Bangladesh J. Fish. (2020) **32**(1): 1-10

Microsatellite marker-based variation in the growth hormone genes of Nile tilapia (*Oreochromis niloticus*)

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Abstract. Polymorphisms in growth hormone genes have been found to cause variation in growth performance of fish. The objective of the study was to reveal variations in microsatellite loci located in the growth hormone genes of Nile tilapia (*Oreochromis niloticus).* Five microsatellite loci namely GH-MS01, IGFII, IGFII-MS01, IGFII-MS03, and STR were analyzed to assess the genetic variation in the growth hormone genes of four stocks of *O. niloticus viz.* FBG-Mini Hatchery, FM-Mini hatchery, Eon Aquaculture Ltd. and BFRI. The microsatellite markers were amplified by polymerase chain reaction, separated by polyacrylamide gel electrophoresis and visualized through ethidium bromide staining. All the five loci were found to be polymorphic. The average number of alleles of FM-Mini hatchery stock (3.8) was found to be highest and that of the FBG-Mini hatchery (2.8) and Eon Aquaculture stocks was found to be lowest. The average observed heterozygosity (H_o) value of the FM-Mini hatchery stock was the highest (0.140) and that of FBG-Mini hatchery stock was the lowest (0.040). On the other hand, the average expected heterozygosity (H_e) was highest in the BFRI stock (0.660) and lowest in the FM-Mini hatchery and FBG-Mini hatchery stock (0.432). The fixation index ($1 - (H_o / H_e)$) values were positive in all the loci (except locus GH-MS01 in Eon Aquaculture stock), which means these stocks (*O. niloticus*) were deficient in heterozygosity. Deviation from Hardy-Weinberg expectation at STR locus in FBG-Mini hatchery and Eon Aquaculture stocks were not significant but in all other cases the deviations were found to be significant. The results provide evidence that genetic variation exists within the growth hormone genes in all four stocks of *O. niloticus*. The polymorphisms that have been detected in the present study can be used to study association with growth and thus selection of fast growing Nile tilapia in Bangladesh.
Key words: Polymorphisms, Microsatellite, *Oreochromis niloticus*, Heterozygosity

Introduction

Aquaculture is the fastest-growing food production sector in the world providing half of the global fish supply. It is estimated that by 2030, aquaculture production will grow by 37% over the production in 2016 (FAO 2018a). The Nile tilapia, *Oreochromis niloticus* was the fourth most contributing aquaculture species in the world (FAO 2018a). Considering its importance worldwide the GIFT strain of *O. niloticus* was developed by the then ICLARM (International Centre for Living Aquatic Resources Management, now WorldFish) through several generations of conventional selective breeding (Eknath *et al.* 2007). The GIFT project played an important role in the expansion of Nile tilapia culture (now reported in 87 countries) by helping to avoid the negative impacts of inbreeding or poor genetic management (Gjedrem 2012). Production of Nile tilapia has quadrupled over the past decade because of its suitability for aquaculture, marketability and stable market prices (Prabhu *et al.* 2019). The GIFT strain was introduced in Bangladesh in 1994 to boost aquaculture and since then there has been tremendous progress in tilapia farming in the country (Hussain *et al.* 2017). In 2016, Bangladesh ranked 4thin Nile tilapia production with 342, 567 MT among the world's total production of 5,898,752 MT (FAO 2018b).

Improvement of performance traits through traditional selection integrated with molecular tools is fast and more accurate and allows us to understand the genetic mechanism affecting performance traits (Haldar 2018).Molecular marker technology is required to sustain the aquaculture industry, promote quality and confirm traceability documentation. Among the molecular markers microsatellites have been commonly used for characterization of fish species, because they are highly reproducible, polymorphic, co-dominant and widely distributed throughout the genome (Liu and Cordes 2004). Though most microsatellite loci are associated with anonymous genomic segments (Liu and Cordes 2004), an increasing body of research has demonstrated that some microsatellites are located within promoter and transcribed regions of the genome and may be involved in regulating gene expression and function (Chistiakov *et al.* 2006). These functional microsatellites may cause distinct phenotypes or influence physiological functioning by directly or indirectly affecting the expression and function of a given gene (Kashi and King 2006) allowing characterization of the phenotypes that result from the expressional variation of a given gene.

