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Jacobs Journal of Aquaculture and Research

Research Article

Estimation of Genetic Variation in Natural and Cultured Stocks of Swimming Crab (*Portunus trituberculatus*) Detected by ISSR Markers

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Received: 07-23-2014

Accepted: 10-28-2014

Published: 11-04-2014

Abstract

Inter simple sequence repeat (ISSR) analysis was performed in order to evaluate the genetic diversity of natural and cultured samples of swimming crab *Portunus trituberculatus*. A group of 180 genotypes belonging to five natural samples, DY, WF, WH, QD, RZ and one cultured population, YT were screened using 13 different ISSR primers. A total of 120 loci were produced in the five studied samples. 42.20%, 40.95%, 45.83%, 43.10%, 43.36% and 39.58% of these loci were polymorphic among the individuals tested in the DY, WF, WH, QD, RZ and YT populations, respectively. The number of polymorphic loci detected by single ISSR primer ranged from 2 to 7. The average heterozygosity of the DY, WF, WH, QD, RZ and YT populations were 0.0757, 0.0730, 0.0839, 0.0814, 0.0802 and 0.0706, respectively. The WH population showed the highest genetic diversity in terms of total number of ISSR bands, total number of polymorphic bands, average heterozygosity and total number of genotypes among all the populations, while the WF population was the lowest among the natural populations. Compared with the natural populations, the cultured population showed a low genetic viability.

Keywords: Swimming Crab; *Portunus trituberculatus;* ISSR Marker; Genetic Variation

Introduction

The swimming crab Portunus trituberculatus, is distributed mainly on sandy and muddy bottoms in the coastal waters of Japan, Korea, and China. It is one of the most common edible crabs in China and Korea and supports a large crab fishery and aquaculture in China [1]. It is now being cultured in North China, especially in Shandong Peninsula, because of its high commercial interest. A few reports are available on molecular phylogeny and population structure in this species using different molecular marker techniques [1-4]. Long-term conservation of genetic diversity is important for any species [5]. Swimming crab resource management and enhancement are a recent practice to maintain long-term resource sustainability. A basic understanding of stock structure among geographical swimming crab samples is thus required.

In general, the effective sizes of founder populations are restrained by farming conditions, in which only a few individuals as broodstock are used. This practice may lead to the erosion of genetic diversity of stocks, thereby compromising industrial performance. Intentional and accidental release of cultured swimming crabs into natural environment could have major ecological consequences. If a large number of cultured swimming crabs escape or are released from aquaculture facilities, they could significantly alter the genetic composition of wild populations by either displacing them or interbreeding with them [6]. Most cultured stocks typically show a reduced

genetic variability, which may possibly result in the reduction of the population's capability to adapt to new environments [7]. Therefore, it is important to establish baseline information on genetic background of the aquaculture population both for genetic enhancement programs as well as protection of the genetic integrity of natural populations. Unfortunately, to date little is known about the population structure of swimming crab in China. Molecular markers provide a solution for the assessment of genetic variations. As a less widely used PCR-based marker, ISSR has a few advantages over other markers. ISSR primers anneal directly to simple sequence repeats [8] and the sequences that ISSR target are abundant throughout the eukaryotic genome and evolve rapidly. Many studies have indicated that ISSR produce more reliable and reproducible bands compared with RAPD because of the higher annealing temperature and longer sequence of ISSR primers [9-12].

Here we report that the genetic diversity among samples of swimming crab from Shandong peninsula in China was assessed using ISSR markers, and that genetic differences were observed among five natural and one cultured samples of swimming crab.

Materials and Methods

Swimming crab sampling

A total of 180 individuals of swimming crab specimens, based on six sample sets, 30 individuals each, were collected in 2009 and genetically screened in the present study. Geographic locations, sample sizes are given in Figure 1. Natural swimming crab were collected at five sites, the coast of Dongying (DY), the coast of Weifang (WF), the coast of Weihai (WH), the coast of Qingdao (QD) and the coast of Rizhao (RZ). Cultured swimming crabs were from a hatchery station in Yantai (YT). The cultured sample was founded using wild caught individuals from Bohai sea. Samples were stored frozen (-20°C) until genetic analysis was performed.

