Genetic differences among varieties of *Saccharina japonica* in northern Japan as determined by AFLP and SSR analyses

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Abstract

The genetic diversity of *Saccharina japonica* inhabiting the Hokkaido coastline and the surrounding area was investigated by AFLP and SSR analyses. A STRUCTURE analysis based on the AFLP data identified two clusters in the species, although a few local populations with genetic structures different from that of the neighboring populations were detected. The two clusters could be identified even within each current variety. In the SSR analysis, genetic variation in nucleotide sequences in all samples was located in four DNA regions (MS10, MS11, MS16, and MS29), and it is thought that these regions can be used as markers to detect individual-level variation in *S. japonica*. The STRUCTURE analysis of the SSR data identified four clusters in the species. Since the geographical distribution of these four different clusters does not correspond exactly to the currently recognized varieties in this species, it will be necessary to re-evaluate the distribution of the varieties of *S. japonica*.

Key words

AFLP; distribution region; fragment analysis; genetic difference; *Saccharina japonica*; SSR

Introduction

Saccharina japonica (J.E. Areschoug) C.E. Lane, C. Mayes, L.D. Druehl & G.W. Saunders, a member of the Laminariales family, grows primarily along the coast of Hokkaido, Japan, with the exception of the Pacific coast of central Hokkaido, and it forms large marine forests. It has been used as a foodstuff in Japan for more than a thousand years and has been one of the major marine resources of Japan.

S. japonica was classified more than a hundred years ago into four independent species on the basis of morphology: *S. japonica*; *S. religiosa* (Miyabe) C.E. Lane, C. Mayes, L.D. Druehl & G.W. Saunders; *S. ochotensis* (Miyabe) C.E. Lane, C. Mayes, L.D. Druehl & G.W. Saunders; and *S. diabolica* (Miyabe) C.E. Lane, C. Mayes, L.D. Druehl & G.W. Saunders (Miyabe 1902, Lane et al. 2006). However, many of the morphological characteristics used for this classification vary greatly with habitat, and molecular phylogenetic analyses have found no differences in the nucleotide sequences of DNA regions such as the ribosomal RNA gene internal transcribed spacer (ITS) region, the ribulose bisphosphate carboxylase/oxygenase (RuBisCo) gene spacer region, and the cytochrome c oxidase subunit 1 (COI) gene, where differences at the species level in seaweeds should generally be found (Yotsukura 2005, Yotsukura et al. 2008). Furthermore, the ranges of the four species are localized but contiguous with each other, and genetic diversity and the results of crossing experiments indicate that natural crossing occurs among the four species. Therefore, at present, the four species are treated as four different varieties (*S. japonica* var. *japonica*, var. *religiosa*, var. *ochotensis*, and var. *diabolica*) (Yotsukura et al. 2008). However, although the four varieties differ greatly in commercial value, they are distinguished mainly by the place of their collection. In addition, most of the distributional data accepted today are based on studies reported about half a century ago (Hasegawa 1959, Kawashima 2004). As the ranges of kelp species inhabiting coastal northern Japan are changing in response to changes in marine environments (Kirihara et al. 2006), objective characterizations of the four varieties are needed, as is an understanding of their distribution.

In addition to the three DNA regions mentioned above, the complete nucleotide sequences of the chloroplast and mitochondrial genomes, as well as nucleotide sequences of other specific regions in the nuclear genome, have been determined for *S. japonica* (e.g. Yotsukura et al. 2006, 2010, Wang et al. 2013). Comparison of the nucleotide sequences, however, has revealed very little sequence variation between the varieties and

between individuals from different localities in Hokkaido, and has not identified a gene region with significant useful variation. In addition, a draft genome of the species has been reported recently and compared to wild populations (Ye et al. 2015); those samples, however, were very few and were collected from a broad area, and they do not represent a detailed population analysis.

Unlike comparison of the nucleotide sequences of specific gene regions, DNA polymorphism analysis allows detection of slight genetic variations in the whole genome. Particularly, amplified fragment length polymorphism (AFLP) analysis and microsatellite marker analysis, also known as simple sequence repeat (SSR) analysis, have been widely used in investigations of the genetic diversity of kelps (Kusumo and Druehl 2000, Kusumo et al. 2006, Shan and Pang 2009, Shan et al. 2011). High-precision DNA polymorphism analysis has been challenging in *S. japonica* because the polysaccharides and polyphenols present in abundance in this species inhibit the reactions of restriction enzymes and DNA polymerases and damage DNA (Crowley et al. 2003, Do and Adams 1991, Fang et al. 1992, Loomis 1974). However, a method to extract high-purity DNA from *S. japonica* has been recently established, and the extracted DNA has been shown to be suitable for AFLP analysis with high reproducibility, as well as for the development of microsatellite markers (Maeda and Yotsukura 2013, Maeda et al. 2013). The present study investigated the genetic diversity of *S. japonica* inhabiting the Hokkaido coastline and the surrounding area using AFLP and SSR analyses of DNA extracted by the method mentioned above.

Materials and methods

Sample collection and DNA extraction

Sporophytes of *S. japonica* were collected from 47 localities along the coast of Hokkaido, 12 localities along the coast of Aomori Prefecture, one locality along the coast of Iwate Prefecture, and two localities along the coast of southern Sakhalin Island, Russia (Table 1, Fig. 1). In addition, sporophytes were collected from seven sites in Oshoro Bay (Otaru City, Hokkaido) in order to analyze the genetic structure of the species in a specific area.

A sample 15 cm^2 in size was cut with a razor blade from an immature section of the lamina around the meristem. The tissue surface was washed repeatedly in sterile seawater and wiped with paper towels to remove attached organisms. The washed tissue was placed in a zippered plastic bag with medium-sized silica gel and dried in a refrigerator. After

drying, the tissue was stored at -80 °C until use. For DNA extraction, 20-30 mg of tissue was cut into fragments about 0.5 mm^2 in size and placed into 2 mL microtubes to be used as samples.

Genomic DNA was extracted by combining the CTAB and SDS methods as described in Maeda et al. (2013), and purification was carried out using a GENECLEAN II Kit (Bio 101, Inc., La Jolla, CA, USA) according to the manufacturer's instructions with slight modifications: (1) 7 μL of GLASSMILK was added regardless of the concentration of the DNA extracts; (2) after GLASSMILK addition, the samples were incubated for 10 min; (3) the samples were washed three times with NEWWASH; and (4) the drying time for GLASSMILK was 8 min at room temperature and 3 min at 65 °C.

