Cell selection technique for establishment of low salinity tolerance strain in Pyropia tenuipedalis

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Cell selection technique for establishment of low salinity tolerance strain in *Pyropia tenuipedalis*†

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Abstract: *Pyropia tenuipedalis* is characterized by reddish thalli and direct budding from the shell substratum. The distribution of this species is limited to coastal areas in the Seto Inland Sea, Ise Bay and Tokyo Bay in Japan. In Yamaguchi prefecture, this species has been locally used for direct human consumption. Moreover, alanine and glutamic acid contents of *P. tenuipedalis* are three times and two times higher, respectively than those of *P. yezoensis* "nori". Yamaguchi Prefectural Fisheries Research Center began development of the mariculture technique of *P. tenuipedalis* in 2002, and succeeded in its commercialization in 2007. However, production has continued to decrease since 2012. More recently, intense disappearance of thalli is observed during January to February. One of the reasons for the production decrease is thought to be low salinity due to recent heavy rain fall supposedly associated with global climate change. The aim of this study was to develop low salinity tolerance strains of *P. tenuipedalis* through a cell selection technique. Punched out disks of thallus were cultured in a 10% seawater medium diluted with distilled water with modified 1/2SWM-III medium for 1 to 4 months. A few cells survived in this medium and were subsequently cultured in a 100% seawater medium with 1/2SWM-III. Surviving cells divided and regrew to thallus, and stock culture strains were established with self-fertilization. A field culture trial of the established low salinity tolerance strain and the conventional strain was carried out in Koto River estuary from December 2018 to February 2019. The low salinity tolerance strain showed better growth than the conventional strain, suggesting the efficacy of the cell selection technique for breeding. However, some of the thalli growing on the culture plates were suddenly shortened or disappeared in early January. Time-lapse observation with an underwater camera revealed that the disappearance of the thalli was due to predation by blackhead seabream, *Acanthopagrus schlegelii*. Immediate establishment of not only strains resistant to changing environments, but also effective measures for countering predation are necessary to increase production of *P. tenuipedalis*.

Key words: *Pyropia tenuipedalis*, breeding, cell selection, low salinity tolerance

Intoduction

Recently, fisheries production of *Pyropia yezoensis*, commonly referred to as "nori" in Japanese language, has decreased due to climate change impacts, such as seawater temperature rise and oligotrophication. Many researchers are developing or seeking new strains that have tolerances to the above-

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† *Pyropia tenuipedalis* was replaced to *Neopyropia tenuipedalis* by Yang *et al.* (2020).
Yang L.-E., Deng Y.-Y., Xu G.-P., Russel S., Lu Q.-Q.,and Brodie J., 2020: Redefining *Pyropia* (Bangiales, Rhodophyta): four new genera, resurrection of *Porphyrella* and description of *Calidia pseudolobata* sp. nov. from China. *J. Phycol.*, **56(4)**, 862-879.

mentioned environmental stresses (Shimada, 2010; Sakaguchi, 2011). In Yamaguchi Prefecture, *Pyropia tenuipedalis*, which is closely related to *P. yezoensis,* is an indigenous species, and is a resource used to promote local industries (**Fig. 1**). As *P. tenuipedalis* has reddish thalli unlike blackish *P. yezoensis* and directly buds from the shell substratum, this species is generally called “Aka-nori” in Yamaguchi Prefecture, and the Japanese vernacular name is “Kaigara-ama-nori”. Aka and Kaigara means red and shell, respectively in Japanese. The distribution of this species is limited to coastal areas in the Seto Inland Sea, Ise Bay, and Tokyo Bay in Japan, and this species is designated as an endangered species. In Yamaguchi Prefecture, this species has been used as an important local food resource for human consumption (Abe *et al*., 2015).

The life cycle of *P. tenuipedalis* is quite different from that of *P. yezoensis* (**Fig. 2**). Matured thalli of *P. yezoensis* releases many carpospores. Carpospores germinate and grow into filamentous conchocelis. Conchosporangia are formed on conchocelis, and many conchospores are released. Conchospores divide and grow into young thalli. Young thalli of approximately 2 mm in blade length release many monospores. Monospores grow into young thalli. After release of monospores, the thalli resumes growth (Yoshida, 1993). Therefore, *P. yezoensis* can produce a large number of spores and thalli. However, matured thalli of *P. tenuipedalis* release

Fig. 1. Photo of *Pyropia tenuipedalis* collected in January 2011.

