

# Development of DNA markers that distinguish male and female haploid germlings of the brown alga, *Cladosiphon okamuranus*

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## SUMMARY

Okinawa mozuku, *Cladosiphon okamuranus*, is one of many edible brown algae, yielding 22 Kton annually and contributing ¥5 billion to the Japanese economy. The life cycle of *C. okamuranus* is complicated, since the alga has self-cloning life cycles in both haploid (N, male and female) and diploid (2N) conditions, but only diploid “seeds” (germlings) become edible sporophytes. Because haploid and diploid germlings are morphologically indistinguishable, haploid germlings are often mistakenly combined with diploid germlings for cultivation, which results in less efficient harvesting of mozuku. Sexual identification of haploid germlings is essential to develop better diploid strains by crossbreeding. With this aim, we performed RNA-seq analysis of haploid germlings of *C. okamuranus*. Using its decoded diploid genome and transcriptomic information, we identified 269 genes that are expressed specifically in male or female haploids. BLAST analysis with *Ectocarpus siliculosus* gene models revealed that nine of 269 genes were putative sex determination-related genes of *C. okamuranus*. A unique set of polymerase chain reaction primers for these nine genes was designed, and DNA amplification using primers enabled us to distinguish male and female haploid and diploid germlings. This tool will enable mozuku farmers to select diploid germlings free of haploid germlings. Using this DNA marker technique, the amount of mozuku cultivated in Okinawa is expected to increase.

Key words: brown alga cultivation, *Cladosiphon okamuranus*, diploid, haploid male and haploid female germlings, marker genes for each phase, polymerase chain reaction primers.

## INTRODUCTION

Brown algae are significant primary producers in marine ecosystems. Since ancient times, they have been an important food resource, and recently they have been cultivated commercially for this purpose. In Japan, many edible brown algae (class Phaeophyceae) include members of the order Laminariales, including “kombu”, *Saccharina japonica* (Areschoug) C.E. Lane, C. Mays, Druehl & G.W. Saunders and “wakame”, *Undaria pinnatifida* (Harvey) Suringar, and the order Chordariales, “Okinawa mozuku”, *Cladosiphon okamuranus* Tokida and

“ito-mozuku”, *Nemacystus decipiens* (Suringar) Kuckuck. Especially in Okinawa, the southwestern prefecture of Japan, *C. okamuranus* (Fig. 1a) and *N. decipiens* (Fig. 1b) have been cultivated since the 1980s, leading to the establishment of several unique strains of mozuku, including the S-strain that was found at Iheya Island, the K-strain from Katsuren, the O-strain from Onna, and the C-strain from Chinen (Fig. 1a). Approximately 22 500 tons of *C. okamuranus* and *N. decipiens* (worth ¥5 billion) were produced in fiscal year (FY)2020. In addition to demand for these algae as food, *C. okamuranus* and *N. decipiens* are sources of fucoidan (Tako *et al.* 1999), a sulfated polysaccharide with anti-coagulant, anti-thrombin-like, and tumor-suppressant activities (Baba *et al.* 1988). For these reasons, cultivation of mozuku (herein, both *C. okamuranus* and *N. decipiens*, unless otherwise mentioned) has been increasing. For the last three or four decades, Okinawan fishermen have expanded cultivation “fields” to produce larger quantities of mozuku along the seashore close to the coral reefs. However, availability of fields is limited. In addition, brown-alga aquaculture has also been threatened by seawater warming and acidification due to global environmental changes.

Mozuku has a unique and complicated life cycle compared to land plants and animals (Shinmura 1977 and Fig. 2a). In Okinawa, preparation for mozuku cultivation generally starts in October. First, diploid germlings, so-called “seeds”, are cultured with cultivation nets in tanks (Fig. 2b). After attaching the germlings to the net and observing budding, mozuku nets are put in the ocean. Two to four months later, mature mozuku sporophytes are harvested using pumps. Mozuku is cultivated and harvested as sporophytes that originate from diploid (2N) germlings (Figs. 2a, 3a, and S1a).

