Short Research Communication

Analysis of Microsatellite DNA Markers Reveals no Genetic Differentiation between Wild and Hatchery Populations of Pacific Threadfin in Hawaii

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Abstract

Pacific threadfin, *Polydactylus sexfilis*, is popular fish in recreational fishing, as well as aquaculture in Hawaii. Its natural population has been continuously declining in the past several decades. Microsatellite DNA markers are useful DNA-based tool for monitoring Pacific threadfin populations. In this study, fifteen Microsatellite (MS) DNA markers were identified from a partial genomic Pacific threadfin DNA library enriched in CA repeats, and six highly-polymorphic microsatellite loci were employed to analyze genetic similarity and differences between the wild population and hatchery population in Oahu Island. A total of 37 alleles were detected at the six MS loci in the two populations. Statistical analysis of fixation index (F_{ST}) and analysis of molecular variance (AMOVA) showed no genetic differentiation between the wild and hatchery populations (F_{ST}=0.001, $CI_{95\%}$= $-$0.01-0.021). Both high genetic diversity (H_o=0.664-0.674 and H_e=0.710-0.715) and Hardy-Weinberg equilibrium were observed in the wild and hatchery populations. Results of genetic bottleneck analysis indicated that the hatchery was founded with sufficient numbers of brooders as inbreeding coefficient is very low (F_{IS}=0.052-0.072) in both wild and hatchery populations. Further studies are needed for comprehensive determinations of genetic varieties of primary founder broodstocks and successive offspring of the hatchery and wild populations with increased number of Pacific threadfin sample collections.

Key words: Pacific threadfin; *Polydactylus sexfilis*; microsatellite; genetic differentiation; Oahu islands.

1. Introduction

Pacific threadfin, *Polydactylus sexfilis*, is locally known as moi by its Polynesian name. It is a popular fish in recreational fishing, as well as aquaculture since recent development of offshore cage culture (1). The natural population of Pacific threadfin has dramatically decreased due to over-fishing and resort developments, which may severely deplete the reproductive stocks and destroy spawning and nursery habitat (2). Since 1990s, the State of Hawaii and the Oceanic Institute have been releasing juvenile threadfin reared by artificial hatchery to the coastal waters of

Oahu Island (1-3). As the broodstock fish for artificial propagation have been taken directly from the coastline of the Oahu Island, one of the challenges in maintaining Pacific threadfin hatchery population is to control loss of genetic variability as a result of limited number of founders (4,5). Pacific threadfin are protandrous hermaphrodites, fingerlings are initially male, change sex to female when grow up. Previously, we have noticed that offspring is mainly produced by only several specific parent pairs in consecutive hatchery events (6) even though sufficient numbers of

breeders are presented in each rearing tank. Consequently, this may lead to inbreeding problems over several generations. We are concerned about genetic variability reduction in a closed population of hatchery operation. Decreased genetic variability may has detrimental effects on commercial traits such as growth rate, survival and disease resistance, which can post a great risk for large-scale offshore aquaculture operations. Therefore, it is vital and critical to monitor the genetic variability in successive generations of broodstock populations.

Among various molecular markers now available to study genetic diversity in different fish species, microsatellite DNA markers are highly polymorphic with codominant inheritance (7-10). Currently, limited microsatellite DNA markers are available for Pacific threadfin (6). The present study is aimed at identifying new microsatellite loci and comparing the genetic similarity and differences of wild and hatchery Pacific threadfin populations in Hawaii.

2. Materials and methods

Sample Collection and DNA extraction

Skeletal muscle tissues were collected from several Pacific threadfin individuals, which were used for genomic DNA isolation and microsatellite enriched partial genomic library construction. For genetic diversity analysis, a non-destructive sampling method was employed with only fin-clips of each sample were collected. A total of 30 wild individual samples of Pacific threadfin were collected from the east coastline of Oahu Island. For the hatchery population, 31 samples were randomly selected from one broodstock population, which is derived from the founders originally collecting from the wild population. All the samples were placed in absolute ethanol and kept frozen at -20°C until DNA extraction. Total genomic DNA from muscle tissue was extracted by proteinase K digestion, followed by standard phenol-chloroform extraction protocol (11). The genomic DNA from fin clips was extracted with QIAamp DNA mini Kit (Qiagen, Valencia, CA, USA).