AFLP analysis

Purified DNA prepared at a concentration of 20 ng/μL was used as template DNA, and the analysis was performed as follows, with reference to Maeda *et al*. (2013). *Mse*I and *Eco*RI adaptors (in an AFLP Ligation and Preselective Amplification Module) (Applied Biosystems, Foster, CA, USA), incubated at 95 °C for 10 min followed by incubation at 22 °C for 10 min, were ligated to the ends of DNA fragments produced by a restriction enzyme reaction. To ligate the adaptors and carry out the restriction enzyme reaction, 8.5 μL of enzyme mixture solution (containing 10× T4 buffer DNA Ligase, 0.1 mg/mL BSA, 2.5 U/μL *Mse*I, 9 U/μL *Eco*RI, 70 U/μL T4 DNA Ligase (New England Biolabs Japan, Inc., Tokyo, Japan), and 0.05 M NaCl), 1 μL of *Mse*I adaptor, and 1 μL of *Eco*RI adaptor were added to 2.5 μL of DNA extract (20 ng/μL), and the resulting solution was incubated at 37 °C for 14 h. The reaction solution was then incubated at 65 °C for 20 min followed by incubation at 22 °C for 20 min, and it then was finally diluted with 0.1 TE buffer to 10-20 times its volume.

The preselective PCR and the following selective PCR were performed according to the AFLP plant mapping protocol; the following five selective primer pairs were used for the selective PCR with a Selective Amplification Start-Up Kit for Regular Plant Genomes, 500-6000 Mb (Applied Biosystems): *Eco*RI ACA-*Mse*I CAG, *Eco*RI ACA-*Mse*I CAT, *Eco*RI AAG-*Mse*I CTA, *Eco*RI AAG-*Mse*I CTG, and *Eco*RI AAC-*Mse*I CTC. The selective PCR products were analyzed with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), and the sizing and genotyping of each fragment was performed with Gene Mapper ver. 4.1 (Applied Biosystems). The band detection threshold was set to 20 relative fluorescence units and the presence or absence of detected fragments larger than 60 bp was assessed.

The genetic diversity parameters genetic similarity (*S*) and degree of genetic diversity (*H*) were calculated according to methods described by Dice (1945) and Nei (1987), respectively. Arlequin 3.11 (Excoffier et al. 2005) was used to calculate coefficients of genetic differentiation (*Gst*) and to perform an analysis of molecular variance (AMOVA) to evaluate genetic differentiation among the populations. Genetic structure based on allele loci was estimated from up to five individuals from each population with STRUCTURE ver. 2.4.3 (Pritchard et al. 2000). For the analysis using STRUCTURE, burn-in was set to 100,000 and Markov Chain Monte Carlo simulation was set to 1,000,000. An optimum number of populations (*K*) was estimated from a comparison of the likelihoods of *K* and ΔK (Evanno et al. 2005) using STRUCTURE HARVESTER web ver. 0.6.93 (Earl and von Holdt 2012).

Microsatellite marker analysis (SSR analysis)

Genomic DNA (500 ng) obtained from an individual from Usujiri was digested using six restriction enzymes (*Alu*I, *EcoR*V, *Hae*III, *Hinc*II, *Rsa*I, and *Ssp*I) (New England Biolabs Japan, Inc.). Reaction solutions and restriction enzyme reactions were prepared and carried out according to recommended protocols for each enzyme. Restriction fragments with unequal-length adaptors were produced by ligating two types of oligonucleotides with known sequences (Lian and Hogetsu 2001) to the blunt ends of DNA fragments produced by digestion using a TaKaRa Ligation Kit ver. 1 (Takara Bio Inc., Shiga, Japan). Then the nucleotide sequence of a one-sided region adjacent to a SSR and of another region adjacent to the SSR were determined according to the method explained in Maeda and Yotsukura (2013). That is, it was advanced by using the dual-suppression-PCR technique in Lian and Hougetsu (2004) and the DNA cloning technique in Denboh et al. (2003), with the following modifications: (a) the restriction enzyme were changed to *EcoR*V, *Hinc*II, and *Ssp*I (New England BioLabs, USA), (b) the PCR master mix was changed to AmpliTaq Gold 360 Master Mix (Applied Biosystems, USA), (c) the amplified fragments produced by PCR were used together for cloning, (d) inserts with a length of 500–800 bp were picked out to ensure a sufficient size of DNA for subsequent primer (IP1 primer and IP2 primer) design, (e) the T_m values of IP1 primer and IP2 primer were set to be ca. 61 and 57, respectively, and (f) the annealing time was changed to 10–20 sec or touchdown PCR was conducted when the previously unknown sequences adjacent to both ends of the SSR region were determined. Using the nucleotide sequences of the regions adjacent to the SSR, an IP3 primer was designed to extend in the direction of the SSR.

Next, with regard to the 10 regions reported in previous studies (Maeda and Yotsukura 2013), the regions containing the SSR were amplified using one of the two primer sets (IP1 primer and IP3 primer, or IP2 primer and IP3 primer) and DNA samples obtained from individuals collected at six localities (Ofune, Usujiri, Yasuura, Kakkumi, Osatsube, and Kinaoshi). The parameters for PCR amplification were as follows: 95 °C for 10 min, followed by 40 cycles of thermal denaturation at 95 °C for 30 s, annealing at the T_m of the primer pair for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min; the products were incubated at 4 °C. The PCR products were electrophoresed on 1.2% agarose gel to confirm the success of the amplification, and primer pairs that yielded one or two bands were used to clone the amplified PCR products following the same protocol except that a mixed solution containing genomic DNA extracted from the sporophytes of 10 individuals was used as template DNA. Next, 20 positive colonies were selected to perform direct colony PCR, and their nucleotide sequences were determined to assess polymorphism. The length of the amplified fragments, estimated by 10% polyacrylamide gel electrophoresis using a constant temperature electrophoresis apparatus NB-1214 (Nihon Eido Co., Ltd., Tokyo, Japan), was compared among the identified polymorphic regions. The power for the electrophoresis system was supplied by POWER PAC 3000 (Bio-Rad Laboratories, CA, USA) at 1500 V, 40 A, and 60 W for 2 h 30 min. After the electrophoresis, the PCR products were stained with ethidium bromide and examined under ultraviolet irradiation with a UV transilluminator (UVP, LLC, Upland, CA, USA) to determine the band size and heterozygosity. For regions with high polymorphism, SSR analysis was performed with up to five individuals from each sampling locality, and gene structures were examined by the STRUCTURE analysis.

Results

AFLP analysis

For the samples analyzed in this study, the number of fragments amplified by selective PCR with the primer pairs ranged from 421 to 595 (Table 2). Three individuals (one each from Sumiyoshi, Onnemoto, and Aikappu) were excluded from the subsequent analyses because their genetic similarity to individuals collected from other localities was markedly low. Nevertheless, no fragments were common to all localities. The genetic similarity (*S*) of individuals from all localities was calculated from the number of fragments; *S* at an individual locality ranged from 0.53 to 0.82 (0.71 ± 0.07) and between different localities ranged from 0.29 to 0.75 (0.46 ± 0.08) (Supplementary Table 1). The

degree of genetic diversity (*H*) was 0.149 ± 0.031 (Table 2) and the coefficient of genetic differentiation (*Gst*) ranged from -0.01 to 0.71 (Supplementary Table 2). Generally, genetic similarity was high between localities that were near to each other and low between localities that were distant from each other; the exceptions were Osatsube, Hyuga, Ohama, Kawashiro, and Ogon-misaki, which showed high genetic similarity to distant localities. The *ΔK* values obtained by the STRUCTURE analysis supported the existence of two clusters (Fig. 2).