Growth is a trait of moderate to high heritability (Gjedrem *et al.* 2012) that displays a complex development and physiology. It involves both the increase of muscle cell number (hyperplasia) and cell size (hypertrophy), and the balance between both processes depends on the developmental stage, but it is also influenced by the environment. Growth is regulated by the hypothalamic-pituitary axis hormones which also control feeding behavior (Kawauchi and Sower 2006. Despite its good response to traditional selection, the sole application of phenotypic and relatedness information to improve growth rate might determine the loss of relevant genetic variation affecting the medium- and long-term performance of breeding programs (Eynard *et al.* 2016). In this sense, identification of allelic variants of moderate to large effect could be useful in marker-assisted selection (MAS). For example, Sultana et al. (2020) identified sex-linked microsatellite DNA markers, ARO172, which facilitated reliable differentiation between male and female genotypes of *O. niloticus*.

Recently MAS became a very popular method of indirect selection for production of the genetically improved fish in aquaculture breeding programme. Polymorphisms in the growth related genes such as Growth hormone (GH), Insulin like growth factors-I and II, Somatolactin (SL), Myostatin (MSTN-1) and Prolactin (PRL) are associated with the growth rate of fish and have been the target of many breeding programmes that significantly improve fish production. Advances in molecular assisted breeding have enhanced the efficiency with which breeders can select superior phenotypes with best gene combinations.

Growth hormone (GH) is a single-chain polypeptide that is synthesized and secreted by the anterior pituitary gland. GHs have a wide range of physiological regulatory functions, as they can regulate the metabolism of three major substances (sugars, lipids and proteins), and their primary effect on animals is to significantly increase the growth rate, promote the growth of muscles and bones, and decrease the fat content, thereby affecting the growth and development (Reinecke *et al.* 2005). GH gene polymorphism is often associated with growth or production performance (Hua *et al.* 2009, Cheng *et al.* 2016) in animals. GHs have significant growth-promoting effects in fish, as they can promote protein synthesis, decrease fat content and accelerate the longitudinal growth of bones (Chatakondi *et al.* 1995). In addition, they can also promote food conversion efficiency and sexual maturity (Tsai *et al.* 1994). Thus, the GH gene

has become an important target gene for the selective breeding of growth traits. GH gene polymorphism studies have been carried out in many fish species, which has resulted in the identification of molecular markers such as microsatellites and SNPs that are significantly associated with growth traits in fishes such as *Oreochromis niloticus* (Jaser *et al.* 2017) and *Cyprinus carpio* (Liu *et al.* 2017) which have been proposed as potential targets for marker-assisted selection in fish (Wang *et al.* 2019). We have characterized five microsatellite DNA markers located in five different growth related genes in selected stocks of the Nile tilapia *O. niloticus* in order to select better performing fish by marker assisted selection.

Materials and Methods

Collection of fish sample and extraction of genomic DNA: A total of 43 Nile tilapia, *O. niloticus* individuals were collected from four different stocks such as FBG-Mini Hatchery and FM-Mini Hatchery of Bangladesh Agricultural University, Eon Aquaculture Ltd. Muktagacha, Mymensingh and Freshwater Station of the Bangladesh Fisheries Research Institute (BFRI). The fish were electronically tagged and fin samples were collected from caudal fin and preserved in microfuge tube containing 95% ethanol and stored at -18 °C. The fish were stocked in cemented cistern at the FBG-Mini hatchery. Genomic DNA was extracted from fin tissues according to the method described by Alam and Islam (2005) with some modifications.

Amplification of microsatellite markers by PCR and electrophoretic separation: Five sets of microsatellite primers for growth hormone genes of *O. niloticus* such as GH- MS01, IGF- II, IGF- II- MS01, IGF-II- MS03, STR were analyzed (Table I). The polymerase chain reaction was conducted in a volume of 20 μl containing 1x buffer with $MgCl_2$, dNTPs, and 0.2 μM dNTPs and 1 unit Taq DNA polymerase. The thermal profile consisted of 3 min initial denaturation at 95°C, followed by 35 cycles each comprising of 30 s denaturation at 95°C , 45 s annealing at 60°C and 1.15 min elongation at 72°C. The last cycle was followed by a single elongation step of 7 min at 72°C. The PCR products were electrophoresed on polyacrylamide gel and stained with ethidium bromide. Photographs of the stained gels were taken using a gel documentation system and saved for further analysis.

