PRISCILA BARRETO DE JESUS

ESTUDOS BIOSSISTEMÁTICOS EM ESPÉCIES DO GÊNERO *HYPNEA* J.V. LAMOUROUX (GIGARTINALES, RHODOPHYTA)

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ESTUDOS BIOSSISTEMÁTICOS EM ESPÉCIES DO GÊNERO *HYPNEA* J.V. LAMOUROUX (GIGARTINALES, RHODOPHYTA)

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SUMÁRIO GERAL

INTRODUÇÃO GERAL

A família Cystocloniaceae é composta por 13 gêneros (sete deles monotípicos) (Guiry & Guiry 2016), a maioria deles sendo bem conhecida pelos polissacarídeos presentes nas suas paredes celulares (Chiovitti et al. 1998). Dentro desta família, o gênero *Hypnea* J.V. Lamouroux apresenta o maior número de espécies (Guiry & Guiry 2016), para o qual já foram descritos aproximadamente 116 táxons, ilustrando o cenário de confusão taxonômica na delimitação dessas espécies.

O gênero *Hypnea* foi estabelecido por Lamouroux em 1813 com base em cinco espécies (*H. charoides* Lamouroux, *H. hamulosa* Lamouroux, *H. musciformis* Lamouroux, *H. spinulosa* Lamouroux e *H. wighii* Lamouroux), a maioria delas tendo sido removida do gênero *Fucus* Linnaeus (Tanaka 1941). Este gênero é caracterizado por apresentar talo ereto ou prostrado, cilíndrico a achatado, com consistência membranácea a cartilaginosa, muito ramificado de forma irregular, dicotômica ou lateral, e com numerosos râmulos curtos laterais (Figura 1). O eixo principal pode ser distinto ou não. A cor da planta é uma característica bastante variável, sendo encontrados espécimes de coloração amarelada, esverdeada, rósea, vermelha, vinácea, marrom ou enegrecida (Abbott 1997, Masuda et al. 1997, Jesus 2012, Jesus et al. 2013a, 2014).

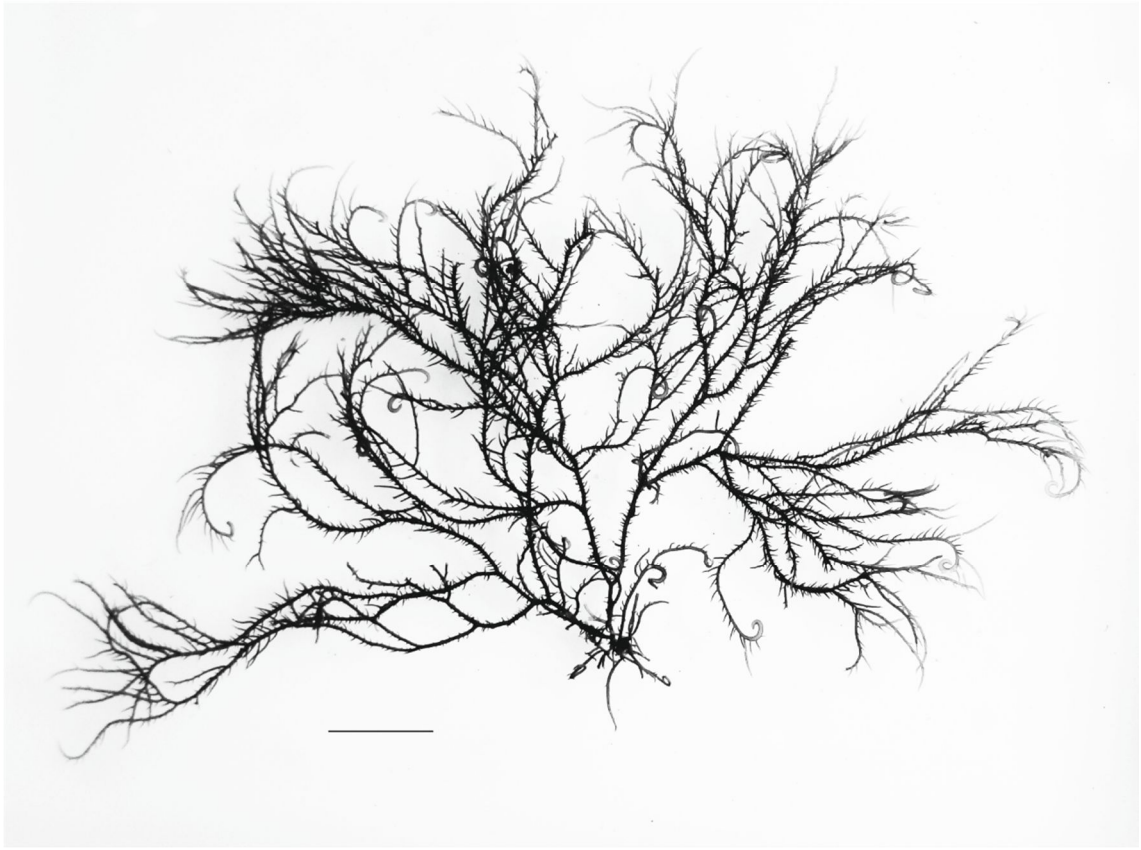

Figura 1: *Hypnea pseudomusciformis* Nauer, Cassano & M.C.Oliveira. Aspecto geral do talo (1 cm).

Após os trabalhos de Lamouroux (1813), muitas outras espécies foram incluídas no gênero. No entanto, o primeiro arranjo sistemático foi feito por J. Agardh em 1852, que descreveu vinte espécies e as classificou dentro de três seções infragenéricas com base no hábito do talo: seção *Virgatae,* cujo talo apresenta eixo principal distinto e densos râmulos laterais que formam tufos eretos, mas não intrincados; seção *Spinuligerae,* com talo formando tufos intricados e ramos e râmulos com pequenos processos do tipo espinho arranjados alternadamente e, a seção *Pulvinatae* cujo talo é prostrado do tipo "almofoda" com ramos inferiores anastomosados e ramos superiores férteis.

Atualmente, são referidas 66 espécies válidas de *Hypnea* (Guiry & Guiry 2016, Jesus et al. 2016 – Capítulo 2, Nauer et al. 2016), amplamente distribuídas nos mares tropicais e subtropicais, com ocorrência nos Oceanos, Índico, Pacífico e Atlântico. No entanto, diversos problemas taxonômicos ainda são recorrentes no gênero, com um elevado número de complexos taxonômicos, espécies pobremente definidas, espécies crípticas e prováveis sinônimos, além de frequentes falhas na identificação de algumas espécies (Price et al. 1992, Abbott 1999, Dawes & Mathieson 2008, Geraldino et al. 2010, Jesus 2012, Jesus et al. 2013, 2014, Nauer et al. 2014, 2015, 2016).

Diversos estudos baseados em dados morfológicos têm contribuído para a compreensão da diversidade do gênero *Hypnea* ao redor do mundo (Tanaka 1941, Cordeiro-Marino 1978, Mshigeni 1978, Schenkman 1986, Mshigeni & Chapman 1994, Masuda et al. 1997, Abbott 1997, 1999, Dawes & Mathieson 2008, Jesus & Nunes 2012, Jesus et al. 2013a, 2013b, 2014 entre outros). A morfologia simples associada à grande plasticidade fenotípica e ampla distribuição do gênero são, provavelmente, as principais causas da dificuldade na delimitação específica em *Hypnea*, demonstrando a importância dos estudos moleculares para resolução dos problemas taxonômicos no gênero (Jesus 2012). A utilização de dados moleculares em estudos taxonômicos e sistemáticos em *Hypnea* é relativamente recente e têm fornecido alguns esclarecimentos sobre a sistemática do gênero (Yamagishi & Masuda 2000, Yamagishi et al. 2003, Geraldino et al. 2006, 2009, 2010, 2015 Nauer et al. 2014, 2015, 2016).

Os primeiros estudos de taxonomia molecular do gênero limitaram-se a espécies asiáticas (Yamagishi & Masuda 2000; Yamagishi et al. 2003; Geraldino et al. 2006, entre outros). A delimitação de espécies do complexo *H. charoides-valentiae* e o reconhecimento do status de *H. stellulifera* (J. Agardh) Yamagishi et Masuda foram realizados com base na sequência do gene plastidial *rbc*L – gene que codifica a subunidade grande da RuBisCo (ribulose bifosfato carboxilase oxigenase) (Yamagishi &

Masuda 2000, Yamagishi et al. 2003). Geraldino *et al.* (2006) analisaram o gene mitocondrial *cox*1(*cytochrome oxidase I*) de 23 espécimes de *H. flexicaulis* Yamagishi & Masuda e concluíram que este gene mitocondrial pode ser utilizado de forma independente para a identificação das espécies de *Hypnea.* Posteriormente, os genes SSU rDNA (nuclear) e *cox*1 foram associados ao *rbc*L para investigação de espécimes da Coréia, Japão e Taiwan (Geraldino et al. 2009), resultando no reconhecimento de *H. asiatica* P.J.L. Geraldino, E.C. Yang & Boo para o nordeste do Pacífico.

No litoral brasileiro, as espécies de *Hypnea* são freqüentes na região entremarés, formando populações densas, crescendo fixas às rochas ou frequentemente como epífitas de outras algas (Jesus 2012). No entanto, trabalhos dedicados exclusivamente aos estudos das espécies deste gênero até pouco tempo eram escassos, e a maioria das informações era proveniente de levantamentos de floras regionais e de listagem de espécies. Estes estudos apresentavam chaves de identificação ou comentários, mas ainda assim, tratavam de poucas espécies e traziam informações escassas, dificilmente esclarecendo as dificuldades de identificação geralmente encontradas para as espécies desse gênero (ex. Cordeiro-Marino 1978, Yoneshigue 1985, Reis-Santos 1990, Amado-Filho 1991 e Nunes 2005). Schenkman (1986) destacou-se como um importante estudo das espécies de *Hypnea* para o estado de São Paulo. Mais recentemente, Jesus (2012) realizou um tratamento taxonômico detalhado das espécies de *Hypnea* no litoral do estado da Bahia, fornecendo informações detalhadas acerca dos caracteres que devem ser levados em conta para uma correta identificação dos táxons e contribuindo de forma significativa para o entendimento do gênero no litoral brasileiro.

Através de dados morfológicos *H. cenomyce* J. Agardh, *H. cervicornis* J. Agardh, *H. cornuta* (Kütz.) J.Agardh, *H. platyclada* P.B. Jesus & J.M.C. Nunes e *H. spinella* (C.Agardh) Kütz. foram referidas para o litoral brasileiro (Nunes 2005, Guimarães 2006, Jesus 2012, 2015, Jesus & Nunes 2012, Jesus et al. 2013b). Estudos moleculares relacionados às espécies de ocorrência no Oceano Atlântico são bastante recentes, e tiveram ênfase na utilização de fragmentos específicos do DNA (região 5' do gene *cox* 1 e o "Universal Plastid Amplicon – UPA) para a delimitação de espécies, denominada DNA barcode (Nauer et al. 2014, 2015, 2016). Tais estudos têm revelado grande diversidade de novos táxons e/ou novas ocorrências para o Brasil: *Hypnea brasiliensis* P.B. Jesus, Nauer & J.M.C. Nunes, *H. edeniana* Nauer, Cassano & M.C. Oliveira, *H. flava* Nauer, Cassano & M.C. Oliveira, *H. pseudomusciformis* Nauer, Cassano & M.C. Oliveira, *H. stellulifera*, *H. wynnei* Nauer, Cassano & M.C. Oliveira, e *H. yokoyana*

Nauer, Cassano & M.C. Oliveira (Jesus et al. 2015 - Capítulo 1, Jesus et al. 2016 - Capítulo 2, Nauer et al. 2015, 2016); elevando para 12 o número de espécies ocorrentes nesta região.

A descoberta desses novos táxons evidencia a gama de espécies crípticas que ocorrem nesta região, e confirma como a conjunção de dados morfológicos, anatômicos e moleculares pode auxiliar na resolução de alguns problemas taxonômicos. Entretanto, os recentes trabalhos realizados na costa brasileira (Jesus 2012, Jesus & Nunes 2012, Jesus et al. 2013a, b, 2014, Nauer 2013, Nauer et al. 2014, 2015, 2016) também demonstraram algumas discrepâncias entre dados moleculares e morfológicos e revelaram a presença de alguns complexos de espécies que ainda carecem de mais estudos. Tais divergências são fortes indícios de que estes complexos deveriam ser estudados do ponto de vista molecular, em um contexto filogenético mais amplo, abrangendo uma maior área de estudo e ampliando-se também a quantidade de marcadores e de análises moleculares utilizadas.

As espécies *Hypnea cornuta* (Kützing) J. Agardh e *H. stellulifera,* ambas ocorrentes no litoral brasileiro (Figuras 2 e 3, respectivamente), constituem um complexo a ser investigado. *H. cornuta* é amplamente distribuída em regiões tropicais e subtropicais (Yamagishi et al. 2003), sendo caracterizada pela presença de processos estrelados típicos a presença de uma cutícula espessa transparente (Tanaka 1941, Taylor 1960, Mshigeni & Chapman 1994, Chiang 1997, Yamagishi & Masuda 1997, Dawes & Mathielson 2008),

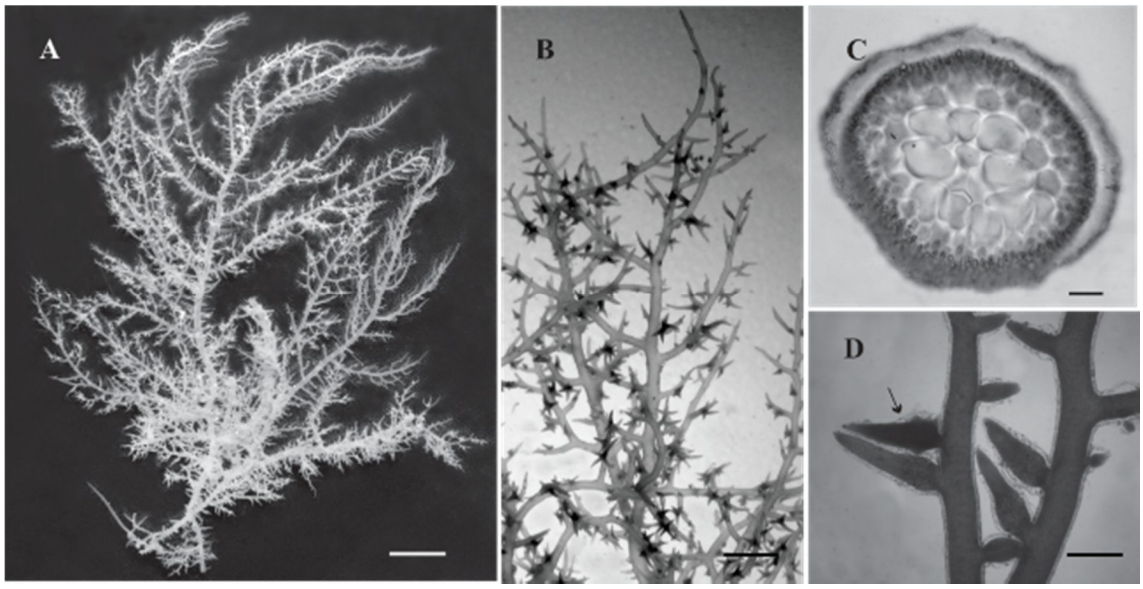

Figura 2: Aspectos morfológicos de *Hypnea cornuta* (Kützing) J. Agardh. A. Aspecto geral (1 cm); B. Detalhe do talo portando râmulos estrelados (0,5 cm); C. Corte transversal na região apical de um ramo. Notar cutícula espessa (100 µm). D. Soros tetrasporangiais desenvolvidos na região basal do talo e um processo estrelado (seta) (500 µm). Modificada de Jesus & Nunes (2012).

evidente tanto em corte transversal quanto em vista superficial (Cecere et al. 2004). *H. stellulifera* apresenta como principais caracteres diagnósticos, o eixo rígido com pequenos e espessos râmulos laterais, além de soros tetrasporangiais e espermatangiais formados na região basal dos râmulos que comumente estendem-se para os ramos e eixos principais (J. Agardh 1852, Lewmanomont 1997, Yamagishi et al. 2003), sendo referida como endêmica da Ásia tropical (Yamagishi et al. 2003).

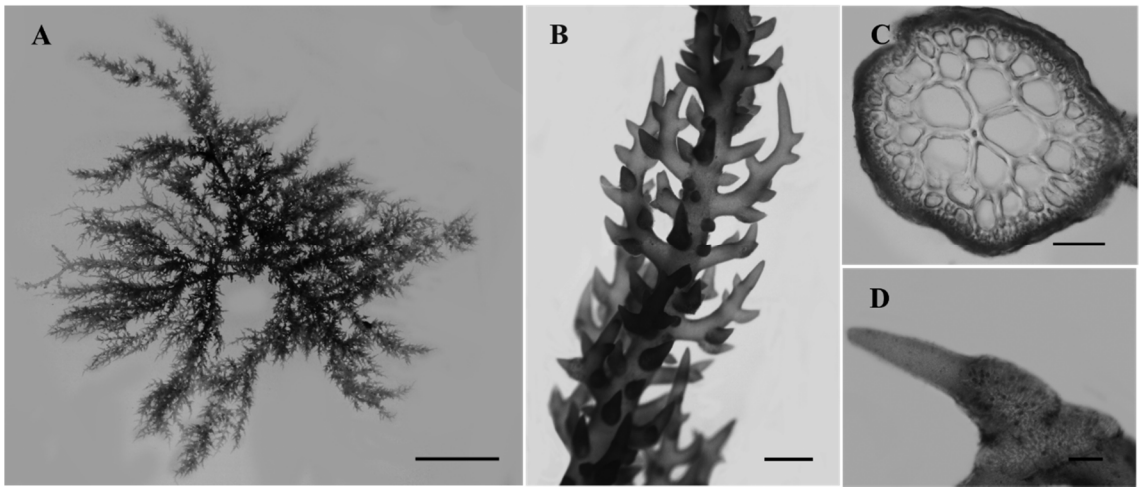

Figura 3: Aspectos morfológicos de *Hypnea stellulifera* (J. Agardh) Yamagishi et Masuda. A. Aspecto geral (2 cm). B. Detalhe do talo portando râmulos espessos curvados em direção adaxial (1 cm); C. Corte transversal na região mediana de um ramo (800 μm). D. Detalhe de um soro tetrasporangial na região basal de um râmulo estendendo-se para o ramo lateral (200 μm).

Hypnea cornuta foi referida pela primeira vez no Brasil com base em exemplares coletados no Rio de Janeiro (Joly et al. 1968) e uma caracterização detalhada dos aspectos morfológicos, anatômicos e reprodutivos desta espécie foram fornecidas por Jesus & Nunes (2012) com base em exemplares coletados no estado da Bahia, nordeste do Brasil. Nauer (2013) analisou sequências moleculares dos marcadores COI-5P e *rbc*L de exemplares coletados no litoral baiano, que apresentavam a morfologia típica de *H. cornuta*, e detectou que os mesmos eram mais proximamente relacionados com espécimes asiáticos de *H. stellulifera* do que com *H. cornuta*. Como divergências moleculares significativas foram encontradas entre os exemplares brasileiros e asiáticos, Nauer (2013) identificou os espécimes brasileiros como "*Hypnea* cf. *stellulifera*" e supôs que os mesmos poderiam tratar-se de uma nova espécie do complexo *H. cornuta-stellulifera* sem, no entanto, propor um tratamento taxonômico definitivo para este complexo.

O complexo *Hypnea cervicornis* é constituído por *H. aspera, H. cervicornis* e *H. flexicaulis* (Figura 4). *H. cervicornis* foi descrita por J. Agardh (1852) com base em amostras do Brasil, Índias Ocidentais e México. Posteriormente, esta espécie foi sinonimizada a *H. spinella* (C. Agardh) Kutzing por Haroun & Prud'Home van Reine (1993), como se estas fossem duas formas de crescimento da mesma espécie induzidas pelo hidrodinamismo. Após a sinonimização, o debate sobre a coespecificidade destas duas espécies continuou (Wynne 1995, 1998, 2005, Chiang 1997, Xia & Wang 1997, Yamagishi & Masuda 1997, 2000, Abbott 1999, Geraldino et al. 2006, Dawes & Mathieson 2008). Sob este cenário, Yamagishi & Masuda (2000) descreveram uma nova espécie de ocorrência no Japão: *H. flexicaulis* Yamagishi et Masuda, ainda que esta fosse morfologicamente idêntica à espécie conhecida como *H. cervicornis* (Yamagishi & Masuda 2000). Entretanto, como esta espécie havia sido reduzida como sinônimo de *H. spinella*, os autores justificaram a descrição da nova espécie com base nas diferenças morfológicas e moleculares (*rbc*L) de *H. spinella*. As características morfológicas usadas na circunscrição de *Hypnea flexicaulis* eram muito similares aquelas usadas na delimitação de *H. cervicornis.* No entanto, como até aquele momento não haviam sequências de DNA de *H. cervicornis* disponíveis em bancos de dados, o *status* taxonômico da nova espécie poderia ser questionado.

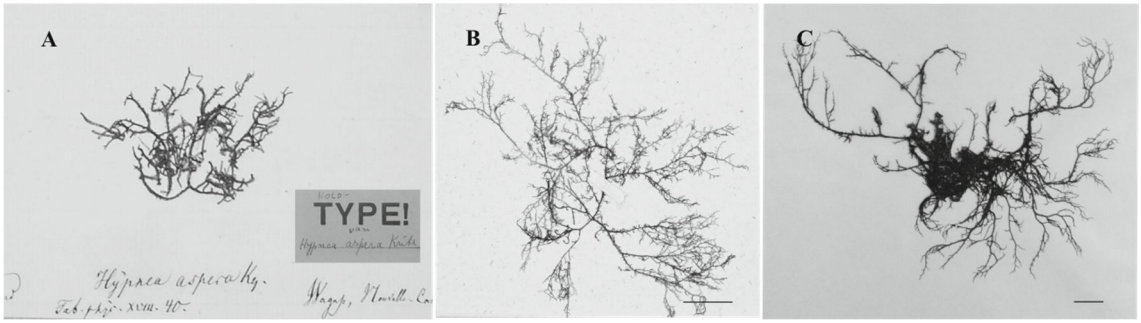

Figura 4: Exemplares do complexo *Hypnea cervicornis* J. Agardh. A. Holótipo de *H. aspera* da Nova Caledônia (L 941). B. Lectótipo de *H. cervicornis* (LD 33878) (1cm). C. Parátipo de *H. flexicaulis* coletado no Japão (SAP 71799) (1cm).

Geraldino et al. (2006, 2009) geraram mais dados que complicaram a história taxonômica destas espécies. Geraldino et al. (2006) realizaram um estudo molecular de *Hypnea flexicaulis* na Coréia e observaram que sequências desta espécie (*rbc*L) eram muito similares a uma sequência do Taiwan gerada por Hommersand &Fredericq (2003) e identificada como *H. boergesenii* T. Tanaka (= *H. aspera* Kutzing; Millar & Prud'homme van Reine 2005). Posteriormente, Geraldino et al. (2009) incluíram mais

sequências em suas análises e observaram novamente um forte relacionamento entre estas espécies, nomeando assim, as sequências geneticamente mais próximas à sequência de Hommersand & Fredericq (2003) como *H. boergesenii* e as mais distantes como pertencendo a *H. flexicaulis*. Nauer et al. (2014), por sua vez, também observaram uma forte similaridade genética entre exemplares coletados no litoral brasileiro e as espécies *H. flexicaulis* e *H. aspera.* Estes autores sugeriram que a coespecificidade entre estas espécies não poderia ser descartada e reportaram a ocorrência de *H. aspera* pela primeira vez no Oceano Atlântico. A morfologia dos espécimes brasileiros, no entanto, é muito similar a observada para *H. cervicornis*, complicando ainda mais a história taxonômica deste complexo de espécies.

Um terceiro complexo, denominado *Hypnea pseudomusciformis*, foi recentemente descrito (Nauer et al. 2015) com base em espécimes coletados no litoral brasileiro. Schenkman (1986) realizou o cultivo *in vitro* de espécies de *Hypnea* como subsídio para estudos morfológicos, reprodutivos e taxonômicos e ressaltou dificuldades taxonômicas para separar algumas espécies. Esta autora fez o primeiro relato de ocorrência de *H. nigrescens* Greville ex J. Agardh (1852) e *H. valentiae* (Turner) Montagne para o litoral brasileiro mas relatou que estas espécies poderiam ser confundidas com *H. musciformis* e a análise conjunta de características de habitat, morfologia, anatomia e crescimento, permitiriam sua separação. Guimarães (2006), Jesus (2012) e Jesus et al. (2013a, 2014) realizaram estudos taxonômicos em espécies do gênero coletadas no litoral do estado da Bahia fornecendo subsídios para a correta identificação destas espécies com base em seus aspectos morfoanatômicos.

Apesar das diferenças morfológicas apontadas entre estas espécies (J. Agardh 1852, Schenkman 1986, Guimarães 2006, Jesus 2012, Jesus et al. 2013a, b, 2014), Nauer et al. (2015) afirmam que os exemplares previamente identificados na costa do Brasil não apresentaram diferenças genéticas entre si e deveriam pertencer a uma única espécie, que eles descreveram como *H. pseudomusciformis* Nauer, Cassano and M.C. Oliveira. Segundo estes autores, os exemplares do litoral brasileiro identificados como *H. musciformis*, *H. nigrescens* e *H. valentiae* (Figura 5) deveriam ser considerados como variações morfológicas de *H. pseudomusciformis* (Nauer et al. 2015), embora estas variedades não tenham sido formalmente propostas.

Nauer et al. (2015) relatam ainda que os exemplares mundialmente conhecidos como *Hypnea musciformis* representam um complexo de espécies e não uma única

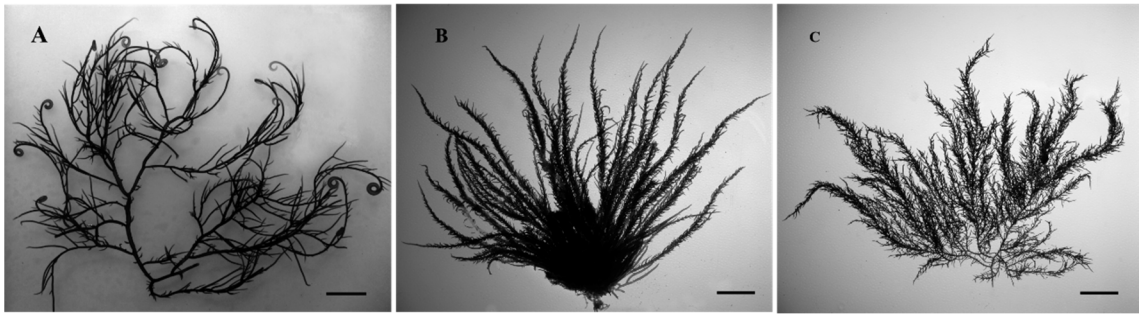

Figura 5: Variedades morfológicas do complexo *Hypnea pseudomuciformis.* A. Morfotipo *musciformis* (1cm). B. Morfotipo *nigrescens* (1cm). C. Morfotipo *valentiae* (1cm). Modificado de Jesus et al. (2013a).

espécie cosmopolita. Uma questão importante avaliada por Nauer e colaboradores foi a relação entre os exemplares sul-americanos (Brasil e Uruguai) previamente identificados como *H. musciformis* e aqueles coletados na América do Norte e Europa, incluindo a localidade tipo de *H. musciformis* (Itália). Estes autores não encontraram diferenças morfológicas entre estes os exemplares analisados, mas afirmaram que eles poderiam ser separados com base nas grandes divergências genéticas encontradas nos dados moleculares. A nova espécie proposta para a América do Sul, *H. pseudomusciformis*, seria proximamente relacionada a *H. musciformis* mas com divergência suficiente para serem consideradas espécies distintas. No entanto, a estrutura populacional destas duas espécies ainda não foi avaliada de modo que os fatores evolutivos que teriam levado à divergência entre estes táxons permanecem desconhecidos.

Apesar da importância econômica e dos diversos problemas taxonômicos acima discutidos, até o presente momento apenas um estudo de filogenia molecular do gênero foi realizado (Geraldino et al. 2010). Neste estudo foram avaliadas as relações entre 23 espécies do gênero, a partir de sequências de *rbc*L e *psa*A (photosystem I P700 apoprotein A1). Conforme os autores, as análises moleculares revelaram que estas espécies agrupam-se em três clados, os quais corresponderam às três seções definidas inicialmente por J. Agardh (1852), com base na morfologia do talo. Estes autores também comprovaram o monofiletismo do gênero com análises baseadas em sequências de *cox1, rbcL* e *psaA*. Entretanto, os autores chamam a atenção para algumas incongruências nos resultados que corroboram a existência de falhas na identificação taxonômica em algumas espécies. O estudo de Geraldino (2010) destaca-se como pioneiro no estabelecimento das relações filogenéticas entre espécies de *Hypnea.* Apesar disso, a maior parte dos exemplares analisados eram provenientes da Ásia e Europa com poucos táxons coletados no Oceano

Atlântico, de modo que as relações filogenéticas entre as espécies sul-americanas não foram avaliadas.

As seções infragenéricas reconstruídas por Geraldino et al. (2010) com base em dados moleculares apresentaram algumas incongruências em relação às seções inicialmente propostas por J. Agardh (1852) com base no hábito do talo. Os autores utilizaram vários caracteres morfológicos para corroborar seus resultados, no entanto, a interpretação do hábito de alguns exemplares parece ter sido equivocada, de modo que inconsistências são observadas nas seções de Geraldino et al. (2010) quando comparadas com a classificação estabelecida por J. Agardh (1852). Como as relações filogenéticas foram estabelecidas com base em espécies de distribuição restrita, essas contradições são um indício de que a validade desta classificação infragenérica deveria ser vista com cautela e que uma revisão destas seções deve ser realizada incluindo uma amostragem mais ampla.

Todos estes exemplos de incongruência entre dados e confusões taxonômicas indicam que estudos mais abrangentes, incluindo múltiplas abordagens, seriam necessários para correta identificação das espécies de *Hypnea* e para a compreensão dos processos que levaram à diversificação do gênero, possibilitando assim uma melhor compreensão das relações entre estas espécies. Uma nova abordagem de estudos denominada Biossistemática, pode fornecer embasamento para sustentar decisões taxonômicas, a qual propõe a delimitação de espécies baseada em um conjunto maior de informações, integrando abordagens como a genética de populações, biologia reprodutiva, morfologia, fitoquímica, entre outros (Stace 1989, Ratnam 2009). Atualmente, esta abordagem tem sido ainda pouco explorada no Brasil, para resolver problemas taxonômicos envolvendo complexos de espécies.

Diante do exposto, considerando a importância econômica do gênero, a grande extensão do litoral brasileiro e a dificuldade na identificação correta das espécies; fazem-se necessários estudos mais amplos (tais como a Biossistemática) que contribuam para a sistemática do gênero através da compreensão da diversidade e estruturação genética e o comportamento de populações naturais, bem como dos padrões de distribuição e das relações filogenéticas entre estas espécies.

OBJETIVOS

Este trabalho teve como objetivo geral a realização de estudos biossistemáticos e filogenéticos em espécies do gênero *Hypnea*, baseados em caracteres morfológicos, ecológicos e moleculares.

Os objetivos específicos foram:

- Aprimorar a base para a sistemática do grupo, por meio de evidências morfológicas, anatômicas, fenológicas e moleculares, contribuindo para que exista maior segurança na delimitação de espécies do gênero *Hypnea*;
- Contribuir para a delimitação das espécies *Hypnea cervicornis, H. pseudomusciformis* e *H. stellulifera,* à luz de novos dados;
- Reavaliar os níveis de divergência molecular intra e inter-específica utilizados para a delimitação de táxons de *Hypnea*;
- Estudar o padrão fenológico de "*Hypnea nigrescens*" e verificar se uma abordagem ecológica pode contribuir para a taxonomia do complexo *H. pseudomusciformis* no Brasil;
- Analisar a diversidade genética e a estrutura populacional das espécies *H. musciformis* e *H. pseudomusciformis*.
- Contribuir para a compreensão das relações filogenéticas entre as espécies de *Hypnea* com ênfase nos táxons sul-americanos.

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CAPÍTULO 1

EXTENSION OF THE DISTRIBUTION RANGE OF *HYPNEA STELLULIFERA* (CYSTOCLONIACEAE, RHODOPHYTA) TO THE SOUTH ATLANTIC: MORPHOLOGICAL AND MOLECULAR EVIDENCE

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Extension of the distribution range of *Hypnea stellulifera* (Cystocloniaceae, Rhodophyta) to the South Atlantic: Morphological and molecular evidence☆

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ABSTRACT

Hypnea stellulifera was, until now, considered endemic to tropical Asia. Here, we report for the first time the expansion of its distribution to the Atlantic Ocean on the basis of collections from the northeast of Brazil. Comparison of morphological features of our specimens with Asian specimens of *H. stellulifera* and molecular data analysis allow us to confirm its identification. Samples analyzed in this study represent the first assessment of *Hypnea* sequences collected in a tropical area from South America. Among the three genes analyzed (the mitochondrial *cox*1 and the plastidial UPA and *rbcL*), UPA was the most conserved, and the *cox*1 was the most variable marker. Despite this, all three markers were efficient as DNA barcoding markers for *Hypnea*. In our phylogenetic analysis, *H. stellulifera* had a sister relationship with the clade that includes *H. cornuta*, *H. musciformis*, *H. flagelliformis*, and *H. chordacea*. Our results demonstrate that the analysis of *Hypnea* species collected at large geographic distances and/or in different tropical areas reveals a higher degree of intraspecific variation as well as decreased interspecific divergence among distinct species from closer areas. These findings corroborate the necessity of a combined analysis of morphology and different genetic markers for a better understanding of taxonomy and phylogeny of the genus *Hypnea*.

1. Introduction

The genus *Hypnea* Lamouroux (1813) includes approximately 60 species (Guiry and Guiry, 2014) and can be found on all tropical and subtropical shores throughout the world (Mshigeni and Chapman, 1994; Geraldino et al., 2009, 2010). According to Wynne (2011), nine *Hypnea* species are present on America's tropical and subtropical coast: *Hypnea cenomyce* (J. Agardh), *Hypnea cervicornis* (J. Agardh), *Hypnea cornuta* (Kützing) J. Agardh, *Hypnea krugiana* (Hauck), *Hypnea musciformis* (Wulfen) J.V. Lamouroux, *Hypnea nigrescens* Grev. ex (J. Agardh), *Hypnea spinella* (C. Agardh) Kütz, *Hypnea valentiae* (Turner) Mont. and *Hypnea volubilis* (Searles). Of these, only *Hypnea krugiana* has not been found on the Brazilian coast (Jesus and Nunes, 2012; Jesus, 2014).

In its original description, *Hypnea cornuta* was distinguished by Agardh (1852) in two varieties: var. *cornuta* from Guinea and St. Thomas, Virgin Islands, in the Atlantic Ocean; and var. *stellulifera* from Vietnam and Manila, the Philippines, in the western Pacific Ocean (Lewmanomont, 1997 Yamagishi et al., 2003). These two entities were characterized as follows: "var. α *cornuta fronde elongate gracili, spinulis stellulaeformibus sparsissimis*" and "var. β *stellulifera fronde breviori rigidiuscula, spinulis stellulaeformibus densis*" (Agardh, 1852; Lewmanomont, 1997).

Yamagishi et al. (2003) studied specimens of these two entities to elucidate their precise taxonomic status. The authors proposed raising the variety *stellulifera* to the rank of species: *Hypnea stellulifera* (J. Agardh) Yamagishi et Masuda; a taxonomic move supported by a detailed examination of the morphology as well as molecular analysis of variations in the *rbcL* gene. They concluded that *H. cornuta* is widely distributed in tropical and subtropical regions

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0304-3770/

while *H. stellulifera* is endemic to tropical Asia. With the results of the study of Yamagishi et al. (2003), Geraldino et al. (2006, 2009) included these two species into their studies. Geraldino et al. (2010) analyzed the phylogenetic relationships among *H. cornuta*, *H. stellulifera* and 14 other *Hypnea* species on the basis of molecular data. As a result, *H. cornuta* and *H. stellulifera* were grouped inside of the section *Virgatae*, together with *H. flexicaulis* Y. Yamagishi and M. Masuda, *H. musciformis*, and *H. tenuis* Kylin.

According to Jesus et al. (2013a), the Brazilian coast can be considered representative of the diversity of American taxa of *Hypnea*. However, despite the various molecular studies recently published for the genus, this tropical region has been completely neglected, constituting a major gap in the taxonomy of the group.

Here, we report the presence of *Hypnea stellulifera* in a tropical region of Brazil, extending the distribution range of this species to the Atlantic Ocean. Morphological comparisons with Asian samples of *H. stellulifera* were carried out to confirm the identification of the Brazilian specimens and for further molecular characterization, analyses were performed using *cox*1 (mitochondrial gene coding for cytochrome oxidase 1) and *rbcL* (plastid gene coding for large subunit of RUBISCO), two genes that have been commonly used at diverse taxonomic levels in *Hypnea* (Yamagishi and Masuda, 2000; Yamagishi et al., 2003; Geraldino et al., 2006, 2009, 2010; Wolf et al., 2011). Mitochondrial *cox*1 has been extensively used for DNA barcoding, and the plastid *rbcL* has proven useful for understanding the phylogeny of red algae (Saunders, 2005; Robba et al., 2006). Another region that has been proposed as a DNA barcode for photosynthetic organisms, the universal plastid amplicon (UPA) (Presting, 2006), was also amplified in this study to be tested as an additional barcode on *Hypnea*.

This is the first time that samples from South America have been included in molecular analyses of the genus, which will allow us to argue the intra- and inter-specific molecular divergence levels in *Hypnea* species at great geographic distances and/or in different tropical areas. Samples of *H. musciformis* (which is the generitype, well known, and more characteristic species of the genus – Jesus et al., 2013b) from the Brazilian and Colombian coast were also included in the analyses to assist us in interpreting the intraspecific range.

The additional goals of this study were as follows: to evaluate the suitability of the *cox*1 and UPA genes for molecular identification (DNA barcoding) of *Hypnea* species; to discuss the intraspecific variation levels used to delimit species in *Hypnea* and; to broaden our understanding of the phylogenetic relationships of *H. stellulifera*, including South American specimens, through *rbcL* data, as well its circumscription within the section *Virgatae*.

2. Materials and methods

2.1. Taxon sampling and morphology

As suggested by Mshigeni and Chapman (1994) and Geraldino et al. (2010), we incorporated additional taxon sampling of *Hypnea stellulifera* from South America and performed intensive molecular observations including samples collected in the type locality. We also analyzed specimens of *H. stellulifera* from Malaysia deposited in the Herbarium of the Graduate School of Science, Hokkaido University (SAP 93004; 93007).

Hypnea stellulifera specimens were collected in the state of Bahia, in northeast Brazil, from the beaches of Enseada do Pedrão, Vera Cruz (13°03′28.35″S, 38°42′10.75″W) and Coroa Vermelha, Santa Cruz de Cabrália (16°19′58.12″S, 39°00′19.5″W). Specimens of *H. musciformis* were collected from several locations of Brazil and from a beach in Colombia (Table 1). Samples were collected during low tides and transported live back to the laboratory, cleaned, and sorted carefully under a stereomicroscope (Leica® Zoom 2000). Three–five pieces of thalli were preserved in silica gel desiccant for DNA extraction.

Materials for morphological observations were preserved in seawater containing 4% formaldehyde. Histological sections were performed by hand with the aid of a razor blade, and reproductive structures were stained with 5% aniline blue solution. Identifications were made in a stereomicroscope and a photonic microscope (Olympus® CBA) with an attached measuring ocular. Photomicrographs of structures were obtained with the aid of an image capture program (QCapture Pro) and a digital camera (QImaging GO-3) attached to the photomicroscope (Olympus trinocular CX31RTS5®). Observations of ecological conditions and the growth habit were noted at the time of collection. All voucher specimens were deposited in the Alexandre Leal Costa Herbarium (ALCB) of the Biology Institute, Federal University of Bahia, Brazil.

2.2. Molecular procedures

2.2.1. DNA extraction, PCR reaction and sequencing

Total DNA was extracted from samples weighing approximately 20–40 mg by maceration in liquid nitrogen using a modified version of the CTAB (Cetyl Trimethyl Ammonium Bromide) procedure of Doyle and Doyle (1987). Molecular markers were PCR amplified under the following conditions: 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 5 ng of genomic DNA, and 1.25 U of Taq DNA polymerase (Invitrogen, Brazil) in a total volume of 25 μL. For the amplification and sequencing reactions for each gene, specific primer pairs were used: for *cox*1, GazF1 and GazR1 (Saunders, 2005) and for UPA, p23Sv_f1 and p23Sv_r1 (Sherwood and Presting, 2007). PCR of the *rbcL* gene was performed with the primers F*rbcL* and R*rbcS* described by Freshwater et al. (1994) and the additional internal primers R753 and F492 described by Freshwater and Rueness (1994).

PCR was performed with an initial denaturation step at 94 °C for 10 min, followed by 35 cycles of 30 s at 90 °C, 30 s at 50 °C (variation of 47 °C for *rbcL* and 55 °C for UPA), and 2 min at 72 °C, with a final 10 min extension cycle at 72 °C. Purifications and sequencing reactions of the PCR products were performed by the company Genewiz, USA.

2.2.2. Molecular analyses

Electropherograms were manually assembled using the Staden package (Staden et al., 2003) and edited in BioEdit 5.0.6 (Hall, 1999). For each marker, a multiple alignment was generated with the ClustalW tool (Thompson et al., 1994) available in BioEdit 5.0.6 (alignments excluded PCR primer sequences). In addition to the sequences of *H. stellulifera* generated in this study and collected from GenBank (www.ncbi.nlm.nih.gov/genbank/), sequences of *H. musciformis* from samples collected on the Brazilian and Colombian coasts (unpublished data) and other *Hypnea* species downloaded from GenBank (searched on May 07, 2014) were included in the analyses. All new consensus *cox*1, UPA, and *rbcL* sequences from *Hypnea* were deposited in the barcode of life data systems (BOLD, http://www.boldsystems.org/) and GenBank (Table 1). For *rbcL* data, outgroups were *Chondrus crispus* Stackhouse (U02984), *Gracilaria tenuistipitata* var. *lui* Zhang and Xia (EF434906), *Griffithsia okiensis* Kajimura (GQ252547), and *Porphyra purpurea* (Roth) C. Agardh (JN831094).

For *cox*1 and UPA data, clustering trees were performed in MEGA 5.0 (Tamura et al., 2011) using the neighbor-joining (NJ) algorithm based on Kimura two-parameter corrected distances (Kimura, 1980) with 2000 bootstraping replicates. The purpose of the barcoding analysis is to group specimens according to sequence similarity, assigning them to a certain species. Therefore, the

Table 1
List of *Hypnea* specimens used in the molecular analysis. Sequences determined in this study are in boldface.

Taxa	Collection site (locality, collector, date, field ID and voucher or reference)	GenBank accession number		
		*cox*1	*rbcL*	UPA
Hypnea asiatica P.J.L. Geraldino, E.C. Yang and S.M. Boo	Gampo, Gyeongju, South Korea (Geraldino et al., 2009)	EU240799	EU346007	–
	Kurohae Beach, Chiba, Japan (Geraldino et al., 2009)	EU240805	EU240839	–
	Lonedome, Keelung, Taiwan (Geraldino et al., 2009)	EU240809	EU346008	–
H. aspera Kützing (as *H. boergesenii* T. Tanaka)	Gimyeong, Jeju, South Korea (Geraldino et al., 2009)	–	EU345994	–
	Taiwan (De Clerck et al., 2005)	–	AF385634	–
H. caespitosa Geraldino et Boo	Bonbon, Cagayan de Oro Philippines (Geraldino et al., 2010)	FJ694902	FJ694937	–
	Cape Rachado, Malaysia (Geraldino et al., 2010)	FJ694899	FJ694943	–
	Sentosa Island, Singapore (Geraldino et al., 2010)	FJ694911	FJ694944	–
H. cervicornis J. Agardh	Hawaii, USA (Sherwood et al., 2010)	–	–	HQ421284
H. charoides J. V. Lamouroux	Point Peron, Perth, Australia (Geraldino et al., 2009)	EU240819 EU240820	EU240844	–
	Pinnaroo Pt., Australia (Barbara et al., 2013)	HM915818	–	–
	Shizuoka, Shimoda, Japan (Yamagishi and Masuda, 2000)	–	AB033159	–
H. chordacea Kützing	Shirahama, Shimoda, Japan (Yamagishi and Masuda, 2000)	–	AB033160	–
H. cornuta (Kützing) J. Agardh	Yuseong-gu, Daejeon, Korea (Geraldino et al., 2010)	GQ141878	–	–
	Teguma, Nagasaki, Nagasaki Prefecture, Japan (Yamagishi and Masuda, 2000)	–	AB033161	–
	Sukuji, Ishigaki Island, Okinawa Prefecture, Japan (Yamagishi et al., 2003)	–	AB095911	–
	Taranto, Italy (Yamagishi et al., 2003)	–	AB095912	–
	Point Peron, Perth, Australia (Geraldino et al., 2009)	–	EU345990 EU345992	–
	Bali, Indonesia (Geraldino et al., 2009)	–	EU345991 EU345993	–
H. flagelliformis J. Agardh	Fukaura, Aomori, Japan (Yamagishi and Masuda, 2000)	–	AB033162	–
H. flexicaulis Yamagishi et Masuda	Gampo, Gyeongju, Korea (Geraldino et al., 2006)	EF136611	EF136622	–
	Wolpo, Pohang, Korea (Geraldino et al., 2006)	EF136610	–	–
	Padova, Italy (Wolf et al., 2011)	FN823052	–	–
	Daisanglan, Keelung, Taiwan (Geraldino et al., 2006)	EF136608	–	–
	Dancalan, Bulusan, Philippines (Geraldino et al., 2006)	EF136591	EF136632	–
	Shomoda, Shizuoka prefecture, Japan (Yamagishi and Masuda, 2000)	–	AB033163	–
H. japonica Tanaka	Gampo, Gyeongju, Korea (Geraldino et al., 2009)	EU345986	EU346003	–
	Keelung, Taiwan (Geraldino et al., 2009)	EU345988 EU345989	EU345995	–
	Ei, Kagoshima prefecture, Japan (Yamagishi and Masuda, 2000)	–	AB033164	–
H. musciformis (Wulfen) Lamouroux	Praia do Fogo, Rio do Fogo, RN, Brazil; coll. E.M. Soriano, 19 Jun. 2012; Field ID: P122; Voucher: ALCB 103598	**KP725279**	–	**KP725303**
	Ponta do Seixas, João Pessoa, PB, Brazil; coll. P.B. Jesus et al., 20 Jul. 2012; Field ID: P97; Voucher: ALCB 110258	**KP725278**	–	**KP725302**
	Mar Grande, Ilha de Itaparica, BA, Brazil; coll. P.B. Jesus et al., 04 Dez. 2010; Field ID: P57; Voucher: ALCB 107007	**KP725277**	–	–
	Imbassaí, Entre Rios, BA, Brazil; coll. P.B. Jesus et al., 23 Oct. 2010; Field ID: P19; Voucher: ALCB 100293	–	**KP725289**	–
	Subaúma, Entre Rios, BA, Brazil; coll. P.B. Jesus et al., 25. Oct. 2010; Field ID: P24; Voucher: ALCB 100295	–	–	**KP725301**
	Cigarras, São Sebastião, SP, Brazil; coll. P.B. Jesus et al., 19 Aug. 2012; Field ID: P115; Voucher: ALCB 110270	**KP725276**	**KP725288**	**KP725300**
	Santa Martha, Magdalena, Caribe Colombiano; coll. M.C. Diaz-Ruiz, 26 Apr. 2013; Field ID: P163; Voucher: ALCB 110412	**KP725275**	–	**KP725299**
	Antibes, France (Geraldino et al., 2009)	–	EU346014	–
	Hawaii, USA (Sherwood et al., 2010)	HQ422612 HQ422876	–	HQ421453 HQ421580
	Japan (Geraldino et al., 2010)	GQ141881	–	–

Table 1 (*Continued*)

Taxa	Collection site (locality, collector, date, field ID and voucher or reference)	GenBank accession number		
	New Hanover, North Carolina, USA (Geraldino et al., 2009)	–	U04179	–
	Spain (Diaz-Tapia et al., Unpublished)	–	KC121141	–
H. nidifica J. Agardh	Sentosa Island, Singapore (Geraldino et al., 2010)	–	FJ694932	–
	Hawaii, USA (Sherwood et al., 2010)	–	–	HQ421469
H. nidulans Setchell	Hedo-misaki, Japan (as *H. pannosa*, Yamagishi and Masuda, 2000)	–	AB033165	–
	Batac, Ilocos Norte, Philippines (Geraldino et al., 2010)	FJ694907	FJ694947	–
	Manjagao, Surigao del Norte, Philippines (Geraldino et al., 2010)	FJ694900	FJ694948	–
H. pannosa J. Agardh	El Sargento, Mexico (Geraldino et al., 2010)	FJ694894	FJ694959	–
H. pannosa J. Agardh	Pangalo Island, Bohol, Philippines (Geraldino et al., 2010)	FJ694910	FJ694951	–
	Hawaii, USA (Sherwood et al., 2010)	HQ422908	–	HQ421470 HQ421272
H. rosea Papenfuss	Durban, Kwazulu Natal, Africa (Geraldino et al., 2010)	–	FJ694935	–
H. spinella (C. Agardh) Kützing	Florida, USA (Hommersand and Fredericq, 2003)	–	AF385635	
	Nha Trang, Vietnam (Geraldino et al., 2009)	EU240818	EU240849	
	Panang Bay Panang, Viet Nam (Geraldino et al., 2009)	EU240817	–	–
	Hawaii, USA (Sherwood et al., 2010)	HQ422681	–	HQ421582
	Sesoko Island, Okinawa prefecture, Japan (Yamagishi and Masuda, 2000)	–	AB033166	–
H. stellulifera (J. Agardh) Yamagishi et Masuda	Coroa Vermelha, Santa Cruz de Cabrália, BA, Brazil; coll. PB. Jesus et al., 07 Nov. 2010			
	Field ID: P02; Voucher: ALCB 103120	KP725281	KP725290	KP725305
	Field ID: P10; Voucher: ALCB 103121	KP725282	KP725291	KP725306
	Coroa Vermelha, Santa Cruz de Cabrália, BA, Brazil; coll. PB. Jesus, T.A. Caires, 03 Jun. 2012			
	Field ID: P83; Voucher: ALCB 110263	**KP725280**	–	–
	Field ID: P88; Voucher: ALCB 110362	**KP725287**	–	–
	Field ID: P90; Voucher: ALCB 110249	–	–	**KP725310**
	Field ID: P91; Voucher: ALCB 110250	**KP725286**	**KP725293**	**KP725309**
	Field ID: P92; Voucher: ALCB 110251	**KP725285**	–	**KP725308**
	Field ID: P93; Voucher: ALCB 110252	**KP725284**	–	**KP725307**
	Field ID: P94; Voucher: ALCB 110253	**KP725283**	**KP725292**	–
	Field ID: P95; Voucher: ALCB 110254	–	–	**KP725304**
	Panglao, Bohol, Philippines (Geraldino et al., 2009)	EU345984 EU345985	EU346004 EU345999	–
	Melaka, Pulau Besar, Malaysia (Yamagishi et al., 2003)	–	AB095913	–
	Sabah, Pulau Sipadan, Malaysia (Yamagishi et al., 2003)	–	AB095914 AB095915	–
	Hawaii, USA (Sherwood et al., 2010)	HQ422721	–	HQ421577
H. tenuis Kylin	Durban, Kwazulu Natal, South Africa (Geraldino et al., 2010)	–	FJ694934	–
H. valentiae (Turner) Montagne	Hawaii, USA (Sherwood et al., 2010)	–	–	HQ421583 HQ421584
	USA (Sherwood and Presting, 2007)	–	–	EF426626
	Bali, Indonesia (Geraldino et al., 2010)	–	FJ694933	–
H. viridis Papenfuss	Lala neck, Durban, Africa (Geraldino et al., 2010)	FJ694908	FJ694930	–
H. volubilis Searles	Los Angeles, USA (Hommersand and Fredericq, 2003)	–	AF385636	–
H. yamadae Tanaka	Nomozaki, Nagasaki Prefecture, Japan (Yamigishi et al., 2003)	–	AB095916	–

algorithms employed in these analyses are simple and do not require a robust model of evolution (Hebert et al., 2003; Kucera and Saunders, 2008; Milstein et al., 2011). To assess the level of variation in *cox*1, UPA and *rbcL* data, estimates of divergence values within and among species were computed using MEGA 5.0.