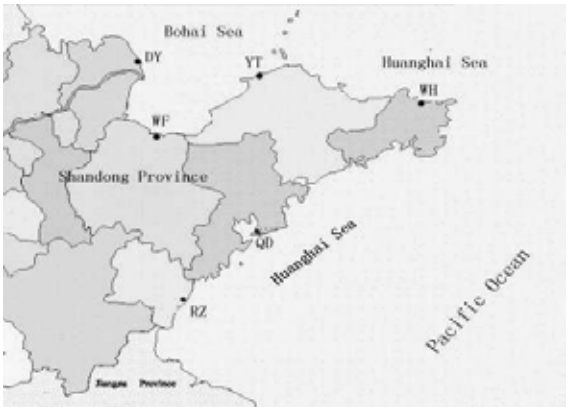

Figure 1. Sampling localities of swimming crab Portunus trituberculatus samples. DY, Dongying (n=30); WF, Weifang (n=30); YT, Yantai (n=30); WH, Weihai (n=30); QD, Qingdao (n=30); RZ, Rizhao (n=30).

Genomic DNA extraction

Genomic DNA was extracted from swimming crab muscle. About 150mg muscle tissue was digested overnight at 37°C in 0.85ml of lysis buffer (6 M urea, 10 mM Tris–HCl, 125 mM NaCl, 1% SDS, 10 mM EDTA, pH 7.5) and 50 µl of proteinase K (20 mg/ml). DNA was extracted twice with phenol and once with chloroform. DNA was precipitated by adding 200 µl of 7.5 M ammonium acetate and 500 µl of ethanol. DNA was collected by brief centrifugation and washed twice with 75% ethanol, air-dried, and dissolved in TE buffer.

ISSR PCR amplification

Twenty primers from Sangon Inc. (Shanghai, China) were tested for PCR. Amplifications were performed on a Peltier Thermal Cycler (PTC-200). PCR amplification was carried out in a 25 µl reaction mixture that included 20 pmol of primer, 100 µM of dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2% formamide, 1 unit of DNA polymerase, and approximately 50ng of template DNA. PCR cycles were as follows: 5min pre-amplification denaturation at 94°C, 40 cycles of 30s at 94°C, 60s at a primer-specific annealing temperature, and 1.5min at 72°C. At a final step, products were fully elongated for 5min at 72°C.

Gel electrophoresis and silver staining

The PCR products were mixed with an equal volume of formamide dye (99% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were heated to 95ºC to denature for 5min and immediately placed on ice. The gel was pre-electrophoresed at 60W for 30min, then 5.0 µl of the amplified DNA was loaded and run through a 5% denaturing polyacrylamide gel (4.75% acrylamide, 0.25% bisacrylamide, 7.5 M urea and 1×TBE buffer) with 1×TBE buffer on a DNA sequencing system (Liuyi Corporation, China) at 80W for 120min.

Silver staining was conducted using the procedures of Liu et al. [13] with modifications. After electrophoresis, the gel was fixed in 10% ethanoic acid for at least 30min. The gel was rinsed in distilled water three times and stained with a mixture of 0.1% silver nitrate and 0.15% formaldehyde for 30min. The stained gel was rinsed again with distilled water and immersed in a developing solution (3% sodium carbonate, 0.15% formaldehyde, 0.02% sodium thiosulphate). The development was subsequently stopped with 10% ethanoic acid when the bands became visualized and reached desirable intensity. Band sizes were estimated by a standard AFLP DNA ladder (Takara Corporation, China).

