Systematics of the genus *Halimeda* (Bryopsidales, Chlorophyta) in Brazil including the description of *Halimeda jolyana* sp. nov.

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**ABSTRACT:** The genus *Halimeda* has a wide geographic distribution in tropical to subtropical regions of the world. Molecular studies have uncovered cryptic and pseudocryptic species as well as strong biogeographic signals in phylogenies. To date, the diversity of Brazilian Bryopsidales has been studied from only a morphological perspective. Here we revised the diversity of Brazilian *Halimeda* based on molecular data (DNA barcode assessments of the *tufA* marker and multigene concatenated phylogenies) as well as morphological observations. Of the seven recognized morphospecies, only three were confirmed by molecular data: *Halimeda opuntia*, *H. simulans* and *H. incrassata*. The remaining four species, referred to as *H. aff. cuneata*, *H. aff. gracilis*, *H. aff. tuna* and *H. aff. discoidea*, showed morphological similarities with known species, but their sequences did not group with sequences of specimens from type localities, indicating the presence of cryptic diversity. Among the four taxa, *H. aff. cuneata* is so far endemic to Brazil and is herein described as a new species, *H. jolyana* sp. nov. The remaining three (pseudo-)cryptic species require further studies using a global sampling design. The evolutionary and biogeographic origins of Brazilian *Halimeda* species are discussed based on new molecular phylogenetic hypotheses.

**KEY WORDS:** Biogeography, DNA Bar coding, Multigene, Phylogeny, *tufA*

**INTRODUCTION**

The marine green macroalgal genus *Halimeda* J.V.Lamouroux has 44 species (Guiry & Guiry 2016) and is an important constituent of calcareous sediment on the continental shelf in tropical and subtropical environments (Ries 2009). *Halimeda* species precipitate calcium carbonate in between their cells walls in the form of aragonite. The thallus is composed of calcified green segments formed by coenocytic filaments. Their segments accumulate after death (Hillis-Colinvaux et al. 2010) to form reef limestone, which can be extracted and commercially exploited to produce lime or used in animal feed (Bandeira-Pedrosa et al. 2004b). *Halimeda* species are conspicuous members of benthic macroalgal communities, often forming clumps of thalli or monospecific meadows and providing shelter for important fishery resources, such as lobster (*Panulirus argus* Latreille, 1804) (Lentner & Ellis 2014). *Halimeda* specimens also produce secondary metabolites or bioactive molecules with antimicrobial activity, conferring them further economic value (e.g. Paul & Fenical 1984). The genus has received much attention by taxonomists due to its ecological and economic importance (Verbruggen et al. 2005a, b; Dijoux et al. 2012). Furthermore, several species present great phenotypic diversity. The genus contains cryptic and pseudocryptic species, making species delimitation on morphological grounds problematic (Kooistra et al. 2002; Verbruggen et al. 2005a, b; Dijoux et al. 2012; Pongparadon et al. 2015; Cremen et al. 2016).

Molecular studies have used techniques such as DNA bar coding to assist in green algal species delineation (e.g. Famâ et al. 2002; Saunders & Kucera 2010; Sauvage et al. 2016). The plastid *tufA* marker has been successfully applied to delimit *Halimeda* species in New Caledonia (Dijoux et al. 2012) and Thai-Malay (Pongparadon et al. 2015) based on its suitable characteristics as both a DNA barcode and a phylogenetic marker (Saunders & Kucera 2010). *TufA* has also provided good phylogenetic resolution for other genera in the Bryopsidales, such as *Caulerpa* (Belton et al. 2014), confirming the effectiveness of this gene to discriminate species in the order. However, caution must be exercised, as the *tufA* marker is not a universal marker in the Chlorophyta. For example, *tufA* does not amplify in PCR reactions in species of Cladophorales, and hence its potential use within that order cannot be assessed (Saunders & Kucera 2010).

Maranhão state (northeastern Brazil, 02°31’47”S) to Rio de Janeiro state (southeastern Brazil, 23°22’05”S) and from intertidal to subtidal habitats up to 166 m deep (Bandeira-Pedrosa et al. 2004b). Due to high levels of morphological plasticity, the presence of cryptic species and the lack of molecular information, the main objective of this study was to revise the Brazilian Halimeda flora based on a combination of molecular and morphological data. Aspects of the biogeography of the Brazilian Halimeda flora are discussed. Our approach consisted of DNA bar code species delimitation methods using the tufA marker, phylogenetic reconstructions based on a concatenated data set of three genes and morphological observations.

