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Phenological shifts and genetic differentiation between sympatric populations of *Sargassum horneri* (Fucales, Phaeophyceae) in Japan

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ABSTRACT: Marked seasonality, especially in sexual reproduction, is common among seaweed species along temperate coasts and increases the possibility of successful fertilization in outcrossing species. A phenological shift in reproductive seasons, therefore, could be an effective isolation barrier between conspecific seasonal populations, although its power has not been verified in algae. *Sargassum horneri*, a major component of seaweed beds along the temperate coast of Japan, is known for variability in its reproductive phenology. To understand the significance of phenological shift as an isolation barrier in seaweed species, phenological investigations of *S. horneri* seasonal populations on the Sea of Japan coast of central Honshu, Japan, were combined with Bayesian cluster analysis based on a nuclear simple sequence repeat genotype. Results from these analyses concordantly suggest a genetic differentiation between the seasonal populations, although almost 20% of field-collected plants were estimated to be hybrids or have a hybrid origin based on results of Bayesian cluster analyses using experimental hybrids. A collapse of seasonal isolation was also detected at the site of the field investigation, and a high percentage of putative hybrids in the following generation at the site (41%) suggested significant seasonal isolation in the differentiation observed in this study.

KEY WORDS: Seasonality · Isolation barrier · Brown algae · Reproductive phenology · Seasonal isolation · Simple sequence repeat · Sea of Japan coast

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1. INTRODUCTION

Life histories with remarkable phenological peaks have evolved in many marine organisms, especially in temperate and cold waters (e.g. Coma et al. 2000, Varpe 2012), in response to seasonal fluctuations of abiotic (such as temperature, nutrients, light, etc.) and biotic environments (Boero 1994, Bertram et al. 2001). Marked seasonality of reproduction, which is observed commonly in seaweed species in temperate waters (Hoffmann 1987), would be responsible for survival of zygotes and indispensable for fertilization success in obligate outcrossing dioecious species through synchronous maturation and gamete release in both female and male plants (Brawley 1992). Phenological shifts between populations, especially in their reproductive peaks, have been reported in some brown algal species, such as *Fucus vesiculosus* (Berger et al. 2001) and *Sargassum* spp. (Norton & Deysher 1989, Honda & Okuda 1990), including

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S. horneri (Okuda 1987, Yoshida et al. 1998, 2001, Yoshida 2005). While a phenological shift could effectively prevent gene flow between populations (Taylor & Friesen 2017), the significance of seasonal isolation in the speciation process is controversial (Coyne & Orr 2004, Rundle & Nosil 2005, Levin 2009, Matsubayashi et al. 2010, Nosil 2012). In seaweed species, seasonal conspecific populations, i.e. populations of a single species exhibiting a phenological shift, have never, to our knowledge, been analyzed in their genetic diversification, except in the case of *F. vesiculosus* in the Baltic Sea (Tatarenkov et al. 2007). In Tatarenkov et al. (2007), however, genetic differentiation was concordant with the geographic origins (e.g. 70 km geographic distance) of the populations rather than reproductive seasonality (summer vs. autumn).

S. horneri is the main component of seaweed beds in eastern Asia. As with other members of the genus, *S. horneri* has a thallus at least 2 m — sometimes as much as 10 m — long and stands up from the seabed due to the buoyancy of its bladders (Yoshida 1983). In Japan, this annual species plays an important role in coastal ecosystems by providing various animals with habitats of complicated spatial structures, especially from winter to early summer (Umezaki 1984).

Owing to its ecological significance, as well as its remarkable biomass, the ecological traits of *S. horneri*, including its phenology, have been studied extensively in Japan. The reproductive peaks are in April to May in southern regions, which gradually shift to July going further north (Marui et al. 1981, Umezaki 1984). However, early-fruiting populations, i.e. those fertile in autumn or winter (November to January), as well as spring-fruiting populations sympatric with early-fruiting populations, have been reported in some regions of Japan (Yoshida 2005). Although monoecious populations have been reported, the species is usually dioecious. However, no pairs of sympatric seasonal populations have been subjected to a genetic comparison, especially one based on nuclear simple sequence repeat (SSR, microsatellite) markers.

A pair of sympatric seasonal populations is present on the Sea of Japan coast of central Honshu, Japan; those around Sado Island have been subjected to genetic analysis, but only of mitochondrial *cox*3 haplotype frequency (Watanabe et al. 2019). In this region, fertile *S. horneri* can be observed continuously from the end of January to early July. Populations fertile in winter (January to March, hereinafter 'winter population') and in spring (April to July, 'spring population') showed clear differences in haplotype frequency, suggesting the presence of 2 seasonal populations (Watanabe et al. 2019). The reproductive phenology of the winter populations has not been clarified to date, because these populations along the island have been targets of fisheries catch, and collections are prohibited except by fishermen. Information on the location of winter populations outside the island is limited.

The aim of the present study was to analyze the genetic diversification between the seasonal populations on the Sea of Japan coast of central Honshu using 12 SSR markers, which can detect gene flow more effectively than mitochondrial markers. Furthermore, the reproductive phenologies of 2 seasonal populations were investigated along mainland Honshu. Plants subjected to phenological investigation were then included in the genetic analyses to investigate the length of the temporal gap between the 2 reproductive seasons; if presence and length of an overlapped reproductive season is irrelative to the amount of hybridization between seasonal populations, the phenological shift does not play a significant role in reproductive isolation. Presence or absence of putative hybrids in the field was estimated based on SSR genotypes of experimental hybrids.

2. MATERIALS AND METHODS

Sargassum horneri is a common, annual species along the temperate coasts of China, Korea, and Japan (Yoshida 1983). As with most populations of the species, both seasonal populations studied were dioecious, and female plants easily identified by receptacles that are shorter and thicker than those of male plants. A single female receptacle releases eggs intermittently a few times from the base to the apex, and as with other members of the genus (Chapman & Chapman 1973), released eggs are kept on the surface of the maternal receptacle for several days until the egg is successfully fertilized and starts developing. In the study populations, degraded, unfertilized eggs were frequently observed on the receptacle surface. Receptacles containing eggs were dark brown in color and easily distinguished from those that had already released and shed fertilized eggs.

2.1. Sample collections and genetic diversity analyses for mitochondrial haplotype

Mitochondrial haplotypic information of 212 samples (plants) from Watanabe et al. (2019) was included in this study, and their DNA was reanalyzed using SSR markers. More than 300 new samples were col-

lected from 20 sites on the Sea of Japan coast of central Honshu (Table 1), were used in the present study. Branchlets of fertile plants, i.e. those with receptacles with eggs on the surface, or those with receptacles of the maximum size class, were collected. However, for Populations 23 and 33, sterile young plants were collected and utilized for analyses, due to difficulties in re-sampling of fertile plants. These sites were revisited, and the reproductive periods were confirmed. When samplings were repeated at a single site, individuals were grouped as separate populations by the date of sampling (Table 1). At the sites of field investigation mentioned below (Section 2.4), samples for DNA analysis were collected only once for each plant, usually when eggs were first observed on the receptacles.

Sample preparation, DNA extraction, and sequencing of mitochondrial *cox*3 were performed as described in Watanabe et al. (2019). Partial *cox*3 sequences of 469 bp were determined, and haplotypic and nucleotide diversities of each population, as well as pairwise N_{ST} between populations, were calculated using DnaSP v.5.10.01 (Librado & Rozas 2009).

2.2. Development and analyses of SSR markers

A male thallus of *S. horneri* was collected from Teradomari, Niigata, Japan, on May 21, 2008. Culture and induction of receptacle formation of *S. horneri* were performed as described by Yoshikawa et al. (2014). Total RNA was extracted from *S. horneri* using cetyltrimethylammonium bromide (Imaizumi et al. 2000). The RNA was precipitated with 2 M LiCl overnight at 4°C. After centrifugation, the RNA was washed with 70% ethanol and dissolved in Tris-EDTA. To further purify the solution, the total RNA was refined by RNA isolation from a plant kit (Macherey-Nagel). The isolated RNAs were used for RNA-Seq analysis. cDNA libraries were constructed using an Agilent SureSelect Strand-Specific RNA Library Prep Kit. Sequencing was carried out with an Illumina HiSeq 2500 system with a 100 bp paired-end read.