Genomic library construction and microsatellite screening

A partial genomic library enriched for CA repeats was constructed by following the microsatellite hybrid capture technique with slight modifications (12,13). In general, genomic DNA extracted from muscle was digested with *Dpn*II and DNA fragments ranging from 300-800 bp were isolated and ligated to the phosphorylated catch linker sequences (oligo C: GTCAAGAATTCGGTACCGTCGAC and oligo D: GATCGTCGACGGTACCGAATTCT denatured and annealed together) with T4 DNA ligase (Fermentas, Hanover, MD, USA). Linker-ligated DNA was amplified in PCR with oligo C as primer and then hybridized to biotinylated $(GT)_{10}$ probes which attached to streptavidin-coated magnetic beads (NEB, Ipswich, MA, USA). After a second round of PCR amplification with enriched fragments, the products were purified and ligated into PCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) followed by transformation into E.*coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA). Plasmids with an insertion of above 300 bp were extracted using QIAGEN Plasmid Mini Kit (Qiagen, Valencia, CA, USA) and sequenced on an ABI PRISM 3730 Sequencer (ABI, Foster, CA, USA) using M13 forward and reverse primers. Microsatellite sequences were screened using TANDEM REPEATS FINDER Version: 4.04 (14). The criteria used in searching of potential microsatellite markers in the DNA sequences are: seven repeats for dinucleotide repeat, five repeats for trinucleotide repeat, four repeats for tetranucleotide repeat (15).

PCR amplification and allele scoring

Primers were designed at the flanking regions of microsatellite loci by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). An initial screening of the polymorphic status of each microsatellite was performed with polyacrylamid gel eletrophoresis (PAGE) and silver staining in 14 individuals randomly selected from the wild population. The forward or reverse primers of the selected highly polymorphic markers were fluorescently labeled with HEX or 6-FAM at 5' end. The PCR amplification was performed in a 25 μl volume by following our previous protocol (6) with specific annealing temperatures listed in Table 1.

Polymorphisms of different microsatellite loci were analyzed by an ABI PRISM 3100 Automated Sequencer (Applied Biosystems, Foster, CA, USA). The size of each allele was visualized and determined by GeneMapper (version 4.0) software (ABI, Foster, CA, USA). Genotyping errors associated with microsatellite analysis such as stutter bands, null alleles and large allele dropout were checked with the software MICRO-CHECKER Version 2.2.3 (16).

Table 1. Primers used to amplify 15 microsatellite loci in Pacific threadfin (*Polydactylus sexfilis*).

Locus	Repeat Motif (5'-3')	Primer sequences (5'-3')	Tm (°C)	Size (bp)	GenBank Accession No.
Ptd11	$(CA)_{30}$	F: AAGATCCTCGTGCCACCTCA; R: GTTATTTAGAGTTGTCACCG	60	239	HM586074
Ptd15	$(CA)_{22}$	F:GCACCCACAAACATGCTCAAAT; R:TGTGACGGTTTCTCCATTG	58	206	HM586075
Ptd16	$(CA)_{31}$	F: CGCAATGGAGAAACCGTCA; R: GATGTTCACCTTGGCTCTC	61	189	HM586088
Ptd20	$(GT)_{35}$	F: AAAGTCTCCCAACAGATGAT; R: ATGCCAATTACAAGAGTCGA	55	267	HM586076
Ptd21	$(AC)_4+(AC)_{13}+(AC)_{24}$	F: CCCTGCTGGTCAAACTACAC; R: CATCAGTGTATGCCTGGTAA	58	167	HM586077
*Ptd33	$(CATT)_8$	F: CCTGCTCATTGACAGATAA; R: GCGTGCAAACGAATGACGGA	58	138	HM586078
Ptd35	$(ATTT)_7$	F: CTGTGAAACTTGTGACTCTCG; R: CTCTTCAATAGGCTTCTCTGC	55	365	HM586079
*Ptd37	$(CA)_{19}$	F: CTAAACTGGAAGAGGCACAA; R: TGACCTGTGAACCTGGGAAA	56	164	HM586080
*Ptd52	$(CT)_{16}$	F: GACTGATGAGCTGACTTCACC; R: CTGGTGATTCTAGATACTGCG	60	297	HM586081
Ptd57	$(TG)_{27}$	F: AAAAGGCTGTGAGTGAATGA; R: GACCTGGTGCTTTATTACTT	59	179	HM586082
Ptd58	$(CA)_{10}$	F: CTTATCATCGCTCCACGCTGT; R: GTACTCTGTTGGCAGGAGCA	58	253	HM586083
*Ptd73	$(GT)_{23}$	F: GTGGACAGTGGGTCAAGTGC; R: GGTGTCAGGAGGGAGGGTTA	58	259	HM586084
Ptd84	$(CT)_8$	F: TGTCAGTCAGTCGACGGTG; R: CGTAGGAACAGACGGAGCA	56	218	HM586085
*Ptd88	$(GT)_{26}$	F: CCTGGAGTTAGAAAGATTGT; R:AACCAAGTCAATATCACAGGAT	55	296	HM586086
*Ptd89	$(CA)_{12}$	F: GTCAGGGACAACGAGCCAT; R: TCTGCCACAAGATGGAAGCT	60	79	HM586087