Phylogenetic analyses of *rbcL* data were inferred by maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis. MP trees were constructed with PAUP* 4.0b.10 (Swofford, 2002) using a heuristic search algorithm with the following settings: starting trees obtained by stepwise addition, using tree bisection–reconnection (TBR) branch swapping algorithm; MulTrees, all characters unordered and with equal weight, accelerated transformation (ACCTRAN) as character-state optimization; branches with a maximum length of zero collapsed to yield polytomies. A strict consensus analysis was computed and bootstrap values for the resulting nodes were assessed using 1000 bootstrapping replicates with simple sequence addition. The consistency (CI), retention (RI), and rescaled consistency (RC) indices resulting from the MP analysis were calculated.

For ML and Bayesian analyses, the best models were assessed under MrModeltest 2.3 (Nylander, 2008). The best model was selected using the akaike information criterion (AIC) as recommended by Posada and Buckley (2004). The ML analyses were conducted using RAxML (randomized axelerated maximum likelihood, version 7.0.4; Stamatakis, 2006) with the GTR GAMMA model. The best-scoring ML tree and 500 bootstrap trees were obtained using the rapid hill-climbing algorithm (Stamatakis et al., 2008).

Bayesian analyses were conducted with MrBayes 3.1 software (Ronquist and Huelsenbeck, 2003) using the Markov chain Monte Carlo method (MCMC) with the selected general-time-reversible model of nucleotide substitution with invariant sites and gamma distributed rates for the variable sites (GTR + I + G). Four million generations in two independent runs were performed with four chains (one hot and three cold), sampling one tree every 10 generations for one million generations starting with a random tree. The burn-in period was identified graphically by tracking likelihoods at each generation to determine whether the likelihood values had reached a plateau. Log-likelihood values were stabilized around 23.500 generations, which were discarded as burn-in. A 50% consensus tree (majority rule as implemented by PAUP) was computed after the burn-in point. Clades were considered supported with Bayesian posterior probability (PP) ≥ 95% or nonparametric bootstraps in NJ, MP and ML ≥ 75%.

3. Results

3.1. Morphology

Hypnea stellulifera (J. Agardh) Yamagishi et Masuda.

Basionym: Hypnea cornuta (Kützing) J. Agardh var. *stellulifera* J. Agardh, Sp. Gen. Ord. Alg. , 2(2): 449. 1852.

Distribution: Tropical regions in the Pacific Ocean (Lewmanomont, 1997; Yamagishi et al., 2003), Indian (Yamagishi et al., 2003) and Atlantic (present study).

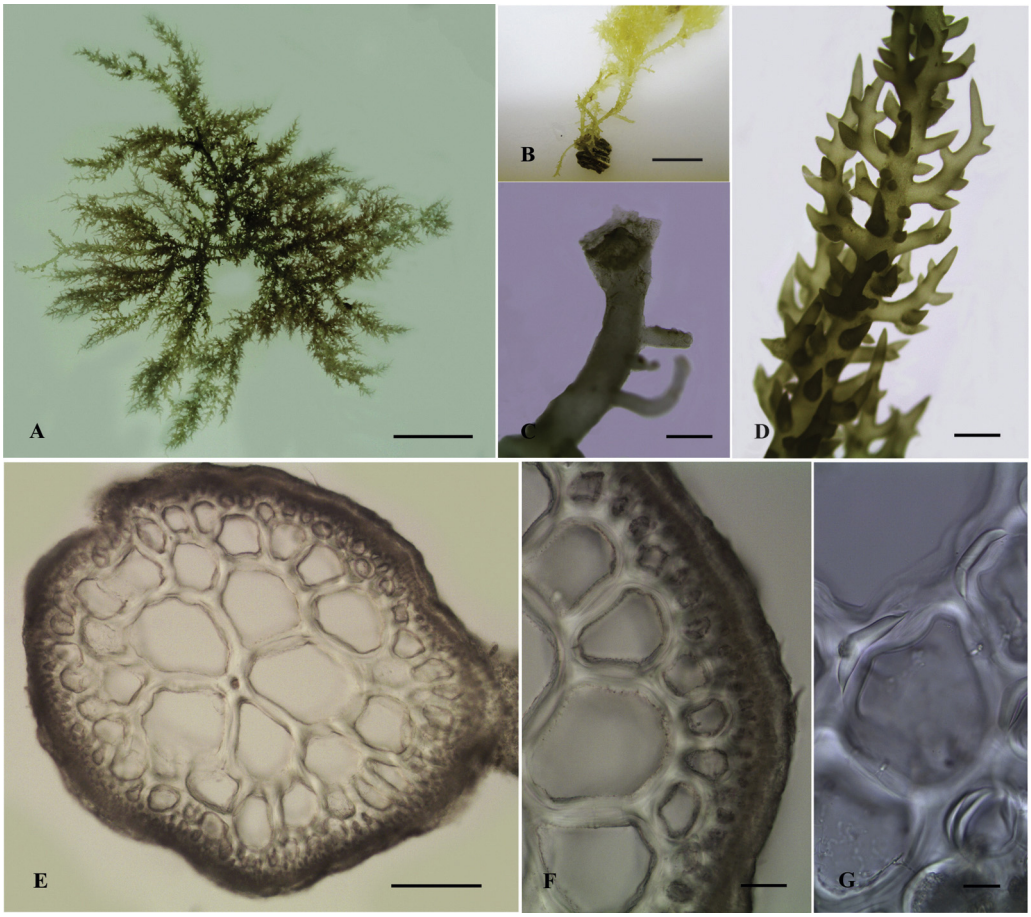

Fig. 1. Vegetative features of *Hypnea stellulifera*. (A) Habit of a female gametophyte (ALCB 110253); scale bar = 2 cm. (B) Basal portion of the thallus; scale bar = 1 cm. (C) Front view of a fixation disk; scale bar = 800 μm. (D) Apical portion of a lateral branch with thick branchlets curved towards the adaxial direction; scale bar = 1 cm. (E) Cross section on the median region of a branch; scale bar = 800 μm. (F) Detail of cortical cells in cross section; scale bar = 50 μm. (G) Lenticular thickenings in medullary cells; scale bar = 20 μm.

Analyzed material: BRAZIL, Bahia: Santa Cruz de Cabrália, Coroa Vermelha Beach, 7.xi.2010, P.B. Jesus, G.M. Lyra, and J.M.C. Nunes (male gametophyte ALCB 103120; female gametophyte ALCB 103121); 3.vi.2012, P.B. Jesus and T.A. Caires, (male gametophyte ALCB 110249, 110251; female gametophyte ALCB 110250, 110253, 110254; tetrasporophyte ALCB 110252); Ilha de Itaparica, Vera Cruz, Enseada do Pedrão, 2.viii.2012, P.B. Jesus et al. (male gametophyte ALCB 110255; tetrasporophyte ALCB 110256). Malaysia, Penang: Pulau Aman, 1.i.1998, Yamagishi et al. (tetrasporophyte SAP 93004); a canal near Pulau Penang 1.i.1998, Yamagishi et al. (tetrasporophyte SAP 93007).

Plants occur solitary or in aggregates, sometimes forming loose tufts. Thallus erect, subcartilaginous, rosy to brownish-red, measuring 5–14.5 cm in height (Fig. 1A). Main axis terete (718–1.073 μm wide) arise from a primary discoid holdfast (Fig. 1B and C) and produce first-order branches irregularly arranged at angles of 50–80°. First-order branches up to 3 cm in length, bearing sparse ordinary branches of up to fourth order, gradually shorter. Ordinary branches and branchlets curve towards the adaxial direction and then grow straight; numerous branchlets, simple or divided, with a broad base (Fig. 1D). Apices of the branches and branchlets are acute, ending in a sharp apical cell. In cross section, the lower portion of axes show a small, circular, and pigmented axial cell (29–73 μm in diameter), surrounded by 4–6 oval periaxial cells (47–264 μm in diameter), 2–3 layers of hyaline medullary cells (45–180 μm in diameter) gradually smaller towards the periphery (Fig. 1E), and 1–2 layers of pigmented cortical cells, 9–38 μm in diameter (Fig. 1F). Lenticular thickenings usually present in periaxial and medullary cells (Fig. 1G). Tetrasporangial sori formed in the lower regions of the branchlets commonly extending to the axes or branches (Fig. 2A), bearing zonate tetrasporangia 24–53 μm in length and 16–30 μm in diameter (Fig. 2B and C). Cystocarps single, or aggregated and globose (Fig. 2D); produced from the base to the apex of the axis and branches (647–1.058 μm in length and 672–1.187 μm in diameter), with thick pericarp and without evident ostioles (Fig. 2E). Carposporangia ovate, 24–34 μm in diameter, arranged in clusters at the apex of the gonimoblasts filaments (Fig. 2F). Spermatangial sori formed in the lower and middle regions of the branchlets, commonly extending to the axes or branches (Fig. 2G). Spermatangia 2.9–4.7 μm in diameter, three to six in number, are arranged in a concatenated manner in the cortical region (Fig. 2H and I).

The samples of *H. stellulifera* were collected growing in the upper intertidal zone in protected sites, on stones covered by a sandy

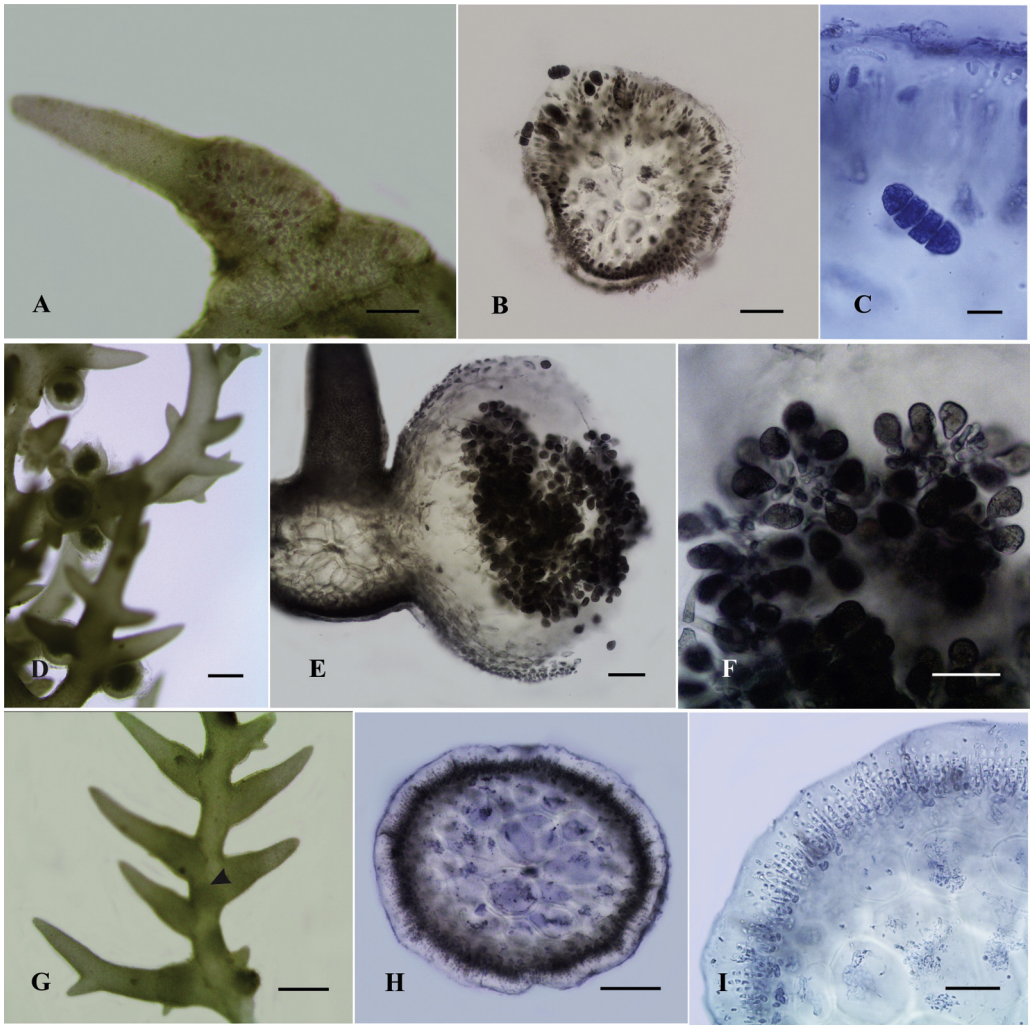

Fig. 2. Reproductive features of *Hypnea stellulifera*. (A) Detail of a tetrasporangial sori in the lower region of a branchlet extending to the axes; scale bar = 200 μm. (B) Cross section of a branch containing tetrasporangia in the cortical region; scale bar = 100 μm. (C) Tetrasporangia zonally divided in detail; scale bar = 20 μm. (D) Cystocarps in branches; scale bar = 400 μm. (E) Longitudinal section through a mature cystocarp; scale bar = 100 μm. (F) Clusters of ovate carposporangia in detail; scale bar = 50 μm. (G) Spermatangial sori formed in the lower regions of the branchlets extending to the axes (arrowhead); scale bar = 600 μm. (H) Cross section of a branch containing spermatangial sori arranged on cortical region; scale bar = 100 μm. (I) Details of spermatangia in chains; scale bar = 20 μm.

substrate. *In situ*, the species is usually found next to *H. cervicornis* and *H. musciformis* specimens, which can easily be distinguished by the robust aspect of the thallus.

3.2. Molecular analyses of data sets

A total of 127 sequences from *cox*1, UPA, and *rbcL* markers were analyzed (Table 1). For each region, a multiple alignment was generated excluding priming regions: *cox*1 included 46 sequences (13 new and 33 published) and 664 base pairs (bp) of which 287 positions were variable (43.2%) and 225 (33.9%) parsimoniously informative; UPA included 23 sequences (12 new and 11 published) and 369 bp, of which 77 (20.9%) were variable and 31 (8.4%) parsimoniously informative; and *rbcL* included 58 sequences (6 new and 52 published) and 1.363 bp, of which 485 (35.6%) were variable and 357 (26.2%) parsimoniously informative.

The *cox*1 and UPA sequences obtained in this study, together with the available sequences from GenBank, were clustered using NJ (Fig. 3). UPA sequences from *Hypnea* available in GenBank are still scarce, and there are no other reliable UPA sequences for *H. stellulifera* available for comparison. For this reason the NJ UPA tree is not shown.

Sequences of *Hypnea stellulifera* specimens collected from Bahia, northeast Brazil, were identical in UPA and *rbcL* data, and had a minor difference of just 1 bp (0.19%) in *cox*1. These sequences formed a cluster with *H. stellulifera* sequences from the Philippines that was supported by high bootstrap values in NJ *cox*1 analysis (99% – Fig. 3). In all phylogenetic trees based on *rbcL*, despite biogeographic lineages, sequences of *H. stellulifera* also were grouped in a strongly supported clade formed by specimens from Malaysia, the Philippines and Brazil (100% for MP, 98% for ML and 1.0 for PP – Fig. 4). The sequences from the Philippines differed from those of Brazilian coast by up to 38 bp (7.41%) in *cox*1 and 20 bp (2.08%) in *rbcL*.

Comparisons with sequences of *Hypnea musciformis* showed similar results. While UPA and *rbcL* analyses revealed a minor difference of only 1 bp between sequences from some locations of Brazil, *cox*1 analysis demonstrated a more defined geographic pattern: sequences from specimens of northeast Brazil (RN, PB, and BA – Table 1) differed by 8–9 bp (1.56–1.75%) from a sample collected in southeast Brazil (SP), over 3000 km away. These Brazilian sequences clustered with samples from Colombia, the USA, France, Spain and Japan with a total intraspecific variation of 27–35 bp (5.26–6.82%) in *cox*1, 16–18 bp (1.6–1.87%) in *rbcL* and 3–4 bp (0,82%–1.9%) in UPA marker.

Interspecific divergences were generally higher than intraspecific levels, ranging from 52 bp (10.14%) to 84 bp (16.37%) among *cox*1 sequences; from 26 bp (2.70%) to 72 bp (7.48%) among *rbcL* sequences; and from 4 bp (1.09%) to 16 bp (4.36%) in UPA data.

Topologies of the *rbcL* phylogenetic trees obtained from MP, ML, and Bayesian analyses are very similar, with a short difference on the relationships of *Hypnea stellulifera.* MP analysis of the data resulted in two optimal trees of 1267 steps with a consistency index of 0.5138, a retention index of 0.7632, and a rescaled consistency index of 0.3921. There were no substantial (or significant) topological differences between the two trees inferred using the parsimony method with a minor variation on the uncertain (and unsupported) position of the samples *Hypnea charoides* (EU2408441) and *H. valentiae* (FJ694933). In the ML analysis, the – ln likelihood score was estimated at 8008.260161 under the GTR + G + I model. The ML tree is presented in Fig. 4 with MP and ML bootstrap values and Bayesian posterior probabilities (PP) plotted above each clade.

In ML and Bayesian analyses *Hypnea stellulifera* was sister to the clade containing *H. cornuta*, *H. musciformis*, *H. flagelliformis* Greville ex J. Agardh, and *H. chordacea* Kützing, with strong support (94% for ML and 1.0 for PP). These taxa were clustered within a major clade (Fig. 4) containing a mix of species belonging to two sections: *Hypnea aspera* Kützing, *H. chordacea*, *H. musciformis*, *H. flagelliformis*, and *H. tenuis* in the section *Virgatae*; and *H. cornuta*, *H. flexicaulis*, and *H. stellulifera* included into section *Spinuligerae* (Agardh, 1852; Tanaka, 1941).

4. Discussion

4.1. Morphology

Features such as rigid axes (Fig. 1A), short and thick adventitious branchlets (Fig. 1D), and tetrasporangial and spermatangial sori formed in the lower and middle regions of the branchlets, commonly extending to the axes or branches (Fig. 2A and G) were described in the literature to *H. stellulifera* (Agardh, 1852; Lewmanomont, 1997 Yamagishi et al., 2003) and observed in the Brazilian and Malaysian specimens.

Lewmanomont (1997, Figs. 5–16) illustrated Agardh's voucher specimens of var. *stellulifera* and pointed out that while some specimens presented abundant stellate branchlets, another had few of these branchlets. The Malaysian material, elevated from variety to the rank of species by Yamagishi et al. (2003), is also at times devoid of these branchlets. Only some tetrasporangial specimens collected from calm regions produced numerous stellate branchlets.

Yamagishi et al. (2003) hypothesized that the production of the stellate branchlets may be limited to a certain stage of the algal life history. In any case, none of the specimens found on the Brazilian coast presented them. As pointed out by Yamagishi et al. (2003), besides the absence of stellate branchlets, the other morphological features do not vary among our specimens. An approach with periodic monitoring would be needed to clarify whether these stellate branchlets are restricted to stages of life, certain environmental conditions or if actually not occur in the Brazilian material.

4.2. Molecular analyses of data sets

Among the three markers analyzed, the plastidial UPA was the most conserved, and the mitochondrial *cox*1 the most variable. Regardless of this difference, all three markers could be used to identify taxonomic groups in *Hypnea*. The *cox*1 intraspecific variations among samples analyzed here were much higher than the empirical 0–0.7% reported previously for DNA barcoding of red algae (Saunders, 2005; Robba et al., 2006) and in Asiatic and European *Hypnea* (Geraldino et al., 2006, 2009, 2010; Wolf et al., 2011). However, recent studies have demonstrated *cox*1 intraspecific divergences ranging from 5.0% to 10% as result of the inclusion of multiple specimens from widely separated localities (Sherwood, 2008; Sherwood et al., 2008; Freshwater et al., 2010; Wiriyadamrikul et al., 2013).

Despite these high *cox*1 divergence levels, specimens analyzed in this study clustered into the *Hypnea stellulifera* clade from type locality, and shared morphological characteristics with the former species from Malaysia, which allowed us to recognize the taxa from Brazil as *Hypnea stellulifera*.

It is noteworthy that, sequences attributed to *Hypnea stellulifera* from the USA (Hawaii) available in GenBank (*cox*1: HQ422721 and UPA: HQ421577 – Sherwood et al., 2010) failed to cluster with our Brazilian and the Asiatic sequences. These sequences should be examined carefully to determine whether it has been correctly identified or, as Hawaiian red algal flora is well known to be diverse and isolated (Sherwood et al., 2010), whether these differences could be due to speciation.

UPA analysis revealed the same clades observed in analysis of *cox*1, indicating that conservation of this marker does not interfere with its potential utility as a DNA barcode, which is easier to

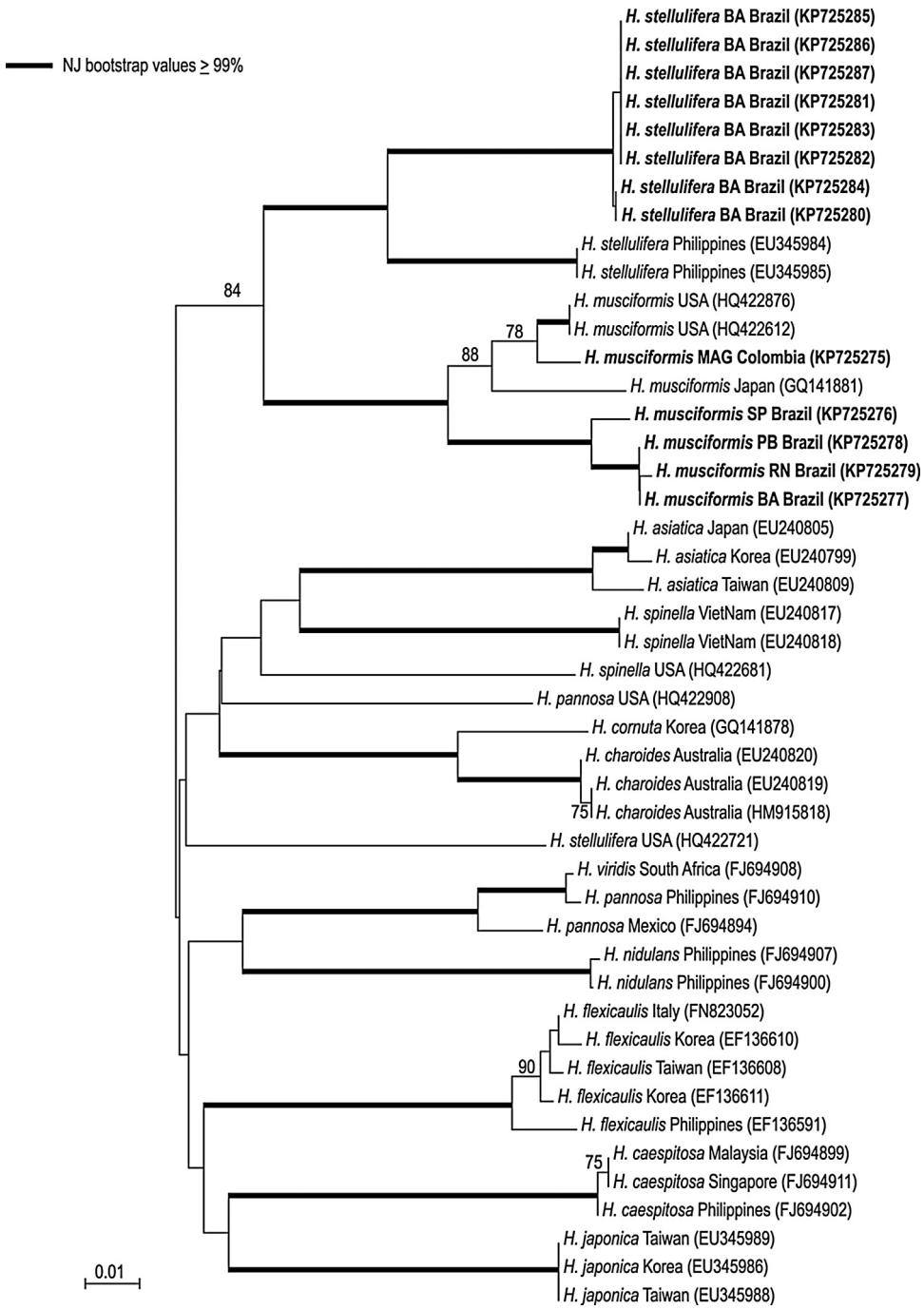

Fig. 3. Neighbor Joining (NJ) phylogram for the *cox*1 marker showing the grouping of the *Hypnea* sequenced in this study (in boldface) and available from GenBank (see Table 1 for information on each sample). Bootstrap support values for 2000 replicates ≥99% are represented by a thick line and ≤98% are indicated above each node, according to legend.

amplify and sequence compared to *cox*1 (Sherwood et al., 2008; Milsten et al., 2011; Costa et al., 2012). Likewise, the *rbcL* data corroborated the results from *cox*1 marker, reinforcing the idea that by performing broader sampling the intraspecific variation in red algae genetic sequences can be more accurately estimated.

Biogeographic separation between Brazilian specimens and those from others regions of the globe is evident both in the NJ phylogram and pairwise nucleotide differences of the *cox*1, UPA, and *rbcL* sequences, as well in phylogenetic analyses of the *rbcL* data. Our results allow us to infer these clusters may be better recognized as genetic lineages within species rather than distinct taxa. Mechanisms that lead to these high intraspecific divergences are not always explained, but some authors have raised interesting possibilities, including the presence of multiple species, cryptic species, or incipient species (Sherwood et al., 2008, 2011; Wiriyadamrikul et al., 2013).

In contrast to enormous intraspecific variation among samples collected from widely separated localities, no substantial differences were observed among *rbcL* sequences of distinct species collected from neighboring localities. It was observed when comparing *H. aspera* with *H. flexicaulis,* and also between *Hypnea asiatica* P.J.L. Geraldino, E.C. Yang and S.M. Boo and *H. charoides* J.V. Lamouroux (Figs. 3 and 4). The grouping of different species collected in nearby areas with a smaller amount of genetic diver-

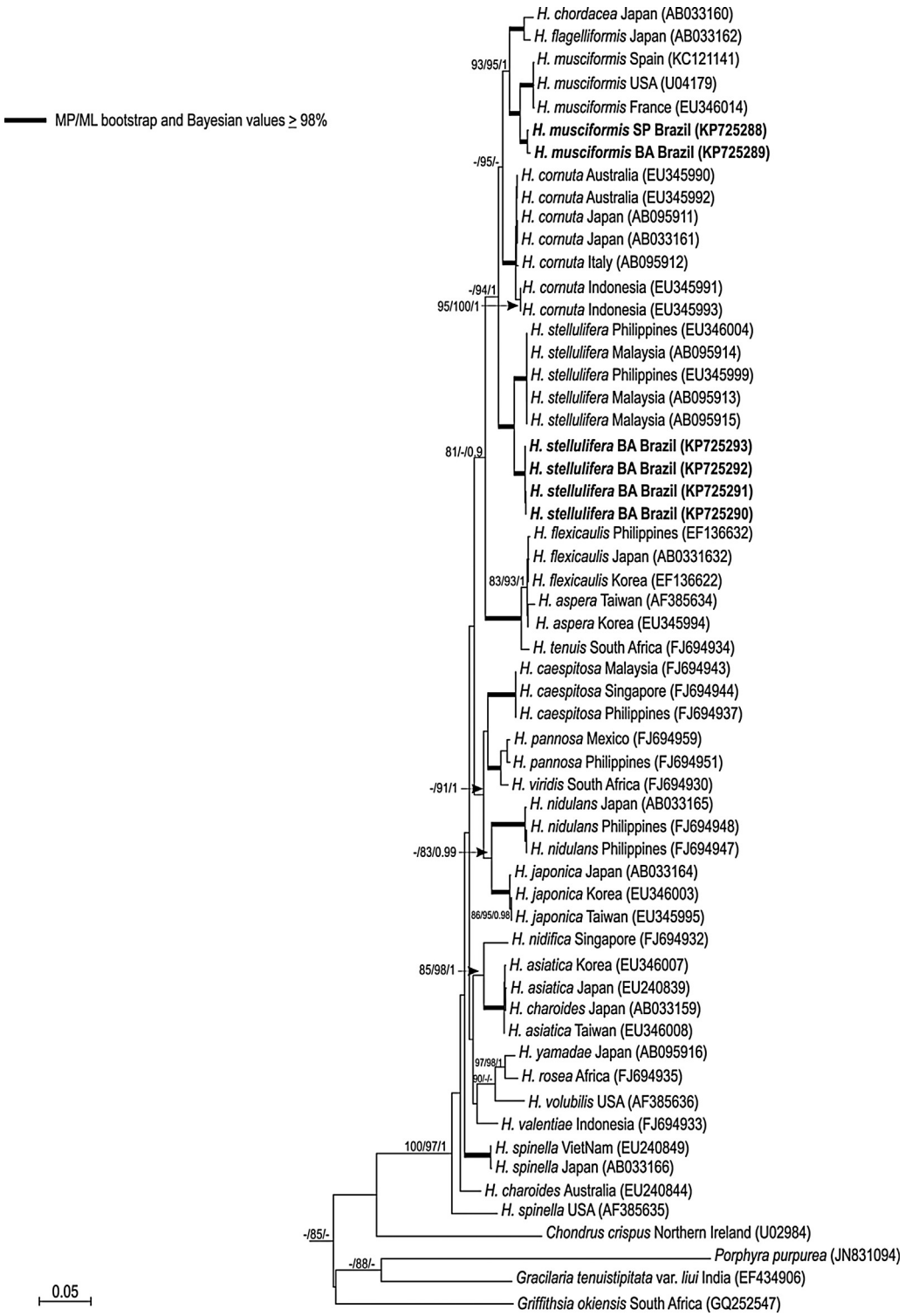

Fig. 4. ML (maximum likelihood) tree inferred from *rbc*L. Sequences of the *Hypnea* species in this study are in boldface. Values above each clade refer to MP (maximum parsimony) and ML bootstrap values and Bayesian posterior probabilities, respectively. Only bootstrap values in MP and ML ≥ 75% and Bayesian posterior probability (PP) ≥ 95% were plotted and nodes with bootstrap and Bayesian support values ≥ 98% are represented by a thick line, according to the legend.

sity raises the hypothesis that hybridization could occur in the genus, as suggested by Boo et al. (2014) for *Gelidium* J.V. Lamouroux.

In view of the great differences within the same species collected from geographically distinct localities and of the high similarities between different species from nearby locations, we believe (like Sherwood et al., 2008) that the recognition of new taxa based only on DNA sequences is not prudent. Instead, a conservative approach to taxonomic assignments should be applied until expanded global samplings coupled with morphoanatomical assessments. In this way, an over- or under-estimation of species diversity within the genus can be avoided.

In previous phylogenetic studies, *Hypnea stellulifera* and *H. cornuta* formed a clade of *Hypnea* species with stellate proliferations (Yamagishi et al., 2003; Geraldino et al., 2006, 2009, 2010), although usually supported only in Bayesian analyses. Even though it was expected that these species would present a sister relationship due to their taxonomic history, in our analyses were in a sister relationship only in the MP analysis, even if without strong support. It is likely that the incorporation of the additional Brazilian specimens of *H. stellulifera* and *H. musciformis* led to this unexpected result.

The clade containing *Hypnea aspera*, *H. chordacea*, *H. cornuta*, *H. flexicaulis*, *H. musciformis*, *H. flagelliformis*, *H. stellulifera* and *H. tenuis* would correspond to the section *Virgatae* as recognized molecularly by Geraldino et al. (2010). However, this circumscription was not the same first determined on the basis of the thallus by Agardh (1852), having as main exceptions *H. cornuta*, and *H. stellulifera*, that belong instead to the section *Spinuligerae.* Our results and those of Geraldino et al. (2010) indicate that there are divergences between morphological and molecular circumscription of the section *Virgatae*, where *H. stellulifera* appears to fit.

4.3. Biogeography of *Hypnea stellulifera*

Guiry and Guiry (2014) mention the occurrence of *H. stellulifera* in the Atlantic Ocean from the Virgin Islands (Caribbean Islands) and Guinea (Africa) according to a report by Lewmanomont (1997). However, a rigorous analysis of this work demonstrates that Lewmanomont's figures (Figs. 1–4, p. 181) were erroneously identified as being specimens of *H. cornuta* var. *stellulifera*. This confusion can be clarified on page 185 of the text where the author explains that Figs. 1–4 refer in fact to var. *cornuta* specimens from Atlantic areas, while specimens of var. *stellulifera* from Pacific and Indian Oceans are shown in figures 5–16.

In this case, *H. stellulifera* would have therefore only been reported in the Pacific and Indian Oceans (Lewmanomont 1997 Yamagishi et al., 2003), and the presence of this species in the Atlantic Ocean is thus confirmed for the first time in the present study. Until now *H. stellulifera* samples were found only in the state of Bahia, a tropical region in the northeast of the country. Despite this, it is perhaps premature to treat this taxon as exotic or invasive.

According to the analysis of Oliveira et al. (2009), the decision to include a species of seaweeds in the category of "invasive" or "non-native" is not trivial, particularly in poorly studied regions. We believe that this taxon could be categorized as a cryptogenic species (Carlton, 1996). This and other reports of red algae, once considered endemic to the Indo-Pacific, originating from recent investigations carried out on the Bahia coast (Nunes and Guimarães 2008; Nunes et al., 2011, 2014) underscore the importance of further studies in this wide yet overlooked tropical area.

5. Conclusions

This is the first record of *H. stellulifera* outside Asia. *cox*1 and UPA markers were suitable to DNA barcoding in *Hypnea*. Our results demonstrate as a broader sampling may impact our understanding of *Hypnea* taxonomy and lead us to reconsider the threshold intra- and inter-specific divergences values taken into account for routine DNA barcoding. These findings also indicate that additional studies are needed to definitively prove the occurrence of hybridization between sympatric *Hypnea* species. Infrageneric sections of *Hypnea*, originally determined on the basis of morphology alone, are not supported by the molecular data, showing that it will be necessary to include more species sampled from different geographical localities to help to elucidate the precise taxonomic status and phylogenetic relationships.

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CAPÍTULO 2

SPECIES-DELIMITATION AND PHYLOGENETIC ANALYSES OF SOME COSMOPOLITAN SPECIES OF *HYPNEA* (RHODOPHYTA) REVEAL SYNONYMS AND MISAPPLIED NAMES TO *H. CERVICORNIS*, INCLUDING A NEW SPECIES FROM BRAZIL

PRISCILA BARRETO DE JESUS, FABIO NAUER, GOIA DE MATTOS LYRA, VALÉRIA CASSANO, MARIANA CABRAL OLIVEIRA, JOSÉ MARCOS DE CASTRO NUNES & ALESSANDRA SELBACH SCHNADELBACH

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SPECIES-DELIMITATION AND PHYLOGENETIC ANALYSES OF SOME COSMOPOLITAN SPECIES OF *HYPNEA* (RHODOPHYTA) REVEAL SYNONYMS AND MISAPPLIED NAMES TO *H. CERVICORNIS*, INCLUDING A NEW SPECIES FROM BRAZIL[1]

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Hypnea **has an intricate nomenclatural history due to a wide pantropical distribution and considerable morphological variation. Recent molecular studies have provided further clarification on the systematics of the genus; however, species of uncertain affinities remain due to flawed taxonomic identification. Detailed analyses coupled with literature review indicated a strong relationship among *H. aspera*, *H. cervicornis*, *H. flexicaulis*, and *H. tenuis*, suggesting a need for further taxonomic studies. Here, we analyzed sequences from two molecular markers (COI-5P and *rbc*L) and performed several DNA-based delimitation methods (mBGD, ABGD, SPN, PTP and GMYC). These molecular approaches were contrasted with morphological and phylogenetic evidence from type specimens and/or topotype collections of related species under a conservative approach. Our results demonstrate that *H. aspera* and *H. flexicaulis* represent heterotypic synonyms of *H. cervicornis* and indicate the existence of a misidentified *Hypnea* species, widely distributed on the Brazilian coast, described here as a new species: *H. brasiliensis*. Finally, inconsistencies observed among our results based on six different species delimitation methods evidence the need for adequate sampling and marker choice for different methods.**

Key index words: **Cystocloniaceae; *Hypnea aspera*; *Hypnea flexicaulis*; *Hypnea tenuis*; single-marker delimitation methods; synonymy; systematics; taxonomy**

***Abbreviations*: ABGD, Automated Barcode Gap Discovery; BOLD, Barcode of Life Database Systems; BP, Bootstrap percentage; COI-5P, the standard DNA barcode region of the mitochondrial cytochrome c oxidase 1 gene (*cox*1); dNTP, triphosphate deoxyribonucleotide; GMYC, generalized mixed Yule-coalescent method; mBGD, Manual Barcode Gap Discovery; ML, maximum likelihood; PP, Bayesian Posterior probability; PSH, primary species hypothesis; PTP, Poisson tree processes; *rbc*L, gene encoding the large subunit of RuBisCo (ribulose**

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bisphosphate carboxylase oxygenase); SPN, Statistical Parsimony Network; SSH, secondary species hypothesis; SSU, small subunit

The red algal family Cystocloniaceae includes 13 genera (seven monotypic) (Guiry and Guiry 2016), most of which are well known for their cell-wall polysaccharides (Chiovitti et al. 1998). The most speciose genus in the family is *Hypnea* J.V. Lamouroux, with 58 valid species (Guiry and Guiry 2016). Despite this, ~116 infrageneric taxa have already been described hitherto, illustrating the scenario of taxonomic confusion in the delimitation of *Hypnea* species. According to Price et al. (1992), this taxonomically challenging genus has an intricate nomenclatural history. Mshigeni and Chapman (1994) further suggested that there are numerous *Hypnea* species passing under more than one name. Additionally, morphological plasticity in the genus is still poorly understood. Therefore, it requires monographic treatment on a global scale (Price et al. 1992) for critical reassessment of species delimitation using extensive preserved collections, including those from the type localities (Masuda et al. 1997).

Several studies based on morphological data have contributed to our understanding of *Hypnea* diversity around the world (Tanaka 1941, Cordeiro-Marino 1977, Mshigeni 1978, Schenkman 1986, Mshigeni and Chapman 1994, Masuda et al. 1997, Abbott 1997, 1999, Dawes and Mathieson 2008, Jesus et al. 2013, 2015, and others). Recent molecular articles have provided further clarification of the systematics of the genus (e.g., Yamagishi and Masuda 2000, Yamagishi et al. 2003, Geraldino et al. 2006, 2009, 2010, Nauer et al. 2014, 2015, Jesus et al. 2015). However, species of uncertain affinities remain (Geraldino et al. 2009), due to flawed taxonomic identification (Geraldino et al. 2010).

J. Agardh (1851) first described *Hypnea cervicornis* based on samples collected from Brazil, West Indies, and Mexico. Later, Haroun and Prud'Homme van Reine (1993) reduced this species to a synonym of *H. spinella* (C. Agardh) Kützing by proposing that these taxa were ecologically induced growth forms of the same species in Macaronesia, northeastern Atlantic Ocean. Even after synonymy, the debate whether to retain *H. spinella* and *H. cervicornis* as distinct species continued (Wynne 1995, 1998, 2005, Chiang 1997, Xia and Wang 1997, Yamagishi and Masuda 1997, 2000, Abbott 1999, Geraldino et al. 2006, Dawes and Mathieson 2008).

Under this scenario of uncertainties, Yamagishi and Masuda (2000) designated a new species occurring in Japan, *Hypnea flexicaulis* Yamagishi et Masuda, morphologically identical to the species known as *H. cervicornis* in Japan (Yamagishi and Masuda 2000). However, as the latter species had been reduced to a synonym of *H. spinella*, the authors justified the description of a new species based on its morphological and molecular (*rbc*L) differences from *H. spinella* (Yamagishi and Masuda 2000). It is noteworthy that morphological features used in the circumscription of *H. flexicaulis* were very similar to the ones used in the delimitation of *H. cervicornis*. However, *rbc*L sequences from specimens identified as *H. cervicornis* were not available in public databases before that study. These findings raised doubts about the taxonomic status of *H. flexicaulis* as a valid species.

Geraldino et al. (2006, 2009) generated further data that complicated the taxonomic status of *H. flexicaulis*. Geraldino et al. (2006) compared an *rbc*L sequence determined by Hommersand and Fredericq (2003) as *H. boergesenii* T. Tanaka from Taiwan (= *H. aspera* Kützing; see Millar and Prud'homme van Reine 2005) with sequences of *H. flexicaulis*. The authors observed that sequences of *H. boergesenii* and *H. flexicaulis* were molecularly closely related. Later, Geraldino et al. (2009) included more sequences of both species in their analyses and a strong relationship was consistently observed, indicating the need for further study on their taxonomy.

Here, we used meticulous morphological and anatomical observations together with molecular analyses from type specimens and/or topotype collections of *Hypnea aspera*, *H. cervicornis,* and *H. flexicaulis* to test the conspecificity of these taxa. In addition, we included sequences of *H. spinella* and *H. tenuis* Kylin previously published in GenBank to expand our knowledge of the phylogenetic relationships and distribution of these overlapping taxa. We analyzed sequences from two molecular markers (COI-5P and *rbc*L) and used several DNA-based delimitation methods to delineate primary species hypothesis (PSHs). We contrasted those PSHs with morphological and phylogenetic evidence, and they served as basis for our taxonomic decisions. Our findings provide new insight into species circumscription within the genus. They also indicate the existence of a species new to science, widely distributed on the Brazilian coast, described here as *Hypnea brasiliensis* P.B. Jesus, Nauer & J.M.C. Nunes.

MATERIALS AND METHODS

Collections and morphological observations. Specimens analyzed in this study were sampled in several locations along the Brazilian coast, Panama, and the Maldives, including representative samples of *Hypnea cervicornis* collected in the type locality (Table S1 in the Supporting Information). Samples were collected during low tides and transported live back to the laboratory, cleaned, and sorted carefully in a stereomicroscope (Leica® Zoom 2000; Leitz Park, Wetzlar, Germany). Observations of ecological conditions and growth habit were noted at the time of collection. Three to five pieces of thalli were preserved in silica gel desiccant for DNA extraction. Material for morphological observations was preserved in seawater containing 4% formaldehyde.

Histological sections were made by hand with the aid of a razor blade, and reproductive structures were stained with 5% aniline blue solution. Identifications were made in a stereomicroscope and a photonic microscope (Olympus® CBA; Shinjuku Tokyo, Japan) with an ocular micrometer. Photomicrographs of structures were obtained with the aid of an image capture program (QCapture Pro; Media Cybernetics Inc., Rockville, Maryland, USA) and a digital camera (QImaging GO-3; QImaging Corporation, Surrey, British Columbia, Canada) attached to the photomicroscope (Olympus trinocular CX31RTS5®; Tokyo).

All voucher specimens were deposited in the herbaria Alexandre Leal Costa (ALCB) of the Universidade Federal da Bahia and at the Universidade de São Paulo (SPF), Brazil. We analyzed paratype specimens of *H. flexicaulis* borrowed from the Herbarium of the Graduate School of Science, Hokkaido University (SAP). We also observed photographs of the type material of *H. aspera* from the National Herbarium Nederland in Leiden (L), *H. cervicornis,* and *H. spinella* from the Botanical Museum Herbarium, Lund, Sweden (LD), and a sample studied by Hommersand and Fredericq (2003) identified as "*Hypnea boergesenii.*"

DNA extraction, PCR reaction, and sequencing. Total DNA was extracted from samples weighing ~20–40 mg by maceration in liquid nitrogen using a modified version of the Cetyl Trimethyl Ammonium Bromide procedure of Doyle and Doyle (1987) or using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), following the kit protocol. Molecular markers were PCR amplified under the following conditions: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 5 ng of genomic DNA, and 1.25 U of Taq DNA polymerase (Invitrogen, São Paulo, Brazil) in a total volume of 25 μL. For the amplification and sequencing reactions for each marker, specific primer pairs were used: for COI-5P, GazF1 and GazR1 (Saunders 2005); for *rbc*L, FrbcL and RrbcS (Freshwater et al. 1994) and R753 and F492 (Freshwater and Rueness 1994). PCR was performed with an initial denaturation step at 94°C for 10 min, followed by 35 cycles of 30 s at 90°C, 30 s at 50°C (47°C for *rbc*L), and 2 min at 72°C, with a final 10-min extension cycle at 72°C. The reactions were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems®, Foster City, CA, USA). Purifications and sequencing reactions of the PCR products were performed by the company GeneWiz (Cambridge, MA, USA, http://www.genewiz.com/).

Molecular analyses. Electropherograms were manually assembled using the Staden package (Staden et al. 2003) and edited in BioEdit 5.0.6 (Hall 1999). For each marker, a multiple alignment was generated with the ClustalW tool (Thompson et al. 1994), available in BioEdit 5.0.6 (alignments excluded PCR primer sequences). In addition to sequences generated in this study, sequences from other *Hypnea* species were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/, searched on October 2015 – Table S2 in the Supporting Information) and included in the analyses. All new consensus sequences of COI-5P and *rbc*L were deposited in the Barcode of Life Data Systems (http://www.boldsystems.org/) and GenBank (Table S1).

In order to resolve this nebulous and long-lasting problem, and to make rationale taxonomic decisions on the species complex comprising *Hypnea aspera*, *H. cervicornis*, *H. flexicaulis*, *H. spinella,* and *H. tenuis*, we applied DNA-based delimitation methods, using separate data sets of COI-5P and *rbc*L: (i) manual Barcode Gap Discovery (mBGD) described by Freshwater et al. (2010); (ii) Automated Barcode Gap Discovery (ABGD) method of Puillandre et al. (2012a); (iii) Statistical Parsimony Network (SPN) analysis developed by Templeton et al. (1992); (iv) the Poisson tree process model (PTP) of Zhang et al. (2013), with a maximum likelihood (ML) implementation; and the general mixed Yule-coalescent model introduced by Pons et al. (2006) that can be run using (v) single and (vi) multiple delimiting thresholds. A ML analysis, as described below, was performed using a concatenated alignment (COI-5P + *rbc*L) to summarize the results of all single-marker species-delimitation methods using *Griffithsia okiensis* Kajimura (*cox*1: EU194973/*rbc*L: GQ252547) as outgroup.

To assess the level of variation in data sets, estimates of divergence values within and among species were computed using MEGA 6.0 (Tamura et al. 2013). mBGD was calculated by dividing the minimum interspecific sequence divergence by the maximum intraspecific sequence divergence (Freshwater et al. 2010). A barcode gap is present when this calculated value is >1, while values equal to and smaller than 1 indicate overlap of genetic diversity (Tables 1 and 2). The ABGD proposes the grouping of the input sequences into several hypothetical species automatically with the sole use of pair-wise distances (Puillandre et al. 2012a). The method was implemented using the Web interface available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. A fasta alignment was used as input file using ABGD default parameters (pmin = 0.001, pmax = 0.10, number of bins = 20), except the relative gap width (X) was set to 10 to avoid the capture of smaller local gaps (as made by Puillandre et al. 2012b and Pardo et al. 2014). Both, JC69 (Jukes and Cantor 1969) and K2P (Kimura 1980) genetic distances were tested.

A SPN analysis was performed using the TCS version 1.21 software (Clement et al. 2000). TCS program calculates the minimal number of mutational steps by which the sequences of the same species can be joined with >95% confidence. In this analysis, different species are displayed as unconnected sequences or networks due to their greater evolutionary distinctness as measured by mutations. PTP is a newly developed method that can delimit species by means of nonultrametric phylogenies by using branch lengths to estimate the mean expected number of substitutions per site between two branching events (Zhang et al. 2013). ML tree files (obtained in the same way as for the phylogenetic analyses described below) were used as input files in the implementation of PTP analyses. The Web server of the Exelixis Lab (http://species.h-its.org/

TABLE 1. Levels of intraspecific genetic variation (in percentage, diagonal in gray), interspecific variation (in percentage, under the diagonal), and DNA barcode gaps (over the diagonal) for COI-5P DNA sequences, based on uncorrected p-distances between taxa in the *Hypnea cervicornis* complex and their respective sister species. Intraspecific variation values are presented in bold. (), number of sequences/species analyzed; –, absent values or barcoding gaps <1.

	"*H. aspera*"	"*H. flexicaulis*"	*H. cervicornis*	*H. tenuis*	*H. brasiliensis* sp. nov.	*H. spinella*
"*H. aspera*" (01)	–	–	–	–	5×	20×
"*H. flexicaulis*" (22)	0–1.9	**0–1.9**	–	3×	5×	6×
H. cervicornis (39)	0.6–3.0	1.0–4.0	**0–3.4**	2×	4×	3×
H. tenuis (01)	5.8	5.4–6.0	5.3–6.5	–	6×	22×
H. brasiliensis sp. nov. (104)	14.0–14.4	13.6–14.9	13.6–14.6	14.9–15.7	**0–2.6**	5×
H. spinella (08)	11.8–12.0	11.4–12.7	11.2–12.5	13.1–13.3	12.5–14.0	**0–0.6**

TABLE 2. Levels of intraspecific genetic variation (in percentage, diagonal in gray), interspecific variation (in percentage, under the diagonal), and DNA barcode gaps (over the diagonal) for *rbc*L DNA sequences, based on uncorrected p-distances between taxa in the *Hypnea cervicornis* complex and their respective sister species. Intraspecific variation values are presented in bold. (), number of sequences/species analyzed; −, absent values or barcoding gaps <1.

	"*H. aspera*"	"*H. flexicaulis*"	*H. cervicornis*	*H. tenuis*	*H. brasiliensis* sp. nov.	*H. spinella*
"*H. aspera*" (04)	**0–0.5**	–	–	2×	9×	12×
"*H. flexicaulis*" (22)	0.1–0.7	**0–0.2**	–	6×	9×	30×
H. cervicornis (09)	0.2–1.4	0–0.9	**0–1.5**	–	4×	4×
H. tenuis (01)	1.1–1.7	1.2–1.3	1.2–1.9	–	9×	60×
H. brasiliensis sp. nov. (12)	6.2–7.1	6.0–6.6	5.9–7.2	6.0–6.3	**0–0.7**	5×
H. spinella (05)	6.0–6.7	6.0–6.2	5.8–6.8	6.0–6.1	3.6–4.2	**0–0.1**

ptp) was used to run the analyses with default settings, except by using a five-digit random seed number.

