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Identification of Growth-related EST-derived Microsatellite Marker in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

This study aimed at investigating growth-related, EST-derived microsatellite markers in Nile tilapia (*O. niloticus*). The link between alleles and genotypes of these makers was particularly examined, as well as the growth performances of the samples of offspring produced from the broodstocks of 5 different fishery stations in Thailand. A total of 25 families of offspring were reared to observe the growth performance for a period of 83 days. Fish samples in this study were divided into two categories according to average body weight: fast (+SD) and slow (-SD) growth. Then, the fins of the fish samples were employed to perform DNA analysis. From the fast and slow growth evaluations, 3 families, namely F1, F24 (from Chumphon) and F11 (from Petchaburi), were chosen for the association analysis. A total of 30 fishes with 5 samples from fast and slow growth of each family were utilized. Nineteen EST-derived microsatellite markers were used to genotype 30 DNA samples of the fast and slow growth fishes. Out of 19 loci, 14 loci (i.e., OMO392, OMO051, OMO097, OMO072, OMO327, OMO277, OMO122, OMO193, OMO198, OMO200, OMO335, OMO374, OMO049, and OMO069) were found polymorphic. Another 4 loci (i.e., OMO059, OMO068, OMO315, and OMO337) were observed as monomorphic. Based on the genotype data, there appeared to indicate a strong, significant relationship between allele and growth of the A3 allele of OMO392 locus in the fast growth group. However, no significant genotypes regarding 19 EST-microsatellite were found to be related with growth. This study suggests that the identified allele A3, which has designated the growth hormone related EST-derived microsatellite primer OMO392, can potentially be used to facilitate marker-assisted selection regarding the fast growth of *O. niloticus*.

Keyword: EST-derived microsatellite marker, *Oreochromis niloticus*, Polymorphic loci, Allele, Genotype

Introduction

Nile tilapia (*O. niloticus*) has become one of the most important fresh water finfish species in aquaculture [1]. With a global production of about 2.8 million metric tons in 2008 [2], it was estimated to increase to 8.89 million metric tons by the year of 2020 [3]. However, Yue *et al.* [4] stated that sustainable aquaculture played an important role in transition to a more environmentally and economically viable production by genetic improvement [5]. The production of tilapia has already been under improvement through breeding [6,7]. However, the aquaculture industries of tilapia are still facing the problem of slow growth fish [8]. With the rapid expansion of next-generation sequencing technologies [9], marker-assisted selection and genomic selection are considered as a potential tool to speed up the genetic improvement of tilapia [10,11]. The marker-assisted selection is an effective method in

determining the interested trait, such as growth [12]. It is ideal for various types of applications in tilapia genetics especially in genomic mapping and discovery of QTL [13]. Therefore, their co-dominant in nature, high allelic variation, and polymorphism are indicating imperative for successful growth rate [14-16]. Polymorphic DNA markers, such as microsatellites or SNPs (Single Nucleotide Polymorphisms), can be used to analyze the association between markers and traits [17]. Although tandem nucleotide repeats are present in coding regions of the genome most microsatellites markers are neutral or Type II markers [18]. Because of their location in well-conserved regions of the genome, cDNA-derived microsatellites are expected to be conserved in across closely related species and represent a potential source of Type I markers [18]; however, EST-derived microsatellite markers are predicted to be relatively less polymorphic than those derived from genomic DNA [19,20]. In this study, we focused on the growth related EST-derived microsatellites marker in Nile tilapia. We studied the frequency of allele and genotype of microsatellite marker concentrating on the association between these makers and growth related allele and genotype to find a satisfactory way regarding growth based genetic improvement of the fish. This study focused on EST-derived microsatellite marker that was selected in consideration of the criteria of linkage group, species and growth related functions [21]. EST sequences are usually derived from cDNA sequence that are highly polymorphic in nature and play significant part in gene expression, developmental stages and under different environmental conditions [22]. In addition, EST sequences provide crucial information for microarray creation, RNA-sequencing analysis, and genome annotation [23]. It has also been specified that ESTs are useful for high-quality reference-guided assembly of next-generation sequencer-generated short reads [24]. Furthermore, it provides a unique idea for understanding of genetic mechanisms [25]. Hagen-Larsen *et al*. [26] stated that one strategy for identifying the genes expressed in specific life stages and tissues is to use expressed sequence tags or ESTs. These are short stretches of single pass sequences obtained from sequencing cDNA, and are widely used for gene discovery, mapping, polymorphism analysis, expression studies and gene prediction. Gene discovery methods using ESTs include hunting for new members of gene families in the same species (paralogues), for functionally equivalent genes in other species (orthologues), or even for alternatively spliced forms of known genes. ESTs are also used to predict or refine computational predictions of the location of genes in genomic DNA. The aim of this study is to identify growth-related EST-derived microsatellite marker in *O. niloticus.*