MATERIAL AND METHODS

Specimens were collected along the Brazilian coast during 2009–2014 from the state of Maranhão (02°31’47”S, 44°18’10”W) to the state of Espírito Santo (20°19’10”S, 40°20’16”W) (Fig. 1). A list of studied specimens with collection details and voucher accession numbers is presented in supplementary data Table S1. Samples were collected in either the intertidal region during low tide or subtidally to a depth of 24 m via SCUBA. Samples for molecular analysis were cleaned, dried in silica gel or preserved in absolute ethanol. Voucher specimens were deposited in the following herbaria: PEUFR (Rural Federal University of Pernambuco, Brazil), MELU (University of Melbourne, Australia) and SPF (São Paulo University, Brazil).

The internal and external morphology were analysed according to Hillis-Conlivaux (1980) and Bandeira-Pedrosa et al. (2004b). For the internal morphology, specimens were decalcified with 20% HCl, and the filaments were dissected using a Leica S6D (Wetzlar, Germany) stereoscopic microscope. Utricle measurements and observations were performed with a Zeiss optical microscope Zeiss Axioskop (Oberkochen, Germany) and a stereoscopic microscope (Leica S6D).

Silica-dried samples were macerated in liquid nitrogen to obtain a fine powder. DNA extraction was performed using a CTAB (cetyl trimethyl ammonium bromide) protocol as described in Oliveira-Carvalho et al. (2012). The plastid tufA gene was amplified with the primer pairs tufAF and tufAR (Famá et al. 2002). A single specimen per species-level cluster recognised in the tufA data was selected for rbcL amplification, which was achieved with two overlapping fragments with the primer pairs: F22-41 (Curtis et al. 2008) + R689–667 (Hanyuda et al. 2000) and F623-603 (Curtis et al. 2008) + R1396–1372 (Lam & Zechman 2006).

The genes were amplified by polymerase chain reaction (PCR). PCR was performed for each marker in a final volume of 50 μl, using 37.5 μl of MilliQ water, 5 μl of 10× PCR buffer, 1.5 μl of 50 mM MgCl2, 1 μl of 0.2 mM dNTP, 1 μl of 0.2 μM each primer, 3 μl of total DNA and 0.25 μl of Taq DNA polymerase (1.25 U; Promega Corp., Madison, Wisconsin USA). The reactions were performed in a Techne TC-4000 thermocycler (Bibby Scientific Ltd, Staffordshire, UK). For the tufA gene, the amplification cycle followed the Famá et al. (2002) protocol and for rbcL followed Curtis et
al. (2008). PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. Purified amplicons were sequenced in both directions using the same primers mentioned above and the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California USA) on an ABI PRISM 3100 or ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

Consensus sequences from the reads and multiple sequence alignments were constructed manually using BioEdit v7.0.4.1 software (Ibis Biosciences, Carlsbad, California USA) (Hall 1999). In order to assess species membership of Brazilian specimens, we ran analyses of the tufA gene with data from previously published studies. For tufA analyses, Penicillus capitatus Lamarck (KU361927) and Udotea sp. (KU361932) were used as outgroups. Neighbour-joining (NJ) analysis was performed for tufA marker in PAUP v4.0b10 (Sinauer Associates Inc., Sunderland, Massachusetts USA) (Swofford 2002) with 2000 bootstrap replicates. NJ was implemented using the Tamura & Nei (1993) substitution model.

The most appropriate model of sequence evolution for maximum likelihood (ML) and Bayesian inference (BI) was selected using jModeltest v2.1.10 (Darriba et al. 2012) under the Akaike information criterion (AIC) as implemented on the online server CIPRES Science Gateway v3.3. The model selected was the general time reversible with invariant sites and gamma-distributed rates for the variable sites (GTR+I+G). ML analysis was performed using RAxML v8.2.0 (Stamatakis 2014) on the CIPRES portal with 100 bootstrap replicates. BI analysis was performed in MrBayes v3.0.4 (Huelsenbeck & Ronquist 2001) using four chains of the Markov chain Monte Carlo runs, sampling one tree every 1000 generations for 5,000,000 generations, starting with a random tree. The analysis was concluded when the average deviation of split frequencies was less than 0.01. We discarded the first 30,000 generations in both runs as the burn-in to build the consensus tree. The range of tufA divergence values within and between species was calculated using uncorrected p-distances in PAUP.

