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Morphological and Molecular Characters Differentiate Common Morphotypes of Atlantic Holopelagic *Sargassum*

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Abstract: Since 2011, massive new strandings of holopelagic *Sargassum* have been reported on the coasts of the Caribbean, northern Brazil, Guiana, and West Africa, causing severe economic and ecological damage. Three common morphotypes (*S. fluitans* III, *S. natans* I, and *S. natans* VIII) were identified as responsible for these catastrophic events, with dominance shifts between them over time. However, the taxonomic status of these holopelagic *Sargassum* morphotypes remains unclear. Using an integrative taxonomy framework, combining a morphological study and molecular analyses, this study aimed to clarify their taxonomic status. Morphological analyses of 54 characters revealed no intermediate form between the three morphotypes, with the overall shape, nature of the axis, and size and shape of blades and vesicles being the most discriminating. An analysis of mitochondrial (*IGS*, *cox2*, *cox3*, *mt16S rRNA*, and *nad6*) and plastid (*rbcL*) markers confirmed the genetic divergence among the three morphotypes, with a lower level of divergence between the two *S. natans* morphotypes. Without additional molecular characterization, these morphotypes cannot be classified as three distinct species. However, due to their distinct morphological characteristics and sympatry within drifting aggregations, a revision of holopelagic species names is proposed, with *Sargassum fluitans* var. *fluitans* (for *S. fluitans* III), *Sargassum natans* var. *natans* (for *S. natans* I), and *S. natans* var. *wingei* (for *S. natans* VIII). This revision provides necessary clarity on the species involved in inundations of the tropical Atlantic.

Keywords: holopelagic *Sargassum*; North Atlantic Ocean; phylogeny; *Sargassum natans* var. *wingei*

1. Introduction

Bloom-forming macroalgae are of global concern, causing ecological, economic, and health issues [1–5]. Among the most widely proliferating seaweeds, several species of the genus *Sargassum* C. Agardh (Phaeophyceae, Fucales) are threatening coastal ecosystems in temperate [6–8] and tropical [9–14] areas. Most *Sargassum* species are benthic, colonizing

rocky reefs, pebbles, detritic sediments, or coral reefs from the sea surface to several dozen meters in depth [15–17]. In the Atlantic Ocean, however, two holopelagic species have been characterized: *Sargassum natans* (Linnaeus) Gaillon and *Sargassum fluitans* (Børgesen) Børgesen [18,19]. Winge [20] described several morphotypes of these species, and their delineation was refined by Parr [21], although still absent from current taxonomic keys.

Holopelagic *Sargassum* species, historically most abundant in the Sargasso Sea and open Gulf of Mexico [21,22], float and grow at the sea surface during their entire lifetime, as they have no benthic stage. Individual thalli decay and sink to depth [23] or can wash ashore, removing them from the pelagic system. At the sea surface, *Sargassum* thalli tend to aggregate and form mats of different structure and density [24], which host a diverse community of marine organisms [25–28]. These drifting aggregations can travel long distances under the action of winds, waves, and currents [22,24,29]. Since 2011, unprecedented massive strandings of holopelagic *Sargassum* have been reported on the coasts of the eastern and western Caribbean, northern Brazil, Guiana, and West Africa (reviewed in Fidai et al. [30]). These recurrent strandings are associated with extensive *Sargassum* aggregations in the tropical North Atlantic, as reported by satellite imagery [31], hydrographic modeling [32], and field campaigns [24,33]. Satellite images and direct observations show that the Great Atlantic *Sargassum* Belt (GASB) phenomenon has been annually variable in magnitude [31]. Thus, the absence of reliable growth and dispersion models for holopelagic *Sargassum* complicates bloom forecasting efforts [34–37] and the establishment of suitable management procedures.

Species identification adds another level of complexity to understanding the origins and composition of these recent massive standings. The growth response [38–43] and morphology-induced windage [44,45] indeed differ between *Sargassum* morphotypes. In addition, in the aggregations, the most abundant morphotype has shifted across annual blooms. Schell et al. [33] reported, in the North Atlantic Ocean between 10 and 30° N, three dominant morphotypes described by Parr [21], namely, *Sargassum natans* VIII, *Sargassum natans* I, and *Sargassum fluitans* III, with *S. natans* VIII noted as the predominant form inundating eastern Caribbean coasts. Later, *S. fluitans* III dominated beached *Sargassum* in Mexico during 2016 and *S. natans* I contributed substantially to the community composition in the same location in 2018 [46]. On a finer temporal scale, the morphotype composition of beached holopelagic *Sargassum* in Barbados switched from *S. fluitans* III dominating during spring/summer 2021 to *S. natans* VIII-dominated during autumn 2021/winter 2022 [27].

Integrative taxonomy [47], combining a morphological study with molecular analyses, can clarify whether the different holopelagic *Sargassum* morphotypes observed in recent years correspond to different species or to intra-specific variants. As Winge [20] and Parr's [21] morphotype descriptions did not follow the Botanical Code (International Code of Nomenclature for algae, fungi, and plants; [48]) for species description and the subsequent discontinuity in classification based on inconsistent character selection [49], a comprehensive morphological description of each common morphotype is warranted. Moreover, molecular analysis challenged traditional taxonomic divisions within the genus and questioned the use of some morphological or chemical characters for species delineation and taxonomy [50,51]. Even the monophyly of the *Sargassum* genus has been disputed [52].

Early sequencing efforts revealed no divergence between holopelagic species nor between holopelagic and some benthic species [53–56]. Such results may have arisen from the selection of markers that, while standard for macroalgal studies, did not capture the regions where polymorphisms among holopelagic *Sargassum* were located [53–56]. Moreover, some included the incorrect identification of the specimens sequenced [54,56]. Subsequent comparisons of full-length mitogenomes, with a documented correct morphotype identification, indicated a closer proximity between *S. natans* I and *S. natans* VIII when compared to *S. fluitans* III; the divergence levels were around 0.02% at the mitogenome level between *S. natans* I and *S. natans* VIII and 0.3% between *S. natans* I/VIII and *S. fluitans* III [57]. In a more extended temporal and geographic analysis of holopelagic *Sargassum*, targeting multiple mitochondrial gene segments each with multiple polymorphisms, genetic distances among

holopelagic morphotypes were in the range of those between pelagic and benthic species and on the same order as divergence among some benthic species [58].

The present study aimed to clarify the taxonomic status of holopelagic *Sargassum* morphotypes presently found in the Atlantic Ocean. A broad suite of morphological characters and multi-gene molecular analyses were conducted across a large sample of individuals representing all three common holopelagic morphotypes. With this deeper understanding of *Sargassum* taxonomy, the scientific record can be rectified and an appropriate level of differentiation can be incorporated into future modeling, forecasting, and ecological studies.

