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Molecular analyses and reproductive structure to verify the generic relationships of *Hypnea* and *Calliblepharis* (Cystocloniaceae, Gigartinales), with proposal of *C. saidana* comb. nov.

Mi Yeon Yang and Myung Sook Kim*

Department of Biology, Jeju National University, Jeju 63243, Korea

The genera *Hypnea* and *Calliblepharis* of the family Cystocloniaceae are discriminated by their female reproductive structure, especially in the formation of carposporangia and gonimoblasts. *Hypnea saidana*, once classified based on obsolete evidence, has not been studied phylogenetically using molecular analysis and detailed reproductive structure though it shares many morphologic features with the genus *Calliblepharis*. To provide better understanding of generic relationship of *H. saidana* with *Hypnea* and *Calliblepharis*, we carried out molecular analyses using the nuclear-encoded small subunit ribosomal DNA (SSU) and chloroplast-encoded large subunit of the RuBisCO (*rbcL*), and exact morphological observations focusing on the reproductive structures of wild specimens. Our molecular phylogeny showed that *H. saidana* is closely related to *Calliblepharis*, but distinct from the clade of *Hypnea*. Female reproductive structure of *H. saidana* characterized by upwardly developing chains of carposporangia, central reticulum of cell, and gonimoblast filaments not connected to the pericarp provides definite evidence to assign the taxonomic position of this species to *Calliblepharis*. Based on our combined molecular and morphological analyses, we have proposed *Calliblepharis saidana* comb. nov., expanding the distribution of *Calliblepharis* habitat from the eastern Atlantic South Africa, the northern Indian Ocean, Australasia, and Brazil to the western Pacific Ocean.

Key Words: *Calliblepharis saidana* comb. nov.; Cystocloniaceae; *Hypnea*; molecular phylogeny; reproductive structure; taxonomy

INTRODUCTION

The family Cystocloniaceae Kützting (Order Gigartinales F. Schmitz) includes ca. 100 species, in 13 genera that are characterized by uniaxial, terete or flat thalli, filamentous or pseudoparenchymatous medulla, zonately divided tetrasporangia, and procarps with 3- or 4-celled carpogonial branches (Womersley 1994, Saunders et al. 2004, Guiry and Guiry 2017). *Hypnea* J. V. Lamouroux, the most speciose genus in the family with 62 species, generally has terete thalli that are erect or entangled (Guiry

and Guiry 2017), but their male and female structures are inadequately understood (Kylin 1930, Mshigeni 1976, Vázquez-Delfín et al. 2016). Recent molecular studies have confirmed *Hypnea* as a strongly supported monophyletic group that is closely related to the genus *Calliblepharis* Kützting (Saunders et al. 2004, Geraldino et al. 2010).

Although the inclusion of molecular data has become commonplace in modern taxonomy (Yang et al. 2015,

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*Corresponding Author

E-mail: myungskim@jejunu.ac.kr

Tel: +82-64-754-3523, Fax: +82-64-756-3541

Calderon and Boo 2016, Won et al. 2016, Yang and Kim 2016), reproductive structures have remained important diagnostic characteristics for differentiating many genera in the order Gigartinales (Hommersand et al. 1999, Díaz-Tapia et al. 2013). For instance, *Hypnea* and *Calliblepharis* can be readily distinguished each other by female reproductive structures even if they share many vegetative features in common. Although carposporangia are formed as terminal cells of each gonimoblast filament in both *Hypnea* and *Calliblepharis*, each gonimoblast filament produce only one carposporangium in *Hypnea*, while *Calliblepharis* exhibits in chains. Also, numerous filaments link the gonimoblasts and the pericarp in *Hypnea*, but these filaments are absent in *Calliblepharis* (Kylin 1928, 1930, 1932).

Hypnea saidana Holmes is a red algae characterized by complanate and more or less dichotomous thalli with alternate branches that are mostly curved bearing short branchlets (Holmes 1895, Okamura 1909, Tanaka 1941). First described based on the material collected from Enoshima, Japan (Holmes 1895), it has remained one of the most taxonomically well characterized species in Korea and Japan. However, recent development of phylogenetic methodology made the need for improving *H. saidana* taxonomy based on obsolete evidence urgent. For example, current classification of *H. saidana* lack the consideration of reproductive structure, according to Shin and Boo (1994) which is essential for proper reassessment due to its similarity to *Calliblepharis* in terms of vegetative features, i.e., complanate thalli, dispersed surface cells, and numerous pit connections between medullary cells. In addition, recently described taxa, *Calliblepharis hypneoides* Díaz-Tapia, Bárbara & Hommersand and *C. psammophilus* D'Archino & W. A. Nelson, were assigned to *Calliblepharis* based on observations of the female structures even though they superficially resembled the species of *Hypnea* (Díaz-Tapia et al. 2013, D'Archino et al. 2015).