In order to reliably infer the relationships between Brazilian and other Halimeda species, we constructed a concatenated alignment of tufA, rbcL, and 18S DNA sequences, including some of our sequences along with previously published data. This data set was subjected to ML and BI phylogenetic analyses. ML analyses were carried out with RAxML v7.2.8 (Stamatakis 2006) with a GTR+I+G model and 100 bootstrap replicates. A chronogram was estimated to draw further biogeographic and evolutionary inferences from the data. The chronogram was constructed using the relaxed molecular method implemented in BEAST v1.8.2 (Drummond et al. 2012), the GTR+I+G model of sequence evolution, a Yule tree prior and a lognormal noncorrelated molecular clock model. The root of the tree, which was inferred by the relaxed molecular clock, was set to have an age of 147 Ma (Verbruggen et al. 2009b). The time calibrations by Verbruggen et al. (2009b) rely only on the fossil record of siphonous green algae, and hence no vicariance events are used to calibrate the molecular clock. The analysis was run for 10 million generations, logging the parameters and tree every 1000th generation. Stationarity of the run was determined visually with Tracer v1.6.0 and the burn-in set at 1 million generations. The post–burn-in sample of trees was summarized into a consensus with TreeAnnotator v1.8.2, calculating median node heights.

RESULTS

Morphological analyses

Seven morphologically distinct taxa were identified among the 37 samples of Halimeda obtained from tropical and warm-temperate coasts of Brazil (Figs 2–8): H. tupa (Fig. 2), H. opuntia (Fig. 3), H. aff. discoidea (Fig. 4), H. simulans (Fig. 5), H. aff. gracilis (Fig. 6), H. jolyana sp. nov. (= H. aff. cuneata) (Fig. 7) and H. incrassata (Fig. 8). A comparison of morphological and anatomical characters among species is shown in Table 1.

Molecular species delimitation (tufA alignment)

The tufA alignment used for species delimitation was 792 nucleotides long and contained a total of 119 sequences: 36 newly generated and 83 downloaded from GenBank (Table S2). The data set consisted of 436 constant characters, of which 307 corresponded to parsimony-informative sites. The phylogeny showed five clades corresponding to distinct sections of the genus; however there was no support for section Halimeda and moderate support for section Microdesiac in the NJ tree (Fig. 9).

Two species of section Rhipsalis – H. simulans and H. incrassata – showed the lowest interspecific differences for tufA (0.9%). Sequences of H. simulans and H. incrassata grouped with previously sequenced samples, confirming their morphological identification. The type locality of H. simulans is Isla de Culebra, Puerto Rico (Hillis-Conlivaux 1980), and the sequences available in the database are from Jamaica, an island near the type locality. The type locality of H. incrassata is ‘West Indies’ (Silva et al. 1996). DNA sequence for tufA of H. incrassata from Brazil is identical to a sequence from the British Virgin Islands (FJ624534), suggesting that our material is true H. incrassata (Fig. 8).

In section Opuntia, only the pantropical species H. opuntia was present in Brazil, with 100% identity between sequences from Brazil, French Polynesia and the Philippines (Fig. 9). There is no sequence from the type locality, Jamaica (Hillis-Colinvaux 1980), for comparison.

For section Pseudo-opuntia, only specimens identified as H. aff. gracilis were found in Brazil (Fig. 6). In the tufA analyses, the Brazilian specimens did not group with sequences from the type locality, Sri Lanka, from which they differed by 3.8%, suggesting that they are not the same species. There was no intraspecific variation among Brazilian tufA sequences of H. aff. gracilis (n = 2). The genetic divergence for H. gracilis from different countries ranged from 0.3% to 7.2%, and a 2.4% sequence divergence was found between Brazilian and US (Florida) specimens. The phylogenetic position and the high genetic divergence values between Brazilian and Floridian specimens of H. gracilis indicate that H. aff. gracilis from Brazil is a new species. Halimeda gracilis from the Philippines...
Figs 2–8. Habit of the studied taxa. Scale bars = 1 cm.