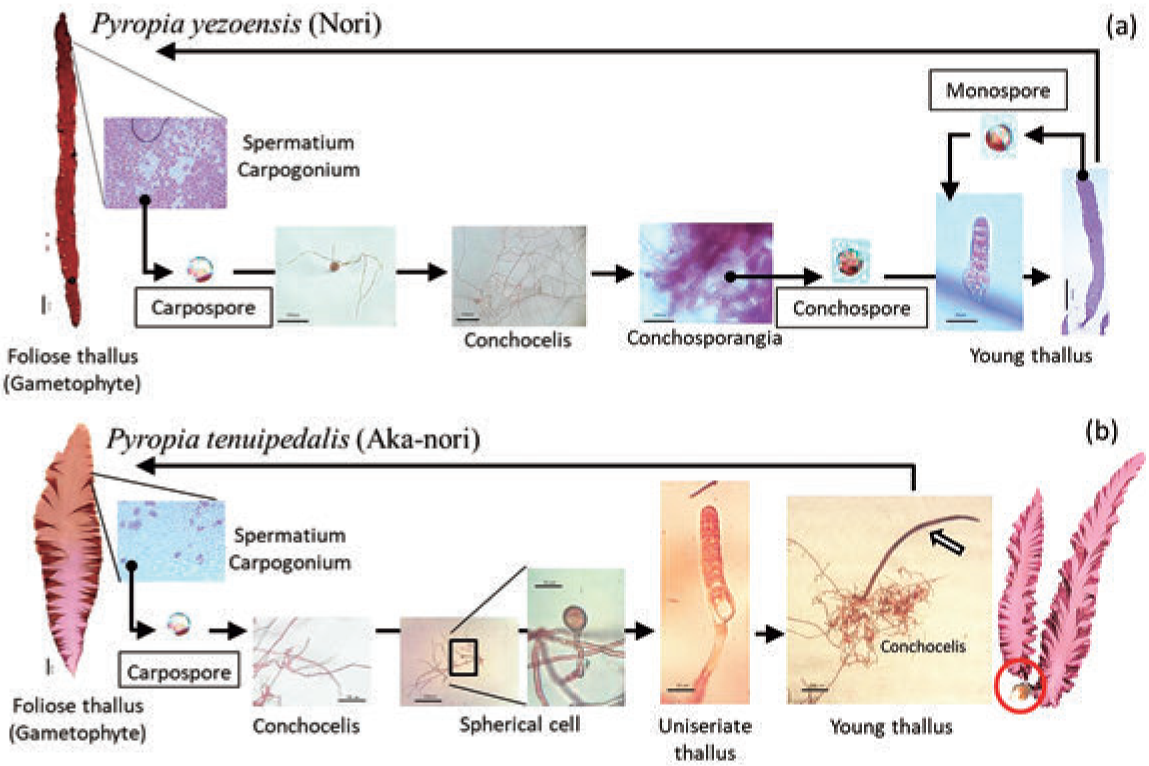

Fig. 2. Life cycles of *Pyropia yezoensis* (a) and *Py. tenuipedalis* (b).

