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Evaluation of the morphological characteristics and culture performance of *Cladosiphon okamuranus* strains

Running title: Morphology of *Cladosiphon* strains

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Abstract

This study aimed to determine whether morphological differences of *Cladosiphon okamuranus* strains at different aquaculture sites were inherent characteristics and to select useful forms for aquaculture production and processing. Three candidate strains with potentially excellent morphological characteristics, i.e.,ON, SY and KT, were selected from six local strains collected from Okinawa Islands. Then, these three candidate strains were transplanted to four aquaculture sites to evaluate their characteristics. The thallus length was significantly larger (e.g., 1.6–2.1 times) in the SY strain than in the ON strain in each area and the density of the primary lateral branches of the latter was significantly (e.g., 2.4–5.9 times) higher than that of the other two strains. The SY and ON strain characteristics were also distinguished by a comprehensive evaluation of eight traits with principal component analysis. Conversely, the KT strain tended to have an intermediate length between ON and SY strains, The biomass yield of the SY and KT strains was higher than that of ON strain. These results, indicating that some morphological differences are intrinsic strain characteristics, will provide information to aquaculture producers using appropriate strains to maximize the unit yield and thalli quality.

Keywords: *Cladosiphon okamuranus,* strain selection, morphological characteristics, seaweed aquaculture, Mozuku

Abbreviations: (Strain name) ON, Onna; KT, Katsuren; IZ, Izena; KM, Kumejima; IG, Ishigaki, SY, Suiken Yamada

Introduction

Cladosiphon okamuranus Tokida (standard Japanese name: Okinawa mozuku) is a subtropical brown alga that is ,endemic to the Ryukyu Islands in Japan (Toma, 1993). In this site, natural seaweeds were traditionally used as an ingredient in home cooking and court cuisine of the Ryukyu Kingdom (Ohno and Largo, 2006; Sudo, 2012). Cultivation of the species has been actively developed since the 1970s to meet increasing national demands for this seaweed as a healthy food with low calories and high in dietary fibers. The alga is an acknowledged source of fucoidan, a sulphated polysaccharide known as a health functional ingredient (Shinmura and Yamanaka, 1974; Toma, 1991, 1993; Moromizato et al., 2005; Sudo, 2012). Consequently, aquaculture of this species in Japan has gradually increased. The amount of Mozuku produced had a fresh weight (FW) of 16,470 tons as of 2018, >90% of which came from *C. okamuranus* (Toma, 1993). Mozuku worth 4.3 billion JPY (40 million USD), and is currently the fourth most cultivated species in Japan after the *Neopyropia* spp.(251,362 tons FW, 94.2 billion JPY = 864 million USD), *Undaria* spp.(45.099 tons FW, 12.1 billion JPY = 111 million USD), and *Laminaria* spp.(21,812 tons FW, 10.4 billion JPY = 95 million USD) (Ohno and Largo, 2006, Hwang et al., 2019, Ministry of Agriculture, Forestry and Fisheries, Japan, 2019, IMF, 2022). In recent years, the consumption of seaweeds has increased due to increased health-consciousness. The aquaculture producers of *C. okamuranus* should increase the unit biomass yields, whilst retaining the quality of the thalli, as demanded by processors and consumers. However, to achieve this goal, two problems must be solved and these are highlighted below.

One of the problems of *C. okamuranus* aquaculture is its unstable production. The annual production of 'Mozuku' has been fluctuating between 8 - 22,000 tonnes FW since 2010 (Ministry of Agriculture, Forestry and Fisheries, Japan 2019). One of the primary causes of these fluctuations are poor growth and loss of cultured thalli from their aquaculture nets. In part this is due to strong wave action during monsoons and typhoons in the aquaculture sites. Cultivated thalli can be broken away from the nets, significantly decreasing the production; strong waves have directly caused these declines (Moromizato, 2005). However, such declines are sometimes observed even without the influence of extreme weather conditions, requiring further studies on causes. Another problem is that in processing, there are significant decreases in the process yield and texture during the salting process owing to variability in the quality of raw thallus material such as thickness, firmness and amount of mucilage (Okinawa Prefecture 2012). The most current processing of Okinawa mozuku uses salting for storage, and when thin and soft thalli are mixed together, tearing and disintegration occurs. Operators involved in the fishery industry are demanding the development of techniques for the stable cultivation of highly productive thalli that can grow well in various environments, whilst also providing high-quality thalli with thicker and firmer branches that can withstand processing with salt. To solve the problems related to the unstable production and inconsistent processing quality, it is considered that the selection of superior strains with high productivity and quality will become a key technique (Moromizato, 2005; Hwang et al., 2019) and has been performed in aquaculture of other seaweeds for their breeding (FAO 2019, Robinson et al. 2013). However, only few studies have been conducted on the selection of *C. okamuranus* strains (Hwang et al., 2019).

