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Crossing test among floating *Ulva* thalli forming 'green tide' in Japan

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Abstract

Crossing tests were made to determine the relationship between the identified *Ulva pertusa*, which commonly grows in Japan as an attached form on exposed rocks, and the floating *Ulva* forming "green tide" inside calm bays. The floating *Ulva* thalli were collected from five major green tide sites in Japan (Yokohama, Mikawa, Miyajima, Kochi and Hakata). Reproductive maturation was induced in *U. pertusa* and the floating thalli from each site. Mating between induced gametes was observed. It is therefore believed that the floating thalli from Yokohama, Mikawa and Miyajima were mainly *U. pertusa*, while those from Kochi and Hakata were of a different species (*Ulva* sp.1). Furthermore, the *Ulva* species found in Mikawa is also a species (*Ulva* sp.2) different from both *U. pertusa* and *Ulva* sp.1.

Introduction

Recently, there has been an increasing number of worldwide reports on the formation of huge mass of benthic algae due to eutrophication. These excessive growths largely consisted of various species of green benthic algae such as *Enteromorpha*, *Ulva*, *Chaetomorpha* and *Cladophora*, and are termed 'green tides' (Fletcher, 1996). In Japan, green tides caused by *Ulva* spp. have been reported since 1970s in many coastal areas where nutrient levels were higher than normal. The accumulation of *Ulva* spp. has caused serious economic and ecological problems (Fig. 1). Green tide phenomenon occurs in the southern and western Pacific coasts around Tokyo, Inland Sea, and the islands of Shikoku and Kyushu. All these areas are affected by the warm Kuroshio Current. There are no reports of green tides in the coasts facing Japan Sea and in the northern Pacific coasts, which are affected by the cold currents.

The conditions which led to green tides in Japan include high nutrient fluxes and the lack of strong currents or wave actions in relatively shallow water (Ohno, 1999). These conditions are similar to the ones reported from other countries (Fletcher, 1996). The development of green tides is most significant at sites with muddy, silty or sandy bottoms. It is a unique characteristic of *Ulva* spp. that they can grow without the need of any attachment to the substratum.

Arasaki (1984) surveyed several green tide phenomena in Japan and found that the *Ulva* species forming green tide were not made up of *Ulva pertusa* Kjellman, a very common species that grows on rocks in lower intertidal zone along almost the entire coast of Japan, but were made up of other species. He thought that these foreign *Ulva* spp. were introduced to Japan via large ships. On the other hand, Migita (1985) described that the green tide at Ohmura bay in western Japan was formed by a sterile mutant of *U. pertusa*. This *Ulva* strain was subsequently recognized as a sterile mutant of *U. pertusa*, which was

Figure 1. Gathering of *Ulva* biomass in Yokohama. The government of this city spends more than 40 million yen every year to eliminate accumulation of this alga on the beach.

Figure 2. *Ulva* from Hakata collected from the floating population.

utilized for some experiments in nearby fishery institutes. There are thus two opinions concerning the identity of *Ulva* species forming green tides in Japan. The taxonomic status of these species remains uncertain. In the present study, to determine the relationship between the identified *Ulva pertusa* and the floating *Ulva* forming 'green tide' and to evaluate the validity of the two opinions, we made crossing tests between the attached *U. pertusa* and the floating *Ulva* spp. from five sites in Japan where green tides have largely occurred using an artificial technique to induce reproductive maturation.

Figure 3. Collection sites of *Ulva* in Japan where green tides are observed.

Table 1. Date and number of collected samples

Collection site	Collection date	Sample number
Hakata	August 28, 1997	5
Kochi	September 19, 1997	25
Mikawa	June 9, 1998	8
Yokohama	June 10, 1998	11
Miyajima	April 19, 1999	26

Materials and methods

Well-developed thalli from floating populations of *Ulva* spp. (Fig. 2) were collected from Yokohama, Mikawa, Miyajima, Kochi and Hakata, Japan, where significant green tides occurred (Fig. 3). The collection date and sample number in each site are shown in Table 1. After collections of these samples, we found out that there were two morphotypes in color and texture. One morphotype was dark green color with comparatively thick thallus (40–100 μm). Other type was light green color and thin thallus (30–50 μm). All samples from Yokohama and Miyajima and five samples from Mikawa belonged to the former. The samples from Kochi and Hakata and three others from Mikawa belonged to the latter. Typical morphotypes in color and texture were selected for crossing tests. The attached *Ulva pertusa* with rhizoid was collected