Finally, the GMYC method combines models of stochastic lineage growth (Yule models) with coalescence theory to determine the point of transition from species-level (speciation and extinction) to population-level (coalescence) evolutionary processes on a time-calibrated ultrametric tree by maximizing the likelihood score of the model (Pons et al. 2006, Zhang et al. 2013). For the GMYC analysis, we removed nonunique haplotypes from the alignments using Geneious® 6.1.6 (Drummond et al. 2011). UPGMA (Unweighted Pair Group Method with Arithmetic mean; Sokal and Michener 1958) trees were generated through a Bayesian analysis implemented in BEAST v1.8.1. (Drummond et al. 2012). BEAST input files were generated using BEAUti v1.8.1 (Drummond et al. 2012) with the best–fitting model (GTR+I+G) identified by MrModeltest 2.3 (Nylander 2008), under an "uncorrelated lognormal relaxed molecular clock," a "coalescent" tree prior (see Monaghan et al. 2009), and each data set was run for 20,000,000 generations sampling every 1,000 steps. All of the other options were kept as the BEAUti default settings. The burn-in of each run was determined using Tracer v1.6 (Rambaut et al. 2013), and TreeAnnotator v1.8.1 was used to summarize all trees after burn-in to give a maximum clade credibility tree with target node heights. Both, the single-threshold and the multiple-threshold versions of the GMYC model were implemented on Web server of the Exelixis Lab (http://species.h-its.org/gmyc/).

All single-marker species delineation methods were compared with phylogenetic results, detailed morphological and anatomical analyses of several specimens, including a broad survey of the historical literature. This multiple-faceted approach allowed us to turn PSHs into conclusive secondary species hypotheses (SSHs) and draw taxonomic conclusions.

Maximum parsimony (MP), ML, and Bayesian analysis, based on single-marker COI-5P and *rbc*L datasets, were performed to infer the phylogenetic relationships of determined SSHs. Outgroups were *Calliblepharis ciliata* (Hudson) Kützing (*rbc*L: AF385653), *Chondrus crispus* Stackhouse (COI-5P: KJ960551/*rbcL*: U02984), *Gracilaria tenuistipitata* var. *liui* Zhang and Xia (*cox*1: EF434924), and *Griffithsia okiensis* (*cox*1: EU194973/*rbc*L: GQ252547). MP trees were constructed with PAUP* 4.0b.10 (Swofford 2002) using a heuristic search algorithm with the following settings: starting trees obtained by stepwise addition, using tree bisection–reconnection branch swapping algorithm; MulTrees, all characters unordered and with equal weight, accelerated transformation as character-state optimization; branches with a maximum length of zero collapsed to yield polytomies. A strict consensus analysis was computed, and bootstrap values for the resulting nodes were assessed using 1,000 bootstrapping replicates with simple sequence addition. The consistency (CI), retention (RI), and rescaled consistency (RC) indices resulting from the MP analysis were calculated.

The best models were assessed under MrModeltest 2.3 (Nylander 2008) and selected using the Akaike Information Criterion, as recommended by Posada and Buckley (2004). The ML analysis was conducted using Randomized Axelerated Maximum Likelihood (version 7.0.4; Stamatakis 2006) with the GTR GAMMA model. The best-scoring ML tree and 500 bootstrap trees were obtained using the rapid hill-climbing algorithm (Stamatakis et al. 2008).

Bayesian analyses were conducted with MrBayes 3.1 software (Ronquist and Huelsenbeck 2003) using the Markov chain Monte Carlo method with the selected general-time-reversible model of nucleotide substitution with invariant sites and gamma distributed rates for the variable sites (GTR+I+G). Seven million generations in two independent runs were performed with four chains (one hot and three cold), sampling one tree every 100 generations starting with a random tree. Each estimation parameter, obtained from two runs, was checked in Tracer v1.6 to ascertain whether the stationary state had been reached. Trees from the first 10% of generations were discarded as burn-in. The remaining trees were combined to build a 50% majority-rule consensus tree. Clades were considered supported with Bayesian posterior probability (PP) ≥95%, or nonparametric bootstrap support ≥75% in MP and ML. This threshold is higher than the commonly used 70% BP cutoff, which is often associated with 95% confidence that the clade is real (Hillis and Bull 1993, Xi et al. 2013). We chose a conservative approach to avoid erroneously considering subclades within a species as separate species.

RESULTS

Single-marker delimitation methods and phylogenetic analyses. We obtained 93 partial sequences (79 COI-5P and 14 *rbc*L) that were combined with 219 sequences downloaded from GenBank (138 COI-5P and 81 *rbc*L). Our COI-5P final alignment comprised 217 sequences of 465 base pairs (bp), with 205 (44%) variable, and 163 (35%) parsimoniously informative characters. Ninety-five *rbc*L sequences were aligned using 1,355 bp, with 842 (62%) variable, and 325 (23%) parsimoniously informative characters. Tables 1 and 2 show levels of intra- and interspecific genetic variation, and DNA barcode gaps among samples of *Hypnea* species used in this study for COI-5P and *rbc*L, respectively.

We used six species-delimitation methods (mBGD, ABGD, SPN, PTP, and GMYC single and GMYC multiple), based on COI-5P and *rbc*L data sets, for circumscribing the taxa previously identified as *Hypnea aspera*, *H. cervicornis*, *H. flexicaulis*, *H. spinella,* and

H. tenuis. The topology of a ML, tree from a concatenated two-markers dataset (COI-5P + *rbc*L) is shown in Figure 1, summarizing all results of species-delimitation methods. There was no incongruence among the main groups, which were recognized as three well-resolved (100% of bootstrap value) and distinct clades: (i) a *H. cervicornis* clade: samples we identified as *H. cervicornis* from Brazil and the Maldives formed a large cluster with public sequences named as *H. aspera*, *H. flexicaulis*, and *H. tenuis* from Asia and Africa; (ii) a *H. spinella* clade: specimens from Brazil and Barbados (Caribbean) and (iii) a Brazilian clade with newly generated DNA sequences from previously misidentified specimens.

Although the number of PSHs varied according to the approach and marker, all delineation methods results for the *Hypnea spinella* clade recovered specimens from Brazil and the Barbados (type locality) as a single entity, attesting the strong affinity between Caribbean and Brazilian flora observed by Horta et al. (2001) and corroborated by Nauer et al. (2014).

On the clade formed exclusively of Brazilian samples, results from COI-5P and *rbc*L were highly congruent, most supporting a single PSH (five of six methods in each marker). This clade contained specimens, which Jesus et al. (2013) identified as *Hypnea spinella* based on morphology, and Nauer et al. (2014) identified as *H. cervicornis*, based on

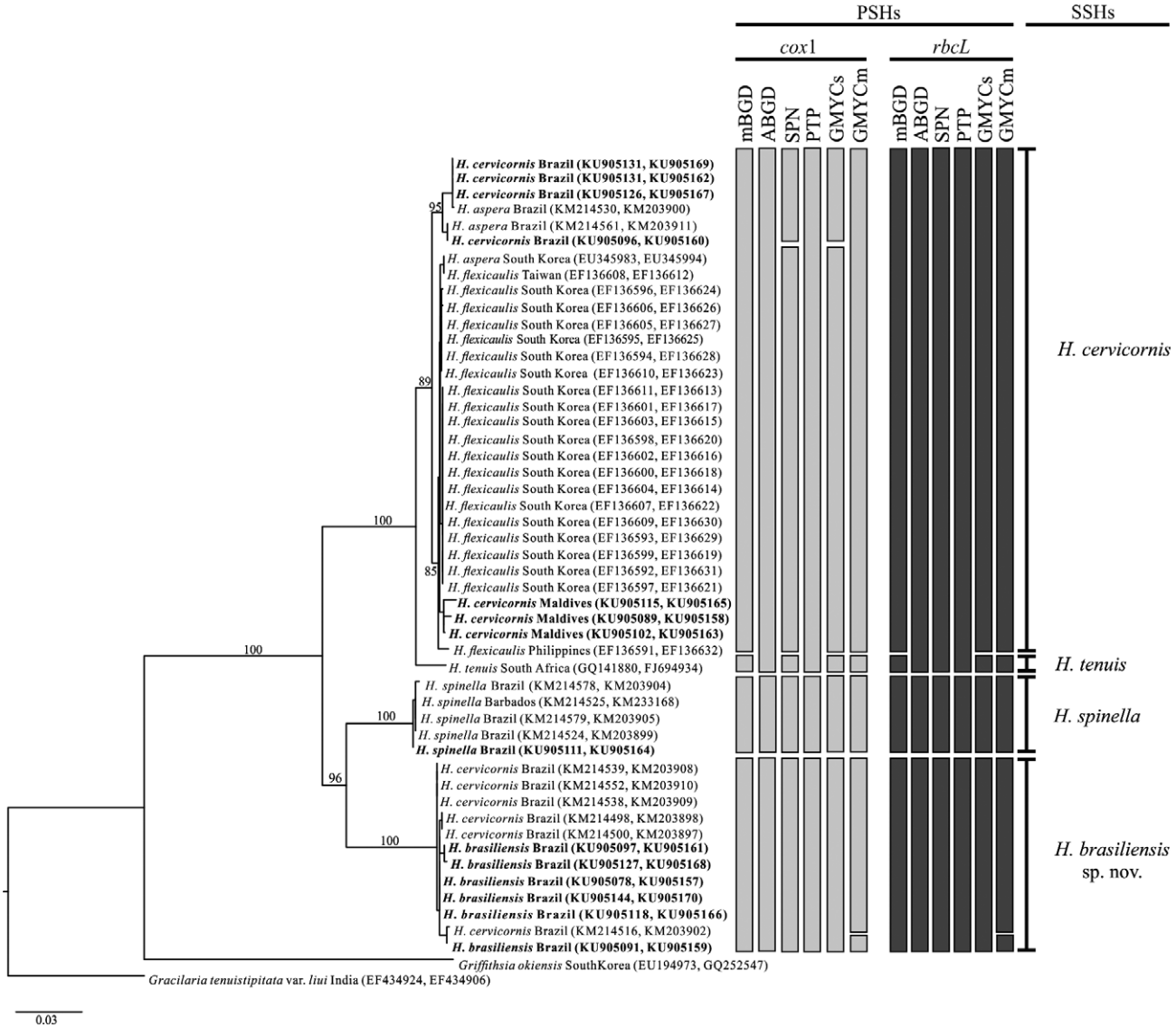

FIG. 1. The optimal maximum likelihood (ML) topology inferred from the combined data set of COI-5P and *rbc*L bringing all results of single-marker species-delimitation methods. Support values at each node are bootstrap values converted to percentages. >75%. New sequences produced in this study are in boldface, others are from GenBank. Vertical thick lines indicate PSHs delineated with mBGD, ABGD, SPN, PTP, GMYCs, and GMYCm methods based on COI-5P (light gray) and *rbc*L (dark gray). At the right edge of the tree are shown the conclusive SSHs obtained from congruent results of delimitation methods.

molecular analyses. However, evidence from several DNA-based delimitation methods (Fig. 1) and phylogeny (Figs. 2 and 3) led us to review the literature and reconsider the morphological features used in the circumscription of these taxa. Our results suggested that specimens in this clade were primarily misidentified and are, in fact, a new species to science, described below as *H. brasiliensis* sp. nov.

When attempting to resolve species delineation within the *Hypnea cervicornis* clade, COI-5P and *rbc*L data sets produced variable results. Some methods had results that are more inclusive, grouping the four putative species into a single PSH. ABGD results from different markers were similar, despite the selected model (JC69 or K2P), but the number of PSHs varied according to the maximum prior distance (*P*) used. Although extreme values lead to uninformative partitions, intermediate prior thresholds from COI-5P ($P = 0.0046$ to 0.01) and *rbc*L ($P = 0.0046$) yielded only one PSH. Species delineation using the PTP based on relative branch lengths of the ML tree also produced the same result for the two data sets, although poorly supported. SPN results from *rbc*L also supported the existence of one PSH, but with high haplotype diversity (15 haplotypes).

Methods such as mBGD and GMYC multiple-threshold (from COI-5P and *rbc*L) recovered two putative species in the *Hypnea cervicornis* clade, splitting *H. tenuis* from other species. Using mBGD, the barcoding gap between the two clusters was 3× and 6× in COI-5P and *rbc*L, respectively. GMYC single threshold also delimited two PSHs, but only based on the *rbc*L data set. GMYC single threshold from COI-5P data (like SPN results) splits *H. cervicornis* clade into three distinct biogeographic groups (Brazil, Asia, and Africa). For both versions of the method for *rbc*L analysis, the GMYC model ($\log L_{\text{GMYC single}} = 121.5305$ and $\log L_{\text{GMYC multiple}} = 121.5669$) was not favored over the null model ($\log L_0 = 120.2241$, $P\text{-value}_{\text{GMYC single}} = 0.271$, $P\text{-value}_{\text{GMYC multiple}} = 0.261$). The same result was observed in the single threshold from COI-5P data ($\log L_{\text{GMYC single}} = 138.2067$, $\log L_0 = 135.7898$, $P = 0.0892$), but in the multiple-threshold version, the likelihood of the GMYC model ($\log L_{\text{GMYC multiple}} = 138.8236$) was significantly higher than that of the null model, although with a $P = 0.0481$. These results indicated that there is no significant evidence for the predicted shift in branching rates from interspecific to intraspecific events (Pons et al. 2006, Leliaert et al. 2009).

For phylogenetic reconstructions (Figs. 2 and 3), COI-5P and *rbc*L sequences from *Hypnea* species available on GenBank were used, together with new sequences generated in this study. Topologies of trees obtained from MP, ML, and Bayesian analyses were very similar and the genus proved to be monophyletic with high support in relation to the outgroup.

MP analysis of COI-5P data resulted in 12 optimal trees of 764 steps with a consistency index of 0.4346, a retention index of 0.9339, and a RC index of 0.4058. The *rbc*L data resulted in six optimal trees of 1,261 steps with a consistency index of 0.5059, a retention index of 0.8538, and a RC index of 0.4320. There were no well-supported topological differences between the trees inferred using the parsimony method with a minor variation on the position of the samples within the same species. In the ML analyses, the final − ln likelihood scores were estimated at 4,053.164103 from COI-5P and 8,315.065429 from *rbc*L under the GTR + G + I model. ML trees from COI-5P and *rbc*L are presented in Figures 2 and 3, respectively, with MP and ML bootstrap values and PP plotted above each clade, respectively.

All phylogenetic analyses revealed the clades *H. brasiliensis* sp. nov., *H. cervicornis,* and *H. spinella* as distinct and well-supported among the remaining species of the genus. *H. brasiliensis* formed, in most analyses, a well-supported sister clade (COI-5P: 91% for ML and 0.9 for PP/*rbc*L*:* 83% for MP, 99% for ML, and 1.0 for PP) with *H. asiatica* P.J.L. Geraldino, E.C. Yang, and S.M. Boo and *H. nidifica* J. Agardh, although showing high divergence ranges (10.78%–12.50% of divergence in COI-5P and 2.96%–3.84% in *rbc*L). *H. spinella* samples from Brazil and the Caribbean (type locality) were grouped with high support in most analyses (COI-5P: 99% for ML and 1.0 for PP/*rbc*L: 100% for MP and ML and 1.0 for PP). The *H. cervicornis* clade (COI-5P: 80% for MP, 97% for ML, and 1.0 for PP/*rbc*L: 100% for MP and ML and 1.0 for PP) included closely related specimens previously identified as *H. aspera*, *H. cervicornis*, *H. flexicaulis,* and *H. tenuis* (Tables 1 and 2 with divergences values). In the *rbc*L analyses, these taxa were clustered within a major clade containing *H. chordacea* Kützing, *H. cornuta* (Kützing) J. Agardh, *H. musciformis* (Wulfen) J.V. Lamouroux, *H. flagelliformis* Greville ex J. Agardh, and *H. stellulifera* (J. Agardh) Yamagishi et Masuda (84% for MP, 96% for ML, and 1.0 PP). *H. brasiliensis* and *H. spinella* were retrieved within a large cluster belonging to the section Spinuligerae, while the *H. cervicornis* clade was at the base of the section Virgatae. Despite clusters within species have been consistent and well-supported for COI-5P, phylogenetic relationships between species resulted in some incongruences and weakly supported groups (Fig. 2).

Morphological data.

Hypnea brasiliensis P.B. Jesus, Nauer & J.M.C. Nunes **sp. nov.** (Fig. 4, A–K)

Diagnosis: *H. brasiliensis* is similar to *H. spinella* by a prostrate habit, being mainly epilithic, and in the number and shape of the periaxial cells; it can be distinguished from this species by a soft texture and by branchlets that are smaller and narrower than branches and principal axes.

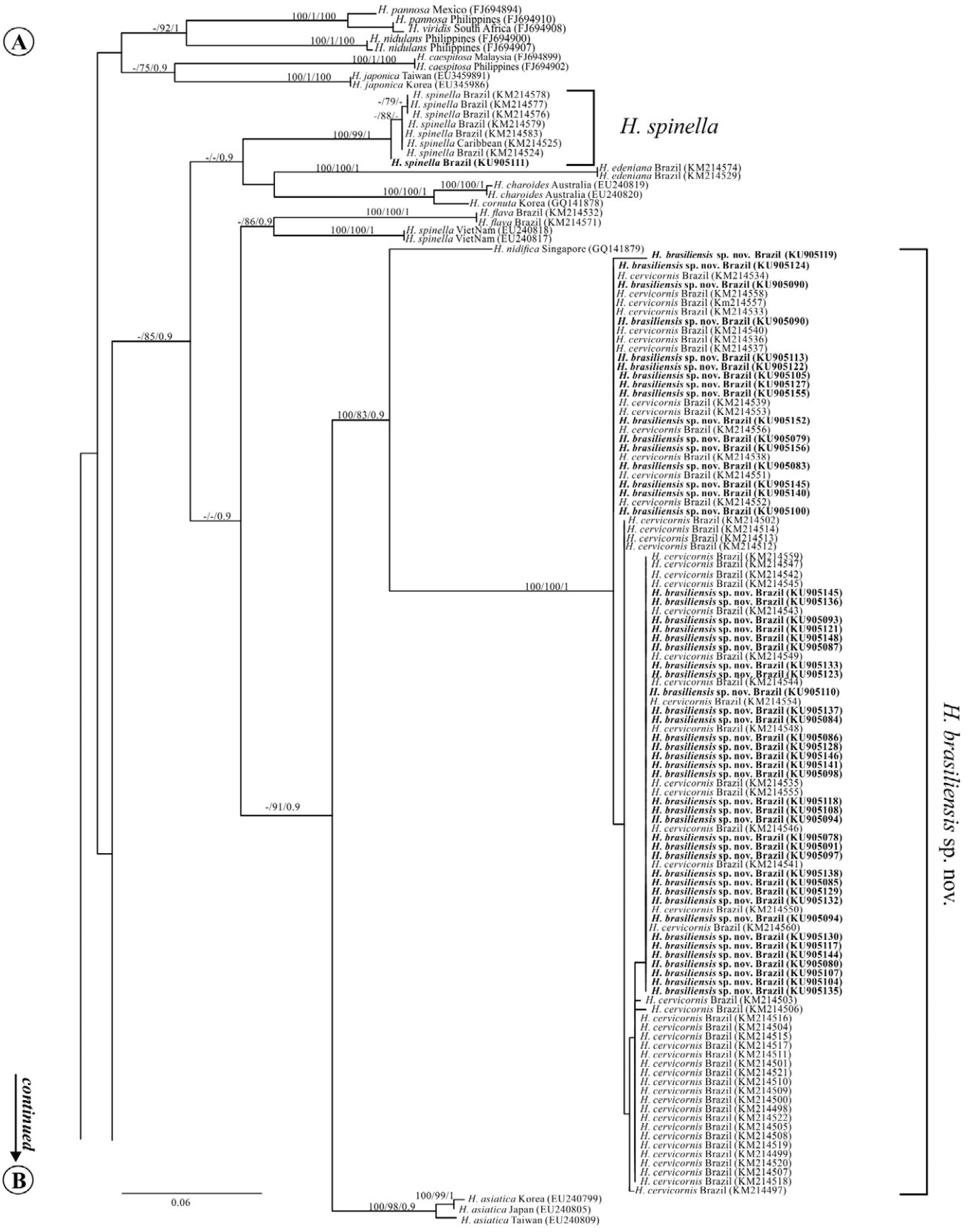

FIG. 2. The optimal maximum likelihood (ML) topology based on the COI-5P data set (ntax = 217). Values above each clade refer to MP (maximum parsimony) and ML bootstrap values converted to percentages and Bayesian posterior probabilities, respectively. Only bootstrap values in MP and ML ≥75% and Bayesian posterior probability (PP) ≥90% were plotted. New sequences produced in this study are in boldface.

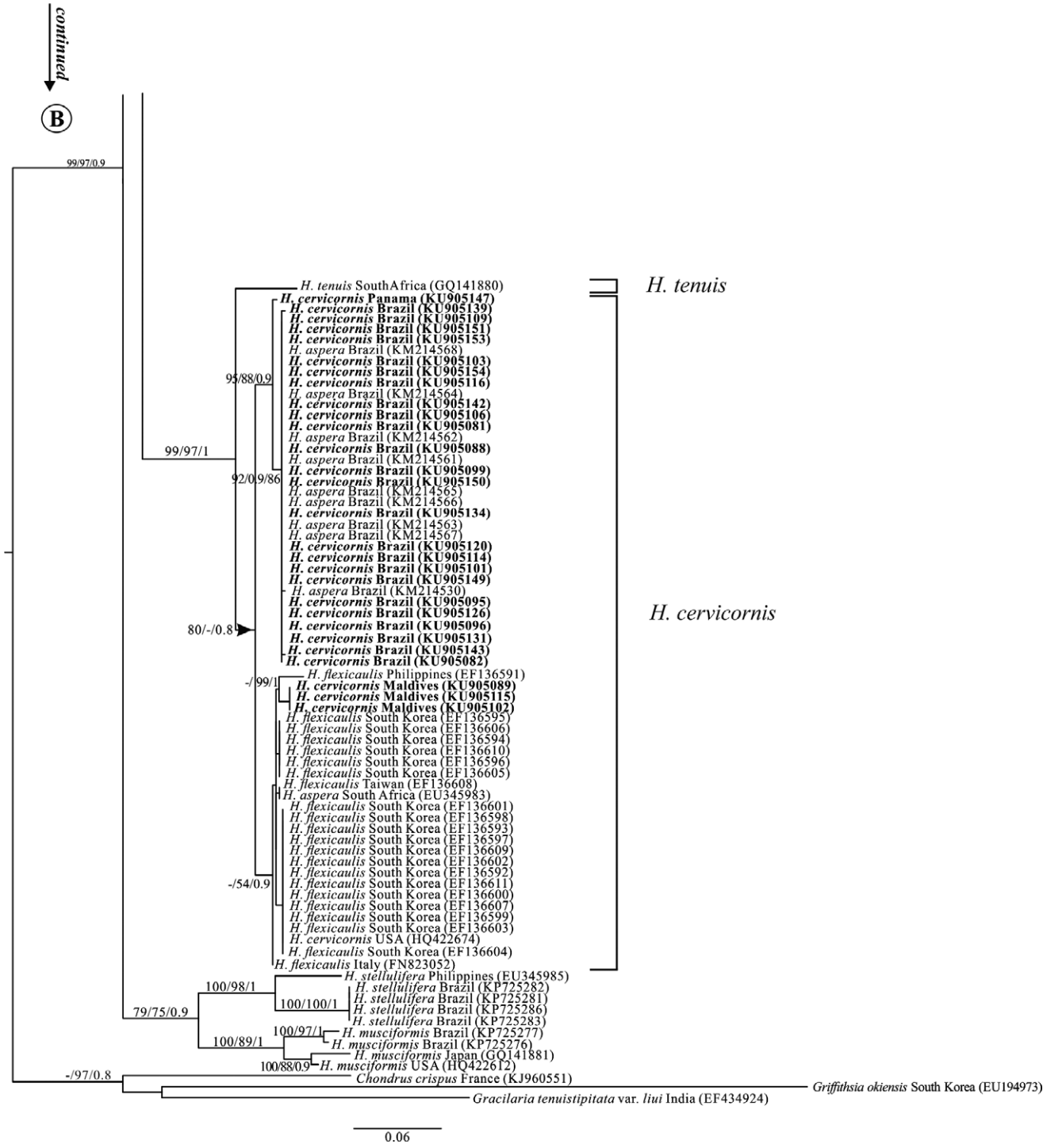

FIG. 2. Continued.

Misapplied names: *Hypnea cervicornis* sensu Nauer et al. (2014) (pp. 562–563, figs. 23-28); *H. spinella* sensu Guimarães (2006) (pp. 158, fig. 10) and Jesus et al. (2013) (pp. 13–14, fig. 12, A–K).

Holotype: Brazil, Bahia State, Porto Seguro, Arraial D'Ajuda, Mucugê beach; 16°29′ 51.67″ S, 39°04′ 10.47″ W; coll. Jesus et al., 06 November 2010; ALCB 100234; Field ID: P14; GenBank accessions: COI-5P – KU905078/*rbc*L – KU905157; vegetative.

Etymology: The epithet refers to the country where this widely distributed species was so far only found.

Distribution: Brazil: States of Ceará, Paraíba, Pernambuco, Alagoas, Bahia, Espírito Santo, Rio de Janeiro, São Paulo, Santa Catarina.

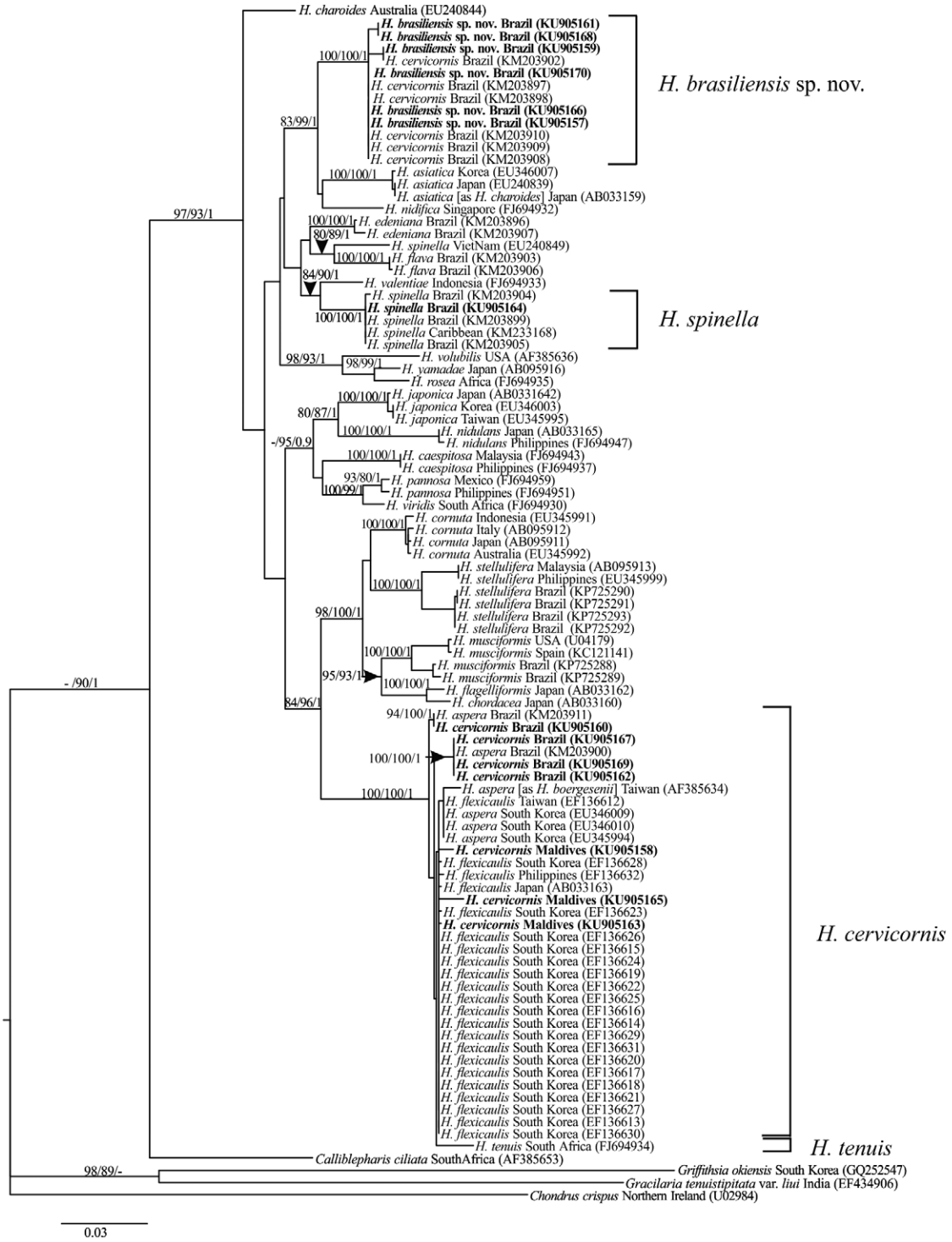

FIG. 3. The optimal maximum likelihood (ML) topology based on the *rbc*L data set (ntax = 95). Values above each clade refer to MP (maximum parsimony) and ML bootstrap values converted to percentages and Bayesian posterior probabilities, respectively. Only bootstrap values in MP and ML ≥75% and Bayesian posterior probability (PP) ≥90% were plotted. New sequences produced in this study are in boldface.

Thalli ranging from prostrate to erect, epilithic or epiphytic, reddish-pink, yellowish to brownish when alive, forming tangled loose tufts (Fig. 4, A and B). Thalli delicate with soft to cartilaginous texture, measuring 1–12 cm in height, attached to the substratum by a primary discoid holdfast and with many secondary holdfasts formed on all portions of branches and branchlets (Fig. 4C). Main axis percurrent (Fig. 4D), terete (350–714 μm in diameter at middle portions), irregularly, alternately or

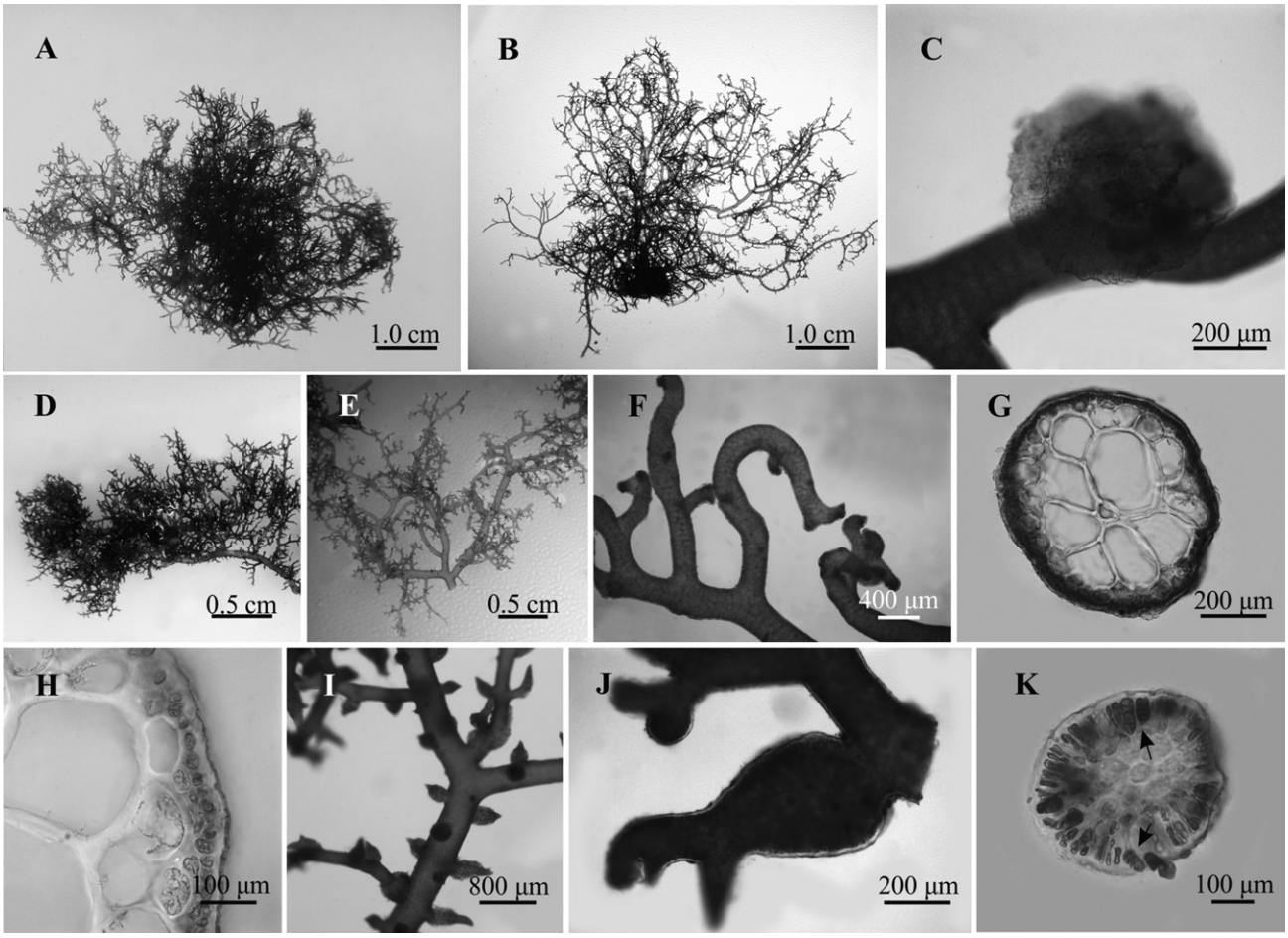

FIG. 4. *Hypnea brasiliensis* sp. nov. (A, B) Habits of tetrasporangial fronds. (C) Front view of a fixation disk. (D, E) Portions of the thalli showing the percurrent main axis, branches, and branchlets. (F) Bent apical portion of the branchlets. (G) Cross-section on the median region of a branch. (H) Detail of cortical cells in cross-section. (I) Tetrasporangial branchlets disposed throughout the thalli. (J) Detail of a tetrasporangial sorus in the basal region of a forked branchlet. (K) Cross-section of a branchlet containing tetrasporangia (arrows) in the cortical region.

dichotomously branched, at right angles in the basal portion and acute near apex. First-order branches 1–4.5 cm long, with equal diameter or slightly more slender than the main axes, loosely intertwined and slightly bent in different directions. Frequent anastomoses between branches. Spine-like branchlets of size (160–1,300 µm) and diameter (90–170 µm) were much smaller than the branches and principal axis, irregularly scattered throughout the thalli (Fig. 4E). Apices of the branches and branchlets acute, straight, bent (Fig. 4F), and sometimes forked, ending in a distinct apical cell. In cross-section, the middle portion of axes show a circular and pigmented axial cell, much smaller than the periaxial cells (36–45 µm in diameter). The axial cell is surrounded by 4–6 periaxial cells, oval to triangular, 74–236 µm in diameter; 1–2 layers of hyaline medullary cells (37–114 µm in diameter) gradually smaller toward the periphery (Fig. 4G), and 1–2 layers of pigmented cortical cells, 5–24 µm in diameter (Fig. 4H). Lenticular thickenings usually present in periaxial and medullary cells at basal and middle portions of the thallus. Tetrasporangial branchlets throughout the thallus (Fig. 4I), with tetrasporangia in sori surrounding the basal swollen portions (248–520 µm in diameter) of simple or forked branchlets (Fig. 4J), zonately divided, 69–136 µm long and 41–99 µm in diameter (Fig. 4K). Gametophytes not found.

Habitat: Pools or areas exposed to waves in the lower intertidal to subtidal zones on rocks or epiphytic on other algae.

Representative specimens examined: BRAZIL, CEARÁ: Caucaia, Pacheco, 3°41′ 09.18″ S, 38°37′ 57.19″ W; coll. P.B. Jesus et al., 26 March 2013; Field ID: P125 (ALCB 110343), BAHIA: Mar Grande, Ilha de Itaparica, 12°58′ 00.70″ S, 38°36′ 30.79″ W; coll. P.B. Jesus et al., 04 December 2010; Field ID: P28 (ALCB 100223); Serra Grande Beach, Uruçuca, 14°28′ 39″ S, 39°01′ 48″ W; coll. P.B. Jesus et al., 18 April 2011; Field ID: P53 (ALCB 103119); SÃO PAULO: Dura Beach, Ubatuba, 23°29′ 37.70″

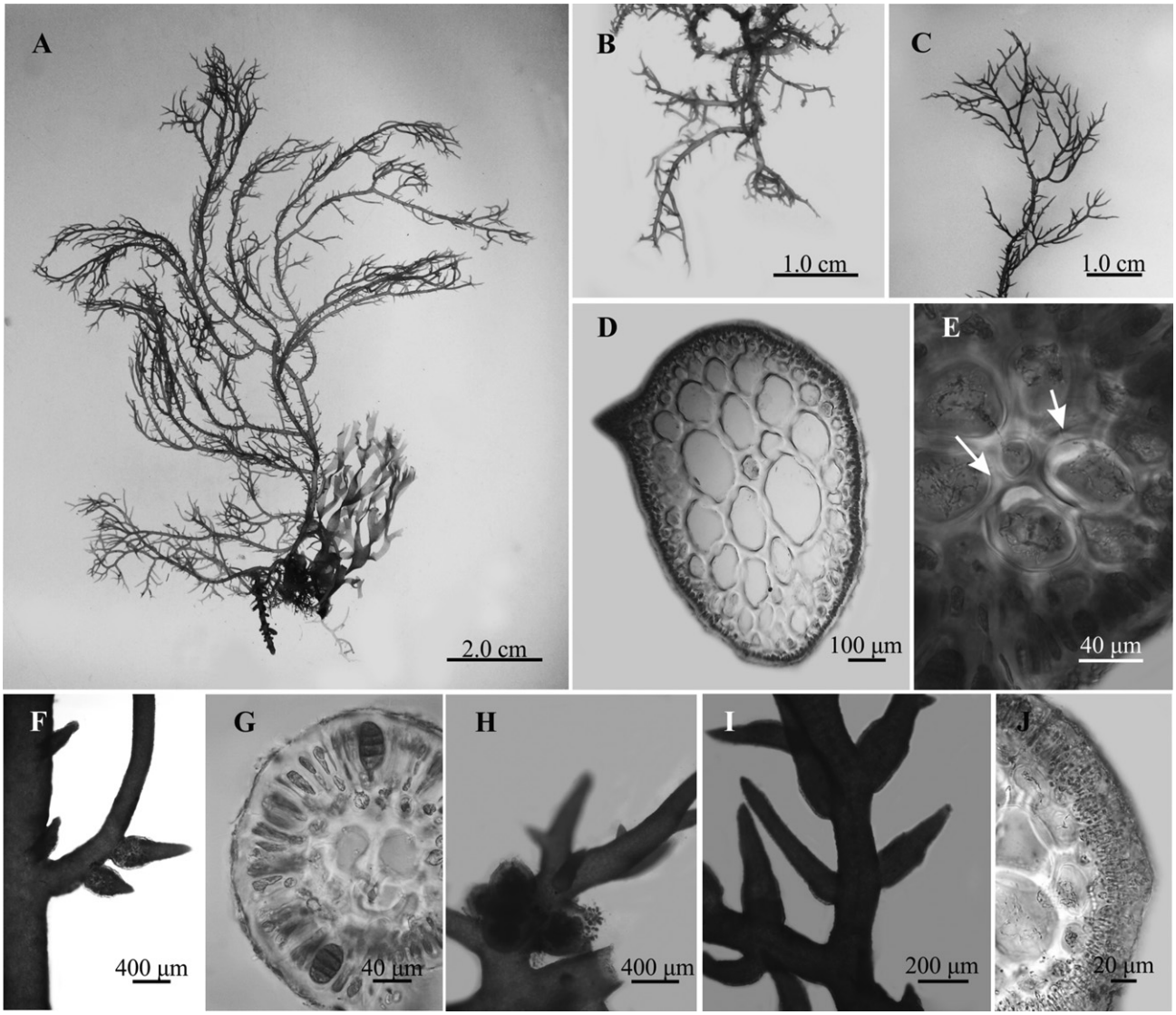

FIG. 5. *Hypnea cervicornis*. (A) Habit of an epiphytic tetrasporangial frond. (B) Basal portion of the thallus showing decumbent branches. (C) Apical portion of the forked branches and branchlets. (D) Cross-section in the median region of the thallus. (E) Lenticular thickenings in periaxial cells (arrows). (F) Tetrasporangial branchlets disposed throughout the thalli. (G) Cross-section of a branchlet containing tetrasporangia in the cortical region. (H) Aggregated cystocarps produced on branches. (I) Spermatangial sori formed in the lower regions of the branchlets. (J) Cross-section of a branchlet containing spermatangial sori arranged on cortical region.

S, 45°9′ 53.41″ W; coll. P.B. Jesus et al., 18 August 2012; Field ID: P110 (ALCB 110264); SANTA CATARINA: Sepultura Beach, Bombinhas, 27°08′ 29.75″ S, 48°28′ 40.22″ W, coll. F. Nauer et al., 28 February 2012, Field ID: IBC0806 (SPF 57511). To additional material analyzed, see Tables S1 and S3 in the Supporting Information.

Hypnea cervicornis J. Agardh (1851, p. 451–452) (Figs. 5, A–J and 6, A–I)

Homotypic synonym: *Hypnophycus cervicornis* (J. Agardh) Kuntze (1891: 900).

Heterotypic synonyms: *Hypnea musciformis* var. *pumila* Harvey (1834: 154); *H. aspera* Kützing (1868: 14); *H. boergesenii* Tanaka (1941: 233–235), *H. flexicaulis* Yamagishi and Masuda (2000: 28).

Lectotype: Botanical Museum Herbarium, Lund, Sweden (LD 33878!, *Sphaerococcus spinellus*; Agardh 1822: 323 var. *laxior*; Haroun and Prud'Homme van Reine 1993: 122).

Type locality: Bahia State, Brazil "*in litore maris prope Bahian.*"

Syntype localities: Brazil, West Indies, Mexico, Mauritius? (Agardh 1851: 451–452, doubtful occurrence for Mauritius)

Thalli prostrate or more frequently erect, epilithic or epiphytic, yellow, rosy to greenish or brownish when alive (Fig. 5A). Thalli delicate with soft to cartilaginous texture, measuring 4–25 cm in height, attached to the substratum by a primary discoid holdfast or by a dense entangled base of creeping

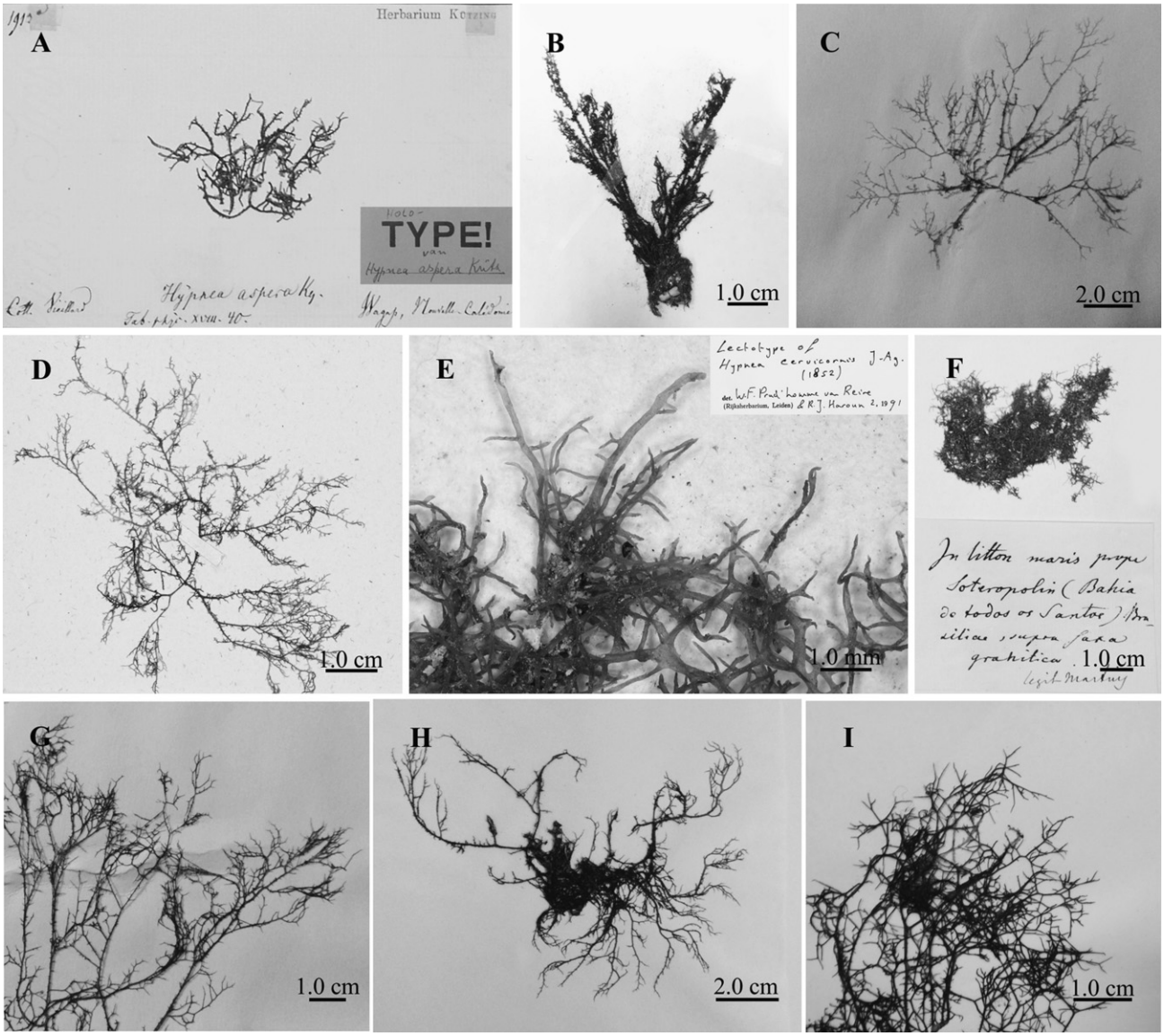

Fig. 6. Voucher specimens analyzed in this study and their morphological variation. (A) Holotype of "*H. aspera*" from New Caledonia (L 941!). (B) Sample from Taiwan collected and identified as "*H. boergesenii*" by Hommersand and Fredericq (2003) (Voucher unspecified). (C) Habit of a *H. cervicornis* sample collected from Brazil (Bahia, Itaparica Island, Penha – ALCB 100218). (D) Detail of apical portion of a syntype of *H. cervicornis* (LD 33876!). (E) Lectotype of *H. cervicornis* (LD 33878!). (F) Syntype of *H. cervicornis* (LD 33872!) with record of location of collection. (G) Apical portion in detail of a *H. cervicornis* sample collected from Brazil (Bahia, Itaparica Island, Mar Grande – ALCB 100219). (H) Paratype of "*H. flexicaulis*" from Japan (SAP 71799!). (I) Detail of apical portion of a paratype of "*H. flexicaulis*" (SAP 71826!).

to decumbent branches (Fig. 5B) with many secondary holdfasts accessories. One or multiple main axes percurrent, terete (529–1,114 µm in diameter at middle portions). Dichotomous to subdichotomous branching, at right angles in the basal portion and acute near apex. First-order branches arising 1 cm above the base, 2–8.5 cm long, with equal diameter or slightly more slender than the main axes. Rare anastomoses between branches. Spine-like branchlets irregularly scattered at basal and middle portions of the thalli and alternately to distichous near to apex, turned upwards, 200–1,328 µm long and 120–160 µm in diameter. Branch apices and branchlets straight, forked (Fig. 5C), ending in a distinct apical cell. Hyaline hairs throughout the thalli. In cross-section, the middle portion of axes showing a circular and pigmented axial cell, much smaller than periaxial ones (27–142 µm in diameter; Fig. 5D). The axial cell is surrounded by 4–6 periaxial cells, oval to triangular, generally of equal size (175–321 µm in diameter) or having only one of similar in size to the axial cell (52–64 µm in diameter). One to two layers of hyaline medullary cells (69–254 µm in diameter) variable in size, and 1–2 layers of pigmented cortical cells, 5–11 µm in diameter. Lenticular thickenings usually present in

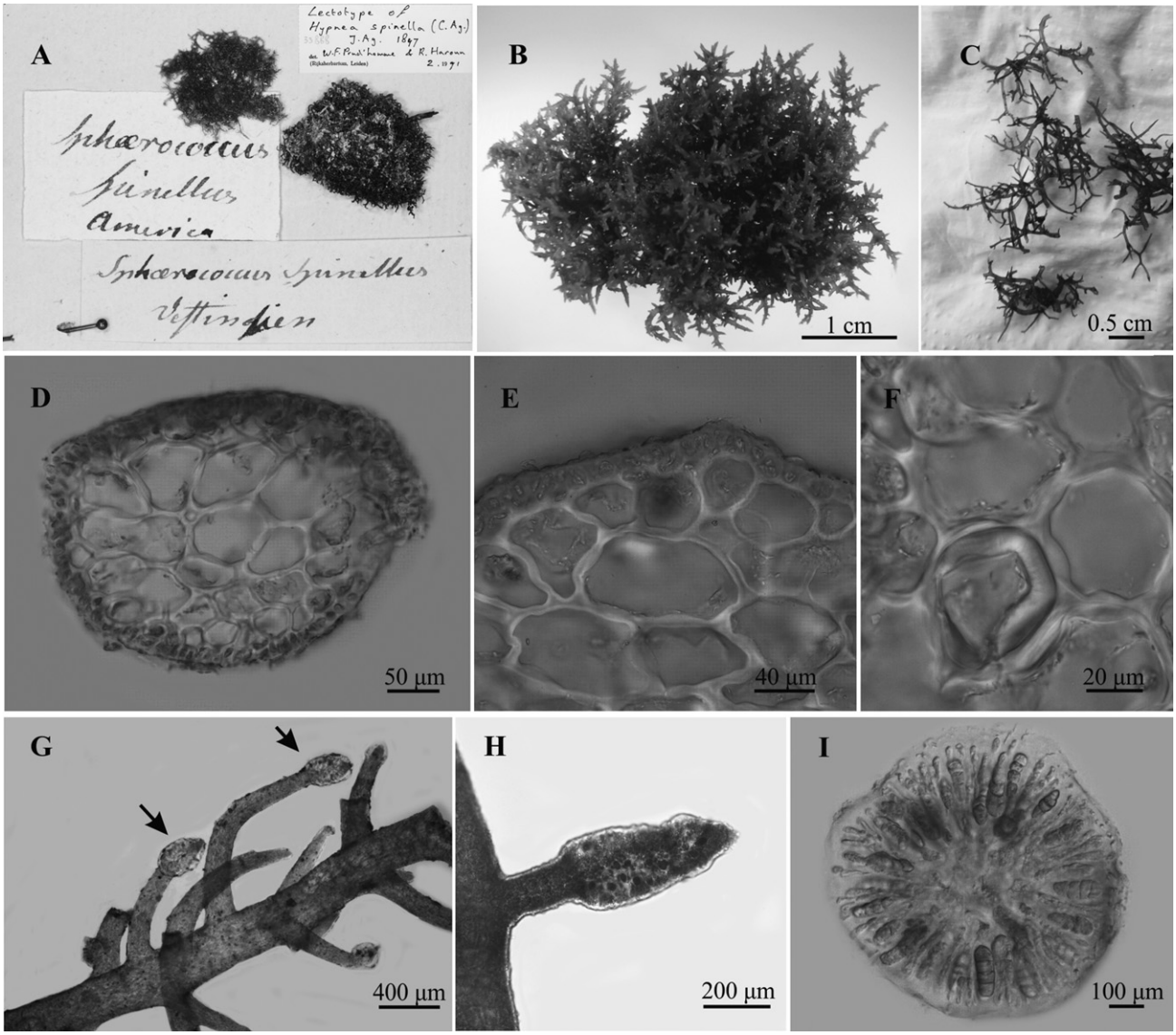

FIG. 7. *Hypnea spinella*. (A) Lectotype (LD 33888!). (B) Habit. (C) Branches and branchlets. (D) Cross-section of a branch. (E) Detail of cortical and medullary cells in cross-section. (F) Lenticular thickenings in a medullary cell. (G) Tetrasporangial sori in the apical regions of the branchlets. (H) Detail of a tetrasporangial sorus. (I) Cross-section of a branchlet containing tetrasporangia in the cortical region at different stages of maturation.

periaxial and medullary cells in all portions of the thallus (Fig. 5E). Tetrasporangial sori formed in the lower (Fig. 5F) and middle swollen portions of the branchlets, 414–1,519 µm in diameter, bearing zonate tetrasporangia 128–173 µm in length and 53–73 µm in diameter (Fig. 5G). Cystocarps single or aggregated (Fig. 5H), globose, produced from the base to the apex of the axis and branches (495–771 µm in length and 672–1,187 µm in diameter). Carposporangia ovate, 18.5–34 µm in diameter, arranged in clusters at the apex of the gonimoblast filaments. Spermatangial sori formed in the lower and middle swollen portions of the branchlets (692–1,153 µm; Fig. 5I). Spermatangia 1.9–4.5 µm in diameter, 3–6 in number, arranged in a concatenated manner in the cortical region (Fig. 5J).