Some studies have reported geographical variation in morphological characteristics and productivity. Shinmura (1977) compared the morphotypes of wild populations collected from five sites in the Ryukyu Archipelago and found differences in thallus length, diameter of the main axis and number of lateral branches from different areas. In addition, Toma (1991, 2004, 2012) showed that the unit biomass yield per cultivation net for this species differed amongst sites, and Moromizato(2005) investigated the environmental differences such as the depth and wave action in those areas. However, these reports focused on morphological differences from a taxonomic perspective, or the influence of environmental factors on the biomass yield. Differences in the characteristics of strains were not considered. Meanwhile, aquaculture producers and processors have empirical knowledge of differences in morphological or processing characteristics that depend on the production area, i.e., thicker thalli being harvested on the east coast of Okinawa main island and longer thalli in deeper aquaculture areas. A possible factor for the difference in these traits is due to potential ecotypes. Some aquaculture producers collected germlings from their cultivated thalli with high unit yields or favourable growth, which were then repeatedly used as local strains for aquaculture. They may have empirically distinguished the differences in ecotype traits and used them as a culture strain. Genome sequencing of *C. okamuranus* revealed some strains have genetic differences (Nishitsuji et al., 2020). However, to the best of our knowledge, there are no reported studies that have quantitatively evaluated trait differences among different of *C. okamuranus* strains. Therefore, aquaculture producers still require information to understand the traits of their strains.

It is essential to determine the differences in traits that are expressed owing to strain-specific characteristics and to select strains with superior productivity and quality. The main evaluation indicators for the strain selection are the thallus length and density of lateral branches, which are related to the unit biomass yield per net, along with thickness and firmness of main axis and lateral branches, which affect the process yield during salt processing(Table 1).

In this study, we first collected six local strains of *C. okamuranus* from representative aquaculture sites in Okinawa Prefecture and selected three candidate strains with potentially superior traits for aquaculture production and processing. Then, we transplanted the candidate strains to major aquaculture areas in order to investigate whether each of the characteristics was expressed as strain-specific characteristics and evaluated how these characteristics could be useful for aquaculture producers.

Material and Methods

Collection of local strains

Specimens of six local strains of *Cladosiphon okamuranus*, namely, IZ, ON, SY, KT, KJ and IG, were collected from five major aquaculture sites, Izena, Onna, Katsuren, Kume Island and Ishigaki Island in Okinawa Prefecture between 7 May 2009 and 17 May 2009 (Fig. 1). Just before harvesting the thalli at each site, a representative sampling was conducted. A 10-cm long piece of the aquaculture net with the entire thallus bodies was cut off from a densely growing part of the net to obtain multiple local strain specimens from each site. Since some wild populations were distributed in some aquaculture areas where nets are installed, the net piece was carefully selected by checking the morphology of the thalli using a diving survey to prevent the collection of wild species attached or mixed on the net. The collected piece with thalli was stored in a plastic bag and promptly transported to the laboratory under refrigeration and then frozen at −30°C (Fig. S1).

Morphological measurements

Before the morphological traits were measured, the frozen net pieces were thawed in a plastic bag under running water, and ten intact individuals were selected to evaluate the form of a complete condition. The thickest branch growing from the base of the thallus was defined as the main axis, and based on this, the branches departing from the main axis with a length of >3 cm were defined as the primary lateral branches, the branches departing from the primary lateral branches were defined as secondary lateral branches and the small branches of <1 cm at the tip of the thallus were defined as branchlets (Fig. 2). Eight characters were measured: thallus length(1); diameter of main axis (2); primary lateral branch (3) and secondary lateral branch(4) as thallus thickness; density of primary lateral branches (5) arising from the main axis and the branchlets (6) at the tip of the primary lateral branch; , and breaking strength of the main axis (7) and primary lateral branch (8) as firmness. Among these characteristics, the thallus length was measured from the main axis using a caliper. The thickness was measured by the diameters of the main axis, primary lateral branch, and secondary lateral branch at the thickest part of each branch magnified using a profile projector (Nikon V-12A) and a caliper. The density of the primary lateral branches was measured by counting the number of primary lateral branches on the basal 10 cm of main axis. The density of branchlets was measured by counting the number of branchlets observed in the apical 5 cm of the longest primary lateral branch. The firmness was measured by the breaking force (N) at the thickest part of the main axis and lateral branches using a texture analyser (Rheo Meter CR-500DX, Sun Scientific Co., Ltd., Tokyo, Japan No. 10 probe). The breaking force was measured as the maximum force recorded in a force-tie curve obtained from the analyser during the thallus compression using a probe. Each trait was measured once per individual.The differences in the measured characteristics of each local strain were compared using analysis of variance and Tukey–Kramer test ($P < 0.05$), and all data were reported as mean ± standard deviation. Grouping of strains based on traits was attempted using principal component analysis (PCA), which is applied as a non-parametric approach (cf. Hayashi et al. 2017) in R ver. 3.4.3 software (R core team 2016).

Transplant experiment

The three candidate strains, ON, KT and SY, considered superior and selected from among six local strains based on the evaluation in the Results and Discussion sections were used for experiments. Neutral zoospores, which were released from plurilocular zoosporangia on a 2n sporophyte (Shinmura 1977), were isolated from a part of the individual that showing the maximum thallus length in each of the collected strains (Fig. 3). The germlings from neutral zoospores were sub-cultured on agar medium following Moromizato (2001, 2003, 2005). Transplant experiments were conducted between December 2009 and May 2012 at four aquaculture sites where this species is primarily cultivated (Fig. 1). Before the experiments, free-living germlings of each strain were cultured in seawater medium enriched with KW21 solution (Daiichi Seimou Co., Ltd., Kumamoto, Japan) for seed collection following Moromizato (2003). The seeding was performed in 500 L polycarbonate tanks on land at each transplanting site. The three tanks, one for each strain, were filled with sterilised seawater. Ten culture nets were placed into each tank and the prepared free-living germlings of each strain were added into the respective tank to allow neutral spores to attach to the nets. The size of the culture net was 1.5m × 20m for Izena, Motobu, and Kumejima, and 1.2m × 18m for Onna Village, which were typical sizes for each aquaculture area. Two polyethylene terephthalate (PET) plates (10 × 20 cm) were also placed in each of the tanks containing the nets in order to observe the number of attached spores and discoidal germlings (microthalli), which are an indicator of seeding density. An air bubbler was placed at the center of the tank to provide gentle aeration. The seed collection period was 10–20 days. After seed collection, two PET plates for each strain were brought to the laboratory under refrigerated conditions. The average seeding density was calculated by counting the number of discoidal microthalli in a total of 20 gridded sites by observing both sides of two PET plates at the five sites each per single side of the plate using a microscope. The grid was established by dividing the plate into five sections in the longitudinal direction, and the center of each grid was observed.