However, taxonomy based on the reproductive characteristics, though effective, poses a serious problem in identifying appropriate specimens during the asexual stage of red algae (Sherwood et al. 2010) and molecular procedures have been used to circumvent this problem. In fact, these molecular procedures have rapidly redefined phylogenetic relationships among Gigartinales taxa recently (Yang et al. 2015, Calderon and Boo 2016, Yang and Kim 2016). Several molecular studies have also been performed to verify the phylogenetic relationships of species within genus *Hypnea* itself (Geraldino et al. 2009, 2010, D'Archino et al. 2015).

These considerations clearly reveal the possibility that current taxonomy of *H. saidana* based on vegetative morphology could be mistaken as Díaz-Tapia et al. (2013) mentioned, although some red algal species can be assigned to *Hypnea* based on morphology, they could be assigned equally well to *Calliblepharis*. This implies that phylogeny regarding species of these two genera requires highly refined morphological characterization and molecular analysis using diverse species enough to allow inter-genera comparison within the order Gigartinales.

In this study, we tried to reexamine *H. saidana* phylogeny (Okamura 1909, Tanaka 1941, Shin and Boo 1994, Yamagishi and Masuda 1997) to put it on a more accurate taxonomic position. As mentioned above, the family Cystocloniaceae requires the concentrated study using both molecular and morphological data to infer their phylogenetic relationships. Therefore, we put special emphasis on precise morphological characterization of reproductive structures, which was possible through direct observation of female and male reproductive structures of wild specimens. We also noted that our morphological characterization tightly supported our molecular analyses using the combined alignments of the nuclear-encoded small subunit ribosomal DNA (SSU) and chloroplast-encoded large subunit of the RuBisCO gene (*rbcL*) sequences.

MATERIALS AND METHODS

Specimen preparation

Samples of *Calliblepharis saidana* were collected in Korea and Japan during 2012-2016 (Table 1). Vegetative specimens were collected year-round, but female / male specimens were sampled only in the Jeju (August 2015) and Wando (January 2016), Korea. Field collections were kept fresh in a cool box containing seawater and transported to the laboratory. Voucher specimens were deposited to the Herbarium of Jeju National University and the National Institute of Biological Resources (NIBR RD0000000005), Incheon, Korea. Sub-samples were dried in silica gel for molecular analyses or in 5% formalin / seawater for morphological observations. Sections were hand cut using a razor blade or prepared using a benchtop freezing microtome (MFS No. 222; Nippon Optical Works, Tokyo, Japan). Section samples were stained with 1% aniline blue, acidified with 1% HCl and mounted in a 40% corn syrup solution. We also treated with Witmann's (1965) aceto-iron-hematoxylin-chloral hydrate, and mounted in 50% Hoyer's mounting medium (Lin et

al. 2009). Photomicrographs were captured using the EOS 600 D digital camera (Canon, Tokyo, Japan) mounted on the BX 43 microscope (Olympus, Tokyo, Japan). Images of pressed specimens were captured using the D 80 camera (Nikon, Tokyo, Japan). The digitized images were imported into Adobe Photoshop (ver. 6.1) software and edited to produce the photographic plates.

DNA extraction and sequencing

DNA was extracted from small portions of dried thalli in liquid nitrogen using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The extracted DNA was stored at -20°C, and used to amplify chloroplast *rbcL* and nuclear SSU rDNA. The primer combination F7-R898 and R762-R1442 was used for *rbcL* (Gavio and Fredericq 2002, Kim et al. 2010) and G01-G14 and G04-G07 for SSU rDNA (Saunders and Moore 2013), respectively. Polymerase chain reaction (PCR) amplification was performed in the Swift MaxPro thermal cycler (ESCO, Singapore) using AccuPower PCR Premix (Bioneer, Daejeon, Korea). PCR products were pu-

rified using the Accuprep PCR Purification Kit (Bioneer) following manufacturer's instructions. Sequencing of the forward and reverse strands of the purified PCR products was performed commercially by Macrogen (Seoul, Korea). Two electropherogram outputs from each sample were processed using Chromas version 1.45 software (Technelysium Pty Ltd., South Brisbane, QLD, Australia).

