Mar Biol (2009) 156:901–911
DOI 10.1007/s00227-009-1136-y

ORIGINAL PAPER

Geographical genetic structure and phylogeography of the *Sargassum horneri/filicinum* complex in Japan, based on the mitochondrial *cox3* haplotype

Shinya Uwai · K. Kogame · G. Yoshida · H. Kawai · T. Ajisaka

Received: 25 June 2008 / Accepted: 12 January 2009 / Published online: 11 February 2009

Abstract The genetic structure and phylogeography of the brown seaweed *Sargassum horneri/filicinum* complex in Japan were studied based on the mitochondrial *cox3* haplotype. The *cox3* haplotypes found were divided into three clades in a statistical parsimony network, among which there were large numbers of steps. Contrary to the reported large amount of drifting *S. horneri* along the Japanese coast, the three clades were dividedly distributed on the Japanese coast: the northern Pacific, the central Pacific, and western Japan. The western Japan *S. horneri* had haplotypes that were phylogenetically closer to those of *S. filicinum* than to the northern and central Pacific *S. horneri* populations. The *S. filicinum* populations were included within the western Japan clade and grouped together with the *S. horneri* samples from western Japan. Taken together with the unstable morphological diagnosis, this result suggests that *S. filicinum* should be reduced into a synonymy of *S. horneri*. The TMRCA analysis suggested that the divergence time of each clade may go back to the last interglacial period and a skyline plot suggested that the last glacial maximum had only a small effect on the population size of *S. horneri*. The geographic subdivision of the three groups, in spite of a large amount of drifting mats, suggests a limited contribution of drifting mats to gene flow on a large geographic scale. On a small geographic scale, a small number of haplotypes were shared between *S. horneri*-type and *S. filicinum*-type populations. This result suggests that populations of these two types are partially, though not completely, isolated from each other, possibly by selfing in *S. filicinum*-type populations or by a difference in peak reproduction.

Communicated by T. Reusch.

S. Uwai · H. Kawai
Kobe University Research Center for Inland Seas,
Rokkodai 1-1, Nada-Ku, Kobe 657-8501, Japan

K. Kogame
Department of Natural History Sciences,
Faculty of Science, Hokkaido University,
Kita 10, Nishi 8, Kita-Ku, Sapporo 060-0810, Japan

G. Yoshida
National Research Institute of Fisheries and Environment
of Inland Sea, Fisheries Research Agency, Maruishi 2-17-5,
Hatsukaichi, Hiroshima 739-0452, Japan

T. Ajisaka
Division of Applied Bioscience,
Graduate School of Agriculture,
Kyoto University, Kitashirakawa-Oiwake-Cho,
Sakyo-Ku, Kyoto 606-8502, Japan

Present Address:
S. Uwai (✉)
Department of Environmental Science,
Faculty of Science, Niigata University,
Ikarashi-2, Nishi-Ku, Niigata 950-2181, Japan
e-mail: uwai@env.sc.niigata-u.ac.jp

Introduction

The geographic distribution of genetic diversity in a species can be a strong tool for estimating historical events such as speciation and contemporary gene flow (Templeton et al. 1995; Hewitt 1996; Comes and Kadereit 1998; Taberlet et al. 1998; Avise 2000). In marine environments, for example, the ocean current pattern can affect contemporary gene flow to some degree if the species has the ability for long-distance dispersal by floating or rafting (Helmuth et al. 1994; Kojima et al. 1997, 2004), which would result in genetic similarity along the ocean current. On the other hand, historical fragmentation can result in a large genetic

divergence within a species (Comes and Kadereit 1998; Avise 2000). The marine environment changed in the late Quaternary on the Japanese coast. The Sea of Japan was covered by cold, low-saline water (by 5–7 psu and 10°C lower than today) and was anoxic due to a strong stratification of the water column during the last glacial maximum (LGM, 18–20 kyr BP, Oba et al. 1991; Gorbarenko and Southon 2000). On the Pacific coast, the Kuroshiwo warm current flowed further south and was colder than present conditions during and soon after the LGM (Chinzei et al. 1987). The Seto Inland Sea, presently located between Honshu and Shikoku, was terrestrial during the LGM due to a decrease in the sea level (Yashima 1994).

The genus *Sargassum,* which is an ecologically significant group for constructing seaweed beds in coastal waters of Japan, forms drifting mats after detachment from the substratum. It is well known that floating *Sargassum* plants can drift for 1–5 months and are found hundreds of km beyond the coastal distribution limits (Yoshida 1963; Okuda 1977), although drifting mats are considered to be of local coastal flora origin and are more frequent in coastal waters or the coastal front of sea currents (Yoshida 1963; Ohno 1984; Uehara et al. 2006). The drifting nature of *Sargassum* species is of great ecological importance for many invertebrates and fishes, but its contribution to gene flow among its own populations has never been evaluated, except on a small geographic scale (Engelen et al. 2001).

Sargassum horneri (Turner) C. Agardh is widely distributed along the Japanese coast and is known as a major component of drifting seaweed mats in Japanese waters (Yoshida 1963; Komatsu et al. 2007). *Sargassum filicinum* Harvey is a close relative of *S. horneri* limited to western Japan (Sawada 1955; Yoshida 1983), though this species has been introduced to California, USA (Miller et al. 2007), and Mexico (Aguilar-Rosas et al. 2007). These species are similar to each other, but distinguished by the shape of their vesicles (terete in *S. horneri* vs. shortly elliptical to spherical in *S. filicinum*) and sexuality. *S. horneri* shows dioecism, while *S. filicinum* has androgynous receptacles, in which male and female gametangia are both formed on a single receptacle (fertile branchlet), but in separate conceptacles (group of gametangia) (Okuda 1977; Yoshida 1983). Peak reproduction of the two species differs by ca. 1 month (Sawada 1955). However, exceptional character status has been reported (Okuda 1987), and phylogenetic analysis using nuclear rDNA-ITS showed that *S. horneri* and *S. filicinum* are monophyletic, indistinguishable, and separate from other clades in the genus (Stieger et al. 2003).

In the present study, we examined sequence polymorphisms of mtDNA among the *S. horneri/filicinum* populations off the Japanese coast to determine the phylogeography of this species complex and the genetic relationships between *S. filicinum* and *S. horneri*. Additionally, we estimated the contribution of drifting mats to gene flow. In modern Japan from 8 to 10 kyr ago, two warm ocean currents and a cold current have affected coastal waters (Fig. 1); the warm Kuroshiwo current flows through southern to central Pacific Japan, and the warm Tsushima current, a branch of the Kuroshiwo current, flows southwards along the Sea of Japan coast. The Tsushima current also affects northern Pacific Japan where it is also influenced by the Oyashiwo cold current. A large drifting mat of *S. horneri* of Chinese origin has been found in the East China Sea and tracked to either the Sea of Japan or the Pacific coast of Japan (Komatsu et al. 2007), transported by sea currents. Furthermore, large amounts of drifting mats have been estimated to have been transferred from the Sea of Japan to northern Pacific Japan by the Tsushima current (Yoshida 1963).

