Chapter 14
Phylogeography of Macroalgal Species Distributed in Brackish Water: *Ulva prolifera* (Ulvophyceae) and *Pyropia tenera* (Bangiophyceae)

Satoshi Shimada, Kensuke Ichihara, Yuka Masakiyo, Riko Kawaguchi and Norio Kikuchi

Abstract Comparative studies of closely related species or populations in contrasting environments can potentially provide insights into adaptive mechanisms. We review the phylogeography and population diversity of brackish water species derived from marine species, *Ulva prolifera* Müller (Ulvophyceae) and *Pyropia tenera* (Kjellman) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi (Bangiophyceae). Brackish *U. prolifera* and marine *Ulva linza* L. have been resolved as closely related species based on phylogenetic analysis of moleclar markers, with *U. linza* apparently parental to *U. prolifera*. Our analyses of 5S rDNA spacer region in samples from an Ulvalean bloom in Qingdao, China, indicate that the species appear to be derived from Japanese *U. prolifera.* Hybridization tests suggest that *U. linza* and the Qingdao bloom samples are probably distinct species, but gene flow is possible between them. The threatened brackish water species, *P. tenera*, is morphologically and phylogenetically related to the coastal species, *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi. One form, *P. yezoensis* Ueda f. *narawaensis* Miura (new combination "*Pyropia yezoensis* f. *narawaensis*" has not yet been proposed), is the largest aquaculture source of "Nori" in Japan. Hybridization between these species has been reported, especially between male *P. tenera* and female *P. yezoensis*. Sequences of the nrITS1 region and *rbc*L gene, and PCR-RFLP (ARP4 gene) analyses suggested that *P. tenera* is distributed across 15 prefectures from Kyushu to Tohoku in Japan; but is

S. Shimada (✉) · Y. Masakiyo · R. Kawaguchi
Division of the Natural/Applied Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2–1–1 Otuka, Bunkyo-ku 112–8610, Tokyo, Japan
e-mail: shimada.satoshi@ocha.ac.jp

K. Ichihara
Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa 277-8562, Chiba, Japan

N. Kikuchi
Coastal Branch of Natural History Museum and Institute, Chiba, Yoshio 123, Katsuura 299-5242, Chiba, Japan

restricted to estuarine and brackish water habitats. Based on SSR analysis on the genetically identified *P. tenera* samples, we conclude that this species spread from Kyushu to Tohoku through the Kanto region.

Keywords Endangered species · Green algae · Green tide · Japan · NrITS1 · Red algae · SSR

14.1 Introduction

Macroalgae comprise three major groups: Ulvophyceae (green seaweeds), Phaeophyceae (brown seaweeds) and Rhodophyceae (red seaweeds), which are found on seashores all over the world (van den Hoek et al. 1995). Comparative studies of related species in different environments can potentially elucidate mechanisms of adaptation. In addition to marine species, each group includes brackish and freshwater species, e.g., *Dichotomosiphon tuberosus* (Braum) Ernst (Bryopsidales, Ulvophyceae), *Heribaudiella fluviatilis* (Areschoug) Svedelius (Sphacelariales, Phaeophyceae) and *Caloglossa continua* (Okamura) King et Puttock (Ceramiales, Rhodophyceae) (Guiry et al. 2015). Molecular phylogenetic studies have revealed that many of these brackish/freshwater species evolved from marine ancestors (Lam and Zechman 2006; Krayesky et al. 2012; Silberfeld et al. 2014). Brackish water species have acquired molecular, functional and physiological characteristics required for adaptation to low-salinity conditions. However, many questions about the biological evolution of brackish and freshwater algae are yet to be resolved. Which genes are responsible for this new environmental adaptation? When and where did the brackish/freshwater species separate from marine ancestral species? What are their distributions relative to environmental gradients?

Phylogeography and population genetics are among the most powerful tools used to understand the provenance, genetic structure, distributional expansion and population diversity of organisms (e.g., dinoflagellates, Nagai et al. 2009; crayfish, Koizumi et al. 2012; humans, Jinam et al. 2012; butterflies, Jeratthitikul et al. 2013; hares, Nunome et al. 2014a, b). Land plants have been particularly well studied (van Inghelandt et al. 2010; Kaya et al. 2013; Xu et al. 2014). For instance, the probable distributions of woody plants at the Last Glacial Maximum (LGM, 0.026–0.019 Ma) has been estimated with palaeodistribution modeling (Sakaguchi et al. 2012), endangered plants were interpreted using population diversity and conservation units (Matsuda and Setoguchi 2012), and extinction risks for wild pollinated conifer populations were results from Simple Sequence Repeat (SSR) marker analyses (Iwasaki et al. 2013). These research techniques are also useful for examining the evolutionary history of seaweeds and phylogenetically related brackish and freshwater macroalgae (Perevra et al. 2009). Phylogeographic research can provide important clues to help understand molecular evolution, as it relates to

environmental adaptation through all three aquatic environments (freshwater, brackish water, and seawater).