Phylogenetic analyses

Sequences generated in this study and sequences obtained from GenBank were aligned using Clustal O software (Sievers et al. 2011), and adjusted visually. We used sequences of 24 Gigartinales taxa for SSU and 41 Gigartinales taxa for *rbcL*, respectively. The sequence alignment of *rbcL* (a protein-encoding gene) was unequivocal due to the absence of indels while ambiguous sites of SSU rDNA were trimmed for the alignment. The SSU and *rbcL* alignments were concentrated and a combined data set were analyzed using maximum likelihood (ML) and Bayesian inference (BI) mentioned the following methods.

To infer phylogenetic relationships, we conducted

Table 1. Collection information of *Calliblepharis saidana* comb. nov. used in this study

Collection information	Voucher	GenBank accession No.
Aedo, Namhae, Korea; May 19, 2012; 34°41'31" S, 128°02'06" E; subtidal	Gigar017	MF083596 ^a
Biyangdo, Jeju, Korea; Jul 15, 2015; 33°24'51" S, 126°13'45" E; subtidal	Gigar425 ^b	MF083583 ^a
♀; Biyangdo, Jeju, Korea; Jul 15, 2015; 33°24'51" S, 126°13'45" E; subtidal	Gigar426 ^b	MF083582 ^a MF083599 ^c
Chujado, Jeju, Korea; Aug 17, 2012; 33°56'40" S, 126°19'51" E; intertidal	Gigar115	MF083594 ^a
Daesambudo, Yeosu, Korea; Jul 31, 2013; 34°03'33" S, 127°23'03" E; subtidal	Gigar195 ^b	MF083595 ^a
Ganjeolot, Ulsan, Korea; Jan 12, 2012; 35°21'35" S, 129°21'43" E; intertidal	Gigar129 ^b	MF083597 ^a
Gijang, Busan, Korea; Jul 26, 2012; 35°11'24" S, 129°13'25" E; intertidal	Gigar134	MF083592 ^a
Jakdo, Yeosu, Korea; Jul 26, 2012; 34°24'53" S, 127°54'14" E; subtidal	Gigar016 ^b	MF083598 ^a
Munseom, Jeju, Korea; Jan 17, 2016; 33°13'40" S, 126°34'02" E; subtidal	Gigar096 ^b (NIBRRD0000000005)	MF083589 ^a
Seopseom, Jeju, Korea; Jan 23, 2014; 33°13'46" S, 126°35'46" E; subtidal	Gigar263	MF083590 ^a
Songjeong, Busan, Korea; Dec 20, 2012; 35°10'39" S, 129°12'09" E; subtidal	Gigar098	MF083593 ^a
⊕; Wando, Korea; Jan 16, 2013; 34°17'46" S, 126°42'05" E; drift	Gigar135 ^b	MF083591 ^a
⊕; Wando, Korea; Jan 16, 2013; 34°17'46" S, 126°42'07" E; drift	Gigar533 ^b	MF083580 ^a
♀; Wando, Korea; Jan 24, 2016; 34°17'46" S, 126°42'07" E; drift	Gigar535 ^b	MF083579 ^a
♂; Wando, Korea; Jan 24, 2016; 34°17'46" S, 126°42'07" E; drift	Gigar534 ^b	MF083578 ^a
Enoshima beach, Fujisawa, Japan; Mar 28, 2014; 35°18'25" S, 139°25'15" E; drift	Gigar308 ^{b,d}	MF083585 ^a MF083600 ^c
Enoshima beach, Fujisawa, Japan; Mar 28, 2014; 35°18'25" S, 139°25'15" E; drift	Gigar309 ^{b,d}	MF083584 ^a
Ohara, Isumi, Chiba, Japan; Mar 24, 2014; 35°15'10" S, 140°24'26" E; drift	Gigar307 ^b	MF083587 ^a
Onjuku beach, Chiba, Japan; Mar 24, 2014; 35°10'56" S, 140°21'21" E; drift	Gigar306 ^b	MF083588 ^a
Ubara beach, Katsuura, Japan; Mar 22, 2014; 35°08'12" S, 140°16'26" E; drift	Gigar305 ^b	MF083586 ^a
Ubara beach, Katsuura, Japan; Mar 22, 2014; 35°08'12" S, 140°16'26" E; drift	Gigar510 ^b	MF083581 ^a

^a*rbcL* sequences.

^bSpecimens for morphological observation.

^cSSU sequences.

^dSpecimens from the type locality.

