

Research Article

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Systematics of *Hypnea* (Cystocloniaceae, Rhodophyta) from coastal North Carolina, with a first report of *Calliblepharis saidana* from the United States Atlantic Coast

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Abstract: Complete and/or partial DNA sequences for the plastid-encoded *rbcL* gene and the 5' end of the mitochondrial cytochrome oxidase I (COI-5P) gene were used to re-examine the systematics of *Hypnea* species (Cystocloniaceae, Rhodophyta) from North Carolina, USA. These data, combined with light microscopic observations, indicate that two species (*Hypnea cryptica* and *H. musciformis sensu stricto*) are present in nearshore waters of coastal North Carolina. Molecular and morphological analyses with topotype material of *Hypnea volubilis* from North Carolina offshore waters revealed that it and *Calliblepharis saidana* are conspecific. *Hypnea volubilis* is proposed as a heterotypic synonym of *C. saidana*. This is the first report of *Calliblepharis* from the United States Atlantic coast and only the second report from the western hemisphere.

Keywords: COI-5P; *rbcL*; seaweeds; systematics; Western Atlantic Ocean.

1 Introduction

The red algal genus *Hypnea* (Cystocloniaceae, Rhodophyta) includes ~60 species that are absent from polar waters but are otherwise circumglobally distributed in

marine ecosystems (Bast et al. 2014; Kang and Nam 2018; Yang and Kim 2017). Some *Hypnea* species are sources of commercial grade kappa-carrageenans and are of considerable economic value (Ganesan et al. 2006; Oliveira 1998; Zemke-White and Ohno 1999). Distinguishing among *Hypnea* spp. in nature is sometimes straightforward but is most often difficult. Sexual reproductive features (e.g., the size, shape, position, development of cystocarps, and spermatangia, etc.) have been used to delimit *Hypnea* from other genera placed in the Cystocloniaceae (Díaz-Tapia et al. 2013). Whereas these data are fundamental for defining *Hypnea*, they do not help define *Hypnea* species (e.g. Geraldino et al. 2010; Jesus et al. 2016; Nauer et al. 2014). The vegetative thalli of many *Hypnea* species exhibit extensive plasticity; phenotypic variation within some species exceeds that observed in others and taxonomically reliable discrete character states for delimiting species are rare (Jesus et al. 2016; Nauer et al. 2015, 2017). In the past, these variants have been recognized as distinct varieties or subspecies (taxonomically) or as ecotypes/ecads (Jesus et al. 2019; Nauer et al. 2015).

A spate of new *Hypnea* species have been described in the last decade based principally, but not exclusively, on DNA sequence analyses, and employing, implicitly or explicitly, the phylogenetic (or 'molecular') concept for species (e.g. Geraldino et al. 2009, 2010; Jesus et al. 2013; Nauer et al. 2014). DNA sequence data for *Hypnea* spp. are tacitly recognized as the only practical means of establishing interspecific boundaries within the genus. Eighteen *Hypnea* species are presently recorded from the tropical and subtropical Western Atlantic (Table 1) and six novel *Hypnea* species have been described from this area in the last half-decade (Jesus et al. 2019; Nauer et al. 2015, 2016, 2019a; Wynne 2011).

Four species of *Hypnea* have been reported from near- and offshore North Carolina, USA habitats including *H. cervicornis* J. Agardh, *H. musciformis* (Wulfen) J. V. Lamouroux, *H. valentiae* (Turner) Montagne, and *H. volubilis* Searles.

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Table 1: Species of *Hypnea* (Cystocloniaceae), with their type localities, recorded from the Western Atlantic Ocean.

Species	Type locality
<i>H. brasiliensis</i> P.B. Jesus, Nauer et J.M.C. Nunes	Brazil, Bahia State, Porto Seguro, Arraial D'Ajuda, Mucugê beach
<i>H. cenomyce</i> J. Agardh	Australia, ad oras Nova Hollandiae
<i>H. cornuta</i> (Kützing) J. Agardh	Guinea Coast, Africa, ad oras Guineae
<i>H. cryptica</i> P.B. Jesus et J.M.C. Nunes	Brazil, Bahia State, Santa Cruz de Cabrália, Coroa Vermelha Beach
<i>H. caraibica</i> Nauer, Cassano et M.C. Oliveira	Jamaica, Montego Bay, Sunset Cove
<i>H. cervicornis</i> J. Agardh	Bahia State, Brazil
<i>H. flava</i> Nauer, Cassano et M.C. Oliveira	Brazil, Rio de Janeiro State, Praia do Carro, Saco do Mamanguá, Parati.
<i>H. krugiana</i> Hauck	Puerto Rico
<i>H. musciformis</i> (Wulfen) Lamouroux	Trieste, Italy
<i>H. nigrescens</i> Geville ex J. Agardh	India
<i>H. platyclada</i> P.B. Jesus et J.M.C. Nunes	Brazil, Bahia State, Santa Cruz de Cabrália, Apua Beach
<i>H. pseudomusciformis</i> Nauer, Cassano et M.C. Oliveira	Brazil, Espírito Santo State, Itapemirim, Praia de Itaoca
<i>H. spinella</i> (C. Agardh) Kützing	West Indies
<i>H. valentiae</i> (Turner) Montagne	Red Sea
<i>H. volubilis</i> Searles in C.W. Schneider et Searles	Atlantic Ocean, North Carolina
<i>H. schneideri</i> Nauer, Cassano et M.C. Oliveira	Dominican Republic, Bayahibe, Isla Saona
<i>H. wynnei</i> Nauer, Cassano et M.C. Oliveira	Brazil, Rio de Janeiro State, Angra dos Reis, Ilha Grande Island, Lagoa Verde
<i>H. yokoyana</i> Nauer, Cassano et M.C. Oliveira	Brazil, Rio de Janeiro State, Angra dos Reis, Ilha Grande Island, Laje Branca