2. Materials and Methods

2.1. Sample Collection

Holopelagic *Sargassum* specimens were randomly collected during two scientific cruises in the North and tropical Atlantic Ocean in 2017, with the goal of studying the composition and characteristics of *Sargassum* aggregations. The Caribbean expedition (http://dx.doi.org/10.17600/17004300, accessed on 28 March 2024) used the R/V Antea from 19th June to 13th July 2017, and extended from the area of extensive *Sargassum* bloom development off northeast Brazil up to the Sargasso Sea (25° N), with a focus along French Caribbean islands. The Transatlantic expedition (http://dx.doi.org/10.17600/17016900, accessed on 28 March 2024) used the M/Y Yersin from 6th to 24th October 2017, and crossed the Atlantic between 8° and 12° N, from Cape Verde to Martinique (see [24] for details).

Holopelagic *Sargassum* aggregations were randomly sampled at 31 of 49 stations during the two oceanographic cruises (Figure 1). The three morphotypes were simultaneously present at all 31 sampling stations, but proportions differed by site. *Sargassum fluitans* III was dominant nearly everywhere (around 70%), with ~15% each for *S. natans* VIII and *S. natans I*. However, at two stations (18 and 20) in the south Sargasso Sea, *S. natans* VIII dominated (~70%) and *S. natans* I and *S. fluitans* III contributed ~15% each.
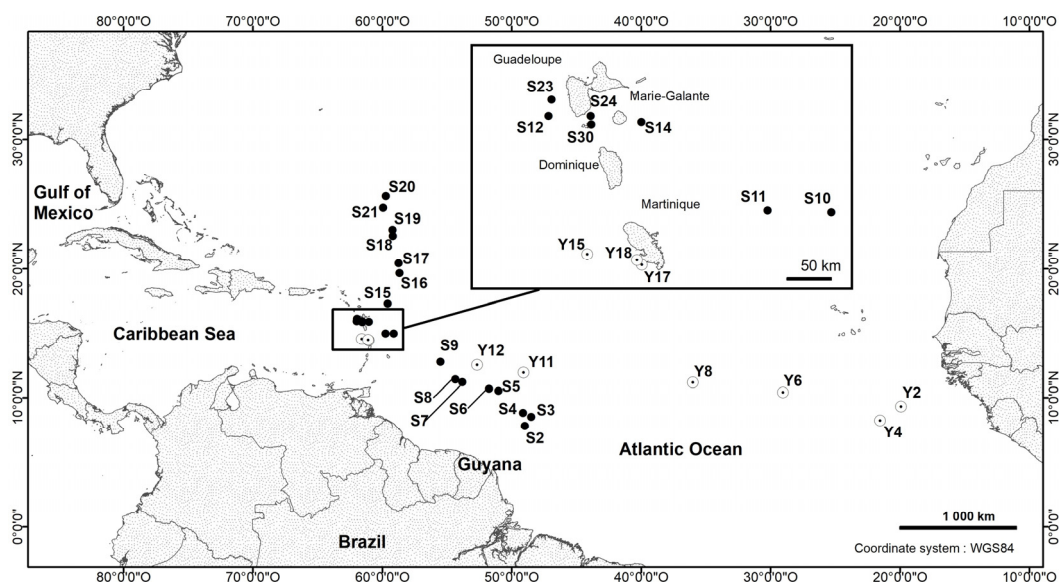
Figure 1. Sampling locations of holopelagic *Sargassum* during the two 2017 expeditions. S: Caribbean expedition; Y: Transatlantic expedition.

At each station, collected *Sargassum* thalli were identified and sorted by morphotype. For each *Sargassum* morphotype, two to three replicate fronds were randomly selected for analysis. A total of 264 individuals of holopelagic *Sargassum* were collected to carry out joint morphological and genetic analyses. All specimens have been kept as vouchers with a corresponding fragment stored in silica gel at room temperature for further molecular analysis. Eight specimens of benthic *Sargassum*, five of *S. hystrix*, two of *S. furcatum*, and one of *S. polyceratium* var. *ovatum* were also collected by scuba-diving and snorkeling in

the French Caribbean islands of Guadeloupe and Martinique and used as out-groups in phylogenetic analyses. All specimens are kept in the Herbarium of the Mediterranean Institute of Oceanography at Aix-Marseille University, Marseille, France (HCOM). Herbarium abbreviations follow Thiers [59].

2.2. Morphological Analyses

Measurements were made on dried specimens for comparison with dried specimens stored in different herbaria housed in HCOM. Fifty-four characters were identified and selected based on analysis of all available descriptions from the literature of *Sargassum* identification keys from the North Atlantic Ocean [49,60–65] (Table S1). These 54 characters were observed and measured on 264 individuals; they correspond to all the specimens collected during the cruises.

Non-Multi-Dimensional Scaling (nMDS) was performed based on the resulting 54 characters (coding 0, 1) and 264 individual matrices (Table S1), using S17 Bray–Curtis similarity distance. PERMANOVA and pair-wise tests were applied to detect significant differences between the three examined morphotypes. P-values were obtained by 999 permutations of residuals under a reduced model [66–68].

2.3. DNA Processing and Phylogenetic Analyses

2.3.1. Amplification and Sequencing of Mitochondrial and Plastid Loci

Total DNA was extracted with the Qiagen DNeasy Plant Mini Kit or Macherey-Nagel NucleoSpin 96 Plant II kit according to manufacturer's instructions. Before extraction, samples were disrupted with a FastPrep®-24 by 2.381 mm using inox beads (CIMAP). Using 1/50 dilution of DNA solutions, five mitochondrial markers (mt spacer IGS, *cox2*, *cox3*, *mt16S rRNA*, and *nad6*) and two plastid markers (*psbA* and *rbcL*) were amplified. Primer names, sequences, and PCR programs for all loci are presented in Table 1.

Table 1. Primer names and sequences, annealing temperature and time, elongation time, expected size of the PCR product, and references for all loci studied in the present study.

Locus	Primer	Sequence	Annealing	Elongation	Size (bp)	Reference
mt spacer IGS	tRNALys-F tRNALys-R	CGTTTGGTGAGAACCTTACC TACCACTGAGTTATTGCTCCC	48 °C, 1 min	1 min	250	[52]
cox2	*cox2*-370F *cox2*-776R	CAAAGATGGATTCGACGGTTGG CCGGTATCAAACTCGCCCTT	57 °C, 1 min	1 min	406	[57]
cox3	*cox3*-467F *cox3*-901R	GGTTCAACGACACCCATTT TAGCGTGATGAGCCCATG	50 °C, 1 min	1 min	434	[57]
16S rRNA	*Sarg-mt16S-F* *Sarg-mt16S-R*	GTAGTCGGTTGGGTTAGGCC GTTTGAACCCCCGCCAATTC	60 °C, 1 min	1 min	616	[58]
nad6	*Sarg-nad6-F* (External) *Sarg-nad6-R* (External)	TATGATTCTTGGGGCTGGT GGGATCATTCAAAGCAGAAGA	56 °C, 1 min	1 min	431	[58]
psbA	*psbA-F* *psbA-R1*	ATGACTGCTACTTTAGAAAGACG GCTAAATCTARWGGGAAGTTGTG	49 °C, 1 min	1 min 30 s	970	[69]
rbcL	*rbcL-F* *rbcL-R*	TATGATTGATTT AGTGGTTGG GTTCGTCAC TTAAATCTGGTA	51 °C, 1 min 30 s	2 min	820	[70]

