博　士　論　文

Physiological and ecological studies on nutrient uptake kinetics and the development of a new cultivation technique of *Saccharina ochotensis* and *Undaria pinnatifida*

（栄養塩吸収特性を中心としたリシリコンブとワカメの
生理生態学的研究および新たな養殖技術の開発）

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Physiological and ecological studies on nutrient uptake kinetics and the development of a new cultivation technique of *Saccharina ochotensis* and *Undaria pinnatifida*

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CONTENTS

ABBREVIATION

Ar: Argon

C: Carbon

CFCS: a cyclone and floating culture system

DIN: dissolved inorganic nitrogen

IMTA: integrated multi-trophic aquaculture

K_s: the substrate concentration at $V_{max}/2$

mt: mitochondria

N: Nitrogen

V_{max}: maximum nutrient uptake rates

α: V_{max}/K_s

PREFACE

The global population will continue to grow, reaching approximately 9 billion by the middle of this century, and recent studies suggest that 70% to 100% more foods will be required by 2050 (World Bank 2008, Royal Society of London 2009). The primary solution to food shortages has been to provide more land for agriculture (Godfray et al. 2010). However, while grain production has more than doubled over the past 50 years, the amount of arable land has globally increased by only about 9% (Pretty 2008). In recent decades, the land that was formerly used for agricultural production has been lost as a result of urbanisation and other human uses, as well as climate change (IPCC 2007, Nelleman et al. 2009). Furthermore, the production of biofuels has led to competitive pressure against high-quality agricultural land (Fargione et al. 2008). Thus, an increasing amount of food needs to be produced using the same amount of (or even less) land (Godfray et al. 2010).

The term ‘seaweed’ traditionally includes only macroscopic, multicellular marine red, green and brown algae (Hurd et al. 2014). Brown algae are placed under the group Heterokontophyta on the basis of characteristic pigment such as carotenoid fucoxanthin as well as chlorophylls *a* and *c* in their chloroplasts, which include various groups of multi cellular organism (Verma et al. 2015). Kelp is a large brown alga that exhibits a heteromorphic annual life cycle with alternating sporophytic and gametophytic generations (Fig. P–1; Akiyama and Kurogi 1982). Sporophytes of kelp have a lifespan ranging from one to several years and form an extensive population in coastal areas of arctic and subarctic regions worldwide, termed ‘marine forest’ (Mann 1972). Marine forest plays an important role in providing habitats and nurseries to wide range of organisms (Tanaka and Leite 2003, Graham 2004).

The majority of kelp species are used globally as industrial resources. *Undaria pinnatifida* (Wakame) and *Saccharina* genus (Kombu) of *Laminariales* are major species of kelp that have commonly been used as edible foods and are cultivated in northeastern Asia (Yamanaka and Akiyama 1993). Kelp contains abundant minerals, polysaccharides and antioxidant compounds; it can be used as a fertilizer as well as in chemicals and animal feed (Holdt and Kraan 2010). It has been recently considered as an ideal feedstock for the production of biofuels, since it does not have an adverse impact on food supplies (because they do not require arable land, fertilizer or fresh water resources; Aitken and Antizar 2012). Moreover, by utilising the inorganic nutrient uptake ability of kelp, attempts have been made to reduce the amount of waste generated by the fish aquaculture industry through the aquaculture of bivalves and seaweeds (referred to as 'Integrated Multi-Trophic Aquaculture' [IMTA]; Buschmann et al. 2001; Troell et al. 2003). Therefore, kelp can directly or indirectly contribute to increased food production, and as such is an important resource.

The production of kelp is decreasing markedly due to higher temperatures and a decrease in inorganic nutrients in seawater. For example, increases in marine water temperature of 4–5°C, as a result of the El Niño oceanographic events during 1982 and 1983, have led to nutrient depletion and deforestation of *Macrocystis pyrifera* along the coast of Alta and Baja California (Paine et al. 1998; Tegner and Dayton 1991). The relationship between ocean warming and decreased kelp abundance has been reported worldwide for several kelp species (e.g. Johnson et al. 2011, Kirihara et al. 2006, Müller et al. 2009). Studies conducted near the British Columbia coast in the 1970–80s showed that depletion of dissolved inorganic nitrogen (DIN) in seawater was negatively correlated with the growth of kelp (Chapman and Craigie 1977;

Chapman and Lindley 1980, Gagnĕ et al. 1982). Recently, nutrient depletion in early spring led to serious damage during the production of *U. pinnatifida*, characterised by discoloration and decreased quality (Dan et al. 2015).

It is difficult to manage the coastal environmental condition. Therefore, to increase natural resources and cultivation amounts of kelp, it is important not only to ensure nutrient conditions based on plant nutrition but also to develop a new cultivar based on breeding technologies. To increase the amount of kelp in the open sea by fertilisation, optimal nutrient conditions are required for individual kelp species based on eco-physiological studies. Furthermore, to improve kelp breeding, it is important to identify genetic or environmental differences among species. Today, studies for the field of kelp are necessary as in studies for agriculture and horticulture of the terrestrial plants.

'The first step in breeding is to list the purposes of breeding. Determining the method with the best efficiency will also influence the aim. Developing new varieties of plants means selecting genetic variation' (Kuckuck, Kobabe and Wenzel, 1985). In the field of kelp breeding, is it possible to achieve a sufficient number of studies focusing on the various breeding strategies?

There is no elite line of kelp used continuously in the industrial field. In the kelp industry, breeding purpose differs according to countries or regions and should be analysed from the perspectives of fishermen, buyers and customers. Genetic variations in kelp are unclear owing to yearly changes in environmental conditions, and it also remains unclear whether the characteristics of kelp species are due to adaptation or acclimatisation to environmental conditions. As kelp breeding methods, although heterosis of *U. pinnatifida* (Hara and Akiyama

1985) and selection breeding of *S. japonica* (Zhang et al. 2011) have been examined, we have never accomplished variety registration of kelp in Japan. Thus, development of the optimum and useful breeding method for kelp is necessary.

To address these issues, I examined two species for this study, *S. ochotensis* and *U. pinnatifida*. It is known that natural resources of *S. ochotensis* have been remarkably declined among *Saccharina* genus (Nabata and Takiya 2003). In contrast, *U. pinnatifida* has successfully invaded many coastal regions worldwide during these only 30 years (Dellatorre et al. 2014), it is speculated its wide range of environmental adaptability. I thought that their ecophysiological and breeding study could provide useful information for the field of kelps to understanding of adaptation for environmental conditions and developing of elite lines. Therefore, I conducted the following five studies focusing on nutrient uptake kinetics and the development of cultivation techniques for breeding:

1) To study on adaptation to environmental conditions through seasonal changes in resource accumulation and nutrient uptake kinetics for the growth of *Saccharina ochotensis.*

2) To develop a new tank culture system for kelp under controllable environmental conditions.

3) To evaluate genetic or environmental differentiation of *U. pinnatifida* by cultivation in the sea and use of a new tank culture system.

4) To demonstrate the practicability with respect to the sea cultivation of earlier (and later) season *U. pinnatifida* crops using the selection breeding method in the new tank culture system on the basis of genetic differentiation.

5) To optimise conditions of heavy-ion beam irradiation for *U. pinnatifida* for mutation breeding and development of elite lines.

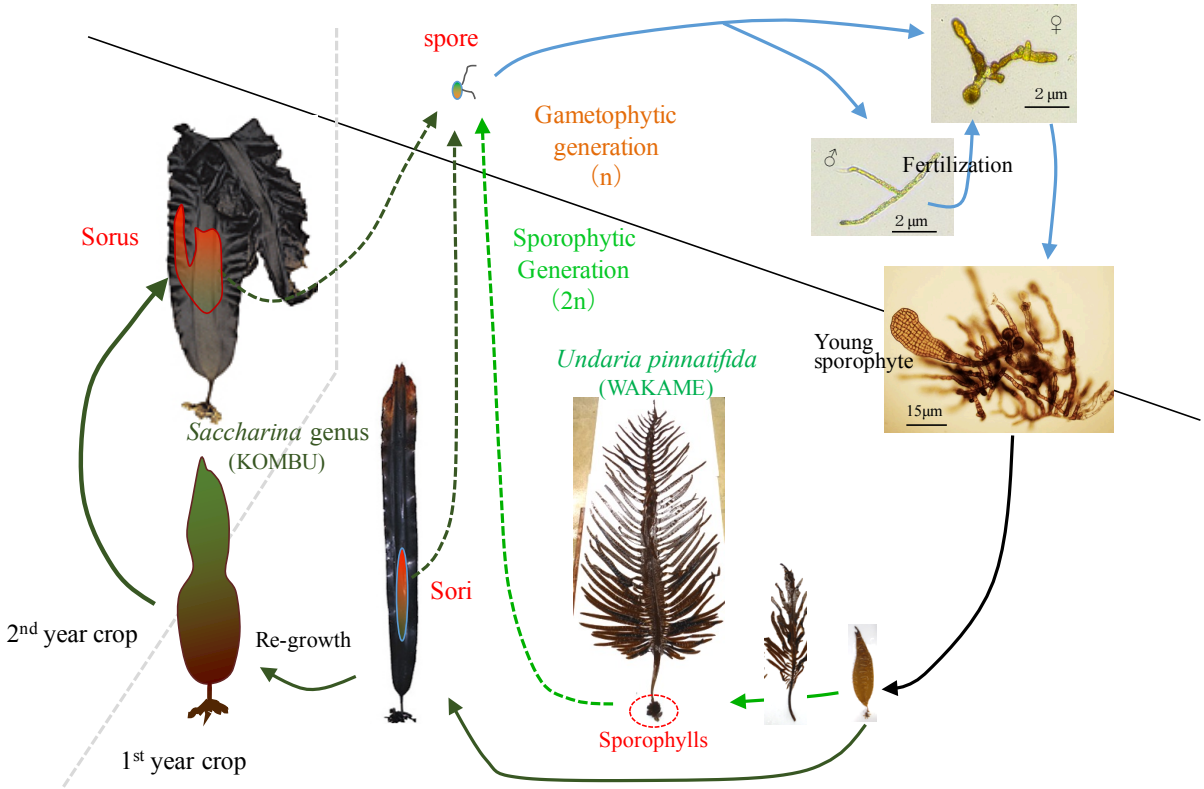


Fig. P-1. Life cycle of *Saccharina* genus and *Undaria pinnatifida*, with alternating sporophytic and gametophytic generations.

CHAPTER I

Physiological and ecological studies on nutrient uptake kinetics of the kelp *Saccharina ochotensis*

SUMMARY

The resource accumulation and nutrients uptake kinetics of *Saccharina ochotensis* were demonstrated and the adaptation for changes in nutrients concentration in the seawater were discussed. The species is known the most decreasing resources amount in the *Saccharina* genus. Its hatchery-raised young sporophytes collected from Rishiri Island, Hokkaido, were cultivated in Matsushima Bay, Miyagi, from November 2000 to July 2001. Seasonal morphologies, C and N content, photosynthetic rates, and nutrient uptake kinetics were examined. The blade length reached a maximum in May, and then decreased as a result of an increase in erosion rate. The blade weight increased gradually from January until July when the carbon and nitrogen content increased markedly at the meristem. The high photosynthetic rate in the part of the blade with the maximum width and in the apical part was maintained from January to June. At the meristem, the uptake rate of NH_4-N increased significantly from May to July. The correlations between NO_3-N and NH_4-N concentrations and the uptake rates of the kelp were fitted to hyperbolic regressions, indicating high uptake ability at high and low nutrient concentrations, in winter and summer, respectively. These results suggest adaptation of the kelp to seasonal nutrient conditions in the northern Sea of Japan. Resource accumulation in the meristem is the

result of marked expansion of the blade area from winter to spring, when sufficient carbon and nitrogen are produced by a high rate of photosynthesis, high nutrient uptake ability, and translocation of resources from the eroding apical part of the blade. This resource accumulation is likely to be essential for regrowth of the kelp the following year.

INTRODUCTION

Global ocean warming has led to a reduction in the supply of nutrients from deep waters as a result of increased vertical thermo–stability of seawater (Sarmiento et al. 2004). The relationship between ocean warming and a decrease in the abundance of the kelps has been reported at many coastal areas in the world (e.g., Johnson et al. 2011, Kirihara et al. 2006, Müller et al. 2009). A culture experiment indicated that nitrogen (N)-limited *L. saccharina* was more susceptible to the deleterious effects of that shortage compared with N-replete plants at high water temperatures, suggesting that the large-scale declines are the result of a synergic effect of N limitation and high water temperature (Gerard 1997).

N content and growth of laminarian kelps are positively correlated with the concentrations of dissolved inorganic nitrogen (DIN) (e.g. Chapman and Craigie 1977). Addition of NO_3-N fertilizer to beds of *L. longicruris* improved the growth rates in summer when N availability was depleted (Chapman and Craigie 1977). A longer period at high NO_3-N prolonged the growth period of this species (Gagnĕ et al. 1982). These researches indicate that the growth of *L. longicruris* is restricted by N concentration.

N and phosphorus fertilizers increased the growth rate of juvenile *M. pyrifera* off southern California during El Niño events (Dean and Jacobsen 1986). Fertilization also promoted the growth of adult *M. pyrifera* under conditions of high water temperatures and low nutrient concentrations (North and Zimmerman 1984; Zimmerman and Kremer 1984; Hernandez-Carmona et al. 2001). By contrast, little is known about the mechanisms behind the differences in seasonal nutrient uptake associated with growth, maturation, resource accumulation and photosynthetic rates. Recently, attempts were made to reduce the amount of waste expelled

from the fish aquaculture industry through the aquaculture of bivalves and seaweeds in Chile and Norway (IMTA, Buschmann 2001; Troell et al. 2003). More information about the nutrient uptake kinetics of seaweeds is needed to maintain a productive aquaculture industry without negative impacts on the surrounding environment.

Saccharina ochotensis (Rishiri Kombu) is an independent species according to taxonomic re-examination by Lane et al. (2006). This kelp is one of the major macroalgae commercially harvested in Japan. However, Saccharina kelp production is influenced by hydrographic conditions (Nishida 1999). In Japan, the production of natural and cultivated kelps decreased from 179,997 t in 1962 to 72,691 t in 2005 and from 72,924 t in 1992 to 43,251 t in 2010 (MAFF 2013). A remarkable decline was reported off northern Hokkaido, where the production of *S. ochotensis* at Rishiri Island in northern Hokkaido has decreased to 12% of its peak during the 1940s (Nabata and Takiya 2003). In particular, the production of second-year plants has decreased markedly (Nabata et al. 2003). A decrease in nutrient concentration (Nakata et al. 2001) resulting from an increase in the flux of the Tsushima Warm Current (Onishi and Ohtani 1997) has been hypothesized as the reason for this decline (Nabata et al. 2003).

The aims of the present research were to investigate (i) If exist a carbon (C) and N accumulation patterns that influence the growth and maturation of S. ochotensis, and the relation with the morphology, photosynthetic rates, and nutrient uptake rates; (ii) Describe the nutrient uptake mechanism based on characteristics of the kinetics through seasonal nutrient uptake; (iii) Analyze the applicability of these results to the use the kelp fertilization to increase the production on the basis of nutrient uptake kinetics.

MATERIALS AND METHODS

Cultivation of sporophytes

In October 2000, mature sporophytes of *S. ochotensis* were collected off the coast of Kutsugata (45°11'N, 141°09'E), Rishiri Island, Hokkaido. Zoospores were released after desiccation in shade for 12 h and then attached to 2-mm diameter and 300 m length plastic-fiber threads coiled itself around the flame of PVC pipe as a substrate base. The settled zoospores were then cultured in tanks 100 L of enriched PESI medium (Tatewaki 1966) at 15ºC with gentle aeration for 3 weeks until the total length of the sporophytes was approximately 0.5 cm. The PESI medium was made using sterilized seawater with a salinity 32 psu. At the first week, a photoperiod of 12 : 12 h (light /dark) and a light intensity of 30 μmol photons m^{-2} s^{-1} were employed. A photoperiod and a light intensity were changed to 14 h and 45 μmol photons m^{-2} s^{-1} at the second week, and 16 h and 60 μmol photons m^{-2} s^{-1} at the third week. The culture medium was renewed every week. In November, the threads were transferred to Matsushima Bay (38°22'N, 141°03'E), Miyagi Prefecture and immersed in the sea at a depth of 50 cm by hanging rope for 10 days at 14–15ºC. The threads on which the young sporophytes grew to 1 cm in length were cut into 3−5 cm sections. Fifty sections were inserted into a rope of 50 m in length and 15 mm in diameter at intervals of 100 cm. The rope was secured at the surface with floats at a depth of 2 m and cultivated from November 2000 to July 2001. The plants derived from one thread section were regarded as one stock and 50 stocks were used for this study, 20 of which were tagged for estimation growth and erosion rates, and 30 of which were used for measurements of morphological and physiological characteristics.

Morphological measurements

One untagged stock was collected randomly each month from December 2000 to July 2001 and the largest 30 individuals within the stock were selected. The blade length, maximum width and thickness were measured. The plants with sori were counted and the percentages of plants with sori were calculated for all plants. The blade area of each plant was measured using an Imaging Analysis Device (Q-600, Leica, Cambridge, UK). The blades were dried in a convection oven at 80°C for one week and the dry weights was recorded.

Given that *S. ochotensis* is characterized by intercalary growth, individual tag marks can be used to estimate seasonal growth and erosion rates. Tagging was done on plants that were more than 30 cm in length from the same 20 stocks every month from December 2000 to June 2001. To distinguish the tag marks from the grazing trails of herbivores, two holes 5 mm in diameter were punched in the margins of the blades 10 cm from the meristem of the plants (Taniguchi et al. 1991). From January to July, one tagged stock was collected randomly every month. For the largest 20 plants, the distance between each hole and the meristem each month was measured each month and then the growth and erosion rates were estimated using Equations 1 and 2:

Growth rate (cm d^{-1}) = (Distance from the holes to the meristem in the current month – distance from the holes to the meristem in the previous month)/days (1)

Erosion rate (cm d^{-1}) = [(Average blade length in the previous month + distance moved by the hole in the current month) – average blade length in the current month]/days (2)

Measurement of photosynthetic rate and nutrient uptake rate

Four plants of average blade length were selected from untagged stocks collected every month and, using a cork borer with 1.6 cm in diameter, 2.01 cm^2 discs were bored from the meristem, the middle part of the blade with the maximum width, and the apical part. The discs were incubated in a tank for 24 h with flowing filtered-seawater pumped up off Sasunohama, Ishinomaki (38º24'N, 141º22'E) to allow recovery from damage caused by cutting (Sakanishi et al. 1988).

An improved 'product-meter' (Yokohama et al. 1986; Li et al. 2009) was used to determine the photosynthesis rate and nutrient uptake rate. This equipment was composed of a light source, a water bath with shaker, a temperature controller, and main parts with reaction and control vessels. Oxygen evolution in the reaction vessel resulting from photosynthesis and respiration of algal tissue causes the changing indicator and it is able to calculate the photosynthesis and respiration rate (Li et al. 2009). Each disc was put into a flask containing 10 ml autoclaved and filtered seawater (GF/F, Whatman, Maidstone, UK) and incubated at each monthly average seawater temperature in a water bath (Taitec CL-150 F, Koshigaya, Japan) at 150 rpm under an irradiance of 180 μmol photons $m^{-2}s^{-1}$. Halogen lamps were used as a light source. The monthly average seawater temperature from 1966 to 1999 had been measured daily at Enoshima Island (38° 24′ N, 141° 35′ E), a pelagic island near the cultivation site, by Miyagi Prefecture Fisheries Research and Development Center, and these recordings were used as the incubation and experimental temperatures.

For measurement of photosynthetic rate, the discs were incubated for 30 min to acclimatize them to the experimental temperature, and the oxygen produced by the discs was then measured every 3 min six to ten times. The uptake rates of NH_4-N, NO_3-N and PO_4-P were obtained from the differences between the concentrations analyzed by an auto-analyzer (TRAACS 800; Bran-Luebbe, Tokyo, Japan) after a 60-min incubation of the discs and those in the blanks as control (Li et al. 2009). For measurement of the photosynthetic rate and nutrient uptake rate in the different parts of the blade, initial concentrations were set to 30 μmol L^{-1} NH_4-N and NO_3-N, and 2 μmol L^{-1} PO_4-P. These concentrations were based on the annual maximum level at Matsushima Bay. For examination of seasonal nutrient requirements in the meristem and the middle part of the blade under different nutrient levels, the nutrient concentrations of NH_4-N and NO_3-N, and for PO_4-P were set to 2, 5, 7.5, 15, 30, 60 μmol L^{-1} and 0.05, 0.25, 0.5, 1.5, 3, 6 μmol L^{-1}, respectively. No uptake rate of PO_4-P was measured in June. Seawater used in this experiment was obtained from the Sea of Japan by the Akita Prefectural Institute of Fisheries. The nutrient concentrations in seawater were almost below the limit of detection. After filtration and sterilization, the seawater was enriched by the addition of NH_4Cl, $NaNO_3$ and $NaH_2PO_4 \cdot 12H_2O$（Wako Pure Chemical Industries Ltd, Tokyo, Japan）.

Nutrient uptake kinetics

The correlations between initial nutrient concentration (S) and the nutrient uptake rate (V) were fitted a hyperbolic regression, similar to Michaeris-Menten equation, using JMP (SAS Institute Inc., Cary, NC, USA) based on a least-squares regression method. The Michaelis–Menten parameter for each individual was calculated with the following equation (3):

$$V = (V_{max}\ S)(K_s + S)^{-1} \quad (3)$$

where V (μmol g d.w.$^{-1}$ h^{-1}) is the uptake rate, V_{max} (μmol g d.w.$^{-1}$ h^{-1}) is the maximum uptake rate at the saturating concentration, S (μmol l^{-1}) is the saturating concentration, and K_s (μmol l^{-1}) is the substrate concentration at $V_{max}/2$. V_{max}, K_s, and V_{max}/K_s (α) are effective indicators of nutrient uptake dynamics that may be used for cross comparisons among populations (Fig.1-1) (Harrison et al. 1989). High V_{max} and K_s values are indicative of rapid nutrient uptake and high affinity at high concentrations (Harrison and Hurd 2001); a low Ks value is indicative of high affinity at low nutrient concentrations (Hurd et al. 2014); high V_{max}/K_s values are indicative of an enhanced uptake capability at low nutrient concentrations (Henley 1980; Bracken et al. 2011; Bracken and Williams 2013, Hurd et al. 2014).

Carbon and nitrogen content

Blade discs were collected after measurements of photosynthesis and nutrient uptake rates and were dried at 80 ºC for 24 h for determination of dry weight. The discs were then mashed up and used for the analysis of their C and N content by an Elemental Analyzer (Eager 200; Fisons Instruments, USA).

Seawater temperature and nutrient concentration in the field

Data of bimonthly seawater temperature and the concentrations of NH_4-N, NO_3-N and PO_4-P from October 2000 to October 2001 at Matsushima Bay and the monthly average seawater

temperature from 1969 to 1999 at Enoshima Island (38° 24′ N,141° 35′ E) near the southernmost geographic range of Saccharina were obtained from the Miyagi Prefectural Fisheries Research and Development Center. Data of monthly seawater temperature and concentrations of NH_4-N, NO_3-N and PO_4-P from October 2000 to October 2001 at Rishiri Island were obtained from the Hokkaido Wakkanai Fisheries Experimental Station.

Statistical analysis

Significant differences in morphological characters among months, and dry weight, C and N content, photosynthetic rate, and nutrient uptake rate among months and parts of the blade were analyzed by the Kruskal-Wallis test followed by Scheffé's multiple comparison test because not all data showed a normal distribution and homogeneous variance. The significance of the correlation between initial nutrient concentration and nutrient uptake rate was analyzed using p-values calculated by the TDIST function of Excel ($p<0.05$).

RESULTS

Environmental factors

Seasonal seawater temperature and concentrations of NH_4-N, NO_3-N and PO_4-P in Matsushima Bay, Rishiri Island, together with the monthly average seawater temperature at Enoshima Island, are shown in Fig. 1-2. Seawater temperature at Enoshima Island and in Matsushima Bay ranged from 7.3°C in March to 21.3°C in August and from 2.6°C in February to 24.7°C in August, respectively. At Rishiri Island, the seawater temperature ranged from 3.5°C in February to 20.3°C in August. The temperature in Matsushima Bay was almost the same as that in Rishiri Island from December to February and was then 3−6°C higher until August. The temperature at Enoshima Island was 2.0−6.7°C higher than that at Rishiri Island from December to April and 3.4−5.9°C lower than that in Matsushima Bay from June to August (Fig. 1-2a).

The concentrations of NH_4-N, NO_3-N and PO_4-P in October, December and August in Matsushima Bay were higher than those at Rishiri Island (Fig. 1-2b, c, d). In contrast, nutrient concentrations in February, April and June were low and the same as those at Rishiri Island, except for NH_4-N in April. At Rishiri Island, the concentrations of NH_4-N were very low at <0.78 μmol L^{-1} all year round (Fig. 1-2b) and were below the detection limit except for December and February. The concentrations of NO_3-N ranged from 1.82 to 3.29 μmol L^{-1} from December to April and were <0.85 μmol L^{-1} in the other months (Fig. 1-2c, d). Similarly, PO_4-P varied from 0.40 to 0.56 μmol L^{-1} from December to April and was about 0.36 μmol L^{-1} in the other months.

Morphological characteristics

Changes in growth and erosion rates, blade length, area, width, weight and thickness, and the percentage of *S. ochotensis* plants with sori are shown in Fig. 1-3. The growth rate increased to a maximum of 4.8 cm d^{-1} in January and then decreased. By contrast, the erosion rate increased from January to a maximum of 3.7 cm d^{-1} in July (Fig. 1-3a). The blade length, area and width increased from January to a maximum of 328.1 cm, 0.3 m^2 and 20.3 cm in May, respectively, and then decreased (Fig. 1-3b,c,d). The blade weight and thickness increased to a maximum of 58.3 g and 2.08 mm in July, respectively (Fig. 1-3e,f). Plants with sori occurred from April to June and peaked at 27% of total plants in May (Fig. 1-3g). Sori were observed at the tip of the blade, occupying <5% of the whole blade area.

Dry weight, and C and N content

Changes in dry weight, and C and N content in the apical, middle and meristem parts of the blade are shown in Fig. 1-4. Little change was detected in the values of these parameters until the summer when the values increased significantly. Among blade parts, the values were lowest in the apical and middle parts and highest in the meristem. But by July the values in the apical and middle parts increased to the levels approaching those in the meristem.

Photosynthetic rate and nutrient uptake rate

Changes in photosynthetic rate and uptake rates of NH_4-N, NO_3-N and PO_4-P at the meristem, middle and apical parts of the blade are shown in Fig. 1-5. Seasonally, the photosynthesis rates in the three blade parts were more or less constant until the onset of

summer in June. At that time the photosynthesis rates declined in the mid and apical parts while remaining constant in the meristem. The photosynthetic rate at the middle and apical part of the blade was >20 μL O_2 cm^{-2} h^{-1} until June, and then significantly decreased to a minimum in July ($p<0.01$). For most of the growing season the photosynthesis rates in the meristem were lower than in the outer parts of the blade until the onset of summer when the mid and apical parts had lower rates. The rates at the meristem were significantly lower than in the other parts from January to May.

There was a general trend in the uptake rates of NH_4-N of increasing uptake from winter through summer (Fig. 1-5). And although there were significant differences among the different blade parts, the differences were small and the trend was similar in all parts

Trends in the uptake rates of NO_3-N are more difficult to determine. Rates of uptake fluctuated throughout the months. Most notable was that the mid-part of the blades displayed the lowest uptake rates and the meristem generally had the highest rates. There was a spike in the uptake rate of NO_3-N in June in the meristem.

The uptake rates of PO_4-P varied little over the growing seasons and also varied little among the parts of the blade (Fig. 1-5). Uptake rates varied from about 1–3 nmol cm^{-2} h^{-1}. It was not until July that the mid-part of the blades showed a marked decrease in the uptake rate to the point of actually losing PO_4-P to the medium.

Nutrient uptake kinetics

The kinetics of the uptake of NH_4-N, NO_3-N and PO_4-P at the meristem and middle part of the blade at six different nutrient concentrations from January to July are shown in Fig. 1-6.

Formula of hyperbolae and the indices of the regression (K_s, V_{max} and V_{max}/K_s) are shown in Table 1-1. The uptake rates of NH_4-N at all the blade parts were correlated with the nutrient concentrations in the media in all months ($p<0.01$). V_{max} at the all blade part indicated the maximum in January, and the value tended to decrease toward July. In contrast, V_{max}/K_s at the all the blade part tended to increase from winter and reached a maximum in July.

There were significant hyperbolic correlations between the uptake rates of NO_3-N and the concentrations at the meristem in all month ($p<0.05$) (Table 1-1). At the middle part of the blade, no significant hyperbolic correlations were detected from May to July ($p>0.05$). Ks values at both blade parts were at a maximum in March. Low values were found in July at the meristem and in April at the middle part of the blade. The values of V_{max} at both parts of the blade were markedly lower than those of NH_4-N. The values of V_{max}/K_s (α) at both parts of blade were low in March, and high from May to July at the meristem, and higher than those of NH_4-N in all months except for that in the middle part of the blade in January.

A marked seasonal change was not found in the uptake rates of PO_4-P. K_s at the all blade part indicated 4.3–5.8. V_{max} and V_{max}/K_s (α) at the all blade part were indicated lower than 0.01 and 0.001, respectively.

DISCUSSION

Environmental condition at Matsushima Bay and Rishiri Island

In Rishiri Island, where *S. ochotensis* grows naturally, the nutrient concentrations are high in winter and decrease towards summer (Fig.1-2), as typical of oceanographic conditions in subarctic and temperate regions (Dotsu et al. 1999). Environmental data indicate that cultivation of kelp occurs at higher water temperatures with a wider annual range and more eutrophic conditions than those seen at Rishiri Island. The water temperature in Matsushima Bay was lower in winter and higher in summer compared with that at Enoshima Island, indicating an environmental characteristic of an 'inner bay' (Fig.1-2)(Li et al. 2007).

Changes in growth and resources accumulation

Results in the present study reveal that cultivated kelp extended its blade area rapidly from December to May, and then accumulated C and N at the meristem in June and July (Fig.1-3,4). The high photosynthetic rates at the middle and apical parts of the blade were maintained from January to June, and then decreased markedly in July (Fig.1-5). By contrast, the uptake rates of NH_4-N and NO_3-N at the meristem were significantly higher than in the other parts of the blade in June. In addition, the uptake rate of NH_4-N increased significantly from May to July (Fig.1-5). These results suggest that C and N are accumulated through photosynthesis and nutrient uptake and then translocated to the basal part of the kelp (Lüning *et al.* 1973; Schmitz and Lobban 1976), particularly because the nutrient resources in the meristem increased from June and July.

Comparison of the resource storage pattern among* Saccharina *genus

The seven kelp species in the genus *Saccharina* in Hokkaido are considered as comprising the *S. japonica* group (*S. japonica*, *S. ochotensis*, *S religiosa*, *S diabolica* and *S. longipedaris*), whose mucilage canals are present in both stipe and blade, and the *S. angustata* group (*S. angustata* and *S. longissima*), whose mucilage canals are present only in the blade (Miyabe 1902). Yabu (1964) confirmed that *S. ochotensis* germinates and grows normally based on an interspecific gametophyte crossing experiment with *S. japonica* and *S. religiosa*, but crossings between each of those species and *S. angustata* result in abnormally shaped sporophytes and high mortality. Although Lane et al. (2006) listed *S. ochotensis* as an independent species, they supported the conclusion that *S. japonica*, *S. ochotensis*, *S. diabolica*, *S. religiosa* and *S. longissima* are conspecific by ITS sequences (Yostukura et al. 1999, Yoon et al. 2001). Selivanova et al. (2007) treated three species (*S. ochotensis*, *S. diabolica* and *S. religiosa*) as synonyms of *S. japonica* from analysis of the DNA nucleotide sequences of ITS and Rubisco spacer. To verify the molecular-phylogenetic data, the growth and resource storage pattern of *S. ochotensis* is compared with other species of the genus *Saccharina*. Li et al. (2009) cultivated *S. diabolica* and *S. longissima* in Matsushima Bay and examined the differences in seasonal morphological characteristics, photosynthetic rate, nutrient uptake rates, and resource contents. Results showed that the blade lengths of *S. longissima* was 1.28 times in April and 4.14 times in July that of *S. diabolica*, and the erosion rate of *S. diabolica* was twice that of *S. longissima*, although the physiological characteristics of both kelp species are similar. The resources are translocated and accumulate in the whole-blade tissue in *S. longissima*, but in the meristem in *S. diabolica* from May to June. These results suggest that morphological differences are

responsible for different resource storage patterns, which are genetically fixed characteristics adapted to specific environments. In the present study, the resource storage pattern of *S. ochotensis*, which accumulates C and N intensively in the meristem (Fig. 1-4), is similar to that of *S. japonica* (Li et al. 2007) and *S. diabolica* (Li et al. 2009) belonging to *S. japonica* group. This supports the inclusion of *S. ochotensis* as one of the variations of *S. japonica*, according to Yotsukura et al. (1999) and Yoon et al. (2001).