Habitat: Epilithic in the intertidal zone, in sheltered bay or epiphytic on other algae.

Representative specimens examined: BRAZIL, BAHIA: "*in litore maris prope Bahiam*," coll. Martius (LD 33878! – Lectotype); Mar Grande, Ilha de Itaparica, 12°58′ 00.70″ S, 38°36′ 30.79″ W; coll. P.B. Jesus et al., 04 December 2010; Field ID: P30 (ALCB 100219). MÉXICO: "*In littus mexicanum*," Vera Cruz, coll. Liebmann (LD 33869! 33881! – Syntypes). JAPAN, Kamo, Yamagata Prefecture, coll. Y. Yamagishi, 11 September 1994 (SAP 071826! – as *H. flexicaulis*). NEW CALEDONIA, Wagap, coll. Vieillard,

1913 (L941 – as *H. aspera*). To additional material analyzed, see Tables S1 and S3.

Hypnea spinella (C. Agardh) Kützing (1847: 23) (Fig. 7, A–I)

Basionym: *Sphaerococcus spinellus* Agardh (1822: 323).

Homotypic synonym: *Gigartina spinella* (C. Agardh) Greville (1830: lviii).

Lectotype: Botanical Museum Herbarium, Lund, Sweden (LD 33888)!, *Sphaerococcus spinellus* (Haroun and Prud'Homme van Reine 1993: 122).

Type locality: West Indies.

Thalli prostrate, epilithic, greenish, pale-red to dark-red when alive, forming dense entangled cushion-like tufts (Fig. 7, A and B) with creeping branches. Thalli cartilaginous in texture, 2–10.5 cm long and 2–4 cm in height, attached to the substratum by several discoid holdfasts formed throughout the thallus. Main axes indistinguishable (Fig. 7C), terete (330–600 μm in diameter at middle portions), irregularly or alternately branched, at right angles in the basal portion and acute near apex. First-order branches slightly more slender than the main axis, 180–400 μm in diameter. The thalli are interwoven by many anastomoses, which may bend in all directions. Spine-like branchlets straight, at acute or right angles, irregular to alternately scattered throughout the thalli, 130–440 μm long and 87–155 μm in diameter. Apices of the branches and branchlets acute, straight, rarely forked, ending in a sharp apical cell. In cross-section, the middle portion of axes show a circular and pigmented axial cell, much smaller than periaxial ones (30–82 μm in diameter; Fig. 7D). The axial cell is surrounded by 4–6 periaxial cells, rounded to elliptical, 55–132 μm in diameter; 1–2 layers of hyaline medullary cells and 1–2 layers of pigmented cortical cells, 5–12 μm in diameter (Fig. 7E). Lenticular thickenings usually present in axial and periaxial cells at basal and middle portions of the thallus (Fig. 7F). Tetrasporangial branchlets disposed throughout the thalli, mainly in their upper third part. Tetrasporangia in sori surrounding the basal, median and apical swollen portions (190–357 μm in diameter) of branchlets (Fig. 7, G and H), zonately divided, 30–40 μm long and 12–25 μm in diameter (Fig. 7I). Gametophytes not found.

Habitat: Thalli growing in sites exposed to the impact of waves or subtidal zone, often associated with Corallinaceae algae, difficult to separate without breaking.

Representative specimens examined: BRAZIL, BAHIA: Penha, Ilha de Itaparica; 12°59′ 08.76″ S, 38°37′ 02.80″ W; coll. P.B. Jesus et al., 05 December 2010; Field ID: P17 (ALCB 100232); SÃO PAULO: Vermelha do Sul Beach, Ubatuba, 13 May 2008, coll. E.C. Oliveira (SPF 56879); RIO DE JANEIRO: Forno Beach, Arraial do Cabo, 28 September 2011, coll. F. Nauer, C. Iha and B. Torrano-Silva (SPF 57423); ESPÍRITO SANTO: Enseada dos Padres, Meaípe, 09 May 2012, coll. F. Nauer (SPF 57542). CARIBBEAN: "*Vettindien*," coll. Liebmann (LD 33888! – Lectotype). To additional material analyzed, see Tables S1 and S3.

DISCUSSION

Comparison of species-delimitation methods. In this study, we used several DNA-based species-delimitation methods on a group of taxonomically challenging *Hypnea* species. According to Carstens et al. (2013), the appropriate way to conduct an investigation on species delimitation is to analyze the data with a wide variety of methods and to delimit lineages that are consistent across the results. In addition to the DNA-based delimitation methods, morphological and phylogenetic evidence coupled with the nomenclatural history of all taxa was taken into account to turn PSHs in conclusive SSHs. A conservative approach was followed to avoid an overestimation of the species diversity and the creation of unwarranted new species names (Puillandre et al. 2012b, Jesus et al. 2015).

Overall, species-delimitation methods recovered the same partitions in the *H. brasiliensis* and *H. spinella* clades, with major inconsistencies observed only in the *H. cervicornis* clade. This clade comprised several specimens collected in Brazil, including Bahia, its type locality (Agardh 1851). It also included COI-5P and *rbc*L published sequences from taxa that have been extensively sampled by Geraldino et al. (2006, 2009, 2010) and Nauer et al. (2014). As this group was the most geographically widely sampled (America, Asia, and Africa) covering a wide genetic variation, incongruences were expected.

Even though they are based on the same principles (differences between intra- and interspecific divergences), ABGD and mBGD differed in their results. The mBGD depends directly on the sampling, and recent studies on the genus (Geraldino et al. 2006, 2009, 2010, Nauer et al. 2014, Jesus et al. 2015) have shown that a larger geographical scale will increase its genetic divergence and smaller the barcoding gap will become. Despite this, the results were congruent for both COI-5P and *rbc*L markers allowing satisfactory species circumscriptions. The ABGD requires a maximum prior intraspecific distance to choose among the different partitions, to ignore unrealistic hypotheses, and to determine PSHs (Puillandre et al. 2012a). Similar to these authors, we observed that the number of species determined by ABGD was more consistent with PSHs in primary partitions ($P = 0.1$ to COI-5P and $P = 0.004$ to *rbc*L). ABGD is often reported as a lumper approach, predicting fewer groups than other methods, such as GMYC (Paz and Crawford 2012, Puillandre et al. 2012a,b), which was also detected in this study.

The PTP model assumes that each substitution has a small probability of generating a speciation

event; hence, the number of substitutions between species is expected to be significantly higher than those within species (Zhang et al. 2013, Dumas et al. 2015). In our analyses, like ABGD, the PTP method lumped more than one morphological group into a single PSH, although poorly supported. These results are in accordance with those Kozak et al. (2015) reported, whereas Zhang et al. (2013) and Modica et al. (2014) considered that PTP may overestimate the number of species when taxon sampling is uneven between species.

The multiple threshold of GMYC model resulted in the largest number of PSHs delineated among all methods. Numerous studies have reported that the GMYC method tends to overestimate the number of species (Esselstyn et al. 2012, Paz and Crawford 2012, Puillandre et al. 2012a,b, Talavera et al. 2013, Modica et al. 2014, Pardo et al. 2014, Tang et al. 2014). Zhang et al. (2013) pointed out that single-threshold GMYC is usually more accurate than the multiple-threshold version. Both versions of the method showed that GMYC was not favored over the null model (except for multiple-threshold version from COI-5P data, $P = 0.0481$). Pons et al. (2006) listed several explanations in rejecting the null model: (i) the clade might in fact represent a single species; (ii) the power to detect the transition was weakened by incomplete sampling (singletons, like *Hypnea tenuis*'s data); and (iii) combination of actual branching processes like fast speciation rate and large population sizes will also make it harder to detect the transition.

Results from SPN included cases of both lumping and splitting in *Hypnea cervicornis* clade, which seems to be more related to the marker than to the approach. By using the COI-5P and *rbc*L data set, our analyses resulted in three and one single PSHs, respectively. These findings supported those of Muangmai et al. (2014), which suggest that incongruent results obtained from different species-delimitation methods may be influenced by the different mutation rates of the COI-5P and *rbc*L markers.

Taxonomic consequences. Our results revealed the need for significant taxonomic changes in a historically intriguing group within *Hypnea* as follows: (i) revised species delineations, (ii) new synonyms, and (iii) the description of a new species long misidentified and widely distributed along the Brazilian coast.

This study provides the first strong evidence that *Hypnea aspera* (as *H. boergesenii*), *H. cervicornis* and *H. flexicaulis* should be treated as a single species: *H. cervicornis* J. Agardh. Jesus et al. (2015) interpreted the low genetic divergence between *H. aspera* and *H. flexicaulis* as potential hybridization occurring within the genus. However, our analyses reveal that these taxa should not be treated as separate species. *H. aspera* was originally described by Kützing (1868) based on samples collected by Vieillard 1913 from New Caledonia and characterized by the presence of many fine proliferous lateral branchlets along the main axes. *H. boergesenii* was described by Tanaka (1941) as a new species from Taiwan also by possessing densely lateral branchlets throughout the frond. By analyzing fragments of *H. aspera*'s holotype (L 941, 97_186/barcode L 0055947), Millar and Prud'homme van Reine (2005) suggested that *H. boergesenii*, described later and widely reported, should be a synonym of *H. aspera*.

Yamagishi and Masuda (2000) described *H. flexicaulis* as a new species from Japan, characterizing it by a flexuous percurrent main axes and antler-like branches with wide branching angles showing an abrupt abaxial bending. This description was made after Haroun and Prud'Homme van Reine (1993) reduced *H. cervicornis* as synonym of *H. spinella*. It probably led Yamagishi and Masuda (2000) to limit the comparison of the new species only with morphological traits of *H. spinella*. These authors made available the first *rbc*L sequences of *H. flexicaulis*, a species that Geraldino et al. (2006, 2009, 2010, 2015) extensively studied. In 2003, Hommersand and Fredericq deposited the first *rbc*L sequence of "*Hypnea boergesenii*" from Taiwan (AF385634). Geraldino et al. (2006, 2009) observed a strong relationship between *H. aspera* (as *H. boergesenii*) and *H. flexicaulis*, but made no decision whether to unite them or not, probably because they did not examine the material studied by Hommersand and Fredericq (2003).

In addition to phylogenetic analyses and multiple species delineation approaches based on COI-5P and *rbc*L, we analyzed images of type materials of *Hypnea aspera* and *H. cervicornis*, as well as topotype collections of *H. cervicornis* and *H. flexicaulis* and photographs of the "*H. boergesenii*" specimen of Hommersand and Fredericq (2003), so that our SSHs are well grounded, which helped us re-delineate these taxa. We observed several Agardh's specimens (LD 33866 – 33878!) used in the original description of *H. cervicornis* and performed an extensive sampling along the Brazilian coast. The specimens collected by us are in accordance with morphological traits of the type. The paratypes of *H. flexicaulis* from SAP Herbarium (SAP 71799, 71814, 71826!) showed the same features reported for *H. cervicornis*, such as percurrent axes, dichotomous branching, widely divaricate branches bending upward ("antler-like"), or curling downward and bifurcate apices.

According to Chiang (1997), the herbarium sheets from Hokkaido University that Tanaka (1941) analyzed when describing the new species "*Hypnea boergesenii*" caused confusion. Chiang (1997) observed that specimens identified by Tanaka as *H. cervicornis* were similar to a plant of "*H. boergesenii*" mounted on the same sheet as the type specimen, indicating that these specimens may present more similarities when alive. *H. aspera* descriptions (Tanaka 1941, Chiang 1997, Xia and Wang 1997)

and photographs resemble tetrasporophyte samples of *H. cervicornis* found growing on rocks in exposed areas from southeastern Brazil, with upright axis and branchlets at narrower angle (see Nauer et al. 2014, figs. 15–22). These morphological similarities (in addition to low pair-wise divergences) led Nauer et al. (2014) to consider the occurrence of *H. aspera* for the first time for the Atlantic Ocean. The authors, however, were not able to analyze specimens from northeastern Brazil, which are genetically similar and quite characteristic of the former *H. cervicornis* species. Based on all the evidences outlined, and following the International Code of Nomenclature for Algae, Fungi, and Plants, we propose that *H. aspera* Kützing (1868) and *H. flexicaulis* Yamagishi and Masuda (2000) represent heterotypic synonyms of *Hypnea cervicornis* J. Agardh (1851), which has priority of publication.

Geraldino et al. (2015) carried out biogeographic studies on "*H. flexicaulis*" (= *H. cervicornis*) specimens occurring in Australia, Hong Kong, Indonesia, India, Mexico, Italy, Japan, Korea, Taiwan, and the Philippines, and they hypothesized an Indo-Pacific origin for this taxon. However, several findings also demonstrate the occurrence of this species in the Brazilian coast, Panama, Hawaii, and the Maldives (Guimarães 2006, Sherwood et al. 2010, Jesus et al. 2013, Nauer et al. 2015 and this work). According to Garbary et al. (2001), a strong taxonomic understanding of the species, their variation, and individual distribution is necessary to clarify biogeographic patterns. Phylogeographic studies can be useful for analyzing the connectivity between Atlantic and Indo-Pacific populations of *H. cervicornis*.

Geraldino et al. (2010) identified the specimen that originated the two *Hypnea tenuis* sequences available to date (COI-5P: GQ141880/*rbc*L: FJ694934) from Africa. Kylin (1938) originally described this taxon, based on material from South Africa, presenting branches with hooked tips. It seems to be rare and restricted to areas near its type locality (Lucio 2006, Guiry and Guiry 2016). Due to inconclusive results and because we did not analyze the specimen of Geraldino et al. (2010), we decided to consider this taxon as a separate species. Note that results from delineation methods can be problematic when species are represented by singletons, as Lohse (2009) and Puillandre et al. (2012a,b) detected. Hence, we recommended that the name *H. tenuis* should be used with caution until more sequences are available from its type locality, preferably together with images allowing detailed morphological comparisons.

Interestingly, numerous individuals who collected along the Brazilian coast did not match any recognized *Hypnea* species sequenced to date. Specimens with entangled habit have been, for a long time, misidentified based on morphology as *H. spinella* in Brazil (Guimarães 2006, Jesus et al. 2013). Considering that no *rbc*L sequence of *H. cervicornis* was available in the databanks, Nauer et al. (2014) identified these specimens as belonging to this species. The sequencing of topotype specimens of *H. cervicornis* (this work) and *H. spinella* (Nauer et al. 2014) can now clarify this taxonomic conundrum, and we here describe a new species: *H. brasiliensis*, characterized by a prostrate habit, irregular branching and with the branchlets more narrow than the branches. All phylogenetic analyses indicated that the new species is not closely related to any other known species, and all delimitation methods corroborated its delineation as a conclusive SSH.

We also included *Hypnea spinella* in our analyses due to the long and conflicted taxonomic history related with *H. cervicornis*. Our results from all methods and markers used and determined *H. spinella* as a distinct and separate entity from the other *Hypnea* taxa. The long-standing conundrum involving *H. cervicornis* (and its synonyms), *H. spinella*, and more recently *H. brasiliensis* can be explained by superficial characterization of these taxa. Our findings suggested that the habit should be the main feature to be analyzed to assist in the circumscription of these species: *H. brasiliensis* forms loose tufts with percurrent axis evident, and broader than branches and branchlets; *H. cervicornis* usually presents a percurrent axis with the same diameter of the branches; and *H. spinella* has no percurrent axis and forms intricate tufts.

In all phylogenetic analyses based on rbcL, the genus was monophyletic with high support in relation to the outgroup. The three main clades would correspond to the sections Virgatae, Pulvinatae, and Spinuligerae, as J. Agardh (1851) proposed. These clusters were the same molecularly recognized by Geraldino et al. (2010), Nauer et al. (2014), and Jesus et al. (2015), even though slightly different from the original circumscription, based on thallus habit (Agardh 1851). Jesus et al. (2015) pointed out the need to extend the sampling of *Hypnea* species to help to elucidate the phylogenetic relationships within infrageneric sections. Phylogenetic relationships based on COI-5P resulted in some incongruences and weakly supported groups, although it has recovered the same clusters as the *rbc*L within species. Our findings indicated that, for the studied group, COI-5P does not generate well-resolved phylogenies when used alone even if, according to Lyra et al. (2015), it could increase resolution and support of phylogenetic trees if analyzed simultaneously with *rbc*L.

CONCLUSIONS

Combining traditional taxonomy, including analyses of type material, phylogenetic evidence, and multiple species-delimitation approaches to set the limits of supposedly related species within the genus *Hypnea*, resulted on the circumscription of four conclusive SSHs: *H. brasiliensis* sp. nov, *H. cervicornis*,

H. spinella, and *H. tenuis*. Among these findings, we redefined a new species widely distributed in the Brazilian coast and synonymized *H. aspera* and *H. flexicaulis* with *H. cervicornis*. Inconsistencies observed among our results based on distinct approaches did not affect the final results of the delimitation, but evidenced the need for adequate sampling and attention to marker choice for different methods. We recommend using various methods and markers with different rates of evolution, in order to compensate for possible sampling errors or artifacts methods.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. List of *Hypnea* species sequenced in this study with voucher information and GenBank accession numbers.

Table S2. Published *Hypnea* specimens included in the analyses, with locality information, reference publication, and GenBank accession numbers.

Table S3. List of additional voucher specimens analyzed in this study.

Table S1. List of *Hypnea* species sequenced in this study with voucher information and GenBank accession numbers.

Taxon	Voucher information	GenBank accessions	
		COI-5P	***rbc*L**
Hypnea brasiliensis sp. nov.	Caucaia, Pacheco, Ceará, Brazil, 3°41'09,18" S, 38°37'57,19"W; coll. P.B. Jesus et al., 26 Mar 2013; Field ID: P125; Voucher: ALCB110343	KU905092	
	Caucaia, Pacheco, Ceará, Brazil, 3°41'09,18" S, 38°37'57,19"W; coll. P.B. Jesus et al., 26 Mar 2013; Field ID: P126; Voucher: ALCB118346	KU905156	
	Ronco do Mar Beach, Paracuru, Ceará, Brazil, 3°24'11,22''S, 39°01' 30.00'' W; coll. F. Nauer, 01 Jun 2012; Field ID: IBC0860, Voucher: SPF57550	KU905090	
	Canoa Quebrada, Aracati, Ceará, Brazil, 4°31'28.62''S, 37°41'58.06'' W; coll. F. Nauer, 05 Jun 2012; Field ID: IBC0881, Voucher: SPF57566	KU905127	KU905127
	Canoa Quebrada, Aracati, Ceará, Brazil, 4°31' 28.62''S, 37°41' 58.06'' W; coll. F. Nauer, 05 Jun 2012; Field ID: IBC0882, Voucher: SPF57567	KU905136	

	Ponta do Seixas, João Pessoa, Paraíba, Brazil, 7°8’47.05” S, 34°47’52.01”W; coll. P.B. Jesus et al., 20 Jul 2012; Field ID: P99; Voucher: ALCB118354	KU905146	
	Carapibus, Conde, Paraíba, Brazil, 7°17’59.76” S, 34°47’55.30”W; coll. P.B. Jesus et al., 22 Jul 2012; Field ID: P102; Voucher: ALCB118350	KU905112	
	Carapibus, Conde, Paraíba, Brazil, 7°17’59.76” S, 34°47’55.30”W; coll. P.B. Jesus et al., 22 Jul 2012; Field ID: P103; Voucher: ALCB118351	KU905129	
	Carapibus, Conde, Paraíba, Brazil, 7°17’59.76” S, 34°47’55.30”W; coll. P.B. Jesus et al., 22 Jul 2012; Field ID: P104; Voucher: ALCB118352	KU905117	
	Carapibus Beach, Conde, Paraíba, Brazil, 7°17’59.63’’S, 34°47’55.31’’ W; coll. F. Nauer & C. A. Azevedo, 19 Jul 2012; Field ID: IBC0888; Voucher: SPF57571	KU905097	KU905161
	Carapibus Beach, Conde, Paraíba, Brazil, 7°17’59.63’’S, 34°47’55.31’’ W; coll. F. Nauer & C. A. Azevedo, 19 Jul 2012; Field ID: IBC0889; Voucher: SPF57572	KU905084	

	Carapibus Beach, Conde, Paraíba, Brazil, 7°17’59.63’’S, 34°47’55.31’’ W; coll. F. Nauer & C. A. Azevedo, 19 Jul 2012; Field ID: IBC0891; Voucher: SPF57574	KU905133
	Coqueirinhos Beach, Conde, Paraíba, Brazil, 7°19’18.86’’S, 34°47’52.28’’ W; coll. F. Nauer & C. A.Azevedo, 19 Jul. 2012; Field ID: IBC0892; Voucher: SPF57575	KU905132
	Coqueirinhos Beach, Conde, Paraíba, Brazil, 7°19’18.86’’S, 34°47’52.28’’W; coll. F. Nauer & C. A. Azevedo, 19. Jul 2012; Field ID: IBC0893; Voucher: SPF57576	KU905123
	Tambaba Beach, Conde, Paraíba, Brazil, 7°21’54.72’’S, 34°47’52.79’’W; coll. F. Nauer & C. A. Azevedo, 20 Jun 2012; Field ID: IBC0900; Voucher: SPF57583	KU905080
	Frances Beach, Marechal Deodoro, Alagoas, Brazil, 9°46’07.79’’S, 35°50’17.44’’ W, coll. T. Vieira-Pinto et al., 10 Mar 2013; Field ID: IBC0954; Voucher: SPF57629	KU905122

	Jequia Beach, Marechal Deodoro, Alagoas, Brazil, 9°46’07.79’’S, 35°50’17.44’’ W, coll. T. Vieira-Pinto et al., 10 Mar 2013; Field ID: IBC0957; Voucher: SPF57632	KU905087
	Tamandaré Beach, Tamandaré, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’ W, coll D. Pupo, 13 Mar 2013, Field ID: IBC0958; Voucher: SPF57633	KU905130
	Tamandaré Beach, Tamandaré, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’ W, coll D. Pupo, 13 Mar 2013, Field ID: IBC0959; Voucher: SPF57634	KU905104
	Tamandaré Beach, Tamandaré, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’ W, coll D. Pupo, 13 Mar 2013, Field ID: IBC0962; Voucher: SPF57636	KU905138
	Tamandaré Beach, Tamandaré, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’ W, coll D. Pupo, 13 Mar 2013, Field ID: IBC0963; Voucher: SPF57637	KU905086

	Mucugê Beach, Arraial D'Ajuda, Porto Seguro, Brazil; 16°29'51, 67"S, 39°04'10,47"W; coll. Jesus et al., 06 Nov 2010; Fiel ID: P14; Voucher: ALCB100234	KU905078	KU905157
	Mar Grande, Ilha de Itaparica, Bahia, Brazil, 12°58'00,70"S, 38°36'30,79"W; coll. P.B. Jesus et al., 04 Dec 2010; Field ID: P28, Voucher: ALCB100223	KU905113	
	Mar Grande, Ilha de Itaparica, Bahia, Brazil, 12°58'00,70"S, 38°36'30,79"W; coll. P.B. Jesus et al., 04 Dec 2010; Field ID: P31, Voucher: ALCB100233	KU905145	
	Emissário Beach, Camaçari, Bahia, Brazil, 12°44'25,70"S, 38° 8'58,97"W; coll. P.B. Jesus et al., 23 Sep 2010; Field ID: P42, Voucher: ALCB100228	KU905118	KU905166
	Emissário Beach, Camaçari, Bahia, Brazil, 12°44'25,70"S, 38° 8'58,97"W; coll. P.B. Jesus et al., 23 Sep 2010; Field ID: P43, Voucher: ALCB100228	KU905098	
	Serra Grande Beach, Uruçuca, Bahia, Brazil, 14°28'39"S, 39°01'48"W; coll. P.B. Jesus et al., 18 Apr 2011; Field ID: P51, Voucher: ALCB100236	KU905152	

	Serra Grande Beach, Uruçuca, Bahia, Brazil, 14°28’39”S, 39°01’48”W; coll. P.B. Jesus et al., 18 Apr 2011; Field ID: P53, Voucher: ALCB103119	KU905100
	Penha, Ilha de Itaparica, Bahia, Brazil,12°59’08,76”S, 38°37’02,80”W; coll. P.B. Jesus et al., 06 Sep 2010; Field ID: P79; Voucher: ALCB95273	KU905119
	Muta Beach, Porto Seguro, Bahia, Brazil, 16°21’19.07’’S, 39°00’46.95’’ W; coll. F. Nauer & C. A. Azevedo, 14 Sep2012; Field ID: IBC0920; Voucher: SPF57599	KU905083
	Muta Beach, Porto Seguro, Bahia, Brazil, 16°21’19.07’’S, 39°00’46.95’’ W; coll. F. Nauer & C. A. Azevedo, 14 Sep 2012; Field ID: IBC0921; Voucher: SPF57600	KU905124
	Muta Beach, Porto Seguro, Bahia, Brazil, 16°21’19.07’’S, 39°00’46.95’’W; coll. F. Nauer & C. A. Azevedo, 14 Sep 2012; Field ID: IBC0924; Voucher: SPF57603	KU905155

	Muta Beach, Porto Seguro, Bahia, Brazil, 16°21’19.07’’S, 39°00’46.95’’W; coll. F. Nauer & C. A. Azevedo, 14 Sep 2012; Field ID: IBC0929; Voucher: SPF57607	KU905135	
	Ponta Grande, Porto Seguro, Bahia, Brazil, 16°22’33.05’’S, 39°00’33.86’’W, coll. F. Nauer & C. A.Azevedo, 16 Sep 2012; Field ID: IBC0941; Voucher: SPF57619	KU905108	
	Dura Beach, Ubatuba, São Paulo, Brazil, 23°29’37.70”S, 45° 9’53.41”W; coll. P.B. Jesus et al., 18 Aug 2012; Field ID: P110; Voucher: ALCB110264	KU905144	KU905170
	Dura Beach, Ubatuba, São Paulo, Brazil, 23°29’37.70”S, 45° 9’53.41”W; coll. P.B. Jesus et al., 18 Aug 2012; Field ID: P111; Voucher: ALCB 110264	KU905148	
	Cigarras Beach, São Sebastião, São Paulo, Brazil, 23°43’55.63”S, 45°23’55.31”W; coll. P.B. Jesus et al., 19 Aug 2012; Field ID: P112; Voucher: ALCB110267	KU905137	

	Cigarras Beach, São Sebastião, São Paulo, Brazil, 23°43’55.63”S, 45°23’55.31”W; coll. P.B. Jesus et al., 19 Aug 2012; Field ID: P113; Voucher: ALCB118349	KU905110
	Brava Beach, Caraguatatuba, São Paulo, Brazil, 23°37’40.04”S, 45°22’3.98”W; coll. P.B. Jesus et al., 19 Aug 2012; Field ID: P116; Voucher	KU905085
	Jabaquara Beach, Ilha Bela, São Paulo, Brazil, 23°44’07.14’’S, 45°17’36.48’’W, coll. M. T. Fujii. 20 May 2008; Field ID: IBT0069; Voucher: SP365640	KU905079
	Jabaquara Beach, Ilha Bela, São Paulo, Brazil, 23°44’07.14’’S, 45°17’36.48’’W, coll. M. T. Fujii. 20 May 2008; Field ID: IBT0070; Voucher: SP365641	KU905121
	Jabaquara Beach, Ilha Bela, São Paulo, Brazil, 23°44’07.14’’S, 45°17’36.48’’W, coll. M. T. Fujii. 20 May 2008; Field ID: IBT0071; Voucher: SP365649	KU905140

	Arvoredo Island, Governador Celso Ramos, Santa Catarina, Brazil, 27°17’19.49’’S, 48°22’05.95’’W; coll. F. Nauer et al., 25 Feb 2012; Field ID: IBT0073; Voucher: SPF57489	KU905105	
	Arvoredo Island, Governador Celso Ramos, Santa Catarina, Brazil, 27°17’19.49’’S, 48°22’05.95’’W; coll. F. Nauer et al., 25 Feb 2012; Field ID: IBC0792; Voucher: SPF57490	KU905091	KU905159
	Arvoredo Island, Governador Celso Ramos, Santa Catarina, Brazil, 27°17’19.49’’S, 48°22’05.95’’W; coll. F. Nauer et al., 25 Feb 2012; Field ID: IBC0793; Voucher: SPF57491	KU905128	
	Sambaqui Beach, Florianópolis, Santa Catarina, Brazil, 27°29’24.55’’S, 48°32’18.89’’W, coll. F. Nauer et al.; 26 Feb 2012, Field ID: IBC0794; Voucher: SPF57493	KU905093	
	Sambaqui Beach, Florianópolis, Santa Catarina, Brazil, 27°29’24.55’’S, 48°32’18.89’’W, coll. F. Nauer et al.; 26 Feb 2012, Field ID: IBC0796; Voucher: SPF57495	KU905141	

	Ganchos de Fora, Governador Celso Ramos, Santa Catarina, Brazil, 27°18’21.82’’S, 48°32’49.83’’W; coll. F. Nauer et al.; 27 Feb 2012; Field ID: IBC0798; Voucher: SPF57503	KU905107
	Sepultura Beach, Bombinhas, Santa Catarina, Brazil, 27°08’29.75’’S, 48°28’40.22’’W, coll. F. Nauer et al., 28 Feb 2012; Field ID: IBC0806; Voucher: SPF57511	KU905125
	Sepultura Beach, Bombinhas, Santa Catarina, Brazil, 27°08’29.75’’S, 48°28’40.22’’W, coll. F. Nauer et al., 28 Feb 2012; Field ID: IBC0814; Voucher: SPF57511	KU905094
H. cervicornis J. Agardh	Dois Coqueiros, Caucaia, Ceará, Brazil, 3°41’08.40’’S, 38°38’00.18’’W, coll. L. Soares, 28 Aug 2011; Field ID: IBT0802; Voucher: SP365646	KU905088
	Rio do Fogo Beach, Rio do Fogo, Rio Grande do Norte, Brazil, 5°16’32.90’’S, 35°22’41.76’’W, coll. C. A. Azevedo, 12 Jan 2013; Field ID: IBC0968; Voucher: SPF57641	KU905139

	Toquinho Beach, Ipojuca, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’W, coll. N. S. Yokoya, 14 Mar 2013; Field ID: IBC970; Voucher: SPF57643	KU905154
	Toquinho Beach, Ipojuca, Pernambuco, Brazil, 8°36’12.37’’S, 35°02’42.25’’W, coll. N. S. Yokoya, 14 Mar 2013; Field ID: IBC971; Voucher: SPF57644	KU905120
	Frances Beach, Marechal Deodoro, Alagoas, Brazil, 9°46’07.79’’S, 35°50’17.44’’W, coll. T. Vieira-Pinto et. al., 10 Mar 2013; Field ID: IBC0956; Voucher: SPF57631	KU905153
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; 16°16’49,89”S, 39°01’12,33”W; coll. Jesus et al., 07 Nov 2010; Fiel ID: P04; Voucher: ALCB100200	KU905106
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; 16°16’49,89”S, 39°01’12,33”W; coll. Jesus et al., 07 Nov 2010; Fiel ID: P09; Voucher: ALCB100221	KU905081

	Mucugê Beach, Arraial D'Ajuda, Porto Seguro, Brazil; 16°29'51, 67"S, 39°04'10,47"W; coll. Jesus et al., 06 Nov 2010; Fiel ID: P12; Voucher: ALCB100222	KU905109	
	Penha, Ilha de Itaparica, Bahia, Brazil,12°59'08,76"S, 38°37'02,80"W; coll. P.B. Jesus et al., 05 Dec 2010; Field ID: P16; Voucher: ALCB100218	KU905150	
	Subaúma, Entre Rios, Bahia, Brazil, 12°14'10,10"S, 37°46'5,60"W; coll. P.B. Jesus et al., 25 Oct 2010; Field ID: P26; Voucher: ALCB100224	KU905131	KU905169
	Mar Grande, Ilha de Itaparica, Bahia, Brazil, 12°58'00,70"S, 38°36'30,79"W; coll. P.B. Jesus et al., 04 Dec 2010; Field ID: P30, Voucher: ALCB100219	KU905142	
	Praia do Forte, Mata de São João, Bahia, Brazil, 12°34'42,33"S, 38°0'6,45"W; coll. P.B. Jesus et al., 25 Sep 2010; Field ID: P37; Voucher: ALCB100217	KU905101	KU905162

	Praia do Forte, Mata de São João, Bahia, Brazil, 12°34’42,33”S, 38°0’6,45”W; coll. P.B. Jesus et al., 25 Sep 2010; Field ID: P38; Voucher: ALCB100296	KU905114	
	Apuã Beach, Santa Cruz de Cabrália, Brazil; 16°16’49,89”S, 39°01’12,33”W; coll. Jesus et al., 05 Nov. 2010; Fiel ID: P40; Voucher: ALCB100230	KU905126	KU905167
	Mar Grande, Ilha de Itaparica, Bahia, Brazil; coll. P.B. Jesus et al., 04 Dec 2010; Field ID: P60, Voucher: ALCB118347	KU905134	
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; 16°16’49,89”S, 39°01’12,33”W; coll. Jesus et al., 03 Jun 2012; Field ID: P89; Voucher: ALCB110274	KU905082	
	Apuã Beach, Santa Cruz de Cabrália, Brazil; 16°16’49,89”S, 39°01’12,33”W; coll. Jesus et al., 04 Jun 2012; Field ID: P155; Voucher: ALCB118353	KU905103	

	Mucugê Beach, Arraial D'Ajuda, Porto Seguro, Brazil; 16°29'51, 67"S, 39°04'10,47"W; coll. Jesus et al., 05 Jun 2012; Field ID: P162; Voucher: ND	KU905143	
	Beach Armação, Florianópolis, Santa Catarina, Brazil; 27°44'58.70''S, 48°30'00.12'' W; coll. M.B. Batista, 25 Feb 2013; Field ID: P133; Voucher: ALCB118348	KU905095	
	Beach Armação, Florianópolis, Santa Catarina, Brazil; 27°44'58.70''S, 48°30'00.12'' W; coll. M.B. Batista, 25 Feb 2013; Field ID: P134; Voucher: ALCB110259	KU905149	
	Ponta das Canas, Florianópolis, Santa Catarina, Brazil, 27°23'38.79''S, 48°26'08.75''W, coll. F. Nauer et al., 26 Feb 2012; Field ID: IBC0801; Voucher: SPF57498	KU905096	KU905160
	Praia de Palmas, Governador Celso Ramos, Santa Catarina, Brazil, 27°18'47.90''S, 48°32'23.01''W, coll. F. Nauer et al., 27 Feb 2012, Field ID: IBC0803; Voucher: SPF57500	KU905151	

	Taquaras, Balneário Camburiu, Santa Catarina, Brazil, 27°00’39.04’’S, 48°34’41.79’’W, coll. F. Nauer et al., 28 Feb 2012, Field ID: IBC0811; Voucher: SPF57508	KU905116	
	Taquaras, Balneário Camburiu, Santa Catarina, Brazil, 27°00’39.04’’S, 48°34’41.79’’W, coll. F. Nauer et al., 28 Feb 2012, Field ID: IBC0812; Voucher: SPF57509	KU905099	
	Bocas del Toro, Panamá, coll. C. A. Azevedo & M. Sissini; Field ID: BOT005; Voucher: SPF57592	KU905147	
	Kuredo, Maldivas, 5°32’53.94”N, 73°27’36.29”L, coll. M. C. Oliveira, 28 Apr 2013, Field ID: MAL1; Voucher: SPF57880	KU905102	KU905163
	Kuredo, Maldivas, 5°32’53.94”N, 73°27’36.29”L, coll. M. C. Oliveira, 28 Apr 2013, Field ID: MAL2; Voucher: SPF57880	KU905115	KU905165
	Kuredo, Maldivas, 5°32’53.94”N , 73°27’36.29”L, coll. M. C. Oliveira, 28 Apr 2013, Field ID: MAL3; Voucher: SPF57881	KU905089	KU905158

H. spinella	Penha, Ilha de Itaparica, Bahia, Brazil,12°59’08,76”S, 38°37’02,80”W; coll. P.B. Jesus et al., 05 Dec 2010; Field ID: P17; Voucher: ALCB100232	KU905111	KU905164

Table S2. Published *Hypnea* specimens included in the analyses, with locality information, reference publication and GenBank accession numbers.

Taxon	Locality	Reference publication	GenBank accessions	
			COI-5P	***rbc*L**
Hypnea asiatica P.J.L.Geraldino, E.C.Yang & S.M.Boo	South Korea, Gampo	Geraldino et al. (2009)	EU240799	EU346007
	Japan, Chiba	Geraldino et al. (2009)	EU240805	EU240839
	Taiwan	Geraldino et al. (2009)	EU240809	
H. aspera Kützing [deposited as *H. boergesenii*]	Taiwan, Long Tung Park	Hommersand & Fredericq (2003)		AF385634
	South Korea, Jeju	Geraldino et al. (2009)		EU346010
	South Korea, Jeju	Geraldino et al. (2009)		EU346009
	South Korea, Jeju	Geraldino et al. (2009)	EU345983	EU345994
	Brazil, São Paulo	Nauer et al. (2014)	KM214561	KM203911

	Brazil, São Paulo	Nauer et al. (2014)	KM214567	
	Brazil, São Paulo	Nauer et al. (2014)	KM214568	
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214530	KM203900
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214564	
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214563	
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM233170	
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214562	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214565	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214566	
	Brazil, São Paulo	Nauer et al. (2014)	KM214497	
	Brazil, São Paulo	Nauer et al. (2014)	KM214498	KM203898

Hypnea brasiliensis. P.B. Jesus, F. Nauer & J.M.C. Nunes sp. nov. [deposited as *H. cervicornis*]	Brazil, São Paulo	Nauer et al. (2014)	KM214499	
	Brazil, São Paulo	Nauer et al. (2014)	KM214500	KM203897
	Brazil, São Paulo	Nauer et al. (2014)	KM214501	
	Brazil, São Paulo	Nauer et al. (2014)	KM214502	
	Brazil, São Paulo	Nauer et al. (2014)	KM214503	
	Brazil, São Paulo	Nauer et al. (2014)	KM214504	
	Brazil, São Paulo	Nauer et al. (2014)	KM214505	
	Brazil, São Paulo	Nauer et al. (2014)	KM214506	
	Brazil, São Paulo	Nauer et al. (2014)	KM214507	
	Brazil, São Paulo	Nauer et al. (2014)	KM214508	

	Brazil, São Paulo	Nauer et al. (2014)	KM214509	
	Brazil, São Paulo	Nauer et al. (2014)	KM214510	
	Brazil, São Paulo	Nauer et al. (2014)	KM214511	
	Brazil, São Paulo	Nauer et al. (2014)	KM214512	
	Brazil, São Paulo	Nauer et al. (2014)	KM214513	
	Brazil, São Paulo	Nauer et al. (2014)	KM214514	
	Brazil, São Paulo	Nauer et al. (2014)	KM214515	
	Brazil, São Paulo	Nauer et al. (2014)	KM214516	KM203902
	Brazil, São Paulo	Nauer et al. (2014)	KM214517	
	Brazil, São Paulo	Nauer et al. (2014)	KM214518	
	Brazil, São Paulo	Nauer et al. (2014)	KM214519	

	Brazil, São Paulo	Nauer et al. (2014)	KM214520	
	Brazil, São Paulo	Nauer et al. (2014)	KM214521	
	Brazil, São Paulo	Nauer et al. (2014)	KM214522	
	Brazil, São Paulo	Nauer et al. (2014)	KM214538	KM203909
	Brazil, São Paulo	Nauer et al. (2014)	KM214539	KM203908
	Brazil, São Paulo	Nauer et al. (2014)	KM214540	
	Brazil, São Paulo	Nauer et al. (2014)	KM214541	
	Brazil, São Paulo	Nauer et al. (2014)	KM214542	
	Brazil, São Paulo	Nauer et al. (2014)	KM214543	
	Brazil, São Paulo	Nauer et al. (2014)	KM214544	
	Brazil, São Paulo	Nauer et al. (2014)	KM214545	

	Brazil, São Paulo	Nauer et al. (2014)	KM214546
	Brazil, São Paulo	Nauer et al. (2014)	KM214547
	Brazil, São Paulo	Nauer et al. (2014)	KM214560
	Brazil, São Paulo	Nauer et al. (2014)	KM214559
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214537
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214533
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214534
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214536
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214535
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214548
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214549

	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214550	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214551	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214552	KM203910
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214553	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214554	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214555	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214556	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214557	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214558	
H. caespitosa Geraldino et Boo	Philippines, Cagayan de Oro	Geraldino et al. (2010)	FJ694902	FJ694937

	Malaysia, Cape Rachado	Geraldino et al. (2010)	FJ694899	FJ694943
H. cervicornis J. Agardh	USA, Hawaii	Sherwood et al. (2010)	HQ422674	
H. charoides J. V. Lamouroux	Japan, Shimoda	Yamagishi & Masuda (2000)		AB033159
	Australia, Perth	Geraldino et al. (2009)	EU240819	EU240844
	Australia, Perth	Geraldino et al. (2009)	EU240820	
H. chordacea Kützing	Japan, Shimoda	Yamagishi & Masuda (2000)		AB033160
H. cornuta (Kützing) J. Agardh	Italy, Taranto	Yamagishi et al. (2003)		AB095912
	Australia, Perth	Geraldino et al. (2009)		EU345992
	Indonesia, Bali	Geraldino et al. (2009)		EU345991
	Korea	Geraldino et al. (2009)	GQ141878	
	Japan, Okinawa Prefecture	Yamagishi et al. (2003)		AB095911

H. edeniana F. Nauer, V. Cassano & M.C. Oliveira	Brazil, São Paulo	Nauer et al. (2014)	KM214529	KM203896
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214574	KM203907
H. flagelliformis J. Agardh	Japan. Aomori,	Yamagishi & Masuda (2000)		AB033162
H. flava F. Nauer, V. Cassano & M.C. Oliveira	Brazil, São Paulo	Nauer et al. (2014)	KM214532	KM203903
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214571	KM203906
H. flexicaulis Y. Yamagishi & M. Masuda	Japan, Shizuoka prefecture	Yamagishi & Masuda (2003)		AB033163
	Italy, Venice Lagoon	Wolf et al. (2011)	FN823052	
	Philippines, Bulusan	Geraldino et al. (2006)	EF136591	EF136632
	South Korea, Geomundo	Geraldino et al. (2006)	EF136592	EF136631
	South Korea, Wolpo	Geraldino et al. (2006)	EF136609	EF136630
	South Korea, Gampo	Geraldino et al. (2006)	EF136593	EF136629

	South Korea, Gampo	Geraldino et al. (2006)	EF136594	EF136628
	South Korea, Gampo	Geraldino et al. (2006)	EF136605	EF136627
	South Korea, Gijang	Geraldino et al. (2006)	EF136606	EF136626
	South Korea, Gampo	Geraldino et al. (2006)	EF136595	EF136625
	South Korea, Pohang	Geraldino et al. (2006)	EF136596	EF136624
	South Korea, Pohang	Geraldino et al. (2006)	EF136610	EF136623
	South Korea, Gampo	Geraldino et al. (2006)	EF136607	EF136622
	South Korea, Geomundo	Geraldino et al. (2006)	EF136597	EF136621
	South Korea, Geomundo	Geraldino et al. (2006)	EF136598	EF136620
	South Korea, Geomundo	Geraldino et al. (2006)	EF136599	EF136619
	South Korea, Geomundo	Geraldino et al. (2006)	EF136600	EF136618

	South Korea, Geomundo	Geraldino et al. (2006)	EF136601	EF136617
	South Korea, Geomundo	Geraldino et al. (2006)	EF136602	EF136616
	South Korea, Geomundo	Geraldino et al. (2006)	EF136603	EF136615
	South Korea, Geomundo	Geraldino et al. (2006)	EF136604	EF136614
	South Korea, Gampo	Geraldino et al. (2006)	EF136611	EF136613
	Taiwan, Keelung	Geraldino et al. (2006)	EF136608	EF136612
H. japonica Tanaka	Taiwan, Keelung	Geraldino et al. (2009)	EU3459891	EU345995
	Korea, Gyeongju	Geraldino et al. (2009)	EU345986	EU346003
	Japan, Kagoshima prefecture	Yamagishi and Masuda (2000)		AB033164
H. musciformis (Wulfen) J.V.	USA, North Carolina	Geraldino et al. (2009)		U04179
Lamouroux	USA, Hawaii	Sherwood et al. (2010)	HQ422612	

	Spain, Cadiz	Diaz-Tapia et al. (Unpublished)		KC121141
	Japan	Geraldino et al. (2010)	GQ141881	
	Brazil, São Paulo	Jesus et al. (2015)	KP725276	KP725288
	Brazil, Bahia	Jesus et al. (2015)	KP725277	KP725289
H. nidifica J. Agardh	Singapore, Sentosa Island	Geraldino et al. (2010)	GQ141879	FJ694932
H. nidulans Setchell	Japan, Hedo-misaki	Yamagishi and Masuda (2000) - as *H. pannosa*		AB033165
	Philippines, Ilocos Norte	Geraldino et al. (2010)	FJ694900	FJ694947
	Philippines, Ilocos Norte	Geraldino et al. (2010)	FJ694907	
H. pannosa J. Agardh	Philippines, Bohol	Geraldino et al. (2010)	FJ694910	FJ694951
	Mexico, El Sargento	Geraldino et al. (2010)	FJ694894	FJ694959
H. rosea Papenfuss	South Africa, Durban	Geraldino et al. (2010)		FJ694935

H. spinella (C.Agardh) Kützing	Caribbean, Barbados	Nauer et al. (2014)	KM214525	KM233168
	Brazil, São Paulo	Nauer et al. (2014)	KM214524	KM203899
	Brazil, São Paulo	Nauer et al. (2014)	KM214578	KM203904
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214576	
	Brazil, Rio de Janeiro	Nauer et al. (2014)	KM214577	
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214579	KM203905
	Brazil, Espírito Santo	Nauer et al. (2014)	KM214583	
	Vietnam, Nha Trang	Geraldino et al. (2009)	EU240817	EU240849
	Vietnam, Nha Trang	Geraldino et al. (2009)	EU240818	
Hypnea stellulifera (J. Agardh) Yamagishi et Masuda	Malaysia, Pulau Besar	Yamagishi et al. (2003)		AB095913
	Philippines, Bohol	Geraldino et al. (2009)	EU345985	EU345999

	Brazil, Bahia	Jesus et al. (2015)	KP725281	KP725290
	Brazil, Bahia	Jesus et al. (2015)	KP725282	KP725291
	Brazil, Bahia	Jesus et al. (2015)	KP725283	KP725292
	Brazil, Bahia	Jesus et al. (2015)	KP725286	KP725293
H. tenuis Kylin	South Africa, Durban	Geraldino et al. (2010)		FJ694934
H. valentiae (Turner) Montagne	Indonesia, Bali	Geraldino et al. (2010)		FJ694933
H. viridis Papenfuss	South Africa, Durban	Geraldino et al. (2010)	FJ694908	FJ694930
H. volubilis Searles	USA, Los Angeles	Hommersand & Fredericq (2002)		AF385636
H. yamadae Tanaka	Japan, Nagasaki Prefecture	Yamagishi et al. (2003)		AB095916

Table S3. List of additional voucher specimens analyzed in this study.

Taxon	Collection Location	Voucher
Hypnea brasiliensis sp. nov.	Aghá Beach, Piúma, Espírito Santo, Brazil, 06 May 2012, F. Nauer, C. Iha and B. Torrano-Silva	SPF 57529
	Enseada Azul, Meaípe, Espírito Santo, Brazil, 09 May 2012, F. Nauer, C. Iha and B. Torrano-Silva	SPF 57546
	Forno Beach, Arraial do Cabo, Rio de janeiro, Brazil, 28 Sep 2011, F. Nauer, C. Iha and B. Torrano Silva	SPF 57422
	Forno Beach, Armação dos Búzios, Rio de janeiro, Brazil, 25 Sep 2011, F. Nauer, C. Iha and B. Torrano-Silva	SPF 57425
	Ilha Grande, Angra dos Reis, Rio de janeiro, Brazil,18 Apr 2012, F. Nauer	SPF 57517
Hypnea cervicornis J. Agardh	"*In littore maris prope Soteropolin*" (Bahia de Todos os Santos), Bahia, Brazil, coll. Martius (Syntypes)	LD 33866-33872!
	Unspecified locality, Bahia, Brazil, coll. Chamisso (Syntype)	LD 33876!

	Ilha das Cabras, Ubatuba, São Paulo, 05 Sep 2011, coll. A. Medeiros, C. Iha and B. Torrano-Silva	SPF 57443
	Saco do Mamanguá, Parati, Rio de Janeiro, Brazil, 20 Nov 2009, coll. E.C. Oliveira	SPF56540
	Farol Beach, Ilha do Farol, Arraial do Cabo, Rio de Janeiro, Brazil, 28 Sep 2011, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF57418
	Atalaia, Arraial do Cabo, Rio de Janeiro, Brazil, 28 Sep 2011, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF57416
	Parati, Anchieta, Espírito Santo, Brazil, 05 May 2012, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF57524
	"*Vettindien*", México, coll. Liebmann (Syntype)	LD 33875!
	"*Ins Jamaica plagis*", West Indies, coll. Mertens (Syntype)	LD 33873!
	"*Ind. occidentali*", West Indies, s/coll. (Probable Syntype)	LD 33874!
	"*in oceano indico ad insulam Mauritii - ad insulam Franciae*", coll. Telfair (Syntype)	LD 33879!