The nets with attached microthalli were then moved to the seabed of the nursery grounds in the aquaculture site for intermediate cultivation until the microthalli grew into upright macrothalli with a length of 1–2 cm (Fig. S2). Next, they were transferred to the main cultivation ground to continue growing until harvest (Fig. S2, Table 2). The period from spore collection, intermediate cultivation, and main cultivation to harvesting was based on the standard aquaculture process in the Okinawa Prefecture (Okinawa Prefecture 2012). At the time of harvesting, 10-cm sections of aquaculture net, with the entire thalli, were removed from the most densely growing parts of the net, for each strain. The net samples with the attached thalli were brought to the laboratory, as previously described in 'Collection of local strains' and the measurements of ten intact thalli for each strain and statistical analysis of the eight characteristics were performed as described in 'Morphological measurements'. In addition, all thalli on the nets were harvested using a suction pump and the fresh weight from ten culture nets of each strain was measured to obtain the total biomass yield per net. To compare the average biomass yield per net reported in previous findings, the total biomass yields obtained in the transplant experiments were divided by the number of nets harvested to calculate the average yield per net for convenience.

Results

Characteristics of the six local strains

The thalli of the IZ and SY strains were significantly larger than those of the ON strain ($p < 0.01$), which had the smallest mean value (Table 3). Therefore, IZ, SY, and ON were classified into two types: large and small. The mean lengths of KT, KM and IG strains were intermediate between the large and small types. The diameters of the main axis and primary and secondary branches of the SY strains were thicker than those of the ON and KM strains, respectively ($p < 0.01$). After the SY strain, KT was significantly thicker in the main axis and primary lateral branches than the ON and KM strains ($p < 0.01$). The mean density of lateral branches and branchlets in the ON strain was significantly greater than those of the other five strains ($p < 0.01$). The breaking force of the main axis of the SY and KT strains was significantly higher than that of the IG strain ($p < 0.01$), and the that of the primary branch of the SY strain was significantly greater than those of the IZ and IG strains ($p < 0.01$).

The cumulative contribution of the PCA was 43.4 and 63.2% for the first and second principal component, respectively (Table S1). In the first principal component, the factor loadings of the density of branchlets and lateral branches indicated high absolute values of 0.71 and 0.54, respectively, in the positive direction, and the factor loadings of the thallus length and the main axis, first lateral branch and secondary lateral branch diameters indicated high values of −0.78, −0.83, −0.83 and −0.69, respectively, in the negative direction (Fig S3). In the second principal component, the factor loadings in the diameter of the secondary lateral branch, breaking force of the main axis and breaking force of the primary lateral branch indicated high values of −0.51, −0.63 and −0.64, respectively, in the negative direction. The distribution of the principal component scores based on these data evidently showed that the ON and SY strains were located on the positive and negative ends of the first principal component axis, respectively (Fig. S3). The KT and IZ strains had smaller distributions of principal component scores in both the first and second principal components than the other four strains, and the KT strain was located more in the negative direction of the second principal component than the IZ strain.

Characteristics of transplanted strains

As a result of transplant experiments using three strains, ON, SY and KT, the thallus length of strain SY was significantly larger than that of ON and KT in the three experimental sites of Izena, Onna and Kumejima, except for Motobu, where the thallus of the ON strain was eventually washed away for some reasons during cultivation (Fig. 4). The most substantial differences were found in the mean values of thallus length in the SY and ON strains, SY, i.e., 2.1, 1.6 and 1.8 times longer than ON in Izena, Onna, and Kumejima, respectively. Conversely, the density of the primary lateral branches of the ON strain was significantly higher than that of the SY and KT strains ($p < 0.001$). The largest differences (i.e., 2.4–5.9 times differences) were found in the mean values of the ON and SY strains. The SY and KT strains had significantly lower branchlet densities than the ON strain in Izena and Onna ($p < 0.01$), although no difference was observed in Kumejima. More specifically, the KT strain had a lower branchlet density than the SY strain in Izena and Motobu ($p < 0.01$). KT and SY strains had thicker main axes, primary and secondary lateral branches than the ON strain in Onna and Kumejima, although no significant difference was observed in Izena ($p < 0.01$). Furthermore, in Motobu, the diameter of each branch of the KT strain was thicker than that of the SY strain ($p < 0.01$). In terms of breaking force, the main axis and primary lateral branches of the KT strain showed higher values than those of the ON and SY strains in Kumejima and Motobu ($p < 0.01$). In addition, the primary lateral branches of the SY and KT strains have significantly higher breaking force than those of the ON strain in Onna. There was no significant differences in Izena. These characteristics were visually recognised based on the external appearance (Fig. 5).