Materials and methods

Sampling, DNA extraction and sequencing

Samples of *S. horneri* were collected at 31 sites and *S. filicinum* at 6 sites during 2002–2005 (Table 1). Samples (3–17 individuals) were collected at random within each population and were identified primarily based on the shape of vesicles. For fertile plants, sexuality was also checked for identification. Apical portions of branches including several leaves and vesicles were put into plastic bags with silica gel for DNA extraction. DNA was extracted using a DNeasy® plant mini Kit (Qiagen) according to the manufacture's protocols. Before DNA extraction, a few vesicles with a coronal leaf were re-hydrated to remove contaminations of epi- and endophytic algae. Extracted DNA was purified

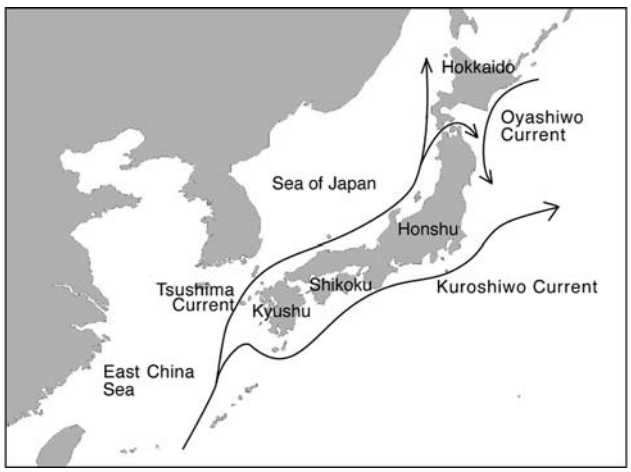

Fig. 1 The approximate routes of ocean currents around Japan based on the web site of the Japan Metrological Agency (http://www.data.kishou.go.jp/kaiyou/db/kaikyo/knowledge/index.html)

Table 1 The populations of *Sargassum horneri* and *S. filicinum* sampled in this study

Species	Pop. code	Locality	Longitude	Latitude	No. samples	Haplotypes (no. of samples)
S. horneri	1	Komoike, Kagoshima Pref.	31°32′N	130°33′E	14	Type1
	2	Tomioka, Amakusa, Kumamoto Pref.	32°31′N	130°02′E	5	Type1 (4), type7 (1)
	3	Matsushima, Amakusa, Kumamoto Pref.	32°32′N	130°25′E	9	Type1 (1), type26 (8)
	4	Aino, Tachibana Bay, Nagasaki Pref.	32°47′N	130°08′E	16	Type1 (8), type2 (2), type5 (4), type6 (1), type19 (1)
	5	Iki Isl., Nagasaki Pref.	33°51′N	129°42′E	14	Type1 (9), type3 (1), type4 (4)
	6	Hirado, Nagasaki Pref.	33°22′N	129°33′E	3	Type1
	7	Tsurumu, Saeki, Oita Pref.	32°56′N	131°59′E	14	Type12 (4), type18 (10)
	8	Rokuren Isl., Shimonoseki, Yamaguchi Pref.	33°56′N	130°55′E	4	Type9
	9	Heigun Isl., Yanai, Yamaguchi Pref.	33°47′N	132°13′E	5	Type1
	10	Azuki, Yanai, Yamaguchi Pref.	33°52′N	132°08′E	13	Type18
	11	Miyajima Isl., Saeki, Hiroshima Pref.	34°16'N	132°15′E	10	Type1 (1), type16 (1), type17 (1), type18 (7)
	12	Mukaijima Isl., Onomichi, Hiroshima Pref.	34°21′N	133°13′E	17	Type13
	13	Yura, Sumoto, Awaji Isl., Hyogo Pref.	34°16′N	134°57′E	10	Type13 (4), type 14 (2), type15 (1), type18 (3)
	14	Gokasyo Bay, Watarai, Mie Pref.	34°19′N	136°41′E	4	Type27
	15	Shimoda, Shizuoka Pref.	34°39′N	138°56′E	3	Type28
	16	Kannonzaki, Yokosuka, Kanagawa Pref.	35°14′N	139°44′E	9	Type28
	17	Uhara, Katsu-ura, Chiba Pref.	35°07′N	140°17′E	4	Type28 (2), type29 (2)
	18	Shioyazaki, Iwaki, Fukushima Pref.	36°59′N	140°59′E	13	Type28
	19	Onagwa, Oshika, Miyagi Pref.	38°26′N	141°27′E	5	Type28 (1), type31 (1), type32 (3)
	20	Shizugawa, Minami-sanriku, Miyagi Pref.	38°38′N	141°28′E	4	Type30 (1), type31 (1), type32 (2)
	21	Sanriku, Ofunato, Iwate Pref.	39°06′N	141°49′E	3	Type32
	22	Otsuchi, Iwate Pref.	39° 20′N	141°56′E	3	Type31
	23	Shiriyazaki, Shimokita, Aomori Pref.	41°25′N	141°27′E	6	Type32
	24	Imabetsu, Tsugaru, Aomori Pref.	41°10′N	140°28′E	7	Type1
	25	Imago-ura, Kasumi, Hyogo Pref.	35° 39′N	134°38′E	4	Type1 (3), type10 (1)
	26	Himi, Toyama Pref.	36° 50′N	136°59′E	9	Type1
	27	Kujiranami, Kashiwazaki, Niigata Pref.	37°21′N	138°30′E	3	Type1
	28	Fukaura, Aomori Pref.	40°38′N	139°55′E	10	Type1
	29	Moheji, Kami-iso, Hokkaido	41°45′N	140°36′E	14	Type33
	30	Oshoro, Otaru, Hokkaido	43°12′N	140°51′E	7	Type1 (6), type11 (1)
	31	Zhoschan, Zhejan, China	30°43′N	122°46′E	12	Type1 (11), type8 (1)
		Total			254	
S. filicinum	32	Aino, Tachibana Bay, Nagasaki Pref.	32°47′N	130°08′E	4	Type19 (3), type21 (1)
	33	Saikaibashi Bridge, Nagasaki Pref.	33°02′N	129°45′E	13	Type19 (5), type20 (6), type22 (1), type23 (1)
	34	Mukaijima Isl., Onomichi, Hiroshima Pref.	34°21′N	133°13′E	15	Type13 (2), type18 (1), type24 (12)
	35	Mukuchijima Isl., Kurashiki, Okayama Pref.	34°25′N	133°46′E	12	Type24 (11), type25 (1)
	36	Iwaya, Awaji Isl., Hyogo pref.	35°34′N	135°01′E	2	Type24
	37	Kanku Airport Isl., Sennan, Osaka Pref.	34°25′N	135°14′E	5	Type24
		Total			51	

The population code, the location, number of samples and haplotypes found are shown

using a GENECLEANE® II kit (Bio101) and was used as a template of PCR amplification.

