

***Calliblepharis yasutakei* sp. nov. and *Hypnea tsudae* sp. nov. (Cystocloniaceae, Rhodophyta): novel diversity from the Hawaiian Islands**

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Abstract

Surveys of Hawaiian macroalgae over the past 15 years have yielded numerous specimens representing species new to science. *Calliblepharis yasutakei* sp. nov. is here described based on a plant collected at a depth of 98 m from Kapou, Papahānaumokuākea Marine National Monument, Hawai‘i. Phylogenetic analyses of three molecular markers (COI, *rbcL*, and SSU) and analyses of morphological features were used to describe the new species in the family Cystocloniaceae. *Calliblepharis yasutakei* sp. nov. grouped with *C. fimbriata*, *C. rammediorum*, *C. occidentalis* and *C. jolyi* in a clade with full support for the *rbcL* analysis, representing a distinct lineage within the genus. Phylogenetic and vegetative morphological comparisons demonstrated that the new Hawaiian species is most closely related to *C. rammediorum* from Israel (*rbcL* similarity of 96.3%), although no female reproductive structures were found to allow a more comprehensive comparison. In order to determine whether *C. yasutakei* represents the first confirmed report of the genus *Calliblepharis* in the Hawaiian Islands, phylogenetic and morphological analysis of the Hawaiian *Hypnea saidana* (= *Calliblepharis saidana*) specimen accessioned at the Bernice P. Bishop Museum was performed. These analyses demonstrated that this specimen belongs to a new species in the genus *Hypnea*, which is here described as *H. tsudae* sp. nov. *C. yasutakei*, in addition to being a new species, is also reported as the first confirmed record of the genus *Calliblepharis* in the Hawaiian archipelago, and the description of *H. tsudae* brings the number of species for this genus in Hawai‘i to eight.

Keywords: COI, Cystocloniaceae, Lisianski, MCE, macroalgae, phylogeny, *rbcL*, SSU

Introduction

The family Cystocloniaceae is one the most diverse in the Gigartinales, with 15 genera and 113 species currently recognized (Guiry & Guiry 2022), and is considered economically important as a source of carrageenan (Chiovitti *et al.* 1998). Morphologically, the family is characterized by uniaxial, terete or flat thalli, with pseudoparenchymatous or filamentous medulla, procarps with 3- or 4-celled carpogonial branches, and zonately divided tetrasporangia (Yang & Kim 2017, Huisman 2018, Guiry & Guiry 2022). The use of molecular markers to investigate phylogenetic relationships in the red algae has become commonplace, improving the accuracy of taxonomic studies, especially in cases where specimens lack reproductive structures for full morphological identification (Saunders 2005, Sherwood *et al.* 2010, Saunders & Moore 2013). Since the emergence of molecular techniques, a number of studies have been conducted on the order Gigartinales, including the genera *Calliblepharis* Kützinger and *Hypnea* J.V. Lamouroux (Saunders & Kraft 1994, Geraldino *et al.* 2010, Díaz-Tapia *et al.* 2013, Yang & Kim 2017, Soares *et al.* 2019, Soares & Fujii 2020, Campbell *et al.* 2022).

The genus *Calliblepharis* was established in 1843 by Kützinger, and is the third most speciose genus in the family Cystocloniaceae, with 11 species currently accepted (Guiry & Guiry 2022). Mostly found in subtidal zones, *Calliblepharis*

species have a wide distribution encompassing the north-eastern Atlantic from the British Isles to Mauritania and the Mediterranean Sea, South Africa, Brazil, Japan, Korea, New Zealand, Australia and USA (Guiry & Guiry 2022). Over the past decade, several taxonomic studies using morphological and molecular data have investigated taxonomic and phylogenetic relationships within *Calliblepharis*; however, the genus remains paraphyletic (Díaz-Tapia *et al.* 2013, Yang & Kim 2017, Soares *et al.* 2019, Soares & Fujii 2020).

Hypnea and *Calliblepharis* are phylogenetically closely related genera, with several vegetative features in common, although they can be easily distinguished based on female reproductive structures: while in *Hypnea* each gonimoblast filament produces only one carposporangium, in *Calliblepharis* they are borne in chains, although in both genera carposporangia are formed by terminal cells of gonimoblast filaments (Díaz-Tapia *et al.* 2013, Yang & Kim 2017). In the Hawaiian archipelago, *Hypnea* is the only reported genus of the family Cystocloniaceae, with seven species recorded for the Main Hawaiian Islands (Abbott 1999, Huisman *et al.* 2007, Sherwood *et al.* 2010, Nauer *et al.* 2019b), including *H. saidana* Holmes. The morphological similarity between *Hypnea* and *Calliblepharis* species led Yang & Kim (2017) to investigate their phylogenetic relationship, and based on morphological and molecular evidence, *Hypnea saidana* from the type locality, Japan, was transferred to the genus *Calliblepharis*, as *C. saidana* (Holmes) M.Y. Yang & M.S. Kim. Additionally, Campbell *et al.* (2022) realized that specimens of *H. volubilis* Searles that had been reported from the Carolinas, Florida, the Gulf of California and the Gulf of Mexico resembled *H. saidana*, and molecular investigations of the type specimen led the authors to synonymize this species with *C. saidana*. Thus, prior morphological records of *H. saidana* in the Hawaiian Islands need to be evaluated to determine whether they truly correspond to the genus *Calliblepharis*, or represent a different taxonomic entity.

Using DNA sequence data and morphological characters, many new red algal species have been recently described from Mesophotic Coral Ecosystems (MCEs) of the Main Hawaiian Islands and the Papahānaumokuākea Marine National Monument (PMNM), including members of Gigartinales (Cabrera *et al.* 2021, Sherwood *et al.* 2021b), Peyssonneliales (Sherwood *et al.* 2020, 2021a), and Ceramiales (Sherwood *et al.* 2019, Paiano *et al.* 2020). In this study, we describe a new species of *Calliblepharis* from PMNM by analyzing the mitochondrial COI barcode region, the *rbcL* plastid gene, and the nuclear SSU gene, in addition to morphological analyses. We also generate molecular data and morphological analysis for a specimen previously identified as *H. saidana* from Hawai‘i, to clarify whether the specimen belongs to the genus *Calliblepharis* or whether it represents another case of misidentification stemming from use of only vegetative morphological characters for identification.

