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High heterozygosity and phenotypic variation of zoids in apomictic *Ulva prolifera* (Ulvophyceae) from brackish environments

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ABSTRACT

Apomictic reproduction without alternation of generations has been reported in *Ulva prolifera* Müller from some brackish environments, and few genetic and phenotypic variations are expected in such apomictic populations. We collected 534 specimens of *U. prolifera* throughout the year from brackish lakes and a river and genotyped all the samples using a nuclear-encoded *hsp90* gene sequence. In total, 14 genotypes were detected, 12 of which were heterozygous ($n = 531$), and the genotype composition differed between the lakes and estuaries as well as between the high and low brackish sites. We tried to induce discharge of zoids from the collected thalli, and the phenotypes of zoids were examined in total 309 specimens. The flagellum number of the zoids differed among the eight most frequently encountered genotypes, i.e., six genotypes released only biflagellate zoids, whereas the other two produced quadriflagellate zoids. The ratio of specimens that produced positively phototactic zoids, which varied among the genotypes regardless of the flagellum number, was relatively high in the high-salinity brackish regions. The parthenogenetic thallus had the same genotype, flagellum number, and direction of phototaxis relative to the parental thallus in all the genotypes examined, which suggested clonal apomictic reproduction. Apomixis induction through outcrossing between genetically different entities has been reported in other algae and this may have occurred many times in *U. prolifera*.

1. Introduction

Ulva prolifera Müller is abundant in estuaries and brackish lakes and is an important edible seaweed in Japan (Nisizawa et al., 1987; Mouritsen, 2013). Recently, large-scale blooms of free-floating *U. prolifera* have been reported from the Yellow Sea, which have caused ecological and social problems (Keesing et al., 2011). Because of its rapid growth, *U. prolifera* has also been considered as a biofuel resource (XiuChen et al., 2012; Zhuang et al., 2012).

Mikata-goko comprises five lakes connected directly or indirectly to the Sea of Japan, thereby producing a large-scale salinity gradient throughout most of the interconnected system (Fig. 1). Previously, we conducted morphological and genetic examinations of *Ulva* spp. from Mikata-goko and the adjacent Minami River during a 2-year period (Ogawa et al., 2013). After analyzing the ribosomal internal transcribed spacer (ITS) of 125 specimens from a marine site and 1169 specimens from seven brackish sites, we detected four marine species and six brackish species. Among the brackish species, *U. prolifera* exhibited the widest distribution and the highest genetic diversity, and furthermore its genetic variation was much higher than that reported in *U. prolifera* from other regions (Huh et al., 2004; Shimada et al., 2008; Zhao et al., 2011; Duan et al., 2012). Recently, Hayakawa et al. (2012) surveyed differentiation of *Cladophora* from different salinity regimes in Mikata-goko and indicated intraspecific variations of salinity response. In *U. prolifera*, growth and reproduction responses to various salinity have been investigated (Young et al., 1987; Wang et al., 2007; Lin et al., 2011; Luo and Liu, 2011), but physiological differentiation has not been reported.

There have been many previous reports of apomictic (asexual) reproduction in *U. prolifera*, i.e., the recycling of haploid gametophytes without fertilization or the recycling of diploid sporophytes without meiosis (Bliding, 1963; Hiraoka et al., 2003a; Lin et al., 2008; Ogawa et al., 2013). Heterozygous ribotypes and their parental ribotypes of the ITS sequences were found frequently in all brackish sites in Mikata-goko, implying the occurrence of sexual reproduction. However, the use of tandem repeat ITS regions is potentially problematic during genotyping because high levels of sequence homogeneity may occur through gene conversion and unequal crossing over (Liao, 1999; Feliner and Rosselló, 2007).

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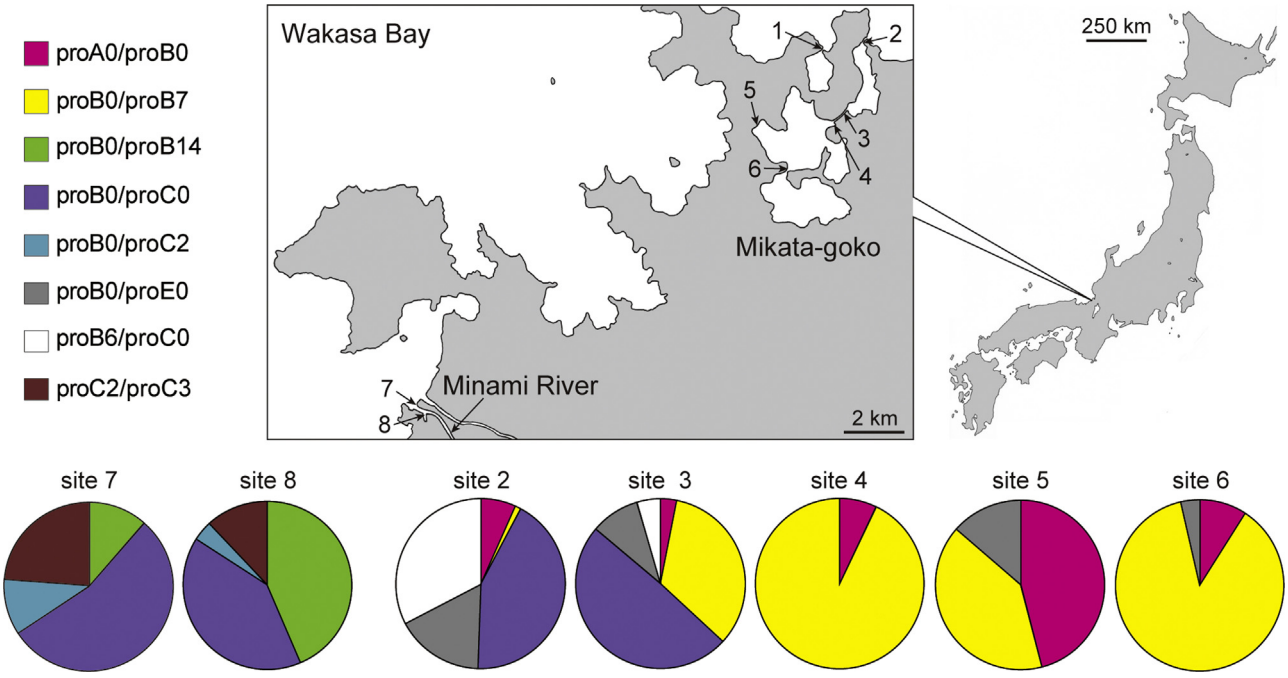

