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Taxonomic note on *Gracilaria articulata* Chang *et* Xia (Gracilariales, Rhodophyta) from Okinawa, Japan[1]

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Abstract — *Gracilaria articulata* Chang *et* Xia, previously known from southern China and Vietnam, is reported for the first time from the Okinawa Island, southern Japan. The species is characterized by the following set of morphological features: 1) terete thalli, 2) the presence of articulated segments with attenuated bases and obtuse apices, 3) the presence of traversing filaments in the cystocarp, and 4) shallow, cup-shaped spermatangial conceptacles. Molecular phylogenetic analyses, using the intergenetic spacer regions between the mitochondria encoded *cox*2 and *cox*3 genes (*cox*2-3 spacer), and the plastid encoded *rbc*L and *rbc*S genes (RuBisco spacer) supported the conspecificity of the Chinese alga and our Japanese entity, but clearly distinguished *G. articulata* from morphologically similar species such as *G. blodgettii* Harvey and *G. salicornia* (C. Agardh) Dawson.

***Gracilaria articulata* / Gracilariaceae / Gracilariales /Japan / marine algae / Rhodophyta / taxonomy**

Résumé — Note taxinomique sur *Gracilaria articulata* Chang *et* Xia (Gracilariaceae, Rhodophyta) d'Okinawa, Japon. *Gracilaria articulata* Chang *et* Xia, précédemment connu du Sud de la Chine et du Vietnam, a été récolté pour la première fois au sud du Japon, dans l'île d'Okinawa. L'espèce est caractérisée par un ensemble de traits morphologiques : 1) des thalles de section circulaire, 2) la présence de segments articulés avec des bases atténuées et des apex obtus, 3) la présence de filaments traversant le cystocarpe et 4) des cryptes mâles superficielles et en forme de coupe. Les analyses phylogénétiques moléculaires, utilisant l'espaceur situé entre les gènes mitochondriaux *cox*2 et *cox*3 (*cox*2-3 spacer), ainsi que l'espaceur situé entre les gènes plastidiaux *rbc*L et *rbc*S (RuBisco spacer), confirment la conspécifité des algues chinoises et de nôtre entité japonaise, en revanche, elles distinguent nettement des espèces morphologiquement semblables telles que *G. blodgettii* Harvey et *G. salicornia* (C. Agardh) Dawson.

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1. Dedicated to Dr Isabella Abbott on the occasion of her 85th birthday

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INTRODUCTION

The red alga *Gracilaria articulata* Chang *et* Xia (1976) was originally described from material collected from Hainan Island, Guangdong Province, southern China, and later reported from Vietnam (Nguyen, 1992). According to the original description (Chang & Xia, 1976), it is similar to *G. blodgettii* Harvey in gross morphology and reproductive features, but differs in the presence of articulations. Withell *et al.* (1994) also suggested that *G. articulata* is similar to the *G. blodgettii* or *G. salicornia* (C. Agardh) Dawson. Therefore, the taxonomic status of this species has been questioned. In this study, we give a detailed description of *G. articulata* based on field-collected and cultured materials from Okinawa Island, the Ryukyu Archipelago, Japan. This also represents the first record of the species outside of China and Vietnam. In addition, we have undertaken molecular phylogenetic analyses to elucidate the relationship between *G. articulata* and other species in the genus, using two intergenetic spacer regions between the mitochondria encoded cytochrome oxidase 2 and cytochrome oxidase 3 genes (*cox*2-3 spacer), and between plastid encoded large and small subunits of the ribulose-1,5-bisphosphate carboxylase/oxgenase genes (RuBisco spacer) (Zuccarello *et al*., 1999; Bynne *et al*., 2002).

MATERIALS AND METHODS

Morphological observation

Specimens examined were collected at Namisato (26°26.806'N 127°56.514'E), Kin Town, Okinawa Prefecture, Japan: (1) Mar. 8, 2004, *SAP 096688* (cystocarpic); *SAP 096689* (tetrasporangial, Fig. 1); *Terada 1601* (spermatangial). (2) Feb. 21, 2003, *Terada 1596* (cystocarpic and tetrasporangial). (3) Dec. 23, 2002, *Terada 1598* (vegetative); *Terada 1597* (tetrasporangial). (4) Jan. 9, 2002, *Terada 1446* (tetrasporangial); *Terada 1447* (spermatangial). Type material of *Gracilaria articulata* collected from Xinying, Dan Xian, Hainan Island, Guangdong Province, China on May 12, 1959 and deposited in the Herbarium of Institute of Oceanology, Academica Sinica, China (*AST 59-2565c*), and in the Bishop Museum, USA (*BISH 531985* (*AST 59-2565d*)), was examined to confirm our identification.