Fig. 2. *H. tuna*.
Fig. 3. *H. opuntia*.
Fig. 4. *H. aff. discoidea*.
Fig. 5. *H. simulans*.
Fig. 6. *H. aff. gracilis*.
Fig. 7. *H. aff. cuneata* (= holotype of *H. jolyana*).
Fig. 8. *H. incrassata*. 
Table 1. Summary of morphoanatomical characteristics of *Halimeda* species from Brazilian coasts.

<table>
<thead>
<tr>
<th>Features</th>
<th><em>H. opuntia</em></th>
<th><em>H. incrassata</em></th>
<th><em>H. simulans</em></th>
<th><em>H. aff. discoidea</em></th>
<th><em>H. aff. gracilis</em></th>
<th><em>H. aff. tuna</em> (as <em>H. aff. cuneata</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape of segments</td>
<td>reniform, ribbed, auriculate, cylindrical, flattened and trilobed</td>
<td>cylindrical, flattened</td>
<td>ribbed, reniform, subcylindrical, discoid and subcuneate</td>
<td>reniform, discoid</td>
<td>reniform, cuneate and subcylindrical</td>
<td>reniform, discoid and subcuneate</td>
</tr>
<tr>
<td>Holdfast</td>
<td>multiple holdfasts</td>
<td>bulbous</td>
<td>bulbous</td>
<td>basal and small</td>
<td>single</td>
<td>basal and single</td>
</tr>
<tr>
<td>Thallus length (cm)</td>
<td>3.5–5.5</td>
<td>3–4</td>
<td>8</td>
<td>2.5</td>
<td>6–17</td>
<td>3.8–5</td>
</tr>
<tr>
<td>Layer of utricles</td>
<td>short with 2 or 3 medullary siphons</td>
<td>single cluster by means of pores connecting adjacent siphons</td>
<td>single cluster by means of pores connecting adjacent siphons</td>
<td>short or complete by 2 or 3 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
<td>short or complete by 2 medullary siphons</td>
</tr>
<tr>
<td>Nodal fusion</td>
<td>short or complete by 2 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
<td>complete by 2 or 3 medullary siphons</td>
</tr>
<tr>
<td><em>H. opuntia</em></td>
<td>12.5–35</td>
<td>40–62.5</td>
<td>22.5–45</td>
<td>47.5–75</td>
<td>47.5–67.5</td>
<td>27.5–50</td>
</tr>
<tr>
<td>Primary utricles diameter (surface view, μm)</td>
<td>12.5–50</td>
<td>45–90</td>
<td>40–100</td>
<td>25–85</td>
<td>40–75</td>
<td>50–125</td>
</tr>
<tr>
<td>Primary utricles length (frontal view, μm)</td>
<td>12.5–87.5</td>
<td>30–72.5</td>
<td>50–87.5</td>
<td>115–225</td>
<td>30–150</td>
<td>52.5–90</td>
</tr>
<tr>
<td>Second utricles length (frontal view, μm)</td>
<td>rounded to polygonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>rounded</td>
<td>polygonal</td>
</tr>
<tr>
<td>Shape of utricles (surface view, μm)</td>
<td>rounded to polygonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>rounded</td>
<td>polygonal</td>
</tr>
</tbody>
</table>
(FJ624727) and South Africa (JN644680) were the most divergent relative to other samples from this species complex (7%–7.2%) (Fig. 9), indicating that Philippine and South African samples are not genuine _H. gracilis_.