Material and Methods

A single specimen of *Calliblepharis* was collected in July 2019 during a survey of mesophotic algal diversity in PMNM at Kapou (Lisianski Island), Hawai‘i by NOAA technical divers, at a depth of 98 m. The specimen was preserved in silica gel desiccant for molecular analyses and pressed as a herbarium voucher for morphological studies. The type specimen was mounted on herbarium paper and accessioned at the Bernice P. Bishop Museum (BISH) under accession BISH 783229. Morphological and anatomical investigations of the sample were conducted by gently rehydrating the thallus in modified Pohl’s Solution (Clark, unpubl.: https://www.eeob.iastate.edu/research/bamboo/pdf/anatomy_protocols.pdf) for 30 min, hand sectioning with a double-edged razor blade, staining with 0.5% aniline blue, and mounting in 30–40% Karo™. Photomicrographs were taken on a Zeiss AxioImager A1 compound light microscope (Pleasanton, CA) with an Infinity2-1RC digital camera (Lumenera Corporation, Ottawa, Canada). To investigate the diversity of the genus *Calliblepharis* in Hawai‘i, we sampled a specimen morphologically identified as *Hypnea saidana* (currently a synonym of *Calliblepharis saidana*) from Bishop Museum herbarium voucher BISH 740394, collected on 03 August 2006, at Pu‘ukohala Heiau National Park, Island of Hawai‘i, for molecular confirmation.

Genomic DNA was extracted from both specimens using the OMEGA E.Z.N.A.® Plant DNA DS Kit (OMEGA Biotek, Norcross, GA, USA) or the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocols. The genes selected for the molecular analyses included the chloroplast-encoded *rbcL* and nuclear SSU rDNA. The *rbcL* gene was amplified using primers for two overlapping fragments: *rbcLF7* and R898 (Gavio & Fredericq 2002, Kang & Kim 2013) for the first fragment, and *rbcLF762* and *rbcLR1442* (Kim *et al.* 2010) for the second fragment, for a total of 1388 bp. The SSU gene was amplified using the following overlapping primer pairs: G04 and G07, and G01 and G14 (Saunders & Moore 2013), for a length of 1872 bp. A 639-bp fragment from the mitochondrial DNA barcode region near the 5’ end of the COI gene was generated using the GazF1 and GazR1 primer pair (Saunders 2005). Successful PCR products were submitted to GENEWIZ (South Plainfield, NJ, U.S.A.) for

bidirectional Sanger sequencing. Raw sequences were assembled and aligned using the MUSCLE v. 3.8.425 plug-in (Edgar 2004) in Geneious Prime 2021.0.3 (<http://www.geneious.com>) with other available sequences for *Calliblepharis* and related genera of the Cystocloniaceae from GenBank and BOLD (Table S1). COI, *rbcL* and SSU alignments were analyzed with PartitionFinder 2 (Lanfear *et al.* 2017). The ends of the SSU alignment were trimmed and gaps were deleted to match the query sequences, resulting in a final alignment length of 938 bp. Maximum Likelihood (ML) analyses were performed on all alignments using RAXML-HP2 on XSEDE v.8.2.10 (Stamatakis 2014) via the CIPRES gateway (Miller *et al.* 2010) with 1,000 bootstrap replicates and using the GTRCAT model. Bayesian Inference (BI) analysis was performed using the MrBayes v.3.2.7a plug-in (Huelsenbeck *et al.* 2003) through CIPRES with four chains of Metropolis-coupled Markov Chain Monte Carlo for 5,000,000 generations (for *rbcL* and SSU) or 1,000,000 generations (COI), sampling every 500 generations and with other parameters set to default. Twenty-five percent of sampled trees were discarded as burn-in in determining posterior probabilities. Tracer v1.7.1 (Rambaut *et al.* 2018) was used to estimate the burn-in cutoff and to check if further runs were required to reach convergence, with the average standard deviation of split frequencies = 0.010123 (COI), 0.005547 (*rbcL*) and 0.008988 (SSU).

Results

Eight new sequences were generated: three for the new mesophotic specimen of *Calliblepharis* and five for the *Hypnea* specimens: one *rbcL* sequence each for ARS03115 and ARS03542 and all three markers for the BISH 740394 (“*H. saidana*”) specimen. Phylogenetic analyses of all markers confirmed that BISH 740394 (“*H. saidana*”) from Hawai‘i is a new species of the genus *Hypnea* (Figs 1, 2 and S1). For each of the three markers, Maximum Likelihood and Bayesian Inference analyses produced similar results. The *rbcL* alignment consisted of 66 sequences and was 1388 bp in length. The Hawaiian mesophotic *Calliblepharis* was resolved in a clade with full support that included *C. fimbriata* (Greville) Kützing, *C. rammediorum* R.Hoffman, M.J.Wynne & W.A.Nelson, *Calliblepharis* sp., *C. occidentalis* Joly & Yamaguishi-Tomita and *C. jolyi* E.C.Oliveira (divergences of 3.9%, 3.7%, 3.7%, 4.0% and 5.7%, respectively between the Hawaiian specimen and sequences of these other species), forming a sister clade with *C. saidana*. A clade including species of the genus *Craspedocarpus* F.Schmitz was positioned, although unsupported, among the remaining *Calliblepharis* species, except for *Calliblepharis psammophila* D’Archino & W.A.Nelson, which grouped as an outside branch in a clade with *Hypnea*. All *Hypnea* species included in the analysis were in a clade with full support, with “*C. saidana*” (BISH 740394) from Hawai‘i positioned within this clade (Fig. 1), in a lineage with *H. asiatica* P.J.L.Geraldino, E.C.Yang & S.M.Boo, *H. brasiliensis* P.B.Jesus, Nauer & J.M.C.Nunes, *H. charoides* J.V.Lamouroux and *H. nidifica* J.Agardh, with genetic divergences of 5.2%, 4.9%, 5.2% and 5.5%, respectively.

