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Characterization of 28 polymorphic microsatellites for Japanese sea urchin (*Strongylocentrotus intermedius*) via mining EST database of a related species (*S. purpuratus*)

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Twenty-eight polymorphic microsatellite markers for Japanese sea urchin (*Strongylocentrotus intermedius*) were developed from the EST database of a related species purple sea urchin (*S. purpuratus*). The characterization results showed the moderate polymorphisms at these loci. The number of alleles ranged from 2 to 5, and the values of H_o and H_e varied from 0.0667 to 0.9655 and 0.0655 to 0.7350, respectively. No significant linkage disequilibrium (LD) between pairs of loci was found and 21 loci conformed to the Hardy-Weinberg equilibrium (HWE). The results in this study well demonstrated the feasibility of microsatellite marker development via mining EST database of a genetically related species.

Introduction

Japanese sea urchin (*Strongylocentrotus intermedius*), which was introduced into China from Japan in 1989, has been successfully domesticated and then cultured along the northern coasts of China. It has been considered to be one of the most valuable commercial sea urchin species during the past decades. With the supports of national and provincial projects, we carried out various genetic studies and breeding practices for this economical species, such as linkage mapping construction, molecular marker-assisted selection (MAS) and assessment of genetic basis of heterosis (Zhou *et al*. 2006). Due to the predominant advantages of microsatellites including reproducibility, hyperpolymorphism, codominance, abundance and good genome coverage, they have been extensively used in genetic researches and breeding practices in aquaculture (Liu & Cordes 2004). However, the availability of few microsatellite markers has hampered the practical genetic improvement for *S. intermedius*. So far, the registration of numerous data of expressed sequence tags (EST) from purple sea urchin (*S. purpuratus*) provided an opportunity for the development of microsatellite markers (EST-SSRs) in other related sea urchins. These markers, which represent transcribed genes and putative functions, are considerably valuable

because of their higher level of transferability to related species, and they can often be used as anchor markers for comparative mapping and evolutionary studies (Varshney *et al*. 2005).

Material and methods

In order to develop microsatellite markers for Japanese sea urchin (*S. intermedius*), the EST database of related species purple sea urchin (*S. purpuratus*) in GenBank (http://www.ncbi.nlm.nih.gov/dbEST/index.html) was analyzed with a homemade mining program, Repeat Reporter (ver. 1.5). The bioinformatic mining was conducted to identify sequences containing simple DNA repeats using the same parameters described by Zhan *et al*. (2005). EST sequences containing SSRs were handled with the Vector NTI suite 8.0 software package (Invitrogen) for clustering analysis. Independent sequences with the long perfect repetitions and suitable GC content in flanking regions were selected for polymerase chain reaction (PCR) primers design using software Primer Premier 5.0 (www.PremierBiosoft.com/faq.html). All the primer pairs commercially synthesized were subject to the temperature gradient PCR system (Eppendorf Mastercycler ep gradient S) for annealing temperature optimization. PCR amplifications were set up in a volume of 20 μl composed of 200 ng of mixed genomic DNA from 5 different individuals, 0.2 μM of each primer, 200 μM of each dNTP, 1 U of *Taq* polymerase (Takara) and 1 x universal PCR buffer. The PCR program was used: 5 min at 95 °C for an initial denaturation; followed by 35 cycles of 30 sec at 95 °C, 30 sec at gradient temperature (50–70 °C), 45 sec at 72 °C and a final extension at 72 °C for 5 min. PCR products were separated using electrophoresis on 10% non-denaturing polyacrylamide gels.

A total of 32 Japanese sea urchin (*S. intermedius*) individuals collected from Lingshui hatchery of Dalian City (Liaoning Province, P. R. China) were used for polymorphism assessment. The total DNA was isolated from the frozen gonad tissue using the traditional phenol/chloroform method. Extracted DNA from different specimen was amplified with PCR in a thermal cycler (Eppendorf Mastercycler ep gradient S). The PCR mix contained about 100 ng of template DNA, 0.2 μM of each primer, 200 μM of each dNTP and 1 U *Taq* polymerase (TaKaRa) with 1 × PCR buffer in a total volume of 20 μl. The amplified PCR products were separated by electrophoresis on 6% denaturing polyacrylamide gel at 60 W constant power for 1.5–2 h, and the bands were visualized using silver staining (Zhou *et al*. 2006). Each gel included two lanes of 20 bp DNA marker (Sigma), and allele size was determined with the software Quantity One ver. 4.4 (Bio-Rad) by comparison to the 20 bp DNA ladder standard.

