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A species dilemma: utilizing biological species concept to reconcile DNA barcode and morphological divergences for *Hypnea pseudomusciformis* (Gigartinales, Rhodophyta)

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Abstract

Taxonomic studies based on molecular markers led to the description of a new species, *Hypnea pseudomusciformis*, to accommodate three morphologically divergent species that occur on the coast of Brazil and have identical DNA barcode sequences (*H. musciformis*, *H. nigrescens*, and *Hypnea valentiae*). These three previous species were then identified as morphological variants of *H. pseudomusciformis*. Later, phylogeographic studies revealed that *H. pseudomusciformis* is subdivided in three distinct haplotype groups with significant intraspecific divergence for the COI-5P marker. Moreover, each haplogroup presents physiological and morphological differences related to each of the three floristic provinces along the Brazilian coast: tropical, transition, and warm temperate. Therefore, these haplogroups were denominated as ecotypes. In this work, we tested the biological species concept (BSC) in two crossing experiments: (i) between specimens belonging to different haplogroups (obtained from tropical and warm temperate provinces) and (ii) between different morphological variants ("musciformis" and "nigrescens") belonging to the same haplogroup. As a result, we observed that all crosses performed were positive and the reach of fertility in all tetrasporophytic plants of the F1 and F2 generations. Therefore, we concluded that (i) specimens from tropical and warm temperate provinces and (ii) specimens identified as "musciformis" and "nigrescens" belong to the same biological entity, despite their intraspecific molecular divergences and physiological differences and their differences in morphology, respectively. When testing and confirming the biological concept of species, we also validate previous works or confirm that the species has great phenotypic and physiological plasticity, which allows its occurrence in different niches and in provinces with different abiotic characteristics. The BSC proved to be a valuable tool for responding to conflicts raised by the DNA barcode technique.

Keywords Crossing experiments · DNA barcode · Species concept · Barcoding gap · Rhodophyta

Introduction

Many species concepts were proposed in the last century due to the difficulty in establishing an universal criterion to define what a species is (De Queiroz 2007). In phycology, species definition and delimitation are problematic since many species of algae exhibit substantial intraspecific morphological variation or cryptic speciation (Logares et al. 2007; Leliaert et al. 2014). Thus, the classification of algal species based on morphological comparisons alone may result in misidentification (Trainor 1998; Macaya and Zuccarello 2010).

In the biological species concept (BSC), a species is based on the property of interbreeding, resulting in viable and fertile offspring (Mayr 1942). This concept has been applied to delimit species boundaries in many red, green, and brown algae, when identification based on morphological variation was not conclusive (Coleman 1977; Zuccarello and West 2003; Hiraoka et al. 2011; Maggs et al. 2011). The first Rhodophyta crossing experiments were conducted by Edwards (1970); later other researches used the technique to study the taxonomic relationships among species (i.e., Rueness 1973; Brodie et al. 1993; Polanshek and West 1975, 2008; Guiry and West 1992; Plastino and Oliveira 1988, 1990, 1996; Guiry 1992). Crossing experiments were also

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used in the biogeographic study of the *Bostrychia radicans* (Montagne) Montagne species complex (Zuccarello and West 1995) and, more recently, to observe possible reproductive barriers among populations of *Gracilaria caudata* J. Agardh (Chiaramonte et al. 2019). One example in the literature of sterility barriers in red algal species is the formation of pseudocystocarps (cystocarpic-like structures that do not produce carpospores), which may indicate a postzygotic barrier (Plastino and Oliveira 1996).

Derived from the evolutionary species concept (Simpson 1951), in the phylogenetic species concept (PSC), a species is represented by a monophyletic group, sharing a common ancestor (Rosen 1979). Nowadays, species delimitation of macroalgae is often based on molecular data, utilizing the monophyletic criteria (Leliaert et al. 2014). As an example, the DNA barcode technique, especially concerning the COI-5P marker, proved to be useful and, in some cases, essential in the identification and delimitation of species, revealing a cryptic diversity that would go unnoticed by morphology alone (Herbert et al. 2003; Saunders 2005). In that technique, species are delimited based on a ratio of intra versus interspecific divergence (the barcoding gap) of short molecular markers (Hebert et al. 2004). However, critics of DNA barcoding technique argue that methodologies that rely solely on one data type and one criterion have higher failure rates than a holistic approach, which also considers data such as morphological frequency, ecological strategies, and haplotype frequency across the distributional range of the organism (Moritz and Cicero 2004).

An example of how species delimitation is made based on DNA barcode is the taxonomy of the red algae *Hypnea* J.V. Lamouroux (1813) on the Brazilian coast, which exhibits substantial intraspecific morphological variation (Jesus et al. 2015, 2016; Nauer et al. 2014, 2015, 2016). In the genus, some taxa are treated as species complexes due to the difficulty of delimitation of each entity, such as the *H. musciformis-pseudomusciformis* complex (Nauer et al. 2015) and the *H. cornuta-stellulifera* complex (Jesus et al. 2019). Other taxa, such as *H. edeniana* and *H. yokoyana*, are morphologically indistinguishable (Nauer et al. 2014, 2016).