For the COI marker, the alignment was 663 bp in length and included 45 sequences. The analysis showed the mesophotic *Calliblepharis* specimen grouped in a strongly supported clade with *C. fimbriata*, *C. occidentalis* and *Calliblepharis* sp., with divergences of 8.6%, 9.0% and 9.2% respectively, while “*C. saidana*” (BISH 740394) from Hawai‘i was resolved in a clade with full support with two sequences of *Hypnea* sp. (HQ422821 and HQ422958), also from Hawai‘i, allied with *H. asiatica*, *H. bullata* P.Kundu & F.Bast, *H. indica* P.Kundu & F.Bast and *H. nidifica*, with genetic divergences of 9.5%, 10.2%, 13.6% and 8.5%, respectively. *Calliblepharis jubata* (Goodenough & Woodward) Kützing and *C. ciliata* (Hudson) Kützing formed a fully supported clade, grouping with all *Hypnea* species, while the remaining *Calliblepharis* species formed a clade, but with low support (Fig. S1).

The nuclear SSU alignment included 40 sequences and was 993 bp in length. The Hawaiian mesophotic *Calliblepharis* specimen grouped with *C. celatospora* Kraft and *C. occidentalis* (1.3% and 1.7%, respectively), although this clade was supported by low bootstrap values (< 70%) and moderate to high posterior probabilities (0.87), and with *C. psammophila* as a sister species (Fig. 2). Unlike COI and *rbcL*, the SSU analysis showed two different clades, both with high support: the first composed of the Hawaiian mesophotic *Calliblepharis*, *C. celatospora*, *C. occidentalis*, *C. psammophila*, *C. ciliata*, *C. jubata*, *C. saidana* and *C. hypneoides* P.Díaz-Tapia, I.Bárbara & M.H.Hommersand, and the second composed of *C. planicaulis* (Harvey) Kylin positioned sister to species of *Hypnea*, which again included “*C. saidana*” (BISH 740394) from Hawai‘i. *Hypnea* was resolved as polyphyletic in the SSU analyses and the Hawaiian specimen of “*C. saidana*” diverged 3.0–3.1% from *H. charoides*, 3.0–5.8% from *H. asiatica* specimens and 3.1% from *H. spinella* (C.Agardh) Kützing. Our molecular results clearly exclude the possibility of the specimen described as *Calliblepharis saidana* from Hawai‘i belonging to *Calliblepharis*. Instead, they show that the specimen represents a new species in the genus *Hypnea*. Combined with morphological analyses, we here describe two new Hawaiian species of the Cystocloniaceae, *Calliblepharis yasutakei* and *Hypnea tsudae*.

TABLE 1. Comparison of main morphological characters used to distinguish *Calliblepharis* species.

Species	Type locality	Thallus	Rosettes	Axial row arrangement	Spermatangia	Placentation	Cystocarp	Reference
<i>C. ciliata</i>	England	compressed	absent	filaments	sori on younger branches	reticulum	ostiolate	Dixon & Irvine (1977)
<i>C. jubata</i>	Devonshire, England	compressed	absent	filaments	unknown	reticulum	ostiolate	Dixon & Irvine (1977)
<i>C. hypneoides</i>	Asturias, Spain	Terete	absent	filaments	sori on branchlets	reticulum	nonostiolate	Díaz-Tapia <i>et al.</i> (2013)
<i>C. psammophila</i>	Castlepoint, New Zealand	terete	absent	filaments	sori on apical branches	reticulum	nonostiolate	D'Archino <i>et al.</i> (2015)
<i>C. celatospora</i>	Port Denison, Australia	compressed	incipient	medullary cells	unknown	columns	nonostiolate	Chiovitti <i>et al.</i> (1998)
<i>C. fimbriata</i>	Cape Town, South Africa	compressed	incipient	unknown	on surface	columns	nonostiolate	Thraissnson (1986)
<i>C. jolyi</i>	Espirito Santo, Brazil	compressed	incipient	medullary cells	scattered on young branches	columns	nonostiolate	Soares & Fujii (2020)
<i>C. occidentalis</i>	Ceará, Brazil	compressed	incipient	medullary cells	sori on apical branchlets	columns	nonostiolate	Soares <i>et al.</i> (2019)
<i>C. rammediorum</i>	Haifa, Israel	compressed	present	medullary cells	unknown	unknown	unknown	Hoffman <i>et al.</i> (2018)
<i>C. saidana</i>	Enoshima, Japan	compressed	incipient	medullary cells	sori on branches and branchlets	columns	nonostiolate	Yang & Kim (2017)
<i>C. planicaulis</i>	George Town, Tasmania	compressed	incipient	medullary cells	sori on branchlets	columns	nonostiolate	Min-Then & Womersley (1976)
<i>C. yasutakei</i> sp. nov.	Kapou (Lisianski), PMNM, Hawai'i, USA	compressed	incipient	medullary cells	unknown	unknown	unknown	This study