Fig. 1. Vegetative morphology of *Calliblepharis saidana* comb. nov. (A) Drift sample from the type locality, Enoshima, Japan (Mar 28, 2014, voucher = Gigar308). (B) Drift sample from Wando, Korea (Jan 24, 2016, voucher = Gigar533). (C) Intertidal sample collected from Ganjeolot, Korea (Jan 12, 2012, voucher = Gigar129). (D) Subtidal sample collected from Munseom, Korea (Jan 17, 2016, voucher = Gigar096). (E) Subtidal sample from Daesambudo, Yeosu, Korea (Jul 31, 2013, voucher = Gigar195). (F & G) Apical part of an erect branch with numerous alternate branchlets and a second one with outward branchletss. (H) Basal part showing a small discoid holdfast (arrow). (I) Several projections (arrows) on a branch. (J) Surface view of a thallus showing angular to rounded cortical cells. (K) Apical cell (arrow) of a thallus tip. (L) Longitudinal section of a thallus median part showing an axial filament (arrow) with several central elongated cells. (M) Cross-section of a branch showing two to four rounded medullary cells. (N) Layer of rounded cortical cells. Scale bars represent: A & B, 2 cm; C-G, 1 cm; H, 5 mm; I, 2 mm; J, K & N, 50 μ m; L & M, 200 μ m.

ML analysis using the GTR + Γ evolutionary model ('-m GTRGAMMA' option) in RAxML (Stamatakis 2006). To identify the best-fit tree, we performed 200 independent tree searches using 'the number of runs' option. We ran 1,000 replications in RAxML software using the same setting to generate bootstrap support value of monophyletic nodes (MLBt). We also performed the BI in MrBayes v.3.2.1 (Ronquist et al. 2012) using the metropolis-coupled Markov Chain Monte Carlo simulations with the GTR + Γ model to select the most appropriate phylogenetic tree. For two independent runs, we computed 2×10^6 generations with four chains, and sampled the trees every 100

generations. The burn-in point was identified graphically by tracking the likelihoods when the plateaued. Eventually, total 29,000 trees sampled in the stationary state were used to infer Bayesian posterior probabilities (BPP).

RESULTS

Morphology of *Calliblepharis saidana*

We collected *Calliblepharis saidana* from lower intertidal to subtidal zones where plants were attached

Fig. 2. Female reproductive structure of *Calliblepharis saidana* comb. nov. (voucher = Gigar425, 535). (A) Cystocarps formed on the base of branches or branchlets. (B & C) Placenta (p) were developed inwardly from the gonimoblast initial (gi) at the center of cystocarp while gonimoblast filaments (gf) were produced at the outward location. (D) Transverse section of a cystocarp showing its sessile shape without ostiole. Central reticulum (r) and basal placenta (p) positions are marked. (E) Central reticulum (r) composed of interconnected filaments. (F) Chains of carposporangia. Scale bars represent: A, 2 cm; B, C & E, 100 μ m; D, 200 μ m; F, 50 μ m.

to small pebbles, shellfish, other seaweeds, or drifted near the shoreline. Thalli of *C. saidana* were erect, compressed, 2-8 cm tall, 0.5-2.0 mm wide, and 400-850 μ m thick, formed cartilaginous rounded clumps (Fig. 1A-E, L & M). Branches appeared dichotomous or alternate of erect thalli or unilaterally in outward side of second thalli. They were usually curved, tapered to points, and bore short branchlets. Branching took place most often in a single plane but sometimes in three dimensional space (Fig. 1F & G). Holdfasts were discoid and multiple thalli arose from a single holdfast (Fig. 1H). Small projections attached to the substratum or adjacent branches were observed in thalli and branches (Fig. 1I).

On a surface view, cortical cells of a thallus were of various convex polygonal shapes and they did not form distinct rosettes (Fig. 1J). A thallus grew uniaxially initiated by a spearheading apical cell (Fig. 1K). It progressed

through oblique divisions producing newly differentiated apical cells in alternating sides of growth axis. Longitudinal sections of the mid-frond (Fig. 1L) showed an axial filament, the trace of apical cell strand during growth, surrounded by elongated cells linked through secondary pit-connections. In the cross-sectional view, the central core of axial filament and periaxial cells were surrounded by two to four layers of large, rounded medullary cells measured 90-280 $\times$ 85-160 μ m (Fig. 1M). The cortex consisted of a layer of rounded or cuboidal cells with 10-30 μ m in diameter (Fig. 1N).

