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Research Article

Characterization of New Polymorphic Microsatellite Markers Derived from Genomic DNA Sequencing in the Larvae of Manila Clam *Ruditapes philippinarum*

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Abstract

In this study, 10 novel microsatellite loci were developed using Illumina paired-end shotgun sequencing and characterized in a population of *R. philippinarum* to study the genetic changes due to the artificial selection. Among the population studied, 39 alleles were detected. The number of alleles (NA) at the DL wild population ranged from 3 to 5 (mean NA=6.897). The observed and expected heterozygosities of DL population ranged from 0 to 0.735 and from 0.377 to 0.758, with an average of 0.404 and 0.595, respectively. The PIC ranged from 0.304 to 0.715 with an average of 0.537. Only one microsatellite loci in DL population deviated significantly from Hardy–Weinberg equilibrium (HWE) after correction for multiple tests. In seven groups (N=5, 10, 20, 40, 60, 80, and 104), significant genetic differences between groups (N=5 and 10) was observed. Information on the genetic variation and differentiation in population genetics is useful for future genetic improvement by selective breeding and design suitable management guidelines for *R. philippinarum*.

Keywords: ***Pomatomus saltatrix*; Size; Length-length; Length-weight relationship; Food; Spawning; Sex ratio; Length at first maturity; Oman; Arabian sea**

Introduction

It is significant to maintain the genetic diversity for population vitality and success reproductive and is a vital consideration for selective breeding programs to produce offspring with excellent characteristics. Furthermore, the breeders and their hereditary basis are vital factors to avoid risk associated with reduced vigor [1]. In hatchery breeding programmes, establishing new generations and avoiding inbreeding depression is highly related to effective population size [2]. The traditional method was to select some individuals with superior economic traits, and chose those individuals as breeders to reproduce in ways as human wish which may cause genetic drift and bottleneck. Consequently, the breeders are at risk of inbreeding because the correlation between individuals is elevated and genetic variation is lost [3]. Due to the large number of shellfish spawning behavior depends on the natural reproductive mating behavior, hatchery managers are often limited control of the contribution of each breeding, producing offspring in each pool. For these reasons, it is important to recognize the impact of selection on founders and to describe the key parameters of the sustainability of the breeding population, since genetic drift can result in a significant change in gene frequency in a short period of time and lower adaptation to changing environments [4].

Microsatellite DNA marker or Simple Sequence Repeat (SSR) was often used as a genetic tool for estimating genetic data (such as heterozygosity and allele diversity) and determining whether the population bottleneck occurs [5]. The application of microsatellite markers in genetics and breeding includes investigating the genetic differentiation of wild and cultured populations, assessing and determining the genetic relationship of the breeding population [6]. Although some microsatellite loci are available in *R. Philippinarum* [7-9], the gene-associated molecular markers is still not enough. The gene-related marker provides an assessment of the genetic diversity of the coding region and may be of greater value than the anonymous marker, because it provides information about the genomic region that is directly responsible for phenotypic variation [10]. Therefore, the gene-associated microsatellite markers were used to detect the diversity of wild populations and the genetic variation of different populations were analyzed at the DNA level to serve for farming work [11].

The Manila clam, *R. Philippinarum*, as an important marine economic shellfish, is widely distributed in the north and the south sea area of China. The farming production of the *R. Philippinarum* accounts for about one-third of China's shellfish production and 90% of world Manila clam production [12]. Nowadays, breeding practices of *R. Philippinarum* always choose traits that are economically important and typically selected for include growth, morphology, processing yields and disease resistance (Yasuda et al. [7]). In recent years, however, some problem in aquaculture of this species has occurred such as poor growth and high mortalities [13]. During artificial breeding process, insufficient number of the breeding population might reduce the genetic diversity of the closed populations by selective pressure and inbreeding [14,15]. According

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to previous reports, successive closed breeding with a limited number of parental founders could lead to a reduction in genetic diversity and the effective population size, which could increase the rate of both inbreeding and genetic drift. Therefore, the analyses of genetic status of the *R. Philippinarum* populations are necessary to maintain the genetic diversity of the valuable resources.

