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- 2 okamuranus strains
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- 4 Running title: Morphology of *Cladosiphon* strains
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21 Abstract

22	This study aimed to determine whether morphological differences of <i>Cladosiphon</i>
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	

Keywords: *Cladosiphon okamuranus*, strain selection, morphological characteristics, seaweed
 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 <i>C. okamuranus</i> (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 <i>Neopyropia</i> 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

causes of these fluctuations are poor growth and loss of cultured thalli from their 68 aquaculture nets. In part this is due to strong wave action during monsoons and typhoons 69 70 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, 71 2005). However, such declines are sometimes observed even without the influence of 72 extreme weather conditions, requiring further studies on causes. Another problem is that in 73 processing, there are significant decreases in the process yield and texture during the 74 salting process owing to variability in the quality of raw thallus material such as thickness, 75 76 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 77 together, tearing and disintegration occurs. Operators involved in the fishery industry are 78 demanding the development of techniques for the stable cultivation of highly productive 79 thalli that can grow well in various environments, whilst also providing high-quality thalli 80 81 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 82 considered that the selection of superior strains with high productivity and quality will 83 become a key technique (Moromizato, 2005; Hwang et al., 2019) and has been performed 84 in aquaculture of other seaweeds for their breeding (FAO 2019, Robinson et al. 2013). 85 However, only few studies have been conducted on the selection of C. okamuranus strains 86 (Hwang et al., 2019). 87

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 93 such as the depth and wave action in those areas. However, these reports focused on 94 95 morphological differences from a taxonomic perspective, or the influence of environmental factors on the biomass yield. Differences in the characteristics of strains 96 were not considered. Meanwhile, aquaculture producers and processors have empirical 97 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 island and longer thalli in deeper aquaculture areas. A possible factor for the difference in 100 101 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 102 repeatedly used as local strains for aquaculture. They may have empirically distinguished 103 the differences in ecotype traits and used them as a culture strain. Genome sequencing of 104 C. okamuranus revealed some strains have genetic differences (Nishitsuji et al., 2020). 105 However, to the best of our knowledge, there are no reported studies that have 106 quantitatively evaluated trait differences among different of C. okamuranus strains. 107 Therefore, aquaculture producers still require information to understand the traits of their 108 strains. 109

It is essential to determine the differences in traits that are expressed owing to strainspecific 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.

122

123 Material and Methods

124 Collection of local strains

Specimens of six local strains of Cladosiphon okamuranus, namely, IZ, ON, SY, KT, KJ 125 126 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 127 (Fig. 1). Just before harvesting the thalli at each site, a representative sampling was 128 conducted. A 10-cm long piece of the aquaculture net with the entire thallus bodies was cut 129 off from a densely growing part of the net to obtain multiple local strain specimens from 130 131 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 132 thalli using a diving survey to prevent the collection of wild species attached or mixed on 133 134 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). 135

136

137 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 143 branches and the small branches of <1 cm at the tip of the thallus were defined as 144 145 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; 146 density of primary lateral branches (5) arising from the main axis and the branchlets (6) 147 at the tip of the primary lateral branch; , and breaking strength of the main axis (7) and 148 149 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 150 151 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 152 caliper. The density of the primary lateral branches was measured by counting the 153 number of primary lateral branches on the basal 10 cm of main axis. The density of 154 branchlets was measured by counting the number of branchlets observed in the apical 5 155 156 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 157 analyser (Rheo Meter CR-500DX, Sun Scientific Co., Ltd., Tokyo, Japan No. 10 158 159 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 160 trait was measured once per individual. The differences in the measured characteristics 161 of each local strain were compared using analysis of variance and Tukey-Kramer test 162 (P < 0.05), and all data were reported as mean \pm standard deviation. Grouping of strains 163 164 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 165 (R core team 2016). 166