The number of fragments amplified by selective PCR with the abovementioned five primer pairs were 212-360 (*S. japonica* var. *japonica*), 264-403 (var. *religiosa*), 178-300 (var. *ochotensis*), and 184-282 (var. *diabolica*). The number of fragments common to every individual was 0-1 (var. *japonica* and var. *religiosa*), 2-9 (var. *ochotensis*), and 0-2 (var. *diabolica*). The genetic similarity (*S*) calculated from these values was 0.53 ± 0.04 within varieties and 0.44 ± 0.02 between varieties (Table 3). The degree of genetic diversity (*H*) of each of the four varieties was 0.068 ± 0.013 (var. *japonica*), 0.075 ± 0.013 (var. *religiosa*), 0.052 ± 0.011 (var. *ochotensis*), and 0.059 ± 0.022 (var. *diabolica*), and the coefficient of genetic differentiation (*Gst*) within varieties (except for *S. japonica* var. *japonica*) was high and comparable to that between varieties (Table 4). Moreover, the result of the STRUCTURE analysis of each variety supported the existence of two clusters.

The number of fragments amplified in samples from individuals collected at the seven sites in Oshoro Bay ranged from 27 to 107. The number of fragments common to the individuals collected at each site ranged from 10 to 60 (Table 2). Genetic similarity (*S*) among individuals from the same site was 0.60 ± 0.02 (0.56 ± 0.06 to 0.64 ± 0.04) and that of individuals between sites was 0.58 ± 0.18 (Supplementary Table 1). Of the seven sites, the genetic similarity between individuals collected in the mouth of the bay and those collected in the inner part of the bay, which are considered the most distant, was low; the degree of genetic diversity (*H*) was low in the inner part of the bay (Table 2). The coefficient of genetic differentiation (*Gst*) ranged from -0.01 to 0.13, and slight genetic differentiation was found between the five sites around the mouth of the bay (Sites 1 to 5) and the two sites around the inner part of the bay (Sites 6 and 7) (Supplementary Table 2). The result of the STRUCTURE analysis supported the existence of two clusters. Although there were no significant differences in gene constitution between the sites, the AMOVA analysis showed significant differences between Site 1 and Site 7, between Site 2 and Site 6, between Site 2 and Site 7, between Site 3 and Site 7, and between Site 5 and Site 7, whichever primer pairs were used.

Microsatellite marker analysis (SSR analysis)

The 10 DNA regions were compared using samples collected from six localities in Hakodate City. Eight of these DNA regions (all except MS24 and MS32) were found to have distinct AFLP patterns.

Of the eight DNA regions, MS10, MS11, MS16, and MS29, which had high frequencies of specific types of AFLP profiles, were selected as the target regions and their fragment lengths in individuals collected from five localities (Osatsube, Hyuga, Ohama, Kawashiro, and Ogon-misaki) were compared with those of individuals from other localities. As a result, three types (in the Osatsube and Hyuga samples), one type (in Ohama samples), and two types (in Kawashiro and Ogon-misaki samples) of AFLP profiles were found in the MS10 region. Comparison of these fragments with those of samples from other localities revealed that 360 bp and 364 bp fragments in particular occurred not only in individuals from Sumiyoshi and Kotaniishi, which are close to Hyuga, but also in individuals from Usu and Kinaoshi. It should be noted that all individuals from Kawashiro were homozygotes. A 393 bp fragment, although it did not reflect the difference in localities, was found in individuals from all localities except Kamaishi, Chogo, Ohama, Kawashiro, Ogon-misaki, Rikibiru, Kutsugata, Shinminato, Onnemoto, Ochiishi, Daikokujima, Aikappu, and Akkeshi-ko, and it was detected in all individuals collected from Horai, Omisaki, Hon-cho, Omu, and Futatsuiwa. In all individuals from Onnemoto, Ochiishi, Daikokujima, Aikappu, and Akkeshi-ko, except for one individual from Aikappu, only amplified fragments that were 400 bp or longer were detected. In the MS11 region, five types (in Osatsube samples), three types (in Hyuga and Ogon-misaki samples), one type (in Kawashiro samples), and four types (in Ohama samples) of AFLP patterns were found. These fragment length polymorphism types were not specific to any locality. Similar to the MS10 region, heterozygosity at the four sampling localities was 0.47, except for individuals from Kawashiro, which were all homozygotes. Comparison of fragments found in individuals from these five localities with those from other localities showed that heterozygosity of individuals from localities within the distributional range of var. *japonica* and var. *religiosa* was 0.46, whereas heterozygosity of individuals from localities within the distributional range of var. *ochotensis* and var. *diabolica* was as low as 0.28. It should be noted that all individuals from Rebunge, Oniwaki, Kutsugata, Shinminato, and Oshidomari, which were used for the comparison, were homozygotes, and only 329 bp fragments were detected in the samples. In the MS16 region, six types (in Osatsube samples), three types (in Hyuga, Ohama, and Ogon-misaki samples), and two types (in Kawashiro samples) of AFLP

patterns were obtained. All of these fragments were found in other localities and these did not reflect the difference in localities. Similar to other regions, all individuals collected from Kawashiro were heterozygotes in the MS16 region. However, unlike other regions, locality-specific bands were not detected in the MS16 region. In the MS29 region, three types (in Osatsube samples), one type (in Ogon-misaki samples), and two types (in Hyuga, Ohama, and Kawashiro samples) of AFLP patterns were obtained. Of these fragments, 172 bp and 181 bp alleles were found in all localities, and a band about 250 bp long, which was found in Osatsube samples, was found only in Utoma, Charatsunai, Usu, Rebunge, Shizukari, Ofune, Usujiri, Yasuura, Kakkumi, Osatsube, Kinaoshi, and Sumiyoshi samples. It should be noted that no amplified products were found in two individuals from Hyuga and one individual from Ohama in the MS10 region, in one individual from Hyuga and one individual from Ogon-misaki in the MS16 region, and in one individual from Hyuga in the MS29 region.

The STRUCTURE analysis based on fragment lengths of the identified microsatellite markers showed the existence of four clusters (Fig. 3).