	Omaezaki, Shizuoka Prefecture, Japan, coll. M. Masuda, 27 Aug 1996 (as *H. flexicaulis*)	SAP 071799
	Shitsumi, Obama, Fukui Prefecture, Japan, coll. Y. Yamagishi, 5 Sep 1998 (as *H. flexicaulis*)	SAP 071814
	Long Tung Park, Taiwan, coll. S Fredericq & S-M Lin, 12 Aug 1993 (as *H. boergesenii*)	s/n
Hypnea spinella (C.Agardh) Kützing	Saco do Poço, Ilhabela, São Sebastião, São Paulo, Brazil, 31 May 2008, coll. M.C. Oliveira	SPF 56884
	João Fernandes Beach, Armação dos Búzios, Rio De Janeiro, Brazil, 27 Sep 2011, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF 57438
	Enseada Azul, Guarapari, Espírito Santo, Brazil, 09 May 2012, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF 57544
	Castelhanos Beach, Anchieta, Espírito Santo, Brazil, 05 May 2012, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF 57523

	Cruz Beach, Marataízes, Espírito Santo, Brazil, 07 May 2012, coll. F. Nauer, C. Iha and B. Torrano-Silva	SPF57534

CAPÍTULO 3

PHENOLOGICAL STUDIES ON THE *HYPNEA PSEUDOMUSCIFORMIS* COMPLEX (CYSTOCLONIACEAE, RHODOPHYTA): AN ECOLOGICAL APPROACH WITH TAXONOMIC RESPONSES?

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Manuscrito a ser submetido ao periódico Botanica Marina

Phenological studies on the *Hypnea pseudomusciformis* complex (Cystocloniaceae, Rhodophyta): an ecological approach with taxonomic responses?

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Abstract

We investigated the phenology of a tropical population of "*Hypnea nigrescens"* in order to describe its biology and verify if this ecological approach could help to elucidate the taxonomic status of the *H. pseudomusciformis* complex. The population analyzed did not show significant fluctuations in total biomass throughout the year. All reproductive stages were frequently recorded during the study, with vegetative fronds occurring only once. Tetrasporophytes had the highest percentage of biomass, followed by female and male gametophytes and vegetative plants, suggesting that asexual predominates over sexual reproduction. Phenological variations found in this study were correlated with some environment variables analyzed, such as air and seawater temperatures, insolation, rainfall and humidity. A surprising finding was the frequent presence of male gametophytes, which has never been reported to the variety *musciformis*. We observed significant differences regarding to thallus size, with the smallest heights occurring in the month with the highest amount of accumulated rainfall. Despite being considered as a single genetic species, we have shown that ecological aspects of "*H. nigrescens*" and "*H. musciformis*" exhibit remarkable differences, which is evident when results are compared with local studies. The recognition of the factors that determine all these differences remains a major challenge for these taxa.

Keywords: Biomass, *Hypnea nigrescens*, Reproduction, Systematics, Species complex

Introduction

Hypnea nigrescens Greville ex J. Agardh (1851) was described based on specimens from the Indian Ocean and, since then, it has been rarely cited in taxonomic studies of the genus (Jesus et al. 2013a, Guiry and Guiry 2016). Nevertheless, this species seemed to be widely distributed on Brazilian Coast (Schenkman 1986, Nunes 2005, Guimarães 2006, Lyra et al. 2007, Jesus 2012, Jesus et al. 2013a), the only region out of the Indian Ocean where it has been reported.

Despite of several morphological differences separating *Hypnea nigrescens* from *H. musciformis* (Wulfen) J.V. Lamouroux and *H. valentiae* (Turner) Montagne (J. Agardh 1851, Schenkman 1986, Guimarães 2006, Jesus 2012, Jesus et al. 2013a), they were recently considered a species complex on the Brazilian coast with basis on molecular data (Nauer et al. 2015). In the lattest study, specimens previously identified as *H. musciformis*, *H. nigrescens* and *H. valentiae* were described as morphological variations of a new species based on their genetic similarity: *H. pseudomusciformis* Nauer, Cassano and M.C. Oliveira. Because these varieties are not formally proposed, here we are referring these Brazilian taxa as "*H. musciformis"*, "*H. nigrescens*" and "*H. valentiae*".

Despite the low genetic divergences (Nauer et al. 2015), besides the marked morphological traits (Jesus 2012, Jesus et al. 2013a), the continuous surveys carried out on Brazilian specimens of the genus, demonstrated that "*Hypnea musciformis*", "*H. nigrescens*" and "*H. valentiae*" also exhibit consistent ecological differences, and rarely occur in sympatry. When it happens, one variety usually form large populations, while the other remains restricted to a few individuals (personal observation). Ecological aspects are generally used to complement the algae description; however, its variation has received little attention. Evaluations of the patterns of phenological variation are not

commonly used at a taxonomic scale in phycological studies. Thus, we wondered if an ecological approach could help to elucidate this taxonomic conundrum.

Espinosa-Avalos (2005) described macroalgae phenology as the study of the timing of recurring biological events and the causes of their timing with regard to abiotic and biotic factors. Several studies have been conducted in order to describe the reproductive biology of *Hypnea* species (Mshigeni 1976a; b; c, Rao 1977, Rangaiah and Rao 1983, Schenkman 1989, Wallner et al. 1992, Reis and Yoneshigue-Valentin 2000, Smith et al. 2002, Kong and Ang Jr. 2004, Mouradi et al. 2008, Caires et al. 2013a).

While the phenology of *H. nigrescens* has not been evaluated before, phenological aspects of *Hypnea musciformis* (Rao 1977, Smith et al. 2002, Mouradi et al. 2008) and *H. valentiae* (Rao 1977, Rangaiah and Rao 1983) have been analyzed in various locations of the globe. These works mostly claim that phenology is highly influenced by the environmental factors in which the alga is found. Despite this, results for *H. musciformis* always displays a prevalence of tetrasporophytes and absence of male gametophytes observed, indicating that there is a very characteristic pattern for this species (Rao 1977, Wallner et al. 1992, Reis and Yoneshigue-Valentin 2000, Mouradi et al. 2008, Caires et al. 2013a). Rao (1977) also reported the absence of male gametophytes in *H. valentiae* on west coast of India, although Rangaiah and Rao (1983) have found male specimens of this species on east coast of India.

On Brazilian coast, phenological analyses were perfomed only with the variety *musciformis* (Schenkman 1989, Wallner et al. 1992, Reis and Yoneshigue-Valentin 2000, Caires et al. 2013a), revealing prevalence of tetrasporophytes and absence of male gametophytes. In this study, we aimed to analyse the phenological pattern of the "*Hypnea nigrescens*" in a tropical area of Brazilian coast and to verify if an ecological approach could contribute to the taxonomy of the *Hypnea pseudomusciformis* complex.

Materials and methods

The study site was a rocky shore of the Emissário Beach (12°44'25.70" S, 38° 8'58.97" W), located at Arembepe, north coast of the Bahia state, Brazil (Figure 1). The shore is fragmented, directly exposed to wave action and mainly occupied by sessile mollusks and incrusting algae. It was chosen because of the banks of "*Hypnea nigrescens*" that occurs strongly attached to substrate, forming clumps.

According to Leão et al. (2010), this part of the Brazilian coast has a tropical climate, with rainfall ranging from 1300 mm y^{-1} in its northernmost portion. Average air temperatures range from 23 ºC in the winter to 28 ºC in the summer and temperature of the surface seawater varies from around 24 ºC (winter) to 28 ºC (summer). Spring tides can reach up to 3.0 m. In this study, seawater temperature and salinity data were measured by using an electronic thermometer (JProlab SH-102) and a portable refractometer (Biobrix 103) respectively. The National Institute of Meteorology provided data on rainfall, humidity, monthly insolation and air temperature – thirty days before sampling (INMET, http://ww/w.inmet.gov.br/portal/index.php?r=bdmep/bdmep, accessed February 2016).

Collections were carried out using a destructive methodology (De Wreede 1985) between August 2013 and July 2014. Samples were collected in the intertidal zone, during spring low tides (0.0 to 0.3), always during the first fortnight of each month. Three transects (20 m) were parallely disposed to the shoreline. In each transect five random points were scored on which squares of 0.04 m^2 were arranged. Each square was considered as one sample. All specimens contained in the square were collected with the aid of a metallic spatula and transferred to lab. To estimate the dry biomass of "*Hypnea nigrescens*", 180 samples were analyzed (15 samples per collection). For

qualitative analyses, individuals were arbitrary collected around each transect, totalizing 250 specimens. We measured the frond lengths from the base to the tip of each clump of "*H. nigrescens*". Samples were fixed in 4% formalin, and analyzed under a stereomicroscope (Leica Zoom® 2000) to remove algae epiphytes and sediment and to identify the reproductive stages. All collected samples were separated into tetrasporophytes, female (with cystocarps) or male gametophytes (Figure 2) and non-reproductive (vegetative or infertile) fronds.

For phenological inferences and estimates of the biomass, seaweeds were dried in glasshouse Fanem® at 60°C by 48hs or until reached constant weight. Dry biomass data are expressed as mean ± standard deviation (SD) ($g.m^{-2}$) or as percentage of dry biomass. Qualitative samples were herborized according to Nunes (2010) and deposited in the Herbarium Alexandre Leal Costa Bahia (ALCB) at Universidade Federal da Bahia (UFBA). Frond's lengths were measured from plants collected during the qualitative sampling.

Dry biomass data and frond's length of samples were tested for normality and homocedasticity of variances with Shapiro-Wilk tests and Levene's test, respectively. The data were non-parametric, so that we used the Kruskal-Wallis to verify if there are significant differences between biomass and frond's length during the months studied. Dunn test was applied a posteriori to verify which periods were significant differences throughout the study. We performed a Spearman's correlation test to determine possible relationships between biomass and thallu's length with the analyzed environmental parameters. For all analyses, significance level at $p \leq 0.05$ was adopted on environment R (R Core Team 2016).

Results

The historical series of abiotic data showed values typical of a tropical region. Mean air temperature ranged from 23°C in July 2014 to 26.4°C in April 2014, and the total monthly insolation ranged from 147 h in August 2013 to 230 h in April 2014 (Figure 3). The rainfall accumulated ranged from 41 mm (December 2013) to 167 mm (June 2014), while the humidity presented an average of 81.4 % to 88 % (April and June 2014, respectively) (Figure 4). Seawater temperature and salinity were measured at the time of sampling and exhibited maximum variation range of 4°C (26 to 30°C – 27 ± 1.4°C) and 9 (31 to 40 – 36 ± 2.3), respectively (Figures 5A and B).

"*Hypnea nigrescens*" specimens were present on Emissário Beach throughout the period of study. Total biomass ranged from 0.14 to 0.50 $g.m^2$, no significant differences occurring among the months throughout the collection period (Table 1, Figure 6). Total biomass showed negative correlation with seawater temperature (Table 2). Biomass of tetrasporophytes was significantly different from vegetative ($p=0.02$), cystocarpic ($p<0.01$) and male fronds ($p<0.01$).

Vegetative fronds were observed only in August (2013). The biomass of male gametophytes throughout the year was so low that did not differ significantly among the months studied, while tetrasporophytes and female gametophytes showed significant variations (Table 1). Biomass of tetrasporophytes varied among the months studied, in which June 2014 differed significantly from April 2014 ($p=0.005$), October 2013 ($p=0.03$) and November 2013 ($p=0.04$). Biomass of female gametophytes differed significantly between August 2013 and April 2014 ($p=0.04$).

Table 2 presents the correlations between biomass and frond's size of "*Hypnea nigrescens*" and environmental variables on Emissário Beach, during the study. A negative correlation was observed between biomass of tetrasporophytes and seawater temperature. Biomass of gametophytes showed positive correlations with air

temperature and negative correlations with relative humidity. Male gametophytes biomass displayed positive correlation with monthly insolation.

Fertile specimens of "*Hypnea nigrescens*" were found during the whole study period (except male gametophytes, absent in August 2013). Percentages of the tetrasporophytic, female and male gametophytes and non-reproductive fronds are shown in Figure 7. Tetrasporophytic fronds accounted for most of the total biomass in all studied months (74.63%), with female and male gametophytes representing 17.10 % and 6.80% of the biomass, respectively. Considering the whole studied period, vegetative fronds were very rare (1.47 %), occurring only in August 2013. We observed the highest frequency of tetrasporophytes in November 2013 (88.79 %) and the lowest in January 2014 (62.82 %), while the occurrence of cystocarpic fronds was lower in October 2013 (7.20 %) and higher in January 2014 (29.85 %). April 2014 was the month with the highest frequency of male fronds (13.95 %).

Thallus' size ranges from 3.64 e 6.77 cm (Table 1). Significant differences were detected throughout the studied period, June being recorded as the month with the smallest fronds' lengths (Table 1, Figure 8). With respect to the mean thallus lengths for the different reproductive stages (Figure 9), tetrasporophytes and female gametophytes reached its maxima on October 2013 and minima on June 2014. Male gametophytes reached its highest lengths on February 2014 and the lowest frond's size on June 2014. Thallus' length of male gametophytes showed positive correlations with air temperature and monthly insolation, and negative correlations with rainfall and relative humidity (Table 2).

Discussion

The analyzed population of "*Hypnea nigrescens*" did not show significant fluctuations in total biomass throughout the year, different from the observed for "*H. musciformis"* on Brazilian coast (Schenkman 1989, Wallner et al. 1992, Faccini and Berchez 2000, Reis and Yoneshigue-Valentin 1998, Caires et al. 2013b). Reis and Yoneshigue-Valentin (1998) related these fluctuations of biomass with environmental factors, although they have pointed out the absence of a seasonal pattern in this variation for populations from the state of Rio de Janeiro. On other hand, data from the coast of Bahia State shows conflicting patterns on the literature: Wallner et al. (1992) reported decreasing of biomass during summer, while Caires et al. (2013b) recorded the highest biomass values occurring during the dry season.

We observed a decline of the total biomass in June 2014, in which occurred the highest rainfall accumulated, however without significant differences. Nonetheless, the total biomass showed a negative correlation with seawater temperature. This also was observed in southeastern and northeastern Brazil (Schenkman 1989, Wallner et al. 1992) to variety *musciformis* and in other tropical and subtropical populations of *Hypnea musciformis* on several locations of the globe (Rao 1970, Guist Jr et al.1982, Kong and Ang Jr 2004). Mshigeni (1977) inferred that the high light intensity and high temperature conditions are among the important factors responsible for the reduction of *Hypnea* populations in summer. Despite this, temperate populations seems to have a different behavior, so that biomass of this alga generally reaches their maximum values during the summer (Friedlander and Zelikovitch 1984, Aziza et al. 2008). Biomass of tetrasporophytes also showed a negative correlation with seawater temperature. As this reproductive phase corresponded to the highest percentage of the total biomass, we expected that one followed the decrease of other.

Regarding the reproductive phenology of "*Hypnea nigrescens*", tetrasporophytes showed the highest biomass proportion observed, followed by female and male gametophytes and by vegetative fronds. According to Cecere et al. (2000), the tetrasporophyte dominance appears to be very common in red algae, which also was generally reported to natural populations of *Hypnea* species (Rangaiah and Rao 1983, Schenkman 1989, Wallner et al. 1992, Faccini and Berchez 2000, Reis and Yoneshigue-Valentin 2000, Kong and Ang Jr. 2004, Mouradi et al. 2008, Caires et al. 2013a). Three mainly hypothesis can explain the predominant occurrence of the tetrasporophytic phase in *Hypnea* populations: (1) tetrasporophytes may produce more upright thalli per plant and are more robust under stressful conditions (Mathielson 1989); (2) this could be resulted from apomeiosis (Reis and Yoneshigue-Valentin 2000, Kong and Ang Jr. 2004); or (3) the populations are maintained itself mainly by vegetative reproduction (Schenkman 1989). This last hypothesis can be the most plausible for the studied population, for two reasons: first, because the investigated population developed under severe wave action, what could cause fragmentation of the fronds and its regeneration in diploid thalli; and second, because normal meiosis seems to occur in the tetrasporangia of *Hypnea* spp. as observed by Schenkman (1986).

Female gametophytes of "*Hypnea nigrescens*" were frequent throughout the studied period. This observation is contradictory to most studies carried out with other *Hypnea* species – especially *H. musciformis* – which reported female plants as rare or absent (Rao 1977, Rangaiah and Rao 1983, Schenkman 1989, Wallner et al. 1992, Reis and Yoneshigue-Valentin 2000, Kong and Ang Jr. 2004, Mouradi et al. 2008, Caires et al. 2013a). Reis and Yoneshigue-Valentin (2000) and Caires et al. (2013a) generally reported the presence of cystocarpic plants in "*H. musciformis*" during the summer. Wallner et al. (1992) interpreted this kind of pattern as a response of the algae to better

light conditions. In this study, we did not confirm this hypothesis for "*H. nigrescens*", once biomass of female gametophytes showed no correlation with insolation. Despite this, we observed a positive correlation of female biomass with air temperature, as well as a negative correlation with relative humidity, indicating that these conditions (usually observed in the summer) could be related with the induction of the fertilization and consequent development of cystocarps.

It is interesting to note that the frequency of female gametophytes increased between January and June 2014, while that tetrasporic plants decreased. The inverse also was observed (July 2014 / August to December 2013), with increase of tetrasporic plants accomplished by decreased of cystocarpic plants. This finding agree with the observations of Rao (1977) for *Hypnea valentiae* from India, who inferred a possible regular alternation of tetrasporic and cystocarpic phases. As we detected male specimens of "*H. nigrescens*", and they followed the fluctuation of the cystocarpic phase, we could extrapolate this alternation to a regular sequence of tetrasporic and sexual phases.

Environments with greater water movement facilitate the meeting of gametes in red algae Searles (1980). Carposporophytic phase is entirely correlated with the rate of fertilization, and the occurrence of female gametophytes (detected by presence of cystocarps) in this study can be related to environment with great exposure to waves (Engel & Destombe 2002, Caires et al. 2013). Reis and Yoneshigue-Valentin (2000) observed differences when compared the phenology of epiphytic and epilithic populations identified as "*H. musciformis*" from Rio de Janeiro, Brazil. These authors verified female gametophytes only on epilithic population, relating its presence to the substratum, degree of exposure to open sea and frequency with which whole plants were emersed/immersed. Knowing the epilithic population studied by Reis and Yoneshigue-

Valentin (2000), we believe that this could refers in fact to what we are treating as "*H. nigrescens*".

We frequently observed male gametophytes during the whole studied period, however with minor biomass. This is a surprising finding, since antheridia were commonly reported as absent or rare in *Hypnea* species (Rao 1977, Rangaiah and Rao 1983, Schenkman 1989, Wallner et al. 1992, Reis and Yoneshigue-Valentin 2000, Kong and Ang Jr. 2004, Mouradi et al. 2008, Caires et al. 2013a). As we noted for female plants, correlations between male biomass and environment variables were characteristic of the dry season. Positive correlations with air temperature and insolation, and negative correlation with relative humidity, reinforce the idea that these factors were acting in synergy, and may have relation with development of gametophytes in "*Hypnea nigrescens*".

Reis and Yoneshigue-Valentin (2000) explain the lack/rarity of male gametophytes in "*H. musciformis*" by the shortest duration of this particular reproductive phase, or by difficulty to recognize them. Schenkman (1986) assigned the difficulty in detecting male specimens, in part, to the absence of descriptions of these structures on bibliographic references. Our recent taxonomic studies carried out on Brazilian coast (Jesus et al. 2013a, b, 2014, 2015) have demonstrated the occurrence of male gametophytes in "*H. nigrescens*" and "*H. valentiae*" among others, confirming that this reproductive stage is much more common that the reported previously. It is noteworthy that, until now, we did not observe them in "*H. musciformis*". In fact, antheridia are hard to distinguish at the first time, but we have provided detailed information so that they may be more easily detected.

Vegetative fronds of "*Hypnea nigrescens*" were found only on August (2013), despite representing a considerable biomass (~20%) on this month's collection. Non-fertile specimens of other *Hypnea* species have been regularly reported, and its presence is attributed to an increase in stressful conditions for plant growth caused by abiotic factors (Reis and Yoneshigue-Valentin 2000). However, August 2013 did not display environmental condition that may be considered as stressful to explain the occurrence of vegetative plants. It is noteworthy that some disturbance may have occurred in the month preceding this collection or that occurrence of vegetative thalli is related to reduction of male plants in this month. The rare incidence of vegetative specimens observed in this study, as well as the frequency of female and male gametophytes, could be an indicative that this population, despite under high hydrodynamic activity, grows in a favorable environment, being able to complete their life cycle and to reproduce sexually.

Concerning to thallus size, we observed significant differences in the smallest heights recorded on June 2014, the highest amount of accumulated rainfall and humidity during this month, as well as low values of air temperature and insolation. These factors, in conjunction with the strong waves observed at the collection site, could contribute to thallus fragmentation (Reis and Yoneshigue-Valentin 2000, Kong and Ang Jr. 2004) and, hence, to the decreased their length. With respect to fronds' size in reproductive stages, thallus length of the tetrasporophytes and female gametophytes showed no correlations with any environment variable recorded. Despite this, male gametophytes' size showed positive correlations with air temperature and insolation and negative correlations with rainfall and humidity, ratifying that the best conditions for the development of this stage were found in the dry season. Cecere et al. (2000) states that gametophytes would be ephemeral; so that they could die soon after reproduction or

could become reproductive when still so small as to be underestimated. Our findings suggested that, at least for genus *Hypnea*, it is not true, once tetrasporophytes and gametophytes displayed similar thallus heights.

Phenological traits might be expected to show little or no relationship with phylogeny because their expression is, for the most part, environmentally determined (Davies et al. 2013). In fact, some of the environmental parameters analyzed seemed to affect the reproductive phenology of "*H. nigrescens*", but it does not mean some form of endogenous regulations is involved in their development. Therefore, if phenology is largely determined by environment, but phenological responses are mediated by biological traits, it is expected that closely related species might then share similar phenologies simply because they grow in – and are adapted to – similar environments (Davies et al. 2013).

We have shown that the phenology of "*Hypnea nigrescens*", when compared with local studies on "*H. musciformis"*, exhibit remarkable differences mainly regarding the expression of sexual phases. Despite being recently reduced to a single genetic species (*H. pseudomusciformis*), these taxa appear to be different not only morphologically but also behaviorally, developmentally and ecologically. Recognizing the factors that determine all these differences remains a major challenge to help in the resolution of the Brazilian species complex. Despite this, we believe that these differences are sufficient to sustain the separation of these taxa in the *H. pseudomusciformis* complex, and propose these these entities are formally accepted at a rank of variety.

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Table 1: Summary of results from Kruskal-Wallis (H) test for the biomass and frond's size of "*Hypnea nigrescens*" on Emissário Beach, Bahia, Brazil among the months studied.

Parameter	(min) mean ± SD (max)	H	*p*
Biomass			
Total	(0.14) 0.35 ± 0.11 (0.50)	15.71	0.15
Tetrasporophyte	(0.21) 0.58 ± 0.21 (0.93)	31.18	0.001
Female gametophyte	(0.07) 0.13 ± 0.07 (0.23)	21.53	0.02
Male gametophyte	(0.01) 0.06 ± 0.04 (0.16)	14.83	0.13
Frond's size			
Total	(3.64) 5.37 ± 0.85 (6.77)	92.79	4.73E-15
Tetrasporophyte	(3.41) 5.44 ± 0.99 (7)	63.70	1.89E-09
Female gametophyte	(3.89) 5.34 ± 0.8 (6.91)	28.56	0.002
Male gametophyte	(4) 5.16 ± 0.96 (6.5)	15.54	0.11

max = maximum value; min = minimum value; *p* = probability; SD = standard deviation

Table 2: Correlations between biomass and frond's size of "*Hypnea nigrescens*" and environmental variables on Emissário Beach, Bahia, Brazil, from August 2013 to July 2014.

	Environmental variable	r value	*p* value
Biomass			
Total	Seawater temperature	-0.69	0.01
Tetrasporophyte	Seawater temperature	-0.65	0.02
Female gametophyte	Air temperature	0.76	0.004
	Relative humidity	- 0.69	0.01
Male gametophyte	Air temperature	0.78	0.004
	Insolation	0.67	0.02
	Relative humidity	- 0.76	0.01
Frond's size			
Male gametophyte	Air temperature	0.70	0.01
	Insolation	0.82	0.002
	Precipitation	-0.65	0.03
	Relative humidity	-0.84	0.001

r = Spearman's rho correlation coefficient, *p* = probability

Figures:

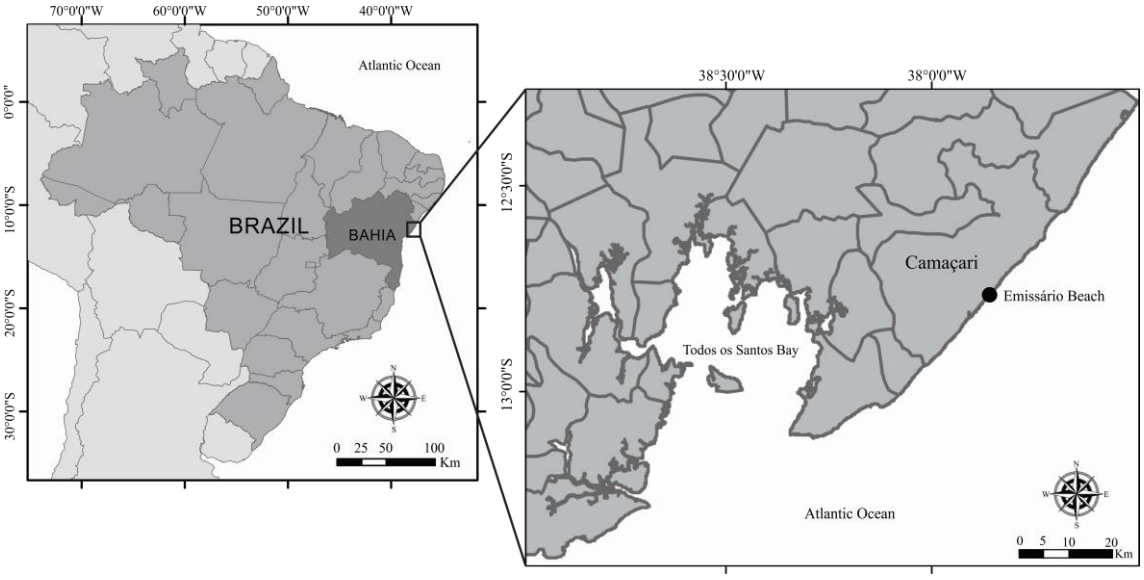
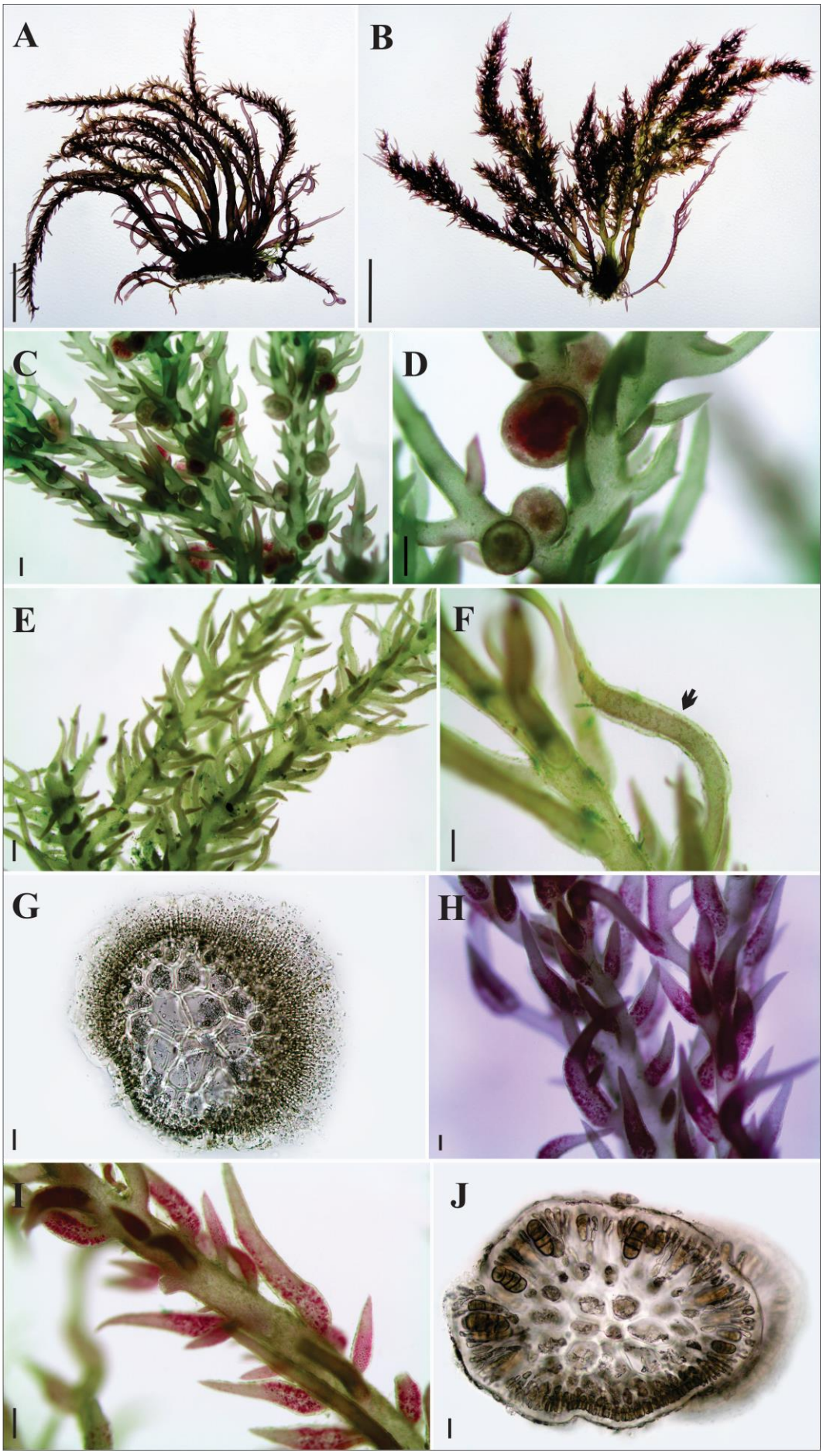


Figure 1: Map showing the study location: Emissário Beach, Bahia State, Brazil.

Figure 2: Morphology and reproduction of "*Hypnea nigrescens*". (A) General aspect of tetrasporophyte. (B) General aspect of female plant. (C) Cystocarps on branches and branchlets. (D) Cystocarps in detail. (E) Spermatangial sori in branchlets. (F) Close up of a spermatangial sori (arrow). (G) Cross section of a branchlet with spermatangia surrounding them incompletely. (H) Tetrasporangial sori in branchlets. (I) Close up of tetrasporangial sori. (J) Cross section of a branchlet with tetrasporangia surrounding them incompletely. Scale bars: A and B, 1 cm; C, 500 μm; D, 300 μm; E, 500 μm; F, 200 μm; G, 100 μm; H, 200 μm; I, 300 μm; J, 100 μm.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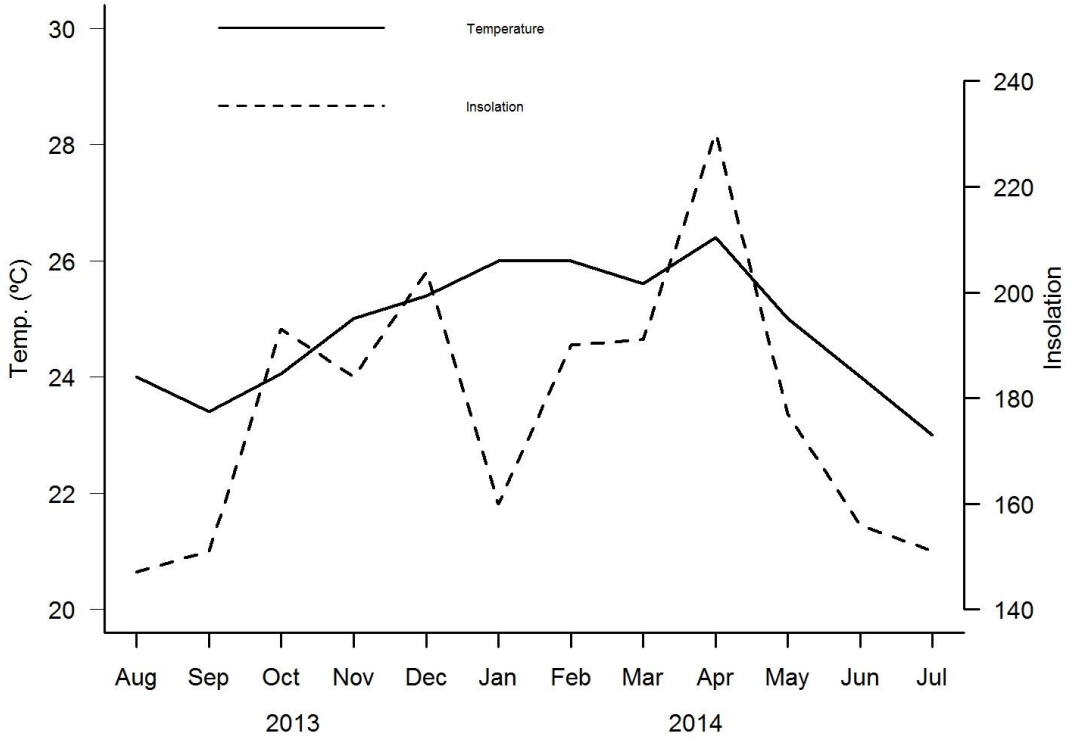

Figure 3: Mean monthly temperature and total insolation on Bahia, Brazil - Environmental variables provided by INMET.

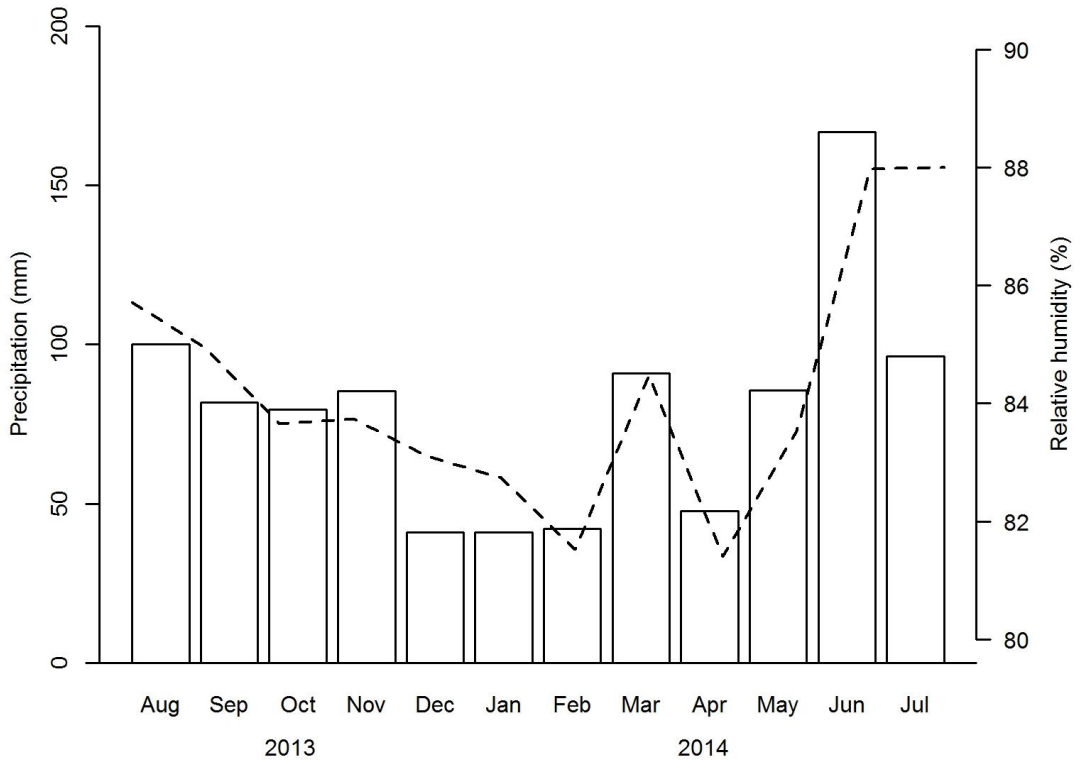

Figure 4: Accumulated rainfall and mean monthly humidity - Environmental variables provided by INMET.

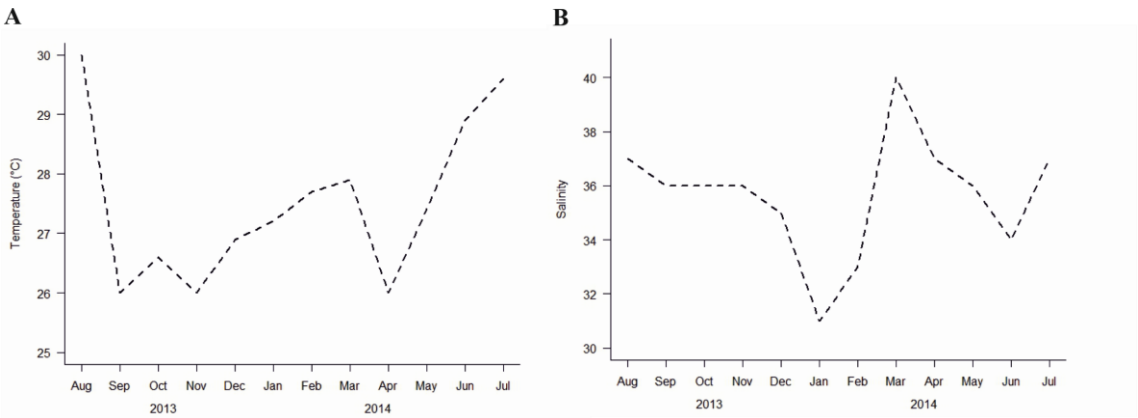

Figure 5: Environmental variables measured on the studied area. (A) Seawater temperature (B) and salinity.

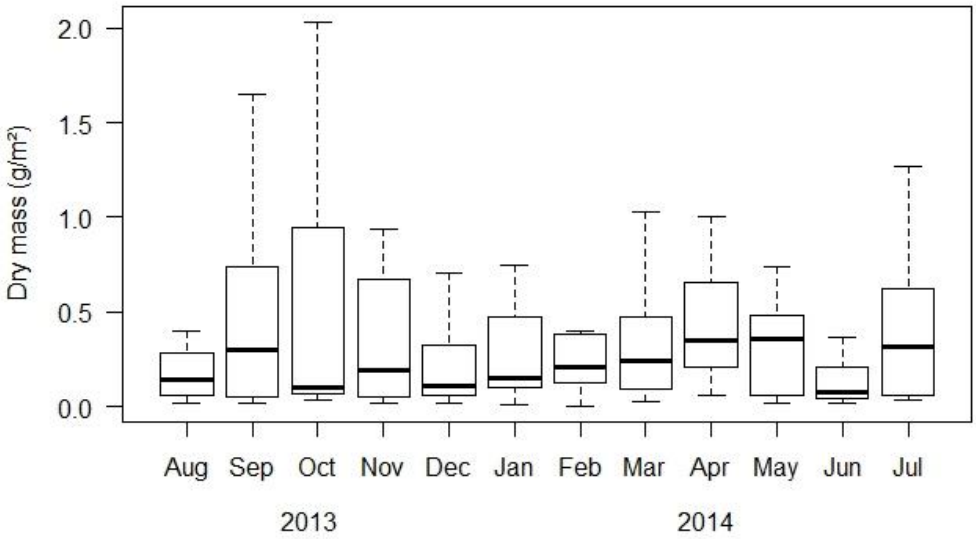

Figure 6: Total biomass (dry mass) of "*Hypnea nigrescens*" at Emissário Beach during the studied period (standard deviations indicated).

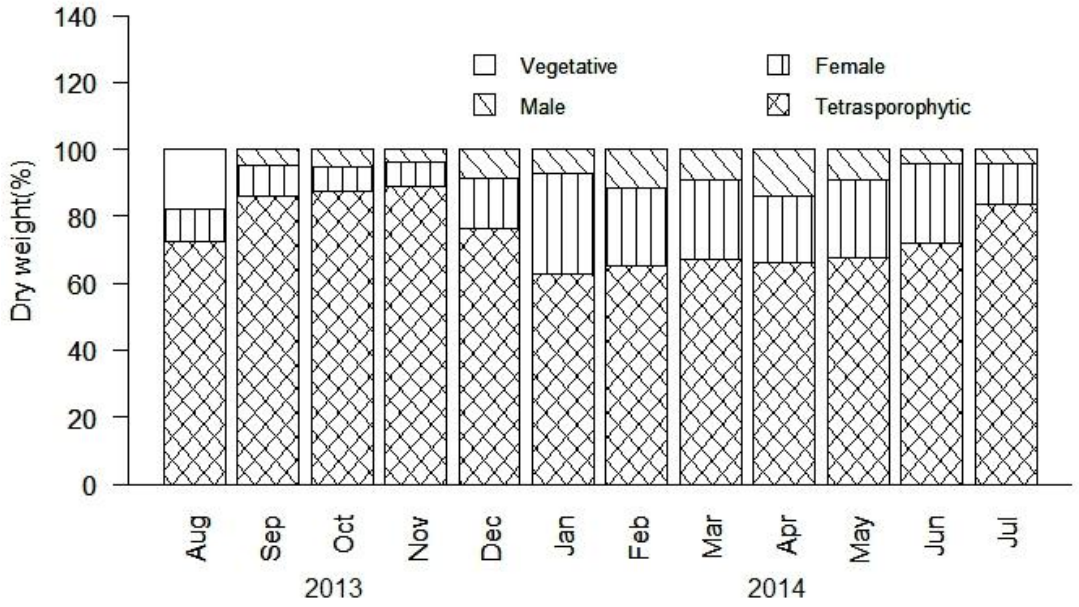

Figure 7: Percentages in biomass (dry mass), corresponding to each reproductive stage (vegetative, tetrasporophytic, female and male gametophytes) of "*Hypnea nigrescens*".

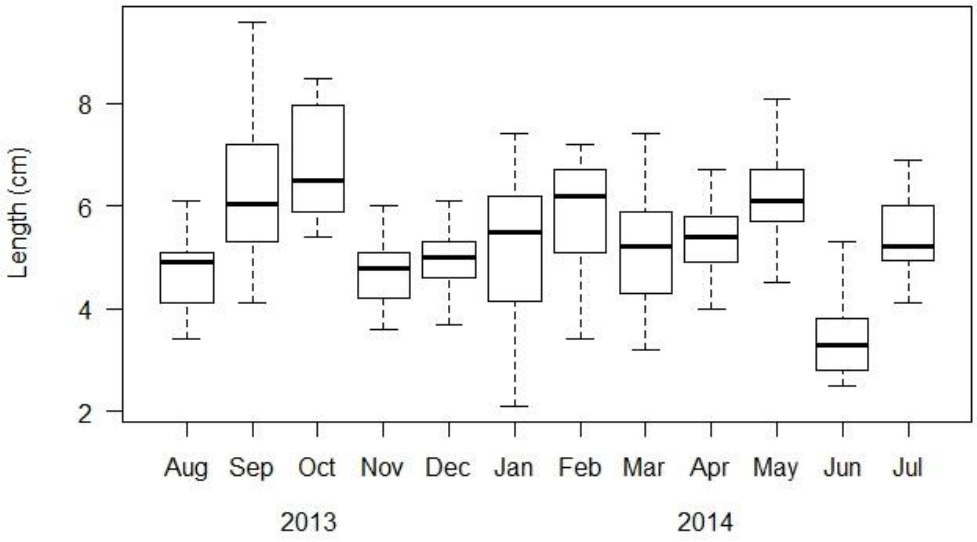

Figure 8. Variation in mean length (standard deviations indicated) of "*Hypnea nigrescens*" during the studied period.

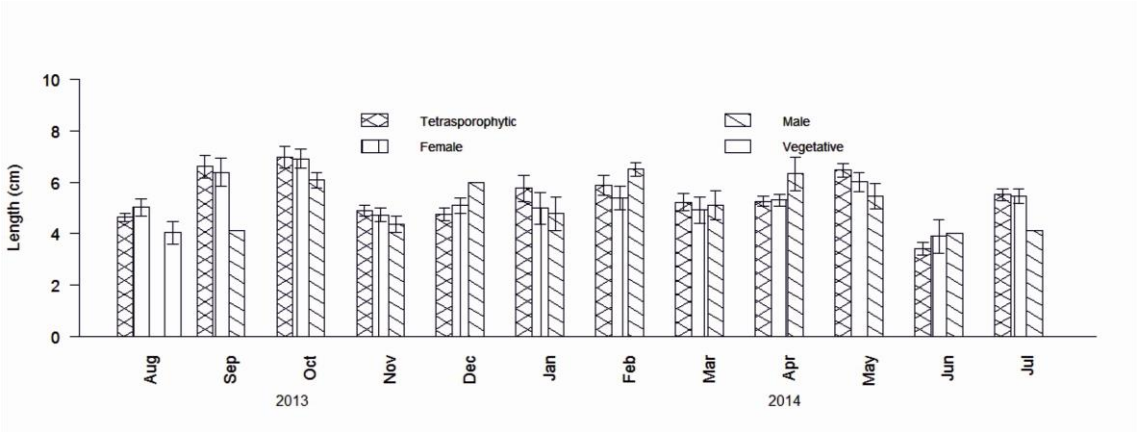

Figure 9. Mean thallus lengths for the different stages (vegetative, tetrasporophytic, female and male gametophytes) of "*Hypnea nigrescens*".

CAPÍTULO 4

PHYLOGEOGRAPHIC PATTERNS IN *HYPNEA MUSCIFORMIS* AND *H. PSEUDOMUSCIFORMIS* (CYSTOCLONIACEAE, RHODOPHYTA): SISTER SPECIES WITH DIFFERENT DEMOGRAPHIC HISTORIES

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Phylogeographic patterns in *Hypnea musciformis* and *H. pseudomusciformis* (Cystocloniaceae, Rhodophyta): sister species with different demographic histories

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Condensed title: Phylogeography of *Hypnea musciformis* and *H. pseudomusciformis.*

Abstract:

In the last years, several studies on the Brazilian coast have revealed a high diversity of the marine benthic flora. Recent discovery of new *Hypnea* species raised questions about how these species have evolved. Here, we investigated the evolutionary history of *H. musciformis* and *H. pseudomusciformis* based on sequences of mitochondrial (COI-5P) and plastidial (*rbc*L) markers. We addressed three main questions: (1) how these lineages evolved; (2) how these populations are structured and what is their demographic history and (3) which processes are driving the actual distribution of these species. We employed several analyses to remake their phylogenetic relationships, population structure and historical demography. Our results splitted the *H. musciformis* clade in two lineages, with interesting distribution patterns: the M1 lineage, with populations from both sides of the Atlantic Ocean, and the M2 lineage, with populations from both sides of the Panama Isthmus. *H. pseudomusciformis* revealed a strong population structure with robust evidences of recent population expansion, contrary to *H. musciformis*, which showed more stable populations. The mitochondrial COI-5P was more sensitive to detect phylogeographic patterns, confirming its suitability for this purpose. Studies are still necessary to estimate the times and divergence rates leading to speciation within the genus *Hypnea*.

Key index words: Barriers, Distribution, Gene Flow, Genetic Diversity, Gigartinales

INTRODUCTION

The number of new algae species described for Brazil during the period between 2010 and 2015 means that around six species were described per year (Menezes et al. 2015), greatly increasing the data on the diversity of this marine benthic flora. In the last three years, molecular data have provided clarifications about the diversity of the genus *Hypnea* J.V. Lamouroux from Brazilian coast, with six new species recently discovered (Jesus et al. 2013, 2016, Nauer et al. 2014, 2015, 2016). These findings raised questions about how *Hypnea* species have evolved, and what would be the processes driving their distribution in South America.

Hypnea musciformis (Wulfen) J.V. Lamouroux is the generitype, well known, and the more characteristic species of the genus (Jesus et al. 2013). According to Nauer et al. (2015), it represents a complex of closely related species rather than a single cosmopolitan species. In fact, epiphyte specimens with numerous tendrils at the apices were, for a long time, identified as *H. musciformis* on the Brazilian coast (eg., Schenkman 1986, Guimarães 2006, Jesus et al. 2013, 2014, Nauer 2014, 2015). Jesus et al. (2015) detected a high degree of genetic divergence between these Brazilian specimens and those from North Atlantic, but interpreted it as intraspecific variation due to isolation by distance. Posteriorly, Nauer et al. (2015) considered this divergence (despite of the close relationship) consistent with interspecific variation, describing a new species *H. pseudomusciformis* Nauer, Cassano & M.C. Oliveira, based on collections from South America.

Phylogeography is a field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those with and among closely related species (Avise 2000). Despite several molecular studies carried out with *Hypnea* species (e.g., Yamagishi and Masuda 2000, Yamagishi et al. 2003,

Geraldino et al. 2006; 2009; 2010, Jesus et al. 2015; 2016, Nauer et al. 2014; 2015; 2016), patterns of population structure and gene flow, historical demography and speciation still remain largely unknown. Geraldino et al. assessed the genetic diversity of *H. asiatica* P.J.L. Geraldino, E.C. Yang & S.M. Boo (2009) and *H. cervicornis* J. Agardh (2015 – as *H. flexicaulis*), although without support of robust analysis.

Despite the evidence's lines demonstrating the genetic divergence of the widespread *Hypnea musciformis* and its morphologically identical sister *H. pseudomusciformis*, their genetic diversity and demography have not been evaluated yet. The aim of the present study was (1) to evaluate how *H. musciformis* and *H. pseudomusciformis* lineages evolved; (2) to investigate their population structure and demographic history and; (3) to suggest the processes driving the actual distribution of these species. Populations of *H. pseudomusciformis* have been broadly sampled throughout the southern Hemisphere (Nauer et al. 2015, Jesus et al. 2015, Ale et al. 2016), allowing us a detailed phylogeographic investigation. Despite biased sampling in *H. musciformis*, we also were able to recover the demographic patterns for this lineage. We employed several analyses to remake their phylogenetic relationships, population structure, summary statistics and historical demography.

MATERIALS AND METHODS

Sampling and molecular procedures

Specimens of *Hypnea pseudomusciformis* were sampled in several locations along the Brazilian coast (Table S1 in the Supporting Information), during low tides. We transported the samples to the laboratory, cleaned, and sorted carefully in a stereomicroscope (Leica® Zoom 2000, Wetzlar, Germany). Three to five pieces of thalli were preserved in silica gel desiccant for DNA extraction. Voucher specimens were

deposited in the herbaria Alexandre Leal Costa Bahia (ALCB) of the Universidade Federal da Bahia, Brazil. Laboratory procedures for extraction and amplification of DNA of the COI-5P and *rbc*L markers followed Jesus et al. (2016). The company GeneWiz (Cambridge, Massachusetts, USA, http://www.genewiz.com/) performed purifications and sequencing reactions of the PCR products.

Sequences' processing

Electropherograms were assembled using Geneious v 6.0.6 (Biomatters, Auckland, New Zealand; Kearse et al. 2012) and edited in BioEdit 5.0.6 (Hall 1999). For each marker, a multiple alignment was generated with the ClustalW tool (Thompson et al. 1994), available in BioEdit 5.0.6 (alignments excluded PCR primer sequences). In addition to sequences generated in this study, sequences from *Hypnea musciformis, H. pseudomusciformis* and other *Hypnea* species were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/, searched on June 2016 - Table S1) and included in the analyses. We deposited all new consensus sequences of COI-5P and *rbc*L in the Barcode of Life Data Systems (http://www.boldsystems.org/) and GenBank (Table S1).