Table I. Primer sequences and repeat motifs of five microsatellites located within growth-related genes

Primer name	Primer sequence (5´-3´)	Position within gene	Repeat	Gene Bank ID/ Reference
GH-MS01	F: CCAGCCATGAACTCAGGTAAGACA	intron 1 of GH	TGTC	M97765
	R: TGCTGAGAGGAGACGCCCAAACA			
IGF-II	F: GGGAGCCGTGATGAAGACTG	Intron-3, Exon-3	-	Khatab *et al.* (2014)
	R: CAAATAGCAATCACG CAG C			
IGF-II-MS01	F: TCCCCAGCTGGAAGATGTGTCACG	promoter of IGF-II	CT	AF033802
	R: CTGGACGCAGCTGAAATCCTGTGG			
IGF-II-MS03	F: ATGCTAGCAAACATCAAAGGTC	3'UTR of IGF-II	ATCT	AF033804
	R: GATATGCTGATGATGCACAGAGTC			
STR	F:TGTAAAACGACGGCCAGTCCAGCATG TTTGCACTGAGTA	Promoter	ATTCT	M97766
	R: GCCTAGCCATGGACACATTTA			

Analysis of microsatellite marker data: 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. A single genotype data matrix was constructed for all loci. The software GenAlEx version 6.503 (Peakall and Smouse 2012) was used to calculate the allelic variations, observed (H_o) and expected (*He*) heterozygosity, fixation index (inbreeding value), and test for Hardy-Weinberg Expectation.

Results

Allele frequency and size variation within stocks: The sizes and frequencies of alleles of the five microsatellite loci in four different stocks are shown in Table II. The sizes of the alleles for all loci in four stocks ranged from 142 to 488bp. Four alleles were found at three loci (GH-MS01, IGFII-MS01 and STR) while five alleles were found at two loci (IGFII and IGFII-MS03) (Table II). Three (142 bp, 148 bp, 158 bp) out of four allelic variants at locus GH-MS01 were absent in the samples of Eon Aquaculture while the other three stocks had lacked one single variant (148bp for BFRI, and 158bp forFBG-Mini hatchery and FM-Mini hatchery). Out of the four alleles in the locus IGFII, alleles 440bp and 457bp were absent in FBG-Mini hatchery stock

Table II. Allele Frequencies at five microsatellite loci in four stocks of *O. niloticus*

Locus	Allele (bp)	FBG-Mini hatchery	FM-Mini hatchery	Eon Aquaculture Ltd.	BFRI
GH-MS01	142	0.700	0.400	0.000	0.000
	148	0.200	0.400	0.000	0.429
	152	0.100	0.200	1.000	0.286
	158	0.000	0.000	0.000	0.286
IGF-II	440	0.000	0.050	0.667	0.429
	457	0.000	0.050	0.333	0.429
	471	0.700	0.300	0.000	0.143
	480	0.200	0.500	0.000	0.000
	488	0.100	0.100	0.000	0.000
IGFII-MS01	206	0.000	0.400	0.333	0.071
	212	0.900	0.500	0.333	0.500
	220	0.100	0.100	0.333	0.357
	233	0.000	0.000	0.000	0.071
IGFII-MS03	194	0.200	0.500	0.111	0.286
	210	0.100	0.400	0.500	0.107
	226	0.600	0.000	0.222	0.321
	232	0.100	0.050	0.167	0.107
	243	0.000	0.050	0.000	0.179
STR	177	0.000	0.200	0.056	0.000
	185	0.000	0.500	0.278	0.357
	194	0.600	0.200	0.611	0.357
	204	0.400	0.100	0.056	0.286
No of allele missed across all loci		8	3	8	4

while allele 471bp and 488 were absent in Eon Aquaculture; the BFRI stock lacked only one allele (488bp) in this locus. In locus IGFII-MS01, the allele 206bp was absent in the FBG-Mini hatchery stock while the allele 233bp was absent in the rest three stocks. Allele 226bp of locus IGFII-MS03 was absent in FM-Mini hatchery stock and the allele 243bp was absent in the FBG-Mini hatchery and Eon Aquaculture stock. The 177 bp and 185 bp alleles of STR were absent in FBG-Mini hatchery and BFRI stocks. Overall a maximum 8 alleles were absent in each of FBG-Mini hatchery and Eon Aquaculture stocks. The numbers of missing alleles in the FM-Mini hatchery and BFRI were 3 and 4 respectively (Table II).