Data analysis

In analyzing the variation in microsatellite loci in wild and hatchery populations, allele frequencies, the number of alleles per locus (N_A), allele size range of each locus (S), observed heterozygosity (H_o), expected heterozygosity (H_e) were determined by using GENEPOP version 4.0 (17). Allelic richness (A_n) as a standardized measure of the number of alleles per locus independent of the sample size was calculated by FSTAT version 2.9.3.2 (18). Deviations from Hardy-Weinberg Equilibrium were tested with GENEPOP version 4.0 (17) with exact *P* values being estimated using the Markov chain algorithm with 10,000 dememorization steps 100 batches and 1,000 iterations. GENEPOP version 4.0 was also used for testing linkage disequilibrium to determine the extent of distortion from independent segregation of loci. To evaluate the extent of differences within and among populations, the fixation index F_{IS} was calculated as an estimator of inbreeding. The significance of the inbreeding coefficients was determined within populations and over loci using FSTAT version 2.9.3.2 (18). The genetic differentiation between wild and hatchery populations was compared by the *F*-statistics of Weir & Cockerham (19) with each parameter tested against zero by a bootstrapping method using FSTAT version

2.9.3.2. The hierarchical genetic variation existing between wild and hatchery populations and within populations was analyzed by Analysis of Molecular Variance (AMOVA) using Arlequin version 3.5 (20). As hatchery populations are often subjected to founder effects and bottleneck that result in lower genetic diversity, Bottleneck software version 1.2.02 (21) was employed to test the bottleneck hypothesis under a two-phased model of mutation (TPM). This method can be used for testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency.

3. Results and discussion

Microsatellite markers isolations and allele frequency

A total of 96 clones were picked up from the enriched partial genomic library for sequencing, 51 of which were found to contain microsatellite repeats with 11 clones found to be duplicates. Twenty-three clones were discarded because the microsatellite sequences were so close to the catch linker sequence that primer sequences can not be designed for amplification. After initial PCR assays, 15 microsatellite loci can successfully be amplified (Table 1). An initial evaluation of the polymorphic status of each locus was done

by PAGE and silver staining in 14 individuals randomly selected from the wild population. Six microsatellite loci (*Ptd33*, *Ptd37*, *Ptd52*, *Ptd73*, *Ptd88* and *Ptd89*) showed great allele varieties with clear band patterns. Allele frequency of each microsatellite allele in the wild and hatchery populations were reported in Table 2. Most common microsatellite alleles have similar frequencies in the wild and hatchery populations. Several private alleles were observed in these two populations (one in the wild population; five in the hatchery population); however their frequencies were very low and only existed in one or two samples genotyped.

Genetic variations within populations

All six microsatellite loci were found to be highly polymorphic in both populations. A total of 37 different alleles were observed and the average number of alleles per locus was 6.17 (ranges from 3 to 8). MICRO-CHECKER software did not detect errors in the genotypic data matrix, indicating that there are not scoring errors associated with null alleles, stuttering bands or large allele dropout in all six loci screened. The number of alleles per locus (N_A), size range of each locus (S), allelic richness (A_n), observed heterozygosity (H_o), expected heterozygosity (H_e),

inbreeding coefficient (F_{IS}) and probability of significant deviation from Hardy-Weinberg equilibrium (P) are presented in Table 3. The mean expected and observed heterozygosity were respectively 0.710 and 0.674 in the wild population while the corresponding parameters were 0.715 and 0.664 in the hatchery population, respectively. There were not significant ($P>0.05$) differences of expected and observed heterozygosity between the wild and hatchery populations. Due to the difference in sample size of the wild and hatchery populations, the parameter allelic richness (A_n) was employed to compare different populations independent of sample size. A higher mean allelic richness was observed in the hatchery population (A_n =5.82) than the wild population (A_n =5.19) based on minimal sample size of 23. The inbreeding coefficient (F_{is}) of the hatchery population and the wild population is 0.072, and 0.052, respectively, both of them are very low. All the loci were found to be in Hardy-Weinberg equilibrium (p=0.093) with a marginal value (p=0.052), which was caused by homozygote excess observed at locus Ptd33 in the wild population. Examination of pair-wise linkage disequilibrium by GENEPOP version 4.0 revealed that all the six microsatellite loci were in linkage equilibrium.