PCR of the mtDNA *cox3* gene was performed using primers and PCR conditions described by Kogame et al. (2005). PCR products purified by PEG precipitation were used as templates for cycle sequencing reaction with the same primers as those of the PCR using a CEQ cycle sequencing kit (Beckman Coulter). Sequencing was accomplished using a CEQ8000 DNA analysis system (Beckman Coulter) according to the manufacture's instructions. Resulted sequences were easily aligned by eye because there were no insertions/deletions in the alignment. Representative sequences were deposited in the international DNA database under accession number AB430550–AB430581 (numerically according to the haplotype code) except for haplotype 24 that was deposited previously (Miller et al. 2007, AB264794).

Phylogenetic analyses

Phylogenetic trees of *cox*3 haplotypes were inferred using maximum likelihood (ML) method as implemented in PAUP* 4.0b10 (Swofford 2002). The ML trees were reconstructed using the HKY model with shape parameter of the gamma distribution estimated by MODELTEST ver. 3.7 (Posada and Crandall 1998). For the ML tree, 50 bootstrap replicates with ten random sequence additions were preformed. Bayesian phylogenetic analysis using the HKY model also was performed by MRBAYES ver. 3.0 (Huelsenbeck and Ronquist 2001). The analysis was initiated with a random starting tree and was run for 5×10^7 generations, sampling trees every 100th generation. *Sargassum muticum* (AB430582) was used as an outgroup.

Genetic structure and phylogeographic analysis

In order to explore nonrandom haplotype distributions and the phylogeographic process that created them, a statistical parsimony network was created using TCS ver. 1.21 (Clement et al. 2000) based on the DNA sequences of *cox3* haplotypes, and the phylogeographic nested clade analysis (PNCA; Templeton et al. 1995; Templeton 1998) was performed using GEODIS ver. 2.4 (Posada et al. 2000) with 1,000 permutations. To explore the results of the GEODIS analysis, the inference key that summarizes patterns of haplotype distributions expected was used from the GEODIS web site (updated in November 11, 2005).

The sequence data of each haplotype and the frequency of the haplotypes in each population were used to define groups of populations that were homogeneous geographically and differentiated maximally to each other (Spatial Analysis of the Molecular Variance, SAMOVA), using SAMOVA ver. 1.0 (Dupanloup et al. 2002). The SAMOVA combines geographical information of each population with AMOVA. A simulated annealing procedure that maximizes proportion of total amount of molecular variance due to difference between groups of geographically homogeneous populations, was done based on pairwise differences and for a varying number of groups ($k = 2$–8). For each simulated annealing, 300 permutations were performed (due to machinery limitation). Gene diversity (h), Nucleotide diversity (π), as well as pairwise ϕ_{st} based on TrN genetic distance, were calculated for each SAMOVA groups using ARLEQUIN ver. 2.001 (Schneider et al. 2000). The TrN genetic distance was selected based on the estimation by MODELTEST ver. 3.7.

TMRCA analysis and skyline plot

The Time to the Most Recent Common Ancestor (TMRCA) was estimated using BEAST ver. 1.4 (Drummont and Rambaut, http://evolve.zoo.ox.ac.uk/beast). Before TMRCA estimation, the mutation substitution rate of the mitochondrial *cox3* was estimated following the method of Hoarau et al. (2007), because available mutation rate of mitochondrial genome of brown algae is that of a spacer region (Hoarau et al. 2007; 2.0–3.4% per myr). The divergence age of the two species was calculated based on the reported mutation rate of *psbA* (0.08–0.12% per myr, Hoarau et al. 2007) and the sequence divergence between *S. horneri* and *Fucus serratus* in *psbA* (5.76%). The mutation rate of *cox3* was estimated based on the divergence time and the sequence divergence of *cox3* (17.7–18.8%) between *S. horneri* and *F. serratus*. The estimated mutation rate of *cox3* was ranged 2.6–4.1% per myr with an average value of 3.4% per myr. The xml-format file for BEAST was prepared using BEAUti ver. 1.4 (Drummont and Rambaut, http://evolve.zoo.ox.ac.uk/beast); we used the HKY + G model with 2 codon partition (1 + 2, 3). Trace files generated by BEAST under the upper and lower mutation rates were analyzed, and TMRCAs under each mutation rate, as well as their median value, were estimated by Tracer ver. 1.4 (Drummont and Rambaut, http://tree.bio.ed.ac.uk/software/tracer/). The demographic change of the Japanese *S. horneri/filicinum* population was also estimated by the Bayesian skyline plot (Drummond et al. 2005).

Results

Alignment of the 469 bp *cox3* sequences revealed 33 haplotypes. The statistical parsimony network (Fig. 2) provided three 4-step clades, four 3-step clades, six 2-step clades, 13 1-step clades, and 32 0-step clades, when the network was nested following the nesting rule of Templeton (1998).

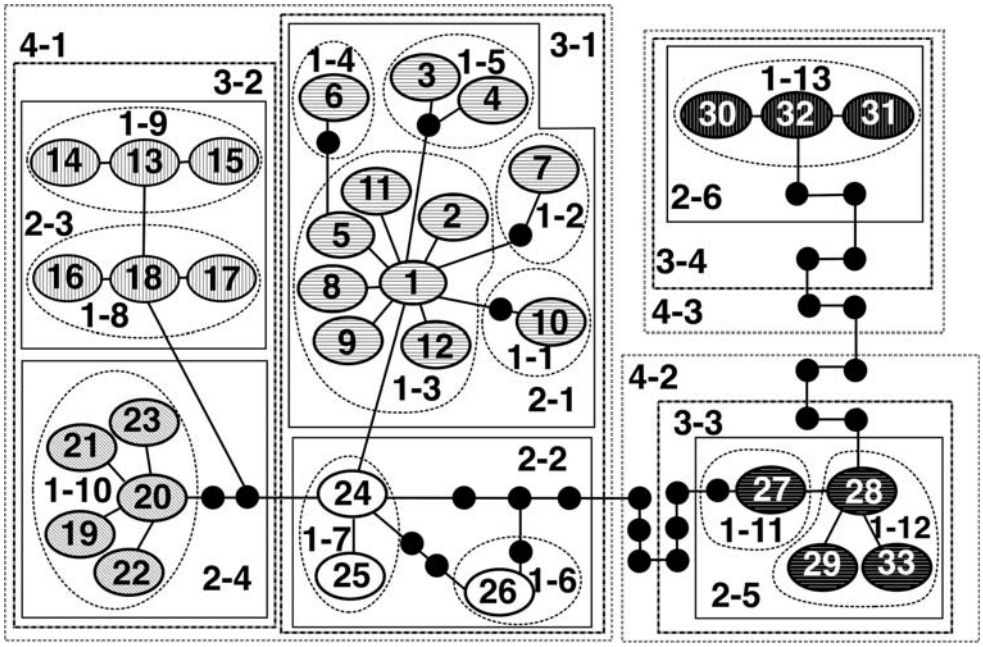

Fig. 2 A result of nesting of the statistical parsimony network of the *cox3* haplotypes in the *Sargassum horneri/filicinum*. Haplotype lineages of each *2-step clade* are shown by different patterns. Numerals of each *0-step clade* represent haplotype codes used in Table 1