In previous works, Nauer et al. (2019a, 2020) detected 14 haplotypes of *H. pseudomusciformis* subdivided into three haplogroups geographically related to the floristic provinces of the Brazilian coast (Horta et al. 2001; Spalding et al. 2007): tropical, transition, and warm temperate. The most abundant haplotype (h29) in the tropical province covers >1800 km of distributional range (5° to 12°S). The haplotype h38 was only found in the transition zone (in the north of Espírito Santo state, 19°S), and the most abundant haplotype (h25) in the warm temperate province covers >1400 km of distributional range (23° to 27°S). These results suggest that strong reproductive barriers probably evolved between the three lineages of *H. pseudomusciformis*, but until now, the presence of reproductive barriers has not been tested between them. These three groups were only evaluated considering physiological aspects in response to irradiance (Nauer et al. 2020) and temperature (Nauer et al., 2021), then being considered as ecotypes.

A second interesting aspect was the discovery that Brazilian specimens morphologically identified as *Hypnea musciformis*, *H. nigrescens*, and *Hypnea valentiae* presented no divergence for COI-5P and *rbc*L markers within the same floristic province (Nauer et al. 2015). These species were then described as morphological variants of *H. pseudomusciformis*, which therefore presents high phenotypic plasticity. These variants could also occupy distinct niches in nature, since "nigrescens" specimens are found growing epilitically in the mid-intertidal and "musciformis"specimens are found growing as epiphytes of some macroalgae (usually *Sargassum* sp.) in the lower intertidal zone (Nauer et al. 2015, 2019b). Physiological studies made under similar controlled conditions of culture evidenced that specimens of "nigrescens" and "musciformis" presented different growth rates but had similar pigment content and also required the same amount of time to complete their life history (Nauer et al. 2019b). The physiological differences detected between both variants indicate that genetic background as well as environmental conditions could play an important role in their occurrence in nature.

Cossing experiments between specimens from different floristic provinces and between different morphological variants were previously suggested by Nauer et al. (2020) to test whether they represent a single biological entity, within the biological species concept (BSC). According to Schenkman (1986), regarding the delimitation of species within the genus *Hypnea* on the Brazilian coast, it is important to carry out holistic studies that combine analyses of habitat, morphology, anatomy, and physiology for the correct identification of a species. In this study, crossing experiments with *H. pseudomusciformis* specimens were conducted under two scenarios to test the biological species concept: (I) between specimens from tropical (haplotype h25) and warm temperate (haplotype h29) provinces, in which a 1.3% genetic divergence is present based on COI-5P marker, and (II) between "musciformis" and "nigrescens" morphological variants, in which no genetic divergence is detected. Our hypothesis is that ecotypic and morphological variants of *H. pseudomusciformis* are in fact the same biological entity.

Materials and methods

Algal material

Specimens of *Hypnea pseudomusciformis* were collected in two different floristic provinces on the Brazilian coast

(Fig. 1, Table 1). Specimens representing the tropical ecotype (haplotype h25) (Fig. 1A and B) and warm temperate ecotype (haplotype h29) (Fig. 1C and D) are ~2940 km distant and were the same specimens utilized by Nauer et al. (2020) to validate the presence of *H. pseudomusciformis* ecotypes along the Brazilian coast. These specimens from the tropical and warm temperate provinces also represent the "musciformis" morphological variant, which was found growing as epiphytes of some macroalgae such as *Dictyota* sp. and *Sargassum* sp. Specimens representing the "nigrescens" morphological variant (Fig. 1E and F) were the same ones utilized by Nauer et al. (2019b) to complete the life cycle of *H. pseudomusciformis* in the laboratory. These specimens were found growing epilitically in the middle intertidal zone of the rocky shore. The COI-5P sequences of those specimens were done previously (Nauer et al. 2020).

The ecotypes can also be separated based on the length of the thallus, number of branches produced, and spinescent branchlet length. The "musciformis" variant from the tropical province displayed a thallus average length of 15.6 cm, varying from 13 to 16 cm (Fig. 1B), while the "musciformis" variant from the warm temperate province displayed thallus average length of 6.2 cm, varying from 5 to 9 cm (Fig. 1D) (Nauer et al. 2020). All specimens, however, had numerous hooks and tendrils in the apical portion of their branches, used to epiphyte other algae. The "nigrescens" morphological variant from the warm temperate province presents a more robust and rigid thalli, forming tufts of multiple main axes of similar height resembling the bristles of a brush, with up to 9 cm in height (Fig. 1F). Tendrils and hooks are scarcer but can be present in both main and lateral apex (Nauer et al. 2015, 2019b).