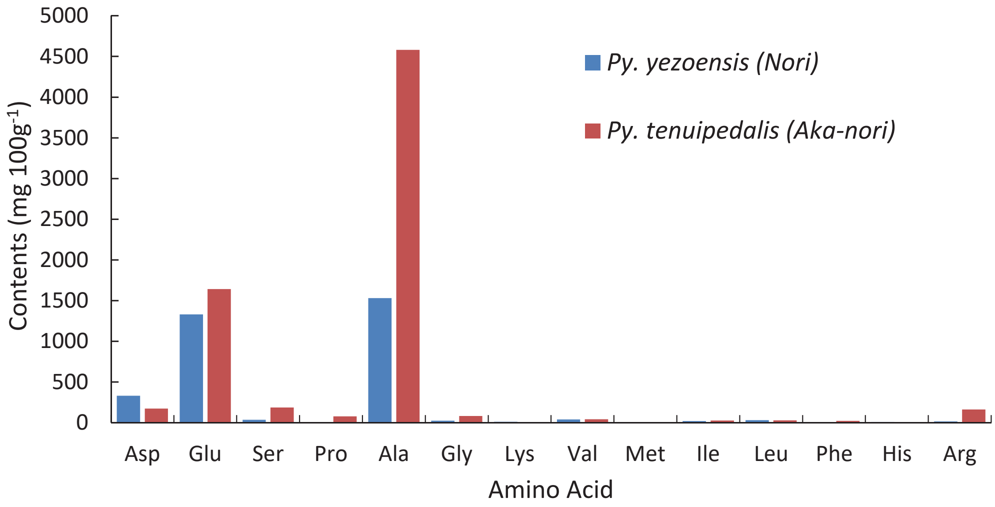

Fig. 3. Amino acid content of *Pyropia yezoensis* and *Py. tenuipedalis*.

many carpospores and becomes conchocelis, as in the case of *P. yezoensis*. However, *P. tenuipedalis* forms a spherical cell on the tip of conchocelis instead of conchosporangia. This spherical cell divides and grows into uniseriate thalli and young thalli. *P. tenuipedalis* does not have conchospore and monospore (Notoya and Kikuchi, 1993). Consequently, one spherical cell grows into one thallus.

Amino acid contents are completely different between *P. yezoensis* and *P. tenuipedalis* (**Fig. 3**). Alanine and glutamic acid contents of *P. tenuipedalis* are higher than those of *P. yezoensis*. This suggests that *P. tenuipedalis* has stronger "umami" and "sweetness" compared with *P. yezoensis*. Yamaguchi Prefectural Fisheries Research Center focused on *P. tenuipedalis* as a new mariculture species in 2002. They developed the culture plate technique for *P. tenuipedalis* mariculture and succeeded in its commercialization in 2007. This culture plate is made from calcium carbonate, and conchocelis are cultured on this plate. These culture plates are set directly on the sea bottom in the field in November every year. Thalli growing on the culture plates are harvested by hand and dried by machine from January to March, and then it become the final product. The price for the product is 1,200 Japanese yen / 10 g in dry weight. Among seaweed products in Japan, this is the most expensive.

Mariculture using the culture plates was previously carried out only in Yamaguchi Bay

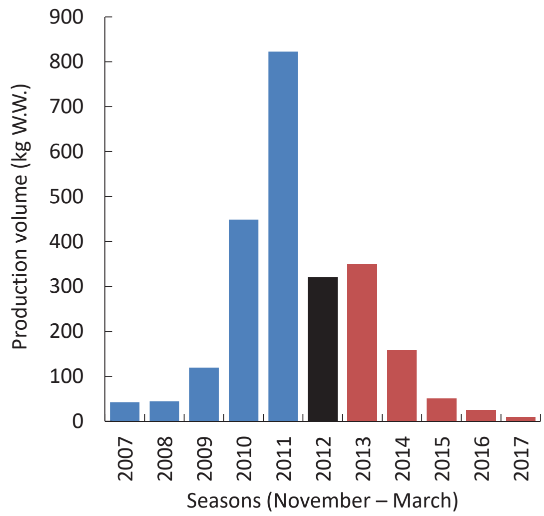

Fig. 4. Production volume of *Pyropia tenuipedalis* mariculture from November to March during 2007–2017. 2007–2011: Yamaguchi Bay (blue bar), 2012: Yamaguchi Bay and Koto River estuary (black bar), 2013–2017: Koto River estuary (red bar).

from 2007 to 2011. In 2012, it was conducted in Yamaguchi Bay and Koto River estuary. Since 2013, it has been conducted only in Koto River estuary. *P. tenuipedalis* production had the highest value in 2011 with the increase in the number of the culture plates (**Fig. 4**). However, production has continued to decrease since 2012 although the number of culture plates was kept constant. More recently, intense disappearance of thalli is observed from January to February. One of the main reasons for the production decrease is thought to be low salinity due to recent heavy rain fall. Water discharge from the Koto River Dam during the period from December to January—the growing season for *P. tenuipedalis*—clearly increased from 2008 to 2016 by about 30% on average and 2 to 3 times in maximum instantaneous flow (Water and Disaster Management Bureau of the Ministry of Land, Infrastructure, Transport and Tourism: Database of Dams: http://mudam.nilim.go.jp/home). Therefore, we identified that low salinity tolerant strains were needed to cope with this problem.

