



Discerning the Putative U and V Chromosomes of *Saccharina japonica* (Phaeophyta) by Cytogenetic Mapping of Sex-Linked Molecular Markers

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Saccharina japonica, an importantly industrial species in brown seaweeds, has a typical alternate life history of heteromorphic generations and UV sex determination system. But up to now, the sex chromosomes, U and V, in this kelp remain to be evidenced cytologically. In the present study, the female-linked (SJ-f_000170 and MSj68-58-2) and male-linked (SJ-13_001840 and MSj68-16-2) molecular markers developed on the basis of *Ectocarpus* sex-determining regions (SDRs) were selected after PCR amplification and Southern blotting analysis. Mono-color fluorescence *in situ* hybridization (FISH) to the kelp metaphase chromosomes with each of these confirmed sex-linked markers as a probe indicated that the hybridization signals were confined to either female or male gametophytes exclusively. The shape and size of hybridized chromosomes and the relative location of these sex-linked markers on them suggested that the marked chromosomes in a sex were the same, which was further confirmed by dual-color FISH observations. SJ-f_000170 and SJ-13_001840, corresponding to each sex, were therefore used to map the sporophyte metaphase chromosomes by dual-color FISH technique, and they were hybridized on different chromosomes as expected. The sporophyte metaphase chromosomes as hybridized by SJ-f_000170 and SJ-13_001840 resembled those in either female or male gametophytes, suggesting that they were the putative U and V, respectively, chromosomes in *S. japonica*. To further demonstrate the reliability of this inference, genomic information of the screened bacterial artificial chromosome (BAC) clones carrying two female-linked markers, for example, from the constructed BAC libraries of the kelp female gametophytes was provided. The insert sequencing of two selected BAC clones revealed that repetitive elements were rich whereas gene density was poor, which was characterized as non-recombining SDR sequences in brown seaweed *Ectocarpus* sp. and liverwort *Marchantia polymorpha*. The present study laid a solid foundation for unveiling the mystery of *S. japonica* sex chromosomes.

Keywords: bacterial artificial chromosome (BAC), fluorescence *in situ* hybridization (FISH), gametophyte, kelp, *Saccharina japonica*, sex-determining region (SDR), sex-linked markers, sporophyte

INTRODUCTION

Saccharina japonica (Areschoug) C. E. Lane, C. Mayes, Druehl et G. W. Saunders (syn. *Laminaria japonica* Aresch.) possesses a heteromorphic haploid-diploid life cycle involving macroscopic phylloidal sporophytes ($2N$) and microscopic filamentous gametophytes (N) (Wu and Suo, 1962). When the sporophytes developed to maturity, zoospores were released from the unilocular sporangia of matured sporophytes and adhered to a substratum. Under appropriate environmental conditions for germination, the attached embryospores developed into female and male gametophytes at a sex ratio of 1 to 1 (Schreiber, 1930). Large eggs and small spermatozooids were then produced from the female and male gametangia, respectively, implying the sexual dimorphism in *S. japonica* (commonly referred to as kelp). This kelp is hence an oogamous species with dioicous gametophytes as proposed by Luthringer et al. (2014); Coelho et al. (2019), and Coelho and Umen (2021). In addition, several experiments on parthenogenesis (Tai and Fang, 1977; Fang et al., 1978; Motomura, 1991; Dai et al., 1993; Lewis et al., 1993) pointed out that parthenogenetic sporophytes which derived from female gametophytes *via* apomixis or apogamy could grow to maturity. All the produced zoospores from the parthenogenetic sporophytes were able to germinate and develop into female gametocytes exclusively. From these findings, Fang et al. (1978) inferred that an XY-like sex-determination system existed in *S. japonica*, and that the diploid sporophyte was XY type whereas the haploid female and male gametophytes were X- and Y-type, respectively. Now, this type of chromosomal sex determination in the haploid phase of life cycle has been termed UV sex-determination system by Bachtrog et al. (2011) to discriminate from diploid XY or ZW systems of animals and higher plants.

In cytological observations of the gametophytes of *S. latissima* and other species of *Laminaria* by an acetocarmine squash method, Evans (1963) found that the female gametophytes had a very large chromosome which was absent in male gametophytes. Since the large chromosome paired with a small one at meiosis of sporophytes in *S. latissima*, it was proposed to be a U (formerly X) chromosome by Evans (1965). This finding was supported by a cytological observation on *Laminaria yendoana* chromosomes (Yasui, 1992). Nonetheless, such a conspicuously large sex chromosome was not observed in *S. japonica*, while both research groups of Tai and Fang (1977) and Yabu and Yasui (1991) cytologically examined the kelp chromosomes. Such an incongruous view of whether there were sex chromosomes in *S. japonica* could be resolved by cytogenetic evidence. The physical features, however, of the kelp chromosomes in number, size, and morphology as reviewed by Lewis (1996) limited further cytogenetic studies. Fortunately, this situation now has improved by applying the DNA-binding fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) and the technique fluorescence *in situ* hybridization (FISH) to the observations on the kelp chromosomes (Liu et al., 2012). Using this established protocol, telomere and rDNAs were successfully localized on the kelp chromosomes (Liu et al., 2017; Yang et al., 2017; Liu Y. et al., 2021). Meanwhile, some female or male gametophyte-specific rather than genetically linked markers

were developed and localized on *S. japonica* chromosomes using FISH technique, too (Liu et al., 2009, 2012; Gu et al., 2014). Accordingly, the available sex-linked molecular markers developed from *S. japonica* would facilitate the application of FISH to discerning U and V chromosomes in this kelp.

By use of several strategies for identification of the sex-determining regions (SDRs) in *Ectocarpus siliculosus*, Ahmed et al. (2014) estimated that the male and female SDRs of *E. siliculosus* were 920 and 929 kbp in size, respectively. While performing a homology search on *S. japonica* genome database (Ye et al., 2015) using the SDR-linked genes of *E. siliculosus*, Lipinska et al. (2017) found that four genes were linked to *S. japonica* U chromosome and four to V one. Zhang et al. (2018) developed four new male gametophyte-specific markers and one female gametophyte-specific marker in *S. japonica* based on the SDR genes of *E. siliculosus* reported by Ahmed et al. (2014). In this case, these female-linked genes and molecular markers are expected to be co-located on the same U chromosome, whereas the male-linked ones are to be on the same V chromosome of *S. japonica*.

In order to visualize these abovementioned sex-linked genes or markers to discern the putative U and V chromosomes of *S. japonica* using FISH technique, at first, we verified these sex-linked genes or markers from the genomic DNA of this kelp by polymerase chain reaction (PCR) amplification and Southern blotting analysis. Based upon mono-color FISH profiles with each of these confirmed sex-linked markers as a probe, two of them associated to each sex were then used as probes to detect if they were co-localized on the same chromosome of male or female gametophytes in *S. japonica* using dual-color FISH technique. In the same manner, at last, two (one each sex) of them were labeled as probes to illustrate their co-localization on the sporophyte metaphase chromosomes of this kelp. In addition, bacterial artificial chromosome (BAC) clones carrying the U chromosome-linked markers were screened, sequenced, and characterized to exhibit their genomic features. The present study suggested that the putative U and V chromosomes in *S. japonica* could be distinguished using FISH with sex-linked molecular markers as probes.

MATERIALS AND METHODS

Algal Material and Culture

Saccharina japonica was selected as the algal material in the present study. The female and male gametophyte clones germinated from zoospores were isolated under a microscope as reported by Zhou and Wu (1998). They were kept under the vegetative growth conditions of 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $17 \pm 1^\circ\text{C}$ with a photoperiod 12 h/12 h (light/dark). PES medium (Starr and Zeikus, 1993) was used for this culture and replaced once every 2 weeks.

To compare the transcript levels of annotated genes from the sequenced BAC clones between male and female gametophytes, quantitative real-time PCR (qRT-PCR) was used. The gametophytes were cut into fragments and cultured in PES medium as previously described by Bi et al. (2021), but under the illumination of 80 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 10 h/14 h

(light/dark) regimen at $10 \pm 1^\circ\text{C}$. Eight days later, the male and female gametophytes were centrifuged as previously described (Zhou and Wu, 1998) for RNA isolation.

Juvenile sporophytes (approximately 2 cm in length), developed from the isolated male and female gametophytes of this kelp as described by Zhou and Wu (1998), were presented kindly by Oriental Ocean Sci-tec Co., Ltd., Yantai, Shandong province, China.