The final number of sequences obtained for each marker depended on PCR and sequencing success, and on the observed levels of variability between *Sargassum* morphotypes; fewer samples for *psbA* were sequenced, because that marker exhibited the lowest level of variability. The numbers of sequences analyzed per marker and morphotype are presented in the resulting phylogenetic trees and haplotype networks (see Results). The PCR reaction mix for all loci was prepared for a final volume of 20 μL with GoTaq® Flexi Buffer 1X, GoTaq® DNA Polymerase 0.02 U/μL, dNTP 0.2 mM, MgCl$_2$ 5 mM, forward

and reverse primers 0.5 μM each, BSA 0.8 mg/mL, and 2 μL of diluted DNA. All PCR programs included 3 min at 94 °C, 35 cycles with [1 min at 94 °C, 1 min or 1 min 30 s at annealing temperature (Table 1), and 1 to 2 min at 72 °C], and 5 min at 72 °C. The PCR products were sent for Sanger sequencing at Eurofins Genomics. Each unique sequence (i.e., haplotype) obtained in this study is available in GenBank under accession numbers MT422788 to MT422805, and the final sequence alignments have been submitted in Zenodo.

2.3.2. Analysis of Sequence Data

Sequences were visualized and aligned with UGENE v 1.31.1 [71]. For each marker, all *Sargassum* sequences available were retrieved from Genbank and aligned with the new sequences obtained here. Sequence alignments were performed with MUSCLE [72] in UGENE. Markers were first analyzed separately, then some were concatenated for tree or network construction. Concatenation was not always possible because of the very unbalanced distribution of *Sargassum* species across markers in Genbank.

For phylogenetic analyses, two datasets were used: a *psbA* alignment allowing a comparison with distant Phaeophyceae taxa, and a *rbcL-cox3* concatenated alignment for the *Sargassum* genus. Other markers were not used because of the lack of corresponding sequences in Genbank or difficulty aligning evolutionary distant taxa (as in the case of mitochondrial IGS). A Maximum Likelihood (ML) phylogenetic reconstruction was performed with IQ-TREE 1.6.8 [73] and a Bayesian Inference (BI) with MrBayes 3.2 [74,75]. With IQ-TREE, 1000 ultrafast bootstraps were used, along with the bnni option to reduce risk of overestimating branch support with ultrafast bootstrap [76]. In the case of combined datasets, the ModelFinder option of IQ-TREE was used to find the best partition scheme [77]. For BI, a sample frequency of 100 over 10^6 generations (burnin 250,000) was used for *rbcL-cox3* and $2 \cdot 10^6$ generations (burnin 10^6) was used for *psbA*. Convergence of the MCMC analysis was assumed when the average standard of split frequency was below 0.01, when the log probability of the data did not show any trend, and when the potential scale reduction factor was close to 1.0. Trees were visualized with FigTree 1.4.4 [78]. For the analyses based on *psbA* sequences, *Cutleria* and *Bachelotia* sequences were used as outgroups [79]. For *rbcL-cox3*, according to the present results and to Mattio and Payri [51], the tree was rooted with the clade containing two species, *Sargassum ringgoldianum* Harvey and *Sargassum thunbergii* (Mertens ex Roth) Kuntze, belonging to the subgenus *Bactrophycus*. Because of different representation in Genbank, the *psbA* and *rbcL-cox3* dataset did not lead to the same species lists; previous *S. fluitans* and *S. natans rbcL* sequences were not included, as available sequences for these taxa were only partially aligned with the ones obtained in this present study.

As a complementary approach, haplotype networks were built with the TCS 1.21 software [80], in order to study relationships among the most closely related sequences, to include indels in the analysis, and to visualize the frequency of the different haplotypes. Indels longer than one base pair were transformed into a single base-pair indel before analysis. Networks were built independently for five datasets: mt spacer IGS, a concatenation of partial *cox2* and *cox3*, *nad6*, and *16S rRNA* mitochondrial sequences, as well as part of the plastidial *rbcL*.

3. Results

3.1. Morphological Analyses

According to the morphological analysis of the 54 characters (Table S1), all individuals of *Sargassum* examined during the oceanographic cruises unambiguously belonged to one of three dominant morphotypes described by Parr [21], namely, *Sargassum fluitans* III, *Sargassum natans* I, and *Sargassum natans* VIII. Importantly, no intermediate morphology was observed. Analysis of all collected individuals (*n* = 264) based on all characters showed three distinct, clearly separated clusters, matching with the three morphotypes (Figure 2). In addition, these clusters differed significantly, as a whole and by pairs (Table 2).
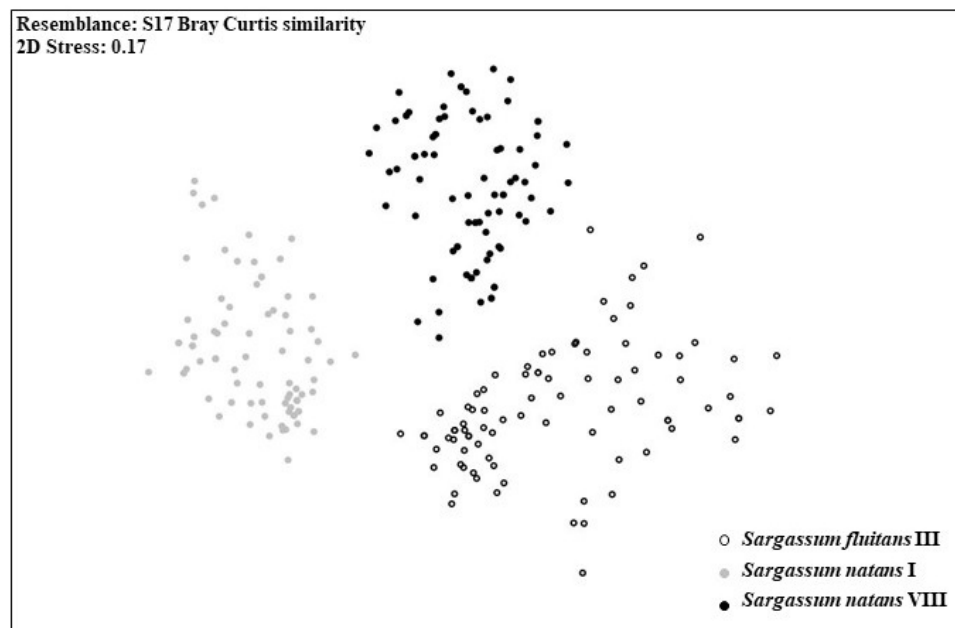
Figure 2. Two-dimensional nMDS ordination plot using S17 Bray–Curtis similarity based on 54 discriminant morphological characters of 264 holopelagic specimens of *Sargassum* collected during the two 2017 campaigns.

Table 2. Permutation multivariate analysis of variance (PERMANOVA) and pair-wise test, based on the morphological characters of holopelagic specimens of *Sargassum* collected during the two 2017 campaigns (after a square root transformation of the data, and using S17 Bray–Curtis similarity).