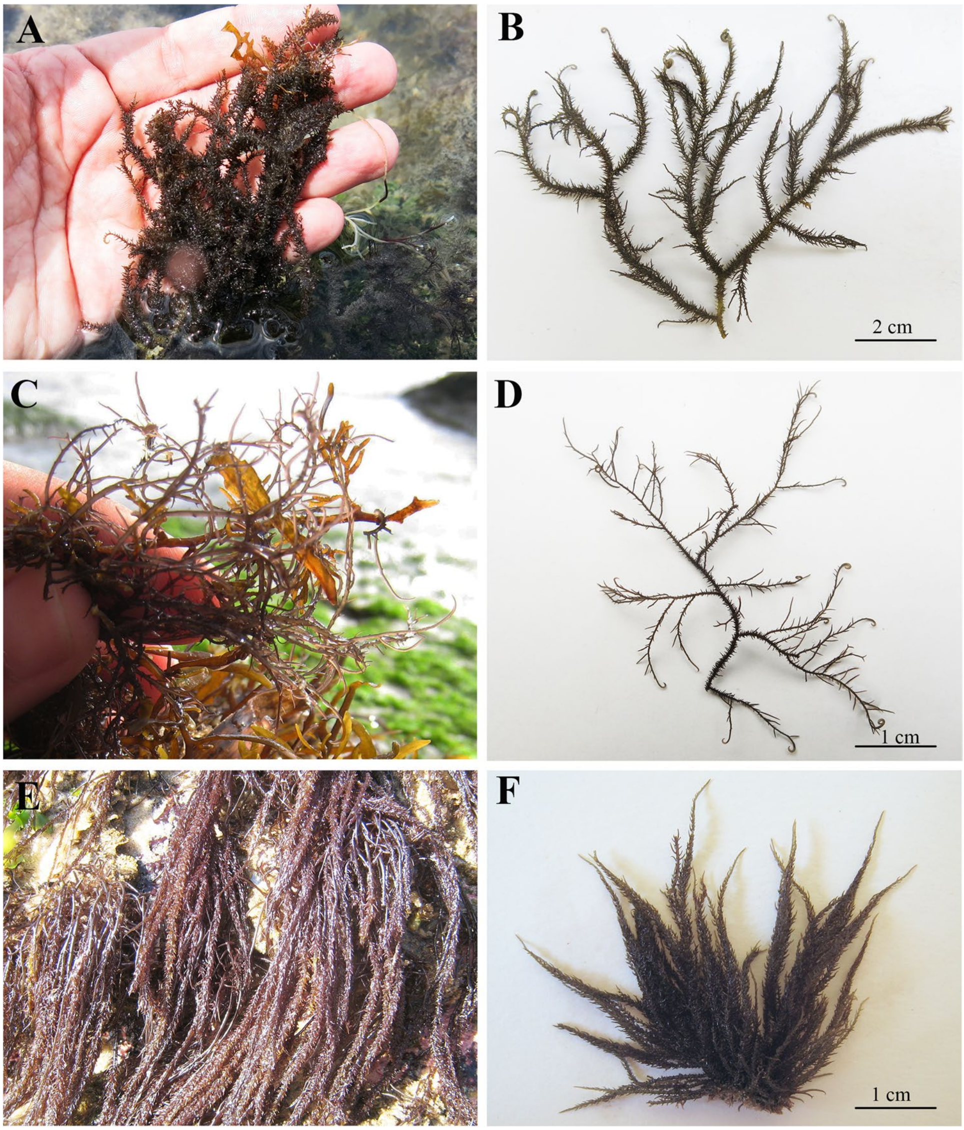


Fig. 1 Specimens of *Hypnea pseudomusciformis* collected in this study. **A** Specimen from the northeast coast (5°S), representing the tropical ecotype epiphyting *Dictyota*. **B** Habit of a tropical ecotype tetrasporophyte. **C** Specimen from the southeast coast (23°S), representing the warm temperate ecotype and the "musciformis" morphological variant epiphyting *Sargassum*. **D** Habit of a warm temperate ecotype "musciformis" tetrasporophyte. **C** Specimen from the southeast coast (23°S), representing the "nigrescens" morphological variant growing epilitically. **D** Habit of a "nigrescens" tetrasporophyte

Table 1 Collection of information of the specimens analyzed in this work. *GB, GenBank accession numbers

Brazilian floristic province/ecotype	COI-5P haplotype	Morphological variant	Collection site, latitude, longitude	Voucher	COI-5P*
Tropical	h25	"musciformis"	Rio do Fogo Beach, Rio do Fogo. RN. F. Nauer & F. Marchi. 25 November 2018. 5°16′18.18″S/35°22′54.27″W	SP513818	MT757410, MT757411, MT757412
Warm temperate	h29	"musciformis"	Cigarras Beach. São Sebastião. SP. F. Nauer & L. M. Ayres. 30. September. 2016. 23°43′41.38″S/45°23′53.84″W	SPF58095	MH523053, MH523054, MH523055
	h29	"nigrescens"	Cibratel Beach. Itanhaém. SP. F. Nauer & M. C. Oliveira. 02 July 2015. 24°12′05.68″S/46°48′38.72″W	SPF58101	MH523058, MH523059, MH523060
		Crossing experiments/F1 generation	Cross between tropical and warm temperate ecotypes (h25 × h29 haplotypes)	SPF58541	OL347694, OL347695, OL347696
			Cross between "musciformis" × "nigrescens" variants (h29 × h29 haplotypes)	SPF58542	OL347700, OL347701, OL347702
		Crossing experiments/F2 generation	Cross between tropical and warm temperate ecotypes (h25 × h29 haplotypes)	SPF58543	OL347691, OL347692, OL347693
			Cross between "musciformis" × "nigrescens" variants (h29 × h29 haplotypes)	SPF58544	OL347697, OL347698, OL347699

Experimental design

In total, nine tetrasporophytes were used (three "musciformis" from the tropical province, three "musciformis" from the warm temperate province, and three "nigrescens" from the warm temperate province). A single apical branch (length 1 cm) of each tetrasporophyte was isolated and kept in unialgal culture under the following conditions: 500 mL of sterilized seawater (32 psu) enriched with 50% von Stosch medium modified according Ursi and Plastino (2001), 14 h light, 24 ± 1 °C, and 70 ± 5 µmol photons m^{-2} s^{-1} (Osram 40 W day light fluorescent tubes). The cultures were bubbled with compressed air for 30 min h^{-1}, and the medium was renewed weekly. After 40 days of cultivation under these general culture conditions, the tetrasporophytes became fertile and released tetraspores after their germination developed into male and female gametophytes. The time required to reach the reproductive maturity of male gametophytes was 30 days, and only after that period, female gametophytes could be identified by the presence of cystocarps along the thallus. Later, from each tetrasporophyte collected in nature, we randomly selected and isolated only one apical segment (1 cm in length) from one male and one female gametophyte to be used in the crossing experiments. They were kept in the general conditions of cultivation until they were the appropriate size for the beginning of the crossings.