Specimens assignable to *H. musciformis*, the type species of *Hypnea*, are by far most abundant and conspicuous in the North Carolina flora; however, DNA-based studies suggest that this phenotypically conservative and widespread taxon is a species-complex (Nauer et al. 2019a, 2019b). Specimens identified as *H. valentiae* were originally identified as *H. cornuta* (Kützing) J. Agardh (Kapraun 1980; Pearce and Williams 1951; Taylor 1960). Schneider and Searles (1991) re-assigned the species to *H. valentiae* following Børgesen's (1943) treatment of *H. cornuta* as a morphotype of *H. valentiae*, but this synonymy has not been universally accepted in the Western Atlantic (e.g. Nauer et al. 2014; Wynne 2011). Based on the type localities for both *H. valentiae* (Red Sea) and *H. cornuta* (West Africa), both may be misapplied names to the North Carolina species. *Hypnea volubilis* was described from offshore North Carolina (Schneider and Searles 1976). The species varied from other *Hypnea* species in its complanate branching and flattened thallus. Schneider and Searles (1976) noted that specimens of *H. volubilis* resembled *Hypnea saidana* Holmes, which has since been reassigned to the genus *Calliblepharis* (Yang and Kim 2017). *Hypnea volubilis* has been reported based solely on morphology from the Carolinas, Florida, Gulf of California, and the Gulf of Mexico (Fredericq et al. 2009; Schneider and Searles 1991) with only one *rbcL* sequence available in the GenBank database. *Hypnea cervicornis* has been recorded once from coastal North Carolina, and the specimen was dredged in offshore waters. This record represents the northern range limit for the species (Schneider and Searles 1976).

This study was undertaken to reevaluate the systematics of *Hypnea* species from coastal North Carolina, reassess *Hypnea* species diversity in the Western Atlantic Ocean, and determine how these species are related to those from other marine biogeographic provinces.

2 Materials and methods

2.1 Taxon sampling

Collection information for *Hypnea* specimens examined in this study are presented in Table 2. Most specimens were dried and preserved in silica gel desiccant; others were preserved in a 10% formalin: seawater solution. DNA sequence and morphological data for some specimens was obtained from herbarium presses made in the field or laboratory.

Sections of formalin-fixed or dried material were made using a razor blade and stained using 1% aniline blue as described in Tsuda and Abbot (1985) for light microscopy study. Observations were made using an Olympus BX60 microscope (Olympus Corp., Lake Success, NY, USA) equipped with a Leica DMC2900 digital camera and Leica LAS (ver. 4.9) imaging software (Leica Microsystems, Wetzlar, Germany), or a Zeiss Axio Imager Z1 compound microscope fitted with an AxioCam MRC 5 camera system (Carl Zeiss Microimaging Inc., Thornwood, NY, USA).

2.2 DNA extraction, PCR amplification and sequencing

Total cellular DNA was extracted from branch apices free of obvious external epiphytes and other potential (eukaryotic) DNA contaminants using the Bioline MyTaq Extract PCR kit (Meridian Life Science, Inc. Memphis, TN USA). The manufacturer's protocol was followed

Table 2: (continued)