Establishment of Low Salinity Tolerant Strain of *P. tenuipedalis*

In this study, we used a cell selection technique for establishment of a low salinity tolerant strain of *P. tenuipedalis* (Abe *et al*., 2019). Punched out disks of thalli were cultured in the modified 1/2SWM-III medium prepared with 10% seawater (approximately 3 psu) diluted with distilled water for one to four months (**Fig. 5**). Under the low salinity conditions, although the majority of normal cells were bleached and eventually died, a few cells survived, which were then cultured in the modified 1/2SWM-III medium with 100% seawater. The surviving cells were allowed to divide and regrow to thalli, and stock culture strains were established with self-fertilization of a large size of single thalli.

Field Culture Trial of Low Salinity Tolerant Strain

A field culture trial of the established low salinity tolerant strain and the conventional strain of *P. tenuipedalis* was carried out in Koto River estuary from December 2018 to February 2019. Conchocelis of both the conventional strain and low salinity tolerant strain attached to the culture plates were set in Koto River estuary at the end of November in 2018. In addition, time-lapse observation at 1-hour intervals of culture plates was carried out with an underwater camera to determine the cause of the intense disappearance of the thalli.

The length of thalli in both strains were measured every 2 weeks from December 2018 to February 2019 (**Fig. 6**). The thallus lengths of the conventional and the low salinity tolerant strain were 1.0±1.0 mm and 1.3±1.4 mm, respectively on December 10th 2018. On December 25th, the low salinity tolerant strain grew larger (13.4±9.5 mm) than the conventional strain (7.0±5.4 mm). However, the

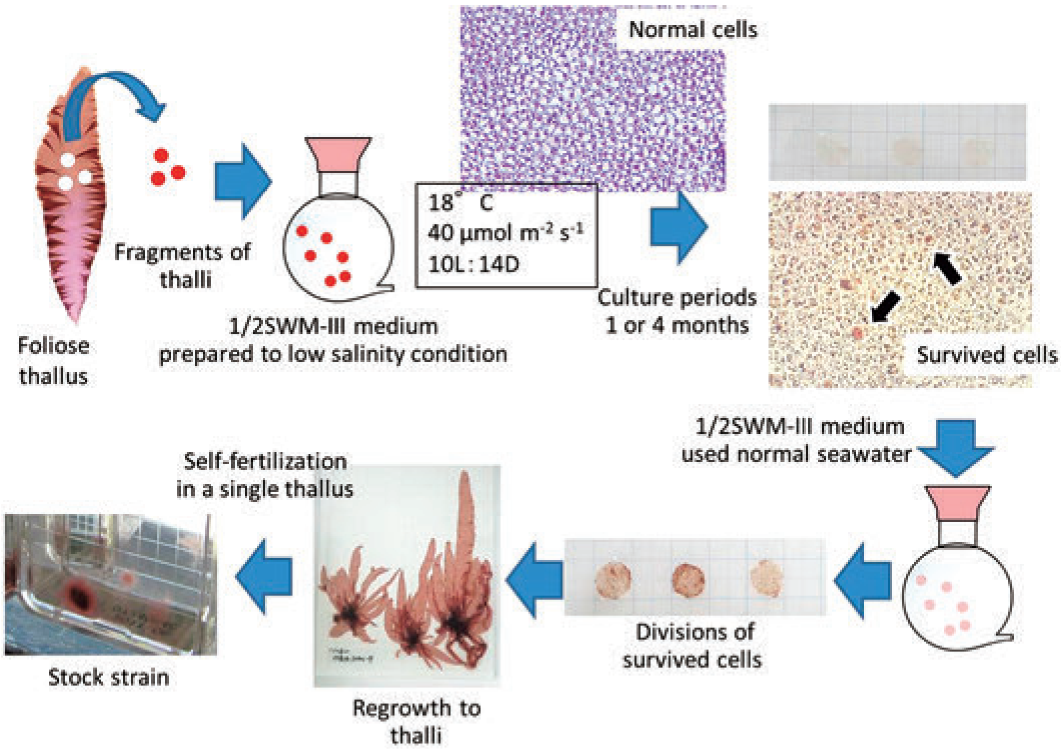

Fig. 5. Procedures of the cell selection technique used in this study.