Crossing test I, between tropical (haplotype h25) and warm temperate ecotypes (haplotype h29), in this experiment, we used a total of three male and three female gametophytes of each ecotype. To test the BSC, an apical segment (~5 cm in length) of each female gametophyte (♀) was incubated in an Erlenmeyer flask with 500 mL of medium in three distinct ways: (1) ♀ alone, to confirm the absence of parthenogenesis in the female gametophyte (negative control); (2) ♀ together with an apex (~5 cm in length) of male gametophyte (♂) of the same ecotype (positive control); and (3) ♀ together with an apex (~5 cm in length) of a male gametophyte (♂) of the different ecotype (crossing test).

Crossing test II, between "musciformis" and "nigrescens" morphological variants, in this experiment, we used three male and three female gametophytes of each morphological variant. To test the BSC in this second scenario, an apical segment (~5 cm in length) of each female gametophyte (♀) was incubated in an Erlenmeyer flask with 500 mL of medium in three distinct ways: (1) ♀ alone, to confirm the absence of parthenogenesis in the female gametophyte (negative control); (2) ♀ together with a ♂ (~5 cm in length) of the same morphological variant (positive control); and (3) ♀ together with a ♂ (~5 cm in length) of the different morphological variant (crossing test). In both crossing experiments (I and II), we used $n = 3$ since each way of cultivation was repeated three times (see Fig. S1 for experimental design).

Branching ratio

The branching ratio was analyzed in this work as an alternative and quantitative data that could be compared between the parental tetrasporophytes (from nature) and the F1 and F2 tetrasporophytic generations, by counting the total number of differentiated branches present in all flasks, excluding spinescent branchlets. All nine tetrasporophytes had their branches counted after 28 days of culture (three "musciformis" from the tropical province, three "musciformis" from the warm temperate province, and three "nigrescens" from the warm temperate province). For F1 and F2 tetrasporophytic generations, a single tetrasporophyte from each flask of the experimental design had their branches counted after 28 days of culture, totalizing 12 specimens from the F1 generation and 12 specimens from F2. The data was tested for normality (Kolmogorov–Smirnov) and homoscedasticity (Bartlett's test). The two-way ANOVA with two independent variables (ecotype, tropical and warm temperate, and morphological variant, "musciformis" and "nigrescens") and one quantitative dependent variable (branching ratio) was performed using the software STATISTICA 10.0 (StatSoft, Inc.). The a posteriori Newman–Keuls test ($p < 0.05$) was used to establish statistical differences.

Carpospore germination

In our crossing experiments I and II, we performed cross-sections of the formed cystocarps, with a total of three cystocarps per repetition belonging to each type of crossing (1, 2 and, 3). In addition, we monitored the germination pattern of the released carpospores. For this, nine cystocarps present in female gametophytes were selected at random from each replication. These cystocarps were then detached from the thallus and placed separately on glass slides inside crystallizers containing 100 mL of culture medium, without aeration and under the other general culture conditions, that is, for each repetition, nine slides were performed. After 2 days from the carpospores releasement, the germination pattern started to be analyzed under an optical microscope and their germination pattern ware recorded (day 2, day 3, day 4, day 5, day 6, day 7, day 8, and day 9 after the releasement).

Molecular analysis

A total of 21 COI-5P sequences (12 new and 9 published) with 466 nucleotides (nt) of *Hypnea pseudomusciformis* species (from Nauer et al. 2019b, 2020; available on GenBank) were aligned using Clustal W within BioEdit 7.0 sequence editor (Hall 1999) and checked visually. Neighbor-joining (NJ) analysis was performed under heuristic search with 2000 bootstrap replicates (Felsenstein 1985), using PAUP 4.0b10 (Swofford 2002). *H. musciformis* (KM523208) was used as an outgroup for COI-5P.

Results

Crossing experiments

For crossing test I, between tropical (haplotype h25) and warm temperate ecotypes (haplotype h29), no cystocarp was observed in the negative control (type 1). Furthermore, crossings performed between the same ecotype (tropical or warm temperate, type 2) and between different ecotypes (tropical × warm temperate, type 3) were positive, evidenced by the appearance of cystocarps bearing carpospores in female plants (Table 2). For crossing test II, between "nigrescens" and "musciformis" morphological variants, no cystocarp was observed in the negative control (type 1). Crossings carried out between the same ("nigrescens" or "musciformis", type 2) and between different ("nigrescens" × "musciformis", type 3) morphological variants were positive, evidenced by the appearance of cystocarps bearing carpospores in female plants (Table 2).

Carpospore germination

Cystocarps become visible to the naked eye approximately 8 days after the contact of the male with the female gametophyte (Fig. 2A). Cystocarps were isolated or grouped in the female gametophyte branchlets (Fig. 2B) and began to release carpospores between 2 and 4 days after cystocarps became visible (Fig. 2C). We could observe three phases in the germination of carpospores: (i) after 2 days of their releasement, the carpospores divided in four cells (after two mitotic divisions) (Fig. 2D), they start increasing in size by mitosis and also their numbers of cells start forming a round mass until the fifth day (Fig. 2E, F, and G); (ii) after 6 days of releasement, the forming thallus is elongated, and the apical cell could be observed (Fig. 2H); (iii) the following days, the thallus continued to elongate (Fig. 2I and J) and could be seen with the naked eyes after approximately 9 days, giving rise to tetrasporophytic plantlets.