In section _Halimeda_, Brazilian specimens identified as _H. aff. tuna_ showed low intraspecific divergence (0%–0.2%, n = 5) and are sister to a cladode formed by _H. tuna_ (Mexico) and _H. scabra_ (Bahamas). The Brazilian specimens of _H. aff. tuna_ diverged from _H. tuna_ from Mexico by 1.2% and from _H. scabra_ by 0.7%. The Brazilian specimens did not group with _H. tuna_ from the Mediterranean Sea, the type locality of the species. The divergence between the Brazilian specimens and those from the type locality was high and ranged from 10.5% to 19.9%. It is noteworthy that samples from the Mediterranean Sea (Italy and France) differed from each other by 10% (79 base pairs). This high divergence may indicate that these are all distinct taxa. Brazilian specimens identified as _H. aff. cuneata_, including a sequence previously published as _H. cuneata_ from Brazil (AY826358), were phylogenetically distinct from well-supported clades of _H. cuneata_ from other parts of the world. The type locality of _H. cuneata_ is South Africa, from which two species-level clusters corresponding to the morphology of _H. cuneata_ are known (Verbruggen et al. 2005a). The intraspecific variation of Brazilian _H. aff. cuneata_ range between 0.2% and 0.7% (n = 7). Brazilian _H. aff. cuneata_ diverged from _H. cuneata_ from different countries by 9.1%–10.3% and from sequences from the type locality (AY826353, AY826354) by 8.5%–12.5%, indicating that the Brazilian specimens are not _H. cuneata_ but a separate species. The sample of Brazilian _H. aff. discoidea_ grouped with a sequence from the Canary Islands, Spain (AY826361), and is sister to a well-supported _H. discoidea_ clade from Jamaica (AY826362). The type locality of _H. discoidea_ is considered Kamtschatka (northeastern Russia), but this information is questionable (Hillis-Conlivaux 1980), and there is no sequence from the type locality for comparison. The Brazilian _H. aff. discoidea_ clade is separated from sequences of _H. discoidea_ from the Indo-Pacific (e.g. New Caledonia, JN644661). _Halimeda discoidea_ showed intraspecific divergences ranging from 1% to 10.1%. The highest observed divergence occurred between samples of _H. aff. discoidea_ from Brazil and New Caledonia (10.1%) and the lowest between Brazil and Canary Islands sequences (1%). Specimens from Brazil and Jamaica differed by 4.3%.

**Molecular phylogeny (concatenated alignment)**

The chronogram (Fig. 10) also showed how Brazilian species (gray boxes) relate to species from elsewhere. A tree with error bars for the inferred node ages is provided as supplementary material (S3). _Halimeda jolyana_ (= _H. aff. cuneata_) was recovered with high confidence in the top lineage of section _Halimeda_, but resolution within that lineage is poor, and the exact position of the species was inconclusive. The chronogram indicates that _H. jolyana_ (= _H. aff. cuneata_) is an early-branching lineage, diverging from other lineages c. 70 Ma (95% HPD: 88–53 Ma). Brazilian _H. aff. discoidea_ and _H. aff. tuna_ are recovered in the same lineage of section _Halimeda_. In section _Opuntia_, the pantropical species _H. opuntia_ was the most closely related to the Indo-Pacific species _H. distorta_ 1 and 2. In section _Pseudo-opuntia_, the chronogram indicates that the Brazilian species _H. aff. gracilis_ is an early-branching species (diverging c. 50 Ma; 95% HPD 59–37 Ma) without close relatives. _Halimeda incrassata_ and _H. simulans_ were inferred as sister species inside a well-supported lineage that also contained the Caribbean species _H. monile_ (J.Ellis & Solander) J.V.Lamouroux and the Pacific species _H. cf. stuposa_ W.R.Taylor.

**Taxonomic changes**

Based on our results, a new species is described to accommodate the taxon previously known as _H. cuneata_ from Brazil:

_Halimeda jolyana_ Ximenes, Bandeira-Pedrosa, Cassano, Oliveira-Carvalho, Verbruggen & S.M.B.Pereira, _sp. nov._

_Figs 7, 11–22_

**DIAGNOSIS**: Thallus with light calcification and dark green colour, composed of very thick segments, 1.7–3.0 mm; segments often rounded, and some of them nearly half as long; nodal region with stalk zone giving loose appearance between the segments; medullary filaments torulose.

**HOLOTYPE**: Brazil, Espirito Santo, Anchicata, Praia de Castelhanos, 20°48’45.4°S, 40°38’05.6°W, leg. F. Scherner, collected 30 March 2012, subtidal zone, 3 m deep, deposited in Rural Federal University of Pernambuco, Brazil (PEUF 52078). GenBank accession number for _tufA_ KT781872.

**ETYMOLOGY**: The species is named in honor of Dr Aylthon Brandão Joly from the University of São Paulo, the first Brazilian phycologist.