Table 2. Percentage frequency of zooid formation (reproductive maturation rate) over 10 days after excision in 100 disks (1.2 mm diam.) obtained from floating *Ulva* and attached *Ulva pertusa* (male and female gametophytes)

Sample no.	Zooid	Reproductive maturation rate (%)										
		Days after excision of thallus disks										
		1	2	3	4	5	6	7	8	9	10	Total
Yokohama 1	Gamete	0	0	1	0	4	0	0	0	0	0	5
Yokohama 2	gamete	0	0	3	0	48	30	13	6	–	–	100
Yokohama 4	Zoospore	0	0	45	10	22	1	11	11	–	–	100
Mikawa 1	Gamete	0	0	26	0	0	16	0	18	9	3	72
Mikawa 2	Zoospore	0	0	33	0	0	17	0	1	13	2	66
Mikawa 6	Zoospore	0	0	41	0	0	17	0	11	3	0	72
Mikawa 7	Zoospore	0	0	19	0	0	0	0	0	0	0	19
Miyajima 1	Zoospore	0	0	45	4	0	0	0	0	0	0	49
Miyajima 3	Zoospore	0	0	49	6	0	0	0	0	0	0	55
Miyajima 4	Zoospore	0	0	2	0	0	0	0	0	0	0	2
Kochi 2	Zoospore	0	0	1	15	0	0	7	0	0	0	23
Kochi 6	Zoospore	0	0	0	4	0	0	30	0	0	0	34
Kochi 11	Zoospore	0	0	7	1	0	0	0	0	0	0	8
Kochi 22	Zoospore	0	0	68	10	0	0	21	0	0	0	99
Hakata 6	Zoospore	0	0	0	11	4	0	0	0	0	0	15
Hakata 9	Zoospore	0	0	0	15	0	0	0	0	0	0	15
U. pertusa	Gamete(♂)	0	100	–	–	–	–	–	–	–	–	100
U. pertusa	Gamete(♀)	0	100	–	–	–	–	–	–	–	–	100

from rocky shores of Kochi. Samples agreeing well with the description of Kjellman (1897) were selected (Fig. 4a–d). Male and female gametes released from the attached *U. pertusa* formed zygotes, which were isolated and cultured up to sporophytes. Male and female gametophytes were established from zoospores released from the sporophytes for crossing test.

In order to obtain zooids (viz. male gametes, female gametes or zoospores), samples of *Ulva* spp. were induced using the 'punching method' described in Hiraoka & Enomoto (1998) and Hiraoka et al. (1998). This method involved the excision of small thallus disks of 1–2 mm diameter and the incubation of these disks in PES medium at 20 °C, 12:12 h L:D cycle and fluorescent light at 100 μmol photons m^{-2} s^{-1} (Hiraoka & Enomoto, 1998). Under such conditions, zooids can normally be formed within several days. Quadriflagellate zoospores were microscopically distinguished from biflagellate gametes. When the floating *Ulva* samples did not release gametes but zoospores, these could not be used directly for crossing tests. Therefore, the zoospores were cultured up to their gametophyte stage, of which gamete formation was induced for crossing test. Successful mating of gametes released from attached *U. pertusa* and the floating *Ulva* was also determined microscopically.

Results and discussion

Ulva spp. in this study produced either quadriflagellate zoospores or biflagellate gametes. The percentage frequency of daily zooid formation is shown in Table 2. Zooid formation in cultured *Ulva pertusa* occurred on the second day after excision. However, it took 3 or more days for zooid formation to occur in the floating thalli. Depending on individuals, a large variation in the degree of zooid formation in the floating thalli was observed. Using the punching method, the zooid formation in the floating thallus occurred in less than 50% of the disks within 3 days (except for Kochi 22), while zooid formation in established male and female gametophyte of *U. pertusa* occurred in 100% of disks 2 days after excision. In some of the floating thalli zooids could not be induced at all by this method. Zooid formation in *Ulva* spp. would normally occur when the thallus is fragmented and the inhibitors of zooid formation leaked out from the fragmented tissue (Nordby, 1977; Stratmann et al., 1996). The difference in maturation time and rate between attached and