Species	Collection site and date	Collector	Collection/silica voucher #	Herbarium voucher #	COI-5P	rbcl
	Middle Marsh, Carteret Co., NC, 34° 41.362' N 76° 37.231' W, 09 September 2019					
	Intracoastal Waterway, Ocean Isle Beach, Brunswick Co., NC, 33° E. Dingess 53.649' N 78° 26.611' W, 25 October 2010		NCweed-1959		-	MZ303640
	Intracoastal Waterway, Topsail Sound, Pender County, NC, 34° F. Montgomery 24.893' N 77° 35.285' W, June 2003		G03-41		MZ303686	MZ303662
	Middle Marsh, Carteret Co., NC, 34° 41.362' N 76° 37.231' W, 09 September 2019	D. W. Freshwater	NCweed-1978		MZ303675	MZ303651
<i>Calliblepharis saidana</i> (Holmes)	210 Rock, Onslow Bay, NC, 34° 14.434' N 76° 35.526' W, 21 May 2008	S. Viehman	NC08-007		MZ303680	MZ303656
M.Y. Yang et M.S. Kim	Dan Spot 2, Onslow Bay, NC, 33° 38.134' N 77° 13.301' W, 21 October 2014	D. W. Freshwater, H. J. Styron	NCweed-1481	WNC2014-S036	MZ303677	MZ303653
	23-mile reef 1, Onslow Bay, NC, 33° 59.470' N 77° 20.630' W, 29 January 2013	J. Idol, C. Slog, D. W. Freshwater	NCweed-1199		MZ303668	MZ303644
	C-Cam site, 23-mile reef complex, Onslow Bay, NC, 33° 59.545' N 77° 21.654' W, 11 July 2005	D. W. Freshwater, J. Souza	Hyp volu NC1		MZ303671	MZ303647

with two exceptions: (1) material was first ground in extraction buffer using a 1.5-ml microcentrifuge tube pestle, and (2) the initial incubation (75 °C) was extended to 1 h. Extracted DNA was used as template in subsequent PCR reactions or was diluted ten-fold using sterile H₂O prior to use. PCR amplifications (25 µL) were performed using a MyTaq HS Red Mix PCR kit (Meridian Life Science, Inc.). The 5' end of the mitochondrial gene encoding cytochrome c oxidase subunit 1 (COI-5P) was PCR amplified and sequenced using primer pairs GAZF1-GAZR1 or GWSFn-GWSRx (Saunders 2005; Saunders and McDevit 2012). The plastid-encoded *rbcl* gene was amplified as two overlapping fragments using primer pairs F57-R1144 and F753-RrbcS Start (Freshwater and Rueness 1994; Iha et al. 2016). PCR parameters were the same for both gene amplifications: a preliminary denature step at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, with a final extension step at 72 °C for 7 min. PCR reaction products were checked for yield on 0.8% agarose gels and cleaned using the Illustra™ ExoProStar™ Enzymatic PCR and Sequencing Clean-up kit according to manufacturer's instructions (GE Healthcare UK Limited, Buckinghamshire, UK). PCR products were sequenced on both strands using BDx64 enhancing buffer (Molecular Cloning Lab [MCLAB], San Francisco, CA, USA) and Big Dye v. 3.1 (ThermoFisher Scientific, Waltham, MA, USA) according to the MCLAB BDx64 protocol and analyzed on an ABI3500 automated DNA sequencer (ThermoFisher Scientific). Sequences were assembled and edited using the Sequencher software package (GeneCodes Corp., Ann Arbor, MI).

2.3 Phylogenetic analyses

The *rbcl* and COI-5P sequences were aligned separately with homologous sequences from GenBank using MUSCLE (Edgar 2004) as implemented in Geneious (v. R8; Biomatters, Auckland, New Zealand). A concatenated dataset was created from both alignments (COI-5P + *rbcl*) with a total length of 2048 bp. Maximum likelihood (ML) and Bayesian Inference (BI) analyses were carried out using the RAXML (Stamatakis et al. 2005) and Mr. Bayes (Huelsenbeck and Ronquist 2001) Geneious plugins. ML analyses were performed using the GTR CAT I model with data partitioned by codon position and Rapid hill-climbing algorithm for 10 random starting trees. ML bootstrap values were obtained using the GTR CAT I model and the rapid bootstrapping algorithm (1000 replicates) with data partitioned by codon position. Bayesian analyses were performed using the GTR model and two simultaneous runs of four Monte-Carlo Markov Chains (three heated and one cold) for 1,000,000 generations, sampling every 750 generations and with a burn-in value of 250,000 generations.

3 Results

3.1 Molecular analyses

Sequence data (*rbcl* and COI-5P) were determined for 26 North Carolina specimens. The lengths of generated *rbcl* sequences ranged from 694 to 1426 bp with the majority being over 1350 bp. The *rbcl*-5P region was only generated for seven of the specimens and the *rbcl*-3P region was only generated for one. Generated COI-5P sequences ranged from 602 to 664 bp with a mean length of 659 bp.

