Development of 101 Gene-based Single Nucleotide Polymorphism Markers in Sea Cucumber, *Apostichopus japonicus*

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**Abstract:** Single nucleotide polymorphisms (SNPs) are currently the marker of choice in a variety of genetic studies. Using the high resolution melting (HRM) genotyping approach, 101 gene-based SNP markers were developed for *Apostichopus japonicus*, a sea cucumber species with economic significance for the aquaculture industry in East Asian countries. HRM analysis revealed that all the loci showed polymorphisms when evaluated using 40 *A. japonicus* individuals collected from a natural population. The minor allele frequency ranged from 0.035 to 0.489. The observed and expected heterozygosities ranged from 0.050 to 0.833 and 0.073 to 0.907, respectively. Thirteen loci were found to depart significantly from Hardy–Weinberg equilibrium (HWE) after Bonferroni corrections. Significant linkage disequilibrium (LD) was detected in one pair of markers. These SNP markers are expected to be useful for future quantitative trait loci (QTL) analysis, and to facilitate marker-assisted selection (MAS) in *A. japonicus*.

**Keywords:** single nucleotide polymorphism (SNP); *Apostichopus japonicus*; high resolution melting (HRM) analysis; marker-assisted selection (MAS)
1. Introduction

The sea cucumber *Apostichopus japonicus* (Selenka 1867), naturally distributes along the coasts of China, Japan, Korea and Russia [1]. Due to their nutritional and medicinal value, they have long been exploited as an important fishery resource in East Asian countries. Over the past decade, the aquaculture of *A. japonicus* has become widespread along the coasts of China, due to increasing market demand and over-exploitation of wild sea cucumbers [2]. However, the rapid expansion and intensification of sea cucumber aquaculture has resulted in some severe problems, such as wide-spread disease and stock deterioration, possibly caused by inappropriate broodstock management and inbreeding depression [2]. In order to properly manage broodstock resources and efficiently enhance aquaculture production, control of inbreeding and selection of broodstock with the desired traits, such as rapid growth and disease resistance, are currently necessary for sustainable development of the *A. japonicus* aquaculture. Recently, marker-assisted selection (MAS) has become a valuable tool for selecting individuals with traits of interest [3]. To perform MAS, a large number of genetic markers are usually needed to determine the quantitative trait loci (QTLs) associated with economically important traits.

Single nucleotide polymorphisms (SNPs) have been shown to be the most abundant type of genetic variations in eukaryotic genomes [4], and are currently the marker of choice in a variety of genetic studies, such as high-density genetic linkage mapping and QTL analysis. However, only a limited number of SNP markers have been reported for *A. japonicus* [5–7]. Moreover, molecular markers developed from the expressed sequence tag (EST) databases offer several advantages over anonymous genomic markers, as (i) they can detect variation in the expressed portion of the genome, so that gene tagging could give “perfect” marker-trait associations; (ii) they could alleviate the problem of null alleles which is usually associated with markers developed from the non-transcribed regions; and (iii) they are expected to have greater transferability between species, since transcribed regions are more conserved among closely related species/genera.

Previously, our group has released a large amount of EST data by 454 sequencing of the *A. japonicus* transcriptome [7]. By mining our EST dataset, more than 54,000 putative SNPs have been identified, 200 of which were selected in this study for marker development. SNP validation was performed using 48 *A. japonicus* individuals collected from four natural populations. Genetic parameters of the validated SNP markers were evaluated using 40 *A. japonicus* individuals from a single natural population. These SNP markers will be useful for future QTL analysis in order to facilitate MAS in *A. japonicus*.

2. Results and Discussion

Transcriptomic sequences represent an important resource for rapid and cost-effective development of gene-based SNPs. For the high resolution melting (HRM)-based SNP marker development, we designed PCR primers for 200 candidate SNPs (Table 1), which were previously identified from the *A. japonicus* transcriptome generated by 454-FLX sequencing [7]. After PCR amplification, 159 (79.5%) amplified strong bands with expected sizes. The others were discarded without further consideration, as they produced bands larger than expected (possibly caused by introns) or resulted in poor amplification (weak or non-specific amplification). During the initial HRM screen, 63.5% (101) of the 159 successfully
amplified loci showed polymorphisms in 48 individuals collected from 4 natural populations, 21.4% (34) generated non-polymorphic curves, and 15.1% (24) displayed unreliable melting curves. In this study, we showed that minor allele frequency (MAF) can serve as an important selection criterion to distinguish true SNPs from sequencing errors when performing SNP mining from 454 sequencing data (Figure 1). For example, most of the validated SNPs usually have a MAF of more than 35%, whereas most non-validated SNPs usually have a MAF of less than 25%. Although our study demonstrated that SNP markers can be efficiently developed from transcriptomic resources, it should be noted that the SNPs obtained may largely represent common genetic variations due to the low coverage of the original transcriptome sequencing, and may suffer from ascertainment bias resulting from simple sample source used in the original transcriptome sequencing.

Table 1. Results of validation and genotyping of candidate single nucleotide polymorphisms (SNPs).

| Categories                  | Number of SNPs |
|-----------------------------|----------------|
| Total number of tested SNPs | 200            |
| Successful PCR              | 159            |
| Successful genotype calling | 135            |
| Polymorphic SNPs            | 101            |
| Monomorphic SNPs            | 34             |
| Failed SNPs                 | 65             |

Figure 1. Distribution of SNP minor allele frequency (MAF) for *Apostichopus japonicus*. The number above each bar was the polymorphic rate in respective MAF categories.

