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Screening of polymorphic microsatellites and their application for *Saccharina angustata* and *Saccharina longissima* population genetic analysis

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Abstract

Saccharina angustata and *S. longissima* are ecologically and economically important seaweeds. So far, no microsatellite marker was available to *S. angustata* and *S. longissima* due to a lack of genomic data. Here, we developed polymorphic simple sequence repeats (SSRs) in *S. longissima* and *S. angustata* and applied these markers for further population genetic analysis. Cross-amplification tests showed that 11 SSR loci exhibited high amplification rate in *S. angustata* and *S. longissima*. Polymorphic information content values (PIC) of 11 loci showed 10 loci (PIC = 0.552–0.908), except for SJ31 (PIC = 0.482), have high polymorphism (PIC > 0.5). Polymorphism tests indicated that 10 loci have high polymorphism in three varieties of *S. japonica* and two related species. Genetic diversity analysis confirmed the validation of these markers in accessing the genetic diversity level of these kelps. STRUCTURE and NJ tree results indicated these 10 microsatellites are effective in evaluating the genetic relationship among different kelp populations. As results, these 10 polymorphic microsatellite markers are valid to *Saccharina* genetic analysis.

Keywords *Saccharina angustata* · *Saccharina longissima* · Phaeophyceae · Simple sequence repeats · Cross amplification

Introduction

The genus *Saccharina* Stackhouse is mainly distributed along northwestern coasts of the Pacific Ocean and has approximately 20 species (Kawashima 2012; Lane et al. 2006; Yotsukura et al. 2010a). These kelps are the primary producer in the marine ecosystem and constitute the marine forest to provide marine animal habitats (Yotsukura et al. 2010a). In addition, most of these kelps could be used for food, alginate manufacture, and other economical usages.

Saccharina japonica (Areschoug) Lane, Mayes, Druehl, and Saunders is a typical intertidal kelp, and *S. religiosa*, *S. ochotensis*, and *S. diabolica* are regarded as varieties of *S. japonica* (Yotsukura et al. 2008, 2010b). *Saccharina angustata* and *S. longissima* are closely related to *S. japonica*. Recently, inter-specific hybridization was used for elite cultivar breeding, such as Rongfu, Dongfang No. 2, and Dongfang No. 3 (Li et al. 2007, 2008; Zhang et al. 2007). Nevertheless, there is less knowledge on the genetic background of *S. longissima* and *S. angustata*, and it is necessary to develop more efficient markers for genetic study of these kelps.

Simple sequence repeats (SSRs) are 1–6 nucleotide tandem repeats randomly distributed throughout the genome and have become the most popular markers for population genetic studies (Kalia et al. 2011). There are many reports on the SSRs developed for *S. japonica* (Liu et al. 2010; Li et al. 2016; Peng et al. 2016; Yotsukura et al. 2016; Zhang et al. 2014, 2018); nevertheless, limited SSR loci are available for other *Saccharina* species. Cross-species amplification of the SSRs

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has been reported in many seaweeds (Martinez et al. 2005; Liu et al. 2010; Akita et al. 2018), and it could save time and resources to develop SSRs. It is feasible to screen polymorphism SSRs for *S. longissima* and *S. angustata* with SSR loci from *S. japonica*.

To enhance the efficiency of genetic analysis for these kelps, we aimed to screen polymorphism SSRs in *S. longissima* and *S. angustata* and assess the suitability of these markers in further genetic analysis.

Materials and methods

Sample collection and DNA extraction

We sampled 10 populations (200 individuals), including three varieties of *Saccharina japonica* (*S. japonica* var. *religiosa*, *S. japonica* var. *ochotensis*, and *S. japonica* var. *diabolica*) and two related species (*S. longissima* and *S. angustata*) from Hokkaido, Japan (Table 1; Fig. 1). We also collected four *S. japonica* populations from China, Korea, and Russia (Table 1; Fig. 1). All samples were preserved with silica gel. Genomic DNA was extracted as indicated in Zhang et al. (2015). The purity and quality of genomic DNA were checked by the A_{260}/A_{280} ratio and visualized by gel electrophoresis.

SSR loci selection and screening

After primarily screening for the 24 polymorphic microsatellites (Table S1), which were originally from *S. japonica* (Li et al. 2016; Zhang et al. 2017), we tested these markers on three varieties of *S. japonica* and two related species (*S. longissima* and *S. angustata*). PCR amplification was followed as previously (Zhang et al. 2017). We selected the successful cross-amplification SSR loci to validate polymorphism in 10 populations (200 individuals) of *Saccharina* (Table 1).