The specimens fixed in 5% formalin/seawater were used for microscopic observations. Sections were made by hand or freezing microtome (Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and stained with 1% cotton blue in 50% glycerol/seawater. Voucher herbarium specimens were deposited in the Herbarium of Graduate School of Science, Hokkaido University, Sapporo (SAP) or the Herbarium of Faculty of Fisheries, Kagoshima University, Kagoshima (Terada).

Culture studies were carried out following the procedures of Yamamoto & Sasaki (1987) and Terada & Yamamoto (2000). Culture conditions were: PES medium (Provasoli, 1968), temperatures of 24°C, 75 μ-mol photon m^{-2} s^{-1} (cool white fluorescent lamps), 12L:12D, and aeration (0.3 L min^{-1}) in unattached cultures. The material for culture studies was collected on January 17, 2003, from the same location as that of the morphological study.

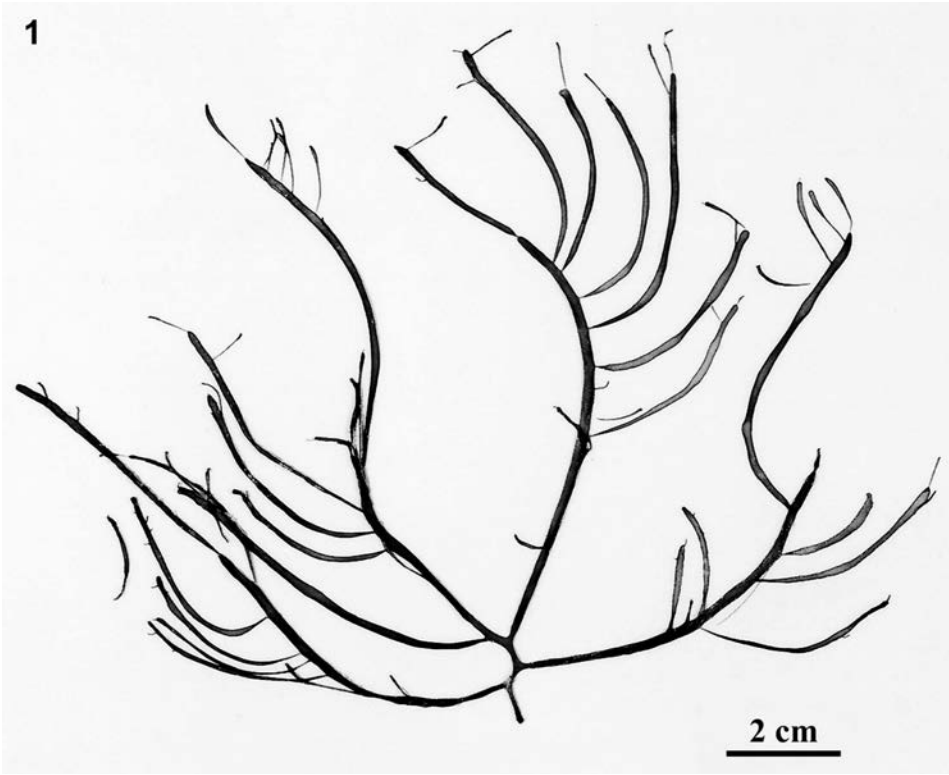


Fig. 1. Herbarium specimen of *Gracilaria articulata* Chang *et* Xia from Namisato, Kin (Okinawa Prefecture, Japan) SAP 096689.

Molecular phylogenetic analysis

Samples used for molecular analysis are listed in Table 1. Portions of field-collected individuals were dried immediately by Silica-Gel for DNA analysis. Total DNA was extracted from the Silica-Gel samples or dried herbarium specimen (eleven samples of five species) using the DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the protocol of the manufacturer. Total DNA was used as template for the polymerase chain reaction (PCR) and the following two pairs of primers were used for amplification: *Cox*2-3 spacer, forward primer 5'-GTACCWTCTTTDRGRRKDAAATGTGATGC-3' and reverse primer 5'-GGATCTACWAGATGRAAWGGATGTC-3' (Zuccarello *et al.,* 1999a); RuBisco spacer, forward primer 5'-TATACTTCTACAGA-CACAGCTGA-3' (*rbc*F1), and reverse primer 5'-ATTTCA-CACAGGAAACAGCTATGACATGTCAAATAATGGTAGTCCCCA - 3' ($rbcR_2$-M_2) (Zuccarello *et al.,* 1999b). PCR products were checked by electrophoresis in a 1 % agarose gel, stained in ethidium bromide, visualized under UV light and photographed. Prior to cycle-sequencing PCR products were purified by PEG precipitation and directly sequenced using the ABI PRISM BigDye

Table 1. Samples used for phylogenic analyses.