The reads were assembled into transcript contigs using the Rnnotator all-in-one package (Martin et al. 2010), in a nonP mode and other default settings, which performs read pre-processing, de novo assembly, and contig post-processing. The MISA tool v.1.0 (Thiel et al. 2003) was applied to the contig sequences (DDBJ accession no. PRJDB4109) to detect SSR sequences with default parameters. Subsequently, primer regions (18–25 bp) whose melting temperature (T_m) was predicted to be 60°C were explored in the upstream and downstream sequences of SSR type p2.

PCR amplifications of SSR loci were confirmed separately for each locus, and multiplex amplifications were performed using the Type-it Microsatellite PCR kit (Qiagen) under the following PCR conditions: denaturation at 95°C for 5 min, followed by 32 cycles of 95 °C for 30 s, 60°C for 90 s, and 72°C for 30 s, and a final extension of 72°C for 30 min. Amplification of targeted SSR loci was confirmed through common TA-cloning and DNA sequencing procedures. In addition to the 11 SSR markers developed in this study, Shorn18 was also included among the SSR loci from Shan et al. (2015), considering the stability of amplification and its T_m. The size of each allele was determined using the 3130 Genetic Analyzer (Thermo Fisher Scientific) and the Peak Scanner v1.0 (Thermo Fisher Scientific). The primer pairs used in this study, number of alleles, and size range for each locus are shown in Table S1 in the Supplement at www.int-res.com/articles/suppl/m642p103_supp.pdf.

Allelic richness and F_{IS} values for each population were estimated by FSTAT v.2.9.3.2 (www2.unil.ch/popgen/softwares/fstat.htm). The significance ($p < 0.05$) of F_{IS} was evaluated using a permutation procedure with 10 000 randomizations implemented in FSTAT. Calculations of Jost's D_{EST}, as well as principal coordinate analysis (PCoA) based on Nei's genetic distance, were performed using GenAlEx (Peakall & Smouse 2012). Populations with a small sample size ($n < 5$) were excluded from these analyses. In addition, Bayesian cluster analysis using STRUCTURE v.2.3.3 (Pritchard et al. 2000), which assigns each individual probabilistically into an assumed number of clusters (K), was performed under the admixture model with 100 000 burn-ins and 500 000 replicates of MCMC after burn-in. K values ranged from 2 to 16. Ten permutations for each K value were run, and the most plausible K was estimated based on delta$K$ analyses (Evanno et al. 2005). NewHybrid v.1.1b (Anderson & Thompson 2002), which can estimate the possibility of hybrids of 2 groups, was also applied, with 100 000 sweeps for both before and after burn-in.

2.3. Crossing experiment

Crossing experiments were aimed to evaluate (1) compatibility between seasonal populations and (2) assignment probability of experimental hybrids under STRUCTURE analysis, the latter of which was then applied to field-collected plants to identify putative

Table 1. Sampling information and genetic diversity of each *Sargassum horneri* population. Sampling dates represent reproductive seasons of each population, unless otherwise indicated. SSR: simple sequence repeat; n: number of samples of each marker; *h*: haplotype diversity; *Pi*: nucleotide diversity; *AR*: allelic richness; H_E: expected heterozygosity; H_O: observed heterozygosity; F_{IS}: inbreeding coefficient. –: not applicable. *significant F_{IS} value ($p < 0.05$)

Population data				Mitochondrial *cox*3				Nuclear SSR				
Month	Population no.	Latitude, longitude	Sampling date	n	Haplotypes (n)	*h*	*Pi*	n	*AR*	H_E	H_O	F_{IS}
Jan	1	38° 7′ 3.4″ N, 138° 26′ 15.0″ E	Jan 20, 2009	30	M (29), A (1)	0.0667	0.0008	27	2.87	0.59	0.48	0.21*
	2	38° 7′ 3.4″ N, 138° 26′ 15.0″ E	Jan 22, 2014	24	M (24)	0	0	10	2.81	0.56	0.46	0.23
	3	38° 4′ 36.7″ N, 138° 26′ 48.5″ E	Jan 28, 2009	12	M (9), A (2), B (1)	0.4394	0.0055	14	2.88	0.58	0.56	0.07
	4	36° 51′ 52.1″ N, 136° 59′ 43.2″ E	Jan 28, 2018	3	M (3)	0	0	5	3.10	0.59	0.43	0.36*
Feb	5[a]	36° 58′ 55.8″ N, 137° 39′ 16.5″ E	Feb 3 and 22, 2017[c]	2	M (2)	0	0	21	3.40	0.66	0.52	0.24*
	6	37° 48′ 44.1″ N, 138° 16′ 28.5″ E	Feb 6, 2016	7	M (7)	0	0	8	3.26	0.64	0.47	0.33*
	7	38° 7′ 3.4″ N, 138° 26′ 15.0″ E	Feb 25, 2013	0	–	–	–	4	–	0.55	0.40	–
	8[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Feb 4 and 28, 2017[c,d]	17	A (15), M (2)	0.2206	0.0027	18	3.61	0.71	0.52	0.30*
	9[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Feb 19, 2018	3	A (3)	0	0	3	–	0.59	0.64	–
	10	37° 58′ 30.2″ N, 138° 15′ 42.2″ E	Feb 21, 2017	4	M (4)	0	0	11	2.39	0.47	0.47	0.05
Mar	11	37° 57′ 36.0″ N, 138° 20′ 0.2″ E	Mar 3, 2009	30	M (30)	0	0	34	3.01	0.62	0.48	0.24*
	12[a]	36° 58′ 55.8″ N, 137° 39′ 16.5″ E	Mar 6, 2017	6	M (5), P(1)	0.3333	0.0123	6	2.79	0.56	0.51	0.20
	13	37° 48′ 44.1″ N, 138° 16′ 28.5″ E	Mar 11, 2016	2	M (2)	0	0	25	3.53	0.69	0.55	0.23*
	14	37° 49′ 31.5″ N, 138° 18′ 46.3″ E	Mar 12, 2013	10	M (10)	0	0	13	3.53	0.69	0.63	0.13
	15	37° 54′ 29.6″ N, 138° 28′ 52.3″ E	Mar 14, 2013	9	M (8), A(1)	0.2222	0.0027	8	3.90	0.72	0.65	0.16
	16[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Mar 10 and 29, 2017[c]	21	A (20), M (1)	0.0952	0.0012	19	3.49	0.68	0.61	0.13*
	17[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Mar 18 and 29, 2018[c]	9	A (8), M (1)	0.2222	0.0027	10	3.15	0.62	0.45	0.33*
Apr	18	38° 4′ 36.7″ N, 138° 26′ 48.5″ E	Apr 5, 2013	14	A (7), M (6), G (1)	0.6044	0.0069	10	4.12	0.74	0.50	0.39*
	19	37° 48′ 37.1″ N, 138° 16′ 40.8″ E	Apr 11, 2016	11	A (11)	0	0	18	3.97	0.75	0.69	0.12*
	20[a]	36° 58′ 55.8″ N, 137° 39′ 16.5″ E	Apr 21, 2017	1	M (1)	–	–	1	–	–	–	–
	21[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Apr 11 and 28, 2017[c]	14	A (14)	0	0	14	3.51	0.68	0.61	0.15*
	22[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Apr 10, 2018	0	–	–	–	2	–	0.54	0.67	–
May	23	38° 4′ 27.5″ N, 138° 14′ 41.2″ E	Mar 3, 2009[e]	15	A (15)	0	0	12	3.75	0.71	0.53	0.30*
	24	38° 4′ 36.7″ N, 138° 26′ 48.5″ E	May 14, 2013	21	A (17), M (2), G (1), R (1)	0.3476	0.0028	16	4.16	0.76	0.75	0.05
	25[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	May 12, 2017	8	A (8)	0	0	8	3.47	0.66	0.65	0.09
	26[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	May 1 and 16, 2018[c]	12	A (11), M (1)	0.1666	0.0020	12	3.51	0.68	0.58	0.19*
	27	37° 20′ 12.1″ N, 138° 27′ 55.6″ E	May 12, 2017	12	A (6), B (4), I (2)	0.6666	0.0024	12	3.03	0.58	0.54	0.11
	28	37° 21′ 5.7″ N, 138° 29′ 17.0″ E	May 12, 2017	7	A (7)	0	0	17	3.80	0.72	0.68	0.09
	29	37° 48′ 37.1″ N, 138° 16′ 40.8″ E	May 18, 2016	9	A (9)	0	0	22	4.00	0.76	0.66	0.16*
	30	37° 50′ 8.4″ N, 138° 21′ 58.5″ E	May 28, 2013	10	A (4), I (2), J (2), B (1), K (1)	0.8222	0.0034	10	4.34	0.77	0.65	0.21*
	31	37° 58′ 16.9″ N, 138° 15′ 28.6″ E	May 28, 2013	29	A (23), C (6)	0.3399	0.0010	34	3.78	0.73	0.61	0.19*
	32	37° 56′ 49.7″ N, 138° 19′ 27.4″ E	May 28, 2013	32	A (29), F (3)	0.1754	0.0005	19	4.01	0.75	0.64	0.17*
	33	36° 58′ 10.0″ N, 137° 34′ 54.7″ E	Mar 19, 2013[f]	6	A (6)	0	0	5	2.56	0.45	0.35	0.35
	34	38° 24′ 17.3″ N, 139° 27′ 44.5″ E	May 28, 2018	4	A (4)	0	0	9	3.40	0.65	0.71	–0.03