In the present study, novel microsatellite marker were developed and characterized in a artificial breeding population. We take advantage of microsatellite markers to measure allelic variation, genetic diversity and polymorphism within the cultured population. The aim of this work is to develop several gene-associated microsatellite markers via high-throughput sequencing (Illumina paired-end shotgun sequencing). Furthermore, investigate different sample size groups in genetic terms in order to know the impact of artificial selection on genetic diversity of artificially cultivated populations.

Materials and Methods

R. Philippinarum are collected from Dalian (121° 38'E, 38° 98'N). Genomic DNA of adult individuals (*n*=8) was extracted from adductor muscle tissue by Marine shellfish Extraction Kit (TIANGEN) DNA and stored in -20°C. Chelex-100 was used to extract the genomic DNA of Manila clam larvae (*n*=96). A total of 145 primers (Illumina paired-end shotgun sequencing by Novogene) were tested and then eight individuals were used to detect the amplification products and polymorphism. Polymerase chain reaction (PCR) amplifications were performed in a 10 μl reaction volume containing 0.5 U rTaq DNA polymerase (Takara, Japan), 1 × PCR buffer, 0.2 mM dNTP, 0.4 μM of each primer set, and about 15 ng template DNA. PCRs were performed using the following cycling conditions: pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 30 sec; and a final extension step of 5 min at 72°C. Amplification products were resolved on an 8% Polyacrylamide gel and visualized by silver staining. 10 bp DNA ladder (Invitrogen) was used to determine allele sizes.

Primers were designed using PRIMER 5.0 program. For the successful amplified primers, the fragment length, the number of alleles (N_A), observed (H_o) and expected (H_e) heterozygosities were estimated using the program MICROSATELLITE ANALYSER [16]. Deviations from Hardy–Weinberg equilibrium (HWE) and Linkage Disequilibrium were performed by GENEPOP 4.0 [17]. Polymorphism information content (PIC) was performed by PIC_CALC 0.6. Sequential Bonferroni corrections [18] were applied for all multiple tests. The MICRO-CHECKER 2.2.3 software [19] was used to check microsatellites for null alleles and scoring errors.

The 104 samples collected from Dalian were used in this experiment, and then the number of different populations were randomly selected from the whole group, and seven groups of different populations were selected (N=5, 10, 20, 40, 60, 80, 104). The genetic diversity statistics were calculated in these groups (repeats 10 times, and then calculated the average values). The mean number of alleles per locus is one of the most indicators of genetic variation [20]. The number of inter-population differences in the average number of alleles per locus is likely to be complex, based on an unequal number of samples. For this reason, we add average observed heterozygosity (H_o) to estimate population differences. Both theory and experiments indicate that mean number of alleles per locus is more sensitive to the effects of short, severe genetic drift. Additionally, heterozygosity may reflect more effectively in population's long-term evolutionary potential [21,22]. Nei's gene diversity index (H) is the most frequently used in analyses and comparisons of diversity within populations. So these three indices are used together to enhance the significance of conclusions and improve the credibility of the results. Results of these simulations were used to help assess the relationship between sample size and population genetic diversity. H is then equal to $H=1-(\Sigma Pi2)/N$ [23].

Results

A set of 10 SSR primers were successfully amplified with polymorphism from 145 primers 104 individuals. The number of alleles (Na) was between 3 and 5, the average number of alleles was 3.9, the observed Heterozygosity (Ho) and the expected heterozygosity (He) were between 0.000-0.735, and 0.337-0.758, with an average value of 0.404 and 0.595, respectively (Table 1). Polymorphic information content (PIC) was in the range of 0.304-0.715, with an average value of 0.537. Seven SSR loci have PIC values greater than 0.5, and the other three loci PIC values are between 0.25 and 0.5. The χ^2 test for Hardy-Weinberg equilibrium showed that there is only one loci with significant deviations ($P<0.05$) and 9 loci were in line with Hardy-Weinberg equilibrium. In this study, 10 microsatellite locis with unambiguous amplification bands were screened by genomic sequencing data in *Ruditapes*, and 2 of the development locis are related to functional genes, that Rpg2765 and Rpg6648 are Oreochromis niloticus G-protein coupled receptor family C group 6 member A-like (LOC100706264) and Gallus gallus ClpB homolog, mitochondrial AAA ATPase chaperonin (CLPB), respectively (Table 2).