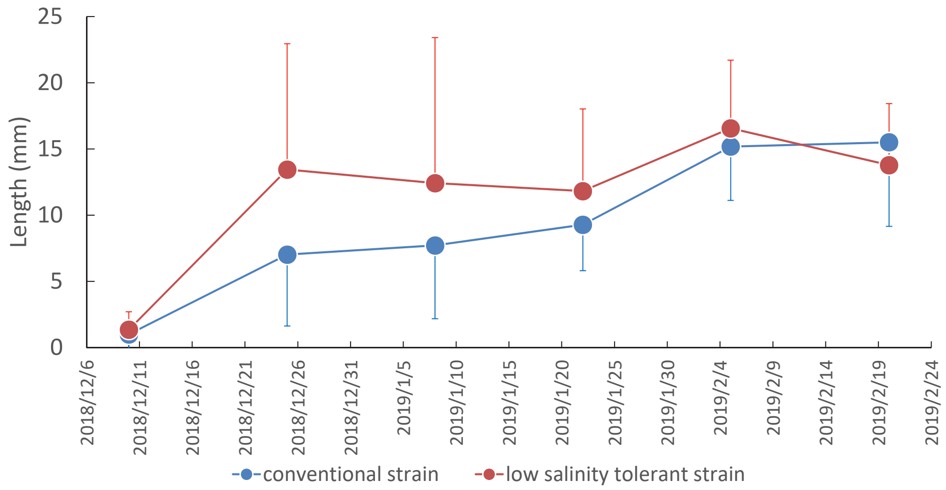

Fig. 6. The growth of thalli in the conventional and low salinity tolerant strains during the field culture trial.

length of the low salinity tolerant strain stopped growing and ranged from 12.4±11.0 mm to 16.6±5.1 mm from January to February. The conventional strain grew gradually through the field culture trial up to 15.5±6.4 mm. Thus, the low salinity tolerance strain showed better growth than the conventional strain, suggesting the efficacy of the cell selection technique for breeding.

Intense Disappearance of the Thalli

Some of the thalli growing on the culture plates suddenly shortened and disappeared in early January. Time-lapse observation with an underwater camera revealed that the disappearance of the thalli was due to predation by blackhead seabream, *Acanthopagrus schlegelii* (**Fig. 7**). Blackhead seabream appeared in 15% of the photos taken in daytime. It was estimated that the population of blackhead seabream was large in this area from the relationship between the interval of time-lapse photography and the appearance of blackhead seabream in the photos. The intense disappearance from January 4th to 5th was remarkable (**Fig. 8**). Thalli on the culture plates were considerably shortened over one night.

We unintentionally found a countermeasure for fish predation in a field trial that was conducted to study work efficiency of the seaweed deployment. Usually, the culture plates are placed directly on the sea bottom. Therefore, activities for mariculture

Fig. 7. Photo of blackhead seabream (*Acanthopagrus schlegelii*) grazing *Pyropia tenuipedalis* growing on culture plates.

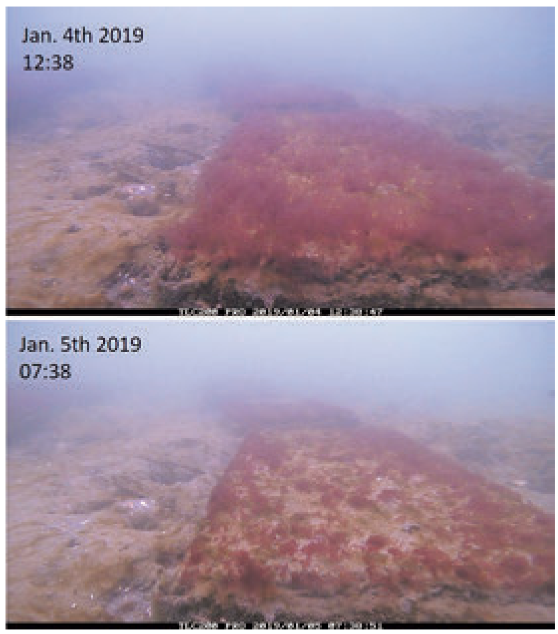

Fig. 8. Photos of *Pyropia tenuipedalis* thalli on a culture plate on January 4th and January 5th 2019.