Genetic parameters of the validated SNP markers were further evaluated using 40 *A. japonicus* individuals from a single natural population. As expected, all 101 SNP loci were polymorphic. The minor allele frequency ranged from 0.035 to 0.489 (Table 2). The $H_o$ ranged from 0.050 to 0.833, while the $H_e$ varied from 0.073 to 0.907. Thirteen loci departed significantly ($p < 0.01$) from Hardy–Weinberg equilibrium (HWE) after Bonferroni correction, suggesting that these loci may be under ongoing natural selection. Significant linkage disequilibrium (LD) was detected in one pair of SNP markers (ApjSNP092_CT and ApjSNP098_CT).
# Table 2. Characterization of 101 SNPs for the sea cucumber *Apostichopus japonicus*.

| Locus ID     | Gene Name                                           | Primers and Probes (5′–3′)                                           | Size (bp) | Ho   | He   | MA   | MAF  | p-Value |
|--------------|-----------------------------------------------------|-----------------------------------------------------------------------|-----------|------|------|------|------|---------|
| ApjSNP001_CT | similar to Mech2 protein                            | F:CCTCAGTCCCAATCACCCACT R:ACACTGGCATACACCCAGCAA P:CAATGACTTCTCTCTACAGCTTCC | 98        | 0.250| 0.431| T    | 0.128| 0.239   |
| ApjSNP002_CT | Iron-sulfur cluster assembly 2 homolog              | F:TGAATCAGGCGAGTTGTGATGA R:GGTCCAGCTAGTCATGTCTTT P:TCAAAGAAATACACTATTTCATCACGATAAAGCAAGCAG | 102       | 0.231| 0.485| C    | 0.397| 0.080   |
| ApjSNP003_AC | Protein strawberry notch homolog 2                  | F:AGCGATTATATCCGATGCGAG R:GCAGCAAGGGTAGGCTCGAC P:TCAAAGAGTCGCAATCAGAGGCGAGC | 108       | 0.431| 0.583| A    | 0.489| 0.482   |
| ApjSNP004_AG | Thiosulfate sulfurtransferase                       | F:CAGTTTGAATCTGACCTAGCAG R:ATGCCTACTTGGATGCGAGA P:ACCACAGGCTGATGCTCGTAGC | 70        | 0.314| 0.342| A    | 0.178| 0.578   |
| ApjSNP005_AG | Thiosulfate sulfurtransferase                       | F:AGGCATCCCTACGGGTATTT R:ACAGGAATGAGTGCTTGAG P:TCCGCTCTAGCTTCCCGCTCAGCAG | 70        | 0.374| 0.312| A    | 0.240| 0.857   |
| ApjSNP006_CT | Dynein heavy chain 6, axonemal                      | F:GGGAGGCTTCAAGGATGGA R:CGAGGAGTCGGAGTCAGTTG P:ACCCCTACCGACGTCCCGCAGAACG | 97        | 0.271| 0.273| C    | 0.384| 0.345   |
| ApjSNP007_AG | Sodium-dependent phosphate transport protein 2B     | F:ACCTTGGTGCGAGATGCGAG R:TTCACTGTCCCGCACCTTTTT P:AGTTGAACTAACGCGGCCGCTCGAGACG | 75        | 0.252| 0.432| G    | 0.287| 0.418   |
| ApjSNP008_GT | Testis-specific serine/threonine-protein kinase 1   | F:CCACAAATTAGCGATGGTTT R:CAGGAGTCCGACCTGTCGAG P:GTGTTGATGCGAGGCTTGTTTAAGGAAAGGAAAC | 102       | 0.349| 0.488| G    | 0.410| 0.058   |
| ApjSNP009_AC | Disintegrin and metalloproteinase domain-containing | F:CTAAAGGGGATCACCCGAC R:ATAAGCGCTCTCTCTCTCAG P:ACCTTCCGGCCAGGACGCAA | 94        | 0.208| 0.289| A    | 0.104| 0.365   |
| Locus ID     | Gene Name                                      | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho   | He   | MA   | MAF   | p-Value |
|--------------|-----------------------------------------------|--------------------------------------------------------------------------------------------|-----------|------|------|------|-------|---------|
| ApjSNP010_GT | Zinc finger protein 62 homolog                | F:CCACACAGATGTCTTTTGATTCG R:TCACGACCAATACTGCTTTGG P:AGATCGGACCCAGCAAGACACAGGT               | 106       | 0.428| 0.512| G    | 0.448| 0.552  |
| ApjSNP011_AG | Kelch-like protein 9                           | F:CACTCGCCTAGCCCTTACCA R:TCGTGGACCTTTGGTACTGATG P:GTGCAAACCAATCGCAAGTCATTGTCG              | 93        | 0.500| 0.498| G    | 0.458| 0.574  |
| ApjSNP012_CT | TATA box-binding protein-associated factor RNA | F:CCCTCCACTGTTATGCGATTTT R:GTGCAAACCAATCGCAAGTCATTGTCG                                      | 88        | 0.314| 0.468| T    | 0.240| 0.045  |
| ApjSNP013_AT | Protocadherin Fat 3                           | F:GTGTTAGCCTCTATCAAGGATGA R:TCCATACCTCCTGCCAATC P:GTGCAAACCAATCGCAAGTCATTGTCG              | 102       | 0.500| 0.454| A    | 0.454| 1.000  |
| ApjSNP014_GT | Seryl-tRNA synthetase, mitochondrial           | F:ATTCGTTGCAGTCTGCAAT R:GAGATCGGCGGATATAACCA P:GTGCAAACCAATCGCAAGTCATTGTCG               | 96        | 0.271| 0.276| G    | 0.386| 0.346  |
| ApjSNP015_CT | Creatine kinase, flagellar                    | F:TCACGCGCTGATGTAGGATGA R:TCCATACCTCCTGCCAATC P:GTGCAAACCAATCGCAAGTCATTGTCG                | 92        | 0.436| 0.502| C    | 0.446| 0.556  |
| ApjSNP016_AG | Fibrinogen-like protein A                     | F:AAAGCCTAAGAAAGGGGA R:TCCAGTACCTAGGTAGGACACAGCA P:GAATTCACTAGGGAGTCATCTCTCAGATC          | 108       | 0.430| 0.583| A    | 0.483| 0.497  |
| ApjSNP017_GT | Abhydrolase domain-containing protein 14B     | F:CCGGGCTGTACCTCATACAGGACC R:TCCCGGCCGCACTACAGTGT       P:GCTATATTGAGCCCATATTGGTGATATG          | 78        | 0.293| 0.444| T    | 0.475| 0.854  |
| ApjSNP018_AG | Apolipoprotein A–I-binding protein            | F:CATAGGTGTTCCAGAAATGTTCCG R:TGGTTGTTCCAGAAATGTTCCG P:GCTATATTGAGCCCATATTGGTGATATG         | 93        | 0.073| 0.083| G    | 0.083| 1.000  |
Table 2. Cont.

