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Prolactin I microsatellite as genetic markers for characterization of five *Oreochromis* tilapia species and two *Oreochromis niloticus* strains

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Abstract

Hybrid tilapia is a major aquaculture food fish species in developing countries. The development of molecular markers to characterize and trace tilapia species is necessary to improve tilapia quality and enhance the competitive advantage of the aquaculture industry. Microsatellite markers have been suggested to assist breeding, species identification, and traceability system for tilapia. We used six microsatellites markers located within growth-related genes to discriminate between several tilapia species. We found that a combination of two microsatellites markers located within the proximal promoter of the prolactin I (PRL I) gene, PRL I-MS01 and PRL I-MS02, were able to discriminate between five Oreochromis tilapia species (*O. mossambicus, O. aureus, O. niloticus, O. hornorum* and *O. spilurus*) and two *O. niloticus* strains that exhibit the distinctive growth traits. Furthermore, we found that PRL I-MS01 microsatellite marker was able to trace parental origin of hybrid tilapia. Thus, this marker is a potentially beneficial tool for a tilapia traceability system. We conclude that the GT tandem repeats in PRL I-MS01 and CA tandem repeats in PRL I-MS02 are useful genetic markers to characterize diverse tilapia species, assist in the genetic tracing and conventional breeding of superior strains and strengthen the management of the tilapia aquaculture industry. There was strong relationship between the cold and both temperature at death and cumulative degree hours represented by negative correlation coefficient for all tested fish. There was no correlation between cold tolerance and fish size for all tested fish. The cooling degree hours were significantly different between the selected and non-selected *O. niloticus* ($P<0.005$). The selected *O. aureus* exhibited greater cold tolerance than the non-selected and death began at 14.1°C, while non-selected occurred at 15.2°C.

Keywords: Prolactin; Tilapia; Microsatellite; Growth; Traceability

Introduction

Tilapia is an important aquacultural fish species globally because of its large size, high reproductive rate, rapid growth and palatability. According to the Food and Agriculture Organization (FAO), global tilapia production exceeded more than 4.2 million metric tons with a sales value of more than $5 billion in 2012. Because tilapia products are known for highly palatable taste and hygienic quality, therefore they have become popular worldwide. As a result, tilapia has become the one of the most important exported fish species and is a major source of income in developing countries. In general, high-quality tilapia products are derived from hybrids of superior tilapia strains that have been developed using advanced aquacultural technology. Most of these superior tilapia strains are bred using traditional generational selection. The characterization and maintenance of these superior tilapia strains are important to enhance the competitive advantage in aquaculture industry. Furthermore, food contamination occurs frequently in fish and fishery products. Consumers are increasingly concerned about what they eat and whether their foods are from high-quality and safe sources. A traceability system for the fishery chain is the most important tool to manage food safety [1]. Such a system can trace fish and fishery products to their farm of origin if toxins, diseases or unsatisfactory qualities are detected in the market. Most traceability schemes involve documentation at every processing step. However, it is difficult to verify theses records in the event of fraudulent activities. The development of molecular marker technology is needed to facilitate conventional selective breeding, sustain the tilapia aquaculture industry, promote tilapia quality and confirm traceability documentation [2].

Several molecular markers have been used to characterize tilapia species and permit tilapia traceability, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) and microsatellites [3-8]. Among these molecular markers, microsatellites have been commonly used because they are highly reproducible, polymorphic, co-dominant and widely distributed throughout the genome [9]. Microsatellites are repeated sequences of DNA throughout the genome. Most of microsatellite loci are associated with anonymous genomic segments [9]. However, an increasing body of research has demonstrated that some microsatellites are located with promoter and transcribed regions of genome and may be involved in regulating gene expression and function [10-13]. These functional microsatellites may cause distinct phenotypes or influence physiological functioning by directly or indirectly affecting the expression and function of a given gene [11,14]. Therefore, functional microsatellites can be used as genetic markers to characterize the phenotypes that result from the expressional variation of a given gene.