The results of the PCA showed that the cumulative contribution of the first and second principal components ranged from 52.7 - 59.8% among the sites (Table S2). For the samples from Izena, factor loadings of the first principal component had high absolute values for the density of primary lateral branches (0.73) as well as density of branchlets (0.65) in the positive direction and for the thallus length (−0.82) as well as diameter of the primary lateral branch (−0.66) in the negative direction (Fig. 6). The second principal component showed high values in the negative direction for the diameter of the secondary lateral branches (−0.51), breaking force of the main axis (−0.63) and breaking force of the primary lateral branches (−0.64). Based on the distribution of the principal component scores, the ON strain was differentiated from the SY and KT strains by their contrasting distributions on the first principal component. Detailed observation showed that the score distribution of the KT strain was included in that of the SY strain.

For the samples from Onna, the factor loadings on the first principal component had high absolute values for the thallus length (0.79), diameter of the main axis (0.77), density of primary lateral branches (−0.89), density of branchlets (−0.70) and breaking force of primary lateral branches (0.60; Fig. 6). The second component had high values for the diameter of second lateral branches (−0.80) and breaking force of the main axis (0.73). The distribution of the principal component scores-showed that the ON strain was evidently distinguished from the overlapping KT and SY strains based on their contrasting distributions on the first principal component.

For the samples from Kumejima, the factor loadings of the first principal component had high absolute values for thallus length (0.55), diameter of the main axis (0.81), density of the primary lateral branches (−0.90) and density of the branchlets (−0.52; Fig. 6). The second component had high values for the thallus length (−0.63), diameter of the second lateral branch (−0.60) and breaking force of the main axis (0.64) and lateral branch (0.67). The principal component scores of the three strains were distinguished from each other by the composite distribution of the first and second principal components.

For the samples obtained from Motobu, the factor loadings of first principal component had high absolute values for the diameter of the main axis (0.73), diameter of the first lateral branch (0.75), diameter of the second lateral branch (0.86), breaking force of the main axis (0.77) and primary lateral branch (0.75; Fig. 6). The second component had high factor loadings for the thallus length (0.86). The principal component scores showed that the SY strain was evidently distinguished from the KT strain by its contrasting distribution on the first principal component.

Seeding density and yield

The mean value of the seeding density of each strain at the end of the collection ranged from 16 to 46 microthalli mm^{-2} at Izena, 95 to 163 microthalli mm^{-2} at Onna, 236 to 331 microthalli mm^{-2} at Kumejima and 97 to 174 microthalli mm^{-2} at Motobu (Table 4).

The harvests were obtained from all ten nets for each strain in Izena, Kumejima and Motobu, except for Onna, where due to grazing damage caused by herbivorous fish , i. e., *Kyphosus* or *Siganus* only five nets with intact thalli for each strain could be selected and harvested. The total biomass yield of the KT strain was the largest in Izena (2,148 kg FW), Kumejima (1,032 kg FW), and Motobu (1,990 kg FW) (Table 5). In Onna, the yield of the SY strain was the largest at 290 kg FW. Simultaneously, the yield of the ON strain was lower than that of the SY and KT strains at all sites.

Discussion

In this study, the morphological characteristics of six local strains collected from representative *C. okamuranus* aquaculture sites were compared, leading to the selection of three candidate strains, SY, ON, and KT, for transplant experiments. First, based on the morphological comparison of six local strains, the thallus length of the IZ, SY, and IG strains reached 35 cm. Previous studies revealed the average length of cultivated and wild thalli to be 20–30 cm (Shinmura, 1977; Toma and Nakama, 1981; Moromizato et al., 2005), indicating that these three strains were classified as large size. In addition to its large size, the SY strain in the breaking force of the main axis and primary branches as well as the diameter of the primary and second lateral branches were higher than those of the IZ and IG strains, which were of similar size. Therefore, SY was characterised as a large thallus type with thick and firm branches and was selected as a candidate strain with excellent productivity and processing traits. In contrast, the ON strain was classified as a small-type in the thallus length with a high density of primary lateral branches and branchlets. In particular, the density of the primary lateral branches (27.4 per 10 cm) was higher than that of the other five strains and considered noteworthy. There is little previous research on the density of lateral branches in cultured thalli, but Shinmura (1977) reported that the highest density of wild thalli was 3.4 branches per 10 cm (converted from 7.0 lateral branches per 20.8 cm average algal length), indicating that density of the lateral branches of the ON strain is unusually high. The high density of lateral branches increases their exposure to hydrodynamic drag, but it is also expected to contribute to higher unit yield, and the ON strain was selected as another candidate strain with superior traits for production. Meanwhile, the KT strain was classified as having a medium-sized thallus length owing to its length falling within the average range of previous findings (Shinmura, 1977; Toma and Nakama, 1981;

