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High genetic diversity and differentiation in three red tilapia stocks revealed by microsatellite DNA marker analysis

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Abstract Red tilapia is thought to be the result of mutant-colored female Mozambique tilapias mating with male Nile, blue, or Zanzibar tilapias and cultured widely in Asia and central and South America. However, there is limited information about its genetic diversity and stock structure. In this study, we investigated the genetic variability of red tilapia stocks to provide fundamental knowledge for genetic improvement by molecular-marker-assisted selective breeding programs. Individuals (n = 180) from three stocks (Chinese Taiwan, Israel, and Malaysia) of red tilapia were genotyped based on 14 microsatellite markers. The results showed that all microsatellite loci were detected with high levels of polymorphism, with a mean number of 14.87 ± 3.85 alleles per locus in all stocks. Taiwan and Israel stocks showed higher heterozygosity than did the Malaysia stock. The F-statistic analysis showed that there was no significant genetic differentiation between the Taiwan and Israel stocks ($P > 0.05$), whereas there was highly significant genetic divergence in the other pairwise stocks ($P < 0.01$), suggesting that Taiwan and Israel stocks could be regarded as a single genetic group distinct from Malaysia stock. This result was in accordance with the UPGMA dendrogram based on Nei's genetic distances of three stocks. The analysis of molecular variances (AMOVAs) revealed highly significant genetic variation among three stocks ($P < 0.01$) and accounted for 8.68% of the total variance. The results reported above were confirmed by Bayesian analysis in genetic

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structure simulation, which indicated a distinct genetic difference between Taiwan and Israel stocks compared with Malaysia stock.

Keywords Red tilapia · Stock · Microsatellite · Genetic diversity · Genetic structure

Introduction

Tilapia (*Oreochromis spp.*), which is the common name of a group of cichlid fish that are native to tropical Africa, consists of *Oreochromis*, the mouthbrooding genera *Sarotherodon*, and the substrate-spawning *Tilapia* (Trewavas 1983). According to data reported by the Food and Agriculture Organization of the United Nations (FAO 2013), the annual aquaculture production of tilapia has continued to show strong growth, increasing almost three times from 1.33 million tons in 2003 to 3.90 million tons in 2013. Currently, tilapia is the second most important fish species in global aquaculture after carp, with China being the largest supplier of tilapia in the world. Red tilapia hybrids were produced from local crossbreeding between rare mutant-colored (reddish-orange) female Mozambique tilapia (*O. mossambicus*) and male Nile (*O. niloticus*), blue (*O. aureus*), or Zanzibar tilapia (*O. urolepis hornorum*) (Galman and Avtalion 1983; Wohlfarth et al. 1990; Sandeep et al. 2012). Red tilapia has many positive characteristics for commercial culture including pure red body color, porgy shape, no black coelarium and off-flavor, fast growth, high feed conversion rate (FCR), high dressing-out percentage, euryhalinity, and high adaptability to different environments (Romana-Eguia and Eguia 1999; Chen and Dai 2003).

Microsatellite DNA markers are distributed evenly throughout the genome at high abundance, exhibiting features of high levels of polymorphism, co-dominance inheritance, and ease for PCR analysis, which were widespread used in studies on genetic diversity and population structure of fish species and even improving stocks through marker-assisted selection (Liu and Cordes 2004; Zhao et al. 2011). Moreover, microsatellites have been used to differentiate between closely related populations of the same *Oreochromis* species and numerous primers corresponding to microsatellites have been developed for this purpose in tilapia (Kocher et al. 1998). Thus, microsatellites represent effective tools for the genetic study of tilapia. Zhang et al. (2010) analyzed the genetic diversity of six tilapia populations and the genetic relationship between Nile tilapia and Blue tilapia based on 20 microsatellites. In an analysis of GIFT (strain Nile tilapia), Li et al. (2009) showed high levels of genetic diversity in the GIFT stock and identified several microsatellite loci associated with body weight in females and one locus associated with body shape in males. However, basic information on the genetic diversity of red tilapia is scarce and the genetic ancestries are not well documented. In this study, we investigated the genetic diversity and genetic structure of three red tilapia stocks (Chinese Taiwan, Israel, and Malaysia) to provide general information that may facilitate genetic improvement by molecular-marker-assisted selective breeding programs.