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms
Morphotype	2	77,756	38,878	175.73	0.001	998
Residual	252	55,752	221.24			
Total	254	1.3351×10^5				

Groups		t	P(perm)	Unique perms	P(MC)
S. natans VIII, *S. natans* I		14.31	0.001	999	0.001
S. natans VIII, *S. fluitans* III		10.59	0.001	999	0.001
S. natans I, *S. fluitans* III		14.93	0.001	999	0.001

Among the 54 characters analyzed, several were particularly discriminating: the overall shape, the nature of the axis (thorny or smooth), the blades (size and shape), and the vesicles (air bladders; size, presence or absence of apical spine-like appendage; length of pedicel) (Figures 3 and 4). Briefly, *Sargassum fluitans* III was characterized by (1) a short to loose, globular or cylindrical, open, and stringy shape, (2) a thorny axis, (3) blades that are lanceolate with an asymmetrical base, and (4) short pedicellate, oblong vesicles without an apical spine-like appendage but frequently with a foliaceous margin. *Sargassum natans* I was characterized by (1) a mostly cylindrical, sometimes pyramidal shape, (2) a smooth axis, (3) linear blades that are narrow with a symmetrical base, and (4) vesicles, frequently with a long pedicel, a foliaceous margin, and an apical spine-like or reduced foliaceous appendage. *S. natans* VIII was well-characterized by (1) its measurements larger than those of the two other morphotypes and a mostly pyramidal shape, (2) a smooth axis, (3) lanceolate and broad blades with a symmetrical base, and (4) vesicles that are shortly pedicellate, usually without an apical spine-like appendage (Figures 3 and 4; see key for identification).
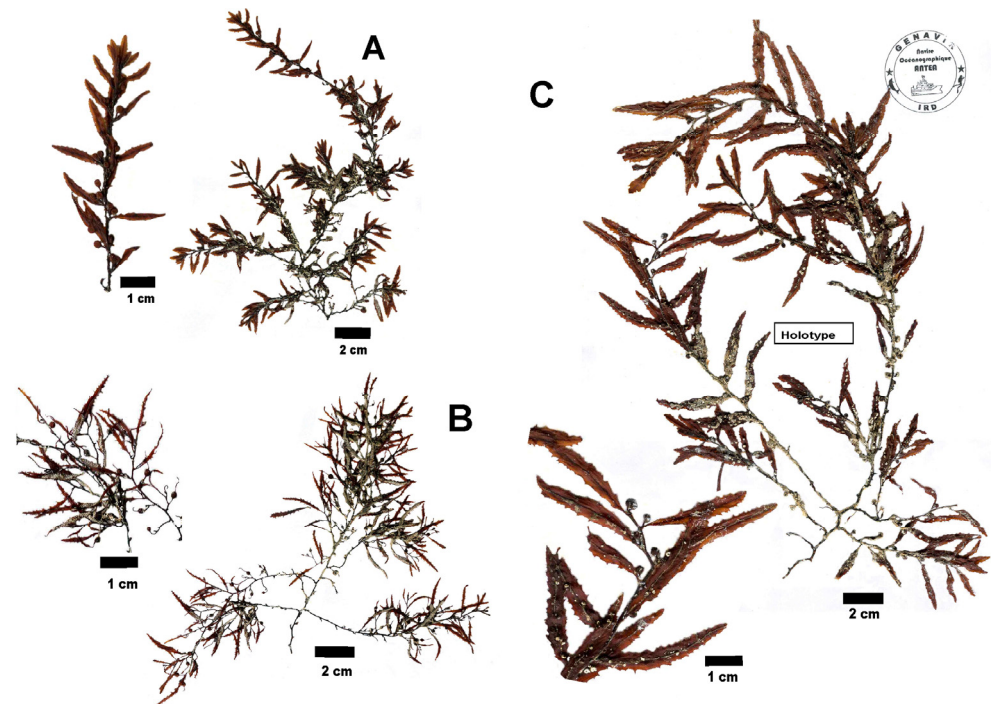
Figure 3. Dried specimens and detail of holopelagic *Sargassum* morphotypes collected at station S10. (**A**) *Sargassum fluitans* III (*S. fluitans* var. *fluitans*, ref. HCOM S10-S3-1). (**B**) *Sargassum natans* I (*S. natans* ref. HCOM S10-S2-2). (**C**) *Sargassum natans* VIII (*S. natans* var. *wingei* nov. var., holotype, ref. HCOM S10-S1-3).
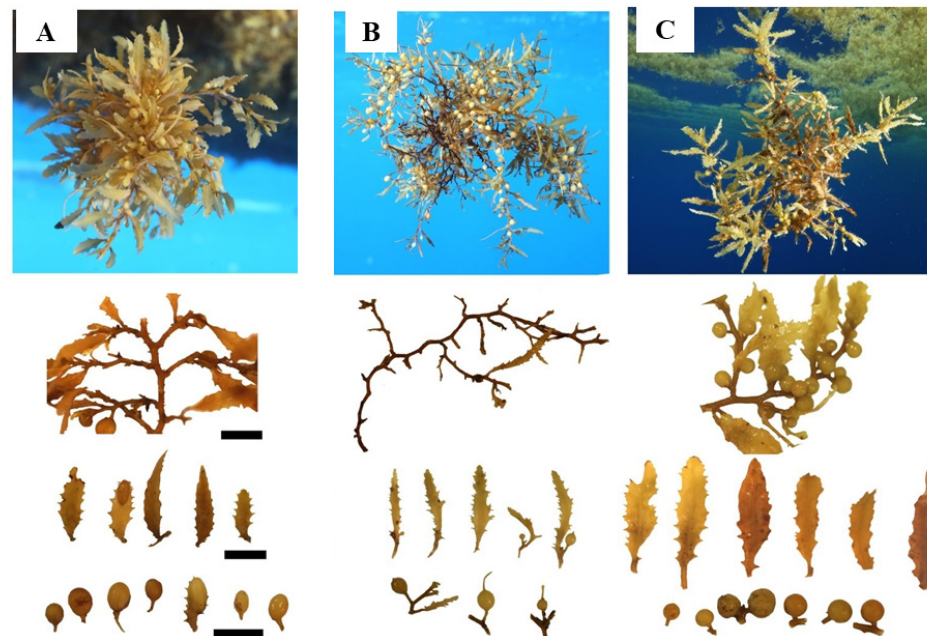
Figure 4. Morphological comparison between *S. fluitans* III, *S. natans* I, and *S. natans* VIII morphotypes of holopelagic *Sargassum* collected during the two expeditions of 2017. (**A**) Habitus, axis, and vesicles of *S. fluitans* III morphotype (*S. fluitans* var. *fluitans*). Scale bars = 1 cm. (**B**) Habitus, axis, and vesicles of *S. natans* I morphotype (*S. natans* var. *natans*). (**C**) Habitus, axis, and vesicles of *S. natans* VIII morphotype (*S. natans* var. *wingei* nov. var.). Each vesicle is attached to the axis by a pedicel. Vesicles have an apical spine-like appendage only for *Sargassum natans* var. *natans*.