However, mozuku cultivation presents three major problems that need to be overcome. As mentioned above, diploid germlings produce edible sporophytes, whereas haploid male and female germlings do not (Fig. 2a). Since diploid and haploid germlings exhibit similar morphology, it is difficult to distinguish them by eye (Figs 3 and S1). To the extent that

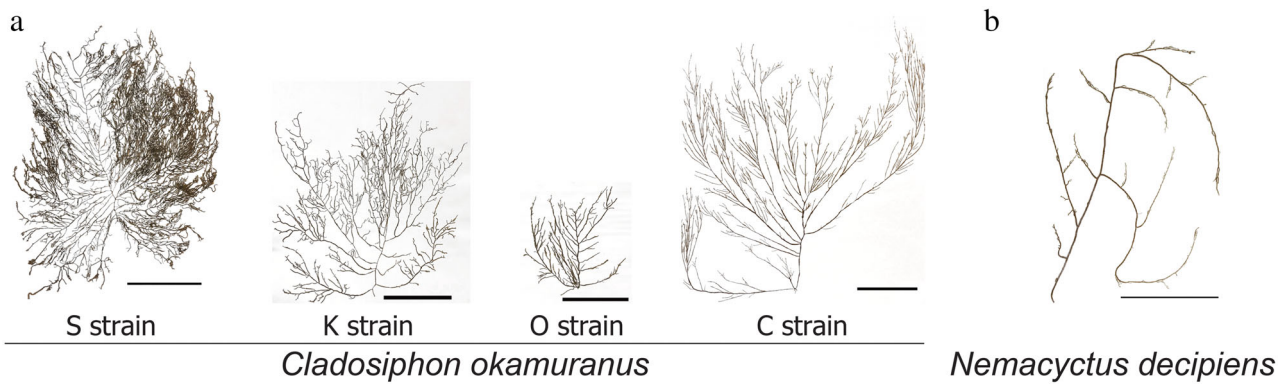
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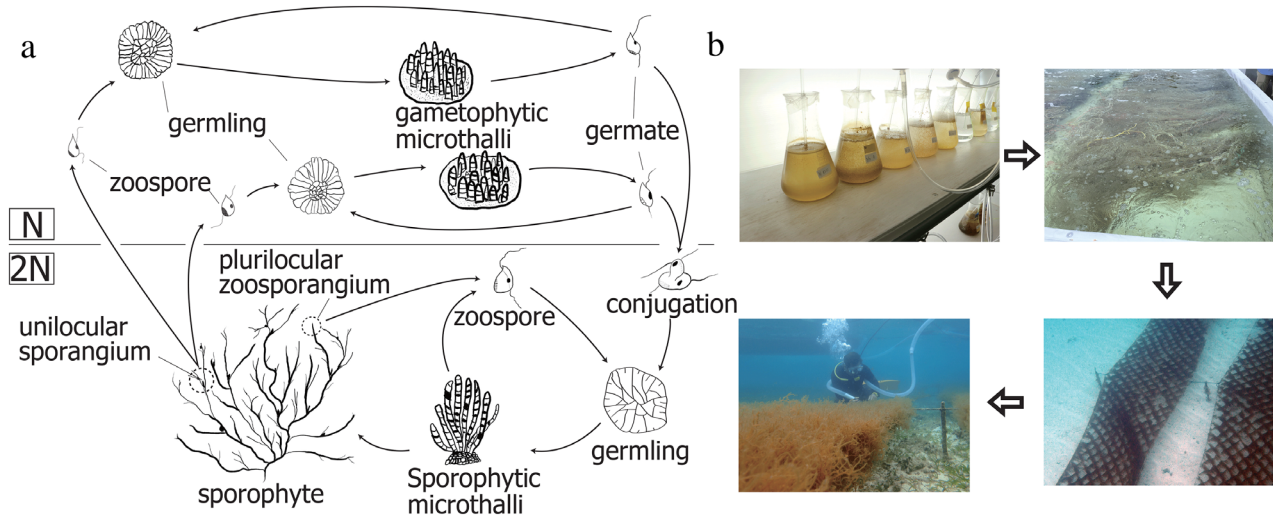
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**Fig. 1.** Two species of mozuku cultivated in Okinawa, (a) *Cladosiphon okamuranus* and (b) *Nemacystus decipiens*. (a) Four strains of *C. okamuranus*, isolated and established since the 1980s, are shown. From left to right, the S-strain from Iheya Island, the K-strain from Katsuren, the O-strain from Onna, and the C-strain from Chinen. These strains have distinctive morphologies. (b) The Onna-1 strain of *N. decipiens*. The morphology of *N. decipiens* is similar to that of *C. okamuranus*, although its cultivation is more difficult. Scale bars: 10 cm.