Genetic variation: Five microsatellite loci namely GH-MS01, IGFII, IGFII-MS01, IGFII-MS03, STR were analyzed in this study and except loci GH-MS01 all of them were found to be polymorphic (P95) in these four stocks. The GH-MS01 locus in Eon Aquaculture stock was found to be monomorphic. The microsatellite profiles of the IGFII-MS01 is shown in Fig. 1. The average number of alleles in FM-Mini hatchery stock (3.8) was found to be the highest and that of FBG-Mini hatchery and Eon Aquaculture stock (2.8) was found to be the lowest.

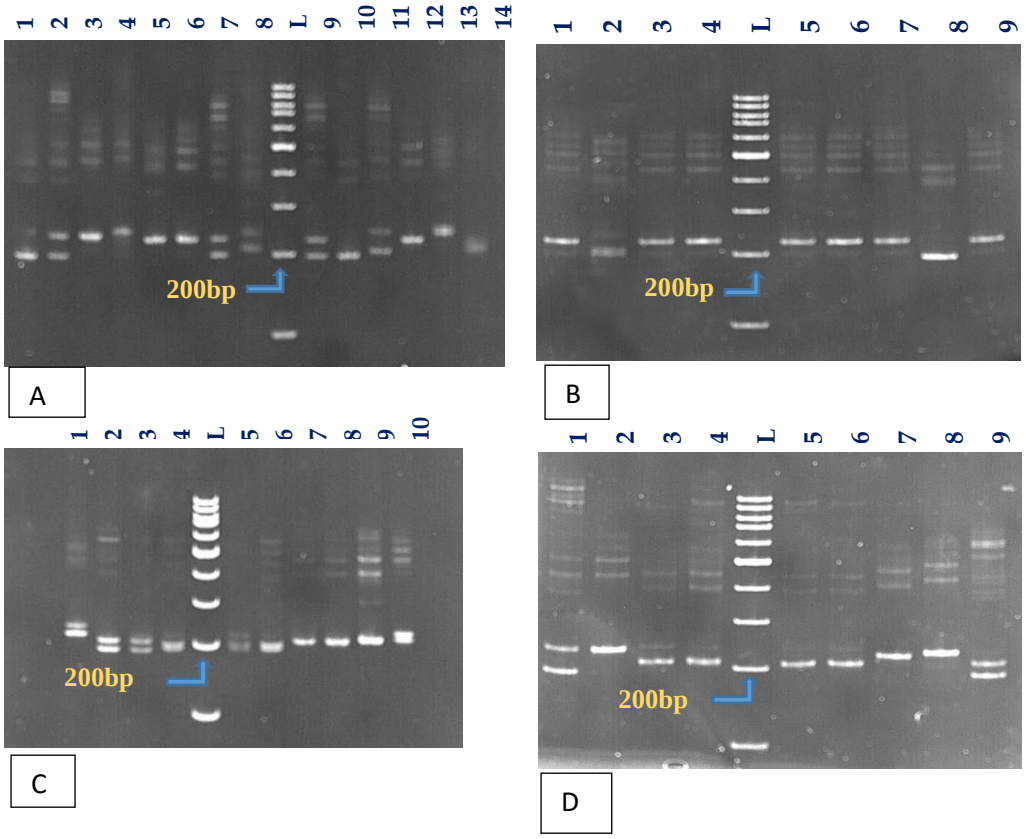

Fig. 1. Microsatellite profile of the locus IGFII-MS01 in BFRI (A), FBG-Mini hatchery (B), FM-Mini hatchery (C) and Eon Aquaculture Ltd. (D) stocks of *O. niloticus*.

Deviation from Hardy-Weinberg expectation: Significant deviations from Hardy-Weinberg Expectation (HWE) were detected in 17 out of 20 tests (Table IV). The test for fit to Hardy-Weinberg expectations revealed that BFRI stock was highly deviated compared to other stocks. These deviations were not systematic rather occurred at different loci for different stocks. The deviation from HWE at loci GH-MS01, IGFII, IGFII-MS03 in FBG-Mini hatchery stock, GH-MS01, IGFII-MS01, IGFII-MS03 in FM-Mini hatchery stock, IGFII-MS01 in the Eon Aquaculture stock and all five loci in the BFRI stock were high ($p<0.001$). STR locus in FBG-Mini hatchery and Eon Aquaculture stocks was not deviated (Table IV).

