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EST-SSR markers derived from *Laminaria digitata* and its transferable application in *Saccharina japonica*

Fuli Liu · Feijiu Wang · Delin Duan

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Abstract The public availability of numerous expressed sequence tag (EST) enables EST-based SSR (simple sequence repeat) markers to be widely used for genetics and breeding studies. In the present study, EST-SSR markers were developed from ESTs of *Laminaria digitata* and were transferred to the non-congeneric species *Saccharina japonica*. Among the 2,668 non-redundant ESTs, 83 (3.1%) ESTs containing SSR were identified totally, with an average of one SSR per 13.6 kb. Analysis of SSR motifs revealed that the trinucleotide and tetranucleotide were major motifs, accounted for 44.58% and 16.87%, respectively. Based on the 83 ESTs containing SSR, we designed 45 pairs of primers in the flanking regions of the SSR, of which 13 pairs showed polymorphism in a wild *S. japonica* population, and the mean alleles per locus was 3.6 (ranging from 2 to 6). The observed (Ho) and expected (He) heterozygosities of these EST-SSRs were 0.234–0.632 and 0.260–0.635, respectively. All loci were in Hardy–Weinberg equilibrium in the wild population and no linkage disequilibrium was detected among loci. The obtained EST-SSR markers can facilitate and promote related research such as ecological investigation, genetic diversity assessment and breeding practice of *S. japonica* as well.

Keywords *Saccharina japonica* · *Laminaria digitata* · EST · SSR · Phaeophyta

F. Liu · F. Wang (✉)
Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences,
Qingdao 266071, China
e-mail: wangfj@ysfri.ac.cn

F. Liu · D. Duan
Institute of Oceanology, Chinese Academy of Sciences,
Qingdao 266071, China

Introduction

Among different kinds of molecular markers, simple sequence repeats (SSR) have been extensively shown to be useful in plant genetics and breeding due to its high reproducibility, multi-allelic nature, codominant inheritance, relative abundance, and good genome coverage (Powell et al. 1996; Nybom 2004). The major drawback of SSR is that for de novo species, it needs to be developed innovatively. The literature shows that the SSR markers have been mainly isolated from genomic sequences using different methods (reviewed by Zane et al. 2002). While these SSR markers are known as genomic SSRs, there is another type of SSR markers typically referred as EST-SSRs or genic microsatellites, which have been isolated from expressed sequence tags (ESTs), genes and cDNA clones. In contrast to genomic SSR markers, EST-SSR characterizes with some intrinsic advantages: (1) developing the procedure is easy and inexpensive; (2) can be transferable to use in congeneric and non-congeneric species; (3) can be used as functional markers (reviewed by Varshney et al. 2005a). During the past decades with the rapid increasing public availability of numerous EST data, EST-SSRs have gradually shown their validity and function in comparative mapping, functional genomics, gene tagging, QTL analysis and population genetics (Varshney et al. 2005a, b; Ellis and Burke 2007).

Saccharina japonica is a common edible kelp species along the northwest coast of Pacific Ocean (Bartsch et al. 2008). It can be used as food and raw industrial materials for algin, mannitol, and iodine extraction, and also plays an important role in maintaining offshore ecological balance and in bioremediation of marine pollution (Yang et al. 2005; An et al. 2010). Because of its important economic and ecological value, *S. japonica* is farmed in large scale in

Table 1 Characterization of SSR distribution and frequency in *L. digitata* ESTs

Repeat type	Number	Frequency	Number of motif type
Dinucleotides	10	12.05%	6
Trinucleotides	37	44.58%	18
Tetranucleotides	14	16.87%	11
Pentanucleotides	9	10.84%	8
Hexanucleotides	13	15.66%	12
Total	83	100%	55

western Pacific region. Especially in China, *S. japonica* aquaculture is a well-developed industry (Tseng 2001; Zemke-White and Ohno 1999). The past decades witnessed considerable interests on ecology, genetics and breeding of this kelp worldwide. Recently RAPD, ISSR and AFLP markers were developed in *S. japonica* and used for germplasm assessment and identification (Wang et al. 2004), parentage analysis (Billot et al. 1999), genetic diversity investigation (Wang et al. 2005; Yotsukura et al. 2001; Xia et al. 2005), population genetics study (Shan et al. 2011; Bi et al. 2011), genetic mapping (Li et al. 2007; Liu et al. 2009; Yang et al. 2009), QTL analysis (Liu et al. 2010, 2011). However, to date, only a few genomic SSRs and EST-SSR are available for *S. japonica* (Shi et al. 2007; Liu et al. 2009), although the SSR has already exhibited merits compared to other markers.