**Fig. 2.** The life cycle and cultivation of *Cladosiphon okamuranus* in Okinawa. (a) The life cycle of mozuku. Both diploid (2N) and haploid (N) germlings reproduce in classical botanical alternation of generations. They cannot be distinguished morphologically. Diploid germlings develop to sporophytes and are used for mozuku cultivation. In contrast, haploid germlings do not develop to sporophytes. (b) Cultivation of mozuku. Diploid germlings are reproduced in tanks (upper left) and attached to nets in the tanks from September to November (upper right). Then, the nets are put into the ocean inside the barrier coral reefs in December and January (bottom right). Finally, mature mozuku sporophytes are harvested using a vacuum pump from March to June (bottom left).

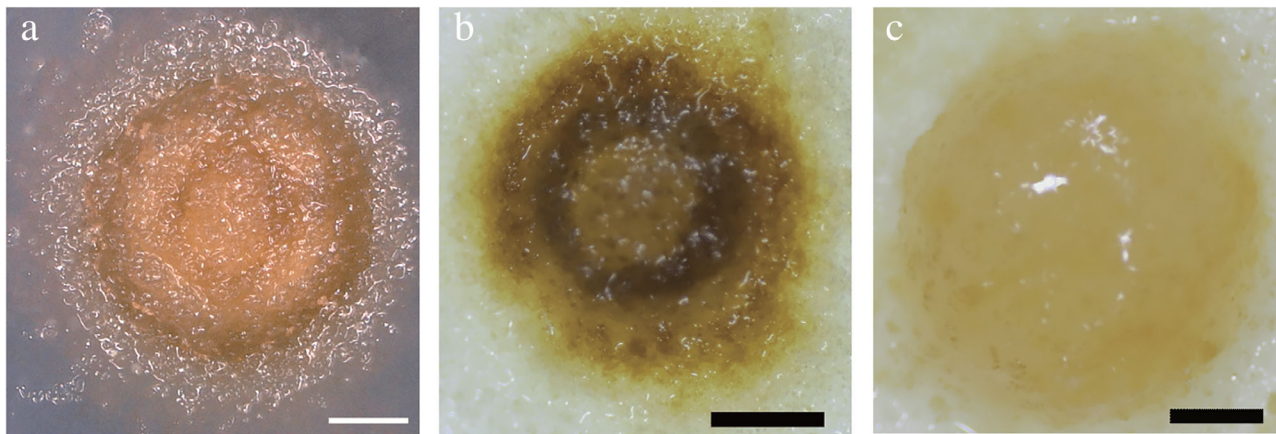
haploid germlings are inadvertently cultivated, the sporophyte harvest is reduced. Therefore, a method to distinguish haploid and diploid germlings would be very useful for mozuku fishermen.

After mozuku nets are put in the ocean, the mozuku sometimes falls off the net (Fig. 2b). Although detached mozuku survives and can produce edible sporophytes, they are impossible to harvest because currents and tides carry them out of the cultivation area. As a result, yields are further reduced, but to date, no method has been developed to prevent detachment or to develop new strains of mozuku that attach more firmly to the nets.