There have been the most divergence in sample size n=104 and n=5. The number of alleles per locus was 2.1 and 3.9, average observed heterozygosity (H_o) was 0.318 and 0.404 and Nei's gene diversity index (H) was 0.512 and 0.594. The size of the population was significantly affected by the genetic structure of the population. Through the comparison of polymorphic loci in each group, it was found that the number of alleles in each group was different due to the population size. By comparing the mean number of alleles per locus, average observed heterozygosity (H_o), and Nei's gene diversity index (H) and population size, when the number of groups was greater than 20, respectively, the difference with the control group disappeared. Considering the genetic diversity parameters among the groups, as long as the number of breeding population is greater than 20, the number of breeding populations will not have a significant effect on the genetic diversity of the population in this wild population. All three genetic parameters are remarkably similar statistical properties. With the increase of sample size, the genetic diversity among populations also increased until the sample size reached 20. However, increasing the sample size (per population) produced diminishing returns: at some loci, sampling more individuals had little effect upon the coefficient of variation of the genetic diversity (Table 3).

Discussion

This work provides 10 microsatellite markers in *R. Philippinarum* for genetic analyze in the influence on artificial selection. Our results show that genetic diversity changed with sample size (n), and the establishment of population is an important factor in the overall level of genetic variability. A small founding population means a lower genetic variation level and then decreases with the passage of time. This reflects an important issue that must be considered when establishing a hatchery program. If some of the founders were used to produce offspring, and a few of these offspring were later used as the founders themselves, this would result in serious genetic bottleneck.

Table 1: Characterization of 10 polymorphic microsatellite markers developed from *R. philippinarum*.

Locus	Accession Number	Repeat motif	Primer sequence (5'-3')	T_a (°C)	functional gene	Size range (bp)
Rpg1504		$(AATTG)_4$	F:GTGAACTCGTGTACGAACACATCG	60	None	146-156
			R: AATCAAACCGTGTTTGCAATATCC		None	
Rpg1520		$(CTGTT)_4$	F:TGTCCAGATTTGGATCTTCTGACC	63	None	102-137
			R:TCAAAATGAAGCATCACTCACTGC		None	
Rpg2765		$(TTTGA)_4$	F:ATGCATGTATGTCGGTCAAAAACT	61	Oreochromisniloticus G-protein coupled receptor family C group 6 member A-like	149-159
			R:TTTTCGACCTTAATGGTCAATGGT		None	
Rpg3487		$(GAGTC)_5$	F:AATTGCAAAATTCATCCAATGACA	63	None	128-138
			R:AAAGAGTGACGGTGACACTAAGGC		None	
Rpg3803		$(TGTAT)_4$	F:TTGTGTTGAATGTTAATTGCTACAAACT	60	None	123-143
			R:TGATGTATATCACCTCACTTGCATTC		None	
Rpg3906		$(ATCTA)_5$	F:CTGCAACAAGCACTTGTATTTGAA	62	None	123-153
			R:GCCTATATAATTGTCCCAATGGTG		None	
Rpg3993		$(TCTTA)_4$	F:GGCCGGGTACCTCTAAATTGTATG	61	None	131-171
			R:CAAAGCAAGACAGAGATGTGTGAAA		None	
Rpg4997		$(ACCAT)_4$	F:CATTGGTGAATCGTCCACATACAT	61	None	97-117
			R:AGCCACAAAATGTGACCACTACAA		None	
Rpg6648		$(CCAAC)_4$	F:ATGCATATCACTCCACTCCACTCA	62	Gallus gallus ClpB homolog, mitochondrial AAA ATPase chaperonin (CLPB)	89-109
			R:TGCCGTATGATGGGATAGAATAGG		None	
Rpg6854		$(TGTTC)_4$	F:GATTGTGTGGCTATAATTGTGTTGTG	63	None	118-148
			R:GACACGGGTTGATATGCACTTG		None	

T_a: Annealing temperature of each primer pair

Table 2: Analysis of genetic diversity in 104 sample size population of *R. philippinarum*.