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Nile tilapia can live longer than 10 years. Food availability and water temperature appear to be the limiting factors to growth for *O. niloticus*. Optimal growth is achieved at 28-36°C and declines with decreasing temperature. The ability to vary their diet may also result in variation in growth. In aquaculture ponds, *O. niloticus* can reach sexual maturity at the age of 5-6 months. Growth enhancement is a desired phenotype for tilapia in the aquaculture industry. Several growth-related genes are involved in cell proliferation and somatic growth, including growth hormone (GH), insulin-like growth factors-2 (IGF-2), prolactin-I (PRL-I) and insulin gene [15-18]. In the present study, we elucidate the ability of six microsatellite markers within these growth-related genes to characterize several tilapia species. We found that five *Oreochromis* species and two strains of *O. niloticus* can be differentiated by the microsatellite markers PRL I-MS01 and PRL I-MS02. The parentage of hybrid tilapia can also be traced by PRL I-MS01. We conclude that PRL I microsatellite markers are useful genetic markers for traceability systems and for breeding superior strains in the tilapia aquaculture industry.

Materials and Methods

Tilapia species and cultivation

Five *Oreochromis* tilapia species, [*O. mossambicus* (M), *O. aureus* (A), *O. hornorum* (H), *O. spilurus* (S), *O. niloticus* (N)] and one *O. niloticus* (N_F) strain with a rapid-growth phenotype were obtained from the Freshwater Aquaculture Research Center (FARC) of the Aquaculture Research Institute, Council of Agriculture (Lukang, Changhua, Taiwan). Fish were maintained in one-ton tanks equipped with circulating water at 25°C.

Genotypic analysis of tilapia using growth-related microsatellite markers

Genomic DNA of diverse *Oreochromis* tilapia species was extracted from 20 mg of muscle tissue using the Master Pure™ Complete DNA Purification Kit (Epicentre Biotechnologies, Wisconsin, WI, USA) according to the manufacturer's protocol. Each genomic DNA sample was quantified using a Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA). Genomic DNA samples were adjusted to a final concentration of 100 ng/L. Primer sequences for growth-related microsatellite loci were designed according to the methods of Yue and Orban [19] and are listed in Table 1. PCR was performed in 50-μl volumes containing 1×PCR buffer, 2 mM $MgSO_4$, 0.2 mM dNTP, 200 nM of primers, 1.0 unit of Platinum *Taq* high-fidelity DNA polymerase and 100 ng of genomic DNA. PCR was carried out as follows: 94°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; and a final cycle at 72°C for 10 min. A 1-μl aliquot of each PCR product was used for genotypic analysis in a 2100 Bioanalyzer equipped with Expert version B.01.02.S1136 software and a DNA 1000 Lab Chip kit (Agilent Technologies, Palo Alto, CA, USA). The lengths of PCR products were calculated by the software based on the product retention times compared to size standards. A total of 25 samples (n=25) of each tilapia species were genotyped.

Bardakci [5] reported that feed treated with 17 alpha methyl testosterone (MT) is used to manipulate the gender of early tilapia fry. A series of experiments demonstrated that the analytical procedure for detecting MT in feeds lacked high precision and on average biased results. Low MT was uniformly mixed with feed by spraying an MT-alcohol solution on feed while it was blended in an industrial ribbon mixer. Alcohol volumes ranging from 15 to 150 ml/kg were equally effective at dispersing MT in feed. The concentrations of MT in feeds consistently declined over time if the storage temperature was 25°C or higher. Freezing preserved the MT in feed, and the refrigeration of feed fabricated to contain 60 mg MT/kg maintained acceptable MT concentrations during 6 months of storage.