Selection of Sex-Linked Molecular Markers

Comparing *Ectocarpus siliculosus*, *E. fasciculatus*, and *Scytosiphon lomentaria* in Ectocarpales, with other three brown seaweeds *S. japonica*, *Macrocystis pyrifera*, and *Undaria pinnatifida* in Laminariales, Lipinska et al. (2017) found that 20 of the 34 *Ectocarpus* sp. SDR genes had orthologs whereas only 9 of the 20 orthologous genes were sex-linked. Among the nine sex-linked genes in *E. siliculosus*, one *gametologue* pair, Ec-13_001840 and Ec-sdr_f_000170, was highly conserved in all the examined seaweeds, implying that these two genes were derived from the same ancestor and located within the non-recombining region of opposite sex chromosomes as defined by Coelho et al. (2018). These two *gametologues* were referred to as SJ-13_001840 and SJ-f_000170, respectively, in *S. japonica* by Lipinska et al. (2017) and therefore chosen for the present study.

Meanwhile, MSj68-16-2 was also chosen for the present study since it was proposed to be a male-linked marker which was developed by Zhang et al. (2018) on the basis of *S. japonica* transcriptomic and genomic sequences. It shared 100% similarity with partial coding sequence (170 bp in length) of the kelp HMG-box-containing gene (*SjHMG*) (Zhang et al., 2019), suggesting that this marker was a part of DNA sequence of this gene. Moreover, Zhang et al. (2019, 2021) provided experimental evidence that the HMG domain-containing protein, a transcription factor (Ec-13_001750) in *Ectocarpus* as annotated by Ahmed et al. (2014), was expressed exclusively in the kelp male gametophytes and was thus proposed to be a candidate male-determining gene. In addition, MSj68-58-2, a developed female-specific marker in *S. japonica* by Zhang et al. (2018), was selected in the present study as a candidate for sex-linked marker. The main reason for this was that MSj68-58-2 was developed based on the sequence of *U. pinnatifida* and *M. pyrifera* female-specific marker, M_68_58_2, which was originally derived from the *Ectocarpus* SDR gene as described by Lipinska et al. (2015).

DNA Isolation

About 0.1 g of *S. japonica* gametophytes were collected by centrifugation. After rinsing thrice with sterilized seawater, the harvested gametophytes were ground into powder in liquid nitrogen. Genomic DNA was extracted from them by use of a modified cetyltrimethyl ammonium bromide (CTAB) method as described previously (Hu and Zhou, 2001). The extracted genomic DNA was dissolved in 100 μL $1 \times$ Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C .

Extraction of genomic DNA from *S. japonica* sporophytes was pre-treated with a mixed enzymatic solution as described by Liu et al. (2012), and the subsequent DNA was extracted in the same way as that from the gametophytes.

Quality and quantity of the extracted nucleic acids were determined by the ratios of measured optical density (OD) at wavelengths of 260 to 280 nm (i.e., $\text{OD}_{260}/\text{OD}_{280}$) and of OD_{260} to OD_{230} using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, United States). Integrity of the extracted nucleic acids was evaluated by 1% agarose gel electrophoresis.

Polymerase Chain Reaction Amplification of Sex-Linked Molecular Markers

Two pairs of primers, f170-F and f170-R, and m1840-F and m1840-R (**Supplementary Table 1**), were designed according to the SDR gene sequences of SJ-f_000170 and SJ-13_001840, which were developed from the female and male gametophytes, respectively, of *S. japonica* by Lipinska et al. (2017). The other two pairs, f58-F1 and f58-R1, and m16-F and m16-R (**Supplementary Table 1**), were used for amplification of the SDR markers MSj68-58-2 (GenBank accession No: MF850255) and MSj68-16-2 (GenBank accession No: MF135546) in the kelp female and male gametophytes, respectively, as developed by Zhang et al. (2018). The amplification was conducted in a total volume of 25 μL reaction system containing 9.5 μL distilled deionized (dd) H_2O , 12.5 μL $2 \times$ Taq PCR MasterMix (100 μM each), 2 μL genomic DNA (200 $\text{ng} \cdot \mu\text{L}^{-1}$), and 1 μL forward and reverse primers (0.2 μM each). PCR was performed in a gradient Mastercycler (Eppendorf, Hamburg, Germany) and programmed as described by Zhang et al. (2018), but the annealing temperature was listed in **Supplementary Table 1**.

The amplified products were purified and ligated into a pMD19-T Simple vector (TaKaRa, Kyoto, Japan) and transformed into the competent cells of *Escherichia coli* DH5 α cells (Tiangen Biotech, Beijing, China). The recombinants were screened *via* blue-white selection on ampicillin-containing Luria-Bertani (LB) plates. White or positive colonies were verified by PCR amplification with the general primers RV-M and M13-20 (TaKaRa). The positive clones were then selected and sent to Genewiz Biological Technology Co., Ltd. (Suzhou, China) for sequencing using an automated DNA sequencer ABI Prism 3730 (Foster City, United States).

Southern Blotting Analysis

Aliquots of the isolated genomic DNA (approximately 20 μg per sample) from the *S. japonica* gametophytes were digested to completion at 37°C for 6 h, independently, with endonucleases either *SpeI* that could not digest the male-linked markers or *XbaI* that could not digest the female-linked markers. The digested DNA samples were fractionated on a 1.0% agarose gel, blotted onto a positively charged nitrocellulose filter membrane (Pall, Exton, United States), and subsequently hybridized with the target markers as probes. The probes which

were inserted into the vector pMD19-T were labeled with biotin-dUTP using a North2South Biotin Random Prime Labeling Kit (Thermo Fisher Scientific, Rockford, United States). Hybridized bands were detected using a North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific) following the manufacturer's guidelines, and signals were visualized by automatic exposure with Amersham™ Imager 600 (GE Healthcare, Uppsala, Sweden).

Chromosome Preparation

Chromosome preparation of the *S. japonica* gametophytes and sporophytes was conducted referring to Liu et al. (2012). In brief, appropriate amount of *S. japonica* gametophytes or sporophytes was added into sterilized sea water containing 0.02% colchicine to arrest metaphase nuclei at 4°C for 8 h and then washed with sterilized sea water twice. The samples were fixed in a freshly prepared Carnoy's fixative fluid (glacial acetic acid/ethanol, 3/1, v/v) at 4°C for more than 24 h (Schweizer, 1976). The fixed samples were washed with deionized water for 2 or 3 times, taken into pieces with a dissecting needle, and added into the mixed enzyme solution (cellulase/pectinase/macerozyme R-10/freshly extracted abalone alginate lyase, 2/1/1/1.5, v/v/v/v) to digest enzymatically at 37°C for 14 – 20 h. The chromosome preparation was obtained by spreading the digested samples on poly-L-lysine coated slides. The slides were screened under an upright phase contrast microscope, and the well-spread slides were selected and stored at – 20°C for FISH experiments.

Fluorescence *in situ* Hybridization Analysis

Prior to FISH experiments, the constructed plasmids carrying the four sex-linked molecular markers were extracted from the transgenic *Escherichia coli* following the protocol of Plasmid Extraction Kit (Tiangen). The extracted plasmids, at a concentration of not less than 200 ng·μL⁻¹, were used as templates to prepare for FISH probes by Nick translation (Rigby et al., 1977) using an ADVANCE™ Nick Translation Kit (Sigma-Aldrich, St. Louis, United States) under the guidance of the manufacturer's instructions. The probes of the sex-linked genes SJ-f_000170 and SJ-13_001840 were labeled with Texas Red-5-dCTP (Invitrogen, Carlsbad, United States), whereas the sex-linked markers MSj68-58-2 and MSj68-16-2 were done with Alex Flour Green-5-dUTP (PerkinElmer, Boston, United States) for the hybridization to gametophyte metaphase nuclei. If for the hybridization to sporophyte metaphase nuclei to discern U and V chromosomes simultaneously with two different colors, the probe of the sex-linked gene SJ-f_000170 was labeled with Alex Flour Green-5-dUTP (PerkinElmer) instead with Texas Red-5-dCTP (Invitrogen) used in observations on gametophyte nuclei.

The FISH experiment was conducted according to the previously described method (Liu et al., 2012). Briefly, the slides containing chromosomes were placed in an ultraviolet cross-linker BLX-E254 (Vilbert Lourmat, France), and they were cross-linked twice by ultraviolet at 254 nm for 15 s each time. The slide was then placed on ice, and hybridization solution containing 2 × saline sodium citrate (SSC; 0.3 M NaCl, 0.03 M sodium

citrate, pH 7.0), 1 × TE buffer, and 10 ng of the labeled probes, was dropped onto it. After that, it was covered with a slip of plastic sheet, and placed in a water bath at 100°C for 5 min. The slides were then transferred to a DNA hybridization chamber for incubation overnight at 55°C. Post-hybridization washes of the slides were carried out with 2 × SSC hybridization buffer in a shaker for 2 min to remove non-specifically unbound hybridized probes. Before microscopic observation, the oven-dried slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 2 μg·mL⁻¹) containing anti-quencher (Vector Laboratories, Burlingame, United States) and left still in the dark for 10 min.

