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

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4 Running title: Morphology of *Cladosiphon* strains

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20

21 **Abstract**

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

38

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

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

43 **Introduction**

44 *Cladosiphon okamuranus* Tokida (standard Japanese name: Okinawa mozuku) is a
45 subtropical brown alga that is ,endemic to the Ryukyu Islands in Japan (Toma, 1993). In
46 this site, natural seaweeds were traditionally used as an ingredient in home cooking and
47 court cuisine of the Ryukyu Kingdom (Ohno and Largo, 2006; Sudo, 2012). Cultivation of
48 the species has been actively developed since the 1970s to meet increasing national
49 demands for this seaweed as a healthy food with low calories and high in dietary fibers.
50 The alga is an acknowledged source of fucoidan, a sulphated polysaccharide known as a
51 health functional ingredient (Shinmura and Yamanaka, 1974; Toma, 1991, 1993;
52 Moromizato et al., 2005; Sudo, 2012). Consequently, aquaculture of this species in Japan
53 has gradually increased. The amount of Mozuku produced had a fresh weight (FW) of
54 16,470 tons as of 2018, >90% of which came from *C. okamuranus* (Toma, 1993). Mozuku
55 worth 4.3 billion JPY (40 million USD), and is currently the fourth most cultivated species
56 in Japan after the *Neopyropia* spp.(251,362 tons FW, 94.2 billion JPY = 864 million USD),
57 *Undaria* spp.(45.099 tons FW, 12.1 billion JPY = 111 million USD), and *Laminaria*
58 spp.(21,812 tons FW, 10.4 billion JPY = 95 million USD) (Ohno and Largo, 2006, Hwang
59 et al., 2019, Ministry of Agriculture, Forestry and Fisheries, Japan, 2019, IMF, 2022). In
60 recent years, the consumption of seaweeds has increased due to increased health-
61 consciousness. The aquaculture producers of *C. okamuranus* should increase the unit
62 biomass yields, whilst retaining the quality of the thalli, as demanded by processors and
63 consumers. However, to achieve this goal, two problems must be solved and these are
64 highlighted below.

65 One of the problems of *C. okamuranus* aquaculture is its unstable production. The
66 annual production of ‘Mozuku’ has been fluctuating between 8 - 22,000 tonnes FW since
67 2010 (Ministry of Agriculture, Forestry and Fisheries, Japan 2019). One of the primary

68 causes of these fluctuations are poor growth and loss of cultured thalli from their
69 aquaculture nets. In part this is due to strong wave action during monsoons and typhoons
70 in the aquaculture sites. Cultivated thalli can be broken away from the nets, significantly
71 decreasing the production; strong waves have directly caused these declines (Moromizato,
72 2005). However, such declines are sometimes observed even without the influence of
73 extreme weather conditions, requiring further studies on causes. Another problem is that in
74 processing, there are significant decreases in the process yield and texture during the
75 salting process owing to variability in the quality of raw thallus material such as thickness,
76 firmness and amount of mucilage (Okinawa Prefecture 2012). The most current processing
77 of Okinawa mozuku uses salting for storage, and when thin and soft thalli are mixed
78 together, tearing and disintegration occurs. Operators involved in the fishery industry are
79 demanding the development of techniques for the stable cultivation of highly productive
80 thalli that can grow well in various environments, whilst also providing high-quality thalli
81 with thicker and firmer branches that can withstand processing with salt. To solve the
82 problems related to the unstable production and inconsistent processing quality, it is
83 considered that the selection of superior strains with high productivity and quality will
84 become a key technique (Moromizato, 2005; Hwang et al., 2019) and has been performed
85 in aquaculture of other seaweeds for their breeding (FAO 2019, Robinson et al. 2013).
86 However, only few studies have been conducted on the selection of *C. okamuranus* strains
87 (Hwang et al., 2019).