Table 2. Frequency of each microsatellite allele in the wild and hatchery populations.

Locus	Allele	Wild	Hatchery	Locus	Allele	Wild	Hatchery
Ptd52	295	0.232	0.117	Ptd89	71	0.017	0.000
	297	0.429	0.667		77	0.367	0.339
	301	0.339	0.217		79	0.000	0.016
					81	0.250	0.210
Ptd73	243	0.000	0.016		83	0.333	0.371
	253	0.037	0.032		85	0.017	0.032
	255	0.574	0.500		87	0.017	0.032
	257	0.148	0.097				
	259	0.019	0.097	Ptd33	128	0.067	0.161
	261	0.111	0.194		138	0.200	0.226
	265	0.111	0.065		140	0.067	0.065
					150	0.300	0.226
Ptd88	286	0.000	0.019		154	0.067	0.081
	294	0.043	0.115		158	0.150	0.145
	296	0.217	0.192		162	0.100	0.048
	300	0.065	0.096		166	0.050	0.048
	302	0.478	0.365	Ptd37	148	0.333	0.250
	304	0.196	0.173		156	0.117	0.117
	308	0.000	0.019		158	0.300	0.317
	312	0.000	0.019		164	0.250	0.317

Table 3. Genetic variability indices of Pacific threadfin in the wild and hatchery populations*.

Population	Microsatellite loci						
	Ptd33	Ptd37	Ptd52	Ptd73	Ptd88	Ptd89	Mean
Wild							
N_A	8	4	3	6	5	6	5.33
A_n	7.98	4.00	3.00	5.83	5.00	5.30	5.19
n	30	30	28	27	23	30	
S	128-166	148-164	295-301	253-265	294-304	71-87	
H_o	0.700	0.767	0.750	0.519	0.609	0.700	0.674
H_e	0.836	0.735	0.659	0.634	0.695	0.703	0.710
F_{IS}	0.165	-0.044	-0.141	0.185	0.126	0.004	0.052
P	0.052	0.236	0.314	0.140	0.372	0.897	
Hatchery							
N_A	8	4	3	7	8	6	6.00
A_n	7.96	4.00	3.00	6.67	7.65	5.61	5.82
n	31	30	30	31	26	31	
S	128-166	148-164	295-301	243-265	286-312	79-87	
H_o	0.677	0.800	0.500	0.677	0.654	0.677	0.664
H_e	0.849	0.736	0.503	0.700	0.791	0.713	0.715
F_{IS}	0.205	-0.089	0.007	0.032	0.176	0.050	0.072
P	0.145	0.633	0.811	0.088	0.099	0.444	
Mean							
N_A	8	4	3	6.5	6.5	6	5.67
A_n	7.90	3.99	3.00	6.24	6.40	5.28	5.47
H_o	0.689	0.783	0.625	0.598	0.631	0.689	0.669
H_e	0.845	0.735	0.580	0.668	0.745	0.708	0.713
F_{IS}	0.185	-0.067	-0.074	0.100	0.155	0.028	0.062

*N_A: number of alleles per locus; A_n: allelic richness; n: Sample size; S: size range of each locus; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: inbreeding coefficient; P: probability of significant deviation from Hardy-Weinberg equilibrium.

In testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency for both populations, bottleneck analysis was conducted using Bottleneck software under the TPM of microsatellites. No population displayed significant heterozygosity excess (P>0.05) through the sign test, standardized differences test and Wilcoxon sign rank test, suggesting that both the wild and hatchery populations have not experienced a recent bottleneck.