To detect the hybridization sites on the *S. japonica* sporophyte chromosomes, the well-spread mitotic chromosomes were pre-treated with an appropriate amount of freshly extracted abalone alginate lyase at 37°C for 1 h. Subsequently, they were subjected to FISH detection as described above.

Fluorescence Microscopy and Image Acquisition

Images were taken with a Leica DM4000B epifluorescent microscope (Wetzlar, Germany) equipped with different filter set and a digital camera DFC550 (Heerbrugg, Switzerland). Three kinds of fluorescent signals, Alex Flour (green), DAPI (blue), and Texas Red (red), were detected at the emission wavelength of 488, 495, and 612 nm, respectively. All images were processed using ImageJ¹ software for color contrast and brightness uniformity. The karyotypic patterns or ideograms of the gametophytes and sporophytes were edited using Adobe Photoshop 7.0² based on the chromosome size.

Polymerase Chain Reaction-Based Screening for Bacterial Artificial Chromosome Libraries

To provide genomic information of the U-linked markers, the strategy of “three-dimensional” screen from the constructed female gametophyte BAC libraries (Liu P. F. et al., 2021) was performed following the established protocol by Yasukochi (2002).

Every ten of 384-well microtiter plates were mixed in equal amounts (2 μL) to generate a primary DNA pool or superpool (**Supplementary Figure 1**). After culture with addition of 50 μL LB medium containing chloramphenicol (12.5 g·L⁻¹) at 37°C for 8 h, the bacterial cells in the superpool were used to isolate the plasmid DNA using a QIAwell 96 Ultra Plasmid Kit (Qiagen, Düsseldorf, Germany) according to the supplier's manual. Using the isolated DNA as a template, PCR was performed to screen the library with the two pairs of primers f170-F and f170-R, and f58-F2 and f58-R2 (**Supplementary Table 1**). The amplification reaction system and program were the same as described above, with exception of annealing temperature at 62°C (**Supplementary Table 1**).

If the target band was electrophoretically detected from a given primary pool, the secondary pools were constructed according to

¹<https://imagej.nih.gov/ij/>

²<https://www.adobe.com/products/photoshop.html>

each row, column, or plate constituting this selected superpool for the “three-dimensional” screen (**Supplementary Figure 1**). After culture and DNA isolation, PCR was performed as mentioned above to screen positive BAC clones.

The screened BAC positive clones harboring the target markers were then sent to Bacgene Biotechnology Co., Ltd. (Wuhan, China) for sequencing analysis using PacBio Sequel System (Pacific Biosciences, Menlo Park, United States).

Sequence Analysis of Inserts in Bacterial Artificial Chromosome Clones

In order to analyze the insert sequence characteristics of BAC clones, VecScreen³ were used to remove the vector sequences. Repetitive sequences in the inserts of screened BAC clones were analyzed using RepeatMasker program.⁴ Physical maps of the insert sequences of BAC clones were drawn using IBS1.0 (Liu et al., 2015) and MapChart2.32 (Voorrips, 2002). Both AUGUSTUS⁵ (Stanke and Waack, 2003) and software TopHat (v.2.0.8)⁶ and Cufflinks (v.2.1.1)⁷ were used to *de novo* annotate gene information. Blastn⁸ was used to estimate the sequence similarity.

Quantitative Real-Time PCR

Total RNA was isolated from the freshly collected gametophytes of *S. japonica* using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the Reverse Transcribed Kit II (TaKaRa). Quantitative RT-PCR amplification was conducted using a SYBR[®] RT-PCR Kit (TaKaRa) in a Bio-Rad iQ[™] 5 multicolor real-time PCR detection system (Bio-Rad) following the previous protocols (Ye et al., 2014). The 18S rRNA gene (GenBank Accession No. EU293553) of *S. japonica* was used as a house-keeping one, and all primers used for qRT-PCR analysis were listed in **Supplementary Table 1**. The relative abundance of gene transcripts from triplicate reactions performed for each sample was expressed as the mean \pm standard deviation (SD) using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The significance was estimated by the two-tailed Student's *t*-test using SPSS 22.0 software.⁹

RESULTS

Polymerase Chain Reaction Amplification of the Selected Sex-Linked Markers and Their Southern Blotting Analyses

Based on the sequences of four selected sex-linked markers SJ-f_000170, MSj68-58-2, SJ-13_001840, and MSj68-16-2,

four pairs of primers (f170-F and f170-R, f58-F1 and f58-R1, m1840-F and m1840-R, and m16-F and m16-R; **Supplementary Table 1**) were designed. With these primers, their corresponding amplified products were obtained by PCR amplification (**Supplementary Figure 2**). Sequencing analyses of these products showed that the sequences and sizes (**Supplementary Table 1**) were the same as expected. It was noteworthy that the markers SJ-f_000170 and MSj68-58-2 were amplified only from the female gametophytes of *S. japonica* (Lanes 2 and 4 in **Supplementary Figure 2**), whereas SJ-13_001840 and MSj68-16-2 were done only from the male gametophytes (Lanes 7 and 9 in **Supplementary Figure 2**). These results pointed out that they were male- and female-specific markers in *S. japonica* as documented by Lipinska et al. (2015) and Zhang et al. (2018). When the genomic DNA extracted from sporophyte was taken as a template, the electrophoresis profiles of amplicons demonstrated that all these markers could be detected (Lanes 12-15 in **Supplementary Figure 2**), suggesting that they all could be inheritable in the sporophyte and its corresponding gametophytes of *S. japonica*.

To further validate the natural existence in the kelp genome, the four sex-linked markers were labeled as probes independently into digested genomic DNA (upper panels in **Figure 1**) extracted from the either female or male gametophytes of *S. japonica*. Southern blotting profiles (lower panels in **Figure 1**) showed that only one signal was present in the female gametophytes after hybridization with the labeled SJ-f_000170 or MSj68-58-2 probes (Lanes 3 or 5 in **Figure 1**, respectively). With these two labeled probes for Southern blotting, on the contrary, there was no any signal in the male gametophytes (Lanes 2 and 4 in **Figure 1**), further suggesting that SJ-f_000170 and MSj68-58-2 were female-specific in *S. japonica*. Similarly, SJ-13_001840 and MSj68-16-2 were unique to the kelp male gametophytes (Lanes 8 and 9 in **Figure 1**, respectively) as shown by Southern blotting profiles. It was of interest to find that each of the four markers had only one hybridization signal, suggesting that they were located on the chromosomes only at one locus in their corresponding gametophytes.

Fluorescence *in situ* Hybridization Mapping of the Sex-Linked Markers to *Saccharina japonica* Chromosomes

Prior to FISH experiments, the quality of synthesized probes was assessed by electrophoresis. Electrophoretic results (**Supplementary Figure 3**) showed that all the labeled probes appeared smear with a peak intensity between 100 and 500 bp, thus being suitable for subsequent experiments. To determine the chromosomal location of each sex-linked marker, a total of at least 20 metaphase nuclei of the female or male samples was examined using mono-color FISH technique. Of the observed mitotic metaphase nuclei, 10 of the 20 female nuclei hybridized with SJ-f_000170 as a probe, 16 of the 25 female nuclei with MSj68-58-2, 16 of the 24 male nuclei with MSj68-16-2, and 12 of the 23 male nuclei with SJ-13_001840, had only one fluorescent signal as shown in **Supplementary Figure 4**. These FISH images illustrated that SJ-f_000170 and MSj68-58-2 were unique to