Growth performance experiment

Five experimental groups [progeny of ♀*O. aureus* (A) mated to ♂*O. aureus* (A), ♀*O. niloticus* (N) mated to ♂*O. niloticus* (N), ♀*O. niloticus* (N_F) mated to ♂*O. niloticus* (N_F), ♀*O. niloticus* (N) mated to ♂*O. aureus* (A) and ♀*O. niloticus* (N_F) mated to ♂*O. aureus* (A) were used to evaluate growth performance. Larvae from each group were grown for one month in temperature-controlled 20-L plastic aquaria. Subsequently, 120 juvenile fish from each group were shifted to a pond (5 m x 3 m x 1 m) with a circulating water system and fed a commercial fodder (23% protein content) at a rate of approximately 1.5-5% of fish body weight twice daily for 20 weeks (n=120; duplicate). Thirty fish from each experimental group were randomly sampled on one monthly basis to determine body length, total body length and body weight. Body length and total body length were measured as the distance from the tip of the mouth to the hypural bone and from the tip of the month to the caudal fin, respectively [20]. Specific growth rate (SGR) was calculated using the following equation: SGR (%/day)=[(ln W_2–ln W_1)/t_2–t_1]×100, where t_1 is the time at the beginning of growth, t_2 is the time at the end of growth, W_1 is the average body weight or body length at t_1, and W_2 is the average body weight or body length at t_2.

Parentage assignment

Hybrid tilapia population obtained by mating *O. niloticus* strains (N or N_F) to *O. aureus* (A) (NxA or N_FxA) were used to study parentage assignment with PRL I microsatellite markers. Genomic DNA aliquots (100 ng) from hybrid tilapia and their parental strains were used as

Primer name	Primer sequence (5'-3')	Position within gene	Repeat motif	References (Gene Bank Accession no.)
PRL I-MS01	F:GTTAGCCCCCTCCTCACTCT	promoter of prolactin	GT	X92380
	R:ACCTTGCTCGTCACACCTG			
PRL I-MS02	F:TCGTGTCTTGTGGGGAAACC	promoter of prolactin	CA	X92380
	R:TGAATGGATGCAACAGGATG			
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			
GH-MS01	F:CCAGCCATGAACTCAGGTAAGACA	intron 1 of GH	TGTC	M97765
	R:TGCTGAGAGGAGACGCCCAAACA			
ISP-MS01	F:GAGCTGAGCAGATGGAGCAGAAG	5'UTR of insulin precursor	CA	AF038123
	F:GAGCTGAGCAGATGGAGCAGAAG			

Table 1: Primer sequences used in this study and repeat motifs of six microsatellites located within growth-related genes.

PCR templates. A 1-µl aliquot of each PCR product was subjected to genotypic analysis using a 2100 Bioanalyzer equipped with Expert version B.01.02.S1136 software and DNA 1000 LabChip kits (Agilent Technologies).

Identification of tandem repeats in PRL I microsatellite loci

Genomic DNA was extracted from the progeny of NxN and N_FxN_F tilapia as described above (Genotypic analysis of tilapia using growth-related microsatellite markers). Genomic DNA from both populations was used in PCR reactions with PRL I-MS01 and PRL I-MS02 primers. The major PCR product bands were separated by electrophoresis on a 3% agarose gel and then purified using a QIAquick gel extraction kit (Qiagen, Düsseldorf, Germany). PCR products containing tandem repeats were ligated into the pGEMT-T Easy vector (Promega, Madision, WI, USA) for sequencing.

Results

PRL I microsatellites can be used to discriminate between tilapia species

Tilapia species differ in growth performance due to their distinct genetic backgrounds. We elucidate the ability of six growth-related microsatellites regions found in the growth-related gene IGF-II, PRL I, GH and insulin to discriminate between five *Oreochromis* tilapia species. Of these microsatellites loci, only two PRL I microsatellites were able to distinguish between different tilapia species. All tilapia species examined except *O. spirulus* could be identified using PRL I-MS01 microsatellite marker. Two major alleles of the PRL I-MS01 microsatellite locus (312 bp and 346 bp) were observed in *O. aureus*. Two alleles (290 bp and 320 bp) were observed in *O. mossambicus*. Two major alleles (302 bp and 338 bp) were observed in *O. hornorum*, and two alleles (260 bp and 288 bp) were observed in *O. niloticus*. Although two major alleles (286 bp and 318 bp) were found in *O. spirulus*; the allele sizes were too similar to distinguish in *O. mossambicus* (Figure 1A). In contrast to PRL I-MS01, the PRL I -MS02 microsatellite marker could differentiate only *O. spilurus* from the other tilapia species. Two major bands (288 bp and 316 bp) were detected in *O. spilurus*. However, the other four species exhibited similar allele patterns that could not be distinguished (Figure 1B). A few additional genotypes were observed in each species, but they did not affect our ability to distinguish each species. These results suggest that the combination of PRL I-MS01 and PRL I-MS02 can efficiently discriminate between the five tilapia species studies.

