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Inheritance pattern of EST-SSRs in self-fertilized larvae of the bay scallop *Argopecten irradians*

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Null allele and marker segregation distortion are considered to be common in bivalve molluscs. To facilitate genetic analysis of the bay scallop, the inheritance patterns of nineteen EST-SSRs were tested in four self-fertilized families. While the filial genotypes in twelve segregations conformed to Mendelian inheritance, three locus-family cases showed the significant departure. Two loci confirmed Mendelian expectations when null alleles were considered. The molecular basis of null alleles was detected by redesigning primers in the external flanking regions, and single nucleotide mutations in primer annealing sites were responsible for the null allele. Based on our results, the inheritance pattern and null allele of novel microsatellite markers should be further tested before using them for population studies and parentage assignment in the bay scallop. Additionally, six markers were recommended as members of a standard marker panel for use in population genetic and parentage studies.

Introduction

Molecular DNA markers have been shown to be some of the most useful tools at the individual, population and species levels (Haig 1998, Parker *et al*. 1998, Maxwell *et al*. 2000). Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated sequences (usually 1–6 bp) that show high polymorphism and an even distribution throughout the whole genome (Toth *et al*. 2000). Due to the prominent advantages of the SSR marker system, such as hypervariability, abundance, neutrality, codominance and unambiguous scoring of alleles, it is widely used in population genetic studies, fertilization analysis, linkage maps construction, parentage investigation and evolutionary analysis (Huvet *et al*. 2001, Selvamani *et al*. 2001, Xu *et al*. 2001, Martin *et al*. 2002, Ohara *et al*. 2005).

Traditionally, microsatellite markers show Mendelian inheritance: independent segregation and random assortment of allele (e.g. Maxwell *et al*. 2000, Rajora *et al*. 2001, Slavov *et al*. 2004). However, contraventions of these laws are continuously reported (e.g. Dobrowolski *et al*. 2002, de Meeus *et al*. 2004), and these seem to be more severe in marine molluscs, such as abalones, clams, mussels and oysters (Launey & Hedge-

cock 2001, Li *et al*. 2003, Hedgecock *et al*. 2004, Baranski *et al*. 2006). However, there is currently no information available on the microsatellite transmission in scallop species until now.

The bay scallop (*Argopecten irradians* Lamarck 1819) is naturally distributed in the western North Atlantic along most of the United States coast (Robert *et al*. 2005). It is considered to be one of the most important economic marine organisms for aquaculture or fishery in many countries. Due to its high fecundity (usually more than 1 000 000 eggs per mature female) and the large population size, the bay scallop is expected to have higher mutation rates, which would lead to high nucleotide diversity causing the widespread of null alleles in different individuals within and among populations (Williams 1975). The presence of null alleles within a pedigree can result in an individual's genotype being apparently inconsistent with classical Mendelian inheritance (Callen *et al*. 1993, Li *et al*. 2003). This phenomenon has been elucidated in some marine molluscs such as the European flat oyster *Ostrea edulis* (Bierne *el al*. 1998) and the Pacific oyster *Crassostrea gigas* (Launey & Hedgecock 200l, Hedgecock *et al*. 2004). The presence of null alleles can lead to confounding results in genetic analyses, such as the erroneous elimination of putative parents or errors regarding the degree of relatedness between individuals in a parentage analysis (Ardren *et al*. 1999). We should also be aware that apparent excesses of homozygotes, incorrect allele frequency estimates and overestimates of inbreeding all might be due to null allele(s) in population genetic studies (Jones *et al*. 1998, Xu *et al*. 2001).

Thus, it is imperative that we should confirm the Mendelian inheritance of microsatellite markers and check the spread of null alleles for their potential use. The direct tests are to pair mates and analyze the genotypic frequencies of their offspring. Due to the hermaphroditism and the simultaneous releases of spermatozoa and ovocyte in the natural spawning cycle, it is therefore challengeable to establish crosses between heterozygous parents to detail the Mendelian segregation in the bay scallop. In the present study, we established four self-fertilized families to test the inheritance patterns of 19 polymorphic EST-SSRs that we recently developed for the bay scallop. Additionally, the molecular basis of null alleles was assessed by redesigning primers in the external flanking regions, and the frequency of the null allele was estimated by a population. Based on the results, we recommend 6 microsatellite markers as part of a standard marker panel for population genetic and parentage studies in the bay scallop.