Materials and methods

Experimental animals

Brood fish of Nile tilapia (*O. niloticus*) of Thai strain (GIFT-derived *Chitralada* 3) were obtained from 5 different fishery stations in Thailand. The stations included Uttaradit, Pathum Thani, Phetchaburi, Buri Ram and Chumphon. The fish were reared in the experimental stations for 4 months to reach the maturation stage. Then, the selection of best male and female pairs with a ratio of 1:1 was conducted to produce 5 family progenies by using single pair mating design crossing. The offspring were primarily reared for 35 days at Chumphon Fishery Station and 60 fry from each family of a total of 25 families were brought to aquaculture laboratory at Walailak University. The fry were, then, grown until they reached the age of 83 days old. The water quality parameters including pH, temperature, and alkalinity were measured and adjusted to obtain the suitable rearing conditions. The monitored pH was 7.2 to 7.5, temperature was 27 to 30 °C, and alkalinity was 80 - 100 ppm.

Quantitative traits measurements

The growth performances of Nile tilapia samples from different families were measured in terms of body weight, number of fishes available and survival rate (%) after culture period. Meanwhile, the final mean weight ($\bar{x}$) and standard deviation (SD) were analyzed and used as selection criteria to evaluate the fast growth (extremely high growth; $\bar{x}$ + SD) and slow growth (extremely less growth; $\bar{x}$ - SD) of each family. However, the number of fish available in each group was also considered as representative families for further DNA analysis.

Sample collection and DNA extraction

The fin samples of 5 fish from fast and slow growth of each family were collected for DNA isolation and EST-derived microsatellites analysis. Fin tissues were used for DNA extraction following method of Wuthisuthimethavee [27] with some modifications. The quantity and quality of extracted DNA were examined by Spectrophotometers (Thermo Scientific NanoDrop 2000, United States) and 1 % Agarose gel electrophoresis, respectively.

Microsatellite analysis

The 19 pairs of EST's microsatellite primer were selected based on growth related to the genes from 125 EST's microsatellite primer [21], providing that the level of heterozygosity and good amplification were obtained. The criteria for selection of EST's microsatellite markers include linkage map of chromosome, polymorphic loci, growth trait related functions, information of *O. niloticus* and associated species, number of repeats and flanking region. However, out of 19 pairs of EST sequences, 18 were directly involved with growth regulatory pathways including growth hormone regulatory primer OMO392. On the other hand, OMO337 is to some extent indirectly linked in terms of growth regulatory functions. The polymerase chain reaction was performed in a 10 µl reaction volume with a final concentration of 0.2 µM forward and reverse primer respectively, 5 x HOT FIREPol® (Solis BioDyne, Tartu Estonia) Blend Master Mix, 1 µl of DNA template, with nuclease free water was added until the final volume of 10 µl. The PCR cycling conditions were started with pre-denaturing with a step of 95 °C for 15 minutes to activate *Taq* polymerase. The processes were then followed by 35 cycles of denaturing with a step of 30 seconds at 95 °C, annealing of 30 s at 50 - 60 °C (depending on the Tm °C of each primer), and extension of 30 seconds at 72 °C. Finally elongation step of 7 min at 72 °C of PCR reaction was performed. The PCR products were then visualized on 1 % agarose gel electrophoresis. Electrophoretic separations of amplification of PCR products were performed using polyacrylamide gel electrophoresis and silver staining. The bands representing alleles at the microsatellite loci were scored based on their position and designated as alphabetical order from the bottom to the top of the gel.

Association study between EST-Microsatellite marker and growth

The associations between each EST-derived microsatellite allele, genotype and growth data were analyzed by using one-way analysis of variance [28,29]. The f-value was used to illustrate the variation between sample means and variation within the samples. The p-value was used to determine statistical value on the basis of significance. The level of significant of p-value was used 0.05 as the rejection or acceptance of the association between microsatellite allele and genotype related to growth of Nile tilapia.