After 28 days of in vitro culture, the thalli of F1 tetrasporophytes were fully developed (Fig. 3A, B, and C, F1 generation from the crossing between "musciformis" and "nigrescens"; Fig. 3G, H, and I, F1 generation from the crossing between tropical and warm temperate ecotypes) and became fertile after approximately 40 days, presenting branches with tetrasporangia. Released tetraspores developed into viable gametophytes (F1 gametophyte), which were grown together (male and females) for 30 days until cystocarps were visible in the female gametophytes. From

Table 2 Crossing experiments' results between the ecotypes (~2940 km distant) and between the morphological variants "musciformis" and "nigrescens" of *Hypnea pseudomusciformis*. *MT*, "musciformis"/tropical ecotype; MW, "musciformis"/warm temperate ecotype; and *NW*, "nigrescens"/warm temperate ecotype

	Crossing experiments					
	Tropical × Warm temperate ecotypes			"musciformis" × "nigrescens"		
	Gametophytes	Cystocarp	Carpospore status	Gametophytes	Cystocarp	Carpospore status
Negative control	♀MT1	Absent	–	♀NW1	Absent	–
	♀MT2	Absent	–	♀NW2	Absent	–
	♀MT3	Absent	–	♀NW3	Absent	–
	♀MW1	Absent	–	♀MW1	Absent	–
	♀MW2	Absent	–	♀MW2	Absent	–
	♀MW3	Absent	–	♀MW3	Absent	–
Positive control	♀MT1 × MT1♂	Present	Viable	♀NW1 × NW1♂	Present	Viable
	♀MT2 × MT2♂	Present	Viable	♀NW2 × NW2♂	Present	Viable
	♀MT3 × MT3♂	Present	Viable	♀NW3 × NW3♂	Present	Viable
	♀MW1 × MW1♂	Present	Viable	♀MW1 × MW1♂	Present	Viable
	♀MW2 × MW2♂	Present	Viable	♀MW2 × MW2♂	Present	Viable
	♀MW3 × MW3♂	Present	Viable	♀MW3 × MW3♂	Present	Viable
Crosses	♀MT1 × MW1♂	Present	Viable	♀NW1 × MW1♂	Present	Viable
	♀MT2 × MW2♂	Present	Viable	♀NW2 × MW2♂	Present	Viable
	♀MT3 × MW3♂	Present	Viable	♀NW3 × MW3♂	Present	Viable
	♀*MW1* × MT1♂	Present	Viable	♀*MW1* × NW1♂	Present	Viable
	♀*MW2* × MT2♂	Present	Viable	♀*MW2* × NW2♂	Present	Viable
	♀*MW3* × MT3♂	Present	Viable	♀*MW3* × MW3♂	Present	Viable

the carposporophytes formed in the thallus of female F1 gametophytes, carpospores were released, germinated, and developed into the F2 tetrasporophytic generation (Fig. 3A, D, E, and F, F2 generation from the crossing between "musciformis" and "nigrescens"; Fig. 3J, K, and L, F2 generation from the crossing between tropical and warm temperate ecotypes).

Branching ratio

The branching pattern of *Hypnea pseudomusciformis* individuals is a result of the interaction between ecotype and morphological variant (Table 3) ($F = 30.50$; $p < 0.01$). Overall, branching ratio (Br) was different between tropical and warm temperate ecotypes ($p < 0.01$) and different between "musciformis" and "nigrescens" ($p < 0.01$). However, no differences in Br were observed within the tropical ecotype and F1 and F2 generations resulting from crosses between ecotypes; and no differences were observed within the "nigrescens" morphological variant and F1 and F2 generations from crosses between morphological variants. The plants from the warm temperate ecotype displayed the lowest values of Br of all specimens analyzed.

COI DNA barcoding divergences

Intraspecific variation between tropical (haplotype h25) and warm temperate ecotypes (haplotype h29) sequences was 9 bp (1.3%). No divergence between "musciformis" and "nigrescens" variants from warm temperate province was found. Because of that, the divergence between "nigrescens" from warm temperate province and "musciformis" from tropical province was 1.3% as well.

Discussion

The results from the crossing experiments confirm that despite the observed intraspecific genetic divergence (1.3 to 1.9% between all COI haplotypes and up to 0.6% for *rbc*L) and physiological differences (Nauer et al. 2015, 2019a, 2020), *Hypnea pseudomusciformis* ecotypes from tropical and warm temperate provinces of Brazil belong to the same biological entity. The DNA barcode technique has been proposed as a fast and efficient way to survey species in an ecosystem, with or without background taxonomic information (Hebert et al., 2003). However, geographically widespread species are likely to display elevated levels of intraspecific variability if the species is sampled across its distributional

