文章编号: 1000- 0615(2006)01- 0015- 06

Preliminary study on applicability of microsatellite primers developed from *Crassostrea gigas* to genomic analysis of *Hyriopsis cumingii*

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Abstract: *Hyriopsis cumingii*, which produces pearl ofthe highest quality, is a kind of freshwater species unique in China. The development of microsatellite markers of *H. cumingii* has not been reported. However, many microsatellite markers have been developed from *Crassostrea gigas*. The conservativeness of the side sequences of the microsatellite in species of close genetic relationship has already been discovered. In order to determine the applicability of microsatellite primers developed from *C. gigas* to genomic analysis in *H. cumingii*, 32 polymorphic microsatellite primers identified in the Pacific oyster were employed to amplify in the genome of mussel. The conditions of polymerase chain reaction(PCR) were optimized for the fidelity of DNA synthesis during PCR amplification. It was found that 19 loci failed to be amplified and 13 loci (about 41%) amplified specific products successfully. Among 13 loci, 3 (Cgi-6, Cgi-27and Cgi-28) were monomorphic and 10 (about 31%) (Cgi-1, Cgi-10, Cgi-18, Cgi-22, Cgi-24, Cgi-25, Cgi-26, Cgi-29, Cgi-30 and Cgi-32) were polymorphic. The analysis of the genetic diversity showed the average heterozygosity of 10 microsatellites loci of *H. cumingii* ranged betwen 0.125 and 0.693 and 7 loci (Cgi-10, Cgi-22, Cgi-24, Cgi-26, Cgi-29, Cgi-30, Cgi-32) are high polymorphic(He> 0.500), whereas 3 loci (Cgi-1, Cgi-18 and Cgi-25) are low polymorphic(He< 0.500). This study confirmed that 10 Pacific oyster pimers could be used for the analysis of the genetic diversity in *H. cumingii*. This result showed some of the microsatellite primers can be used for genetic analysis of mussel without high coat costing and time consuming. And it suggested that this method could be useful in genetic analysis of other species of mussel.

Key words: *Hyriopsis cumingii*; *Crassostrea gigas*; microsatellite primer; genomic analysis

CLC number: S917 **Document code:** A

Microsatellite, with a short length of dozens to hundreds bps among the molecular of DNA, is a highly repeated sequence that covers in an even way in the whole genome, through some nucleotides of 1- 6 bp tandem sequence ranging from head to rear[1, 2]. The alleles of microsatellite have some traits, such as quick mutation, high level of polymorphism, high quality of heterozygosity, informativeness, common dominance, easy-getting material, little effort of samplings, the screening of motifs by polymerase chain reaction (PCR) and the recognition of the sequence of the alleles. So, it has a wide application in genetic structure analysis of population, in genetic diversity study of population, in construction of the genetic map and the analysis of the linkage of the productive loci[3-6]. The common way to obtain microsatellite sequence is through construction and screening of genomic library, but it has the disvantage as it is time-consuming and costly. Meanwhile, the conservativeness of the side sequences of the

Received date: 2005-03-11

Foundation items: This study was supported by the grants from the Basic Research Priorities Program of the Science and Technology Committee of Shanghai (03JC14063); Shanghai Leading Academic Discipline Project(Y1101); E-Academy of Aquaculture of University in Shanghai(03E009); Sunlight Reaseach Program of the Education Committee of Shanghai (01SG42).

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microsatellite in the species of close genetic relationship has already been discovered[7-9]. And this kind of conservative sequences has provided a convenient way for the fast development of target organism, by using molecular markers of model organism or other organisms. USDA has already begun the research on five kinds of aquaculture species. *Crassostrea gigas* is the only kind of mussel. There have already been some reports about the development of the microsatellite markers in *C. gigas*, which establish a very stable basis for the development of microsatellite markers for other kinds of mussels. *Hyriopsis cumingii*, which produces pearl of the highest quality, is a kind of species unique in China[10]. The development of microsatellite markers of *H. cumingii* is still a blank. This experiment is dedicated to the possibility of microsatellite primers developed from *C. gigas* for the genomic analysis of *H. cumingii*.

1 Materials and Methods

1.1 Collection and treatment of samples

H. cumingii was sampled from pearl culture factory in Wangjiajing, Zhejiang province, which came from F1 generation of the inbred population of Dongting Lake. The mantles of the living *H. cumingii* were stored in ethanol at 4 ℃ for the extraction of total DNA.