Phylogenetic relationships

For each separate dataset of COI-5P and *rbc*L, clustering trees were performed in MEGA 6.0 (Tamura et al. 2013) using the Neighbor-Joining (NJ) algorithm based on Kimura two-parameter corrected distances (Kimura 1980) with 2000 bootstrapping replicates. Phylogenetic relationships between *Hypnea musciformis* and *H. pseudomusciformis* were estimated using a maximum likelihood (ML) analysis, with a concatenated alignment (COI-5P + *rbc*L). Several *Hypnea* species (*H. brasiliensis* P.B. Jesus, Nauer & J.M.C. Nunes, *H. cervicornis*, *H. caespitosa* P.J.L. Geraldino & S.M. Boo,

H. japonica , *H. nidulans* Setchell, *H. pannosa* J.Agardh, *H. spinella* (C.Agardh) Kützing, *H. viridis* Papenfuss) were used as outgroup, besides *Griffithsia okiensis* Kajimura (*cox*1: EU194973 / *rbc*L: GQ252547) *Gracilaria tenuistipitata* var. *liui* (*cox*1: EF434924 / *rbc*L: EF434906). The ML analysis was conducted using RAxML (Randomized Axelerated Maximum Likelihood, version 7.0.4; Stamatakis 2006) with the GTR GAMMA model. The best model was assessed under MrModeltest 2.3 (Nylander 2008) and selected using the Akaike Information Criterion (AIC), as recommended by Posada and Buckley (2004). The best-scoring ML tree and 500 bootstrap trees were obtained using the rapid hill-climbing algorithm (Stamatakis et al. 2008).

Population structure

We generated median-joining networks (Bandelt et al. 1999) using NETWORK 5.0.0.0 (www.fluxus-engineering.com) for each marker to study the relationships between haplotypes and their geographic distribution. In addition, a Bayesian inference of the genetic structure was accessed using BAPS 5.4 (Bayesian Analysis of Population Structure, Corander et al. 2008) to verify the number of populations without prior information of the sampling location for both datasets, separately. Initially, we assumed mixture model to run the analysis and to determine the most probable number of populations (*k*) given the data. Posteriorly, we used the resulting mixture clustering for an admixture analysis to identify the proportion of each individual's genome assigned to the resulting genetic clusters. We carried out 500 iterations and repeated the admixture analysis 500 times per individual. Each analysis was repeated five times to check for convergence among different runs.

For each major lineage recovered by NETWORK and BAPS, we implemented a hierarchical analysis of molecular variance (AMOVA) in Arlequin v 3.5 (Excoffier &

Lischer 2010) to determine the level of population structure within and among the defined groups. Populations were grouped according to their geographic location and the significance was obtained using 1000 permutations. For this analysis, only localities with at least two individuals were assigned to populations.

We performed a Mantel test for isolation-by-distance (IBD) with 30.000 permutations to assess the relationships between genetic and geographic distances between populations. According to Jensen et al. (2005), IBD could be defined as a decrease in the genetic similarity among populations as the geographic distance between them increases. We calculated uncorrected genetic distances (p-distance) using MEGA 6.0, while geographic distances (minimum coastline distance) were estimated with DIVA-GIS 7.5 (Hijmans et al. 2012). The Web server of the San Diego State University (http://ibdws.sdsu.edu/~ibdws/) was used to run the analyses.

Summary statistics and historical demography

In order to test signs of demographic expansion, Tajima's *D* and Fu's F_s neutrality tests, besides population size change R_2 test (Ramos-Onsins & Rozas 2002) were calculated for each marker using DnaSP 5.10 (Librado & Rozas 2009). The significances were achieved considering 10.000 coalescent simulations. We also estimated haplotype (Hd) and nucleotide (π) diversities with DnaSP 5.10. Historical demographic of the each group furthermore reconstructed by applying the Mismatch distribution analysis (the distribution of the observed pairwise nucleotide site differences, Rozas & Rozas 1999) as implemented in DnaSP 5.10. Populations at equilibrium are expected to show a multimodal distribution of haplotype frequencies, while populations having recently passed through a demographic expansion or a range expansion are predicted to have an unimodal distribution (Fratini et al. 2016).

RESULTS

Phylogenetic relationships between Hypnea musciformis and H. pseudomusciformis

Our COI-5P final alignment comprised 180 sequences of *Hypnea musciformis* and *H. pseudomusciformis* consisting of 465 base pairs (bp), with 54 (12%) variable, and 48 (10%) parsimoniously informative characters. Thirty-three *rbc*L sequences were aligned using 1.355 bp, with 61 (4.5%) variable, and 31 (2.28%) parsimoniously informative characters. NJ trees of COI-5P and *rbc*L (Figure S1 and S2 in the Supporting Information), as well the ML tree from the concatenated two-markers dataset (Figure 1), consistently recovered three major clades and a showed strong geographic structure among the populations. Because lower number of *rbc*L sequences, on concatenated alignment the M2 group was represented by an unique node sharing COI-5P and *rcb*L sequences from the same locality (Barbados, Caribbean).

Population structure

The COI-5P haplotype network revealed three major lineages on the analyzed *Hypnea* species (Figure 2). The first lineage included *H. pseudomusciformis* populations from South America (Brazil and Uruguay) and Africa (Ghana), with 23 haplotypes of high diversity (Hd= 0.8082), and strongly structured in three internal groups. In *H. musciformis*, two unexpected and highly divergent lineages were observed: a phylogroup containing samples from Africa (Namibia), Europe (Italy and France) and North America (USA), with five haplotypes highly diverse (M1 – Hd= 0.9048); and other including three haplotypes of medium diversity (M2 – Hd= 0.5556), with samples from North, Central and South America (Colombia, Mexico, Barbados and Hawaii). On *rbc*L haplotype network, the same lineages were recovered, but with a smaller sampling and internal

geographic structuration (Figure 2). Ten haplotypes were recovered in *H. pseudomusciformis* (Hd= 0.8889), while *H. musciformis* had one and eight haplotypes in two lineages, respectively.

BAPS analyses for COI-5P indicated five genetics groups (k = 5) with the log of marginal likelihood of optimal partition -879.873 (p = 0.01). These clusters were broadly correlated with their taxonomic and geographical distributions as previously recovered as in network analysis: *Hypnea musciformis* into two groups (M1 and M2 lineages) and *H. pseudomusciformis* segregated in three groups: Northeastern Brazil; Southern and Southeastern Brazil and Uruguay; and Ghana (PM1, PM2 and PM3 groups, respectively). Overall, the admixture analysis assigned each individual's genome almost entirely to its original cluster, with only a very little degree of admixture observed between the Northeastern and Southeastern Brazil. On *rbc*L, the BAPS analyses recovered three groups (k=3) as optimal partition, once all samples from *H. pseudomusciformis* were grouped as one genetics group (log of marginal likelihood -598.2851; p = 0.01). The admixture analysis for this marker also revealed genetic admixture between the two lineages of *H. musciformis*.

AMOVA analyses for COI-5P (Table 1) showed that genetic variation of *Hypnea musciformis* and *H. pseudomusciformis* lineages was mainly due to differentiation among regions, with the highest FST values representing the existence of strong genetic structure ($p < 0.001$). Analyses of the designated groups within lineages, revealed differentiation (not always significant) both among regions and within populations. Despite the lower sampling, AMOVA analyses based on *rbc*L significantly revealed that most of the genetic variance was among populations of the two lineages within regions. Nonetheless, significant FST values to *H. musciformis* (0.26818) and *H. pseudomusciformis* (0.63361)

revealed different levels of the structuration, with high gene flow occurring on the former and restrict gene flow on the last.

Based on COI-5P, the Mantel test applied to *Hypnea musciformis* lineages indicated a positive and significant correlation (r = 0.5702 / p = 0.003) between genetic distance and geographical distance to M1 group, while no significant correlation (r = 0.0211 / p = 0.35) was detected to M2 group. To *H. pseudomusciformis*, a positive and significant isolation by distance model (r = 0.5522 / p < 0.0001) was observed (Figure 3A). By analyzing the *rbc*L marker, none significant correlation was detected to *H. musciformis* (r = 0.1076 / p = 0.2595) and *H. pseudomusciformis* (r = 0.3572 / p = 0.0222) (Figure 3B), possible due small sizes samples.

Historical demography

Due to the limited size of the samples in *rbc*L alignment, demographic history analyses were based only on COI-5P, revealing marked differences among populations of *Hypnea musciformis* and *H. pseudomusciformis* (Table 2). Patterns of population expansion was detected in all groups to *H. pseudomusciformis* as evidenced by negative values Tajima's *D* and Fu's F_s, although not always being significant. Neutrality tests applied to *H. musciformis* lineages revealed positive and negative values to each test in each group, not allowing an evaluation of expansion or equilibrium of these populations through these parameters. Surprisingly, population size change tests (R_2), used to evaluate signs of population expansion, were always lower and significant in all groups and to both markers analyzed. To further characterize the expansion pattern, the mismatch distributions were calculated for each group of *Hypnea musciformis* and *H. pseudomusciformis*. In general, *H. musciformis* lineages exhibited bi or multimodal distributions, while groups of *H. pseudomusciformis* (PM1 and PM2) displayed unimodal

distributions (Figure 4). The PM3 group (from Ghana) was composed of only one haplotype, not allowing to calculate the mismatch distributions.

DISCUSSION

The increase in molecular studies on regional scale has allowed us to infer evolutionary patterns on a global scale. Despite lacking morphological variation, *Hypnea musciformis* and *H. pseudomusciformis* showed considerable genetic distinction (Nauer et al. 2015). Starting from recent studies carried out with the genus *Hypnea*, we could investigate the phylogeographic patterns of *Hypnea pseudomusciformis* and their sister *H. musciformis*. Our analyses (NJ and ML trees, haplotype network and BAPS) revealed presence of unexpected three major well-supported and mutually monophyletic lineages within this group.

According to Lessios (2008), sister species represent initially similar genomes placed into separate environments, giving hypotheses about evolutionary divergence and its causes. The disjunct distribution of haplotypes across physical and biogeographical barriers can indicate the direction of speciation in *Hypnea* species. *H. musciformis* distribution is concentrated on North Hemisphere, while *H. pseudomusciformis'* seem to be restricted to the Southern Hemisphere. The Amazon River outflow can be a putative barrier to vertical dispersion between these two species (Horta et al. 2001). Historical geographic and genetic events leading to this differentiation could suggest vicariance as the main factor driving the evolution of these lineages. According to Garbary (2001), the Western Atlantic would strongly influence the African flora, which could explain the presence of *H. musciformis* in Namibia, if this was not introduced by anthropogenic activity. We suggest investigating the *Hypnea* populations from this locality in order to understand their demographic history.

Hypnea musciformis comprised two lineages with interesting distribution patterns. The division of the continent of Laurasia resulted in the separation of Eurasian and North American. Despite this, AMOVA analysis showed that genetic variation in M1 group (Namibia, Italy, France and USA) was due to differentiation within populations (COI-5P) and among populations within groups (*rbc*L), demonstrating occurrence of gene flow among populations (Pearse & Crandall 2004). The positive correlations between genetic and geographic distances on Mantel tests results also confirm this assumption, once isolation-by-distance is expected between populations if gene flow is prevalent but dependent on geographical distance (Zeller et al. 2006). Recent studies also demonstrated occurrence of transatlantic migration of red algae between Europe and North America in *Chondrus crispus* Stackhouse (Hu et al. 2010), and *Mastocarpus stellatus* Stackhouse (Li et al. 2016). Summary statistics was not significant to this group; nevertheless, a multimodal mismatch distribution was an indicative that this population is in equilibrium (Rogers & Harpending 1992, Muangmai et al. 2015).

We founded another intriguing distribution pattern in M2 lineage of *Hypnea musciformis*, with shared haplotype on samples of the two sides of the Panama Isthmus. The complete closure of the Isthmus occurred by 3 mya, definitely separating the waters of the tropical western Atlantic the tropical eastern Pacific (Lessios 2008). Even without statistical significance, all variation found in this group was due to differentiation among regions. Despite small sampling, the isolation-by-distance was not confirmed and a bimodal mismatch distribution was observed on this lineage. This can be resulting from low gene flow (Pearse & Crandall 2004) or constant population size among an old population or an admixture of populations (Hu et al. 2010). This is easily explained by the geographical barrier, indicating that populations of the two sides of Isthmus were connected in past and now presents signals of differentiation. Populations from this

lineage were genetically (phylogeny, p-distances and haplotype network) well differentiated from M1 lineage, despite having no vertical apparent physical barrier between them. Admixture analysis of the assignment of individuals through BAPS assigned each individual's genome to its original cluster in COI-5P, but revealed a degree of admixture in *rbc*L clusters, indicating admixed ancestry in the two *H. musciformis* lineages.

Hypnea pseudomusciformis was recently segregated from *H. musciformis* having its range distribution recovered to Brazilian and Uruguayan coasts (Nauer et al. 2015). Sequences from Ghana (as *H. musciformis* – Ale et al. 2016) were included in our analyses and have confirmed that this species' distribution extends to the coast of Africa. COI-5P molecular divergences (p-distances) between samples from South America and Africa were consistent with expected to intraspecific level in *Hypnea* (Geraldino et al. 2006; 2009; 2010, Nauer et al. 2014; 2015, Jesus et al. 2015; 2016). Despite this, haplotype from this locality was unique, indicating absence of gene flow and differentiation among the amphi-Atlantic populations. Absence of haplotype diversity in the thirty-one sequences from Ghana hindered population structure and historical demography analyses in this group (PM3). Horta et al. (2001) and Hommersand & Fredericq (2003) suggested that Benguela Upwelling Province influences distribution of red algae between South Africa and South America, represented by related species in both. We suggested this system might be one via of dispersion to occidental Africa, explaining the recent distribution of *H. pseudomusciformis* to Ghana. Increase sampling of *Hypnea* species for the southern coast of Africa should help explaining the genetic diversity and demographic history in *H. pseudomusciformis.*

Our findings revealed a strong latitudinal geographic structuration in *Hypnea pseudomusciformis*. Haplotype network and BAPS analyses based on COI-5P indicated

the presence of three genetic groups (PM1 – Northeastern Brazil; PM2 – South, Southeastern Brazil and Uruguay; PM3 – Ghana). The genetic composition observed in the South America group showed a strong geographic structure, but with subtle degree of admixture between Northeastern (PM1) and Southern (PM2) phylogroups, indicating some gene flow. Until now, the State of Bahia, northeastern Brazil, seems to represent the southern limit of distribution of PM1, while PM2 phylogroup occurs starting at Espírito Santo State. These findings corroborate those of Horta et al. (2001) relative to the lower limit of Brazil's tropical region. Regarding the absence of *rbc*L sequences from Ghana, analyses based on this marker consistently grouped all *H. pseudomusciformis* samples in a unique cluster. Due to the lower number of *rbc*L sequences and to the degree of conservation of this marker, it was not so sensitive to rescue population structure of this species. We observed positive and significant correlations on Mantel tests, showing that an isolation-by-distance pattern drives genetic variation on group (Jensen et al. 2005). Negative values of neutrality tests for both South America group are in agreement with a recent demographic expansion. Star-like topologies in haplotype networks, coupled with unimodal mismatch distributions, confirmed this hypothesis. The lowest R_2 values were observed in this *H. pseudomusciformis* lineage, which is expected to recent severe population growth (Ramos-Onsins & Rozas 2002). This pattern is distinct from the one observed to *H. musciformis*, which revealed deeper demographic histories consistent with older relationships and populations in equilibrium.

Concordance between taxonomy-based biogeography and genetic-based phylogeography would indicate a continuum from population isolation to morphological divergence to evolutionary innovation (Bowen et al. 2016). Our evidences suggested that the split between *Hypnea musciformis* and *H. pseudomusciformis* was recent. According to Pante et al. (2015), evaluating this divergence may not be easy when the emergent

lineages are in "grey zone", i.e. the temporal zone of the genealogical network during which two lineages are definitively diverging (de Queiroz 1998). In such cases, a phylogeographic approach can help understanding the evolutionary forces driving this differentiation.

In conclusion, our findings clearly revealed distinct demographic histories to *Hypnea musciformis* and *H. pseudomuciformis*, with the former having more stable populations and the last experiencing a sudden expansion. Isolation-by-distance model drove the transatlantic populations of M1 lineage, whereas the M2 lineage presented major source of their genetic variation among regions. These are strong evidence that populations in each lineage of *H. musciformis* were separated a long time ago, and despite barriers, are in equilibrium. We were not able to get samples from Indian Ocean, which could provide more detail in their evolutionary history, if coupled with a wide sampling in other Pacific areas. The wide sampling in the Southern Hemisphere allowed us to investigate in detail the genetic variation, population structure and demographic history of *H. pseudomusciformis.* Mitochondrial COI-5P was more sensitive to detect phylogeographic patterns, confirming its suitability for this purpose. Studies are still necessary to estimate the times and divergence rates leading to speciation within the genus *Hypnea*.

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Table 1. Hierarchical analysis of molecular variance (AMOVA) for *Hypnea musciformis* and *H. pseudomusciformis*. *H. musciformis* - M1 group: samples from USA, Namibia, Italy and France; M2 group: samples from Colombia, Mexico, Punta Cana, Barbados and Hawaii; *H. pseudomusciformis*: HPM1 group: Samples from northeastern Brazil; HPM2 group: Samples from southern and southeastern Brazil and Uruguay; HPM3 group: Samples from Ghana.

Structure	Among regions			Among populations within regions			Within populations		
	d.f.	% var	FCT	d.f.	% var	FSC	d.f.	% var	Fst
COI-5P									
H. musciformis	1	84.83	0.84835 n.s.	2	8.32	0.54839*	6	6.85	0.93151**
HM1 group	1	25.00	-	-	-	-	2	75.00	0.25000 n.s.
HM2 group	1	100	-	-	-	-	4	0.0	1.0 n.s.
H. pseudomusciformis	2	56.68	0.56684**	36	33.43	0.77187**	76	9.88	0.90119**
HPM1 group	13	59.14	-	-	-	-	22	40.86	0.59136*
HPM2 group	17	42.62	-	-	-	-	32	57.38	0.42621**
HPM3 group	6	0.0	-	-	-	-	22	0.0	0 n.s.
*rbc*L									
H. musciformis	1	-67.32	-0.67316 n.s.	1	94.13	0.56261*	7	73.18	0.26818*
H. pseudomusciformis	1	23.91	0.23912 n.s.	3	39.45	0.51847**	9	36.64	0.63361**

n.s.: not significant; % var: percentage of variance; FCT: genetic variance among regions; FSC: genetic variance among populations within regions; FST: genetic variance within populations; * $p < 0.05$; ** $p < 0.001$.

Table 2. Summary statistics and historical demography estimates for *Hypnea musciformis* and *H. pseudomusciformis* based on COI-5P. *H. musciformis* - M1 group: samples from USA, Namibia, Italy and France; M2 group: samples from Colombia, Mexico, Punta Cana, Barbados and Hawaii; *H. pseudomusciformis*: HPM1 group: Samples from northeastern Brazil; HPM2 group: Samples from southern and southeastern Brazil and Uruguay; HPM3 group: Samples from Ghana.

Lineage / Group	Tajima's *D*	Fu's F_s	R_2	Hd	θπ
H. musciformis					
HM1 group	0.72232 n.s.	-0.282 n.s.	0.20446 **	0.905	0.00799
HM2 group	-1.29379 n.s.	0.824 n.s.	0.21379 **	0.556	0.00275
H. pseudomusciformis					
HPM1 group	-2.30644 **	-6.182 n.s.	0.11349 **	0.345	0.00135
HPM2 group	-1.59779 n.s.	-8.091 n.s.	0.10394 **	0.677	0.00278
HPM3 group	-	-	-	0.0	0.0

n.s.: not significant; * $p < 0.05$; ** $p < 0.001$.

FIGURES

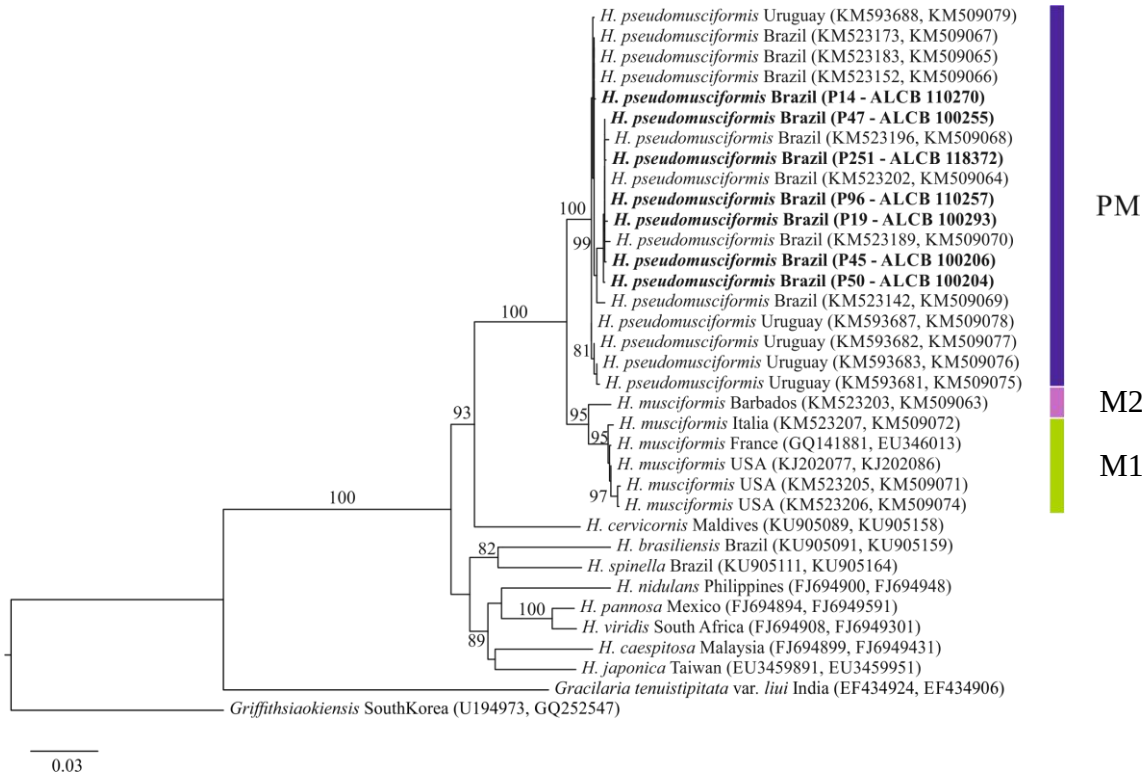


FIGURE 1: The optimal maximum likelihood (ML) topology inferred from the combined dataset of COI-5P and *rbc*L. Support values at each node are bootstrap values > 75. New sequences produced in this study are in boldface, others are from GenBank. Color-coding relates individuals to one of major lineages inferred by haplotype network and BAPS: Green - M1 lineage of *Hypnea musciformis*; Rose – M2 lineage of *H. musciformis;* Blue - lineage of *H. pseudomusciformis* (PM).

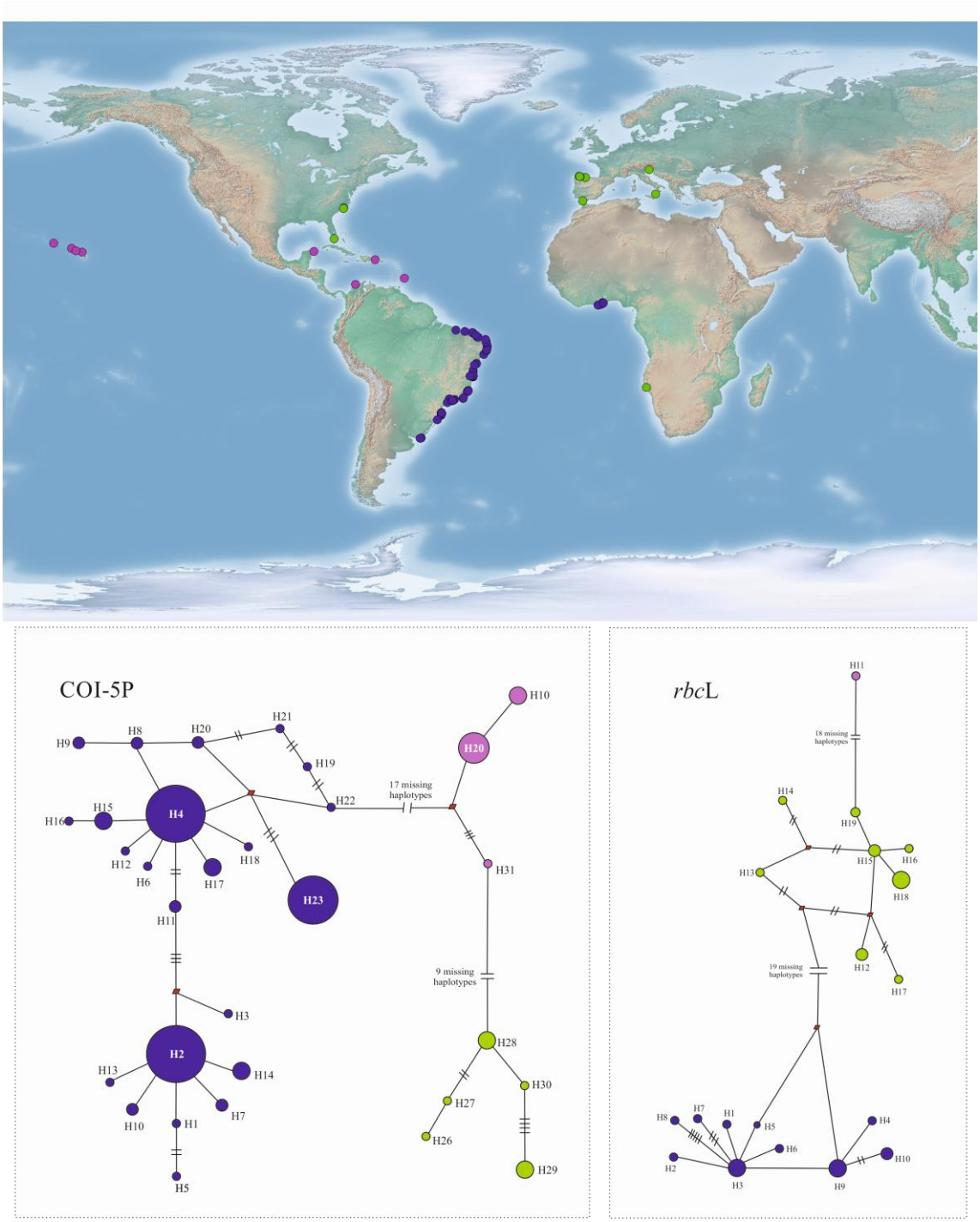


FIGURE 2: Sampling sites of *Hypnea musciformis* and *H. pseudomusciformis* and median-joining network for COI-5P and *rbc*L. Circles sizes reflect haplotype frequency and lines represent single mutational steps. Color-coding are the same in all figures and indicates relates individuals to one of major lineages inferred by haplotype network and BAPS: Green - M1 lineage of *Hypnea musciformis*; Rose – M2 lineage of *H. musciformis;* Blue - lineage of *H. pseudomusciformis* (PM).

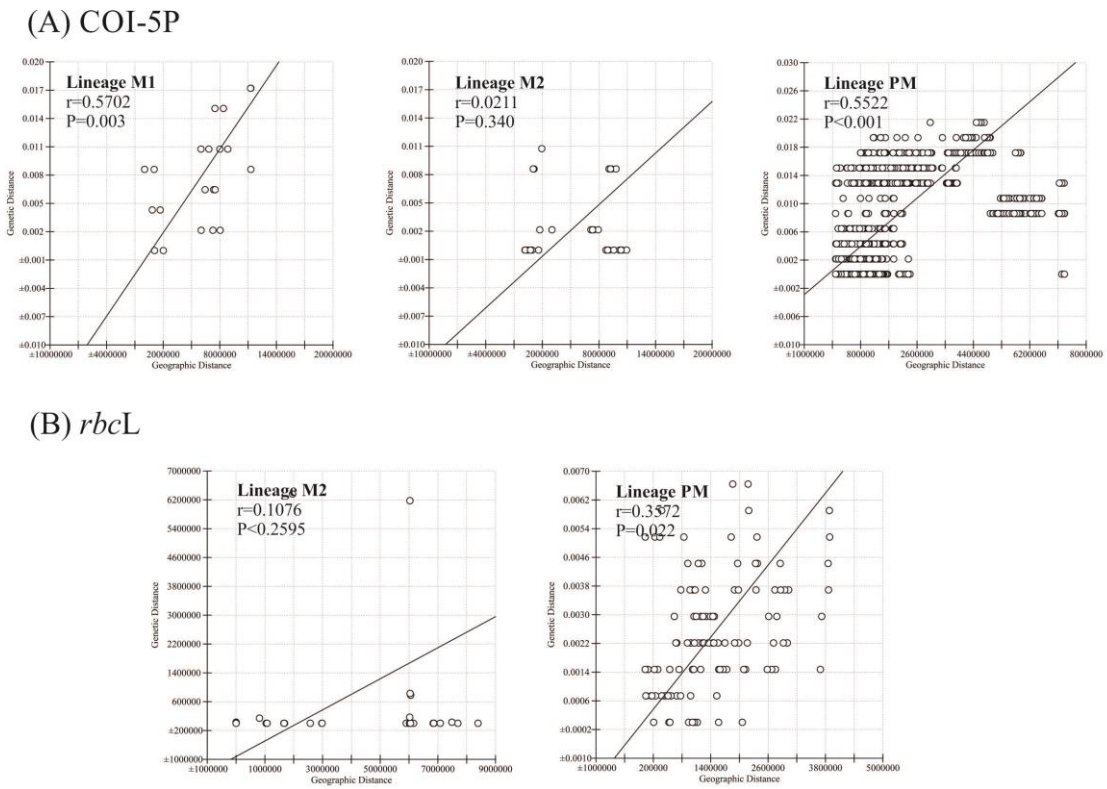


FIGURE 3: Relationship between genetic (uncorrected p) distance and geographical distance (in km) in *Hypnea musciformis* and *H. pseudomusciformis*, based on COI-5P and *rbc*L. The line represents the fitted linear regression. M1 lineage: *H. musciformis* samples from USA, Namibia, Italy and France; M2 lineage: *H. musciformis* samples from Colombia, Mexico, Punta Cana, Barbados and Hawaii; PM lineage: *H. pseudomusciformis* samples from Brazil, Uruguay and Ghana.

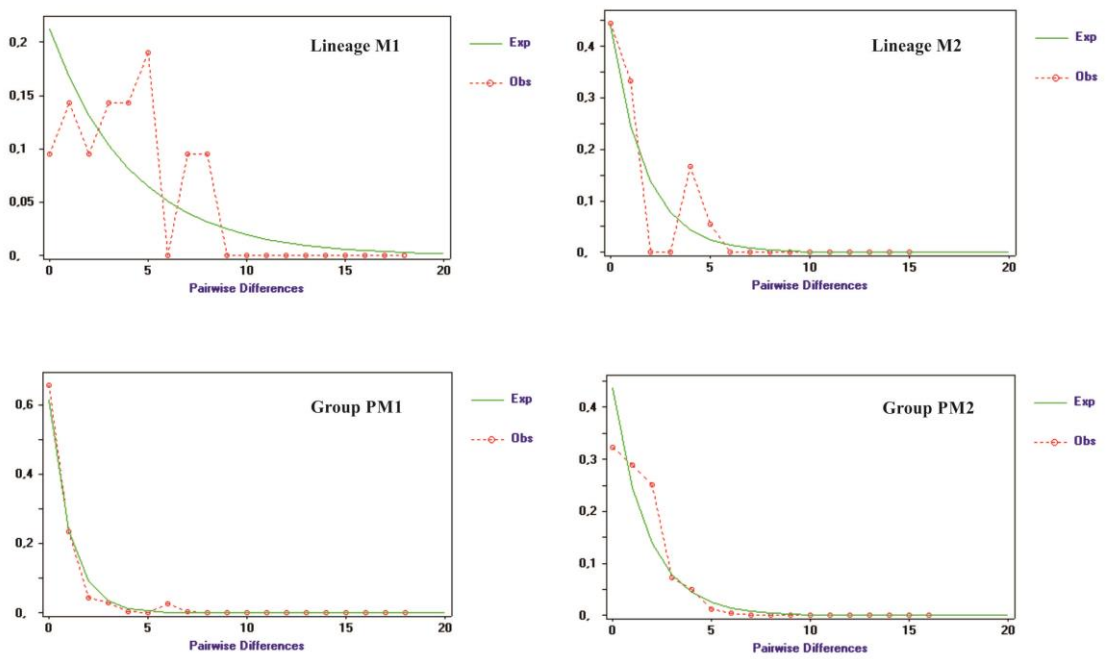


FIGURE 4: Mismatch distribution patterns to *Hypnea musciformis* and *H. pseudomusciformis*. Observed (dotted line) and expected (continuous line) distributions. M1 lineage: *H. musciformis* samples from USA, Namibia, Italy and France; M2 lineage: *H. musciformis* samples from Colombia, Mexico, Punta Cana, Barbados and Hawaii; HPM1 group: samples from northeastern Brazil; HPM2 group: samples from southern and southeastern Brazil and Uruguay.

SUPPLEMENTARY MATERIAL:

Table S1. List of *Hypnea* species analyzed in this study with voucher information and GenBank accession numbers. *Accession numbers (***) will be added to the table before the publication

Taxon	**Voucher information**	**GenBank accessions**	
		COI-5P	***rbc*L**
Hypnea pseudomusciformis	Olho D'água, Maranhão, Brazil, 2°28'16.83"S, 44°13'4.49"W coll. P.B. Jesus et al., 06 Dec 2013		
	Field ID: P185; Voucher: ALCB 110405	***	***
	Field ID: P186; Voucher: ALCB 110406	***	
	Peito de Moça, Luís Correia, Piauí, Brazil. 2°54'14.51"S, 41°32'40.65"W; col. R. Petti, 16 Jan 2013.	KM523224	
	Caucaia, Pacheco, Ceará, Brazil, 3°41'09,18" S, 38°37'57,19" W; col. P.B. Jesus et al., 26 Mar 2013; Field ID: P125; Voucher: ALCB110343	KU905092	
	Caponga, Ceará, Brazil; 4^{0}02'17,88"S, 38^{0}11'35,68"W; col. P.B. Jesus et al		
	Field ID: P190; Voucher: ALCB 110446	***	

	Field ID: P191; Voucher: ALCB 110446	***	
	Field ID: P192; Voucher: ALCB 110447	***	
	Field ID: P194; Voucher: ALCB 110449	***	
	Field ID: P195; Voucher: ALCB 110456	***	
	Ronco, Parcuru, Ceará, Brazil. 3^{0}24’11.22’’ S, 39^{0}01’30.00’’W; col. . F. Nauer. 01 Jun 2012	KM523216	
	Ronco, Parcuru, Ceará, Brazil. 3^{0}24’11.22’’ S, 39^{0}01’30.00’’W; col. . F. Nauer. 01 Jun 2012	KM523188	
	Lagoinha, Paraipaba, Ceará, Brazil. 3^{0}20’42.37’’S, 39^{0}08’09.24’’W; col. . F. Nauer. 02 Jun 2012	KM523189	KM509070
	Lagoinha, Paraipaba, Ceará, Brazil. 3^{0}20’42.37’’S, 39^{0}08’09.24’’W; col. . F. Nauer. 02 Jun 2012	KM523190	
	Lagoinha, Paraipaba, Ceará, Brazil. 3^{0}20’42.37’’S, 39^{0}08’09.24’’W; col. . F. Nauer. 02 Jun 2012	KM523191	
	Flecheiras, Trairí, Ceará, Brazil. 3^{0}13'25.99"S, 39^{0}15'00.63"W; col. F. Nauer. 03 Jun 2012	KM523192	

	Guajiru, Trairí, Ceará, Brazil. $3^0$13’51.70’’ 3S, $39^0$14’38.04’’W; col. F. Nauer. 03 Jun 2012	KM523193	
	Guajiru, Trairí, Ceará, Brazil. $3^0$13’51.70’’$39^0$14’38.04’’W; col. F. Nauer. 03 Jun 2012	KM523194	
	Guajiru, Trairí, Ceará, Brazil. $3^0$13’51.70’’$39^0$14’38.04’’W; col. F. Nauer. 03 Jun 2012	KM523195	
	Mundaú, Trairí, Ceará, Brazil. $3^0$10’44.22’’S, $39^0$22’21.81’’W; col. . F. Nauer. 03 Jun 2012	KM523196	KM509068
	Pedra Rachada, Parcuru, Ceará, Brazil. $3^0$23’57.65’’S, $39^0$00’46.49’’W; col. F. Nauer	KM523197	
	Pedra Rachada, Parcuru, Ceará, Brazil. $3^0$23’57.65’’S, $39^0$00’46.49’’W; col. F. Nauer. 04 Jun 2012	KM523234	
	Ponta Grossa, Icapuí, Ceará, Brazil. $4^0$37’36.24’’S, $37^0$29’57.87’’W; col. F. Nauer. 05 Jun 2012	KM523235	
	Ponta Grossa, Icapuí, Ceará, Brazil. $4^0$37’36.24’’S, $37^0$29’57.87’’W; col. F. Nauer. 05 Jun 2012	KM523236	

Lagoa do Mato, Aracati, Ceará, Brazil. $4^0$37’35.17’’S, $37^0$38’10.06’’W; col. F. Nauer. 05 Jun 2012	KM523237
Canoa Quebrada, Aracati, Ceará, Brazil. $4^0$31’28.62’’S, $37^0$41’58.06’’W; col. F. Nauer. 05 Jun 2012	KM523238
Morro Branco, Beberibe, Ceará, Brazil. $4^0$09’16.08’’S, $38^0$06’30.90’’W; col. F. Nauer. 05 Jun 2012	KM523239
Fontes, Beberibe, Ceará, Brazil. $4^0$10’16.24’’S, $38^0$05’23.78’’W; col. F. Nauer. 06 Jun 2012; Voucher: SPF57569	KM523162
Ponta Negra, Natal, Rio Grande do Norte, Brazil. $5^0$52’55.23’’S, $35^0$09’52.39’’W; col. M.C. Oliveira & E. C. Oliveira; 17 May 2011	KM523162
Ponta Negra, Natal, Rio Grande do Norte, Brazil. $5^0$52’55.23’’S, $35^0$09’52.39’’W; col. M.C. Oliveira & E. C. Oliveira; 17 May 2011	KM523164
Tabatinga, Nísia Floresta, Rio Grande do Norte, Brazil. $6^0$04’12.15’’S, $35^0$05’51.40’’W; col. C. A. Azevedo & R. Menezes; 12 Jan 2013	KM523218
Rio do Fogo, Rio do Fogo, Rio Grande do Norte, Brazil. $5^0$16’32.90’’S, $35^0$22’41.76’’W; col. C. A. Azevedo & R. Menezes, 12 Jan 2013	KM523219

Camurupim, Nísia Floresta, Rio Grande do Norte, Brazil. 6^{0}04’12.15’’S, 35^{0}05’51.40’’W; col. C. A. Azevedo & R. Menezes; 12 Jan 2013	KM523220
Rio do Fogo, Rio do Fogo, Rio Grande do Norte, Brazil. 5^{0}16’32.90’’S, 35^{0}22’41.76’’W; col. C. A. Azevedo & R. Menezes, 12 Jan 2013	KM523230
Rio do Fogo, Rio Grande do Norte, Brazil; 5^{0}16’32.90’’S, 5^{0}22’41.76’’W; col. P.B. Jesus et al.; Field ID:P222	KP725279
Cabo Branco, João Pessoa. Paraíba, Brazil. 7^{0}08’44.75’’S, 34^{0}48’28.90’’W; col. F. Nauer; 18 Jul 2012	KM523240
Carapibus, Conde, Paraíba, Brazil. 7^{0}17’59.63’’S, 34^{0}47’55.31’’W; col. F. Nauer; 19 Jul 2012	KM523242
Coqueirinhos, Conde, Paraíba, Brazil. 7^{0}19’18.86’’ S, 34°47’52.28’’W; col. F. Nauer; 19 Jul 2012	KM523241
Tambaba, Conde, Paraíba, Brazil.; 7^{0}21’54.72’’S, 34^{0}47’52.79’’W; col. F. Nauer & C. A. Azevedo; 20 Jul 2012;	KM523200
Tambaba, Conde, Paraíba, Brazil.; 7^{0}21’54.72’’S, 34^{0}47’52.79’’W; col. F. Nauer & C. A. Azevedo; 20 Jul 2012;	KM523217

	Tambaba, Conde, Paraíba, Brazil.; 7^{0}21’54.72’’S, 34^{0}47’52.79’’W; col. F. Nauer & C. A. Azevedo; 20 Jul 2012;	KM523243	
	Tambaba, Conde, Paraíba, Brazil.; 7^{0}21’54.72’’S, 34^{0}47’52.79’’W; col. F. Nauer & C. A. Azevedo; 20 Jul 2012;	KM523298	
	Tambaba, Conde, Paraíba, Brazil.; 7^{0}21’54.72’’S, 34^{0}47’52.79’’W; col. F. Nauer & C. A. Azevedo; 20 Jul 2012;	KM523299	
	Ponta do Seixas, João Pessoa, Paraíba, Brazil; 7°8'47.05"S, 34°47'52.01" W; col. P.B. Jesus et al.;		
	Field ID: P97	KP725278	
	Field ID: P96, Voucher: ALCB 110257	***	***
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; 8^{0}18’13.10’’S, 34^{0}56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523160	
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; 8^{0}18’13.10’’S, 34^{0}56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523161	
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; 8^{0}18’13.10’’S, 34^{0}56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523226	

	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; $8^0$18’13.10’’S, $34^0$56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523227	
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; $8^0$18’13.10’’S, $34^0$56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523228	
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; $8^0$18’13.10’’S, $34^0$56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523229	
	Cabo de Santo Agostinho, Recife, Pernambuco, Brazil; $8^0$18’13.10’’S, $34^0$56’35.99’’W col. N. S. Yokoya & S. M.P.B. Guimarães; 27 Sep 2011	KM523225	
	Toquinho, Ipojuca, Pernambuco, Brazil; $8^0$36’12.37’’S,$35^0$02’42.25’’W; col. N. S. Yokoya; 13 Mar 2013	KM523223	
	Francês, Marechal Deodoro, Alagoas, Brazil; $9^0$46’07.79’’S, $35^0$50’17.44’’W; col. T. Vieira-Pinto, C. A. Azevedo & B. Torrano-Silva; 10 Mar 2013	KM52322	
	Imbassai, Bahia, Brazil; 12°30'11,10"S, 37°57'36,36"W; col. P.B. Jesus et al.; Field ID:P19 ; Voucher: ALCB100293	***	KP725289
	Gravatá, Bahia, Brazil; 14°55'51"S, 39°00'55"W, col. P.B. Jesus et al.; Field ID:P45; Voucher: ALCB 100206	***	***

	Gravatá, Bahia, Brazil; 14°55'51"S, 39°00'55"W, col. P.B. Jesus et al.; Field ID:P47; Voucher: ALCB 100255	***	
	Serra Grande, Bahia, Brazil; 14°28'39" S, 39°01'48"W, col. P.B. Jesus et al.; Field ID:P50; Voucher: ALCB 100204	***	***
	Praia do Forte, Bahia, Brazil; 12°34'42,33"S, 38°0'6,45"W; col. P.B. Jesus et al.; Field ID:P117; Voucher: ALCB110262	***	
	Penha, Bahia, Brazil; 12°59'08,76"S, 38°37'02,80"W; col. P.B. Jesus et al.; Field ID: P251; Voucher: ALCB118372	***	***
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; $16^0$19’57.02’’S, $39^0$00’13.59’’W; col.F. Nauer & C. A. Azevedo; 15 Sep 2012	KM523202	KM509064
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; $16^0$19’57.02’’S, $39^0$00’13.59’’W; col.F. Nauer & C. A. Azevedo; 15 Sep 2012	KM523209	
	Coroa Vermelha, Santa Cruz de Cabrália, Bahia, Brazil; $16^0$19’57.02’’S, $39^0$00’13.59’’W; col.F. Nauer & C. A. Azevedo; 15 Sep 2012	KM523210	
	Ponta Grande, Porto Seguro, Bahia, Brazil; $16^0$22’33.05’’S, $39^0$00’33.86’’W; col. F. Nauer & C. A. Azevedo; 16 Sep 2012	KM523201	

Tartaruga, Santa Cruz de Cabrália, Bahia, Brazil; 16^{0}13’42.83’’S, 39^{0}59’51.70’’W; col. F. Nauer & C. A. Azevedo; 17 Sep 2012	KM523212
Guaiú, Santa Cruz de Cabrália, Bahia, Brazil; 16^{0}09’22.85’’S, 38^{0}56’53.80’’W; col. F. Nauer & C. A. Azevedo; 17 Sep 2012	KM523211
Guaiú, Santa Cruz de Cabrália, Bahia, Brazil; 16^{0}09’22.85’’S, 38^{0}56’53.80’’W; col. F. Nauer & C. A. Azevedo; 17 Sep 2012	KM523213
Apuã, Santa Cruz de Cabrália, Bahia, Brazil; 16^{0}16’56.48’’S, 39^{0}01’15.62’’W; col. F. Nauer & C. A. Azevedo; 17 Sep 2012	KM523214
Mucugê, Arraial D’Ajuda, Bahia, Brazil; 16^{0}30’09.69’’S, 39^{0}04’17.62’’W; col. F. Nauer & C. A. Azevedo; 18 Sep 2012	KM523215
Mar Grande, Ilha de Itaparica, Bahia, Brazil; 12°58'00,70"S, 38°36'30,79" W; col. P.B. Jesus et al.; Field ID:P57	KP725277
Guarapari, Espirito Santo, Brazil; 20°43'40.86"S, 40°31'19.32"W; col. P.B. Jesus et al.; Field ID:P18	***
Castelhanos, Anchieta, Espirito Santo, Brazil; 20^{0}50’04.18’’S, 40^{0}37’21.57’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 05 May 2012	KM523182

	Itaoca, Itapemirim, Espirito Santo, Brazil; 20^{0}54’20.32’’S, 40^{0}46’42.72’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 06 May 2012	KM523183	KM509065
	Ponta das Raias, Marataízes, Espirito Santo, Brazil; 21^{0}01’57.43’’S, 40^{0}48’44.43’’W;col. F. Nauer, C. Iha & B. Torrano-Silva; 07 May 2012	KM523184	
	Namorados, Guarapari, Espirito Santo, Brazil; 20^{0}40’19.65’’S, 40^{0}29’47.22’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 08 May 2012	KM523187	
	Setiba, Guarapari, Espirito Santo,Brazil; 20^{0}38’06.83’’S, 40^{0}26’13.25’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 08 May 2012	KM523186	
	Ponta das Raias, Marataízes, Espirito Santo, Brazil; 21^{0}01’57.43’’S/40^{0}48’44.43’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 07 May 2012	KM523185	
	Prainhas do Atalaia; Arraial do Cabo; Rio de Janeiro; Brazil; 22^{0}59’17.09’’S, 42^{0}00’46.14’’W; col. F. Nauer, C. Iha & B. Torrano-Silva. 28 Sep 2011	KM523151	
	Prainha, Arraial do Cabo, Rio de Janeiro, Brazil; 22^{0}57’22.06’’S, 42^{0}01’36.30’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523135	
	Forno, Búzios, Rio de Janeiro, Brazil; 22^{0}45’42.56’’S, 41^{0}52’29.11’’W; col.. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523138	

	Forno, Búzios, Rio de Janeiro, Brazil; 22^{0}45’42.56’’S, 41^{0}52’29.11’’W; col.. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523137	
	Forno, Búzios, Rio de Janeiro, Brazil; 22^{0}45’42.56’’S, 41^{0}52’29.11’’W; col.. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523136	
	Ferradura, Búzios, Rio de Janeiro, Brazil; 22^{0}46’09.62’’S, 41^{0}52’59.81’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523139	
	Brava, Búzios, Rio de Janeiro, Brazil; 22^{0}45’14.35’’S, 41^{0}52’24.45’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 27 Sep 2011	KM523142	KM509069
	Brava, Búzios, Rio de Janeiro, Brazil; 22^{0}45’14.35’’S, 41^{0}52’24.45’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 27 Sep 2011	KM523141	
	Ferradura, Búzios, Rio de Janeiro, Brazil; 22^{0}46’09.62’’S, 41^{0}52’59.81’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 25 Sep 2011	KM523140	
	Brava, Búzios, Rio de Janeiro, Brazil; 22^{0}45’14.35’’S, 41^{0}52’24.45’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 27 Sep 2011	KM523143	
	Ponta do Costa, Saco do Mamanguá, Paraty, Rio de Janeiro, Brazil; 23^{0}15’32.17’’S, 44^{0}37’16.67’’W; col. F. Nauer, C. Iha & B. Torrano-Silva. 10 Dec 2011	KM523145	

	Carro, Saco do Mamanguá, Paraty, Rio de Janeiro, Brazil; 23^{0}14’21.95’’S, 44^{0}37’06.85’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 10 Dec 201	KM523144	
	Itaoca, Pouso da Cajaíba, Paraty, Rio de Janeiro, Brazil; 23^{0}16’11.50’’S, 44^{0}34’29.07’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 11Dec 2011	KM523148	
	Itaoca, Pouso da Cajaíba, Paraty, Rio de Janeiro, Brazil; 23^{0}16’11.50’’S, 44^{0}34’29.07’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 11Dec 2011	KM523147	
	Itaoca, Pouso da Cajaíba, Paraty, Rio de Janeiro, Brazil; 23^{0}16’11.50’’S, 44^{0}34’29.07’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 11Dec 2011	KM523146	
	Praia Grande, Pouso da Cajaíba, Paraty, Rio de Janeiro, Brazil; 23^{0}15’49.31’’S, 44^{0}35’04.43’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 11Dec 2011	KM523150	
	Praia Grande, Pouso da Cajaíba, Paraty, Rio de Janeiro, Brazil; 23^{0}15’49.31’’S, 44^{0}35’04.43’’W; col. F. Nauer, C. Iha & B. Torrano-Silva; 11Dec 2011	KM523149	
	Cigarras, São Paulo, Brazil; 23°43'55.63"S, 45°23'55.31"W; col. P.B. Jesus et al.; Field ID:P114; Voucher: ALCB110270	***	KP725288
	Cigarras, São Sebastião, São Paulo, Brazil; 23°43'55.63"S, 45°23'55.31"W; col. P.B. Jesus et al.; Field ID:P115	KP725276	