Mozuku growth is disturbed by seawater temperature changes, especially warmer seawater. Due to recent global warming, the amounts of harvested green, red, and brown algae have decreased by 1% every year, including mozuku

(Shendure *et al.* 2017). In particular, higher winter sea temperatures damaged the mozuku yield in FY2010. To develop more heat-tolerant strains of mozuku, selective breeding has just been started, resulting in establishment of four distinct strains of *C. okamuranus* (Fig. 1a). Although crossbreeding of strains is a common technique with land plants, it is difficult to apply to algae. This is mainly because it is impossible to collect male and female germlings separately, so artificial conjugation has not been achieved. Methods to distinguish haploid male and female germlings would enable crossbreeding and could improve cultivation efficiency.

Genomic information facilitates many types of biological research. Since the genome of influenza virus was decoded in 1995, genomes of more than 50 000 species have been submitted to the NCBI database 2019. However, available brown



**Fig. 3.** Germlings with microthalli of *Cladosiphon okamuranus* maintained at the Okinawa Prefectural Fisheries Research and Extension Center (OPFREC). (a) A diploid (2N) germling with microthalli. (b) A haploid germling (tentatively named N-1) with microthalli, which proved to be male. (c) A haploid germling with microthalli (N-2 that proved to be female). Scale bars: 1 mm.

algal genomes comprise only 12 species or strains to date (Nishitsuji 2019). Of those, genomes of four strains of *C. okamuranus* and that of *N. decipiens* have been decoded by our research group (Nishitsuji *et al.* 2016; Nishitsuji 2019; Nishitsuji *et al.* 2020). Although those genomes were decoded in order to understand mozuku biology, we wish to use the genomic information to innovate methods to resolve the three problems of mozuku cultivation mentioned above. Here, we report development of DNA markers that can distinguish germlings of haploid male and female germlings, and diploid germlings of *C. okamuranus*. Genes expressed specifically in haploid or diploid germlings represent markers to identify haploid male or female germlings and diploid germlings. This development provides the first step for effective cultivation of not only mozuku, but also other brown algae.

## MATERIALS AND METHODS

### Brown algal samples

The Okinawa Prefectural Fisheries Research and Extension Center (OPFREC) has isolated and maintained a strain of diploid (2N) mozuku germlings (Figs 3a and S1a) and two strains of haploid germlings tentatively named N-1 (Figs 3b and S1b) and N-2 (Figs 3c and S1c). Diploid and haploid germlings (N-1 and N-2) were distinguishable since the former produce sporophytes. However, sexes of haploid germlings could not be determined at the beginning of this study. OIST was provided with these strains by OPFREC under a material transfer agreement between the two institutions.

### RNA sequencing and identification of genes that are expressed in haploid male and female germlings

Total RNA was isolated from N-1 and N-2 haploid germlings using an RNeasy Plant Mini Kit (Qiagen). RNA-seq libraries of extracted RNAs were prepared using a True-seq stranded mRNA library preparation kit (Illumina). The Illumina MiSeq

platform was used for sequencing with MiSeq reagent V3 kit (600 cycles).

In a previous study, we carried out nuclear genome sequencing, as well as RNA-seq of diploid germlings of *C. okamuranus* (Nishitsuji *et al.* 2016). The transcriptome data and gene models of *C. okamuranus* were downloaded from DRA accession no. DRA004654 and [https://marinegenomics.oist.jp/okinawa\\_mozuku\\_v2/viewer/info?project\\_id=67](https://marinegenomics.oist.jp/okinawa_mozuku_v2/viewer/info?project_id=67), respectively. Adapter sequences of RNA-seq reads were trimmed using trimmomatic software with seed mismatches, 2; palindrome clip threshold, 30; simple clip threshold, 10 parameters (version 0.30) (Bolger *et al.* 2014). Trimmed RNA-seq reads were mapped to *C. okamuranus* mRNA data using Salmon software (version 0.8.2) (Patro *et al.* 2017). Transcriptome abundance was estimated as transcripts per million (TPM). Genes that were expressed in diploid germlings and those of haploid male or female germlings were collected for development of DNA markers.

### BLAST analysis for gene annotation

Candidate marker genes of *C. okamuranus* were annotated using NCBI-BLAST+ software (2.7.1) with *Ectocarpus siliculosus* (Dillwyn) genome data (Camacho *et al.* 2009). Data were downloaded from <https://bioinformatics.psb.ugent.be/gdb/ectocarpusV2/>.