Nutrient uptake kinetics and adaptation to seasonal nutrient condition

Espinoza and Chapman (1983) reported that the nutrient uptake rates of *L. longicruris* were fitted to a hyperbolic regression, and showed that V_{max}/K_s was higher for plants in the nitrate-poor St. Margaret's Bay than in those in nitrate-rich Bay of Fundy, Nova Scotia, Canada. This result suggests adaptation to each local nitrate environment. Druehl et al. (1989) demonstrated that sporophytes of *L. groenlandica* exposed to relatively high summer ambient N levels had lower uptake rates than those maintained in low N conditions. The authors suggested that kelp accommodates a range of ambient nitrate conditions by altering its uptake kinetics through genetic ecotypic differentiation, plastic phenotypic differentiation and ontogenetic differentiation. In the present study, the correlations between initial NO_3-N and NH_4-N concentrations and the uptake rates of *S. ochotensis* were fitted to a hyperbolic regression except for NO_3-N from May to July at middle part and PO_4-P in May and July at meristem part (Fig.1-6). Decreases in V_{max} and K_s and an increase in α values were detected from winter to summer. High V_{max} and K_s in winter, and high α values from spring onwards indicate a high uptake ability at high and low nutrients concentrations, respectively (Table 1-1). It is likely that these

physiological characteristics result from adaptation to seasonal nutrient conditions in the northern Sea of Japan (Dotsu et al. 1999). It is thought that this seasonal adaptation of nutrient uptake kinetics supports accumulation of C and N at the meristem for maturation after summer and regrowth to form a second plant after autumn, which is the life cycle of *S. ochotensis* (Sakai et al 1967; Yanagida et al. 1971; Nabata et al. 1981).

Apical erosion is suggested to be a mechanism to recycle nutrients under serious N deficiency (Mizuta et al. 1994). However, persistent erosion of *S. ochotensis* cultivated in Matsushima Bay from January onwards indicates a continuous accumulation of C and N in the meristem for maturation without any relation to ambient nutrient concentrations.

A wave-exposed environment diffuses the boundary layer on the surface of macroalgae and leads to the transfer of essential nutrients (Hurd et al. 1996). The total length and weight of plants cultivated in Matsushima Bay reached a maximum at 2−3 months and 3−4 months, respectively than those in Rishiri Island (Sakai et al. 1967; Yanagida et al. 1971). This could be caused by the 10°C higher water temperature and effective uptake of NH_4-N at the higher concentration than at Rishiri Island. In addition, it is likely that sea surface cultivation which is agitated by constant wave action (Neushul et al. 1992) improves the nutrient availability for the kelp, resulting in sorus formation in spring from April to June, as seen for *S. japonica* (Mizuta et al. 1998; Li et al. 2007). Generally, sorus formation by *S. ochotensis* occurs in autumn, when accumulation of C and N peak under short-day conditions (Mizuta et al. 1998). However, *S. ochotensis* cultivated in Matsushima Bay decay at high water temperatures in the summer. In conclusion, resource accumulation in the meristem is caused by marked expansion of the blade area from winter to spring, when sufficient C and N are produced by high photosynthesis and

high nutrient uptake, respectively, and translocation of the resources from the apical part occurs through erosion of the blade.

Application for increasing resources of S. ochotensis

The uptake rates of NH_4-N of V_{max} in a hyperbolic regression were 16 times higher than one of NO_3-N (Table 1-1). Braga and Yoneshigue-Valentin (1996) reported that the uptake of NH_4-N by young *L. abyssalis* sporophytes was more rapid than that of NO_3-N. They suggested that NH_4-N would be an efficient N fertilizer to use for cultivation. The highest uptake rates of NH_4-N and NO_3-N, for *L. saccharina* and *Nereocystis luetkrana* grown in a salmon sea-cage farm in Canada, were 40 μmol L^{-1} and 30 μmol L^{-1}, respectively, suggesting that the integrated cultivation of salmon and kelp is an effective means of production (Ahn et al. 1998). Agatsuma et al. (2014) enhanced *Saccharina* kelp production by supplying ammonium sulfate and dihydrogen phosphate *in situ* in the Sea of Japan off southwestern Hokkaido and demonstrated that the production of *Saccharina* kelp is restricted by low nutrient concentrations. The concentration of NH_4-N ranged from 35.2 to 173.2 μmol L^{-1}. In the Sea of Japan, in northern Hokkaido, decrease in the production of *S. ochotensis* is suggested to be associated with a decrease in nutrient concentrations (Akaike et al. 1998). In the present study, the high NH_4-N uptake ability of *S. ochotensis* supports a positive correlation between a temporal increase in nutrient concentration and thallus growth. Based on these characteristics of nutrient uptake kinetics, studies on the enhancement of kelp production and the impact on environment are needed.

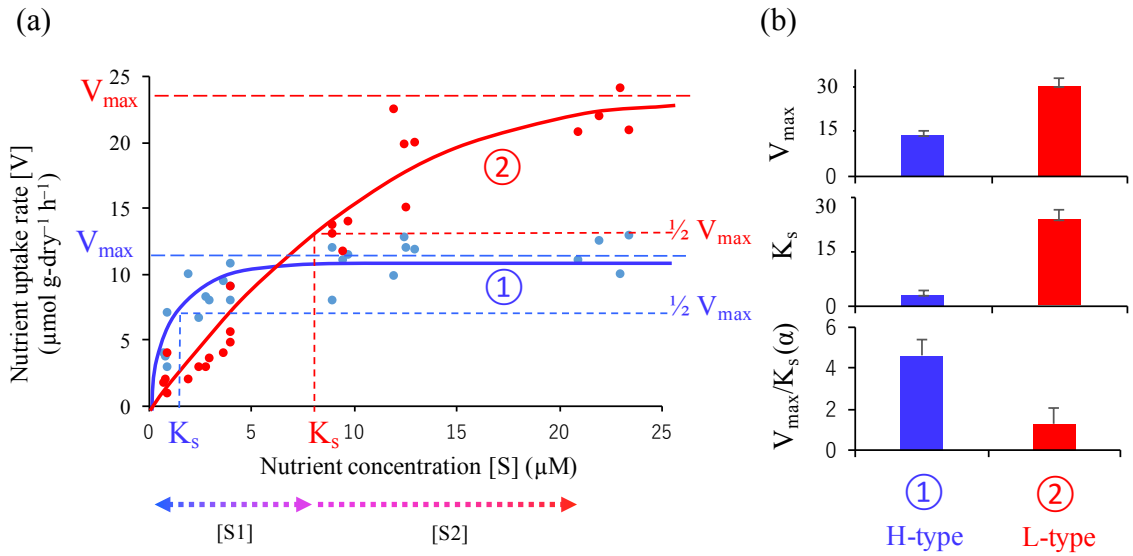


Fig. 1-1. Schematic representation of nutrient uptake rate depending on nutrient concentration (a) and parameter of nutrient uptake kinetics (b).

①: H-type, nutrient uptake kinetics of high affinity at low nutrient concentration (S1 range),

②: L-type, nutrient uptake kinetics of high affinity at high nutrient concentration (S2 range),

S1: An ordinary nutrient level in the ocean, S2: An eutrophic nutrient level or temporary nutrient loading in the coastal area.

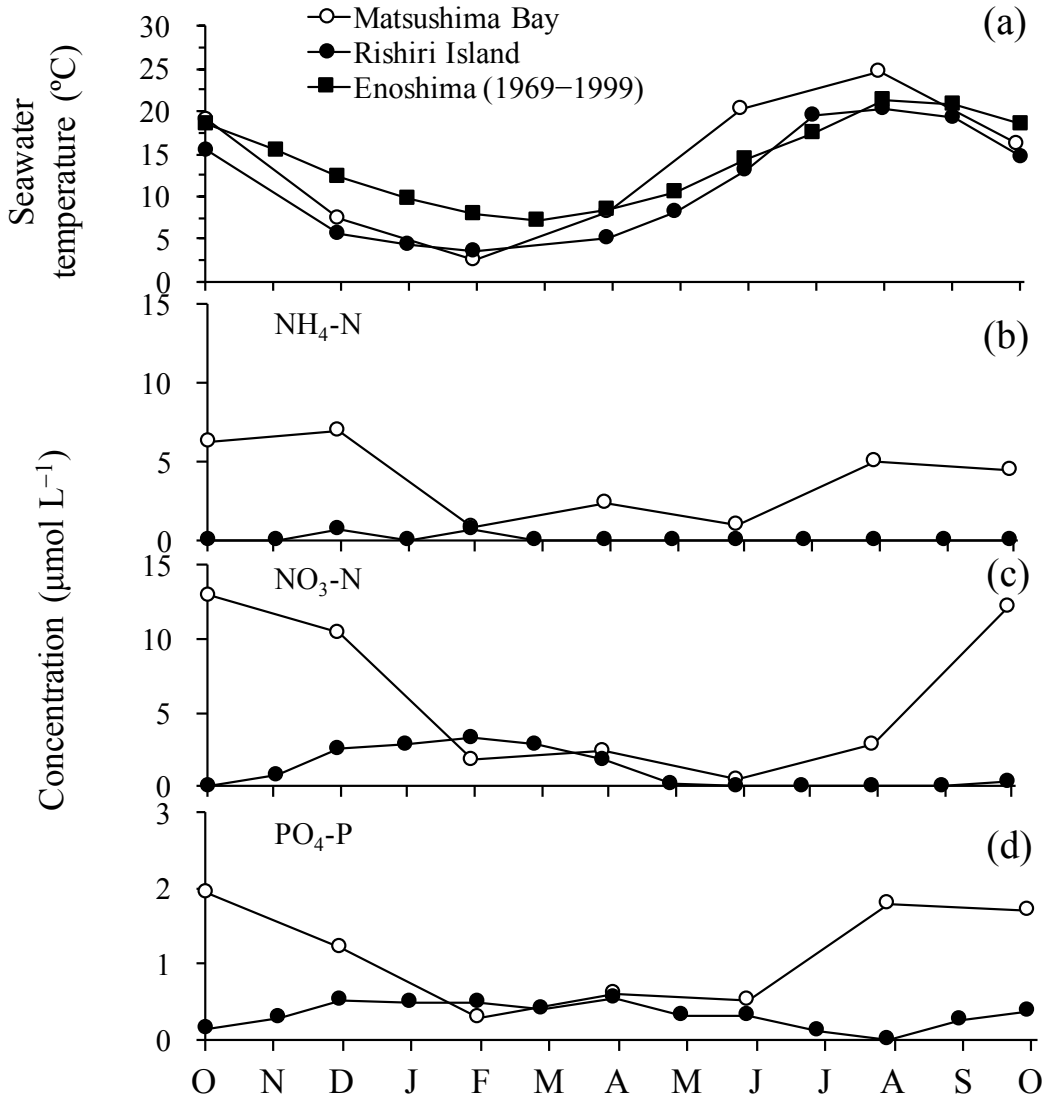


Fig. 1-2. Seasonal seawater temperature (a) and concentrations of NH_4-N (b), NO_3-N (c) and PO_4-P (d) in Matsushima Bay from October 2000 to October 2001, together with those at Rishiri Island (b,c,d) and the average seawater temperature at Enoshima Island and Rishiri Island (a).

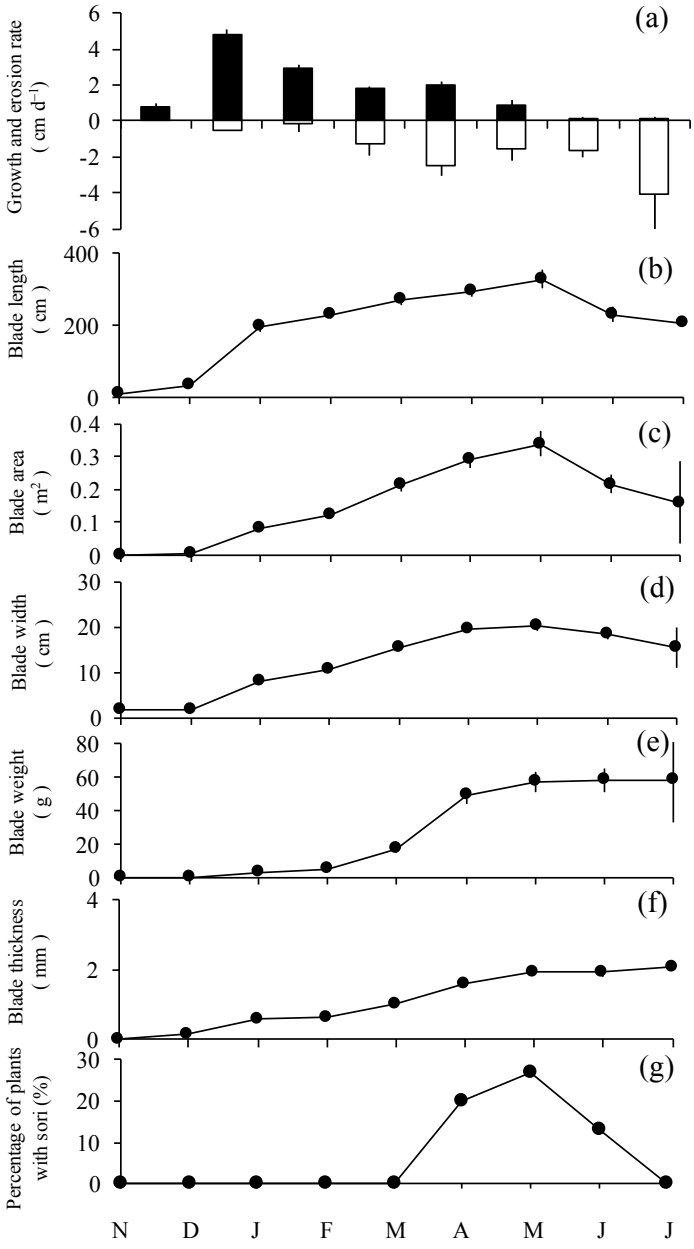


Fig. 1-3. Changes in growth rates (shaded bars), erosion rates (open bars) (a), blade length (b), blade area (c), blade width (d), blade weight (e), blade thickness (f) and percentages of plants with sori (g) of *Saccharina ochotensis* from November 2000 to July 2001. Vertical bars indicate 95% confidence interval.

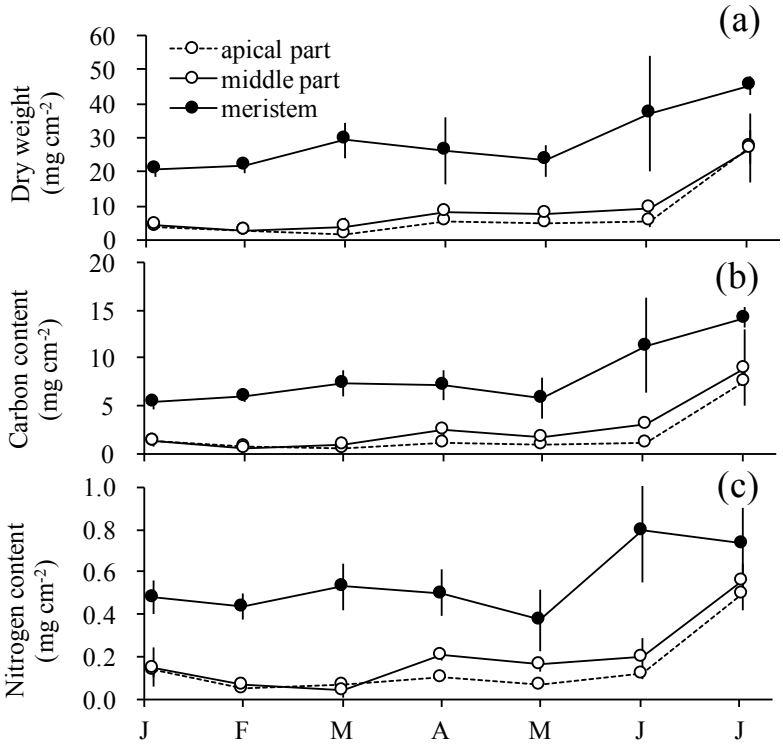


Fig. 1-4. Changes in dry weight (a), carbon (b) and nitrogen content (c) at the meristem, the middle part of the blade with maximum width and the apical part of the blade of *Saccharina ochotensis* from January to July 2001. Vertical bars indicate 95% confidence interval.

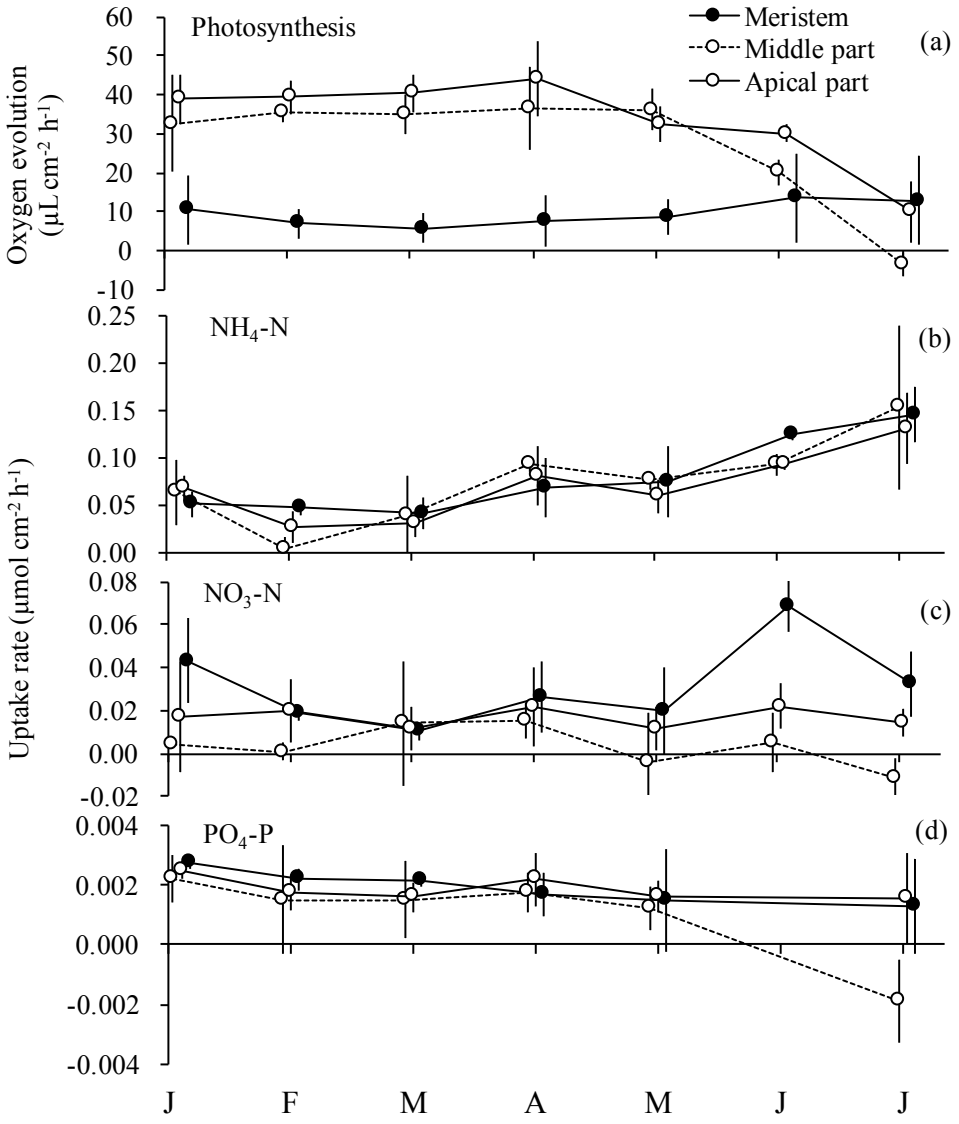


Fig. 1-5. Changes in photosynthetic rate (a) and uptake rates of NH_4-N (b), NO_3-N (c) and PO_4-P (d) at the meristem, the middle part of the blade with maximum width and the apical part of the blade of *Saccharina ochotensis* from January to July 2001. Vertical bars indicate 95% confidence interval.

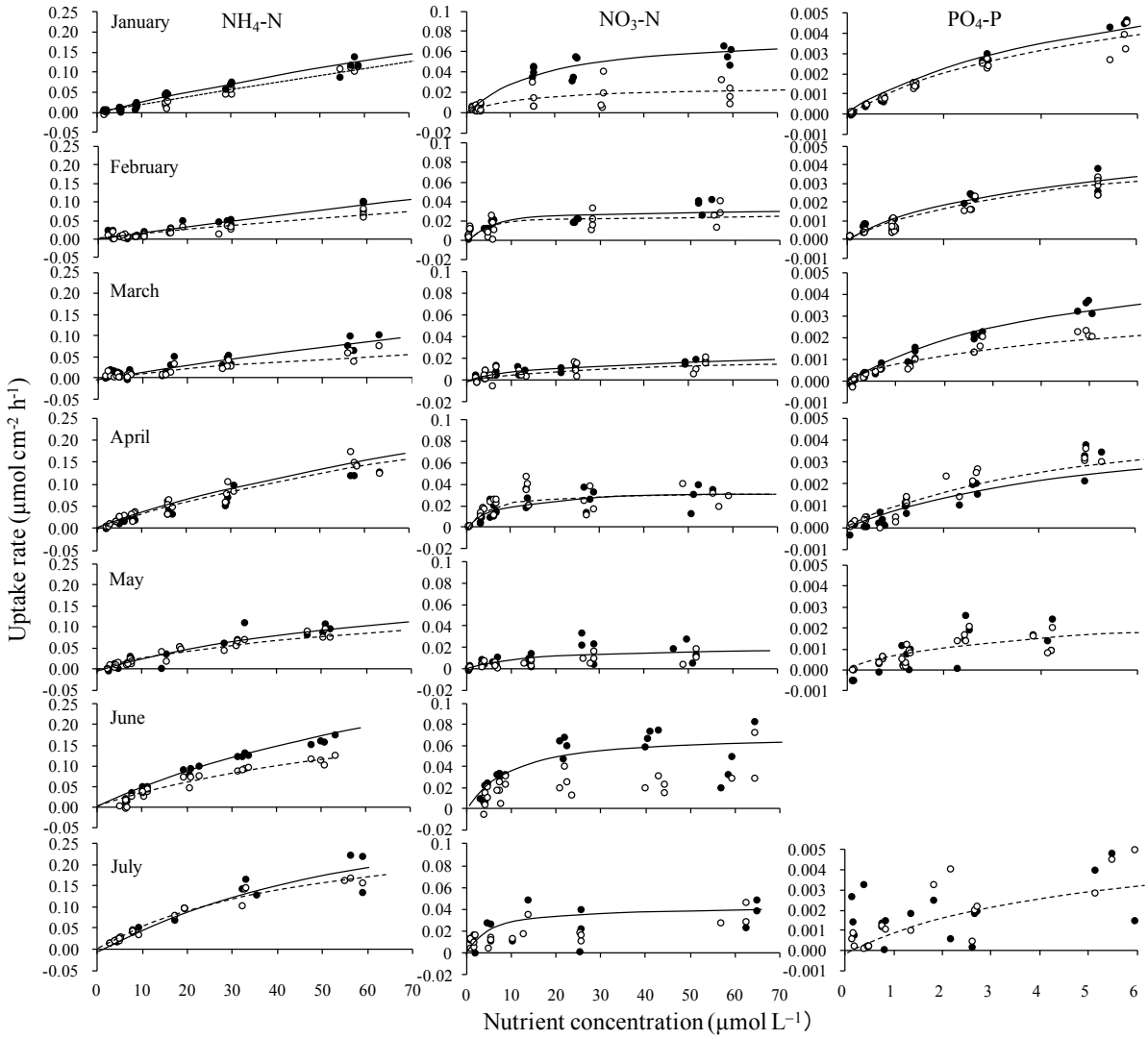


Fig. 1-6. Uptake rate of NH_4-N, NO_3-N and PO_4-P, and the hyperbolic lines of the correlations at the meristem (solid circles and solid lines) and the middle part of the blade with maximum width (open circles and dashed lines) of Saccharina ochotensis at six different nutrient concentrations from January to July 2001.

Table 1-1. Values of *p*, R^2, Ks, Vmax and α of NH_4-N, NO_3-N and PO_4-P at meristem and middle part with maximum blade width of *S. ochotensis* cultivated in Matsushima Bay

Nutrients	Blade parts	Month	*p*	R^2	Ks [a] [μM]	Vmax [b] [μmol/cm^2/h]	α[c]
NH_4-N	Meristem	Jan.	0.00	0.96	666.5	1.29	0.002
		Feb.	0.00	0.92	337.7	0.61	0.002
		Mar.	0.00	0.87	301.7	0.51	0.002
		Apr.	0.00	0.95	214.0	0.57	0.003
		May	0.00	0.89	117.0	0.31	0.003
		Jun.	0.00	0.97	107.8	0.52	0.005
		Jul.	0.00	0.92	68.5	0.42	0.006
	Middle	Jan.	0.00	0.97	208.0	0.55	0.003
		Feb.	0.00	0.87	334.5	0.42	0.001
		Mar.	0.00	0.85	147.6	0.20	0.001
		Apr.	0.00	0.92	132.5	0.47	0.004
		May	0.00	0.93	81.4	0.22	0.003
		Jun.	0.00	0.93	82.2	0.31	0.004
		Jul.	0.00	0.97	47.6	0.30	0.006
NO_3-N	Meristem	Jan.	0.00	0.91	19.3	0.08	0.004
		Feb.	0.00	0.67	6.9	0.03	0.005
		Mar.	0.00	0.63	53.9	0.04	0.001
		Apr.	0.00	0.58	8.1	0.03	0.004
		May	0.04	0.43	18.2	0.24	0.013
		Jun.	0.00	0.60	9.6	0.07	0.007
		Jul.	0.01	0.53	6.1	0.05	0.007
	Middle	Jan.	0.03	0.44	13.8	0.02	0.002
		Feb.	0.01	0.50	5.6	0.03	0.005
		Mar.	0.04	0.42	53.9	0.03	0.001
		Apr.	0.02	0.46	3.7	0.03	0.008
		May	0.05	0.41	n.s.	n.s.	n.s.
		Jun.	0.08	0.36	n.s.	n.s.	n.s.
		Jul.	0.13	0.33	n.s.	n.s.	n.s.
PO_4-P	Meristem	Jan.	0.00	0.98	5.8	0.01	0.001
		Feb.	0.00	0.89	5.2	0.01	0.001
		Mar.	0.00	0.96	5.1	0.01	0.001
		Apr.	0.00	0.84	5.3	0.01	0.001
		May	0.05	0.40	n.s.	n.s.	n.s.
		Jul.	0.93	0.02	n.s.	n.s.	n.s.
	Middle	Jan.	0.00	0.95	5.8	0.01	0.001
		Feb.	0.00	0.92	5.2	0.01	0.001
		Mar.	0.00	0.96	5.1	0.00	0.001
		Apr.	0.00	0.91	5.3	0.01	0.001
		May	0.00	0.61	4.3	0.00	0.001
		Jul.	0.00	0.68	5.1	0.01	0.001

CHAPTER II

Development and evaluation of a novel tank system designed for macroalgae

SUMMARY

Kelps are an invaluable primary producer with high productivity. Although breeding studies have attempted to reduce instability in this natural resource, almost all cultivation tests were carried out in the ocean, which has limited the development of new cultivars. To address this limitation, I developed a new tank culture system, referred to as a cyclone and floating culture system (CFCS), for macroalgae. In the CFCS, kelps can be cultivated under controlled environmental conditions. Water flow velocity in the CFCS can be regulated by changing the angle of a seawater inlet spout without changing the amount of seawater in the tank. *Undaria pinnatifida* and *Saccharina japonica* cultivated in the CFCS exhibited morphological features identical to those of plants grown naturally in the ocean. Two hundred and thirty individually identified *U. pinnatifida* plants of the same strain that were cultivated in the CFCS exhibited significant variation in relative growth rates.

INTRODUCTION

Kelps are an invaluable primary producer in the coastal ecosystem with high productivity (Mann 1972). Their productivity has been estimated to be 2.8 times greater than that of sugarcane, the most productive land plant under cultivation (Gao and McKinley 1993). Kelp species are used as edible foods and industrial materials (Jensen 1993) and the total global production in 2008 was estimated to be 1.5 million MT (FAO 2010). Furthermore, brown macroalgae have become the focus of attention as ideal feedstocks for biofuel production because they do not require arable lands, fertilizers, or fresh water resources (Wargacki et al. 2012).

A relationship between decreased kelp abundance and ocean warming has been reported for several species at many coastal regions (Johnson et al. 2011, Kirihara et al. 2006, Müller et al. 2009). As I described in Chapter I, a remarkable decline in *Saccharina* was reported in Hokkaido, northern Japan, where the production of *S. ochotensis* has decreased to 12% of its peak level during the 1940s (Nabata et al. 2003). Because of this instability in natural kelp resources, market demand is driving the development of new cultivation technologies (FAO 2010). To increase kelp productivity, progress in new breeding techniques is required.

Previous kelp breeding studies identified heterosis in *Undaria pinnatifida* (Hara and Akiyama 1985) and *M. pyrifera* (Westermeier et al. 2010). By breeding for five generations, Zhang et al. (2011) obtained a new variety of *S. japonica*, Rongfu, which attained yields that were 24–27% higher than those of other varieties. To reduce the duration of breeding periods and confirm cultivar phenotypes, a new tank culture system in which kelps can be cultivated through an entire life cycle under controlled environmental conditions was needed. Previous

studies showed that water motion impacts the morphological characteristics and productivity of kelps (Hurd et al. 1996, Nanba et al. 2011, Peteiro and Freire 2011); therefore, the ability to control water flow velocity was needed in the new tank system. Tank culture systems that were suitable for the small perennial macroalga *Chondrus crispus* (Neish et al. 1977), the small annual macroalga *Ulva prolifera* (Hiraoka and Oka 2008), and the young sporophyte stage of the large perennial macroalgae, *M. pyrifera* and *Lessonia trabeculata* (Westermeier et al. 2006) were previously demonstrated. While such tank systems have been developed, a tank system which can be used to cultivate large numbers of mature kelp sporophytes under controlled environmental conditions has not yet been put into practical use.

In this study, I developed a new tank system in which temperature, light intensity, and water flow velocity can be controlled. The new system was designated as a cyclone and floating culture system (CFCS) for macroalgae based on the characteristic water motion in the tank. I cultivated *U. pinnatifida* and *S. japonica* sporophytes in the CFCS and evaluated their morphological responses to water flow velocity. The growth responses of *U. pinnatifida* and *S. japonica* to water flow velocity observed in the CFCS were the same as those of plants grown in the ocean. Sporophytes of *U. pinnatifida* cultivated in the CFCS exhibited variation in growth rates among individuals germinated from the same pair of male and female gametophytes. These results suggest that the CFCS is useful for investigating the responses of kelps to environmental conditions and for selecting cultivars with higher productivity.

MATERIALS AND METHODS

Development of a new tank system

A polycarbonate circular tank (SPS-2000, Earth Co., Ltd., Tokyo, Japan) 90 cm tall and 180 cm in diameter with a capacity of 2000 L was used as the main component of the CFCS (Fig. 2-1a). A hole 30 mm in diameter was made in the center of the tank bottom and connected to a circular base (CB-2000, Earth Co., Ltd.) for drainage (Fig. 2-1b, c). A vertical PVC pipe 100 cm in length was connected to the hole in the center of the tank (Fig. 2-1d). A conical structure 85 cm in length was installed around the PVC pipe (Fig. 2-1e). The entire surface of the conical structure was covered with holes 2 mm in diameter at 20 mm intervals. To supply air to the tank, eight porous stones were placed around the narrow end of the conical structure at the bottom of the tank (Fig. 2-1f). The porous stones were connected to an air compressor by silicon tubing 5 mm in diameter.

Seawater was filtered through a 1 μm microfiber filter (TCW-CS, Advantec MFS, INC., California, US) and sterilized using ultraviolet (UV) lamps (FLONLIZER 4L, Chiyoda-Kouhan Co., Ltd., Tokyo). The sterilized seawater was added to the tank through an angled PVC pipe located at the edge of the tank. By changing the direction of the angled pipe at the edge of the tank (Fig. 2-1i), the water flow in the tank could be regulated (Fig. 2-1g). The volume of the seawater introduced into the tank was controlled by a ball valve. Seawater was removed from the tank by flowing through holes in the conical structure, through the top of the central vertical PVC pipe (Fig. 2-1j), and through a 1 μm mesh filter (CUNO filter bag, 10EA, 3M, Tokyo, Japan) (Fig. 2-1h). Three fluorescent lights were installed above the tank and the light intensity level at the surface of the seawater could be regulated from 0 to 200 μmol photons $m^{-2}s^{-1}$ by

adjusting the distance of the lights from the surface. A switch timer was used to regulate photoperiods. The seawater temperature was regulated between 5 and 25ºC using a heater/cooler (BFL-80F, MITSUBISHI, Tokyo, Japan).

Measurement of water flow velocity in the CFCS

Water motion influences the morphological characteristics and productivity of kelps (Hurd 2000). To evaluate the effects of water flow velocity in the tank, the direction of the spout was set at angles of 0, 45, and 90º relative to the diameter of the tank (Fig. 2-1i). The water flow velocity was measured at each angle for ten minutes using an electromagnetic current meter (INFINITY-EM, JFE Advantec Co., Ltd., Tokyo, Japan). Measurements of water flow velocity in the CFCS were taken at three positions: an upper position 5 cm below the surface of the seawater, a middle position, and a lower position 5 cm above the tank bottom. The average water flow velocity at each position in the tank was calculated from 600 values recorded during a period of 10 minutes. The flow rate of seawater into the tank was set at 1500 L h^{-1} using a ball valve.