	Ilha das Palmas, Ubatuba, São Paulo, Brazil; 23^{0}32’45.30’’S, 45^{0}01’44.85’’W; col. A. Medeiros, C. Iha & B. Torrano-Silva; 29 Aug 2011	KM523152	KM509066
	Ilha das Cabras, Ubatuba, São Paulo; Brazil; 23^{0}31’01.41’’S, 45^{0}02’29.36’’ W; col. A. Medeiros, C. Iha & B. Torrano-Silva;. 30 Aug 2011	KM523153	
	Lagoinha; Ubatuba; São Paulo; Brazil; 23^{0}31’20.35’’S, 45^{0}11’26.65’’W; col. F. Nauer & F. Kino; 22 Oct 2011	KM523155	
	Cigarras, São Sebastião, São Paulo, Brazil; 23^{0}31’20.35’’S, 45^{0}11’26.65’’W; col. L. Ayres; 16 Jun 2011	KM523163	
	Jabaquara, Ilha Bela, São Paulo, Brazil; 23^{0}44’07.14’’S, 45^{0}17’36.48’’W; col. M. T. Fujii; 20 May 2008	KM523154	
	Cibratel, Itanhaém, São Paulo; Brazil; 24^{0}12’05.68’’S, 46^{0}48’38.72’’W; col. M. T. Fujii; 30 Oct 2001	KM523159	
	Praia do Éden, Guarujá, São Paulo, Brazil; 23^{0}59’05.10’’S, 46^{0}11’11.64’’W; col. F. Nauer, A. Medeiros & C. Iha. 12 Sep 2011	KM523158	
	Praia do Éden, Guarujá, São Paulo, Brazil; 23^{0}59’05.10’’S, 46^{0}11’11.64’’W; col. F. Nauer, A. Medeiros & C. Iha. 12 Sep 2011	KM523157	

	Praia do Éden, Guarujá, São Paulo, Brazil; 23^{0}59’05.10’’S, 46^{0}11’11.64’’W; col. F. Nauer, A. Medeiros & C. Iha. 12 Sep 2011	KM523156	
	Prainha, Florianópolis, Santa Catarina, Brazil; 27°34'36.20"S, 48°25'14.51"W; col. M. C. Oliveira & E. C. Oliveira; 11 Oct 2011	KM523165	
	Praia do Molhe, Florianópolis. Santa Catarina, Brazil; 27°34'25.64"S, 48°25'21.27"W, col. M. C. Oliveira & E. C. Oliveira; 11 Oct 2011	KM523169	
	Praia do Molhe, Florianópolis. Santa Catarina, Brazil; 27°34'25.64"S, 48°25'21.27"W, col. M. C. Oliveira & E. C. Oliveira; 11 Oct 2011	KM523168	
	Prainha, Florianópolis, Santa Catarina, Brazil; 27°34'36.20"S, 48°25'14.51"W; col. M. C. Oliveira & E. C. Oliveira; 11 Oct 2011	KM523167	
	Praia do Molhe, Florianópolis. Santa Catarina, Brazil; 27°34'25.64"S, 48°25'21.27"W, col. M. C. Oliveira & E. C. Oliveira; 11 Oct 2011	KM523166	
	Ponta das Canas, Florianópolis, Santa Catarina; Brazil; 27°23'38.79"S , 48°26'08.75"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 26 Feb 2012	KM523173	KM509067
	Ponta das Canas, Florianópolis, Santa Catarina; Brazil; 27°23'38.79"S , 48°26'08.75"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 26 Feb 2012	KM523172	

	Sambaqui, Florianópolis, Santa Catarina; Brazil; 27°29'24.55"S , 48°32'18.89" W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 26 Feb 2012	KM523171
	Armação, Florianópolis, Santa Catarina, Brazil; 27°44'58.70"S, 48°30'00.12"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 26. Feb 2012	KM523170
	Ganchos de Fora, Governador Celso Ramos, Santa Catarina, Brazil; 27°18'21.82"S, 48°32'49.83"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 27 Feb 2012	KM523176
	Palmas, Governador Celso Ramos, Santa Catarina, Brazil; 27°18'47.90"S, 48°32'23.01"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 27 Feb 2012	KM523175
	Laranjeiras, Balneário Camburiu, Santa Catarina, Brazil; 27°18'46.30"S, 48°34'41.93"W; col. F. Nauer, C. Azevedo & B. Torrano-Silva; 28 Feb 2012	KM523180
	Laranjeiras, Balneário Camburiu, Santa Catarina, Brazil; 27°18'46.30"S, 48°34'41.93"W; col. F. Nauer, C. Azevedo & B. Torrano-Silva; 28 Feb 2012	KM523179
	Laranjeiras, Balneário Camburiu, Santa Catarina, Brazil; 27°18'46.30"S, 48°34'41.93"W; col. F. Nauer, C. Azevedo & B. Torrano-Silva; 28 Feb 2012	KM523178

	Ganchos de Fora, Governador Celso Ramos, Santa Catarina, Brazil; 27°18'21.82"S, 48°32'49.83"W; col. F. Nauer, C. Azevedo, M. Sissini & B. Torrano-Silva; 27 Feb 2012	KM523177
	Bombas, Bombinhas, Santa Catarina, Brazil; 27°08'42.44"S, 48°29'57.47"W; col. F. Nauer, C. Azevedo & B. Torrano-Silva; 28. Feb 2012	KM523181
	Praia da Cal, Rio Grande do Sul, Brazil; 29°20'41.5"S, 49°43'35.8"W; col. P.B. Jesus et al.; Field ID: P227; Voucher: ALCB 114354	***
	Prainha, Rio Grande do Sul, Brazil; col. P.B. Jesus et al.;	
	Field ID: P226, voucher: ALCB114359	***
	Field ID: P224, voucher: ALCB114362	***
	Field ID: P223, voucher: ALCB114363	***
	Praia Grande, Rio Grande do Sul, Brazil; col. P.B. Jesus et al	
	Field ID: P222, voucher: ALCB114371	***
	Field ID: P221, voucher: ALCB114369	***
	Field ID: P220, voucher: ALCB114370	***

	Los Ingleses Beach, Punta del Este, Maldnonado, Uruguay; 34°58'03.02"S, 54°56'48.39"W; col. F. Nauer; 20 Mar 2014	KM593681	KM509075
	Los Ingleses Beach, Punta del Este, Maldnonado, Uruguay; 34°58'03.02"S, 54°56'48.39"W; col. F. Nauer; 20 Mar 2014	KM593682	
	Los Ingleses Beach, Punta del Este, Maldnonado, Uruguay; 34°58'03.02"S, 54°56'48.39"W; col. F. Nauer; 20 Mar 2014	KM593683	
	El Emir Beach, Punta del Este, Maldnonado, Uruguay; 34°57'46.60"S, 54°56'25.64"W; 34°57'46.60"S / 54°56'25.64"W; col. F. Nauer. 20 Mar 2014	KM593684	
	El Emir Beach, Punta del Este, Maldnonado, Uruguay; 34°57'46.60"S, 54°56'25.64"W; 34°57'46.60"S / 54°56'25.64"W; col. F. Nauer. 20 Mar 2014	KM593685	
	Brava Beach. Jose Ignacio, Maldonado,Uruguay; 34°50'45.39"S, 54°37'57.53"W; col. F. Nauer, 21 Mar 2014	KM593688	KM509079
	Brava Beach. Jose Ignacio, Maldonado,Uruguay; 34°50'45.39"S, 54°37'57.53"W; col. F. Nauer, 21 Mar 2014	KM593687	
	Brava Beach. Jose Ignacio, Maldonado,Uruguay; 34°50'45.39"S, 54°37'57.53"W; col. F. Nauer, 21 Mar 2014	KM593686	
	Mighty Beach, Gahna; 5°36'29.9"S, 0°03'20.5"W	KT899516	

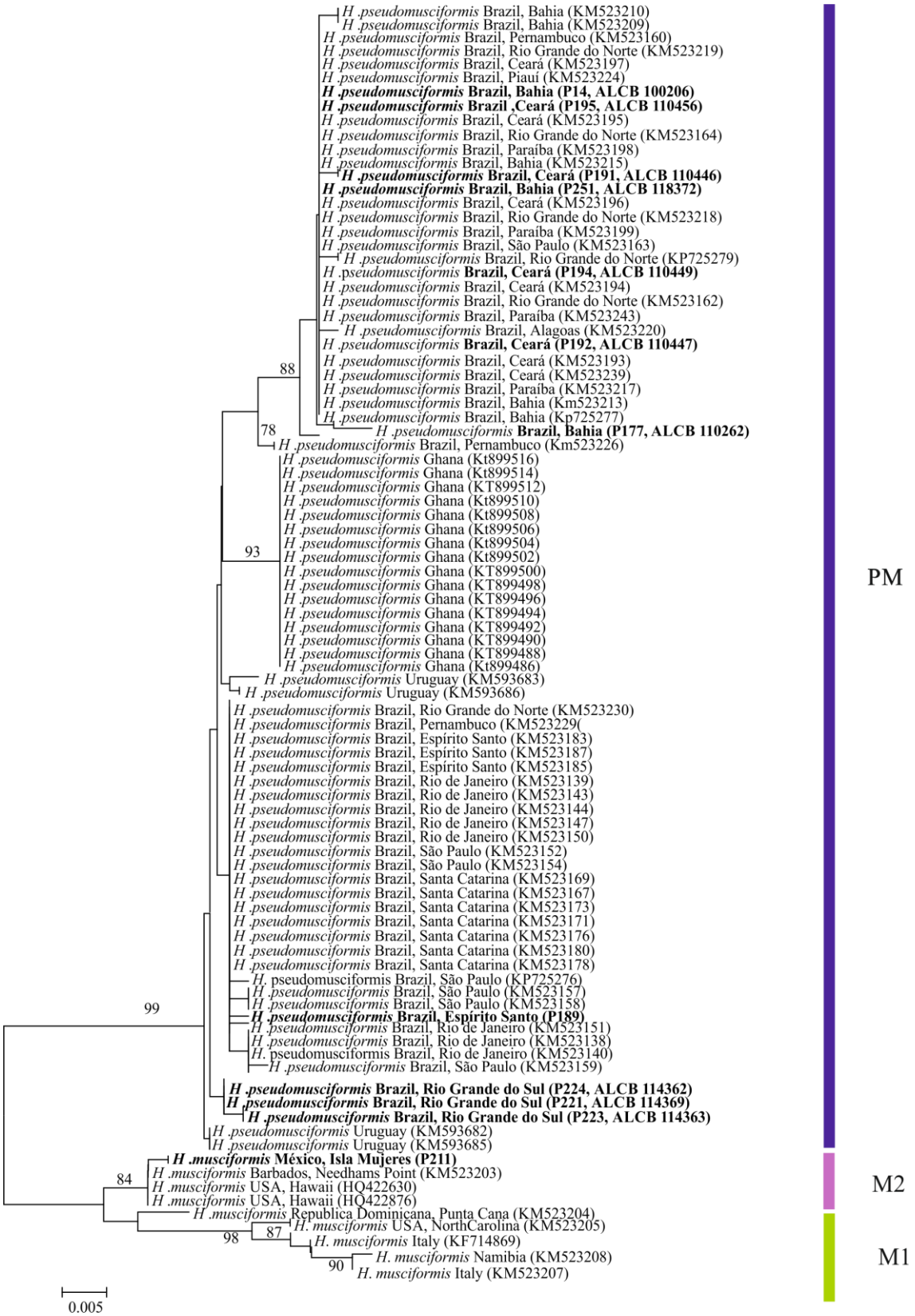
Tema New Town, Ghana, Africa, 5°39'03.2"S, 0°02'01.4"W	KT899515
Ahwiam, Ghana, Africa; 5°45'00.0"S, 0°14'00.0"W	KT899514
Old Ningo, Ghana, Africa; 5°44'38.8"S, 0°11'39.3"W	KT899513
Prampram, Ghana, Africa; 5°42'18.2"S , 0°07'32.3"W	KT899512
Komenda; Ghana, Africa; 5°02'54.7"S, 1°29'10.3" W	KT899511
Apam, Ghana, Africa; 5°17'37.2"S, 0°43'44.4"W	KT899510
Mumford, Ghana, Africa; 5°15'51.0"S, 0°45'16.3" W	KT899509
Shama, Ghana, Africa; 5°01'08.9"S, 1°37'27.8"W	KT899508
Apam, Ghana, Africa; 5°17'37.2"S, 0°43'44.4"W	KT899507
Apam, Ghana, Africa; 5°17'37.2"S, 0°43'44.4"W	KT899506
Apam, Ghana, Africa; 5°17'37.2"S, 0°43'44.4"W	KT899505
Old Ningo, Ghana, Africa; 5°44'38.8"S, 0°11'39.3"W	KT899504
Old Ningo, Ghana, Africa; 5°44'38.8"S, 0°11'39.3"W	KT899503
Old Ningo, Ghana, Africa; 5°44'38.8"S, 0°11'39.3"W	KT899502

Komenda, Ghana, Africa; 5°02'54.7"S, 1°29'10.3" W	KT899501
Komenda, Ghana, Africa; 5°02'54.7"S, 1°29'10.3" W	KT899500
Komenda, Ghana, Africa; 5°02'54.7"S, 1°29'10.3" W	KT899499
Ahwiam, Ghana, Africa; 5°45'00.0"S, 0°14'00.0"W	KT899498
Ahwiam, Ghana, Africa; 5°45'00.0"S, 0°14'00.0"W	KT899497
Ahwiam, Ghana, Africa; 5°45'00.0"S, 0°14'00.0"W	KT899496
Ahwiam, Ghana, Africa; 5°45'00.0"S, 0°14'00.0"W	KT899495
Mumford, Ghana, Africa; 5°15'51.0"	KT899494
Mumford, Ghana, Africa; 5°15'51.0"	KT899493
Mumford, Ghana, Africa; 5°15'51.0"	KT899492
Mumford, Ghana, Africa; 5°15'51.0"	KT899491
Shama, Ghana, Africa; 5°01'08.9"S, 1°37'27.8"W	KT899490
Shama, Ghana, Africa; 5°01'08.9"S, 1°37'27.8"W	KT899489
Shama, Ghana, Africa; 5°01'08.9"S, 1°37'27.8"W	KT899488

	Prampram, Ghana, Africa; 5°42'18.2”S , 0°07'32.3"W	KT899487	
	Prampram, Ghana, Africa; 5°42'18.2”S , 0°07'32.3"W	KT899486	
Hypnea musciformis	Namibia; Africa, 19°37'47.35"S, 12°50'32.96"W	KM523208	
	Gulf of Triest, Ancona, Italy; 45°39'42.27"S, 13°44'17.67"W	KM523207	KM509072
	Lago Ganzirri, Messina, Italy; 38°15'68.5"S, 15°36'93.9"W	KF714869	
	Florida, USA; 24°52'46.53"S, 80°41'40.58"W	KM523206	KM509072
	New Hanover, North Carolina, USA; 34°12'16.80"S, 77°47'42.59"W	KM523205	
	Fort Fisher rocks, Onslow Bay, North Carolina,USA; 33°58'07.6"S, 77°54'52.4"W	KJ202077	KJ202086
	Cap Ferrat, Ville Franche, France; 43°40'24.3"S, °19'46.8"W	GQ141881	
	Kauai, Hawaii, USA; 22°02'33.2"S, 159°18'09.3"W	HQ422876	
	Maui, Hawaii, USA; 20°55'06.9"S, 156°11'32.9"W	HQ422646	
	Necker, Hawaii, USA; 23°34'28.9"S, 164°41'60.0"W	HQ422630	
	Oahu, Hawaii, USA; 21°17'39.4"S, 157°59'14.1"W	HQ422612	
	New Hanover, North Carolina, USA; 34°12'16.80"S, 77°47'42.59"W		U04179

	Punta Cana, Dominican Republic; 18°36'21.9"S, 68°25'41.2"W	KM523204	
	Needham's Point, Barbados; Caribean, 13°04'41"S, 59°36'43"W	KM523203	KM509063
	Cadiz, Cala Encendida, Spain; 36°18'31.9"S, 6°09'11.1"W		KC121142
	Archipeldu Frioul, Marselha, Framce; 43°16'39.2"S,5°18'45.0"W		KC121141
	Cap Ferrat, Ville Franche, France; 43°40'24.3"S, °19'46.8"W		EU346013
	Cannes, Theole, France; 43°32'46.2" S, 7°01'17.9"W		EU346012
	Antibes_France; 43°34'41.8" S,7°08'23.5"W		EU346014
	Santa Martha, Magdalena, Colombia; 11°14'40.2"S, 74°13'14.4"W; col. P.B. Jesus et al.; Field ID:P163	KP725275	
	Isla Mujeres, Mexico; 21°8'11.93"S, 86°44'34.15"W, col. P.B. Jesus et al.; Field ID:P211	***	
	Isla Mujeres, Mexico; 21°8'11.93"S, 86°44'34.15"W, col. P.B. Jesus et al.; Field ID:P213	***	

FIGURE S1: Neighbor Joining (NJ) phylogram for the COI-5P marker showing the grouping of the *Hypnea* sequenced in this study (in boldface) and available from GenBank (see Table S1 for information on each sample). Bootstrap support values for 2000 replicates ≥75% are indicated above each node.


H .pseudomusciformis Brazil, Bahia (KM523210)
H .pseudomusciformis Brazil, Bahia (KM523209)
H .pseudomusciformis Brazil, Pernambuco (KM523160)
H .pseudomusciformis Brazil, Rio Grande do Norte (KM523219)
H .pseudomusciformis Brazil, Ceará (KM523197)
H .pseudomusciformis Brazil, Piauí (KM523224)
H .pseudomusciformis Brazil, Bahia (P14, ALCB 100206)
H .pseudomusciformis Brazil ,Ceará (P195, ALCB 110456)
H .pseudomusciformis Brazil, Ceará (KM523195)
H .pseudomusciformis Brazil, Rio Grande do Norte (KM523164)
H .pseudomusciformis Brazil, Paraíba (KM523198)
H .pseudomusciformis Brazil, Bahia (KM523215)
H .pseudomusciformis Brazil, Ceará (P191, ALCB 110446)
H .pseudomusciformis Brazil, Bahia (P251, ALCB 118372)
H .pseudomusciformis Brazil, Ceará (KM523196)
H .pseudomusciformis Brazil, Rio Grande do Norte (KM523218)
H .pseudomusciformis Brazil, Paraíba (KM523199)
H .pseudomusciformis Brazil, São Paulo (KM523163)
H .pseudomusciformis Brazil, Rio Grande do Norte (KP725279)
H .pseudomusciformis Brazil, Ceará (P194, ALCB 110449)
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H .pseudomusciformis Brazil, Paraíba (KM523243)
H .pseudomusciformis Brazil, Alagoas (KM523220)
H .pseudomusciformis Brazil, Ceará (P192, ALCB 110447)
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H .pseudomusciformis Brazil, Ceará (KM523239)
H .pseudomusciformis Brazil, Paraíba (KM523217)
H .pseudomusciformis Brazil, Bahia (Km523213)
H .pseudomusciformis Brazil, Bahia (Kp725277)
H .pseudomusciformis Brazil, Bahia (P177, ALCB 110262)
H .pseudomusciformis Brazil, Pernambuco (Km523226)
H .pseudomusciformis Ghana (Kt899516)
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H .pseudomusciformis Ghana (KT899488)
H .pseudomusciformis Ghana (Kt899486)
H .pseudomusciformis Uruguay (KM593683)
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H .pseudomusciformis Brazil, Rio Grande do Norte (KM523230)
H .pseudomusciformis Brazil, Pernambuco (KM523229(
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H .pseudomusciformis Brazil, São Paulo (KM523154)
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H .pseudomusciformis Brazil, Santa Catarina (KM523171)
H .pseudomusciformis Brazil, Santa Catarina (KM523176)
H .pseudomusciformis Brazil, Santa Catarina (KM523180)
H .pseudomusciformis Brazil, Santa Catarina (KM523178)
H. pseudomusciformis Brazil, São Paulo (KP725276)
H .pseudomusciformis Brazil, São Paulo (KM523157)
H .pseudomusciformis Brazil, São Paulo (KM523158)
H .pseudomusciformis Brazil, Espírito Santo (P189)
H .pseudomusciformis Brazil, Rio de Janeiro (KM523151)
H .pseudomusciformis Brazil, Rio de Janeiro (KM523138)
H. pseudomusciformis Brazil, Rio de Janeiro (KM523140)
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H .pseudomusciformis Brazil, Rio Grande do Sul (P224, ALCB 114362)
H .pseudomusciformis Brazil, Rio Grande do Sul (P221, ALCB 114369)
H .pseudomusciformis Brazil, Rio Grande do Sul (P223, ALCB 114363)
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H .pseudomusciformis Uruguay (KM593685)
H .musciformis México, Isla Mujeres (P211)
H .musciformis Barbados, Needhams Point (KM523203)
H .musciformis USA, Hawaii (HQ422630)
H .musciformis USA, Hawaii (HQ422876)
H .musciformis Republica Dominicana, Punta Cana (KM523204)
H. musciformis USA, NorthCarolina (KM523205)
H. musciformis Italy (KF714869)
H. musciformis Namibia (KM523208)
H. musciformis Italy (KM523207)
88
78
93
99
84
98
87
90
0.005
PM
M2
M1

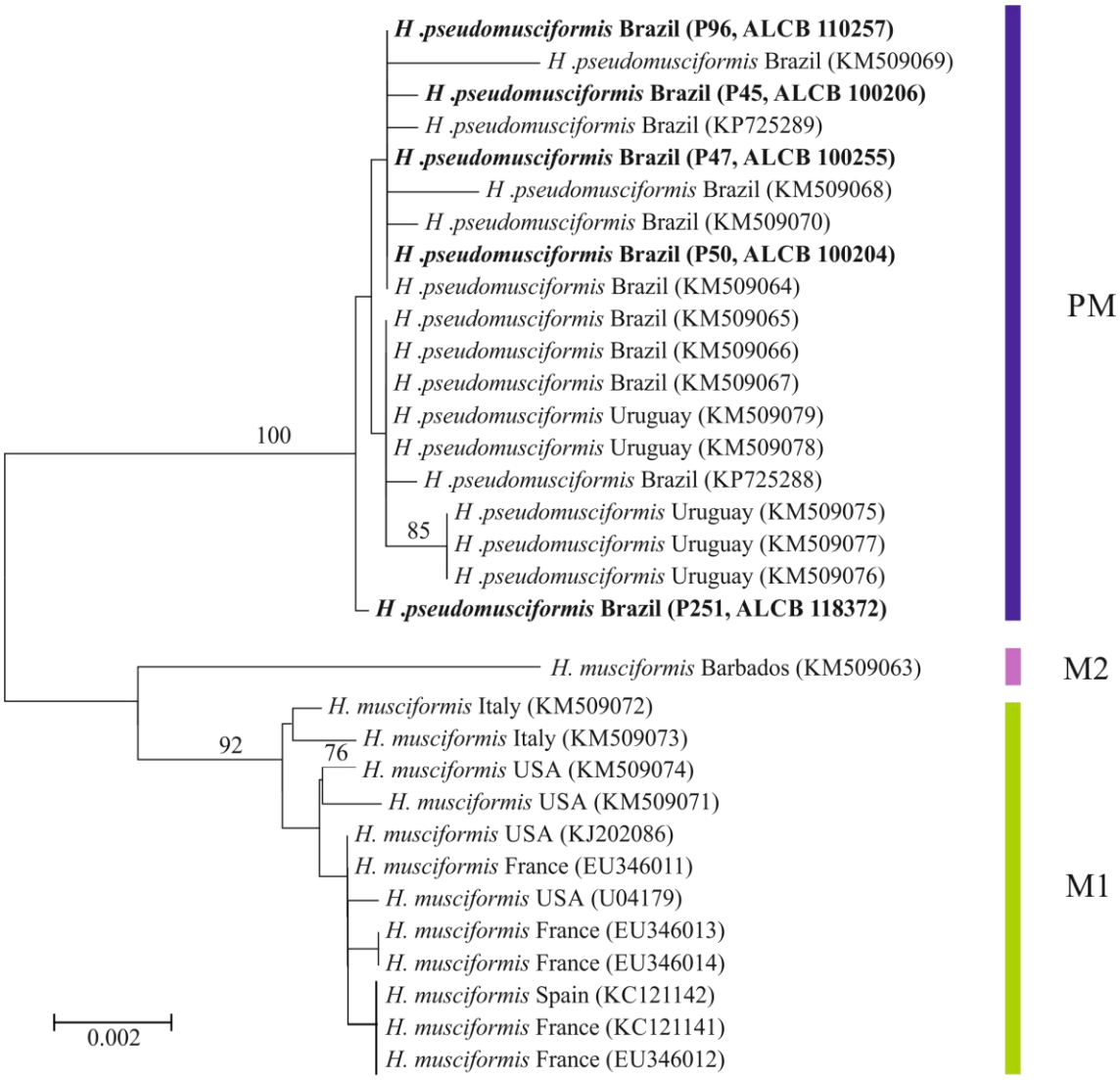

FIGURE S2: Neighbor Joining (NJ) phylogram for the *rbc*L marker showing the grouping of the *Hypnea* sequenced in this study (in boldface) and available from GenBank (see Table S1 for information on each sample). Bootstrap support values for 2000 replicates ≥75% are indicated above each node.

CAPÍTULO 5

NEW INSIGHTS INTO THE PHYLOGENETIC RELATIONSHIPS AND INFRAGENERIC CLASSIFICATIONS OF *HYPNEA* (CYSTOCLONIACEAE, RHODOPHYTA)

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Condensed title: Phylogeny of *Hypnea*

Abstract:

Hypnea J.V. Lamouroux is a monophyletic genus with complex nomenclatural history and taxonomy and an important source of carrageenan. Species delimitation in *Hypnea* is complicated by the high degree of morphological variation within species. Phylogeny of the genus *Hypnea* have been accessed based primarily on Asian species, but recent studies carried out South America revealed a great diversity of species, which phylogenetic relationships need to be evaluated. Three infrageneric sections are recognized in the genus: *Pulvinatae*, *Spinuligerae*, and *Virgatae*, but morphological and molecular circumscriptions within each one of them appears to differ. In this study, we analyzed three distinct markers to establish the phylogenetic relationships between *Hypnea* species. We identified one species complex that require taxonomic and evolutionary studies at regional and global scale, and three unidentified/doubtful species demand additional morphological and molecular studies. The combination of three markers increased resolution and support, resulting in the largest and best-resolved phylogeny of the genus to date. Isolated and combined analyses proved that the three sections of the genus *Hypnea* are invalid, as currently recognized, probably due to convergent evolution.

Key index words: Gigartinales, *Pulvinatae*, Relationships, *Spinuligerae*, *Virgatae*

INTRODUCTION

Hypnea J.V. Lamouroux is a monophyletic genus with complex nomenclatural history and taxonomy (Geraldino et al. 2010), and is an important source of carrageenan (Yamagishi and Masuda 2000). This genus was established based on five species (*H. charoides* Lamouroux, *H. hamulosa* Lamouroux, *H. musciformis* (Wulfen) J.V.Lamouroux, *H. spinulosa* Lamouroux e *H. wighii* Lamouroux), most of them removed from the genus *Fucus* Linnaeus (Tanaka 1941).

J. Agardh (1852) made the first systematic arrangement of the genus, classifying 19 species in three infrageneric sections based on the morphology of the thallus. These sections were characterized as follows: <u>*Virgatae*</u> - *frondes steriles caespitosae virgato-ramulosae,ramulis adultioribus basi constrictis; sporigerae conformes, in ramulis siliquosis, basi attenuata sterili pedicellatis, sphaerosporas foventes; capsuligeae divaricato-ramosissimae;* <u>*Spinuligerae*</u> - *frondes steriles intricato-caspitosae, ramis patentibus alterne ramosae, ramulisque subulatis, a basi latiore acuminatis, obsilae; sporiferae conformes in ipsa basi aut media parte ramulorum intumescente sphaerosporas gerentes;* and <u>*Pulvinatae*</u> - *frondes steriles pulvinatim expansae intricatoramosissimae, ramis concretis cohaerentes; fertiles extra pulvinar emergentes, invicem liberae.*

Currently, 66 valid *Hypnea* species are recognized (Guiry and Guiry 2016, Nauer et al. 2015, 2016, Jesus et al. 2016), widely distributed in tropical and subtropical waters, on the Indian, Pacific and Atlantic Oceans. Despite its widespread occurrence and economic importance, several taxonomic problems are recurrent in the genus, with a large number of taxonomic complexes, poorly defined and cryptic species, synonyms and frequent failure to identify some species (Price et al. 1992, Abbott 1999, Dawes and Mathieson 2008, Geraldino et al. 2006, 2009, 2010, Jesus et al. 2013, 2015, 2016, Nauer

et al. 2014, 2015). Species delimitation in *Hypnea* is complicated by the high degree of morphological variation within individual species, which may be influenced by environmental factors occurring in specific habitats (Yamagish and Masuda 2000). Furthermore, different circumscription of species might cause taxonomic confusion (Yamagish and Masuda 2000, Geraldino et al. 2010, Jesus et al 2015), demonstrating the importance of molecular studies on the genus.

The first molecular taxonomic studies of the genus were limited to Asian species (Yamagishi and Masuda 2000, Yamagishi et al. 2003, Geraldino et al. 2006, among others). The delimitation of the *Hypnea charoides-valentiae* complex and the discrimination of *H. stellulifera* (J. Agardh) Yamagishi et Masuda from *H. cornuta* var. *stellullifera* J. Agardh were based on sequences of the plastid *rbcL,* gene encoding the large subunit of RuBisCo (ribulose biphosphate carboxilase oxigenase) (Yamagishi and Masuda 2000, Yamagishi et al. 2003, respectively). Subsequently, nuclear SSU rDNA (small subunit ribosomal RNA - SSU rRNA) and mitochondrial *cox*1 (gene encoding cytochrome oxidase I - COI) were associated with *rbc*L for study of specimens from Korea, Japan and Taiwan (Geraldino et al. 2009), resulting in the recognition of a new species to the northeast Pacific: *H. asiatica* P.J.L. Geraldino, E.C. Yang & Boo. Geraldino et al. (2010) evaluated the phylogenetic relationships among species of the *Hypnea*, most of them Asiatic, by using the genes *cox*1, *rbc*L and *psa*A (photosystem I P700 apoprotein A1). Despite the small number of species, this molecular study revealed the species clustered into three major clades, would correspond to three infrageneric sections previously defined by J. Agardh (1852).

Molecular studies in Atlantic *Hypnea* species are quite recent, and have employed two specific DNA fragments (COI-5P, the standard DNA barcode region of the mitochondrial cytochrome c oxidase 1 gene (*cox*1) and the UPA, the Universal Plastid

Amplicon) for the delimitation of species, called DNA barcode (Jesus et al. 2015, 2016, Nauer et al. 2014, 2015, 2016). These studies revealed six new species in the Brazilian coast (*Hypnea brasiliensis* P.B. Jesus, F. Nauer and J.M.C. Nunes; *Hypnea edeniana* Nauer, Cassano & M.C. Oliveira; *H. flava* Nauer, Cassano & M.C. Oliveira; *H. pseudomusciformis* Nauer, Cassano & M.C. Oliveira, *H. wynnei* Nauer, Cassano & M.C. Oliveira and *H. yokoyana* Nauer, Cassano & M.C. Oliveira) bringing to 12 the number of species on this coast.

Nauer et al. (2014) and Jesus et al. (2015, 2016) accessed phylogenetic relationships among some Brazilian species; however based only on the sequence of *rbc*L. Jesus et al. (2015) pointed out some divergences between morphological and molecular circumscriptions of the *Hypnea* sections, indicating that this could be due to the inclusion of the South American species on the analyses. These authors indicated that further studies are required to understand the processes that led to the diversification of the genus. Studies should use greater numbers of markers and South American species, which would allow a better understanding of the relationships among *Hypnea* species.

In order to analyze the validity of the three morphological (J. Agardh 1852) and molecular (Geraldino et al. 2010) recognized sections, we analyzed one mitochondrial and two plastidial markers commonly used for the genus: COI-5P, *psa*A, and the *rbc*L. Extensive sampling on Brazilian coast, coupled with molecular (combined and isolated) analyses recovered a robust phylogeny of the genus and provided new insights on the infrageneric circumscription, taxonomic status, distribution limits and relationships among and within *Hypnea* species.

MATERIALS AND METHODS

Collections. One hundred and five specimens were collected in several locations along the Brazilian coast, increasing existing data in GenBank (Table S1 in the Supporting Information). Three to five thalli fragments were preserved in silica gel desiccant for subsequent DNA extraction. Materials for morphological observations were preserved in 4% formalin in seawater. All voucher specimens were deposited in the herbaria Alexandre Leal Costa Bahia (ALCB) of the Universidade Federal da Bahia, Brazil. We sequenced some voucher specimens borrowed from the Herbarium of the Graduate School of Science, Hokkaido University (SAP), the Natural History Museum (BM), the Herbarium of Trinity College Dublin (TCD) and the Herbarium Nacional of the Mexico (MEXU).

DNA extraction, PCR reaction and sequencing. Total DNA was extracted from samples with approximately 20–40 mg, by maceration in liquid nitrogen using a modified version of the CTAB (Cetyl Trimethyl Ammonium Bromide) procedure of Doyle and Doyle (1987). Molecular markers were PCR amplified under the following conditions: 1× PCR Buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 μM each primer, 5 ng of genomic DNA, and 1.25 U of Taq DNA polymerase (Invitrogen, São Paulo, São Paulo, Brazil or Applied Biological Materials Inc, BC, Canada) in a total volume of 25 μL. For the amplification and sequencing reactions for each marker, specific primer pairs were used: for COI-5P, GazF1 and GazR1 (Saunders 2005); for *rbc*L, FrbcL and RrbcS (Freshwater et al. 1994) and R753 and F492 (Freshwater and Rueness 1994); for *psa*A: psaA130F and psaA1760R (Yoon et al. 2002), and psaA971F and psaA1110R (Yang & Boo 2004). PCR was performed with an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C (37ºC for FrbcL+ R753 primers), and 1.30 min at 72°C, with a final 5 min extension cycle at 72°C. The reactions were

performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems®, Foster City, California, USA). The company Gene Wiz (Cambridge, Massachusetts, USA, http://www.genewiz.com/) performed purifications and sequencing reactions of the PCR products.

Molecular analyses. Electropherograms were assembled using Geneious v6.0.6 (Biomatters, Auckland, New Zealand; Kearse et al. 2012) and edited in BioEdit 5.0.6 (Hall 1999). For each marker, a multiple alignment was generated with the ClustalW tool (Thompson et al. 1994), available in BioEdit 5.0.6 (alignments excluded PCR primer sequences). In addition to sequences generated in this study, other *Hypnea* species that have been sequenced for all the three markers were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/, searched on June 2016 - Table S1 in the Supporting Information) and included in the analyses. We deposited all new consensus sequences of COI-5P, *rbc*L and *psa*A in the Barcode of Life Data Systems (http://www.boldsystems.org/) and GenBank.

To assess the level of variation in data sets, estimates of divergence values within and among species were computed using MEGA 6.0 (Tamura et al. 2013). We evaluated the congruence between the markers by comparing clade supports between individual data partitions (Wiens 1998, Cardoso et al. 2013). Incongruent clades with nonparametric bootstrap supports >80% were taken as evidence for conflicts between markers.

Maximum parsimony (MP) and Maximum likelihood (ML) analyses of individual and combined data sets (COI-5P, *rbc*L and *psa*A) were performed to infer the phylogenetic relationships within the genus *Hypnea*. As outgroups we selected sequences of *Calliblepharis ciliata* (Hudson) Kützing (*rbc*L: AF385653), *Chondrus crispus* Stackhouse (COI-5P: KJ960551 / *rbc*L: U02984), *Gracilaria tenuistipitata* var. *liui*

Zhang and Xia (*cox*1: EF434924) and *Griffithsia okiensis* (*cox*1: EU194973 / *rbc*L: GQ252547). MP trees were constructed with PAUP* 4.0b.10 using a heuristic search algorithm with the following settings: starting trees obtained by stepwise addition, using tree bisection–reconnection (TBR) branch swapping algorithm; MulTrees, all characters unordered and with equal weight, accelerated transformation (ACCTRAN) as character-state optimization; branches with a maximum length of zero collapsed to yield polytomies. A strict consensus analysis was computed, and bootstrap values for the resulting nodes were assessed using 1000 bootstrapping replicates with simple sequence addition. We calculated the consistency (CI), retention (RI), and rescaled consistency (RC) indices resulting from the MP analysis.

The best models to each dataset were assessed under MrModeltest 2.3 (Nylander 2008) and selected using the Akaike Information Criterion (AIC), as recommended by Posada and Buckley (2004). ML analysis was conducted using RAxML (Randomized Axelerated Maximum Likelihood, version 7.0.4; Stamatakis 2006) with the GTR + GAMMA + I model. The best-scoring ML tree and 500 bootstrap trees were obtained using the rapid hill-climbing algorithm (Stamatakis et al. 2008).

RESULTS

Based on a combination of morphological and molecular data, we identified ten *Hypnea* species sampled in several locations along the Brazilian coast: *H. brasiliensis, H. cervicornis, H. cornuta, H. edeniana, H. flava, H. pseudomusciformis, H. spinella, H. stellulifera, H. wynnei, H. yokoyana* (Figures 1 and 2; Figures S1 and S2 in the Supporting Information).

We generated 80, 29 and 23 new *Hypnea* sequences for COI-5P, *psa*A and *rbc*L, respectively. A multiple alignment was generated to each individual dataset: COI-5P included 113 sequences with 465 bp, of which 189 were variable and 164 parsimony

informative; *psa*A included 50 sequences with 1.295 bp, of which 497 were variable and 352 parsimony informative; and *rbc*L included 98 sequences with 1.355 bp, of which 450 were variable and 338 parsimony informative.

The individual gene trees topologies from COI-5P (Figure S1 in the Supporting Information), *psa*A (Figure S2 in the Supporting Information) and *rbc*L were not in conflict (data for COI-5P and *psa*A not shown). Despite this, the single datasets did not showed good resolution and support in order to clarify the phylogenetic relationships among the sampled groups. For this reason, we chose to analyze and describe the relationships from the analysis of combined data matrix (Figure 1). We additionally presented the *rbc*L tree (Figure 2) because it includes the densest taxon sampling.

We did not perform the incongruence length difference test (IDL test; Farris et al. 1994) to test the phylogenetic incongruence among data partitions, since this has been target from many criticisms (e.g., Cunningham 1997; Barker and Lutzoni 2002, Darlu and Lecointre 2002). The phylogenetic incongruence between data sets was analyzed by comparing clade bootstrap supports, according to Cardoso et al. (2013).

Analyses of combined mitochondrial and plastidial data. The combined matrix included 35 accessions for the three mitochondrial and plastidial DNA markers, with 3.115 characters, in which 465 corresponded to COI-5P, 1.295 to *psa*A and 1.355 to *rbc*L (Table 1).

The Maximum Parsimony (MP) analysis of the combined datasets resulted in a single optimal tree of 2.990 steps. The consistency (IC), retention (RI) and rescaled consistency (RC) indices were described in detail for all individual and combined datasets in Table 1. Of 3.115 total characters, 765 (24.5%) were informative to the Parsimony. In the Maximum Likelihood (ML) analysis, the – ln likelihood score was 18275.923398

under the GTR + G + I model, with the following parameters: base frequencies A = 0.3086, C = 0.1581, G = 0.1936 and T = 0.3395, rate matrix A–C = 1.5025, A– G = 6.1719, A–T = 1.7954, C–G = 0.8370, C–T = 11.4911 and G–T = 1.0000. We presented the topology of the most likely tree in Figure 1 with MP and ML nonparametric bootstrap values plotted above each clade. We discussed the main clade differences among the infrageneric sections morphologically established by J. Agardh (1852), and recognized by Geraldino et al. (2010) based on molecular data.

The genus *Hypnea* emerged as monophyletic in both analysis with high bootstrap supports (100% for ML and MP), including two large clades highly supported (Figure 1). The first clade to diverge (Clade 1, Sub-clade A - 100% for ML, 95% for MP) included *H. asiatica, H. brasiliensis, H. charoides, H. edeniana, H. flava, H. nidifica, H. rosea, H. spinella, H. valentiae* and *Hypnea* sp. 1 arranged in two minor clusters. In the first, *H. brasiliensis* was sister to *H. nidifica* and *H. asiatica* with high bootstrap supports (100% for ML, 96% for MP). In ML analysis, *H. rosea* formed a sister group with *H. brasiliensis*, *H. asiatica* and *H. nidifica* with 95% bootstrap, although this relationship was not sustained in MP analysis. On the second cluster (100% for ML, 97% for MP), *H. spinella* samples from Brazil and Caribean were grouped with *H. valentiae* (100% for ML, 99% for MP), *H. edeniana,* H. *charoides*, and these with *H. flava* and *Hypnea* sp.1 from Vietnam (100% for ML, 96% for MP).

The second clade (95% for ML, 96% for MP – Clade 2) assembled a mix of species belonging to the four small sub-clades (B, C, D and E). *Hypnea japonica* was recognized as basal into the sub-clade E (100% for ML and MP), being clustered with *H. cervicornis* and *H. tenuis* (100% for ML and MP). *H. musciformis* and *H. pseudomusciformis* (100% for ML and MP – sub-clade D) were sisters to *H. stellulifera* and *H. cornuta* (100% for ML, 100% for MP – sub-clade C). In this cluster, *H. stellulifera* seems to be paraphyletic,

since Brazilians *H. stellulifera* and *H. cornuta* were more related to each other than to the Philippine *H. stellulifera*. In the clade correspondent to the Group B (100% for ML, 93% for MP), the subclade including *Hypnea caespitosa* + *H. nidulans* (93% for ML) were in a sister relationship with subclade including *H. viridis* + *H. pannosa* (100% for ML and MP).

Analyses of rbcL data. Maximum Parsimony (MP) analysis of *rbc*L data resulted in a single optimal tree of 1.133 steps. Of 1.355 characters, 338 (24.9%) were informative to Parsimony. In the Maximum Likelihood (ML) analysis, the – ln likelihood score was 7829.990689 under the GTR + G + I model, with the following parameters: base frequencies A = 0.3049, C = 0.1514, G = 0.2073 and T = 0.3361, rate matrix A–C = 1.0420, A– G = 6.4339, A–T = 2.1918, C–G = 1.0807, C–T = 14.1373 and G–T = 1.0000. We presented the topology of the most likely *rbc*L tree in Figure 2 with MP and ML nonparametric bootstrap values plotted above each clade.

The *rbc*L tree showed three main groups within *Hypnea* (Clades 1, 2 and 3 – Figure 2), although not always with strong bootstrap supports. As in the concatenated analysis, the Cluster 1 (Sub-clade A) was identified as the first to appear. This section has no support in the ML analysis, and several minor groups collapsed in the MP. It included almost the same species circumscription observed in the concatenate analyses (*H. asiatica, H. brasiliensis, H. charoides, H. edeniana, H. flava, H. nidifica, H. spinella, H. valentiae* and *Hypnea* sp.1) including a small group formed by *H. rosea, H. volubilis, H. yamadae, H. yokiana* and *Hypnea* sp.2 from Brazil.

The Clade 2 (90% for ML) included four small highly supported sub-clades (C, D, E and F). The sub-clade C (98% for ML, 100% for MP) consisted of *Hypnea stellulifera* and *H. cornuta* from Brazil (98% for ML, 99% for MP) as sister to *H. stellulifera* from

Asia. The sub-clade F (99% for ML and MP) was formed exclusively by samples of *H. cornuta* from Europe, Asia and Australia. The Group D (90% for ML, 92% for MP) included *H. chordacea* + *H. flageliformis* (100% for ML and MP) as sister to *H. musciformis* from Caribbean, USA and Europe + *H. pseudomusciformis* from South America (100% for ML and MP). The sub-clade E (100% for ML and MP) included *H. cervicornis* and *H. tenuis*, and occupied a basal position on the Clade 2, being grouped with C, D and F sub-clades.

In the Clade 3, the sub-clade B (96% for ML, 86% for MP) included *H. japonica* and *Hypnea nidulans* (85% for MP), as sisters to the subclade containing *H. viridis* + *H. pannosa* (100% for ML, 99% for MP) and *H. caespitosa*. Both ML and MP analyses based on *rbc*L revealed *Hypnea* sp. 3 from USA (AF3856351 – as *H. spinella*) as a sister group of other *Hypnea* species. *H. wynney* occupied an undefined position in all single analyzes; not being possible to infer their position in any of the clades described for the genus through molecular data.

DISCUSSION

In this study, we analyzed three distinct markers to infer phylogenetic relationships between *Hypnea* species. Our phylogeny is the most complete to date; including 255 sequences from 28 *Hypnea* species. All phylogenetic analyses based on independent and combined COI-5P, *psa*A and *rbc*L datasets confirmed the monophyletism of the genus, as Geraldino et al. (2010), Nauer et al. (2014, 2015) and Jesus et al. (2015, 2016) observed.

J. Agardh (1852) classified *Hypnea* species into three sections based on thallus habit. *Hypnea ceramioides* Kützing, *H. flagelliformis* Greville ex J.Agardh, *H. musciformis*, *H. nigrescens* Greville *ex* J.Agardh, *H. ramentacea* (C.Agardh) J.Agardh

and *H. spicifera* (Suhr) Harvey were arranged in the section *Virgatae* by its main axis and dense lateral branchlets, caespitose and erect, but not intricate. *H. cervicornis* J.Agardh, *H. charoides*, *H. cornuta* (Kützing) J.Agardh, *H. divaricata* (C.Agardh) Greville, *H. hamulosa* (Esper) J.V.Lamouroux, *H. nidifica* J.Agardh, *H. stellulifera* (J.Agardh) Yamagishi & Masuda and *H. valentiae* (Turner) Montagne were characterized by fronds intricate-caespitose, alternately arranged with branches and branchlets with short spine-like processes and were classified into the section *Spinuligerae*. *Hypnea cenomyce* J.Agardh, *H. pannosa* J.Agardh and *H. spinella* (C.Agardh) Kützing presented a cushion-like habit with anastomosing branches and fertile upper branchlets, and were grouped in the section *Pulvinatae*.

Geraldino et al. (2010) accessed the validity of these infrageneric sections via molecular data (*rbc*L + *psa*A) and found three major clades, which they interpreted as belonging to the first established morphological section. We identified some divergences among the infrageneric sections recognized in this study and those from J. Agardh (1852) and Geraldino et al. (2010). The COI-5P, *psa*A and *rbc*L trees had three large clades, although with low nonparametric ML and MP bootstrap supports. Our concatenate tree revealed only two main large and well-supported clades, which are at odds with both the proposed by J. Agardh (1852) and Geraldino et al. (2010). Jesus et al. (2015) indicated that there were divergences between morphological and molecular circumscriptions of the *Hypnea* sections, demonstrating that the inclusion of the South American sequences could lead to this result.

In our analyses (Figures 1 and 2), the first clade to diverge (Clade 1 – sub-clade A) consisted of *Hypnea asiatica*, *H. brasiliensis*, *H charoides*, *H. edeniana*, *H. flava*, *H. nidifica*, *H. rosea*, *H. spinella*, *H. valentiae*, and *H. volubilis*. These species fit well within the section *Pulvinatae* (J. Agardh 1852) because of its prostrate and intricate tufts - except

for *H. asiatica, H charoides* and *H. valentiae* (Mshigeni 1978, Jesus et al. 2014, Jesus et al. 2016). Probably because of the presence of these latter species, Geraldino et al. (2010) classified this clade as belonging to the section *Spinuligerae*. These findings indicated that molecular data did not corroborate *Pulvinatae* and *Spinuligerae* sections as morphologically recognized. All *Hypnea* species recently described from the Brazilian coast (Nauer et al. 2014, 2016, Jesus et al. 2016) were included in this clade, and relationships among these mat-forming species were accessed in this study by the first time. Another group of species that fit with morphological delineation of the section *Pulvinatae* was recovered in the clade B, consisting of *Hypnea caespitosa, H. nidulans, H. pannosa* and *H. viridis*. Geraldino et al. (2010) also recognized this group as belonging to the section *Pulvinatae*. This section was paraphyletic with two sub-clades (A and B), highly supported but distantly related.

The section *Spinuligerae* comprises species with subulate branches alternately arranged on the thalli, bearing short spine-like processes (J. Agardh 1852). On our analyses, three different sub-clades (C, E and F) presented species with these features, demonstrating that section *Spinuligerae* also is paraphyletic. Moreover, the relationship among species within each group requires attention. On ML analysis based on *rbc*L, *Hypnea japonica* was grouped into Group B (section *Pulvinatae*). Based on *rbc*L + *psa*A, Geraldino et al. (2010) also observed this clustering, but argued that it belongs to the section *Spinuligerae* (Tanaka et al. 1941). In our combined COI-5P + *rbc*L + *psa*A analyses, *H. japonica* took a highly supported basal position in sub-clade E (*Spinuligerae* section), which is consistent with Tanaka (1941) delineation. *H. tenuis* was sister to *H. cervicornis* in all our analyses, another incongruence, because this species should be in the section *Virgatae* (Geraldino et al. 2010). Species identified as *H. cornuta* and *H. stellulifera* from the Brazilian coast were more related to each other than with their

European, Asian and Australian sisters (sub-clades C and F). Jesus et al. (2012, 2015) provided a detailed morphological characterization of these Brazilian species and we were able to compare their features with type and topotype specimens, so there is no doubt of its morphological delimitation. Even though it was expected that these species would present a sister relationship due to their taxonomic history (Jesus et al. 2015), these results raises the hypothesis that reproductive isolation could not be complete on the some *Hypnea* species, as suggested by Jesus et al. (2015). Further studies involving Brazilian and European, Asian and Australian specimens of this species complex should be performed in order to establish their population structure and historical demography.

The *Virgatae* section also appeared as paraphyletic in all our analysis (sub-clade D) because, according to Tanaka (1941), it should to include *H. tenuis*. This clade of species with percurrent axis densely branched (J. Agardh 1852, Tanaka 1941, Yamagish and Masuda 1997) included *H. chordaceae, H. flagelliformis, H. musciformis* and *H. pseudomusciformis.* Tanaka (1941) recovered the same circumscription in this section, while Geraldino et al. (2010) found in this group species belonging to the section *Spinuligerae*. The placement of *H. tenuis* outside the section *Virgatae* should be treated with caution, since this species had inconclusive results in several delimitation tests performed by Jesus et al. (2016).

Hypnea sp.1 from Japan (AB0331662, Yamagishi and Masuda 2000) and Vietnam (EU240849, Geraldino et al. 2009) were sister to *H. flava* from Brazil into a group of mat-forming species (Group A). *Hypnea* sp.3 from USA (AF3856351, Hommersand and Fredericq 2003) have been constantly grouped as sister of all other *Hypnea* species (Geraldino et al. 2010, Jesus et al. 2015). These *rbc*L sequences were identified as belonging to the *H. spinella* species, but it has been established that they do not belong to this taxon (Nauer et al. 2014, Jesus et al. 2016) and result from flawed taxonomic

identification (Geraldino et al. 2010). In fact, they should constitute novel species for science, which need to be described. To achieve this goal, the available molecular data should be combined with data from new markers and also with morphological descriptions in order to provide an accurate delimitation.

Hypnea sp.2 from the Brazilian coast was grouped with *H. yokoyana* and *H. volubilis* in the *rbc*L analyses (subclade A). In COI-5P (Figure S1), *Hypnea* sp.2 sequence was clustered with *H. yokoyana* and two new sequences generated in this study, without divergence. These COI-5P sequences were produced from old herbaria specimens previously identified as *H. divaricata* from Guadeloupe, Caribbean (P181 - BM904832) and *H. valentiae* from Kenya, Africa (P183 - BM904833). We were not able to generate *rbc*L and *psa*A sequences from these specimens, probably due to poor DNA quality. Under these circumstances, we have doubts about the taxonomic status of the newly described species *H. yokoyana* (Nauer et al. 2016) and *H. volubilis* (Schneider and Searles 1976) and new sequences are necessary to clarify this issue.