Moromizato et al., 2005). However, its diameter and breaking force of the lateral branches were the second largest following the SY strain. In addition, the morphological variation of the KT strain was considered less than that of other strains because of the result of its smaller standard deviation in the measurement of thallus length and breaking force and the narrower distribution of PCA scores. Length, thickness (diameter) and firmness breaking force are used as indicators of traits to determine quality in terms of processing (Okinawa Prefecture 2012), and homogeneous raw materials are required. Therefore, the KT strain was considered a potential superior strain for processing. The IZ, IG and KM strains presented no remarkable superior characteristics because the IZ and IG strains were thinner and softer than the SY strain while they were classified as large, and the KM strain was intermediate in length but had thin branches. Overall, these results suggest the morphological characteristics of three local strains —SY, ON, and KT— potentially have advantages in each aspect of aquaculture and/or processing. The transplant experiments using the three selected candidate strains—ON, SY, and KT—showed that each strain had several intrinsic characteristics inherited from their mother plant. The ON and SY strains were distinguishable visually and statistically. The result of the morphological measurements showed that the SY strain thalli were significantly longer than those of the ON and KT strains in Izena, Onna and Kumejima. Moreover, the mean length of the SY strain thalli was 30–55 cm, which is longer than the mean value of 20–30 cm found in previous findings (Shinmura, 1977; Toma and Nakama, 1981; Moromizato et al., 2005). These findings suggested that the thallus length characteristics of the SY strain were inherited from the mother thalli to the transplanted thalli and the SY is classified as a strain with a trait of large thalli. Meanwhile, the ON strain had significantly denser lateral branches than the SY and KT strains in the three areas, except for Motobu, indicating that the high branch density of the ON cultivar strain is an endemic characteristic. In the case of *Nemacystus decipiens* (Suringar) Kuckuck, a species of Spermatochnaceae cultured using a method similar to that used in the culture of *C. okamuranus*, Yotsui (1980) reported that morphological characteristics, such as the density of branching and mucus content, were inherited from mother plants when transplanting two types of seedlings from different habitats to the same aquaculture site. Regarding the morphology of *Chordaria flagelliformis* (O.F.Müller) C. Agardh, belonging to Chordariaceae, the same family as that of *C. okamuranus*, Kim and Kawai (2002) suggested that there are different ecotypes with different lateral branch densities. These studies support the finding that the characteristics of lateral branch density in *C. okamuranus* are also inherited from mother thalli to transplanted thalli. The factor loadings of the PCA showed high correlations in the thallus length for the SY strain and the number of primary lateral branches and branchlets for the ON strain, revealing that these traits are important indicators that characterise both strains. Moreover, the PC score distribution of SY and ON strains indicates the two strains were distinct from each other in the three areas, except Motobu where ON strain thalli were washed away. On the contrary, the morphological characteristic value of the KT strain was higher in the diameter and breaking force and lower in the density of branchlets than that of the ON and SY strains, thereby the PC score distribution of the KT cultivar was evidently distinct from the other strains in Kumejima and Motobu. Besides, the standard deviation of the KT strain in thallus length was smaller than that of the other two strains, which resulted in the narrow distribution of the principal component scores, particularly in Izena. This indicates that the KT strain is also characterized by a small variation in thallus length. Therefore, the KT strain is expected to have superior characteristics in terms of processing quality, depending on the aquaculture site. These results indicate that the three strains had inherited characteristics, particularly the thallus length and the density of primary lateral branches, which were stable in different aquaculture areas. Nishitsuji *et al.* (2020) conducted a genomic analysis using microthalli of these three selected strains that were subcultured onagar medium and suggested that they were indeed different at the sub-species level. This analysis genetically supported the results of the morphological characteristics in this study. These morphological and genetic data will be important information for future studies to accurately identify the strains.

Among the three strains, the expression of some characteristics such as branch diameter and breaking force in the KT strain varies to different extent depending on the aquaculture area, and the influence of environmental factors on the expression of these characteristics needs to be further investigated. Choi et al. (2018) compared several cultivars of *Undaria pinnatifida* (Harvey) Suringar and reported that the phenotype of the cultivars varied depending on the individual genotype and its aquaculture environment, for example, long-growing cultivars differed in different sea areas. Molis et al. (2015) suggested that the cell hardness of *Fucus vesiculosus* Linnaeus is affected by environmental factors, such as wave strength, and Demes et al. (2013) suggested that the algal tissue toughness of *Egregia menziesii* (Turner) Areschoug, a species of the kelp family, is affected by environmental factors, such as wave strength, as well as genetic differences between individuals within the species. Further investigation of the relationship between the morphological characteristics of *C. okamuranus* and environmental factors will help us identify suitable strains for respective aquaculture areas. Hwang et al (2020) described the techniques combining morphological characteristics and SSR data to identify kelp cultivars, and it will be necessary to apply those techniques for *C. okauranus* as well.

The results showed that yields of the SY and KT strains tended to exceed that of the

ON strain, suggesting that these two strains were superior in terms of production. Toma (2001) investigated the average yield per net of various aquaculture sites in Okinawa Prefecture—except for Kumejima—and reported that it was approximately 100 kg FW in Izena, 20–30 kg FW in Onna and 100–170 kg FW in Motobu. In our research, the highest yield per net (converted from total biomass yield)at each transplant site was estimated to be 214.8 kg FW in Izena for the KT strain, 58 kg FW in Onna for the SY strain and 199 kg FW in Motobu for the KT strain, which were higher than those reported by Toma (2001). These results also supported that the yields of the KT and SY strains were superior to these average yields. In terms of traits, thallus length was longer in the SY than in the KT strain at Izena and Onna. Nevertheless, the yield of KT tended to be higher than that of SY, suggesting that the adhesion of the KT strain to the nets was stronger. The ON strain, characterised by high lateral branch density, was expected to present traits associated with high yield, but its yield was lower than that of the other two strains. Particularly in Motobu, no yield was obtained because thalli had been washed away. The aquaculture site in Motobu was located in the channel between the islands, where fast currents are generated by the ebb and flow of the tide. Therefore, the main reason was inferred that the ON strains with high lateral branches were exposed to the force of the current and were washed away; thus, it may have been at a disadvantage in terms of its yield in areas subjected to fast currents or strong wave action. Future research is necessary to elucidate the differences in adherence strength among strains and the relationship between productivity and environmental factors.