Results

Experimental animals

At the age of 83 days old, the mean final weight ($\bar{x}$), standard deviation (SD), number of fishes available for fast and slow growth and survival rate were analyzed as shown in **Table 1**. From the selection criteria, only 2 families (F1, F11) met the criteria as representative families regarding to the numbers of fish for fast growth and slow growth at least 5 individuals.

In case of F24, the standard deviation was found higher than that of mean that affected the slow growth sample selection. Regarding this issue, no fish was grouped for slow growth of family F24 and the weight lower than 1 g was potentially selected for family F24 for slow growth. Finally, a total of 30 samples from 3 families of F1, F11 and F24 (5 fish from fast and slow growth) were chosen for DNA analysis and association study.

Table 1 Selection of fast and slow growth of *O. niloticus* samples using final weight (mean and SD), numbers of fish available and survival rates at 83 days old.

Source	Family	Final mean weight (g)	SD	No. of fish assigned as Fast	No. of fish assigned as Slow	No. of fish at 83 days old	Survival Rate (%)
Chum	**F_1**	**5.65**	**4.68**	**8**	**5**	**46**	**95.83**
Buri	F_2	11.03	9.48	5	3	36	97.30
Utta	F_3	10.94	8.55	6	4	33	91.67
Pat	F_4	7.63	8.86	3	0	24	68.57
Pat	F_5	7.42	7.56	5	0	31	91.18
Chum	F_6	10.65	10.89	4	0	33	89.19
Chum	F_7	8.97	10.54	5	0	28	73.68
Chum	F_8	7.51	9.14	5	0	23	79.31
Pet	F_9	11.46	10.95	4	0	30	96.77
Utta	F_{10}	9.36	8.49	6	2	26	89.66
Pet	**F_{11}**	**9.32**	**6.98**	**6**	**8**	**34**	**85.00**
Buri	F_{12}	5.82	5.77	4	0	27	100.00
Pet	F_{13}	4.56	4.60	3	0	29	85.29
Utta	F_{14}	4.09	4.91	2	0	20	68.97
Buri	F_{15}	8.14	9.51	4	0	25	78.13
Chum	F_{16}	5.83	5.05	5	1	31	72.09
Chum	F_{17}	9.25	9.07	3	0	17	51.52
Pet	F_{18}	3.07	2.90	3	0	26	59.09
Pat	F_{19}	3.84	3.58	4	0	25	73.53
Chum	F_{20}	3.41	3.33	2	0	29	67.44
Utta	F_{21}	8.90	7.67	4	2	14	36.84
Utta	F_{22}	7.39	6.13	5	0	33	64.71
Buri	F_{23}	8.24	5.41	6	3	45	88.24
Chum	**F_{24}**	**6.12**	**8.10**	**5**	**0**	**34**	**79.07**
Chum	F_{25}	6.78	5.40	6	2	36	75.00

Chum = Chumphon, Buri = Buriram, Utta = Uttaradit, Pat = Patumtanni and Pet = Petchaburi.

PCR amplification

The 27 EST-derived microsatellite primers were screened with Nile tilapia DNA samples. Among them, 19 EST-derived microsatellite were successfully optimized. The size of PCR products varied from 133 to 444 bp and the banding pattern of 2 EST-microsatellite primers, OMO049 and OMO193 having PCR product lengths of 224 bp and 349 bp as shown in **Figure 1**.