3.2. Genetic Analyses

Molecular phylogenetic analyses were conducted for *pbsA* and *rbcL-cox3* concatenated sequences. The phylogenetic tree made with *psbA* did not reveal any variation among the three morphotypes, and the sequences were identical to benthic species such as *Sargassum vulgare* (Figure S2). Thus, the tree did not clarify the phylogenetic position of the holopelagic samples but confirmed that they belong to the *Sargassum* genus. Identical *psbA* sequences were also observed between close species within the Sargassaceae family, like between *Polycladia indica* (Schiffner) Draisma, Ballesteros, F. Rousseau & T. Thibaut and *P. heinii* [52] or *Ericaria amentacea* (C.Agardh) Molinari & Guiry and *E. selaginoides* (Hudson) Molinari & Guiry. With the concatenated fragment *rbcL-cox3*, sequences from the holopelagic samples were placed, with good support, in the same monophyletic group as *Sargassum yendoi* Okamura & Yamada, *S. vachellianum* Greville and *S. piluliferum* (Turner) C. Agardh (Figure 5). The separation of holopelagic morphotypes *S. natans* VIII and *S. natans* I was well-supported (bootstraps of 96%). The morphotype *S. fluitans* III appeared in the sister position to this group, though with lower support (76/0.89).
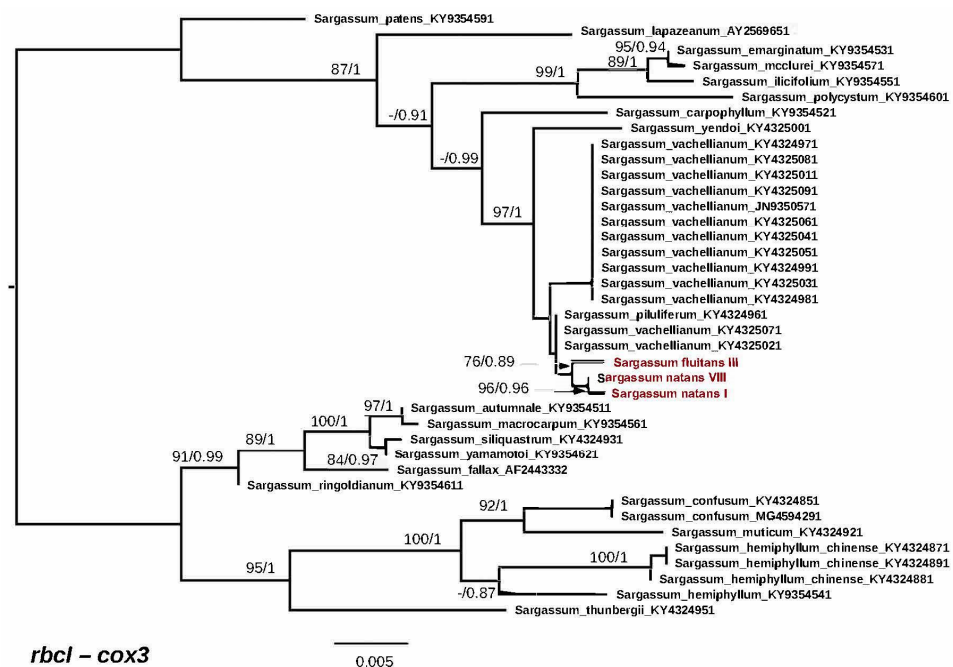
Figure 5. Phylogenetic tree based on the concatenation of *rbcL* and *cox3* sequences. The topology shown here corresponds to the output of the Maximum Likelihood analysis. Bootstrap values higher than 80%, and posterior probabilities higher than 0.9 are indicated at the left of the corresponding group. Newly generated sequences are in red. For the sequences retrieved from GenBank, the accession number is indicated together with the species name (corrected with current taxonomic status).

The molecular identification of the three holopelagic *Sargassum* morphotypes, among each other and in comparison with benthic species, was dependent upon the marker used for analysis. The new mt spacer IGS for the *S. fluitans* III morphotype was identical to previous sequences from *S. fluitans* III. New mt spacer IGS sequences for the *S. natans* VIII and *S. natans* I morphotypes were identical to each other and to *S. natans* VIII and *S. natans* I sequences from Amaral-Zettler et al. [57]. The TCS network showed a divergence of six mutations between the *S. natans* complex and *S. fluitans* III, including two indels of two base pairs (bps) each (Figure 6). For plastidial *rbcL*, the fragment sequenced did not completely match with the ones previously sequenced for *S. natans* and *S. fluitans*. Therefore, no comparisons could be made beyond this dataset. The morphotypes *S. natans* VIII and *S. natans* I shared the same haplotype with the benthic *Sargassum* analyzed in this study. This haplotype was separated by two substitutions in *rbcL* and a 9 bp deletion in the

spacer (not shown) from the morphotype *S. fluitans* III haplotype (Figure 6). One haplotype of morphotype *S. fluitans* III (station 20) was separated from other morphotype *S. fluitans* III samples by four mutations, and from the other multi-species haplotypes by one mutation and a 6 bp indel. The mitochondrial *16S rRNA*, *cox2-cox3* concatenated, and *nad6* sequences separated the three morphotypes and confirmed matches between the sequences obtained in this study and earlier published sequences for all three holopelagic morphotypes. The *16S rRNA* sequences for *S. fluitans* III differed from the benthic species and from *S. natans* I by a single substitution and from *S. natans* VIII by two substitutions (Figure 6). Two *S. natans* VIII sequences from the current dataset matched the *S. natans* I sequences, and one *S. hystrix* sequence matched the *S. fluitans* III sequences. In the *cox2-cox3* network, haplotypes from morphotypes *S. natans* VIII and *S. natans* I were only separated by one substitution in the *cox3* partial fragment (Figure 6). Haplotypes from morphotypes *S. natans* VIII and *S. fluitans* III were separated by four substitutions, one in *cox3* and three in *cox2*. The *nad6* network was composed of multiple grouped haplotypes representing each holopelagic *Sargassum* morphotype, with one dominant haplotype in each group (Figure 6). Two *S. natans* VIII and one *S. fluitans* III matched the dominant *S. natans* I haplotype, which differed from the dominant *S. fluitans* III haplotype by two nucleotide substitutions and from the dominant *S. natans* VIII by one nucleotide substitution.

Figure 6. Sequence networks for mt spacer IGS, *rbcL*, *16S rRNA* gene, concatenation of *cox2* and *cox3*, and *nad6*. Sequences of *S. polyceratium*, *S. vachellianum*, *S. spinuligerum*, and *Sargassum* sp., as well as dot-filled wedges correspond to sequences retrieved from GenBank. Number of sequences per haplotype is noted.