Both the wild and hatchery populations were found in Hardy-Weinberg equilibrium for all the six loci (Table 3), indicating minimum selective forces acting on the six microsatellite loci or on loci closely linked to them in the wild and hatchery populations. This may suggest that the mating strategy used in the hatchery population had similar effects on the genotypic frequencies to those expected under panmixia (4). The mean allelic richness (A_n) and mean number of alleles per locus (N_A) for the two populations of Pacific threadfin were 5.47 and 5.67 respectively, which is dramatically lower than the reported N_A for freshwater fish (N_A=9.1±6.1 averaged from 13 species) and marine fish (N_A=19.9±6.6 averaged from 12 species) (22). However, the mean observed (H_o=0.669) and expected heterozygosity (H_e=0.713) of Pacific threadfin was comparable to other marine fish species (H=0.77±0.19 averaged from 12 species) and slightly higher than that of freshwater fish species (H=0.54±0.25 averaged from 13 species) (22). Analysis of potential recent bottleneck showed no evidence of recent bottleneck in either the wild or hatchery population. The relatively high H_e observed in both populations, HWE seen at all the six loci and relatively low inbreeding indexes (0.052-0.072) indicate that the wild and hatchery populations of Pacific threadfin contain high genetic diversity with large population size. As allele number is positively related to sample size (23), the number of alleles observed at all the six loci is certainly related to the relatively small size of the sampling of the two populations.

In the hatchery populations, homozygote excess is commonly caused by a limited number of founders or founder effects. However, in this study the hatchery population seem to have sufficient effective population size that the observed F_{IS} can not simply attribute to the founder effect. As Pacific threadfin are communal spawners, the communal spawning behavior could easily lead to inbreeding in Pacific threadfin and other fish species (6,25). The homozygote excess observed in hatchery populations is probably caused by unequal contributions of a limited number of pair mating to the next generation. In the wild populations, homozygote excess could be explained by a small population bottleneck (dramatic decrease

of natural population), whose consequences are similar to those of the founder effect or by over-generation stocking effects or Wahlund effect, inbreeding or possibly assortative mating by repeated interbreeding between the natural population and mass-released hatchery strains (4). As the number of fish released over the years of stock enhancement program, the number of wild fish brought in for broodstock are not available, we can not determine more details about the founder effects on genetic drift in non-random mating.

Genetic diversity between the wild and hatchery populations

No genetic differentiation between the wild and hatchery populations was detected by F_{ST} over all six microsatellite loci (F_{ST}=0.001; $CI_{95\%}$=−0.01-0.021). This is confirmed by the similar frequency distributions of common alleles in each microsatellite locus between the wild and hatchery populations (Table 2). A F_{ST} value (0.001, P=0.970) far less than 0.05 was observed in the wild and hatchery populations, suggesting that the two populations can be regarded as one population (24).

The analysis of molecular variance (AMOVA) of all the six microsatellites revealed the similar results to FSTAT analysis with the variation within individuals, among individuals within populations, among populations being 93.71% (P=0.029), 6.23% (P=0.024), and 0.06% (P=0.970), respectively (Table 4). F_{IS}, F_{ST} and F_{IT} were 0.062, 0.001 and 0.063, respectively. AMOVA showed that the variation between the wild and hatchery populations explained only 0.06% of the total variance while the variation from among individuals within populations and within individuals explained 6.23% and 93.71% of the total variation, respectively. The limited genetic differentiation between the wild and hatchery populations may suggest a relatively short domestication history of hatchery broodstock fish, which was initially started in 1990s.

In conclusion, genetic studies on Pacific threadfin with microsatellite DNA markers are very rare with only one report on the development of new microsatellites for parentage assignment available from our previous work (6). No detailed information is available hitherto on the genetic diversity of wild and cultured broodstocks of Pacific threadfin. In this study we reported more new microsatellite loci for Pacific threadfin identified. Six highly polymorphic and quality microsatellite loci were characterized and used for studying genetic differences of the wild and hatchery populations. A total of 37 alleles were detected from these two populations with 32 alleles detected in the wild population and 36 detected in the hatchery population. No sign of linkage disequilibrium (P>0.18) was observed among the six microsatellite loci at 95% confidence levels with Markov chain correction. Genetic difference was not detected between the wild and hatchery populations of Pacific threadfin in Oahu Island based on analysis of six microsatellite loci. The high genetic heterozygosity observed in both populations implied the relatively minor effects of inbreeding. Monitoring genetic variance of successive generations of Pacific threadfin released to the wild through DNA markers is essential for the successful implementation of Pacific threadfin stock enhancement program.

Table 4. Analysis of molecular variance (AMOVA) of 6 microsatellite loci in the wild and hatchery populations of Pacific threadfin

Source of Variation	Sum of squares	Variance components	Percentage variation (%)	P-value
Among Populations	2.352	0.001	0.06	0.969
Among individuals within populations	126.825	0.134	6.23	0.024
Within individuals	116.500	2.009	93.71	0.029
Total	245.678	2.143		

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Conflict of Interests

The authors have declared that they have no conflict of interest.

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