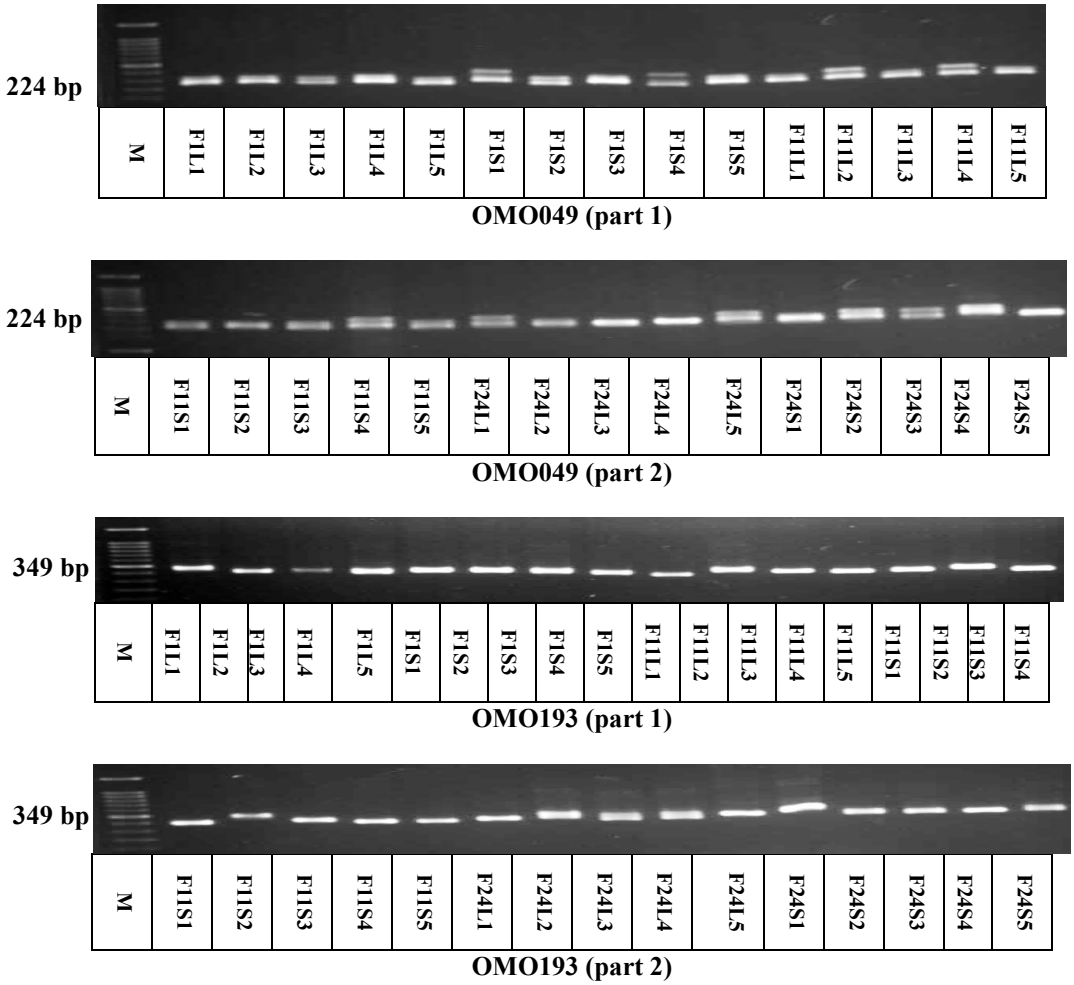
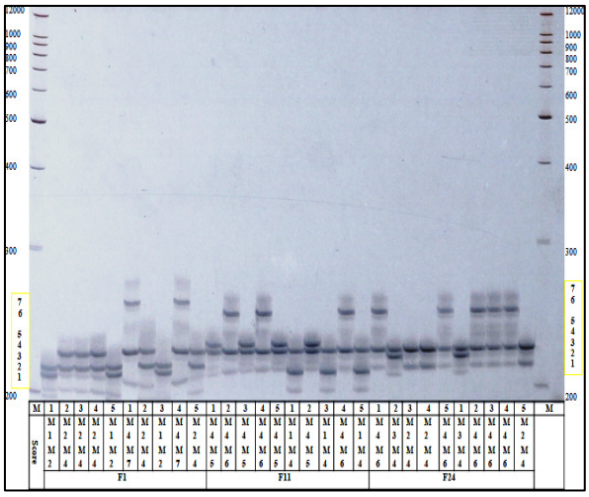


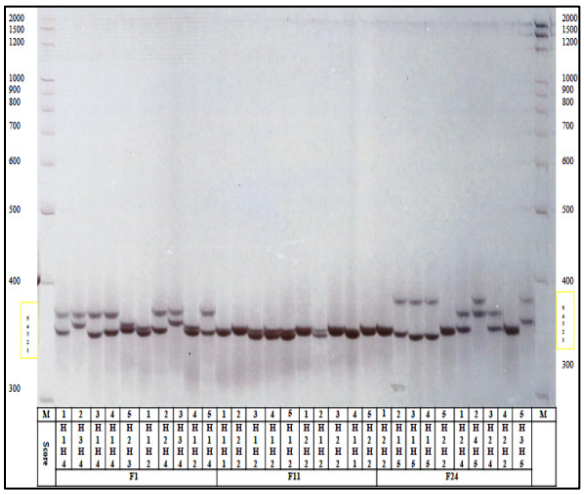
Figure 1 PCR amplification products on 1 % agarose gel electrophoresis; OMO049 and OMO193 are EST-derived microsatellite primers, 100 bp DNA ladder (M) and F1L1-F24S5 are DNA of *O. niloticus* samples. Amplicon sizes are indicated on the left.