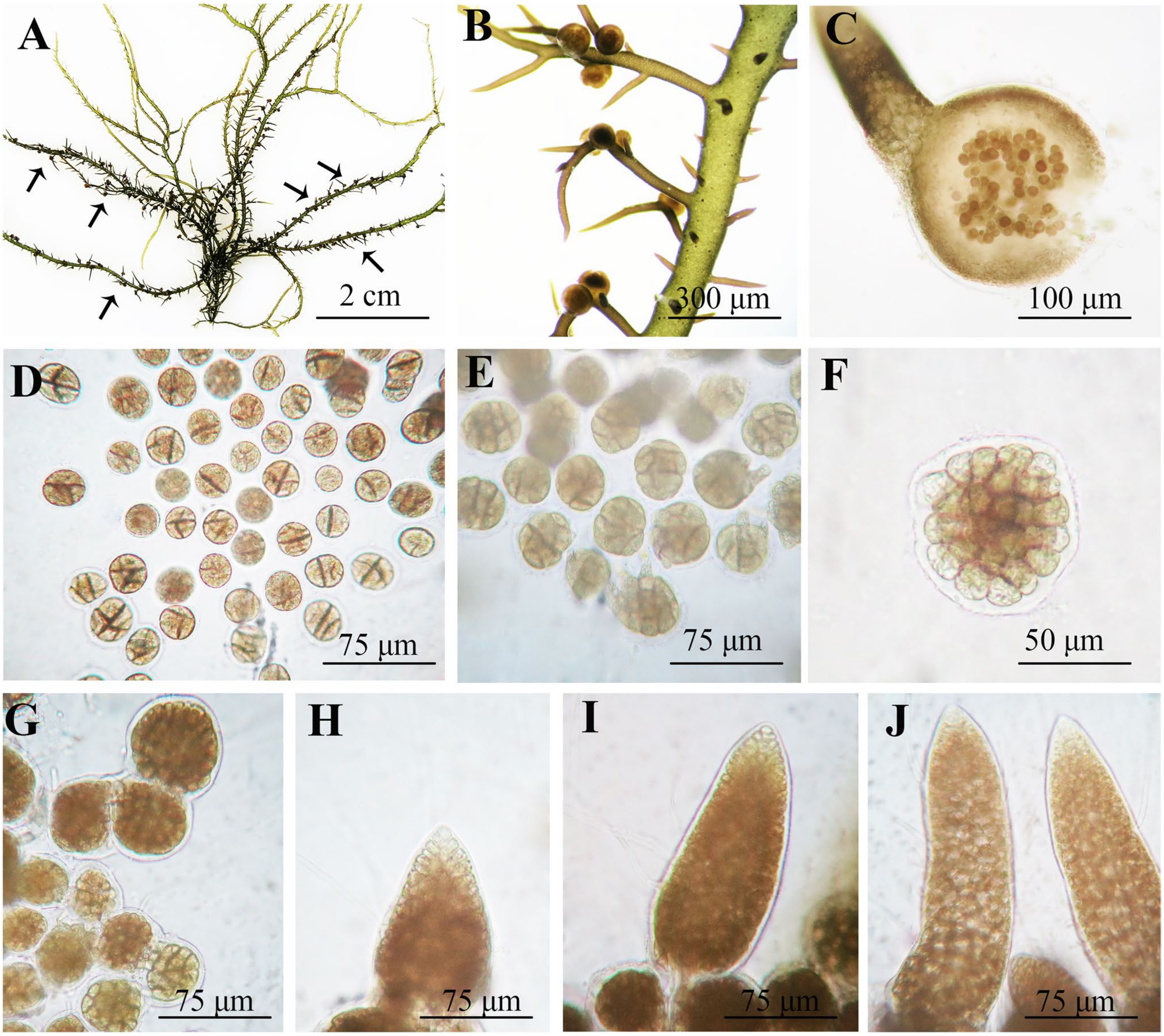
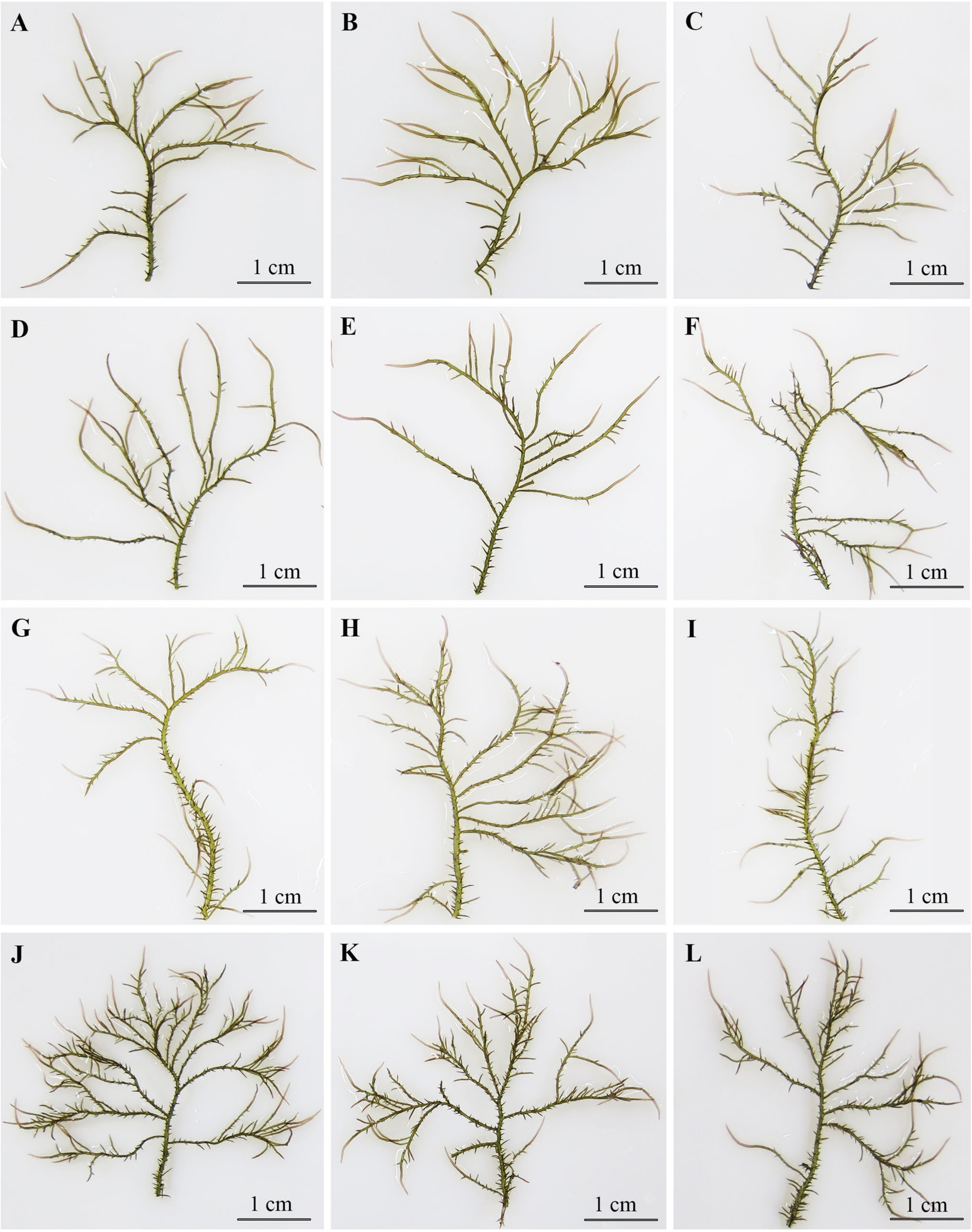