Materials and methods

Family establishment

The parental scallops were sampled from the Yantai Scallop Hatchery (Shandong Province, P. R. China). Healthy individuals were randomly selected as the progenitors for this study. Gametes were procured from these scallops during their natural spawning cycle, and zygotes were separately cultured in 1-μm-filtered seawater at 24 °C. After about 30 hours, the larvae were harvested in the swimming D-larvae stage by sieving with filters and preserved in 100% ethanol at 4 °C. Four specimens were self-fertilized to create 4 separated families (family A, B, C and D) for the microsatellite transmission study.

Microsatellite analysis and individual genotyping

The DNA of parental scallop was extracted from adductor muscle using the standard phenol/chloroform method described by Sambrook *et al*. (1989). A larva was individually picked out with the Micro-Operation System (Nikon) and then lysed in 20 μl LoTEPA buffer (Tris-HCl, 3 mM, pH 7.5; EDTA, 0.2 mM, pH 7.5; proteinase K, 0.5 mg ml^{-1}). The mixture was performed in a thermal cycler (GeneAmp PCR System 9700) using the program of 56 °C for 1 h and followed by 95 °C for 10 minutes to inactivate the proteinase K, and 1.5 μl DNA solution was directly used as the template for PCR amplification.

A total of 19 polymorphic EST-SSRs (Zhan *et al*. 2005, 2006) were screened in 4 families by PCR amplification. Heterozygous loci identified in parents were selected for further segregation analysis. Five additional homozygous loci were

randomly selected and used for the detection of null allele. The characteristics of these loci are given in Table 1.

PCR amplification was performed in a thermal cycler (GeneAmp PCR System 9700). The reaction mixture contained about 40 ng of DNA extracted from adult scallop or 1.5 μl of larvae DNA solution, 0.2 μM of each primer, 200 μM of each dNTP, 1.5 mM of Mg^{2+}, and 0.5 U *Taq* polymerase (Takara) with 1 × PCR buffer in a total volume of 10 μl. The PCR program was 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at the locus-specific annealing temperature (Table 1) and 45 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were separated on 10% non-denaturing polyacrylamide gel at 250 V for 2–3 h. The gels were stained with ethidium bromide and photographed under UV light. The genotypes of different loci in parents and offspring were scored with Quantity One Version 4.4 (Bio-Rad) by comparing with the DNA molecular standard (100 bp DNA ladder marker, TaKaRa) in the gels.

The nonamplification in PCR for individual larvae was repeated to exclude technical problems, such as larvae sampling or larvae lysis for DNA release, rather than PCR amplification. Finally, 88 individual templates that showed good amplification in each family were prepared for further analysis.

Segregation analysis

The inheritance patterns of microsatellites were checked in at least one of the 4 families established. All observed filial genotypes of each locus obtained from 88 offspring in each family were tested against the expected Mendelian segregation ratio using the χ^2-test with $n - 1$ degrees of freedom, where n = number of genotypic classes. The significant criteria were adjusted for the number of simultaneous tests using the sequential Bonferroni technique (Rice 1989).

Table 1. Locus name, accession number in GenBank, repeat motif, primer sequences and annealing temperature for 8 EST-SSRs selected in the bay scallop.