Polyacrylamide gel electrophoresis

Amplified PCR products were then run on 5 % denaturing polyacrylamide gel and subsequent visualization were performed using silver staining methods. Among 19 EST-derived microsatellite primers, 14 loci were resulted in clear banding pattern and visualized through staining. Banding patterns of OMO049 and OMO193 on 5 % denaturing polyacrylamide gel were shown in **Figure 2**.



OMO049



OMO193

Figure 2 Polymorphism of PCR amplification products on 5 % polyacrylamide gel electrophoresis; OMO049 and OMO193 are EST-microsatellite primers, 100 bp DNA ladder (M) and F1L1F24S5 are DNA of *O. niloticus* samples. Amplicon sizes are indicated on the left.

Allele frequency

A total of 51 alleles were resulted from 14 EST-derived microsatellite loci. The number of allele varied from 2 to 7 (**Table 2**). The average number of allele in each locus was estimated as 3.65. From allele frequency, 25 alleles leaded to the allele frequency of 0.25 in each locus whereas alleles 10 were estimated as the frequency of less than 0.1. Moreover, the highest number of allele was observed in OMO049 and the lowest numbers of allele were detected in primer OMO198, OMO335 and OMO374.

Table 2 Allele frequency of 14 EST-derived microsatellite primers **(**allele frequency ≥ 0.25 were highlighted).

Primers	Number of allele						
	1	**2**	**3**	**4**	**5**	**6**	**7**
OMO392	0.130	**0.310**	**0.560**				
OMO051	0.084	**0.500**	**0.250**	0.166			
OMO072	**0.310**	0.160	0.180	**0.350**			
OMO097	0.084	0.050	**0.450**	**0.416**			
OMO327	0.200	**0.250**	0.050	**0.400**	0.100		
OMO277	0.150	**0.590**	0.100	0.160			
OMO122	**0.817**	0.016	0.167				
OMO193	**0.283**	**0.400**	0.067	0.167	0.083		
OMO198	**0.360**	**0.640**					
OMO200	0.174	**0.576**	**0.250**				
OMO335	**0.600**	**0.400**					
OMO374	**0.570**	**0.430**					
OMO049	0.100	0.184	0.033	**0.450**	0.067	0.133	**0.033**
OMO069	0.184	**0.400**	**0.416**				

Genotype frequency

The total number of the observed genotype was 79 from 14 EST-derived microsatellite loci. The genotype number was ranged from 3 to 10. The average number of the genotype was estimated to be 5.65. Maximum numbers of genotype were found in OMO327 and OMO193 whereas minimum numbers of genotype were resulted in OMO392, OMO97, OMO198, OMO335 and OMO374 (**Table 3**).

Table 3 Genotype frequency of 14 polymorphic EST-microsatellite loci.

Name of primer	Name of genotype	No. of genotype		Frequency of genotype
OMO 392	A1A1	4	3	0.133
	A2A3	19		0.633
	A3A3	7		0.233
OMO 051	B1B2	3	6	0.100
	B1B3	2		0.067
	B2B2	10		0.333
	B2B3	5		0.167
	B2B4	2		0.067
	B3B4	8		0.266
OMO 072	C1C1	3	9	0.100
	C1C2	6		0.200
	C1C3	1		0.033
	C1C4	7		0.233
	C2C3	2		0.067
	C2C4	2		0.067
	C3C3	2		0.067
	C3C4	4		0.133
	C4C4	3		0.100
OMO 193	H1H1	2	10	0.067
	H1H2	6		0.200
	H1H4	4		0.133
	H1H5	3		0.100
	H2H2	7		0.233
	H2H3	1		0.033
	H2H4	3		0.100
	H3H4	2		0.067
	H3H5	1		0.033
	H4H5	1		0.033
OMO 198	I1I1	3	3	0.100
	I1I2	16		0.533
	I2I2	11		0.367
OMO 200	J1J1	3	6	0.115
	J1J2	1		0.038
	J1J3	2		0.077
	J2J2	12		0.462
	J2J3	5		0.192