| Locus ID     | Gene Name                                           | Primers and Probes (5′–3′)                                      | Size (bp) | Ho  | He  | MA  | MAF  | p-Value |
|--------------|-----------------------------------------------------|----------------------------------------------------------------|-----------|-----|-----|-----|------|---------|
| ApjSNP019_AG | N-acyl-phosphatidylethanolamine-hydrolyzing         | F:CGTGTCGGTTTAAATGTTG R:CATGGTGAACCTGGAAGCGG P:CCAAGCAACAAGAAGGAGGATTCCA | 91        | 0.688 | 0.505 | A   | 0.354 | 0.498   |
|              |                                                     | F:AAAGAGGTATCGACCTTGGCAA R:TGCTCGGACTTGATGTCATC P:GCTGAGGATCTCCAAAGAATGAGATCGAAGGATC  | 109       | 0.250 | 0.256 | A   | 0.328 | 0.857   |
| ApjSNP021_AG | Hyalin                                              | F:TTCAAGTGGTATGCACGAAAGC R:CGTCTATGCTGTGGCTGTCTCAATGCTGAG         | 92        | 0.108 | 0.333 | A   | 0.290 | 0.557   |
| ApjSNP022_CG | Transmembrane protein 129                          | F:TGGACTGAACTACACCAACCA R:TTGACACCACCCAGCCAATCTC G:GCTGAGGACAGATGACATCC  | 80        | 0.442 | 0.364 | G   | 0.483 | 0.381   |
| ApjSNP023_CT | Mediator of RNA polymerase II transcription subunit | F:GCTGATGCAGCAACTTCCACACT R:CAAGTTTCAGACGCGGACCTG G:GCTGAGGATCTGGATGACACTCA  | 95        | 0.146 | 0.505 | T   | 0.489 | 0.051   |
| ApjSNP024_AG | AF339450_1 hillari                                  | F:TCCATGGAAACGGAAGGACCTTC R:CAAGACATGTTCCGCTTGG P:GCTGAGGATCTGGATGACACTCA  | 108       | 0.419 | 0.484 | A   | 0.395 | 0.376   |
| ApjSNP025_AC | Proteasome subunit beta type-5                      | F:TCCAGATGCTAGCTGCCAGTTC R:ACGACCATGTTTACGTTCAAGAG P:GCTGAGGATCGGATGACATCC  | 81        | 0.250 | 0.250 | A   | 0.423 | 0.125   |
| ApjSNP026_AG | Dynactin subunit 5                                 | F:GCTGCTGGTGAACCTTGGACTTC R:CTGGGACATGTTAATGCTGGAACCTTGGACTTC  | 110       | 0.316 | 0.365 | A   | 0.461 | 0.724   |
| ApjSNP027_AG | Apoptosis-inducing factor 2                         | F:AGAGAAAGCAACGGAAGGATGAGA R:ATGATTTCAACTGGGCTCATC P:GATGATGAAACGAGGAGATTGCA  | 88        | 0.516 | 0.467 | A   | 0.361 | 0.324   |
| Locus ID   | Gene Name                          | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho     | He     | MA  | MAF   | p-Value |
|-----------|------------------------------------|------------------------------------------------------------------------------------------|-----------|--------|--------|-----|-------|---------|
| ApjSNP028_CT | Uncharacterized protein C6orf163  | F: ATAGTTGGGTGTGGCTTTGC  
R: CCGATGCAGTGATGGAATA  
P: AAAAAATGTCAATACGTGATTCATCCTGCTCCTGCC | 104       | 0.209  | 0.190  | C   | 0.105 | 0.698  |
| ApjSNP029_AT | F-box/LRR-repeat protein 2        | F: CGGTGATCTCTAAATGAGGGTA  
R: CGCTAAGGTAAGAGAAAGAAGCA  
P: GCCTAACCATACGTGATGCTAGCAGT | 98        | 0.271  | 0.237  | A   | 0.135 | 0.762  |
| ApjSNP030_CT | TBC1 domain family member 10B    | F: CCGGAGAGCTAAAGACACTC  
R: TCGTCGTGCTCTGATCCAC  
P: AAGTCTGGACAGCTTTAGCTAAGGGC | 91        | 0.191  | 0.174  | T   | 0.095 | 0.754  |
| ApjSNP031_CG | Stej aggregin-A subunit alpha    | F: ATCGGTGCTAGACCCAAGA  
R: TCCTTCTCTTGTAATGATTG  
P: CATCCAACAGCCGACCGTATGTA | 81        | 0.150  | 0.245  | G   | 0.264 | 0.358  |
| ApjSNP032_AC | Lysine-specific demethylase 6A   | F: CGAAGGCAAACACAGTGGAC  
R: TGCCACCTCGATCATTTTCT  
P: CGCTGTTGTTAATAACTTCATAGTCCGTTAC | 91        | 0.138  | 0.833  | C   | 0.383 | 0.497  |
| ApjSNP033_AG | ATP synthase subunit beta, mitochondrial | F: GAGTAAACACGCCCAGAAA  
R: TACAGTGCCTACACGCCGTC  
P: GCGTGCAGGGCTTTAGGCTGCTGAATCCTGCA | 76        | 0.458  | 0.467  | A   | 0.232 | 0.854  |
| ApjSNP034_GT | Ubiquitin carboxyl-terminal hydrolase 8 | F: GGCTTGGAAGAAACATGGTAA  
R: CCGATTAGATTGCATCTTCCTTCCATC  
P: TACATGTCATTCCATCTTCCTTATACCAACACGATGACAT | 110       | 0.292  | 0.314  | G   | 0.035 | 1.000  |
| ApjSNP035_CT | Uncharacterized protein C7orf26 homolog | F: CCGTTGAGGTGGGTCTACATT  
R: GGAATAGGCCAATCTCGAGGAA  
P: GTCGGTGAACTGAAAGCCTTATGGAATCCTTGCAG | 76        | 0.449  | 0.367  | T   | 0.485 | 0.498  |
| ApjSNP036_AC | hypothetical protein             | F: AAGTGCAGCCAGCAGCAAACAA  
R: CATGACTGCTCTCCTGCTC  
P: CAGGAATCTTCACAAGACGAGGGGAACACT | 100       | 0.545  | 0.413  | C   | 0.264 | 0.857  |
| Locus ID   | Gene Name                                      | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho   | He   | MA    | MAF   | p-Value |
|-----------|-----------------------------------------------|--------------------------------------------------------------------------------------------|-----------|------|------|-------|-------|---------|
| ApjSNP037_AG | DNA replication licensing factor MCM8         | F: GGAACCCGGAGAGATAGACAGA  
R: CCAGCGTCGTCACCTTTTAC  
P: AGAGCAAGTCAACGAGATGGACAAAGTA | 95        | 0.492 | 0.502 | A     | 0.458 | 0.557  |
| ApjSNP038_AG | LRP2-binding protein                          | F: GATGAAAGATCTCGGGAGGAA  
R: AGCTGATCATCGGTCCCATCT  
P: GGAGATGAGATGATCCTCCACTGACAAACTC | 83        | 0.750 | 0.625 | G     | 0.147 | 0.381  |
| ApjSNP039_AG | Endoplasm                                       | F: ATACGTCGGAGCGAGGATTC  
R: AGCAACCCATCTCTCGAC      
P: AAGGCTTGGAGATACACAGTCGGATGCC | 76        | 0.409 | 0.479 | G     | 0.387 | 0.051  |
| ApjSNP040_CT  | heat shock protein 90 kDa beta                | F: CCTTGAGAATGATGAGCACAAG  
R: TTGTGGTCTGCAAGGGTTT      
P: ACTCCTGACCTGCCTCTCAATGTGA | 102       | 0.348 | 0.291 | C     | 0.174 | 0.084  |
| ApjSNP041_CT  | Titin                                         | F: AGCCATCGAGATGAGAAGC  
R: ATGATGGTCTGATCCACAC      
P: GGTCACCGACTCAGAAGATCGGATGCC | 82        | 0.382 | 0.314 | T     | 0.192 | 0.091  |
| ApjSNP042_AG | Midasin                                        | F: CAGCCTGGAGACACCTCAG   
R: TGTGACTACCAACCAGACC     
P: AACCAGCTCAATCCGATGGACCGT | 88        | 0.800 | 0.691 | G     | 0.291 | 0.635  |
| ApjSNP043_CT  | Scavenger receptor cysteine-rich type 1 protein M130 | F: GGTTCACAACCTCATGGATGAC  
R: CTTCGACACCGACCTTTT      
P: GAAATACACTCTGCTTTAGTGTCAGGATAG | 95        | 0.317 | 0.505 | C     | 0.476 | 0.200  |
| ApjSNP044_AC  | FK506-binding protein 15                      | F: TCATACACTCGGACATCCA  