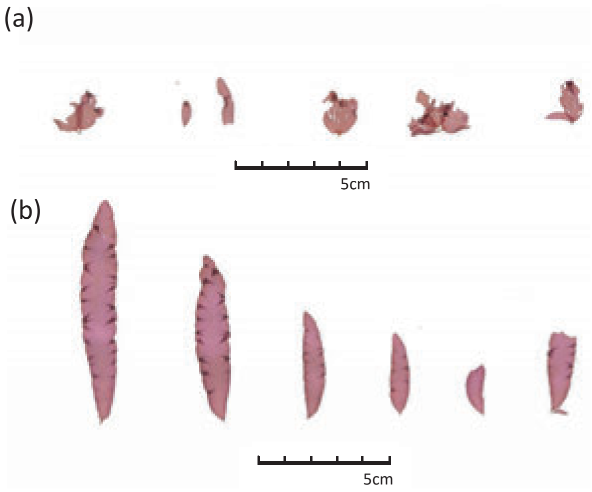

Fig. 9. Specimens of *Pyropia tenuipedalis* collected on January 7th 2019, including: (a) thalli on the culture plates set directly on the sea bottom, and (b) thalli on the culture plates placed within a crab basket. Bars indicate 5cm.

management, such as cleaning sediments on the culture plates and harvesting thalli, are restricted during low tide at the spring tide due to limited accessibility. In order to enable the operator to perform the activities regardless of the tidal height, the culture plates were placed within a crab basket that can be lifted up on the boat. As part of the study, we compared the growth between thalli set directly on the sea bottom and those placed within a crab basket (**Fig. 9**). We found that the thalli within a crab basket grew better and that the crab basket was effective in physically protecting the thalli from fish predation.

Conclusion

In Yamaguchi Prefecture, *P. tenuipedalis* has been used as a local fisheries resource, and mariculture of this species was intended as a unique local industry. However, intense disappearance of the thalli has been observed from January to February for the past few years, which is a hindrance to the successful industrialization. One of the reasons for the production decrease was thought to be low salinity due to recent heavy rainfall. We established low salinity tolerant strains by developing the cell selection technique for breeding. A field culture trial showed the established low salinity tolerant strain had better growth than the conventional strain in the early culture period.

Intense disappearance of *P. tenuipedalis* due to predation by omnivorous fish were observed by time-lapse observation with underwater camera set in the field culture trial site. For *P. tenuipedalis* mariculture using culture plates, it is advisable to physically protect the seaweed from fish predation in a cage.

Reference

Abe M., Murase N., Hatama T., Shikano Y., and Kanai T., 2015: New record of *Pyropia tenuipedalis* from Koto river estuary, Yamaguchi prefecture. *J. Nat. Fish. Univ.* **63**, 244–248. (in Japanese with English abstract)

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Notoya M., and Kikuchi N., 1993: *Porphyra tenuipedalis* Miura, in "An illustrated atlas of the life history of algae" (ed. by Hori T.), Vol. 2, Uchida Rokakuho Publishing Co., LTD., Tokyo. pp.214–215. (in Japanese)

Sakaguchi K., 2011: New nori strain with high water temperature tolerance in Mie prefecture "Mie no AKARI" (Mie ken no kousuion taisei kuro nori no shin hinshu "MIE NO AKARI"). *Research journal of food and agriculture,* **34**, 42–43. (in Japanese)

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Yoshida T., 1993: *Porphyra yezoensis* Ueda, in "An illustrated atlas of the life history of algae" (ed. by Hori T.), Vol. 2, Uchida Rokakuho Publishing Co., LTD., Tokyo. pp.216–217. (in Japanese)

Annotated Bibliography of Key Works

(1) Abe M., Murase N., Hatama T., Shikano Y., and Kanai T., 2015: New record of *Pyropia tenuipedalis* from Koto river estuary, Yamaguchi prefecture. *J. Nat. Fish. Univ.,* **63**, 244–248. (in Japanese with English abstract)

We collected reddish foliose thalli from Koto River estuary, Yamaguchi Prefecture. In order to identify the species, we carried out field collections of the foliose thalli, morphological observation in culture and PCR-RFLP analysis using the two regions of the partial mitochondrial DNA. As for the morphological survey, the spherical cells were formed at the tips of conchocelis and developed to foliose thalli. The fragment patterns of this species in the PCR-RFLP analysis matched with *Pyropia tenuipedalis*. In the present study, the foliose thalli collected at Koto River were identified as *P. tenuipedalis*, which is an endangered species distributed along the coast of Japan. This species was newly recorded from Koto River estuary, Yamaguchi Prefecture.