167

168 Transplant experiment

The three candidate strains, ON, KT and SY, considered superior and selected from 169 170 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 171 zoosporangia on a 2n sporophyte (Shinmura 1977), were isolated from a part of the 172 individual that showing the maximum thallus length in each of the collected strains 173 174 (Fig. 3). The germlings from neutral zoospores were sub-cultured on agar medium following Moromizato (2001, 2003, 2005). Transplant experiments were conducted 175 176 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 177 strain were cultured in seawater medium enriched with KW21 solution (Daiichi Seimou 178 Co., Ltd., Kumamoto, Japan) for seed collection following Moromizato (2003). The 179 seeding was performed in 500 L polycarbonate tanks on land at each transplanting site. 180 181 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 182 were added into the respective tank to allow neutral spores to attach to the nets. The 183 184 size of the culture net was $1.5 \text{m} \times 20 \text{m}$ for Izena, Motobu, and Kumejima, and $1.2 \text{m} \times$ 18m for Onna Village, which were typical sizes for each aquaculture area. Two 185 polyethylene terephthalate (PET) plates $(10 \times 20 \text{ cm})$ were also placed in each of the 186 tanks containing the nets in order to observe the number of attached spores and 187 discoidal germlings (microthalli), which are an indicator of seeding density. An air 188 bubbler was placed at the center of the tank to provide gentle aeration. The seed 189 collection period was 10-20 days. After seed collection, two PET plates for each strain 190 were brought to the laboratory under refrigerated conditions. The average seeding 191 density was calculated by counting the number of discoidal microthalli in a total of 20 192

gridded sites by observing both sides of two PET plates at the five sites each per single 193 side of the plate using a microscope. The grid was established by dividing the plate into 194 five sections in the longitudinal direction, and the center of each grid was observed. 195 The nets with attached microthalli were then moved to the seabed of the nursery 196 grounds in the aquaculture site for intermediate cultivation until the microthalli grew 197 into upright macrothalli with a length of 1-2 cm (Fig. S2). Next, they were transferred 198 199 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 200 201 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 202 net, with the entire thalli, were removed from the most densely growing parts of the net, 203 for each strain. The net samples with the attached thalli were brought to the laboratory, 204 as previously described in 'Collection of local strains' and the measurements of ten 205 206 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 207 nets were harvested using a suction pump and the fresh weight from ten culture nets of 208 each strain was measured to obtain the total biomass yield per net. To compare the 209 average biomass yield per net reported in previous findings, the total biomass yields 210 obtained in the transplant experiments were divided by the number of nets harvested to 211 calculate the average yield per net for convenience. 212

213

214 **Results**

215 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 218 were intermediate between the large and small types. The diameters of the main axis and 219 220 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 221 main axis and primary lateral branches than the ON and KM strains (p < 0.01). The mean 222 density of lateral branches and branchlets in the ON strain was significantly greater than 223 those of the other five strains (p < 0.01). The breaking force of the main axis of the SY and 224 KT strains was significantly higher than that of the IG strain (p < 0.01), and the that of the 225 primary branch of the SY strain was significantly greater than those of the IZ and IG 226 strains (p < 0.01). 227

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

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) ,

- 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.

304

305 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.

317

318 Discussion

In this study, the morphological characteristics of six local strains collected from 319 320 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 321 the morphological comparison of six local strains, the thallus length of the IZ, SY, and 322 IG strains reached 35 cm. Previous studies revealed the average length of cultivated and 323 wild thalli to be 20-30 cm (Shinmura, 1977; Toma and Nakama, 1981; Moromizato et 324 al., 2005), indicating that these three strains were classified as large size. In addition to 325 326 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 327 those of the IZ and IG strains, which were of similar size. Therefore, SY was 328 characterised as a large thallus type with thick and firm branches and was selected as a 329 candidate strain with excellent productivity and processing traits. In contrast, the ON 330 331 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 332 (27.4 per 10 cm) was higher than that of the other five strains and considered 333 334 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 335 3.4 branches per 10 cm (converted from 7.0 lateral branches per 20.8 cm average algal 336 length), indicating that density of the lateral branches of the ON strain is unusually high. 337 The high density of lateral branches increases their exposure to hydrodynamic drag, but 338 339 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 340 was classified as having a medium-sized thallus length owing to its length falling within 341 the average range of previous findings (Shinmura, 1977; Toma and Nakama, 1981; 342