Cultivation of* Undaria pinnatifida *and* Saccharina japonica *in the CFCS and morphological measurements

Parental sporophytes of *U. pinnatifida* and *S. japonica* were collected from wild populations growing on the coast near Hirota Bay (38º95’ N, 141º67’ E) in southern Iwate Prefecture in July 2012 and in Shukunohe (40º37’ N, 141º76’ E) in northern Iwate Prefecture in August 2012, respectively. Pieces (3 × 3 cm) were excised from *U. pinnatifida* sporophylls

and from *S. japonica* sori, cleaned in sterilized seawater, and kept in paper towels at 4ºC for induction of zoospores. After 12 h, the pieces were washed three times in sterilized seawater and placed in a 1 L beaker containing 800 ml of sterilized seawater at 15ºC followed by zoospore induction after approximately 15 min. The zoospores were collected under a microscope using a micropipette and placed into plastic dishes containing 30 ml of PESI medium (Tatewaki 1966). Approximately 2000 individual zoospores were counted in each dish using a hemocytometer. The dishes were incubated under a 12 h light:12 h dark cycle with a light intensity of 20 μmol photons $m^{-2}s^{-1}$ and a temperature of 20ºC. After 50 d, one male and one female gametophyte were transferred to a 15 ml glass vial containing 10 ml of sterilized seawater and homogenized at 5,000 rpm for 2 min using a micro blender (T 18 digital ULTRA-TURRAX, IKA, Osaka, Japan). Fragments of the gametophytes were incubated under a 10 h light:14 h dark cycle at 50 μmol photons m^{-2} s^{-1} and 20ºC for 2 weeks to promote maturation.

Germinated sporophytes were cultivated in 3 L Erlenmeyer flasks containing 2 L of 1/4-strength PESI medium with aeration and a 12 h light:12 h dark cycle at 90 μmol photons m^{-2} s^{-1} and 15ºC. The culture medium was changed at 3 day intervals. After the average total length of the sporophytes reached 20 mm, the 300 longest, healthy juvenile sporophytes were selected and transferred to the CFCS. Sporophytes were precultured in the CFCS under 180 μmol m^{-2} s^{-1} at 10ºC and the spout angle was set at 0º for 2 weeks after which the longest 200 sporophytes were selected and divided equally into two groups. One hundred sporophytes were then cultivated in the CFCS with the angle of the inlet spout set at 0 or 90º. The sporophytes were collected and the longest 30 individuals were selected from each group after 15–30 d. The total lengths and sporophyte weights of the *U. pinnatifida* and *S. japonica* were measured (Fig. 2-2).

In addition, the number of *U. pinnatifida* plants with sporophylls was counted. For *S. japonica*, the maximum blade width was measured (Fig. 2-2).

Cultivation of* U. pinnatifida *germinated from a single strain in the CFCS.

Efficient breeding requires analysis of the phenotypes of many individuals cultured under the same environmental conditions. *U. pinnatifida* sporophytes tagged with small plastic plates on plastic bands were cultured in the CFCS and the growth rates of all plants were measured. Juvenile sporophytes were induced from one pair of male and female gametophytes and precultured according to the method described above. After the average total length of the sporophytes reached 20 mm on 24 June, 2013, all plants were transferred to the CFCS and cultivated at 10ºC under 12 h photoperiods, a light intensity of 180 μmol photons $m^{-2}s^{-1}$, and an inlet angle of 90º. After 36 d (30 July, 2013), the 230 longest plants were selected and tagged for individual identification and cultivated in the CFCS for 20 d. Fresh weights of all sporophytes were measured after being blotted dry both prior to the experiments and at the end of the experiments. Relative growth rates (RGR) were calculated according to the equation:

$$RGR\ (\%\ day^{-1}) = 100 \ln (Wt\ Wo^{-1})\ t^{-1}$$

where Wo is the initial fresh weight, Wt is the final fresh weight at the end of the experiment, and t is the number of days of cultivation.

Statistical analysis

The statistical significance of differences in water flow velocities in the CFCS among water inlet angles was analyzed using ANOVA followed by Scheffe's multiple comparison test. The statistical significance of differences in morphological characteristics of *U. pinnatifida* and *S. japonica* during the measurement periods was analyzed using the Kruskal-Wallis test followed by Scheffé's multiple comparisons test, because not all data exhibited a normal distribution and homogeneous variance. The statistical significance of differences in morphological characteristics of the two species cultivated at various water flow velocities was analyzed using the Mann-Whitney U test.

RESULTS

Water motion and water flow velocity in the CFCS

Water motion in the CFCS was circumferential along the inner wall of the circular tank. Compressed air rose from air stones at the center of the bottom of the tank to the surface of the seawater along the outer wall of the central conical structure with circumferential movement followed by movement of the air bubbles to the edge of the tank in a spiral motion (Fig. 2-1k). The water flow velocity varied significantly ($p < 0.01$) with the inlet angle at all measuring positions (Fig. 2-3). Average water flow velocity values were 4.81–7.81, 8.41–13.14, and 14.93–18.05 cm h^{-1} at 0, 45, and 90º, respectively, in the CFCS.

Changes in morphological characteristics of **U. pinnatifida** *and* **S. japonica** *cultivated under various water flow velocities in the CFCS*

The total lengths of *U. pinnatifida* exhibited significant increases to maximum values of 145.68 cm on the 68th day of cultivation under the fast flow velocity and 98.67 cm on the 50th day under the slow flow velocity (Fig. 2-4a, $p < 0.01$). The weights of sporophytes grown under the fast flow velocity were significantly higher than those grown under the slow velocity ($p < 0.05$), except on the 50th day, and reached maximum values on the 99th day under both water flow velocities (Fig. 2-4b). The proportion of plants with sporophylls peaked at 96% of all plants on the 68th day under the fast flow velocity, while the ratio reached 60% of all plants under the slow flow velocity condition (Fig. 2-4c). Spores released from the *U. pinnatifida* sporophylls cultivated in the CFCS germinated normally into gametophytes and then germinated into sporophytes. The sporophytes then grew and formed sporophylls.

The total lengths of *S. japonica* grown under the fast and slow flow velocities increased significantly to maximum values of 227.6 and 227.2 cm, respectively, on the 70th day (Fig. 2-5a, $p < 0.01$). The maximum width of *S. japonica* blades grown under the slow flow velocity increased significantly until the 70th day (Fig. 2-5b, $p < 0.01$) and reached a maximum value of 7.63 cm (Fig. 2-5b,d), while significant increases in the maximum width under the fast flow velocity stopped at the 29th day (Fig. 2-5b,e). The maximum widths of blades grown under the slow flow velocity were significantly higher than those of blades grown under the fast flow velocity after the 29th day ($p < 0.01$). The sporophyte weights of *S. japonica* cultivated under the fast and slow flow velocities increased significantly until the 70th day (Fig. 2-5c, $p < 0.01$). The individual weights were significantly higher under the slow flow velocity than the fast flow velocity, except for the 48th day (Fig. 2-5c, $p < 0.05$).

Variation in relative growth rates of individual **U. pinnatifida** *plants derived from a single strain*

The relative growth rate (RGR) of 229 plants, except for one eroded plant, indicated average 6.47% day^{-1} and ranged from a minimum value of 3.32% day^{-1} to a maximum value of 9.65% day^{-1} (Fig. 2-6), although all of the plants were germinated from a single pair of gametophytes.

DISCUSSION

Macroalgae cultivation in the CFCS

With the water agitation, the movement of *U. pinnatifida* and *S. japonica* cultivated in the CFCS was the same as that of the bubbles, and the plants drifted in the tank without tangling in the circumferential current. *Sargassum fusiforme* and *U. prolifera* could also be cultivated in the CFCS without tangling (data not shown) suggesting that almost all macroalgae species may be cultivated in the CFCS.

The water flow velocity in CFCS is changed significantly by inlet angles without regulating water volume. This result showed that the CFCS is useful for cultivating kelps under significantly different water flow velocities (Fig. 2-3). For the subsequent cultivation tests of *U. pinnatifida* and *S. japonica* in the CFCS, spout angles of 90º and 0º were defined as fast and slow water flow velocities, respectively (Fig. 2-4,5). The water velocity with a spout angle of 90º was higher than that of a *U. pinnatifida* and *S. japonica* cultivation site at Okirai Bay, Iwate Prefecture (Nanba et al. 2011), which is one of the major *U. pinnatifida* cultivation sites in Japan. The CFCS made the cultivation of kelps under a water flow velocity similar to that of typical cultivation sites possible.

Changes in morphological characteristics of U. pinnatifida *and* S. japonica *cultivated under various water flow velocities in the CFCS*

Neushul et al. (1992) reported that fast wave motion provides increased water velocity and turbulence, which enhances the uptake of nutrients and carbon dioxide by reducing the diffusion boundary layer along the algal surface. In this study, the fast flow velocity probably enhanced

U. pinnatifida sporophyte growth due its effect on the diffusion boundary layer. *U. pinnatifida* growth is affected by water motion, with fast flow velocities leading to larger, thicker fronds (Nanba et al. 2011) and higher biomass (Peteiro and Freire 2011). Our results show that the growth characteristics of *U. pinnatifida* sporophytes cultured in the CFCS exhibited the same dependency on water flow velocities as shown in previous studies carried out in the ocean (Fig. 2-4) (Nanba et al. 2011, Peteiro and Freire 2011) indicating that cultivation of *U. pinnatifida* in the CFCS under significantly different water flow velocity conditions makes the evaluation of the effects of water flow velocity on morphological characteristics and productivity of kelps possible.

Kelps have relatively smooth, flat blades and narrow, thick morphology when exposed to wave action (Gerard and Mann 1979, Hurd et al. 1996). A narrow, thick morphology reduces drag forces that would otherwise damage or remove blades, and increases blade strength (Gerard and Mann 1979). *S. japonica* cultivated under the fast flow velocity in the CFCS exhibited a narrower shape than those grown under the slow flow velocity, demonstrating that the water flow velocities in the CFCS reproduced the growing conditions of *S. japonica* grown in the ocean (Fig. 2-5).

Variation in relative growth rates of individual U. pinnatifida *plants derived from a single strain*

The relative growth rates (RGR) of *U. pinnatifida* sporophytes could be measured by the CFCS cultivation. The values were represented variable, although all of the plants were germinated from a single pair of gametophytes. This result shows that higher-growth

individuals can be selected based on the weight data of individual kelps cultivated in the CFCS (Fig. 2-6). The ability to cultivate and select kelps in the CFCS will be useful for breeding new cultivars to be used as feedstock for biofuel production, for which biomass productivity is the most important factor. Previously, kelp breeding studies were conducted in the ocean using a rope set horizontally on the surface of the seawater (Li et al. 2007, Zhang et al. 2007, Zhang et al. 2011). These studies could be carried out at only once per year, highlighting the need to reduce the length of time required for breeding studies. The results of our study suggest that the selective breeding of kelps can be carried out independent of the cultivation schedule in the ocean.

Land-based cultivation of macroalgae has recently attracted attention because it may be the most viable means for generating biomass suitable for high-value functional products (Hafting et al. 2012). Because the water flow velocity can be regulated in the CFCS (Fig.2-3), the device is useful for identifying conditions of water motion suitable for increasing the production of any species. The results also suggest that the CFCS is useful for research on the physiological ecology of macroalgae because large numbers of individuals can be cultivated in the tank under a controlled environment.

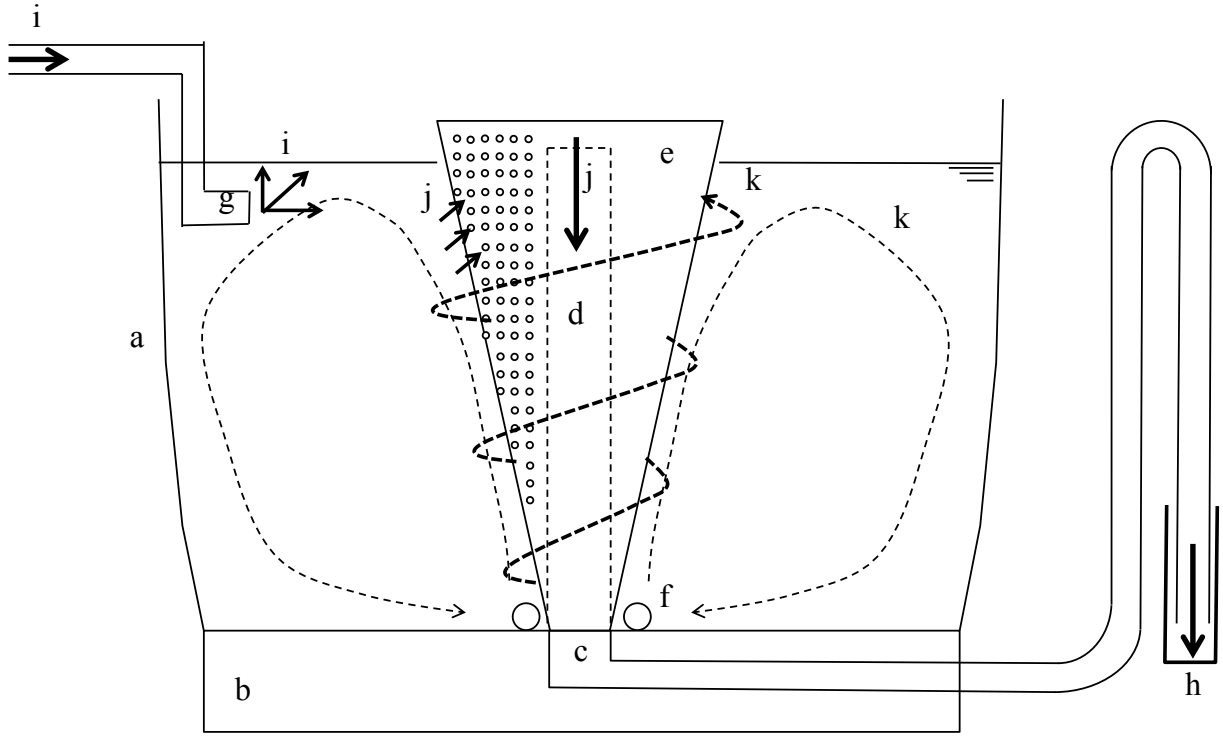


Fig. 2-1. Schematic view of a new tank system, a cyclone and floating culture system (CFCS) for macroalgae: (a) Circular polycarbonate tank. (b) Circular base. (c) Drainage hole. (d) PVC pipe for seawater drainage. (e) Conical polystyrene structure. (f) Aeration stone. (g) PVC inlet pipe. (h) Filtration bag. (i) Seawater flow from inlet pipe. (j) Seawater flow to drainage. (k) Air flow.

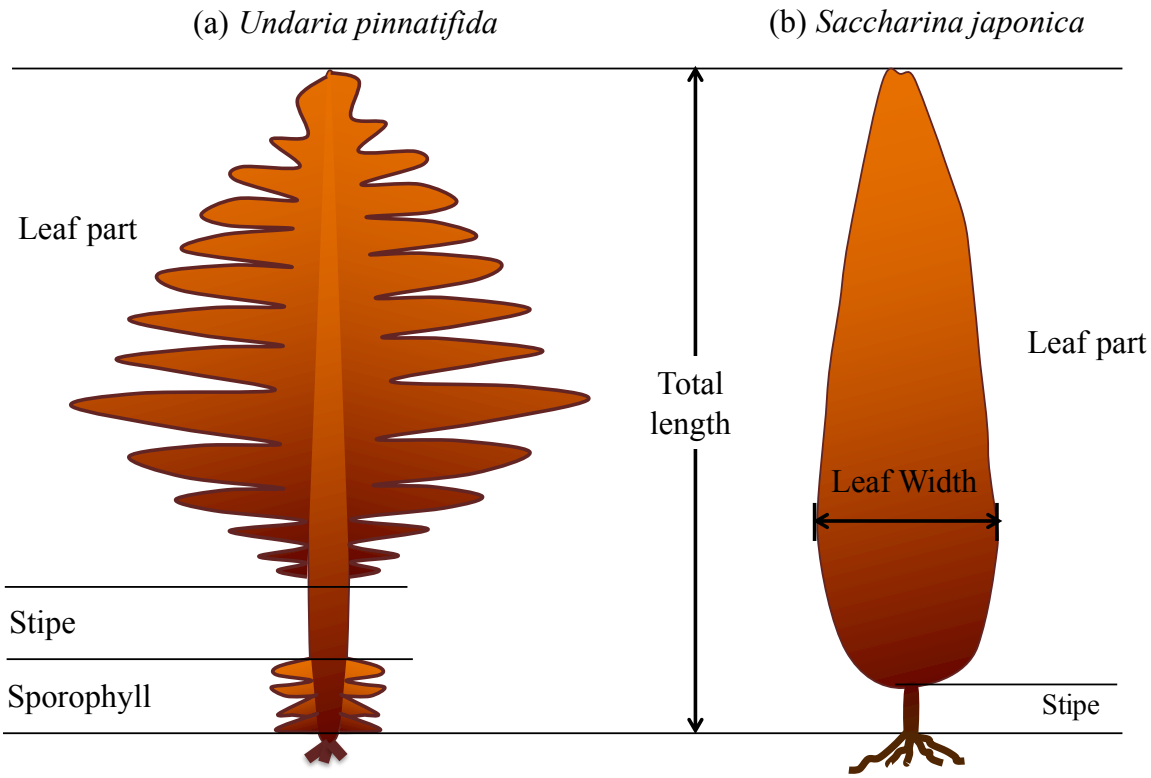


Fig. 2-2. Morphological parameters of *Undaria pinnatifida* and *Saccharina japonica*.

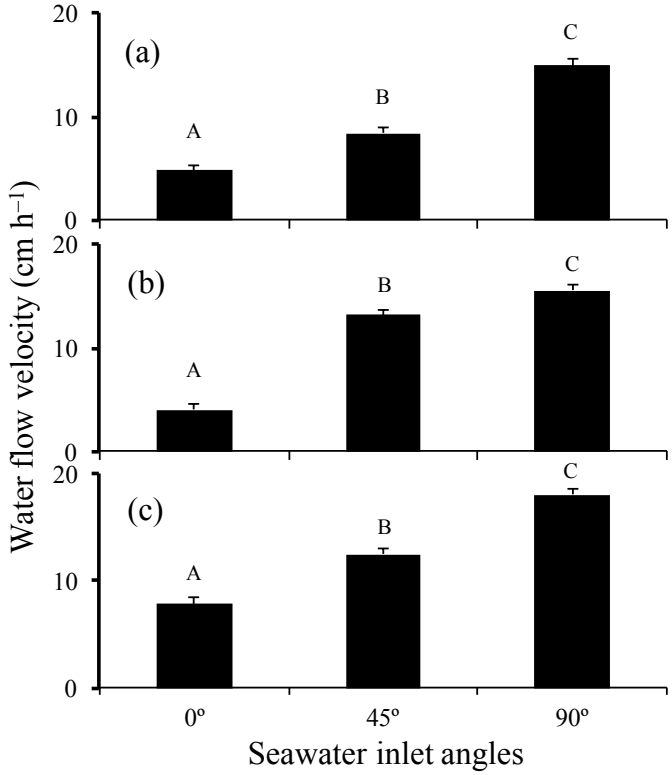


Fig. 2-3. Comparison of water flow velocities of various seawater inlet angles at upper (a), middle (b), and lower (c) measurement sites in the CFCS. Inlet angles to introduce seawater into the circular tank were set at 0, 45, and 90º relative to the tank diameter. Vertical bars indicate standard deviations. Different letters indicate significant differences ($p < 0.05$) between seawater inlet angles.

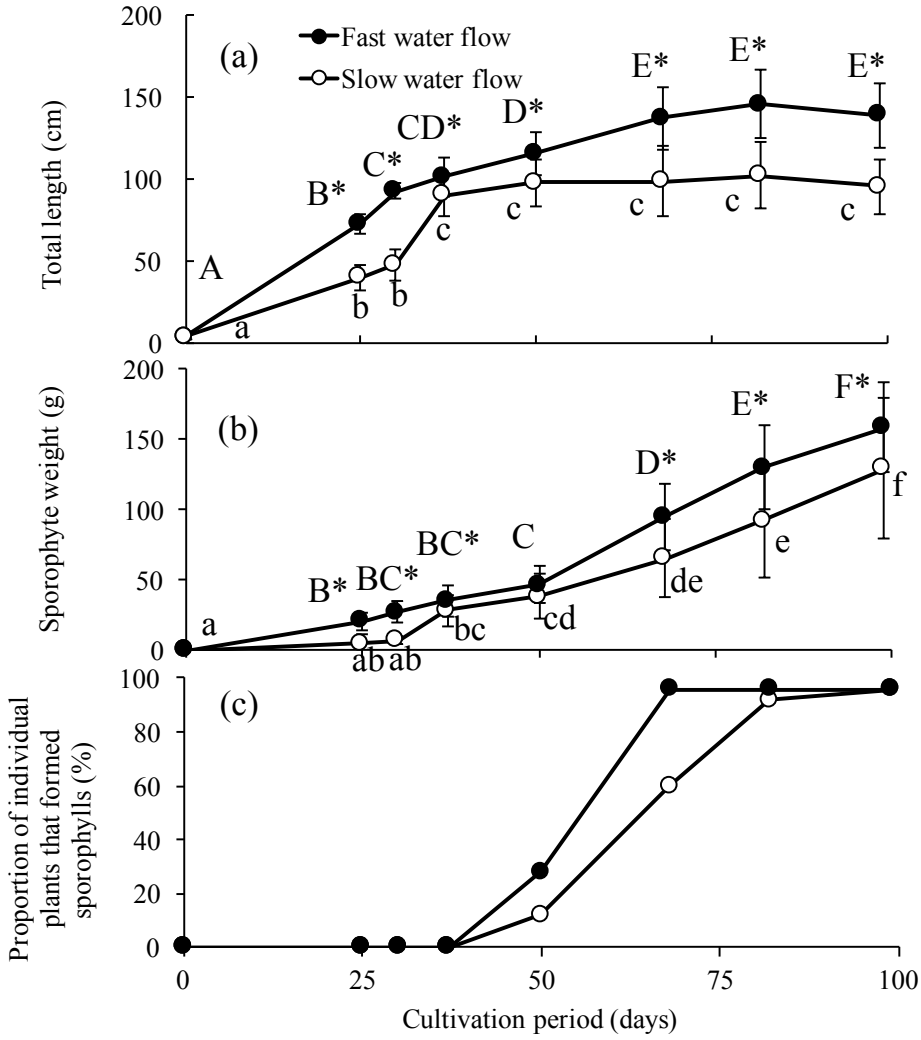


Fig. 2-4. *Undaria pinnatifida.* Effect of water flow velocity on morphological characteristics and maturation of *U. pinnatifida* cultivated in the CFCS. (a) Total length. (b) Sporophyte weight. (c) Proportion of individual plants that formed sporophylls. Vertical bars indicate standard deviations. Different letters and asterisks indicate significant differences ($p < 0.05$) between cultivation periods and water flow velocities, respectively.

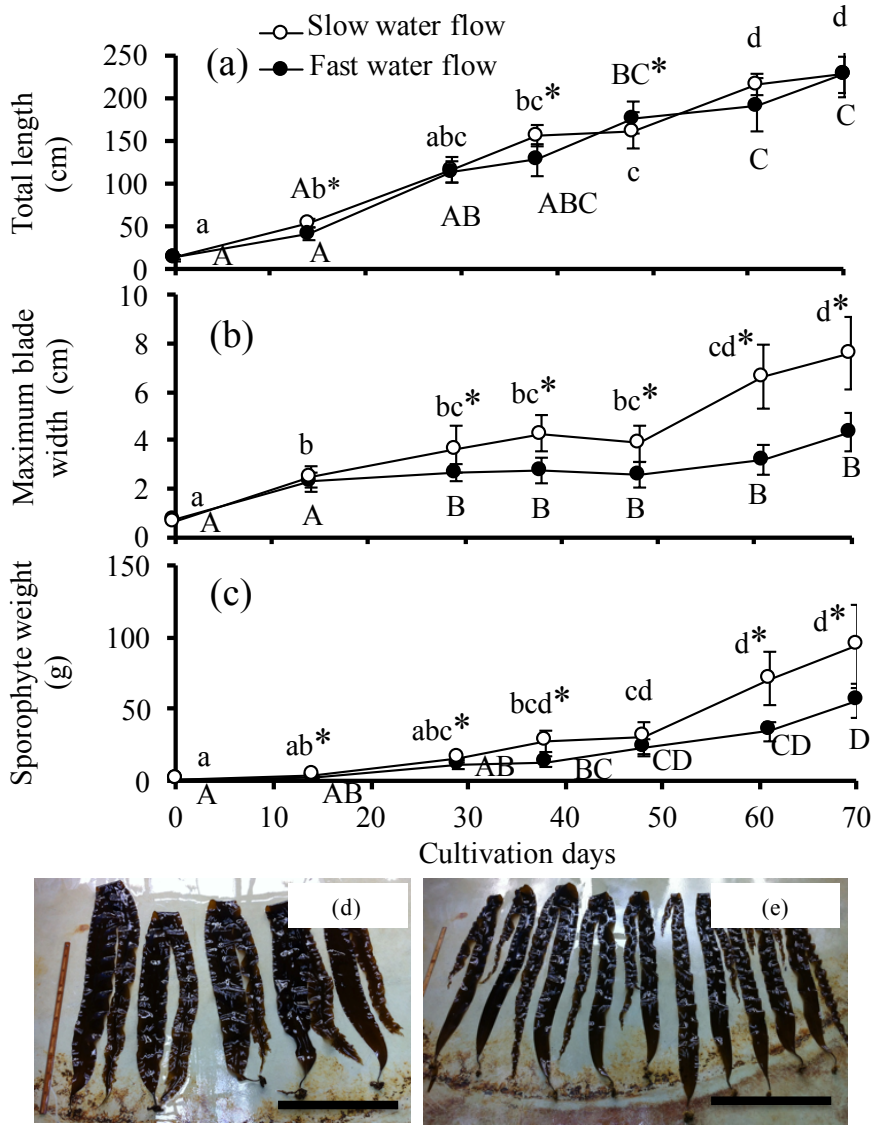


Fig. 2-5. *Saccharina japonica*. Effect of water flow velocity on morphological characteristics of *S. japonica* cultivated in the CFCS. (a) Total length. (b) Maximum blade width. (c) Sporophyte weight. (d) Photograph of *S. japonica* cultured under the slow water flow velocity. (e) Photograph of *S. japonica* cultured under the fast water flow velocity. Vertical bars indicate standard deviations. Different letters and asterisks indicate significant differences ($p < 0.05$) between cultivation periods and water flow velocities, respectively. Bars in photographs indicate 50 cm.

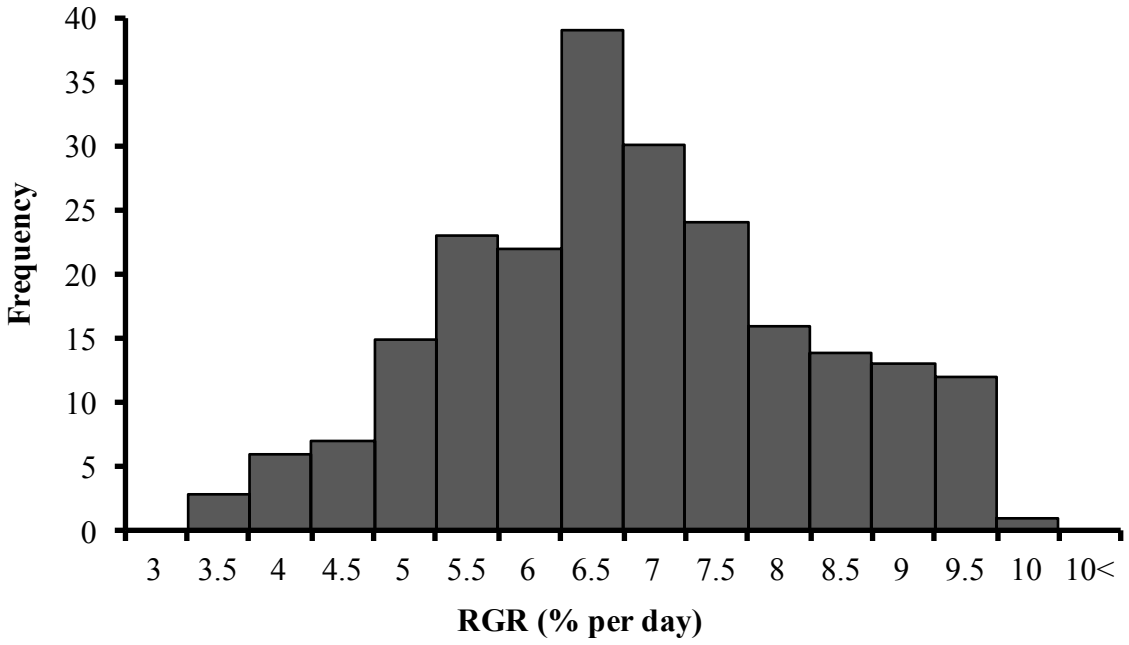


Fig.2-6. Variation in the relative growth rates of individual *Undaria pinnatifida* plants cultivated in the CFCS.

CHAPTER III

Phenotypic and genetic differentiation in the morphology and nutrient uptake kinetics among *Undaria pinnatifida* cultivated at six sites in Japan

SUMMARY

Undaria pinnatifida is grown for food and industrial materials worldwide; advanced breeding is needed to meet quality and productivity requirements, and to promote adaptation to site-specific environmental changes. In this study, I selected six cultivation sites in Japan with different environmental conditions: Hirota Bay (HRT, north-eastern Pacific coast), Matsushima Bay (MAT, north-eastern Pacific coast), Naruto (NAR, Seto Inland Sea coast), Akashi (AKA, Seto Inland Sea coast), Shimonoseki (SIM, southern Sea of Japan coast), and Oga (OGA, northern Sea of Japan coast). I first cultivated sporelings originating from the natural population at each site in seawater. Then, we cultivated the next generation in the CFCS (Chapter II) under controlled environmental conditions. I measured the morphological characteristics and nutrient uptake kinetics (V_{max}, K_s, and V_{max}/K_s) of the plants. Plants from MAT grew faster and those from SIM were smaller than those from other sites, characteristics that were observed in both cultivation stages. Although plants cultivated at HRT had double the blade thickness of those cultivated at OGA, there were no significant differences among plants from the various sites after cultivation in tanks. Nutrient uptake kinetics of NAR and AKA plants (V_{max} and K_s) and OGA plants (V_{max}/K_s) cultivated in seawater were greater than those of plants from the other

sites. This suggests that the NAR and AKA, and OGA plants had adapted to temporarily high nutrient loading and low-nutrient conditions, respectively. In tank cultivation, although the V_{max} and K_s values of NAR plants were similar to those of plants from the other sites, OGA plants had the greatest V_{max}/K_s values, similar to the result from seawater cultivation. Thus, the morphological characteristics of MAT and SIM plants, and nutrient uptake kinetics of OGA plants probably have a genetic basis, and may provide sources for mother plant selection. We can use, for example, MAT plants for faster growth and OGA plants for growth in low-nutrient conditions.

INTRODUTION

Most of the cultivated crop of *Undaria pinnatifida* (Harvey) Suringar (Laminariales; Phaeophyta) is produced in Japan, Korea, and China, in which it ranks among the major commercial species of seaweeds (Yamanaka and Akiyama 1993). Since the 1970s, *U. pinnatifida* has successfully invaded many coastal regions worldwide, including the south-western Atlantic (Casas and Piriz 1996; Dellatorre et al. 2014), north-eastern Atlantic (Castric-Fey et al. 1993), north-eastern Pacific (Silva et al. 2002), the Mediterranean Sea (Cecere et al. 2000), and the waters off Australia (Sanderson 1990) and New Zealand (Hay and Luckens 1987). This species has long been part of the cuisine of north-eastern Asia, and in recent years, it has begun to be consumed in Europe. Several cultivation trials have been performed off the Brittany coast of France (Perez et al. 1984) and in Spanish Galician waters (Pérez-Cirera et al. 1997).

Although the industrial demand for *U. pinnatifida* has been increasing globally, its annual production in Japan has been in decline, accompanied by a decreasing number of fishermen and an increasing proportion of seniors (MAFF 2013). The variable quality and raw material prices are regarded as major factors in the production decrease. For instance, the low nutrient level in seawater after February caused discolouration of *U. pinnatifida* in Tokushima Prefecture, southern Japan (Dan et al. 2015), which affected its quality and price. Furthermore, fishermen must work hard from early morning to midnight to check product quality for harvesting during the short cultivation period. Therefore, cultivars with higher productivity despite low nutrient levels or an extended cultivation period are required. On the other hand, the cultivation industry in China developed quickly, and the level of production is no longer a critical problem.

Cultivars with distinct characteristics, such as elevated contents of fucoidan or alginic acid, have been bred to meet market demands (Shan et al. 2015). Therefore, further breeding is needed to meet the requirements and the site-specific environmental characteristics of the locations in which the species is cultivated.

Transplantation experiments have identified genetically based geographical differentiation in the morphological characteristics and growth patterns of *U. pinnatifida* (Kato and Nakahisa 1962; Kito et al. 1981; Ishikawa 1994). The critical upper temperature for the growth of *U. pinnatifida* young sporophytes originating from Mugisaki (central Japan) is 27°C (Morita et al. 2003), whereas the critical upper limit for sporophytes from Matsushima Bay, northern Japan, is 25°C (Akiyama and Kurogi 1982). Gao et al. (2013a) transplanted *U. pinnatifida* from a warm location (Naruto) to a colder one (Matsushima), and compared the physiological characteristics between the populations at these sites. Sporophytes from Naruto had significantly higher photosynthetic activity at high temperatures and a larger N accumulation capacity than those from the northern population. They concluded that the different tolerance levels to high temperatures in juvenile sporophytes from geographically separated populations resulted from genetic differentiation of ecotypes rather than phenotypic plasticity. Ecotypes that are adapted to different ranges of environmental factors provide source material for selective breeding; however, research into whether the morphological and physiological characteristics of *U. pinnatifida* plants in different regions are due to genetic factors or environmental differentiation through the growth period is limited. Moreover, from the viewpoint of genetic resource protection at the different sites, to examine seedling transplantation from originating sites to other sites is generally discouraged.