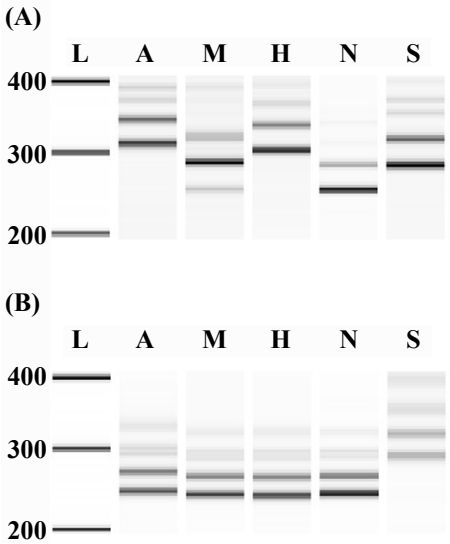

Figure 1: Characterization of *Oreochromis* tilapia species by PRL I microsatellite markers. PRL I-MS01 (A) and PRL I-MS02 (B) genotypes were determined using an Agilent 2100 Bioanalyzer. L: molecular weight ladder; A:*O. aureus*; M:*O. mossambicus*; H: *O. hornorum*; N: *O. niloticus*; S: *O. spilurus*.

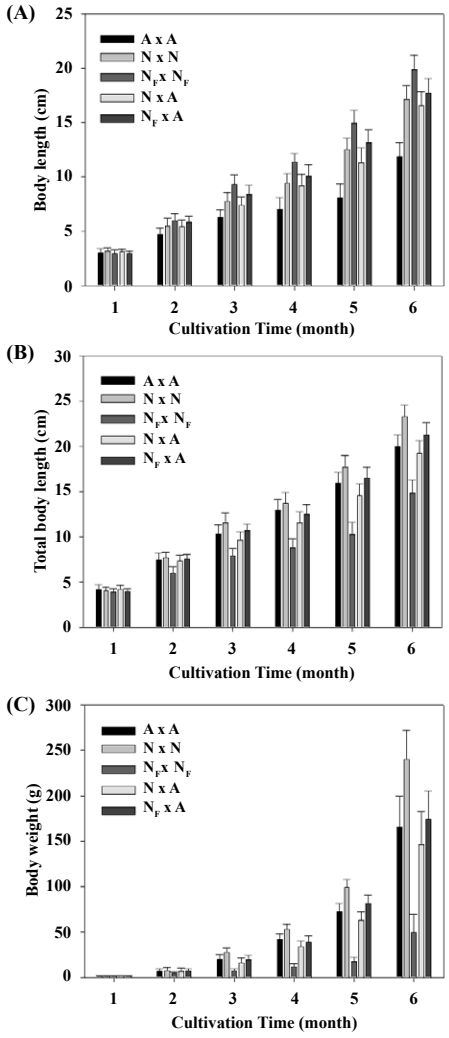

Figure 2: Comparison of growth performance between *O. aureus* (A), *O niloticus* (N), *O niloticus* (N_F) and hybrid tilapia. The phenotypic traits (mean ± SD) of body length (A), total body length (B) and body weight (C) were evaluated at one-month intervals for six months (n=60). AxA: progeny of ♀ *O. aureus* (A) mated to ♂ *O. aureus* (A). NxN: progeny of ♀ *O. niloticus* (N) mated to ♂ *O. niloticus* (N). N_FxN_F: progeny of ♀ *O. niloticus* (NF) mated to ♂ *O. niloticus* (NF). N x A: progeny of ♀ *O. niloticus* (N) mated to ♂ *O. aureus* (A). N_F x A: progeny of ♀ *O. niloticus* (N_F) mated to ♂ *O. aureus* (A).