³<https://www.ncbi.nlm.nih.gov/tools/vecscreen/>

⁴<http://repeatmasker.org/>

⁵<http://augustus.gobics.de>

⁶<http://ccb.jhu.edu/software/tophat/index.shtml>

⁷<http://cole-trapnell-lab.github.io/cufflinks/>

⁸<https://blast.ncbi.nlm.nih.gov/Blast.cgi>

⁹<https://www.ibm.com/products/spss-statistics>

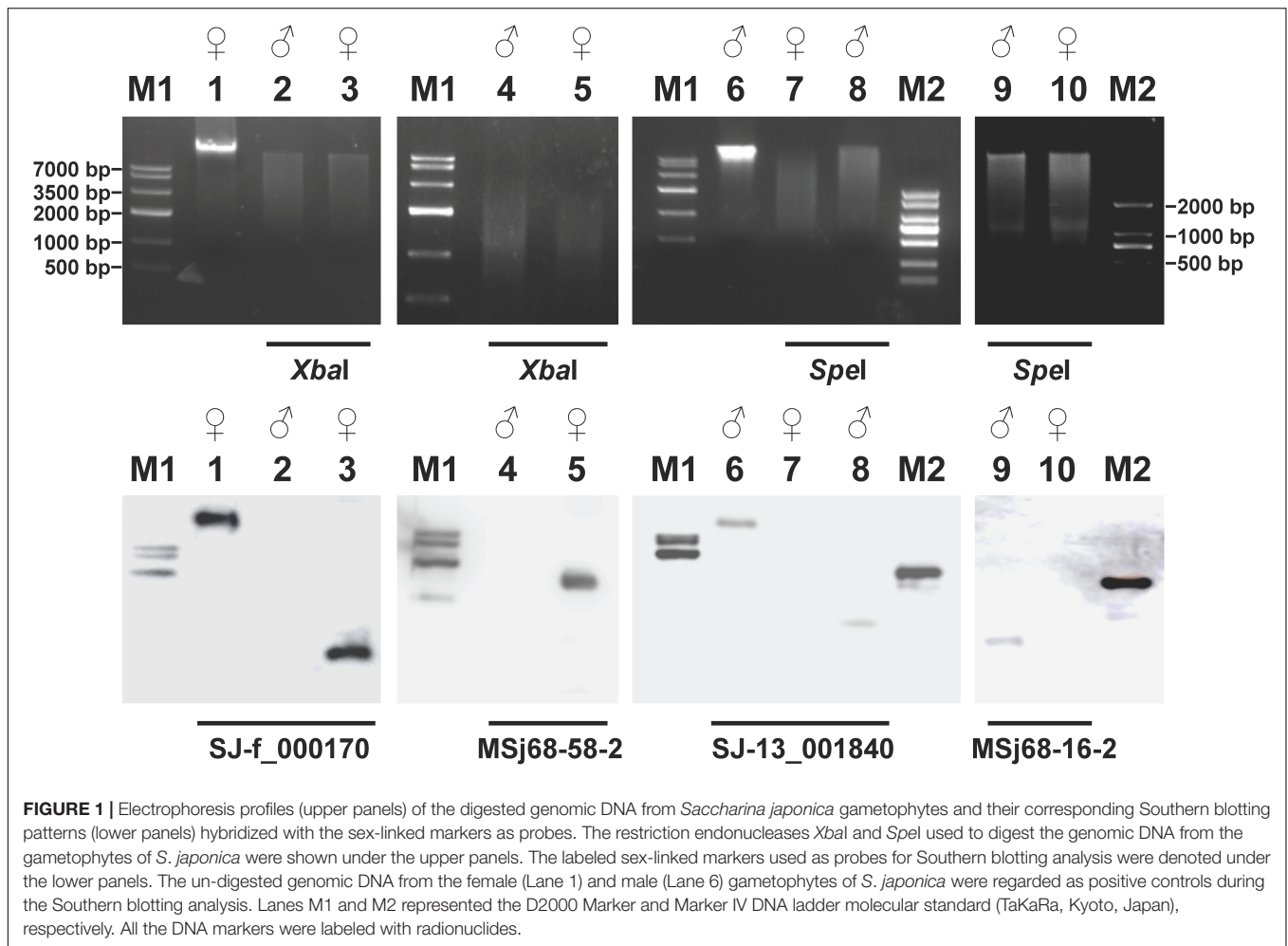


FIGURE 1 | Electrophoresis profiles (upper panels) of the digested genomic DNA from *Saccharina japonica* gametophytes and their corresponding Southern blotting patterns (lower panels) hybridized with the sex-linked markers as probes. The restriction endonucleases *XbaI* and *SpeI* used to digest the genomic DNA from the gametophytes of *S. japonica* were shown under the upper panels. The labeled sex-linked markers used as probes for Southern blotting analysis were denoted under the lower panels. The un-digested genomic DNA from the female (Lane 1) and male (Lane 6) gametophytes of *S. japonica* were regarded as positive controls during the Southern blotting analysis. Lanes M1 and M2 represented the D2000 Marker and Marker IV DNA ladder molecular standard (TaKaRa, Kyoto, Japan), respectively. All the DNA markers were labeled with radionuclides.

TABLE 1 | Localization of the labeled probes on chromosomes and length of the hybridized chromosomes prepared from the female and male gametophytes and the sporophytes of *Saccharina japonica*.

Metaphase chromosome	Labeled probe	Location of the labeled probe	Length (μm) of the labeled chromosome (mean ± SD)*
Gametophyte (mono-color FISH)			
Female	SJ-f_000170	Interstitial region of short arm	2.068 ± 0.180 ^a (n = 10)
	MSj68-58-2	Interstitial region of short arm	2.106 ± 0.272 ^a (n = 16)
Male	SJ-13_001840	Pericentromeric region	1.662 ± 0.305 ^b (n = 12)
	MSj68-16-2	Pericentromeric region	1.584 ± 0.162 ^b (n = 16)
Gametophyte (dual-color FISH)			
Female	SJ-f_000170 and MSj68-58-2	Interstitial region of short arm	2.138 ± 0.351 ^a (n = 15)
Male	SJ-13_001840 and MSj68-16-2	Pericentromeric region	1.632 ± 0.272 ^b (n = 15)
Sporophyte (dual-color FISH)			
	SJ-f_000170	Interstitial region of short arm	2.223 ± 0.411 ^a (n = 15)
	SJ-13_001840	Pericentromeric region	1.737 ± 0.305 ^b (n = 15)

*The superscript different letters indicate that there is a significant difference ($P < 0.05$) between each two set of chromosome length.

female chromosomes whereas SJ-13_001840 and MSj68-16-2 to male chromosomes. When the probes SJ-f_000170 and MSj68-58-2 were used to hybridize the female gametophyte metaphase nuclei, it was interesting to find that each signal was situated at the interstitial region of the short arm of hybridized chromosomes (Supplementary Figure 4). Meanwhile, the

probes SJ-13_001840 and MSj68-16-2 were hybridized to the male gametophyte chromosomes near the centromeres (Supplementary Figure 4). In the latter case, both the arms of hybridized chromosomes were nearly equal to each other in length. On the average, the length of chromosome hybridized by the probe SJ-f_000170 (2.068 ± 0.180 , $n = 10$) was nearly

equal to that by MSj68-58-2 (2.106 ± 0.272 , $n = 16$) in the female gametophytes (Table 1). Similarly, in the male gametophytes of *S. japonica*, the size of chromosome probed by SJ-13_001840 (1.662 ± 0.305 , $n = 12$) was not significantly different from that by MSj68-16-2 (1.584 ± 0.162 , $n = 16$) (Table 1). Although the mono-color FISH images showed the similar appearance and signal locations of the hybridized chromosomes within the female or male gametophytes (Supplementary Figure 4 and Table 1), it was not understood that whether the chromosome hybridized by the labeled SJ-f_000170 as a probe might be the same as that by MSj68-58-2, and whether the labeled SJ-13_001840 might be hybridized to the same chromosome as the marker MSj68-16-2.

To provide experimental evidence, dual-color FISH technique was employed. With the probes labeled by different colors, the hybridized metaphase chromosome image (upper panels in Figure 2) showed that SJ-f_000170 and MSj68-58-2 were co-localized at the same chromosome of the kelp female gametophytes. They occurred in the interstitial region of the short arm of the hybridized chromosomes (Figure 2), consistent with the mono-color FISH results (Supplementary Figure 4). In the same manner, the male-specific markers SJ-13_001840 and MSj68-16-2 were observed to be present near the centromeres of the hybridized chromosome of the kelp male gametophytes (upper panels in Figure 3). The arranged chromosomes by their decreasing length indicated that the markers SJ-f_000170 and MSj68-58-2 were situated on Chromosome 2 of the kelp female gametophytes (lower panel in Figure 2) while the markers SJ-13_001840 and MSj68-16-2 resided on Chromosome 8 of the male gametophytes (lower panel in Figure 3). As aforementioned, these selected markers were developed on the basis of the sequences of several sex-linked markers from the SDRs of *Ectocarpus* (Lipinska et al., 2015; Zhang et al., 2018), and MSj68-16-2 was the partial DNA sequence of the gene *SjHMG* that was reported to be expressed only in the male gametophytes of *S. japonica* (Zhang et al., 2019, 2021). Taken together with the dual-color FISH images (Figures 2, 3), SJ-f_000170 and MSj68-58-2 were proposed to be located at the female SDR while SJ-13_001840 and MSj68-16-2 at the male SDR of *S. japonica*. As a result, one of SJ-f_000170 and MSj68-58-2 or of SJ-13_001840 and MSj68-16-2 could be chosen as reliable markers to discern the putative sex chromosomes in the sporophyte metaphase nuclei of *S. japonica*.