**OTHER REPRESENTATIVE SPECIMENS**: Brazil. Espirito Santo, Anchicata, Praia de Castelhanos, subtidal zone; (PEUF 52081), intertidal reef (MELU HV05153), intertidal reef (MELU HV05229), Bahia, Prado, Praia Ponta do Corumbau, intertidal reef (PEUF 52076).


**Morphology and anatomy**

Plants were dark green and attached to rock by an extensive filamentous holdfast disc that spreads out for 1–2 cm (Figs 11–15). Mature plants stand 4–25 cm tall. The thallus branched dichotomously or in trifurcations. Segments were very lightly calcified. Basal segments were commonly cylindrical or subcylindrical. Segments higher up the thallus were often discoid, sometimes cuneate or subcuneate and 5–19 mm broad, 4–14 mm long, and 1.8–3.0 mm thick. Segments were remarkably thick set (Fig. 14), with a thickness-to-length ratio...
Fig. 10. Bayesian chronogram for the genus *Halimeda* resulting from the BEAST analysis, showing the relationship of species present in Brazil (grey boxes) with those from elsewhere. Branch support is given as Bayesian posterior probabilities (BEAST analysis, shown if ≥ 0.90) and ML bootstrap values (RAxML analysis, shown if ≥ 50).
Figs 11–15. Habit of the new species *Halimeda jolyana*. Scale bars = 1 cm.

Fig. 11. Patch of plants growing on a rocky shore in the low intertidal (HV05229, Espírito Santo, Marataizes, Praia da Cruz).

Fig. 12. Dense plant growing in a rock pool in the mid-intertidal (HV05091, Espírito Santo, Ubu, Praia de Parati).

Fig. 13. Appearance of live plant in field laboratory, showing common discoid segments (HV05086, Espírito Santo, Ubu, Praia de Parati).

Fig. 14. *In situ* photograph showing very thickset appearance of segments (Espírito Santo, Marataizes, Praia da Cruz).

Fig. 15. Live plant showing very thick segments (HV05221, Espírito Santo, Marataizes, Praia da Cruz).
of 0.25–0.55 measured in fresh and wet-preserved material. Some segments were separated by an uncorticated region of medullary filaments extending for up to 1 mm. The cortex consisted of three (up to five) layers of utricles. Secondary utricles were 75–175 μm long, giving rise to two primary utricles. The primary utricles were 60–165 μm long and 50–75 μm wide and were elongate cup-shaped, and some fuse laterally with adjacent utricles. In surface view, primary utricles appeared in a regularly polygonal (mostly hexagonal) pattern, tightly pressed against one another. Nodal fusions were complete, with two medullary filaments joined to form a single one above the node. The spacer zone present between some segments consisted of joined siphons, showing thick cell walls and occasional tufts of rudimentary cortication (Bandeira-Pedrosa et al. 2004a). Gametangia were formed along the distal edge of the segment as well as on the segment surface, often in tufts (Figs 16–22). Gametangia were obovoid-pyriform and borne per one to four on gametophores (Figs 19–22). The species also had lenticular thickenings of the external cell wall of primary utricles (Bandeira-Pedrosa et al. 2004a). For detailed figures see also Bandeira-Pedrosa et al. (2004b) as H. cuneata.

**DISCUSSION**

**Species diversity in Brazil**

Our molecular and morphological results showed that several taxonomic revisions of the genus *Halimeda* at the species level are needed in Brazil. Of the seven taxa initially identified on morphological grounds, three were later assigned to the correct species based on molecular results: *H. opuntia*, *H. incrassata* and *H. simulans*. The remaining four species had morphological matches to known species, but molecular data showed that they represented cryptic diversity and hence are part of species complexes.

The first well-defined species, *H. opuntia*, is easily recognized by the presence of multiple holdfasts that allow for the formation of large meadows in tropical reef flat areas (Hillis-Colinvaux 1980; Bandeira-Pedrosa et al. 2004b; Dijoux et al. 2012). Specimens of *H. opuntia* analysed in this study had high phenotypic plasticity, mainly in segment shape, but DNA sequence data confirmed that this variation is only at the morphological level. Morphological variation in shape, size and form have historically been attributed to environmental influences by Hillis-Colinvaux (1980), Bandeira-Pedrosa et al. (2004b) and Kooistra & Verbruggen (2005).