Species		*Location*	*Collecting date*	*GenBank No.*	
				Cox2-3 spacer	*RuBisco spacer*
Ptilophora prolifera		Garden Is., Australia		AY131313	AY131312
Gracilaria aculeata					AY241158
Gracilaria arcuata	Gr-15	Namisato, Kin, Okinawa, Japan	23-déc-02	AB193448	AB193456
Gracilaria arcuata	Gr-19	Kayo, Nago, Okinawa, Japan	16-mai-03	AB193449	AB193457
Gracilaria articulata	Gr-13	Namisato, Kin, Okinawa, Japan	21-fév-03	AB193442	AB193445
Gracilaria articulata	Gr-20	Xinying, Dan Xian, Hainan Island, China	12-mai-59	AB193443	AB193446
Gracilaria articulata	Gr-22	Namisato, Kin, Okinawa, Japan	21-fév-03	AB193444	AB193447
Gracilaria beckeri					AY241136
Gracilaria blodgettii	Gr-12	Namisato, Kin, Okinawa, Japan	23-déc-02	AB193450	AB193458
Gracilaria canaliculata					AY241147
Gracilaria capensis					AY241171
Gracilaria caudata					AY241133
Gracilaria chilensis		Marion Bay, Tasmania, Australia		AY131316	AY131299
Gracilaria chilensis		North Island, New Zealand			U21356
Gracilaria cliftonii		Portland, Victoria, Australia		AY131321	AY131304
Gracilaria corticata					AY241160
Gracilaria denticulata					AY241161
Gracilaria eucheumatoides		Lombok, Indonesia		AY131318	AY131305
Gracilaria gigas	Gr-24	Nagahama, Hayato, Kagoshima, Japan	08-mai-01	AB193451	AB193459
Gracilaria gigas	Gr-25	Jogashima, Misaki, Miura, Kanagawa, Japan	06-avr-01	AB193452	AB193460
Gracilaria gracilis					AY241173
Gracilaria millardetii					AY241156
Gracilaria pacifica		San Juan Is.,Western Australia, Australia			U21353
Gracilaria perplexa		Bare I., Botany Bay, NSW, Australia		AY131325	AY131306
Gracilaria preissiana		Carnac Is., Western Australia, Australia		AY131324	AY131309
Gracilaria robusta		Monterey Co., CA			U21355
Gracilaria salicornia					AY241153
Gracilaria cf. *salicornia*	Gr-14	Namisato, Kin, Okinawa, Japan	09-jan-03	AB193453	AB193461
Gracilaria secundata		Kurnell, Botany Bay, NSW, Australia		AY131323	AY131311
Gracilaria tenuistipitata		Hainan Is., China		AY131317	AY131310
Gracilaria tikvahiae		Pomquet Harbor, Antigonish, Nova Scotia			U21357

Gracilaria verrucosa		DNA provided by C. Bird, NSERC, Halifax			U21358
Gracilaria vermiculophylla	Gr-16	Jogashima, Misaki, Miura, Kanagawa, Japan	06-avr-01	AB193454	AB193462
Gracilaria vermiculophylla	Gr-23	Shinori, Hakodate, Hokaido	11-juil-03	AB193455	AB193463
Gracilaria sp.JAW 3448		Dumaguete, Philippines		AY131322	AY131298
Gracilariopsis longissima					AY241174
Gracilariopsis tenuifrons		Venezuela			U21351
Gracilariopsis funiculus					AY241169
Gracilariopsis lemaneiformis		Central California, Oregon and B.C.			U21347
Gracilariopsis cf. lemaneiformis		culture from M. Pedersen, Uppsala Sweden			U21350

Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems, CA, USA) according to the manufacture's protocol. Both forward and reverse strands were sequenced using a DNA autosequencer (ABI PRISM, 310 Genetic Analyzer, Applied Biosystems, CA, USA).

Sequences of 8 samples of *Cox*2-3 spacer and 28 samples of RuBisco spacer belonging to *Gracilaria sensu lato* were downloaded from GenBank and included in the alignments, respectively. *Ptilophora prolifera* (Harvey) J. Agardh was used as an outgroup (Bynne *et al.*, 2002). Sequences were first aligned with the CLUSTAL W computer program (Thompson *et al*., 1994) and then refined by eye. The alignments are available from the second author upon request.

The maximum likelihood (ML) method was used to construct phylogenetic trees with PAUP 4.0 b10 (Swofford, 2002). Gaps in the alignments were treated as missing data. Takahashi & Nei (2000) mentioned that a simple model is better when short sequences are used. Thus, we used JC model (Jukes & Cantor, 1969) with TBR full heuristic search in the both spacer analyses. Bootstrap supports (100 replicates) were computed with the following settings: full heuristic search with TBR, starting tree = obtained by neighbor joining.