88 Some studies have reported geographical variation in morphological characteristics and
89 productivity. Shinmura (1977) compared the morphotypes of wild populations collected
90 from five sites in the Ryukyu Archipelago and found differences in thallus length, diameter
91 of the main axis and number of lateral branches from different areas. In addition, Toma
92 (1991, 2004, 2012) showed that the unit biomass yield per cultivation net for this species

93 differed amongst sites, and Moromizato(2005) investigated the environmental differences
94 such as the depth and wave action in those areas. However, these reports focused on
95 morphological differences from a taxonomic perspective, or the influence of
96 environmental factors on the biomass yield. Differences in the characteristics of strains
97 were not considered. Meanwhile, aquaculture producers and processors have empirical
98 knowledge of differences in morphological or processing characteristics that depend on the
99 production area, i.e., thicker thalli being harvested on the east coast of Okinawa main
100 island and longer thalli in deeper aquaculture areas. A possible factor for the difference in
101 these traits is due to potential ecotypes. Some aquaculture producers collected germings
102 from their cultivated thalli with high unit yields or favourable growth, which were then
103 repeatedly used as local strains for aquaculture. They may have empirically distinguished
104 the differences in ecotype traits and used them as a culture strain. Genome sequencing of
105 *C. okamuranus* revealed some strains have genetic differences (Nishitsuji et al., 2020).
106 However, to the best of our knowledge, there are no reported studies that have
107 quantitatively evaluated trait differences among different of *C. okamuranus* strains.
108 Therefore, aquaculture producers still require information to understand the traits of their
109 strains.

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

116 In this study, we first collected six local strains of *C. okamuranus* from representative
117 aquaculture sites in Okinawa Prefecture and selected three candidate strains with

118 potentially superior traits for aquaculture production and processing. Then, we transplanted
119 the candidate strains to major aquaculture areas in order to investigate whether each of the
120 characteristics was expressed as strain-specific characteristics and evaluated how these
121 characteristics could be useful for aquaculture producers.

122

123 **Material and Methods**

124 **Collection of local strains**

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

136

137 **Morphological measurements**

138 Before the morphological traits were measured, the frozen net pieces were thawed in
139 a plastic bag under running water, and ten intact individuals were selected to evaluate
140 the form of a complete condition. The thickest branch growing from the base of the
141 thallus was defined as the main axis, and based on this, the branches departing from the
142 main axis with a length of >3 cm were defined as the primary lateral branches, the

143 branches departing from the primary lateral branches were defined as secondary lateral
144 branches and the small branches of <1 cm at the tip of the thallus were defined as
145 branchlets (Fig. 2). Eight characters were measured: thallus length(1); diameter of main
146 axis (2); primary lateral branch (3) and secondary lateral branch(4) as thallus thickness;
147 density of primary lateral branches (5) arising from the main axis and the branchlets (6)
148 at the tip of the primary lateral branch; , and breaking strength of the main axis (7) and
149 primary lateral branch (8) as firmness. Among these characteristics, the thallus length
150 was measured from the main axis using a caliper. The thickness was measured by the
151 diameters of the main axis, primary lateral branch, and secondary lateral branch at the
152 thickest part of each branch magnified using a profile projector (Nikon V-12A) and a
153 caliper. The density of the primary lateral branches was measured by counting the
154 number of primary lateral branches on the basal 10 cm of main axis. The density of
155 branchlets was measured by counting the number of branchlets observed in the apical 5
156 cm of the longest primary lateral branch. The firmness was measured by the breaking
157 force (N) at the thickest part of the main axis and lateral branches using a texture
158 analyser (Rheo Meter CR-500DX, Sun Scientific Co., Ltd., Tokyo, Japan No. 10
159 probe). The breaking force was measured as the maximum force recorded in a force-tie
160 curve obtained from the analyser during the thallus compression using a probe. Each
161 trait was measured once per individual. The differences in the measured characteristics
162 of each local strain were compared using analysis of variance and Tukey–Kramer test
163 ($P < 0.05$), and all data were reported as mean $\pm$ standard deviation. Grouping of strains
164 based on traits was attempted using principal component analysis (PCA), which is
165 applied as a non-parametric approach (cf. Hayashi et al. 2017) in R ver. 3.4.3 software
166 (R core team 2016).