Fig. 2 Carpospore germination stages. **A** Female gametophyte with cystocarps. **B** Detail of a branch with cystocarps. **C** Cross-section of a cystocarp with carpospores. **D** Carpospores germinaion after 2 days. **E** Carpospores germination after 3 days. **F** Carpospores germination after 4 days. **G** Carpospores germination after 5 days. **H** Formation of the first apexes containing one apical cell after 6 days. **I** Plantlet growth after 7 days. **J** Plantlet growth after 8 days

range (Hamsher et al. 2011; Hoef-Emden 2012). Consequently, barcoding gaps are usually blurred with the overlaps between the genetic distance among closely related species and the variability within a species (Meyer and Paulay 2005; Bittner et al. 2010; Bergsten et al. 2012). As a result, the decision on whether a sequence divergence is high enough to consider a distinct species often relies on intuition (Leliart et al., 2014). Overall, the intraspecific divergence for *Hypnea* species ranged from 0 to 0.9%, while the divergence between species was greater than 4.5% (Yokoya et al. 2020). Based on the DNA barcode gap for *H. pseudomusciformis* ecotypes, the intraspecific divergence of 1.9% could be considered a gray zone. Thus, the use of the biological species concept is important to validate *H. pseudomusciformis* ecotypes as the same biological entity, despite being putatively isolated in different floristic provinces along the Brazilian coast.

Plastino and Oliveira (1990) attempted crossings among populations of the same species of *Gracilaria*. In that study, monospecific crossings were positive even between isolated populations, with production of cystocarps and carpospores, a similar scenario to *H. pseudomusciformis* ecotypes. However, a pseudocystocarp was produced by female *Gracilaria mammillaris* in the presence of male branches of *Gracilaria cervicornis*. These cystocarpic-like


A
1 cm
B
1 cm
C
1 cm
D
1 cm
E
1 cm
F
1 cm
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1 cm
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1 cm
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1 cm
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1 cm
L
1 cm

◂**Fig. 3** Tetrasporophytes of *Hypnea pseudomusciformis* resulted from crossing experiments after 28 days of culture. **A**, **B**, and **C** F1 generation from the crossing between "musciformis" and "nigrescens" morphological variants. **D**, **E**, and **F** F2 generation from the crossing between "musciformis" and "nigrescens". **G**, **H**, and **I** F1 generation from the crossing between tropical and warm temperate ecotypes. **J**, **K**, and **L** F2 generation from the crossing between tropical and warm temperate ecotypes

structures did not produce carpospores, and a section showed only an empty cavity surrounded by a pericarp. The authors concluded that the presence of sterility barriers in species of *Gracilaria* was expected for many of the Brazilian species that occur sympatrically. In our study, which details the formation and release of carpospores and the growth of the subsequent generation, we excluded the presence of sterility barriers for *H. pseudomusciformis* ecotypes in vitro. In nature, these haplotypes/ecotypes are geographically isolated along the Brazilian coast by putative marine biogeographic barriers, leading to their current distribution pattern (Nauer et al. 2020).

Plastino and Oliveira (1996) attempted crosses of *Gracilariopsis* sp. from southeastern Brazil and Venezuela, populations separated by thousands of kilometers, and production of viable F1 generation was observed. Chiaramonte et al. (2019) attempted crosses of distinct populations of *G. caudata* from northeastern to southeastern Brazil and did not observe any reproductive barriers against the formation of diploid hybrid genotypes or postzygotic isolating mechanisms. Reproductive barriers are not always complete and have been reported between sexually reproducing species of algae before (Brodie et al. 1993; Coyer et al. 2002; Casteleyn et al. 2009; Zardi et al. 2011). Moreover, laboratory crosses do not take into account extrinsic reproductive barriers (e.g., differences in timing of sexual reproduction or ecological preferences), only intrinsic barriers (Leliaert et al. 2014). In this scenario, population genetic studies are necessary for *H. pseudomusciformis* along the Brazilian coast to confirm if genetic flow occurs between these ecotypes in nature or if there are efficient barriers separating those populations as the phylogeographic studies suggest (Nauer et al. 2019a).