To aim at simultaneously discerning the putative sex chromosomes, SJ-f_000170 and SJ-13_001840 were taken as probes into *S. japonica* sporophyte chromosomes using dual-color FISH technique. The FISH images (Figure 4 and Supplementary Figure 5) illustrated that the two markers SJ-f_000170 and SJ-13_001840 were located on different chromosomes in the sporophyte metaphase nuclei as expected. Upon karyotype analysis only according to the decreasing length of chromosomes, it was found that SJ-f_000170 and SJ-13_001840 were situated on the paired Chromosomes 2 and 8 (data not shown), the same order as those in the gametophyte nuclei (Figures 2, 3, respectively). It should be pointed out that, however, when homologous chromosomes paired in the prophase stage of meiotic nuclei, SJ-f_000170

and SJ-13_001840 should reside on the independently paired chromosomes as shown in the lower panel of Figure 4 if the hybridized chromosomes were the putative sex ones U and V in *S. japonica*. In addition, the locations of these markers on the sporophyte metaphase chromosomes hybridized by SJ-f_000170 and SJ-13_001840 were observed in the interstitial region of short arm and pericentromeric region (Figure 4 and Table 1), respectively, which was similar to the observations in the gametophyte nuclei (Figures 2, 3). Length of the hybridized chromosome by the labeled probe SJ-f_000170 ($2.223 \pm 0.411 \mu\text{m}$, $n = 15$) was different from that by SJ-13_001840 ($1.737 \pm 0.305 \mu\text{m}$, $n = 15$), also corresponding well to the obtained data from the gametophytes (Table 1). These pieces of information demonstrated that the putative sex chromosome for the determination of female was larger than that for male in *S. japonica*, thus consistent interestingly with the observations by Evans (1963, 1965) and Yabu and Yasui (1991) in several species of the Laminariales, even if this putative large U chromosome was not too conspicuous.

Genomic Features of the Selected Bacterial Artificial Chromosome Clones Containing the Kelp U-Linked Markers

As suggested by Ming and Moore (2007) and confirmed experimentally by Lipinska et al. (2017), genomic sequence comparisons of sex chromosomes could uncover their common features. Accordingly, several pieces of genomic information on the aforementioned four sex-linked markers and their surroundings in *S. japonica* were searched and compiled in Supplementary Table 2. Blast searching the assembled genomes of *S. japonica* (Ye et al., 2015; Shao et al., 2019; Fan et al., 2020) with each of the U-linked markers as a query indicated that SJ-f_000170 was not able to be searched from the kelp gametophyte genome (Ye et al., 2015; Fan et al., 2020), but it matched with Contig4871 (6,269 bp) from the sporophyte one (Shao et al., 2019) with 100% sequence identity (639 bp) (Supplementary Table 2). In the same way, MSj68-58-2 was found to match with Contig3481 (188,527 bp) from the sporophyte one (Shao et al., 2019) with 100% sequence identity (1,105 bp), whereas it matched with Scaffold220 (444,739 bp) from the kelp gametophyte genome (Ye et al., 2015) only with 52% (573/1,105 bp) sequence identity (Supplementary Table 2). Furthermore, while aligning one searched Contig3481 with the other Scaffold220 (Supplementary Figure 6A), it was found there was no identity between them. The uncertainty of the screened two assembled sequences Contig3481 and Scaffold220 prevented them from applying to the genomic feature elucidation. The present study therefore provided some pieces of information inferred from a BAC library of *S. japonica*. Since this BAC library has been constructed only from the kelp female gametophytes (Liu P. F. et al., 2021), the genomic feature related to U chromosome was elucidated.

With the designed primers based on the sequences of SJ-f_000170 and MSj68-58-2, two BAC clones, BAC669-A11 and BAC652-P6 encompassing the two markers, respectively, were screened using PCR technique (Supplementary Figure 7). Using

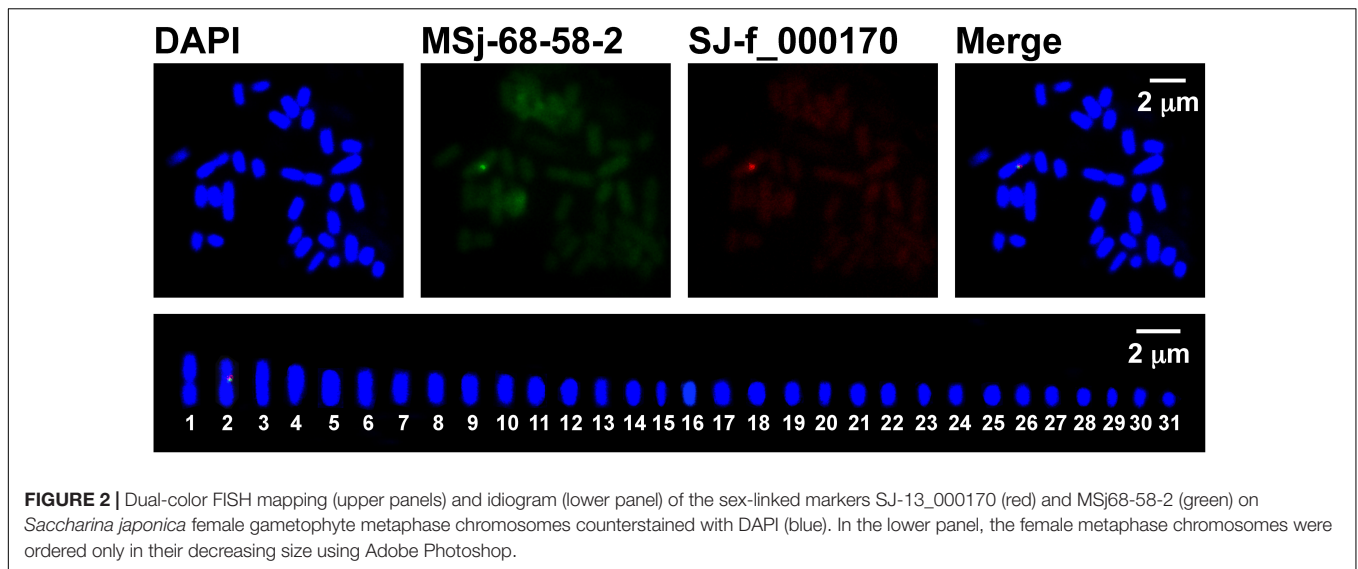


FIGURE 2 | Dual-color FISH mapping (upper panels) and idiogram (lower panel) of the sex-linked markers SJ-13_000170 (red) and MSj68-58-2 (green) on *Saccharina japonica* female gametophyte metaphase chromosomes counterstained with DAPI (blue). In the lower panel, the female metaphase chromosomes were ordered only in their decreasing size using Adobe Photoshop.