In this chapter, we review the phylogeography and population diversity of brackish water species derived from marine species in two case studies: *Ulva prolifera* Müller (Ulvophyceae) and *Pyropia tenera* (Kjellman) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi (Bangiophyceae).

14.2 *Ulva* (Ulvophyceae, Chlorophyta)

The genus *Ulva* is one of the most common seaweeds (Canter-Lund and Lund 1995; van den Hoek et al. 1995; Shimada et al. 2007). New species from the genus have been described year after year (Horimoto et al. 2011; Masakiyo and Shimada 2014; Matsumoto and Shimada 2015). This genus includes brackish species (*U. prolifera*) (Hiraoka and Shimada 2004) and freshwater species (*Ulva limnetica* Ichihara et Shimada) (Ichihara et al. 2009a). Due to the euryhaline nature of *Ulva*, low-salinity tolerance and adaptation can be found, as reported in many studies (Reed and Russell 1979; Martins et al. 1999; Ohno et al. 1999; Kamer and Fong 2000; McAvoy and Klung 2005; Ichihara et al. 2009b, 2011, 2013).

Ulva prolifera (Fig. 14.1a), which was originally described from Nebbelund, Lolland Island, Denmark (Müller 1778), has branched tubular thalli and is commonly distributed in estuarine and brackish waters (Burrow 1991; Shimada et al. 2008; Hiraoka et al. 2003, 2011). This species is of major importance to the

Fig. 14.1 Cultured thalli of molecular-identified *Ulva prolifera* (**a**) and *Ulva linza* (**b**) under 20 °C, 16:8 h LD, 15–25 μE m^{-2} s^{-1} photon flux with PES medium (Provasoli 1968)

1 cm
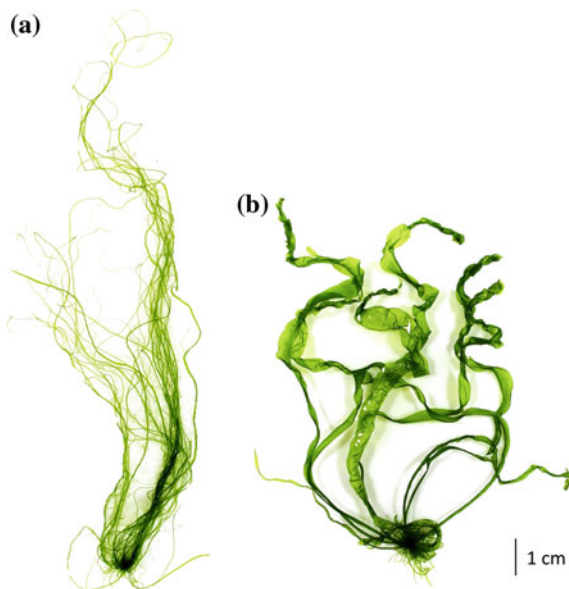
Japanese fisheries industry, as it is a food for human consumption and has a regulated IQ (import quota) under Japanese domestic law (Kawashima et al. 2014). Due to an increasing demand, the supply of *U. prolifera* is also supplemented by land-based culture techniques in tanks using seawater (Hiraoka and Oka 2008). This species and *Ulva linza* L. are closely related based on phylogenetic studies (Shimada et al. 2003).

Ulva linza (Fig. 14.1b) is distributed in marine habitats, and possesses unbranched distromatic folious thalli without a stipe or margin (Brodie et al. 2007). In freshwater conditions, the cell viability of *U. linza* dropped to approximately 20 % after 7 days, while that of *U. prolifera* remained at almost 100 % (Ichihara et al. 2013). These physiological differences seem to be the source of their distinct distributions, with *U. prolifera* inhabiting estuaries with fluctuations in salinity, while *U. linza* occurs in marine environments (Ohno et al. 1999; Hiraoka and Shimada 2004). *Ulva linza* has higher intraspecific diversity than *U. prolifera*, suggesting that the latter brackish species is derived from marine *U. linza* (Shimada et al. 2008).