Discussions

Although a problem in the reproducibility of results obtained by AFLP analyses has been pointed out (Bonin et al. 2004), that problem can be addressed by isolating specific polymorphic bands from the amplified fragments and by using the isolated bands as sequence-tagged-site (STS) markers with high reproducibility (e.g. Griffiths and Orr 1999). This study found some amplified fragments with high reproducibility in individuals of *S. japonica* from specific regions or localities. Therefore, the use of these amplified fragments as STS markers will allow us to identify the geographic origin of *S. japonica* specimens by simple operations.

The STRUCTURE analysis based on the AFLP identified two clusters. One cluster comprised individuals from Nakanogawa, Kotaniishi, and Siogama along the coastline of the southern Oshima Peninsula, Hokkaido; from Ohama, Kawashiro, and Ogon-misaki along the coastline of the Japan Sea; from Misaki-cho, Tomosiri-gyoko, and Akkeshi-ko in the eastern part of Hokkaido; from the coast of Aomori Prefecture; and from Kamaishi. The other cluster comprised the individuals collected in the area ranging from the coastline of the Japan Sea of Hokkaido to Utoro on the Shiretoko Peninsula, excluding Ohama and Kawashiro. In the area ranging from the Pacific coastline west of Utoma, Hokkaido, to Sumiyoshi on the coastline of the southwestern Oshima Peninsula, both types of populations were found: those in which one cluster was dominant over the other,

and those in which the two clusters were equally present.

The four varieties of *S. japonica* presently recognized are classified primarily on the basis of their morphological features, and these varieties have distinct localized areas of distribution (Kawashima 2004). Although local population structure was identified in the present study, it did not map clearly onto the existing varieties. Previous studies reported that genetic similarity (*S*) inferred from the AFLP analysis reflects the geographical relations of *S. japonica* (Shan et al. 2011). Therefore, if high genetic similarity is found between individuals collected from distant localities, the two individuals are considered to belong to the same gene cluster. In this study, individuals from Ofune, Hakodate City, differed from the neighboring localities but were genetically similar to individuals from Shitatehama, Aomori Prefecture. Furthermore, individuals from Sarugamori, Aomori Prefecture, also showed higher genetic similarity to individuals from Siogama, Fukushima-cho, Hokkaido, than to individuals from neighboring localities. Because these results do not conform to the expected correlation between localities and genetic similarity, and because they are different from the case where individuals are genetically similar to those collected from distant localities such as Osatsube, Hyuga, Ohama, Kawashiro, and Ogon-misaki, which are regarded as local populations in this study, it was supposed that genes had been introduced from other localities by artificial means, such as hull fouling.

Previous AFLP analyses have detected community-level variation in *Alaria marginata* (Laminariales), suggesting that AFLP analysis can detect genetic variation between populations that have been separated by changes in habitat (Kusumo and Druehl 2000). Therefore, AFLP analysis is expected to be an effective method to understand local populations of *S. japonica*. In this study, individuals collected from communities at the seven sites in Oshoro Bay were classified into two clusters. Previous studies on the Oshoro Bay reported the presence of local communities of *S. japonica* in the mouth and inner parts of the bay (Funano 1983). Although there is no detailed information about the seawater currents in the regions of the bay inhabited by communities of *S. japonica*, the rate of exchange of seawater inside and outside the bay is not considered high because salinity decreases markedly at the site near the mouth of the bay during the snow-thawing season from April to May (Nakata et al. 2001). Furthermore, while the diffusion of spores of Laminariales, including *S. japonica*, is affected by transfer of seawater, the diffusion distance is not very great (Akino et al. 2005, Coyer et al. 1997), suggesting that the genetic difference observed between populations at the mouth and those in the inner part of the Bay is the result of factors related to seawater flow inside the bay (cf. Kusumo and Druehl 2000). Accordingly, it is inferred that the distribution of local populations of *S. japonica*

can be influenced even by small changes in the physical environment, and it is feared that the decline of these small scale communities may lead to a disappearance of genetic diversity in this species.

In contrast, the microsatellite marker analysis (SSR analysis) of the 10 DNA regions investigated in this study demonstrated that variations in the nucleotide sequences in all samples were located in the MS10, MS11, MS16, and MS29 regions. The number of polymorphisms and the heterozygosity levels indicate that these regions can be used as markers to detect individual-level variation in *S. japonica*. Although MS18 does not have many types of fragment polymorphisms and has low heterozygosity, it contains a 12 or 21 bp repetitive sequence that allows us to easily determine the size of fragments by electrophoresis on agarose gel or acrylamide gel. Accordingly, MS18 can be used for preliminary discrimination of strains at aquaculture sites.

The STRUCTURE analysis based on fragment lengths of the identified microsatellite markers of the MS10, MS11, MS16, and MS29 regions supported the existence of four clusters. Cluster 2 is dominant in individuals from Ogon-misaki, which are regarded as a local population by the AFLP analysis, and individuals having this cluster existed in significant numbers in Gokibiru, Hamamasu, Shokan-kaigan, Rikibiru, and Oniwaki. This suggests that individuals from Ogon-misaki are not genetically isolated from the surrounding area. In contrast, Cluster 3 is dominant in individuals from Kawashiro, and it is also found in significant numbers in individuals from Hyuga, Kaitorima, Shimamakimura-ko, Ohama, and the inner part of Oshoro Bay. Some individuals from Ohama have both Cluster 4 and Cluster 1; however, Cluster 1 is dominant in all individuals, as it is in individuals from the neighboring localities. These results indicate that, like the individuals from Ogon-misaki, the individuals from Kawashiro and those from Ohama are not genetically isolated. It should be noted that individuals from Kawashiro (Cluster 3) and from Ohama (Cluster 1), two localities where different clusters are dominant, were found to be genetically close to var. *japonica* in the AFLP analysis. This results suggests that the existing two varieties, var. *japonica* and *var. ochotensis,* are genetically close to each other, although their areas of distribution are not adjacent. The microsatellite marker analysis, however, did not detect any genetic differences between individuals from Osatsube and those from the adjacent localities. Although the cause of the difference between the SSR and AFLP analyses not clear, AFLP analysis yields the highest effective multiplex ratio among various polymorphism analysis methods (Powell et al. 1996), and therefore the difference between its ability to detect polymorphism frequencies and the ability of the microsatellite marker analysis may have affected the results. Individuals inhabiting the coast of Aomori Prefecture are classified as var.

japonica. However, in this study, many individuals were found to consist of mixed clusters. This may be due to the effect of breeding transplantation experiments performed actively in Aomori Prefecture (e.g. Kirihara et al. 1989, 1992).