Data analysis

MICRO-CHECKER 2.2.3 was applied for checking the potential genotyping errors caused by stuttering or large allele dropout and the presence of null alleles (Van Oosterhout et al. 2004). Linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were estimated for each SSR locus and kelp population using GENEPOP 4.2.2 with 20 batches with 5000 iterations per batch (Raymond and Rousset 1995; Rousset 2008). For polymorphism evaluation of each SSR locus, the number of alleles (N_A), the effective number of alleles (N_E), the mean observed and expected heterozygosity (H_E and H_O), and the numbers of private alleles (N_P) were calculated using GENALEX 6.41 (Peakall and Smouse 2006). Polymorphic information content (PIC) was calculated using POPGENE 1.3.1 (Yeh et al. 1999). Allelic richness (A_R) across all loci based on the minimal sample size with the rarefaction method was calculated in FSTAT 2.9.3.2 (Goudet 1995). Microsatellite data files were converted into the formats for the various analysis software using PGDSPIDER 2.0.1.0 (Lischer and Excoffier 2012) and CONVERT 1.3.1(Glaubitz 2004).

Population genetic differentiation was estimated by calculating the F_{ST} in ARLEQUIN 3.5 (Excoffier and Lischer 2010). The significance of the F_{ST} value was tested by 10,000 permutations for each pairwise comparison. A hierarchical molecular variance analysis (AMOVA) was conducted to partition the genetic variance using ARLEQUIN 3.5.

The phylogenetic tree was generated based on Nei's standardized genetic distance Da using the neighbor-joining (NJ) algorithm with 1000 bootstrap replicates in POPTREE (Takezaki et al. 2010). Tree topologies were viewed and adjusted with FIGTREE 1.4.2 (available at http://tree.bio.ed.ac.uk/software/figtree/). To visualize the pattern of genetic clustering of populations, we used STRUCTURE 2.3.4 (Pritchard et al. 2000) to assess genetic relatedness of 10 populations. Twenty independent analyses were performed for $K = 1–10$ based on 1,000,000 Markov chain Monte Carlo iterations following a

Table 1 Sampling information of *Saccharina japonica* and its two closely related species

Code	Locations	Coordinates	Genetic background	Sampling time
SJ	Muroran, Japan	N 42° 21′ E 140° 59′	Wild *S. japonica* population	2017.05
SD	Akkeshi, Japan	N 43° 01′ E 144° 45′	Wild *S. japonica* var. *diabolica* population	2017.05
SR	Tomari, Japan	N 43° 04′ E 140° 29′	Wild *S. japonica* var. *religiosa* population	2014.11
SO	Tomamae, Japan	N 44° 13′ E 141° 38′	Wild *S. japonica* var. *ochotensis* population	2014.11
SA	Muroran, Japan	N 42° 21′ E 140° 59′	Wild *S. angustata* population	2017.05
SL	Akkeshi, Japan	N 43° 01′ E 144° 45′	Wild *S. longissima* population	2017.05
C	Dalian, China	N 39° 05′ E 122° 47′	Wild *S. japonica* population	2014.07
K	Gangneung, Korea	N 37° 47′ E 128° 55′	Wild *S. japonica* population	2013.06
R	Amgu, Primorsky, Russia	N 45° 48′ E 137° 41′	Wild *S. japonica* population	2014.12
RU	Aniva Bay, Sakhalin, Russia	N 46° 06′ E 143° 18′	Wild *S. japonica* population	2011.09