R: GCGTAGGCATATGACGAGA     
P: CAGTTTGTAGGTCTTGACAGTATGG | 90        | 0.583 | 0.473 | A     | 0.332 | 0.149  |
| ApjSNP045_AG | Titin                                         | F: CGTTGAGATCCAACTGCAAG  
R: TGATGGTGAGTTGTAAGACG     
P: TAGAAAGAATGGCAAGCGTCCCTGGAGT | 105       | 0.512 | 0.502 | A     | 0.456 | 0.897  |
| Locus ID     | Gene Name                                      | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho     | He     | MA   | MAF   | p-Value |
|-------------|-----------------------------------------------|-------------------------------------------------------------------------------------------|-----------|--------|--------|------|-------|---------|
| ApjSNP046_AG | Radial spoke head protein 4 homolog A          | F:GGGGAAGATGAGGTAGAAACG<br>R:GCTCATACCGATTCTCTGTT<br>P:ACTCCCAAACCTACCQGAACCTTATGTTTAGA | 81        | 0.113  | 0.109  | G    | 0.056 | 0.623   |
| ApjSNP047_CT | Phenylalanyl-tRNA synthetase beta chain        | F:TGGAATAATCAATCGGATTCT<br>R:ACCGTCAAATGTATACCTCTAGG<br>P:CCTAAAAGTTGAGCTTCCAAAACCATGTGGA | 102       | 0.326  | 0.300  | T    | 0.178 | 0.653   |
| ApjSNP048_AG | Mitochondrial inner membrane protein           | F:CCGATGAGAGGGGTATTCGA<br>R:CCCCCATTCTGCTATCAC<br>P:GGAGAGGAGGAAATGATCCAGAGATA | 98        | 0.222  | 0.468  | A    | 0.361 | 0.002 * |
| ApjSNP049_CT | Sulfotransferase family cytosolic 1B member 1 | F:CCAGGGAAGTCAAGGTCA<br>R:ACTGAGCTTCCGTACAG<br>P:CTGAAAGTTTCCACACCTTAGA | 82        | 0.524  | 0.479  | T    | 0.278 | 0.401   |
| ApjSNP050_CT | RalA-binding protein 1                         | F:GGTTGGAGAGTCTGAGGAGT<br>R:CATCCAGCTCATCCACACA<br>P:CTGAAATGATTTGCCCCACCTACACCTTAGA | 105       | 0.250  | 0.408  | C    | 0.275 | 0.018   |
| ApjSNP051_GT | Alpha-amylase B                                | F:TTCGATCCATCTCGTCTTTG<br>R:CTTGACCTTGAGGCGTGGTTT<br>P:GGGAGAGGAGAATCCCTGCTACATGGAATTG | 107       | 0.096  | 0.481  | T    | 0.390 | 0.005 * |
| ApjSNP052_GT | Putative vitellogenin receptor                 | F:CCGGCTTCAAGGAACCCACTGAGA<br>R:CGATTAGGAGGAGCTTCTGGAAAACG<br>P:GGCAGAGGATACAGCCTCGGATTTCTGCTGGTTAATGG | 98        | 0.411  | 0.485  | G    | 0.400 | 0.758   |
| ApjSNP053_CT | UDP-N-acetylglucosamine--peptide              | F:TCAAGGCTTAGTCTGAGCA<br>R:TGTGAAGGATTTGAGGAGCA<br>P:TGATTTGGAGGCGTCTGTGATGAGCACTCA | 101       | 0.071  | 0.503  | T    | 0.404 | 0.000 * |
| ApjSNP054_CT | Kanadaptin                                     | F:CAAGCCGTACATGAAGCA<br>R:TGTCCAGGTACAGTGTCATCG<br>P:AGAAGAGAAGAAGGATGGGCGGACGATCT | 88        | 0.585  | 0.506  | C    | 0.489 | 0.307   |
| Locus ID   | Gene Name                                      | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho     | He     | MA  | MAF    | p-Value |
|-----------|-----------------------------------------------|--------------------------------------------------------------------------------------------|-----------|--------|--------|-----|--------|---------|
| ApjSNP055_GT | Epidermal growth factor receptor              | F: TCACGTTCACCAGATTTTG                                                                       | 104       | 0.253  | 0.435  | G   | 0.424  | 0.518   |
|           |                                               | R: ATGATGGGGTTAATGGCATA                                                                      |           |        |        |     |        |         |
|           |                                               | P: TGACCAATAGCATATTCGATGATGTCACCA                                                            |           |        |        |     |        |         |
| ApjSNP056_CT | hypothetical protein                          | F: ATGCCACCTCTTAATCTGG                                                                       | 107       | 0.125  | 0.117  | T   | 0.318  | 0.442   |
|           |                                               | R: CTTGCCCTGGTTTTCATAC                                                                     |           |        |        |     |        |         |
|           |                                               | P: TCAAGACCCTGCTGCCTGACAGTACATT                                                            |           |        |        |     |        |         |
| ApjSNP057_CG | RuvB-like 2                                   | F: CCATAACACCGATGACACCA                                                                     | 108       | 0.295  | 0.388  | C   | 0.258  | 0.159   |
|           |                                               | R: GAAGCTGATAAGATGGAAGTAGGCC                                                                |           |        |        |     |        |         |
|           |                                               | P: CATGTCAAGGCAGTCATCCTGTGACAGGA                                                            |           |        |        |     |        |         |
| ApjSNP058_AG | Eyes absent homolog 1                         | F: CGTATCCGCACCACCAACCT                                                                   | 79        | 0.400  | 0.501  | G   | 0.247  | 0.485   |
|           |                                               | R: AACCCGTAGGAACCTGACT                                                                     |           |        |        |     |        |         |
|           |                                               | P: GGTGTCACCAACCAACGCTGGGTACGG                                                            |           |        |        |     |        |         |
| ApjSNP059_CT | WD repeat and FYVE domain-containing protein 3 | F: TCCAGGGATTTGACAGAGG                                                                     | 110       | 0.530  | 0.500  | C   | 0.446  | 0.984   |
|           |                                               | R: TGGCATAAAGCTGCTAGTCT                                                                   |           |        |        |     |        |         |
|           |                                               | P: TCCAGGGAGAGATCCTAGGGGTACTGGA                                                            |           |        |        |     |        |         |