Table continued on next page

Table 1 (continued)

Month	Population no.	Latitude, longitude	Sampling date	Mitochondrial *cox*3 n	Haplotypes (n)	*h*	*Pi*	Nuclear SSR n	*AR*	H_E	H_O	F_{IS}
Jun	35	38° 15′ 51.4″ N, 139° 26′ 41.1″ E	Jun 7, 2017	3	E (3)	0	0	11	2.51	0.49	0.49	0.05
	36	38° 19′ 22.1″ N, 138° 28′ 27.9″ E	Jun 17, 2009	13	A (12), D (1)	0.1538	0.0005	11	3.36	0.64	0.54	0.21*
	37	38° 4′ 36.7″ N, 138° 26′ 48.5″ E	Jun 7, 2013	12	A (11), O (1)	0.1667	0.0067	12	3.90	0.72	0.58	0.24*
	38	37° 6′ 42.2″ N, 137° 59′ 59.6″ E	Jun 2, 2018	5	A (5)	0	0	8	3.67	0.69	0.66	0.10
	39	38° 33′ 29.3″ N, 139° 32′ 26.6″ E	Jun 17, 2018	10	A (10)	0	0	21	3.39	0.66	0.58	0.15*
	40	37° 20′ 10.2″ N, 138° 27′ 54.8″ E	Jun 4, 2018	5	A (5)	0	0	15	2.79	0.53	0.40	0.27*
	41[b]	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Jun 4, 2018	2	A (2)	0	0	2	–	0.41	0.54	–
No info[g]	42	36° 54′ 57.2″ N, 137° 1′ 36.2″ E	Feb 25, 2004	9	A (9)	0	0	7	3.99	0.73	0.61	0.24*
	43	36° 58′ 25.0″ N, 137° 35′ 12.4″ E	Nov 8, 2018	4	M (2), A (2)	0.6667	0.0082	4	–	0.59	0.44	–
Total				457	14 haplotypes	0.5262	0.0061	548		0.63	0.56	

[a]Sampling site corresponds to Site 1 in field investigation and Table 3; [b]Sampling site corresponds to Site 2 in field investigation and Table 3; [c]Samples were collected twice within a month, and all samples from a single site are treated as a single population in all analyses for simplicity; [d]Including 3 plants fertile in January, because they were collected on February 4; [e]Young, sterile plants were collected, and the fruiting season was confirmed later on May 14, 2013; [f]Fruiting season was confirmed on May 31, 2013; [g]Fruiting season has not been confirmed

hybrids in the field. Crossing a female plant collected in January 2018 (P1 in Table 2) at Ichiburi, Itoigawa, Niigata Prefecture (36° 58′ 55.8″ N, 137° 39′ 16.5″ E) with a male collected in July 2018 (P2) at Kamomegahana, Kashiwazaki, Niigata Prefecture (37° 21′ 5.7″ N, 138° 29′ 17.0″ E) was performed in a 90 mm Petri dish using PESI medium at 15°C, with an 18 h light:6 h dark photoperiod. Thallus of the P1 that formed receptacles was maintained at 15°C and an 18 h:6 h photoperiod until a fertile male plant of the spring population (P2) was obtained. Zygotes (fertilized eggs) that underwent cell division were isolated using sterilized Pasteur pipettes to obtain unialgal culture strains. For a few experimental F1 plants, formations of receptacles were induced successfully, and F2 plants were obtained. There was no evidence of parthenogenesis of either egg or sperm throughout the experiment. For the F1 and F2 plants, as well as both parental individuals, Bayesian cluster analyses based on SSR genotype as performed for the field-collected samples were applied.

2.4. Field investigation of reproductive phenology

The reproductive period of each plant was investigated at 6 sites (Table 3). Female plants with liberated eggs on their receptacles, or those with empty conceptacles (bright-colored part of receptacle), were labeled by tagging a small, numbered plastic float (approximately 15 mm in diameter) and/or by mapping their approximate location based on constructions near the coastline. Each site was visited once a month throughout the investigation period and roughly every 2 wk during reproductive seasons. The conditions of the tagged plants, i.e. presence or absence of fresh eggs (either fertilized or not) and young receptacles, were recorded until the plant was washed out or the plant had sunk to the seabed by losing buoyancy due to senescence. Short tips of branchlets were collected from each tagged plant at the time when the release of eggs was first confirmed, which were then used for genetic analyses, as described above (Section 2.1).

3. RESULTS

3.1. Genetic diversity

All haplotypes found in this study, including the newly found haplotype R (DDBJ accession no. LC500855), were classified into 1 of 3 mitochondrial

Table 2. Membership probability of the strains used in the *Sargassum horneri* crossing experiments in analyses using STRUCTURE software (K = 2) and NewHybrid software. Genetic groups 'Group I' and 'Group II' in the NewHybrid analyses were provisionally related to those in STRUCTURE based on the resulting membership probabilities of the parental plants. The 3 F1 strains were sired from P1 and P2, and 4 F2 were from F1-1 and F1-2. Probability of F1, F2, and backcross (BC) in NewHybrid analysis represent probability of F1 hybrid between Groups I and II, that of F2 hybrid and BC origin to parental genetic group (either Group I or II), respectively. None of the hybrids showed a high membership probability to either group. Mt-haplotype: mitochondrial haplotype

Strain	Sex	Mt-haplotype	Membership probability							
			STRUCTURE (K = 2)		NewHybrid					
			Group I	Group II	Group I	Group II	F1	F2	BC to Group I	BC to Group II
P1	Female	M	0.984	0.016	0.96	0.00	0.00	0.00	0.04	0.00
P2	Male	A	0.02	0.98	0.00	0.99	0.00	0.00	0.00	0.01
F1-1	Female	M	0.595	0.405	0.00	0.00	0.03	0.52	0.39	0.06
F1-2	Male	M	0.674	0.326	0.00	0.00	0.01	0.58	0.37	0.04
F1-3	Unknown	M	0.551	0.449	0.00	0.00	0.03	0.60	0.29	0.07
F2-1	Unknown	M	0.506	0.494	0.00	0.00	0.00	0.94	0.00	0.05
F2-2	Unknown	M	0.807	0.193	0.05	0.00	0.00	0.64	0.29	0.01
F2-3	Unknown	M	0.495	0.505	0.00	0.00	0.00	0.82	0.08	0.09
F2-4	Unknown	M	0.789	0.211	0.01	0.00	0.00	0.31	0.67	0.01

haplotype groups (Mt-groups 1–3) based on phylogeny (Fig. S1). Eleven of 14 haplotypes found in this study were included in Mt-group 2; type R was 1 bp different from type A, the major haplotype of Mt-group 2 (Fig. S1). Haplotypic and nucleotide diversities of each population are shown in Table 1.