The N content and growth of laminarians are positively correlated with the environmental concentrations of dissolved inorganic nitrogen (DIN) (Chapman and Craigie 1977). Torres et al. (2004) analysed the nutrient uptake kinetics of *U. pinnatifida* collected in Nueva Bay, central Argentina, which is close to the city of Puerto Madryn. Sporophytes benefited from the high nutrient concentrations found near the sewage outfall in Nueva Bay. Dean and Hurd (2007) examined N uptake kinetics, C and N contents, and the photosynthetic rates of *U. pinnatifida* collected in New Zealand. The measured ecophysiological parameters of this species more closely resembled those of small seaweeds, such as *Ulva*, than those of other members of the Laminariales. These studies have defined the nutrient uptake kinetics of *U. pinnatifida* in areas where it is considered an invasive species. Comparatively little is known about the nutrient uptake physiology characteristics of the *U. pinnatifida* populations cultivated in East Asia, where the species is native. *U. pinnatifida* is cultivated across Japan, except in north-eastern Hokkaido and Okinawa Prefecture (Saito 1972; MAFF 2013). The processing procedures vary among regions (Fujiwara 2012) because of differences in the morphological characteristics of each population. Hence, a comprehensive understanding of the morphological and physiological characteristics of *U. pinnatifida* sporophytes grown across a range of sites in Japan would provide a valuable knowledge base of the phenotypic differentiation and environmental adaptation in this species.

The goal of this chapter is to clarify the genetic and environmental differentiation of *U. pinnatifida* among plants from various regions and the kinetics of adaptation to environmental conditions for mother plant selection for selection breeding. Therefore, I initially examined the morphological characteristics and nutrient kinetics of *U. pinnatifida* fronds cultivated at six

major aquaculture sites (Fig. 3-1), to understand the differentiation of these characteristics of *U. pinnatifida* in Japan. Secondly, I obtained sporelings induced from the sporophytes cultivated at five regions where this was permitted, I cultivated these sporelings in the CFCS (Chapter II) under the same environmental conditions and compared their morphological characteristics and nutrient uptake kinetics.

MATERIALS AND METHODS

1. Cultivation in coastal waters in various regions in Japan and experiments

Study sites and environmental variables of mother plants

The six cultivation sites, which I selected due to their differences in environmental conditions, are major industrial production areas of *U. pinnatifida* in Japan: 1) Hirota Bay (HRT, 38° 98′ N, 141°71′ E), Iwate Prefecture; 2) Matsushima Bay (MAT, 38° 32′ N, 141° 31′ E), Miyagi Prefecture; 3) Akashi (AKA, 34° 64′ N, 134° 97′ E), Hyogo Prefecture; 4) Naruto (NAR, 34° 20′ N, 134° 63′ E), Tokushima Prefecture; 5) Haedomari, Shimonoseki (SIM, 33° 95′ N, 130° 88’ E), Yamaguchi Prefecture; and 6) the Unosaki coast, Oga (OGA, 39° 86’ N, 139° 80′), Akita Prefecture. A temperature logger (Stowaway TidbiT, Onset Computer, Bourne, MA, USA) was deployed near the surface (0.5 m deep) at each study site. Daily mean water temperature was calculated from measurements made at 2 h intervals. The daily data were averaged for the first, second, and third 10-day periods in each month. A surface seawater sample was collected in a 500 mL plastic bottle once or twice a month at each study site. The samples were filtered through 0.8 μm cellulose acetate filters (DISMIC, 25CS080AS, Advantec, Tokyo, Japan) into 15 mL acid-washed plastic vials, and frozen until analysis. The concentrations of NO_3^-, NH_4^+, and PO_4^{3-} in water samples were measured with an autoanalyser (QuAAtro 2-HR, BLTEC, Osaka, Japan).

Cultivation of **U. pinnatifida** *in coastal waters in various regions in Japan*

U. pinnatifida individuals bearing mature sporophylls were collected from wild populations growing along the coasts of the prefectures where the cultivation sites were located (Fig. 3-2a). Sporophylls were dried in the shade for several hours, and then immersed in

sterilised seawater; zoospores were released via this procedure. At NAR and SIM, the zoospores were settled on plastic fibre threads (2 mm diameter and 50 m long) coiled around a PVC pipe frame, which acted as an attachment substratum (Fig. 3-2b). The settled zoospores were cultured in a land-based tank for approximately 1 month, after which the visible gametophytes had grown and attached to the fibres (Fig. 3-2d). I used the free-living gametophyte methodology at HRT, MAT, AKA, and OGA to produce sporelings (Kito et al. 1981; Kaas and Perez 1989; Pang and Wu 1996). Released zoospores were transferred to plastic dishes containing sterilised seawater. Spores were germinated under a 12 h light/12 h dark photoperiod (20–40 μmol photons m^{-2} s^{-1} during the light phase) at a temperature of 20°C. After gametophytes had grown to a visible size (Fig. 3-2c), they were homogenised in an electronic blender and attached to plastic fibre threads, which were incubated in a land-based tank containing filtered seawater close to the cultivation sites (Fig. 3-2d).

After approximately 1–2 months, sporelings reached visible size at all of the sites. The plastic threads were transferred into the sea at each site and attached to hanging ropes at depths of 50–150 cm (Fig. 3-2e). Sporelings reached lengths of approximately 2 cm by the autumn/early winter of 2013 (October–December). At this point, the threads on which the sporelings were attached were cut into sections 3−5 cm long; 150 sections were threaded into the coils of a rope 50 m long and 15 mm in diameter at intervals of 30 cm. Each rope was secured at the surface with floats, and hung at a depth of 1 m to allow the sporophytes to grow (Fig. 3-2f). Sporophytes originating from one thread section were treated as one stock, and for each stock the average growth of 30 individuals was determined to provide measurements of morphological and physiological characteristics (Fig. 3-2g). Table 3-1 lists the starting dates of

cultivation, the start and completion dates of harvest, and the seawater temperatures for all of the cultivation sites.

Morphological measurements

One stock was randomly collected from each of the six cultivation sites and transferred to a laboratory in each month from December 2013 to April 2014. Sporophytes at OGA for morphological measurements were only collected in April due to a prohibition by the bad weather. Single sporophyte holdfasts were excised, and the longest 30 individuals within the stock were selected for measurements. The total length, blade thickness, sporophyte weight, and sporophyll weight excluding the stipe for each sporophyte were determined. Blade thickness was measured at the widest point in the intercalary region adjacent to the central stipe; the blade was folded into quarters (Ishikawa 1991) and the total thickness was measured with an electronic calliper (Mitsutoyo, NTD13, Kanagawa, Japan). Blade thickness was calculated by dividing the thickness of the folded portion by four.

Carbon and nitrogen

I selected the five longest (total length) sporophytes at each cultivation site for measurements of morphological characteristics. Using a 2.3 cm diameter cork borer, I punched discs (each 4.15 cm^2) from the mid portion of each divided blade at the point of maximum width. Discs were dried at 80°C for 48 h, ground, and weighed to determine dry weights. After weighing, I determined C and N contents using a CHN analyser (Flash2000 Eager Xperience Ver1.02, Thermo Fisher Scientific, Waltham, MA, USA).

Nutrient uptake kinetics

Four sporophytes were selected from the stocks collected in January and March 2014, and immediately transferred to the laboratory in a cool box containing seawater. The blade discs were punched from the mid sections of the blades at the widest point using a 2.3 cm diameter cork borer (4.15 cm^2). Discs from each of the cultivation sites were pre-incubated in 1.0 L flasks containing aerated sterilised seawater at a temperature of 10°C, and provided with 100 μmol photons $m^{-2}s^{-1}$ illumination and a 12 h light/12 h dark photoperiod for 48 h to allow recovery from the shock caused by cutting (Sakanishi and Iizumi 1988; Hurd and Dring 1990). The seawater was changed at 12 h intervals. The seawater used for pre-incubation was collected from the Sea of Japan by the Akita Prefectural Institute of Fisheries. The natural nutrient concentrations in seawater were close to the limits of detection.

Nutrient uptake was measured based on the difference between initial and end concentrations in the vessels (Hurd and Dring 1990; Li et al. 2007; Li et al. 2009). Each disc was transferred to a 200 mL flask containing 100 mL autoclaved seawater that had been filtered (GF/F filters, Whatman, Maidstone, UK). The controls were flasks containing seawater without kelp discs. NO_3^--N and NH_4^+-N were added to the flasks (as $NaNO_3$ and NH_4Cl, respectively) to provide six initial concentrations: 0.5, 2, 5, 10, 15, and 30 μmol L^{-1}. The flasks were incubated at 10°C and shaken at 80 rpm on a multi shaker (MMS-4010, Rikakikai Co., Ltd., Tokyo, Japan) under a photon irradiance of 180 μmol m^{-2} s^{-1}. White fluorescent lamps provided the light source. After a 60-min incubation, seawater samples were collected into plastic tubes and frozen until analysis; blade discs were dried in a convection oven (NDO-600ND, Eyela, Tokyo, Japan) at 80°C for 48 h, after which their dry weights were measured. The uptake rates (V; μmol g d.w.$^{-1}$ h^{-1}) of NO_3^--N and NH_4^+-N were calculated for each disc from the difference

in concentration between the control and experimental flask at each of the six nutrient levels. The nutrient concentrations were determined in an auto-analyser (QuAAtro 2-HR, BLTEC). The seawater used in these experiments was also collected from the Sea of Japan by the Akita Prefectural Institute of Fisheries.

NO_3^--N and NH_4^+-N uptake rates (V) were plotted against mean nutrient concentrations in the control flasks (S, μM) for the four discs from each of the six cultivation sites (Supplemental Fig. 3-1). I fitted a rectangular hyperbola to each plot for each of the cultivation sites using data collected in January and March. The curves were fitted with a least-squares regression procedure using JMP software (SAS Institute Inc., Cary, NC, USA). Parameters of nutrient uptake kinetics were calculated by the same method in Chapter I.

2. Cultivation in the CFCS and experiments

Cultivation of* U. pinnatifida *in the CFCS

Spores from five of the regions, excluding AKA due to a prohibition against dividing from regional mother plants of *U. pinnatifida*, were induced from sporophylls and incubated in a plastic dish containing 30 mL of PESI medium (Fig. 3-3a). After a colony of visible gametophytes was obtained after one month (Fig. 3-3b), male and female gametophytes were separated and incubated individually in the wells of a micro-dish. After one month, wells in which a gametophyte pair was growing were selected and the gametophytes were induced to become sporophytes by the same method described in Chapter II. All sporophytes were cultured in a 300-mL marine flask until their total length reached 20 mm (Fig. 3-3c), and then the sporophytes of the strains from each region were cultivated in a 7-L aquarium with running

seawater and aeration (Fig. 3-3d). After 2 weeks, the 30 longest individuals for each strain were selected and transferred to the CFCS in a volume of 30 L (Fig. 3-3e), and these were subsequently transferred to the CFCS in a volume of 500 L after 2 weeks (Fig. 3-3f), and finally in a volume of 2,000 L after 1 more weeks (Fig. 3-3g). The cultivation conditions of the CFCS were seawater at a temperature of 10°C, illumination of 200 μmol photons m^{-2} s^{-1}, natural photoperiods, DIN concentration of 2–5 μM, and a water velocity of 15 cm s^{-1}.

Morphological measurements

All plants cultivated in the CFCS were collected approximately once every 15 days, and their total lengths and sporophyte weights were measured. The plants were then returned to the CFCS and cultivated until the next measurement time. On the 133rd day after the selection of 30 individuals, the blade thickness was measured by the same method used for those cultivated in the sea.

Nutrient uptake

On the 50th day after the selection of 30 individuals, the five longest plants for each strain were collected and blade discs were excised using a cork borer. Nutrient uptake rates were measured and parameters were estimated using the same methods described for the plants cultivated in the sea.

Photosynthetic rates

On the 68th day after the selection, blade discs from the five longest plants of each regional strain were obtained by the same method used for determining the nutrient uptake rates. The discs were incubated with running seawater at a temperature of 10°C for 24 h in order for them to recover from the cutting injury (Sakanishi and Iizumi 1984). With a seawater temperature

range of 5–25°C and illumination of 200 μmol photons m^{-2} s^{-1}, the photosynthetic rate was measured as the rate of oxygen production using an oxygen electrode meter (Hansatech DW2, King's Lynn, UK). The measurement was started from the lowest temperature in the selected range, and the temperature in the chamber was regulated using a circulating pump.

Phylogenetic analysis

The gametophytes of the strains from each region were weigh approximately 100 mg, they were collected and dried. Total genomic DNA was extracted from approximately 10 mg of dried tissue using the MagExtractor Plant Genome (Toyobo Co., Ltd., Osaka, Japan), in accordance with the manufacturer's instructions. The purified DNA was used as a template for PCR to amplify two regions encoded in the mitochondrial genome: (i) between the *atp8* and *trnS* genes and (ii) between the *trnW* and *trnI* genes, hereafter called atp8-S and W-I, respectively. These were previously used for a phylogenetic study of *U. pinnatifida* in France (Voisin et al. 2005). For atp8-S, 239 base pairs were amplified using atp8-trnS-F (3' end of atp8, 5'-TGTACGTTTCATATTACCTTCTTTAGC-3') and atp8-trnS-R (5' end of trnS, 5'-TAGCAAACCAAGGCTTTCAAC-3') primers and, for W-I, 292 base pairs were amplified using trnW-trnI-F (3' end of trnW, 5'-GGGGTTCAAATCCCTCTCTT-3') and trnW-trnI-R (5' end of trnI, 5'-CCTACATTGTTAGCTTCATGAGAA-3') primers. PCR was carried out as follows: After an initial denaturation step (95°C, 5 min), touchdown PCR was carried out for five cycles of 10 s at 98°C, 15 s at 60°C, reduced by 1°C in each subsequent cycle, and 30 s at 72°C, followed by 30 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 30 s.

Sequencing was performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730xl DNA Analyzer (Applied Biosystems) with the same primers. Sequences were aligned using Genius ver. R9 software (http://www.geneious.com/) and an unrooted phylogenetic tree was established by the neighbour joining method, using TCS ver. 1.21 software (Clements et al. 2000).

Statistical analyses

Significant differences in the morphological characters among months and cultivation sites, the contents of C and N, and the C:N ratio among months were identified by the Kruskal-Wallis test followed by Scheffé's multiple comparison tests. I selected a nonparametric procedure because not all of the data were normally distributed or homoscedasticity. Significant differences in the nutrient uptake kinetic parameters (V_{max}, K_s and V_{max}/K_s) for NH_4^+-N and NO_3^--N among cultivation sites in January and March were identified by the Kruskal-Wallis test followed by Scheffé's multiple comparison tests. Again, not all of the data were normally distributed or homoscedasticity.

RESULTS

Environmental factors at the six cultivation sites

Seawater temperatures at all of the cultivation sites decreased after October/November, and reached minimum values in the period from February to April (Fig. 3-4a). Seawater temperatures at MAT sites were lower than at all other sites until the beginning of March, and decreased to a minimum of 3.6°C in the beginning of February. The temperature at HRT fell to 5.5°C in the beginning of April. At the other four locations, temperatures reached minimum values (7.1–10.6°C) in the period from mid-February to mid-March.

Seasonal trends in the concentrations of NH_4^+-N, NO_3^--N, and PO_4^{3-}-P differed among the six cultivation sites (Fig. 3-4b–d). The concentrations at MAT markedly increased after the beginning of October, and reached values of 15.2 μM NO_3^--N, 7.4 μM NH_4^+-N, and 1.03 μM PO_4^{3-}-P by the end of the month, which were the highest measured across all of the sites. Thereafter, the concentrations decreased, and remained below 3 μM NO_3^--N and NH_4^+-N and 0.1 μM PO_4^{3-}-P after January.

At HRT and NAR, the concentrations of NO_3^--N ranged between 3.6 and 7.8 μM until mid-March and between 3.3 and 8.3 μM until the beginning of March, respectively; thereafter, values fell steeply to <1 μM except for middle of April in NAR. The concentrations of NO_3^--N and PO_4^{3-}-P at both sites varied little among months. The concentrations of NO_3^--N at AKA were similar to those at HRT and NAR (2.1–3.6 μM until the end of January, after which they fell; values were <1 μM after March). The concentrations of NH_4^+-N and PO_4^{3-}-P peaked sharply in December and February, with maximum values of 7.4 μM and 0.5 μM, respectively. The concentrations at SIM varied little during the measurement period; the values of NO_3^--N,

NH_4^+-N, and PO_4^{3-}-P were in the ranges of 2.1–4.2 μM, 0.4–2.6 μM, and 0–0.1 μM, respectively. At OGA, nutrient concentrations were close to the limits of detection, except in December (NO_3^--N: 2.3 μM) and November (NH_4^+-N: 1.3 μM).

Morphological characteristics

1）Sporophytes cultivated in the sea

I found significant differences in total length, blade thickness, sporophyte weight, and sporophyll weight among months at all of the cultivation sites, other than OGA (Fig. 3-5a–d, $p < 0.01$). Total lengths significantly increased with time (Fig. 3-5a, $p < 0.01$). After reaching a maximum, values at MAT and SIM significantly decreased ($p < 0.01$). Total lengths did not significantly change with time at AKA and NAR after March ($p > 0.05$), but significantly increased at HRT until April ($p < 0.01$).

Across cultivation sites, there were significant differences in morphological characteristics in all months, with the exception of sporophyll weight in December (Fig. 3-5a–d, $p < 0.01$); sporophylls were formed only at MAT. The total length was significantly higher at MAT than at other locations from December to February ($p < 0.01$). With the exception of SIM, the maximum total lengths ranged between 168 and 225 cm. Sporophytes at SIM were 104.71 cm in length in February, and total lengths were significantly shorter than at other locations after March ($p < 0.01$; Fig. 3-5a). Blade thickness at HRT was significantly higher than at the other sites in January and February ($p < 0.05$), reaching a maximum value of 0.35 mm in April. The blade thicknesses at NAR in March and at OGA in April were both 0.19 mm, significantly lower than at HRT, MAT, and SIM (Fig. 3-5b; $p < 0.01$). Sporophyte weight was significantly

higher at MAT than at other sites in each month ($p < 0.01$), with the exception of December and March. The maximum weight per individual at MAT reached 668.32 g in April (Fig. 3-5c). Sporophyll weights were significantly higher at MAT than at other sites in February and April ($p < 0.05$). The maximum weight at MAT (265.34 g) was reached in April (Fig. 3-5d).

2）Measurement of sporophytes cultivated in the CFCS

The total length and sporophyte weight of the MAT strain were found to be greater than those of the other strains until the 89th and 111th days, respectively (Fig. 3-6 a,b). After the 89th day, the total length of MAT decreased with erosion from the apical part, but the sporophyte weight did not change significantly until the end of the cultivation (Fig. 3-6a). On the other hand, sporophytes of the HRT, OGA, and NAR strains continued to grow after those of MAT began to exhibit shortening (Fig. 3-6a,b). The SIM strain was significantly smaller than the other strains after the 89th day (Fig. 3-6a,b). Finally, blade thickness did not differ significantly among these five strains (Fig. 3-6c).

Carbon and nitrogen contents of sporophytes cultivated at the six sites in Japan

Among months, the C contents of sporophytes did not significantly vary at any of the cultivation sites, as determined by multiple comparison test, other than at NAR ($p > 0.05$, Fig. 3-7a,b). N contents decreased significantly after February or March, as determined by multiple comparison test ($p < 0.05$, Fig. 3-7b,c). C:N ratios at MAT and NAR were significantly increased after February and March, respectively, reaching a maximum value of 21.2 in February at MAT. Among the cultivation sites, C and N contents and C:N ratio varied significantly, with the exception of C in March and C:N ratio in January ($p < 0.05$). By multiple

comparison test, C content showed no clear tendency according to month. Since the N content at MAT was significantly lower than at other sites in February, the C:N ratio was significantly higher at MAT than at other sites in February, and at HRT, AKA, and SIM after March ($p < 0.05$).

Nutrient uptake kinetics

1）Measurement of sporophytes cultivated in the sea at each site

The uptake rates of NH_4^+-N and NO_3^--N in January and March were correlated with the nutrient concentrations in the media for specimens collected from all of the cultivation sites, other than HRT and SIM, these correlation curves were fitted to a rectangular hyperbola (Supplemental Fig. 3–1). No significant correlations were detected between NH_4^+-N uptake rates and media concentrations for specimens collected from these two sites in March (Supplemental Fig. 3–1). The parameters of uptake kinetics for NO_3^--N and NH_4^+-N for specimens collected from the six sites in January and March are depicted in Fig. 3-8. The parameters significantly differed among sites ($p < 0.05$). V_{max} and K_s values for NO_3^--N uptake in specimens collected at AKA in January were 254.94 μmol g d.w.$^{-1}$ h^{-1} and 149.25 μmol L^{-1}, respectively, the highest values among the sites. These values were significantly higher than those at other sites, with the exception of NAR ($p < 0.01$). The V_{max} value for AKA specimens in March (34.09 μmol g dw^{-1} h^{-1}) significantly exceeded that of OGA ($p < 0.01$, Fig. 3-8a). There were no significant differences in K_s values for NO_3^--N among sites ($p > 0.05$; Scheffé's multiple comparison test, Fig. 3-8b). The V_{max}/K_s ratios for NO_3^--N of specimens collected at OGA in January and March were 6.06 and 2.39, respectively, the highest among cultivation

sites (Fig. 3-8c). At the other sites, the ratios ranged between 1.41 and 2.47 in January and between 1.04 and 1.50 in March, and did not significantly differ among these five locations ($p > 0.05$).

Similar to the NO_3^--N parameters in January, the V_{max} (349.83 μmol g d.w.$^{-1}$ h^{-1}) and K_s (172.34 μmol L^{-1}) values for NH_4^+-N were significantly higher in AKA specimens than in specimens from MAT and OGA ($p < 0.05$; Fig. 3-8d,e). The V_{max} values for NH_4^+-N in March were 114 μmol g d.w.$^{-1}$ h^{-1} for SIM specimens, and were 0.3 μmol g d.w.$^{-1}$ h^{-1} for OGA specimens ($p < 0.05$, Fig. 3-8d). K_s values in March were not significantly different among cultivation sites, as determined by multiple comparison test ($p > 0.05$, Fig. 3-8e). The V_{max}/K_s ratios for NH_4^+-N were not significantly different among sites in January, as determined by multiple comparison test ($p > 0.05$, Fig. 3-8e), but in March the ratios at MAT and AKA (both 2.7) were significantly higher than at HRT and OGA ($p < 0.05$; Fig. 3-8f).

2) Comparison of cultivation in the sea and in the CFCS

NO_3-N and NH_4-N uptake rates at various concentrations of all five strains were fitted to the Michaelis-Menten equation; the V_{max}, K_s, and V_{max}/K_s values for these were calculated and are shown in Fig. 3-9. For NO_3-N, the V_{max} and K_s values of NAR were found to be 6.7 μmol g^{-1} h^{-1} and 10.2 μM, respectively (Fig. 3-9a,b); the values were markedly decreased in comparison with the results in January at the sea (Fig. 3-8a,b). V_{max} and K_s of SIM showed peaks of 67.7 μmol g^{-1} h^{-1} and 152.3 μM, respectively, within strains (Fig. 3-9a,b). V_{max}/K_s of OGA was the highest among the strains, with a value of 6.3; the next highest was 3.5 for MAT, while the others were in the range of 0.6–0.8. For NH_4-N, the V_{max} value of NAR was 57.7 μmol g^{-1} h^{-1} (Fig. 3-9d), which was markedly lower the result for cultivation in the sea (Fig. 3-

8d), similar to NO_3-N. The V_{max} value for SIM was the highest among the strains, at 212.2 μmol g^{-1} h^{-1}. The K_s value was the lowest for MAT, at 7.2 μM; the values of the other strains ranged from 75 to 131 μM (Fig. 3-9e). The V_{max}/K_s value of MAT was the highest among the strains; the values of the others ranged from 0.5 to 0.7 (Fig. 3-9f).

Phylogenetic analysis

An unrooted phylogenetic tree was constructed using the Atp8-trnS and trnW-trnI sequences of five strains from different regions (Fig. 3-10). The HRT and OGA strains belonged to the same branch, but NAR, SIM, and MAT were positioned separately. The distances from HRT and OGA to SIM and MAT were the same, and the distances from HRT and OGA to NAR were greater.

DISCUSSION

Environmental conditions at the six cultivation sites

At HRT, the seawater temperatures fell as nutrient concentrations increased (Fig. 3-4). This seasonal relationship is typical of temperate regions in which *U. pinnatifida* grows (Yoshikawa et al. 2001, Dean and Hurd 2007, Nanba et al. 2011). Our measurements of rapid declines in seawater temperature at MAT in the autumn to winter period coincided with high values for nutrient concentrations (Fig. 3-4). Li et al. (2007, 2009) and Sato and Agatsuma (2015) reported a similar pattern for MAT, which was considered typical of the inner bay. The mineralisation of rotting algae and intrusions of nutrient-rich deep seawaters were responsible for the high nutrient concentrations (Li et al. 2007). AKA and NAR are located in the Seto Inland Sea, where the frequency and scale of red tides dramatically increased during a period of serious eutrophication in the 1960s and 1970s; the highest frequency (per year) of red tides occurred in 1976 (299 per year) (Imai et al. 2006). After the 1970s, DIN levels decreased, and the current values are approximately 40% of those measured 40 years ago (Tanda et al. 2014). Most of the mineral nutrients in the Seto Inland Sea are supplied from the Pacific Ocean (Ishii and Yanagi 2006), but near the coast of AKA, which faces the eastern side of the Seto Inland Sea (Osaka Bay), it is thought that nutrients are supplied by onshore/offshore movements of the Kuroshio Current, influxes of river water, and upward nutrient fluxes from coastal sediments (Nakayama 2011). Thus, short-lived peaks in NH_4^+-N at AKA and NO_3^--N at NAR (Fig. 3-4b,c) were probably originated from land. The SIM and OGA sites faced the Sea of Japan and were influenced by the Tsugaru Warm Current; the nutrient values at OGA were close to the limits of detection (Fig. 3-4).

Changes in growth and maturation

1) Cultivation in the sea

For the sea cultivation in each region, seasonal changes in the total length of *U. pinnatifida* at all of the sites tracked changes in seawater temperature. Blade thickness and sporophyte weight significantly increased through April, and sporophylls appeared after January, but seasonal changes in these morphological characteristics were unrelated to changes in seawater temperature and nutrient concentrations (Fig. 3-4,5). Sporophytes at MAT were significantly larger than those at other sites from December to February, and the sporophyll weights were 5- to 6-fold higher than those at other locations (Fig. 3-5). NO_3^--N fertilisation of *U. pinnatifida* sporelings and juvenile sporophytes promoted the earlier growth of sporophytes and sporophylls (compared to controls) (Gao et al. 2012). At MAT, the combination of low seawater temperatures and high nutrient concentrations in the period from autumn to winter promoted the growth of sporophytes and sporophylls, and advanced the harvesting period compared to other sites (Fig. 3-4,5, Table 3-1).

I found two-fold differences in blade thickness between HRT and OGA (Fig. 3-5d). Blade thickness is relevant to the nutrient physiology of individual sporophytes. In New Zealand, late-recruiting wild sporophytes of *U. pinatifida* developed thin blade, thereby increasing their surface area/volume ratios and N uptake rates per unit dry matter, and decreasing N requirements (Dean and Hurd 2007). Therefore, it is possible that these results indicate the preferential uptake of nutrients by *U. pinnatifida* in various regions on the basis of wide morphological plasticity.

2）Comparison of cultivation in the sea and in the CFCS

Based on comparison of the common morphological characteristics of plants cultivated in the sea and in the CFCS, the five strains from the different regions were divided into three groups (Fig. 3-5,6): 1) MAT, which grew and eroded faster than the other strains; 2) SIM, which had smaller blades than the other strains; and 3) HRT, NAR, and OGA, which continued to grow after the erosion of MAT. These groups reflected the photographic findings for each plant (Fig. 3-11) and were supported by the unrooted phylogenetic tree produced using mitochondrial DNA sequences (Fig. 3-10). These results suggest that these morphological characteristics used to categorize the plants into three groups are due to genetic differentiation. In contrast, although the blade thickness of plants cultivated in the sea were significantly different among the regions, there were no significant differences among the strains cultivated in the CFCS (Fig. 3-5,6). These results show that the differences in blade thickness were due to environmental differentiation, depending on the environmental factors in each region.

With regard to the consumption of *U. pinnatifida*, the thickness and texture of blades are important features, so blade thickness is one of the most important morphological characteristics for classifying its quality. This indicates the importance of the above results showing that the difference in quality in terms of the blade thickness depends on the regional environment.

Changes in C and N contents

The N content of sporophytes decreased after February at MAT, AKA, and NAR concurrently with seasonal changes in seawater NO_3^--N concentrations (Fig. 3-7). This

synchronised pattern is related to the size of the N storage pool in this species, which is smaller than that of other laminarians (Dean and Hurd 2007). The blade N content at MAT was significantly lower than that at other sites. The sporophytes cultivated at MAT may have preferentially allocated N content to their sporophylls, thereby promoting the early growth of these reproductive organs (Skriptsova et al. 2004). Other than in the month of February at MAT, the C:N ratio was <20 (Fig. 3-7), which is at the borderline of N limitation for growth in *U. pinnatifida* (Dean and Hurd, 2007). Furthermore, mature sporophylls were formed at all of the study sites, and I think that sporophytes were not N limited and had adapted to nutrient conditions at each of the locations.

Nutrient uptake kinetics

1）Cultivation at the six sites

There were significant differences in the nutrient uptake kinetics of *U. pinnatifida* sporophytes among the sites (Fig. 3-8). V_{max} and K_s values were the highest in January at AKA. The measured V_{max} values were higher than those reported in previous studies (Dean and Hurd 2007; Torres et al. 2004), suggesting that Japanese sporophytes are extremely effective competitors under naturally high nutrient concentrations and in eutrophicated/sewage-enriched environments (Campbell et al. 1999; Torres et al. 2004). I found that *Undaria* sporophytes cultivated at AKA in January, which is located in the Seto Inland Sea, had higher V_{max} and K_s values than sporophytes cultivated at the other sites (Fig.3–8). At NAR, there were no significant differences in these parameters compared to AKA (Fig.3–8). These locations had periodically high levels of nutrients (Fig. 3–4), and I propose that the sporophytes at AKA and

NAR had acclimated to such conditions. The high DIN concentration periods in the Seto Inland Sea from the 1960s through the 1970s (Imai et al. 2006) and in 1990 (Fujiwara et al. 2006) probably acted as selection pressures for an elevated nutrient affinity when concentrations are high. Among the sporophytes from HRT, MAT, SIM, and OGA, the values for these two parameters were lowest in OGA sporophytes, whose V_{max}/K_s ratios for NO_3^--N were significantly higher in January and March compared to sporophytes from other sites. A high V_{max}/K_s ratio is indicative of an enhanced affinity for nutrients at low concentrations (Harrison and Hurd 2001), and therefore this parameter is used to measure nutrient uptake efficiency (Perini et al. 2014). The coast at OGA is under the influence of the Tsushima Warm Current, which has low nutrient concentrations to which the sporophytes at OGA appear to have adapted.

The ranges of the uptake parameters that I measured were as follows: V_{max} for NO_3^--N, 0.1–254; V_{max} for NH_4^+-N, 0.1–349.8; K_s for NO_3^--N, 0.1–149; K_s for NH_4^+-N, 7.8–182; V_{max}/K_s for NO_3^--N, 1.13–6.06; and V_{max}/K_s for NH_4^+-N, 0.3–3.08 (Fig. 3-8). These ranges are broader than those reported in previous studies at sites where *U. pinnatifida* is an invasive species (Campbell et al. 1999; Torres et al. 2004; Dean and Hurd 2007). Gao et al. (2013a) showed that juvenile sporophytes from Naruto had a greater capacity to accumulate N reserves at high levels than those from colder locations; the authors definitively demonstrated the existence of temperature ecotypes in *U. pinnatifida*, which were genetically stable. I showed that ecotypes with different nutrient uptake kinetics may also exist, and are probably adaptive to diverse environments with different concentrations of nutrients. Dean and Hurd (2007) reported that the ecophysiological parameters of *U. pinnatifida* growing in New Zealand closely resembled those of small, ephemeral seaweeds, which may partially account for the invasive

success of this seaweed. I encountered a wide range of nutrient uptake capabilities in the populations I studied, which may also contribute to successful adaptation of the species to a diversity of environmental conditions.

U. pinnatifida sporophytes growing in Golfo Nuevo, Argentina, preferentially take up NH_4^+ as a source of DIN (Torre et al. 2004), whereas sporophytes in New Zealand show no preference among ions (Dean and Hurd 2007). In one study, when *Saccharina latissima* (L.) sporophytes were provided NO_3^--N and NH_4^+-N at the same concentration, the two ions were taken up simultaneously and at similar rates (Ahn et al. 1998). In other studies, *L. abyssalis* (Braga and Yoneshigue-Valentin 1996), *S. japonica* (Li et al. 2007), and *S. ochotensis* (Sato and Agatsuma 2015) preferentially took up NH_4^+-N. I found similar NO_3^--N and NH_4^+-N uptake parameters in sporophytes from AKA and NAR but not in sporophytes collected from OGA. OGA sporophytes had V_{max} and K_s values for NO_3^--N that were lower than those for NH_4^+-N; furthermore, the V_{max}/K_s ratio for NO_3^--N was higher than that for NH_4^+-N. The NH_4^+-N concentration in the seawater at OGA was below detection limits. Therefore, these results indicate that the preferential uptake of nutrients may vary among *U. pinnatifida* populations.

2) Comparison of cultivation in the sea and in the CFCS

The V_{max}/K_s values were high at OGA and MAT with cultivation in the sea and in the CFCS (Fig. 3-9). This shows that the particular H-type nutrient uptake kinetics at OGA and MAT are due to genetic differentiation. On the other hand, although the V_{max} and K_s values at NAR were higher than at the other sites of sea cultivation, these values decreased in the CFCS cultivation. This shows that the L-type nutrient uptake kinetics at NAR were due to environmental differentiation.