| ApjSNP060_AT | similar to LOC398543 protein                  | F: CCACTACACATCGGTGACCA                                                                    | 110       | 0.095  | 0.433  | A   | 0.309  | 0.008   *
|           |                                               | R: CATCTCCCTCCGATACACAGTT                                                                |           |        |        |     |        |         |
|           |                                               | P: AGATGAAGAATGTAATAACTACGCTGCACACT                                                         |           |        |        |     |        |         |
| ApjSNP061_CT | Coiled-coil domain-containing protein C6orf97  | F: GCTGTGGCCGATGAAACAAAT                                                                  | 110       | 0.479  | 0.447  | T   | 0.329  | 0.489   |
|           |                                               | R: CAAATTTGACGATGAGAC                                                                      |           |        |        |     |        |         |
|           |                                               | P: AGAATATCTCGCTGGGATACGCAAACC                                                              |           |        |        |     |        |         |
| ApjSNP062_CT | Uncharacterized gene 48 protein               | F: CAGAAGGATTAAGTCGCAAAGAGACC                                                              | 86        | 0.182  | 0.220  | T   | 0.198  | 0.809   |
|           |                                               | R: TCTCTCTCTGTGGACATCTG                                                                    |           |        |        |     |        |         |
|           |                                               | P: ACAGGCTCTAGCATGACATCGAAGAATCG                                                           |           |        |        |     |        |         |
| ApjSNP063_AT | Uncharacterized protein C2orf73 homolog      | F: CACATGTGTCACCTCGCTGTA                                                                  | 73        | 0.479  | 0.586  | A   | 0.311  | 0.252   |
|           |                                               | R: ACTGGAACAGCGCTTTTTAGA                                                                   |           |        |        |     |        |         |
|           |                                               | P: CAGCTCAAACCCCTGACAACACTATGCAAG                                                        |           |        |        |     |        |         |
| Locus ID     | Gene Name                        | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho  | He  | MA  | MAF  | p-Value |
|-------------|----------------------------------|--------------------------------------------------------------------------------------------|-----------|-----|-----|-----|------|---------|
| ApjSNP064_AT| Methionine synthase              | F: TCGATACCTCACCAAAAGAAT  
R: CGAGGGTCTTGGGAAGGA  
P: CCAAGCTTACATCAACAAGGTCTTTA | 103       | 0.486 | 0.495 | T   | 0.432 | 0.654   |
| ApjSNP065_CT| Tubulin alpha chain              | F: CATAGCTTCGGTGGGAAC  
R: GCTTCTTCTGACTGGTGAG  
P: GGATTTGCAAGCTCTCTTTGACGCG  | 85        | 0.061 | 0.091 | T   | 0.091 | 1.000   |
| ApjSNP066_AG| TATA element modulatory factor   | F: TGGTGCTCATCAGTGAATCTGT  
R: TGGTCCTTCTTGAGCCCTTCT  
P: GAAAGCAAGACAGCTCGAGAGGCTT  | 86        | 0.415 | 0.100 | G   | 0.321 | 0.007 * |
| ApjSNP067_AG| TATA element modulatory factor   | F: GCAACTGGAGGCGAGAGAG  
R: GGCTGTCCGAGTCTACCT  
P: AGAGACAAAGAAAGACAGCTCGAGGAGGAC  | 79        | 0.315 | 0.400 | A   | 0.206 | 0.486   |
| ApjSNP068_CT| Uncharacterized protein KIAA1704 homolog | F: TGGACTTATGCGAGCATGCTCT  
R: GGAGGATTGTTGGACCAA  
P: AGAGACAAAGAAAGACAGCTCGAGGAGGAC  | 90        | 0.412 | 0.504 | C   | 0.477 | 0.135   |
| ApjSNP069_CT| WD repeat-containing protein KIAA1875 | F: GGTCTTCTCAGCCACATGATA  
R: ACCAGCGCTACGTCTGAGTC  
P: TACCTGGTTATCGCTCTCGAGAAGCAGGAGAAGAAG  | 103       | 0.326 | 0.225 | C   | 0.471 | 0.390   |
| ApjSNP070_AG| Glycoprotein 3-alpha-L-fucosyltransferase | F: CCGAGAGGCTGTAGACCCTTGC  
R: ATCTCGGCTTCAGGGTTGGT  
P: CTAGAGATGCTCGTTCCAGGAGGAGGATGTC  | 102       | 0.528 | 0.469 | G   | 0.334 | 0.051   |
| ApjSNP071_AG| N/A                              | F: CGAAACTATAGTGACCTTGGTCT  
R: CAAAGCCTAGTCCTCTTCTCAG  
P: CAGAATTTTCTCGAGAGCATGTCCCTGAGAGCAGAG  | 104       | 0.364 | 0.470 | A   | 0.364 | 0.189   |
| ApjSNP072_AG| N/A                              | F: GTGTTAGACCCCTCGGGCTAGTTA  
R: GCAAGAGGCTAGCTCTTTGAAGT  
P: TGCATCGACTAGCGCATGGAAACT  | 87        | 0.388 | 0.412 | G   | 0.333 | 0.247   |
| Locus ID  | Gene name | Primers and probes (5′–3′)                                                                 | Size (bp) | Ho   | He   | MA | MAF     | p-Value |
|----------|-----------|--------------------------------------------------------------------------------------------|-----------|------|------|----|--------|---------|
| ApjSNP073_AG | N/A       | F:AAATGTACAGACCCGCATGA R:TGTAATAATGACGCCGACCAAGAG | 107       | 0.225| 0.309| A  | 0.188  | 0.104   |
| ApjSNP074_AT | N/A       | F:GATGGTGAAAATCACGGAGAA R:TTCTATTGTTGATGCAGAGAC | 103       | 0.300| 0.404| A  | 0.275  | 0.108   |
| ApjSNP075_AT | N/A       | F:GCCACCGATGACACGGAGTA R:CTGGCCAACTGCGGAAAG | 95        | 0.630| 0.879| T  | 0.450  | 0.328   |
| ApjSNP076_CT | N/A       | F:AACCTCTGATGGAATGCAAAG R:ACAGACTCCTGTCGACATCTC | 108       | 0.175| 0.392| C  | 0.263  | 0.001   *|
| ApjSNP077_CT | N/A       | F:GACCATCCCTGTAGGCAGTA R:TGTTGGAATGACCATCGTTCG | 103       | 0.175| 0.339| T  | 0.213  | 0.004   *|
| ApjSNP078_GT | N/A       | F:TGGGCAGAAGAAAATTTGGA R:GAGTGGCACATGACTTGGTG | 99        | 0.475| 0.469| G  | 0.375  | 0.084   |
| ApjSNP079_AG | N/A       | F:TGGGCAGAAGAAAATTTGGA R:GAGTGGCACATGACTTGGTG | 99        | 0.475| 0.469| G  | 0.375  | 0.084   |
| ApjSNP080_CT | N/A       | F:GAGGACGTATCAGCACTCTTGA R:TCTTGTGGATGCTGTCGCTCCA | 110       | 0.200| 0.292| C  | 0.175  | 0.062   |
| ApjSNP081_CT | N/A       | F:CTGTTGCAATAGGTTATTTTG R:TGAATACATCCGTTTCTGA R:GTTGATGCGAAACAGTGCATTC | 103       | 0.075| 0.073| C  | 0.038  | 0.780   |
Table 2. Cont.