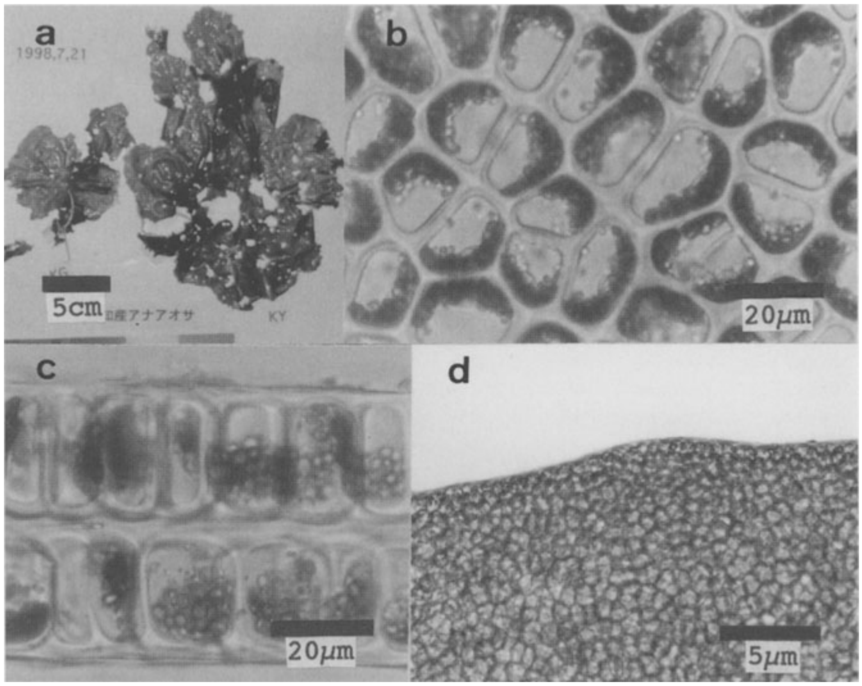

Figure 4. Morphological characteristic of attached *Ulva pertusa*. (a) Two gametophytes of different sizes with rhizoidal holdfast. (b) Surface view of cells from apical region. (c) Cross section from apical region. (d) Marginal part of apical region showing an absence of tooth-like protuberances.