Cystocarps with gonimoblast initial, gonimoblast filaments, and placenta were formed inside branches and branchlets first and protruded through the cortex near their bases or sprouting points during maturation (Fig. 2A-D). When fully matured, they were sessile, subglobose with 600-800 μ m in diameter encased in a thick pericarp

Fig. 3. Male reproductive structure of *Calliblepharis saidana* comb. nov. (voucher = Gigar534). (A) Surface view of a thallus forming spermatia dyed dark. (B) Cross-section of a spermatangial branchlet covered by gelatinous matrix. (C) Modified cortical cells (arrows) producing spermatangial mother cells. (D & E) Spermatangial mother cells (arrows) produce spermatia after cleavage. (F) Spermatangial filaments with up to seven spermatia stacked up at respective terminals. (G) Mature spermatangia enclosed by antheridial walls (arrows). (H) Release of spermatia accumulated in the upper part of antheridial wall (arrow). Scale bars represent: A & C-H, 20 µm; B, 100 µm.

without any ostiole (Fig. 2D). Nutritive placentae were generated at inward position from gonimoblast initial (Fig. 2B). Gonimoblast filaments were developed outwardly from gonimoblast initial and progressively turned into carposporangial chains (Fig. 2B-D). Small, interconnected cells between gonimoblasts and placenta formed a reticulum (Fig. 2D & E). Mature globose carposporangia of 15-30 µm diameter piled up just outside the gonimoblast reticulum (Fig. 2F).

The spermatangia formed sori on branches and branchlets. Surface views of male plants showed rounded spermatia with 3-5 µm in diameter (Fig. 3A). A gelatinous matrix covered the reproductive portion of the thallus (Fig. 3B). Some of cortical cells became elongated (Fig. 3C), formed spermatangial mother cells (Fig. 3D), and produced spermatia (Fig. 3D-F). Spermatia existed in chains stacked up to seven cells (Fig. 3F). The mature antheridium was enclosed in a thick wall cushioned by an interposed gelatinous layer (Fig. 3G). Matured spermatia broke free from spermatangial chains, aggregated at the apex of the wall, and released through an apical split in the antheridial wall (Fig. 3G & H).

Tetrasporangia formed sori covering the basal and mid portions of short cylindrical branches (Fig. 4A-C). Tetrasporangia were initiated from modified cortical cells

which bore lateral cells connected through pit connections (Fig. 4D & E). After rounds of successive zonate division, tetrasporangia with 4 compartments appeared (Fig. 4F & G). Mature tetrasporangia were 15-30 µm in diameter and 60-70 µm in length (Fig. 4H & I).

Molecular phylogeny of *Calliblepharis saidana*

Our nuclear SSU sequence dataset included 24 sequences from 10 genera of the Cystocloniaceae and two outgroup genera of the order Gigartinales (Fig. 5). The ML and BI trees based on SSU sequences were identical and two specimens of *C. saidana* collected in Korea and Japan were also identical. The clade that included *Hypnea* was strongly supported (MLBt = 100 / BPP = 1.0), but *Calliblepharis* was not monophyletic. To our surprise, *C. saidana* formed a group with five species of *Calliblepharis*, including the type species *C. ciliata* (AY437668), support values = 59/0.88. Interestingly, *C. planicaulis* (AY437670) was more closely related to the *Hypnea* clade, support values = 75/0.97 than to *Calliblepharis*.

The plastid *rbcL* sequence dataset comprised 41 sequences representing six genera from the Cystocloniaceae

Fig. 4. Tetrasporangia of *Calliblepharis saidana* comb. nov. (voucher = 135, 533). (A–C) Tetrasporangial sori (arrows) on basal or middle portions of short branchlets. (D) Cross-section of a tetrasporangial sorus showing tetrasporangia (arrows) immersed in the cortex. (E) Tetrasporangial primordium (arrow) differentiating in a modified cortical cell with a pit connection. (F) First division to form two cells (arrow) with a pit connection to its bearing cell. (G) Formation of tetraspores (arrow) through successive rounds of zonate division with a pit connection to its bearing cell. (H & I) Mature tetrasporangia of varying morphology. Scale bars represent: A, 2 mm; B & C, 1 mm; D, 100 µm; E & G–I, 20 µm; F, 30 µm.

ae and three outgroup genera from the Areschoiugiaceae of the order Gigartinales. The ML and BI trees constructed from *rbcL* sequences of the Cystocloniaceae were also identical (Fig. 6). All *rbcL* sequences from 21 specimens of *C. saidana* collected at 15 sites around Korea and Japan were identical as well. Twenty species of *Hypnea* were well clustered, but *C. saidana* was not included in the *Hypnea* clade again. Rather, *C. saidana* was consistently placed between *C. fimbriata* (AF385654) and a clade consisted four other *Calliblepharis* species with the type species *C. ciliata* (KC121116).

