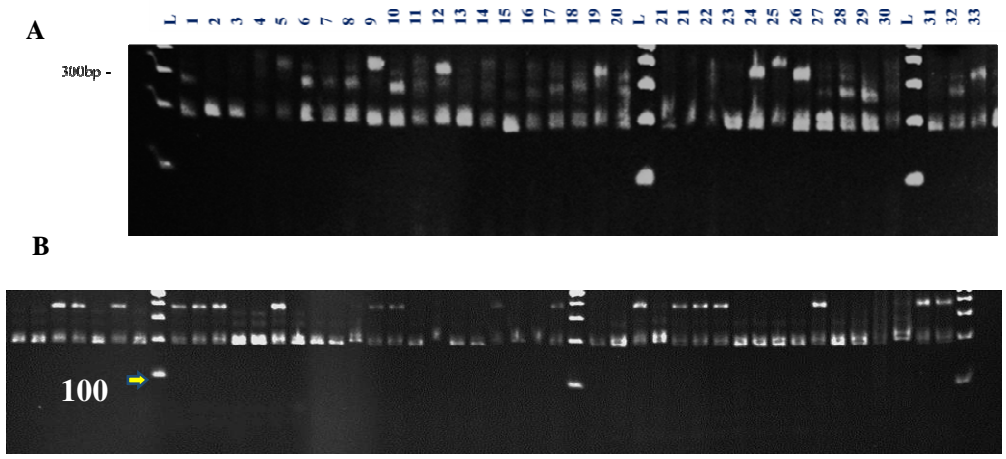

Fig. 1. Microsatellite marker profiles ofthe locus TFB in*O. niloticus* (A) and*O. mossambicus* (B); L: 100bp DNA Ladder.

Table II. Allele frequencies at five gene specific microsatellite loci in *O. niloticus*and *O. mossambicus*

Locus	Allele Size (bp)	*O. niloticus*	*O. mossambicus*
TFA	296	0.727	0.000
	310	0.000	0.671
	320	0.273	0.000
	340	0.000	0.329
TFB	166	0.788	0.000
	188	0.000	1.000
	254	0.212	0.000
Prl1(S-K)	487	1.000	0.000
	500	0.000	1.000
Prl1(L-K)	253	0.188	1.000
	281	0.812	0.000
Prl I-MS01	260	0.641	0.000
	285	0.359	0.578
	320	0.000	0.422

The allelic variations at four microsatellites loci, TFA and TFB, Prl1(L-K) and Prl1-MS01 in *O. niloticus* and *O. mossambicus* are presented in Table III. The *O. niloticus* samples have two alleles at each of the four loci while *O. mossambicus* samples have two alleles at TFA and Prl1-MS01 and one allele at TFBand Prl1(L-K) locus.

Table III. Allelic variations at four microsatellite loci (N= No. of individuals, N_a= No. of alleles, H_o= Heterozygosity observed, H_e= Heterozygosity expected, F_{IS} (inbreeding coefficient), #N/A= Not Applicable) HWEP= probability of conformity in Hardy-Weinberg equilibrium) in samples of *O.niloticus* and *O. mossambicus*

Locus	Parameters	*O. niloticus* (N= 33)	*O. mossambicus* (N= 41)
TFA	N_a	2	2
	H_o	0.545	0.659
	H_e	0.397	0.442
	F_{IS}	-0.375	-0.491
	HWEP	0.031*	0.002**
TFB	N_a	2	1
	H_o	0.424	0.000
	H_e	0.334	0.000
	F_{IS}	-0.269	#N/A
	HWEP	0.122NS	-
Prl1(L-K)	N_a	2	1
	H_o	0.188	0.000
	H_e	0.305	0.000
	F_{IS}	0.385	#N/A
	HWEP	0.03*	-
Prl I-MS01	N_a	2	2
	H_o	0.719	0.756
	H_e	0.374	0.488
	F_{IS}	-0.561	-0.549
	HWEP	0.002**	0.000**
Average number of alleles		2.00	1.5
Average H_o over loci		0.469	0.354
Average H_e over loci		0.365	0.233
Polymorphism (P_{95})		100%	50%

*$p < 0.05$; NS=Not significant

The average observed and expected heterozygosity values of the four polymorphic microsatellite loci of *O. niloticus* (0.460 and 0.365) were higher than those of *O. mossambicus*(0.354 and 0.233) samples. The F_{IS} values of three loci of *O. niloticus* and two loci of *O. mossambicus* were found to be negative. The deviations in Hardy-Weinberg expectations were found to be significant ($p < 0.05$ and $p < 0.01$) in three loci of *O. niloticus* and two loci of *O. mossambicus* (Table III).

Discussion

The genetic variation in a population can be affected by five major forces such as mutation, selection, genetic drift, migration and non-random mating. The genetic structure may change over time through over-exploitation, inbreeding, habitat degradation, environmental pollutions, contaminations of gene pools through introgression, climate changes, mutation etc. Therefore, the molecular characterization of two salt-regulatory genes in tilapia has been studied using gene-located microsatellite DNA markers to identify polymorphism of prolactin and transferrin

genes and genetic variation in *O. niloticus* and *O. mossambicus* populations. As associations have been detected between polymorphism in SSR loci located in prolactin and transferrin gene, any polymorphism detected in the present study would allow selection of salt tolerance individuals of *O. niloticus* based on the polymorphism.