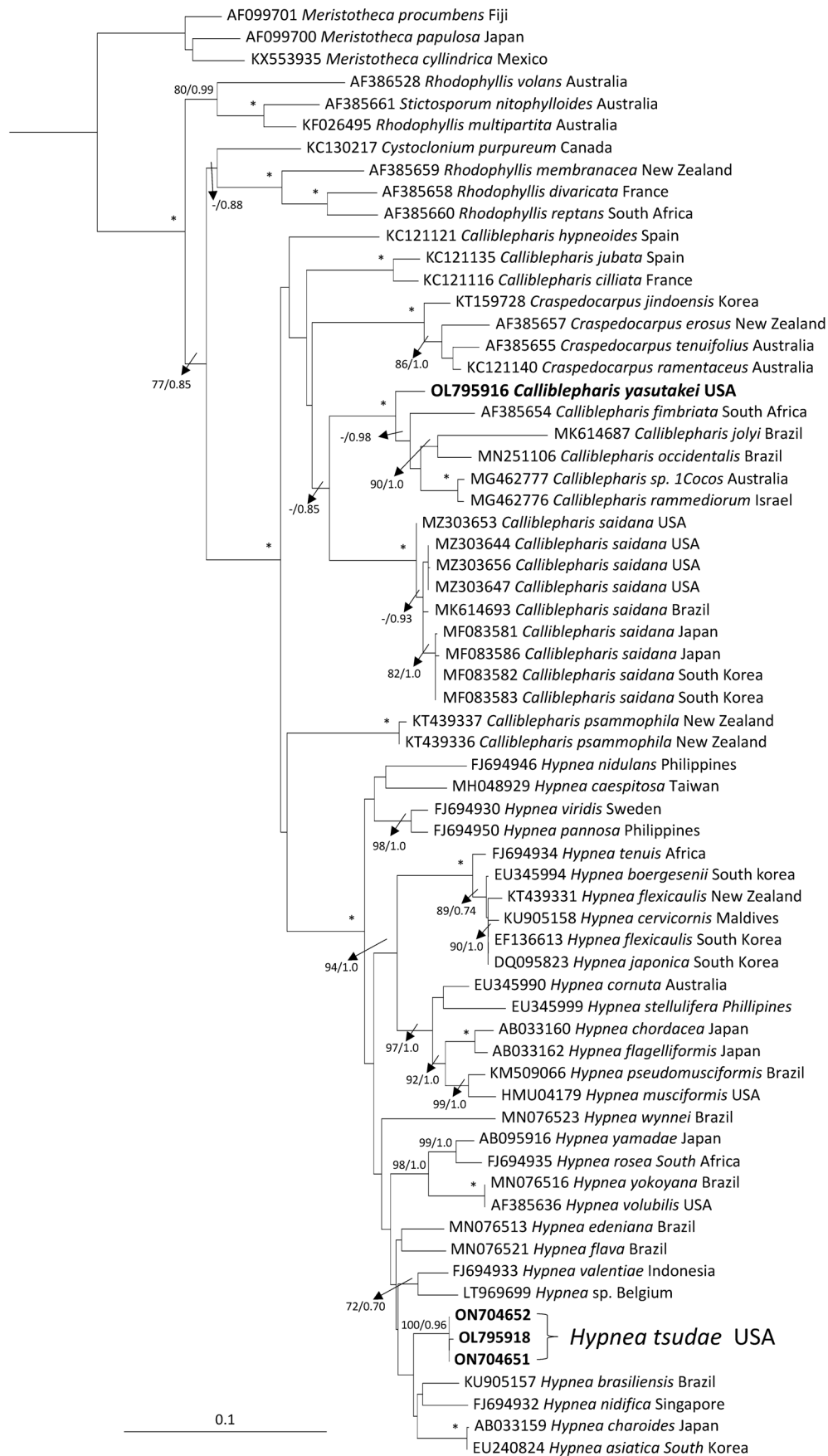


FIGURE 1. Maximum likelihood phylogeny of *rbcL* sequences for the red algal family Cystocloniaceae. Numbers along branches indicate nodal support (first value = bootstrap support, second value = Bayesian posterior probabilities). Nodes with full support are indicated with an asterisk. Scale bar = substitutions per site.