The ML and BI using the combined alignment of SSU and *rbcL* sequences yielded almost concordant tree, with little difference at node (Fig. 7). The combined tree resolved monophyletic node of the genus *Calliblepharis* including type species, *C. ciliata*, even though the node was supported by low bootstrap values. *C. saidana* formed weak sister relationships with *C. fimbriata* and *C. celatospora*.

Based on our morphological and molecular data, we

conclude *C. saidana* is more closely related to the genus *Calliblepharis* than to the genus *Hypnea* and propose a new combination:

***Calliblepharis saidana* (Holmes) M. Y. Yang & M. S. Kim comb. nov.**

Basionym. *Hypnea saidana* Holmes 1895: 256 [New marine algae from Japan. J. Linn. Soc. Lond. Bot. 31:248–260, 6 plates].

Type locality. Enoshima, Japan (Holmes 1895).

DISCUSSION

Calliblepharis saidana has been assigned to the genus *Hypnea* since its first description because it resembles members of the genus *Hypnea* superficially: it has uniaxial, cartilaginous thalli, alternate branches with short branchlets, rounded medullary cells surrounding

Fig. 5. The maximum likelihood phylogeny of *Calliblepharis saidana* comb. nov. based on the nuclear small subunit rDNA sequences data (-lnL = 4,548.176818). Support values shown on branches are maximum likelihood bootstrap (MLBt) and Bayesian posterior probability. MLBt values of <50% are indicated by hyphens (-). The sequence in bold was generated in this study.

the central axial filaments, and zonately divided tetrasporangia (Holmes 1895, Okamura 1909, Tanaka 1941). However, with the continued refinement of conventional morphology-based taxonomic approach and the introduction of novel molecular analysis-based methodology, the red algal taxonomy, especially between *Hypnea* and *Calliblepharis* of the order Gigartinales, has undergone substantial restructuring recently and the need for reassessing *C. saidana* has been raised. For instance, the feature of a complanate thallus in vegetative morphology suggested a relationship with *Calliblepharis* which typically has compressed or flattened thallus (Min-Thein and Womersley 1976, Shin and Boo 1994). Furthermore, there

were reports on *Calliblepharis* species having completely terete thallus as well: *C. hypneoides* and *C. psammophilus* collected from the Atlantic Iberian Peninsula and New Zealand, respectively (Díaz-Tapia et al. 2013, D'Archino et al. 2015).

Our molecular analyses using sequences of both SSU and *rbcL* demonstrated the phylogenetic position of *C. saidana* within the family Cystocloniaceae. The combined tree derived from SSU and *rbcL* alignments showed that *C. saidana* was grouped with the clade including the type species *C. ciliata*. They shared inner structure, an axial filament with surrounding elongated periaxial cells (Díaz-Tapia et al. 2013). The structure such as periaxial

Fig. 6. The maximum likelihood phylogeny of *Calliblepharis saidana* comb. nov. based on the plastid *rbcL* data ($-\ln L = 9,663.853378$). Support values shown on branches are maximum likelihood bootstrap (MLBt) and Bayesian posterior probability from DNA data. MLBt values of <50% are indicated by hyphens (-). Sequences in bold were generated in this study. Asterisk means specimens from type locality.

Fig. 7. The maximum likelihood phylogeny of *Calliblepharis saidana* comb. nov. derived from combined alignment (*rbcl* and SSU rDNA). Support values shown on branches are maximum likelihood bootstrap (MLBt) and Bayesian posterior probability from DNA data. MLBt values of <50% are indicated by hyphens (-). Asterisk means the type species of *Hypnea* and *Calliblepharis*. To right of tree, diagrams of cross section of the vegetative thallus (1-6) and female cystocarp structures (7-9) for the family Cystocloniaceae. 1. Axial cell with medullary cells in the genus *Hypnea*. 2. Axial cell surrounded by several periaxial cells and medullary cells in the genus *Calliblepharis*. 3. Axial cell surrounded by periaxial cells and medullary cells with some rhizoidal filaments in the genus *Craspedocarpus*. 4. Axial cell with medullary cell in the genus *Rhodophyllis*. 5. Inconspicuous axial cell and medulla of elongated cells with rhizoids in the genus *Stictosporum*. 6. Axial cell and medulla of elongated cells with rhizoids in the genus *Austroclonium*. 7. Single carposporangia and numerous filaments connecting the gonimoblast and the pericarp in the genus *Hypnea*. 8. Chains of carposporangia and reticulum of gonimoblast filaments in the *Calliblepharis* species including type species and *C. saidana*. 9. Chains of carposporangia and parallel columns of gonimoblast filaments in *C. fimbriata* and *C. celatospora*.