In the present study, the regional gene structures of *S. japonica* were revealed by the AFLP analysis and the following distributional regions of four different clusters with high genetic similarity were identified by the microsatellite marker analysis: Cluster 1, distributed in the coast of Aomori Prefecture, in Kamaishi of Iwate Prefecture, and in the area ranging from the Pacific coast west of Utoma, Hokkaido, to Ohama; Cluster 2, sparsely distributed in the area ranging from Kaitorima to Oshoro in the southern part of the coast of the Japan Sea in Hokkaido; Cluster 3, distributed in Gokibiru along the coast of the Japan Sea in Hokkaido and Rishiri Island and in Rebun Island, reaching southern Sakhalin Island; and Cluster 4, distributed in the area ranging from the coast of the Sea of Okhotsk in Hokkaido to the Pacific coastline in eastern Hokkaido. Although the areas of distribution of these clusters do not correspond clearly to the distributional ranges of the existing four varieties, taking into consideration the inherited characters of the identified clusters, it reasonable to assume that Cluster 1 is var. *japonica*, Cluster 2 is var. *religiosa*, Cluster 3 is var. *ochotensis*, and Cluster 4 is var. *diabolica*.

The microsatellite markers obtained in this study showed a high degree of genetic diversity (heterozygosity), complementing the results of the AFLP analysis, and therefore revealed the existence of more gene clusters than any other method. Consequently, it has been revealed that each of the varieties of *S. japonica* along the coast of Hokkaido has specific genetic features. Therefore, appropriate conservation of the respective gene resources is needed. In this study, the number of samples collected from each locality was limited because of the distance restriction between collection sites. In future studies, more detailed analyses will be performed by increasing the number of collection localities and the number of samples collected from each locality.

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Fig. 1. Distribution of the varieties of *Saccharina japonica* as determined by Kawashima (2004), and the collecting sites (1-62) of the samples used in this study.

Fig. 2. Genetic structure of *Saccharina japonica* estimated by Bayesian clustering analysis based on the AFLP data. Each column corresponds to one individual. The two clusters obtained are indicated by two different colors. The vertical axis shows the ratio that each cluster occupies.

Fig. 3. Genetic structure of *Saccharina japonica* estimated by Bayesian clustering analysis based on microsatellite analysis. Each column corresponds to one individual. The four clusters obtained are indicated by four different colors: yellow, Cluster 1; red, Cluster 2; blue, Cluster 3; green, Cluster 4. The vertical axis shows the ratio that each cluster occupies.

Table 1 Collectoin date and sites of four varieties of *Saccharina japonica* used for this study

Collection site number	Collection date	Collenction site	Number of individuals	Variety supposed	Additional statement
1	2012.9.27	Utoma, Samani	5	unknown	
2	2010.3.19	Charatsunai, Muroran	10	*S. japonica* var. *japonica*	
3	2013.6.19	Usu, Date	5	*S. japonica* var. *japonica*	
4	2013.6.19	Rebunge, Toyoura	5	*S. japonica* var. *japonica*	
5	2013.6.19	Shizukari, Oshamanbe	5	*S. japonica* var. *japonica*	
6	2011.8.7	Ofune, Hakodate	5	*S. japonica* var. *japonica*	
7	2010.3.18	Usujiri, Hakodate	10	*S. japonica* var. *japonica*	
8	2011.8.7	Yasuura, Hakodate	5	*S. japonica* var. *japonica*	
9	2011.8.15	Kakkumi, Hakodate	5	*S. japonica* var. *japonica*	for SSR analysis
10	2011.8.15	Osatube, Hakodate	4	*S. japonica* var. *japonica*	
11	2011.8.9	Kinaoshi, Hakodate	5	*S. japonica* var. *japonica*	
12	2011.8.16	Sumiyoshi, Hakodate	7	*S. japonica* var. *japonica*	
13	2013.6.21	Nakanogawa, Shiriuchi	5	*S. japonica* var. *japonica*	
14	2013.6.21	Kotaniishi, Shiriuchi	5	*S. japonica* var. *japonica*	
15	2013.6.21	Shiogama, Fukushima	5	*S. japonica* var. *japonica*	
16	2013.6.21	Hyuga, Fukushima	5	*S. japonica* var. *japonica*	
17	2013.7.16	Kamaishi, Iwate	6	*S. japonica* var. *religiosa*	
18	2011.8.15	Tomari, Aomori pref.	5	*S. japonica* var. *religiosa*	
19	2010.10.30	Sarugamori, Aomori pref.	5	*S. japonica* var. *religiosa*	
20	2011.8.15	Shitsukari Aomori pref.	4	*S. japonica* var. *religiosa*	
21	2011.8.15	Shiriya, Aomori pref.	4	*S. japonica* var. *religiosa*	

22	2011.11.9	Kinoppu, Aomori pref.	5	*S. japonica* var. *religiosa*	
23	2011.8.15	Shimotehama, Aomori pref.	5	*S. japonica* var. *religiosa*	
24	2010.10.7	Oma, Aomori pref.	2	*S. japonica* var. *religiosa*	for SSR analysis
25	2011.8.16	Isoya, Aomori pref.	5	*S. japonica* var. *religiosa*	
26	2011.8.16	Chogo, Aomori pref.	3	*S. japonica* var. *religiosa*	
27	2010.10.7	Tairadate, Aomori pref.	5	*S. japonica* var. *religiosa*	
28	2011.8.17	Imabetsu, Aomori pref.	5	*S. japonica* var. *religiosa*	
29	2010.10.18	Minmaya, Aomori pref.	5	*S. japonica* var. *religiosa*	
30	2011.7.22	Kaitorima, Setana	5	*S. japonica* var. *religiosa*	
31	2011.9.12	Shimamakimura-ko, Shimamaki	6	*S. japonica* var. *religiosa*	
32	2013.5.22	Ohama, Iwanai	4	*S. japonica* var. *religiosa*	
33	2013.6.30	Kawashiro, Kamoenai	4	*S. japonica* var. *religiosa*	
34	2010.3.16	Oshoro, Otaru	35	*S. japonica* var. *religiosa*	
35	2013.7.21	Gokibiru, Ishikari	5	*S. japonica* var. *ochotensis*	
36	2013.7.20	Hamamasu, Ishikari	5	*S. japonica* var. *ochotensis*	
37	2013.7.19	Shokan-kaigan, Mashike	5	*S. japonica* var. *ochotensis*	
38	2013.7.18	Ogon-misaki, Rumoi	5	*S. japonica* var. *ochotensis*	
39	2011.11.16	Rikibiru, Tomamae	5	*S. japonica* var. *ochotensis*	
40	2011.10.13	Oniwaki, Rshirifuji	5	*S. japonica* var. *ochotensis*	
41	2011.10.13	Kutsugata, Rishirifuji	5	*S. japonica* var. *ochotensis*	
42	2011.10.13	Shinminato, Rishirifuji	5	*S. japonica* var. *ochotensis*	
43	2011.10.13	Oshidomari, Rishirifuji	5	*S. japonica* var. *ochotensis*	
44	2011.11.28	Kafuka, Rebun	5	*S. japonica* var. *ochotensis*	