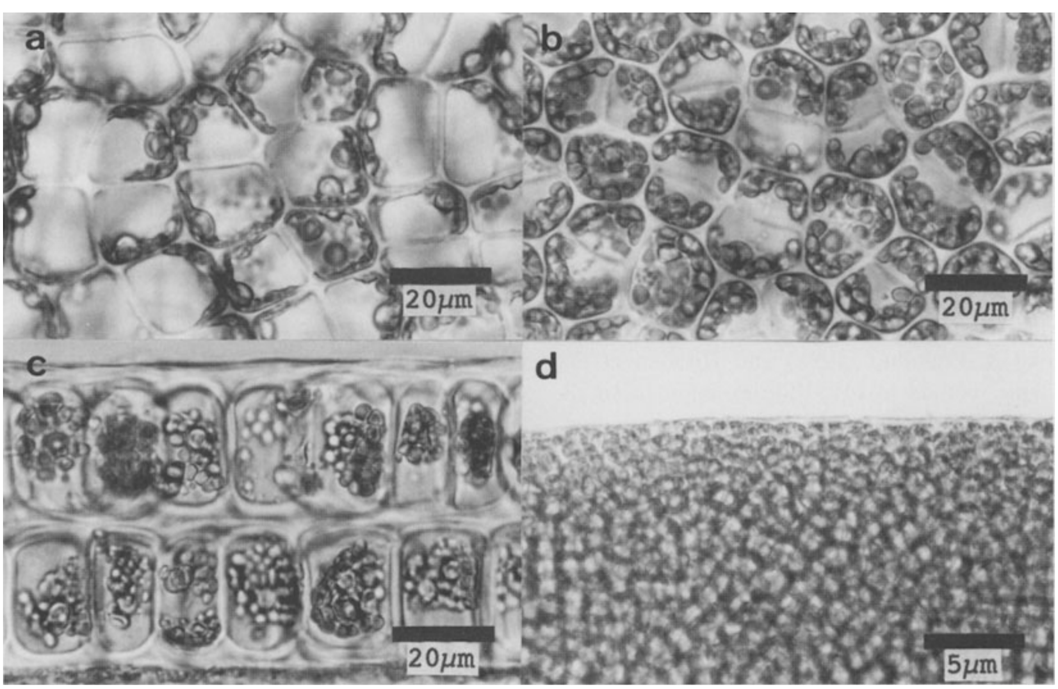

Figure 5. Morphological characteristic of floating *Ulva* crossed with *Ulva pertusa*. (a) Surface view showing cells with 1–3 pyrenoids. (b) Surface view showing cells with many small granules. (c) Cross section. (d) Surface view of marginal part.

Table 3. Crossing tests between floating *Ulva* and attached *Ulva pertusa* thalli. All samples from Yokohama and Miyajima and Mikawa 1, 2 and 6 were dark green color with comparatively thick thallus. Samples from Kochi and Hakata and Mikawa 7 were light green color with thin thallus. Male and female gametophytes were established from the zoospores released from the floating *Ulva* during *in vitro* culture. Success and failure of copulation between male and female gametes are indicated by + and – respectively

Sample no.	Zooid	Sex of gamete from cultured Gametophyte	*Ulva pertusa* attached to rock		Floating *Ulva* from Kochi	
			Male	Female	Male	Female
Yokohama 1	Gamete		+	–	–	–
Yokohama 2	Gamete		–	+	–	–
Yokohama 4	Zoospore	Male	–	+	–	–
		Female	+	–	–	–
Mikawa 1	Gamete		+	–	–	–
Mikawa 2	Zoospore	Male	–	+	–	–
		Female	+	–	–	–
Mikawa 6	Zoospore	Male	–	+	–	–
		Female	+	–	–	–
Mikawa 7	Zoospore	Male	–	–	–	–
		Female	–	–	–	–
Miyajima 1	Zoospore	Male	–	+	–	–
		Female	+	–	–	–
Miyajima 3	Zoospore	Male	–	+	–	–
		Female	+	–	–	–
Kochi 22	Zoospore	Male	–	–	–	+
		Female	–	–	+	–
Hakata 9	Zoospore	Male	–	–	–	+
		Female	–	–	+	–

Table 4. Ulva species occurring in each sites determined by crossing test

Collection site	*Ulva* species
Yokohama	*Ulva pertusa*
Mikawa	*Ulva pertusa*, *Ulva* sp. 2
Miyajima	*Ulva pertusa*
Kochi	*Ulva* sp.1
Hakata	*Ulva* sp.1

floating *Ulva* spp. may depend on the concentration or distribution of inhibitors in the thalli.

Because zooid formation in floating thalli was normally delayed as mentioned above, male and female gametophytes of *U. pertusa* had to be induced to form gametes everyday in order to ensure the availability of gametes of both *U. pertusa* and the floating *Ulva* for crossing tests. The release of zooids from mature disks occurred in the morning both in *U. pertusa* and the floating *Ulva*. The results of crossing test are shown in Table 3 and summarized in Table 4. Dark green and thick morphotype *Ulva* spp. from Yokohama, Mikawa and Miyajima successfully crossed with *U. pertusa*, but the light green and thin morphotype from Kochi and Hakata did not. The thin morphotypes from Kochi and Hakata crossed with each other, the resulting offspring is designated herein as *Ulva* sp.1. The light green and thin *Ulva* morphotype from Mikawa (Mikawa 7) did not cross successfully with either *U. pertusa* or *Ulva* sp.1 and was considered as a different species, designated herein as *Ulva* sp.2.