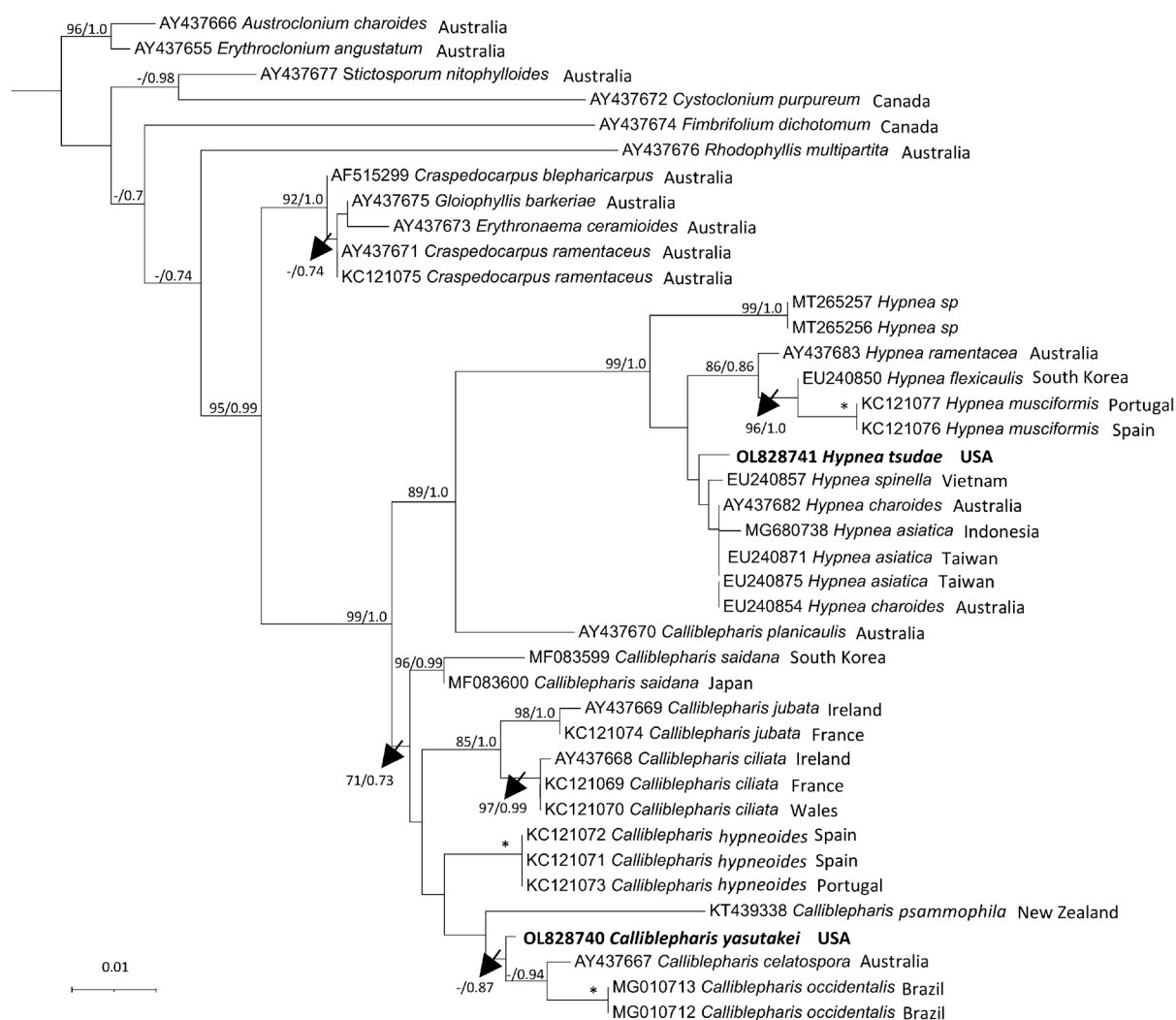


FIGURE 2. Maximum likelihood phylogeny of SSU sequences for the red algal family Cystocloniaceae. Numbers along branches indicate nodal support (first value = bootstrap support, second value = Bayesian posterior probabilities). Nodes with full support are indicated with an asterisk. Scale bar = substitutions per site.

***Calliblepharis yasutakei* M.O.Paiano & A.R.Sherwood, sp. nov. (Fig. 3A–H)**

Holotype:—U.S.A. Hawai‘i, Papahānaumokuākea Marine National Monument, Kapou (Lisianski), 26.08363°N, 174.16647°W, 98 m depth, 30 July 2019, *R. Kosaki* (holotype BISH 783229; ARS 10483; field code NWHI-761).

Description: Thallus erect, delicate, pinkish-red when living, drying to dark red along the main axis and pink along some branches, slightly compressed, plant 3.8 cm tall × 2.6 cm wide (Fig. 3A–B), attached to the substratum by a small and inconspicuous holdfast. Thallus irregularly or dichotomously branched, at narrow angles, with branches up to 3.1 mm wide (Fig. 3B). Upper part of thallus sparsely branched; lacking anastomoses. Thallus organization uniaxial, apex consisting of a single apical cell (Fig. 3C). Surface view of cortical cells irregular to polygonal, 10–20 µm × 5–15 µm, with rosette cells weakly developed around the cortical cells (Fig. 3D). Pit-connections absent. Lenticular thickenings frequently observed in cortical cells (Fig. 3E–F). Cross sections 250–295 µm thick, with medulla consisting of one layer of large, rounded cells, 66.7–74.1 µm long, 85.2–92.6 µm wide, and one outer layer of pigmented, rounded to irregular small cells, 11.1–22.1 µm long, 7.4–22.2 µm wide (Fig. 3G). Central axial filament evident in surface view, consisting of elongate cells (arrows), 20–40 µm long × 8–15 µm wide, surrounded by 2–3 layers of medullary cells and one or two layers of rounded cortical cells. (Fig. 3H). Unicellular hairs not observed. Reproductive characters were not observed.

Etymology:—*C. yasutakei* is named in memory of Mr. Yumi Yasutake, a long-time educator and scientist for the Papahānaumokuākea Marine National Monument. Through the use of algal pressings as a student activity, Yasutake

shared his love of phycology, marine science, and Papahânaumokuâkea with legions of young people throughout the state of Hawai'i.

Distribution and Habitat:—Known only from the type locality, at 98 m depth.

Identification using DNA sequence data:—GenBank accessions OL795915 (COI), OL795916 (*rbcL*) and OL828740 (SSU).