Name of primer	Name of genotype	No. of genotype		Frequency of genotype
OMO 097	D1D3	5	3	0.167
	D2D4	3		0.100
	D3D4	22		0.733
OMO 327	E1E1	1	10	0.033
	E1E2	3		0.100
	E1E4	7		0.233
	E2E2	3		0.100
	E2E3	1		0.033
	E2E4	4		0.133
	E2E5	1		0.033
	E3E5	2		0.067
	E4E4	5		0.167
	E4E5	3		0.100
OMO 277	F1F2	2	7	0.067
	F1F3	3		0.100
	F1F4	4		0.133
	F2F2	13		0.433
	F2F3	3		0.100
	F2F4	4		0.133
	F4F4	1		0.033
OMO 122	G1G1	20	4	0.667
	G1G2	1		0.033
	G1G3	8		0.267
	G3G3	1		0.033
OMO 335	J3J3	3	3	0.115
	K1K1	14		0.466
	K1K2	8		0.267
	K2K2	8		0.267
OMO 374	L1L1	12	3	0.400
	L1L2	10		0.333
	L2L2	8		0.267
OMO 049	M1M2	3	7	0.100
	M1M4	3		0.100
	M2M4	8		0.267
	M3M5	2		0.066
	M4M5	4		0.133
	M4M6	8		0.267
	M4M7	2		0.067
OMO 069	N1N2	5	5	0.167
	N1N3	6		0.200
	N2N2	1		0.033
	N2N3	17		0.567
	N3N3	1		0.033

Allele association

Out of 51 alleles from 14 polymorphic loci, 25 alleles were used to analyze one-way ANOVA considering the frequency of allele exceeding 0.25. From them, only 1 significant allele A3 representing OMO392 was shown association with growth of *O. niloticus* (**Table 4**).

Table 4 25 selected allele associated with growth of *O. niloticus* among 3 different families, f-value represents the comparison of the joint effect of all variables whereas as p-value indicates the level of significant (≤ 0.05).

Allele	Growth	
	F	P
A2	1.217	0.279
A3	**5.244**	**0.030**
B2	0.039	0.845
B3	0.010	0.922
C1	0.183	0.672
C4	0.290	0.595
D3	0.472	0.498
D4	0.026	0.872
E2	1.608	0.215
E4	0.076	0.785

Allele	Growth	
	F	P
F2	0.639	0.431
G1	0.852	0.364
H1	3.438	0.074
H2	0.830	0.370
I1	0.041	0.840
I2	0.620	0.438
J2	0.418	0.523
J3	1.895	0.180
K1	0.068	0.796
K2	0.091	0.766
L1	0.011	0.918
L2	0.486	0.492
M4	0.071	0.792
N2	2.736	0.109
N3	0.004	0.947

Genotype association

Genotype association study was further carried out for 14 polymorphic loci with frequency exceeding 0.25 from a total number of 79 genotypes. Among them, no significant genotype was observed in growth of *O. niloticus*. This suggests that the genotype regarding 19 EST-microsatellite markers are not associated with *O. niloticus* growth.

Table 5 19 selected genotype associated with growth of *O. niloticus* among 3 different families, f- value represents the comparison of the joint effect of all variables whereas as p-value indicates the level of significant (≤ 0.05).

Genotype	Growth	
	F	**P**
A2A3	1.217	0.279
B2B2	0.097	0.757
B3B4	0.034	0.854
D3D4	0.106	0.747
F2F2	0.128	0.723
G1G1	0.010	0.919
G1G3	0.419	0.523
I1I2	0.074	0.787
I2I2	0.041	0.840
J2J2	4.060	0.054
K1K1	0.091	0.766
K1K2	0.006	0.938
K2K2	0.068	0.796
L1L1	0.486	0.492
L1L2	0.390	0.537
L2L2	0.011	0.918
M2M4	0.002	0.961
M4M6	0.167	0.686
N2N3	2.112	0.157

Identification of EST-Microsatellite Marker

Out of total 14 polymorphic loci, 1 EST-derived microsatellite marker namely OMO392 was found to be related in terms of significant allelic association. Alternatively, no primer was found to be associated regarding genotypic association with the growth of *O. niloticus* (**Table 5**). However, A3 allele of OMO392 was considered specific to fast growth group ($P < 0.05$) and overall growth of *O. niloticus* ($P < 0.05$). That means, OMO392 was further indicated a potential DNA marker for the fast growth of Nile tilapia. On the other hand, it was found insignificant in slow growth group of *O. niloticus* ($P > 0.05$). In contrast, genotypic association of 19 genotypes was specified no significant association with growth of *O. niloticus*. Accumulating all allelic and genotypic association, it proposes that, OMO392 can be used effectively for the identification of the fast growth of *O. niloticus*.

