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Full Length Article

Development of SSR Markers Using RNA-Seq Approach and Genetic Diversity of Two Populations of Asian Moon Scallop *Amusium pleuronectes*

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Abstract

In this study, we obtained a batch of simple sequence repeats (SSRs) from the transcriptome data of Asian moon scallop *Amusium pleuronectes* and analyzed the distribution and frequency of these SSRs. A total of 7,315 SSRs were obtained from 159,521 unigenes. Bioinformatics tools were employed to design appropriate primers. A total of 4,038 SSR loci had flanking sequences suitable for polymerase chain reaction primer design. One hundred SSR primers were validated and the rate of successful amplification was 78.0%. Fourteen randomly chosen primer pairs were amplified in Beibu Bay population (BP) and Hainan Baimajing population (HP). The number of alleles at each locus ranged from 2 to 3 in two populations, with mean values of 2.214 and 2.143, respectively. The observed heterozygosity, expected heterozygosity and polymorphism information content of BP were 0.463, 0.646 and 0.281, respectively, while those of HP were 0.309, 0.320 and 0.259, respectively. The developed SSR markers will be helpful for further studies on population genetics, genetic linkage construction and chromosome linkage mapping in the species. © 2017 Friends Science Publishers

Keywords: *Amusium pleuronectes*; SSR development; Genetic diversity; Wild populations

Introduction

Asian moon scallop *Amusium pleuronectes* (Linnaeus) is a traditional economic shellfish species in China, Philippines, Thailand and Australia (Minchin, 2003). However, catch production in the species has recently decreased because wild populations severely suffer from slow growth and mass mortalities. Over the last decades, the studies have been focused on spawning and larval rearing (Morton, 1980; Belda and Del Norte, 1988; Chaitanawisuti and Menasveta, 1992), growth and reproduction (Del Norte, 1988), population ecology (Mcduff, 2001) and genetic diversity (Mahidol *et al*., 2007). Transcriptome sequencing in the species has recently been studied (Huang *et al*., 2015).

Molecular markers are commonly applied to the studies on genetic mapping construction, molecular marker-assisted selection, chromosome linkage mapping and comparative genomics (Deng *et al*., 2014). SSR markers are preferred over RAPD and AFLP, due to their advantages that is involved in genetic co-dominance, abundant sequence dispersed throughout most eukaryotic genomes and high polymorphism (Wang *et al*., 2011). SSRs markers can be generated using several techniques without single locus isolation, such as employing oligonucleotide primers (Zietkiewicz *et al*., 1994), SAMPL (Witsenboer *et al*., 1997) and transcriptome SSRs (Guo *et al*., 2015). When compared with genomic SSRs, transcriptome SSRs are more efficient (Wang *et al*., 2009; Marguerat and Bähler, 2010; Deng *et al*., 2014) with relatively higher transferability (Varshney *et al*., 2005).

We constructed the mantle tissue transcriptome of *A. pleuronectes* using Illumina HiSeq 2000 paired-end sequencing technology in the previous studies (Huang *et al*., 2015). Herein, we developed valuable SSR markers by mining EST sequences and evaluated genetic diversity of two populations of *A. pleuronectes*. The objectives of the present study were to (1) develop a large number of EST-SSRs; (2) test the amplification of a subset of primer pairs and search for polymorphic EST-SSR markers and (3) detect genetic diversity of wild populations. Results reported here will provide valuable resources for further studies on genetic diversity, genetic linkage and chromosome linkage mapping of *A. pleuronectes*.

Materials and Methods

Experimental Animals

Thirty animals were separately sampled from Beibu Bay population (BP) and Hainan Baimajing population (HP).

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Adductor muscle of each animal was sampled and preserved in 70% ethanol.

Transcriptome data originated from the preliminary work (Huang *et al*., 2015). All unigenes were used for searching potential SSR markers using the MISA tool (http://pgrc.ipk-gatersleben.de/misa/). The minimum repeat motifs was dinucleotide and the maximum repeat motifs was penta-nucleotide. The primer pairs were designed using Perl scripts allowing the interaction with Primer3.0 (Rozen and Skaletsky, 2000). The presence of at least 50-bp sequence on both sides of the microsatellite repeats were considered sufficient for primer design by Primer 3.0, and were considered as the potentially amplifiable loci (Guo *et al*., 2015).