Locus name Accession no.	Core repeats	Primer pair name	Primer sequences (5´–> 3´)	Annealing temperature (°C)	Expected size (bp)
AIMS011[a] CV660848	$(GAT)_{10}$	AIMS011P	F: GACAGCAGAACAGTCAGTAGTTGTG R: GCACGTCTGCTTTCTCTGTATTAAC	63	256
AIMS012[a] CN783420	$(TTAT)_{10}$	AIMS012P	F: GAGAGTACAAGCACTGTTCTCATG R: GGTGCTATATCGACCTATATCTGAG	63	256
		AIMS012S[c]	F: TGGGCCTTTAAGCCAGTAAG R: TATCGCCAATTACTGACCTC	56	363
AIMS019[a] CN782436	$(GA)_{14}(AG)_5$	AIMS019P	F: CTCCACCTTCAGAACCATCC R: CGAAAGAAAATATCAAGCACAC	60	214
AIMS020[b] CB416269	$(AGG)_7$	AIMS020P	F: AGTAGAGCGGAACGGATGTGC R: GAAGTTTGAGATAATGAGGTAGGG	61	186
AIMS022[b] CB413627	$(CAAA)_6$	AIMS022P	F: GACCCTGGATACCAATAAGACG R: TTGTATTCCGGGTGAGCGATAG	61	202
		AIMS022S[c]	F: GCACTTCATACACGCAACAC R: TATTAGGCAATGCGATGAACC	58	346
AIMS025[b] CB413181	$(AT)_{10}$	AIMS025P	F: AAGCCACGTGTCCCTTGTGCG R: CGGCGGGTCAATTCTCATGTCG	66	204
AIMS026[b] CB416920	$(GAT)_6$	AIMS026P	F: CACTTCAGACACAAGTTACCGC R: TGAACCACCAAAGGTGACGGGG	66	153
AIMS028[b] CB412414	$(TA)_{11}$	AIMS028P	F: ATCTTATCCTGTGCCATTGGAC R: CTAAATCCTGAAACAAGATGCC	63	136

[a] from Zhan *et al.* 2005.
[b] from Zhan *et al.* 2006.
[c] primers re-designed to elucidate the molecular basis of null alleles.

Elucidation of the molecular basis of null alleles

Based on the results of the segregation analyses, the primers of loci which suggested null alleles were redesigned in the external flanking regions. The microsatellites were re-amplified using the new primer pairs and a high-fidelity DNA polymerase KOD DASH (TOYOBO). The PCR products were cloned into pMD18-T vector with the TA-clone kit (Takara). Plasmid DNAs were isolated and sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit using an Applied Biosystems ABI 3730 Automatic Genetic Sequencer. The sequences obtained were multiple-aligned by the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to elucidate of the molecular basis of null allele.

Estimation of null allele frequency

Thirty bay scallop individuals sampled from the cultured population were randomly collected from Huangdao Scallop Hatchery (Shandong Province, P. R. China) to assess the observed frequency of null alleles. DNA extraction, PCR reaction and genotype determination were performed according to the methods stated above. The genetic data analysis was performed according to Zhan *et al*. (2006), and the allele frequency was estimated using the online version of GENEPOP (http://wbiomed.curtin.edu.au/genepop/).

Results

Inheritance patterns of EST-SSRs

Of the 19 microsatellites developed in the bay scallop, loci AIMS011 in families B and D; AIMS012 in families A and C; AIMS019 in family B; AIMS020 in families A, B and C; AIMS025 in families A and D; AIMS026 in family C and AIMS028 in family D showed heterozygosity in the parents. These loci, together with five loci that showed homozygosity in the parents (Table 2), were adopted for further Mendelian inheritance tests. The genotypes of the parents and offspring in each family at each locus are presented in Table 2. Of 17 genotypic ratios examined, the filial genotypes in 12 segregations conformed to Mendelian inheritance according to the χ^2-test. For example, the parent was heterozygous for the genotype of 183/186 in family B at locus AIMS020, and the offspring produced 3 genotypes (183/183, 183/186 and 186/186) that segregated according to a 1:2:1 Mendelian expectation (Table 2). However, three genotypic ratios (loci AIMS012 and AIMS019 in family B, and locus AIMS022 in family D) did not conform to the expected Mendelian inheritance due to unexpected genotypes in the progenies or severe departure from the Mendelian ratio.