| Locus ID    | Gene Name | Primers and Probes (5’–3’) | Size (bp) | Ho    | He    | MA   | MAF   | p-Value |
|-------------|-----------|-----------------------------|----------|-------|-------|------|-------|---------|
| ApjSNP082_CT| N/A       | F:CAGAAACGGCATGTATTTCAAC   | 94       | 0.500 | 0.404 | C    | 0.275 | 0.098   |
|             |           | R:CCCCGACCACAAGGAAGAGATA   |          |       |       |      |       |         |
|             |           | P:AGGGGAGTTTGATGACAAATTTGTGCAG |          |       |       |      |       |         |
| ApjSNP083_AC| N/A       | F:ACAGATGCCTGTGTGTAAT      | 108      | 0.325 | 0.453 | C    | 0.338 | 0.073   |
|             |           | R:GTCGGCCTCCTGACTAACAG     |          |       |       |      |       |         |
|             |           | P:GCAGCAGAGAAGCGGTGGA      |          |       |       |      |       |         |
| ApjSNP084(CG)| N/A      | F:GGGTGTGGCATTTTCCTCAT     | 75       | 0.150 | 0.444 | G    | 0.325 | 0.000 * |
|             |           | R:TGGCTTCAGTTACACCATCCT    |          |       |       |      |       |         |
|             |           | P:ATCCCTTTGTCGCTGATCCTTGTT |          |       |       |      |       |         |
| ApjSNP085_AG| N/A       | F:GTCATTTGCCTCCAAATACC     | 110      | 0.833 | 0.896 | A    | 0.487 | 0.093   |
|             |           | R:GTCGTAGAGAGACATAACGATAACTGA |          |       |       |      |       |         |
|             |           | P:CCATATGGCTATGCGCTGAACGATAA |          |       |       |      |       |         |
| ApjSNP086_AG| N/A       | F:GACAAATATACATCAAAATGCCTGT | 83       | 0.050 | 0.461 | G    | 0.350 | 0.000 * |
|             |           | R:GATGATGAAATGGTGTGTGGT    |          |       |       |      |       |         |
|             |           | P:CAAGGCGAGTTCGACCAGAAAATG |          |       |       |      |       |         |
| ApjSNP087_AG| N/A       | F:CACCTTGCTGCCCTGCACCTTT   | 109      | 0.450 | 0.353 | C    | 0.225 | 0.252   |
|             |           | R:GTGGAACAACATAGGTCACAGGT  |          |       |       |      |       |         |
|             |           | P:GGGCAAATCGATGTCATGTTACAGGTATGT |          |       |       |      |       |         |
| ApjSNP088_AG| N/A       | F:ATGAAAGCTGCGTGAATGAG     | 83       | 0.250 | 0.222 | G    | 0.125 | 0.256   |
|             |           | R:CGATTTCATCGCTGTCATCAA    |          |       |       |      |       |         |
|             |           | P:AACTGTGGAGATGGAACATATCTATCATATGAAAGAGAA |          |       |       |      |       |         |
| ApjSNP089_AG| N/A       | F:GACTGTGGAGAGATGGAACATCCACAGA | 93       | 0.325 | 0.468 | A    | 0.363 | 0.051   |
|             |           | R:GTGTTTTTGAAGGCACCTGATGA |          |       |       |      |       |         |
|             |           | P:AAAGTTTTAAATGCAAGACTGGGTCAAGACA |          |       |       |      |       |         |
| ApjSNP090_CT| N/A       | F:TTGTACCGGAAAAGGGATGTGTT | 110      | 0.161 | 0.373 | T    | 0.242 | 0.002 * |
|             |           | R:CCTGAAACCACATCTCGCTGA |          |       |       |      |       |         |
|             |           | P:AGAGTATATTTCACCAGAAAAACCGGGAGTAGGT |          |       |       |      |       |         |
| Locus ID   | Gene Name | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho   | He   | MA  | MAF  | p-Value |
|----------|-----------|--------------------------------------------------------------------------------------------|-----------|------|------|-----|------|---------|
| ApjSNP091_CT | N/A       | F:TGCCTCATTTCTAACCAACCA  
R:AAACCTTATGAGGCGAAGCTTGA  
P:CAAAAGCCCTCAATTTTCACAGCACTAA  | 102       | 0.200 | 0.380 | C   | 0.250 | 0.004 * |
| ApjSNP092_CT | N/A       | F:TGAAGGACTCTAGATGTTG  
R:GTGGGCTTCAGACACAGAT  
P:GAGTTCATCAAGGGAACCTGTTGACATACA  | 81        | 0.075 | 0.073 | C   | 0.038 | 0.780   |
| ApjSNP093_AG | N/A       | F:TGAATTGTGTTGACCTTCG  
R:GTGGTACCTTCAGCATCTCTGT  
P:GAATTGGATATACTGTGTTGACTT  | 80        | 0.222 | 0.282 | G   | 0.167 | 0.227   |
| ApjSNP094_GT | N/A       | F:TCTGCTAAGTGTGGAGAGAT  
R:CGAAGCGTGTTTATGTTG  
P:TTCTGGTCACCTGCCCAGGTTCCAC  | 108       | 0.171 | 0.358 | T   | 0.229 | 0.003 * |
| ApjSNP095_AG | N/A       | F:ATAATGCCTTCAGCTTGTCA  
R:GTAAGTGACAAGTCACCAACGA  
P:AAACTTGGCAACGAGATCATGCAT  | 110       | 0.225 | 0.367 | A   | 0.238 | 0.018   |
| ApjSNP096_CT | N/A       | F:TCATTCTGTATATGCGTACCTGTG  
R:GTGGTATGCCCATCGATT  
P:TTAACAATGATCTTAAAGGCTATGGCGAAGCAACACAAAC  | 109       | 0.333 | 0.491 | C   | 0.409 | 0.060   |
| ApjSNP097_CG | N/A       | F:CAACAGGTAGTATGATGACTTCCG  
R:GACCCTGCTTTGTCCTAC  
P:ACACACATGATATACCAAGAATCTGGAATTATGTT  | 94        | 0.316 | 0.337 | C   | 0.211 | 0.698   |
| ApjSNP098_CT | N/A       | F:CTGTGTCAAGAGAAGAAGTGC  
R:CGAAGCTTTCTCAACCCAGT  
P:GGTACTATCAAAGTGACTCAACAAAGCGAC  | 107       | 0.158 | 0.147 | C   | 0.079 | 0.512   |
| ApjSNP099_AG | N/A       | F:GACTCTCAGCTCTGCCCTGAC  
R:CGGATATCACAACACAGAGGC  
P:TCCTCATTCTGCAGTGCTTTCGCAAC  | 97        | 0.075 | 0.162 | G   | 0.088 | 0.080   |
Table 2. Cont.