Clades corresponding to the 2-step clades of the network (Fig. 2) were also recognized as monophyletic clades within a ML tree (data not shown) except for clade 2-2; the clade 2-2 was paraphyletic to clades 2-1, 2-2, and 2-3. The *S. horneri* samples and *S. filicinum* samples had haplotypes belonging to the different clades; haplotypes of *S. filicinum* were harbored in either clades 2-2 (haplotypes 24 and 25) or 2-4, neither of which were found in most *S. horneri* samples. There were, however, a few exceptions; population 4 of *S. horneri* had a haplotype of clade 2-4; *S. filicinum* population 34 had clade 2-2 haplotypes as a majority, but also had a haplotype of clade 2-3, which was otherwise found in *S. horneri* populations. Haplotype 26 in clade 2-2, though two other haplotypes of the clade were found in *S. filicinum*, was found only in a single *S. horneri* population (population 4).

Geographic structure and phylogeographic nested clade analysis on the Japanese coast

The geographic distributions of each 2-step clade are shown in Fig. 3 and Table 1. Clades 2-1, 2-2, 2-3, and 2-4 were found in western Japan and on the coast of the Sea of Japan; clade 2-2 was found in populations 34–37 (the Seto Inland Sea) and distantly located population 3 (the East China Sea); clade 2-3 was also found in the Seto Inland Sea in population 7, a majority in populations 10–13 and a minority in population 34. Group 2–4 was found in populations 4, 32, and 33 (the East China Sea), the first of which includes only one plant having the haplotype of this clade. The haplotypes harbored in clade 2-1 were found in populations on the East China Sea, the Sea of Japan coast, and the Seto Inland Sea. Clades 2-5 and 2-6 were limited to the central (14–19) and northern (19–23) Pacific coast, respectively. Population 29, from northern Japan, was exceptional in that it had a haplotype belonging to clade 2-5.

In the SAMOVA, geographic groups of populations with a high degree of genetic differentiation (ϕ_{ct} = 0.7718–0.883, k = 2–6) were found (Table 2). In k = 2 and 3, populations having haplotypes of clades 2-1, 2-2, 2-3, and 2-4, which have overlapping distributional ranges (Fig. 3) and similar DNA sequences, grouped together; populations belonging to either clades 2-5 or 2-6 grouped together when k = 2, but separated when k = 3. When k = 5, populations having haplotypes of the same 2-step clades grouped together; groupings of populations were similar to that shown in Fig. 3. The major haplotype lineage, if multiple haplotypes lineages were in a single population, decided the population affiliation in SAMOVA; population 4, having both clade 2-1 and clade 2-4 haplotypes, belonged to the group of populations having clade 2-1 haplotypes. Clade 2-2 did not form a separate group, but was grouped together with the group of clade 2-1.

The number of effective haplotypes, gene diversity (h), and nucleotide diversity (π) of each SAMOVA group are shown in Table 3; among the three major SAMOVA groups, both gene and nucleotide diversity were highest for western Japan populations, though the number of samples was also the largest. The gene diversities of three subgroups in western Japan were higher than those of central and northern Pacific Japan.

Geographically nonrandom distributions of the haplotypes were detected in nine clades and in the total cladogram, of which eight clades yielded significant conclusive

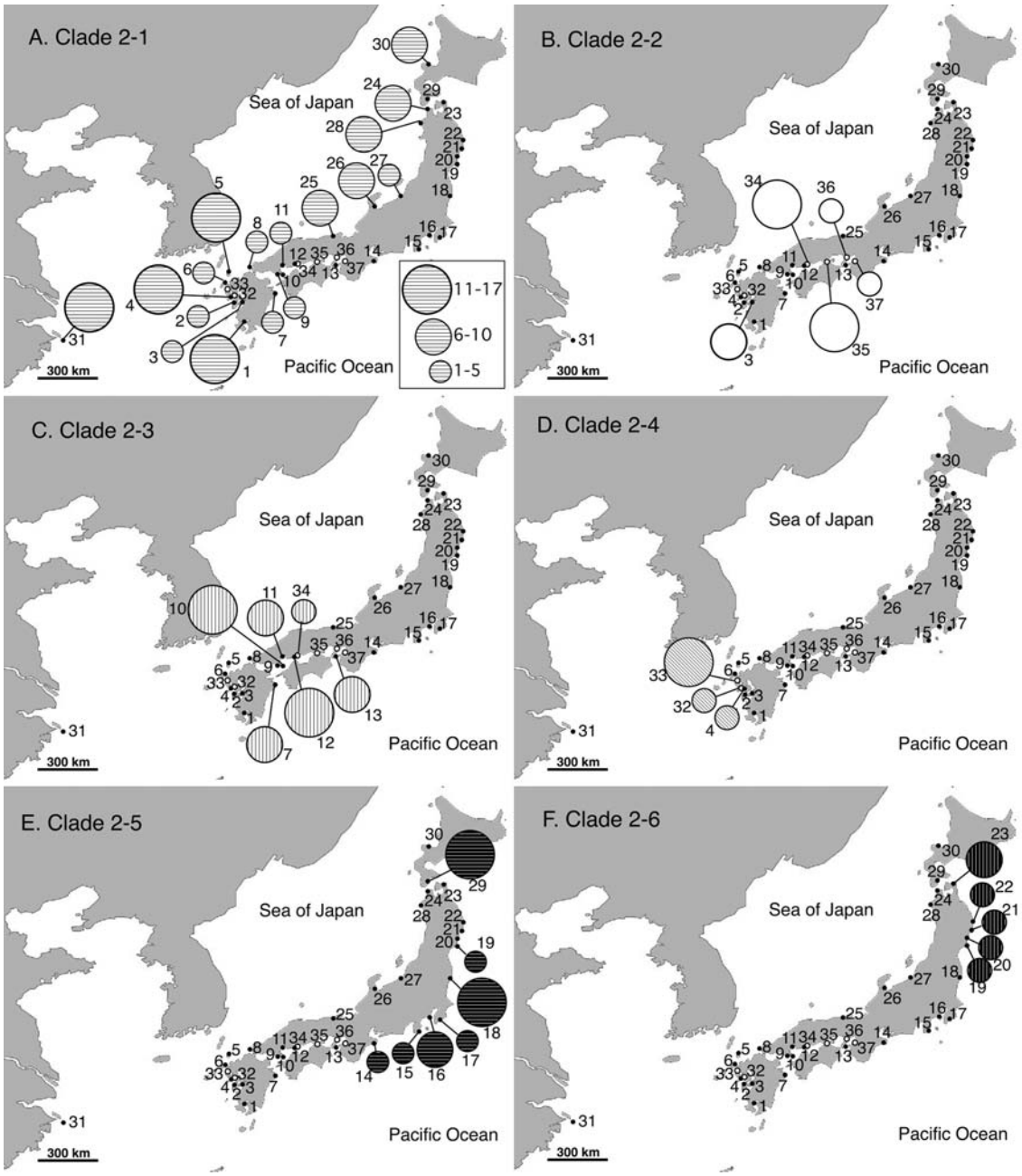

Fig. 3 The geographic distributions of each *2-step clade* in the Japanese *Sargassum horneri/filicinum* populations. *Numerals* represent the population code shown in Table 1. The patterns correspond to those used in Fig. 2