Among these three genotypic ratios that were inconsistent Mendelian segregation, two (locus AIMS012 in family B and locus AIMS022 in family D) confirmed Mendelian expectations when unexpected offspring genotypes were considered heterozygotes for null alleles (Table 2). The genotypes of 26 and 22 offspring could not be scored because of a lack of amplification at locus AIMS012 in family B and at locus AIMS022 in family D. The χ^2-test showed the conformation to the Mendelian inheritance ratio of 3:1 (Table 2), and therefore it is assumed that the parents carry null alleles. The genotypes of the parents should be 259/null and 202/null, and the expected genotypes of the offspring were 259/259, 259/null and null/null, and 202/202, 202/null and null/null, respectively. The null allele heterozygotes (259/null and 202/null) were mis-scored as the homozygotes (259/259 and 202/202) in microsatellite analysis. The primers (AIMS012S and AIMS022S, Table 1) were re-designed in the external flanking regions to examine this assumption and to elucidate the molecular basis of null alleles.

The fact that all of the 88 progenies in family B successfully amplified at locus AIMS019 indicated that the distorted segregation was not due to the null allele. However, the parent transmitted 61 alleles of 210 bp and 115 alleles of 214 bp to the offspring, and the χ^2-test showed that both allelic and genotypic frequencies significantly deviated from Mendelian ratios. To exclude the possibility of deviation caused by sampling

errors, additional 100 larvae were used to check the allele transmission, however, the departure phenomenon was not improved (Table 2).

Molecular basis of null alleles

Amplification using the new primer pair AIMS022S showed heterozygosity in the parent of family D. Sequencing results revealed that three single nucleotide mutations were responsible for this null allele. Two nucleotide mutations were detected in the forward primer, a single nucleotide mutation T to C in the 5´-end and C to A in the middle of the primer, and a single nucleotide mutation, A to T, was identified close to the 5´-end of the reverse primer (Fig. 1). Additionally, 8 single nucleotide mutations were detected in the flanking regions of 322 bp in length (Fig. 1).

PCR amplifications using the newly designed primer pair (AIMS012S) yielded the genotype

Table 2. Inheritance of 8 EST-SSR loci in the bay scallop (*Argopecten irradians*) from 4 self-fertilized families. The genotypes of 258/*x* and 202/*x* at loci AIMS012 and AIMS022 represent the genotype combinations of 258/258 and 258/null, and 202/202 and 202/null, respectively, detected as the "homozygous band" in PAGE.

Locus	Family	Parental genotype	Observed number of offspring in each genotypic class (theoretical number of offspring in each genotypic class)				χ^2
AIMS011	B	246/252	246/246 20 (22)		246/252 40 (44)	252/252 28 (22)	1.091
	C	255/255			255/255 88 (88)		–
	D	252/258	252/252 28 (22)		252/258 42 (44)	258/258 18 (22)	1.227
AIMS012	A	252/272	252/252 30 (22)		252/272 36 (44)	272/272 22 (22)	2.182
	B	258/null		258/*x* 60 (66)		null/null 28 (22)	1.091
	C	260/272	260/260 26 (22)		260/272 36 (44)	272/272 26 (22)	1.455
AIMS019	B	210/214	210/210 18 (46)		210/214 78 (92)	214/214 92 (46)	32.59
	D	214/214			214/214 88 (88)		–
AIMS020	A	180/186	180/180 32 (22)		180/186 38 (44)	186/186 18 (22)	3.045
	B	183/186	183/183 26 (22)		183/186 40 (44)	186/186 22 (22)	0.545
	C	180/186	180/180 28 (22)		180/186 40 (44)	186/186 20 (22)	1.091
AIMS022	D	202/*x*		202/*x* 70 (66)		null/null 18 (22)	0.485
		346/350	346/346 24 (22)		346/350 46 (44)	350/350 18 (22)	0.500
AIMS025	A	189/205	189/189 22 (22)		189/205 50 (44)	205/205 16 (22)	1.227
	D	191/197	191/191 24 (22)		191/197 48 (44)	197/197 16 (22)	1.091
AIMS026	C	144/150	144/144 28 (22)		144/150 46 (44)	150/150 16 (22)	1.682
AIMS028	D	130/134	130/130 20 (22)		130/134 48 (44)	134/134 20 (22)	0.364
	B	140/140			140/140 88 (88)		–