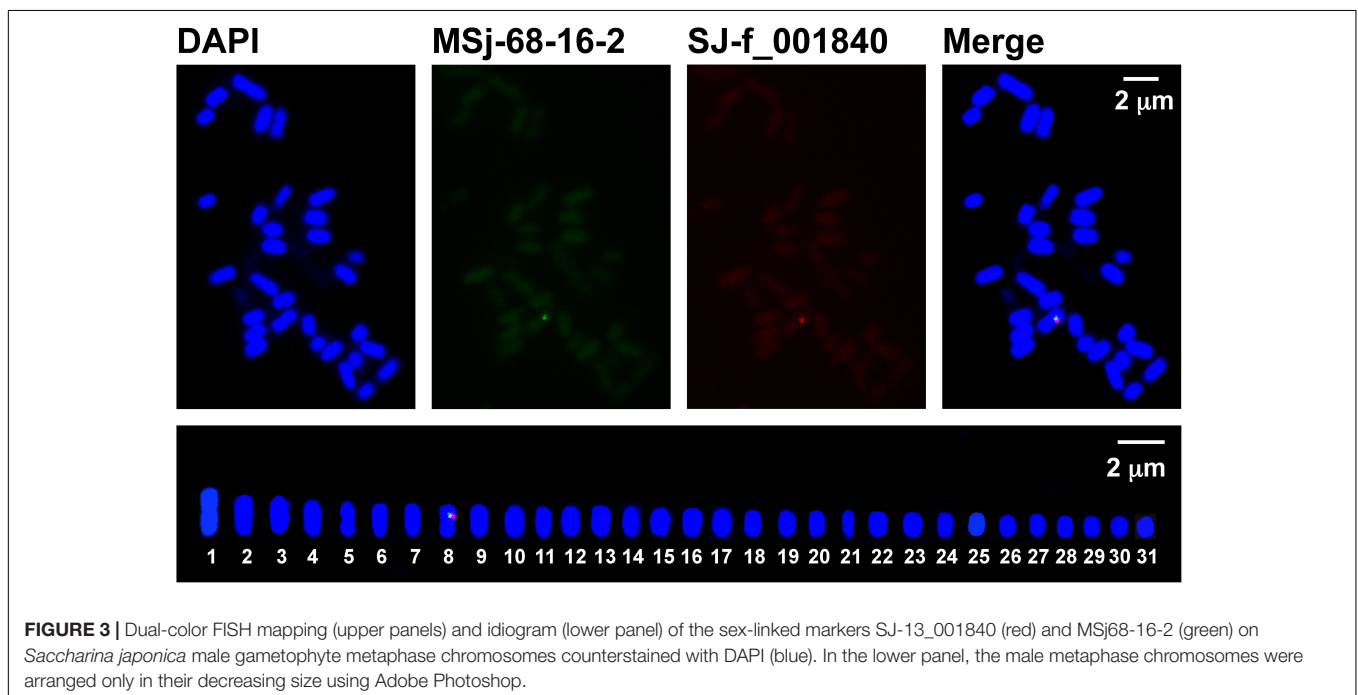
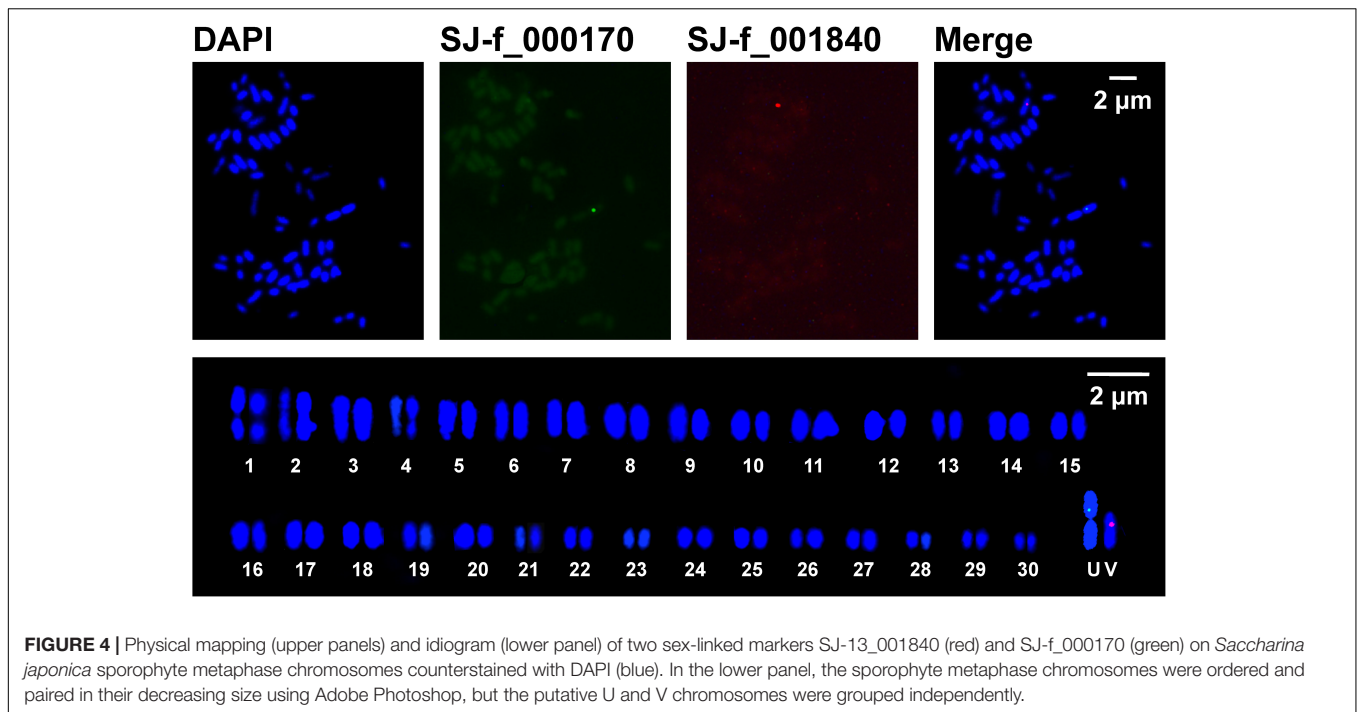


FIGURE 3 | Dual-color FISH mapping (upper panels) and idiogram (lower panel) of the sex-linked markers SJ-13_001840 (red) and MSj68-16-2 (green) on *Saccharina japonica* male gametophyte metaphase chromosomes counterstained with DAPI (blue). In the lower panel, the male metaphase chromosomes were arranged only in their decreasing size using Adobe Photoshop.

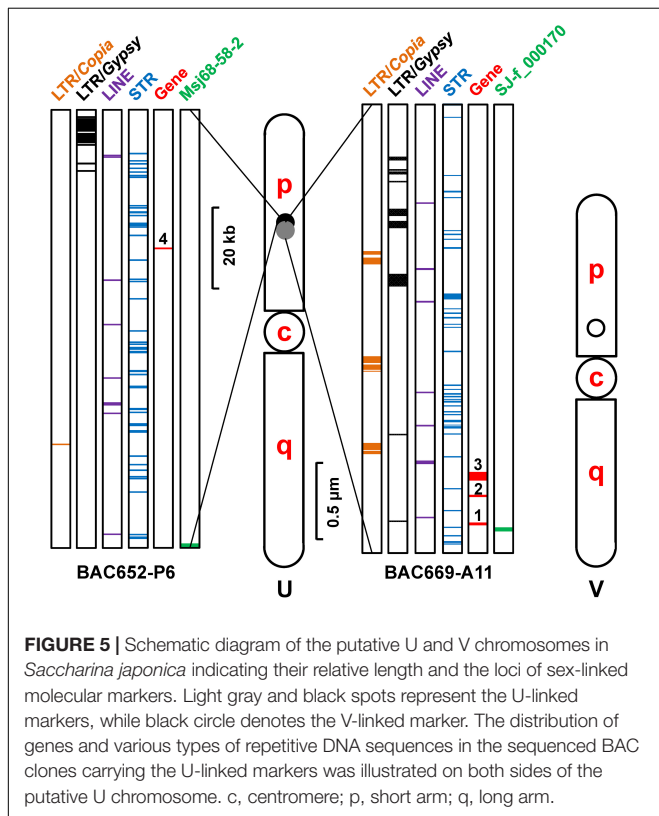
PacBio RS II sequencing platform, total 1,600,988,320 and 1,500,167,532 bases (**Supplementary Table 3**) corresponding to the BAC clones BAC669-A11 and BAC652-P6 were obtained. Through raw reads assembling, full-length sequences of these two inserts were sequenced, and they were 110,085 and 104,111 bp in length (**Supplementary Table 3**). While querying these two sequences to the primarily assembled genome of *S. japonica* (Ye et al., 2015), it was found that 55.69% of BAC669-A11 (from 10,880 to 72,183 bp) could be assigned to several scaffolds of this kelp genome whereas only 25.81% BAC652-P6 (from 88 to 28,496 bp) could be done (**Supplementary Table 4** and **Supplementary Figures 8A,C**). Excitedly, the coverage was evidently increased while searching the target sequences

in another currently released genome of *S. japonica* (Shao et al., 2019). The coverage was up to 82.59 and 92.56% for BAC669-A11 (from 1 to 85,983 bp) and BAC652-P6 (from 1 to 96,362 bp), respectively, even if each BAC clone was still assigned to a number of different contigs (**Supplementary Table 4** and **Supplementary Figures 8B,D**). The difference of the blast search results between the two assembled kelp genomes possibly resulted from the number and length of gaps as shown in **Supplementary Figure 8**. While searching the assembled 31 pseudo-chromosomes of the *S. japonica* gametophyte genome (Fan et al., 2020), the coverage was up to 94.13 and 98.76% for BAC669-A11 (from 1,601 to 105,224 bp) and BAC652-P6 (from 1 to 102,820 bp), respectively, even if each BAC clone



was still assigned to at least 9 and 12 pseudo-chromosomes (Supplementary Table 4). From these blast results, it was inferred that our sequencing data from the selected BAC clones might be

authentic, though there was no well match in the 580.5 million base pairs (Mbps) of genome sequence of *S. japonica* (Liu et al., 2019) due to the only available raw data deposited under the Bioproject accession of PRJNA280905. The insert sequences of the two BAC clones have been deposited in GenBank under the accession Nos. MZ285758 and MZ285759.