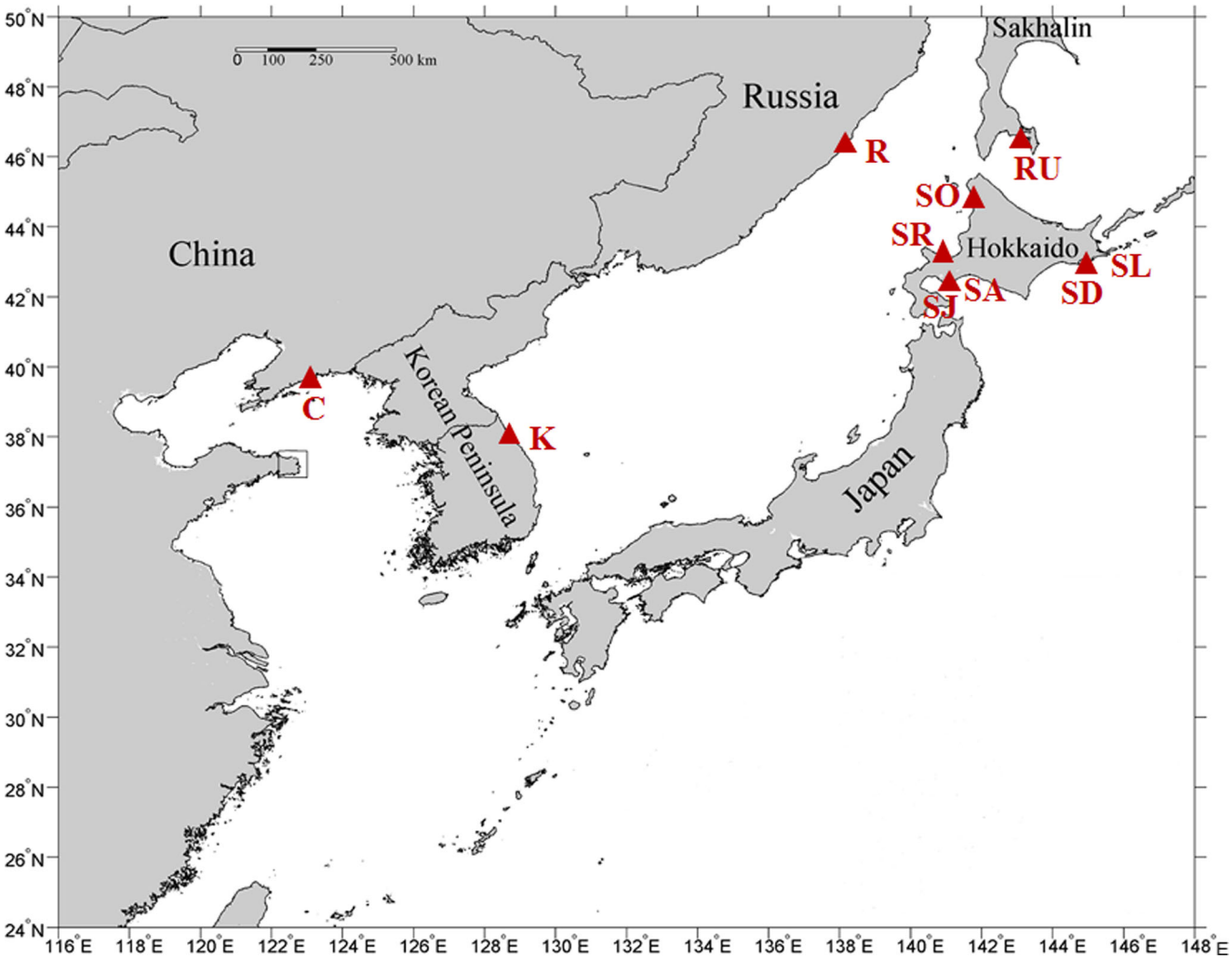

Fig. 1 Geographic locations of 10 *Saccharina* populations used in this study. The abbreviation has been explained in Table 1

burn-in period of 500,000 steps. An admixture model was used after assuming correlated allele frequencies among the populations. The best K value was determined by the delta K (ΔK) method (Evanno et al. 2005) in STRUCTURE HARVESTER (Earl and Vonholdt 2012). The final results were summarized in CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) and displayed with DISTRUCT 1.1 (Rosenberg 2004).

Results

Evaluation of 11 SSR loci

In the amplification tests, 24 SSR loci exhibited high applicability in three varieties of *S. japonica*, but 13 SSR loci have a low amplification rate (below 75%) in the two relatives of *S. japonica* (Table S2). Hence, only 11 SSR loci (SJ3, SJ20, SJ24, SJ31, SJ80, SJ93, SJ101, SJ106, SJ110, SJ133, and SJ167) showed high cross-amplification efficiency to each individual (Table S2). We therefore applied these 11 SSR loci to validate polymorphism for the 10 kelp populations (200 individuals). The percentage of successful amplification (P_A) of all loci is above 98.5% in 10 populations (Table 2). MICROCHECKER detected no evidence for scoring error due to stuttering, large allele dropout, and null alleles. Linkage disequilibrium (LD) tests for each pair of loci indicated that 11 pairs (3.3%) were significantly in disequilibrium after false discovery rate (FDR) correction. Because these loci did not share corresponding disequilibria in all samples, we speculated that none of the loci were physically linked.