The acclimatizing the fry was done at 20°C for 48 hours. After that aquarium water temperature was monitored each hour from the beginning to the end of the experiment. The temperature measurements were done hourly, while the dissolved oxygen (DO), pH, total ammonia, nitrate and nitrite were measured once a day, using a WTWR multi 340i meter and HACH kits. Dissolved oxygen ranged between 6.1 and 10.3 mg/l; pH, 7.9-8.3; ammonia, 0.01-0.1 mg/l; nitrate, 0.5-2 mg/l; and

nitrite, 0.01-0.02 mg/l. Aquaria were cleaned twice daily by suction to remove faeces. Water that was removed during aquarium cleaning was replaced with clean water pre-cooled to the same temperature with ice cubes.

Growth performance of *O. niloticus* strains

The N_F strain, which exhibited rapid growth compare to the original N strain, was developed through generational selective breeding. To demonstrate that the N_F strain possessed the rapid-growth phenotype, we measured the growth curve of N_F and N strain over one month. Most commercial tilapia is derived from hybrids of *O. niloticus* and *O. aureus*. Therefore, we also evaluated the growth performance of the hybrid progeny of N_FxA and NxA. The N_FxN_F progeny population exhibited better growth performance in body length and body weight than NxN population. Among hybrid populations, the N_FxA population showed better growth performance in body length and body weight than the NxA population. Growth performance traits increased in the following order: N_F x N_F>N_FxA>NxN>NxA>AxA (Figure 2). The order of SGR value based on body length or weight for each population was consistent with the growth performance. As shown in Table 2, N_F×N_F progeny exhibited the highest SGR value. Weight-based SGR values in decreasing order were as follows: 3.47% g/day for N_F×N_F; 3.18% g/day for N_F×A; 3.16% g/day for N×N; 2.94% g/day for N×A group; and 2.44% g/day for A×A. SGR values based on body length were also calculated. The N_F×N_F also exhibited the highest length-based SGR value. These results suggest that the rapid growth performance of N_F compared N is due to differences in genetic background.

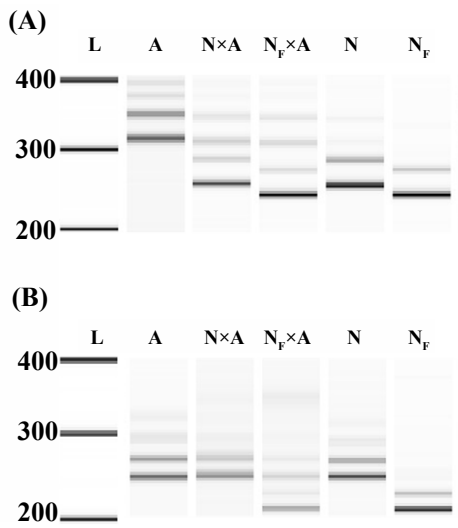

Figure 3: Traceability of hybrid tilapia using PRL I microsatellite markers. PRL I-MS01 (A) and PRL I-MS02 (B) genotypes were determined using an Agilent 2100 Bioanalyzer. NxA: progeny of ♀ *O. niloticus* (N) mated to ♂ *O. aureus* (A). N_FxA: progeny of ♀ *O. niloticus* (N_F) mated to ♂ *O. aureus*(A).A: paternal *O. aureus* (A); N: maternal *O. niloticus* (N); N_F: maternal *O. niloticus* (N_F).