The microsatellite locus Prl1 (S-K) of the prolactin gene of both *O. niloticus* and *O. mossambicus* was found to be monomorphic containing only one allele in each with a size of 487bp and 500bp respectively. Kocher *et al.* (1997) observed polymorphism atPrl1 (S-K) locus in the haploid progeny of *O. niloticus* identifying a single diploid locus which were used for genome mapping. On other hand, Lee and Kocher (1998) recorded a homozygous Prl1 (S-K)locus (254bp/254bp) in a single *O. mossambicus*. Velan *et al.* (2015) found polymorphism in *O. mossambicus* and *O. niloticus* at the Prl1 (S-K) promoter containing genotype 253/263, 247/257 respectively. After cross breed, they found four genotypes 247/253, 247/247, 247/263, 253/263 and observed fish carrying the allelic combination 247/253 grew better in saline water than freshwater and 13% phenotypic variance for growth rate ($p < 0.05$) was noted for this allele. The sizes of alleles (487 and 500bp) obtained in the present study are higher than those of other studies. The size of a PCR fragment can be increased if an insertion takes place between the two priming sites. This can be confirmed by sequencing the PCR fragment. The different length of Prl1 (S-K) microsatellite alleles with CA repeats can affect gene expression as claimed by Streelman and Kocher (2002). At Prl1(L-K) locus, we have found two alleles of sizes 253bp and 281 bp in *O. niloticus* but one allele of 253bp in *O. mossambicus*. At locus Prl1-MS01, we have found two alleles of sizes 260bp and 285 bp in *O. niloticus* and two alleles of sizes 285bp and 320 bp in *O. mossambicus*. Yue and Orban (2002) found polymorphism in Prl1-MS01 locus of *O. mossambicus* and *O. niloticus*. The allele sizes at Prl1-MS01 of *O. mossambicus* were 230bp and 302bp while the allele sizes of Prl1-MS01 of *O. niloticus* were 230bp and 310bp.So, the allele sizes we have observed in the present study are different from those reported by Yue and Orban (2002).

The two microsatellite loci, TFA and TFB located in the salt regulatory gene transferrin were found to be polymorphic in *O. niloticus* but in *O. mossambicus*one locus (TFA) was found to be polymorphic. The polymorphism of transferrin have been detected in many fish species such as goldfish (Yang et al., 2004), coho salmon (Van Doornik*et al.* 1995), European common carp (Jurecka *et al.* 2009), and Nile tilapia (Rengmark and Lingaas 2007) which is caused due to gene duplication, gene loss and horizontal gene transfer (Uribe *et al.* 2011; Bai*et al.* 2016). In the present study, the genotypes in TFA and TFB loci of *O. niloticus* were found to be 296/320 and 166/254, respectively. Whereas, the genotypes in TFA and TFB loci of *O. mossambicus* were 310/340 and 188/188 respectively. Rengmark and Lingaas (2007) found two closely linked haplotype of transferrin (TFA and TFB) having 287/184 and 289/188 where 289/188 was more salt-tolerant than 287/184 in *O. niloticus*.

The average observed heterozygosity (H_o) value of *O. niloticus* (0.469) was higher than that in *O. mossambicus* (0.354) population. The average expected heterozygosity of *O. niloticus* (0.365) was also higher than that in *O. mossambicus* (0.233) samples. Yue and Orban (2002) reported varied average expected and observed heterozygosity in *O. mossambicus* and *O. niloticus* due to selective breeding.

The F_{IS} value of both TFA and TFB loci in *O. niloticus*, TFA in *O. mossambicus,* Prl1 (L-K) and Prl I-MS01 in *O. niloticus* and *O. mossambicus* were negative which means that this population had excess of heterozygosity. Excess of heterozygosity is occurred due to breakdown of isolation and Wahlund effect (Wahlund 1928).The two polymorphic microsatellite loci (TFA and Prl1-MS01) tested in *O. mossambicus* in the present study, were found to be highly deviated ($p < 0.001$) from Hardy-Weinberg expectation. Deviations from Hardy-Weinberg expectation at the locus TFB of *O. niloticus* was insignificant ($p > 0.05$). A population may cause departure from Hardy-Weinberg expectation due to non-random mating, mutation, gene flow, genetic drift and small sample size. The deviation from Hardy-Weinberg expectation observed in the present study was due to excess of heterozygosity. Excess of heterozygosity in a population is caused by Wahlund effect which indicates the mixing of undetected genetically divergent stocks with the samples (Halliburton 2004).

In conclusion, we have detected polymorphism in three microsatellite loci of the salt regulatory genes prolactin and transferrin in *O. niloticus* and two loci in *O. mossambicus* through our preliminary investigation. The fishes carrying different alleles in the salt regulatory genes will allow to select salt tolerant fish. The fish have already been PIT tagged so they can be grouped according to their genotypes and reared in different salinity to see which genotypes tolerate salinity levels better than the others. Thus a stock of salinity tolerant *O. niloticus* can be developed through marker assisted selection.

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