The number of the alleles per locus ranged from 2.8 to 7.8 (Table 2). Polymorphic information content values (PIC) of 11 loci showed SJ31 (PIC = 0.482) has moderate polymorphism ($0.25 < \text{PIC} < 0.5$), and other 10 loci (PIC = 0.552–0.908) has high polymorphism (PIC > 0.5). The allelic richness (A_R), number of all alleles (N_A), and effective number of all alleles (N_E) in SJ167 have lower values; however, PIC and A_R indices indicated SJ167 has high polymorphism (PIC = 0.797; A_R = 7.136) (Table 2). The successful rate of amplification in SJ20 was 85% in *S. angustata*; however, SJ20 has higher

Table 2 Characteristics of 11 SSR loci in 10 populations of *Saccharina*

SSR	P_A (%)	N_A	N_E	N_P	PIC	A_R	H_E	H_O
SJ3	99.5	4.9	2.739	13	0.754	8.484	0.506	0.525
SJ20	98.5	5.9	3.433	36	0.834	11.697	0.516	0.505
SJ24	100	7.8	4.074	3	0.871	10.897	0.698	0.695
SJ31	100	2.9	1.431	3	0.482	4.451	0.252	0.230
SJ80	100	7.0	3.644	42	0.908	13.7	0.627	0.608
SJ93	100	3.2	1.886	2	0.642	4.506	0.356	0.328
SJ101	99	5.3	2.671	33	0.830	10.346	0.522	0.436
SJ106	99.5	3.8	2.072	8	0.796	7.904	0.448	0.390
SJ110	100	6.4	3.243	13	0.856	10.978	0.610	0.548
SJ133	100	3.4	1.934	30	0.552	6.176	0.353	0.329
SJ167	99	2.8	1.640	14	0.797	7.136	0.331	0.296

P_A percentage of successful amplification per locus, N_A number of all alleles, N_E effective number of all alleles, N_P number of private alleles, *PIC* polymorphic information content, A_R allelic richness based on 17 samples per population, H_E mean expected heterozygosity, H_O mean observed heterozygosity

polymorphism in *S. angustata* ($N_A = 20$; $N_E = 13.762$; $A_R = 20$) than that in other five populations ($N_A = 1–8$; $N_E = 1.000–3.791$; $A_R = 1–7.391$) (Table S3). In summary, the 11 loci, except for SJ31, have high applicability with high polymorphism (Table 2) and 10 loci were finally screened for futher population genetic analysis (Table 3).

Genetic diversity and population differentiation

Genetic diversity was evaluated for 10 populations (Table S3). At the population level, the mean number of alleles across loci (N_A) varied from 2.455 for C (*S. japonica* populations from China) to 7.455 for SA (*S. angustata* populations). Allelic richness (A_R) based on 17 samples per population was highest ($A_R = 7.236$) in the SA population and lowest ($A_R = 2.407$) in the C population. The mean observed heterozygosity across loci (H_O) ranged from 0.173 for SD (*S. japonica* var. *diabolica*) to 0.614 for SR (*S. japonica* var. *religiosa*) and expected heterozygosity across loci (H_E) ranged from 0.175 for SD to 0.668 for SA. We compared the mean values of all genetic diversity indices (N_A, A_R, H_O, and H_E) at the population level (Table S3) and found that population SA has higher genetic diversity than other populations. On the contrary, population SD has lower genetic diversity than other populations.

Pairwise F_{ST} test exhibited that all the 10 populations have significant genetic differentiation from each other (Table S4; Fig. 2). F_{ST} values ranged from 0.087 (SR and *K*) to 0.732 (*R* and SD) (Table S4). SD has a deep divergence from other populations ($F_{ST} > 0.452$; $P < 0.001$). F_{ST} values indicated that *R* from Russia significantly diverged from other populations (except RU) ($F_{ST} > 0.372$; $P < 0.001$).