The "green tide" phenomenon occurs under conditions when an extensive biomass of free-floating green algae accumulates on shallow beaches. Free-floating *Ulva* causes green tide at several locations around the world (Fletcher 1996; Ohno 1999). In June 2008, it was reported worldwide that a vast algal bloom had occurred in Qingdao, China. This massive green-tide covered about 600 km^2 along the coast of Qingdao, the host city for the Olympic sailing regatta. An army of more than 10,000 recruits was deployed for a period of one week to help remove the algal bloom. Qingdao city reported that the total mass of the bloom was about 10,000 ton wet weight (BBC NEWS 2008).

Based on satellite imaging data, Liu et al. (2009) proposed the possibility that nonlocal sources were responsible for the Qingdao bloom. According to these authors, on the 15th of May 2008, some small green patches covering a total surface area of about 80 km^2 were observed near the coasts of Yancheng and Lianyungang, Jiangsu province. Soon, they grew rapidly and moved into the middle of the Yellow Sea. Finally, a large quantity of biomass formed the famous green tide along the coasts of Qingdao.

The world's-largest super floating macroalgal bloom of *U. prolifera* has lasted eight years so far, reoccurring every summer in the Yellow Sea (Xing et al. 2014). The inter-annual variability in human-induced nutrient pollution from 2001 to 2012 was assessed, and a significant increase in nutrient uptake was found in the macroalgal bloom phase (2007–2012). Annual in situ nutrient concentrations increased rapidly from 2000 to 2011 in the Jiangsu Shoal, which was the origin of the drifting macroalgae (Xing et al. 2014).

Shimada et al. (2010) carried out a phylogeographic study of *U. prolifera* using samples from Japan and the Qingdao bloom. Figures 14.2 and 14.3 show the geographical distribution of sequence types of the 5S rDNA spacer region and the statistical parsimony network of *U. prolifera*, respectively. Type-A (red circle) individuals are widely distributed along the Pacific coast of Japan from Okinawa to the Kanto region. The blue lineage, derived from type-A in Kanto, radiated to the
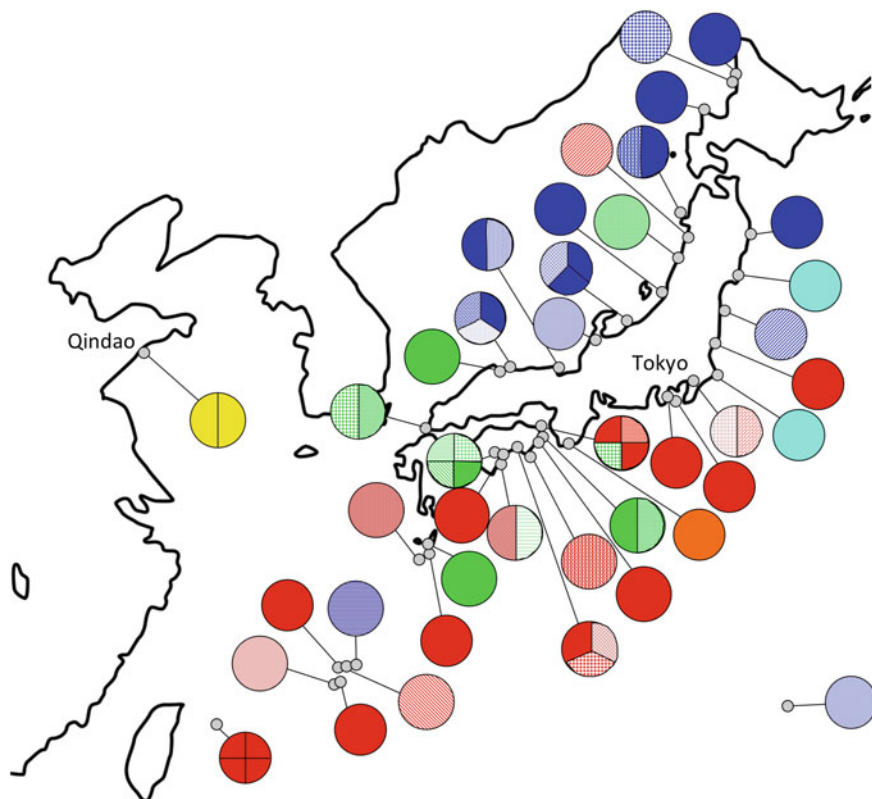
Fig. 14.2 Geographical distributions of sequence types of the 5S rDNA spacer region. Combinations of color and pattern represent different types and are same as Fig. 14.3

northern part of Honshu, and spread to the Sea of Japan through the Tsugaru channel (type-N: blue circle). The Qingdao bloom samples (type-QB: yellow circle) were derived from the blue lineage. There are 54 indels between type-QB and type-N. These results indicate that the individuals causing the Qingdao bloom might have recently derived from the Japanese *U. prolifera,* but are not the same taxon as the latter.