RESULTS

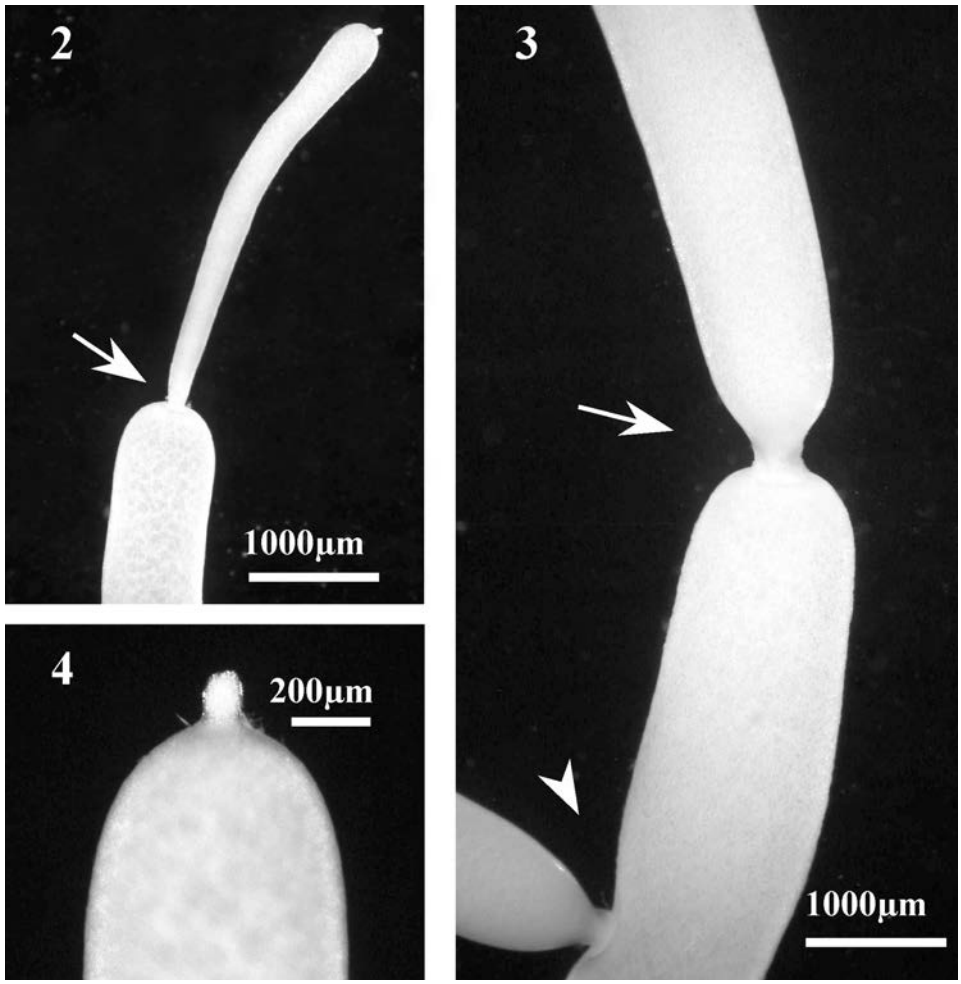
Morphology

Plants grow from November to April on pebbles or dead coral in the lower intertidal zone of a reef flat. Reproductively mature plants appears during late December and March, but mature gametophytes are not common in natural populations.