Genetic structure

STRUCTURE analysis revealed three distinct genetic groups with the highest value of ΔK for $K = 3$ (Fig. S1; delta $K = 3.277$) and detected high genetic admixture in several populations (SJ, K, SR, R, and SD) (Fig. 3). Population SD was divided from other populations with $K = 4$ (delta $K = 0.716$). Populations RU and R were formed one distinct group with $K = 5$ (delta $K = 2.199$). A second highest level of genetic division was identified with $K = 6$ (delta $K = 2.645$), indicating that population SJ formed a genetic group and all the genetic groups has little genetic admixture (Fig. 3). NJ tree showed five genetic clades: clade 1 containing populations C and SJ; clade 2 including populations K and SR; clade 3 containing populations SO, RU, and R; clade 4 including population SD; and clade 5 containing populations SL and SA (Fig. 3).

AMOVA analysis showed that only 14.55% of the variation occurred among three STRUCTURE groups and as much as 56.43% of the variation occurred within populations (Table S5). In addition, the majority of variation was

Table 3 Characteristics of 10 polymorphism and universal SSR loci

Locus	Motif	Forward primer (5′-3′)	Reverse primer (5′-3′)	*Ta* (°C)	Range size (bp)
SJ3	$(CTG)_{10}$	CTTATTCGTGCTGCGTTCATAC	AGTTTGAGCCTCTGCGTCAT	55	291–321
SJ20	$(TTGCT)_7$	AGAGAACGCGGAGTGGAACT	CGCACTCGTCACAACGTGTAT	58	264–299
SJ24	$(GAACC)_7$	CACGTCTGCTTCTGCTCTACAAC	CGTAGATGATGAACAGGCTGAAC	57	271–306
SJ80	$(GCA)_{10}$	CTCCGACTGCCATACTCTTTGAC	CTTCGTGCTTACGTATATCCGAGTT	55	291–321
SJ93	$(AAAC)_7$	CACCCTTATCATCCCTGTTCAAG	TTCGGGACAAGAGTGATACATAGTT	57	288–316
SJ101	$(CGTA)_8$	ACAACGCTCCCAAGAGACTG	GGTCGCATGGTAAGCTCTTCT	57	294–326
SJ106	$(TGT)_7$	ACAAGAAGAGCGCACAGAGAAT	ATACGTACGTGATAGCTGGACTTC	58	260–281
SJ110	$(GTA)_7$	GGACGAAGTTATGGACTCGTTTC	GTATCACCCGTAGTTTCCTCACC	57	270–291
SJ133	$(CAG)_8$	AGCGATAATCAGGAAATCGTCTC	GTTTGGGTTGTTCTGTCACATTC	58	295–319
SJ167	$(GCT)_6$	AAGGAGAGGAGCAGTGTGTGTT	GATGTGCGAACGCTGAGAC	57	250–268

Ta optimized annealing temperature

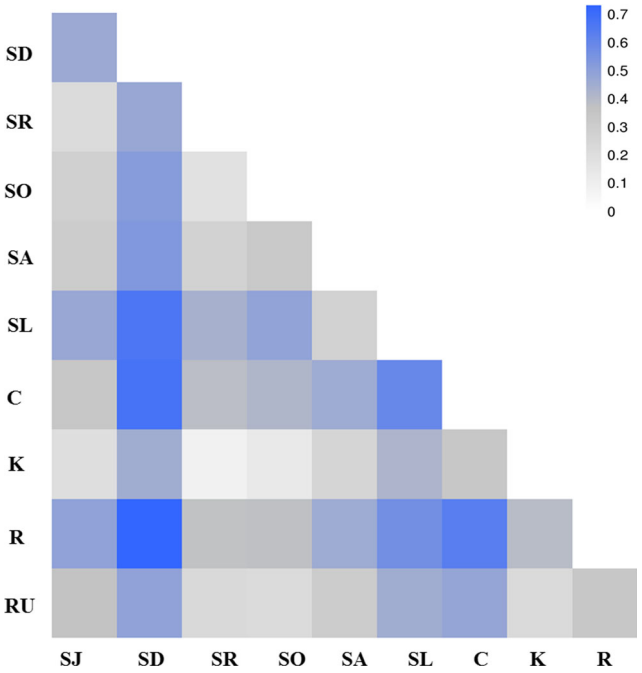

Fig. 2 The average F_{ST} matrix estimated based on SSR loci (lower left). Dark blue and light gray colors indicate high and low F_{ST} values, respectively