Discussion

We examined 19 EST-derived microsatellite primers with the focus on allelic and genotypic association in relation with growth trait of *O. niloticus*. Out of 19 loci, it was found that EST-microsatellite marker namely OMO392 was associated in terms of significant allelic association. An EST-derived microsatellite locus OMO392 was represented 1 significant allele that is A3, considering fast growth group and growth of Nile tilapia. Yet, it was found insignificant in case of slow growth of Nile tilapia. Then again, genotypic association indicated no significant genotype. However, considering the functions of identified locus OMO392, it contributes the regulation of energy homeostasis resulting from the secretion of GH via pituitary gland. The growth hormones-Insulin-like Growth Factor I is another most important growth axis in fish. In this study, OMO392 has identified as growth hormone related EST-derived microsatellite marker. According to our results, OMO392 indicated significant A3 in both fast and overall growth. It suggests that OMO392 potentially facilitates EST-derived microsatellite assisted selection for improving slow growth. In this regard, a significant association was revealed in growth with a major QTL and GHR2 in tilapia. In contrast, the variations of GH, GHR and other genes in the growth hormone pathway bring about serious abnormal growth identified in fishes [30]. It is therefore discovered that endocrine control of growth and metabolism is interrelated [31]. Loci affecting traits of economic value have been detected and mapped in a variety of livestock species [32]. In other aquaculture species such as *Scylla paramamosain*, the study found the EST-derived microsatellite marker and their association with the growth performance [33]. A similar trend was found in other aquatic animals such as GIFT in common carp and Japanese scallop. Pereira *et al*. [34] observed association of GH and IGF-1 polymorphisms with growth traits in synthetic beef cattle breed effects of growth hormone and insulin-like growth factor 1 polymorphisms. Sánchez-Ramos *et al*. [35] also found significant association between MSTN-1 gene polymorphism and growth traits in gilthead seabream considering the growth hormone, IGF-1, MSTN-1, PRL, and SL genes. An association was also found in MSTN-1 gene polymorphism and growth traits. Velan *et al.* [36] reported the association between polymorphism in the Prolactin I promoter and growth of tilapia grown in saline water, but no association was observed between the polymorphism in the PRL I promoter and the expression of the gene. Among fish of the similar genus, *O. mossambicus* is considered more salt tolerant than *O. niloticus* in term of the improved growth performance in saline water [37]. Li *et al.* [38] also focused on the mapping QTL for sex and growth traits in salt-tolerant Tilapia. In salt-tolerant tilapia, different set of genes 'switches' control the growth in males and females. Ma *et al*. [39] characterized ZFAND3 gene mapped in the sex-determining locus in hybrid tilapia. Manzon [40] found that MSTN-1 was expressed mainly in skeletal muscle, at both adult and juvenile stages, and MSTN-2 was expressed almost exclusively in the central nervous system. An association between this candidate gene and growth is not surprising as myostatin acts as a negative regulator of skeletal muscle growth [41]. Accumulating of all the reviews, it suggests that growth hormone modulatory pathway is associated with allele A3 of EST-derived microsatellite marker OMO392 in *O. niloticus*. As far as we know, growth traits are quantitative traits and several to numerous genes possibly control them. These genes may have segregated and/or recombined among different generations. Hence, we should investigate the replicability of this marker in different families and

populations, and evaluate the correlation across different generations. This target microsatellite locus will thus be applied for the practical selection of *O. niloticus* for growth performance.

Conclusions

This study focused on 19 EST-microsatellite primers resulting 14 polymorphic EST-microsatellite loci for *O. niloticus*, and revealed significant ($P < 0.05$) allelic association with growth and fast growth group. As for the association between alleles and growth, allele A3 was found to relate with growth and fast growth group, whereas insignificant association was identified in slow growth group suggesting the EST-microsatellite loci OMO392. On the other hand, from genotypic association, no significant genotypes of the mentioned 14 polymorphic EST-microsatellite loci were found in growth of *O. niloticus*. It can be concluded that the identified allele A3, which designated the growth hormone related EST-derived microsatellite primer OMO392, can potentially be used to facilitate marker-assisted selection regarding fast growth of *O. niloticus*.

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