Representative North Carolina specimen sequences were combined with GenBank sequences in alignments of both loci. The *rbcl* alignment included 51 sequences with a mean length of 1302 bp. The COI-5P alignment included 40 sequences with a relatively short mean length of 556 bp because many of the available GenBank sequences are only 465 bp in length. ML analyses of both genes (Supplementary Figures S1 and S2) resulted in similar topologies with only minor variations likely the result of some species in GenBank lacking data for one locus or the other.

Our phylogenetic reconstructions using combined COI-5P and *rbcl* sequence data identified three distinct species among the specimens sampled in this study

(Figure 1). Eleven individuals field identified as *Hypnea musciformis* from North Carolina had only 0–1 bp (0.0–0.1%) and 0 bp (0.0%) variations across *rbcl* and COI-5P sequences respectively, and they formed a strongly supported clade (maximum likelihood bootstrap [MLB] = 99; Bayesian posterior probability [BPP] = 1.00) with other members of *H. musciformis* including specimens from the type locality (Italy). Sequence variations within this clade were 0–3 bp (0.0–0.4%) for *rbcl* and 0–12 bp (0.0–2.6%) for COI-5P. Twelve individuals identified as *H. valentiae* based on Schneider and Searles' (1991) description of the species from North Carolina were resolved with strong support (MLB = 88; BPP = 1.00) as members of the recently described species