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....*....| .*..|....| ....|....| ....|....|
                        10         20         30         40         50         60         70         80         90
AIMS0224A       GCACTTCATA CACGCAACAC ACCACATACA TGGAAGGTCG TCCTTTCATG ACCCCGGATA CAAATAAGAC GTTAATCAAT TAATCCAACA
AIMS0224B       GCACTTCATA CACGCAACAC ACCACATACA TGGAAGGTCG TCCTTTCATG ACCCTGGATA CCAATAAGAC GTTAATCAAT TAATCCAACA
CB413627        GCACTTCATA CACGCAACAC ACCACATACA TGGAAGGTCG TCCTTTCATG ACCCTGGATA CCAATAAGAC GTTAATCAAT TAATCCAACA

                ....|....| ....|....| ....|....| ....|....| .*..|....| *...|....| ....|....| ....|....| ....|....|
                       100        110        120        130        140        150        160        170        180
AIMS0224A       AACAAACAAA CAAACAAACA AACAAAGATT CCCATGACGC TCTAATGTCG TGTTCCGATG ATATCCCTAA TCGTCATCAT TTGTAATGTC
AIMS0224B       AACAAACAAA CAAACAAACA AA----GATT CCCATGACGC TCTAATGTCG TGTTCCGATG ATATCCCTAA TCGTCATCAT TTGTAATGTC
CB413627        AACAAACAAA CAAACAAACA AA----GATT CCCATGACGC TATAATGTCG AGTTCCGATG ATATCCCTAA TCGTCATCAT TTGTAATGTC

                .*..|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....* ....|....| ....|....|
                       190        200        210        220        230        240        250        260        270
AIMS0224A       AGTCTCACAT TAATTTATTC TTTTTGTGGT GATATATTAG CGGTATGTAG CGGCTATCGC TCACCCGGAT TACAAACATG TATATTTTTG
AIMS0224B       AATCTCACAT TAATTTATTC TTTTTGTGGT GATATATTAG CGGTATGTAG CGGCTATCGC TCACCCGGAA TACAAACATG TATATTTTTG
CB413627        AATCTCACAT TAATTTATTC TTTTTGTGGT GATATATTAG CGGTATGTAG CGGCTATCGC TCACCCGGAA TACAAACATG TATATTTTTG

                ....|...*| ....|....| ....|....* ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       280        290        300        310        320        330        340        350
AIMS0224A       CGGTAATCTG AATGCATTTT TGTTTTTAAT TAATTCTTAA AATGACTAGC TTGATGAAAG GTTCATCGCA TTGCCTAATA
AIMS0224B       CGGTAATCTG AATGCATTTT TGTTTTTAAT TAATTCTTAA AATGACTAGC TTGATGAAAG GTTCATCGCA TTGCCTAATA
CB413627        CGGTAATCCG AATGCATTTT TGTTTTTAAC TAATTCTTAA AATGACTAGC TTGATGAAAG GTTCATCGCA TTGCCTAATA
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Fig. 1. Nucleotide sequences multiple-aligned by BioEdit, indicating the nucleotide differences between the 'normal' (AIMS0224B and CB413627) and 'null' (AIMS0224A) alleles. Primers pairs used to determine the molecular basis of the null allele are underlined. Nucleotides in boldface and asterisks (*) indicate the single nucleotide mutation detected in the sequences.

363/363 in the parent and all of the progenies in family B, suggesting that the "null" and "normal" alleles detected by the primer pair of AIMS012P did not show any length polymorphism in microsatellite region. Further amplifications using recombinations of primer pairs (forward of AIMS012P and reverse of AIMS012S, and forward of AIMS012S and reverse of AIMS012P) indicated that un-annealing of the forward primer of AIMS012P was the cause of this null allele. Due to the homozygosity generated by AIMS012S and primer recombinations, we did not clone and sequence this null allele for the molecular basis assessment.