Materials and methods

Sampling and DNA extraction

In this study, the stock of Malaysia red tilapia (designated MY) was introduced from Malaysia in 2009, and the stocks of Taiwan (China) red tilapia (designated TW) and Israel (designated IL) red tilapia were transferred from Fujian Province, China, in 2014 by the Freshwater

Fisheries Research Center (FFRC) of the Chinese Academy of Fishery Sciences (Yang et al. 2015). All stocks were domesticated and bred at a local experimental aquaculture farm in Wuxi City (Jiangsu Province, China).

Fin clips (approximately 10 × 10 mm^2) from 180 red tilapia individuals (60 individuals from each stock) were collected in 2014 and preserved in 75% ethanol. Genomic DNA was extracted using a standard phenol-chloroform method. The concentration of DNA in each sample was adjusted to 20 ng/μl and arrayed into 96-well PCR plates, which were stored at −20 °C for later PCR amplification.

Microsatellite selection and genotyping

Fourteen primer sets were selected from the National Center for Biotechnical Information (NCBI) database (Table 1) for amplification and identification of polymorphic products in tilapia based on the success of PCR amplification and heterozygosity levels. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and the 5′-ends of the forward primers were labeled with a fluorescent dye (HEX or 6FAM). The PCR amplification was performed on an Eppendorf Mastercycler Pro 384 PCR thermocycler (Eppendorf, Germany). Each PCR sample (reaction volume, 25 μl) contained 10 μl Taq PCR MasterMix (2×) (TianGen Biotech Co., Ltd., Beijing) with 1 U Taq Polymerase, 0.5 mM of each dNTP, 20 mM Tris–HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 2 μl primer mixture (10 μM), 1 μl genomic DNA

Table 1 Primer sequences of 14 microsatellite loci of red tilapia

Locus	Primer sequence (5′-3′)	Annealing temperature (°C)	Accession no.
UNH899	F: HEX-ACGTCACATGGAGGTGCTTA R: GCTAGACCTCTGTCCCCTGA	48	G68202
UNH178	F: 6FAM-GTCACACCTCCATCATC R: AGTTGTTTGGTCGTGTAAG	51	G12330
UNH880	F: HEX-GGCAGCAGTATAACAATCACCA R: TTCTGACATCCATCCAGCAG	46	G68207
UNH222	F: HEX-CTCTAGCACACGTGCAT R: TAACAGGTGGGAACTCA	47	G12373
UNH932	F: 6FAM-AGCGCTAAATGAGCCAGTGT R: TTCTTTAATGCCTGCCAGTG	50	G68238
UNH974	F: HEX-GCACGTCTGAGAGTGTGGAA R: CAGCTTTCACACCAGCCTAA	53	G68261
UNH997	F: 6FAM-ATTCCCCAACATTTGTGTGC R: ACAGCAGCATCCCTGAAAAG	51	G68276
UNH214	F: HEX-TTCCATAATTGCTTTCTG R: GCACGTTTTCCATCACTTCAA	50	G12365
UNH999	F: 6FAM-TGCAAAGTCACAAATCCACAA R: CTCCCATTCATTACCCCAAA	51	G68278
UNH1007	F: HEX-TTCTCTACCTGTGAACATTTGC R: AAGGCAGTCCTGCTCTATGC	42	G68283
UNH106	F: HEX-CCTTCAGCATCCGTATAT R: GTCTCTTTCTCTGTCACAAG	46	G12259
UNH176	F: 6FAM-GATCAGCTCTCCTCTACTTA R: GATCTGATTTCTTATTACTACAA	45	G12328
UNH846	F: HEX-TGGAGCAGCTTCTTCTACATCA R: CACATGATGGAAGCCGTGTA	46	G68185
UNH985	F: 6FAM-GCGTCTTGATGCAGGATACA R: TCCCGACGAGCAACTGTTAT	46	G68266