To overcome the lack of enough SSR markers, the genomic SSR markers developed by Billot et al. (1998) from *Laminaria digitata*, one of the species related to *S. japonica*, were successfully transferred to *S. japonica* (personal communication). This provides some insights for us that the EST-SSR markers derived from *L. digitata* also can be transferred to apply in *S. japonica*. In this study, we developed 13 EST-SSR markers from *L. digitata* ESTs and testified their transferability in a wild *S. japonica* population.

Materials and methods

To validate the transferability of EST-SSR markers from *L. digitata* to *S. japonica*, a total of 48 wild *S. japonica* sporophytes were collected on March 17, 2010, from

Table 2 Characterization of 13 polymorphic EST-SSR primers derived from *L. digitata* in *S. japonica* wild population

Locus	EST accession no.	Motif	Primer sequence(5′-3′)	Ta (°C)	Size range (bp)	Na	Ho	He
LD1	AW400672	(tgct)11	F: GTCCTCCCTCGGCGTAGTAT R: CATCCGTGAGTTGCGAAAGC	55	176–200	5	0.526	0.537
LD2	AW400749	(ctg)6	F: GCTTTCACGCCGCCGACC R:GCCGCCTTAGTTCAAGACGA	58	324–336	3	0.275	0.296
LD3	AW400904	(agg)6	F: AAAAAGCACGGCATAGTAGC R:ACATCCGATTCTCAAAACGA	55	266–381	3	0.345	0.356
LD4	AW401243	(ct)10	F: ATGCTCCTCGGCAGATTG R: CAGTTTGTTTACCCAGAAGT	53	178–190	5	0.612	0.623
LD5	CN466545	(ggtagc)5	F: GGAGTTGATGGCGATGGT R: AATAACAAAAAGTCGTGCGT	53	464–488	3	0.386	0.391
LD6	CN467174	(ggaa)9	F: AACAAAGGACGGTTGGACG R: TAGCCTGGTATTTACGGGTG	55	252–280	5	0.632	0.635
LD7	CN467331	(ttttg)4	F: ACGACAGCGATACAAACG R: GAGTGCTACCAGAACAACCA	50	342–362	3	0.256	0.268
LD8	CN467351	(cgaggc)3	F: GAAACCTTCTACATCGCAG R: ATCACACACTTCCTCCCC	50	500–530	3	0.328	0.360
LD9	CN467886	(agc)6	F: GGGAGGGGACATCCTGAGTA R: AGAAAGAGACCAAGCACGGC	55	284–299	4	0.419	0.426
LD10	CN468120	(tgc)7	F: TTCGCAAGAAGGGACACC R: AAAGCAGGAAAAGACGCA	53	312–318	2	0.234	0.260
LD11	AW401303	(tgc)14	F: AACCCAGTATCTACCTCGTG R: GCACCAAGCAGTATCAGCA	50	205–229	6	0.568	0.576
LD12	AW400562	(at)10	F: TGCGAAGATAATAAATGGGA R: CGTTGTAGCATAGGTTGGAG	50	314–322	3	0.326	0.341
LD13	CN468232	(ggaa)10	F: CGAGGGGTTTGAGAAGCA R: TACCGAAAGTTCTGCCGC	55 55	200–212	2	0.128	0.152

Ho observed heterozygosity, *He* expected heterozygosity, *Ta* annealing temperature, *Na* number of alleles

Hokkaido of Japan (N 42°18′, E 140°59′). *S. japonica* is a brown seaweed; it reproduces sexually by the dioecious gametophytes. As it occurs in sublittoral zone attaching to rocks or other solid substrata, sampling was conducted when the tide is falling. Sporophytes of *S. japonica* (about 6 cm in width and 70 cm in length) about 2 m apart from each other were randomly collected along the coastline. Small pieces of blade (about 50 g fresh weight) were cut from each individual sporophyte, rinsed with clear seawater and dried quickly by silica gel for future genomic DNA extraction.

EST-SSR identification and primer design

L. digitata EST sequences developed by Crépineau et al. (2000), Roeder et al. (2005), and Cosse et al. (2009) were downloaded from the dbEST database at NCBI, and then preprocessed to exclude redundant EST sequences. Next, SSRIT software (Temnykh et al. 2001) was used to identify SSRs with the criteria of minimum of ten, six, five, four and three repeats for di-, tri-, tetra-, penta- and hexanucleotide motifs, respectively.

Primer pairs were designed in the flanking regions of the SSRs with PRIMER 5.0 software. The parameters for primer design were as follows: product length 150–500 bp (optimum 300 bp), primer size 18–24 bp (optimum 20 bp), primer melting temperature of 50–65°C (optimum 55°C), and none secondary structure.