4. Discussion

Morphology strongly supports the delineation between the three holopelagic *Sargassum* morphotypes (*S. fluitans* III, *S. natans* I, and *S. natans* VIII). No individuals displayed intermediate characters, and individuals defined by their morphotype were clearly separated into groups specific to each (Figure 2). *S. fluitans* III, described by Parr [21], morphologically corresponds to the description of *S. fluitans* (Børgesen) Børgesen [18,19]. Consequently, *S. fluitans* III can be renamed as *Sargassum fluitans* var. *fluitans*. *S. fluitans* X, the other described *S. fluitans* morphotype [21], is rare; upon further analysis, it should be assigned the appropriate nomenclature. Moreover, *S. natans* I and *S. natans* VIII are syntopic, i.e., living together in the same habitat at each station, which ruled out the hypothesis that the observed morphological differences were related to their habitat differences. These two taxa, namely, *S. natans* I and *S. natans* VIII, could be in an early stage of speciation (i.e., the gray zone following de Queiroz's terminology [81]). Thus, we conservatively propose their formal separation into varieties as *Sargassum natans* var. *natans* (*S. natans* I) and *Sargassum natans* var. *wingei* (*S. natans* VIII).

Molecular results confirmed the phylogenetic positioning of Atlantic holopelagic *Sargassum* species, in close proximity to each other and among benthic species within a group corresponding to the section *Sargassum* of the subgenus *Sargassum*, as previously suggested (e.g., [51,54]). Nevertheless, within the section *Sargassum*, the low divergence of the sequences used as well as the reduced taxonomic sampling were not sufficient to unambiguously resolve the phylogenetic affinities of holopelagic species and, therefore, do not allow the discussion of the question of a single or multiple evolutionary origin of holopelagic *Sargassum*. The difficulty in resolving the phylogeny of this group could be due to a combination of a relatively recent evolution of the pelagic lifestyle, along with the slow molecular evolution of mitochondrial and plastidial genes considered.

In other *Sargassum* species, mitochondrial *cox3* or plastidial *rbcL* showed intraspecific variation, sometimes useful for phylogeographic studies [50,70,82]. Therefore, the lack of diversity inside holopelagic *Sargassum* species and observed with our marker set may not be a fixed rule. The discrepancy between morphological differentiation and low molecular divergence may be explained by this slow rate of molecular evolution of cytoplasmic genomes in *Sargassum* species. More generally, the observation of morphological differences can usually be explained by the interaction between genetic and environmental factors, but not in this case. There are also cases where species that are very similar genetically may exhibit significant morphological differences due to environmental influences or other factors (e.g., [83,84]), but holopelagic *Sargassum* taxa live in sympatry and are always found mixed together, even if their abundance can vary from year to year and/or place to place [27].

Regarding species/morphotype delineation using molecular markers, the examined genes exhibited only a few mutations separating the three morphotypes, as reported by Amaral-Zettler et al. [57] and Dibner et al. [58] and demonstrated in previous studies on nuclear, mitochondrial, and chloroplastic markers dedicated to the genus *Sargassum* [85–87]. The present study confirmed weak but clear genetic differences (three to six mutations) for the mitochondrial *IGS*, *cox2*, *cox3*, and plastid *rbcL* genes, between morphotypes *S. natans* I and VIII, and *S. fluitans* III [57]. An additional analysis of the mitochondrial *16S rRNA* and *nad6* genes also confirmed weak but clear genetic differences between *S. natans* I and *S. natans* VIII [58]. In all cases, genetic data from these 2017 samples were consistent with previous sampling efforts between 2012 and 2016 [57] and between 2015 and 2019 [58], which seems to indicate a relative stability of composition at that scale. The fact that observed genetic differences between *S. natans* I and *S. natans* VIII were low may be the result of the aforementioned low rate of evolution for cytoplasmic genomes. In future studies, other more polymorphic markers may reveal a higher divergence between the two morphotypes.

Within the genus *Sargassum*, similarities in molecular marker sequences between distinct species are frequent. Yoshida et al. [86] demonstrated similarities in the nuclear

ITS2 sequences of four allied *Sargassum* species belonging to the subgenus *Bactrophycus*: *Sargassum boreale*, *S. confusum*, *S. microceratium,* and *S. pallidum*. In that work, *S. boreale* was identified as a new species because of the presence of a large deletion of 28 bp, which was stable in all specimens of *S. boreale* studied [86]. No nucleotide difference was observed in the ITS2 sequences of *Sargassum mcclurei* and *S. quinhonense* belonging to the subgenus *Sargassum* although their morphology is totally different [85]. The separation of the two species was not supported by the ITS2 phylogeny; nevertheless, *S. mcclurei* and *S. quinhonense* are distinct species as no intermediate morphological form has been reported [85]. On another Sargassaceae genus, Rohfritsch et al. [88] demonstrated that a divergence of almost 17 mutations on 2102 concatenated base pairs was enough to confirm that *Turbinaria ornata* and *T. conoides* were separate species.

The proposed revision is particularly critical at this time, as considerable confusion exists regarding the morphological identification of holopelagic *Sargassum* species in the literature. To address and resolve these inconsistencies, a thorough historic review of holopelagic *Sargassum* taxonomy is presented here. Early taxonomic classification of holopelagic *Sargassum* recognized two species, *Sargassum natans* (L.) and *S. hystrix* (Agardh) var. *fluitans*, based on several morphological traits that clearly distinguished one species from the other [18,19]. In addition to differences in the shape of blades and vesicles, *S. hystrix* var. *fluitans* was notable for branches that are "provided with short spinules." Winge [20], based on hundreds of preserved samples collected from numerous cruises across the North Atlantic between 1911–1922, identified "8 different drifting *Sargassum*-species." Parr [21], based on several cruises in the North Atlantic from 1933–1935, advanced Winge's [20] *Sargassum* taxonomy and provided the first dichotomous key for drifting *Sargassum* species, as well as detailing their unique distributional patterns. Notably, the second couplet in Parr's [21] key addresses the nature of *Sargassum* branches as 'thorny' for all *S. fluitans* morphological types (III and X) versus branches as 'smooth' for all *S. natans* morphotypes (I, II, VIII, and IX). *S. natans* morphotypes are further distinguished by the presence (*S. natans* I) or absence (*S. natans* II, VIII, and IX) of an apical spine on the vesicles, and then the size and shape of blades.

Parr [21] is recognized as the definitive source on holopelagic *Sargassum* taxonomy. Indeed, early reviews of Sargasso Sea science always referenced Parr's [21] taxonomic and distribution studies [89–91]. Investigations examining the changing distribution and abundance of holopelagic *Sargassum* [22,92,93] compared their findings to Parr [21]. Investigations of the epibionts [94–97] and motile fauna [98–100] that live on different types of holopelagic *Sargassum* looked to Parr [21] for proper species identification. Studies examining the growth and production of different holopelagic *Sargassum* species [101,102] also reference Parr [21]. Even though these studies were familiar with the true diversity of holopelagic *Sargassum* morphotypes, in practice, *S. natans* I and *S. fluitans* III were consistently the most abundant, and the need to consider rare morphotypes (*S. natans* II, VIII, and IX, and *S. fluitans* X) waned over time. Eventually, Parr's [21] numeric system was dropped and *S. natans* I and *S. fluitans* III were simply referred to as *S. natans* and *S. fluitans*.