(20 ng/μl), and 12 μl DNase/RNase-free deionized water. PCR amplification was conducted under the following conditions: 5-min pre-denaturation at 94 °C followed by 30 cycles of denaturing 30 s at 94 °C, annealing 30 s at specific annealing temperatures (Table 1), and prolonging 50 s at 72 °C, with a final prolonging at 72 °C for 10 min; subsequently, the reaction products were held at 4 °C for further detection. All individual genotypes were scored after the PCR products were resolved on Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, USA) and sized relative to an internal size standard (GeneScan-500 ROX) using GeneMapper Version 3.5 software (Applied Biosystems, USA) (Fig. 1).

Statistical analysis

The number of alleles (N_a), effective number of alleles (N_e), expected and observed heterozygosities (H_e and H_o, respectively), and polymorphic information content (*PIC*) were calculated using Cervus v3.0 software (Kalinowski et al. 2007). The Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were analyzed by Genepop v4.4 software (Rousset 2008) using a Markov chain of 1,000,000 steps and 100,000 dememorization steps. The Shannon diversity index of alleles and Nei's genetic distances (Nei 1978) were compiled using the Popgene v1.32 software (Yeh et al. 1997). *F*-statistics (F_{ST}) calculation and analysis of molecular variances (AMOVAs) were performed by using Arlequin v3.1 software (Excoffier et al. 2005). Phylogenetic trees were constructed using Nei's genetic distance based on unweighted pair group methods with arithmetic (UPGMA) averages using MEGA v5.1 software (Tamura et al. 2011). Analysis of the genetic structure of populations was performed using STRUCTURE v2.3.3 software (Pritchard et al. 2000) with Bayesian methods. The parameters used in the structure analysis were assumed by an admixture model, with a burn-in of 50,000, with 100,000 Markov chain-Monte Carlo (MCMC) repetitions and five iterations per K (K = 2~3).

Results

Polymorphisms of microsatellite loci

The characteristics of the microsatellite loci identified in this study are listed in Table 2. All 14 microsatellite loci were found to be polymorphic based on the genotypes of 180 individuals. Only

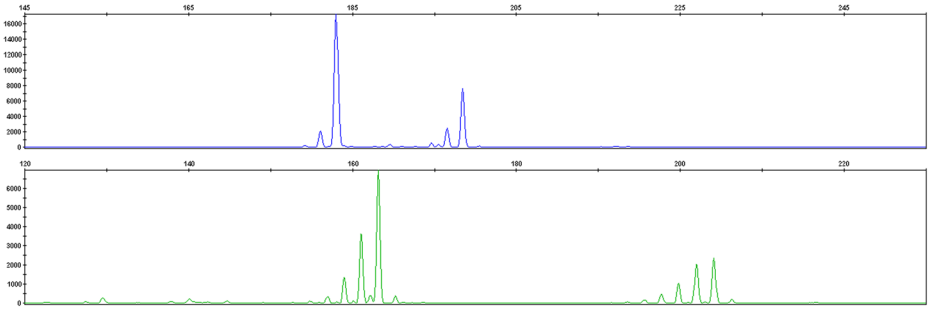

Fig. 1 Example of genotyping results obtained using an Applied Biosystems 3730XL sequencer. *Blue line*, UNH178 locus; *green line*, UNH899 locus. *X-axis* indicates the size of DNA fragments (bp); *Y-axis* indicates the signal intensity of the fluorescent reporter

Table 2 The number of observed and effective alleles, expected and observed heterozygosity, and polymorphism information content for microsatellite loci of red tilapia