Results

When data mining was performed, a total of 141 836 EST sequences were registered in the NCBI database, the bioinformatic search of 2–6 nucleotide motifs showed that about 5% of the sequences were conformed to our mining criteria. Considering the results of clustering analysis and the length and GC content of the flanking regions, 104 primer pairs were selectively designed for commercial synthesis. The results of the temperature gradient PCRs indicated that 35 (33.7%) primer pairs could successfully amplify scorable products. The remaining primer pairs failed to amplify any PCR product, which was probably due to the primer sequences spanning across introns, and/or containing mutations and/or indels (insertion or deletion), and/or the difference of the nucleotide makeup between these two related sea urchins. Actually, nonamplifications (null alleles) caused by nucleotide mutations were directly observed in other species such as Pacific oyster (*Crassostrea gigas*) (McGoldrick *et al*. 2000). Studies on the development of EST-SSRs approved the larger fragments than expected was a common phenomena (e.g. Zhan *et al*. 2005). The presence of long introns between the primers in the genomic DNA might explain the large DNA fragment at some locus (e.g. INTS06 in Table 1). The successful ratio in this study was comparable to those using some strategies in marine organisms such as SSR-enriched libraries in Yesso scallop (*Mizuhopecten yessoensis*) (Sun *et al*. 2007). However,

Table 1. Characterization of 28 novel microsatellite loci for Japanese sea urchin (*S. intermedius*). T_a = optimal annealing temperature (°C); S = allele size range (bp); A = number of alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; P = exact P value for Hardy-Weinberg equilibrium test.