Data analysis

ISSR bands were scored for presence (1) or absence (0)

using the Crosscheck freeware 8 [14], and transformed into a 0/1 binary character matrix. Fragments that could not be scored unambiguously were not included in the analysis. The data matrix was analyzed for population genetic diversity using POPGENE software package 1.3.1 [15]. Population genetic relationships were estimated by constructing a UPGMA tree based on Nei's standard genetic distance [16]. Analysis of Molecular Variance (AMOVA) was performed to analyze genetic distance among samples using ARLEQUIN 3.1 [17]. Average heterozygosities and percent polymorphic loci were estimated using the TFPGA program 1.3 [18]. Average heterozygosity estimates were calculated for each locus and then averaged over loci according to Nei's [16] unbiased heterozygosity formula. The Shannon index, sometimes referred to as the Shannon-Wiener Index, is one of several diversity indices used to measure diversity in categorical data. It is calculated using the computer program POPGENE software package 1.3.1 [15]. The percentages of polymorphic loci were estimated based on the percent of loci not fixed for one allele. Confidence intervals were generated by bootstrapping analysis at the 99% confidence level with 1000 replications. All the above significances were tested using t-test ($P<0.05$ and $P<0.01$). Estimation of pairwise Fst values for all sample combinations were also performed using ARLEQUIN program and were evaluated by a test analyogous to the Fisher's exact test using the Markov-Chain method. Significance value was adjusted for multiple comparisons using the sequential Bonferroni correction [19].

Results

ISSR polymorphism

Twenty ISSR primers were screened on two randomly selected swimming crab individuals. By comparing the effects of magnesium concentrations and annealing temperature during amplification, 13 primers that produced clear and reproducible fragments were selected for further analysis (Table 1). These 13 selected primers generated 120 bands ranging in size from 250 to 2000 bp, corresponding to an average of 9.23 bands per primer. Every primer produced polymorphic bands when all of the six samples were considered. Genetic diversity varied greatly among samples with PPB values ranging from 39.58 to 45.83. The average heterozygosity of the DY, WF, WH, QD, RZ and YT populations were 0.0757, 0.0730, 0.0839, 0.0814, 0.0802 and 0.0706, respectively. The Shannon index (I) of DY, WF, WH, QD, RZ and YT samples were 0.1125, 0.1107, 0.1205, 0.1146, 0.1139 and 0.1059, respectively (Table 2).

The total number of bands and polymorphic bands produced by each primer varied. The highest number of bands was produced by SSR 9, and the highest number of polymorphic bands also by SSR 9. The number of ISSR genotype within each sample distinguished by each primer is shown in Table 3.

Table 1. Primers used for ISSR amplification.

Primer	Sequence of primer	Annealing temperature
SSR1	GT GT GT GT GT GT GT GT A	48
SSR2	AC AC AC AC AC AC AC AC T	46
SSR3	ACC ACC ACC ACC ACC ACC	49
SSR4	CTC CTC CTC CTC CTC CTC	55
SSR5	AG AG AG AG AG AG AG AG C	54
SSR6	AG AG AG AG AG AG AG AG G	50
SSR7	TG TG TG TG TG TG TG TG RA	51
SSR8	AC AC AC AC AC AC AC AC YT	50
SSR9	AC AC AC AC AC AC AC AC YA	50
SSR10	TG TG TG TG TG TG TG TG RT	48
SSR11	TC TC TC TC TC TC TC TC RG	47
SSR12	AG AG AG AG AG AG AG AG YC	49
SSR13	CA CA CA CA CA CA CA CA RG	51

Y=C/T; R= A/G

Table 2. A summary of ISSR analysis of genetic variations in swimming crab *Portunus trituberculatus*.