### Primer design and PCR amplification

Using *C. okamuranus* genome information, unique primers to amplify some of the candidate marker gene were designed using Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). To extract *C. okamuranus* genomic DNA, haploid (male/female) and diploid germlings were boiled for 10 min at 95°C. Polymerase chain reaction (PCR) with designed primers and extracted DNA was performed using Tks Gflex DNA Polymerase (TaKaRa). Cycling parameters included an initial denaturation at 94°C for 1 min, denaturation at 98°C for 10 s, annealing at 60°C for 15 s, extension at 68°C for 1 min, 30 cycles, additional extension at 68°C for 1 min,

**Table 1.** RNA-seq summary of *Cladosiphon okamuranus*

Sample type	Number of reads	Number of bp*
Diploid**	68 377 906	12 505 379 634
Haploid (N-1)	24 507 100	3 446 703 366
Haploid (N-2)	25 522 946	3 755 662 054

\*bp, base pairs.

\*\*DRA004654.

**Table 2.** Numbers of expressed genes in *Cladosiphon okamuranus* germlings

Expression stage	Number of expressed genes
Diploid and haploids (N-1 and N-2)	11 220
Diploid and haploid (N-1)	114
Diploid and haploid (N-2)	155
Diploid	115

then a hold at 12°C. PCR products were electrophoresed on 1% agarose gels and were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics). The sequencing reaction for Sanger sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with the default protocol. Then, extra dye was removed using a FastGene Dye Terminator Removal Kit (NIPPON Genetics). Samples were sequenced using SeqStudio Genetic Analyzer (Thermo Fisher Scientific).

## RESULTS

### Gene expression in haploid and diploid germlings

The Illumina MiSeq platform yielded approximately 3.4 and 3.8 Gb of reads for N-1 and N-2 haploid germlings, respectively (Table 1). In addition, approximately 15 Gb of diploid transcriptome reads were obtained in our previous study (Nishitsuji *et al.* 2016). Analyses of RNA-seq data employing the decoded genome of *C. okamuranus* (Nishitsuji *et al.* 2020) showed that 11 220 of 12 999 protein-coding genes of *C. okamuranus* were expressed in both diploid and haploid germlings (Tables 2 and S1). In addition, we found that 114 genes are specifically expressed in diploid + N-1

(Tables 2 and S2), 155 genes in diploid + N-2 (Tables 2 and S3), and 115 genes in 2N germlings, respectively (Tables 2 and S4). Since the aim of this study was to develop primers that distinguish haploid germling genes from diploid germling genes, we hypothesized that these genes with different expression profiles might provide DNA markers to distinguish whether germlings are haploid or diploid.

### Selection and annotation of nine marker genes specific to haploid germlings

The genome of another brown alga, *Ectocarpus siliculosus*, had been decoded, and genes in that genome were well annotated (Ahmed *et al.* 2014; Cormier *et al.* 2017). In *E. siliculosus*, genes involved in male or female determination are clustered in the V and U regions of the genome, respectively (Ahmed *et al.* 2014, Cormier *et al.* 2017). Further reciprocal BLAST-hit analysis with the *E. siliculosus* genome indicated that sequences of the five N-1-containing and four N-2-containing genes are likely orthologs of *E. siliculosus* genes (Table 3). For example, g11150 corresponded to the *E. siliculosus* gene for omoaconitate hydratase and g11151 for the gene for RING-type zinc finger domain protein (Table 3). On the other hand, the four N-2-like genes of *E. siliculosus* encode proteins with no sequence similarity (Table 3). Because the five genes (g11150, g11151, g12375, g12376, and g12381) corresponding to N-1 are genes clustered in the V region of *E. siliculosus*, a haploid (N-1) *C. okamuranus* that expresses these five genes is likely male. On the other hand, because the four genes (g13772, g19792, g19793, and g20120) corresponding to N-2 are genes clustered in the U region of *E. siliculosus*, a haploid (N-2) that expresses these four genes is probably female.