*Halimeda incrassata* and *H. simulans* belong to section *Rhipsalis*. They are distinguished from other species by the presence of bulbous holdfasts and nodal fusions of siphons into a single cluster by means of pores connecting adjacent siphons (Hillis-Colinvaux 1980; Bandeira-Pedrosa et al. 2004b; Verbruggen & Kooistra 2004). Both species differ in size and shape of the holdfast as well as the shape of segments.
Halimeda incrassata has a larger holdfast and segments with different shapes. Halimeda simulans has subcuneate basal segments and reniform ribbed segments nearer to the apices. The utricle shape also differs between these species: H. simulans has rounded primary utricles; whereas, those of H. incrassata are elongated. Despite the fairly low genetic divergence between H. simulans and H. incrassata, they are clearly distinguishable by morphological features, molecular sequences, and timing of reproduction (Clifton 1997; Verbruggen et al. 2005 a, b).

In Halimeda section Halimeda, H. tuna, H. discoidea and H. cuneata are overall morphologically consistent with the descriptions given by Hillis-Colinvaux (1980) and Bandeira-Pedrosa et al. (2004a, b). However, the Brazilian specimens are genetically distinct from topotype sequences. Thus, we have opted to call them H. aff. tuna and H. aff. discoidea and describe H. aff. cuneata as a new species, H. jolyana. Kooistra et al. (2002), Verbruggen et al. (2005a) and Dijoux et al. (2012) previously reported similar results where each of these morphospecies was recovered as multiple distinct clades, indicating cryptic diversity. Many of these species are geographically separated from one another, and each of the sections in Halimeda shows a biogeographic separation between Indo-Pacific and Atlantic sublineages, suggesting that vicariance may have occurred either across the Isthmus of Panama (Kooistra et al. 1999) or, as molecular clocks would suggest, across the Middle Eastern Isthmus (Verbruggen et al. 2009a, b).

Halimeda jolyana (as H. cuneata) was reported initially from the Atlantic Ocean by Bandeira-Pedrosa et al. (2004a) extending along the Brazilian coast from Rio do Grande do Norte (northeastern Brazil) to Espírito Santo (southeastern Brazil). Hillis-Colinvaux (1980) considered the presence of cuneate segments and small ‘cushion’ intercalary segments (or stalk) as diagnostic features distinguishing H. cuneata from related species, such as H. discoidea and H. tuna. Bandeira-Pedrosa et al. (2004a), using diagnostic features suggested by Hillis-Colinvaux (1980), originally recognized the Brazilian specimens as H. cuneata. However, our new collections from Brazil (Espírito Santo and Bahia) show specimens bearing mostly discoid segments (Figs 23–29; supplementary material S4). In this regard, H. jolyana differs from authentic H. cuneata, whose segments are more wedge shaped. Furthermore, our sequenced samples of H. jolyana are phylogenetically distinct from H. cuneata from the type locality (South Africa) with high genetic divergence between them.

A similar case was recently described by Cremen et al. (2016), who renamed H. cuneata from the southwestern coast of Australia as H. versatilis J.Agardh. The sequence of H. versatilis (as H. cuneata, AY826355) from Australia positioned in a distinct lineage (Fig. 9) with 9.4%-9.5% of divergence regarding our H. jolyana. The Brazilian H. jolyana, the Australian H. versatilis and the authentic South African H. cuneata share some morphological similarities. These include the presence of a stalked nodal region (Figs 7, 28, S4), the presence of commonly cylindrical basal segments and the cushion zones at the nodal region (Cremen et al. 2016; this study). Morphologically, H. versatilis differs from H. jolyana by its broadly cuneate segments, often longer than broad, dichotomous branching pattern, but with two to three (up to five daughter segments), and nodal siphons adhering in pairs or clusters of three or more. Halimeda jolyana, in turn, has often discoid segments, dichotomously branched or in trifurcations and nodal siphons adhering in pairs.

Halimeda aff. tuna showed great sequence similarity with sequences of Caribbean specimens belonging to the same species as well as with H. scabra. In turn, H. tuna from the Mediterranean Sea (type locality) formed a separate group from the Caribbean and Brazilian H. tuna sequences, indicating that this species is restricted to the Mediterranean Sea. It is clear that a new epithet is needed for H. aff. tuna from the Caribbean and Brazil, but due to the close molecular affinities between this entity, H. scabra and H. hummii D.L.Ballantine, more detailed work on this species complex across the geographic ranges of these species will be needed.