Frequency of the null allele

Fortunately, when the PCR reactions were carried out with genomic DNA, all of the 30 individuals derived from a cultured population could be amplified at the two candidate loci that suggested null alleles. At locus AIMS022 in family D, four individuals were homozygous for the same genotype of 202/*x* (202/202 or 202/null, unidentified by PAGE) when amplified with the primer pair of AIMS022P, however, the inconsistent genotype (346/350) was detected when amplified with the primer pair AIMS022S. The PCR products of the 4 individuals were cloned and sequenced, and the results indicated that non-amplified alleles were the same as in Fig. 1. The program GENEPOP calculated that the frequency of the null allele was 0.0667 in the cultured population. However, a new null allele that showed a lower frequency of 0.0333 was generated and detected in two of the 30 individuals at locus AIMS012. These two individuals were heterozygous (259/267) when amplified by the primer pair AIMS012P, whereas an inconsistent genotype (363/*x*) was detected using the primer pair AIMS012S. Due to the sequence length limitation in GenBank, primers with a longer expected PCR product could not be designed to detect the molecular basis of this null allele.

Discussions

There are three indispensable steps in the analysis of inheritance patterns using bivalve larvae: isolation of a single larva, DNA extraction and

analysis with microsatellite markers using PCR amplification (Huvet *et al*. 2001). There are two main sources of error in such an experiment: the difficulty of isolating of a single larva and poor DNA extraction. Compared with a method used to estimate of the larva concentration with a micro-titer plate, the micro-operation system used in this study was more efficient and more accurate, and we were up to 100% successful in separating a single larva. Additionally, the LoTEPA buffer method developed in this study does not require special or expensive reagents. The DNA extracted from a single larva provided enough templates for PCR amplification in more than 10 reactions. This method allowed the rapid genotyping of large numbers of early-stage larvae.

Deviations from Mendelian segregation have been reported in molecular markers in different analyses, especially in linkage map construction: 26% of RFLP markers in the potato (Gebhardt *et al*. 1989), 40% of RAPD markers in the *Medicago* (Jenczewski *et al*. 1997) and 65% of AFLP in the clubroot (Voorrips *et al*. 1997). In the linkage map construction for Zhikong scallop (*Chlamys farreri*) using AFLP markers, the segregation distortion ratio is about 35.24% when all polymorphic bands in F1 progeny were considered (Wang *et al*. 2004). Segregation distortion has also been extensively reported when microsatellite markers were used for genetic analyses in different species, especially in marine bivalves (Beaumont *et al*. 1983, Launey & Hedgecock 2001). In studies on 2- or 3-month-old spats of the pacific oyster (*C. gigas*) obtained by controlled crosses, about 36% of segregations showed significant departures from Mendelian expectations according to the normal significance level $\alpha = 0.05$ (Launey & Hedgecock 2001). Of 8 EST-SSRs loci tested in the present study, segregation distortion was observed at 3 loci (37.5%) in controlled families. Such a high percentage of loci showing segregation distortion makes it much more important to test Mendelian inheritance when using new microsatellite markers for population studies or parentage assignment in the bay scallop.

There are many possible reasons for the distorted segregation of molecular markers. Events that engender distorted transmission or distorted segregation include nonrandom segregation of chromosomes during meiosis, differential viability or functionality of gametes, competition among gametes for preferential fertilization, the product of linkage to another locus with a deleterious dominant allele, and gene conversion (Pardo-Manuel de Villena & Sapienza 2001, Hurst & Schilthuizen 1998). These can be summarized as two generally accepted notions that have been confirmed in bivalve microsatellites: the widespread presence of null alleles and the large genetic load of recessive deleterious mutations (Callen *et al*. 1993, Launey & Hedgecock 200l, Hedgecock *et al*. 2004).