167

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

193 gridded sites by observing both sides of two PET plates at the five sites each per single
194 side of the plate using a microscope. The grid was established by dividing the plate into
195 five sections in the longitudinal direction, and the center of each grid was observed.

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

213

214 **Results**

215 **Characteristics of the six local strains**

216 The thalli of the IZ and SY strains were significantly larger than those of the ON strain
217 ($p < 0.01$), which had the smallest mean value (Table 3). Therefore, IZ, SY, and ON were

218 classified into two types: large and small. The mean lengths of KT, KM and IG strains
219 were intermediate between the large and small types. The diameters of the main axis and
220 primary and secondary branches of the SY strains were thicker than those of the ON and
221 KM strains, respectively ($p < 0.01$). After the SY strain, KT was significantly thicker in the
222 main axis and primary lateral branches than the ON and KM strains ($p < 0.01$). The mean
223 density of lateral branches and branchlets in the ON strain was significantly greater than
224 those of the other five strains ($p < 0.01$). The breaking force of the main axis of the SY and
225 KT strains was significantly higher than that of the IG strain ($p < 0.01$), and the that of the
226 primary branch of the SY strain was significantly greater than those of the IZ and IG
227 strains ($p < 0.01$).

228 The cumulative contribution of the PCA was 43.4 and 63.2% for the first and second
229 principal component, respectively (Table S1). In the first principal component, the
230 factor loadings of the density of branchlets and lateral branches indicated high absolute
231 values of 0.71 and 0.54, respectively, in the positive direction, and the factor loadings of
232 the thallus length and the main axis, first lateral branch and secondary lateral branch
233 diameters indicated high values of -0.78 , -0.83 , -0.83 and -0.69 , respectively, in the
234 negative direction (Fig S3). In the second principal component, the factor loadings in
235 the diameter of the secondary lateral branch, breaking force of the main axis and
236 breaking force of the primary lateral branch indicated high values of -0.51 , -0.63 and
237 -0.64 , respectively, in the negative direction. The distribution of the principal
238 component scores based on these data evidently showed that the ON and SY strains
239 were located on the positive and negative ends of the first principal component axis,
240 respectively (Fig. S3). The KT and IZ strains had smaller distributions of principal
241 component scores in both the first and second principal components than the other four
242 strains, and the KT strain was located more in the negative direction of the second

243 principal component than the IZ strain.

244

245 **Characteristics of transplanted strains**

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

268 The results of the PCA showed that the cumulative contribution of the first and
269 second principal components ranged from 52.7 - 59.8% among the sites (Table S2). For
270 the samples from Izena, factor loadings of the first principal component had high
271 absolute values for the density of primary lateral branches (0.73) as well as density of
272 branchlets (0.65) in the positive direction and for the thallus length (-0.82) as well as
273 diameter of the primary lateral branch (-0.66) in the negative direction (Fig. 6). The
274 second principal component showed high values in the negative direction for the
275 diameter of the secondary lateral branches (-0.51), breaking force of the main axis
276 (-0.63) and breaking force of the primary lateral branches (-0.64). Based on the
277 distribution of the principal component scores, the ON strain was differentiated from the
278 SY and KT strains by their contrasting distributions on the first principal component.
279 Detailed observation showed that the score distribution of the KT strain was included in
280 that of the SY strain.

281 For the samples from Onna, the factor loadings on the first principal component had
282 high absolute values for the thallus length (0.79), diameter of the main axis (0.77),
283 density of primary lateral branches (-0.89), density of branchlets (-0.70) and breaking
284 force of primary lateral branches (0.60; Fig. 6). The second component had high values
285 for the diameter of second lateral branches (-0.80) and breaking force of the main axis
286 (0.73). The distribution of the principal component scores showed that the ON strain
287 was evidently distinguished from the overlapping KT and SY strains based on their
288 contrasting distributions on the first principal component.

289 For the samples from Kumejima, the factor loadings of the first principal component
290 had high absolute values for thallus length (0.55), diameter of the main axis (0.81),
291 density of the primary lateral branches (-0.90) and density of the branchlets (-0.52 ; Fig.
292 6). The second component had high values for the thallus length (-0.63), diameter of

293 the second lateral branch (-0.60) and breaking force of the main axis (0.64) and lateral
294 branch (0.67). The principal component scores of the three strains were distinguished
295 from each other by the composite distribution of the first and second principal
296 components.