The contemporary definitive source for benthic algae taxonomy in the western North Atlantic and Caribbean has been Taylor [49], which naturally includes a thorough account of benthic *Sargassum* species. However, Taylor's [49] approach to the taxonomy of holopelagic *Sargassum* was overly simplified and only recognized two species: *S. natans* and *S. fluitans*, making no mention of the other morphotypes. Critically, Taylor [49] did not distinguish the two *Sargassum* species in his dichotomous key based on the presence or absence of thorns on the branches, the well-established historical precedent, but instead used the blade and vesicle shape. In fact, Taylor [49] describes *S. fluitans* as having a "...stem smooth or very sparingly spinulose," which contradicts the historical descriptions of *S. fluitans III* [18–21], and the accompanying illustration of *S. fluitans* (see Plate 39, Figure 2) shows smooth branches and spherical vesicles that more closely resembles Winge's [20] and Parr's [21] descriptions and illustrations of *S. natans* VIII. A more recent authority on benthic algae in the Caribbean, Wynne's [103] checklist, also adopted Taylor's [49] simplified view of

holopelagic *Sargassum* diversity and did not reference Winge [20] or Parr [21]. Schneider and Searles [104] and Littler and Littler [63] also described two species, making no mention of additional holopelagic *Sargassum* morphotypes, and simplified their dichotomous keys by removing any mention of thorns along the branches. Notably, Littler and Littler [63] provide an accurate photograph of *S. fluitans* III with thorns evident on the branches.

When Wynne [103] and subsequent revisions are consulted as the authoritative catalog of currently accepted nomenclature for species in the region and Taylor [49], Schneider and Searles [104], or Littler and Littler [63] are the only source material used for holopelagic *Sargassum* identification, specimens of *S. natans* VIII, with smooth branches and large, spherical vesicles lacking an apical spine-like appendage, can be mistakenly identified as *S. fluitans* III. de Szechy et al. [105] collected drift *Sargassum* off the coast of Brazil during the first GASB bloom and misidentified *S. fluitans* as *S. natans* (see Table 1, Figures 4 and 5). The extensive morphological and molecular assessment of benthic *Sargassum* in the Caribbean by Camacho et al. [54] appears to have used a specimen of *S. natans* VIII for their analysis of *S. fluitans* (see Figures 16 and 17 in [54]). Other studies at the time provided photographs or illustrations of *S. natans* VIII specimens but identified them as *S. fluitans* [106,107]. This is not surprising, considering sample collection occurred during the 2014–15 GASB event, when the composition of the bloom was dominated by *S. natans* VIII [33]. Sissini et al. [55] in their analysis of *Sargassum* washing up on Brazilian beaches in 2014–15 used Taylor [49], de Szechy et al. [105], and Camacho et al. [54] to make their morphological determinations (*S. natans* and *S. fluitans*), thus calling into question their morphological identification of *Sargassum* specimens and the validity of their molecular results. Similarly, Gonzalez-Nieto et al. [56], in their molecular assessment of *Sargassum* in the Gulf of Mexico and Caribbean region, included a misidentification of *S. natans* VIII, labeled as *S. fluitans* (see Figure 4, Panel D; open habitus, spherical vesicles, and low vesicle-to-blade ratio indicate *S. natans* VIII). Given the likelihood that their holopelagic *Sargassum* samples have been improperly categorized, the merits of their molecular analyses cannot be critically assessed.

These errors in identification have persisted without much cause for concern due to the rarity of several of Parr's [21] *Sargassum* morphotypes and, thus, had little meaningful consequence for misidentification. However, the landscape has changed since 2011, with the development of the GASB and re-emergence of *S. natans* VIII [33]. The improper identification of holopelagic *Sargassum* samples now has significant consequences and limits our ability to understand the driving mechanisms of the GASB, as recent studies have observed annual and seasonal fluctuations in the composition of holopelagic *Sargassum* morphotypes [27,33,46]. For example, the proposal that ten species with their varieties should be synonymized under *S. cymosum* [56], a recognized benthic species, was accepted by Wynne [108], resulting in the removal of *S. fluitans* from the checklist.

We strongly reject the conclusions of Gonzalez-Nieto et al. [56] and Wynne [108] that holopelagic *Sargassum* are in a polytomy with benthic *Sargassum* species. First, all holopelagic *Sargassum* morphotypes are considered sterile, as no receptacles have ever been observed; thus, grouping them with sexually reproductive benthic species with well-documented receptacles is nonsensical. Second, the three most common holopelagic *Sargassum* morphotypes (*S. fluitans* III, and *S. natans* I and VIII*)* co-occur across broad geographic and environmental ranges and, yet, their morphological and molecular differences are conserved as demonstrated in the present study and Dibner et al. [58]. Given the new research presented in this study, we maintain the continued species delineation between *S. fluitans* III and *S. natans* I and now formally recognize *S. natans* VIII as a separate variety of *S. natans*.

Recognition of a greater, not less, amount of diversity in holopelagic *Sargassum* is further supported by the research across ecological, biochemical, and physiological fields of study. Govindarajan et al. [109] observed population-level differences in the hydroid *Aglaophenia latecarinata* among *S. fluitans* III and *S. natans* VIII samples, despite the significant geographic overlap in their distribution. Martin et al. [110] observed differences in motile fauna abundance, diversity, and species composition across the three morpho-

types. Garcia-Sanchez et al. [46] observed shifts in the abundance of holopelagic *Sargassum* morphotypes reaching beaches in Mexico over several years. Similarly, seasonal shifts in *Sargassum* morphotype abundance were observed in Barbados [27]. McGillicuddy et al. [111] observed differences in the nutrient and arsenic biogeochemistry among *S. fluitans* III and *S. natans* I morphotypes. Finally, several studies have documented the different growth and environmental tolerance among holopelagic *Sargassum* morphotypes to varying nutrient, temperature, and salinity conditions [39–43].

Taxonomy is not an end in itself, but a tool allowing researchers to propose species hypotheses [47,112]. Most often, accepted species are distinguished by a set of morphological characters and/or specific characteristics. In addition to the need for name agreement among scientists to help in research communications, environmental managers also need definitive and consistent names to designate the populations they administer, even if the biodiversity is generally more complex than nomenclatural partitioning, notably because speciation processes are gradual. Terrestrial botany, especially studies carried out on Magnoliophytes, offers thousands of examples of taxa that are distinguished even though intermediate forms, in particular hybrids, unite them. In the case of *S. natans* VIII and *S. natans* I, we showed that no intermediate forms exist. In addition, hybridization is, a priori, irrelevant insofar as sexual reproduction has never been observed in these holopelagic macroalgae, despite the major sampling effort that has occurred. Nevertheless, the discord between the genetic differentiation (i.e., very low) and the morphological differences (i.e., high) for *S. natans* I and *S. natans* VIII makes it challenging to propose that the two morphotypes correspond to different species.