1.2 DNA extraction

The mantles stored in ethanol, were washed in double dehydrated H_2O. The genomic DNA was extracted individually from the F1 inbred population following the chloroform/phenol method. For each mussel, about 100 mg of tissue was digested in 500 μL buffer (50 mmol·L^{-1} Tris-HCl, 100 mmol·L^{-1} EDTA, 1% SDS, 0.2 mg proteinase K) at 65 ℃ for 1 hour. DNA was extracted once with (phenol-chloroform-isoamyl alcohol (25∶24∶1) and twice with phenol-isoamyl alcohol (24∶1), then it was precipitated using isopropanol. Pellets were washed in 70% ethanol twice, dried, and suspended in 200 μL autoclaved ddH_2O. Extracted DNA was stored at 20 ℃. Then the value of OD is checked, to measure the purity and density of DNA. Total genomic DNA was analysed by 1% agarose gel electrophoresis.

1.3 PCR amplification and data analysis

Thirty-two pairs of microsatellite markers[11,12] of *C. gigas* were synthesized by Shanghai Biological Project Company (SBPC). *Taq* DNA polymerase and nucleotides were bought from Dalianbao Biological Company, molecular markers pUC19DNA/MspI from SBPC, and some other common chemicals like proteinase *K*, phenol, etc. from Beijing Dingguo Biology and Technology Limited Company.

PCR was performed in 20 μL reaction, containing 2 μL of 10 × buffer, 10 pmol of each primer, 80 μmol·L^{-1} of each dNTP, 0.5 unites of taq DNA polymerase, 100 ng of the template DNA, and the density of the Mg^{2+} varies depending on the locus (Tab. 1). Thirty PCR procedures were as follows: 2 min at 94 ℃ for denaturing, then thirty PCR cycles (1 min at 94 ℃, 1 min at Tm, 1 min and 15 s at 72 ℃), then 5 min elongation step at 72 ℃. PCR products were electrophoresed on 3% agarose gel, using 0.5 × TBE buffer. Results were visualized by scanning the gel using the fluorescence scanner. And heterozygosity was calculated by software TFPGA.

2 Result

2.1 Optimization of PCR procedure

In the PCR reaction, not only the unspecific amplified products appeared in the PCR reaction, but also two other types of unspecific amplified fakes and positive bands-shadow band and heteroduplex, which were related to the microsatellite, appeared[13]. Through experiment, with 20 μL reaction (10 pmol of each peimer, 80 μmol·L^{-1} of each dNTP, 0.3 unites of *Taq* DNA polymerase, 100 ng of the template DNA), and with the optimum annealing temperatures to each of the primers, the general random amplified fake bands were elimimated. Moreover, the two types of fake and positive bands mentioned above were efficiently elimimated by modifying the density of the Mg^{2+}.

2.2 Microsatellite primers of *C. gigas* selected for *H. cumingii*

Thirty-two microsatellite primers of *C. gigas* were used in the PCR amplification of *H. cumingii* genome DNA. It was found that 19 loci failed to be amplified and 13 primers (about 41%) amplified specific products successfully (about 41%). Among 13 loci, 3 (Cgi-6, Cgi-27 and Cgi-28) were monomorphic and 10 (about 31%) (Cgi-1, Cgi-10, Cgi-18, Cgi-22, Cgi-24, Cgi-25, Cgi-26, Cgi-29, Cgi-30 and Cgi-32) were polymorphic (Fig. 1, 2). The amplificatim conditions of these polymorphic primers are seen in Tab. 1.

Tab. 1 The amplification conditions and results of *Crassostrea gigas* microsatellite primers tested for *H. cumingii*

primer	primer sequences	annealing temperature	Mg^{2+} ($mmol \cdot L^{-1}$)	length of PCR product	allele no.
Cgi-1	TTGCAGGAAGCAAGAGATGA CTTGTTAACTGCCGGTGAGG	57℃	1	30-80	2
Cgi-10	TGCACCAATTTGAGATGTGA ACTGAGTTTGAAAATGTCACCG	50℃	1.5	70-600	4
Cgi-18	TCCATGTTTACTGCTACTTTTGG AAATGCTGTGCAGAGAAGCC	50℃	1.5	50-100	3
Cgi-22	GGAAGAGGAATAGTCTACTTATGC GTCAGACGTTCCTAACTCTTC	43℃	1.75	100-240	3
Cgi-24	CAGAGAGCCGGACTATTTC GCTCTTTGACACTATGCCGA	45℃	1	100-550	6
Cgi-25	CATCAGGGGTAAATTAAAGTAAGC CCACAGACGATTTCATATATCCTG	55℃	2	90-120	2
Cgi-26	ATATGTAATGATTACGAAACTC GTATGAGATTTGGTTCCACC	55℃	1.5	90-600	3
Cgi-29	TCAAACCATCTGCTCGTCTACG TCCGAAAATCCAGGAATACCGG	60℃	1.5	90-600	7
Cgi-30	TCGTCACCTCCCTCTCAGAG GCTGTATTTCCATCAATTCGAG	60℃	1.5	60-600	6
Cgi-32	TTGCAGGAAGCAAGAGATGA CTTGTTAACTGCCGGTGAGG	60℃	2	40-620	5