Another factor that may affect the length and morphology of lateral branches is thallus density. Arenas *et al*. (2002) investigated the effect of plant density on the growth of *Sargassum muticum* (Yendo) Fensholt and reported that, within juvenile plant densities of 200–6400 plants m^{-2} (0.0002–0.0064 plants mm^{-2}), a higher plant density resulted in longer algal body length and lower branch density. In the present study, experiments were started at a density of 16–331 micro thalli mm^{-2} of the discoidal microthalli, which is extremely high compared with the density reported in the former study. Shinmura (1977) reported that the diameter of discoidal micro thalli—the initial germlings of *C. okamuranus*—reached 0.5 mm within 2 weeks. With the density assessed in our experiment, the discoidal microthalli began to overlap each other and we observed that microthalli formed a mat-like covering in the intermediate cultivation stage. This density was intended for practical purposes to prevent contamination by miscellaneous algae when the nets are moved to the seabed. Since the erect macrothalli grew from the discoidal microthalli mat, it can be inferred that the density of each strain was determined by the maximum density at which each strain can form erect macrothalli. Thus, the density among the three strains was considered the same for practical use by treating each as having the maximum density. Further research is warranted to determine how the different densities of erect macrothalli affect their morphology in low-density conditions.

In this study, we provide the first detailed morphological data on the differences among the ON, SY and KT strains of *C. okamuranus*, which had only been analysed in terms of their genetic differences to date. Moreover, we revealed that some morphological differences of these strains are their intrinsic characteristics, indicating that the differences in productivity and processing quality of this species are not only due to differences in the environmental factors as previously reported, but also the characteristics of strains or ecotypes in each aquaculture site. We believe that the findings of this study will provide information to aquaculture producers and processors and encourage them to use the strains with appropriate characteristics for each production purpose. The current aquaculture production of *C. okauranus* remains unstable, probably due in part to the recent climate change. To address this situation, aquaculture producers are expected to develop new cultivars with superior growth, thallus quality and tolerance to weather fluctuations. The stability of morphological characteristics of ON, SY, and KT obtained in this study has been confirmed by some aquaculture producers in their subsequent practical experiments on a pilot scale. Thus, the foundation for future breeding is now being established. Based on the study findings, further search for strains and the development of new cultivars through crossbreeding will be promoted to facilitate stable production in the future.

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Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest Statement

The authors declare no conflicts of interest associated with this manuscript.

Ethical Approval

This study does not involve any animal experimentation and therefore no ethical approval was needed.

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Tables

Table.1 Desirable characteristics for biomass yield and salt processing

	For biomass yield	For salt processing
Thallus length	High	Middle to high
Thickness (diameter)	Middle to high	Middle to high
Firmness (Breaking strength)	Middle to high	High
Density of primary lateral branches	To be confirmed	N/A
Branchlets	N/A	Low

N/A : Not applicable

Table 2. Duration and location of transplant experiments in each aquaculture site.

Aquaculture site			Izena	Onna	Kumejima	Motobu
Seed collection	Date		9 Dec 2009	Dec 8, 2011	9 Dec 2011	8 Jan 2012
Intermediate cultivation	Date		23 Dec 2009	28 Dec 2011	19 Dec 2011	28 Jan 2012
	Location	Lat.	127° 54’56”E	127° 50’14”E	126° 50’39”E	127° 53’19”E
		Lon.	26° 55’43”N	26° 28’56”N	26° 19’54”N	26° 27’02”N
	Seabed		Coral rubble	Seagrass bed	Coral rubble	Coral rubble
Main cultivation	Date		27 Jan 2010	20 Mar 2012	27 Jan 2012	7 Mar 2012
	Location	Lat.	127° 55’20”E	127° 47’50”E	126° 54’43”E	127° 52’13”E
		Lon.	26° 54’10”N	26° 26’33”N	26° 20’51”N	26° 38’19”N
	Seabed		Seagrass bed	Seagrass bed	Coral rubble	Sand
Harvest	Date		28 Apr 2010	1 May 2012	27 Apr 2012	25 May 2012

Each date indicates the start of aquaculture schemes.

Table 3. Morphological traits of the six local strains of *Cladosiphon okamuranus*.

Area		IZ	ON	SY	KT	KM	IG
Thallus length	(cm)	41.8 ± 7.6 a	19.0 ± 3.7 c	40.6 ± 9.9 a	25.8 ± 3.0 bc	26.0 ± 6.2 bc	35.7 ± 12.4 ab
Diameter							
Main axis	(mm)	2.5 ± 0.3 a	1.5 ± 0.2 b	2.6 ± 0.3 a	2.3 ± 0.2 a	1.8 ± 0.2 b	2.5 ± 0.6 a
Primary lateral branch	(mm)	1.8 ± 0.2 bc	1.5 ± 0.1 c	2.5 ± 0.4 a	2.1 ± 0.3 ab	1.6 ± 0.3 bc	2.0 ± 0.5 ab
Secondly lateral branch	(mm)	1.3 ± 0.3 b	1.3 ± 0.2 b	1.8 ± 0.5 a	1.7 ± 0.2 ab	1.3 ± 0.3 b	1.4 ± 0.3 ab
Density							
Primary lateral branches		4.5 ± 2.9 b	27.4 ± 14.5 a	3.8 ± 1.8 b	7.0 ± 2.5 b	8.5 ± 3.8 b	8.8 ± 6.5 b
Branchlet		4.0 ± 1.2 c	17.9 ± 5.2 a	5.8 ± 2.4 bc	6.2 ± 2.2 bc	9.3 ± 4.3 b	8.7 ± 3.4 b
Breaking force							
Main axis	(N)	11.2 ± 3.7 ab	11.1 ± 3.7 ab	14.7 ± 5.5 a	14.7 ± 2.4 a	12.9 ± 5.2 ab	9.1 ± 2.9 B
Primary lateral Branch	(N)	6.4 ± 1.8 b	7.1 ± 3.6 ab	11.1 ± 4.0 a	9.2 ± 2.1 ab	8.6 ± 4.7 ab	5.6 ± 2.3 b

Different letters in the same row indicate significant statistical differences ($p < 0.01$) among strains ($n = 10$).