PRL I microsatellite markers are useful to distinguish between *O. niloticus* strains

To distinguish between the two strains of *O. niloticus* (N_F and N), we analyzed the same six microsatellite loci within the growth related-genes IGF-II, PRL I, GH and insulin. The polymorphism and genotypic proportions in the N and N_F strains are shown in Table 3. IGF-II-MS03 was highly variable. A total of 19 genotypes were found in the two strains. We found eleven genotypes for PRL I-MS02, nine genotypes for GH-MS01, seven genotypes for PRL I-MS01, seven genotypes for ISP-MS01 and a six-allele locus for IGF II-MS01. Each genotype consists of two alleles at each locus, one smaller and one larger. Among these microsatellite markers, only PRL I-MS01 and PRL I-MS02 were able to discriminate between the N and N_F strains of *O. niloticus*. PRL I-MS01 genotypes included 258/284 bp, 260/286 bp, 260/288 bp, 264/288 bp and 264/290 bp in the N strain and 248/274 bp and 250/276 bp in the N_F strain. For PRL I-MS02, genotypes of the N strain group included 238/258 bp, 240/260 bp, 240/262 bp, 242/262 bp, 244/262 bp and 246/266 bp, while genotypes of the N_F strain included 202/222 bp, 204/222 bp, 206/222 bp and 208/224 bp. For example, PRL I-MS01 exhibited a genotype consisting of 248-bp and 274-bp alleles in the N_F strain and a genotype consisting of 260-bp and 288-bp alleles in N strain (Figure 3A). For PRL I-MS02, a genotype consisting of 206-bp and 222-bp alleles was observed in the N_F strain, while a genotype consist of 240-bp and 260-bp alleles was observed in the N strain (Figure 3B). These results demonstrate that PRL I microsatellite markers can efficiently discriminate between *O. niloticus* strains (N and N_F) based on electrophoretic genotype.

Tracing the parentage of hybrid tilapia

High proportions of commercial tilapia from Taiwan consist of hybrids of *O. aureus* and *O. niloticus*. To assess the practical ability of PRL I-MS01 and PRL I-MS02 to trace the parentage of hybrid tilapia, the genotypes of hybrid tilapia derived from mating of N and N_F mating with A were analyzed to confirm whether the alleles at each locus matched those of the parental strains. In hybrid tilapia derived from female N and male A parents, the PRL I-MS01 locus exhibited 260-bp and 312-bp alleles. In hybrid tilapia derived from female N_F and male A parents, the PRL-MS01 locus exhibited 248-bp and 312-bp alleles. PRL I-MS02 was not able to distinguish between the A and N strains. Therefore, this marker can be used to trace hybrid tilapia progeny derived only from N_F and A. In the latter population, PRL

Exp. group	Mean initial weight (g)[a]	Mean final weight (g)[b]	SGR % g/day[c]	Mean initial body length [32][a]	Mean final body length [32][b]	SGR % cm/day[c]
A x A	1.29 ± 0.35	49.85 ± 19.46	2.44	3.02 ± 0.38	11.84 ± 1.36	0.91
N x N	1.44 ± 0.37	165.27 ± 34.80	3.16	3.16 ± 0.32	17.18 ± 1.25	1.27
N_F x N_F	1.47 ± 0.26	240.47 ± 32.24	3.47	2.98 ± 0.34	19.88 ± 1.31	1.27
N x A	1.78 ± 0.27	146.67 ± 35.73	2.94	3.14 ± 0.22	16.55 ± 1.30	1.11
N_F x A	1.47 ± 0.33	174.02 ± 31.09	3.18	2.97 ± 0.23	17.77 ± 1.29	1.19

[a]Measured at the beginning of the growth period (one month).
[b]Measured at the end of the growth period (six month).
[c]Calculated over the growth period from one to six months.

Table 2: Comparison of specific growth rates (SGR%) based on body length and weight difference between*O. aureus* (A), *O. niloticus* (N), *O.niloticus* (N_F) and hybrid tilapia over a six-month periods.