Locus name (accession no.)	Primer sequence (5´-3´)	T_a	Repeats	S	A	H_o	H_e	P
INTS01	F: TTTGGGTGGATCCTGTCGTG	57	(AG)$_{10}$	163–183	3	0.5806	0.5309	0.0093
EC439591	R: TCACAATTCCGTCAGGGCTC							
INTS02	F: TGTATGGTCTGTCGGAAAGC	53	(TA)$_{19}$	293–305	2	0.5312	0.5035	0.7511
EC438789	R: GATGCAACAATTGACGGAGC							
INTS03	F: TTTCCAAAGGGTCGGGTGT	55	(TA)$_{10}$	304–330	2	0.6452	0.4442	0.0101
CX697849	R: TATGCCCACACAATGGCGA							
INTS04	F: GCGATTTGTAAACCTGGGGA	57	(TGT)$_{13}$	164–173	2	0.5938	0.5074	0.3282
CD320652	R: AGGTAGGAGTCATGTCGTCG							
INTS05	F: AAGTTGGTTGGCAGGTGCTC	53	(ATA)$_{17}$	176–185	3	0.3125	0.4980	0.0638
CX687827	R: ACACGTACTGGCTGGAATGC							
INTS06	F: TGTAGATGCTGTGCAGGTGT	55	(ATC)$_{9}$	773–1080	3	0.5625	0.5164	0.9461
CX552920	R: TCGACTAGCTGACTGTGCA							
INTS07	F: ATGACAGCTGTGCGAAAGC	53	(TAA)$_{9}$	228–253	3	0.7188	0.5184	0.0226
CX552589	R: GATCTGGAGTGGCAATGTGG							
INTS08	F: CAATGTGCTTGGCGTGGTAG	56	(TCA)$_{20}$	215–267	3	0.8500	0.5853	0.0000
CD291460	R: GCAGCTTACTTCTGGAGGCA							
INTS09	F: GGTGATCATACTGCCTGTGC	54	(TG)$_{14}$	116–132	5	0.5714	0.6994	0.0888
CD338402	R: GACCATGTCACACATGGCTG							
INTS10	F: TGATGGTTTGGGGCATGA	57	(CT)$_{12}$	193–199	2	0.9474	0.5121	0.0001
CD341401	R: TGGTATGTCGGGAGTGTGA							
INTS11	F: ATTACTGGGGCATGCGTGT	57	(AG)$_{18}$	177–249	4	0.5938	0.6156	0.7428
CD340974	R: TAGATGAGGGAGCTGTGCT							
INTS12	F: TCTAGCGTGTGTCAAGCACG	53	(ATA)$_{7}$	195–228	3	0.9655	0.6243	0.0000
CD336118	R: TCGGAGTTGAAGCCGTTGTC							
INTS13	F: GAGTGTGTTTGCATGAGCCA	55.5	(TC)$_{10}$	202–232	3	0.7894	0.5511	0.0000
CD334278	R: AGAAAAGAGAGTGGGGAACG							
INTS14	F: GGGAAGTTTTCCCCACTGAC	58	(AG)$_{12}$	291–301	2	0.1562	0.2455	0.0639
DN808464	R: TGTCCATAACGCCACATTCG							
INTS15	F: ACATCACATGCCAACCCA	59	(CCA)$_{8}$	206–278	3	0.4667	0.5316	0.8904
CX555808	R: GATGAAGGATGTGCACCTGG							
INTS16	F: TCGTCATGAGATGGTCGCT	57	(CT)$_{12}$	231–283	4	0.8667	0.7350	0.1962
DN785343	R: CATTTTACCGTGGTGGGGTC							
INTS18	F: TCTGAGCCCAAAATGCCTGC	54	(AAT)$_{12}$	286–310	2	0.2000	0.1831	0.5814
DN788257	R: TTGATCTGGCGCTGCTCAGT							
1NTS19	F: TCCATAGCAACCATGCAGC	57	(TCA)$_{9}$	232–247	3	0.6800	0.4824	0.1031
DN580000	R: CCCTCGATAACAGCATCAGC							
INTS20	F: GGTCTACAGACATCCAGTGC	58	(AAC)$_{8}$	197–302	4	0.4839	0.4532	0.2633
CX682646	R: GCAAATGTTCAGGCTTGTGG							
INTS22	F: TCCCATATGATTGCTCGTGC	54	(AC)$_{10}$	164–172	4	0.6875	0.6939	0.8133
EC438212	R: AGCATTCACCGCGAAACTG							
INTS23	F: TGGTGGATACAGTCGTGGAG	63	(GGC)$_{6}$	215–221	2	0.0667	0.0655	0.8946
EC439442	R: TTGTCATACCCATCGCGACC							
INTS24	F: TCAGGTGGTAGTTCACGCT	52	(AG)$_{8}$	244–260	3	0.5333	0.5554	0.0002
EC439480	R: ACAGTCACAATTCCGTCAGG							
INTS25	F: GAAAGTTTGCCTCGCTGGTC	52	(AAG)$_{5}$	543–546	2	0.2333	0.2593	0.5632
CX689232	R: CCTATCTTCGAATCGGCCAC							
INTS26	F: AAGAGAGGAAAGCTGGCAC	54	(TAG)$_{8}$	404–521	4	0.2581	0.6066	0.0000
CD341745	R: GGAGAGAAAACACCTCCTGG							
INTS27	F: CACTGGAACAAGTACGCTGG	57	(CTT)$_{5}$	200–209	2	0.1034	0.0998	0.8120
CD341523	R: CATACACCATGGCTGCTCAG							
INTS28	F: GCATGCTAGTCACAACGGGA	59	(ACA)$_{5}$	207–225	3	0.5312	0.5615	0.6261
CD341295	R: AATGACGCACTGACTCGACG							
INTS29	F: AGACCAAATGCAGAGCTGC	54	(ATT)$_{6}$	235–277	3	0.2812	0.3775	0.0000
CD340211	R: TGATTGAGAGCCAAGGGAGC							
INTS30	F: CTAATAGCCCTATGCCGCGT	55	(AAG)$_{6}$	144–162	4	0.8125	0.6741	0.7331
CD335578	R: ATACACCACACGATTCGCAC							

as compared with the other strategies such as screening SSR enrichment libraries, mining EST database is more economical because it avoids several boring procedures such as genomic library constructing, library screening and positive clone sequencing (Zhan *et al*. 2005, 2006).

Among these 35 functional primer pairs, 28 loci showed polymorphism in the 32 sea urchin individuals tested (Table 1) with the allele number ranging from 2 to 5. The observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.0667 to 0.9655 and 0.0655 to 0.7350, respectively. The Markov chain method was employed to estimate the probability of significant deviation from Hardy-Weinberg equilibrium (HWE) using the software GENEPOP (online version http://genepop.curtin.edu.au/). The results showed that 21 loci (Table 1) conformed to HWE after sequential Bonferroni corrections. The null allele(s) and/or aquaculture practices may be responsible for the deviated cases in this study. The linkage disequilibrium (LD) tests by the GENEPOP software indicated that none of the comparisons showed the significant LD between any pairs of loci. The results in this study gave the experimental evidences for the feasibility of microsatellite marker development via mining EST database of genetically related species. The polymorphic microsatellite markers presented in this study will be beneficial in population diversity assessment, reproductive ecology analysis, phylogenetics and comparative genomics studies and SSR-based analysis in aquaculture practice for Japanese sea urchin *S. intermedius*.

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