### Primer design and PCR amplification

Therefore, this set of PCR primers can be used not only to distinguish diploids from haploids, but also male from female haploids. Unique primers were designed for each of the nine genes (Table 4). As expected, PCR amplification of diploid germling DNA with these primers followed by electrophoresis, resulted in appearance of bands showing fragments of the nine genes (Fig. 4a). This indicates that the nine genes are present in the diploid genome. Then, when we carried out the same experiments against N-1 DNA, distinct PCR bands appeared for only five genes of the diploid + N-1 group, but not for four genes of the diploid + N-2 group (Fig. 4b). Sizes

**Table 3.** BLAST search results for nine genes of *Cladosiphon okamuranus* versus *Ectocarpus siliculosus* genes

Gene ID for <i>C. okamuranus</i>	Gene ID for <i>E. siliculosus</i>	Annotation in <i>E. siliculosus</i>	Region in <i>E. siliculosus</i>
g11150 (scaffold74)	Ec-13_001950	Homoaconitate hydratase	Male
g11151 (scaffold74)	Ec-13_002070	RING-type Zinc finger domain protein	Male
g12375 (scaffold100)	Ec-13_001910	STE20-like serine/threonine kinase	Male
g12376 (scaffold100)	Ec-13_002060	Protein phosphatase 2C	Male
g12381(scaffold100)	Ec-13_001800	Glycosyltransferase domain	Male
g13772 (scaffold137)	Ec-sdr_f_000090	Hypothetical protein	Female
g19792 (scaffold436)	Ec-sdr_f_000030	Hypothetical protein	Female
g19793 (scaffold436)	Ec-sdr_f_000150	Hypothetical protein	Female
g20120 (scaffold476)	Ec-sdr_f_000010	Hypothetical protein	Female

**Table 4.** Primers designed for the nine genes

Gene ID	Forward primer	Reverse primer	Estimated product size
g11150	ACACCCTTGAATCGGGAT	GACCACTACATTTTCACCGC	453 bp
g11151	CGCAGGCCATTTTCGGTTTAG	GCTACACGCTCTTCTGGAGG	524 bp
g12375	GGTCGTGAAAGTACTTGAGG	TTCATGAGCAAAGGATCGT	975 bp
g12376	CTGGAGTTGGGAGACACGAG	CCGAACCCATGAACGGATGA	762 bp
g12381	AGGTGAACGCACGAAGGAAA	CGGAAGGACATGGTACACCG	911 bp
g13772	TGCACCGTTGAGAGAGTTGT	TTTCGGTCAAGGAGTCCACC	359 bp
g18829	CCGGTAGCACCAGCAATACT	GCTTCTCGAATTTACCGCC	1950 bp
g19792	GACGTGCTGATGACTCACGA	GTTACGGTTAGCACGAGGGT	476 bp
g19793	AAGCGGGGTTTCCAGAGATG	GTTTTTGTCTGGGGCCGTC	717 bp
g20120	GGTAATGCGAATGTGCGACC	AAGATGGGTATGCTCGGACG	769 bp

of amplicons corresponded closely to the fragment sizes expected from the designed primers (compare Fig. 4b with Table 4). These results suggest that the five genes, g11150, g11151, g12375, g12376, and g12381, are specific to N-1 haploid male germlings.