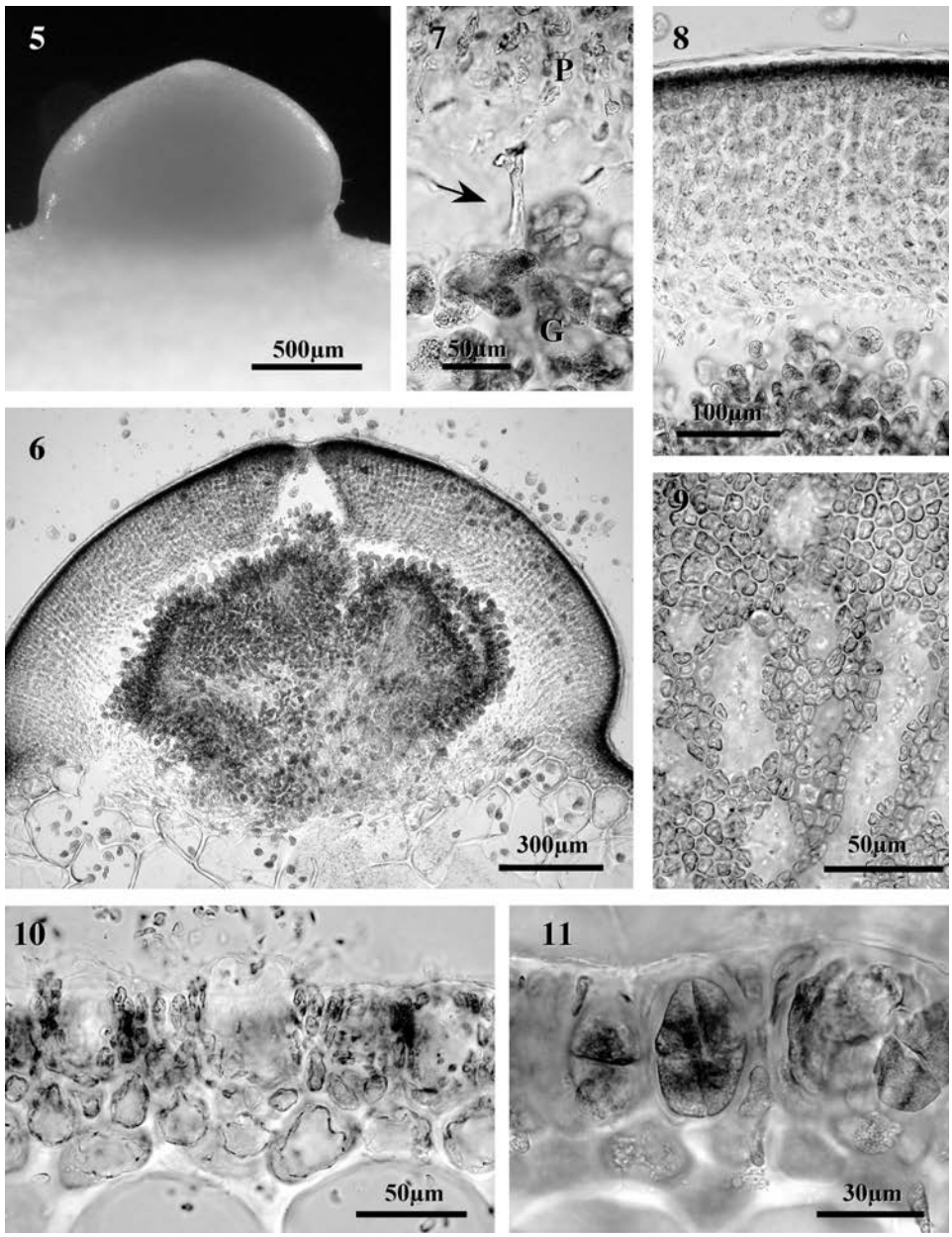
Thalli are solitary or caespitose and generally pale brown or somewhat reddish brown in color. Erect axes arise from a small discoid basal disc and are terete, cartilaginous, and reach up to 15 cm long. They are alternately, sometimes secundly or dichotomously branched. Branches are markedly constricted at the

bases and possess one or two (sometimes three) articulations. The branches and branchlets taper gradually into blunt apices (Figs 1-4). The apical portion has a small segment or sometimes not. Specimens do not adhere to herbarium paper when dried.

The thallus is structurally multiaxial with a pseudoparenchymatous medulla and cortex. The medulla consists of up to six layers of unpigmented, spherical, vacuolated cells, up to 600 μm diam., and the cortex consists of up to two layers of pigmented, globular cells with dense cytoplasm, up to 8 μm high and 7 μm wide. Medullary cells are frequently connected with surrounding cells by secondary pit-connections. Cortical cells are connected with only their parental cells by primary pit-connections.



Figs 2-4. Liquid-preserved material of *Gracilaria articulata* from Namisato, Kin, Okinawa Prefecture. **2, 3**. Upper part (Fig. 2) and lower part (Fig. 3) of main axis (male gametophyte) showing abruptly constricted branch bases (arrowhead) and articulations (arrows). **4**. Closer view of apical portion of branch showing blunt apices with small segment.



Figs 5-11. *Gracilaria articulata* from Namisato, Kin, Okinawa Prefecture. **5**. Globular cystocarp with slight constriction at the base. **6**. Vertical section of the cystocarp showing gonimoblast consisting of large cells. **7**. Traversing filament (arrow) connecting from the gonimoblast (G) to the pericarpic cells (P). **8**. Vertical section of pericarp. **9**. Surface view of main axis of male gametophyte showing mature spermatangial conceptacles. **10**. Vertical section of spermatangial conceptacles (*Textorii*-type). **11**. Vertical section showing cruciately divided tetrasporangia.

Cystocarps are formed on the surface of female gametophytes except for basal and apical parts. Mature cystocarps are protuberant, globose, up to 1.4 mm high by 1.6 mm wide, with a slightly rostrate or not rostrated ostiole and a slightly constricted base (Figs 5-8). Gonimoblasts consist of large cells up to 60 μm wide (Fig. 6). Traversing filaments are present in the cystocarp (Fig. 7). The pericarp consists of 10 layers of cells (Fig. 8) and reaches up to 150 μm thick.