Table 4. Seeding density (number of microthalli per mm^2, mean ± SD, n = 20) in transplant experiments. The density in each aquaculture site was measured at the end of seed collection periods.

Culture site	Izena	Onna	Kumejima	Motobu
ON	21 ± 22	163 ± 115	236 ± 100	174 ± 52
SY	16 ±10	99 ± 53	268 ± 79	171 ± 85
KT	46 ±48	95 ±55	331 ± 92	97 ± 44

Table 5. Biomass yield of transplant experiments. Each yield is represented by the total amount (in kg fresh weight) collected from five or ten nets during harvesting. Value in parentheses indicate the average yield per net converted from the total biomass yield divided by five or ten nets. The size of culture nets was 1.5m × 20m in Izena, Motobu, and Kumejima, 1.2m × 18m in Onna corresponding with the representative size in each site.

Culture site	Izena*1	Onna*2	Kumejima*1	Motobu*1
ON	120 (12.0)	126 (25.2)	428 (42.8)	0 (0)
SY	1370 (137.0)	290 (58.0)	854 (85.4)	1318 (131.8)
KT	2148 (214.8)	266 (53.2)	1032 (103.2)	1990 (199.0)

*1: ten nets, *2: five nets

Figure Legends

Fig. 1. Location of aquaculture sites where local strains of *Cladosiphon okamuranus* were collected and where transplant experiments were conducted.

Abbreviations in parentheses indicate the local strains collected at each site. ON, KT, IZ, KM, and IG were strains used in Onna, Katsuren, Izena, Kumejima, and Ishigaki, respectively. SY was a strain collected in Iheya and used in Onna. The sites where transplant experiments were conducted are underlined.

Fig. 2 Schematic view of *Cladosiphon okamuranus* and its associated terminology. MA, main axis; PB, primary lateral branch; SB, secondary lateral branch; BL, branchlet.

Fig. 3. The life cycle of *Cladosiphon okamuranus*. The diagram is based on Shinmura (1977).

The neutral zoospores from sporophyte(2n) were corrected, and their germlings were subcultured as strains.

Fig. 4 Morphological characteristics of the three *Cladosiphon okamuranus* strains (n = 10) after transplantation in four aquaculture sites. TL, thallus length; Di. MA, diameter of main axis; Di. PB, diameter of primary lateral branch; Di. SB, diameter of secondary lateral branch; De. PB, density of primary lateral branches; De. BL, density of branchlets; Br. MA the breaking force of main axis; Br. PB, breaking force of primary lateral branch. Different letters in the same graph indicate a significant difference ($p < 0.01$) between the strains.

Fig. 5 *Cladosiphon okamurnaus*: Habitat of the mother plant (A) and cultured plants in the transplant experiments (B). Scale bar = 30 cm.

Fig. 6 Scatter diagram of factor loading and plots of principal component scores for eight morphological characteristics of the three *Cladosiphon okamuranus* strains. Definitions of the characteristics are same as in Fig. 3.

Table S1 Eigenvectors of the first (PC1), second (PC2), and third (PC3) principal components for eight characteristics of six local strains collected from five aquaculture sites.

	EV[1]	PV[2]	CC[3]
PC1	1.863	0.434	0.434
PC2	1.257	0.197	0.632
PC3	0.927	0.108	0.739

[1]eigenvalue

[2]proportion of variance

[3]cumulative contribution

Table S2. Eigenvectors of the first (PC1), second (PC2), and third (PC3) principal components for eight characteristics of three strains collected from four transplant sites.

Site	Izena			Onna			Kumejima			Motobu		
	EV[1]	PV[2]	CC[3]	EV	PV	CC	EV	PV	CC	EV	PV	CC
PC1	2.572	0.322	0.322	3.022	0.378	0.378	2.506	0.313	0.313	3.289	0.411	0.411
PC2	1.807	0.226	0.547	1.760	0.220	0.598	1.713	0.214	0.527	1.116	0.140	0.551
PC3	1.261	0.158	0.705	1.242	0.155	0.753	1.301	0.163	0.690	1.065	0.133	0.684

[1]eigenvalue

[2]proportion of variance

[3]cumulative contribution

Fig. S1 The process of freezing and thawing a net piece sample. (a) Transported a net piece sample into the laboratory in the refrigerated condition with ice packs, (b) Sealed in a zipper bag and frozen at -30°C, (c) Thawed under running water.