Hiraoka et al. (2011) experimentally hybridized individuals of *U. linza,* Japanese *U. prolifera* and the Qingdao bloom strain. The sexually reproducing Qingdao strains were successfully crossed with *U. prolifera* with no evidence of a reproductive barrier, but could not be crossed with *U. linza* due to gamete incompatibility. However, the results of *U. prolifera* × *U. linza* showed an unusual mating activity: male gametes of *U. prolifera* cannot fuse with female gametes of *U. linza,* but female gametes of *U. prolifera* successfully fuse with male gametes of *U. linza.* The hybrid zygotes can normally develop into sporophytes, and the F1 sporophyte produces zoospores via meiosis. Additionally, the zoospores grow into F1
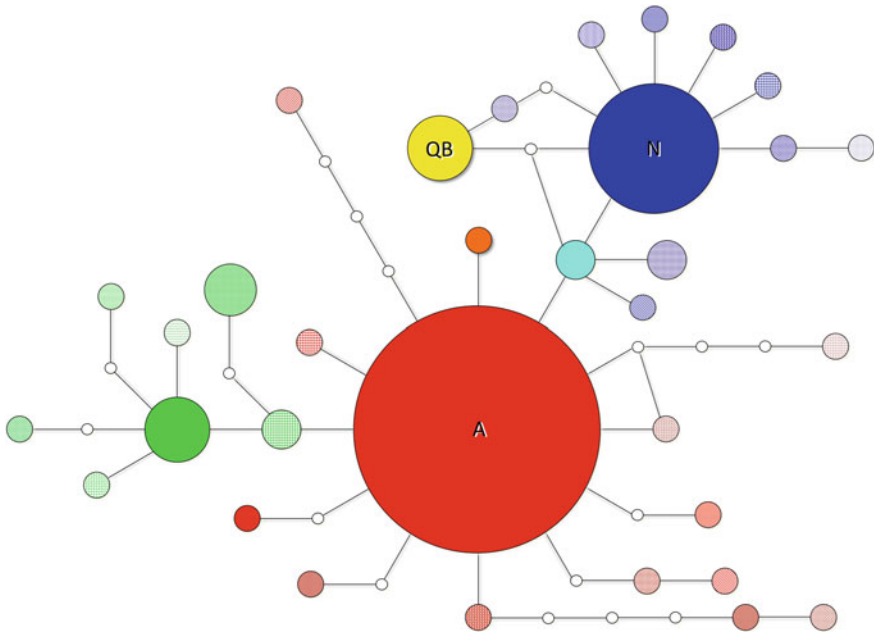
Fig. 14.3 Statistical parsimony network of 5S rDNA spacer region. Indels were treated as missing data. Each line connecting sequence types corresponds to one base mutation. A *white circle* in the parsimony network represents a missing sequence type. The size of circles corresponds to the frequency of each type

gametophytes which can produce normal gametes. These results indicate that *U. linza* and the Qingdao bloom samples are probably different species, but gene flow is nonetheless still possible between them through the Japanese *U. prolifera*.

Microsatellite/SSR markers have been developed for many organisms due to their unique advantages such as being codominant, highly polymorphic, well distributed throughout the genome, and their simplicity of application for analysis and consistency (Varshney et al. 2002; Tanaka et al. 2011). Recently, Expressed Sequence Tag (EST)-SSR markers were developed for *U. prolifera* from the south Yellow Sea (Zhang et al. 2014). The analysis of genetic variation using the SSR markers indicated that most individuals collected at the same site clustered together in a tree produced by the unweighted pair-group mean analysis method (UPGMA). The clusters in the tree showed some consistency with the geographical origins of the samples. In addition, 12 of 13 free-floating samples were grouped as a single clade separated from the attached samples. These free-floating samples were collected from different sites at different dates. These results indicate that the free-floating masses of *U. prolifera* may share the same origin and have been dispersed along coastal areas by wind and ocean currents (Zhang et al. 2014).