Adaptation and acclimation of U. pinnatifida *to environmental conditions*

The information on genetic and environmental differentiation in morphology and nutrient uptake kinetics obtained in this study can increases our understanding of the adaptation and acclimation of *U. pinnatifida* to environmental conditions around Japan. For instance, on the Oga Coast in Akita Prefecture facing the Sea of Japan, where there is a low nutrient concentration (Fig. 3-4), the OGA strain exhibits nutrient uptake kinetics of the H-type and has adapted to a low nutrient concentration via genetic differentiation (Fig. 3-8,9). Furthermore, the thin blades of OGA contribute to its increased nutrient uptake efficiency as a form of environmental differentiation by increasing the ratio of blade surface area to volume (Harrison et al. 2001). The MAT mother plant was collected near Matsushima Bay, where the Oyashio current (cold and high in nutrients) and the Kuroshio current (warm and low in nutrients) mix. The environmental conditions in the above-mentioned area are unstable due to variability of the Oyashio current (Nishida 1999). I propose that MAT has achieved earlier growth and maturation and the H-type nutrient uptake kinetics via genetic differentiation in order to adapt to the unstable temperature and nutrient conditions.

On the other hand, the L-type nutrient uptake kinetics at NAR differed between the plants cultivated in the sea and cultivated in the CFCS (Fig. 3-8). I propose that this is due to environmental differentiation, involving useful acclimation to temporary increases in the nutrients in the environment. The ability to acclimate to environmental changes is adaptive to unstable conditions in general (Terashima 2013). It is thus suggested that a broad range of nutrient uptake kinetics has contributed to the wide spread of *U. pinnatifida* in the last 30 years.

Implications for breeding programmes

Understanding genetic differentiation is necessary for improving breeding (Kuckuck et al. 1998). Based on the genetic differentiation identified in this study, the earlier growth and maturation of MAT and the continued growth of HRT after erosion in MAT could be used for the development of crops suited for earlier and later growth in the season. Moreover, the H-type nutrient uptake kinetics of OGA and MAT could be used for the development of a strain with tolerance to low nutrient concentration. In recent years in Japan, the price and quality of *U. pinnatifida* have decreased after early spring in association with its discolouration, due to a decrease in nutrients in seawater (Dan et al. 2015). The genetic differentiation identified in this study could be applied to overcome this problem by breeding.

Recent attempts have been made to reduce the pollution from Norwegian and Chilean fish farms using the particulate and dissolved nutrient uptake capabilities of bivalves and seaweeds to clean up the waste (Buschmann et al. 2001; Troell et al. 2003). Skriptsova and Miroshnikova (2011) tested the nutrient uptake capabilities of *U. pinnatifida* and *Gracilaria vermiculophylla* (Ohmi) Papenfuss in a biofiltration procedure. They proposed alternating the use of *U. pinnatifida* and *G. vermiculophylla* during cold- and warm-water seasons, respectively. I found that the nutrient uptake kinetics of *U. pinnatifida* varied regionally. Sporophytes with elevated V_{max} and K_s parameters and high V_{max}/K_s ratios may provide a useful source of genetic material for developing cultivars with a high biofiltration capacity and for growth in oligotrophic waters, respectively.

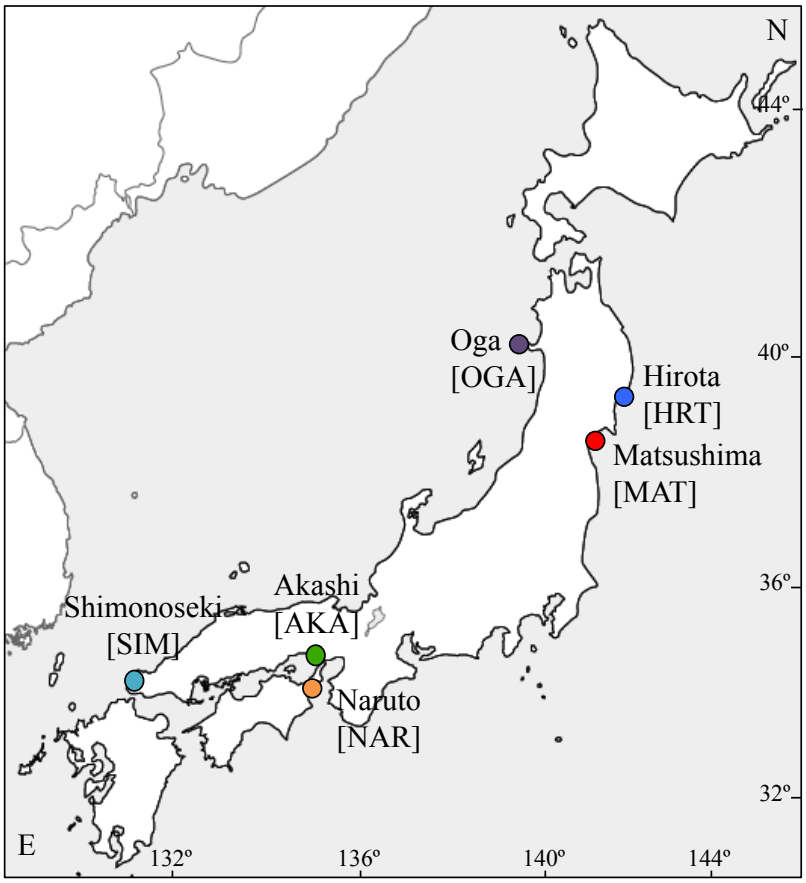


Fig. 3-1. Map of Japan showing the six aquaculture sites where *Undaria pinnatifida* was collected.

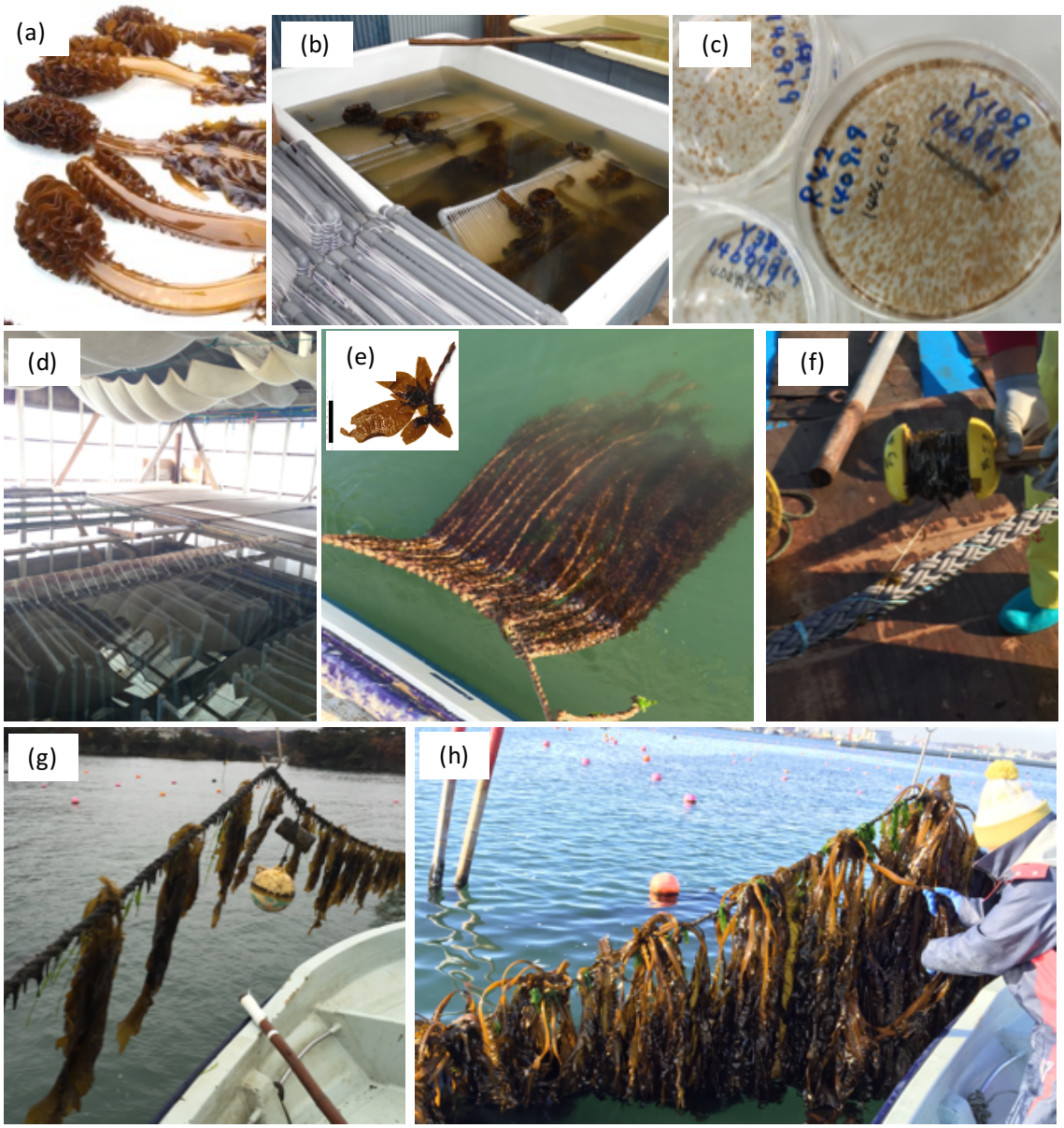


Fig. 3-2. The cultivation method for *Undaria pinnatifida* in the sea: (a) mature sporophylls collected from wild population, (b) zoospores released from sporophylls and attachment substrates of PVC pipe with plastic fiber frame, (c) gametophytes germinated in plastic dishes, (d) the substrates settled zoospores or gametophytes were cultured in a land-based tank, (e) the plastic threads attached to hanging ropes, bar is 2cm, (f) the seedlings was coiled to mother rope, (g) the sporophytes cultivated after 2 months, (h) the sporophytes cultivated after 4 months.

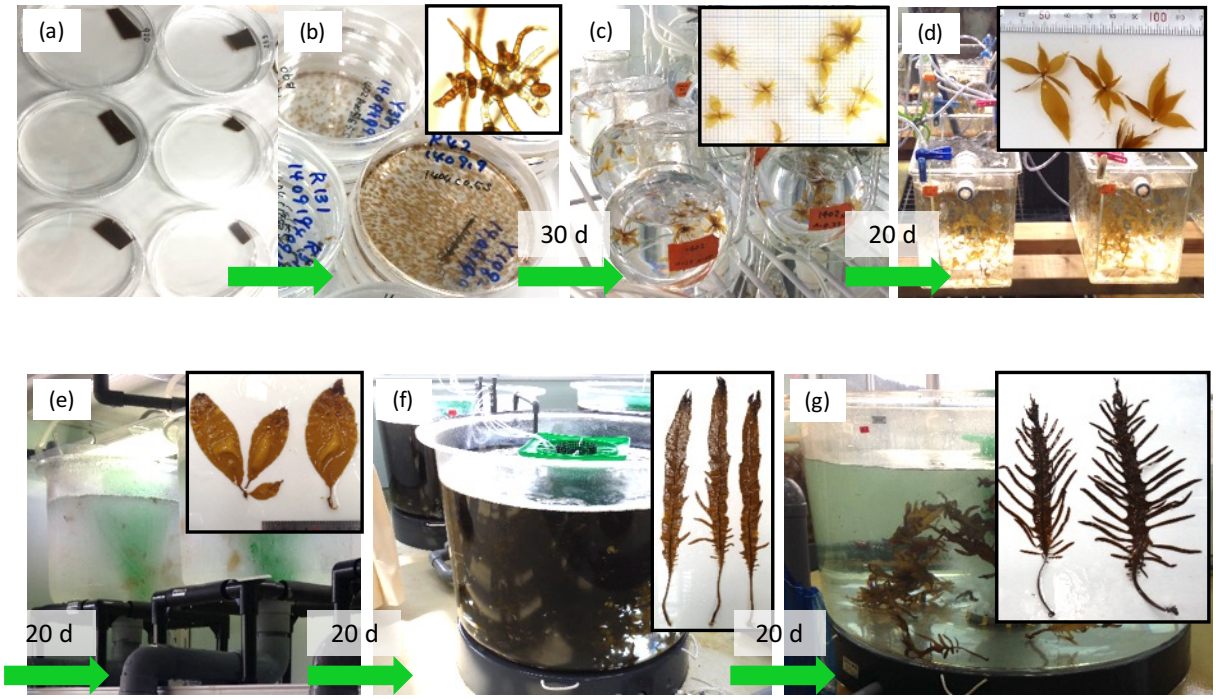


Fig. 3-3. The culture method for *Undaria pinnatifida* using the CFCS: (a) inducing spores from sporophylls of mother plants, (b) culture of gametophytes, (c) culture of sporophytes germinated from gametophytes in a marine flask (total length, 1–5 mm), (d) culture in the 7-L aquarium (total length, 5 mm–3 cm), (e) culture in the 30-L CFCS (total length, 3–10 cm), (f) culture in the 500-L CFCS (total length, 10–100 cm), (g) culture in the 2,000-L CFCS (total length, 1–3 m, until sporophyll formation).

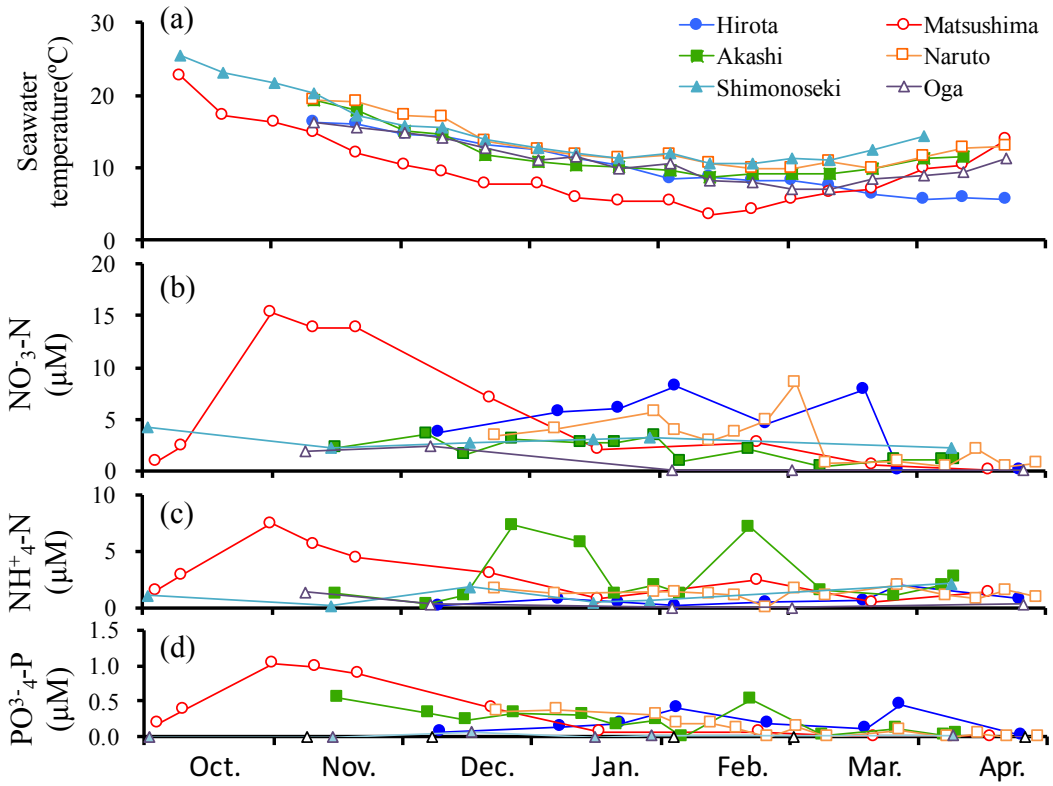


Fig. 3-4. Seasonal fluctuations in seawater temperature (a) and concentrations of NO_3^--N (b), NH_4^+-N (c), and PO_4^{3-}-P (d) at six sites where *Undaria pinnatifida* was cultivated.

Fig.3

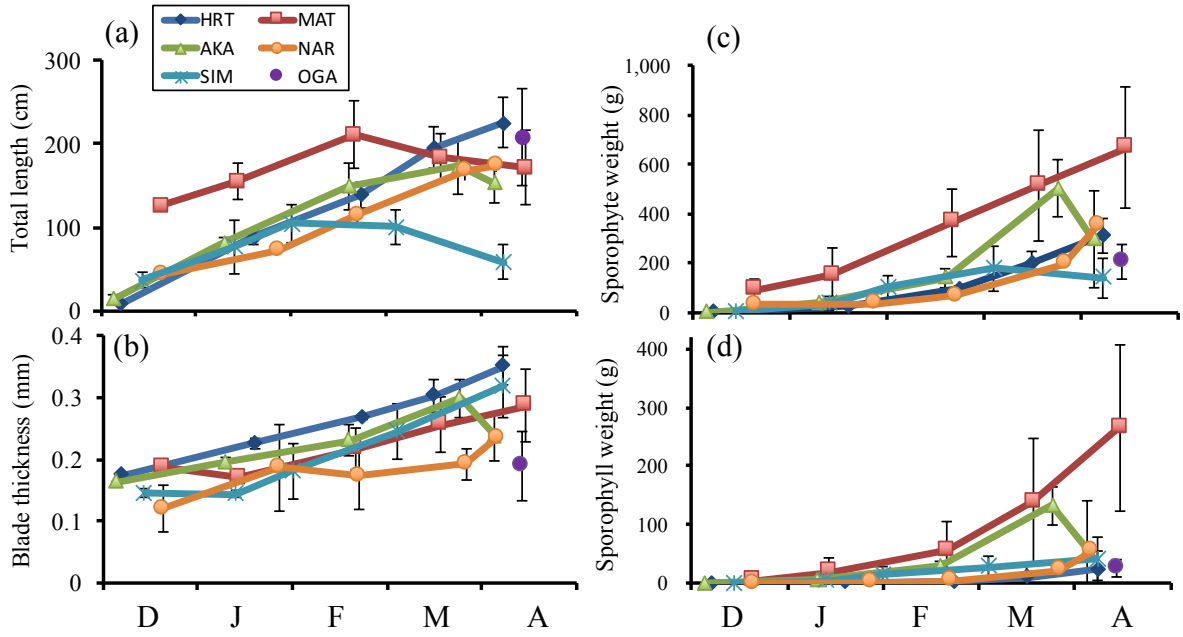


Fig. 3-5. Changes in total length (a), blade thickness (b), sporophyte weight, (c) and sporophyll weight (d) of *Undaria pinnatifida* cultivated at six sites in Japan from November 2012 to April 2013. See Table 1 for an explanation of site codes. Values are means ± SDs.

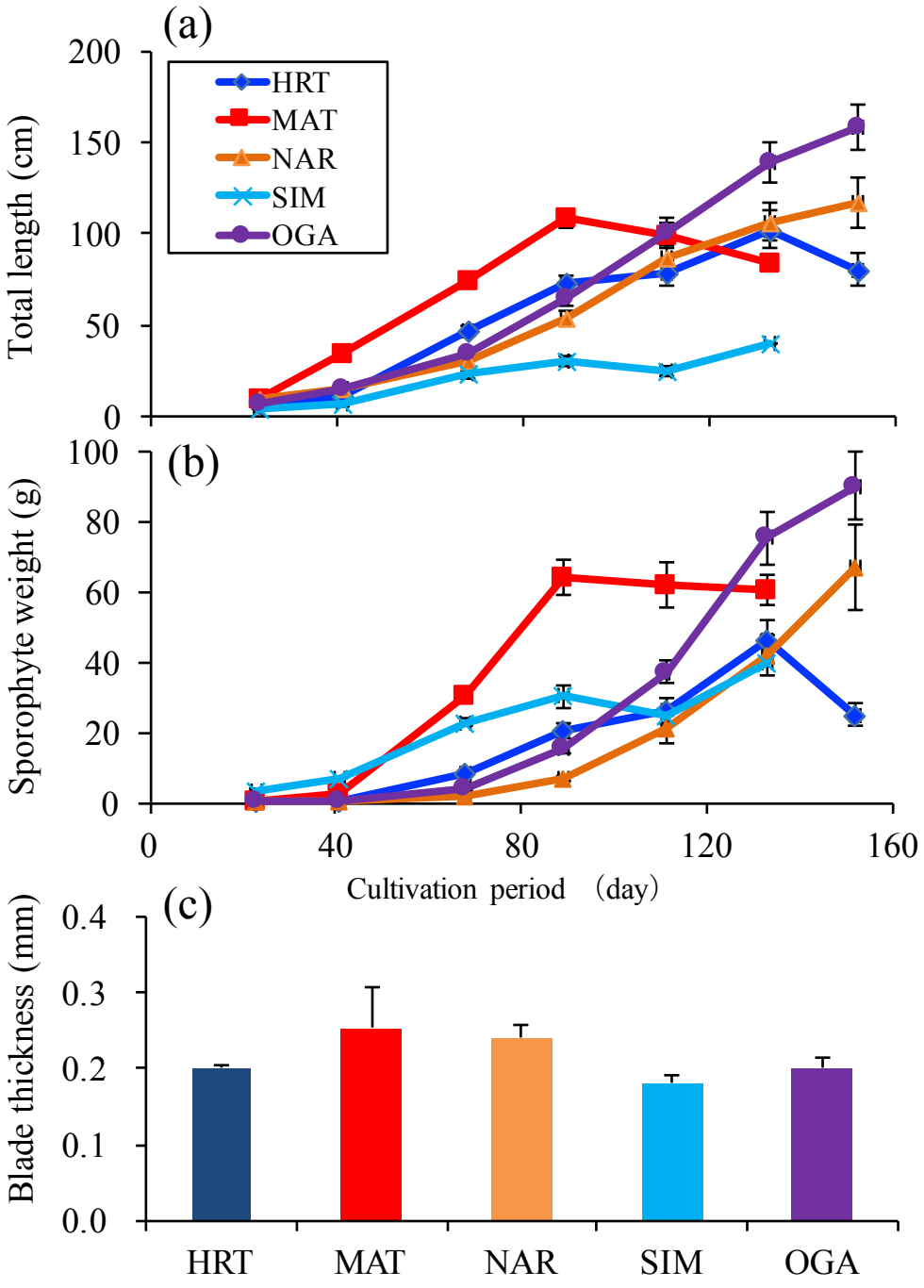


Fig. 3-6. Changes in total length (a), sporophyte weight (b), and blade thickness of *Undaria pinnatifida* cultivated in the CFCS. See Table 1 for an explanation of the site codes. Values are means ± SDs.

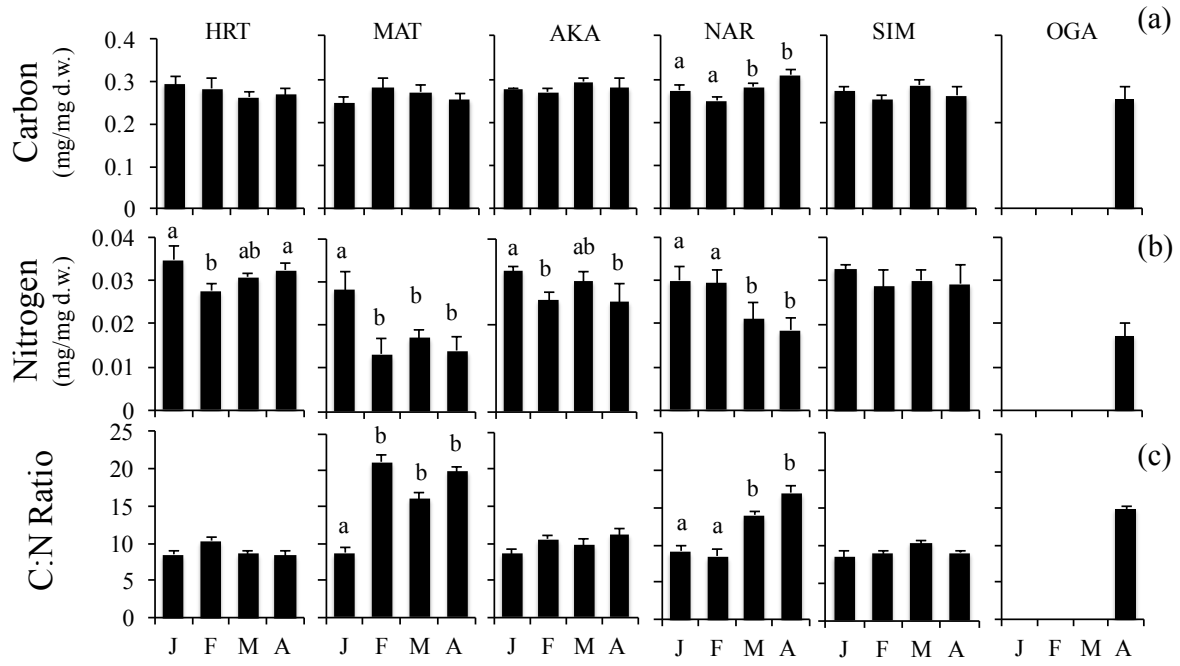


Fig. 3-7. Changes in carbon (C) content (a), nitrogen (N) content (b), and C:N ratio (c) of *Undaria pinnatifida* sporophytes cultivated at six Japanese sites from January to April 2013. Values are means + 95% confidence intervals. Different lowercase letters indicate significant differences ($p < 0.05$) among months. See Table 1 for an explanation of site codes. d.w., dry weight.

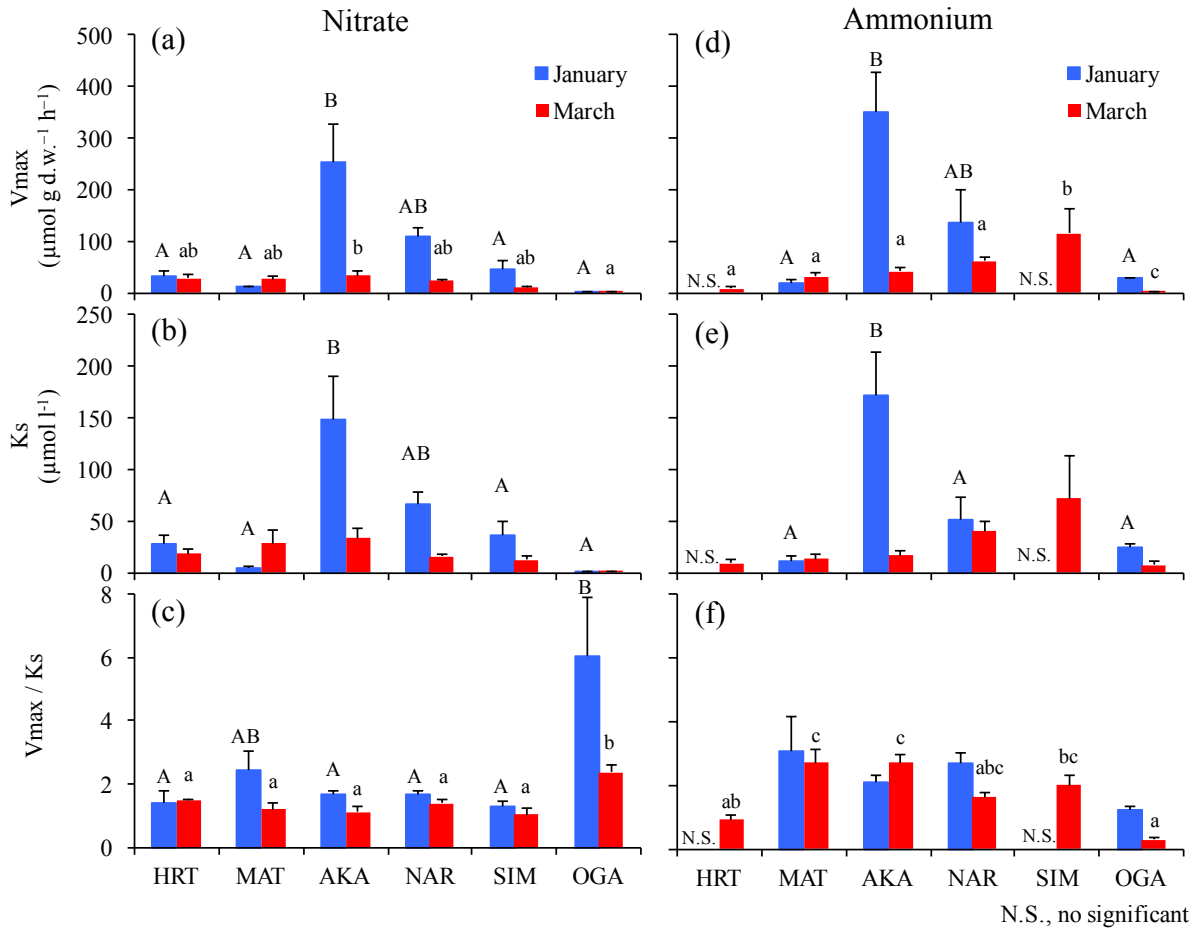


Fig. 3-8. *Undaria pinnatifida*. Kinetic parameters for nitrate and ammonium uptake in January and March. V_{max}, maximum uptake rate; K_s, half-saturation concentration; V_{max}/K_s, nutrient uptake efficiency. Values are means + 95% confidence intervals. Different upper and lower case letters indicate significant differences ($p < 0.05$) among cultivation sites in January and March. See Table 1 for an explanation of site codes. d.w., dry weight.

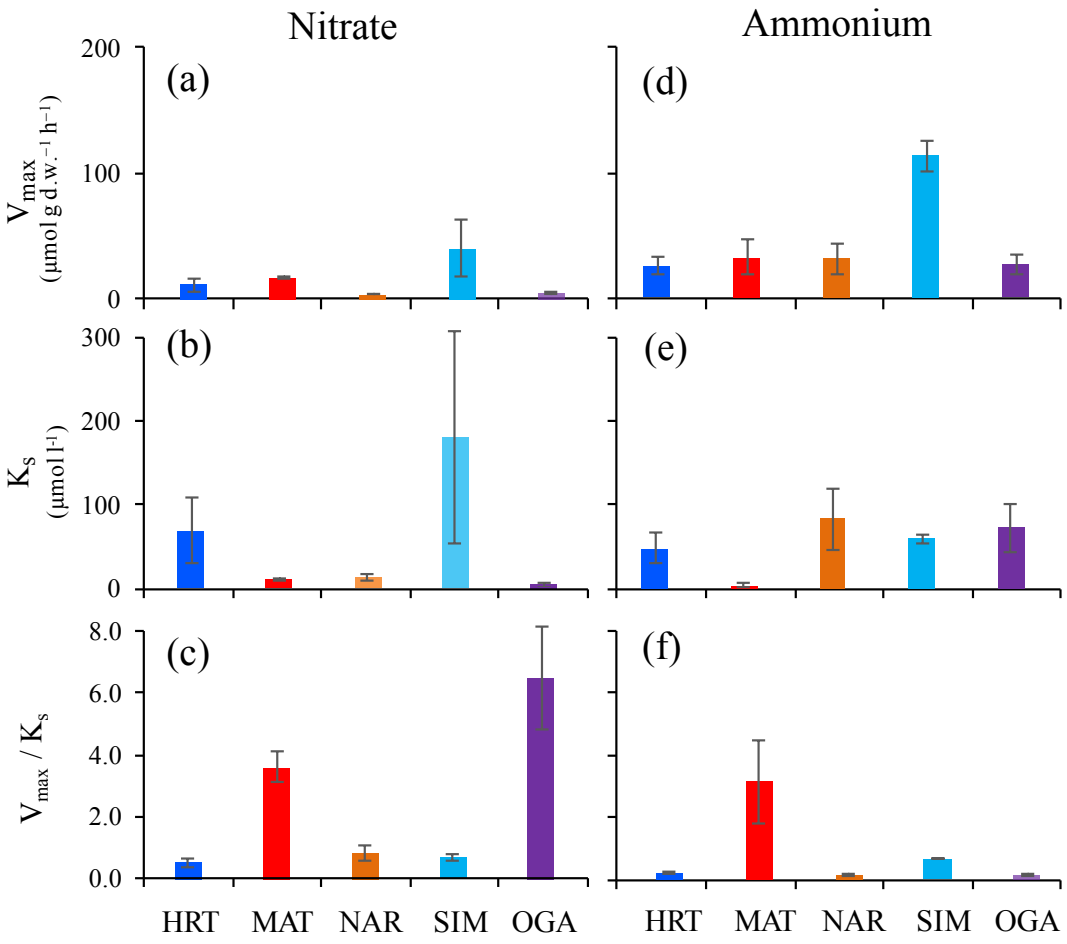


Fig. 3-9. *Undaria pinnatifida* cultivated in the CFCS. Kinetic parameters for nitrate and ammonium uptake on the 50th day after starting cultivation in the CFCS. V_{max}, maximum uptake rate; K_s, half-saturation concentration; V_{max}/K_s, nutrient uptake efficiency. Values are means ± 95% confidence intervals. Different upper- and lower-case letters indicate significant differences ($p < 0.05$) among strains from the various regions. See Table 1 for an explanation of the site codes. d.w., dry weight.

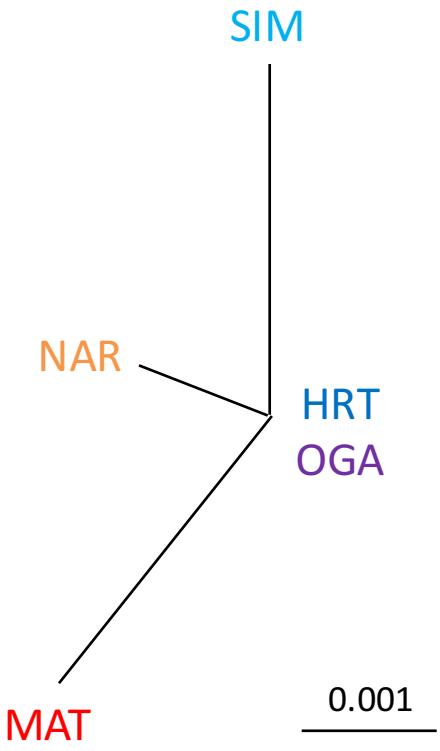


Fig. 3-10. *Undaria pinnatifida*. Unrooted phylogenetic tree for five of the strains from various regions. See Table 1 for an explanation of the site codes.