Table III. Allelic variations at five microsatellite loci in four different stocks of *O. niloticus* (N= No. of individuals, Na= No. of alleles, Ho= Heterozygosity observed, He= Heterozygosity expected, 1 - (Ho / He) = Fixation index)

Microsatellite loci	Parameters	FBG-Mini hatchery (N = 10)	FM-Mini hatchery (N = 10)	Eon Aquaculture Ltd. (N = 9)	BFRI (N = 14)
GH-MS01	N_a	3.000	3.000	1.000	3.000
	H_o	0.000	0.000	0.000	0.000
	H_e	0.460	0.640	0.000	0.653
	$1 - (H_o / H_e)$	1.000	1.000	#N/A	1.000
IGFII	N_a	3.000	5.000	2.000	3.000
	H_o	0.000	0.200	0.000	0.000
	H_e	0.460	0.645	0.444	0.612
	$1 - (H_o / H_e)$	1.000	0.690	1.000	1.000
IGFII-MS01	N_a	2.000	3.000	3.000	4.000
	H_o	0.000	0.000	0.000	0.000
	H_e	0.180	0.580	0.667	0.612
	$1 - (H_o / H_e)$	1.000	1.000	1.000	1.000
IGFII-MS03	N_a	4.000	4.000	4.000	5.000
	H_o	0.000	0.300	0.222	0.357
	H_e	0.580	0.585	0.660	0.760
	$1 - (H_o / H_e)$	1.000	0.487	0.664	0.530
STR	N_a	2.000	4.000	4.000	3.000
	H_o	0.200	0.200	0.222	0.143
	H_e	0.480	0.660	0.543	0.663
	$1 - (H_o / H_e)$	0.583	0.697	0.591	0.785
Average number of alleles		2.8	3.8	2.8	3.6
Average H_o over loci		0.040	0.140	0.089	0.100
Average H_e over loci		0.432	0.622	0.463	0.660
Polymorphism (P_{95})		100%	100%	80%	100%

Discussion

In the present study, we have characterized five growth regulatory genes by microsatellite DNA markers and detected a total of 22 alleles in 43 individuals of four sources. The average number of alleles ranged from 2.8 to 3.8 in the four stocks. With one exception, locus GH-MS01 in Eon Aquaculture stock, all the loci were found to be polymorphic meaning there exist variations in all the loci in all the stock. As many as five alleles have been found at locus IGFII in FM-Mini

hatchery stock and at locus IGFII-MS03 in BFRI stocks. The allelic variations observed in the four stocks of *O. niloticus* suggest that there is scope for improvement in growth performance if the alleles are associated with growth performance. Detection of polymorphisms and characterization of growth traits in Nile tilapia using microsatellite DNA markers have also been reported by a few authors (Yue *et al.* 2002, Chi *et al.* 2014, Khatab *et al.* 2014, Dias *et al.* 2019). The previously identified microsatellite markers located in the growth hormone genes have been found to be effective in the present study to detect genetic variation in the Bangladesh stocks of *O. niloticus*.

Table IV. Deviation from Hardy–Weinberg genotype frequency expectations in four different stocks of *O. niloticus* ($\chi 2$ values, followed by degrees of freedom in parenthesis)

Pop	Locus	ChiSq	DF	Prob.	Signific.
FBG-Mini hatchery	GH MS01	20.000	3	0.000	***
	IGFII	20.000	3	0.000	***
	IGFII MS01	10.000	1	0.002	**
	IGFIIMS03	30.000	6	0.000	***
	STR	3.403	1	0.065	NS
FM-Mini hatchery	GH MS01	20.000	3	0.000	***
	IGFII	20.400	10	0.026	*
	IGFII MS01	20.000	3	0.000	***
	IGFIIMS03	23.025	6	0.001	***
	STR	20.000	6	0.003	**
Eon Aquaculture limited	GH MS01	Monomorphic			
	IGFII	9.000	1	0.003	**
	IGFII MS01	18.000	3	0.000	***
	IGFIIMS03	15.111	6	0.019	*
	STR	9.434	6	0.151	NS
BFRI	GH MS01	28.000	3	0.000	***
	IGFII	28.000	3	0.000	***
	IGFII MS01	42.000	6	0.000	***
	IGFIIMS03	31.755	10	0.000	***
	STR	18.235	3	0.000	***

Key: NS=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

We found the average expected heterozygosity values (0.432, 0.622, 0.463, 0.660) higher the average observed heterozygosity values (0.040, 0.140, 0.089, 0.100) in all the studied stocks. Yue and Orban (2002) reported that average expected heterozygosity was higher than average observed heterozygosity in Nile tilapia due to selective breeding. An *et al.* (2011) also found higher average expected heterozygosity values than average observed heterozygosities values in the hatchery stock. The fixation index (1 - (H_o/H_e) values were mostly found to be positive in all the loci which means these stocks (*O. niloticus*) were deficient in heterozygosity indicating occurrence of inbreeding in the stocks.