Marker	Genotypes	N strain	N_F strain	Marker	Genotype	N strain	N_F strain
PRL I-MS01	248/274	0.00	0.68	IGFII-MS01	218/218	0.00	0.12
	250/276	0.00	0.32		218/238	0.08	0.00
	258/284	0.12	0.00		220/220	0.36	0.24
	260/286	0.20	0.00		220/238	0.00	0.04
	260/288	0.40	0.00		222/222	0.56	0.56
	264/288	0.16	0.00		222/238	0.00	0.04
	264/290	0.12	0.00				
PRL I-MS02	202/222	0.00	0.12	IGFII-MS03	192/234	0.00	0.04
	204/222	0.00	0.16		200/222	0.00	0.05
	206/222	0.00	0.36		214/214	0.00	0.14
	206/224	0.00	0.32		214/234	0.00	0.19
	208/224	0.00	0.04		216/216	0.04	0.05
	238/258	0.04	0.00		216/224	0.12	0.00
	240/260	0.28	0.00		216/236	0.00	0.14
	240/262	0.08	0.00		216/256	0.12	0.05
	242/262	0.20	0.00		220/220	0.04	0.14
	244/262	0.20	0.00		220/230	0.28	0.00
	246/266	0.20	0.00		220/240	0.00	0.05
GH-MS01	158/158	0.36	0.08		220/262	0.04	0.00
	158/226	0.00	0.04		220/276	0.08	0.00
	160/160	0.00	0.28		222/230	0.00	0.10
	160/218	0.00	0.16		226/242	0.00	0.05
	160/222	0.40	0.16		228/272	0.04	0.00
	160/224	0.24	0.00		228/276	0.12	0.00
	160/226	0.00	0.16		230/274	0.08	0.00
	160/230	0.00	0.04		272/272	0.04	0.00
	164/164	0.00	0.08				
ISP-MS01	230/248	0.44	0.00				
	230/250	0.20	0.00				
	232/252	0.08	0.00				
	234/254	0.00	0.28				
	236/246	0.28	0.24				
	236/254	0.00	0.28				
	238/256	0.00	0.20				

Table 3: Genotypic distribution of six microsatellite markers between *O. niloticus* (N) and *O. niloticus* (N_F) tilapia strains (N=25).

Strain	Locus	Allele size	Microsatellite copy numbers
N×N	PRL I-MS01	264 bp	$(GT)_{35}$
$N_F \times N_F$	PRL I-MS01	248 bp	$(GT)_{27}$
N×N	PRL I-MS02	240 bp	$(CA)_{33}$
$N_F \times N_F$	PRL I-MS02	206 bp	$(CA)_{16}$

Table 4: Copy numbers of PRL I-MS01 and PRL I-MS02 microsatellites in *O. niloticus* (N) and *O. Niloticus*.

I-MS02 exhibited 206-bp and 246-bp alleles derived from the maternal N_F strain and a 246-bp allele derived from the paternal A strain (Figure 3B). These results demonstrate that the PRL I-MS01 microsatellite marker can be used to trace the origin of hybrid tilapia products.

Characterization of tandem repeats within PRL I microsatellite loci

Based on sequences obtained from Gene Bank database (NCBI Accession no. X92380), GT and CA tandem repeats are known to exist in the PRL I-MS01 and PRL I-MS02 microsatellite loci. However, the copy numbers of GT and CA tandem repeats in the N and N_F strains are unclear. Most microsatellite genotypes in the N strain consisted of 260-bp and 288-bp alleles (PRL I-MS01) and 240-bp and 260-bp alleles (PRL I-MS02). In the N_F strain, the PRL I-MS01 and PRL I-MS02 microsatellite genotypes were 248/274 bp and 206/222 bp, respectively. To clarify differences in tandem repeats between N and N_F, small alleles of PRL I-MS01 and PRL I-MS02 were cloned and sequenced to identify the copy numbers of tandem repeats. As shown in Table 4, the numbers of GT tandem repeats in N and N_F were 35 and 27, respectively, while the numbers of CA tandem repeats in N and N_F were 33 and 16, respectively. These results further demonstrate that PRL I-MS01 and PRL I-MS02 are useful genetic markers to characterize *O. niloticus* tilapia strains.