Locus	N_a	*Ne*	*Ho*	H_e	*PIC*
UNH178	9	3.21	0.60	0.69	0.65
UNH899	14	2.35	0.52	0.58	0.55
UNH880	22	3.87	0.71	0.74	0.71
UNH932	12	4.24	0.74	0.77	0.73
UNH222	10	4.55	0.75	0.78	0.75
UNH997	14	3.50	0.70	0.72	0.69
UNH974	15	5.00	0.89	0.80	0.78
UNH999	17	7.18	0.89	0.86	0.85
UNH214	21	5.75	0.79	0.83	0.81
UNH1007	19	5.34	0.85	0.82	0.79
UNH176	12	1.95	0.43	0.49	0.46
UNH106	11	5.19	0.68	0.81	0.78
UNH985	17	6.10	0.79	0.84	0.82
UNH846	14	3.44	0.75	0.71	0.69
Mean ± SD	14.87 ± 3.85	4.43 ± 1.42	0.72 ± 0.13	0.75 ± 0.10	0.72 ± 0.11

N_a number of alleles, N_e effective number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, *PIC* polymorphic information content

one locus (UNH106) showed a highly significant deviation from Hardy–Weinberg equilibrium ($P < 0.01$). The number of alleles (N_a) ranged from nine (UNH178) to 22 (UNH880), with an average of 14.87 per locus, and the effective number of alleles (N_e) ranged from 1.95 (UNH176) to 6.10 (UNH985), with an average of 4.43 per locus. The observed/expected heterozygosity (H_o/H_e) ranged from 0.43/0.49 (UNH176) to 0.89/0.86 (UNH999), with an average of 0.72/0.75. *PIC* ranged from 0.46 (UNH176) to 0.85 (UNH999), with an average of 0.72, indicating a high level of polymorphism in all the microsatellite loci ($PIC > 0.5$).

Genetic diversity among stocks

Data for all parameters of genetic diversity for the three red tilapia stocks are shown in Table 3. Three loci (UNH880, UNH997, and UNH106) in Malaysia stock showed significant divergence from Hardy–Weinberg equilibrium ($P < 0.05$), whereas the other two stocks did not. Israel stock presented the highest genetic diversity parameters followed by the Taiwan and Malaysia stocks. However, Taiwan stock showed the highest H_o followed by Israel and Malaysia stocks, while Israel stock exhibited the highest *PIC* followed by Taiwan and Malaysia stocks.

Table 3 Summary of genetic diversity in three red tilapia stocks

Stock	N_a	N_e	H_o	H_e	*PIC*
TW	11.20 ± 2.04	4.04 ± 1.10	0.76 ± 0.10	0.74 ± 0.08	0.71 ± 0.08
IL	12.53 ± 3.44	4.27 ± 1.31	0.74 ± 0.13	0.75 ± 0.10	0.72 ± 0.10
MY	7.00 ± 1.73	2.97 ± 0.97	0.66 ± 0.22	0.62 ± 0.19	0.57 ± 0.18

N_a number of alleles, N_e effective number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, *PIC* polymorphic information content

Genetic divergence and genetic distance

The pairwise F_{ST} estimates presented in Table 4 showed a significant difference between MY and the other two stocks, indicating a moderate genetic differentiation ($0.05 < F_{ST} < 0.15$) (Balloux and Lugon-Moulin 2002), whereas there was no genetic divergence between TW and IL. Nei's genetic distance (D_A) between the stocks ranged from 0.023 to 0.396. The UPGMA dendrogram constructed according to Nei's genetic distance is shown in Fig. 2. TW and IL formed a cluster that did not include MY. The AMOVA revealed genetic variation within and among stocks of 91.32 and 8.68%, respectively, with the variation found to be significant among stocks ($P < 0.01$) (Table 5).

Genetic structure

The logarithm probabilities *Ln P* (*X/K*) associated with different numbers of genetic clusters *K*, calculated from structure analysis of 180 individuals of red tilapia showed the highest value at $K = 2$ and the lowest value at $K = 3$. As shown in Fig. 3, the individuals in Taiwan and Israel stocks were mixed at some levels and were clearly distinct from the individuals in Malaysia stock.