Moromizato et al., 2005). However, its diameter and breaking force of the lateral 343 branches were the second largest following the SY strain. In addition, the morphological 344 345 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 346 breaking force and the narrower distribution of PCA scores. Length, thickness 347 348 (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 materials are required. Therefore, the KT strain was considered a potential superior 350 351 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 352 while they were classified as large, and the KM strain was intermediate in length but 353 had thin branches. Overall, these results suggest the morphological characteristics of 354 three local strains —SY, ON, and KT— potentially have advantages in each aspect of 355 356 aquaculture and/or processing. The transplant experiments using the three selected candidate strains—ON, SY, and KT—showed that each strain had several intrinsic 357 characteristics inherited from their mother plant. The ON and SY strains were 358 distinguishable visually and statistically. The result of the morphological measurements 359 showed that the SY strain thalli were significantly longer than those of the ON and KT 360 strains in Izena, Onna and Kumejima. Moreover, the mean length of the SY strain thalli 361 was 30–55 cm, which is longer than the mean value of 20–30 cm found in previous 362 findings (Shinmura, 1977; Toma and Nakama, 1981; Moromizato et al., 2005). These 363 findings suggested that the thallus length characteristics of the SY strain were inherited 364 from the mother thalli to the transplanted thalli and the SY is classified as a strain with a 365 trait of large thalli. Meanwhile, the ON strain had significantly denser lateral branches 366 than the SY and KT strains in the three areas, except for Motobu, indicating that the 367

high branch density of the ON cultivar strain is an endemic characteristic. In the case of 368 Nemacvstus decipiens (Suringar) Kuckuck, a species of Spermatochnaceae cultured 369 370 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 371 content, were inherited from mother plants when transplanting two types of seedlings 372 from different habitats to the same aquaculture site. Regarding the morphology of 373 374 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 375 376 different ecotypes with different lateral branch densities. These studies support the finding that the characteristics of lateral branch density in C. okamuranus are also 377 inherited from mother thalli to transplanted thalli. The factor loadings of the PCA 378 showed high correlations in the thallus length for the SY strain and the number of 379 primary lateral branches and branchlets for the ON strain, revealing that these traits are 380 381 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 382 areas, except Motobu where ON strain thalli were washed away. On the contrary, the 383 384 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, 385 thereby the PC score distribution of the KT cultivar was evidently distinct from the 386 other strains in Kumejima and Motobu. Besides, the standard deviation of the KT strain 387 in thallus length was smaller than that of the other two strains, which resulted in the 388 389 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. 390 Therefore, the KT strain is expected to have superior characteristics in terms of 391 processing quality, depending on the aquaculture site. These results indicate that the 392

three strains had inherited characteristics, particularly the thallus length and the density 393 of primary lateral branches, which were stable in different aquaculture areas. Nishitsuji 394 395 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 396 different at the sub-species level. This analysis genetically supported the results of the 397 morphological characteristics in this study. These morphological and genetic data will 398 399 be important information for future studies to accurately identify the strains. Among the three strains, the expression of some characteristics such as branch 400 401 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 402 characteristics needs to be further investigated. Choi et al. (2018) compared several 403 cultivars of Undaria pinnatifida (Harvey) Suringar and reported that the phenotype of 404 the cultivars varied depending on the individual genotype and its aquaculture 405 406 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 407 by environmental factors, such as wave strength, and Demes et al. (2013) suggested that 408 409 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 410 differences between individuals within the species. Further investigation of the 411 relationship between the morphological characteristics of C. okamuranus and 412 environmental factors will help us identify suitable strains for respective aquaculture 413 414 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 415 those techniques for C. okauranus as well. 416

417 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 418 (2001) investigated the average yield per net of various aquaculture sites in Okinawa 419 420 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 421 highest yield per net (converted from total biomass yield)at each transplant site was 422 estimated to be 214.8 kg FW in Izena for the KT strain, 58 kg FW in Onna for the SY 423 424 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 425 426 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 427 to be higher than that of SY, suggesting that the adhesion of the KT strain to the nets 428 was stronger. The ON strain, characterised by high lateral branch density, was expected 429 to present traits associated with high yield, but its yield was lower than that of the other 430 431 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 432 islands, where fast currents are generated by the ebb and flow of the tide. Therefore, the 433 434 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 435 in terms of its yield in areas subjected to fast currents or strong wave action. Future 436 research is necessary to elucidate the differences in adherence strength among strains 437 and the relationship between productivity and environmental factors. 438 Another factor that may affect the length and morphology of lateral branches is 439 thallus density. Arenas et al. (2002) investigated the effect of plant density on the 440 growth of Sargassum muticum (Yendo) Fensholt and reported that, within juvenile plant 441