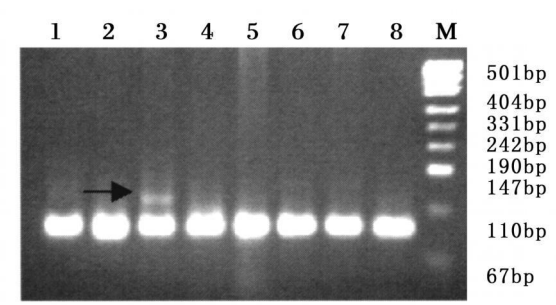

Fig. 1 Electrophoretic patterns of PCR products amplified by primer Cgi-25 in 8 mussels

M: pUC19DNA/MspI maker, 1-8: mussel

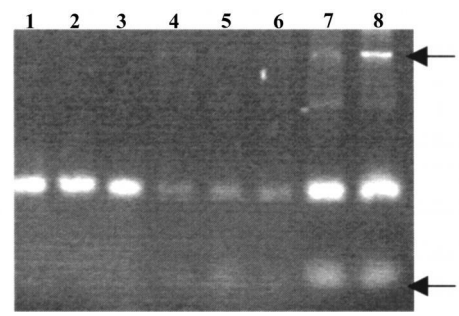

Fig. 2 Electrophoretic patterns of PCR products amplified by primer Cgi-10 in 8 mussels

1-8: mussel, arrow: polymorphic band

2.3 The analysis of the genetic diversity of the microsatellite primers of *C. gigas* to Dongting Lake's *H. cumingii* population

Ten microsatellite primers mentioned above were applied in different individuals of *H. cumingii* from Dongting Lake, using the PCR procedure programs. Heterozygosity was calculated by software TFPGA(Tab. 1). The analysis of the genetic diversity showed the average heterozygosity of 10 microsatellites loci of *H. cumingii* ranged betwen 0.125 and 0.693. Among 10 microsatellite loci, 7 loci such as Cgi-29, Cgi-24, Cgi-30, Cgi-32, Cgi-10, Cgi-22 and Cgi-25, are high polymorphic (He> 0.500), whereas other microsatellite loci, such as Cgi-1, Cgi-18, Cgi-25, are low polymorphic(He< 0.500). Primer Cig-29 has the highest quality of polymorphosis which produced 7 bands in the eight amplified individuals from Dongting Lake and the heterozygosity is 0.693. However, Primer Cgi-1 was less polymorphic, which produced 2 bands in the small population and the heterozygosity is 0.125.

3 Discussion

3.1 The optimization of PCR procedure, elimination of fake and positive bands

To keep the specificity of PCR reaction for the correct differentiation and analysis of the amplified products, it is very important to have the PCR procedure optimized. Generally, as far as the diploid is concerned, the primers are mostly designed directly to the specific microsatellite loci. So, each primer will bring the result of two bands in the heterozygote PCR amplification, and only one band in the homozygote PCR amplification[14]. And that is the criterion to decide whether the STR-PCR reaction is specific or not. In the PCR reaction, except for the common unspecific bands caused by some external factors, such as, the density of polymerase and Mg^{2+}, the optimum annealing temperature, the time of cycling, there is another stutter band which is caused by the trait of the microsatellite itself, and is the allele bands in the electrophosis picture and which is not a single band, but a successive band. This happens mainly in dinucleotide repeated sequences. This is because of the relative slipping when the PCR amplifies, which leads to the addition or diminishing of one or two repeated units. However, by the optimization of PCR procedure, that will not have any negative effect on the analysis of the result[15]. Additionally, what should be mentioned here is that, with different quantities of (G+C), there will be different length of the amplified products. So, different primers have different PCR procedures. The PCR procedure of each of the primers should be ascertained through experiment.