The floating *Ulva* spp., which crossed with attached *U. pertusa*, have rounded cells in surface view. In many cases the chloroplasts are pressed against the cell walls (parietal) in surface view and have 1–3 pyrenoids (Fig. 5a). These may be replaced by numerous small granules (Fig. 5b). The marginal part of the thallus is comparatively thick (40–100 μm; Fig. 5c) without tooth-like protuberances in the margin of the thallus (Fig. 5d). These characters are in agreement with those of typical *U. pertusa* with rhizoidal holdfast (Fig. 4b–d; Kamiya et al., 1993). These morphological similarities also support the suggestion that the floating *Ulva* spp. have been derived from *U. pertusa*.

Hiraoka et al. (1998) have earlier suggested that the floating *Ulva* in Hakata is a different taxon from *U. pertusa* based on crossing test. In this report, we suggest that the floating *Ulva* from Kochi, which crossed with the ones from Hakata, belongs to the same unidentified *Ulva* (*Ulva* sp.1). In our field observations, we found some *Ulva* thalli attached to rock which possibly crossed with *Ulva* sp.1. The samples obtained on this species were morphologically similar to *Monostroma* sp. for their thin, easily torn blade and light green color. These characteristics, however, are more in agreement with those of the floating thalli of *Ulva* sp.1. We believe that these attached thalli belong to *Ulva* sp.1, and the floating type of *Ulva* sp.1 could have been derived from the attached type. The cells of *Ulva* sp.1 are polygonal in surface view (Fig. 6a). The thallus is comparatively thin, only 30–50 μm thick (Fig. 6b). In many cases, tooth-like protuberances are found microscopically in the thallus margin (Fig. 6c). These characters separate *Ulva* sp.1 from *U. pertusa*.

Ulva sp.2, which did not cross with *U. pertusa* and *Ulva* sp.1, is similar to *Ulva* sp.1 in having thin thallus, being easily torn, and light green in color and having polygonal cells in its surface view (Fig. 7a). *Ulva* sp.2 is different from *Ulva* sp.1 in having cells at the marginal part of the thallus being oval in shape in transverse sections (Fig. 7b). The microscopic tooth-

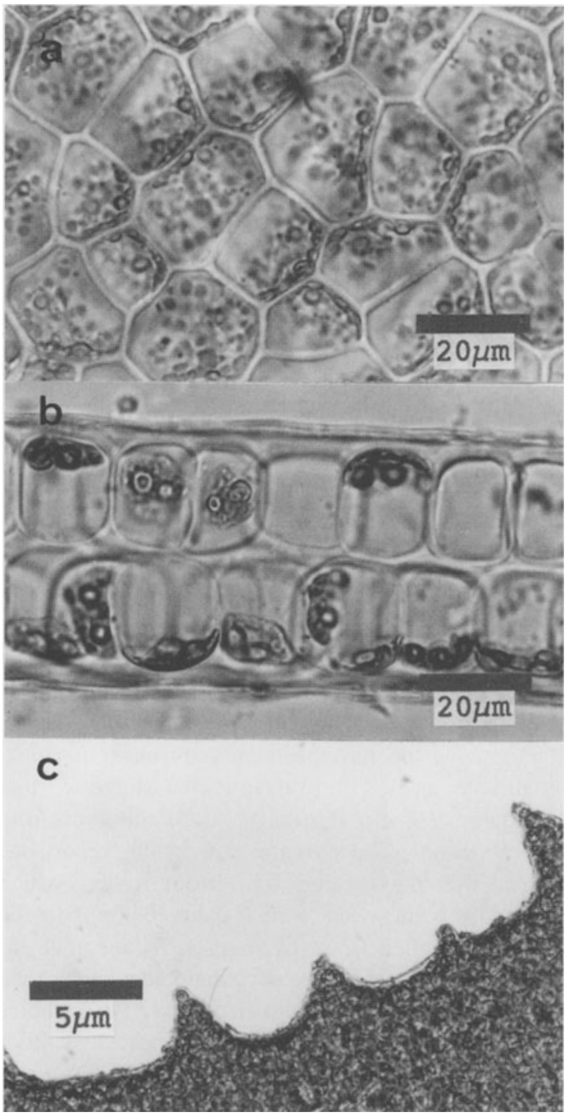

Figure 6. Morphological characteristic of *Ulva* sp.1. (a) Surface view of polygonal cells. (b) Cross section. (c) Marginal part showing tooth-like protuberances of polygonal cells.