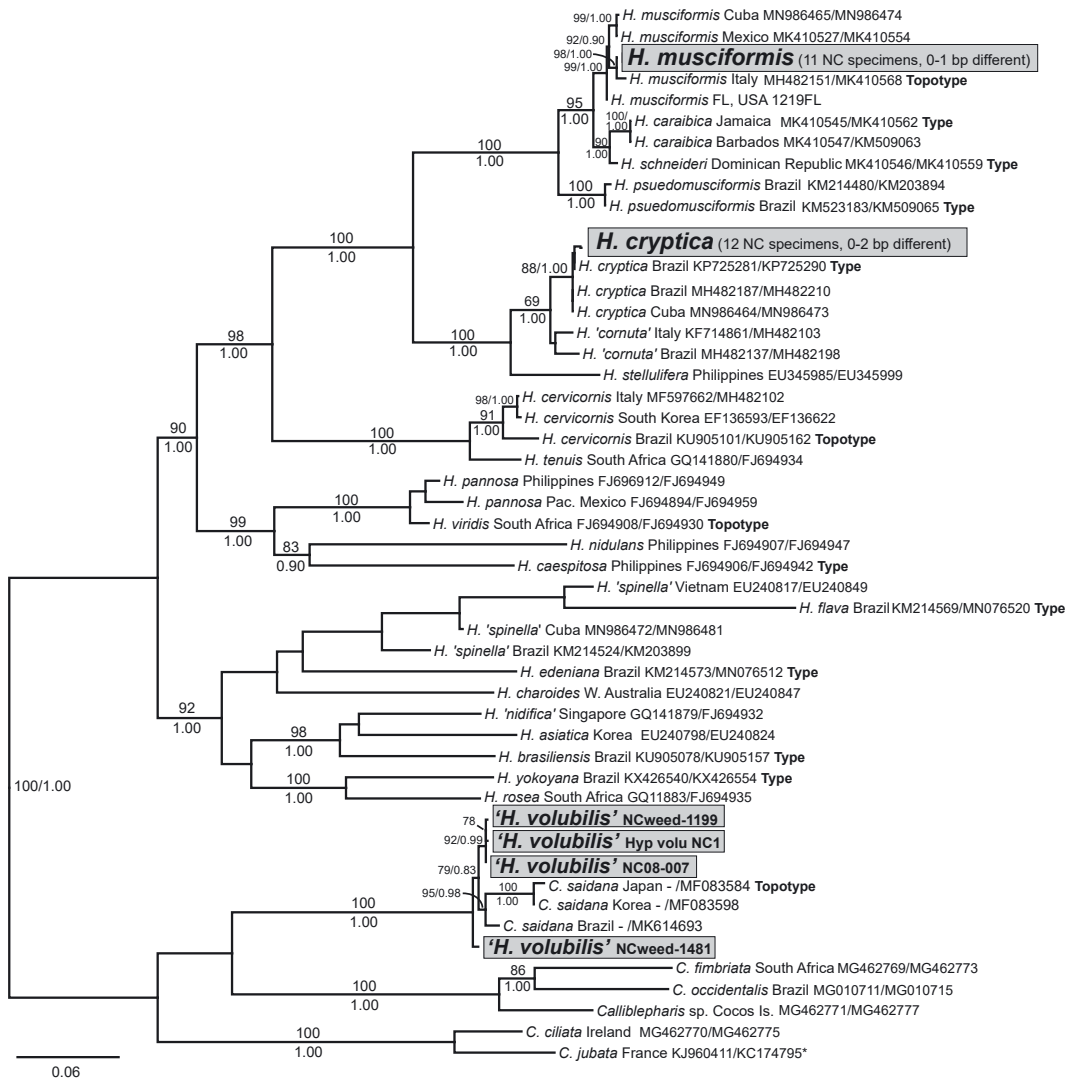


Figure 1: Maximum likelihood (ML) tree depicting phylogenetic relationships inferred among *Hypnea* and *Calliblepharis* species based on combined analyses of plastid *rbcl* and mitochondrial COI-5P sequences. For each specimen, GenBank accession numbers (COI-5P/*rbcl*) are provided, and its geographic origin is noted. 'Type' indicates DNA sequences derived from type material, whereas 'Topotype' denotes DNA data from specimens from the type locality. ML bootstrap values (BS) are shown above branches; Bayesian posterior probabilities (BPP) are shown below.

Hypnea cryptica. There was no *rbcL* sequence variation and only 0–2 bp (0.0–0.2%) variations in COI-5P among these specimens. Our North Carolina sequences differed from the holotype COI-5P and *rbcL* sequences by 0–2 bp, and variations among all available *H. cryptica* COI-5P and *rbcL* sequences were 0–6 bp (0.0–0.8%) and 0–2 bp (0.0–0.1%), respectively. Analysis of *rbcL* and COI-5P sequences from four individuals of *H. volubilis* from the type locality (North Carolina, USA) revealed those individuals were placed in a clade with *Calliblepharis saidana* (Holmes) M.Y. Yang et M.S. Kim that received full support (MLB = 100; BPP = 1.00). Sequence variations among the North Carolina specimens ranged from 0 to 8 bp (0.0–0.6%) for *rbcL* and 0 to 6 bp (0.0–0.9%) for COI-5P. Comparisons with *rbcL* sequences of East Asian and Brazil *C. saidana* specimens revealed variations of 0–13 bp (0.0–1.0%).

3.2 Morphological observations

Multiple specimens representing the three species identified as present in coastal North Carolina by our phylogenetic analyses were examined. Specimens of *H. musciformis* were 5–20 cm tall and predominately displayed loose branching patterns with percurrent main axes; specimens of *H. musciformis* larger than those we examined are not uncommon. Simple to branched branchlets were sometimes present on the surface of main and lateral axes. The tips of axes were generally unbranched and often curved with at least some possessing crozier tips. Axes were terete in transverse section with a central axial cell surrounded by multiple larger hyaline oval to polygonal periaxial cells (Figure 2). This central structure was further surrounded by medullary cells that transitioned to a two-layered, pigmented cortex of smaller cells with a complete outer cortex of polygonal cells (Figure 3).

Hypnea cryptica specimens were 5–30 cm tall with multiple percurrent main axes and multiple orders of branches that became progressively shorter in length. Short lateral axes and branchlets were adaxially curved. The branchlets were spur-like, simple to cornute, and sometimes densely spaced on the surface of main and lateral axes. Transverse sections of axes were terete with a central axial cell surrounded by multiple larger hyaline oval to polygonal periaxial cells, which were surrounded by progressively smaller outer medullary cells. The cortex was composed of two layers of pigmented cells with a complete outer cortex of polygonal cells (Figure 4).

North Carolina specimens of *C. saidana* were small (<5 cm) and complanately branched. The axial cells in living and stained specimens were typically, but not always,