Most of the SSR markers used in this study were hyperpolymorphic, and 14–52 alleles (28.8 on average) were found in each locus, in addition to the single monomorphic locus Shorn18 (Table S1).

3.2. Genetic structure between populations

Mt-group 1, which includes only 1 haplotype, type M, was dominant in winter populations fertile from January to March, and mt-group 2 prevailed among the spring populations, for which the reproductive period runs from April to June. Pairwise N_{ST} values between pairs of a winter and a spring population were larger (mean ± SD: 0.678 ± 0.384) than those between winter population pairs (0.354 ± 0.423) as well as those between spring population pairs (0.145 ± 0.261), with a few exceptions (Fig. S2).

The pairwise D_{EST} (Fig. S2) between different seasonal populations (0.462 ± 0.131), based on nuclear SSR markers, was slightly larger than those between populations showing similar reproductive phenology; those between winter populations were 0.335 ± 0.111 on average; those between spring populations were 0.321 ± 0.131. In the PCoA (Fig. 1), the populations were separated into 2 clusters along

Table 3. Sites and periods of the *Sargassum horneri* field investigation in this study. The field investigations were performed over a few years at 3 of the sites listed, but DNA samples were collected in a single season, except for Site 2. The reproductive period observed at Sites 1–4 and the membership probability of each sample are shown in Figs. 2 & S5. Numerals under 'Populations sampled' are the population numbers in Table 1 and Fig. 3

Site no.	Latitude, longitude	Period		DNA analyses	
		Investigation	Egg release	Populations sampled	n
1	36° 58′ 55.8″ N, 137° 39′ 16.5″ E	Dec 9, 2016–Mar 18, 2018	Feb–Apr[a]	5, 12, 20	28
2	37° 20′ 12.7″ N, 138° 28′ 5.1″ E	Jan 18–May 26, 2017	Jan–May	8, 16, 21, 25	59
		Jan 5–Jun 20, 2018	Feb–Jun	9, 17, 22, 26, 41	29
3	37° 21′ 5.7″ N, 138° 29′ 17.0″ E	Apr 20, 2016–Jun 20, 2018	May–Jun	28	17
4	38° 15′ 51.4″ N, 139° 26′ 41.1″ E	May 10–Jun 28, 2017	Jun	35	11
5	37° 6′ 42.2″ N, 137° 59′ 59.6″ E	Jan 5–Jun 20, 2018	May[b]–Jun	38	8
6	38° 24′ 17.3″ N, 139° 27′ 44.5″ E	May 27–Jun 17, 2018	May–Jun	34	9

[a]The reproductive period of most plants observed had finished by late March; however, only 1 female plant matured in April 2017; [b]Three plants became fertile on May 16, 2018; however, genetic analysis for those plants failed

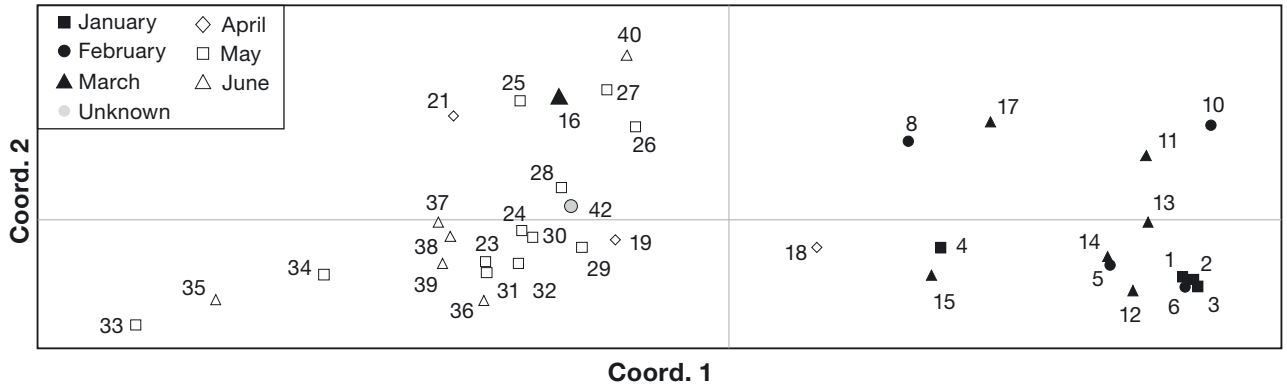

Fig. 1. Principal coordinate analysis based on Nei's genetic distance between the *Sargassum horneri* populations. Coord. 1 and 2 are the first and second principal factors of variability that explain 25.29 and 13.50% of total variations, respectively. Numerals are the population numbers in Table 1 and Fig. 3. Populations 7, 9, 20, 22, and 41 were excluded from this analysis due to small numbers of samples

the first coordinate (25.3% variation), with Population 18 maturing in April at an intermediate position. An exception was Population 16, which was located among the spring populations, although all plants of the population were fertile in March (see Section 3.4).

In the STRUCTURE analysis, $K = 2$ was the most plausible number of genetic groups among samples based on the delta$K$ analysis (Figs. S3 & S4). At $K = 2$, we found that 444 of 548 total plants were clearly assigned to either 1 of the 2 genetic groups (Groups I and II), with a membership probability of 0.9 or higher (Table 4, Fig. 2A). Among the plants with such high probability, 178 plants were fertile from January to March (98.3% of the 181 plants in Group I), while 234 plants matured from April to June (89.0% of the 263 plants in Group II). Therefore, 92.8% of the 444 plants (with a membership probability > 0.9) matched their reproductive season with their genetic group. Some samples of Populations 8 and 16, on the other hand, had high membership probability for Group II, despite a winter maturation. Furthermore, an admixture of Groups I and II was strongly suggested: 104 plants had a membership probability below 0.9 for Group I or II, and some approached 0.5:0.5. In Population 26, for example, a high ratio of samples (7 of 12) exhibited equal membership probability for both genetic groups (see Section 3.4).

3.3. Crossing experiment and probability of membership of the hybrids

Cell divisions of fertilized eggs were observed 2 d after the female (P1 in Table 2) and the male plants (P2) were put together in a Petri dish. No clear difference was observed between the growth of hybrid zygotes and that of zygotes isolated from field samples or obtained from crosses within a single genetic group (data not shown). The F1 plants become fertile approximately 1 yr after fertilization, and F2 zygotes were formed successfully. Further, the growth of F2 plants did not differ from either that of F1 plants or field samples.