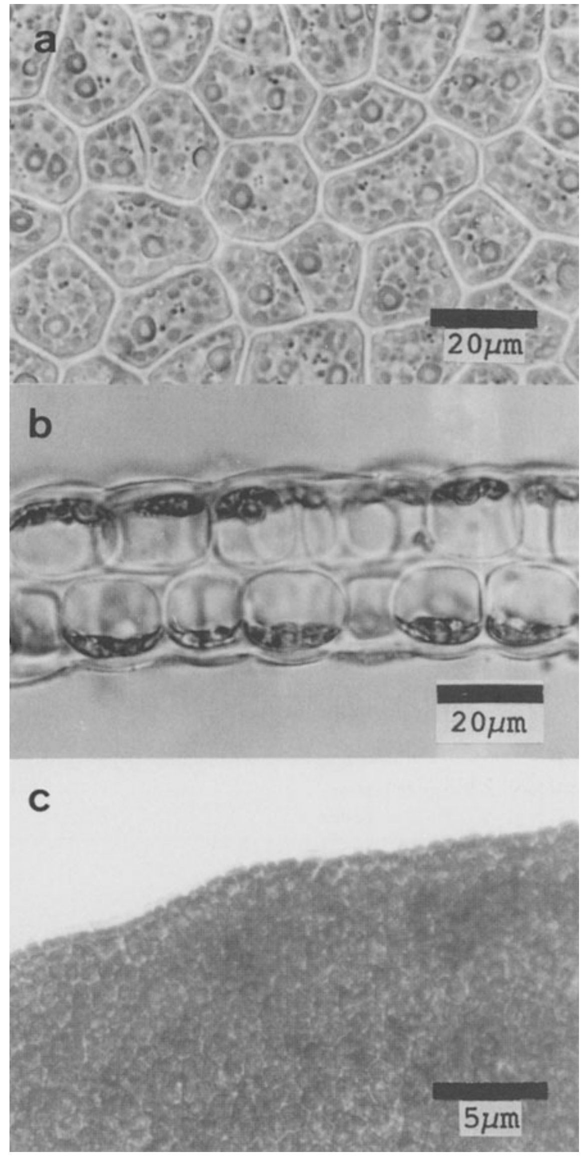

Figure 7. Morphological characteristic of *Ulva* sp.2. a: Surface view of polygonal cells. b: Cross section. c: Marginal part showing an absence of tooth-like protuberances.

like protuberances could not be found in the margin of *Ulva* sp.2 thallus (Fig. 7c). A more detailed description of *Ulva* sp.1 and *Ulva* sp.2 will be presented elsewhere.

Recently, it has been reported that algae forming the green tides of Brittany, France belong to a new species *Ulva armoricana* Dion, de Reviers & Coat (1998). *U. armoricana* is very similar to *Ulva* sp.1 in having a thin, easily torn blade, and polygonal cells in surface view. However, the frequency of tooth-like protuberances at the blade margin of the blade seems to be higher in *U. armoricana* than in *Ulva* sp.1. At present, crossing tests and morphological comparisons between *Ulva* sp.1 and *U. armoricana* from France are being conducted in our laboratory in order to determine the relationship of French and Japanese green tide *Ulva* species.

Based on the summary of crossing tests shown in Table 4, *Ulva* populations occurring in Yokohama, Mikawa and Miyajima could be those of *U. pertusa*, while those in Kochi and Hakata were of a different species (*Ulva* sp.1). Yokohama, Mikawa and Miyajima are located in the temperate zone of Japan and are influenced only weakly by the Kuroshio Current. On the other hand, Kochi and Hakata are in the warm temperate zone and are affected more strongly by the Kuroshio (Tokuda et al., 1994). When the relationship between the marine flora and the distribution of floating *Ulva* is considered, it would be found that *U. pertusa* occurs only in temperate waters while *Ulva* sp.1 occurs in warm temperate waters affected more strongly by the Kuroshio Current. In Mikawa, *Ulva* sp.2 was collected with *U. pertusa*. This indicates that it is possible for more than two species of *Ulva* to occur in the same water. In this study, only one collection was made in each site. Therefore, there remains a possibility that more than two *Ulva* species may be present in the same site if more collections are to be made at different times in a year.

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