filaments of main axis is a synapomorphic feature of the genus *Calliblepharis* but it is absent in other genera of Cystocloniaceae as well as most species of *Hypnea* (Table 1) (Kylin 1930, Tanaka 1941, Díaz-Tapia et al. 2013). Other genera such as *Craspedocarpus*, *Rhodophyllis*, *Cystoclonium*, and *Stictosporum* have an axial cell surrounding by medullary cell and frequently have rhizoidal filaments in medulla (Fig. 7) (Min-Thein and Womersley 1976). The rhizoidal filaments are absent in both genera *Calliblepharis* and *Hypnea*. The genus *Craspedocarpus* is similar with *Calliblepharis* in having several periaxial filaments surrounding axial cell, but *Craspedocarpus* differs by its rhizoids in the medulla and well developed rosette in cor-

tical cells (Womersley 1994).

Contrary to thallus morphology, female reproductive structures reliably discriminate *Calliblepharis* from *Hypnea* despite their convoluted taxonomic history. Both genera were originally placed in the family Hypneaceae because of the procarp structure (Kylin 1930). *Calliblepharis* was later moved to the family Cystocloniaceae (as Rhodophyllidaceae) based on the female reproductive structures (Kylin 1932). However, Hypneaceae and Cystocloniaceae were recently merged based on the phylogenetic analysis of SSU sequences (Saunders et al. 2004), putting *Calliblepharis* and *Hypnea* in the same family again. Notwithstanding, *Calliblepharis* is readily distin-

Table 2. Comparisons of morphological characteristics of *Calliblepharis saidana* with related species in the genus *Calliblepharis*

	<i>C. saidana</i> (Holmes) M. Y. Yang & M. S. Kim	<i>C. ciliata</i> (Hudson) Kützinger	<i>C. fimbriata</i> (Gréville) Kützinger	<i>C. hypneoides</i> P. Díaz-Tapia, I. Bárbara & M. H. Hommersand	<i>C. jubata</i> (Goodenough & Woodward) Kützinger	<i>C. occidentalis</i> Joly & Yamaguishi-Tomita	<i>C. psammophilus</i> D'Archino & W. A. Nelson
Thallus	Compressed; grow up to 8 cm in high	Compressed, terete fibrous proliferations; grow up to 30 cm in high	Compressed, grow up to 23 cm in high	Terete; grow up to 7 cm in high	Compressed, terete fibrous proliferations; grow up to 30 cm in high	Compressed, terete small proliferations; grow up to 20 cm in high	Terete; grow up to up to 12 cm in high
Holdfast	Discoid	Fibrous	Fibrous	Fibrous	Fibrous	Small inconspicuous	Fibrous
Filaments surrounding axial filament	Present	Present	-	Present	Present	-	Present
Rosettes	Absent	Absent	Absent	Absent	Weakly developed	Absent	Present
Gonimoblast	Reticulum	Reticulum	Parallel columns	Reticulum	Reticulum	-	Fusion cell
Carposporangia	Chains	Chains	-	Chains	Chains	-	Chains
Cystocarp	On branchlets without ostiole	On proliferation with ostiole	On proliferation without ostiole	On main axes and branches without ostiole	On proliferation with ostiole	On branchlets without ostiole	On branches without ostiole
Spermatangia	Sori on branchlets; up to 7-celled spermatangial filaments	Large surface sori on the younger parts	-	Sori on branchlets; 3-4-celled spermatangial filaments	Short spermatangial filaments	Sori on upper branches; formed directly by the cortical cells	Sori on apical branches; 2-3-celled spermatangial filaments
Tetrasporangia	Sori on branches	Sori on blade surface and margins	Scattered over blades and proliferations	Sori on branchlets	Sori on blade surface and margins	Scattered on the marginal branches	Scattered on branches
Type locality	Japan	England	South Africa	Atlantic of the Iberian Peninsula	England	Brazil	New Zealand
Distribution	Korea, Japan	Ireland to Mauritania	South Africa, Flakland Islands	Atlantic of the Iberian Peninsula	Ireland to Mauritania	Brazil	New Zealand
References	Okamura (1909), Tanaka (1941), this study	Dixon and Irvine (1977), Díaz-Tapia et al. (2013)	Guimarães and Pereira (1993), Chiovitti et al. (1998)	Díaz-Tapia et al. (2013)	Dixon and Irvine (1977), Díaz-Tapia et al. (2013)	Joly et al. (1965)	D'Archino et al. (2015)