Genomic DNA extraction and PCR analysis

The genomic DNA was extracted from sporophytes using a Plant Genomic DNA kit (Tiangen Biotech CO., Ltd, China) according to the manufacturer instructions. The polymerase chain reactions (PCRs) were carried out in a total volume of 20 μL containing 0.5 U Taq DNA polymerase (MBI), 1× PCR buffer, 0.2 mM dNTP mix, 0.5 μM of each primer set, 2.0 mM $MgCl_2$ and about 50-ng template DNA. The mixture was subjected to 94°C for 4 min, following by 35 cycles of 30 min at 94°C, 30 s at annealing temperatures (refer to Table 2), 40 s at 72°C, and a final step at 72°C for 10 min. Amplified products were resolved via 6% denaturing polyacrylamide gel, and visualized by silver-staining according to Bassam et al. (1991).

Statistical analysis

The availability of the identified EST-SSR loci was validated in a *S. japonica* wild population of 48 individuals. The observed number of alleles (Na), the mean observed heterozygosity (Ho), and the mean expected heterozygosity (He) for each locus in the tested population were calculated by genetic analysis package POPGENE version 1.3 (Yeh et al. 1999). Tests of the Hardy–Weinberg equilibrium and linkage disequilibrium for each locus in the wild population were performed using GENEPOP (Raymond and Rousset 1995).

Results and discussion

Identification and characterization of EST-SSR in *L. digitata*

A total of 3,124 EST sequences of *L. digitata* were gathered from the dbEST database at NCBI and 2,668 non-redundant sequences were obtained after preprocessing. Using the SSRIT software, we identified 83 (3.1%) EST sequences containing SSR, with an average of one SSR per 13.6 kb. Some studies showed that 2–11% EST sequences contain SSR in plants (Cordeiro et al. 2001); e.g., maize (1.5%), *Medicago truncatula* (3.0%), wheat (3.2%), barley (3.4%), rice (4.7%), and sorghum (3.6%) (Kantety et al. 2002. The variation of overall frequency of EST-SSR depends on the criteria used to identify SSRs in the database (Varshney et al. 2005a). Another reason probably is the small number of ESTs explored in our study. It was not convincing statistically to compare the EST-SSR frequency of *L. japonica* with other plants listed above. Nevertheless, the SSR frequency in ESTs obtained in this study still contains potential references for further study on SSR in *L. digitata* genome.

Analysis of the results shows that tri- and tetranucleotide repeats were the most abundant types (44.58% and 16.87%, respectively), followed by hexanucleotide repeats (15.66%). Two other types of repeats were detected in the frequency of 12.05% (di-) and 10.84% (penta-), respectively (Table 1). Although the frequencies and distribution of different repeat motifs vary substantially in different species, trinucleotide repeats are the most common, followed by either dinucleotide repeats or tetranucleotide repeats (Varshney et al. 2002; Morgante et al. 2002; Kantety et al. 2002). Our result is also congruent with this general pattern. The abundance of trimeric SSRs in ESTs may be attributed to the absence of frameshift mutations due to length variation in these SSR (Metzgar et al. 2000).

Validation of transferability of EST-SSR marker in *S. japonica*

Based on the 83 ESTs containing SSR, a total of 45 pairs of primer were designed in the flanking regions of the SSR. Firstly, three individuals were selected randomly and used to test the availability of the designed primers. The results showed that 9 pairs of primer generate no amplified product, whereas 36 pairs of primer could amplify successfully. Among the 36 pairs of primer, 13 pairs gave rise to non-

specific amplifications, 23 pairs of primer could produce the unambiguous amplicons, and their sizes were either similar or larger than those of expected amplicons.

One wild *S. japonica* population containing 48 individuals was used to test the amplified polymorphism of 23 screened pairs of primer. The results showed that 13 pairs could amplify polymorphism in the wild population. Herein, we defined these 13 (56.5%) pairs of primer as EST-SSR loci that could be transferable to use in *S. japonica*. It has been confirmed that the EST-SSRs show higher transferability to relative species compared to genomic SSRs (Cordeiro et al. 2001; Varshney et al. 2005a, b). This phenomenon may be attributed to the conserved nature of coding sequences in contrast to non-coding sequences. The higher transferability of EST-SSR from *L. digitata* to *S. japonica* implied that the two species probably shared the same or similar nucleotide sequence flanking the SSR region. These EST-SSRs markers could produce different number of alleles ranging from 2 to 6 in the wild population, with an average of 3.6 (Table 2). The observed and expected heterozygosities ranged from 0.234 to 0.632 and from 0.260 to 0.635, respectively (Table 2). All loci are in Hardy–Weinberg equilibrium in the wild population and no linkage disequilibrium was detected among loci.

In conclusion, EST-SSR markers from *L. digitata* were developed and transferred to use in *S. japonica*. The results showed that 13 markers derived from *L. digitata* could be across used in *S. japonica*. These obtained EST-SSR markers will facilitate ecological investigation, genetics study and molecular marker assisted breeding of *S. japonica* in the future.

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