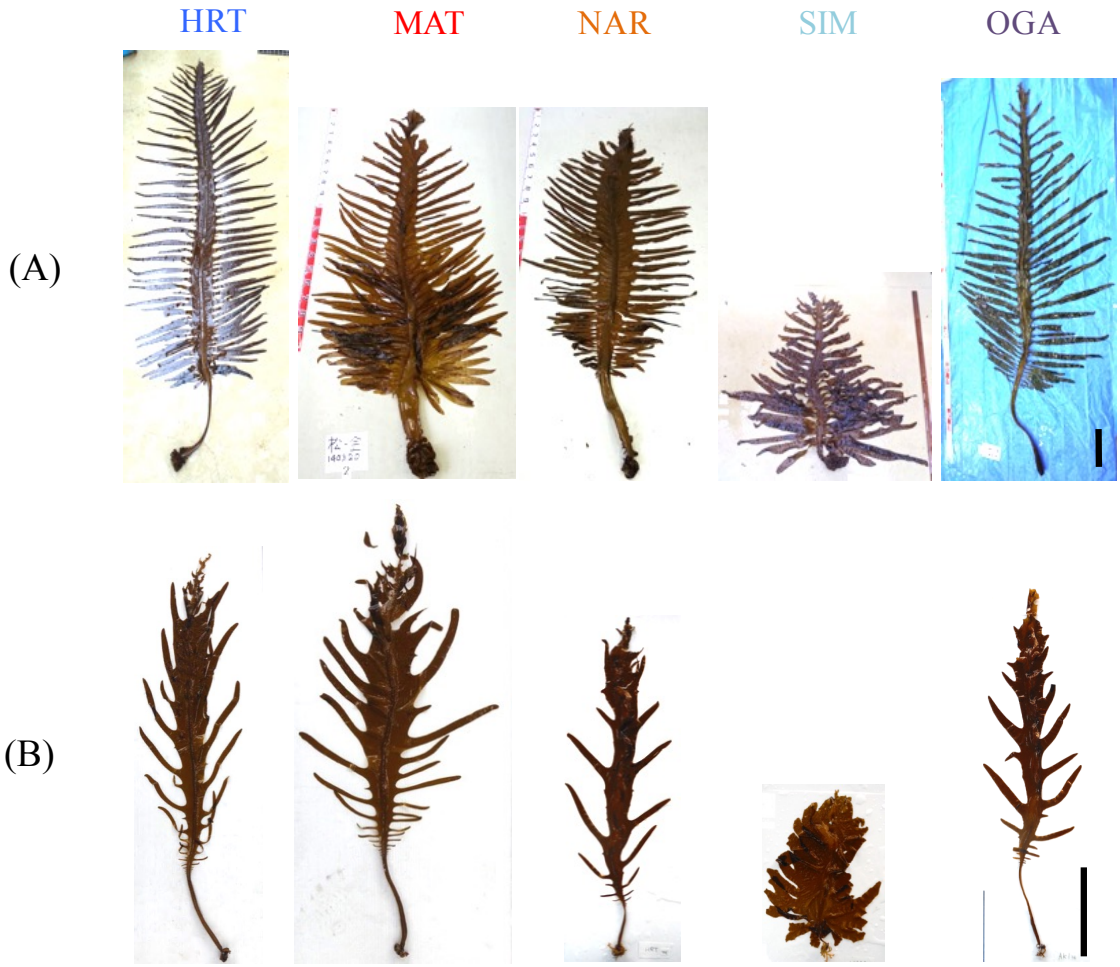
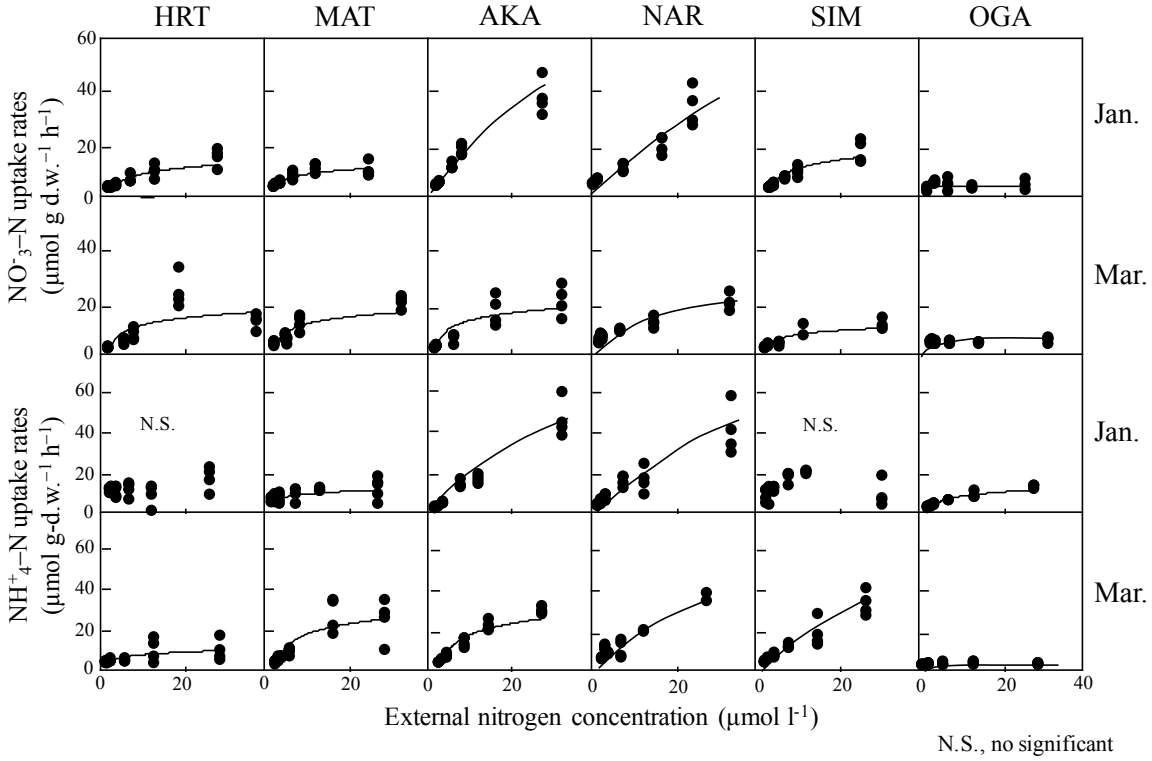


Fig. 3-11. Photographs of typical *Undaria pinnatifida* individuals cultivated in the sea at the five sites (A) and cultivated in the CFCS. Bars indicates 20 cm.

Table 3-1. *Undaria pinnatifida*. Start dates of cultivation, start dates of harvest, completion dates of harvest, and temperatures at six sites. See Figure 1 for geographical locations of the sites

Site	Start date of cultivation	Seawater Temperature in start date of cultivation (ºC)	Start date of harvest	Completion date of harvest
Hirota (HRT)	20 November 2013	16.0	1 March 2014	23 April 2014
Matsushima (MAT)	2 November 2013	16.3	30 December 2013	16 April 2014
Akashi (AKA)	15 November 2013	17.2	1 March 2014	6 April 2014
Naruto (NAR)	26 November 2013	17.3	1 March 2014	5 April 2014
Shimonoseki (SIM)	15 October 2013	21.8	15 February 2014	20 March 2014
Oga (OGA)	7 December 2013	14.7	1 April 2014	13 April 2014



Supplemental Fig. 3-1. *Undaria pinnatifida*. Nitrate and ammonium uptake rates of sporophytes collected in January and March from six sites in Japan. See Table 1 for an explanation of site codes. dw, dry weight.

CHAPTER IV

Development of early and late season crops based on genetic differentiation of *Undaria pinnatifida* and cultivation tests in the sea

SUMMARY

On the basis of genetic differentiation of regional plants of *Undaria pinnatifida*, I derived strain R1 from the MAT (Matsushima Bay) strain as an earlier season crop candidate and R2 from the HRT (Hirota Bay) strain as a later season crop candidate. At Hirota Bay in Iwate Prefecture, cultivation of sporelings of R1 and R2 strains was started at five different dates between September and December 2014. By January, when the seawater temperature was 21.4°C, both R1 and R2 strains for which cultivation was started on 17th September had withered. Although the R1 strain plants for which cultivation began on 9th October and 19th October were larger than the R2 strain plants started on those dates, the R2 strain plants for which cultivation began on 6th November and 12th December were larger than the R1 strain plants started on those dates. These results indicated that characteristics of R1 and R2 reflected genetic differentiation of the two strains, with R1 an earlier and R2 a later season crop. Because the yields of both strains were higher than the strain usually used in Iwate Prefecture, an improvement in productivity can be expected. Proper cultivation of these strains should help growers to avoid an excessive concentration of the harvesting periods and allow the industrial-scale cultivation of *U. pinnatifida*.

INTRODUCTION

The production amount of *U. pinnatifida* in Japan has been decreased since the 1990s (MAFF 2013). This change can be attributed to many factors, such as environmental changes and a decrease in the number of fishery workers. For instance, in Iwate Prefecture, Japan's most important centre for production of *U. pinnatifida*, the number of fishery employees has decreased by approximately 70% in recent decades, from 12,334 in 1998 to 8,948 in 2008. However, during the same decade, the percentage of fishery workers over 65 years of age has increased from 23.6% to 30.3% (Iwate Prefecture 2013). It is to be expected that, with the continuing trend of a decrease in the number of fishery workers and increase in their average age, both an increase in productivity and a decrease in the workload per employee will be required to meet the demand for production of *U. pinnatifida* in Japan. For instance, if an earlier season crop becomes possible, fishery employees could start to harvest within a year and ship to the new year market at a higher price. Moreover, an extended harvesting period could permit a reduction in the working load for elderly fishery workers, as the period of harvest and production could be decentralised. The current working period for harvest and production of *U. pinnatifida* is concentrated for only 1 month from the middle of March to end of April. However, an elite line for the practical use of *U. pinnatifida* has not yet been developed in Iwate Prefecture.

In this chapter, the development of earlier and later season crops is described. MAT and HRT strains were used as mother plants of earlier and later season crop candidates, respectively. The characteristics of both strains developed by genetic differentiation were described in Chapter III. An individual of each strain that exhibited greater growth was selected for the CFCS described in Chapter II. These strains were cultivated in the sea beginning on five dates

in September and December 2014 to confirm their characteristics as earlier and later season crops. The morphological characteristics, photosynthesis rates, and nutrient uptake rates of the elite candidates were compared.

MATERIALS AND METHODS

Selection of earlier and later season crop candidates

Sporophytes of the MAT strain (cultivated in Matsushima Bay) and HRT strain (cultivated in Hirota Bay) were collected in December 2013 as mother plants. As suggested in Chapter III, the characteristics of MAT and HRT could be utilised for an earlier and later season crop, respectively, on the basis of their genetic differentiation by their morphological and physiological characteristics. The selected plants of MAT and HRT strains were transferred to CFCS and cultivated at 10°C and 80–100 μmol m^{-2} s^{-1} until sporophyll formation. Spores of each strain were induced from its sporophylls, and sporophytes were obtained by the method described in Chapter III as the first generation. After reaching an average total length of 20 mm, sporophytes were transferred to a 7-L aquarium with running seawater and cultivated for 3 weeks. The longest 20 plants originating from MAT and HRT strains were selected, and plastic tags were affixed to a portion of their holdfast for individual identification. Sporophyte weights of all plants were measured, and the plants transferred to CFCS in 30-L. After 7 days the sporophyte weights were again measured, and the daily relative growth rate (RGR) was calculated by the same equation used in Chapter II. The individual of each strain with the highest RGR value was selected as an elite candidate in the first generation (Fig. 4-1). Cultivation of these elite candidates was continued, and their gametophytes were obtained from spores in sporophylls. These gametophytes, originating from MAT and HRT strains, were named R1 and R2 strain, respectively. R1 and R2 strains were used for the cultivation test in the sea as elite candidates.

Sporelings production

R1 and R2 gametophytes were incubated in a plastic dish with 30 ml PESI medium (Tatewaki 1966) at 21°C and 20 μmol m^{-2} s^{-1} with 12 h/12 h light/dark condition to promote growth (Fig. 4-2a). Sporelings production from gametophytes was conducted by the method of Iwate Fisheries Technology Center. The method is summarized as follows. After transfer of each gametophyte of R1 and R2 to a 300-ml Erlenmeyer flask with 200 ml PESI medium, the flasks were covered with aluminium foil and incubated at 10°C for 3 days to promote maturation. The gametophytes were shredded to about 10 cells by a homogenizer at 1,500 rpm. To create a substrate for sporelings, nylon fibre thread was wound around a 13-cm-square stainless-steel frame, which was sterilised by boiling and then dried thoroughly. Shredded gametophytes were poured with PESI medium into a plastic vat, and the substrate was soaked with the medium. Subsequently, the gametophytes attached to the fibre by absorption, and the substrates were placed into an aquarium with 7 L of 1/4 PESI medium. During the first week, substrates were incubated at a seawater temperature of 20°C, 40 μmol m^{-2} s^{-1} and illumination with 12-h photoperiods. Each week thereafter, the seawater temperature was increased by 1°C and the light intensity by 20 μmol m^{-2} s^{-1}. Photoperiods were reduced to 11 h at week 3 and 10 h at week 4. At week 5, after visible sporophytes were observed on the fibre, substrates were transferred to an aquarium with running seawater (Fig. 4-2b). Approximately 10 days before starting cultivation in the sea, sporelings were transferred to a cultivation farm in the sea. Fibres were tied on ropes from the stainless-steel frame and these ropes hung at a depth of 50 cm (Fig. 4-2c).

Cultivation in the sea

A rope measuring 150 m in length and 50 mm in diameter was used for cultivation in the sea as the mother rope. This type of rope is generally used in Iwate Prefecture. The fibres with sporophytes attached were cut into 3-cm sections and inserted at 30-cm intervals into a 3-mm-diametre nylon rope; these were used for seedlings. At five different dates, (14th September, 9th October, 19th October, 6th November and 12th December), seedlings of R1 and R2 were set at 10-m intervals on the mother rope from both ends and the cultivation was started begun (Fig. 4-2d,f). Groups I, II, III, IV and V delineate the plants beginning cultivation on these five dates.

Measurements of environmental factors

Seawater temperature was measured at the cultivation farm every 15–20 days. At the time of measurement, a 300-ml seawater sample was collected and concentrations of NO_3-N, NH_4-N and PO_4-P were measured by the same method described in Chapter III. The average seawater temperature every 10 days during the cultivation season of *U. pinnatifida* (September-April) from 2012 to 2015 at Matsushima Bay and from 1995 to 2013 at Hirota Bay were obtained from the Miyagi Prefectural Fisheries Research and Development Center and Iwate Fisheries Technology Center, respectively.

Morphological measurements

All plants of R1 and R2 strains grown on mother rope of 1 m length were collected from every group once every 2–3 weeks (Fig. 4-2e,f). These samples were transferred to the

laboratory, and the total amount of sporophytes, total length, sporophyte weight and sporophyll weight were measured.

Photosynthetic and nutrients uptake rates

Fifty individual sporophytes were cut with a razor from seedlings of group III and cultivated in CFCS. After 1 month, 30 plants of total length of approximately 30 cm were collected and pre-incubated for 1 h in 30 L of CFCS. This CFCS was installed with a thermostatic circulator pump to regulate the seawater temperature. After the pre-incubation, a dark condition was maintained at the central part of the maximum blade width by leaf clip (attachment of PAM-2000, WALZ, Effeltrich, Germany) for 20 min to fully oxidize the electron transport chain before measurement (White and Critchley 1999). While continuing the seawater soaking of sporophytes, the saturating-light method by PAM (MINI-PAM II, WALZ, Effeltrich, Germany) was used to determine the effective quantum yield, *F*v/*F*m, using the following equation (Genty et al. 1989):

$$Y = (\mathrm{Fm} - \mathrm{Fo})/\mathrm{Fm}$$

Through this measurement, using the same 30 sporophytes, the seawater temperature was set at 5, 10, 15, 20 and 25°C, and these tests were conducted for each temperature setting.

Another five individuals were selected, and blade discs of 3.3-cm diameter were cut off from the part of maximum blade width. By the method described in Chapter III, the nutrients uptake rates of R1 and R2 strains were measured. The seawater temperature during the 1-h

incubation was set at 5, 10, 15, 20 and 25°C. The initial concentrations of NO_3-N, NH_4-N and PO_4-P were set at 15, 15, 2 μM, respectively.

Statistical analysis

Significant differences in the morphological characters and physiological characteristics among R1 and R2 strains were identified by Student's *t*-test.

RESULTS

Environmental measurements

The average seawater temperature at Matsushima Bay ranged from 25.3 ºC in the beginning of September to 4.1 ºC in the beginning of January. The average seawater temperature at Hirota Bay ranged from 20.1 ºC in the middle of September to 6.3 ºC in the middle of March (Fig. 4-3a). The seawater temperatures of the starting cultivation date in each case were measured as 21.4°C on 17th September, 20°C on 9th October, 18.2°C on 19th October, 15.2°C on 6th November and 13.1°C on 12th December (Fig. 4-3a). The NO_3-N concentration was measured at 0.29 μM on 21st October and then increased, reaching a maximum value of 7.66 μM on 6th December (Fig. 4-3b). From January to April, NO_3-N concentration decreased and ranged between 3.55 and 5.15 μM. The NH_4-N concentration reached a maximum value of 13.32 μM on 17th September and then decreased to undetectable levels, except for 0.69 μM on 9th October and 2.15 μM on 6th December (Fig. 4-3b). The PO_4-P concentration showed a maximum value of 0.59 μM on 6th December and showed a tendency similar to NO_3-N (Fig. 4-3c).

Growth and production amount of R1 and R2 strains

The changes in the total length, sporophyte weight and sporophyll weight of R1 and R2 for every starting cultivation date (groups I–V) are given in Fig. 4-4. Neither of the group I strains showed visible sporophytes until 30th December, owing to being covered by other algae and diatoms. Sporophytes of R1 were observed only on 30th December, although sporophytes of R2 were observed on 30th December and 15th January. Although sporophytes were observed

in groups II and IV of R1 until 3rd April and in group III of R1 until 28th April, sporophytes were not observed in group V of R1 during the cultivation periods. In contrast, sporophytes were observed in groups II–V of R2 until 28th April. There were significant differences in the total length, sporophyte weight and sporophyll weight between R1 and R2 in every group during the cultivation periods (Fig. 4-4, $P < 0.05$). These characteristics of R1 were significantly higher than R2 in groups II and III, except for the total length of group III ($P < 0.05$, Fig. 4-4II, III). The sporophyte weight and sporophyll weight of R1 in group III were measured at 2,391.32 g and 447.02 g on 28th April, respectively; these values were maximum during the test (Fig. 4-4III). On the other hand, in group IV the total length, sporophyte weight and sporophyll weight of the R2 strain were significantly higher than those of R1 during the cultivation period ($P < 0.05$, Fig. 4-4IV). In group V, only the R2 strain grew (Fig. 4-4V).

Photographs of typical plants of the R1 and R2 strains collected in March are shown in Fig. 4-5. These morphological forms showed the same features as plants cultivated in each place of origin in Chapter III: R1 originating in the MAT strain had large blades and sporophylls, whereas R2 originating in the HRT strain had deeply-cut blades and its sporophylls were smaller than those of R2.

The production amount of both R1 and R2 per mother rope in a 1-m length was higher than ordinary sporelings in Iwate Prefecture (Table 4-1). Although differences in quality were not observed between R1 and R2 strains in the early period of growth, the blades of R1 showed wrinkles on the blade surface and a coarse texture in the later cultivation period in all groups. In contrast, the blade quality of R2 strain plants remained smooth and had good texture until the final harvesting on 28th April (data not shown).

Photosynthesis and nutrient uptake rates

The *F*v/*F*m value was indicated as maximum at 10°C and 5°C in R1 and R2, respectively. Values for both strains decreased with increasing seawater temperature (Fig. 4-6a). The values of R1 were significantly higher than those of R2 at all temperatures above 10°C ($P < 0.05$, Fig. 4-6a).

The NO_3-N uptake rate of R1 was indicated as minimum of 9.15 μmol g^{-1} h^{-1} at 5°C and maximum of 26.88 μmol g^{-1} h^{-1} at 20°C. There were no clear changes in the values of R2 NO_3-N uptake rate with temperature changes; these values ranged from 15.9 to 21.3 μmol g^{-1} h^{-1} (Fig. 4-6b). Significant differences in this value were found between R1 and R2; the value of R1 and R2 was higher than another strain at 20°C and 5°C, respectively ($P < 0.05$, Fig. 4-6b).

The NH_4-N uptake rate of R1 was minimum at 5°C and maximum at 20°C. As for the NO_3-N uptake rate, no clear changes were observed in the values of R2 at the different temperatures ($P < 0.05$, Fig. 4-6c). There were significant differences between R1 and R2, and the value for R1 was higher than R2 above 15°C ($P < 0.05$, Fig. 4-6c).

The trend of PO_4-P uptake rates of both R1 and R2 showed a higher value with increasing temperature; maximum values of R1 and R2 were 0.52 and 0.75 μmol g^{-1} h^{-1}, respectively, at 25ºC (Fig. 4-6d). The values of R2 were higher than those of R1 above 10°C ($P < 0.05$, Fig. 4-6d).

DISCUSSION

Environmental conditions at the Hirota Bay

The seawater temperatures during the present study period reflected approximately those occurring in an average year in the Hirota Bay. It reached 19°C in the middle of October, and this temperature was an index for the start of cultivation of *U. pinnatifida* at that location. The ranges of seawater temperature in Hirota Bay was narrower than one in Matsushima Bay. Although it was needed 7 months for changing the seawater temperature from maximum to minimum values at Hirota Bay, the period was 5 months at Matsushima Bay. Therefore, the seawater temperature condition at Matsushima Bay is more unstable and sharply change than at Hirota Bay.

The nutrient concentration was low in September and October, but increased towards spring. The typical decreasing nutrients in early spring at *Undaria* farm in the Sanriku region is caused by the spring bloom of phytoplankton (Nanba et al. 2011), but it was not observed during the present study.

Comparison of growth between R1 and R2 strain

The R1 strain of groups II and III showed better growth than the R2 strain (Fig. 4-4II,III). In contrast, R2 plants were larger than R1 in group IV, and only the R2 strain grew in group V (Fig. 4-4 IV,V). These results showed that an optimum temperature for growth of R1 was higher than R2, whereas that for R2 was lower than that of R1. It would appear that these results with R1 and R2 can be attributed to the characteristics of an earlier season crop of MAT and a later season crop of HRT, respectively (Chapter III, Fig. 3-5,6). The average seawater temperatures

at Matsushima Bay from September to the middle of October was higher than those at Hirota Bay (Fig. 4-3a). The period corresponds to early growth stage of sporophytes, it would appear that this result is caused high temperature tolerance to R1 strain. MAT indicated H-type nutrient kinetics, which has high affinity for low nutrient concentrations and is considered to indicate genetic differentiation (Chapter III, Fig. 3-8,9). It appears that this characteristic was caused by higher growth of R1 than R2 during the low-nutrient condition in autumn.

At Hirota Bay in Iwate Prefecture, it is generally known by fishery workers that starting cultivation of *U. pinnatifida* at seawater temperatures above 19°C leads to withering of sporelings originating in Iwate Prefecture. Therefore, the fishery workers' cooperative association offers seawater temperature information to the producers to assure that cultivation is started at temperatures below 19°C. In the present study, cultivation of R2 strain originating from the HRT strain of Iwate Prefecture could be started from 9th October (Fig.4-4II), when the temperature was 20°C (Fig. 4-3a). It is expected that the effect of selection in CFCS appeared after incubating at 20°C. Gao et al. (2013a) collected *U. pinnatifida* from Iwate, Miyagi and Naruto (Tokushima Prefectures, southern Japan) and cultivated them in Matsushima Bay. They demonstrated that juvenile sporophytes from Naruto exhibited the greatest capacity to accumulate high nitrogen reserves at higher temperature; therefore, it was used as a high-temperature-tolerance strain. However, the substitution in and out of strains was prohibited in principle at Iwate Prefecture, as it was deemed impractical to introduce new strains from other prefectures into Iwate Prefecture. In the present study, R2 originating from the HRT strain was shown to grow at 20°C; thus, an effective selection in an incubator and CFCS for two generations could be used on a practical scale.

Physiological characteristics of R1 and R2

The photosynthetic rate and nutrients uptake rates of NO_3-N and NH_4-N were shown to be higher in R1 than R2 at higher temperature and higher in R2 than R1 at a lower temperature (Fig. 4-6). These results reflected the characteristics of an earlier and later season crop of R1 and R2, respectively. Little research has been performed in the order Laminariales on the nutrients uptake rates at different temperatures. In the present study, although the uptake rates of NO_3-N and NH_4-N indicated a peak in 20°C of R1 and unclear changes in R2, the PO_4-P value for both strains increased with increasing temperature. These results suggest that there are different optimum temperatures for enzyme-controlled nitrogen and phosphate uptake kinetics.

Implications for earlier- and later- season crop of R1 and R2 strain

The production amounts of both R1 and R2 strains were higher than the strain usually used in this area (Table 4-1). This result also indicated a selection effect in CFCS. In the present study, a selection effect on the basis of genetic differentiation was exhibited.

In the Sanriku region, a standard harvest amount of *U. pinnatifida* is estimated as 10 kg per mother rope from a 1-m rope length. On the basis of this amount and quality of raw material, the cultivation period is determined from the middle of March to 25th April by agreement. In the present study, the yield of R1 had reached 10 kg by February or approximately 2 months earlier than the ordinary strain (Table 4-1). Moreover, the R2 strain maintained good quality until 28th April, which is later than the harvesting limit date of 25th April. Therefore, the

appropriate utilisation of these strains should help growers to avoid an excessive concentration of the harvesting periods and allow the industrial-scale cultivation of *U. pinnatifida*.

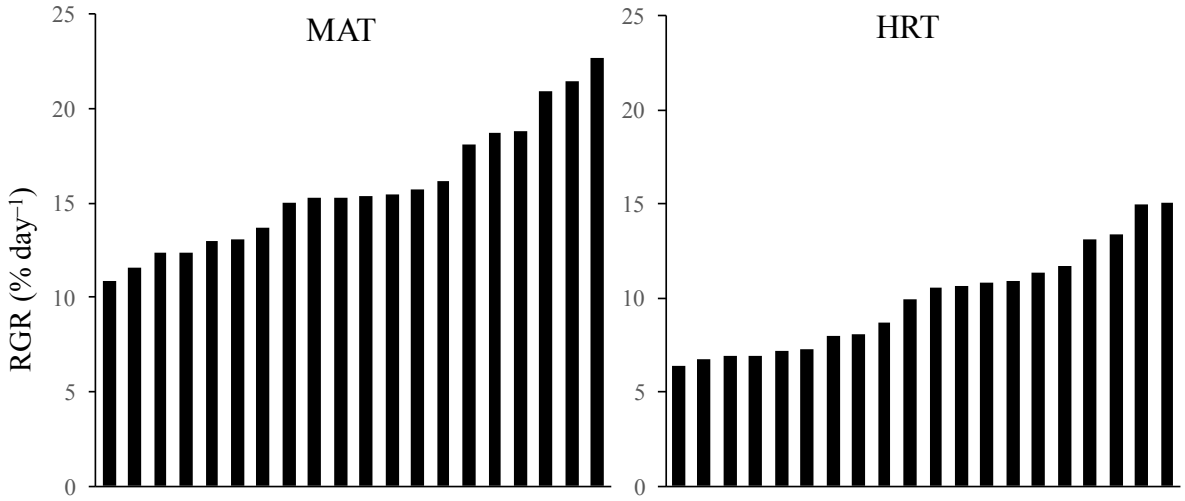


Fig. 4-1 Relative growth rates of 20 individuals originating from MAT and HRT strains of *U. pinnatifida*.

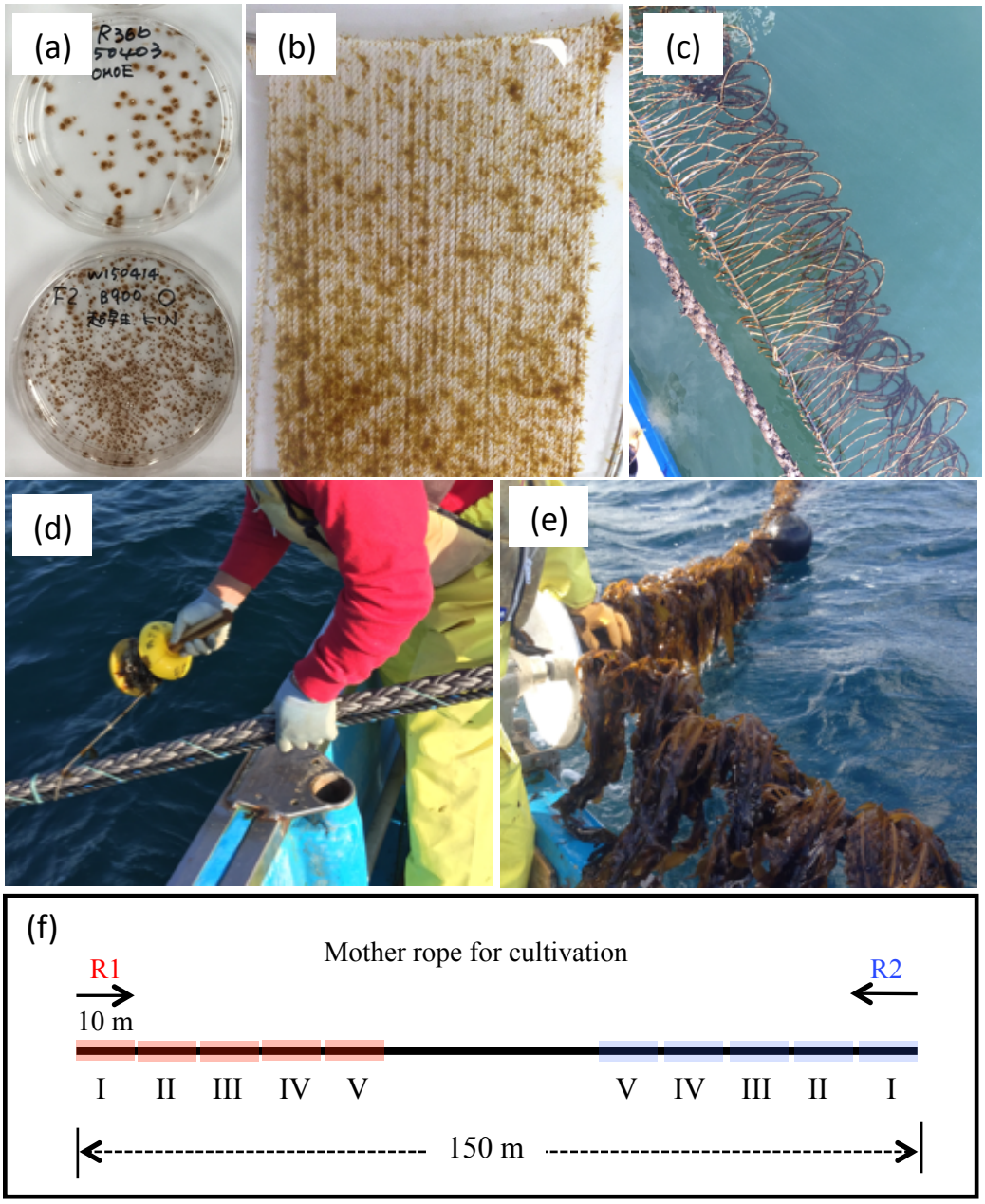


Fig. 4-2. Photographs and a schematic diagram of the cultivation tests. (a) Gametophytes of elite candidates; (b) Sporelings on fibre wound around stainless-steel frame; (c) acclimatisation of sporelings to the cultivation farm in the sea; (d) setting of seedlings on the mother cultivation rope; (e) Harvest amount per 1 m on mother rope; (f) bird's-eye view of 150-m mother rope and starting cultivation of R1 and R2 strains from each end of mother rope. I–V indicate groups I–V at the start of cultivation.