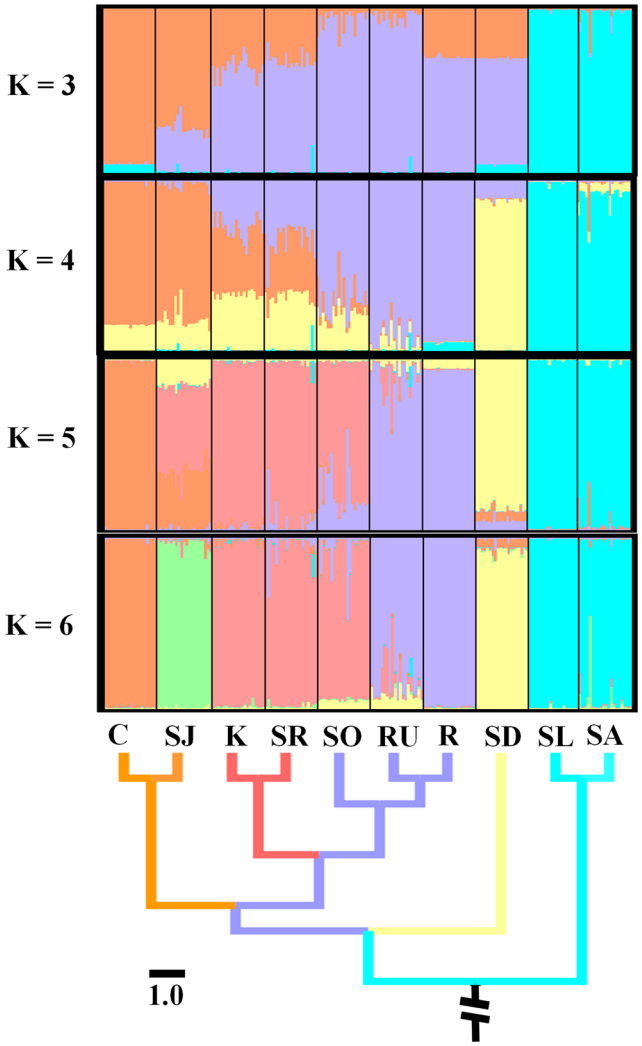

Fig. 3 Genetic structure based on STRUCTURE analysis and neighbor-joining tree

partitioned within populations (58.34%), with only 20.80% of the variation among the six STRUCTURE groups (Tables S6). All variance components were statistically significant ($P < 0.0001$).

Discussion

It is proved that the screened 10 universal and polymorphism microsatellites for three varieties of *S. japonica*, *S. longissima*, and *S. angustata* are valid in accessing the genetic diversity. *Saccharina angustata* population has higher genetic diversity than other kelp populations (Table S3), and it would be potential genetic germplasm for breeding and cultivation. *Saccharina japonica* var. *diabolica* is an important cultivar in Japan due to its wide blade (Kawashima 2012), but exhibited lower genetic diversity (Table S3). We infer that selection and cultivation might reduce allelic variation and genetic diversity, and this phenomenon also has been observed in other cultivated seaweeds (Voisin et al. 2005; Zhang et al. 2017). Taxonomically, *S. japonica* var. *diabolica* is a variety of *S. japonica* (Yotsukura et al. 2010b); however, it has deep divergence from *S. japonica* with a separate genetic lineage (Fig. 2; Table S4).

Based on the NJ tree (Fig. 3), *S. longissima* and *S. angustata* formed a distinct genetic cluster and *S. japonica* and its three varieties clustered together, which was consistent with a previous result with 5S rDNA spacer (Yotsukura et al. 2010c). The wild kelp population from Dalian (C) and *S. japonica* from Hokkaido (SJ) clustered together and have higher genetic similarity, possibly due to artificial introduction during the cultivation. Likewise, population from Korea (K) and *S. japonica* var. *religiosa* from Hokkaido has closer genetic relationship (Fig. 3), which supports the previous assumption that Korean kelp populations might be introduced from Japan (Jang and Gweon 1970; Hwang et al. 2018). Here, the kelp populations from Russia might be the *S. japonica* var. *ochotensis* based on the NJ tree (Fig. 3) and genetic differentiation analyses (Fig. 2). These SSR loci exhibited high resolution for discrimination of genetic relatedness between different kelp populations.

In conclusion, the newly screened 10 polymorphic microsatellite markers from *S. japonica* exhibited high applicability in the two related *Saccharina* species and are valid in the population genetic analysis. It will be positive to evaluate the genetic background to these kelps in the future.

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