(2) Nakayama T., Abe M., Murase N., and Shikano, Y., 2017: Influence of salinity on growth of red alga *Pyropia tenuipedalis* and *Pyropia yezoensis* foliose thallus. *Aquacult. Sci.,* **65**, 321–330. (in Japanese with English abstract)

Pyropia tenuipedalis is a new culture species in Yamaguchi Prefecture, Japan. The habitat of this species is more brackish as compared with that of *P. yezoensis*, which is the common species in Japanese *nori* mariculture. In this paper, the authors investigated the relationship between salinity and growth of *P. tenuipedalis* and *P. yezoensis*. It was revealed that *P. tenuipedalis* tolerates lower salinity in comparison with *P. yezoensis*.

(3) Abe M., Murase N., Hatama T., Shikano Y., and Kanai T., 2017: Environmental characteristics of *Pyropia tenuipedalis* (Miura) Kikuchi et Miyata growing at Yamaguchi Bay, Yamaguchi Prefecture. *J. Nat. Fish. Univ.,* **65**, 19–29. (in Japanese with English abstract)

This study surveyed environmental characteristics of *Pyropia tenuipedalis* foliose thallus growing in Yamaguchi Bay, Yamaguchi Prefecture from November to March, 2010–2014. Young foliose thallus of this species appeared in December and grew to their maximum length from January to March. In March, mature thalli were observed. Water temperature from November to March was usually within the range of 6–16°C, but the temperature was lower in early December in 2012 than other years. Light reaching the growing depth of this species was 6.2 ± 3.1% of the level at water surface at high tide. The concentration of dissolved inorganic nitrogen at the sampling site was within the range of 8.8–68.3 μmol L^{-1}, which was approximately 10 times higher than the level that is assumed to cause the discoloration of *P. yezoensis*. Water temperature reduced at a rate of 0.33°C day^{-1} from November to December in 2012–2013, which was faster than the rate in other years. In this study, it was suggested that the growth of thalli from spherical cells was inhibited by a long- or short-term rapid water temperature decrease that occurred from November to December.

(4) Abe M., Murase N., Nakae M., Nakayama T., Nakagawa M., and Shikano Y., 2018: Water temperature characteristics in growth of filamentous thallus and formations of spherical cell, uniseriate and foliose thallus of *Pyropia tenuipedalis* (Miura) Kikuchi et Miyata. *J. Nat. Fish. Univ.,* **66**, 81–88. (in Japanese with English abstract)

We investigated the water temperature effects on growth of filamentous thallus and formations of spherical cell, uniseriate and foliose thallus of *Pyropia tenuipedalis* with culture experiments. Optimal growth of filamentous thalli was observed at 20°C. Moreover, optimal water temperatures for formations of spherical cells, uniseriate and foliose thalli were 20°C, 15–20°C and 15°C, respectively. Optimal water temperatures of each life stage were different. At 10°C, the formations of uniseriate and foliose thalli were suppressed. At 25°C, almost all thalli had morphological abnormalities. Furthermore, decreasing temperature to 15°C from 20°C enhanced formation of uniseriate and foliose thalli. It was thought that low production in 2012 resulted from suppressed formation of uniseriate and foliose thalli at less than 10°C.

(5) Murase N., Abe M., Fukudome K., Nakagawa M., and Shikano Y., 2018: Influence of temperature on the growth of red alga *Pyropia tenuipedalis* thalli. *J. Nat. Fish. Univ.,* **66**, 215–220. (in Japanese with English abstract)

This study was designed to clarify the optimal temperatures for growth in uniseriate thalli and foliose thalli of *Pyropia tenuipedalis* under laboratory culture at 5°C intervals from 10°C to 25°C or 30 °C. The optimal temperature for uniseriate thalli developed from a spherical cell at the two-cell stage were 15°C and 20°C. The optimal temperature for foliose thalli allowed to develop from young blades with a length of approximately 6 cm was 15 °C. It was suggested that the shift in the optimal temperatures from uniseriate to foliose thalli stages was related to the seasonal reduction of water temperature from autumn to winter.