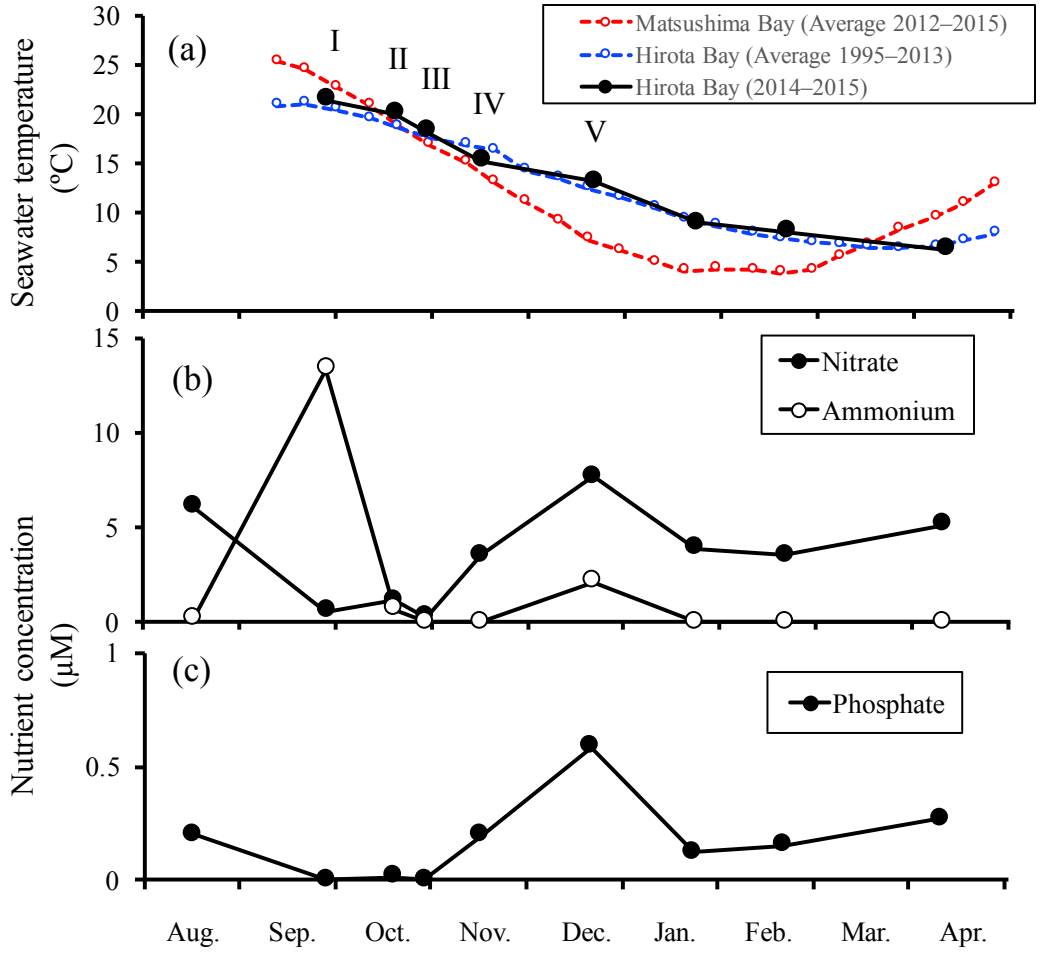


Fig. 4-3. Changes in environmental factors in Hirota Bay, Iwate Prefecture. (a) The average seawater temperature at Matsushima Bay (2012-2015) and at Hirota Bay (1995-2013), and seawater temperature at Hirota Bay during cultivation period; (b) NO_3-N and NH_4-N concentrations; (c) PO_4-P concentration. I–V indicate groups I–V at the start of cultivation.

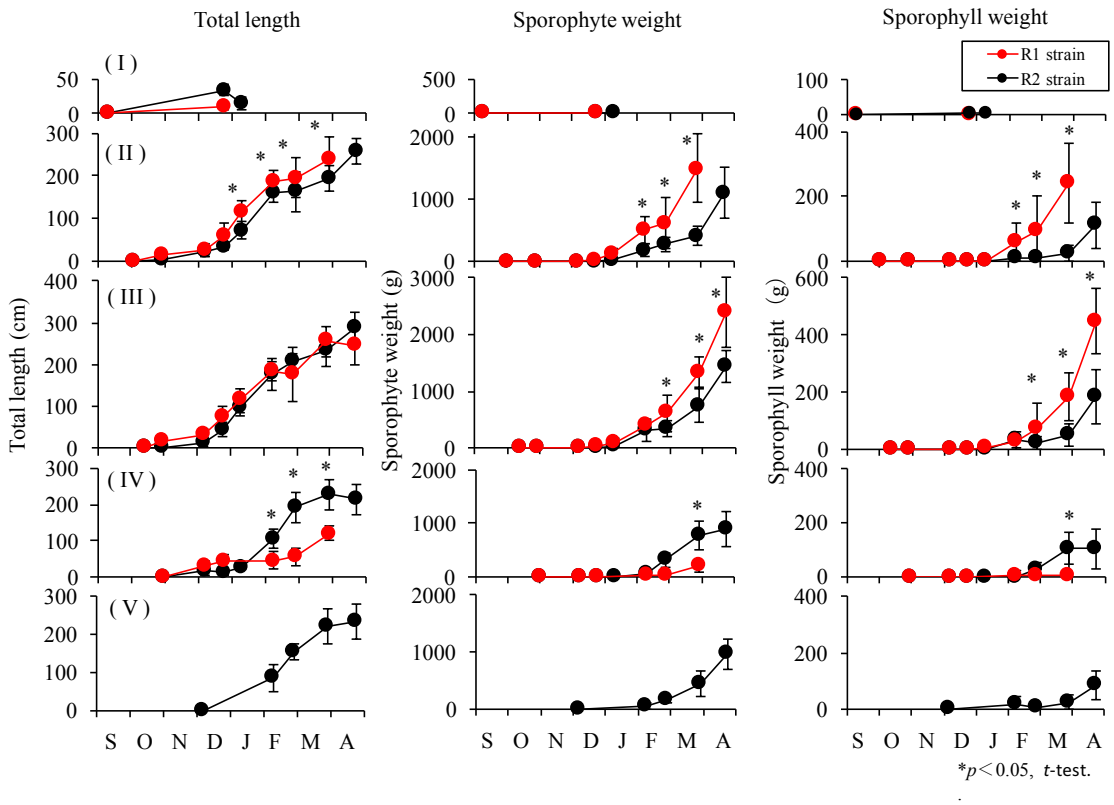


Fig. 4-4. Changes in total length, sporophyte weight and sporophyll weight of R1 and R2 strains. I–V indicate groups I–V at the start of cultivation.

Fig. 4-5. Photographs of typical individuals of R1 and R2 strain harvested in March 2015.

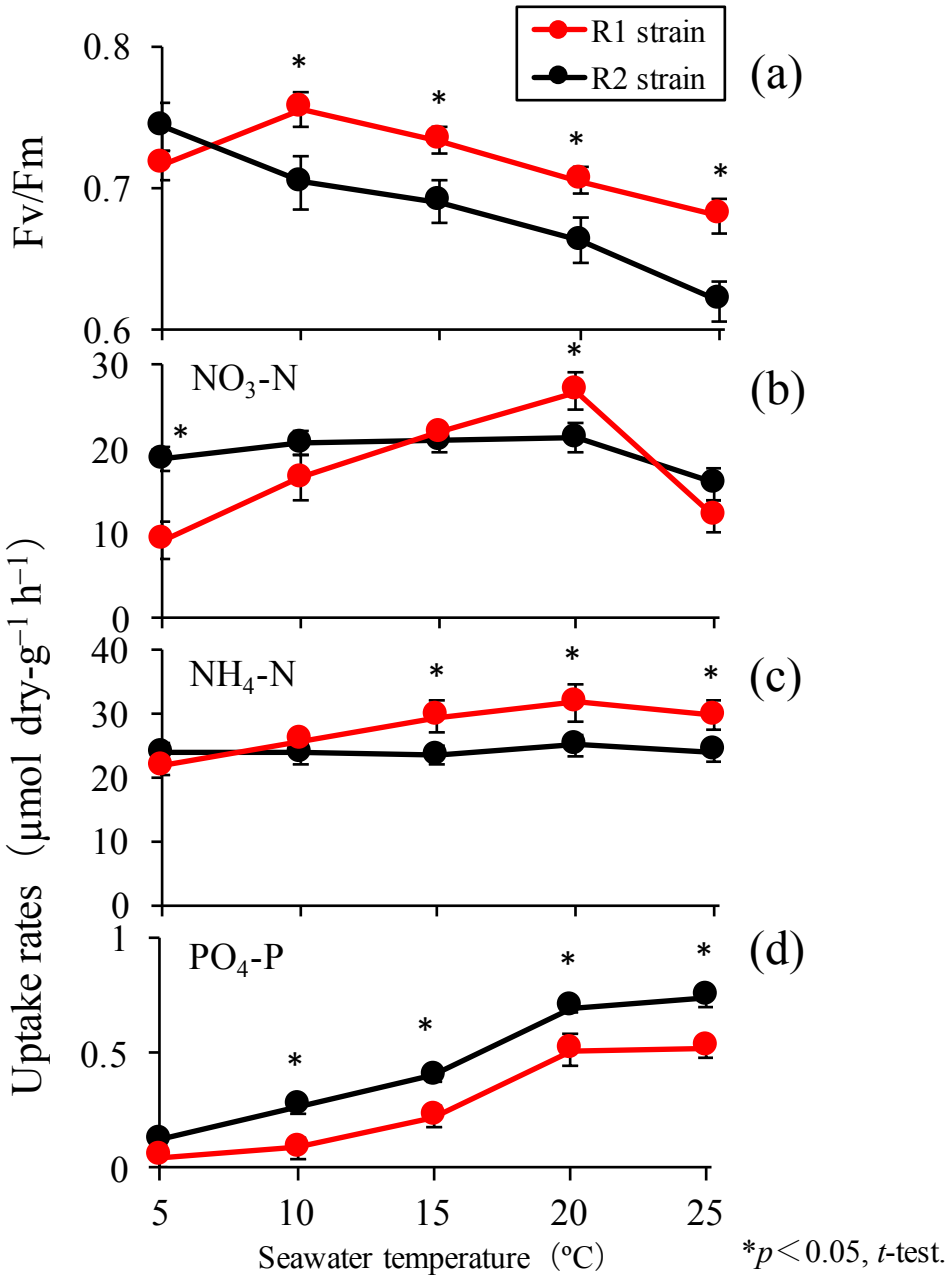


Fig. 4-6. *Fv*/*Fm* and nutrients-uptake rates of R1 and R2 strain according to the different seawater temperatures. (a) *Fv*/*Fm*; (b) NO_3-N; (c) NH_4-N; (d) PO_4-P.

Table 4-1. Changes in the production amount of R1, R2 and control per 1m of the mother rope. Control was ordinary strain used in Iwate Prefecture

	December	January	February	March	The beginning of April	The end of April
R1	0.7	2.1	14.0	11.0	24.6	35.6
R2	0.1	0.9	4.8	8.6	21.8	25.3
Control	0.1	0.3	3.0	8.2	12.2	18.3

CHAPTER V

Optimization of heavy-ion beam irradiation in *Undaria pinnatifida* and development of elite lines

SUMMARY

For the purpose of advancing heavy-ion beam breeding for large brown algae, the effectiveness of heavy-ion beams for mutagenesis in *Undaria pinnatifida* was initially investigated. From the survival rates of *Undaria* gametophytes and sporophytes irradiated heavy-ion beams, the optimum doses were estimated to be 2–12.5 Gy of C ions and 0.2–2.5 Gy of Ar ions. Second, a screening method for mutant selection of *U. pinnatifida* was developed by combining the CFCS (a cyclone and floating culture system) with a previous culture method. By using this method, screening for increased growth and high-temperature tolerance was demonstrated. In the M_2 generation obtained from brother–sister inbreeding of M_1 generation, in which gametophytes and young sporophytes of an HRT strain irradiated with C and Ar ions, candidate mutants with higher growth and high-temperature tolerance than the wild type were obtained. Every mutant candidate was confirmed to have the same taste and texture as the wild type. It is suggested that these candidates could be used as elite lines.

INTRODUCTION

In Chapter IV, an earlier-season crop (R1) and later-season crop (R2) were selected by use of the CFCS culture method on the basis of genetic differentiation of *U. pinnatifida*. Although R1 culture could be started at ≤20°C and the production amount of R2 could be obtained approximately 1.3 times higher than ordinary strain, elite lines allowing starting culture at ≥20°C or producing higher amounts are required in *Undaria* farms. In previous studies of breeding for large brown algae, heterosis in *U. pinnatifida* (Hara and Akiyama 1985) and *M. pyrifera* (Westermeier et al. 2010), mating breeding of *S. japonica* and *S. longissima* for 5 generations (Zhang et al. 2011) were studied. In Tokushima and Hyogo prefectures in Japan, an earlier variety was obtained by crossing 1 male and 1 female gametophyte originating in 1 spore (Dan et al. 2015, Niwa 2015). In contrast, in northern Japan, withering of cultivated juvenile sporophytes of *U. pinnatifida* has led to reduced its production, a cultivar of greater tolerance to higher temperature and low nutrient conditions are required (Gao et al. 2013b). Furthermore, in the Sanriku region in Iwate and Miyagi prefectures, of where the production amount of *U. pinnatifida* accounts for approximately 70% in Japan, an elite strain used continuously for industrial-scale production has not been developed. In the fishery community in Japan, a cultivar with higher productivity and quality during a shorter growing period is strongly needed because the number of fishery workers is decreasing and they are aging.

Recently, mutagenesis using heavy-ion beams as mutagen has received attention. This method is able to induce mutations with high frequency at a relatively low dose at which almost all plants survive, and with a wide range of variation (Tanaka et al. 2010; Abe et al. 2012). Many elite lines have been obtained by heavy-ion beam mutagenesis. For instance, using the

heavy-ion beam of the RIKEN RI-Beam Factory, commercial varieties of 26 lines of terrestrial plants and 2 lines of sake yeast have been derived (Abe et al. 2015). For macroalgae, Niwa et al. (2009) obtained a color mutant of *Pyropia yezoensis* by irradiation with an Ar beam. However, this technique has not been applied to large brown algae. In the present study, an optimum irradiation dose of heavy-ion beam for *U. pinnatifida* was identified and elite lines with increased growth and high-temperature tolerance were selected using a new screening method. The growth and physiological characteristics of these elite lines were analyzed. The optimum heavy-ion beam irradiation dose for *U. pinnatifida* was estimated at 2–5 Gy of C and 0.2–2.5 Gy of Ar. A M_1 generation of the HRT strain irradiated with C and Ar beams was cultured at the CFCS, its sporophylls were collected, and spores were obtained. Spores from sporophytes of the M_1 generation were cultured and sporophytes of the M_2 generation showed segregation for total length. Their morphological and physiological characteristics were confirmed in the M_3 generation.

MATERIALS AND METHODS

Preparation of irradiation sample

U. pinnatifida follows a heteromorphic annual cycle with alternating sporophytic and gametophytic generations (Fig. P-1, Akiyama and Kurogi 1982). Gametophyte fragments and sporophytes <2 mm in length were used for heavy-ion beam irradiation. The mother plant for the irradiation was HRT, which was collected in Hirota Bay, Iwate prefecture, as described in Chapter III.

Effect of heavy-ion beam irradiation on gametophytes and measurement of germination and survival rate of sporophytes

Zoospores of HRT were induced and cultured to gametophytes by the general method described in Chapter III (Fig. 3-3a, b). After 6 months, gametophytes that had grown to 2 cm in diameter as a colony were irradiated with C ions at a dose of 0–25 Gy or Ar ions at a dose of 0–10 Gy (Sato et al. 2012). The irradiated gametophytes were cut into fragments with a homogenizer and fragments of sizes ranging 42–100 μm were collected by sieving through micromeshes. One male gametophyte and 1 female gametophyte were picked up and placed into 1 well of a 48-well micro-plate with 1 ml PESI medium (Tatewaki 1966). Gametophytes were incubated at 20°C with a 12-h photoperiod at a photon flux density of 10 μmol m^{-2} s^{-1}. Gametophyte size was measured after 8 weeks of culture. Gametophytes with longest cell filament <100 μm in length with all cells whitened were defined as dead, and survival rates of irradiated male and female gametophytes were calculated. Gametophytes were then incubated at 15°C with 10-h light/14-h dark photoperiods at a photon flux density of 50 μmol m^{-2} s^{-1} for

induction of sporophytes. Gametophytes with sporophytes were counted after 4 weeks of culture and the sporophyte formation rate was calculated.

Heavy-ion beam irradiation and measurement of sporophytes

Sporophytes of *U. pinnatifida* were derived from fertilization between male and female gametes. Sporophytes 1 mm in length were transferred into 15-ml plastic tubes for C ion irradiation and into plastic bags (Hybri-Bag Hard; Cosmo Bio, California, USA) for Ar ion irradiation in sterilized seawater and were irradiated with C ions (LET: 30.0 keV μm^{-1}) in a dose range of 0–25 Gy or Ar ions (LET: 280 keV μm^{-1}) in a dose range of 0–10 Gy (Sato et al. 2013) at E5 beam line in a RIKEN RI-beam factory (RIBF). Each tube or plastic bag contained 50 sporophytes. After irradiation, the sporophytes were cultured in 300-ml aeration flasks (Marine Flask, Biomedical Science, Tokyo, Japan) with 1/4 PESI medium and aeration at 15°C with a photoperiod of 12-h/12-h (light/dark), and a photon flux density of 90 μmol photons m^{-2} s^{-1} (Fig. 3–3 c). The survival rate was measured after 3 weeks of culture.

The growth of sporophytes in the M_1 generation

All sporophytes germinated from gametophytes subjected to heavy-ion beam irradiation and all sporophytes with heavy-ion beam irradiation with all ions and doses were cultured in a 7-L aquarium for approximately 2 weeks (Fig. 3-3d). With some improvements in aquarium drainage, sporophytes could be cultured with running seawater. The seawater temperature and light intensity were set at 10°C and 100 μmol photons m^{-2} s^{-1}, respectively. Then, sporophytes were cultured in the 30-L CFCS (Chapter II) for 3 weeks (Fig. 3-3e), in the 500-L CFCS for a

further 3 weeks (Fig. 3-3f), and finally in the 2,000-L CFCS until sporophyll formation (Fig. 3-3g). The total length and sporophyte weight of all sporophytes were measured every 2 weeks during culture in the CFCS, plants were selected for length and spores were induced from their sporophylls to yield gametophytes. These were used for screening in the M_2 generation.

The screening for mutant candidates with higher growth in the M_2 generation

Spores induced from sporophylls in the M_1 generation were poured into plastic dishes filled with 30 ml of PESI medium. These dishes were incubated at 20°C with 12-h photoperiods and 20 μmol photons m^{-2} s^{-1} illumination. After 1 month, when germinated gametophytes reached 1 cm in diameter as a colony, 10 colonies of females and 1 colony of males were selected and transferred to a well of a plastic petri dish with 6 wells. One well was treated as 1 test section for every ion and dose. The 6-well dishes were incubated at a light intensity of 20 μmol photons m^{-2} s^{-1} with 12-h photoperiods. The seawater temperature for germination was set at 19°C. The test sections that germinated to sporophytes were forwarded to the next examination. Sporophytes were cultured in the 300-ml marine flask in 1/4 PESI for 3 weeks at a light intensity of 20 μmol photons m^{-2} s^{-1} with a 12-h photoperiod (Fig. 3-3c). The seawater temperature was decreased by 1°C a week from 19°C to mimic culture conditions in the ocean. Sporophytes were then cultured in a 7-L aquarium at 10°C and a light intensity of 50 μmol photons m^{-2} s^{-1} for 20 days (Fig. 3-3d). All sporophytes were placed in a 17 × 23-cm plastic tray filled with sterilized seawater without overlapping and photographed with a digital camera. The total length of all individuals in the image were measured with imaging-analysis software (NDS-900, OLYMPUS, Tokyo, Japan). A box plot was made from the measurement data using

JMP. The test section with individuals having values 3 times higher than IQR indicator as the result of segregation for total length was selected because the elite lines included mutant candidates with increased growth. The candidates of mutant lines and control plants (not irradiated) in the M_2 generation were cultured in the 30-L, 500-L, and 2000-L CFCS (Fig.3-3e–g), and sporophyte weights were measured 100 days after germination. After sporophylls were formed, spores were induced from them and gametophytes were prepared for study in M_3. At the time of sporophyll harvesting, tasting of raw and boiled (for 30 s) sporophyte blades was performed as in ordinary production.

Screening of mutant candidates with high-temperature tolerance in M_2 generation

Six-well dishes containing 1 male and 10 female gametophytes in each well were prepared by the same method used for screening for increased growth. The gametophytes were incubated at a light intensity of 20-μmol photons m^{-2} s^{-1} with 12-h photoperiods. The germination temperature was started at 23°C. Ordinary cultivars of *U. pinnatifida* in Iwate prefecture do not germinate at above 19°C. After 20 days, the only test section that germinated into sporophytes proceeded to the next examination (primary screening). The sporophytes were cultured by the method described above except for temperature; the seawater temperature was decreased 1°C a week from 23°C. After 3 weeks, all sporophytes were photographed. The test section with individuals showing values 3 times higher than the IQR indicator were selected as elite lines including mutant candidates (secondary screening). The subsequent process was similar to that for screening for mutant candidates with higher growth.

Progeny testing in the M_3 generation

The M_3 generation of mutant candidate lines in the M_2 (Y163 and Y164) and wild type were allowed to germinate into sporophyte and cultured in the CFCS after culture in an incubator by the same method used for the M_2. To confirm the growth of the initial stage, total length and sporophyte weight 65 days after germination were measured.

Comparison of nutrient uptake rates between mutant candidates and wild type in M_3 generation

The nutrient uptake rate of Y164 40 days after germination was measured. As wild types, strains of HRT, MAT, and OMOE (Omoe Bay, Iwate prefecture, Japan) were selected. Ten sporophytes of each strain cultured in the CFCS were selected. All sporophytes were pre-incubated in a marine flask filled with 1 L of sterilized seawater from Akita prefecture (Chapter I) at 15°C at a light intensity of 50 μmol photons m^{-2} s^{-1} and 12-h photoperiod for 48 h. For each strain, 5 plants without injuring and discoloration were selected from the 10 plants. Their total length was 2 ± 0.3 cm. These sporophytes were incubated at different NO_3-N and NH_4-N concentrations and nutrient uptake rates were estimated by the method described in Chapters I and III. The nutrient uptake rates were fitted to Michaelis–Menten equations and the parameters of nutrient uptake kinetics, V_{max}, K_s, and V_{max}/K_s, were estimated as also described in Chapters I and III.

RESULTS

Survival rates of gametophytes and sporophytes irradiated with C and Ar ion beams

Although the survival rates of female gametophytes of the HRT strain irradiated with heavy-ion beams with C and Ar ions decreased with increasing dose, the numbers of male gametophytes did not show a similar tendency (Fig. 5-1a). Thus, survival rates of female gametophytes were also shown to correlated with the formation rates of sporophytes germinated from irradiated gametophytes (Fig. 5-1b). The survival rates of sporophytes irradiated with heavy-ion beams were decreased at >12.5 Gy of C and >0.2 Gy of Ar (Fig. 5-2). The individual that withered and died after a blade curled during culture in the marine flask was observed with irradiation dose elevation (data not shown).

Growth of sporophytes in the M_1 generation

The total length and sporophyte weight of sporophytes irradiate with C-ion and Ar-ion beams 100 culture days after induction of germination in M_1 generation are shown in Fig. 5-3. Figure 5-3a shows these values for sporophytes derived from gametophytes irradiated with C and Ar beams. The total length ranges following C and Ar irradiation were 34–125 and 23–118 cm, respectively. The sporophyte weight ranges following C and Ar irradiation were 8.3–72.9 and 3.3–66.4 g, respectively. Figure 5-3b shows the values for sporophytes irradiated with C and Ar beams. The total length ranges of sporophytes irradiated with C and Ar beams were 42–104 and 3.1–69 cm, respectively. The sporophyte weight ranges of sporophytes irradiated with C and Ar beams were 10.4–69.3 and 6.4–75 g, respectively. Sporophytes derived from

gametophytes and sporophytes irradiated with Ar beams at 10 Gy were dead by 50 days after germination. Smaller plants were observed at higher doses of irradiation with both C and Ar.

The screening of the mutant with a higher growth in M_2 generation

As a part of the result, photographs of sporophytes cultured for 40 days after germination at 19°C are shown in Fig. 5-4. The average total length reached 5–10 mm (Fig. 5-4a, b, c, d). Lines including large individuals were observed, as indicated by red arrows (Fig. 5-4c, f). From the results of box plot analysis on the basis of photographic data, 1302G-R68 irradiated with C at 5 Gy and 1302G-B60 irradiated with Ar at 0.2 Gy were selected as elite line candidates in order to include mutant candidates with total lengths markedly higher than those of others (Table 5-1, Fig. 5-5-1a, b). These 2 lines were cultured in the CFCS and 4 plants of 1302G-R68 and 2 plants of 1302G-B60 were observed to be markedly larger than other plants in each line (Fig. 5-6a, b). Finally, as mutant candidates for higher growth, 4 plants of 1302G-R68 (B169–172) and 2 plants of 1302G-B60 (Y163,164) were selected. The sporophyte weight of every mutant candidate was higher than that of the wild type 100 days after germination (Figs. 5-7). The taste and texture of these mutant candidates were similar to those of the wild type (data not shown).

Screening of mutants with high-temperature tolerance in the M_2 generation

Among the lines germinated at 23°C, lines containing large individuals were observed, as in the screening of mutants with higher growth in the M_2 generation. From the results of box plot analysis on the basis of photographic data, the 1302S-R40 strain irradiated with C at 2 Gy

and the 1302G-W40 strain irradiated with Ar at 2.5 Gy were selected as elite line candidates in order to include mutant candidates with high-temperature tolerance (Table 5-1, Fig. 5-5-2c, d). The irradiated stages for 1302S-R40 and 1302G-W40 were sporophytes and gametophytes, respectively. These 2 lines were cultured in the CFCS and 7 plants of both lines were markedly larger than other plants (Fig. 5-6c, d). Finally, as mutant candidates for higher temperature tolerance, B222 of 1302S-R40 and B235 of 1302G-W40 were selected. The sporophyte weight of every mutant candidate was higher than that of the wild type 100 days after germination (Fig. 5-7). The texture of B222 was smooth and better than that of the wild type. The texture and taste of B235 were similar to those of the wild type (data not shown).

Progeny testing in the M_3 generation

The total length and total weight of Y163 and Y164 of the mutant candidates with higher growth were compared to those of the wild type in the M_3 generation (Figs. 5-8). These values were significantly ($p < 0.001$) higher for Y163 and Y164 than for the wild type 65 days after inducing germination, indicating that both lines were mutants with higher growth.

Comparison of nutrient uptake rates between Y164 and wild type in M_3 generation

The NO_3-N and NH_4-N uptake rates of Y164 in M_3 generation showed a tendency of not decreasing at higher concentration (Figs. 5-9). The parameters of nutrient uptake kinetics are presented in Table 2. The V_{max}, K_s, and V_{max}/K_s of NO_3-N in the wild types (HRT, OMOE, MAT) were in the ranges 78.89–89.42 μmol g^{-1} h^{-1}, 6.92–10.76 μM and 7.32–12.91, respectively. The V_{max}, K_s and V_{max}/K_s of NO_3-N in Y164 were in the ranges 4741.37 μmol g^{-}

[1] h^{-1}, 2028.34 μM and 2.33, respectively, and the values of V_{max} and K_s of Y164 were approximately 50 and 30 times higher, respectively, than those of the wild types. The V_{max}, K_s, and V_{max}/K_s of NH_4-N in wild types (HRT, OMOE, MAT) were in the ranges 192.35–365.77 μmol g^{-1} h^{-1}, 21.46–66.87 μM and 5.47–8.96, respectively (Table 5-2). In contrast, the nutrient uptake rates of Y164 were increased and not saturated at a high nutrient level and did not fit a Michaelis-Menten equation.

DISCUSSION

The sensitivity of U. pinnatifida *to heavy-ion beams irradiation and estimation of optimum irradiation dose*

In heavy-ion mutagenesis, a shoulder dose of survival curve in the M_1 generation was suitable for mutant screening in the M_2 generation (e.g. Kazama et al. 2008). Based on the survival rates for female gametophytes and sporophytes irradiated with heavy-ion beams, the optimum irradiation dose was expected to be 2–5 Gy of C and 0.2–1 Gy of Ar for female gametophytes, and 12.5 Gy of C and 0.2 Gy of Ar for sporophytes (Fig.5-1,2). Hirano et al. (2014) studied the survival rates and morphological mutants of *Undaria* gametophytes germinated from spores irradiated with C and Ar ion beams and observed some mutants with respect to cell shape, cell size and intracellular structure. Survival rates were decreased at 2–5 Gy with C ion irradiation and 0.2–2.5 Gy with Ar ion irradiation, dose ranges almost the same as those in the present study.

Screening in M_1 generation and screening using the CFCS

In the M_1 screening, higher growth of sporophytes was observed, at irradiation beam ranges of 0.5–2 Gy of C and 1–5 Gy of Ar (Fig. 5-3). Chen (2011) irradiated *Ulva fasciata* with ^{60}Co gamma radiation and obtained an individual with higher protein content, concluding that this change represented hormesis effect. In the present study, the higher growth in the M_1 generation was also suggested to represent hormesis effect.

In the present study, although the screening in the M_2 generation was performed on large plants, mutant candidates of elite lines in the M_2 generation were obtained not only from large

plants in the M_1 generation. Thus, it was necessary to cultivate large numbers of plants in the M_1 generation and to collect large numbers of gametophyte lines in the M_2 generation, in order to apply mutagenesis, similarly to the case of terrestrial plants. In the present study, it is suggested that the screening method using the CFCS is useful for mutagenesis because all plants irradiated with heavy-ion beams could be cultured for every ion and dose from young plants to plants with sporophylls without loss due to the strong wave motion experienced in ocean culture.

Screening of mutant candidates in the M2 generation

Mutant candidates with high growth and high-temperature tolerance, as elite lines, were obtained in the present study by screening in the M_2 generation (Fig. 5-7). The irradiation doses inducing these mutants were 2 and 5 Gy for C and 0.2 and 2.5 Gy for Ar, values within the optimum ranges expected from the survival rates. The progeny test in the M_3 generation showed that Y163 and Y164, originating in 1302G-B60, were high-growth mutants (Fig. 5-8). These originated from the same line in the M_2 generation and their phenotypes in M_3 generation were similar, suggesting that Y163 and Y164 are the same mutant line. Other mutant candidates should also be subjected to progeny testing and phenotypic analysis in the M_3 generation. Moreover, it is desirable to determine, using a cross-breeding test, whether B 169–172 from 1302G-R68 and Y163 and Y164 from 1302G-B60 were the same mutant lines in each strain.

It is expected that lines B169–172 and Y163–164, which germinated at 19°C and showed higher growth, could be used in industry for higher productivity. Y163–164 would be a highly practical line because of the confirmation by progeny testing in the M_3 generation.

B222 of 1302S-R40 and B235 of 1302G-W40 germinated at 23°C and showed high growth. In Chapter IV, an earlier-season crop (R1) was derived from the MAT strain originating in Miyagi prefecture by selection breeding, but its starting culture temperature was ≤20°C. Thus, B222 and B235 would be useful for earlier-season crops. In Iwate prefecture, *Undaria* culture is generally started at the end of October and the beginning of November, when the seawater temperature is ≤19°C, to prevent death of sporelings (Chapter IV). A seawater temperature of 23°C corresponds to the temperature in mid-September in Hirota Bay in Iwate prefecture, so that these mutants would be able to start culture half a month earlier than ordinary strains.

NO_3-N and NH_4-N uptake kinetics of Y164

The nutrient uptake kinetics of Y164 showed a markedly L-type characteristic owing to its high value of V_{max} and K_s in NO_3-N and lack of saturation at the highest nutrient concentration in NH_4-N (Fig. 5-9, Table 5-2). The high value of V_{max} is similar to those of small macroalgae, which are *Ulva lactuca*, *Enteromorpha prolifera*, *Chaetomorpha linum*, and *Cladophora sericea* of green algae and *Gracilaria tikvahiae* and *Ceramium rubrum* of red algae (Fujita 1985, O'Brien and Wheeler 1987, Pedersen and Borum 1997). It is suspected that these nutrient characteristics similar to those small macroalgae give Y164 its early growth characteristic.

In terrestrial plants, 2 NO_3^- transporters with different affinity for the ion, named HATS (high-affinity transport system) and LATS (low-affinity transport system), control uptake at low (≤0.5 mM) and high (>0.5 mM) nutrient concentrations, respectively (Forde 2000). The genes encoding LATS and HATS are called NRT1 and NRT2, respectively, and mutants

deficient for each gene have been obtained in model plants (Liu et al. 1999, Wang et al. 1998). It is likely that a deficiency in a gene controlling the affinity for ions is responsible for the nutrient uptake kinetics of Y164. Recently, the whole plastid genome of *U. pinnatifida* was analyzed (Zhang et al., 2015). The combination with the CFCS tank system, screening methods for mutant candidates, phenotypic analysis and genome information would advance the breeding and molecular biology of *U. pinnatifida*.

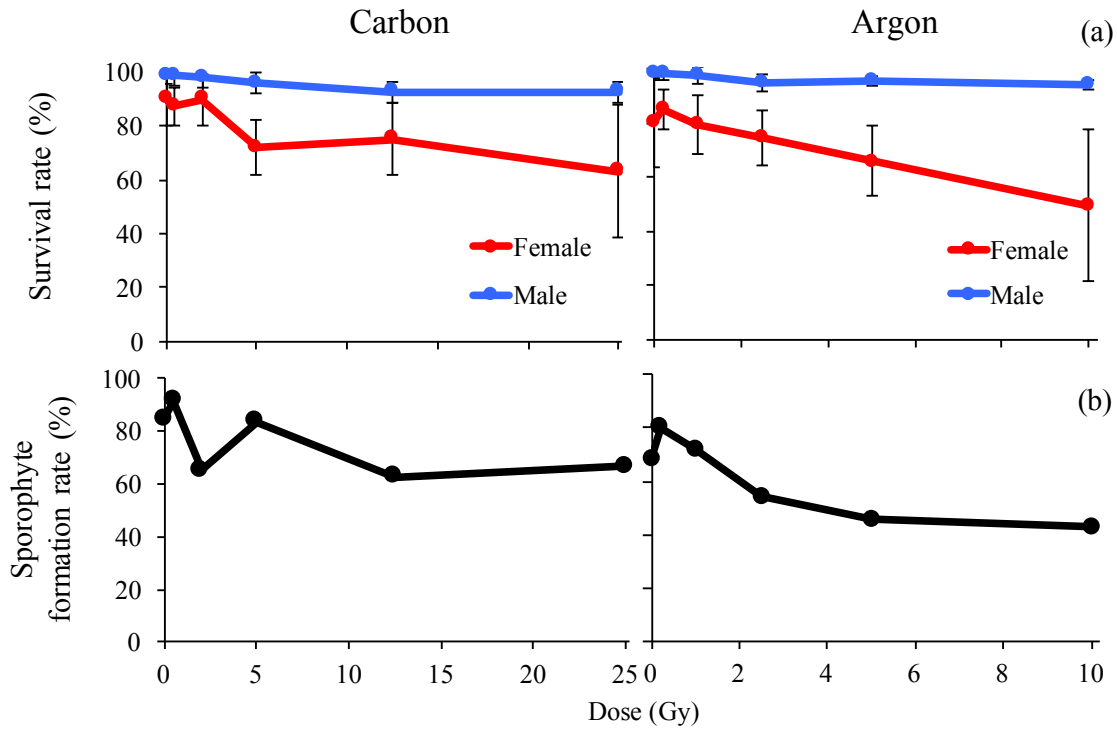


Fig. 5-1. Survival rate of gametophyte and sporophyte formation rate. (a) survival rate of male and female gametophytes irradiated with C-ion and Ar-ion beams, (b) Sporophyte formation rate following germination from gametophytes irradiated with C-ion and Ar-ion beams.