| Locus ID     | Gene Name | Primers and Probes (5′–3′)                                                                 | Size (bp) | Ho    | He    | MA   | MAF   | p-Value |
|--------------|-----------|--------------------------------------------------------------------------------------------|-----------|-------|-------|------|-------|---------|
| ApjSNP100_GT | N/A       | F:TCCACTGAGCCATCCTGATT<br>R:GAAGAAAAACATGTCCCGATG<br>P:AGTGGCTCCCCGGGAATGTAATCCTG          | 103       | 0.505 | 0.547 | T    | 0.458 | 0.279   |
| ApjSNP101_GT | N/A       | F:CTGCTGAAGTGACACATTAGAGAC<br>R:CTAGTACCTTTCTTCTTCAGTAGTTGG<br>P:CTATTGAAAGCTCGATAGGCACATCCTG   | 109       | 0.075 | 0.240 | T    | 0.138 | 0.000 * |

The underlined bases in the probe sequences indicated the positions of the SNPs; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity; MA, minor allele; MAF, minor allele frequency; $P_{HWE}$, $P$ values for Hardy–Weinberg equilibrium (HWE) test; *, statistically significant after sequential Bonferroni correction.
As the gene-derived SNPs reside in or are immediately next to protein-coding sequences, they stand a better chance for identifying functional genes that are responsible for complex traits as well as simply inherited traits [8,9]. In our study, 70 SNP markers (Table 2) were developed from the EST sequences showing significant similarity to an entry in the NCBI nr database [10]. Among the annotation information, genes potentially involved in growth or immunity (e.g., epidermal growth factor receptor, Zinc finger protein 62 homolog and heat shock protein 90 kDa beta) were identified. It would be interesting to see whether any of these growth- or immune-related SNPs are highlighted in future QTL mapping of economically important traits, such as high growth rate and disease resistance.