outcomes in phylogeographic nested clade analysis (PNCA, Table 4) based on the inference key. For clades 1-13 and 4-1, restricted gene flow/dispersal was inferred rather than a historical event, and a restricted gene flow/dispersal or range expansion was inferred for clade 1-3. Past fragmentation followed by range expansion was inferred for the total cladogram, supported by a large number of nucleotide substitutions between the 4-step clades. The results of the PNCA are summarized in Table 4.

TMRCA analysis and the skyline plot

The median values of TMRCA (time to most recent common ancestor) of most of the one- or two-step clades were 0.078–0.15 myr ago (Table 5). The TMRCA of clade 4-1 was 0.216 myr, which corresponds to the values of the TMRCA of *S. horneri* and *S. filicinum*. The TMRCA of the whole *S. horneri/filicinum* population was 0.644 myr ago with a range from 0.498 to 0.789 myr,

Table 2 Summary of SAMOVA

k	Groups and populations included	ϕ_{ct}	ϕ_{sc}	ϕ_{st}
2	[14-23 and 29; clade 2-5 and 2-6] and [1-13, 24-28 and 30-37; clade 4-1]	0.7718	0.8294	0.8294
3	[19-23; clade 2-6[1]], [14-18 and 29; clade 2-5] and [1-13, 24-28 and 30-37; clade 4-1]	0.8523	0.7535	0.964
4	[19-23; clade 2-6[1]], [14-18 and 29; clade 2-5], [32 and 33; clade 2-4] and [1-13, 24-28, 30-31 and 34-37[2]; clade 2-1, 2-2 and 2-3]	0.8527	0.7158	0.9581
5	[19-23; clade 2-6[1]], [14-18 and 29; clade 2-5], [32 and 33; clade 2-4], [10-13; clade 2-3[3]] and [1-9, 24-28, 30-31 and 34-37[2, 4, 5]; clade 2-1 and 2-2]	0.8711	0.5824	0.9462
6	[19-23; clade 2-6[1]], [14-18 and 29; clade 2-5], [29; clade 2-5], [32 and 33; clade 2-4], [7, 10-13; clade 2-3[3]] and [1-6, 8, 9, 24-28, 30-31 and 34-37[2, 4, 5]; clade 2-1, 2-2 and 2-3][6]	0.883	0.5244	0.9443

The *k* values represent number of groups hypothesized

The populations included in each SAMOVA group are described using the population code in Fig. 3 and Table 1

The clade number showed the majority of the haplotype found in the group. The exceptional minor haplotypes, if present, are described

[1] Population 19 had a haplotype of clade 2-5

[2] Population 4 included a haplotype of clade 2-4

[3] Population 11 had a haplotype of clade 2-1

[4] Population 7 included a haplotype of clade 2-3

[5] Population 34 had a haplotype of clade 2-3

[6] Affiliation of the population 7 changed from that of $k = 5$

Table 3 Comparisons of genetic diversity between the SAMOVA groups

The populations included in each SAMOVA group are described using the population code in Fig. 3 and Table 1

The clade number showed the majority of the haplotype found in the group

Group	No. sample	No. hap.	No. effective alleles	Gene diversity (h)	Nucleotide diversity (π)
[1-13, 24-28 and 30-37; clade 4-1]	237	26	4.8061	0.7953 ± 0.021	0.0052 ± 0.0031
[1-9, 24-28, 30-31 and 34-37; clade 2-1 and 2-2]	170	18	2.9448	0.6643 ± 0.036	0.0031 ± 0.0021
[10-13; clade 2-3]	50	7	2.5562	0.6212 ± 0.041	0.0018 ± 0.0014
[32 and 33; clade 2-4]	17	5	2.8058	0.6838 ± 0.081	0.0019 ± 0.0016
[14-18 and 29; clade 2-5]	47	4	2.3376	0.5846 ± 0.055	0.0014 ± 0.0012
[19-23; clade 2-6]	21	4	1.9776	0.5190 ± 0.105	0.0033 ± 0.0023
Total	305	33	7.2062	0.8641 ± 0.0135	0.0152 ± 0.0079

Table 4 Summary of the phylogeographic nested clade analysis (PNCA)

Clade	Chain of inference	Inferred key conclusion	Geographic distribution
1-3	1-2-3-5-6; too few	Insufficient genetic resolution between range expansion and restricted gene flow	Sea of Japan coast
1-12	1-2-3-5; yes for the Moheji population-15; no	Past fragmentation and/or long-distance colonization.	Pacific coast of Central Japan, and the Moheji population (Hokkaido)
1-13	1-2-3-4; no	Restricted gene flow with isolation by distance	Pacific coast of northern Honshu
2-3	1-2-11-12; no	Continuous range expansion	Seto Inland sea
3-1	1-2-11-12; no	Continuous range expansion	Sea of Japan coast
3-2	1-2-11-12-13; yes	Long-distance colonization with fragmentation	Seto Inland sea and northern Kyushu
4-1	1-2-3-4; no	Restricted gene flow with isolation by distance	Western Japan and Sea of Japan coast
Total cladogram	1-2-11-12-13; yes	Past fragmentation followed by range expansion	

corresponding to the estimated upper (4.1% per myr) and lower (2.6% per myr) mutation rates. The Bayesian skyline plot showed that population expansions occurred during 0.1–0.05 myr ago and that there was no apparent oscillation of population size during this 0.04–0.025 myr (Fig. 4).