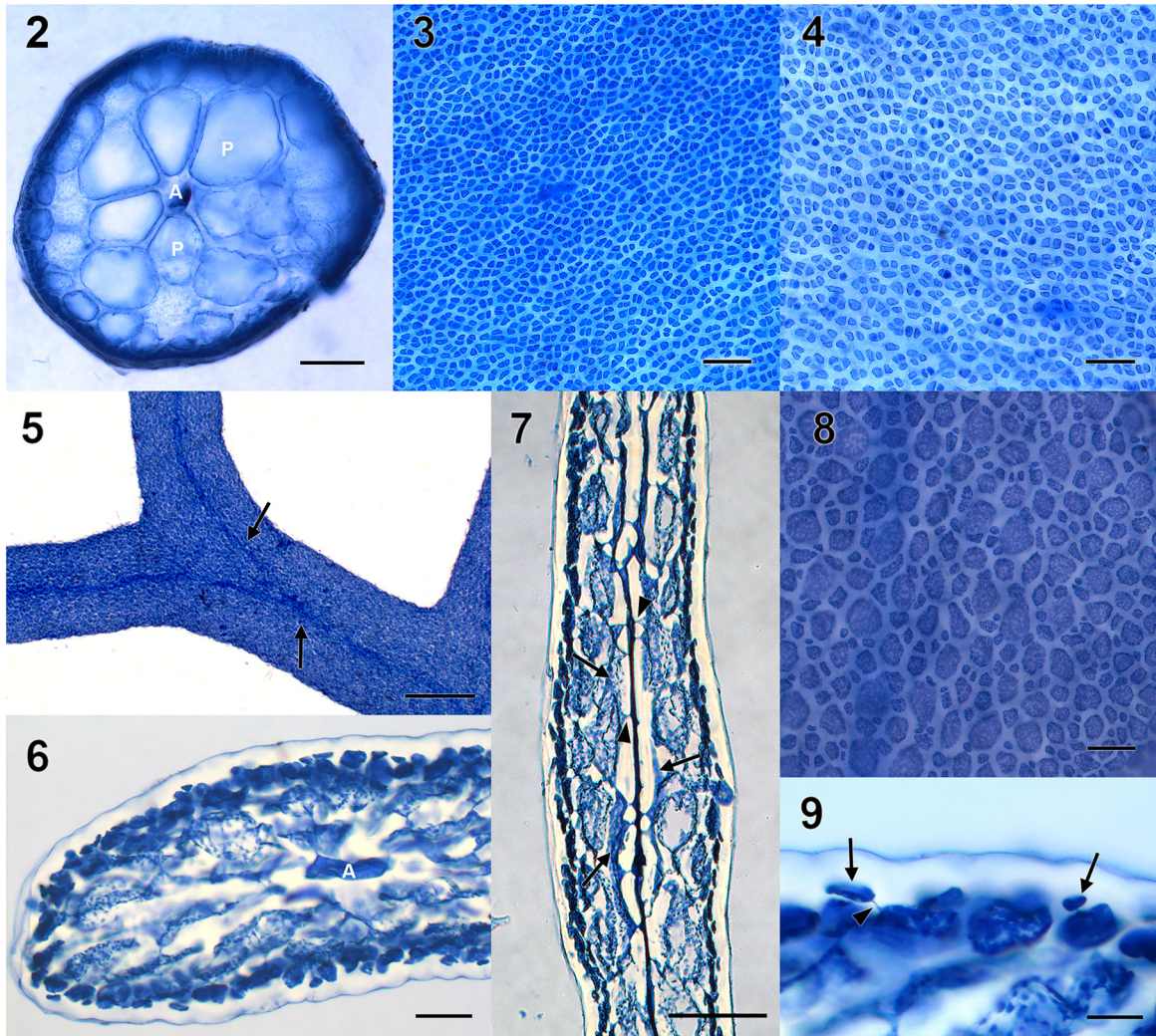
visible in surface view (Figure 5). The thalli of *C. saidana* were compressed to flattened, sometimes with one surface more flattened than the other, and a central axial cell filament was clearly visible in transverse and longitudinal sections (Figures 6 and 7) and linked by multiple secondary pit-connections to adjacent elongated medullary cells (Figure 7). Specimens had an incomplete outer cortex layer of small rounded to polygonal cells forming partial rosettes around the larger rounded to ovoid inner cortical cells from which they are derived (Figures 8 and 9).

Detailed descriptions and illustrations of all three species are included in Jesus et al. (2019), Nauer et al. (2019a), and Yang and Kim (2017).

4 Discussion

Three species were found in our studies of *Hypnea* from coastal North Carolina: *H. musciformis*, *H. cryptica*, previously identified in North Carolina as *H. cornuta* (Kapraun 1980; Taylor 1960) or *H. valentiae* (Schneider and Searles 1991), and *Calliblepharis saidana*, which was previously identified in North Carolina as *H. volubilis* (Schneider and Searles 1976, 1991). A fourth species, *Hypnea cervicornis*, was previously reported from North Carolina based on a single dredge collection made offshore (Schneider and Searles 1976). Schneider and Searles (1976) described this specimen as being lax, perhaps as a product of growing at 27 m depth. No specimens fitting the description of *H. cervicornis* have since been collected in North Carolina, suggesting that it is not present or is a rare and/or transient member of the flora.

The three verified species can be identified in the area by both morphological and habitat differences. *Hypnea musciformis* was most often found on rocks, floating docks, and jetties in the channels and sounds near inlets where there was oceanic influence. Plants varied in pigmentation from greenish-brown to purplish-red and usually possessed the classic crozier tips used to identify members of the *H. musciformis* species complex. *Hypnea cryptica* was seasonally abundant in sounds and coastal waterways. It was found growing attached to hard substrata or seagrass or drifting free, sometimes in large tangled clumps. Specimens varied from straw-colored to dark brownish-red and exhibit characteristic simple to cornute branchlets reported for this species (Jesus et al. 2019). Some specimens of *H. musciformis* also had cornute branchlets similar to those observed in *H. cryptica* but maintained the crozier tips common for the species. *Calliblepharis saidana* was only found in North Carolina offshore waters where it grew as an epiphyte on other seaweeds. It differed from both