DNA Isolation, SSR Amplification and Validation

Genomic DNA was extracted from adductor muscle samples using Universal Genomic DNA Mini-Isolation Kit (Sangon Biotech Shanghai, China), according to the manufacturer's protocol. Genomic DNA was assessed by gel electrophoresis using 1% agarose gel. A total of 100 SSR primers were randomly picked and validated in the two populations. Fourteen SSR primers showing polymorphism in the two populations were used to evaluated genetic diversity.

The PCRs reactions were performed in 10 μL of reaction mixture. The detailed reaction system and process were done according to Deng *et al.* (2014). The PCR products were separated on 8% (w/v) polyacrylamide gels using Takara 20 bp DNA ladder marker by silver staining. Following electrophoresis, gels were stained with silver and imaged using a Gel DocTM XR+ system. Genetic diversity values were calculated with the software GenAlEx 6.4 (Peakall and Smouse, 2006).

Results

SSR Identification and Repeats Distribution

We identified 7,315 potential EST-SSRs from 159,521 unigenes. The unigene number of SSR-containing sequences was 7,025 and the number of sequences contained more than one SSRs was 820 (Table 1). A total of 478 EST-SSRs were present in compound formation (Table 1). On an average, every 14.6 kb unigenes contained one EST-SSR. The most abundant type of repeat motif was di-nucleotide (71.62%), followed by tri-nucleotide (23.96%), tera-nucleotide (4.31%) and penta-nucleotide (0.11%) (Table 1). We also calculated the EST-SSRs frequencies of different numbers of repeat unit. Among these SSRs, 43 motif sequence types were identified (Fig. 1). Among these types, AT/AT (47.07%) was dominant, followed by AC/GT (16.50%), AG/CT (7.75%), ATC/ATG (6.29%), and AAT/ATT (4.24%). A total of 4,038 SSR primers were successfully designed using Primer3.0 (Table 2).

Table 1: Summary of expressed sequence tag-simple sequence repeat (EST-SSR) search results

Total number of sequences examined	159,521
Total size of examined sequences (bp)	106,655,673
Total number of identified SSRs	7,315
Number of SSR-containing sequences	7,025
Number of sequences containing more than 1 SSR	820
Number of SSRs present in compound formation	478
Di-nucleotide	71.62
Tri-nucleotide	23.96
Tetra-nulecotide	4.31
Penta-nucleotide	0.11

Table 2: The numbers of SSR loci and the subset of these that are potentially amplifiable (containing suitable PCR priming sites) in 159,521unigenes using RNA-Seq

Repeat motifs	Number of repeats	Number of loci identified	Number of loci designed	Percentage (%)
Di-nucleotide	5-12	5,240	2,956	56.41
Tri-nucleotide	5-19	1,753	995	56.76
Tetra-nulecotide	5-19	315	85	26.98
Penta-nucleotide	5-9	7	2	28.57
Total	-	7,315	4,038	55.21

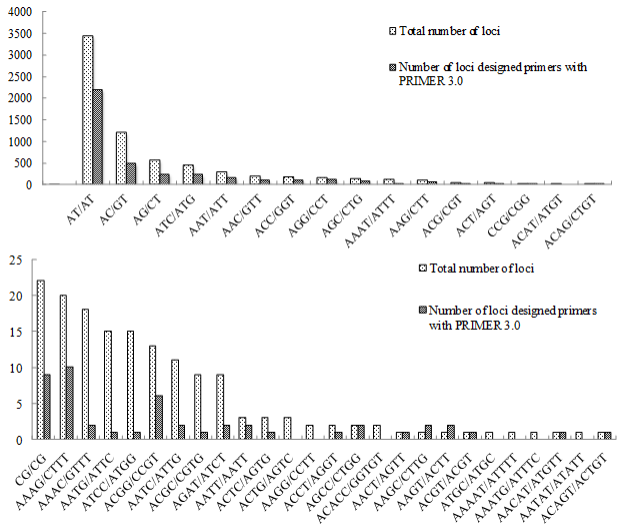

Fig. 1: Number of SSR loci and the subset of these designed primers with Primer3.0. A part of tetranulecotide repeats are listed

SSR Amplification and Polymorphism Validation

We randomly selected 100 SSR primers with optimal expected product sizes for validation in two populations to evaluate the successful amplification proportion and polymorphism of the potential SSR markers. Approximately 78.0% of these pairs were successfully amplified in the 100 SSR primers. Sixty five of the SSR loci showed polymorphisms. A representative profile for 1,152 locus is shown in Fig. 2.