Table 4. Monthly summary of number of *Sargassum horneri* plants analyzed (n), and number of plants assigned to each simple sequence repeat (SSR) genetic group and *cox*3 haplotypic groups. Putative hybrid: plants with membership probability below 0.9 in Bayesian clustering analysis

Month	No. of populations	Nuclear SSR in STRUCTURE software				Mitochondrial *cox*3			
		n	Group I	Group II	Putative hybrid	n	Mt-group 1	Mt-group 2	Mt-group 3
Jan	4	56	49	0	7	69	65	4	0
Feb	6	65	50	7	8	33	15	18	0
Mar	7	115	79	15	21	87	57	29	1
Apr	5	45	2	26	17	40	7	33	0
May	12	176	0	138	38	165	3	162	0
Jun	7	80	0	70	10	50	0	49	1
Unknown	2	11	1	7	3	13	2	11	0
Total	43	548	181	263	104	457	149	306	2

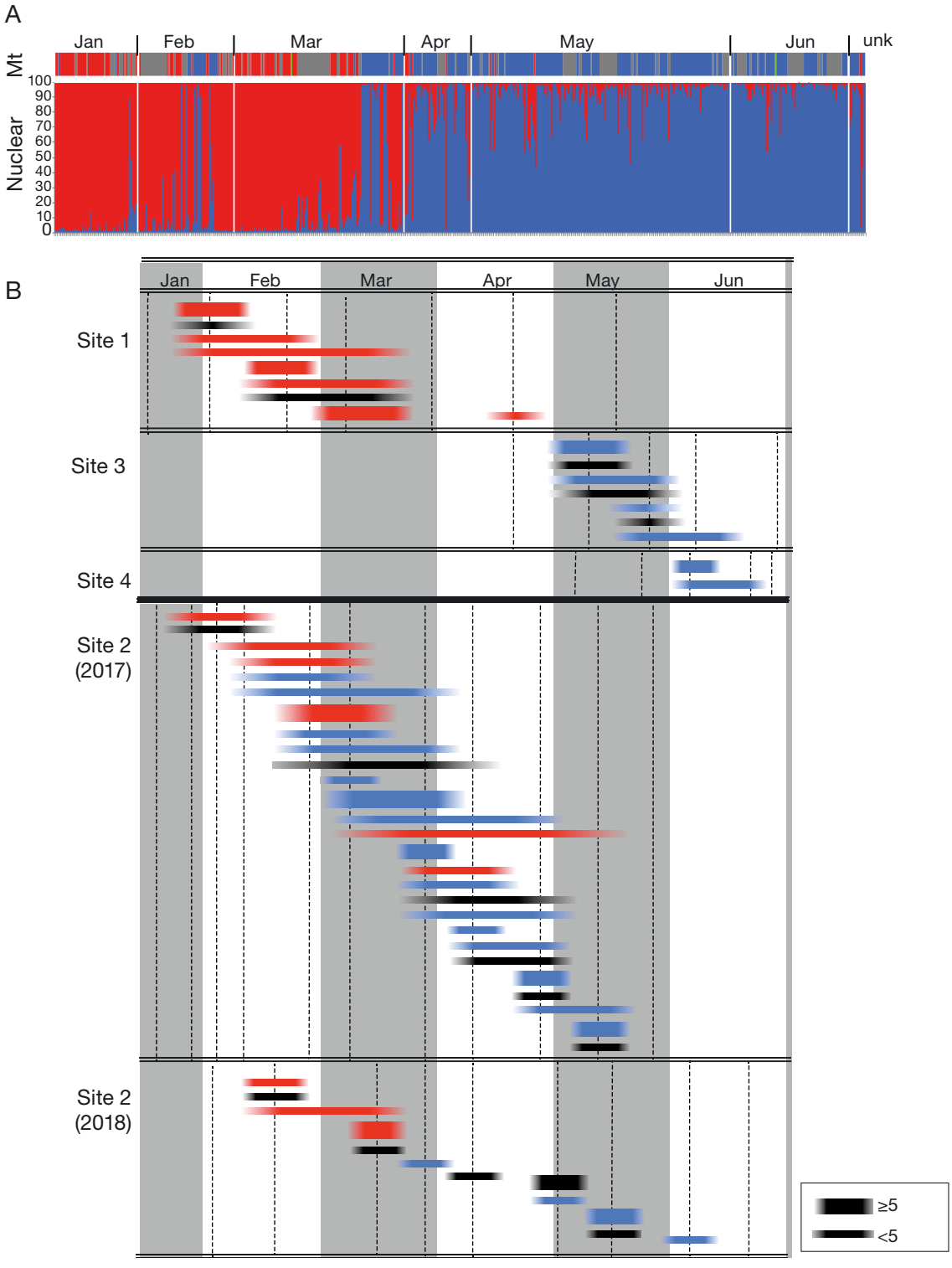

Fig. 2. Genetic structures between the seasonal *Sargassum horneri* populations. (A) Genetic structure in field-collected samples (n = 548) based on mitochondrial haplotype group (Mt) and membership probability in STRUCTURE software ($K = 2$) (Nuclear). Samples are arranged by reproductive month, except for samples for which reproductive seasons are not clear (unk). Red: Mt-group 1 for mitochondrial haplotype, and Group I for nuclear simple sequence repeat (SSR); and blue: Mt-group 2 and Group II, respectively. Grays and yellow-greens in mitochondrial haplotypes: unanalyzed samples and Mt-group 3, respectively. (B) Approximate reproductive periods of the female plants investigated at Sites 1–4. Vertical dashed lines: the day of investigation. Gray backgrounds are shown to separate months. Color and thickness of horizontal bars: the genetic assignment (Group I or II in SSR) and number of individuals, respectively. Black bars: putative hybrids

Membership probabilities ($K = 2$) of the F1 and F2 plants, as well as both parents, are listed in Table 2. Although alleles at each locus were not incompatible with the parenthood between these samples, and although 2 parental plants showed high probability of belonging to either Group I or Group II, the membership probabilities of hybrids varied widely, especially in the F2 generation (0.505–0.807). High probability as 'F1' in the NewHybrid analysis was not indicated, even for experimental F1 individuals.

Based on the membership probabilities of the experimental F1 and F2 generations, plants exhibiting such membership probability (<0.9) are assumed to be hybrids and/or to have recent hybrid origins. If this is true, 18.9% of the field plants analyzed (104 of 548 plants) could be classified into this category (Table 4). In the NewHybrid analysis, high probabilities of F2 or backcross (BC) were estimated for the samples that were supposed to be hybrids (or to have hybrid origins) based on the membership probability in the STRUCTURE analysis (data not shown).

3.4. Field investigation of reproductive phenology

In the field observations at 6 sites, most female plants investigated demonstrated egg liberation for 2 to 4 wk. Some plants had a protracted fertile period of >2 mo. Either one of 2 seasonal populations was observed at 5 of 6 sites investigated (Figs. 2B & S5; for Sites 5 and 6, data not shown); a match between the reproductive season and the genetic group was confirmed for these 5 sites.

Site 2 was the exception. The maturation period at this site began in late January (2017) or February (2018) and lasted until May (2017) or June (2018). Furthermore, genetic analysis showed that 19 plants of Group II, which usually become fertile in spring, matured in February (5 of 18 samples in Population 8) and March 2017 (14 of 19 samples in Population 16). As a result, plants of 2 genetic groups were fertile synchronously (Fig. 2B). Such an exceptional mismatch between the genetic group and the reproductive season, i.e. winter maturation of Group II plants, was not observed in 2018. However, 12 of 29 plants analyzed from this site in 2018 (41% of samples in Populations 9, 17, 22, 26, and 41) showed equivocal membership probability, i.e. <0.9, and were estimated as putative hybrids based on the assignment probability of experimental hybrids. The percentage of putative hybrids observed at Site 2 in 2018, i.e. 41%, is far larger than 17% (10 of 59 samples) observed at this site in 2017.