Physical mapping (Figure 5) of the inserts of the BAC clones indicated that the U-linked marker SJ-f_000170 was located upstream of BAC669-A11, while the U-linked marker MSj68-58-2 was at the beginning of BAC652-P6. A great number of repetitive sequences (Supplementary Table 5), such as short tandem repeats (STRs) and retrotransposons including long terminal repeat (LTR)/Gypsy, LTR/Copia, long interspersed nuclear element (LINE)/L1, and LINE/RTE-BovB, scattered in the inserts of these two BAC clones. The STRs mainly contained mono-(e.g., A, G, C, and T), di-(e.g., GT, AT, and AC), tri-(e.g., CAG, GCA, TGC, ACA, CTG, and GCT), and tetra-(e.g., ATGT, GTAC, TGTT, and GGTG) nucleotide repeats (Supplementary Table 5). The repetitive elements including LTR, LINE, and STR, accounted for 45% of the full-length sequences of inserts (Table 2), which was higher than the average one that was 39 or 43% of *S. japonica* genome as estimated by Ye et al. (2015) and Liu et al. (2019), respectively, suggesting they were non-randomly distributed in the kelp genome. In addition, four genes were *de novo* annotated in these two sequenced inserts of BAC clones, and they were designated as Genes 1, 2, and 3 in BAC669-A11, while only one gene (Gene 4) was annotated in BAC652-P6 (Figure 5). The gene density (0.78%, Table 2 and Supplementary Table 6) corresponding to 18.69 genes per Mbp in these two BAC clones, therefore, was significantly poorer than the average one that was 3.66% (i.e., 34.07 genes per Mbp) or 6.99% (i.e., 61.54 genes per Mbp) (Table 2) as surveyed by

TABLE 2 | Information comparison between SDR of U chromosome and its corresponding genome from various eukaryotes.

	<i>Saccharina japonica</i>			<i>Ectocarpus</i> sp.		<i>Marchantia polymorpha</i>	
	BAC clones	Genome		SDR	Genome	SDR	Genome
Gene density (proportion of coding sequence)	0.78%	3.66%	6.99%	1.84%	12.98%	1.27%	9.50%
Proportion of LTR elements	8.8%	8.4%	8.4%	5.8%	7.0%	29.3%	9.7%
Proportion of repetitive elements	45%	39%	43%	33%	22.7%	70.78%	23.4%
GC content	50.99%	49.1%	48.7%	44.74%	54.02%	42.5%	42.2%
References	This work	Ye et al., 2015	Liu et al., 2019	Cock et al., 2010; Ahmed et al., 2014		Bowman et al., 2017	

Ye et al. (2015) and Liu et al. (2019), respectively, in their reported kelp genomes.

In short, both relatively high proportion of repetitive elements and poor gene density in the sequenced inserts of the BAC clones shared the genomic features about the SDR of *Ectocarpus* sp. or *Marchantia polymorpha* U chromosome (Table 2). Taken together with the similar location of the two markers SJ-f_000170 and MSj68-58-2 on the same chromosome in the kelp female gametophyte nuclei (Figure 2), the region where the two markers located was proposed to be the non-recombining SDR of the *S. japonica* U chromosome.

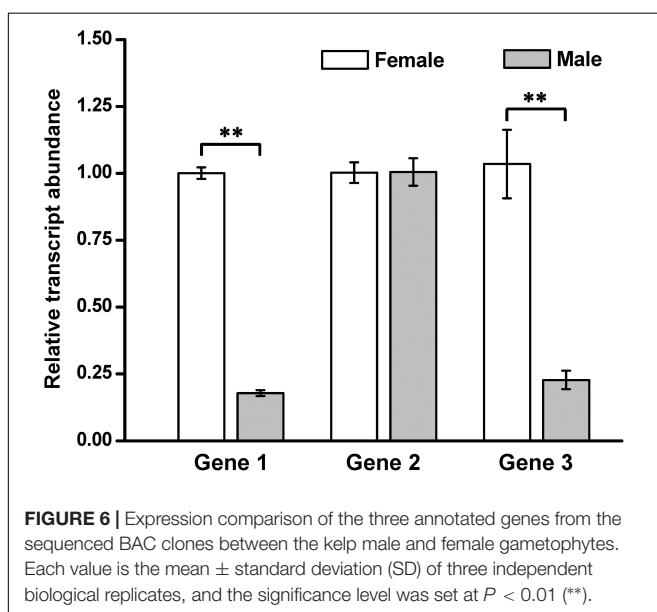
Annotated U-Linked Genes and Their Transcription in the Male and Female Gametophytes

As described above, four genes were *de novo* annotated using AUGUSTUS program from these two sequenced BAC clones. Among them, Genes 1 and 2 were also annotated by Ye et al. (2015) and Shao et al. (2019) in the released *S. japonica* genomes. Using the genomic DNA extracted from *S. japonica* female gametophyte as a template (Supplementary Figure 9), these four genes could be amplified successfully. Sequencing analyses of the amplified products demonstrated that Gene

1 had one intron whereas all the other three genes had no intron (Supplementary Table 7). The results suggested that all the four annotated genes would be active in the kelp female gametophyte genome, but their functions remained to be characterized. Surprisingly, Genes 1, 2, and 3 were also amplified (Supplementary Figure 9 and Supplementary Table 7) from the male gametophyte of *S. japonica*. Comparative analysis of these amplicons showed that these annotated three genes have conserved intron and exon structures but varied sequences between the kelp male and female gametophytes (Supplementary Table 7).

Gene 1, locating just downstream (from 5,400 to 6,034 bp) of the sex-linked marker SJ-f_000170 in BAC669-A11 (Figure 5), was found to completely overlap the DNA sequence of SJ-f_000080 in *S. japonica*. SJ-f_000080, an ortholog of Ec-sdr-f_000080 in *Ectocarpus* sp., has been already described as one of the sex-linked ancestral genes in *S. japonica* by Lipinska et al. (2017). Genes 2 and 3 also annotated from BAC669-A11 were 258 and 2,163 bp in length, respectively, while Gene 4 from the other BAC clone, BAC652-P6, was 252 bp long. Function of these gene products was unknown. However, it was of interest that part (from 77 to 134 bp) of Gene 4 shared highly similarity with the partial sequence (from 401 to 458 bp) of a sex discriminating marker m68_58_3f (GenBank accession No. KP994179) which was developed from *Laminaria digitata* by Lipinska et al. (2015).

To understand transcription of these annotated four genes in the male and female gametophytes of *S. japonica*, qRT-PCR was employed in the present study. The conserved coding sequences of these genes were used to design the qRT-PCR primers. Genes 1 and 3 were expressed fivefold more abundant at the transcription level in the female gametophytes than them in the male ones ($t_2 = 17.183$, $P = 2.02 \times 10^{-5} < 0.01$ for Gene 1; $t_2 = 3.562$, $P = 0.009 < 0.01$ for Gene 3) whereas Gene 2 was transcribed in the similar abundance or constitutively ($t_2 = -2.58$, $P = 0.975 > 0.05$) between the male and female gametophytes (Figure 6). Interestingly, Gene 4, annotated from the insert sequence of BAC652-P6, was found to be expressed exclusively in the female gametophytes (Supplementary Figure 10). It was thus referred to a sex-specific gene as defined by Ellegren and Parsch (2007), while Genes 1 and 3 were the sex-biased genes. Combining the transcription of Gene 4 with its un-amplified product with the extracted genomic DNA as a template from the kelp male gametophytes suggested that Gene 4 was a potential candidate for the female sex-determining factor. In contrast to this, the other three genes, being transcribed differentially (for Genes 1 and 3) or similarly (for Gene 2) in abundance between



the male and female gametophytes (**Figure 6**), were proposed to be *gametologue* pairs in *S. japonica* according to the termed definition by Coelho et al. (2018) and Umen and Coelho (2019). These annotated genes were thereby characterized as SDR genes in transcription as suggested by Lipinska et al. (2015).

DISCUSSION

For more than half a century since the finding of a large chromosome in the laminarian nucleus (Evans, 1963), the existence of sex chromosomes in *S. japonica* has been an open problem to be solved (Tai and Fang, 1977; Yabu and Yasui, 1991). But now, the developed sex-linked markers has provided us with an opportunity to understand whether *S. japonica* has sex chromosomes by use of FISH technique. Two sex-linked markers SJ-f_000170 and SJ-13_001840 have been proved present in the kelp genome by Southern blotting analysis (**Figure 1**) and then co-localized on different chromosomes in the sporophyte metaphase nuclei of *S. japonica* by dual-color FISH technique (**Figure 4** and **Supplementary Figure 5**). The two different chromosomes hybridized by the two sex-linked markers are suggested to be the putative sex chromosomes U and V in this kelp for the following three reasons.