The high intraspecific divergence between tropical and warm temperate ecotypes could indicate that these *H. pseudomusciformis* ecotypes are population lineages evolving separately that could lead to a speciation event. In the evolutionary process, speciation is the first step to interrupt gene flow between isolated populations, leading to divergence and ultimately separated evolving population lineages (Coyne and Orr 2004). When two population lineages separate, different secondary properties (e.g., become sexually incompatible or morphologically distinct) could eventually appear and serve as diagnostic evidence relevant to species delimitation. However, these secondary properties may not appear at all or could appear in different orders and/or times, leading to conflicts between species concepts in many cases, especially between recently formed species (De Queiroz 2007; Leliaert et al. 2014). Our data on branching ratio could be evidence of the morphological differences between *H. pseudomusciformis* ecotypes, in addition to physiological and molecular differences (Nauer et al. 2020), and are indications of an ongoing speciation process.

Several researchers critical to the DNA barcode technique argue that the appropriate delimitation of species should be based on different data sources, including morphological, ultrastructural, biochemical, geographic, ecological, and/or breeding data and not only in DNA divergences (e.g., Pröschold et al. 2001; Walker et al. 2009; Bendif et al. 2011; Milstein and Saunders 2012). Our combined data on morphology, molecular markers, and crossing experiments between the two morphological variants ("musciformis" and "nigrescens") of *H. pseudomusciformis* lead to different interpretations regarding species delimitation based on distinct species concepts. *H. musciformis* and *H. nigrescens* for the coast of Brazil were identified as different species, with overlapping distribution, based on differences in morphology and habitat (Schenkman 1986) but later were united

Table 3 *Hypnea pseudomusciformis* tetrasporophyte branching ratio of specimens utilized in this work and for F1 and F2 generations resulting from crosses between tropical and warm temperate ecotypes and between "musciformis" and "nigrescens" variants. The total number of differentiated branches (T) present in all flasks of each variation (three flasks with three apical apices per flask, $n=3$) and the average (A) of apices per flask were calculated counting the apices on the 1st day (T1, A1) and on the 28th day (T28, A28). Branching ratio (BR) was calculated dividing T28 by T1. Different letters on the BR column represent significant differences among ($p<0.05$)

Ecotype	Morphological variant	T_1	T_{28}	A_1	A_{28}	B_R
Tropical	"musciformis"	9	288	3	96 ± 7	32^{a}
Warm temperate	"musciformis"	9	57	3	19 ± 4	6.3^{c}
	"nigrescens"	9	165	3	55 ± 6	18.3^{b}
F1 generation	tropical × warm temperate	9	243	3	81 ± 9	27^{a}
	"musciformis" × "nigrescens"	9	186	3	62 ± 7	20.6^{b}
F2 generation	tropical × warm temperate	9	309	3	103 ± 8	34.3^{a}
	"musciformis" × "nigrescens"	9	156	3	52 ± 6	17.3^{b}

under the same species, *H. pseudomusciformis*, using the phylogenetic species concept (Nauer et al. 2015). In fact, our crossing experiment results evidenced that "musciformis" and "nigrescens" were able to cross and generate fertile offspring, corroborating the molecular data. Considering the combined evidence, we favor the use of the BSC that corroborated the PSC, and we consider that "musciformis" and "nigrescens" are morphological variants of a single biological entity, *H. pseudomusciformis*. Our data also emphasize that the use of crossing experiments to test the BSC is an important tool to solve taxonomic problems and that the use of *rbc*L and the DNA barcode COI-5P is good proxy to establish and delimit species in red algae.

The geographically distributional range of *H. pseudomusciformis* is approximately 4500 km, from North of Brazil to the coast of Uruguay, which increases the probability of finding elevated levels of intraspecific variability, as detected by the DNA barcode technique. If, however, all specimens of *H. pseudomusciformis* were sampled from the same locality, the total absence or much less intraspecific variability is observed (Nauer et al., 2019). This is the scenario of the morphological variants of *H. pseudomusciformis*, in which the differences in distribution are not latitudinal but vertical, in different zones of the rocky shore, where different environmental conditions operate. In Nauer et al. (2015), 97 specimens were collected along the coast of Brazil spanning 25° degrees of latitude (2°S to 27°S). All the specimens previously identified as *H. nigrescens* were found exclusively on rocky shores with strong wave impact, growing epilitically in the mid-intertidal zone. Specimens previously identified as *H. musciformis* were collected in the lower intertidal zone, epiphytically on other algae, being found in protected areas and reefs. Nauer et al. (2018) described part of the phenotypic plasticity present in *H. pseudomusciformis* as a consequence of environmental factors, since differences in morphology of "musciformis" and "nigrescens" are attenuated in culture, despite their different physiological responses in some of the parameters measured. Here, a distinct branching ratio was observed between the *H. pseudomusciformis* morphological variants despite the identical sequence of selected molecular markers (COI, *rbc*L, and UPA) (Nauer et al. 2015). Although morphological differences are attenuated in culture when compared to specimens in nature, branching ratio differences could still be observed in F1 generation.

In conclusion, biological species concept confirms that the tropical and warm temperate *H. pseudomusciformis* ecotypes belong to the same species, despite the high intraspecific divergence. The BSC also confirms that the morphological variants "nigrescens" and "musciformis" found on the coast of Brazil belong to *H. pseudomusciformis*, which presents high morphological and physiological plasticity, allowing this species to occupy different niches in the marine intertidal zone. Furthermore, our results clearly indicate that crosses between different variants and a polyphasic taxonomy approach represent a very useful tool for recognizing specific entities in a taxonomically complex genus. Lastly, due to its phenotypic plasticity, *H. pseudomusciformis* proved to be an interesting model organism to study genetic and environmental aspects, acting to promote physiological and morphological differences that allow the expansion of a species niche.

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