Due to the high rates of mutation (10^{-2}–10^{-5}) (Weber & Wong 1993) and replication slippage (10^{-3}–10^{-4}) (Schlotterer & Tautz 1992), it is common for microsatellite loci to have null allele. Null alleles have been detected in many species, such as humans (30%; Callen *et al*. 1993), swallow (25%; Primmer *et al*. 1995), and rainbow trout (16%; Ardren *et al*. 1999). Recent experiments have shown higher percentages in marine molluscs, e.g. 71.4% in the pacific abalone (*Haliotis discus hannai*) (Li *et al*. 2003) and 51.0% in the pacific oyster (*C. gigas*) (Hedgecock *et al*. 2004). A null allele can be detected by the apparent non-inheritance of an allele if the family structure has been confirmed by Mendelian inheritance at other loci (Holm *et al*. 2001). Based on the loci tested to date, the design of a new primer is effective for detecting loci containing null alleles (e.g. Jones *et al*. 1998, Holm *et al*. 2001).

A null allele, which cannot be amplified by PCR, implies that the microsatellite may be absent or mutations such as substitutions, insertions or deletions appeared within one or both primer annealing sites (Ede & Crawford 1995, Li *et al*. 2003). Elucidation of the molecular basis of null allele in rainbow trout microsatellite showed that a 4-bp deletion in the 3´-end of the upper primer was the cause of null allele, and the deletion consists of a repetitive-like sequence (Holm *et al*. 2001). Similar phenomena were also observed by Jones *et al*. (1998) in white sand pupfish. Vos *et al*. (1995) reported that failure of primer annealing in a PCR reaction is probably caused by a single base mismatch in the last 3 to 4 bases in the 3´-end of one of the primers. The results in the present study showed

that the null allele at locus AIMS022 contributed to a total of 3 single nucleotide mutations in the primer annealing sites. Although the mutations were located in the middle of or close to the 5´-end of the primers, the non-amplification also occurred under the same annealing temperature as for the 'normal' allele.

Despite the conservative nature of EST, 8 single nucleotide mutations were detected in the flanking regions, which indicated a high rate of nucleotide variation. The rate of nucleotide mutation is much higher than that in some vertebrate species, e.g. the frequency of variable nucleotides in noncoding regions is 1% in some parts of the human chromosome (Nickerson *et al*. 1998). However, this high mutation rate is consistent with those reported in marine bivalves, e.g. one single nucleotide polymorphism (SNP) every 82 base pairs in the flanking regions of microsatellites in the Pacific oyster (Hedgecock *et al*. 2004). This implies a high percentage of primer pairs containing variable nucleotides to cause the widespread appearance of null alleles in bivalves.

In the present study, the extreme transmission distortion was observed at locus AIMS019 in family D. Although all of the offspring inherited the alleles from the parent, the allelic and genotypic ratios strongly deviated from the Mendelian expectations (Table 2). This kind of segregation distortion has been recorded in marine gastropods (Li *et al*. 2003) and bivalves (Foltz 1986, McGoldrick & Hedgecock 1997, Launey & Hedgecock 2001, Hedgecock *et al*. 2004). In oysters such as the European flat oyster and the Pacific oyster, dozens of highly deleterious recessive mutations have recently been confirmed to explain widespread observations of the distorted Mendelian segregation at marker loci (Bierne *el al*. 1998, Launey & Hedgecock 200l, Hedgecock *et al*. 2004). Meanwhile, negative interaction between chromosomal segments has been suggested to explain marker distortions (McGoldrick *et al*. 2000). More experimental evidences should be obtained to clarify the mechanisms of distorted segregation in the bay scallop.

In some species with a wide geographical distribution or high economic value, extensive studies are being conducted in many laboratories. For molecular genetic analysis, various laboratories use different microsatellites for the same genetic analysis, which makes it difficult to compare and share the data among different laboratories. Under appropriate conditions, a panel of markers, which is polymorphic, easily amplified and reproducible, should be selected as standard markers to facilitate genetic studies, e.g. in common carp (*Cyprinus carpio*), 21 microsatellite makers have been recommended for standardized use in population genetic studies (Yue *et al*. 2004). Considering the result of the linkage relationship estimated by Burrows' composite measure (Zhan *et al*. 2005, 2006), and the inheritance pattern and spread of null allele in this study, we strongly recommend that 6 loci (AIMS011, AIMS019, AIMS020, AIMS025, AIMS026 and AIMS028) should be included in a standard marker panel for genetic analyses in the bay scallop.

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