Phylogenetic relationships based exclusively on COI-5P resulted in weakly supported groups; therefore, it was not possible to establish deeper relationships among species based solely on this marker. According to Jesus et al. (2016), COI-5P does not generate well-resolved phylogenies when used alone, and Lyra et al. (2015) suggest that it could increase resolution and support of phylogenetic trees if analyzed simultaneously with *rbc*L. Our phylogeny based on concatenate matrix of COI-5P + *rbc*L + *psa*A was the best resolved one for the genus, with highly supported major clades. These results improved the phylogenetic study of Geraldino et al. (2010) based on *rbc*L + *psa*A dataset, suggesting that the inclusion of COI-5P increased resolution and support of the phylogenetic analyses. In this study, we did not recover the three sessions previously

established by J. Agardh (1852), and detected inconsistencies with the molecular recognition made by Geraldino et al. (2010).

Here, we proved that the traditional infrageneric classification of the genus *Hypnea* (J. Agardh 1852) is not valid. According this first arrangement, *Hypnea* species were organized in three sections with basis on thallus habit. However, our analyses did not recovered clades exclusively formed by species consisting of (1) main axis erect and densely branched – *Virgatae;* (2) thalli intricate-caespitose, alternately branched – *Spinuligerae*; or (3) cushion-like habit with anastomosing branches – *Pulvinatae*. These findings clearly suggests the lack of positive synapomorphic (shared derived) traits amongst them, and reinforce the great phenotypic plasticity found in the genus (Yamagish and Masuda 1997, Geraldino et al. 2010, Jesus et al. 2013, 2014). Thus, the grouping observed on our molecular analysis can be due to convergent morphologies among distantly related species, in response to analogous environmental pressures (Cianciola et al. 2010). Because this, we not able to describe new infrageneric classifications, and suggest that the formally sections proposal by J. Agardh (1851) be rejected since it does not reflect the true evolutionary history of the genus.

CONCLUSIONS

Until now, the phylogeny of the genus *Hypnea* was fundamentally based on Asiatic species. We incorporated taxa extensively sampled along the Brazilian coast, and several previously published sequences from diverse locations in the globe, which allowed the discussion of the legitimacy of the infrageneric *Hypnea* sections. Our analyses showed that the three sections of the genus *Hypnea* are invalid, as currently recognized, due to incongruity between morphologically and molecularly delimited groups. The combination of three markers increased resolution and support, resulting in

the largest phylogeny of the genus. We identified a species complex formed by *H. cornuta* and *H. stellulifera* that require taxonomic and evolutionary studies at regional and global scale. Three unidentified/doubtful species, whose limits demand additional morphological and molecular studies, also deserve further investigation.

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Table 1: Summary of the Maximum Parsimony analyses for independent and combined datasets.

	COI-5P	***psa*A**	***rbc*L**	**Combined Data**
Aligned matrix (bp)	465	1295	1355	3115
Number of constant sites	276	798	905	2029
Number of variables sites	189	497	450	1.086
Number of parsimoniously informative characters	164	352	338	765
Number of most parsimonious trees	6	36	128	1
Tree length (L)	853	1260	1133	2990
N in-group taxa	111	48	96	33
CI	0.38	0.55	0.51	0.50
RI	0.87	0.80	0.87	0.67
RC	0.33	0.44	0.45	0.34

CI= consistency index; RI= retention index; RC = rescaled consistency index

FIGURES:

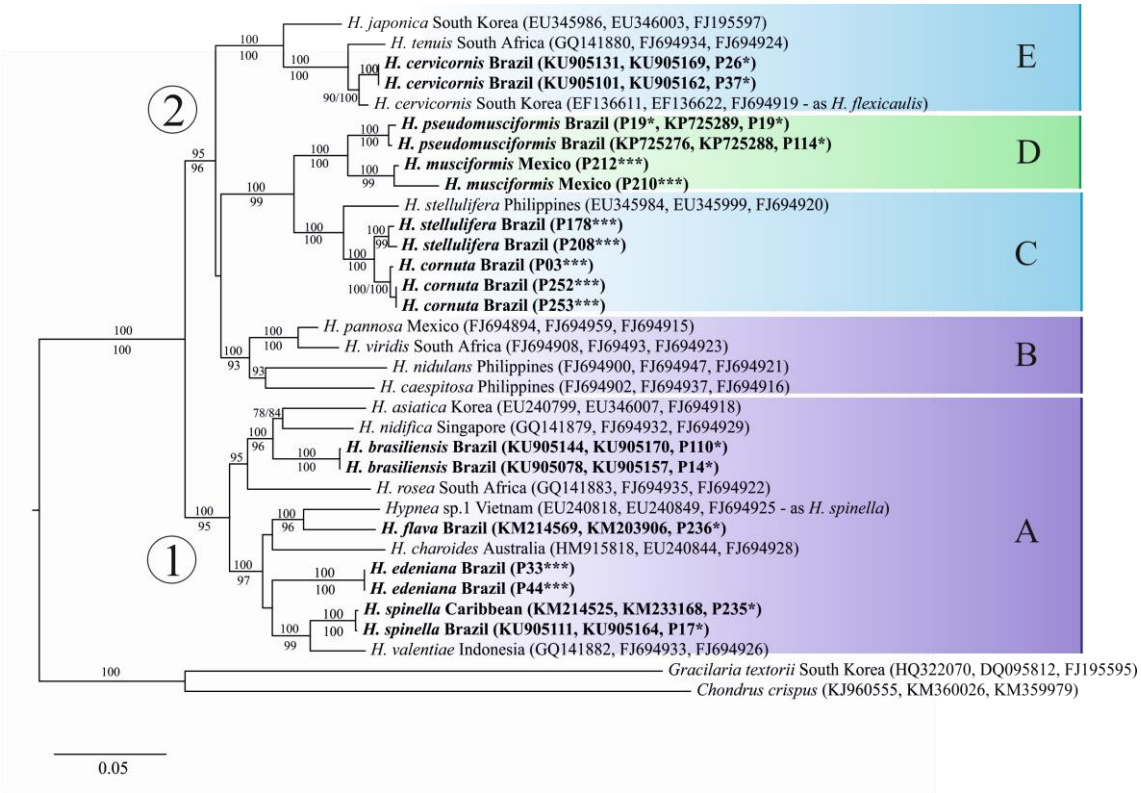
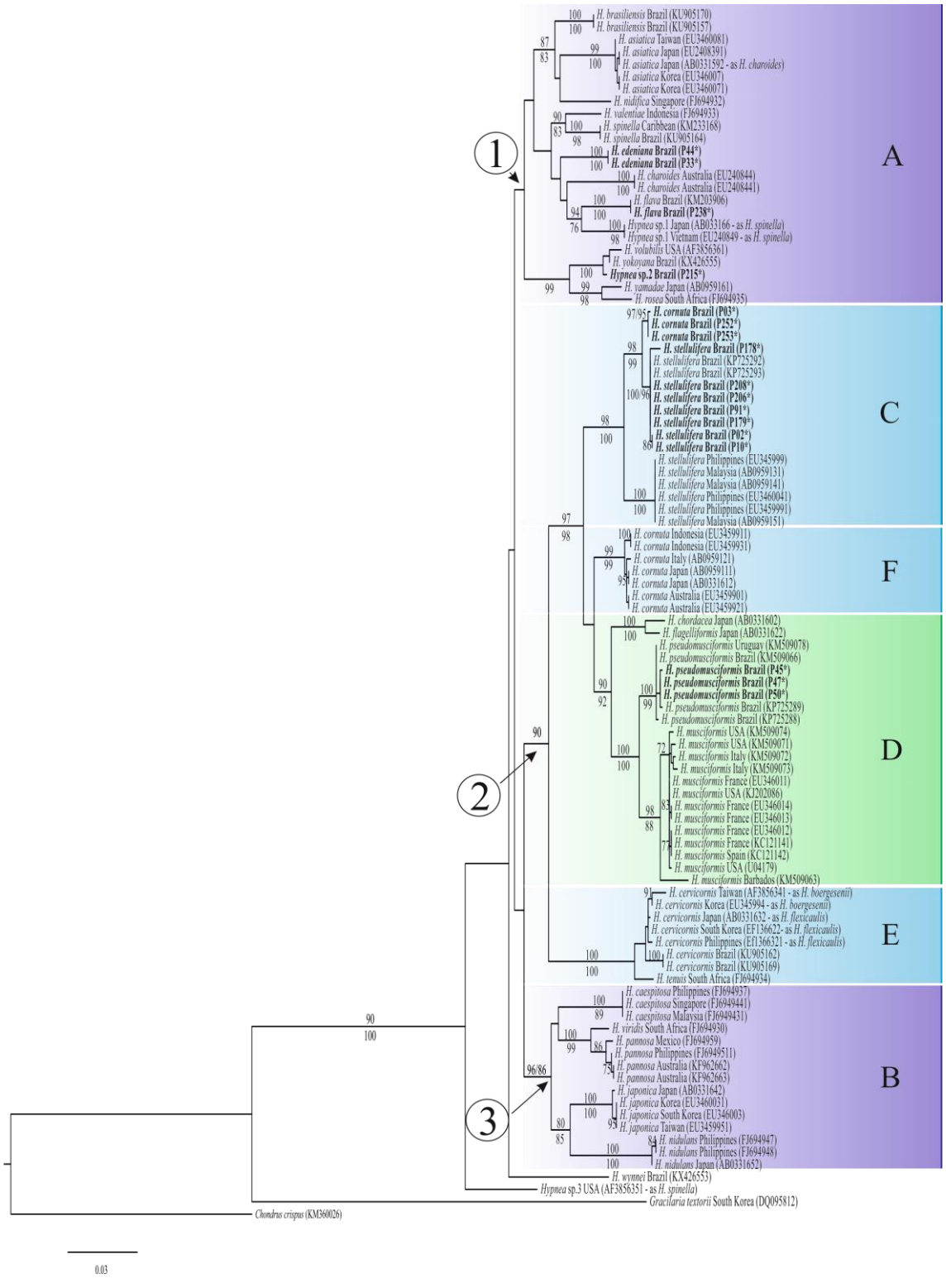


Figure 1. The optimal maximum likelihood (ML) topology based on the concatenate COI-5P + *rbc*L + *psa*A data set (ntax = 35). ML and MP (maximum parsimony) bootstrap values were plotted above and below branches, respectively. Only bootstrap values in MP and ML bootstrap support values branches >75% were plotted. Taxa with new sequences produced in this study are in boldface. New sequences are signed with an asterisk. Colored clades indicate the morphological sections according to thallus habit: blue= *Spinuligerae*, green= *Virgatae* and lilac= *Pulvinatae.*

Figure 2. The optimal maximum likelihood (ML) topology based on the *rbc*L data set (ntax = 98). ML and MP (maximum parsimony) bootstrap values were plotted above and below branches, respectively. Only bootstrap values in MP and ML bootstrap support values branches >75% were plotted. Taxa with new sequences produced in this study are in boldface. New sequences are signed with an asterisk. Colored clades indicate the morphological sections according to thallus habit: blue= *Spinuligerae*, green= *Virgatae* and lilac= *Pulvinatae.*


A
B
C
D
E
F
1
2
3
H. brasiliensis Brazil (KU905170)
H. brasiliensis Brazil (KU905157)
H. asiatica Taiwan (EU3460081)
H. asiatica Japan (EU2408391)
H. asiatica Japan (AB0331592 - as H. charoides)
H. asiatica Korea (EU346007)
H. asiatica Korea (EU346071)
H. nidifica Singapore (FJ694932)
H. valentiae Indonesia (FJ694933)
H. spinella Caribbean (KM233168)
H. spinella Brazil (KU905164)
H. edeniana Brazil (P44*)
H. edeniana Brazil (P33*)
H. charoides Australia (EU240844)
H. charoides Australia (EU2408441)
H. flava Brazil (KM203906)
H. flava Brazil (P238*)
Hypnea sp.1 Japan (AB033166 - as H. spinella)
Hypnea sp.1 Vietnam (EU240849 - as H. spinella)
H. volubilis USA (AF3856361)
H. yokoyana Brazil (KX426555)
Hypnea sp.2 Brazil (P215*)
H. yamadae Japan (AB0959161)
H. rosea South Africa (FJ694935)
H. cornuta Brazil (P03*)
H. cornuta Brazil (P252*)
H. cornuta Brazil (P253*)
H. stellulifera Brazil (P178*)
H. stellulifera Brazil (KP725292)
H. stellulifera Brazil (KP725293)
H. stellulifera Brazil (P208*)
H. stellulifera Brazil (P206*)
H. stellulifera Brazil (P91*)
H. stellulifera Brazil (P179*)
H. stellulifera Brazil (P02*)
H. stellulifera Brazil (P10*)
H. stellulifera Philippines (EU345999)
H. stellulifera Malaysia (AB0959131)
H. stellulifera Malaysia (AB0959141)
H. stellulifera Philippines (EU3460041)
H. stellulifera Philippines (EU3459991)
H. stellulifera Malaysia (AB0959151)
H. cornuta Indonesia (EU3459911)
H. cornuta Indonesia (EU3459931)
H. cornuta Italy (AB0959121)
H. cornuta Japan (AB0959111)
H. cornuta Japan (AB0331612)
H. cornuta Australia (EU3459901)
H. cornuta Australia (EU3459921)
H. chordacea Japan (AB0331602)
H. flagelliformis Japan (AB0331622)
H. pseudomusciformis Uruguay (KM509078)
H. pseudomusciformis Brazil (KM509066)
H. pseudomusciformis Brazil (P45*)
H. pseudomusciformis Brazil (P47*)
H. pseudomusciformis Brazil (P50*)
H. pseudomusciformis Brazil (KP725289)
H. pseudomusciformis Brazil (KP725288)
H. musciformis USA (KM509074)
H. musciformis USA (KM509071)
H. musciformis Italy (KM509072)
H. musciformis Italy (KM509073)
H. musciformis France (EU346011)
H. musciformis USA (KJ202086)
H. musciformis France (EU346014)
H. musciformis France (EU346013)
H. musciformis France (EU346012)
H. musciformis France (KC121141)
H. musciformis Spain (KC121142)
H. musciformis USA (U04179)
H. musciformis Barbados (KM509063)
H. cervicornis Taiwan (AF3856341 - as H. boergesenii)
H. cervicornis Korea (EU345994 - as H. boergesenii)
H. cervicornis Japan (AB0331632 - as H. flexicaulis)
H. cervicornis South Korea (EF136622- as H. flexicaulis)
H. cervicornis Philippines (Ef1366321 - as H. flexicaulis)
H. cervicornis Brazil (KU905162)
H. cervicornis Brazil (KU905169)
H. tenuis South Africa (FJ694934)
H. caespitosa Philippines (FJ694937)
H. caespitosa Singapore (FJ6949441)
H. caespitosa Malaysia (FJ6949431)
H. viridis South Africa (FJ694930)
H. pannosa Mexico (FJ694959)
H. pannosa Philippines (FJ6949511)
H. pannosa Australia (KF962662)
H. pannosa Australia (KF962663)
H. japonica Japan (AB0331642)
H. japonica Korea (EU3460031)
H. japonica South Korea (EU346003)
H. japonica Taiwan (EU3459951)
H. nidulans Philippines (FJ694947)
H. nidulans Philippines (FJ694948)
H. nidulans Japan (AB0331652)
H. wynnei Brazil (KX426553)
Hypnea sp.3 USA (AF3856351 - as H. spinella)
Gracilaria textorii South Korea (DQ095812)
Chondrus crispus (KM360026)
0.03

SUPPLEMENTARY MATERIAL

Table S1. List of *Hypnea* species analyzed in this study with voucher information and GenBank accession numbers. *Accession numbers (***) will be added to the table before the publication.

		GenBank accessions		
Taxa	**Country**	**COI-5P**	***rbc*L**	***psa*A**
Hypnea asiatica Geraldino, Yang et Boo	Gampo, Gyeongju, Korea (Geraldino et al. 2009)	EU240799	EU3460072	FJ694918
	Gampo, Gyeongju, Korea (Geraldino et al. 2009)		EU346007	
	Hengchun, Pintung, Taiwan (Geraldino et al. 2009)		EU346008	
	Shizouka, Shimoda, Japan (as *H. charoides* – Yamagishi & Masuda 2000)		AB033159	
	Chiba, Kurihae Beach, Japan (Geraldino et al. 2009)		EU240839	
H. brasiliensis P.B. Jesus, Nauer & J.M.C. Nunes	Dura Beach,Ubatuba, São Paulo, Brazil (Jesus et al. 2016)	KU905144	KU905170	***
	Mucuge Beach,Porto Seguro, Bahia, Brazil (Jesus et al. 2016)	KU905078	KU905157	***
	Paraíba, Brazil - 103	***		***
	Bahia, Brazil - 214	***		***
H. caespitosa Geraldino et Boo sp. nov.	Philippines (Geraldino et al. 2010)	FJ694902	FJ694942	FJ694916
	Bonbon, Cagayan de Oro, (Geraldino et al. 2010)		FJ694937	
	Cape Rachado, Malaysia (Geraldino et al. 2010)		FJ694943	
	Sentosa Island, Singapore (Geraldino et al. 2010)		FJ694944	
H. cervicornis J. Agardh	Subauma, Entre Rios, Bahia, Brazil Brazil (Jesus et al. 2016)	KU905131	KU905169	***
	Mata de São João, Praia do Forte, Bahia, Brazil (Jesus et al. 2016)	KU905101	KU905162	***
	Gampo, Gyeongju, Korea (Geraldino et al. 2006)	EF136611	EF136622	FJ694919
	Korea - as H. *boergesenii* (Geraldino et al. 2010)	FJ694919	EU345994	
	Taiwan (as *H. boergesenii*) Hommersand & Fredericq.		AF385634	

	Shomoda, Shizuoka prefecture Japan (as *H. flexicaulis* – Yamagishi & Masuda)		AB033163	
	Dancalan, Bulusan, Philippines (as *H. flexicaulis* – Geraldino et al. 2006)	EF136611	EF136632	
	Espírito Santo, Brazil - P166	***		
	Espírito Santo, Brazil - P167	***		
	Espírito Santo, Brazil - P187	***		
	Espírito Santo, Brazil - P188	***		
	Ceará, Brazil - P193	***		
	Rio Grande do Norte - P196	***		
	Rio Grande do Norte - P197	***		
	Rio Grande do Norte - P198	***		
	Piauí, Brazil - P199	***		
	Piauí, Brazil - P200	***		
	Piauí, Brazil - P201	***		
	Piauí, Brazil - P202	***		
	Piauí, Brazil - P203	***		
	Bahia, Brazil - P216	***		
	Bahia, Brazil - P217	***		
	Bahia, Brazil - P218	***		
	Bahia, Brazil - P219	***		
	Bahia, Brazil - P233	***		
	Bahia, Brazil - P234	***		
	Bahia, Brazil - P241	***		
	Bahia, Brazil - P242	***		***
	Bahia, Brazil - P243	***		***
	Bahia, Brazil - P244	***		***
	Bahia, Brazil - P246	***		
	Bahia, Brazil - P248	***		
H. charoides J. V. Lamouroux	Point Peron, Perth, Australia (Geraldino et al. 2009)	EU240818	EU240844	FJ694928
	Chiba, Ashikajima, Japan (Geraldino et al. 2009)		EU240841	
H. chordacea Kützing	Shirahama, Shimoda, Japan (Yamagishi & Masuda 2000)		AB033160	
H. cornuta (Kützing) J. Agardh	Point Peron, Perth, Australia (Geraldino et al. 2009)		EU345990	FJ694927

	Point Peron, Perth, Australia (Geraldino et al. 2009)		EU345992	
	Bali, Indonesia (Geraldino et al. 2009)		EU345991	
	Bali, Indonesia (Geraldino et al. 2009)		EU345993	
	Taranto, Italy (Yamagishi et al. 2003)		AB095912	
	Okinawa Prefecture, Ishigaki Island, Sukuji, Japan (Yamagishi et al. 2003)		AB095911	
	Taranto, Italy (Yamagishi et al. 2003)		AB095912	
	Bahia, Brazil - P03	***	***	***
	Bahia, Brazil - P152	***		***
	Rio de Janeiro, Brazil - P252	***	***	***
	Rio de Janeiro, Brazil - P253	***	***	***
H. edeniana Nauer, Cassano & M.C. Oliveira	Eden Beach, São Paulo, Brazil (Nauer et al. 2014)	KM214573		
	Cruz Beach, Espírito Santo, Brazil (Nauer et al. 2014)	KM214574		
	Pacuiba Beach, São Paulo, Brazil (Nauer et al. 2014)	KM214575		
	Bahia, Brazil - P33	***	***	***
	Bahia, Brazil - P44	***	***	***
H. flagelliformis J. Agardh	Fukaura, Aomori, Japan (Yamagishi et al., 1999)		AB033162	
H. flava Nauer, Cassano & M.C. Oliveira	Carro Beach, Rio de Janeiro, Brazil (Nauer et al. 2014)	KM214569	KM203906	***
	Picinguaba Beach, São Paulo, Brazil (Nauer et al. 2014)	KM214531		
	Rio de Janeiro, Brazil - P237	***		
	Espírito Santo, Brazil - P238	***		
H. japonica Tanaka	Gampo, Gyeongju, Korea (Geraldino et al. 2009)	EU345986	EU346003	FJ694917
	Gampo, Gyeongju, Korea (Geraldino et al. 2009)		EU346003	
	Dali, Keelung, Taiwan (Geraldino et al. 2009)		EU345995	
	Kagoshima prefecture, Japan (Yamagishi et al. 2000)		AB0331642	
H. musciformis (Wulfen) Lamouroux	Cap Ferrat, Villefranche, France (Geraldino et al. 2009)		EU346011	FJ694914

	Theoule, Cannes France (Geraldino et al. 2009)		EU346012	
	Antibes, France (Geraldino et al. 2009)		EU346013	
	Antibes, France (Geraldino et al. 2009)		EU346014	
	Cadiz, Spain (Diaz-Tapia et al. 2013 – unpublished)		KC121141	
	Marsella, France (Diaz-Tapia et al. 2013 – unpublished)		KC121142	
	Fort Fisher, New Hanover Co., USA (Frederic et al. 1996)		U04179	
	North Carolina, Onslow Bay, Fort Fisher Rocks, USA (Freshwater et al., Unpublished)		KJ202086	
	North Carolina, USA (Nauer et al. 2014)		KM509071	
	North Carolina, USA (Nauer et al. 2014)		KM509074	
	Ancona, Italy (Nauer et al. 2014)		KM509072	
	Ancona, Italy (Nauer et al. 2014)		KM509073	
	Barbados, Caribbean (Nauer et al. 2014)		KM509063	
	Magdalena, Colombia - P163	***		
	Isla Mujeres, Mexico - P210	***	***	***
	Isla Mujeres, Mexico - P211	***		***
	Isla Mujeres, Mexico - P212	***	***	***
	Texas, EUA - P182	***		
H. nidifica J. Agardh	Sentosa Island, Singapore (Geraldino et al. 2010)	GQ141879	FJ694932	FJ694929
H. nidulans Setchell	Hedo-misaki, Okinawa prefecture,Japan (Yamagishi et al.,2000)		AB033165	
	Batac, Ilocos Norte, Philippines (Geraldino et al. 2010)		FJ694948	
	Manjagao, Surigao del Norte, Philippines (Geraldino et al. 2010)	FJ694900	FJ694947	FJ694921
H. pannosa J. Agardh	El Sargento, Mexico (Geraldino et al. 2010)	FJ694894	FJ694959	FJ694915
	Pangalo Island, Bohol, Philippines (Geraldino et al. 2010)		FJ694951	
	Australia (Gurgel et al., 2013)		KF962662	

	Australia (Gurgel et al., 2013)		KF962663	
H. pseudomusciformis Nauer, Cassano & M.C. Oliveira	Imbassaí, Entre Rios, Bahia, Brazil (Jesus et al., 2015)	***	KP725289	***
	Cigarras, São Sebastião, São Paulo, Brazil (Jesus et al., 2015)	KP725276	KP725288	***
	Jose Ignacio, Uruguay (Nauer et al. 2014)		KM509078	
	São Paulo, Brazil (Nauer et al. 2014)		KM509066	
	Bahia, Brazil - P45	***	***	
	Bahia, Brazil - P47	***	***	
	Bahia, Brazil - P50	***	***	
	Bahia, Brazil - P96	***		
	Bahia, Brazil - P177	***		
	Maranhão, Brazil - P185	***		
	Maranhão, Brazil - P186	***		
	Espírito Santo, Brazil - P189	***		
	Ceará, Brazil - P190	***		
	Ceará, Brazil - P191	***		
	Ceará, Brazil - P192	***		
	Ceará, Brazil - P194	***		
	Ceará, Brazil - P195	***		
	Rio Grande do Sul, Brazil - P220	***		
	Rio Grande do Sul, Brazil - P221	***		
	Rio Grande do Sul, Brazil - P222	***		
	Rio Grande do Sul, Brazil - P223	***		
	Rio Grande do Sul, Brazil - P224	***		
	Rio Grande do Sul, Brazil - P225	***		
	Rio Grande do Sul, Brazil - P226	***		
	Rio Grande do Sul, Brazil - P227	***		
	Bahia, Brazil - P230	***		
	Bahia, Brazil - P245	***		
	Bahia, Brazil - P251	***		
	Ghana (Ale et al., 2016)	KT899491		
	Ghana (Ale et al., 2016)	KT899492		
H. spinella (C. Agardh) Kützing	Needham's Point, Barbados, Carribeean - P235	KM214525	KM233168	***
	Penha, Ilha de Itaparica, Bahia, Brazil (Jesus et al. 2016)	KU905111	KU905164	***
	Santa Catarina, Brazil - P239	***		***

H. rosea Papenfuss	Durban, South Africa (Geraldino et al. 2010)	GQ141883	FJ694935	FJ694922
H. stellulifera J. Agardh	Panglao, Bohol, Philippines (Geraldino et al. 2009)	EU345984	EU346004	FJ694920
	Panglao, Bohol, Philippines (Geraldino et al. 2009)		EU345999	***
	Bali, Indonesia (Geraldino et al. 2009)		EU345991	
	Pulau Besar, Melaka, Malaysia (Yamagishi et al. 2003)		AB095913	
	Pulau Sipadan, Sabah Malaysia (Yamagishi et al. 2003)		AB095914	
	Pulau Sipadan, Sabah Malaysia (Yamagishi et al. 2003)		AB095915	***
	Santa Cruz de Cabralia, Bahia, Brazil		KP725292	
	Santa Cruz de Cabralia, Bahia, Brazil (Jesus et al., 2015)		KP725293	
	Bahia, Brazil - P02		***	
	Bahia, Brazil - P10		***	
	Bahia, Brazil - P91	***	***	***
	Bahia, Brazil - P178	***	***	***
	Bahia, Brazil - P179	***	***	
	Piauí, Brazil - P206	***	***	***
	Piauí, Brazil - P207	***		***
	Piauí, Brazil - P208	***	***	***
H. tenuis Kylin	Durban, South Africa (Geraldino et al. 2010)	GQ141880	FJ694934	FJ694924
H. valentiae (Turner) Montagne	Bali, Indonesia (Geraldino et al. 2010)	GQ141882	FJ694933	FJ694926
H. viridis Papenfuss	Lala neck, Durban, South Africa (Geraldino et al. 2010)	FJ694908	FJ694930	FJ694923
H. volubilis Searles	Los Angeles, USA (Fredericq et al. 2001)		AF385636	
H. wynnei Nauer, Cassano & M.C. Oliveira	Ilha Grande Island, Angra dos Reis, Rio de Janeiro, Brazil (Nauer et al. 2016)	KX426547	KX426553	
H. yamadae Tanaka	Nomozaki, Nagasaki Prefecture, Japan (Yamagishi et al. 2003)		AB095916	
H. yokoyana Nauer, Cassano & M.C. Oliveira	Laje de Santos, Santos, São Paulo, Brazil (Nauer et al. 2016)		KX426555	
Hypnea sp. 1.	Nha Trang, Panang, Vietnam – as *H. spinella* (Geraldino et al. 2009)	EU240818	EU240848	FJ694925

	Panang, Panang Bay, Vietnam (Geraldino et al. 2009)		EU240849	
Hypnea sp. 2	Bahia, Brazil - P215	***	***	***
	Bahia, Brazil - P264	***		
	Guadeloupe, France - P181 – as *H. divaricata*	***		
	Kenya, Africa - P183 – as *H. valentiae*	***		
Hypnea sp. 3	Florida, USA (as *H. spinella*, Fredericq et al. 2001)		AF385635	

Figure S1. The optimal maximum likelihood (ML) topology based on the COI-5P data set (ntax = 113). ML and MP (maximum parsimony) bootstrap values were plotted above and below branches, respectively. Only bootstrap values in MP and ML bootstrap support values branches >75% were plotted. Taxa with new sequences produced in this study are in boldface. New sequences are signed with an asterisk.

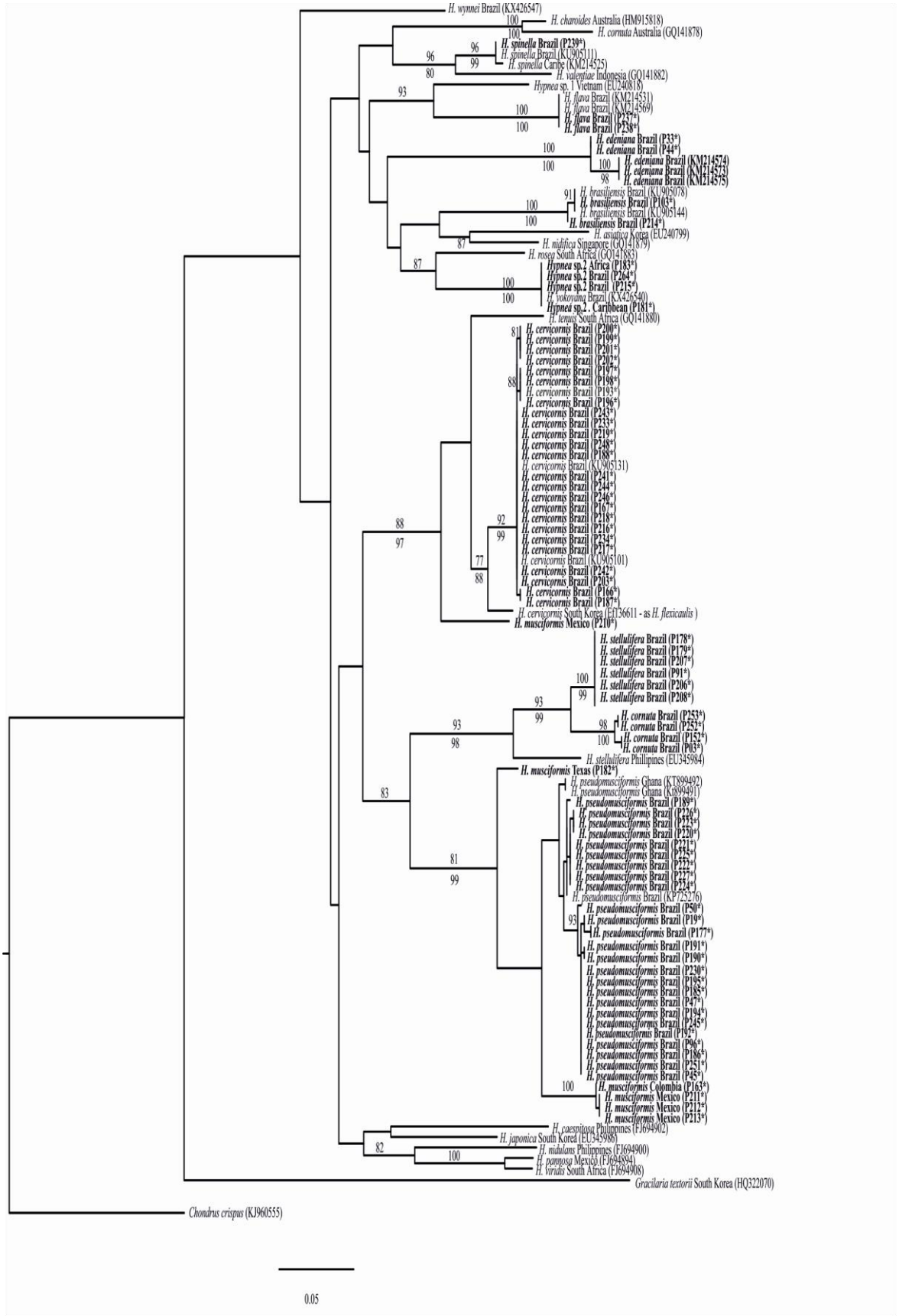

H. wynnei Brazil (KX426547)
H. charoides Australia (HM915818)
H. cornuta Australia (GQ141878)
H. spinella Brazil (P239*)
H. spinella Brazil (KU905111)
H. spinella Caribe (KM214525)
H. valentiae Indonesia (GQ141882)
Hypnea sp. 1 Vietnam (EU240818)
H. flava Brazil (KM214531)
H. flava Brazil (KM214569)
H. flava Brazil (P237*)
H. flava Brazil (P238*)
H. edeniana Brazil (P33*)
H. edeniana Brazil (P44*)
H. edeniana Brazil (KM214574)
H. edeniana Brazil (KM214573)
H. edeniana Brazil (KM214575)
H. brasiliensis Brazil (KU905078)
H. brasiliensis Brazil (P103*)
H. brasiliensis Brazil (KU905144)
H. brasiliensis Brazil (P214*)
H. asiatica Korea (EU240799)
H. nidifica Singapore (GQ141879)
H. rosea South Africa (GQ141883)
Hypnea sp.2 Africa (P183*)
Hypnea sp.2 Brazil (P264*)
Hypnea sp.2 Brazil (P215*)
H. yokoyana Brazil (KX426540)
Hypnea sp.2 . Caribbean (P181*)
H. tenuis South Africa (GQ141880)
H. cervicornis Brazil (P200*)
H. cervicornis Brazil (P199*)
H. cervicornis Brazil (P201*)
H. cervicornis Brazil (P202*)
H. cervicornis Brazil (P197*)
H. cervicornis Brazil (P198*)
H. cervicornis Brazil (P193*)
H. cervicornis Brazil (P196*)
H. cervicornis Brazil (P243*)
H. cervicornis Brazil (P233*)
H. cervicornis Brazil (P219*)
H. cervicornis Brazil (P248*)
H. cervicornis Brazil (P188*)
H. cervicornis Brazil (KU905131)
H. cervicornis Brazil (P241*)
H. cervicornis Brazil (P244*)
H. cervicornis Brazil (P246*)
H. cervicornis Brazil (P167*)
H. cervicornis Brazil (P218*)
H. cervicornis Brazil (P216*)
H. cervicornis Brazil (P234*)
H. cervicornis Brazil (P217*)
H. cervicornis Brazil (KU905101)
H. cervicornis Brazil (P242*)
H. cervicornis Brazil (P203*)
H. cervicornis Brazil (P166*)
H. cervicornis Brazil (P187*)
H. cervicornis South Korea (EF536611 - as H. flexicaulis)
H. musciformis Mexico (P210*)
H. stellulifera Brazil (P178*)
H. stellulifera Brazil (P179*)
H. stellulifera Brazil (P207*)
H. stellulifera Brazil (P91*)
H. stellulifera Brazil (P206*)
H. stellulifera Brazil (P208*)
H. cornuta Brazil (P253*)
H. cornuta Brazil (P252*)
H. cornuta Brazil (P152*)
H. cornuta Brazil (P03*)
H. stellulifera Phillipines (EU345984)
H. musciformis Texas (P182*)
H. pseudomusciformis Ghana (KT899492)
H. pseudomusciformis Ghana (KT899491)
H. pseudomusciformis Brazil (P189*)
H. pseudomusciformis Brazil (P226*)
H. pseudomusciformis Brazil (P253*)
H. pseudomusciformis Brazil (P220*)
H. pseudomusciformis Brazil (P221*)
H. pseudomusciformis Brazil (P222*)
H. pseudomusciformis Brazil (P223*)
H. pseudomusciformis Brazil (P227*)
H. pseudomusciformis Brazil (P224*)
H. pseudomusciformis Brazil (KP725276)
H. pseudomusciformis Brazil (P50*)
H. pseudomusciformis Brazil (P19*)
H. pseudomusciformis Brazil (P177*)
H. pseudomusciformis Brazil (P191*)
H. pseudomusciformis Brazil (P190*)
H. pseudomusciformis Brazil (P230*)
H. pseudomusciformis Brazil (P195*)
H. pseudomusciformis Brazil (P185*)
H. pseudomusciformis Brazil (P47*)
H. pseudomusciformis Brazil (P194*)
H. pseudomusciformis Brazil (P245*)
H. pseudomusciformis Brazil (P192*)
H. pseudomusciformis Brazil (P96*)
H. pseudomusciformis Brazil (P186*)
H. pseudomusciformis Brazil (P251*)
H. pseudomusciformis Brazil (P45*)
H. musciformis Colombia (P163*)
H. musciformis Mexico (P211*)
H. musciformis Mexico (P212*)
H. musciformis Mexico (P213*)
H. caespitosa Philippines (FJ694902)
H. japonica South Korea (EU345988)
H. nidulans Philippines (FJ694900)
H. pannosa Mexico (FJ694894)
H. viridis South Africa (FJ694908)
Gracilaria textorii South Korea (HQ322070)
Chondrus crispus (KJ960555)
0.05

Figure S2. The optimal maximum likelihood (ML) topology based on the *psa*A data set (ntax = 50). ML and MP (maximum parsimony) bootstrap values were plotted above and below branches, respectively. Only bootstrap values in MP and ML bootstrap support values branches >75% were plotted. Taxa with new sequences produced in this study are in boldface. New sequences are signed with an asterisk.

CONSIDERAÇÕES FINAIS

Este estudo constitui a primeira abordagem biossistemática realizada em espécies do gênero *Hypnea*, reunindo ampla amostragem e a combinação de diversas técnicas e análises moleculares, morfológicas e ecológicas. A complexidade na delimitação intraespecífica em *Hypnea* é devida, em parte, à grande plasticidade fenotípica, a ocorrência de diversas espécies crípticas, e a escassez de estudos biossistemáticos no gênero. Desta forma, a história nomenclatural do gênero tem sido confusa, e alguns táxons são tratados como complexos morfológicos devido à difícil separação das espécies.

No primeiro capítulo, a divergência molecular intra e interespecífica em espécies sul americanas foi avaliada pela primeira vez, utilizando os genes COI-5P (mitocondrial), UPA e *rbcL* (plastidiais), análise de distância genética e algoritmo NJ, e análises filogenéticas de Máxima Parcimônia, Máxima Verossimilhança e Bayesiana. Foram detectados altos níveis de diversidade intraespecífica, indicando a possibilidade de ocorrência de espécies crípticas ou incipientes entre os táxons amostrados. Os espécimes pertencentes a um mesmo táxon apresentaram, de forma geral, padrão de agrupamento preferencialmente biogeográfico, e os clusters foram interpretados como linhagens genéticas. *Hypnea stellulifera* foi referida pela primeira vez para o Oceano Atlântico. Nossos resultados indicam que os limites de divergência intraespecífica utilizados na delimitação de espécies de *Hypnea* devem ser reconsiderados, principalmente em táxons de distribuição cosmopolita (COI-5P: 0-8%, *rbc*L: 0-2,5%).

No capítulo 2, foi realizada a delimitação molecular de complexo de espécies de *Hypnea* com história taxonômica bastante confusa. Diversos métodos delimitação específica (mBGD, ABGD, SPN, PTP, GMYCs, e GMYCm) foram realizados com base nos marcadores COI-5P e *rbcL.*A análise conjunta de dados morfológicos e moleculares

resultou em algumas mudanças de *status* e em novidades taxonômicas: *Hypnea brasiliensis* foi descrita como uma nova espécie amplamente distribuída no litoral brasileiro e *H. aspera* (*H. boergesenii*) e *H. flexicaulis* foram sinonimizadas a *H. cervicornis*.

O estudo fenológico da variedade "*Hypnea nigrescens*" (complexo *H. pseudomusciformis*) é apresentado no capítulo 3. O estágio reprodutivo, o comprimento do talo e o percentual de biomassa de cada estágio foram estimados durante o período de um ano. Os dados fenológicos indicam que as variedades *musciformis* e *nigrescens* (= *H. pseudomusciformis*) apresentam várias diferenças ecológicas, além das diferenças morfológicas já reportadas na literatura. Gametófitos femininos foram muito frequentes, ocorrendo em todo o período do estudo diferentemente do relatado para as populações brasileiras de "*H. musciformis*", com as quais *"H. nigrescens*" foi sinonimizada e descrita como uma nova espécie, *H. pseudomusciformis*. Outra diferença importante é a alta frequência de gametófitos masculinos observada em *"H. nigrescens*", fase que geralmente é reportada como rara ou ausente em estudos realizados com exemplares da variedade *musciformis* no Brasil. Em "*H. nigrescens*", a biomassa dos gametófitos femininos apresentou forte correlação positiva com a temperatura do ar e negativa com a umidade relativa, demonstrando que estas condições (geralmente observadas no verão) são responsáveis pela indução da fertilização e consequente aparecimento dos cistocarpos. Estes resultados também demonstram que este tipo de abordagem pode constituir um importante marcador taxonômico em complexos de espécies de algas.

Hypnea pseudomusciformis é uma espécie recentemente descrita, restrita às costas brasileiras e uruguaias, segregada de *H. musciformis*, restrita ao Hemisfério Norte. No capítulo 4 é apresentada a investigação da história evolutiva destas duas espécies estreitamente relacionadas, baseada em abordagem filogeográfica utilizando uma região

mitocondrial (COI-5P) e outra plastidial (*rbcL*). *H. musciformis* possui duas linhagens, a primeira formada (M1) por populações que ocorrem em ambas regiões costeiras do Oceano Atlântico, e a segunda (M2) composta por populações distribuídas em ambos os lados do Istmo do Panamá. Em *H. pseudomusciformis* ocorreram três linhagens, Nordeste do Brasil (PM1), Sudeste do Brasil ao Uruguai (PM2) e Ghana (PM3), no continente Africano. Os estudos filogeográficos corroboraram a diferenciação entre as populações de *H. musciformis* e *H. pseudomusciformis* indicando que a primeira espécie encontra-se em equilíbrio, enquanto a segunda divergiu mais recentemente e encontra-se em expansão. Neste estudo nós confirmamos a ampliação da distribuição geográfica de *H. pseudomusciformis* para a costa da África, enquanto *H. musciformis* parece ser restrita ao Hemisfério Norte, indicando padrão vicariante de especiação.

No capítulo 5, a análise combinada de sequências de três marcadores moleculares (COI-5P, *rbc*L e *psa*A), bem como o grande número de espécimes amostrados, principalmente ao longo do litoral brasileiro, resultou na mais completa e bem resolvida filogenia do gênero até o momento. Neste estudo foram utilizados 255 terminais para inferir as relações filogenéticas de 28 espécies do gênero. As seções infragenéricas de *Hypnea*, conforme reconhecidas atualmente, não foram validadas no presente estudo, uma vez que todas apresentaram padrão parafilético e/ou polifilético. Nossas análises não recuperaram nenhum clado formado exclusivamente por espécies consistindo de eixo principal ereto e densamente ramificado (*Virgatae*); talo ereto e intricado ramificado de forma alterna (*Spinuligerae*) ou com hábito prostrado, com ramos anastomosados formando tapetes (*Pulvinatae*). Esses resultados sugerem a falta de sinapomorfias que sustentem as seções, muito provavelmente devido à grande plasticidade fenotípica observada no gênero. Por este motivo, sugeriu-se que as seções formalmente propostas

com base no hábito do talo sejam abandonadas uma vez que não refletem a história evolutiva do gênero.

Este estudo revelou ainda a necessidade de revisão de algumas espécies com poucas sequências disponíveis, às quais nenhum dado morfológico foi associado e que apresentaram padrão para ou polifilético. Entre essas espécies estão *Hypnea japonica*, *H. charoides* e *Hypnea* sp.1 e sp.3, que foram identificadas inicialmente como *H. spinella* mas provavelmente tratam-se de identificações errôneas. As relações filogenéticas de *H. wynnei* não puderam ser analisadas porque esta espécie ocupou uma posição indefinida nas análises individuais baseadas no COI-5P e no *rbc*L. Problemas taxonômicos ainda permanecem em alguns clados que reúnem espécies crípticas e outras nas quais, o isolamento reprodutivo parece não ser completo. Amostragem de mais marcadores deve ser realizada para definitivamente comprovar a validade da espécie recentemente descrita *H. yokoyana*. Estudos populacionais devem ser realizados para detectar a estruturação genética e populacional das variedades morfológicas de *H. pseudomusciformis*, bem como para verificar a ocorrência de fluxo gênico entre os exemplares brasileiros de *H. cornuta* e *H. stellulifera*.

Os estudos aqui apresentados demonstraram a complexidade e a importância dos estudos biossistemáticos para a delimitação específica e a compreensão das relações evolutivas em *Hypnea*.

RESUMO

O gênero *Hypnea* caracteriza-se por apresentar talo ereto ou prostrado, muito ramificado de forma irregular, dicotômica ou lateral, e com numerosos râmulos curtos laterais. Atualmente, o gênero apresenta 66 espécies amplamente distribuídas nos mares tropicais e subtropicais, com ocorrência nos Oceanos Atlântico, Índico e Pacífico. No entanto, diversos problemas taxonômicos ainda são recorrentes no gênero, como por exemplo, um elevado número de complexos taxonômicos, espécies pobremente definidas, espécies crípticas e prováveis sinônimos, além de frequentes falhas na identificação específica. Diante disto, este estudo teve como objetivo geral a realização de estudos biossistemáticos e filogenéticos em espécies do gênero *Hypnea*, baseados em caracteres morfológicos, ecológicos e moleculares (COI-5P, *rbc*L e *psa*A). Neste estudo, *H. stellulifera* foi referida pela primeira vez para o Oceano Atlântico. Os resultados indicam que os limites de divergência intra e interespecífica utilizados na delimitação de *Hypnea* devem ser reconsiderados, principalmente em táxons de distribuição cosmopolita. Diversos métodos de delimitação específica (mBGD, ABGD, SPN, PTP, GMYCs, e GMYCm) também foram realizados com a finalidade de delimitar as espécies do complexo *H. cervicornis*. Estas análises resultaram na descrição de uma nova espécie amplamente distribuída no litoral brasileiro: *H. brasiliensis*, e na sinonimização de *H. aspera* (*H. boergesenii*) e *H. flexicaulis* a *H. cervicornis*. Recentemente, exemplares identificados como *H. musciformis*, *H. nigrescens* e *H. valentiae* foram considerados variações morfológicas do complexo *H. pseudomusciformis*. Com o intuito de descrever o padrão fenológico de "*H. nigrescens*" e verificar se uma abordagem ecológica poderia auxiliar na delimitação deste complexo, o estágio reprodutivo, o comprimento do talo e o percentual de biomassa de cada estágio foram estimados durante o período de um ano em uma população do litoral do estado da Bahia. "*H. nigrescens*" apresentou alta frequência de gametófitos femininos masculinos, o que representa uma grande diferença ecológica quando comparada com os relatos literários para "*H. musciformis*", indicando que esta abordagem pode se constituir um importante marcador taxonômico. As histórias evolutivas de *H. pseudomusciformis* e *H. musciformis* foram investigadas com base em abordagem filogeográfica. Os estudos filogeográficos corroboraram a diferenciação entre as populações de *H. musciformis* e *H. pseudomusciformis*, indicando que a primeira espécie encontra-se em equilíbrio, enquanto que a segunda divergiu mais recentemente e encontra-se em expansão. Neste estudo nós confirmamos a ampliação da distribuição

geográfica de *H. pseudomusciformis* da América do Sul para a costa da África, enquanto que *H. musciformis* parece ser restrita ao Hemisfério Norte, indicando padrão vicariante de especiação. A análise combinada das sequências de três marcadores moleculares, bem como o grande número de sequências analisadas (255 de 28 espécies), resultou na mais robusta e bem resolvida filogenia do gênero até o momento. As seções infragenéricas de *Hypnea* não foram validadas no presente estudo, uma vez que todas apresentaram padrão parafilético e/ou polifilético. Problemas taxonômicos ainda permanecem em alguns clados com espécies crípticas ou nas quais o isolamento reprodutivo parece não ser completo. Os resultados aqui apresentados demonstraram a complexidade e a importância dos estudos biossistemáticos para a delimitação dos limites específicos e compreensão das relações evolutivas em *Hypnea*.

Palavras-chave: *Hypnea*, Biossistemática, Filogenia, Delimitação molecular, Rhodophyta.

ABSTRACT

The genus *Hypnea* presents erect or prostrate thallus, abundantly and irregularly branched (dichotomously or laterally branched), with several short branchlets. Currently, the genus has 66 species widely distributed in tropical and subtropical waters, occurring in the Atlantic, Indian and Pacific Oceans. However, taxonomic problems are recurrent in the genus, with a large number of taxonomic complexes, poorly defined and cryptic species, probable synonyms and frequent failure to identify some species. In view of these difficulties, this study aimed to conduct biosystematic and phylogenetic studies in the genus *Hypnea*, based on morphological, ecological and molecular characters (COI-5P, *rbc*L and *psa*A). In this study, *H. stellulifera* was referred to the Atlantic Ocean for the first time. Our results indicate that the limits of intra- and interspecific molecular divergence levels in *Hypnea* species should be reconsidered, especially in cosmopolitan taxa. Several DNA-based delimitation methods (mBGD, ABGD, SPN, PTP, GMYCs and GMYCm) were carried out in order to delimit the *H. cervicornis* complex. These analyzes indicate the existence of a new specie to science, widely distributed on the Brazilian coast, described here as *H. brasiliensis*. We propose that *H. aspera* and *H. flexicaulis* represent synonyms of *H. cervicornis*. Recently, Brazilian specimens identified as *H. musciformis*, *H. nigrescens* and *H. valentiae* were considered morphological variations of *H. pseudomusciformis*. We investigated the phenology of a tropical population of "*Hypnea nigrescens*" in order to describe its biology and verify if this ecological approach would be helpful to elucidate its taxonomic status. Biomass, thallus length and reproductive stage were estimated over the period of a year. "*H. nigrescens*" showed remarkable ecological differences from "*H. musciformis*". High frequency of female and male gametophytes indicated that this approach can be an important taxonomic marker. The evolutionary history of *H. musciformis* and *H. pseudomusciformis* were investigated based in a phylogeographic approach. Results confirmed the differentiation between *H. musciformis* and *H. pseudomusciformis* populations, indicating that the first specie is in equilibrium, while the second diverged more recently and is expanding. We confirmed the extension of the distribution range of *H. pseudomusciformis* from South America to Africa, while *H. musciformis* seems to be restricted to the Northern Hemisphere. These findings suggest vicariance as the main speciation pattern. Combined analysis of three molecular markers, as well as the large number of sequences analyzed (255 from 28 species), resulted on the more robust and well-resolved phylogeny of the genus to date.

Our analyses proved that the three sections currently recognized of the genus *Hypnea* are invalid, since they all appeared as paraphyletic or polyphyletic. Taxonomic problems remain in those clades with cryptic species and in those in which the reproductive isolation seems to be incomplete. Our data demonstrated the complexity and importance of biosystematic studies for the delimitation of species limits and a better understanding of evolutionary relationships in *Hypnea*.

Keywords: Biosystematics, *Hypnea*, Molecular Delimitation, Phylogeny, Rhodophyta.

ANEXO 1:

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