Figures 2–9: North Carolina *Hypnea* and *Calliblepharis* species. (2) *H. musciformis* transverse section showing the central axial cell (A) and surrounding periaxial cells (P). Scale bar = 100 μm . (3) *Hypnea musciformis* surface view of outer cortex. Scale bar = 50 μm . (4) *Hypnea cryptica* surface view of outer cortex. Scale bar = 50 μm . (5) *Calliblepharis saidana* surface view of compressed axes with visible central axial cell filament (arrows). Scale bar = 500 μm . (6) *Calliblepharis saidana* transverse section showing compressed axial cell (A). Scale bar = 50 μm . (7) *Calliblepharis saidana* longitudinal section showing elongated central axial and adjacent periaxial cells (arrows) linked by multiple secondary pit connections (arrowheads). Scale bar = 100 μm . (8) *Calliblepharis saidana* surface view showing incomplete outer cortex of small rounded to polygonal cells forming partial rosettes around larger inner cortical cells. Scale bar = 50 μm . (9) *Calliblepharis saidana* transverse section showing small outer cortex cells (arrows) pit connected (arrowhead) to the larger inner cortical cells around which they form partial rosettes. Scale bar = 20 μm .

H. musciformis and *H. cryptica* in having flattened to compressed thalli with complanate branching. The central axial cells of *C. saidana* were often visible in surface view as a ‘midvein’ (Hoffman et al. 2018; Schneider and Searles 1976; Yang and Kim 2017), and the outer cortex was incomplete in contrast to the complete outer cortex of *H. musciformis* and *H. cryptica* (Figures 3, 4 and 8).

Hypnea musciformis has recently been divided into four separate species, *H. musciformis*, *H. pseudomusciformis* Nauer, Cassano et M.C. Oliveira, *H. caraibica* Nauer, Cassano

et M.C. Oliveira, and *H. schneideri* Nauer, Cassano et M.C. Oliveira, all of which are present in the Western Atlantic (Table 1). Available phylogenetic data and complementary range information (Nauer et al. 2019a, 2019b; this study) suggest that *H. musciformis* is found in warm- to cool-temperate waters of the Northwestern Atlantic, *H. caribica* and *H. schneideri* are restricted to the tropical Western Atlantic and Caribbean Sea, and *H. pseudomusciformis* is found in warm- to cool- temperate waters of the Southwestern Atlantic.

Hypnea cryptica was described from Brazil (Jesus et al. 2019) as a cryptic species within the *H. cornuta* complex and was reported to be endemic to Brazil. *Hypnea cryptica* has been recently reported based on molecular data from Cuba (Cabrera et al. 2020), and our results show that its range extends throughout the tropical and subtropical Western Atlantic. *Hypnea valentiae* and *H. cornuta* represent misapplied names to the North Carolina taxon. These species have been considered to be synonymous by some authors (Børgesen 1943; Cribb 1983; Lawson and John 1982) but based on detailed descriptions and drawings of these taxa, they display different forms of the stellate processes that have traditionally been used to identify them (Jesus et al. 2019; Littler et al. 2008; Schneider and Searles 1991).

It appears that the past concept of *H. cornuta* encompasses multiple species. Recent molecular analyses within the *H. cornuta* complex revealed four distinct and phylogenetically unrelated monophyletic groups of specimens identified as *H. cornuta*, with *H. cryptica* being the first to be separated into a novel taxon. The remaining three clades all displayed the same form of the stellate processes attributed to *H. cornuta* (Jesus et al. 2019). These often darkly pigmented stellate processes are peltate and constricted at their attachment point so that they easily detach, and have been referred to as stellate propagules (Huisman et al. 2021). The different '*H. cornuta*' clades appear to exhibit different biogeographic affinities and one has recently been described as *H. corona* Huisman et Petrocelli, but the absence of sequence data from the West African type locality makes the true identity of *H. cornuta* uncertain (Huisman et al. 2021; Jesus et al. 2019). *Hypnea valentiae* was described from the Red Sea and has been reported from warm seas around the world (e.g. Lewmanomont 1997; Verlaque et al. 2015; Womersley 1994). However, Tsiamis and Verlaque (2011) warn that many reports of *H. valentiae* may be inaccurate because of misidentifications and confusion surrounding its status as a species.

North Carolina specimens of *H. cryptica* fit Schneider and Searles (1991) description of *H. valentiae* with alternately branched axes with simple spine-like to cornute branchlets of shorter length along the axis. The currently studied North Carolina specimens did not have peltate to sessile stellate branchlets described for some specimens by Schneider and Searles (1991) and Kapraun (1980, as *H. cornuta*). However, neither the illustrations of this species by both authors, nor North Carolina specimens labeled as *H. cornuta* or *H. valentiae* in the University of North Carolina at Wilmington herbarium (WNC) displayed these features as they are depicted for *H. cornuta* and the newly described *H. corona* (see Børgesen 1920;

Huisman et al. 2021; Jesus et al. 2019; Littler et al. 2008). Morphologically the North Carolina *H. cryptica* specimens match the original description of *H. cryptica* from Brazil (Jesus et al. 2019).