45	2010.3.10	Horai, Wakkanai	10	*S. japonica* var. *ochotensis*	
46	2011.9.1	Omisaki, Wakkanai	10	*S. japonica* var. *ochotensis*	
47	2011.10.19	Hon-cho, Esashi	5	*S. japonica* var. *ochotensis*	
48	2011.10.19	Omu, Monbetsu	5	*S. japonica* var. *ochotensis*	
49	2011.10.19	Futatsuiwa, Abashiri	5	*S. japonica* var. *ochotensis*	
50	2011.10.27	Utoro, Shari	5	*S. japonica* var. *ochotensis*	
51	2011.5.13	Misaki-cho, Rausu	10	*S. japonica* var. *diabolica*	
52	2011.5.12	Barajima, Nemuro	5	*S. japonica* var. *diabolica*	
53	2012.8.28	Onnemoto, Nemuro	7	*S. japonica* var. *diabolica*	
54	2011.10.26	Futaoki, Nemuro	5	*S. japonica* var. *diabolica*	for SSR analysis
55	2013.8.28	Tomoshiri, Nemuro	10	*S. japonica* var. *diabolica*	
56	2013.8.28	Tomoshiri-gyoko, Nemuro	4	*S. japonica* var. *diabolica*	
57	2012.8.23	Ochiishi, Nemuro	7	*S. japonica* var. *diabolica*	
58	2012.10.29	Daikokujima, Akkeshi	5	*S. japonica* var. *diabolica*	
59	2010.5.10	Aikappu, Akkeshi	10	*S. japonica* var. *diabolica*	
60	2012.10.29	Akkeshi-ko, Akkeshi	5	*S. japonica* var. *diabolica*	
61	2013.8.16	Aniwa, Sakhalin	5	unknown	
62	2013.8.14	Gornozavodsk, Sakhalin	5	unknown	

Table 2 Genetic polymorphism and the degree of genetic diversity of individuals in a locality of *Saccharina japonica*

Locality	Number of total bands	Number of common bands	Number of polymorphic bands	*P*(%)	*H*
Utoma	613	180	433	0.173	0.072
Charatsunai	564	170	394	0.157	0.058
Usu	607	202	405	0.161	0.070
Rebunge	599	209	390	0.155	0.065
Shizukari	633	204	429	0.171	0.073
Oofune	568	148	420	0.167	0.067
Usujiri	591	168	423	0.169	0.058
Yasuura	485	158	327	0.130	0.057
Osatube	467	149	318	0.127	0.053
Kinaoshi	548	169	379	0.151	0.061
Sumiyoshi	708	74	634	0.253	0.099
Nakanogawa	571	181	390	0.155	0.067
Kotaniishi	610	194	416	0.166	0.075
Shiogama	672	138	534	0.213	0.094
Hyuga	436	131	305	0.122	0.051
Kamaishi	610	182	428	0.171	0.072
Tomari	617	188	429	0.171	0.074
Sarugamori	729	69	660	0.263	0.116
Shitsukari	590	194	396	0.158	0.074
Shiriya	604	192	412	0.164	0.076
Kinopppu	638	120	518	0.206	0.090

Shimotehama	555	170	385	0.153	0.068
Isoya	559	169	390	0.155	0.068
Chogo	487	203	284	0.113	0.057
Tairadate	558	155	403	0.161	0.074
Imabetsu	598	189	409	0.163	0.071
Minmaya	702	111	591	0.235	0.101
Kaitorima	612	125	487	0.194	0.066
Shimamakimura-ko	593	66	527	0.210	0.079
Ohama	574	196	378	0.151	0.069
Kawashiro	464	222	242	0.096	0.046
Oshoro 1	580	104	476	0.190	0.075
Oshoro 2	548	108	440	0.175	0.072
Oshoro 3	577	78	499	0.199	0.080
Oshoro 4	537	95	442	0.176	0.071
Oshoro 5	531	80	451	0.180	0.073
Oshoro 6	546	106	440	0.175	0.071
Oshoro 7	572	100	472	0.188	0.077
Gokibiru	442	152	290	0.116	0.045
Hamamasu	440	142	298	0.119	0.050
Shokan-kaigan	517	133	384	0.153	0.068
Ogon-misaki	569	225	344	0.137	0.062
Rikibiru	428	156	272	0.108	0.047
Oniwaki	597	158	439	0.175	0.075

Kutsugata	429	192	237	0.094	0.039
Shinminato	424	185	239	0.095	0.042
Oshidomari	449	176	273	0.109	0.046
Kafuka	504	151	353	0.141	0.060
Horai	468	172	296	0.118	0.044
Omisaki	408	116	292	0.116	0.042
Hon-cho	535	196	339	0.135	0.056
Omu	539	188	351	0.140	0.060
Futatsuiwa	446	187	259	0.103	0.043
Utoro	424	140	284	0.113	0.049
Misaki-cho	540	194	346	0.138	0.046
Barajima	438	92	346	0.138	0.051
Onnemoto	696	81	615	0.245	0.095
Tomoshiri	569	140	429	0.171	0.061
Tomoshiri-gyoko	433	206	227	0.090	0.038
Ochiishi	705	93	612	0.244	0.095
Daikokujima	490	213	277	0.110	0.048
Aikappu	554	190	364	0.145	0.052
Akkeshi-ko	429	172	257	0.102	0.042
Aniwa	425	154	271	0.108	0.044
Gornozavodsk	411	156	255	0.102	0.043

Common bands indicate the bands shared by all individuals in a locality. Polymorphic bands indicate the bands with the exception of common bands.