3. Experimental Section

3.1. Sampling and DNA Extraction

A total of 48 *A. japonicus* individuals used for SNP marker validation were collected from four natural populations (Dalian, Yantai, Qingdao and Wendeng) in China. Genetic parameters of the validated SNP markers were further evaluated using 40 *A. japonicus* individuals from the Rongcheng (Shandong, China) population. Genomic DNA was extracted from the muscles of sea cucumbers by following the protocol developed by Zhan *et al.* [11]. The quantity and integrity of genomic DNA was determined using an Ultraspec™ 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences, Uppsala, Sweden) and gel electrophoresis, respectively.

3.2. SNP Discovery and Genotyping

Our group has recently released a large amount of transcriptomic data by 454 sequencing of eight cDNA libraries constructed using more than 200 sea cucumber individuals. Potential SNPs were detected from the assembled contigs using the program GS Reference Mapper (version 2.6, Roche 454 Life Sciences: Branford, CT, USA, 2011) with default parameters (cDNA mode). More than 54,000 putative SNPs were identified from the dataset, 200 of which were selected in this study for marker development with the selection criteria of at least 3× occurrence of the minority allele and at least 6× contigs coverage (number of reads forming the contig). SNP genotyping was performed using a recently developed cost-effective HRM method [12]. For each locus, three non-modified oligonucleotides were used, corresponding to two PCR primers and one probe, primers were designed using Primer3 [13] with the following rules: (1) primer length should be at least 20 bases; (2) product size should not exceed 120 bp in order to decrease the probability of intron interference; (3) the primer Tm should be between 59 °C and 61 °C; (4) the primer GC% should be 40%–60%; and (5) the amplicon contains only one SNP site. Probes were designed using OligoCalc [14] with the following criteria: (1) SNP site locates in the middle of the probe; (2) the length of probe is between 20 and 35 bases; (3) Tm is about 60 °C; (4) the 3’ end of each probe is blocked by two mismatch bases; and (5) no overlap between primes and probe. Each SNP locus was first amplified by an asymmetrical PCR with HRM fluorescent dye in the PCR master mix and then interrogated by an unlabeled probe. The 48 individuals of *A. japonicus* collected from four natural populations were used for SNP marker validation. PCR amplifications were carried out in a 10 µL reaction mixture containing 20 ng of genomic DNA, 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 U Taq DNA polymerase (Takara,
Dalian, China), 0.1 µM forward primer, 0.5 µM reverse primer and 1× LCGreen Plus (Idaho technology inc., Salt Lake City, Utah, USA). The amplification was programmed as: an initial denaturation at 95 °C for 5 min, followed by 55 cycles of 95 °C for 40 s, 60 °C for 40 s and 72 °C for 40 s, finishing with a final elongation at 72 °C for 5 min. The PCR products were checked by gel electrophoresis, and those with correct PCR product sizes were then subjected to probe testing. An aliquot of the appropriate probe was added in each reaction to a final concentration of 5 µM. The PCR product and probe mixture were denatured at 95 °C for 15 min and then slowly cooled to 4 °C. HRM genotyping was immediately performed on a Light Scanner instrument (HR96 model, Idaho technology inc., Salt Lake City, Utah, USA) with continuous melting curve acquisition (10 acquisitions per °C) during a 0.1 °C/s ramp from 40 to 95 °C.

3.3. Data Analysis

Data were retrieved and analyzed using the Light Scanner software followed by manual curation of the obtained genotype calls. POPGENE [15] was used to analyze allele frequency, expected (He) and observed (Ho) heterozygosities, and tests for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD).

4. Conclusions

In summary, 101 gene-based SNPs were successfully developed from the transcriptome sequences of A. japonicus. These developed markers are expected to be useful for future QTL analysis, and to facilitate MAS in A. japonicus.

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