Ulva linza and *U. prolifera*, including the Qingdao blooms, is an ideal species-complex to study speciation and environmental adaptation to low (from

U. linza to Japanese *U. prolifera*) and high (from Japanese *U. prolifera* to the Qingdao bloom samples) salinities. Kawashima et al. (2013) reported that the 5S rDNA spacer analysis of Japanese and Chinese *U. prolifera* commercial products detected the potential for intercrossing between *U. linza* and *U. prolifera*. Therefore, hybrids of *U. linza* and *U. prolifera* are likely to appear in nature. We are now analyzing gene flow between field populations in both species to gain an accurate understanding of their taxonomic status. RNA-seq analysis is being conducted using next generation sequencing (NGS) to elucidate the molecular evolution of low-salinity adaptation in *U. prolifera*, by comparing the gene expression levels of *U. prolifera* and *U. linza* cultured in seawater, brackish water and freshwater conditions. NGS allows us to study gene expression in nonmodel organisms without previous genomic resources (e.g., species-specific primers), and RNA-seq is a very promising application for the study of environmental adaptation (Ekblom and Galindo 2011). RAD-seq (Restriction Site Associated DNA Sequence) from NGS analysis is also useful in studies of speciation, population genetics and phylogeography (Davey and Biaxter 2010; Wagner et al. 2013). NGS analyses will undoubtedly provide a better understanding into algal problems such as green tides. These studies also present new insights into speciation and environmental adaptation, which is the major driver of biological evolution.

14.3   *Pyropia* (Bangiophyceae, Rhodophyta)

Pyropia is a foliose red algal genus, consisting of 60 species (Guiry et al. 2015). It is currently recognized as a distinct genus from the closely related genera *Porphyra*, *Boreophyllum*, *Miuraea*, *Lysithea*, *Fuscifolium*, and *Wildemania* (Sutherland et al. 2011). "Nori" (laver), used in sushi, is an edible seaweed product made using thalli of *Pyropia* species obtained from the field and/or aquaculture, including *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi, *Pyropia haitanensis* (T.J. Chang & B.F. Zheng) N. Kikuchi, M. Miyata, *Pyropia pseudolinearis* (Ueda) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi and others (Park et al. 2003; Touhata et al. 2013; Xia et al. 2013).

Pyropia yezoensis was originally described from Hokkaido, Japan (Ueda 1932) (Fig. 14.4a). Its type locality is "Muroran?" based on the label of the holotype specimen (Sutherland et al. 2011). One form of this species, *P. yezoensis* Ueda f. *narawaensis* Miura (new combination "*Pyropia yezoensis* f. *narawaensis*" has not yet been proposed), is the largest aquaculture source of laver in Japan (Miura 1988). Recently, Nakamura et al. (2013) determined a symbiont-free genome sequence of this species using NGS of purified protoplasts. The *P. yezoensis* genome could represent a model genome for examining red algal life history, and will provide insights into Nori aquaculture in the near future (Nakamura et al. 2013).

Pyropia tenera (Kjellman) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi was described in Japan based on dried Nori sheets (Kjellman 1897) (Fig. 14.4b). Although this species was used for Nori cultivation from the Edo period

Fig. 14.4 Cultured young thalli of molecular-identified samples of *Pyropia yezoensis* (**a**) and *Pyropia tenera* (**b**) under 15 °C, 8:16 h LD, 15–25 μE m^{-2} s^{-1} photon flux with PES medium (Provasoli 1968)

(17 century) to the 1960s, *P. yezoensis* is better than *P. tenera* in color, gloss and tolerance of strong waves and windy conditions, and thus almost all cultivators now use *P. yezoensis* in Japanese aquaculture (Miura 1988; Notoya 2004). *Pyropia tenera* has been categorized as an endangered species (CR + EN) by the Ministry of the Environment in Japan (Environment Agency of Japan 2000). The habitat of this species thought to be limited primarily to the vicinity of the mouths of large and small rivers (brackish water), and only eight localities of *P. tenera* in Japan were known in 2002 (Kikuchi et al. 2002).