P percentage of polymorphic loci

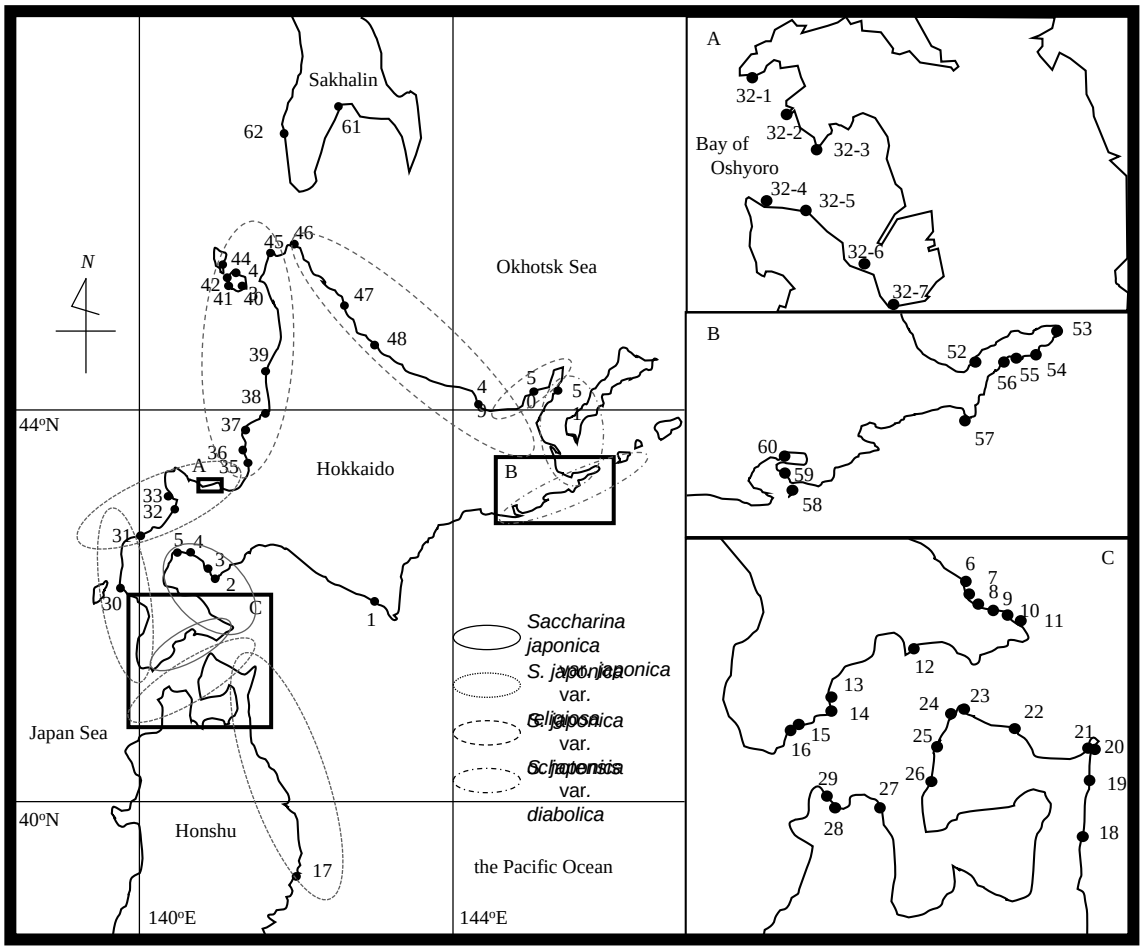
Table 3 Dice similarity coefficient (S) of *Saccharina japonica* within a variety and between varieties

	var. *japonica*	var. *religiosa*	var. *ochotensis*	var. *diabolica*
Saccharina japonica var. *japonica*	0.53 ± 0.10	0.46 ± 0.08	0.45 ± 0.06	0.42 ± 0.05
Saccharina japonica var. *religiosa*		0.47 ± 0.12	0.44 ± 0.06	0.40 ± 0.05
Saccharina japonica var. *ochotensis*			0.57 ± 0.10	0.45 ± 0.05
Saccharina japonica var. *diabolica*				0.55 ± 0.12

Table 4 Pairwise (Gst) value of *Saccharina japonica* within a variety and between varieties

	var. *japonica*	var. *religiosa*	var. *ochotensis*	var. *diabolica*
Saccharina japonica var. *japonica*	0.27 ± 0.11	0.41 ± 0.08	0.51 ± 0.05	0.53 ± 0.06
Saccharina japonica var. *religiosa*		0.40 ± 0.08	0.47 ± 0.09	0.52 ± 0.07
Saccharina japonica var. *ochotensis*			0.43 ± 0.11	0.55 ± 0.06
Saccharina japonica var. *diabolica*				0.51 ± 0.09

Fig. 1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61
Okhotsk Sea
N
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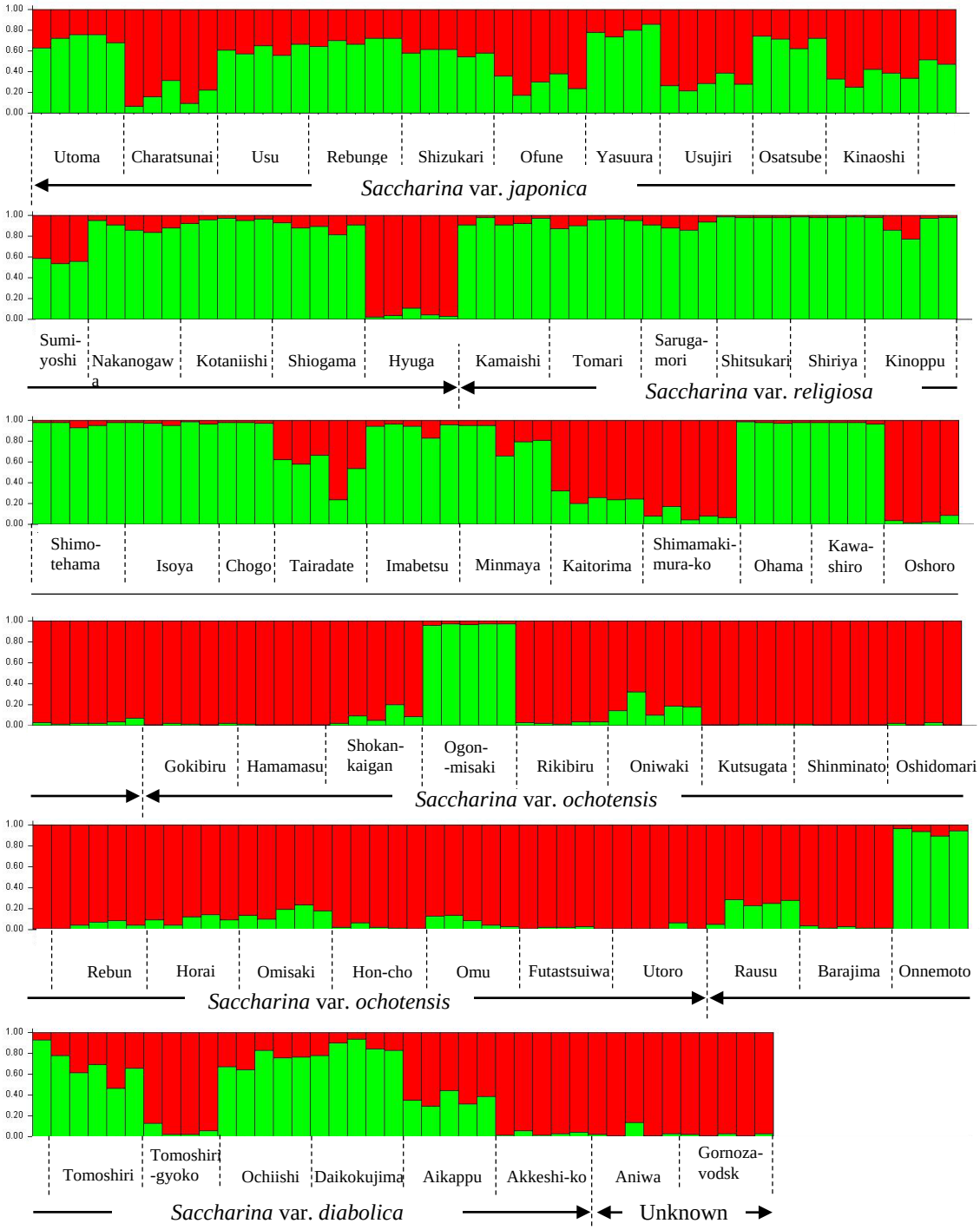
Hokkaido
Japan Sea
40°N
Honshu
the Pacific Ocean
140°E
144°E
A
B
C
Bay of
Oshyoro
32-1
32-2
32-3
32-4
32-5
32-6
32-7
Saccharina
japonica
S. japonica
var.
diabolica