Locus	N_A	H_O	H_E	PIC	*P*
Rpg6854	5	0.5	0.687	0.632	0.984
Rpg3993	5	0.615	0.532	0.467	0.719
Rpg4997	5	0.471	0.758	0.715	1
Rpg1504	3	0.357	0.661	0.582	1
Rpg3803	4	0.642	0.61	0.557	0.106
Rpg3906	4	0.735	0.63	0.58	0.029*
Rpg1520	4	0.333	0.698	0.636	1
Rpg2765	3	0	0.603	0.515	1
Rpg6648	3	0.255	0.337	0.304	0.992
Rpg3487	*3*	0.13	0.438	0.377	1
Mean	*3.9*	0.404	0.595	0.537	

N_A: Number of Alleles; H_O: Observed Heterozygosity; H_E: Expected Heterozygosity. *Indicates significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction ($P<0.05$)

Comparisons between different simple size populations showed differing patterns. The general pattern found in our simulations started to converge on the basis of the wild population values approaching 20 and 40 sample sizes. We suggest that when the nuclear genetic diversity of a population is unknown, researchers should try to obtain a sample size of at least 20, preferably 40 individuals. Estimates based on these sample sizes should provide a useful measure of the genetic diversity of all populations, whether they are hereditary or hereditary or diverse.

The mean number of alleles per locus is thought to reflect the evolutionary potential of a population [24]. However, when samples less than 40 the number of alleles per locus is a poor measure of diversity. These findings correspond well with theory [25]. Error is caused by sampling bias, a lack of precision, and inaccuracy. This leads one to wonder whether or not allelic-based estimators can provide useful measures of diversity. Increasing the number of individuals in a study is not the only way to decrease the coefficient of variation of estimates of genetic diversity. Increasing the number of loci will also improve the precision of estimates of genetic distance [26-30]. In this study, population differentiation is small, and increasing loci may be the better way to estimate genetic diversity. Therefore more locis need to be developed to pave the way for farming work.

Genetic variation in the population is the main source of good breeding material. By studying the genetic diversity structure of *Ruditapes philippinarum* population, analyzing the degree of genetic variation in the population can keep the variability of the population in the maximum extent. Furthermore, the genetic diversity of the breeding species population and the maintenance of sex and variation provide the basis for maintaining a wealth of variation material in breeding work. Reproductive population size is a very important factor affecting the genetic diversity of germ plasm. In theory, if the breeding population is too small to maintain the genetic characteristics of the original species, it will lead to serious genetic drift. The greater breeding population greater the possibility of genetic drift. But in the actual operation, due to human, material and financial resources, it is impossible to reproduce too much of the population. In order to solve this contradiction, finding a suitable breeding population is

Table 3: Genetic change due to population size of 10 polymorphic microsatellite markers developed from *R. philippinarum*.

Population size (n)	the mean number of alleles per locus	average observed heterozygosity (Ho)	Nei's gene diversity index (H)
5	2.10 ± 0.22*	0.318 ± 0.028*	0.512 ± 0.017*
10	2.90 ± 0.29*	0.347 ± 0.019*	0.538 ± 0.009*
20	3.70 ± 0.27	0.385 ± 0.007	0.578 ± 0.011
40	3.80 ± 0.26	0.396 ± 0.005	0.582 ± 0.005
60	3.90 ± 0.18	0.401 ± 0.005	0.591 ± 0.003
80	3.90 ± 0.12	0.403 ± 0.002	0.593 ± 0.002
104	3.9	0.404	0.594

*Indicates significance of the number of alleles per locus, average observed heterozygosity (H_o), and Nei's gene diversity index (H) estimates for different population sizes of *R. philippinarum* measured using SPSS 17.0 (P<0.05)

important to prevent the occurrence of genetic drift in the process of reproduction and preservation. Moreover, more microsatellite loci associated with functional genes will be explored, which will provide more molecular markers for artificial marker-assisted breeding, and lay necessary foundation for the important economic traits such as growth, disease resistance, meat quality.

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