This threatened species, *P. tenera*, is morphologically and phylogenetically related to *P. yezoensis* (Yoshida 1998; Sutherland et al. 2011). Hybridization between *P. tenera* and *P. yezoensis* has been reported, especially between male *P. tenera* and female *P. yezoensis* (Niwa et al. 2009). The reverse combination (hybridization between female *P. tenera* and male *P. yezoensis*) also exists, but does not lead to normal development, and thus it is thought that there are few occurrences in the field (Niwa et al. 2010; Niwa and Sakamoto 2010). Although hybrids between female *P. tenera* and male *P. yezoensis* showed *P. tenera*-type in both nrITS (nuclear encoded Internal Transcribed Spacer) and *rbc*L (ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit) because of concerted evolution, all four types of individuals (1: pure *P. tenera,* 2: pure *P. yezoensis*, 3: hybrids between male *P. tenera* and female *P. yezoensis,* and 4: hybrids between female *P. tenera* and male *P. yezoensis*) can be clearly distinguished by a combination of Niwa's methods using the direct sequence of nrITS and *rbc*L, and PCR-RFLP (Restriction Fragment Length Polymorphism) of the ARP4 gene (Niwa et al. 2009, 2010; Niwa and Sakamoto 2010).

We first determined the distribution of *P. tenera* and the intercross of *P. tenera-yezoensis* in 165 samples identified morphologically as *P. tenera* collected at 46 localities in 17 prefectures around Japan (Ohnishi et al. 2013). Sequences of the nrITS1 region and *rbc*L gene, and PCR-RFLP (ARP4 gene) indicated that *P. tenera*
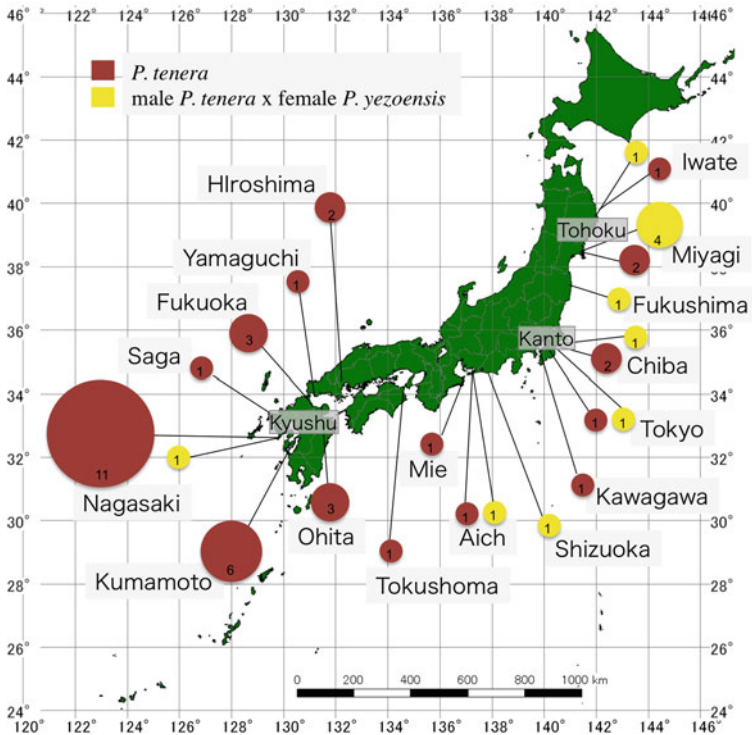
Fig. 14.5 Distribution map of *Pyropia tenera* (*red*) and hybridization between male *P. tenera* × female *P. yezoensis* (*yellow*) based on molecular identification. The number in the *circle* indicates locality numbers in each prefecture. The size of circles corresponds to the frequency of each type

occurred at 38 localities in 15 prefectures (Fig. 14.5). *Pyropia tenera* was restricted to estuarine and brackish water habitats, mainly in the Kyushu region (western part of Japan) and in the Tohoku region, but not in the Sea of Japan. *Nemalionopsis tortuosa* Yoneda et Yagi (Thoreales, Rhodophyta), designated as an endangered species (CR + EN), is also found in many localities around Kyushu (Shimada et al. 2012). Since several threatened algal species have been reported from Kyushu (Environment Agency of Japan 2000), it is one of the most important regions for the conservation of macroalgae in Japan.