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

304

305 **Seeding density and yield**

306 The mean value of the seeding density of each strain at the end of the collection
307 ranged from 16 to 46 microthalli mm^{-2} at Izena, 95 to 163 microthalli mm^{-2} at Onna,
308 236 to 331 microthalli mm^{-2} at Kumejima and 97 to 174 microthalli mm^{-2} at Motobu
309 (Table 4).

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

317

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;

343 Moromizato et al., 2005). However, its diameter and breaking force of the lateral
344 branches were the second largest following the SY strain. In addition, the morphological
345 variation of the KT strain was considered less than that of other strains because of the
346 result of its smaller standard deviation in the measurement of thallus length and
347 breaking force and the narrower distribution of PCA scores. Length, thickness
348 (diameter) and firmness breaking force are used as indicators of traits to determine
349 quality in terms of processing (Okinawa Prefecture 2012), and homogeneous raw
350 materials are required. Therefore, the KT strain was considered a potential superior
351 strain for processing. The IZ, IG and KM strains presented no remarkable superior
352 characteristics because the IZ and IG strains were thinner and softer than the SY strain
353 while they were classified as large, and the KM strain was intermediate in length but
354 had thin branches. Overall, these results suggest the morphological characteristics of
355 three local strains —SY, ON, and KT— potentially have advantages in each aspect of
356 aquaculture and/or processing. The transplant experiments using the three selected
357 candidate strains—ON, SY, and KT—showed that each strain had several intrinsic
358 characteristics inherited from their mother plant. The ON and SY strains were
359 distinguishable visually and statistically. The result of the morphological measurements
360 showed that the SY strain thalli were significantly longer than those of the ON and KT
361 strains in Izena, Onna and Kumejima. Moreover, the mean length of the SY strain thalli
362 was 30–55 cm, which is longer than the mean value of 20–30 cm found in previous
363 findings (Shinmura, 1977; Toma and Nakama, 1981; Moromizato et al., 2005). These
364 findings suggested that the thallus length characteristics of the SY strain were inherited
365 from the mother thalli to the transplanted thalli and the SY is classified as a strain with a
366 trait of large thalli. Meanwhile, the ON strain had significantly denser lateral branches
367 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 Spermatocnaceae 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

393 three strains had inherited characteristics, particularly the thallus length and the density
394 of primary lateral branches, which were stable in different aquaculture areas. Nishitsuji
395 *et al.* (2020) conducted a genomic analysis using microthalli of these three selected
396 strains that were subcultured on agar medium and suggested that they were indeed
397 different at the sub-species level. This analysis genetically supported the results of the
398 morphological characteristics in this study. These morphological and genetic data will
399 be important information for future studies to accurately identify the strains.

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

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

418 ON strain, suggesting that these two strains were superior in terms of production. Toma
419 (2001) investigated the average yield per net of various aquaculture sites in Okinawa
420 Prefecture—except for Kumejima—and reported that it was approximately 100 kg FW
421 in Izena, 20–30 kg FW in Onna and 100–170 kg FW in Motobu. In our research, the
422 highest yield per net (converted from total biomass yield) at each transplant site was
423 estimated to be 214.8 kg FW in Izena for the KT strain, 58 kg FW in Onna for the SY
424 strain and 199 kg FW in Motobu for the KT strain, which were higher than those
425 reported by Toma (2001). These results also supported that the yields of the KT and SY
426 strains were superior to these average yields. In terms of traits, thallus length was longer
427 in the SY than in the KT strain at Izena and Onna. Nevertheless, the yield of KT tended
428 to be higher than that of SY, suggesting that the adhesion of the KT strain to the nets
429 was stronger. The ON strain, characterised by high lateral branch density, was expected
430 to present traits associated with high yield, but its yield was lower than that of the other
431 two strains. Particularly in Motobu, no yield was obtained because thalli had been
432 washed away. The aquaculture site in Motobu was located in the channel between the
433 islands, where fast currents are generated by the ebb and flow of the tide. Therefore, the
434 main reason was inferred that the ON strains with high lateral branches were exposed to
435 the force of the current and were washed away; thus, it may have been at a disadvantage
436 in terms of its yield in areas subjected to fast currents or strong wave action. Future
437 research is necessary to elucidate the differences in adherence strength among strains
438 and the relationship between productivity and environmental factors.