The test for fit to HWE revealed that BFRI stock is highly deviated than other stocks. The deviation from HWE at loci GH-MS01, IGFII, IGFII-MS03 in FBG-mini hatchery stock, at loci GH-MS01, IGFII-MS01, IGFII-MS03 in FM-mini hatchery stock, at loci IGFII-MS01 in Eon Aquaculture stock and at loci GH MS01, IGFII, IGFII MS01, IGFII-MS03, STR in the BFRI stock were highly significant ($p<0.001$). Deviation from HWE in STR locus in FBG-mini hatchery stock and Eon Aquaculture stock were not significant ($p>0.05$). There are many causes for disequilibrium such as inbreeding, selection, mutation, and migration. Samples used for this analysis were taken from hatcheries. In these hatcheries, the stocks of most Nile tilapia strains originated from a small number of parent stock and have been gradually enlarged. As a result, their genetic bases are narrow and founder effects could have had a great influence on these stocks. Secondly, many breeding schemes executed in these hatcheries might give rise to an increase in the inbreeding coefficient. Alam and Islam (2005) reported that all the studied stocks of *Catla catla* deviated from Hardy-Weinberg equilibrium at a number of loci, mostly due to deficiency in heterozygosity. Lal *et al.,* (2004) detected significant heterogeneity in allele frequencies that indicated analyzed samples did not belong to homogenous stock.

Molecular marker-assisted selection increases the selection precision and reduces the number of generations required to achieve a desired improvement. Khatab *et al.* (2014) found nucleotide sequence variation in DNA sequencing of 397 bp fragment of IGF-II gene between large size and small size tilapia in Egypt. Juhua *et al.* (2010) demonstrated that two sites, G161A in exon 3 and the microsatellite locus in intron 3, were significantly associated with male growth of genetically improved farmed tilapia (GIFT). In fish, IGF-II is expressed in many tissues (Peng and Luo 2007) and molecular markers in IGF-IIare associated with back fat, fat deposition, body weight or other characteristics in chicken, goat and cattle (Li *et al.* 2004, Zhang *et al.* 2007, Helal *et al.* 2014). Therefore, our study revealed that the microsatellite markers in the growth related genes in *O. niloticus* can be used for marker assisted selection (MAS) for developing fast growing fish.

Dias *et al.* (2019) observed association of polymorphisms of short tandem repeats loci located in the growth hormone-I promoter and first intron with growth performance in introgressive crossbreed between Red-Stirling and Chitralada strains of the Nile tilapia. They found six alleles in the promoter, which were arranged in 18 of the 21 genotypic possible combinations. Jaser *et al.* (2017) in a study involving association between SNPs in growth hormones gene and growth rate of two strains of *O. niloticus* identified 10 SNPs, nine in the proximal promoter and one located in the 5´ UTR, forming 10 genotype blocks. They observed five genotype blocks which were significantly associated with the highest weights. These findings may be used as part of marker-assisted selection in tilapia breeding programmes.

Growth rate is an important trait for aquaculture species which is influenced by genotype and environmental factors (Schwartz *et al.* 2006). Polymorphisms present in a functional gene would create variation in the genotype and phenotype that can be exploited through selective breeding for stock improvement. Polymorphisms in the growth hormone gene that is associated with the growth rate of farmed fish have been the target of many breeding programmes. We have identified a total of 22 alleles in five growth related genes in *O. niloticus*. As the previous studies have detected significant association between the polymorphisms in SNPs and/or microsatellite loci in the growth hormones genes with growth performance, the alleles that we

have identified may be used to study association between growth performance and the alleles. This will allow to select faster growth rate fish by marker assisted selection.

Acknowledgements: The study was conducted with a Special Allocation fund for the year 2018-2019 of the Ministry of Science and Technology, Government of the People's Republic of Bangladesh (Grant No. BS-57/68/2018-2019). We acknowledge BFRI and Eon Aqiaculture Ltd. For generously providing samples of *O. niloticus*.

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(Manuscript received: 24 May 2020)