Spermatangia are formed in shallow, cup-shaped *Textorii*-type (Yamamoto, 1978) conceptacles up to 40 μm deep, on the entire surface of male gametophytes except for apical and basal parts (Figs 9, 10).

Tetrasporangia are scattered on the entire surfaces of tetrasporophytes except for basal and apical portions. Tetrasporangia, with cruciately arranged spores, are up to 40 μm high by 60 μm wide (Fig. 11).

Culture experiments

Gracilaria articulata showed a *Polysiphonia*-type life history in culture. Released carpospores attached to the bottom of glass bottles within 12 hours and developed into discoid sporelings in five to seven days at 24°C. Sporelings grew to plants with erect axes 3 mm long at 24°C after 30 days. In one-and-a-half-month-old cultures, 10-15 individuals with 10-mm axes were detached from the substratum and transferred to 300 or 500 ml flasks as unattached cultures at the same water temperatures with aeration. They grew to 5 cm in length in three months and formed tetrasporangia after a further month (Fig. 12). Released tetraspores showed a developmental pattern similar to that of carpospores, and grew into mature dioecious gametophytes that formed spermatangial conceptacles or cystocarps in four months.

Fig. 12. *In vitro* cultured plants of *Gracilaria articulata* (mature tetrasporophyte, 24°C, 12L:12D) in five-month culture.

Reproductively mature cultured plants were 4-6 cm long with a morphology similar to that of field-collected plants 10-15 cm long, particularly in the terete axes with intercalary articulations and basal constrictions, and blunt apices (Fig. 18). Reproductive organs formed on the cultured plants were identical to those of field-collected plants.

Molecular phylogenetic analyses

The *Cox*2-3 spacer and RuBisco spacer sequences for the three samples of *G. articulata* from China (type material, *AST 59-2565c*) and Japan were found to be identical, respectively. The phylogenetic trees obtained from the *Cox*2-3 spacer region indicated that *G. articulata* clustered with the clade consisting of *G. blodgettii* and *Gracilaria* sp. with less than 50% bootstrap sup-

port (Fig. 13). In the RuBisco spacer analysis, *G. articulata, G. aculeata* (Hering) Papenfuss, *G. salicornia* (C. Agardh) Dawson, *G. blodgettii* and *Gracilaria* sp. were resolved as monophyletic with 56% bootstrap support (Fig. 14).

DISCUSSION

Gracilaria articulata was first described by Chang & Xia (1976) from Hainan Island. Chang & Xia (1976) and Xia (1985) characterized this species as having the following features: 1) 10 to 20 times articulated thalli with club-shaped segments, 2) presence of lateral branches, 3) presence of nutritive filaments (= traversing filaments) between the gonimoblast and pericarp in the cystocarp, 4) a thick pericarp, 60-85 μm deep, and 5) "oval" spermatangial conceptacles 41 μm deep. Our Okinawan alga agrees well with these features of *G. articulata*. Our molecular data indicated that no sequence differences in the *Cox*2-3 spacer and RuBisco spacer regions are found in our Japanese specimens and type material (*AST 59-2565c*) of *G. articulata*. Accordingly, we can conclude that our Okinawan alga is *G. articulata* reported from Japan for the first time.

Spermatangial conceptacles of *G. articulata* are deeper (about 40 μm deep) than those (less than 25 μm deep) of other Asian species with shallow *Textorii*-type spermatangial conceptacles, such as *G. gigas* Harvey and *G. textorii* (Suringar) De Toni, with the exception of *G. blodgettii* (Yamamoto, 1978; Terada & Yamamoto, 2000). However, spermatangial conceptacles of *G. articulata* should be regarded as the *Textorii*-type because of the confluence of conceptacles when fully mature. Terada & Yamamoto (2000) pointed out that the difference in the depth of conceptacles is due to the presence or absence of the confluence of multiple conceptacles, depending on growth stages as observed for both field-collected and cultured plants of *G. blodgettii*. A similar developmental pattern was also observed in *G. articulata*.

Gracilaria articulata is closely related to the Japanese taxon *G. blodgettii* in vegetative morphology, especially in the remarkable constrictions at the bases of branches and branchlets (Yamamoto, 1978; Terada & Yamamoto, 2000). It is also similar to the latter in reproductive morphology, especially in the large gonimoblast cells, the presence of traversing filaments between the pericarp and gonimoblasts, a thick pericarp consisting of 8-12 layers of cells, and the arrangement of spermatangia. However, intercalary articulations are present only in *G. articulata*. Blunt apices of branches and branchlets of *G. articulata* are also different from the acute apices of *G. blodgettii*. These features of *G. articulata* were also represented in the cultured plants, whereas they were entirely absent in cultured plants of *G. blodgettii* (Terada & Yamamoto, 2000). According to our molecular phylogenetic trees, Chinese and Japanese *G. articulata* form a clade separate to that including Japanese *G. blodgettii*. This result suggests that the presence of articulations and the characteristics of the apical portion of *G. articulata* are critical features that can be used to distinguish it from Japanese *G. blodgettii*. *Gracilaria articulata* also differs from the circumscription of genuine *G. blodgettii* (*sensu stricto*) as originally described by Harvey (1853) from Florida in the aforementioned features. However, articulations are present in Caribbean and Australian *G. blodgettii* (*sensu lato*) (Fredericq & Norris, 1992; Withell *et al.*, 1994), and further studies are required to determine their taxonomic relationships.