Samples	NB	NPB	PPB	H	I
DY	109	46	0.4220±0.05	0.0757±0.02	0.1125±0.06
WF	105	43	0.4095±0.07	0.0730±0.04	0.1107±0.05
WH	120	55	0.4583±0.06	0.0839±0.03	0.1205±0.03
QD	116	50	0.4310±0.08	0.0814±0.02	0.1146±0.02
RZ	113	49	0.4336±0.07	0.0802±0.02	0.1139±0.06
YT	96	38	0.3958±0.05	0.0706±0.02	0.1059±0.03

NB, No. of bands; NPB, No. of polymorphic bands; PPB, Percentage of polymorpic bands; H, Average heterozygosity; I, Shannon index.

Table 3. Number of bands per primer and number of ISSR genotypes determined for each sample per primer.

Primer	NB	NPB	ISSR genotypes					
			DY	WF	WH	QD	RZ	YT
SSR1	9	5	5	5	6	6	5	5
SSR2	7	2	2	2	3	3	2	2
SSR3	7	5	5	4	5	5	4	3
SSR4	12	5	4	5	5	4	5	4
SSR5	10	5	4	4	4	5	5	4
SSR6	9	3	3	3	4	3	3	3
SSR7	10	3	3	2	3	2	3	3
SSR8	8	4	4	4	6	5	5	4
SSR9	11	7	7	7	9	8	7	6
SSR10	9	4	3	2	4	3	3	2
SSR11	8	3	3	3	3	4	4	3
SSR12	11	4	4	4	4	5	4	4
SSR13	9	5	3	3	3	3	3	2
Total	120	55	50	48	59	56	53	45

NB, No. of bands; NPB, No. of polymorphic bands.

Population structure and genetic differences between natural and hatchery populations

The WH population showed the largest number of total ISSR bands, total polymorphic bands, average heterozygosity and total number of genotypes among all the populations, while the YT population had the smallest number. The WF population displayed the smallest number of total ISSR bands, total polymorphic bands, average heterozygosity and total number of genotypes in natural populations. A greater number of total ISSR bands were observed from the natural populations than from the cultured population. There were 109, 105, 120, 116 and113 ISSR loci detected in the DY, WF, WH, QD and RZ populations, while it was 96 in the YT population, respectively (Table 2). The total number of polymorphic bands was also higher (P<0.05) in the natural populations than in the cultured population. The total polymorphic loci were 46 in the DY population, 43 in the WF population, 55 in the WH population, 50 in the QD population, 49 in the RZ population and 38 in the YT population. However, no significant difference was found in the proportion of polymorphic bands among the five populations.

The UPGMA dendrogram constructed on the basis of the inter sample genetic similarity is shown in Figure 2. Genetic distances between samples are summarized in Table 4. Significant genetic differentiation was detected using AMOVA among samples (P<0.05). The pairwise Fst values in Table 5 also indicated significant differentiation among the six samples except between DY and WF, WF and YT, WH and YT, and QD and RZ. These results suggested that genetic divergence between these samples may have arisen.

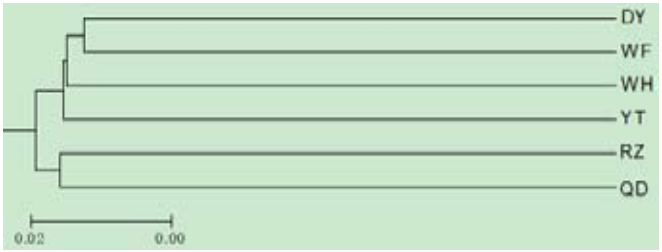

Figure 2. UPGMA dendrogram showing the phylogenetic relationship among six samples of swimming crab Portunus trituberculatus.

Table 4. Genetic distance of the six populations of swimming crab *Portunus trituberculatus.*

Pop ID	DY	WF	WH	QD	RZ	YT
DY	***					
WF	0.0881	***				
WH	0.0886	0.0884	***			
QD	0.0912	0.0907	0.0892	***		
RZ	0.0903	0.0925	0.0905	0.0847	***	
YT	0.0889	0.0882	0.0887	0.0890	0.0893	***

Table 5. Fst values for pairwise comparison among different samples of *Portunus trituberculatus.*

Pop ID	DY	WF	WH	QD	RZ	YT
DY	***					
WF	0.012	***				
WH	0.015*	0.014*	***			
QD	0.031*	0.029*	0.024*	***		
RZ	0.030*	0.026*	0.022*	0.011	***	
YT	0.022*	0.012	0.011	0.021*	0.021*	***

Asterisk indicates significant genetic differentiation test by Fisher's technique after a sequential Bonferroni correction (P<0.0125).