4. DISCUSSION

Genetic structures between conspecific populations, or closely related species, have been reported for various algal taxa, including brown algae, for the past 20 yr (Billot et al. 2003, Coyer et al. 2003, 2006, Engel et al. 2005, Alberto et al. 2011, Zardi et al. 2011, Neiva et al. 2012, Wang et al. 2019). Degrees of genetic differentiation range widely by species. Various factors, such as historical expansion (Coyer et al. 2003, Alberto et al. 2011, Neiva et al. 2012), sea currents (Billot et al. 2003), mating systems (Engel et al. 2005), recent introductions (Coyer et al. 2006, Wang et al. 2019), and microallopatry in the intertidal zone (Zardi et al. 2011), have been explicitly considered or evaluated as a causal barrier of the observed diversification. Tatarenkov et al. (2007) evaluated the genetic differentiation between seasonal populations of *Fucus vesiculosus* in the Baltic Sea, but reported no clear differentiation concordant with seasonality. However, Watanabe et al. (2019) reported strong mitochondrial haplotypic differentiation congruent with the seasonality of the populations, rather than with geographical distributions, in *Sargassum horneri*. This is supported by cluster analyses using nuclear SSR genotypic data in the present study.

Little is known about the effect and significance of phenological shifts in reproductive isolation barriers in algal species. Phenological shifts have been reported between populations (Okuda 1987, Norton & Deysher 1989, Honda & Okuda 1990, Yoshida et al. 1998, Berger et al. 2001) and between closely related species of seaweeds (Yoshida 1983). However, genetic differentiation has been evaluated only in a few cases (Tatarenkov et al. 2007, Watanabe et al. 2019). Genetic diversification driven by a phenological shift has been reported in various land plants and animals (Hendry & Day 2005, Savolainen et al. 2006, reviewed by Taylor & Friesen 2017). However, seasonal isolation has been considered relatively unimportant compared with those of pollinator and/or habitat isolation (Coyne & Orr 2004, Levin 2009, Matsubayashi et al. 2010, Nosil 2012). The individuals found at Site 2 in 2017 (Fig. 2B) showed a mismatch between their winter seasonality and their genetic cluster (Group II). The coexistence of fertile plants of 2 genetic groups may be responsible for the observed high ratio (41%) of putative hybrids in the next generation, i.e. plants at Site 2 in 2018; a collapse of seasonal isolation could result in an increase of hybridization. In the case studied here, a phenological shift would therefore be a key

driver of diversification between the seasonal populations, rather than an ancillary mechanism. Viable hybrids obtained from experimental crossings suggest that no intrinsic post-fertilization barrier would be expected between the seasonal populations. Furthermore, a phenological shift would have a genetic background. Receptacle formations of *S. horneri* have been known to be induced by long-day treatment (Yoshikawa et al. 2014), but culture strains of the winter population did not show a clear photoperiodicity under laboratory culture conditions (Y. Homma & S. Uwai unpubl. data). In the field, microallopatry has been known between seasonal populations. Microallopatry could cause environmental differences such as temperature, nutrients, and light, etc. between habitats and therefore cannot be excluded as a possible trigger of the phenological shift observed. A transplantation experiment would be straightforward; however, no photoperiodic response of winter plants under laboratory conditions suggests that the phenological shift observed here would be due to genetic background rather than environmental factors. Circannual control of reproductive maturation has been considered as a possible cause of marked reproductive seasonality of some seaweed species that did not show a clear photoperiodic response (e.g. Yoshida 2005); however, molecular backgrounds of marked reproductive seasonality of seaweed species have not been clarified so far. Furthermore, whether the genetic differentiation observed between the seasonal populations was derived initially from the phenological shift is not clear, and past allopatric differentiation cannot be excluded, given the genetic distance between the haplotypes dominant in each seasonal population. Diversification of the haplotypes (4 bp different among 469 bp determined) could have taken place 2 million years ago, based on a recalculated molecular clock of *cox*3 (0.25–0.39% per million years; Kantachumpoo et al. 2014).

Differentiation in the face of gene flow is considered common throughout the speciation continuum (Nosil et al. 2008, Abbott et al. 2012, Nosil 2012). Gene flow could work as an enforcement for differentiation, or collapse speciation, or maintain ongoing speciation at the stage. As a clear gap in reproductive peaks between seasonal populations would be a more effective impediment to gene flow (Taylor & Friesen 2017), a large number of putative hybrids and those with possible hybrid origins could be the result of continuous reproductive seasons of the 2 seasonal populations studied here or the sporadic occurrence of early-fruiting spring plants. The reason for the 'early maturation' of the spring plants at Site 2 in 2017 is not clear. Our investigation in 2018 suggests that early maturation could be accidental, unlike those observed commonly and repeatedly every year. Seven plants assigned to the winter population based on their nuclear genomes at Site 2 in 2017 had a mitochondrial haplotype that is usually observed in the spring population (Fig. S5). Such an introgression of an organelle's genome could be the result of past hybridization, which is known in land plants (McKinnon et al. 2010, Palma-Silva et al. 2011) as well as in seaweed species (Neiva et al. 2010). The number of putative hybrids and mismatches between seasonality and mitochondrial haplotypes (Fig. 3) suggests that bidirectional gene flow has occurred and that allochronic isolation, as well as other barriers if present, are permeable. The ecophysiological characterization of both seasonal population, as well as hybrid plants, would be required to determine the presence or absence of isolation barrier(s) other than the seasonal barrier.

The genetic differentiation documented here may have been promoted or prevented by other factors. In land plants, for example, phenological shifts have been discussed frequently in association with habitat preference (Lamont et al. 2003, Rundle & Nosil 2005, Levin 2009, Matsubayashi et al. 2010). Although the habitats of each seasonal population studied here have not been evaluated in detail, the seasonal populations reportedly show a subtle habitat preference, with plants of the spring populations apt to grow on bedrock or a wave-dissipating block at shallower sites, and winter populations preferring pebbles or shells at deeper sites. The 2 habitats tend to be 10s to 100s of meters from each other. As the dispersal ability of zygotes of fucalean species are limited to a few meters (Kendrick & Walker 1991, Serrão et al. 1996), microallopatry could prevent gene flow to some degree. However, the recent addition of coastal infrastructure developments, such as fishery ports and breakwaters, could bring the habitats of the 2 seasonal populations closer; e.g. if 2 seasonal populations can be fertile simultaneously from late March to early April, a breakwater could increase the chance of crossing between a winter population growing up from a deeper site around the breakwater and a spring population at the shallower part of it. Contrarily, gene flow may be prevented by the intensive harvest of the winter populations during March, leaving a clear reproductive gap between seasonal populations.

Maturation in the winter season is uncommon among *Sargassum* species in the studied area; repro-