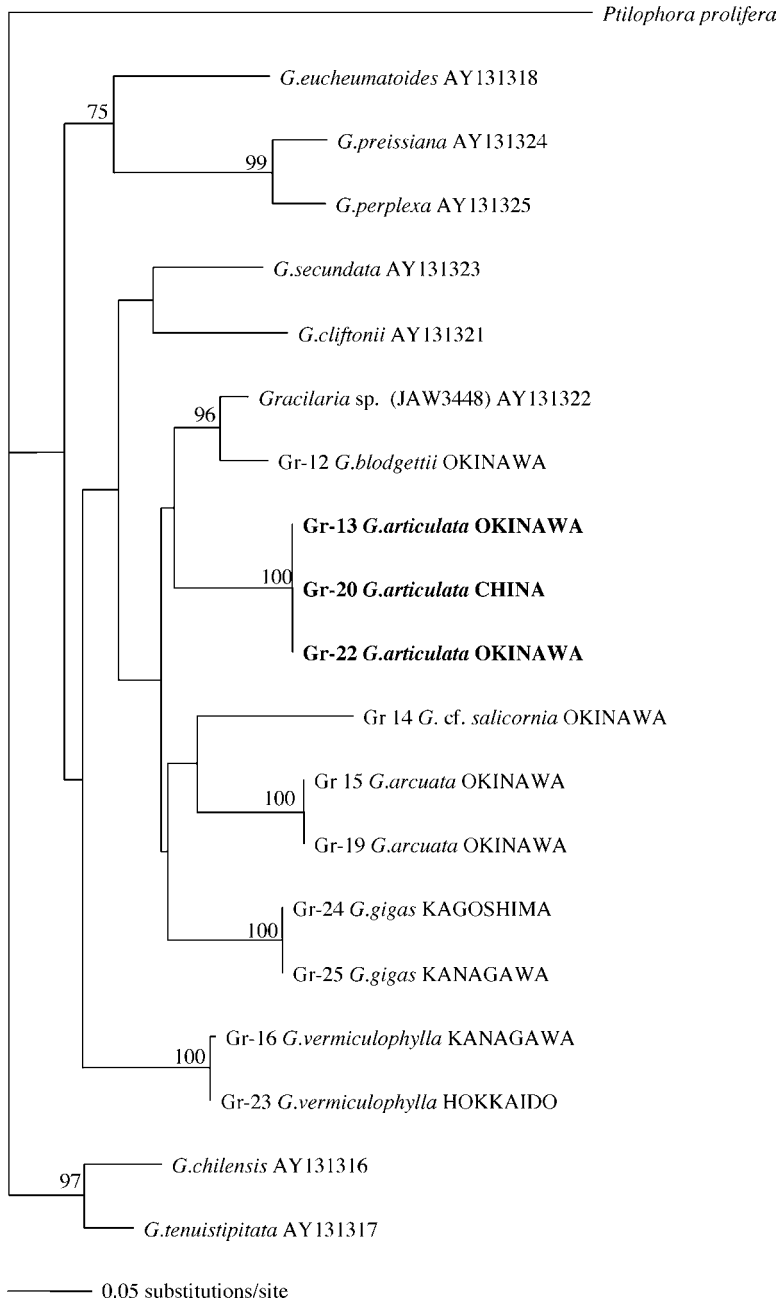


Fig. 13. Maximum likelihood tree constructed from ML analysis of the *cox2-3* spacer sequences. *Ptilophora prolifera* was used as an outgroup. The numbers under the branches represent full heuristic bootstrap values (100 replicates) greater than 50%. Branch lengths are proportional to the Jukes-Cantor distances, which are indicated by the scale bar below the tree.