Current morphotype naming, associated with the lack of a formal description, leads to uncertainties in the description of the holopelagic *Sargassum*. We, therefore, propose a taxonomic framework, with the description of a new variety and an identification key. The type specimen of *Sargassum natans* belongs to the morphotype *Sargassum natans* I (Figure S1). The *Sargassum natans* VIII morphotype was well-identified by Winge [20] and Parr [21], but neither with a formal diagnosis nor a creation of different taxon. Although this name was taken up later (e.g., [33,57,58] and in most of the recent papers on *Sargassum* inundation since 2015), it remains undescribed according to the rules of botanical taxonomy (International Code of Nomenclature of algae, fungi, and plants [48]). It is, therefore, described below, under the name of *Sargassum natans* var. *wingei*, in tribute to Professor Ö. Winge who first recognized the originality of this form.

Species description
We propose the following new name for the morphotype *Sargassum natans* VIII.
Sargassum natans* var. *wingei Thibaut, Blanfuné, Boudouresque, Siuda & Verlaque var. nov.
Figures: Figures 3 and 4.
Holotype: Specimen HCOM S10-S1-3 (Holotype deposited in HCOM Herbarium)
Isotypes: Specimens S10-S1-1 and S10-S1-2, deposited in HCOM Herbarium.
Type locality: Station 10, tropical Atlantic Ocean, geographic co-ordinates: 14°58.440′ N 59°07.230′ W.
Etymology: Named for Professor Öjvind Winge, who was the first scientist to distinguish this taxon (as *Sargassum* sp. VIII) among the pelagic *Sargassum* specimens.
Representative material: All samples of *Sargassum natans* var. *wingei* collected during the two expeditions (Caribbean and transatlantic expeditions). All samples are deposited in HCOM Herbarium (Table S2).
Specimens sequenced and Genbank references: New haplotypes produced here are available in GenBank under accession numbers MT422788 to MT422805, and final sequence alignments have been submitted in Zenodo.
Other descriptions and illustrations: Winge [20] (1923, Figure 13, as *Sargassum* sp. VIII); Parr [21] (1939, figs 9-10, 12-17, as *S. natans* (VIII) Winge); Schell et al. [33] (2015, Figure 1 in ref [33], as *S. natans* VIII Parr); Amaral-Zettler et al. [57] (2017, figs 2c and 2d, as *S. natans* form VIII); Martin (2016); Govindarajan et al. [109] (2019, Figure 1c, as *S. natans* VIII) (Table S1).

Websites: as *S. natans* VIII: Sea Education Association, Woods Hole (https://www.sea.edu/sea_research/sargassum_ecosystem, accessed on 28 March 2024). Sailors for the Sea (https://sailorsforthesea.org/blog/conservation-heading-towards-bermuda/, accessed on 28 March 2024). Center for Resource Management and Environmental Studies (CERMES), University of West Indies Cave Hill, Barbados.
(https://www.cavehill.uwi.edu/cermes/research-projects/sargassum/tools-and-guidance.aspx, accessed on 28 March 2024)

Diagnosis: Plant holopelagic, brown to yellow-brown, without a holdfast or a distinct main axis; branches smooth without cryptostomata, 1–1.5 mm diam., ramified several times; blades simple, shortly pedicellate, flat, lanceolate to broadly lanceolate, without cryptostomata (13) 30–55 (–65) mm long and 3–10 mm wide (L/W ratio: 3–10, typically: 7–8), with coarsely irregularly serrated margins, symmetrical base, and midrib; vesicles (air bladders), more, less, or as much as the blades, smooth, without cryptostomata, spherical, rarely oblong, 2–5 (–7) mm diam., usually without foliaceous margin or apical spine-like appendage; pedicels short, 0.5–2.5 mm long, cylindrical, sometimes foliaceous with toothed margins; receptacles never observed.

Distribution: Tropical Atlantic Ocean, between 7° and 26° N.

Habitat: It is one of the three holopelagic taxa of the tropical Atlantic Ocean, with *S. fluitans* (Børgesen) Børgesen and *S. natans* (Linnaeus) Gaillon.

Key for the identification of Atlantic *Sargassum* species

1a. Plants fixed to the substrate; main axis distinct; cryptostomata and receptacles present. . . . . **Benthic species** (not treated here)

1b. Plants floating, without holdfast and distinct main axis; receptacles absent and cryptostomata absent. . . . . **Pelagic species** (2)

    2a. Branches thorny; blades lanceolate, 3–8 mm wide and 11–60 mm long (L/W: 4–13), with asymmetrical base; vesicles oblong without apical spine-like or foliaceous appendage but frequently with foliaceous margin, usually more numerous than blades, 1–6 mm diam., with a short pedicel, 0.5–2.5 mm long, cylindrical to winged, sometimes with toothed margins . . . .*S. fluitans* **(Børgesen) Børgesen**

    2b. Branches smooth; blades with symmetrical base; vesicles more spherical, fewer or as numerous as blades . . . . (3)

        3a. Blades linear, narrow, 1–4 mm wide and 13–70 mm long (L/W: 5–22); vesicles, 2–5 mm diam., frequently with a foliaceous margin and an apical spine-like or reduced foliaceous appendage, and with a long pedicel, 1–5 mm long . . . .*S. natans* var. *natans* **(Linnaeus) Gaillon**

        3b. Blades lanceolate, broad, 3–10 mm wide and 13–65 mm long (L/W: 3–10); vesicles, 2–7 mm diam., usually without foliaceous margin and apical spine-like appendages, and with a short pedicel, 0.5–2.5 mm long . . . .*S. natans* **var. *wingei* nov. var.**

The decision to place *S. natans* VIII at the level of variety, and not to elevate the morphotype to the status of a new species, is somewhat conservative. Beyond the question of renaming these taxa, the clear morphological differences between the three holopelagic *Sargassum* and the weak genetic differences raise significant doubts about the speciation stage of these taxa; their assignment to a species rank is even more complex as they most likely rely on asexual reproduction as their primary method of propagation, so that the biological species concept can hardly be used for delineation. Our genetic results on *S. natans* suggest that the two varieties could correspond to different fixed clones from a single species, where asexual lineages fixed morphological differences. However, the use of mitochondrial and plastidial DNA together is not sufficient to investigate this hypothesis. Fucales are oogamous [113], and, in oogamous brown algae, chloroplasts and mitochondria are thought to be maternally inherited [114]. Therefore, these two cytoplasmic genomes are not independently inherited, and, alone, they do not correspond to independent markers. Further investigation of the species' evolutionary history could be accomplished by using

nuclear markers, polymorphic within each of the two varieties. This approach would allow for a more in-depth analysis of the extent of clonality, as well as species delineation and speciation scenarios. For example, it would be interesting to test if the pelagic way of life originated once or several times in the *Sargassum* genus. Finally, the phylogeny of the genus *Sargassum* must be profoundly clarified considering that the holotype specimen defining the genus *Sargassum* was *Sargassum bacciferum* Turner (C. Agardh) currently named for the type species as *S. natans* (Agardh, 1820), a clonal holopelagic taxa lacking sexual reproductive apparatus.

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