Fig. 2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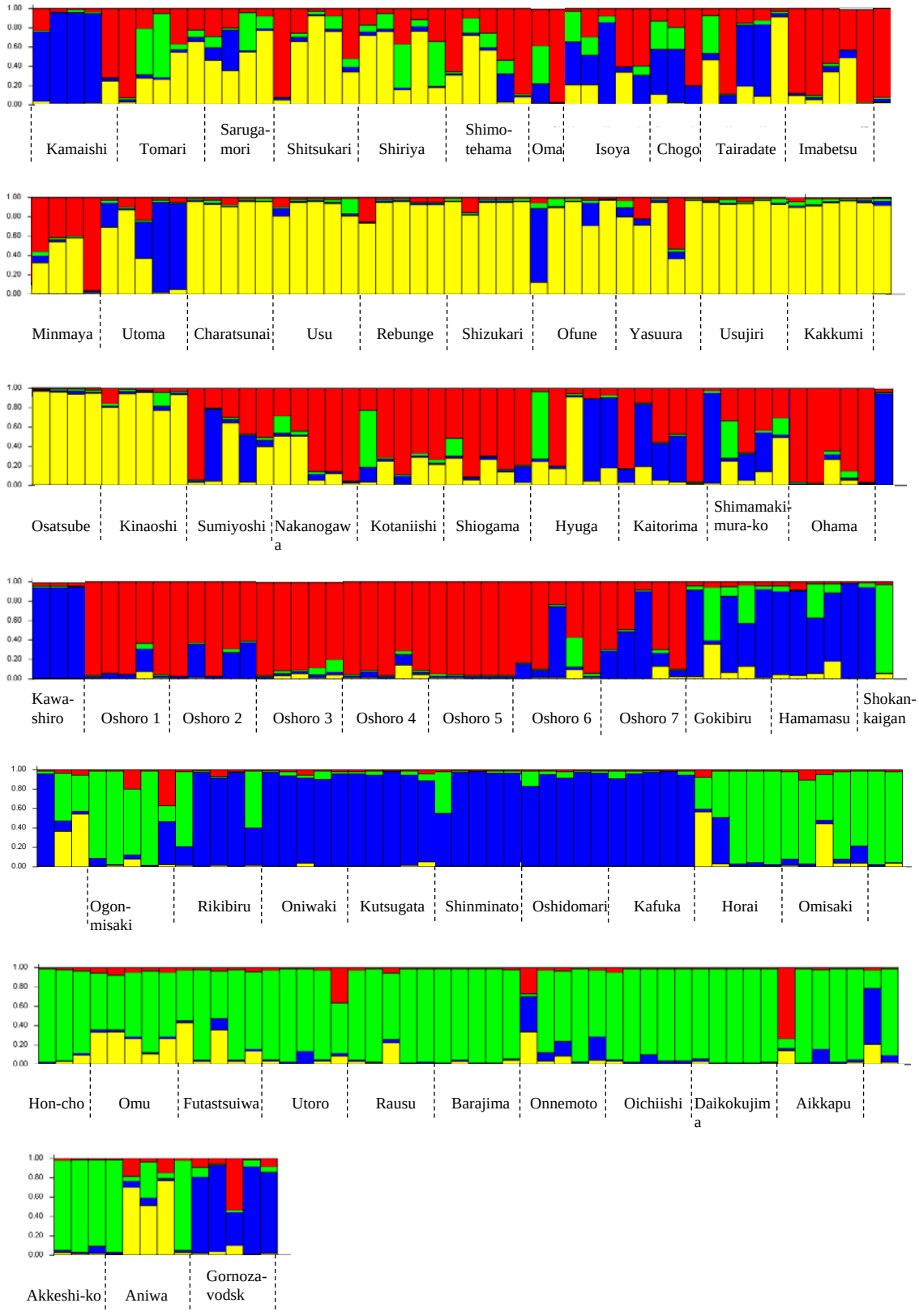
Yasuura
Usujiri
Osatsube
Kinaoshi
Saccharina var. japonica
Sumi-yoshi
Nakanogawa
Kotaniishi
Shiogama
Hyuga
Kamaishi
Tomari
Saruga-mori
Shitsukari
Shiriya
Kinoppu
Saccharina var. religiosa
Shimo-tehama
Isoya
Chogo
Tairadate
Imabetsu
Minmaya
Kaitorima
Shimamaki-mura-ko
Ohama
Kawa-shiro
Oshoro
Gokibiru
Hamamasu
Shokan-kaigan
Ogon--misaki
Rikibiru
Oniwaki
Kutsugata
Shinminato
Oshidomari
Saccharina var. ochotensis
Rebun
Horai
Omisaki
Hon-cho
Omu
Futastsuiwa
Utoro
Rausu
Barajima
Onnemoto
Saccharina var. ochotensis
Tomoshiri
Tomoshiri-gyoko
Ochiishi
Daikokujima
Aikappu
Akkeshi-ko
Aniwa
Gornoza-vodsk
Saccharina var. diabolica
Unknown

Fig. 3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Utoma
Charatsunai
Usu
Rebunge
Shizukari
Ofune
Yasuura
Usujiri
Kakkumi
Osatsube
Kinaoshi
Sumiyoshi
Nakanogawa
Kotaniishi
Shiogama
Hyuga
Kaitorima
Shimamaki-mura-ko
Ohama
Kawa-shiro
Oshoro 1
Oshoro 2
Oshoro 3
Oshoro 4
Oshoro 5
Oshoro 6
Oshoro 7
Gokibiru
Hamamasu
Shokan-kaigan
Ogon-misaki
Rikibiru
Oniwaki
Kutsugata
Shinminato
Oshidomari
Kafuka
Horai
Omisaki
Hon-cho
Omu
Futastsuiwa
Utoro
Rausu
Barajima
Onnemoto
Oichiishi
Daikokujima
Aikkapu
Akkeshi-ko
Aniwa
Gornoza-vodsk