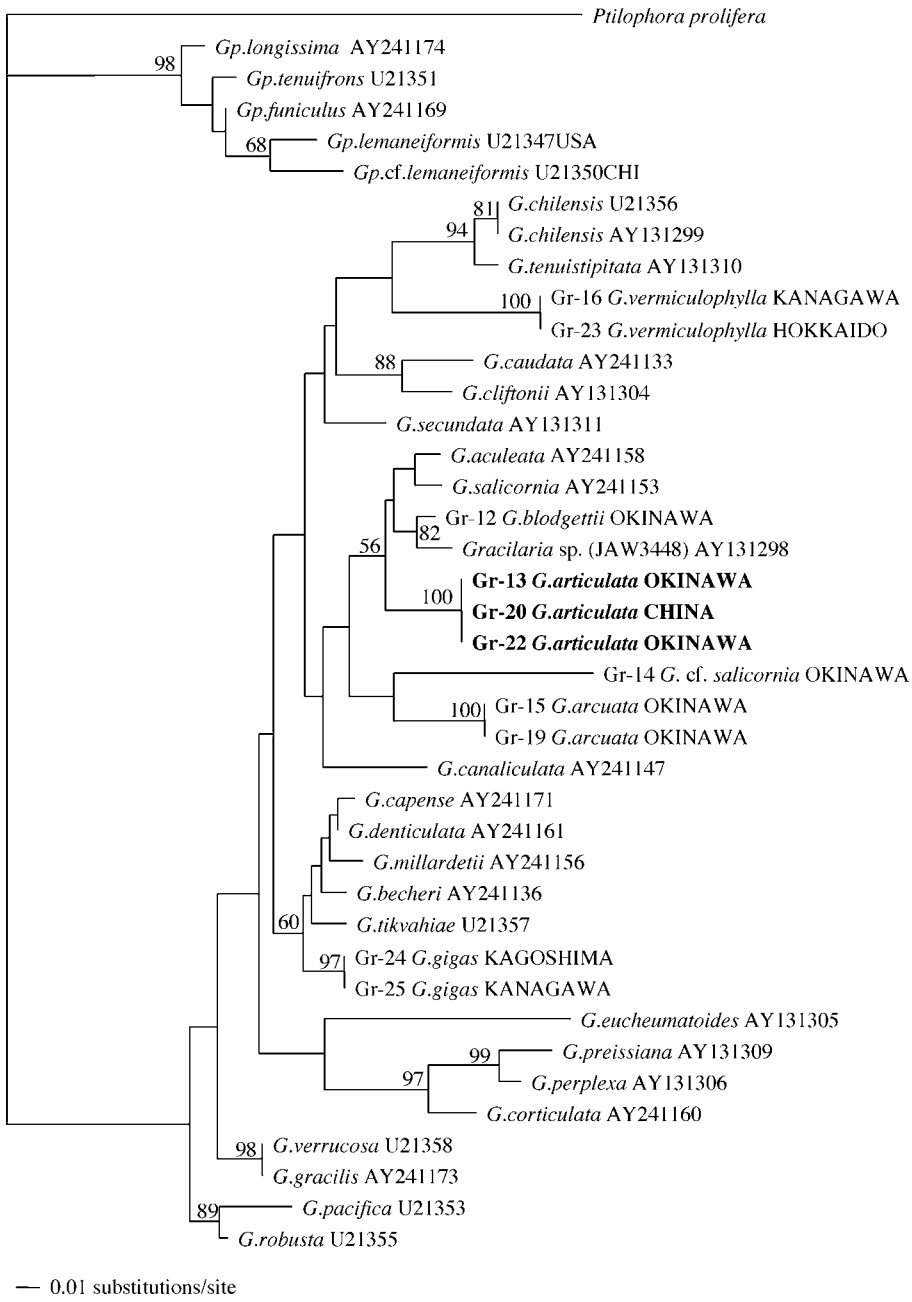


Fig. 14. Maximum likelihood tree constructed from ML analysis of the *RuBisco* spacer sequences. *Ptilophora prolifera* was used as an outgroup. The numbers under the branches represent full heuristic bootstrap values (100 replicates) greater than 50%. Branch lengths are proportional to the Jukes-Cantor distances, which are indicated by the scale bar below the tree.

Gracilaria articulata is also similar to the Pacific species *G. salicornia* (C. Agardh) Dawson, which was originally described from the Philippines (Dawson, 1954), in gross morphology, including conspicuous basal constrictions of branches and branchlets, articulated club-shaped segments, and blunt apices (Yamamoto, 1978). Withell *et al.* (1994) also suggested that *G. articulata* resembles *G. blodgettii* or *G. salicornia* in the presence of articulations. However, *G. articulata* has *Textorii*-type spermatangial conceptacles and therefore differs from *G. salicornia*, which has deep pot-shaped, *Verrucosa*-type spermatangial conceptacles (Yamamoto, 1991). The molecular phylogenic tree by the *cox2-3* spacer region shows that *G. salicornia* occurs in a different clade to that of *G. articulata*.

In this study, we used type materials of *G. articulata* for molecular phylogenic study to avoid misidentification of this species. We suggest that judicious selection of materials is one of the most important aspects of these molecular studies.

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