Soares and Fuji (2020) identified three species of *Calliblepharis* in the Western Atlantic: *C. jolyi* E.C. Oliveira, *C. occidentalis* Joly et Yamaguishi-Tomita, and *C. saidana*. *Calliblepharis saidana* was originally placed in *Hypnea* when it was described (Holmes 1895), and Holmes noted its similarity to *H. pannosa* J. Agardh. These species varied from other *Hypnea* species by their complanate branching and flattened thalli (Schneider and Searles 1976). *Hypnea saidana* was recently transferred to *Calliblepharis* based on molecular and morphological analyses (Yang and Kim 2017).

Hypnea volubilis was tentatively placed in the genus *Hypnea* by Searles (in Schneider and Searles 1976) based upon its vegetative structure, zonate tetrasporangia and presence of multiple pit connections between axial cells and adjacent medullary cells. Searles noted the similarity of *H. volubilis*' slender complanate branches to those of *H. pannosa* and *H. saidana*, but distinguished it from *H. pannosa* based on outer cortex differences (Schneider and Searles 1976). Schneider and Searles (1976) considered *H. saidana* to differ from *H. volubilis* in having elongated periaxial cells, but these were found to be present in our North Carolina specimens (Figure 7) and also those from Brazil (Soares and Fujii 2020).

Hypnea volubilis has been reported from Brazil (Nauer et al. 2019c) where *C. saidana* was reported for the first time in the Western Atlantic (Soares and Fujii 2020). Our results confirmed the similarities noted by Searles in his diagnoses of the species, and placed representatives of *H. volubilis* in a fully supported monophyletic group with *C. saidana*. Cross sections of vegetative thallus material revealed similarities to the Brazilian specimens of *C. saidana* shown in Soares and Fuji (2020), with a cortex comprising two layers of rounded or polygonal cortical cells and the outer cortex being incomplete. The axial cells were clearly visible, transversely flattened, and elongated. Based on the molecular and morphological analyses of topotype *H. volubilis* specimens, this species is here synonymized under *C. saidana*. These results along with the results of Soares and Fuji (2020) expand the range of *C. saidana* from Japan to the broader Western Atlantic, potentially classifying it as a cosmopolitan species. *Hypnea volubilis* has been reported from the Gulf of California, Brazil, and the southeastern United States (Fredericq et al. 2009; Hanisak and Blair 1988; Nauer et al. 2019c; Schneider and Searles 1991), but only one sequence previously existed for a specimen identified as this species. This *rbcL* sequence from a Gulf of

Mexico specimen was not resolved with sequences generated from our topotype specimens; instead forming a monophyletic group with sequences from specimens of *H. yokoyana* Nauer, Cassano et M.C. Oliveira, a recently described species from Brazil with a range that extends north to the Gulf of Mexico (Nauer et al. 2016).

Four species of *Hypnea* had been previously reported from North Carolina waters. This study has shown that one, *H. cervicornis*, is most likely not part of the flora. It also verified the presence of *H. musciformis*, the earliest reported species from North Carolina (Hoyt 1920), and demonstrated that the species previously identified as either *H. cornuta* or *H. valentiae* (e.g. Kapraun 1980; Schneider and Searles 1991) is *H. cryptica*. *Hypnea volubilis*, originally described from North Carolina offshore waters, was shown to be a synonym of *Calliblepharis saidana*, the first record of this species and genus in the northwest Atlantic. Our results have demonstrated again the necessity of using DNA sequence data for systematic studies of *Hypnea* and *Calliblepharis*.

The North Carolina coast is a marine transition zone where elements of both the Western Atlantic cool- and warm-temperate floras meet in nearshore waters, and tropical species occupy offshore habitats influenced by the warm Gulf Stream current (Freshwater et al. 2016; Searles and Schneider 1980). Numerous marine algal species have been reported to have Western Atlantic ranges extending from North Carolina through the tropics to Brazil (Schneider 1976), but only a few, such as *Lobophora dispersa* O. Camacho, Freshwater et Fredericq (Camacho et al. 2019), have been molecularly verified. Here we have shown that two more species *H. cryptica* and *C. saidana* fit this distribution pattern within the Western Atlantic. Continued molecular systematic investigations of marine algae will help reveal if morphologically-based species ranges such as this are real and consistent patterns. This is needed to understand the historical biogeography and physiological requirements leading to these distributions and how they might change in the future.

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