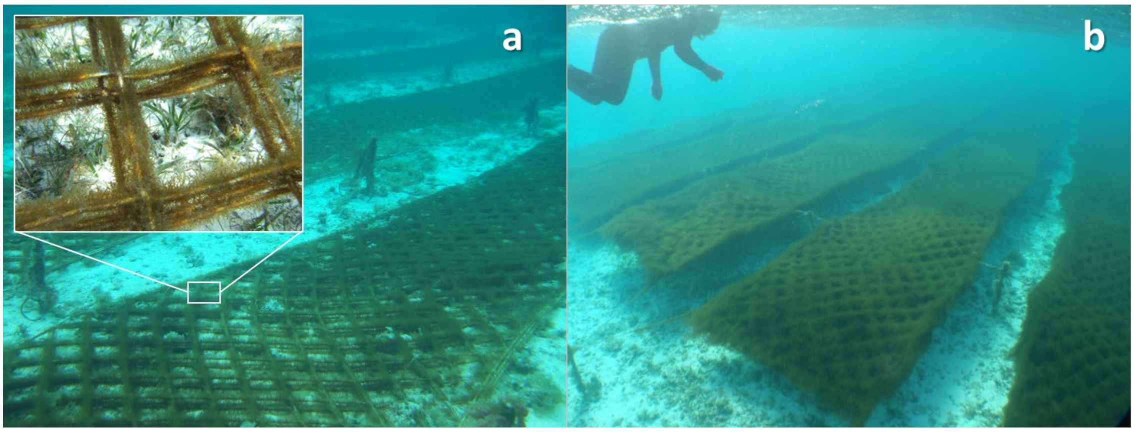

Fig. S2 Aquaculture process of *Cladosiphon okamuranus*, intermediate cultivation (a) and main cultivation (b)

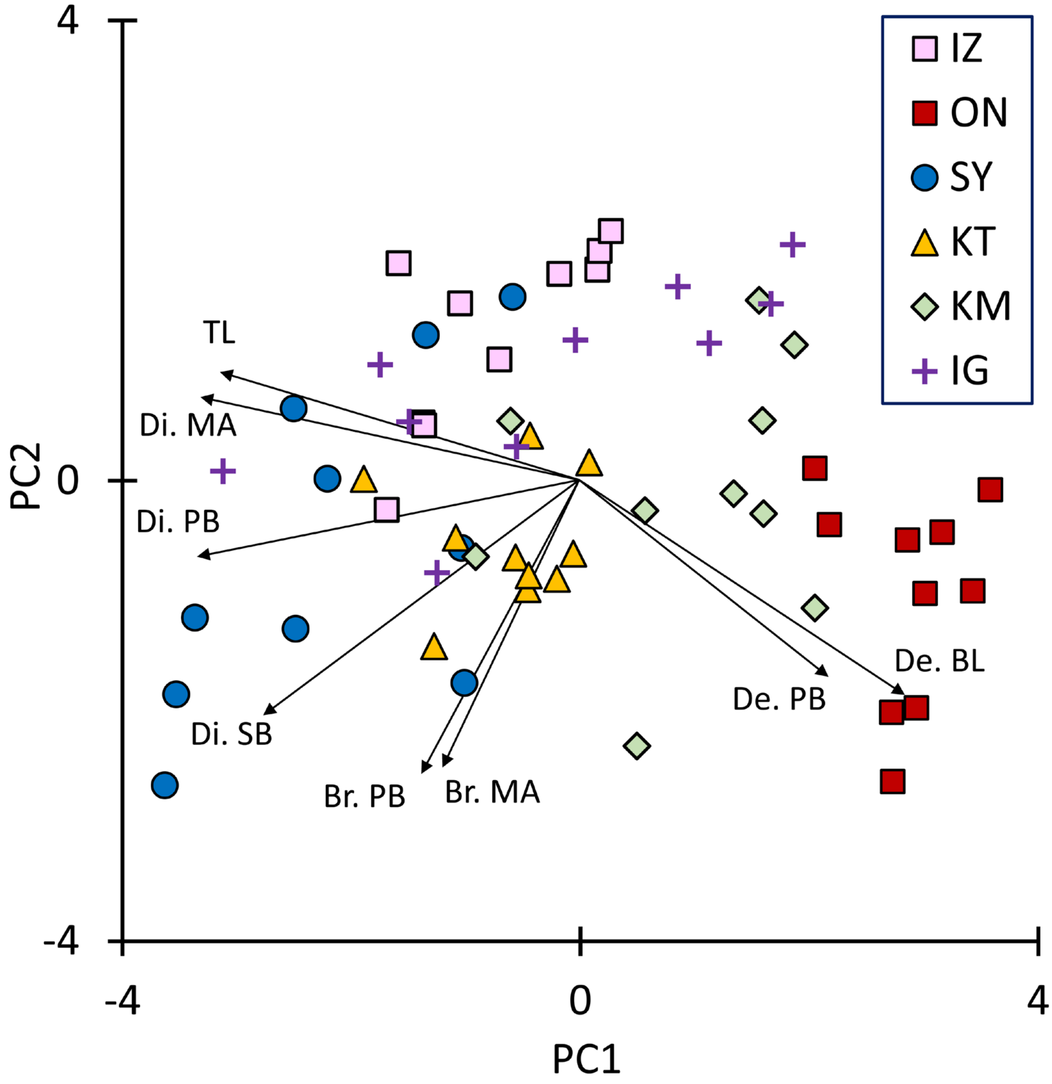

Fig. S3. Scatter diagram of the factor loading and plots of principal component scores for eight morphological characteristics from six local strains of *Cladosiphon okamuranus*. TL, thallus length; Di. MA, the diameter of the main axis; Di. PB, the diameter of the primary lateral branch; Di. SB, the diameter of the secondary lateral branch; De. PB, the density of primary lateral branches; De. BL, the density of branchlets; Br. MA, the breaking force of the main axis; Br. PB, the breaking force of the primary lateral branch.