First, the two sex-linked markers SJ-f_000170 and SJ-13_001840 employed in the present study were developed by Lipinska et al. (2017) on the basis of one *gametologue* pair, Ec-sdr_f_000170 and Ec-13_001840 in the *Ectocarpus* SDRs, respectively. The latter two were found to be highly conserved in six brown seaweeds including *S. japonica* by Lipinska et al. (2017) *via* genomic comparison. In the present study, the hybridization signals on the chromosomes by the labeled sex-linked markers SJ-f_000170 and SJ-13_001840 (**Figure 4** and **Supplementary Figure 5**) illustrated that such a chromosomal SDR could be visualized first by cytogenetic technique, thus suggesting that it would be present evidentially in *S. japonica*. Dual-color FISH mapping of the other two markers, MSj68-58-2 and MSj68-16-2, showed that they were co-localized at the similar loci with SJ-f_000170 (**Figure 2**) and SJ-13_001840 (**Figure 3**), respectively. Moreover, the marker MSj68-58-2 was originally derived from the *Ectocarpus* SDR genes as described by Lipinska et al. (2015). It was hence inferred that all the four sex-linked markers used for FISH mapping were located at the chromosomal SDRs of *S. japonica*. Since SDRs carried a set of genes that determined the sex of individuals (Coelho et al., 2018), the chromosomes where these sex-linked genes resided were referred to U and V sex chromosomes of *S. japonica*.

Second, SDR is a non-recombining region of sex chromosomes (Hobza et al., 2015; Umen and Coelho, 2019) though the size of this region differs between species. The absence of recombination leads to gene degeneration and repetitive DNA sequence accumulation on plant (Kejnovsky et al., 2009) and brown seaweed (Ahmed et al., 2014) SDRs. The present study exemplifies only the U chromosome SDR for lack of information about large fragment of V chromosome so far in *S. japonica*, even if 59 V-linked scaffolds have been identified from the *S. japonica* female gametophyte reference genome sequence (Ye et al., 2015)

by Lipinska et al. (2017) *via* data mining. The repetitive elements distributed in the inserts of two sequenced BAC clones (**Figure 5**) account for 45%. This percentage is intermediate between *Ectocarpus* SDR (33%) and liverwort SDR (70.78%), but higher than the average one of the *S. japonica* genome (**Table 2**). These data at least suggests that the inserts of the two BAC clones from *S. japonica* female gametophytes share a common genomic feature with other SDRs in repetitive sequences. In addition, Fraser and Heitman (2005) and Hobza et al. (2015) proposed that rapid accumulation of repeat sequences especially of the transposable elements as reviewed by Gschwend et al. (2012) and Charlesworth (2013) contributed well to SDR expansion. As a result of accumulation of more repeat sequences (**Table 2** and **Figure 5**), the SDR of *S. japonica* U chromosome is expected to be expanded in size as compared to the U SDR of *Ectocarpus* (see below for more details).

Third, it is generally accepted that sex chromosomes evolve from autosomes through the acquisition of genes involved in sex determination (Kejnovsky et al., 2009; Coelho et al., 2018). In the process of evolution, the gene density in SDRs tends to decline accompanied by repetitive elements acquisition with concomitant gene loss. This tendency has been found in several eukaryotes such as *Volvox carterii* (Ferris et al., 2010), *Ectocarpus* sp. (Ahmed et al., 2014), and *Marchantia polymorpha* (Bowman et al., 2017) with UV or UV-like (mating-type loci same as SDRs) sex determination system. Compared to the genomic data of *S. japonica* as documented by Ye et al. (2015) and Liu et al. (2019), the gene density of the two sequenced inserts of BAC clones in the present study is nearly one fifth or even down to approximately one ninth, respectively, of that in genome (**Table 2**). The low gene density from these sequenced BAC clones is comparable to that in either *Ectocarpus* or *M. polymorpha* U chromosome SDR (**Table 2**), thus exhibiting the common feature of the SDRs of U chromosomes in this regard.

In brief, both the employed sex-linked markers in the cytogenetic mapping and genomic features of the insert sequences of two BAC clones showed that they have the SDR characteristics, thus implying that the chromosomes where they colonized might be sex chromosomes of *S. japonica*.

Now let's go back to the question of estimating the size of *S. japonica* U chromosome SDR. As mentioned above, either U-linked genes or markers SJ-f_000170, MSj68-58-2, SJ-f_000010, and SJ-f_000080 are, respectively, corresponding to the Ec-sdr_f_000170, Esi_0068_0058, Ec-sdr_f_00010, and Ec-sdr_f_00080 in *Ectocarpus* SDR (Lipinska et al., 2017) by synteny analysis (**Supplementary Figure 11**). Of them, SJ-f_000170, SJ-f_000080 (or Gene 1), and MSj68-58-2 are found to be present in the sequenced inserts of the screened two BAC clones BAC669-A11 and BAC652-P6 (**Figure 5** and **Supplementary Figure 8**). Although SJ-f_000010 is not screened from these two BACs, it is co-located with MSj68-58-2 in Contig3481 screened from the assembled kelp sporophyte genome (Shao et al., 2019) as shown in **Supplementary Figure 11**. The sequence of Contig3481 indicates that SJ-f_000010 is downstream of MSj68-58-2 approximately 0.028 Mbp. Therefore, the size between SJ-f_000170 and SJ-f_000010 is estimated to be 0.238 (= 0.102 + 0.108 + 0.028) Mbp (**Supplementary Figure 11**), regardless of the physical gap

present between the two BAC clones. This size is thus larger than that of the syntenic one which has been estimated by Ahmed et al. (2014) to be around 0.176 Mbp apart from one homologous marker Ec-sdr_f_000170 to the other Ec-sdr_f_000010 in the female SDR of *Ectocarpus*. In addition, Pedersen and Lind-Laursen (1995) suggested that a minimum of 5 – 10 Mbp distance would be necessary to resolve FISH signals of two DNA probes on metaphase chromosomes in a FISH mapping profile. From both the similar site but in-completely overlapping location of SJ-f_000170 and MSj68-58-2 on the hybridized metaphase chromosome (**Figure 2**) and the real presence of physical gap between these two sequenced BAC clones, SDR of *S. japonica* U chromosome is inferred to be unambiguously larger than the *Ectocarpus* one. This is also reflected by the aforementioned prediction based upon the higher proportion of repetitive elements in the inserts of the two selected BAC clones (**Table 2**). The complete SDR sequence and its size of the kelp U chromosome will be unveiled, once it is assembled by screening and sequencing of more BAC clones.

With respect to V chromosome, the unavailable BAC library constructed from the male gametophytes of *S. japonica* limits the exploration in the present study. Nevertheless, while searching the assembled genomes of *S. japonica* (Ye et al., 2015; Shao et al., 2019; Fan et al., 2020) with each of the V-linked markers as a query, SJ-13_001840 was found to match with both Contig4772 (10,276 bp) from the sporophyte one (Shao et al., 2019) and Scaffold3074 (24,639 bp) from the kelp gametophyte genome (Ye et al., 2015; **Supplementary Figure 6B**). In the same way, Scaffold1744 and Contig2278 have been searched online with the V-linked marker MSj68-16-2 as a query. Upon aligning, several identical regions are found to be present between Contig4772 and Scaffold3074 or between Scaffold1744 and Contig2278. These regions look matched better than the alignment with the searched sequences (**Supplementary Figure 6A**) using each of the U-linked markers as a query, even if there remained a lot of gaps (**Supplementary Figures 6B,C**). In addition, the V-linked marker MSj68-16-2 is a part of DNA sequence of this gene *SjHMG* which has been expressed exclusively in the kelp male gametophytes (Ahmed et al., 2014; Lipinska et al., 2017; Zhang et al., 2019, 2021), and the cumulative size of 59 V-linked identified scaffolds is up to approximately 4.91 Mbp (Lipinska et al., 2017). These data are expected to help uncover the genomic features of V chromosome SDR, once the BAC library is constructed from the male gametophytes of *S. japonica*. It is pointed out here, however, that the reference genomic draft of *S. japonica* has been sequenced and assembled from the female gametophytes rather than male ones as stated by Ye et al. (2015). Since the gene *SjHMG*, which has been exclusively expressed in male gametophytes as experimentally proved by Zhang et al. (2019, 2021), could be screened from the genomic

data by Lipinska et al. (2017), the female gametophyte materials used for the obtaining of the kelp genomic data are at least supposed to be contaminated by male gametophyte.

In a word, the present study offers first insights into cytogenetic localization of sex-linked molecular markers, thus enabling us to distinguish the putative sex chromosomes U and V from the autosomes in *S. japonica*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, MZ285758; <https://www.ncbi.nlm.nih.gov/genbank/>, MZ285759.

AUTHOR CONTRIBUTIONS

YD carried out most experiments and written the original draft. P-FL searched sex-linked markers in *S. japonica* and screened the target BAC clones. ZL and QZ repeated the FISH experiment. Y-HB analyzed all these data with YD and confirmed the results in addition to the primary writing of this manuscript. Z-GZ made a significant contribution to the conceptual design of this work and the writing and polishing of this manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.821603/full#supplementary-material>

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