Halimeda discoidea also falls into multiple species-level clades, including the lineage H. aff. discoidea present in Brazil. It is not possible to compare the Brazilian sequence to collections from the type locality in order to confirm the identification of the species because the precise information about the type locality remains questionable in the literature (Hillis-Colinvaux 1980). In this case, sequencing the type specimen may be the only option to resolve the issue.

Halimeda aff. gracilis is a good morphological match with the isotype from Sri Lanka examined by Bandeira-Pedrosa et al. (2004b). This indicates that it could be a cryptic species; although, more detailed morphometric analysis may reveal differences (e.g. Verbruggen et al. 2006, 2016).

### Biogeographic considerations

Our results also permit us to discuss the biogeography of the genus in more detail from the perspective of species distributions, biodiversity patterns along the Brazilian coast and the historical origins of the Brazilian species.

Bandeira-Pedrosa et al. (2004b) mapped the geographic distributions of the seven Brazilian Halimeda species. They recorded H. cuneata, H. opuntia and H. discoidea on the Espírito Santo coast. The present study recorded H. simulans for the first time in Espírito Santo as well, extending its distribution along the Brazilian coast (Fig. 1). The distribution of many Halimeda species extend from northeastern to southeastern Brazil having distribution limits in Bahia or Espirito Santo states. However, H. jolyana appears to be restricted to the tropical waters from the south-central region of Bahia to Espirito Santo, being the only intertidal species that occurs in Espirito Santo state.

The greatest species diversity of the genus occurred on the coasts of Bahia and Espirito Santo. They are in the tropical warm province and transition zone, respectively, according to the classification of Horta et al. (2001).

All seven species of Halimeda from Brazil occur in Bahia state (Bandeira-Pedrosa et al. 2004b; Santos & Nunes 2015), which belongs to the tropical warm province, and is characterised by oligotrophic waters and sandstone reefs encrusted with algae and stony corals. Espirito Santo, in the transition between tropical and the so-called warm-temperate zone (sensu Horta et al. 2001), has unique features and high specific diversity. This is attributed to the wide range of environments, including reef formations, bedrock of the crystalline massif, consolidated substrates of calcareous algae and extensive rhodolith banks. These consolidated limestone
substrata extend below 100-m depth under the influence of the Brazil Current, which with its high transparency provides adequate habitat for colonizing macroalgae (Horta et al. 2001).

From a historical biogeographical perspective, the time-calibrated multigene results showed that the Brazilian Halimeda flora is formed by phylogenetically distinct lineages (i.e. four distinct sections). In Brazil, H. jolyana and H. aff. discoidea are the two phylogenetically closest species, yet the chronogram identified their most recent common ancestor at 70 Ma. Most recent common ancestors among all Brazilian Halimeda species are 70 Ma or older, which is indicative of a complete absence of local recent radiations. The relatively species-poor Halimeda flora (i.e. only seven species across ~ 7387 km of tropical and subtropical coastline) and the origin of this flora composed of phylogenetically distinct lineages suggest that Brazil is a sink and not a source of Halimeda species from other parts of the world. Many of the Halimeda species found in Brazil are derived from a more diverse pool of species in the Caribbean Sea and other subtropical Atlantic Ocean localities. Five out of the seven species – H. aff. discoidea, H. aff. tuna, H. opuntia, H. incrustata and H. simulans – are Caribbean species that extend their distribution south into Brazil. The close phylogenetic relationship in terms of the Halimeda flora between Brazil and the Caribbean agrees with a wider analysis of the origin of the Brazilian macroalgal flora that recognizes the Caribbean as one of the main source of species (Oliveira 1977).

The Brazilian coasts also have an endemic element, represented here by the species H. jolyana and H. aff. gracilis. Regrettably, their evolutionary and biogeographic origins are difficult to establish. Our chronogram suggests that both are the sole descendants of early-branching lineages (> 45 Ma), and without close relatives for comparison, it is difficult to be conclusive about their historical biogeographic origins.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/16-77.1.s1.
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