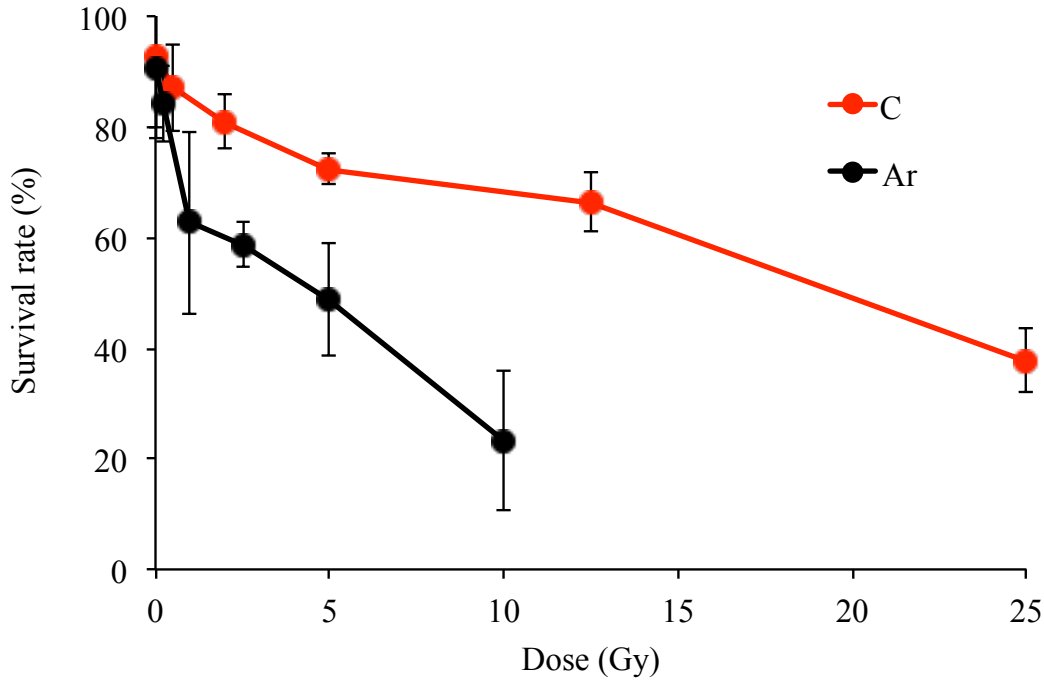


Fig. 5-2. Survival rate of sporophytes irradiated with C-ion and Ar-ion beams.

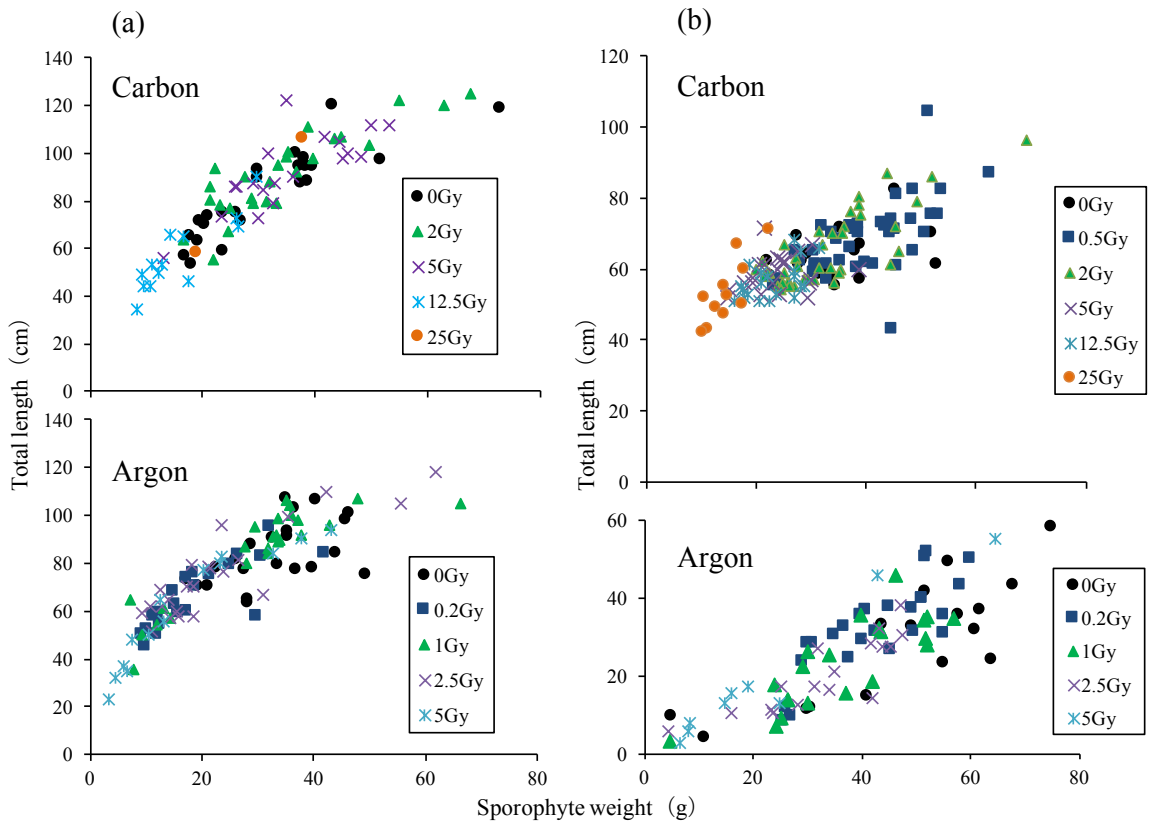


Fig. 5-3. Total length and sporophyte weight of sporophytes irradiated with C-ion and Ar-ion beams 100 culture days after induction of germination in M_1 generation. (a) sporophytes derived from gametophytes irradiated with C-ion and Ar-ion beams, (b) sporophytes irradiated with C-ion and Ar-ion beams.

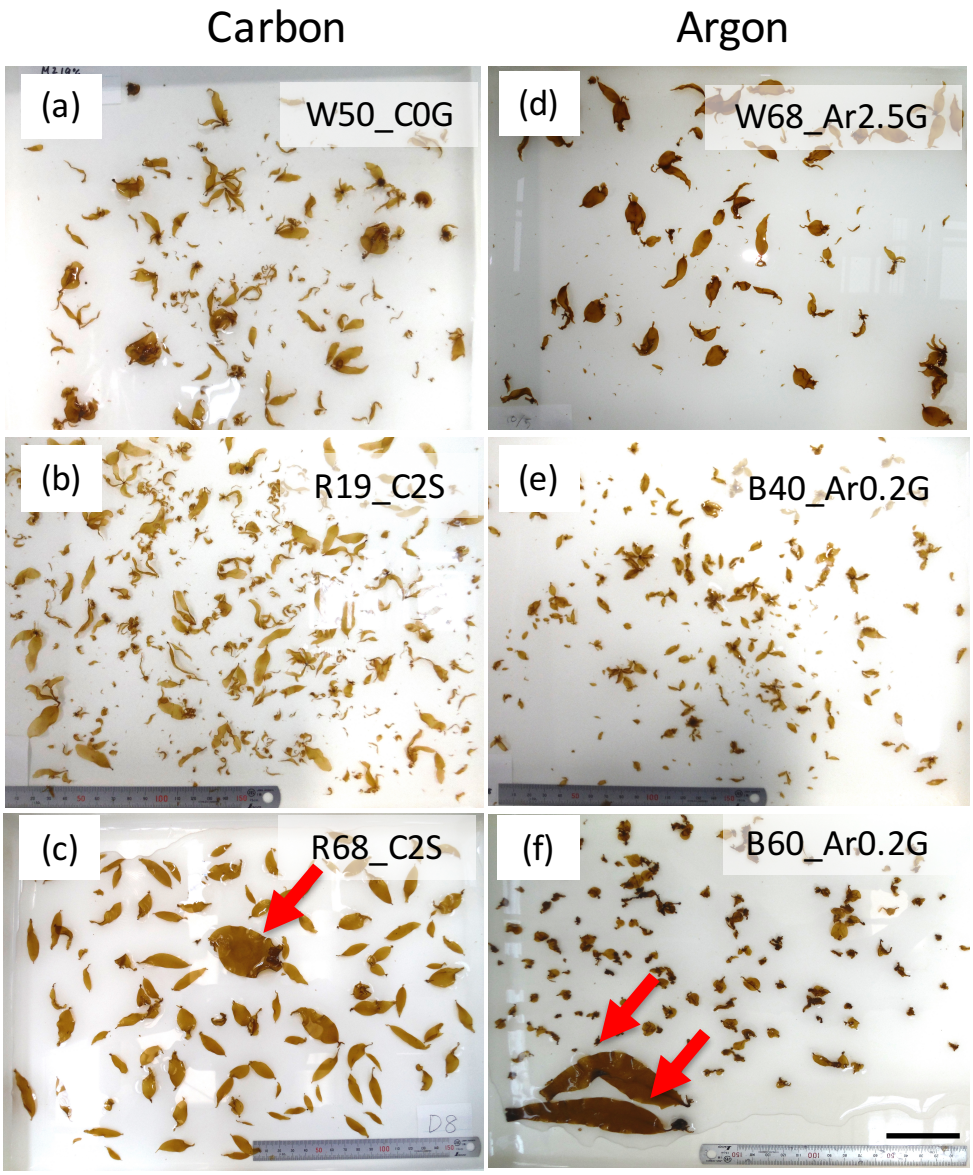


Fig. 5-4. An example photograph of M_2 sporophytes 40 days after germination. (a, b, d, e): ordinary lines of average total length 5–10 mm, (c, f): elite lines including markedly growing individuals. Bar = 50mm.

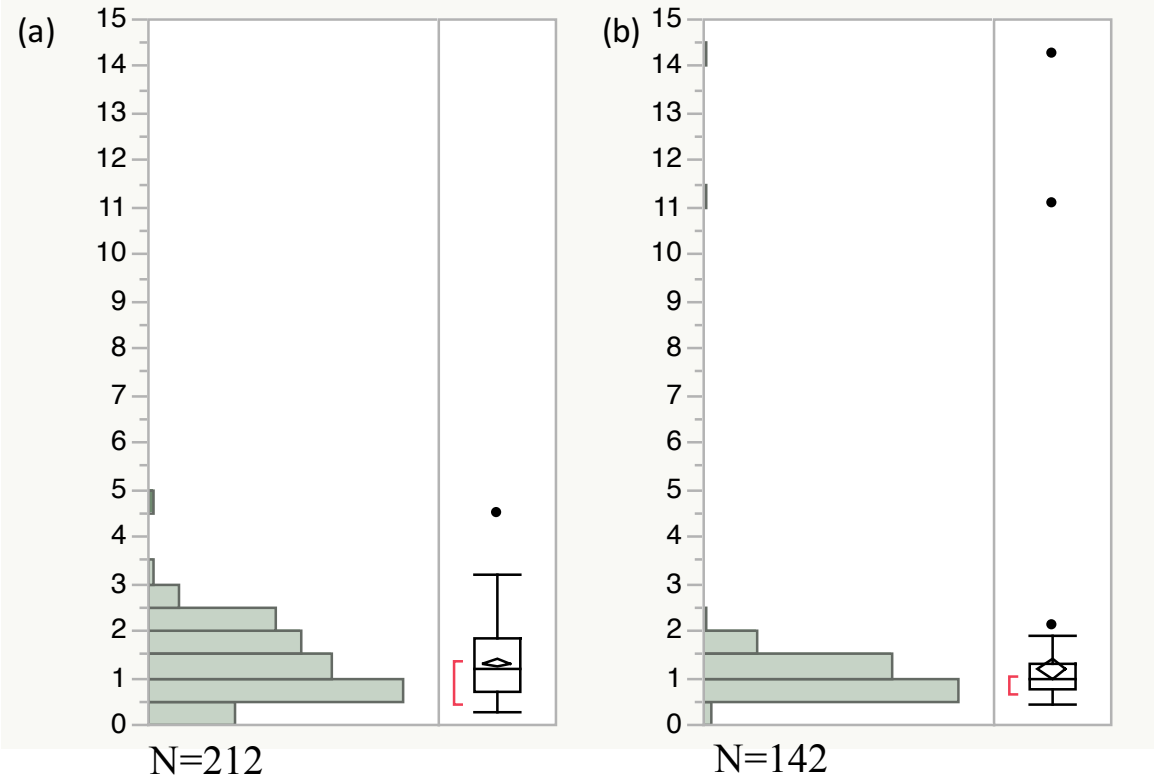


Fig. 5-5-1 Box plot of sporophytes with trait segregation 40 days after germination. (a) C5Gy-1302G-R68, (b)Ar0.2Gy-1302G-B60.

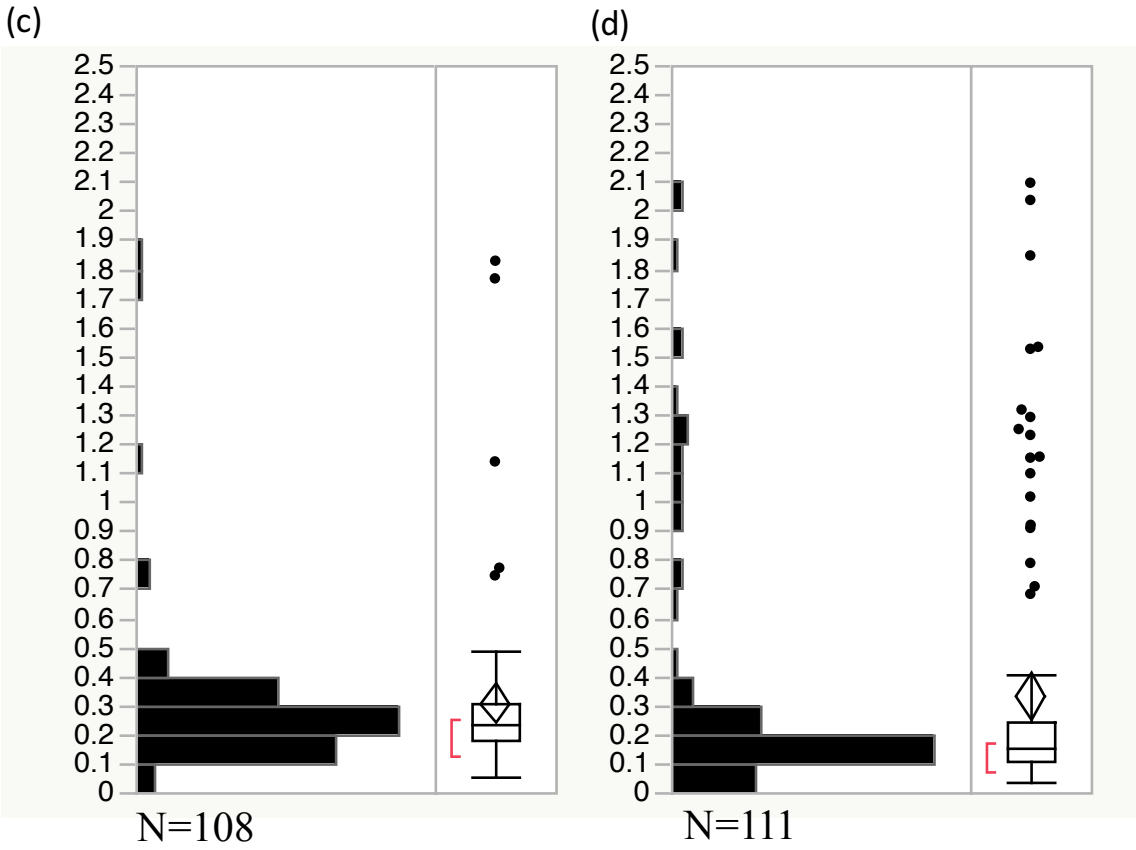


Fig. 5-5-2. Box plot of sporophytes with trait segregation 40 days after germination. (c) C2Gy-1302S-R40, (d)Ar2.5Gy-1302G-W40.

Fig. 5-6. Photographs of elite lines showing segregation of total length of sporophytes 80 days after germination. (a): C5Gy-1302G-R68, (b): Ar0.2Gy-1302G-B60, (c): C2Gy-1302S-R40, (d) Ar2.5Gy-1302G-W40. Bar=5cm.

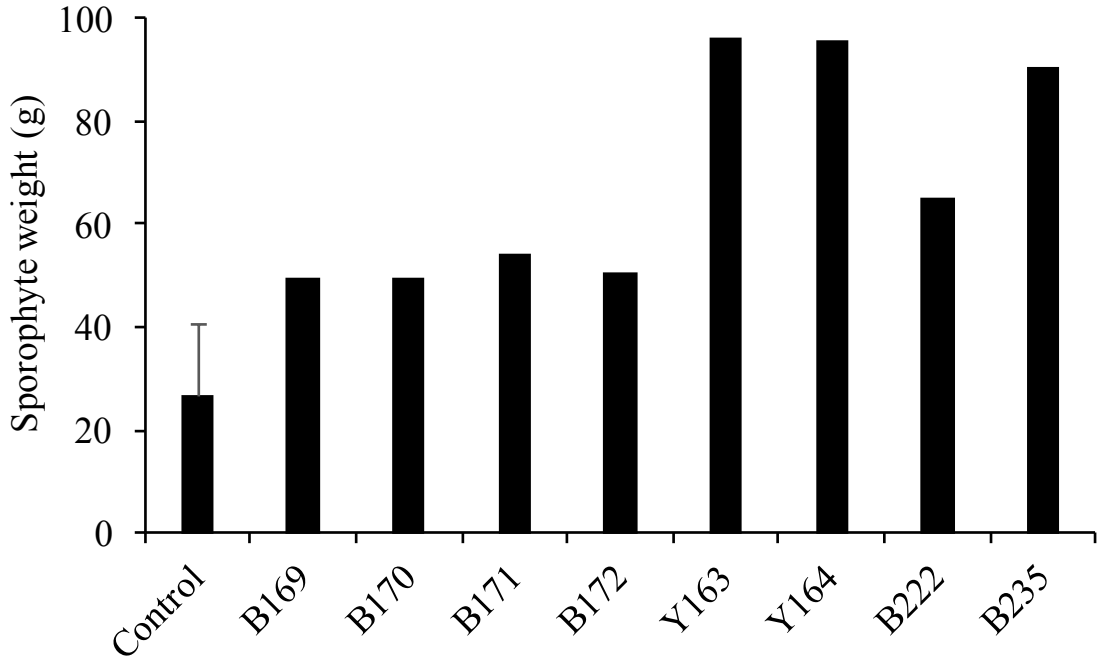


Fig. 5-7. Sporophyte weight of wild type and mutant candidates 100 days after germination in the M_2 generation. B169-172: C5Gy-1302G-R68, Y163, Y164: Ar0.2Gy-1302G-B60, B222: C2Gy-1302S-R40, B235: Ar2.5Gy-1302G-W40.

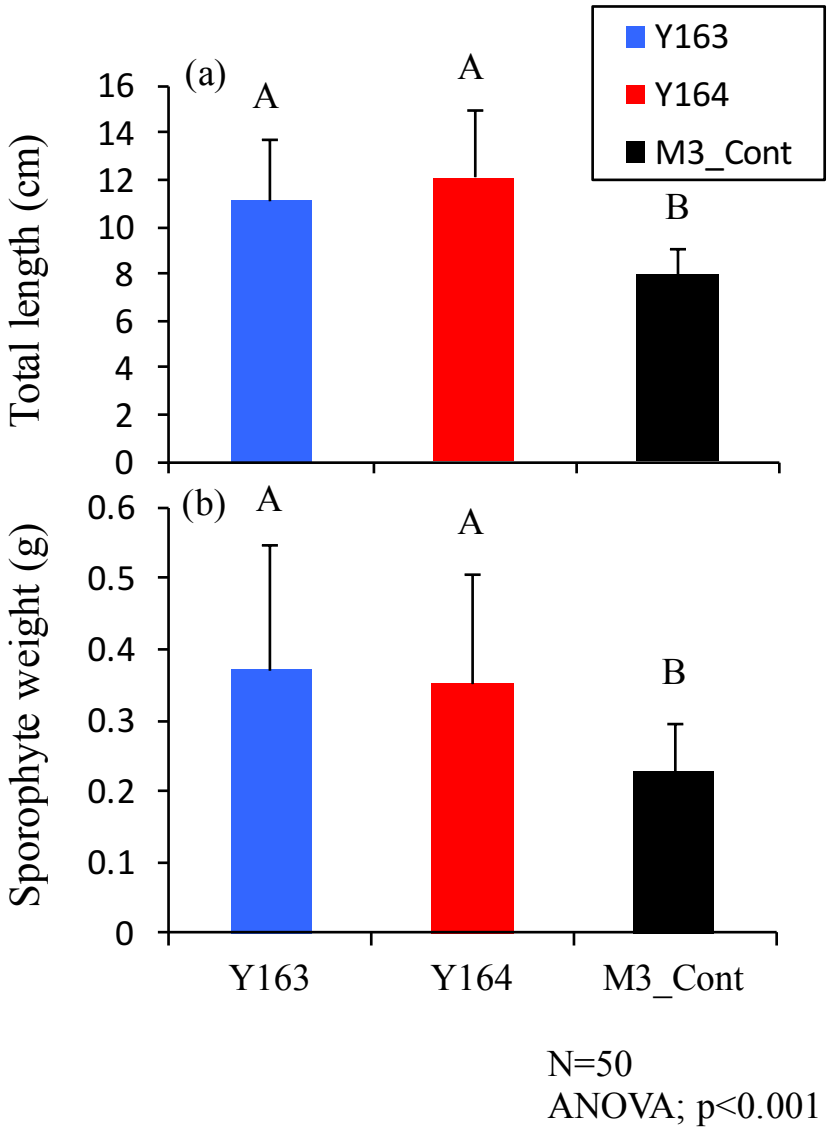


Fig. 5-8. Comparison of total length and sporophyte weight among wild type (M_3_Cont.) and mutant candidates Y163 and Y164 65 days after germination in the M_3 generation. (a): Total length, (b) Sporophyte weight.

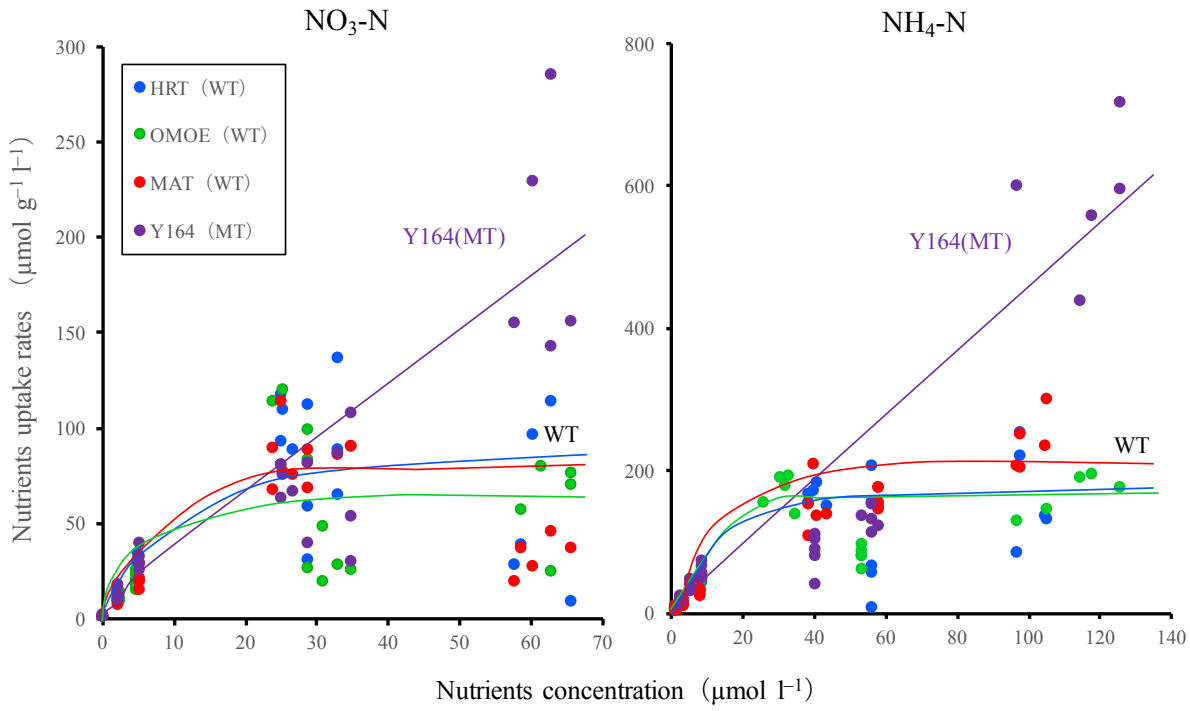


Fig. 5-9. Nutrient uptake rates of wild types and the mutant candidate Y164. (a) NO_3-N, (b) NH_4-N.

Table 5-1. Number of lines screened and of mutant candidates obtained at every germination temperature, ion and dose

Temperature	Ion	Dose (Gy)	Number of M_2 strain	Number of lines included mutant candidates as observation the separation trait in total length of M_2 plants
19	Control	0	14	0
	$^{12}C^{6+}$	0.5	2	0
		2	17	0
		5	10	1
		12.5	2	0
	$^{40}Ar^{17+}$	0.2	4	1
		1	7	0
		2.5	5	0
		5	1	0
23	Control	0	14	0
	$^{12}C^{6+}$	0.5	2	0
		2	17	1
		5	10	0
		12.5	2	0
	$^{40}Ar^{17+}$	0.2	4	0
		1	7	0
		2.5	5	1
		5	1	0

Table 5-2. Uptake kinetics of NO_3-N and NH_4-N of wild types and the mutant candidate Y164 in the M_3 generation

Nutrients	Parameters	HRT WT	OMO WT	MAT WT	Y164 MT
NO_3-N	V_{max}	89.42	78.89	81.99	4741.37
	K_s	6.92	10.76	8.51	2028.34
	V_{max}/K_s	12.91	7.32	9.63	2.33
NH_4-N	V_{max}	192.35	365.77	212.42	n.s.
	K_s	21.46	66.87	33.16	n.s.
	V_{max}/K_s	8.96	5.47	6.41	n.s.

n.s.: not significant

CONCLUSIONS

I demonstrated the following five points concerning the study of the physiological ecology and for developing a new cultivation technique for the purpose of understanding adaptation to environmental conditions and increasing the productivity of kelps:

1) The growth pattern of *S. ochotensis* could be divided into two stages: elongation periods from November to May and a maturation period in June and July. C and N produced by high photosynthetic rates and nutrient uptake rates until May were intensively translated to the meristem with the progression to summer. The nutrient uptake kinetics indicated a high affinity at high and low nutrient concentrations in winter and summer, respectively. These results suggest that *S. ochotensis* adapted to seasonal nutrient conditions in the northern Sea of Japan by changing nutrient uptake kinetics.
2) The cyclone and floating culture system (CFCS) was developed. In the CFCS, kelp can be cultivated successfully under controlled environmental conditions. *U. pinnatifida* and *S. japonica* cultivated in the CFCS exhibited morphological features identical to those grown naturally in the ocean. Plants tagged for individual identification could be cultivated in the CFCS. This system could be useful for kelp screening tests and ecophysiological study.
3) Characteristics of genetic or environmental differentiation in regional strains of *U. pinnatifida* were evaluated by the comparison between cultivation in the sea and the CFCS. On the basis of comparison of the morphological characteristics, the five strains were divided into three groups: (i) MAT, which grew and eroded faster than other strains; (ii) SIM, which had smaller blades than the other strains and (iii) HRT, OGA and NAR, which continued to grow after the erosion of MAT. These classifications were supported by the un-rooted molecular phylogenic tree produced using mitochondrial DNA sequences; these

results suggest that the characteristics had genetic differentiation. On the basis of comparison of nutrient uptake kinetics among cultivation sites, it was revealed that the nutrient uptake kinetics of *U. pinnatifida* were different. A high affinity at low nutrient concentrations in OGA and MAT indicated genetic differentiation by comparison between cultivation in the sea and the CFCS. As a new cultivation technique, I propose the mother plant selection on the basis of genetic differentiation of morphological characteristics and nutrient uptake kinetics by the evaluation of them under the CFCS cultivation.

4) Earlier (R1) and later (R2) season *U. pinnatifida* crops were selected by cultivation in the CFCS on the basis of the genetic differentiation of MAT and HRT in Chapter III. The cultivation tests in the sea were started at five different dates between September and December (Group I–V). Although the R1 and R2 strains in group I were eroded by December, these strains were larger than another strain in groups II and III and groups IV and V, respectively. These results indicated that characteristics of R1 and R2 reflected genetic differentiation of the two strains, with R1 being an earlier and R2 a later season crop. Photosynthetic rates and nutrient uptake rates of MAT and HRT were higher than for other strains at higher and lower temperatures, respectively; the physiological results supported the genetic characteristics of each strain. I propose that a breeding method using selection in the CFCS on the basis of genetic differentiation evaluated in this system is available for developing elite lines for macroalgae.
5) The optimum doses of heavy–ion beam irradiation were estimated to be 2–12.5 Gy of C ions and 0.2–2.5 Gy of Ar ions. A screening method was developed by combining the CFCS with a previous culture method. In the M_2 generation, mutant candidates with higher growth and higher-temperature tolerance than wild type were obtained. Two mutant candidates of

higher growth (strains Y163 and Y164) confirmed the characteristics in the M_3 generation; it was speculated that they were mutant characteristics. The nutrient uptake rates of Y164 were not satisfied in spite of a high concentration over 60 μM, V_{max} of NO_3-N indicated levels approximately 50 times higher than for the wild type.

PERSPECTIVE

In recent years, depletion of nutrient concentration in seawater has led to a decrease in the productivity and quality of kelps (e.g. Nishida 1999, Dan et al. 2015). It is expected that both global climate change and local environmental changes from economic and human activities has been responsible for this situation. In Europe and South America are advancing on the resource recycling cultivation system on the basis of IMTA is advancing. Therefore, evaluation of nutrient uptake kinetics for every species become increasingly important. Furthermore, the proper management of nutrient conditions at all sea sites will be needed, according to the estimation of nutrient uptake capability by macroalgae and confirmation of the balance between fisheries productivity and environmental loading. In the present study, it was demonstrated that OGA and MAT have a nutrient uptake kinetics of high affinity at low nutrient concentration on the basis of genetic differentiation. These strains will be useful for transplantation or mother plant selection in order to develop elite cultivars with low nutrient tolerance. Although characteristics of a high affinity at high nutrient concentration of NAR and AKA were expected in environmental differentiation in the present study, it is possible to obtain these characteristics as genetic differentiation. The establishment of 'algal nutrition' based on ecological physiology similar to 'plant nutrition' for terrestrial plants is required.

The results in this study may be applied to industry, and one of them has been already been put to practical use. The NH_4-N uptake kinetics categorised as a high affinity at high nutrient concentration in *S. ochotensis* has evidence from field research, where a *Saccharina* population was reforested by supplying inorganic nutrients included $(NH_4)_2SO_4$ at the sea desertification area in Hokkaido, northern Japan (Agatsuma et al. 2014). Early- and late-season crops selected

from regional mother plants of MAT and HRT in Chapter III and IV have been cultivated on the large scale for practical use in Hirota Bay, Iwate Prefecture, Japan.

Chapter II to V in the present study were investigated through the project of 'Tohoku Marine Science' founded by MEXT, Japan. After the Great Higashi-Nihon Earthquake on the 11th March in 2011, I fully realised the concern of the fishermen, 'The number of fishermen has decreased considerably. If nothing is changed in the industrial structures and the cultivation method, the *Undaria* and *Saccharina* cultivation may not be possible in decades to come.' For the *Undaria* and *Saccharina* industry, all fishery workers are faced with hard work during the short cultivation periods from harvesting to processing. Through the practical use of early- and late-season crops in this study, it will be possible to increase the productivity within the limited cultivation period and develop a new cultivation method of harvesting several times in a season. I aim to progress cultivation trials in sea farms for industrial use.

Mutation breeding had never been applied to large brown alga until the present study. From the viewpoint of the present market trend in macroalgae, it is difficult to immediately use the elite mutant cultivar for industry due to the lack of understanding of seaweed mutant breeding by customers. In the present study, heavy–ion beam irradiation could induce an elite mutant in *U. pinnatifida*; this technique will be a powerful tool in the development of basic science and industry in kelp as well, similar to in terrestrial plants. I would like to undertake phenotypic and molecular biological analysis of mutants in *U. pinnatifida* as a whole genome study of *U. pinnatifida*.

The industrial promotion of *Saccharina* and *Undaria* will bring sustainable development for food production, human health and the coastal environment. On the basis of algal nutrition

and algal breeding science, I hope to demonstrate the ecophysiological characteristics of kelps and progress the industrial study while focusing on its high productivity for social contribution.

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