439 Another factor that may affect the length and morphology of lateral branches is
440 thallus density. Arenas *et al.* (2002) investigated the effect of plant density on the
441 growth of *Sargassum muticum* (Yendo) Fensholt and reported that, within juvenile plant
442 densities of 200–6400 plants m⁻² (0.0002–0.0064 plants mm⁻²), a higher plant density

443 resulted in longer algal body length and lower branch density. In the present study,
444 experiments were started at a density of 16–331 micro thalli mm^{-2} of the discoidal
445 microthalli, which is extremely high compared with the density reported in the former
446 study. Shinmura (1977) reported that the diameter of discoidal micro thalli—the initial
447 germlings of *C. okamuranus*—reached 0.5 mm within 2 weeks. With the density
448 assessed in our experiment, the discoidal microthalli began to overlap each other and we
449 observed that microthalli formed a mat-like covering in the intermediate cultivation
450 stage. This density was intended for practical purposes to prevent contamination by
451 miscellaneous algae when the nets are moved to the seabed. Since the erect macrothalli
452 grew from the discoidal microthalli mat, it can be inferred that the density of each strain
453 was determined by the maximum density at which each strain can form erect
454 macrothalli. Thus, the density among the three strains was considered the same for
455 practical use by treating each as having the maximum density. Further research is
456 warranted to determine how the different densities of erect macrothalli affect their
457 morphology in low-density conditions.

458 In this study, we provide the first detailed morphological data on the differences
459 among the ON, SY and KT strains of *C. okamuranus*, which had only been analysed in
460 terms of their genetic differences to date. Moreover, we revealed that some
461 morphological differences of these strains are their intrinsic characteristics, indicating
462 that the differences in productivity and processing quality of this species are not only
463 due to differences in the environmental factors as previously reported, but also the
464 characteristics of strains or ecotypes in each aquaculture site. We believe that the
465 findings of this study will provide information to aquaculture producers and processors
466 and encourage them to use the strains with appropriate characteristics for each
467 production purpose. The current aquaculture production of *C. okauranus* remains

468 unstable, probably due in part to the recent climate change. To address this situation,
469 aquaculture producers are expected to develop new cultivars with superior growth,
470 thallus quality and tolerance to weather fluctuations. The stability of morphological
471 characteristics of ON, SY, and KT obtained in this study has been confirmed by some
472 aquaculture producers in their subsequent practical experiments on a pilot scale. Thus,
473 the foundation for future breeding is now being established. Based on the study
474 findings, further search for strains and the development of new cultivars through
475 crossbreeding will be promoted to facilitate stable production in the future.
476

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484

485 **Data Availability Statement**

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

488

489 **Conflict of Interest Statement**

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

491

492 **Ethical Approval**

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

495

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- 588

589 **Tables**

590 **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

591 N/A : Not applicable

592 **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	

593 Each date indicates the start of aquaculture schemes.

594

595 **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

596 Different letters in the same row indicate significant statistical differences ($p < 0.01$)

597 among strains ($n = 10$).

598

599 **Table 4.** Seeding density (number of microthalli per mm², mean $\pm$ SD, n = 20) in
600 transplant experiments. The density in each aquaculture site was measured at the end of
601 seed collection periods.

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

602

603 **Table 5.** Biomass yield of transplant experiments. Each yield is represented by the total
604 amount (in kg fresh weight) collected from five or ten nets during harvesting. Value
605 in parentheses indicate the average yield per net converted from the total biomass
606 yield divided by five or ten nets. The size of culture nets was 1.5m × 20m in Izena,
607 Motobu, and Kumejima, 1.2m × 18m in Onna corresponding with the
608 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)

609 *1: ten nets, *2: five nets

610 **Figure Legends**

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

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

617

618 **Fig. 2** Schematic view of *Cladosiphon okamuranus* and its associated terminology.