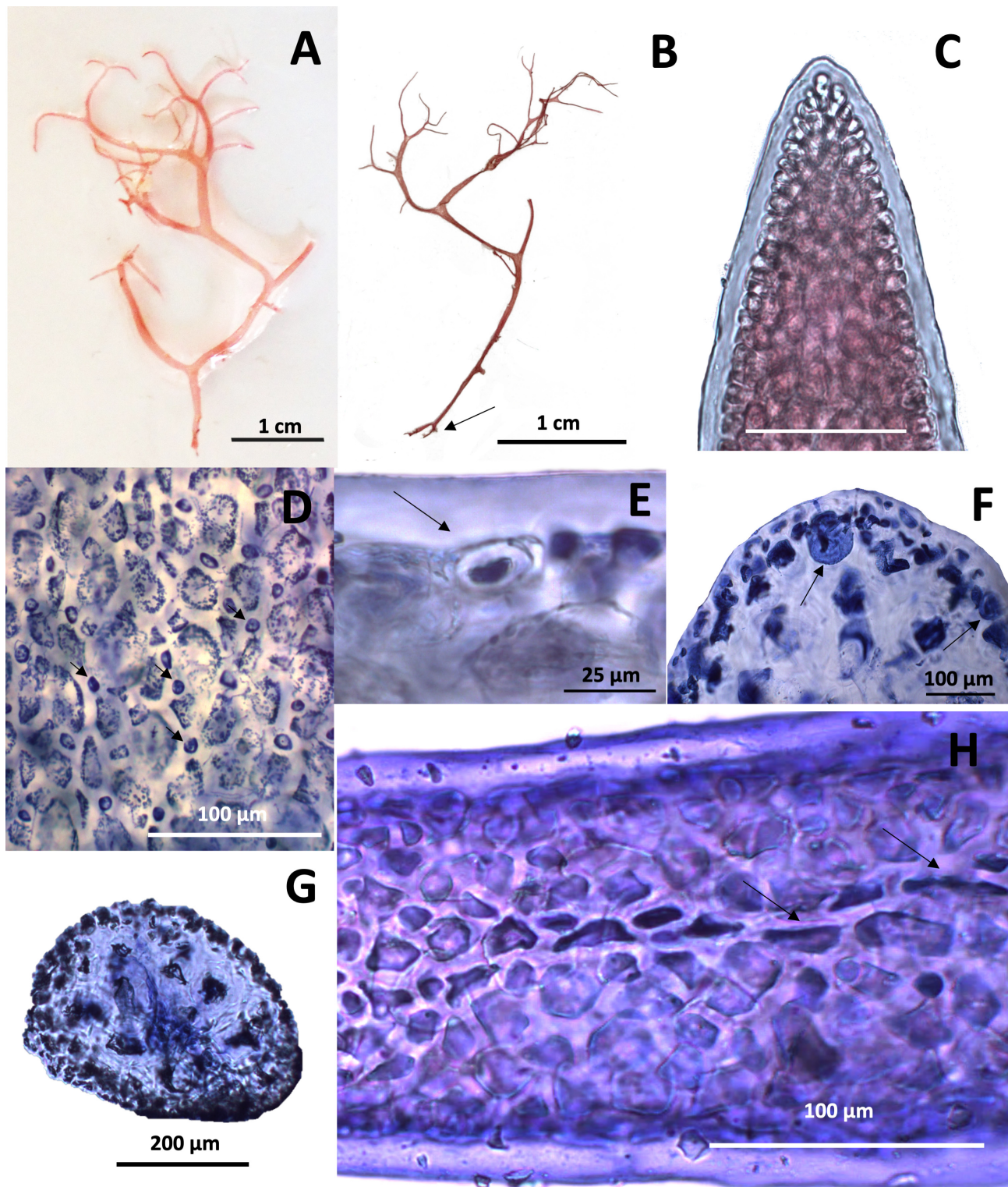


FIGURE 3. General morphology of *Calliblepharis yasutakei* sp. nov. (BISH 783229). **A.** The freshly collected holotype. **B.** Pressed holotype showing the small holdfast (arrow). **C.** Surface view of a branch apex showing the apical cell. **D.** Surface view showing weakly developed rosette cells (arrows) around medullary and cortical cells. **E.** Cross section showing lenticular thickening (arrow) in a cortical cell. **F.** Cross section of basal region of thallus showing lenticular thickenings in cortical cells (arrows). **G.** Cross section of a middle portion of the thallus. **H.** Surface view showing axial filament surrounded by medullary cells (arrows).

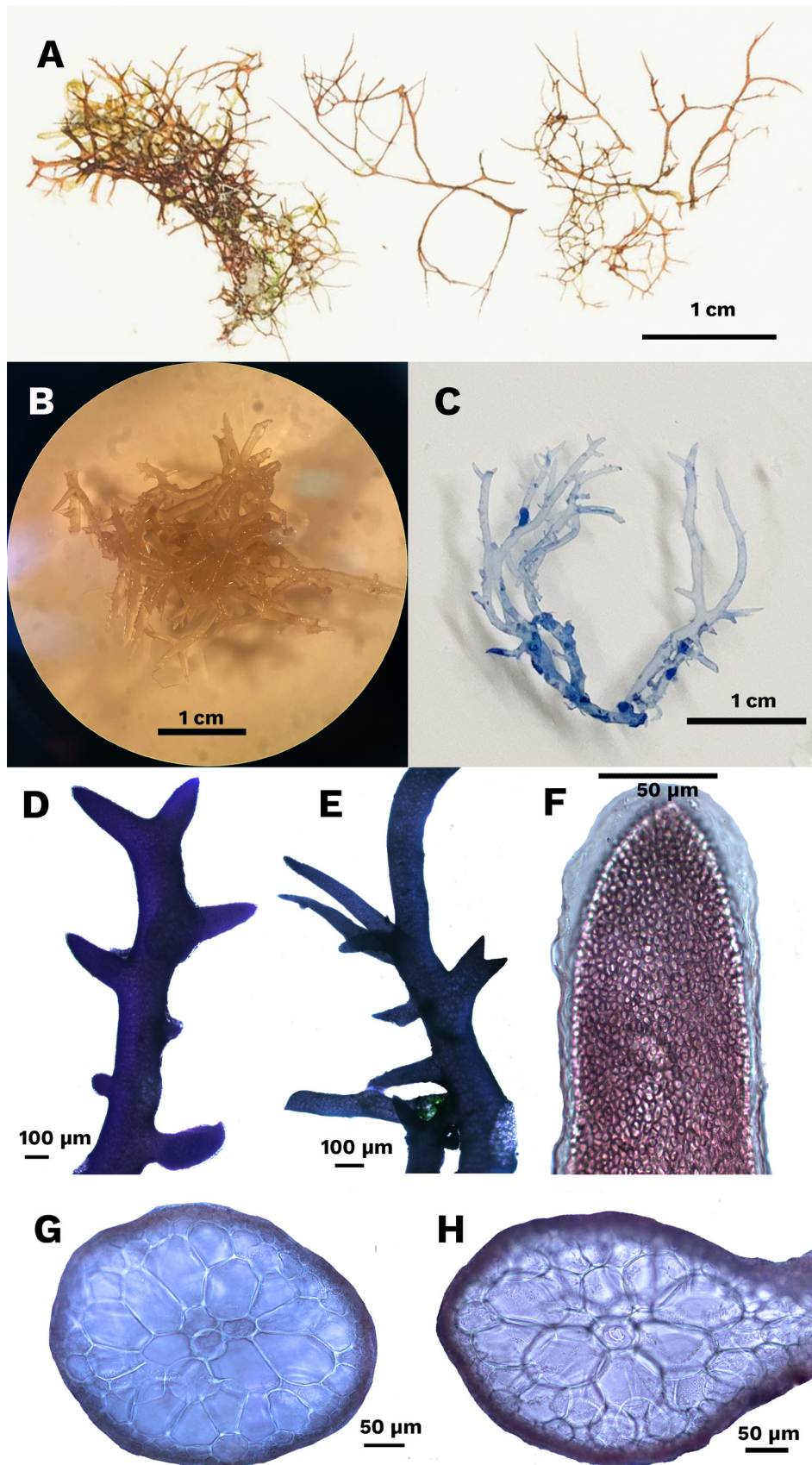


FIGURE 4. General morphology of *Hypnea tsudae* sp. nov. (BISH 740394). **A.** Pressed holotype, image provided by Bishop Museum (BISH 740394). **B.** Paratype ARS 03115 from a formalin voucher showing habit of the thallus. **C.** Stained paratype ARS 03542 from a formalin voucher showing apical portion of the branch. **D.** Detail of an apical branch (ARS 03115) **E.** Detail of a lateral branch (ARS 03542). **F.** Surface view of the apical portion of the thallus. **G.** Transverse section on the basal portion of the thallus. **H.** Transverse section of a middle portion of the thallus (ARS 03115).