Table 5 The estimated time to most recent common ancestor (TMRCA)

Clade	TMRCA (myr ago, lower and upper values)[1]	95% lower-upper confidence limit	Effective sample size
Whole *S. horneri/filicinum*	0.644 (0.498–0.789)	0.322–1.021	72,220
2-5	0.088 (0.067–0.108)	0.034–0.153	9,676
2-6	0.078 (0.060–0.095)	0.030–0.135	7,385
4-1	0.216 (0.167–0.265)	0.093–0.363	24,260
2-3	0.097 (0.074–0.119)	0.042–0.163	8,729
2-4	0.083 (0.064–0.103)	0.037–0.138	5,626
2-1	0.153 (0.118–0.188)	0.064–0.265	18,070

[1] Values correspond to the upper (4.1% per myr) and lower (2.6%) range of mutation rate of *cox3*, and their median value

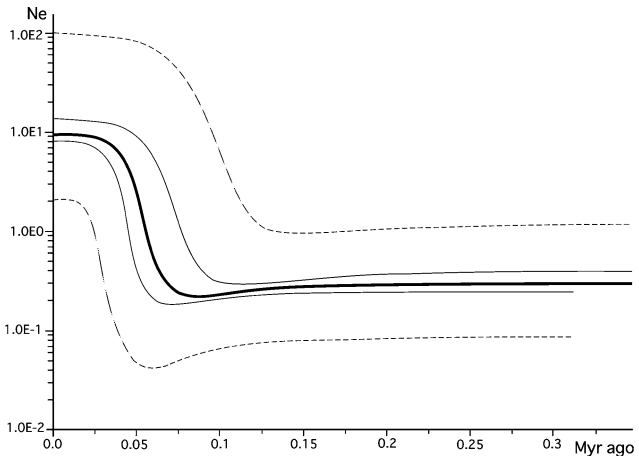

Fig. 4 Bayesian skyline plot of the Japanese *Sargassum horneri/filicinum* populations. The *thick solid line* represents the change of effective population size (*Ne*), based on the average value of the mitochondrial *cox3* mutation rate (3.4% per myr). The *upper and lower thin solid lines* represent the skyline plots based on the slow (2.6%) and the fast (4.1%) mutation rates, respectively. *Dashed lines* above and below the *solid lines* show the 95% confidence limits under the slow and fast mutation rates, respectively

Discussion

Taxonomic status of *S. horneri* and *S. filicinum*

Our analysis of mtDNA *cox3* sequences suggests that *S. filicinum* should be reduced to a synonym of *S. horneri* because of the closer phylogenetic relationship of *S. horneri* populations in western Japan to *S. filicinum* than to *S. horneri* populations in central and northern Pacific Japan. Furthermore, *S. filicinum* populations are not monophyletic in spite of the morphological diagnosis (i.e., spherical vesicles and the mating type, Okuda 1977; Yoshida 1983). Because the divergence among the western Japan haplotypes is shallow, incomplete lineage sorting (Takahata and Nei 1985; Wu 1991) could also cause the observed polyphyly of *S. filicinum*. Ajisaka and Uwai (2005) measured vesicles and receptacles of some populations of *S. horneri* and *S. filicinum* in western Japan; his result showed that the two *S. filicinum* lineages (i.e., clades 2-2 and 2-4) are morphologically indistinguishable. Furthermore, Ajisaka and Uwai (2005) concluded that *S. filicinum* and *S. horneri* are difficult to distinguish morphologically. In the present study, some, although not all, *S. horneri* plants in clade 2-3 showed androgynous receptacles. Similar androgynous receptacles have also been reported in *S. horneri* on the coast of the East China Sea (Okuda 1987). These facts suggest that key morphological characters are not strongly stable. Experimental hybrids (Okuda, unpublished data) and the shared haplotypes in the present study, though small in number, suggest crossability between species under experimental conditions and in the field; therefore, the reproductive isolation between these species is possibly incomplete. Therefore, *S. horneri* and *S. filicinum* appear to be separate from each other, but synonymous.

Alternatively, the Pacific central and northern *S. horneri* could be separated from the western Japan populations based on their deep divergence; at present, however, it is difficult to find a diagnosis between these populations. Given *S. filicinum* is a species independent from *S. horneri*, morphological diagnosis among each 2-step clade, that is, each separate monophyletic clade, is also difficult to find; previous studies have shown large morphological variations even within a single population of *S. horneri* (Yoshida 1983; Ajisaka and Uwai 2005). There are already two synonyms for *S. horneri*, *S. polyodonthum* J. Agardh and *S. spathulatum* J. Agardh, and two intraspecific variations, *S. horneri* f. *furcatodentatum* Kuntze and var. *densum* C. Agardh. However, Yoshida (1983) considered that the morphological variations among those specimens are continuous and that these entities are difficult to separate from each other. For example, Yoshida (1983) described that the pinnae of the leaves are sometimes shallower in the Pacific populations than in the Sea of Japan populations, though not all plants have leaves with shallow pinnae. Hereafter, we consider that *S. horneri* includes both *S. horneri*-type and *S. filicinum*-type populations.

Phylogeography and genetic structure of *S. horneri*

The estimated TMRCA of each of the three groups may go back to the last interglacial period or before, and the skyline plot showed that the last glacial period did not affect the population size of Japanese *S. horneri*, given that the estimated mutation rate is correct. The estimated mutation rate of *cox3* was curiously faster than that of a spacer region of the brown algal mitochondrial genome (2.7% on average,

Hoarau et al. 2007). However, the TMRCAs also go back to the last interglacial period or before, even when the analysis was performed under the spacer mutation rate (data not shown). During the LGM, the sea surface temperature (SST) was about 3–5°C lower than today on both the southern (Xu and Oda 1999; Ijiri et al. 2005) and northern (Oba and Murayama 2004) Pacific coast of Japan, as well as the Sea of Japan (Oba et al. 1991). The modern environment was established about 8–10 kya for both the Pacific and the Sea of Japan coasts; however, any effects of such paleoenvironments were not observed in the skyline plot. *S. horneri* can grow rapidly under the lower temperature of the winter season (10–15°C) in the Seto Inland Sea (Yoshida et al. 1998, 2004). Its growth and maturation (receptacle formation) depend on the day-length rather than the temperature (Uchida 1993; Yoshida et al. 2004). Therefore, plants could possibly survive under SSTs 3–5°C lower than today; the lower SST might shorten the growth period or slow the growth rate but might have no effect on survival. Alternatively, it is possible that populations moved southwards or northwards depending on the environmental oscillations, which would not decrease the genetic variation of populations if slow and steady (Hewitt 1996). Populations in the Seto Inland Sea and Sea of Japan may have been established after the LGM. The continuous distribution of clade 2-1 suggests that the populations on the Sea of Japan coast may have originated from the East China Sea coast, where a wide coastal water existed during the LGM (Ujiie and Ujiie 1999; Xu and Oda 1999; Ijiri et al. 2005).