Hybrids between male *P. tenera* and female *P. yezoensis* were detected at 11 localities in 8 prefectures, mostly located in Tokai, and Kanto to Tohoku regions (Fig. 14.5). Hybridization was restricted to *P. tenera*'s brackish water habitat. The original distribution of *P. yezoensis* is thought to have been from Kanto to Hokkaido (Niwa et al. 2009), thus it is reasonable that the hybrids are most common in the eastern to northern parts of Honshu. As these species are cultivated on the Pacific coast of Japan from Kyushu to Tohoku region (Miura 1988), hybrids in
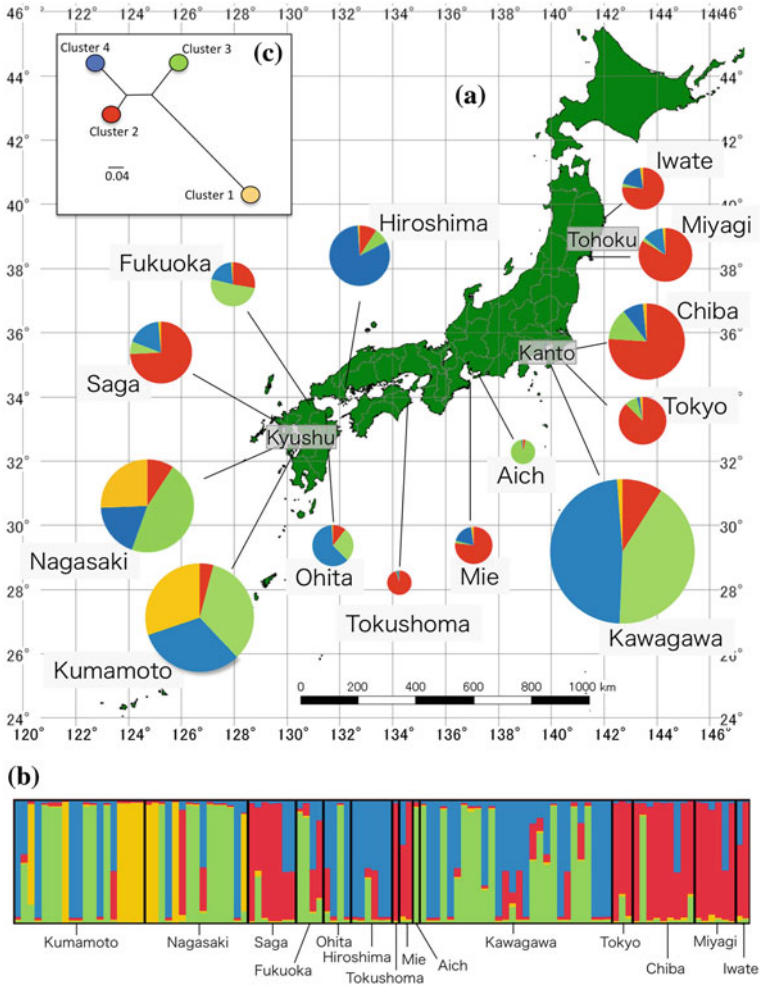
Fig. 14.6 Population tree of Japanese *Pyropia tenera* constructed by neighbor-joining method with D_A data from three SSR markers. Modified from Ohnishi et al. (2013)
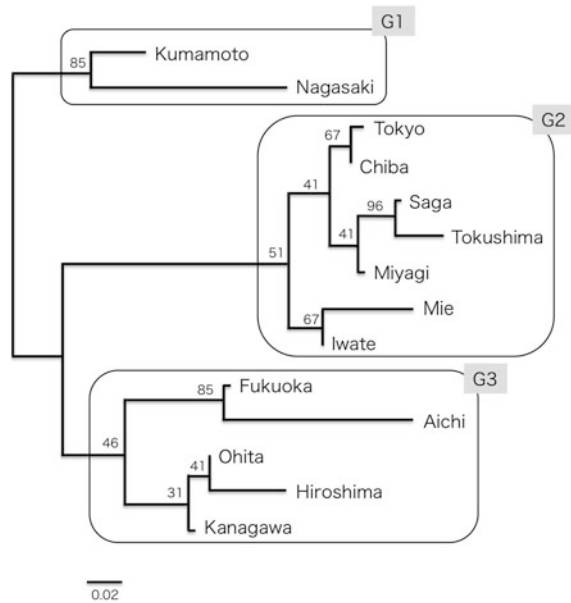
the Nagasaki Prefecture may be the result of crosses between wild *P. tenera* and cultivated *P. yezoensis*. Crossing between the two species apparently occurs relatively easily.

Next, from SSR analysis of the molecular-identified *P. tenera* samples, we determined genotypes of three microsatellite markers (Pye13, Pye41, Pye53 of Niwa et al. 2010). Three genetic groups (G1, G2 and G3) appeared in neighbor-joining trees of inter-population genetic distances (Nei and Tajima 1983) computed with POPULATION 1.2.31 (http://bioinformatics.org/~tryphon/populations/) (Fig. 14.6). G1 comprised Kumamoto and Nagasaki populations (Kyushu region) with a high bootstrap value (85 %). Populations of G2 (51 % bootstrap value) were mainly distributed along the Pacific coast from the Kanto to Tohoku region. G3 was supported by a bootstrap value of only 46 %, and the populations in this cluster were geographically scattered (Fig. 14.6).