Genetic Diversity Analysis of Two Populations

Genetic diversity of the two populations was evaluated by 14 SSR primers (Table 3). The results showed that the number of allele at each locus ranged from 2 to 3. There existed evident differences in genetic diversity between the two populations. The observed heterozygosity, expected heterozygosity and polymorphism information content of BP were 0.463, 0.646 and 0.281, respectively, while those of HP were 0.309, 0.320 and 0.259, respectively (Table 4).

Discussion

A. pleuronectes is precious seafood because of its large size, rapid growth, well-developed adductor muscle and delicious taste (Fu *et al*., 2012). It also was a functional hermaphrodite that probably promotes its selfing and profoundly influences the mode of population genetic structure (Llana and Aprieto, 1980). It is now widely recognized that a large number of molecular markers achieved through molecular genetic techniques are used to evaluate the genetic variability and population structure (Mahidol *et al*., 2007). These molecular markers include sequence-related amplified polymorphism (Zhang and He, 2009), SSR (Tong *et al*., 2007; Shi *et al*., 2013), inter-SSR (Jiang *et al*., 2007), and amplified fragment-length polymorphism (Yu and Chu, 2006; Shi *et al*., 2009). The SSRs are widely and abundantly dispersed in most nuclear eukaryotic genomes. In our studies, we screened a batch of SSR markers from *A. pleuronectes* transcripts. A total of 7,315 potential EST-SSRs in 159,521 unigenes were obtained, which accounted for 4.59% (Table 1). The frequency of SSRs detected in this study was higher than 1.53% in pearl oyster *P. maxima* (Deng *et al*., 2014) and 3.10% in hard clam *Meretrix meretrix* (Li *et al*., 2011), but lower than 10.22% in clam *Paphia textile* (Chen *et al*., 2016) and 4.7% in pearl oyster *P. martensii* (Guo *et al*., 2015). A possible explanation for the case might be the differences in SSR search tools and criteria used. Hence the large number of EST-SSRs obtained from *A. pleuronectes* transcriptome will be useful for population genetics analysis and linkage mapping construction.

The distribution and frequency of the EST-SSRs were calculated. Among these EST-SSRs, di-nucleotide repeat motifs were the most frequent repeat type, followed by tri-nucleotide, tetra-nucleotide and penta-nucleotide (Table 1). The repeat motifs were evidently different from those reported in other shellfish mentioned above. We speculated that the difference of SSR frequency among the different species were due to the following reasons: (1) difference of the genome structure or composition; (2) different parameter settings can also dramatically cause the results and (3) selection of different softwares in detecting SSRs. The most common di- and tri-nucleotide repeats were the motif (AT)n (24.81%) and (GAT)n (1.63%) in the EST-SSRs (Fig. 1).

Table 3: Primer sequence and amplification information of 14 SSR loci

Locus	Primer Sequence(5'-3')	Annealing temperature (ºC)
1152	F: CCT CCC TTT GTTGCA TTC TC R: CTGGAA AGGTTC CCT CAC TG	53.8
46806	F: AACATTTTCGGAGGTTGAACA R: GTTTGTAAGGGGTGAGCCAA	50.8
22332	F: GTCACG TGGGCATAACCTTT R: GTCGTTTGTACCGCTAAGCC	53.8
47806	F: ATG AAA AAGCACGGG TTC TG R: ATTGGTAAGCGAGATGCCAC	51.9
50232	F:ACATTCACGGGTACGCTGTT R:TTC TCTCTCCGAGGAAGCAC	53.8
65095	F:TCACCAACATCGGTAAGGCT R:GAGCTCGTGTTCCTTAATGTGA	53.1
66493	F:ATC ATGATCTCCTGC CCAAC R:CCTTCACATCTG ACT TGGCA	52.8
68197	F:GAC AAG CAGCTATGA ACCTGG R:GAGCCA ACA ATA ACGGGGAA	52.9
70996	F:GCT TGG GTA CAA CAAAACCAA R:ACACAG CGT GTG TTA GCCTG	52.9
79101	F:GGAAAT TCCAAC CGC AATAA R:CAAGGTCGTTCATTT AATTCACA	49.4
96160	F:AACAGGGGCAGT GTG AAATC R:CCTTCCAGGCTGGTACAG AA	53.8
97508	F:CAATGCAGAACT GTGAAGGG R:ACA TGACCTTGACCTTTGCC	52.8
98592	F:AAA ATTCCCCTTTAGCTCCG R:TGC TTTTTGTTTGTT TCTTTGTG	49.4
130155	F:GAA CCGATATTTGGACCCCT R:GTG TAA AAGGCTGCT TTTCC	52.9

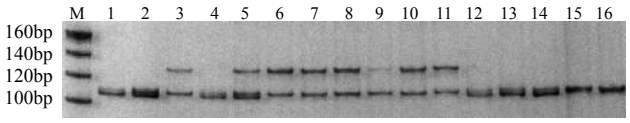

Fig. 2: Amplified profile at 1152 locus in BP (1-8) and HP (9-16) of *A. pleuronectes*

However, the lowest di-nucleotide repeats were the motif (CG)n (0.14%) and (GC)n (0.16%), which is in accordance with many organism genomes, such as Chinese shrimp *Fenneropenaeus chinensis* (Kong and Gao, 2005) and Japanese Pufferfish *Fugu rubripes* (Edwards *et al*.,1998).