Fig. 1. Map showing the eight collection sites at Mikata-goko (numbers 1–6) and Minami River (numbers 7 and 8). The circle graphs indicate the total frequencies of the eight major genotypes of *U. prolifera* collected from each site between April 2011 and March 2012.

Therefore, re-examination of genotype structures using a single- or low-copy nuclear-encoded gene as well as identification of the genotypes in the progeny are necessary to clarify reproductive mode in *U. prolifera*.

In typical ulvacean algae, haploid gametophytes produce biflagellate gametes with positive phototaxis, and diploid sporophytes discharge larger quadriflagellate meiospores (zoospores) with negative phototaxis (e.g. Hoxmark, 1975; Tanner, 1986). Hiraoka et al. (2003a) reported that apomicts of *U. prolifera* produced negatively phototactic biflagellate zoids, which were larger than normal gametes, or produced negatively phototactic quadriflagellate zoids, which were smaller than normal meiospores. These atypical zoids have been also reported in various ulvacean species (Bliding, 1963; Koeman and van den Hoek, 1982, 1984; Ogawa et al., 2013), but it is unknown whether these phenotypic variations are genetically determined.

Microsatellite loci are considered to be one of the most valuable molecular markers for exploring neutral genetic variation in nuclear genomes and have been used for various population genetic studies (Goldstein and Schlötterer, 1999). Kostamo et al. (2008) reported that nine microsatellite markers developed for *Ulva intestinalis* Linnaeus are applicable for other ulvacean algae including *U. prolifera*. We tried to use all of these markers for our samples, but no microsatellite locus was amplified. Instead, our preliminary examinations indicated that *hsp90* gene is a good candidate for genotyping of *U. prolifera*. Among the heat shock proteins (Hsps) that function as molecular chaperones involved with the stabilization of proteins, hsp90 is a widespread family of chaperone proteins in prokaryotes and eukaryotes with functions in the folding, maintenance of structural integrity, and appropriate regulation of a subset of cytosolic proteins (Boston et al., 1996). The amino acid sequence of hsp90 has been used to detect macro-evolutionary relationships (Kim et al., 2006), but the DNA sequences are also useful for supplementing inter- and intraspecific analyses of other nuclear-encoded genes (Kauserud et al., 2006; Steele et al., 2008; Kato et al., 2011; Madani et al., 2011). The full-length cDNA sequences of *Ulva fasciata* Delile and *Ulva pertusa* Kjellman are available (Sung et al., 2011; Tominaga et al., 2012), and only two copies of the *hsp90* gene are encoded in the nuclear genome of *U. pertusa* (Tominaga et al., 2012). Thus, these *hsp90* sequences are considered useful markers for estimating the genetic diversity and structure among/within populations of *Ulva*.

In the present study, we addressed the following questions specifically: (1) Does *hsp90* gene show high frequency of heterozygous genotypes of *U. prolifera* detected in the previous study using the ribosomal ITS region; (2) if so, is this high heterozygosity maintained by frequent gene exchange or apomictic reproduction; and (3) are there any phenotypic variations of zoids among the different genotypes? In order to solve these issues we developed novel nuclear-encoded primers to amplify the sixth exon of the *hsp90* gene and sequenced 534 *U. prolifera* samples, which were collected on a monthly basis from various salinity regimes in Mikata-goko and an adjacent estuary throughout the year. Next, release of zoids was induced using the collected thalli, and the flagellum number, direction of phototaxis, and the size of zoids were recorded in each thallus. The zoids were also isolated and cultured from some samples to confirm the genotypes of the progeny and the phenotypes of their zoids. Furthermore, we examined the optimal salinity condition for germination of zoids to determine the physiological variation among strains isolated from different salinity environments. Finally, comparing the environmental conditions with the genetic structures, zoid phenotypes, and physiological properties, reproductive strategies of *U. prolifera* were discussed.

2. Materials and methods

2.1. Sampling

We attempted to collect 10 specimens of *Ulva* spp. haphazardly from April 2011 to March 2012 at six sites in Mikata-goko and two sites in Minami River (Fig. 1), where the environmental conditions, such as the salinity fluctuation and water currents, appeared to be different. The salinity, pH, and water temperature were recorded at each sampling time using a U-52 multi-parameter water quality meter (Horiba, Kyoto, Japan) (Table 1). Site 1 was near the seashore in a marine habitat and the salinity regime was almost marine with