Holotype:—U.S.A. Hawai‘i, Pu‘ukohala Heiau National Park, Island of Hawai‘i 20.748°N, 24.384°W, 1.5 m depth, 03 August 2006, C. Squair (holotype BISH 740394).

Paratypes:—ARS 03115 (BISH 786150) from U.S.A. Hawai‘i, Island of Maui, Hana, Kaihalulu Beach, 20.7589°N, 155.985°W, intertidal, 10 December 2007, K. Conklin; ARS 03542 (BISH 786151) from U.S.A. Hawai‘i, Island of Kauai, 22.2208°N, 159.583°W, intertidal, 17 March 2007, A. Kurihara.

Description: Thalli upright, terete, ranging from 1.2–4.5 cm in length (Figs 4A–C), usually rich red in color when living, drying to a light pink to orange when pressed (Fig 4A). Thalli are compressed with thorn-like branchlets that are often dichotomously branched at the apices and alternately branched in the mid to basal portions (Figs 4D–E). Branching most often in three-dimensional space (Figs 4A–E). In surface view, thallus is composed of small cortical cells, elongated, 4.0–7.5 µm in length and 2.0–10.8 µm in diameter (Fig 4F). Axes uniaxial, rounded. Transverse sections 310–440 µm in diameter (Figs 4G–H). Each axial cell surrounded by three to five periaxial cells (Figs 4G–H), 38–45 µm in diameter, surrounded by two to four layers of large, rounded to cuboidal medullary cells, 30–98 µm in length and 20–60 µm in width. Holdfast not observed. Only sterile plants observed.

Etymology:—This species is named in memory of our colleague, Dr. Roy Tsuda, who made many contributions to our understanding of the flora of the Western Pacific, and who collaborated with us extensively on the taxonomy of the mesophotic flora of the Hawaiian Islands and the Papahānaumokuākea Marine National Monument.

Distribution and Habitat:—subtidal from Island of Hawai‘i, intertidal coastal waters of Maui and Kaua‘i, in the Main Hawaiian Islands, USA.

Identification using DNA sequence data:—GenBank accessions OL795917 (COI), OL795918 (*rbcL*) and OL828741 (SSU) for the holotype, ON704651 (ARS 03115) and ON704652 (ARS 03542) (*rbcL*) for the paratypes.

Discussion

Based on DNA sequence analyses of the COI, *rbcL* and SSU markers, combined with morphological comparisons, *Calliblepharis yasutakei* and *Hypnea tsudae* are proposed as new species from the Hawaiian Archipelago. Phylogenetic analyses for all three molecular markers, compared with type and topotype sequences of all other recognized species of the genus demonstrated that the mesophotic Hawaiian specimen of *Calliblepharis* is distinct from all other species, with the range of interspecific divergences 2.0–9.5% for *rbcL*, 5.4–14.8% for COI and 0.9–8.2% for SSU. Both COI and *rbcL* phylogenies grouped *C. yasutakei* within *C. fimbriata*, *C. jolyi* (in the *rbcL* analyses), *C. occidentalis* and *C. rammediorum* (*Calliblepharis* sp. from Cocos Island - MG462771 in the COI analyses), and with the Hawaiian species being most closely related to *C. rammediorum* from Israel, corroborating the findings of Soares *et al.* (2019) and Soares & Fujii (2020), which they called group III. Species in this clade share the same vegetative features: compressed thalli, incipient rosettes (except for *C. rammediorum*) and medullary cells surrounding the axial filament. In the SSU phylogeny, *C. yasutakei* grouped with *C. occidentalis* and *C. celatospora*. Although there are no available SSU sequences for *C. rammediorum* and *C. fimbriata*, the SSU phylogeny aided in resolving phylogenetic relationships with *C. planicaulis*. Corroborating previous studies using the nuclear SSU for *Calliblepharis* species and the Cystocloniaceae (Díaz-Tapia *et al.* 2013, Yang & Kim 2017, Soares *et al.* 2019), *C. planicaulis* was more closely related to *Hypnea* than to *Calliblepharis*, which is also supported by vegetative characteristics, supporting the idea presented by Hoffman *et al.* (2018) that this species should probably form the core of a new sister genus to *Hypnea*. According to Soares & Fujii (2020), the main morphological features distinguishing *Calliblepharis* species are: thallus type, presence and development of rosette cells, axial filament arrangement, type of placentation and position of the cystocarp. *Calliblepharis yasutakei* was small and sterile, and based on vegetative features such as the presence of lenticular thickenings and the thallus width, resembles *C. jolyi* from Brazil. Other observed characters, such as the compressed thallus, incipient rosettes around cortical cells, and the axial filament surrounded by medullary cells are in accordance with vegetative features for the remaining *Calliblepharis* species in the same clade.

In 1930, Kylin brought to attention the similarity among genera of the Cystocloniaceae based on procarp structures and vegetative morphology, and these observations were reaffirmed by Díaz-Tapia *et al.* (2013) when they proposed *C. hypneoides*. Recently, Soares & Fujii (2020) investigated flat Cystocloniaceae species from Brazil, recovering *Craspedocarpus jolyi* (E.C.Oliveira) C.W.Schneider as a member of *Calliblepharis* based on a phylogenetic analysis of *rbcL* sequences. Thus, the phylogeny of *Calliblepharis* became unresolved, with *C. ciliata*, *C. jubata*, *C. psammophila*

and *C. hypneoides* positioned in the same clade as *Hypnea*, and the remaining *Calliblepharis* species positioned in a sister clade, which they identified as *Calliblepharis sensu lato* clade A. The polyphyly of the genus has been a recurring problem over the past decade for most studies that have included all available sequences on GenBank (Díaz-Tapia *et al.* 2013, Yang & Kim 2017, Soares *et al.* 2019, Soares & Fujii 2020). In our molecular analyses, except for the SSU phylogeny, *Calliblepharis* was resolved as monophyletic (albeit without acceptable bootstrap support values but with posterior probabilities of 0.84 for the *rbcL* phylogeny). With the description of *C. yasutakei*, the total number of species for the genus increases to 12, and it is likely that the discovery of additional species will provide increased support values for the *Calliblepharis* clade.