To determine the polymorphism of the selected SSR markers, we validated 100 primers in two wild populations. Among the 100 pair primers randomly selected for PCR validation, 78 primers produced clear bands. The PCR success rate (78.0%) was higher than those obtained in other shellfish species. For example, the success rates of were 65.0% in clam *M. meretrix* (Li *et al*., 2011), 50.0% in clam *Mercenaria mercenaria* was 65.0% (Wang *et al*., 2010) and 36.0% in freshwater mussel *Villosa lienosa* (Wang *et al*., 2015). These results showed that the screening EST-SSRs can be used for the subsequent genetic diversity research.

The genetic diversity of species reflects its ability to adapt to the environment. The more abundant the variation within species is, the greater the ability it has to adapt to the environment (Beardmore *et al*., 1997).

Table 4: The numbers of SSR loci identified and the subset of these that are potentially amplifiable (containing suitable PCR priming sites) in 159,521unigenes

Population	Locus	N_a	N_e	H_o	H_e	*PIC*
BP	1152	2	1.997	0.654	0.509	0.375
	46806	3	1.238	0.210	0.198	0.181
	22332	3	1.843	0.667	0.467	0.370
	47806	2	1.383	0.333	0.284	0.239
	50232	2	1.471	0.400	0.325	0.269
	65095	3	1.275	0.103	0.220	0.199
	66493	2	1.724	0.333	0.427	0.332
	68197	2	1.973	0.885	0.503	0.371
	70996	2	1.384	0.333	0.283	0.239
	79101	2	1.220	0.200	0.184	0.164
	96160	2	1.965	0.867	0.499	0.371
	97508	2	1.800	0.667	0.453	0.346
	98592	2	1.166	0.154	0.145	0.132
	130155	2	1.814	0.680	0.458	0.348
	Mean	2.214	1.590	0.463	0.646	0.281
HP	1152	2	1.554	0.464	0.363	0.293
	46806	3	1.826	0.238	0.463	0.384
	22332	3	1.780	0.621	0.446	0.356
	47806	2	1.827	0.308	0.462	0.350
	50232	2	1.147	0.138	0.131	0.120
	65095	2	1.355	0.241	0.267	0.228
	66493	2	1.105	0.100	0.097	0.090
	68197	2	1.923	0.400	0.488	0.365
	70996	2	1.470	0.400	0.325	0.269
	79101	2	1.166	0.154	0.145	0.131
	96160	2	1.427	0.367	0.304	0.255
	97508	2	1.890	0.759	0.479	0.360
	98592	2	1.113	0.107	0.103	0.096
	130155	2	1.684	0.033	0.423	0.324
	Mean	2.143	1.519	0.309	0.320	0.259

N_a, the number of allele; N_e, the number of effective allele; H_o, observed heterozygosity; H_e, expected heterozygosity; *PIC*, polymorphism information content

The evaluation index of genetic diversity includes expected heterozygosity (H_e), observed heterozygosity (H_o) and polymorphic information content (*PIC*). There are a large number of reports about the analysis of the genetic structures of shellfish species by using SSR molecular markers (e.g., Li *et al*., 2007; Liu *et al*., 2014). However, there existed few studies on *A. pleuronectes* by using SSR molecular marker. In the present studies, the average H_o, H_e and *PIC* of BP were 0.463, 0.646 and 0.281, respectively. The average H_o, H_e and *PIC* of HP were 0.283, 0.299 and 0.259, respectively (Table 4). The results indicated that BP has higher genetic diversity than HP.

Conclusion

Our results showed that 14 loci had moderate polymorphism ($0.25<PIC<0.5$) in the two populations. These molecular tags can be regarded as effective genetic markers using genetic diversity analysis of *A. pleuronectes*. This is a first attempt to analyze the genetic diversity of Asian moon scallop populations. The developed markers may be valuable for the studies on genetic resource conservation of the species.

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