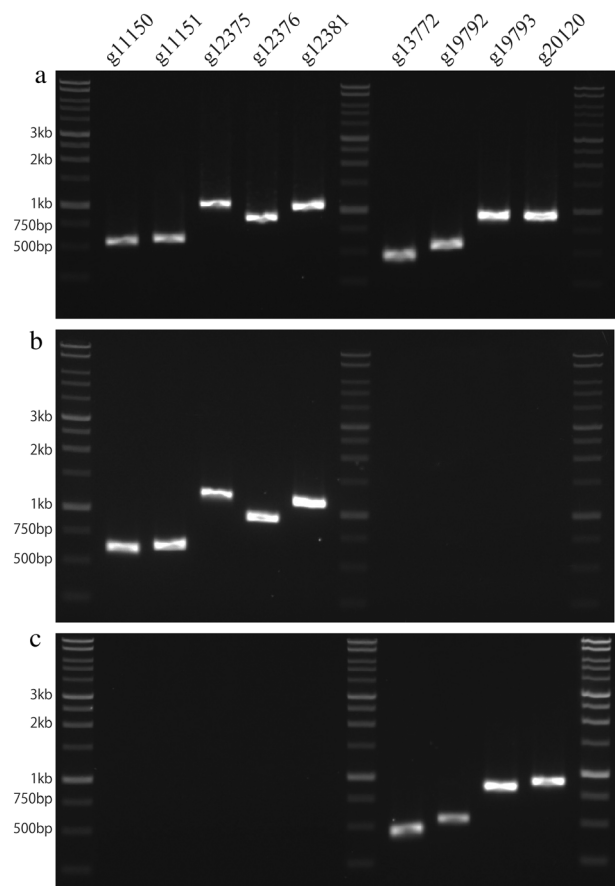
On the other hand, PCR amplification with primers designed for four genes of the diploid + N-2 resulted in clear bands against DNA only from N-2, but not DNA from N-1 (Fig. 4c). In addition, the sizes of amplicons corresponded closely to the fragment sizes expected from the designed primers (compare Fig. 4c with Table 4). Therefore, it is highly likely that the four genes, g13772, g197792, g19793, and g20120, are specific to haploid, female N-2 germlings.

These results indicate that the nine genes are expressed in diploid germlings and in appropriate groups of haploid germlings. Therefore, the nine primers provide DNA markers to distinguish haploid from diploid germlings and are useful for mozuku cultivation. The primers also can differentiate male and female haploid germlings.

## DISCUSSION

### Sex determination regions and genes of *Cladosiphon okamuranus*

Reports of sex determination regions (SDRs) and genes in brown algae are limited to *E. siliculosus* and *S. japonica* so far (Ahmed *et al.* 2014; Lipinska *et al.* 2015; Cormier *et al.* 2017; Lipinska *et al.* 2017; Zhang *et al.* 2018). In this research, five male genes and four female genes were identified in *C. okamuranus*; thus, this additional information could be important to understand evolutionary processes of SDRs in brown algae. In addition, the nine genes were found on five scaffolds of published *C. okamuranus* genome sequences (Table 3). This suggests two possibilities. First, SDRs of *C. okamuranus* may be really fragmented and located on various chromosomes. Second, SDRs are chimeric in published *C. okamuranus* genome sequences; therefore, genes that could be involved in sex determination are distributed on different scaffolds. Furthermore, nine of 269 genes are recognized as sex determination-related genes of *C. okamuranus* (Tables 2 and 3). This also suggests that additional sex determination-related genes could be among the remaining 260 genes. Hence, SDRs and additional sex determination-



**Fig. 4.** Results of polymerase chain reaction (PCR) amplification of germling DNA using primers developed specifically to amplify nine putative marker genes. (a) PCR results with the diploid (2N) genome. All nine marker genes were amplified with each of the primers. (b) PCR results of the N-1 haploid genome. Five genes yielded amplicons that corresponded to marker genes selected from N-1 germlings. Later, the N-1 genome was shown to be male, with male-specific sex determination-related genes. (c) PCR results of the N-2 haploid genome. Four genes yielded amplicons that corresponded to marker genes selected from N-2 germlings. Later, the N-2 genome was shown to be female, with female-specific sex determination-related genes. Sizes of amplicons corresponded almost exactly to sizes of DNA fragments expected from the designed primers.

related genes of *C. okamuranus* should be further analyzed in future studies to understand their evolution.

### DNA markers for male and female haploid germings of *C. okamuranus*

As mentioned above, the set of nine genes provides a molecular means of distinguishing 2N, male N, and female N germings. The set of nine gene primers was used for DNA amplification of unidentified germings. If amplicons appeared in all lanes, the germling in question is 2N, whereas if they appear against the five-gene set (g11150, g11151, g12375, g12376, and g12381), the germling is a male haploid. On the other hand, if amplicons appeared in lanes for g13772, g19792, g19793, and g20120, the germling is a female haploid. Since it is impossible to determine the sex of a haploid germling morphologically, this method is the first tool to distinguish not only haploid and diploid, but also male and female haploids. In *C. okamuranus*, the functions of the nine genes are still unknown.