Discussion

Analysis of microsatellite polymorphisms

Most of the microsatellite loci analyzed in this study were highly polymorphic ($PIC > 0.5$, Botsein et al. 1980) and did not exhibit divergence from Hardy–Weinberg equilibrium, which indicates that these microsatellites can be used efficiently to estimate the genetic variations of red tilapia stocks. All 14 microsatellite markers were detected with a higher degree of polymorphism than that reported by previous studies of the genetic diversity of red tilapia stocks based on microsatellite analysis, such as $N_a = 10$ in a farmed red hybrid tilapia stock (Romana-Egui et al. 2004) and $N_a = 4.90$ in the Florida red tilapia stock (Sandeep et al. 2012). These differences might be due to differences in the study sample (e.g., population size and genetic background), the microsatellite loci analyzed, and detection techniques. Most of the previous studies were conducted using small sample sizes (e.g., 8 individuals, Sandeep et al. 2012), which may lead to bias in the estimation of genetic diversity. Furthermore, the detecting technology with automatic DNA sequencers used in this study might provide higher resolution for genotyping, compared

Table 4 Nei's genetic distance (D_A, below diagonal) and pairwise F_{ST} estimates (F_{ST}, above diagonal) among red tilapia stocks

Stock	TW	IL	MY
TW	****	−0.002	0.137**
IL	0.023	****	0.128**
MY	0.396	0.391	****

*significant level of differentiation ($P < 0.05$); **highly significant level of differentiation ($P < 0.01$).

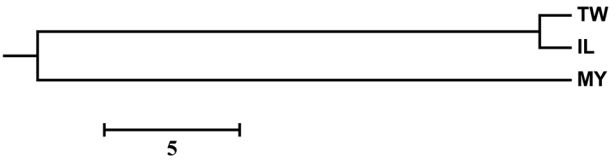

Fig. 2 Unweighted pair group methods with arithmetic (UPGMA) dendrogram based on Nei's genetic distance among the three red tilapia stocks. Stocks of red tilapia: *TW* Taiwan (China), *IL* Israel, *MY* Malaysia. The digit *5* indicates the scale of branch lengths in the UPGMA dendrogram

to that achieved by traditional silver-staining method, such as electrophoresed through 8% polyacrylamide gels (Romana-Egui et al. 2004).

Genetic diversity within stocks

The mean expected heterozygosity in three red tilapia stocks (0.62–0.75) was similar to that reported in farmed red hybrid tilapia stocks (0.70) by Romana-Egui et al. (2004) and in Florida red tilapia (0.74) by Sandeep et al. (2012) but much higher than that in the red tilapia (0.35) reported by Zhang et al. (2009). As mentioned previously, these discrepancies may be caused by differences in microsatellite markers and samples used in the studies. However, previous studies have shown significant positive correlations between genetic diversity and population size (Frankham 1996; Ha et al. 2009). Moreover, domestication is often accompanied by a decline in genetic variation due to genetic drift, selection, and inbreeding (Ha et al. 2009). The lower genetic diversity of Malaysia stock might be due to the small effective population size and possible inbreeding over several generations. Therefore, the level of inbreeding, genetic diversity, and broodfish population size should be considered in red tilapia breeding programs, especially in the selective breeding of independent Malaysia stock. Furthermore, our results indicate that appropriate breeding methods, such as cross-breeding, can be used to maintain genetic diversity.

Relationships among red tilapia stocks

The highly polymorphic microsatellite loci used in this study were selected to provide a reliable genetic evaluation of the stocks under investigation (Kalinowski 2002, 2005). The negative F_{ST} value for Taiwan and Israel stocks indicates there is no significant genetic differentiation between these two stocks, which confirms their common origin. In contrast, the moderate differences in the F_{ST} values for the other pairs of stocks indicate differences in the origin of parental populations and/or the occurrence of genetic drift during breeding. Similar results were found in three paddlefish (*Polyodon spathula*) stocks (Kaczmarczyk

Table 5 Analysis of molecular variances (AMOVAs) of microsatellite loci among three red tilapia stocks