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, 443 experiments were started at a density of 16–331 micro thalli mm⁻² of the discoidal 444 microthalli, which is extremely high compared with the density reported in the former 445 study. Shinmura (1977) reported that the diameter of discoidal micro thalli-the initial 446 germlings of C. okamuranus—reached 0.5 mm within 2 weeks. With the density 447 assessed in our experiment, the discoidal microthalli began to overlap each other and we 448 449 observed that microthalli formed a mat-like covering in the intermediate cultivation stage. This density was intended for practical purposes to prevent contamination by 450 451 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 452 was determined by the maximum density at which each strain can form erect 453 macrothalli. Thus, the density among the three strains was considered the same for 454 practical use by treating each as having the maximum density. Further research is 455 warranted to determine how the different densities of erect macrothalli affect their 456 morphology in low-density conditions. 457 In this study, we provide the first detailed morphological data on the differences 458 among the ON, SY and KT strains of C. okamuranus, which had only been analysed in 459 terms of their genetic differences to date. Moreover, we revealed that some 460

461 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

465 findings of this study will provide information to aquaculture producers and processors

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.

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588	

589 Tables

	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

590 **Table.1** Desirable characteristics for biomass yield and salt processing

591 N/A : Not applicable

Aquaculture site			Izena	Onna	Kumejima	Motobu	
Seed collection	Date		9 Dec 2009	Dec 8, 2011	9 Dec 2011	8 Jan 2012	
	Date		23 Dec 2009	28 Dec 2011	19 Dec 2011	28 Jan 2012	
Intermediate	Location	Lat.	127° 54'56"E	127° 50'14"E	126° 50'39"E	127° 53'19"E	
cultivation	Location	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	
	Date		27 Jan 2010	20 Mar 2012	27 Jan 2012	7 Mar 2012	
Main cultivation	I Location I	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	

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

593 Each date indicates the start of aquaculture schemes.

Area		IZ	ON	SY	KT	KM	IG
Thallus length	(cm)	41.8 ± 7.6 a	$19.0 \pm 3.7 \text{ c}$	40.6 ± 9.9 a	$25.8\pm3.0~bc$	26.0 ± 6.2 bc	35.7 ± 12.4 ab
Diameter							
Main axis	(mm)	2.5 ± 0.3 a	$1.5\pm0.2\;b$	2.6 ± 0.3 a	$2.3\pm0.2~a$	$1.8\pm0.2~b$	$2.5\pm0.6~a$
Primary lateral branch	(mm)	1.8 ± 0.2 bc	$1.5\pm0.1\ c$	$2.5\pm0.4~a$	$2.1\pm0.3~ab$	1.6 ± 0.3 bc	$2.0\pm0.5~ab$
Secondly lateral branch (1		$1.3\pm0.3\ b$	$1.3\pm0.2\ b$	$1.8\pm0.5~a$	$1.7\pm0.2\ ab$	$1.3\pm0.3\ b$	$1.4\pm0.3~ab$
Density							
Primary lateral branches		$4.5\pm2.9\ b$	27.4 ± 14.5 a	$3.8\pm1.8\ b$	$7.0\pm2.5~b$	$8.5\pm3.8\ b$	$8.8\pm6.5~b$
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	$11.1 \pm 3.7 \text{ ab}$	14.7 ± 5.5 a	14.7 ± 2.4 a	12.9 ± 5.2 ab	9.1 ± 2.9 B
Primary lateral Branch	(N)	$6.4\pm1.8~b$	$7.1 \pm 3.6 \text{ ab}$	11.1 ± 4.0 a	$9.2\pm2.1~ab$	8.6 ± 4.7 ab	$5.6\pm2.3\ b$

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

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

597 among strains (n = 10).

Table 4. Seeding density (number of microthalli per mm², mean \pm SD, n = 20) in 599

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

transplant experiments. The density in each aquaculture site was measured at the end of 600

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	126	428	0
ON	(12.0)	(25.2)	(42.8)	(0)
CV	1370	290	854	1318
51	(137.0)	(58.0)	(85.4)	(131.8)
ИT	2148	266	1032	1990
<u> </u>	(214.8)	(53.2)	(103.2)	(199.0)

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

610 Figure Legends

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.
- 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
- 622 Shinmura (1977).
- The neutral zoospores from sporophyte(2n) were corrected, and their germlingswere subcultured as strains.
- 625
- 626 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 < p
- 0.01) between the strains.

633

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

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

- 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

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

8 components for eight characteristics of three strains collected from four transplant sites.

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.



- **Fig. S2** Aquaculture process of *Cladosiphon okamuranus*, intermediate cultivation (a)
- 7 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.