The results indicate that artificial crossbreeding of *C. okamuranus* using male and female haploids could be possible after the sexes are determined using these DNA markers. Selective breeding at least in *C. okamuranus*, *U. pinnatifida*, and *S. japonica* has been performed in Japan, South Korea, and China (Nishitsuji *et al.* 2020; Wang *et al.* 2020; Graf *et al.* 2021). On the other hand, no one has succeeded in establishing new varieties using crossbreeding with green, red, and brown algae, except with three varieties of *Pyropia yezoensis* (Ueda) M.S.Hwang & H.G.Choi (<http://www.hinshu2.maff.go.jp>). At the least, no variety has been registered in Japan. Hence, DNA markers can contribute not only to effective cultivation, but also to the first crossbreeding of algae in the near future.

### Possible increased efficiency of mozuku cultivation in Okinawa

In Okinawa, many local fisheries have cultivated mozuku for a long time. Although OPFREC has provided fishermen with mozuku germings, the judgement of whether germings are diploid or haploid depends upon the experience of OPFREC members. In addition, OPFREC has attempted crossbreeding to produce new mozuku varieties with improved characteristics. Although isolation of haploid germings has been established, artificial conjugation has not succeeded because of a lack of information about male and female germings. These DNA markers could solve such problems.

In addition to the diploid germling supply from OPFREC, several local groups have their own systems of mozuku cultivation, keeping germings by themselves. This may be one of the reasons why at least four strains of *C. okamuranus*, the S-strain from Iheya, the K-strain from Katsuren, the O-strain from Onna, and the C-strain from Chinen, have been established. Under present culture conditions, most germings are a mixture of diploid and haploid. As sporophytes develop only from diploid germings, harvested mozuku yield could be increased if diploid germings could be selected. In order to solve this problem, the DNA marker technique may prove useful.

### Application of the method to other brown algae

These marker genes are very likely involved in sex determination in *C. okamuranus*, and they have been designed for sex identification of haploid germings. This means that the markers may be applicable to other brown algae. For example, the genome of *N. decipiens*, a close relative of *C. okamuranus*, should contain orthologs, so these markers may also improve cultivation of that species. They may also be useful for *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl and G.W. Saunders and *U. pinnatifida*. At least, the concept of such DNA markers is applicable to those species.

### CONCLUSIONS

Using genome information, DNA markers of *C. okamuranus* were designed and used to distinguish diploid and haploid germings and to determine the sex of haploid germings. These DNA markers may be used in the future by many fisheries, companies, and researchers, and may help increase the yield of mozuku. In addition, these markers make possible the creation of new strains by crossbreeding. Taking advantage of this tool, marine algal cultivation will be enhanced not only in Okinawa, but also throughout Japan and in other countries.

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### AVAILABILITY OF DATA

Sequence data have been deposited in DDBJ as PRJDB12532 and LC703133 to LC703150.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** The germling colonies of *Cladosiphon okamuranus* maintained at Okinawa Prefectural Fisheries Research and Extension Center (OPFREC). These germlings are stocked in 9-cm plastic shales with agarose gel. (a) A diploid germling colony. (b) A haploid germling (N-1) colony. Later, this was proved to be male haploid. (c) A haploid germling (N-2) colony. Later, this was proved to be female haploid.

**Table S1.** TPM values of genes expressed in three germlings of *Cladosiphon okamuranus*

**Table S2.** TPM values of genes expressed in diploid and male haploid germlings of *Cladosiphon okamuranus*

**Table S3.** TPM values of genes expressed in diploid and female haploid germlings of *Cladosiphon okamuranus*

**Table S4.** TPM values of genes expressed in diploid germlings of *Cladosiphon okamuranus*

**Table S5.** TPM values of genes expressed in haploid germlings of *Cladosiphon okamuranus*.