The cluster analysis generated using STRUCTURE (Pritchard et al. 2000) and Structure Harvester v. 2.3.1 (Earl and von Holdt 2012) revealed four genetically differentiated clusters in the Japanese *P. tenera* (Fig. 14.7). The clustering patterns for $K = 4$ were highly consistent for 10 independent runs. The result was consistent with the previous POPULATION analysis. Cluster 1 (yellow) was present in only Kumamoto and Nagasaki populations, which is consistent with the G1 POPULATION analysis. Cluster 2 (red) appeared mostly in G2 populations. Clusters 3 (green) and 4 (blue) were detected in almost all populations, indicating that these lineages in these clusters might be ancestral. G3 populations (Fukuoka, Aichi, Ohita, Hiroshima, and Kanagawa populations) overlapped at least 50 % with clusters 3 and 4 and were absent cluster 1.

Fig. 14.7 Best clustering result (*K* = 4 clusters) for the 14 populations of the Japanese *Pyropia tenera* based on the STRUCTURE method. **a** Cluster distribution in each prefecture, **b** bar plot showing each individual. Each individual is represented by a *vertical line* partitioned into *colored segments*; the segment length is proportional to the individual's estimated *K* cluster membership coefficient. The sampled populations are separated by *black vertical lines*. **c** Neighbor-joining tree of the four clusters. Modified from Ohnishi et al. (2013)

The populations of Kumamoto Prefecture had higher levels of genetic diversity (Allele Richness (AR): 1.542, H_E (expected heterozygosity): 0.508, F_{IS} (inbreeding coefficient of an individual with respect to the local subpopulation): 0.556) and Nagasaki (AR: 1.615, H_E: 0.582, F_{IS}: 0.575) than the average of all the other populations (AR: 1.444, H_E: 0.278, F_{IS}: 0.239). This large genetic diversity may indicate a long stable history during which diversity has accumulated. However, the high F_{IS} indicated inbreeding or subpopulations. On the other hand, the populations in the

region extending from Kanto to Tohoku region consisted largely of cluster 2 (red) and had moderately low levels of genetic diversity (AR: 1.291–1.389, H_E: 0.167–0.323) (Table 6 in Ohnishi et al. 2013). This result and the direction of the Kuroshio Current, flowing from the southern to the northern Pacific coast of Japan, indicate that *P. tenera* probably spread from Kyushu to Tohoku through the Kanto region.

Recently, *P. tenera* has been rediscovered at the Tamagawa River (Kanagawa Prefecture, Kanto Region), on the border between Tokyo and Kanagawa, near Haneda International Airport, (Kikuchi and Niwa 2006). In Tokyo Bay, the mouth of the Tamagawa River, *P. tenera* was farmed from the seventeenth century until the 1950s (Okamura 1909; Miyashita 2003). However, since the 1950s, aquaculture of *P. tenera* has not been carried out in the vicinity of Tokyo Bay due to increased landfill and deterioration of farming environments (Kawasaki City Museum 1995). The samples from the Tamagawa River site often showed ancestral clusters 3 and 4 in our SSR analysis. These results indicate that the populations of *P. tenera* at Tamagawa River may include remnant descendants from the Edo Period. Also, a population-specific allele has been observed in this area (allele 229 of pye53) (Table 7 in Ohnishi et al. 2013). In recent years, however, the number of individuals in this region has declined, increasing the risk of extinction (Kikuchi and Niwa 2006). In order to protect this area-specific genetic diversity, specific conservation activities are a high priority.

There are a few reports about differences between the two species: *P. tenera* blades are softer in texture and considered better in taste and flavor (Ueda 1932), and it easier to infect with a virus than *P. yezoensis* (Tatyana et al. 2012). However, it is very difficult to identify *P. tenera, P. yezoensis* and their hybrids by gross morphology alone (Niwa et al. 2005; Kikuchi 2014). By using molecular-identified samples, researchers can better investigate differences in their gross morphology and color, growth rate, adaptation to marine/brackish water habitats, taste and flavor, and resistance to viral infections. It is hoped that such studies can contribute to elucidating the mechanisms of morphological/physiological evolution of seaweeds.

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