3.2 The feasibility of microsatellite primers of *C. gigas* in the application to the analysis of *H. cumingii* genome

The microsatellite marker is widely used for the conservativeness in genetics. Therefore, with the database and papers published, it is an effective and easy way to find the microsatellite locus, by using the microsatellite primers of a species in related taxonomic taxa and the PCR amplification. Many scholars have tried in this way, such as, by using 47 pairs of microsatellite primers of *Cyprinus carpio*, David *et al*. reported that they have got 23 (about 49%) microsatellite loci in the mutated genome of *Cyprinus carpio*, which can be used for the analysis of the *Ctenopharyngodon idellus* genome[16]. Shao *et al*. have done some tests of applying 21 pairs of the microsatellite primers of Acipensersinensis to the *Scaphirhynchus platorynchus*[17]. The genetic linkage map of *Cyprinus carpio* (Linnaeus) constructed by Sun &Liang has 70 SSLP markers of zebrafish and 19 SSLP of *Carassius auratus*, respectively[18].

C. gigas used in this experiment belongs to the *Lamellibranchia pterimorphia* Ostreoida, and the *H. cumingii* belongs to *L. palaeoheterodonta* Unionoida. There is some kinship between the two species. So, the side sequences are probably somewhat of same originality, and this is proved in this experiment. Thirty-two pairs of microsatellite primers of *C. gigas* were used in the PCR amplification of the *H. cumingii* genome, and 13 pairs of the primer can give the PCR amplified product (about 41% of the total)

and 10 of them (about 31%) have clear electriphosis bands, implying that these are specific PCR amplification. Based on the analysis of the genetic diversity in *H. cumingii* from Dongting Lake and 13 pairs of primers mentioned above, the result shows that: in the eight individuals of *H. cumingii* tested, ten pairs of the primers (31%) reveal polymorphsis of alleles among different individuals, the other three pairs of the primer show no polymorphsis bands. This is probably because the sample of the population is too small. Although these primers are not polymorphosis primers, they can be used in the analysis of genome purity of *H. cumingii* individual or population. These initial results show that: some of the microsatellite primer selected can be used in the analysis of *H. cumingii* genome. And till now, the way we obtain the microsatellite sequences is through the construction and the selection of GenBank deposited sequences. But omning to the longtime cycling and great financial cost, the popularity of this technique is hampered. The conservativeness of side sequence of microsatellite in species of close genetic relationship has provided a way for developing the research on populations of organisms, by using the microsatellite primers of some model organisms. *C. gigas*, as a kind of model organism in mussel, has been the reports in relation to the development of microsatellite markers. The results in this experiment establishes a good basis for the microsatellite marker development in related species of mussel.

ACKNOWLEDGEMENTS

Thanks to Zhang-hua ZHU for help with modification of the English paper.

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太平洋牡蛎微卫星引物对三角帆蚌的适用性研究

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摘要: 三角帆蚌(*Hyriopsis cumingii*)是我国特有种, 它形成的珍珠具有珠质光滑细腻、色泽鲜艳等方面的优点, 是淡水蚌中育珠质量最佳者, 已成为最主要的淡水养殖珍珠蚌。本研究选取已发表的32对太平洋牡蛎的微卫星引物在三角帆蚌基因组DNA中进行PCR扩增, 探讨太平洋牡蛎的引物用于三角帆蚌基因组微卫星分析的可能性。通过优化PCR反应条件, 筛选13对引物可在三角帆蚌基因组中扩增出特异性条带(占总数的41%); 其中10对引物(占总数的31%)在洞庭湖三角帆蚌小群体中即检测到了个体间等位基因的多态性, 共出现40条多态性条带。10个位点杂合度大小在0.125到0.693之间, 其中7个微卫星位点(Cgi-10, Cgi-22, Cgi-24, Cgi-26, Cgi-29, Cgi-30, Cgi-32)为高度多态性位点, 杂合度大于0.500, 而其余3个微卫星位点(Cgi-1, Cgi-18 and Cgi-25)杂合度小于0.500, 为低度多态性位点。初步的结果表明, 部分太平洋牡蛎的微卫星引物可以用于三角帆蚌基因组的分析, 这为其它贝类微卫星标记的开发奠定了基础。

关键词: 三角帆蚌; 太平洋牡蛎; 微卫星引物; 基因组分析
中图分类号: S917 文献标识码: A

收稿日期: 2005-03-11
资助项目: 上海市科委重点基础项目(03JC14063); 上海高校水产养殖E-研究院(03E009); 上海市教育基金会曙光计划项目(01SG42); 上海市教委水产养殖重点学科资助(Y1101)
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