Discussion

Tilapia has become the second most important aquaculture fish in the world. Therefore, researchers worldwide have endeavored to improve its growth characteristics. From an economic benefits perspective, farm-raised tilapia selected for large body size are almost exclusively male because of the xenogenic hybridization technology used. Taiwan is one of the major tilapia-producing countries and is an important supplier to the global market. In Taiwan, five major *Oreochromis* tilapia species, including *O. aureus* (A), *O. mossambicus* (M), *O. hornorum* (H), *O. spilurus* (S), and *O. niloticus* (N), and one *O. niloticus* strain (N_F) with a rapid-growth phenotype have been conserved at the Freshwater Aquaculture Research Center (FARC). Most farmed hybrid tilapia is derived from crosses between A and N or N_F. Characterization of tilapia species using genetic analysis

to supplement conventional approaches is required to strengthen the tilapia aquaculture industry and facilitate optimal breeding. Microsatellite markers have been used previously to identify tilapia species, trace parentage, measure genetic diversity and facilitate genetic mapping [4,21-25].

We used six microsatellite regions located within four growth-related genes, including IGF-II, PRL-I, GH and insulin, to discriminate between five tilapia species. Only two microsatellite markers located in the promoter region of the PRL I gene could be used to identify species of tilapia. We found that the genotypes of A, M, H and N could be differentiated using PRL I-MS01. However, the genotypes of these four strains could not be differentiated using PRL I-MS02. The genotype of S could be uniquely distinguished from the other four species using PRL I-MS01. These results demonstrate that the combination of the PRL I-MS01 and PRL I-MS02 microsatellite markers can characterize the five tilapia species examined.

Prolactin (PRL) is a hormone released by the pituitary gland. It is similar in structure and function to growth hormone (GH) and somatolactin (SL). In fish, PRL is involved in the regulation of electrolyte homeostasis and immune function [26,27]. Furthermore, knockdown of the PRL gene in zebrafish embryos leads to phenotypes of shorter body length and smaller head and eye size, suggesting that PRL is also involved in growth [18]. The N and N_F strains of *O. niloticus* exhibit significant differences in body size, body weight and specific growth rate. Farmed hybrid tilapia produced by crossing A with N or N_F also show differences in body size, body weight and specific growth rate. To assess whether the difference in growth performance between N and N_F can be predicted by PRL I microsatellites, the genotypes of N and N_F were analyzed using PRL I-MS01 and PRL I-MS02. Our findings reveal that both microsatellite regions are efficient genetic markers to distinguish between N and N_F tilapia strains. Traceability of farmed hybrid tilapia is important to ensure the overall quality and safety of farm-raised tilapia. PRL I microsatellites were also used to verify whether the genotypes of hybrid tilapia match those of their parents. Our findings reveal that the progeny of hybrid tilapia inherit PRL I alleles from the paternal and maternal genotypes at this locus. Thus, the origin of hybrid tilapia can be traced based on PRL I microsatellite genotypes.

Recent studies have demonstrated that functional microsatellites located in protein-coding regions, untranslated regions (UTRs) and introns can regulate gene expression and function by affecting transcription and translation [10,11,28]. For example, the microsatellite in the 5' flanking region of the GH gene of teleosts is associated with growth [29]. Furthermore, CA tandem repeats within intron 1 of the IGF-I gene affect circulating concentrations of IGF-I in swine [30]. Sequence data confirms that the copy numbers of GT tandem repeats in PRL I-MS01 and CA tandem repeats in PRL I-MS02 vary significantly between the N and N_F tilapia strains. Streelman and Kocher [24] have demonstrated that different lengths of PRL I microsatellite alleles with CA repeats can affect gene expression [31,32]. The difference in copy number of PRL I microsatellites may affect the diversity of PRL I gene expression, further influencing the observed growth performance differences between N and N_F. This hypothesis requires further study.

Nevertheless, it is not clear whether selection for low cortisol response is always recommendable. Further analysis will be carried out on the consistency of stress/cortisol response on this and the next generation to see whether the selection for this characteristic may help to decrease husbandry problems and improve the profitability of aqua cultured fish.

We found that PRL I microsatellites, including PRL I-MS01 and PRL I-MS02, are useful genetic markers to characterize *Oreochromis* tilapia species and to distinguish between the N and N_F strains of *O. niloticus*. Microsatellite markers will provide an efficient tool to strengthen the management of the tilapia aquaculture industry and facilitate the conventional breeding of superior strains.

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