Discussion

Genetic variability is an important attribute of the species under domestication, since those with higher levels of variation are most likely to present high additive genetic variance for productive traits. Wild populations represent the primary source of genetic variability for aquacultured stocks. The YT population presented the smallest number of ISSR bands, total polymorphic bands, average heterozygosity and total number of genotypes compared to the natural populations, suggesting that the genetic variability of the hatchery strain seems likely to have been substantially reduced. This would be caused by losses of many low-frequency alleles due most likely to the small effective number of parents when the strain was founded, suggesting that the cultured strain was bottlenecked. The YT population was founded using about 36 individuals, but the number of effective parents may be smaller than that. This suggests that the genetic diversity in the natural swimming crab is still not being fully exploited. In view of this, there will be a higher chance of enhance diversity viability by frequent outcrossing of the cultivated swimming crab population with the wild population rather than by selective breeding followed by inbreeding among cultivated varieties. In this way, the genetic variability among the cultivated varieties would also be enhanced, thus preventing the occurrence of further different inbreeding that will probably take place if the present situation of unplanned breeding persists. Therefore, for high sustainability of the culture of swimming crab, proper breeding programs must be implemented with careful management and monitoring such that there is frequent outcrossing with the wild forms as well as maintenance of any newly emerged traits by inbreeding. Liu et al [1] found that significant genetic differentiation existed among the SS, ZS, DT, DH, and QZ populations of swimming crab in China, suggesting that gene flow might be reduced, even between the geographically close sites. Therefore, the genetic diversity declined, resulting from over fishing. They also discussed the potential applications of the mtDNA COI marker in the artificial breeding and fisheries management of swimming crab in their research. Many cultured aquatic stocks represent genetically exogenous populations, thus, the intra-specific hybridization with wild stocks may result in reduction of fitness in wild populations [20-22]. Even cultured populations that originated from the same local population may threaten the fitness of the local population through the reduction of its effective population size [23], especially when the absolute size of the wild population is small [24]. Hence, caution should be exercised to avoid significant release of cultured stocks of swimming crab into the wild, either intentionally, or accidentally.

The WH population showed the highest diversity among all the populations while the YT population was the lowest. The WF population displayed the lowest diversity among natural populations. This phenomenon could be explained that the WF population lives in the Bohai sea, a semi-closed water that can prevent genetic exchange with other populations. Our previous report also confirmed this result that a half-smooth tongue sole population lived in the Bohai sea had the lowest diversity than other populations in open waters [25]. QD and RZ population are near geographically and have more chance for gene flow. In the dendrogram, the QD and RZ populations clustered together and were clearly separated from the DY, WF, WH and YT populations. The UPGMA dendrogram showed that the WH population clustered closer to the WF population than to the QD population. The reason is presumably due to WH and WF populations have more chance for gene flow than WH and QD populations with the presence of physical barriers to migration between WH and QD populations. Some differences in the percentage of polymorphic loci among populations were also found in the present study. The question is whether the polymorphic loci distribution maintained in each population temporally is stable or not. The present study did not address this question, and the only way to improve the robustness of the data presented here is repetitive sampling taking into consideration sample size, sampling time and the life stage of sample. Beside such molecular approaches, accumulation of biological and ecological data is crucial. This is because high levels of migration among populations would provide sufficient gene flow to prevent remarkable genetic population differentiation.

Acknowledgements

This work was supported by a grant from Yantai University (SM13B22).

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