The geographic subdivision among the haplotype groups (clades 2-5, 2-6, and 4-1) suggests that the three groups are isolated from each other, and that long-distance dispersal by drifting would be quite rare; no shared haplotype was found between the western Japan (clade 4-1) and central Pacific Japan (clade 2-5) populations, despite the amount of *S. horneri* included in floating seaweed along the Kuroshiwo warm current (Yoshida 1963; Komatsu et al. 2007). Similarly, shared haplotypes between the Sea of Japan coast (clade 2-1) and northern Pacific Japan (clade 2-6) were not detected, even though these regions are connected by the Tsushima current based on a study using drifting buoys (Yoshida 1963). Genetic similarity between the northern Pacific and the Sea of Japan populations has been reported for an intertidal gastropod *Batillaria cumingi* (Kojima et al. 2004), for which dispersal might be accomplished by rafting on algal beds or floating during the early juvenile stage. The observed geographic structure of *S. horneri* populations is, however, similar to that of *Undaria pinnatifida* (Uwai et al. 2006), which does not have buoyancy bag like vesicles and could be expected to have a limited dispersal ability, as estimated for other members of the Laminariales (Reed et al. 1988). Significant isolation-by-distance was reported in *Sargassum polyceratium* even on a small geographic scale (<50 km) by RAPD (Engelen et al. 2001). Therefore, the long-distance drifting ability of *Sargassum* species does not contribute significantly to gene flow. Possibly, drifting *S. horneri* plants are permanently exported from the habitable intertidal zone and/or the gametes/eggs of transported plants are unable to survive or reach the appropriate habitat.

In western Japan (clade 3-1), the genetic differentiation between *S. horneri*-type and *S. filicinum*-type populations may be due to limited contemporary gene flow, rather than historical geographical fragmentation, as suggested in PNCA. Small amounts of gene flow between sympatric *S. horneri*-type and *S. filicinum*-type populations are suggested by shared haplotypes (haplotype 19 in population 4, and haplotypes 13 and 18 in population 34), which is concordant with hybridization under experimental conditions (Okuda, unpublished data). The major haplotypes are, however, different between *S. horneri*-type and *S. filicinum*-type populations, which suggests that hybridization is limited despite the geographical proximity. Selfing in the androgynous *S. filicinum*-type populations could explain the present result; high rates of selfing were reported for the hermaphroditic (having both male and female gametangia in a single conceptacle) brown alga *Fucus spiralis* (Engel et al. 2005). A difference in the fruiting seasons between *S. horneri*-type and *S. filicinum*-type populations (Sawada 1955) could prevent gene flow between them; plants maturing at similar times will be more likely to mate with each other than with those maturing at different times ("isolation by time", Hendry and Day 2005). Isolation by time has been suggested for other species of *Fucus* with positive heterozygote deficiency observed in microsatellite analyses (Coyer et al. 2003; Engel et al. 2005). Differences in peak reproduction have been reported among *S. horneri*-type populations in the Seto Inland Sea (Okuda1987; Yoshida et al. 2004); population 10 becomes fertile in April and population 9 in January, and these populations, though both are dioecious, show high degrees of genetic differentiation in spite of their geographic proximity (ca. 30 km).

In conclusion, a large amount of genetic diversity was found between Japanese *S. horneri* populations. We found three distinct genetic groups, which have been separated from each other for a long period, even before the LGM. One of the three groups, distributed in western Japan, includes both *S. horneri*-type and *S. filicinum*-type populations, which have heretofore been classified as different species. Possibly the androgynous nature of the *S. filicinum*-type populations and the differences in the reproductive peaks explain the genetic differentiation between *S. horneri*-type and *S. filicinum*-type populations on small geographic scales (ca. 30 km). Although previous reports and the sea currents suggest that drifting plants could be transferred between the three groups, the present results suggest that

drifting *S. horneri/filicinum* are permanently exported from the habitable intertidal zone and/or that gametes/eggs are unable to survive or reach the appropriate habitat.

Acknowledgments We would like to express our sincere gratitude to the late Professor emeritus Takeo Okuda who re-recognized *S. filicinum* and gave us many suggestions at the beginning of this study. We are also grateful for the critical comments to our manuscript by J.A. Coyer. We also thank to following for their help in sampling; M. Aoki, Y. Haga, M. Iima, A. Kijima, S. Kirihara, T. Komatsu, K. Kuwano, A. Mikami, H. Shimabukuro, A. Tanaka, K. Tatsukawa, R. Terada, and W.D. Wang.

References

Aguilar-Rosas LE, Aguilar-Rosas R, Kawai H, Uwai S, Valenzuela-Espinoza E (2007) New record of *Sargassum filicinum* Harvey (Fucales, Phaeophyceae) in the Pacific Coast of Mexico. Algae 22:17–21

Ajisaka T, Uwai S (2005) On the morphological variations of vesicles and receptacles in *Sargassum horneri/filicinum*-group. Kaiyo Mon 37:460–465 (in Japanese)

Avise JC (2000) Phylogeography. The history and formation of species. Harvard University Press, Cambridge, Massachusetts, London

Chinzei K, Fujioka K, Kitazono H, Koizumi I, Oba T, Oda M, Okada H, Sakai T, Tanimura Y (1987) Postglacial environmental change of the Pacific Ocean off the coasts of central Japan. Mar Micropaleontol 11:273–291. doi:10.1016/0377-8398(87)90002-8

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659. doi:10.1046/j.1365-294x.2000.01020.x

Comes HP, Kadereit J (1998) The effect of Quaternary climatic changes on the plant distribution and evolution. Trends Plant Sci 3:432–438. doi:10.1016/S1360-1385(98)01327-2

Coyer JA, Peters AF, Stam WT, Olsen JL (2003) Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucales) populations in Northern Europe. Mol Ecol 12:1817–1829. doi:10.1046/j.1365-294X.2003.01850.x

Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol 22:1185–1192. doi:10.1093/molbev/msi103

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. Mol Ecol 11:2571–2581. doi:10.1046/j.1365-294X.2002.01650.x

Engel C, Daguin C, Serrao EA (2005) Genetic entities and mating system in hermaphroditic *Fucus spiralis* and its close dioecious relative *F. vesiculosus* (Fucaceae, Phaeophyceae). Mol Ecol 14:2003–2046. doi:10.1111/j.1365-294X.2005.02558.x

Engelen AH, Olsen JL, Breeman AM, Stam WT (2001) Genetic differentiation in *Sargassum polyceratium* (Fucales: Phaeophyceae) around the island of Curaçao (Netherlands, Antilles). Mar Biol (Berl) 139:267–277. doi:10.1007/s002270100586

Gorbarenko SA, Southon JR (2000) Detailed Japan Sea paleoceanography during the last 25 kyr; constrains from AMS dating and δ^{18}O of planktonic foraminifera. Palaeogeogr Palaeoclimatol Palaeoecol 156:177–193. doi:10.1016/S0031-0182(99)00137-6

Helmuth B, Veit RR, Holberton R (1994) Long-distance dispersal of a subarctic brooding bivalve (Gaimardia trapesina) by kelp-rafting. Mar Biol (Berl) 120:421–426. doi:10.1007/BF00680216

Hendry AP, Day T (2005) Population structure attributable to reproductive time: isolation by time and adaptation by time. Mol Ecol 14:901–916. doi:10.1111/j.1365-294X.2005.02480.x

Hewitt GM (1996) Some genetic consequence of ice age, and their role in divergence and speciation. Biol J Linn Soc Lond 58:247–276

Hoarau G, Coyer JA, Veldsink JH, Stam WT, Olsen JL (2007) Glacial refugia and recolonization pathways in the brown seaweed *Fucus serratus*. Mol Ecol 16:3606–3616. doi:10.1111/j.1365-294X.2007.03408.x