guished from *Hypnea* by its female structures (Fig. 7) (Kylin 1928, 1930) and by its molecular phylogeny (Díaz-Tapia et al. 2013, D'Archino et al. 2015). The central clusters of cystocarps in *Calliblepharis* congeners either form a reticulum or arrange themselves into parallel columns (Chiovitti et al. 1998, Díaz-Tapia et al. 2013). *C. saidana* and the type species *C. ciliata* (Díaz-Tapia et al. 2013) have reticula, but *Hypnea* congeners lack this structure. On the other hand, central clusters of cells without large fusion cells have been observed previously in *Calliblepharis* and *Hypnea* (Hommersand and Fredericq 1990, Chiovitti et al. 1998, Díaz-Tapia et al. 2013, Vázquez-Delfín et al. 2016). The clusters of small cells produce radial gonimoblast filaments, each of which bears only one terminal carposporangium in *Hypnea* (Kylin 1930, Vázquez-Delfín et al. 2016), while that in *Calliblepharis* bears a chain of carposporangia at the end as shown in this study for *C. saidana*. Finally, gonimoblasts generate elongated filaments attached to the pericarp and placenta (Kylin 1930, Vázquez-Delfín et al. 2016) in *Hypnea* while no such cells were observed in *Calliblepharis*. *C. psammophilus* is an interesting exception in that it possesses a central fusion cell develops chains of gonimoblast filaments (D'Archino et al. 2015). The post-fertilization events of *C. psammophilus* should be further analyzed in detail. In the combined tree, *C. saidana* was sister with *C. fimbriata* and *C. celatospora* even though the clade was weakly supported. These two sister species have gonimoblasts in a series of parallel column oriented placenta in distinct from *C. saidana* and *C. ciliata* (Chiovitti et al. 1998). This gonimoblast characteristics were considered the possibility for creating a new genus (Chiovitti et al. 1998), but it was still unresolved in this study, meaning that a wider taxon sampling is necessary to infer the phylogenetic relationships in the genus *Calliblepharis*. Interestingly, *Calliblepharis planicaulis* was positioned between two genera, *Calliblepharis* and *Hypnea*, as found previously (Saunders et al. 2004, Díaz-Tapia et al. 2013). Combined results of molecular data and female structures of *C. planicaulis* (chains of carposporangia in *Calliblepharis*-type and filaments between gonimoblast and pericarp in *Hypnea*-type) (Min-Thein and Womersley 1976) were raised an intriguing taxonomic questions about generic relationship which is required additional discussion in future study.

The male structure of *C. saidana* was quite distinct from those of its congeners (Table 2). *C. saidana* formed spermatangial sori on branchlets. The spermatangia

cleaved off from a spermatangial mother cell that had differentiated from a cortical cell, and formed transversely divided spermatial filaments of up to seven rounded cells. This is longer than those of *C. hypneoides* and *C. psammophilus* (Díaz-Tapia et al. 2013, D'Archino et al. 2015). *C. saidana* exhibited distinctive antheridial walls in the mature spermatangium. This lead to the accumulation of spermatia in one place before their release in *C. saidana*, which is the first observation in the family Cystocloniaceae. Piled up spermatia of *C. saidana* were then released en masse from the antheridial wall unlike other species in Cystocloniaceae where single spermatium was released (Min-Thein and Womersley 1976, Díaz-Tapia et al. 2013, D'Archino et al. 2015).

Gametophytes of *Hypnea* have rarely been found in the wild. Reis and Yoneshigue-Valentin (2000) even claimed that sexual reproduction in these plants has been replaced by asexual and vegetative forms of reproduction, perhaps as a strategy, to survive under difficult environmental conditions. We were very fortunate to collect specimens having female and male reproductive structures that provided morphological evidence supporting our molecular analysis. Our combination of morphological and molecular approaches strongly suggested new taxonomic entity that can extend the understanding of this group. Apparently, further information on the reproductive structures and molecular studies of other species in *Calliblepharis* are required to improve our knowledge on the phylogenetic relationships within this group. Introducing *C. saidana* in this study, we expand the range of *Calliblepharis* habitat to the northwestern Pacific (Korea and Japan) from the eastern Atlantic, South Africa, the northern Indian Ocean, Australasia, and Brazil.

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