Red algal taxonomic assessments based on reproductive characteristics are known to be effective, but relying solely on these characters can become a worrisome problem when only sterile specimens are collected (Sherwood *et al.* 2010). Just one individual of *C. yasutakei* was collected during the survey, and it is possible that the lack of reproductive characters was due to the plant being immature, which reinforces the need for a combination of morphological and molecular data to infer phylogenetic relationships among and within the genera of the Cystocloniaceae.

The molecular investigation of the specimen previously described as *C. saidana* from Hawai‘i (BISH 740394) demonstrated that it is in fact a member of the genus *Hypnea*, here described as *H. tsudae*, rather than *Calliblepharis*. *Calliblepharis saidana* was first described from Enoshima, Japan, as *Hypnea saidana*, and was subsequently transferred to *Calliblepharis* after investigation of topotype specimens (Yang & Kim 2017). Later, Soares & Fujii (2020) recorded *C. saidana* in Brazil with intraspecific genetic divergences of 0.7–0.9% from specimens from Japan for the *rbcL* gene. Recently, Campbell *et al.* (2022) also described *C. saidana* from North Carolina (USA), and sequence divergences among North Carolina and Japan specimens ranged from 0.8–1.2% for *rbcL*. Our *rbcL* phylogeny grouped all species of *Hypnea* in a monophyletic clade, with *H. tsudae* from Hawai‘i placed in a clade within *H. asiatica*, *H. brasiliensis*, *H. charoides* and *H. nidifica*. Morphological comparisons using vegetative characters for species in this clade did not highlight any features that could be used to distinguish *H. tsudae* from related species.

Over the past 20 years, a number of taxonomic studies have been carried out on the genus *Hypnea* (Yamagishi & Masuda 2000, Geraldino *et al.* 2009, Nauer *et al.* 2015, Jesus *et al.* 2016, Nauer *et al.* 2019a, Nauer *et al.* 2019b, Cabrera *et al.* 2020), which demonstrated that morphological identification based on vegetative specimens can be challenging or even impossible (Nauer *et al.* 2019b). Cabrera *et al.* (2020) forwent the use of morphological data altogether and confirmed the presence of *H. spinella*, *H. musciformis* (Wulfen) J.V.Lamouroux, and *H. cryptica* P.B.Jesus & J.M.C.Nunes in Cuba based on *rbcL* and COI data alone. Currently, including *H. tsudae*, nine *Hypnea* species are known from the Hawaiian Archipelago: *H. caraibica* Nauer, Cassano & M.C.Oliveira, *H. cervicornis* J.Agardh, *H. chordacea* Kützinger, *H. musciformis*, *H. pannosa*, *H. esperi* Bory, *H. spinella*, *H. tsudae* and *H. valentiae*. The morphology of several *Hypnea* species is highly variable (Huisman 2018), and vegetative features alone are frequently not enough to characterize different species. Compared to its Hawaiian congeners, in *H. tsudae* plants are small (less than 5 cm high), a characteristic only attributed to *H. pannosa* and *H. spinella* from the Hawaiian Islands. These three taxa can also present the same branching pattern, which is accepted as a diagnostic character for *Hypnea* species (Abbott 1999). Nauer *et al.* (2019b) described specimens known as “*H. musciformis*” from Hawai‘i as *H. caraibica*, using COI sequences from Sherwood *et al.* (2010). *H. musciformis* was introduced in Hawai‘i in 1974, in Kāne‘ohe Bay (O‘ahu) as part of an aquaculture project that was later abandoned (Russell & Balaz 1994, Smith *et al.* 2002). The Hawaiian Biodiversity Survey (Sherwood *et al.* 2010), using LSU, COI and UPA markers, placed sequences of “*H. musciformis*” in more than one clade, not excluding the possibility that both species may exist in the Hawaiian Islands, and although *H. caraibica* is present on O‘ahu, none of the sequences are from Kāne‘ohe Bay. This demonstrates that the use of molecular tools to delimitate *Hypnea* species is more efficient than morphological characters.

Our molecular results support the recognition of *C. yasutakei* not only as a new species, but as the first record of the genus *Calliblepharis* from mesophotic depths. *Calliblepharis* species are distributed worldwide, from cold to warm temperate waters (Chiovitti *et al.* 1998, Díaz-Tapia *et al.* 2013, Soares & Fujii 2020), with records only from drift, intertidal or subtidal habitats, while the Hawaiian *Calliblepharis* is thus far only known from 98 m depth (at a water temperature of 26.1°C). Collections of algae from mesophotic depths in the Hawaiian Islands have yielded a number of other deep water specialist species in recent years (Spalding *et al.* 2016, Sherwood *et al.* 2019, Paiano *et al.* 2020, Sherwood *et al.* 2020, Cabrera *et al.* 2021, Sherwood *et al.* 2021a&b). Based on recent investigations of the *Calliblepharis/Hypnea* complex as well as our findings regarding the identification of *C. saidana* from Hawai‘i, we highly recommend using a molecular approach combined with morphological comparisons for more accurate identification of *Hypnea* species (and Cystocloniaceae, more broadly), especially from Hawai‘i, since most species records thus far for the Hawaiian Archipelago are based only on morphological comparisons (Abbott 1999, Huisman *et al.* 2007, Tsuda 2014) or solely using DNA sequences (Sherwood & Presting 2007, Sherwood *et al.* 2010). For this reason, further taxonomic clarification of Hawaiian members of the Cystocloniaceae is likely.

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