619 MA, main axis; PB, primary lateral branch; SB, secondary lateral branch; BL, branchlet.
620

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

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

625

626 **Fig. 4** Morphological characteristics of the three *Cladosiphon okamuranus* strains (n =
627 10) after transplantation in four aquaculture sites. TL, thallus length; Di. MA, diameter
628 of main axis; Di. PB, diameter of primary lateral branch; Di. SB, diameter of secondary
629 lateral branch; De. PB, density of primary lateral branches; De. BL, density of
630 branchlets; Br. MA the breaking force of main axis; Br. PB, breaking force of primary
631 lateral branch. Different letters in the same graph indicate a significant difference ($p <$
632 0.01) between the strains.

633

634 **Fig. 5** *Cladosiphon okamuraus*: Habitat of the mother plant (A) and cultured plants in

635 the transplant experiments (B). Scale bar = 30 cm.

636

637 **Fig. 6** Scatter diagram of factor loading and plots of principal component scores for
638 eight morphological characteristics of the three *Cladosiphon okamuranus* strains.

639 Definitions of the characteristics are same as in Fig. 3.

1 **Table S1** Eigenvectors of the first (PC1), second (PC2), and third (PC3) principal
 2 components for eight characteristics of six local strains collected from five aquaculture
 3 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

4 ¹eigenvalue

5 ²proportion of variance

6 ³cumulative contribution

7 **Table S2.** Eigenvectors of the first (PC1), second (PC2), and third (PC3) principal
8 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