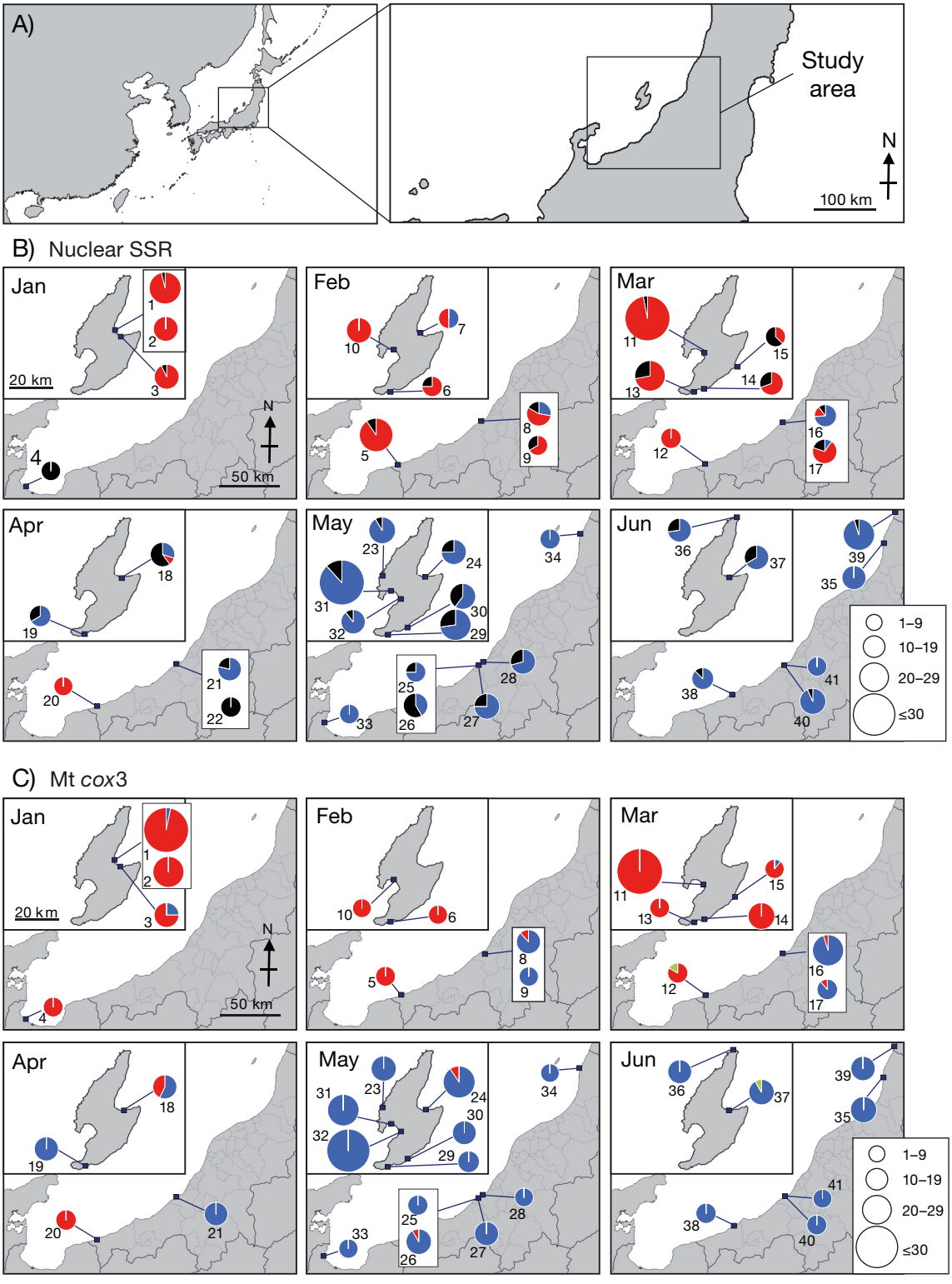

Fig. 3. Seasonal and geographical distributions of the genetic groups based on all field-collected *Sargassum horneri* samples. (A) Study area. (B,C) Frequency of plants of each genetic group (membership probability 0.9 or higher) based on (B) nuclear genotype and (C) mitochondrial haplotype, shown by month. Colors: same as in Fig. 2. Numerals: population numbers shown in Table 1. Black in the pie charts in (B): frequency of putative hybrids or plants having a hybrid origin. Insets in (B,C): Sado Island. SSR: simple sequence repeat

ductive seasons of this group on the Sea of Japan coast of Honshu generally begin in late spring or in early summer (Yoshida 1983). Increasing water temperatures during spring to summer (15–17°C in May; www1.kaiho.mlit.go.jp/KAN9/suion/suion.htm) are likely to be more favorable for the early development of zygotes than the lower temperatures in winter (8–11°C in February). Furthermore, stormy and turbulent conditions are common during winter in the studied region, i.e. along the Sea of Japan coast of Japan, due to a northerly monsoon; extensive wave action has been known to prevent successful recruitment (Vadas et al. 1990) and/or decrease the chance for encounters between sperm and eggs (Serrão et al. 1996). Still, the length of winter plants can be 10 m or more, and they frequently become entangled with neighboring plants, which would enhance fertilization even under continuous turbulent and stormy conditions. Berger et al. (2001) reported the coexistence of 2 seasonal populations of *F. vesiculosus* in the Baltic Sea, and suggested that a temperature lower than that of the vigorous season of filamentous algae could be advantageous for reproduction in terms of competition for space. This could be true in the present case, because the winter populations frequently grow on small pebbles or shells, which are common substrata for small filamentous algae. In addition, a spring bloom of phytoplankton in the Sea of Japan (e.g. Kim et al. 2000) would result in increased sedimentation rates as estimated in the Baltic Sea (Eriksson & Johansson 2003), which would impede the recruitment of juveniles (Schiel & Foster 1986, Terawaki et al. 1996, Berger et al. 2003, Eriksson & Johansson 2003).

In conclusion, genetic differentiation between the seasonal populations of *S. horneri* on the Sea of Japan coast of central Honshu, Japan, has been verified in the present study by analysis of nuclear SSR markers as well as mitochondrial *cox*3 haplotypes. Increased numbers of putative hybrids after the collapse of the phenological shift, i.e. from 17% in 2017 to 41% in 2018, suggest that an allochronic isolation barrier played a key role in the observed diversification. An ecophysiological comparison between seasonal populations is required to determine the presence or absence of other isolation barrier(s) and the fate of the hybrids. The coexistence of 2 seasonal populations is likely to play a significant role in coastal ecosystems, given their impact on primary production and habitat creation for animals. The documented genetic and phenological differences should be considered in coastal development and conservation in this area.

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LITERATURE CITED

Abbott R, Albach D, Ansell S, Arntzen JW and others (2012) Hybridization and speciation. J Evol Biol 26:229–246

Alberto F, Raimondi PT, Reed DC, Watson JR and others (2011) Isolation by oceanographic distance explains genetic structure for *Macrocystis pyrifera* in the Santa Barbara Channel. Mol Ecol 20:2543–2554

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217–1229

Berger R, Malm T, Kautsky L (2001) Two reproductive strategies in Baltic *Fucus vesiculosus* (Phaeophyceae). Eur J Phycol 36:265–273

Berger R, Henriksson E, Kautsky L, Malm T (2003) Effects of filamentous algae and deposited matter on the survival of *Fucus vesiculosus* L. germlings in the Baltic Sea. Aquat Ecol 37:1–11

Bertram DF, Mackas DL, McKinnell SM (2001) The seasonal cycle revisited: interannual variation and ecosystem consequences. Prog Oceanogr 49:283–307

Billot C, Engel CR, Rousvoal S, Kloareg B, Valero M (2003) Current patterns, habitat discontinuities and population genetic structure: the case of the kelp *Laminaria digitata* in the English Channel. Mar Ecol Prog Ser 253:111–121

Boero F (1994) Fluctuations and variations in coastal marine environments. Mar Ecol 15:3–25

Brawley S (1992) Fertilization in natural populations of the dioecious brown alga *Fucus ceranoides* and the importance of the polyspermy block. Mar Biol 113:145–157

Chapman VJ, Chapman DJ (1973) Algae, 2nd edn. Macmillan, London

Coma R, Ribes M, Gili JM, Zabala M (2000) Seasonality in coastal benthic ecosystems. Trends Ecol Evol 15:448–453

Coyer JA, Peters AF, Stam WT, Olsen JL (2003) Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. Mol Ecol 12:1817–1829

Coyer JA, Hoarau G, Skage M, Stam WT, Olsen JL (2006) Origin of *Fucus serratus* (Heterokontophyta; Fucaceae) populations in Iceland and the Faroes: a microsatellite-based assessment. Eur J Phycol 41:235–246

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA

Engel C, Daguin C, Serrão EA (2005) Genetic entities and mating system in hermaphroditic *Fucus spiralis* and its close dioecious relative *F. vesiculosus* (Fucaceae, Phaeophyceae). Mol Ecol 14:2033–2046