Source of variation	d. f.	Sum of squares	Variance component	Percentage of variation
Among stocks	2	99.92	0.39**	8.68
Within stocks	357	1438.52	4.03	91.32
Total	359	1538.44	4.41	

d.f. degrees of freedom

**highly significant source of variation ($P < 0.01$)

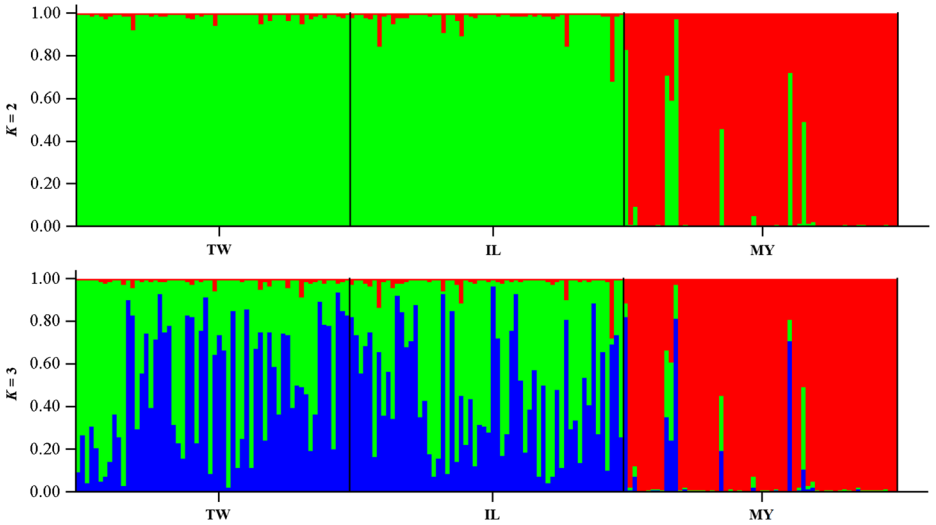

Fig. 3 Bayesian clustering of the three stocks of red tilapia based on 14 microsatellite loci. Each individual fish is represented by a *vertical column*, with lengths proportional to the individual estimate membership coefficient, assuming two ($K = 2$) and three ($K = 3$)

et al. 2012). Thorp (1982) reported genetic distances ranging between 0.03 and 0.20 in a single stock. Based on this observation, we infer that the genetic distance between Taiwan and Israel stocks is very small and typical for closely related populations, while the distance between one of these two stocks and Malaysia stock is large and typical for genetically disparate populations. This hypothesis is consistent with the UPGMA dendrogram constructed based on Nei's genetic distance. The scale of the genetic distances between the stocks suggests that Taiwan stock is most similar to Israel stock, while the genetic differences between Taiwan and Malaysia stock reflect the substantial genetic distance between these two stocks.

AMOVA revealed a highly significant genetic variation among the three stocks ($P < 0.01$). Based on the correlation of genetic differentiation and gene flow (Nei 1987), it can be speculated that the divergence between Malaysia stock and the other two stocks is due to long-term geographic separation and limited natural gene flow. However, it is also possible that artificial gene flow might have contributed to the lack of divergence between Taiwan and Israel stocks because the two stocks had been farmed together in Fujian Province for several years before introduction into the FFRC. These results were confirmed by Bayesian analysis in genetic structure simulations, which indicate that Taiwan and Israel stocks are obviously different from Malaysia stock.

Conclusions

The three red tilapia stocks contained a high level of genetic diversity revealed by microsatellite marker analysis. However, the genetic variation in Taiwan and Israel stock was significantly higher than that in Malaysia stock. UPGMA dendrogram and genetic structure analysis showed that Taiwan and Israel stock can be considered as a single genetic group, which is separated from Malaysia stock. The high genetic variation in red tilapia stocks provides the basis of genetic improvement by molecular-marker-assisted selective breeding.

TW Taiwanese red tilapia; IL Israel red tilapia; MY Malaysia red tilapia; N_a Number of alleles; N_e Effective number of alleles; H_o Observed heterozygosity; H_e Expected heterozygosity; *PIC* Polymorphic information content; HWE Hardy–Weinberg Equilibrium; F_{ST} F-statistics; AMOVA Analysis of molecular variances; UPGMA Un-weighted pair group methods with arithmetic; MCMC Markov Chain-Monte-Carlo

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