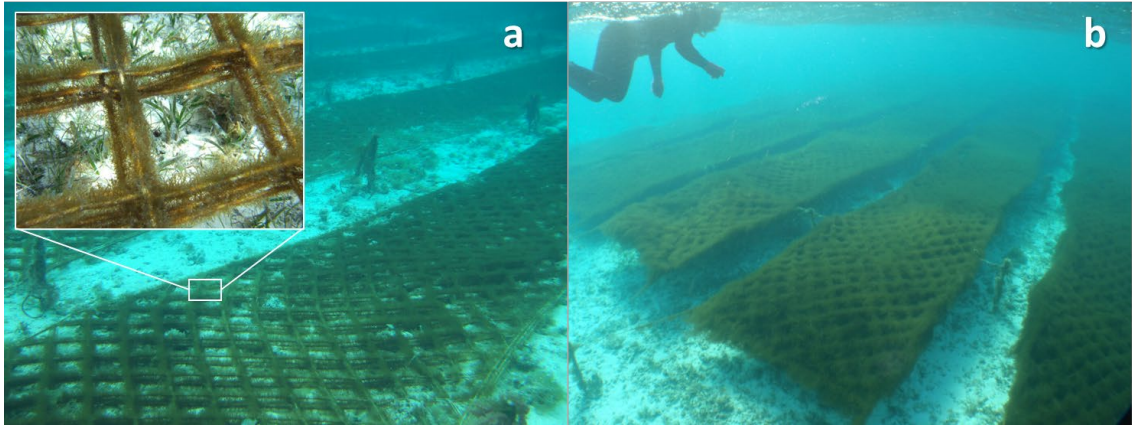
9 ¹eigenvalue

10 ²proportion of variance

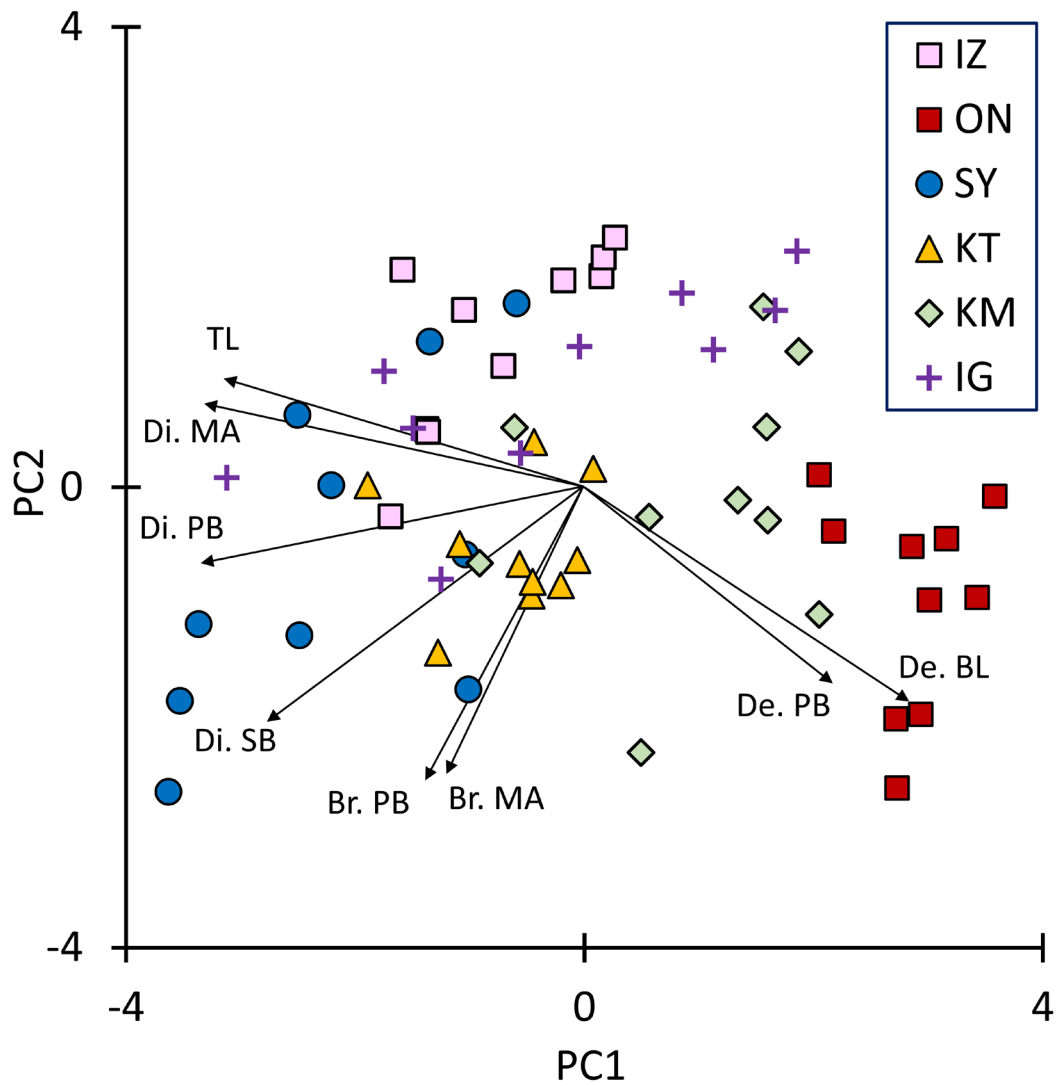
11 ³cumulative contribution



1
2 Fig. S1 The process of freezing and thawing a net piece sample. (a) Transported a net
3 piece sample into the laboratory in the refrigerated condition with ice packs, (b) Sealed
4 in a zipper bag and frozen at -30°C , (c) Thawed under running water.



5
6 **Fig. S2** Aquaculture process of *Cladosiphon okamuranus*, intermediate cultivation (a)
7 and main cultivation (b)



8

9 **Fig. S3.** Scatter diagram of the factor loading and plots of principal component scores

10 for eight morphological characteristics from six local strains of *Cladosiphon*

11 *okamuranus*. TL, thallus length; Di. MA, the diameter of the main axis; Di. PB, the

12 diameter of the primary lateral branch; Di. SB, the diameter of the secondary lateral

13 branch; De. PB, the density of primary lateral branches; De. BL, the density of

14 branchlets; Br. MA, the breaking force of the main axis; Br. PB, the breaking force of

15 the primary lateral branch.

16