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Baysian inference of phylogeny. Bioinfomatics 17:754–755. doi:10.1093/bioinformatics/17.8.754

Ijiri A, Wang L, Oba T, Kawahata H, Huang C-Y, Huang C-Y (2005) Paleoenvironmental changes in the northern area of the East China Sea during the past 42,000 years. Palaeogeogr Palaeoclimatol Palaeoecol 219:239–261. doi:10.1016/j.palaeo.2004.12.028

Kogame K, Uwai S, Shimada S, Masuda M (2005) A study of sexual and asexual populations of *Scytosiphon lomentaria* (Scytosiphonaceae, Phaeophyceae) in Hokkaido, northern Japan, using molecular markers. Eur J Phycol 40:313–322. doi:10.1080/09670260500193008

Kojima S, Segawa R, Hayashi I (1997) Genetic differentiation among populations of the Japanese turban shell Turbo (*Batillus*) cornutus corresponding to warm currents. Mar Ecol Prog Ser 150:149–155. doi:10.3354/meps150149

Kojima S, Hayashi I, Kim D, Iijima A, Furuta T (2004) Phylogeography of an intertidal direct-developing gastropod *Batillaria cumingi* around the Japanese islands. Mar Ecol Prog Ser 276:161–172. doi:10.3354/meps276161

Komatsu T, Tatsukawa K, Filippi JB, Sagawa T, Matsubaga D, Mikami A, Ishida K, Ajisaka T, Tanaka K, Aoki M, Wang W-D, Liu H-F, Zhang S-D, Zhou M-D, Sugimoto T (2007) Distribution of drifting seaweeds in eastern East China Sea. J Mar Syst 67:245–252. doi:10.1016/j.jmarsys.2006.05.018

Miller KA, Engel J, Uwai S, Kawai H (2007) First Report of the Asian Seaweed *Sargassum filicinum* Harvey (Fucales) in California, USA. Biol Invasions 9:609–613. doi:10.1007/s10530-006-9060-2

Oba T, Murayama M (2004) Sea-surface temperature and salinity changes in the northwest Pacific since the Last Glacial Maximum. J Quat Sci 19:335–346. doi:10.1002/jqs.843

Oba T, Kato M, Kitazato H, Koizumi I, Omura A, Sakai T, Takayama T (1991) Paleoenvironmental changes in the Japan Sea during the last 85,000 years. Paleoceanography 6:499–518. doi:10.1029/91PA00560

Ohno M (1984) Observation on the floating seaweed on the near-shore waters of the southern Japan. Hydrobiologia 116/117:408–412. doi:10.1007/BF00027711

Okuda T (1977) *Sargassum filicinum*; its new findings in sexuality and distribution around Japan. Bull Jap Soc Phycol 25(Suppl):265–269

Okuda T (1987) Monoecism and autumn-fruiting of *Sargassum horneri*. Jap J Phycol 35:221–225 (in Japanese with English summary)

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818. doi:10.1093/bioinformatics/14.9.817

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for cladistic nested analysis of the geographical distribution of genetic haplotypes. Mol Ecol 9:487–488. doi:10.1046/j.1365-294x.2000.00887.x

Reed DC, Laur DR, Abeling AW (1988) Variation in algal dispersal and recruitment: the importance of episodic event. Ecol Monogr 58:321–335. doi:10.2307/1942543

Sawada T (1955) Some observations on *Sargassum filicinum* Harvey. Sci Bull Fac Agr Kyushu Univ 15:71–76 (in Japanese with English abstract)

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN: a software for population genetics data analysis. ver. 2.00. Genetics and Biometry Lab, Department of Anthropology, University of Geneva

Stieger V, Horiguch T, Yoshida T, Coleman AW, Masuda M (2003) Phylogenetic relationships within the genus *Sargassum* (Fucales, Phaeophyceae), inferred from ITS-2 nrDNA, with an emphasis on the taxonomic subdivision of the genus. Phycol Res 51:1–10. doi:10.1111/j.1440-1835.2003.tb00164.x

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony (and other methods). ver. 4.0b. Sinauer Associates, Sunderland, Massachusetts

Taberlet P, Fumagalli L, Wust-Saucy A-G (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol 7:453–464. doi:10.1046/j.1365-294x.1998.00289.x

Takahata N, Nei M (1985) Gene genealogy and variance of interpopulational nucleotide differences. Genetics 110:325–344

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Mol Ecol 7:381–397. doi:10.1046/j.1365-294x.1998.00308.x

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. Genetics 140:767–782

Uchida T (1993) The life cycle of *Sargassum horneri* (Phaeophyceae) in laboratory culture. J Phycol 29:231–235. doi:10.1111/j.0022-3646.1993.00231.x

Uehara S, Taggart CT, Mitani T, Suthers IM (2006) The abundance of juvenile yellowtail (*Seriola quinqueradiata*) near the Kuroshio: the roles of drifting seaweed and regional hydrography. Fish Oceanogr 15:351–362. doi:10.1111/j.1365-2419.2005.00382.x

Ujiie H, Ujiie Y (1999) Late quaternary course changes if the Kuroshio Current in the Ryukyu Arc region, northern Pacific Ocean. Mar Micropaleontol 37:23–40. doi:10.1016/S0377-8398(99)00010-9

Uwai S, Yotsukura N, Serisawa Y, Muraoka D, Hiraoka M, Kogame K (2006) Intraspecific genetic diversity of *Undaria pinnatifida* in Japan, based on the mitochondrial cox3 gene and the ITS1 of nrDNA. Hydrobiologia 553:345–356. doi:10.1007/s10750-005-0883-0

Wu C-I (1991) Inferences of species phylogeny in relation to segregation of ancient polymorphisms. Genetics 127:429–435

Xu X, Oda M (1999) Surface-water evolution of the eastern East China Sea during the last 36,000 years. Mar Geol 156:285–304. doi:10.1016/S0025-3227(98)00183-2

Yashima K (1994) A geomorphological study of the caldrons in the Seto Inland Sea. Rep Hydrogr Res 30:237–327 (in Japanese with English summary)

Yoshida T (1963) Studies on the distribution and drift of the floating seaweeds. Bull Tohoku Reg Fish Lab 23:141–186 (in Japanese with English summery)

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

Yoshida G, Arima S, Terawaki T (1998) Growth and maturation of the 'autumn-fruiting type' of *Sargassum horneri* (Fucales, Phaeophyceae) and comparisons with the 'spring fruiting type'. Phycol Res 46:183–189. doi:10.1111/j.1440-1835.1998.tb00112.x

Yoshida G, Murase N, Arai S, Terawaki T (2004) Ecotypic differentiation in maturation seasonality among *Srgassum horneri* (Fucales, Phaeophyta) populations in Hiroshima Bay, Seto Inland Sea, Japan. Phycologia 43:703–710