Eriksson BK, Johansson G (2003) Sedimentation reduces recruitment success of *Fucus vesiculosus* (Phaeophyceae)

in the Baltic Sea. Eur J Phycol 38:217–222
Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620
Hendry AP, Day T (2005) Population structure attributable to reproductive time: isolation by time and adaptation by time. Mol Ecol 14:901–916
Hoffmann AJ (1987) The arrival of seaweed propagules at the shore: a review. Bot Mar 30:151–165
Honda M, Okuda T (1990) Egg liberation, germling development and seasonal changes in photosynthetic rates of autumnal *Sargassum micracanthum.* Jpn J Phycol 38: 263–268 (in Japanese with English Abstract)
Imaizumi T, Kanegae T, Wada M (2000) Cryptochrome nucleocytoplasmic distribution and gene expression are regulated by light quality in the fern *Adiantum capillus-veneris*. Plant Cell 12:81–95
Kantachumpoo A, Uwai S, Noiraksar T, Komatsu T (2014) Levels and distribution patterns of mitochondrial *cox*3 gene variation in brown seaweed, *Sargassum polycystum* C. Agardh (Fucales, Phaeophyceae) from Southeast Asia. J Appl Phycol 26:1301–1308
Kendrick GA, Walker DI (1991) Dispersal distances for propagules of *Sargassum spinuligerum* (Sargassaceae, Phaeophyta) measured directly by vital staining and venture suction sampling. Mar Ecol Prog Ser 79: 133–138
Kim SW, Saitoh S, Ishizaka J, Isoda Y, Kishino M (2000) Temporal and spatial variability of phytoplankton pigment concentrations in the Japan Sea derived from CZCS images. J Oceanogr 56:527–538
Lamont BB, He T, Enright NJ, Krauss SL, Miller BP (2003) Anthropogenic disturbance promote hybridization between *Banksia* species by altering their biology. J Evol Biol 16:551–557
Levin DA (2009) Flowering-time plasticity facilitates niche shifts in adjacent populations. New Phytol 183:661–666
Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452
Martin J, Bruno VM, Fang Z, Meng X and others (2010) Rnnotator: an automated *de novo* transcriptome assembly pipeline from stranded RNA-Seq reads. BMC Genomics 11:663
Marui M, Inai S, Yoshida T (1981) Growth and maturation of six species of *Sargassum* and *Cystoseira* (Phaeophyta, Fucales) in Oshoro Bay, Hokkaido, Japan. Jpn J Phycol 29:277–281 (in Japanese with English Abstract)
Matsubayashi KW, Ohshima I, Nosil P (2010) Ecological speciation in phytophagous insects. Entomol Exp Appl 134: 1–27
McKinnon GE, Smith JJ, Potts BM (2010) Recurrent nuclear DNA introgression accompanies chloroplast DNA exchange between two eucalypt species. Mol Ecol 19: 1367–1380
Neiva J, Pearson GA, Valero M, Serrão EA (2010) Surfing the wave on a borrowed board: range expansion and spread of introgressed organellar genomes in the seaweed *Fucus ceranoides* L. Mol Ecol 19:4812–4822
Neiva J, Pearson GA, Valero M, Serrão EA (2012) Drifting fronds and drifting alleles: range dynamics, local dispersal and habitat isolation shape the population structure of the estuarine seaweed *Fucus ceranoides.* J Biogeogr 39:1167–1178
Norton TA, Deysher LE (1989) The reproductive ecology of *Sargassum muticum* at different latitudes. In: Ryland JS, Tyler PA (eds) Reproduction, genetics and distributions of marine organisms. Olsen & Olsen, Fredensborg, p 147–152
Nosil P (2012) Ecological speciation. Oxford University Press, Oxford
Nosil P, Harmon LJ, Seehausen O (2008) Ecological explanation for (incomplete) speciation. Trends Ecol Evol 24: 145–156
Okuda T (1987) Monoecism and autumn-fruiting of *Sargassum horneri*. Jpn J Phycol 35:221–225
Palma-Silva C, Wendt T, Pinheiro F, Barbara T, Fay MF, Cozzolino S, Lexer C (2011) Sympatric bromeliad species (*Pitcairnia* spp.) facilitate tests of mechanisms involved in species cohesion and reproductive isolation in Neotropical inselbergs. Mol Ecol 20:3185–3201
Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28:2537–2539
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959
Rundle HD, Nosil P (2005) Ecological speciation. Ecol Lett 8: 336–352
Savolainen V, Anstett MC, Lexer C, Hutton I and others (2006) Sympatric speciation in palms on an oceanic island. Nature 441:210–213
Schiel DR, Foster MS (1986) The structure of subtidal algal stands in temperate waters. Oceanogr Mar Biol Annu Rev 24:265–307
Serrão EA, Peason G, Kautsky L, Brawley S (1996) Successful external fertilization in turbulent environments. Proc Natl Acad Sci USA 93:5286–5290
Shan T, Pang S, Li J, Su L (2015) Isolation and characterization of eight novel microsatellite loci from the brown alga *Sargassum horneri*. J Appl Phycol 27:2419–2421
Tatarenkov A, Jonsson RB, Kautsky L, Johannesson K (2007) Genetic structure in populations of *Fucus vesiculosus* (Phaeophyceae) over spatial scales from 10 m to 800 km. J Phycol 43:675–685
Taylor RS, Friesen VL (2017) The role of allochrony in speciation. Mol Ecol 26:3330–3342
Terawaki T, Yoshida G, Yoshikawa K, Arima S (1996) Observation on *Sargassum* vegetation in relation to substratum height above the sea bottom in the western Seto Inland Sea. Bull Nansei Natl Fish Res Inst 29:49–58
Thiel T, Michalek W, Varshney R, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley. Theor Appl Genet 106:411–422
Umezaki I (1984) Ecological studies of *Sargassum horneri* (Turner) C. Agardh in Obama Bay, Japan Sea. Bull Jpn Soc Sci Fish 50:1193–1200
Vadas RL, Wright WA, Miller SL (1990) Recruitment of *Ascophyllum nodosum*: wave action as a source of mortality. Mar Ecol Prog Ser 61:263–272
Varpe Ø (2012) Fitness and phenology: annual routines and zooplankton adaptations to seasonal cycles. J Plankton Res 34:267–276
Wang X, Shan T, Pang S, Su L (2019) Assessment of the genetic relationship between the recently established benthic population and the adjacent floating populations of *Sargassum horneri* (Phaeophyceae) in Dalian of China by newly developed trinucleotide microsatellite markers. J Appl Phycol 31:3989–3996

Watanabe K, Homma Y, Karakisawa H, Ishikawa R, Uwai S (2019) Haplotypic differentiation between seasonal populations of S*argassum horneri* (Fucales, Phaeophyceae) in Japan. Phycol Res 67:59–64

Yoshida T (1983) Japanese species of *Sargassum* subgenus *Bactrophycus* (Phaeophyta, Fucales). J Fac Sci Hokkaido Univ Ser V (Botany) 13:99–246

Yoshida G (2005) Phenological differentiation among populations of *Sargassum horneri* (Fucales, Phaeophyta) in Hiroshima Bay, Seto Inland Sea, Japan. Bull Fish Res Agency 15:27–126 (in Japanese with English Abstract)

Yoshida G, Arima S, Terawaki T (1998) Growth and maturation of the 'autumn-fruiting type' of *Sargassum horneri* (Fucales, Phaeophyta) and comparisons with the 'spring-fruiting type'. Phycol Res 46:183–189

Yoshida G, Yoshikawa K, Terawaki T (2001) Growth and maturation of two populations of *Sargassum horneri* (Fucales, Phaeophyta) in Hiroshima Bay, the Seto Inland Sea. Fish Sci 67:1023–1029

Yoshikawa S, Kamiya M, Ohki K (2014) Photoperiodic regulation of receptacle induction in *Sargassum horneri* (Phaeophyceae) using clonal thalli. Phycol Res 62: 206–213

Zardi GI, Nicastro K, Canovas F, Costa JF, Serrão EA, Pearson GA (2011) Adaptive traits are maintained on steep selective gradients despite gene flow and hybridization in the intertidal zone. PLOS ONE 6:e19402

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