








RESEARCH ARTICLE

Consistent genetic divergence observed among pelagic *Sargassum* morphotypes in the western North Atlantic

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Abstract

Sargassum natans and *Sargassum fluitans* are uniquely holopelagic macroalgae, providing open ocean nursery and foraging habitat for commercially and ecologically important species. Recent basin-wide changes in pelagic *Sargassum* diversity and distribution have manifested in proliferation of a previously rare morphotype, *Sargassum natans VIII*, to rival biomass levels of historically dominant *S. natans I* and *S. fluitans III*. Precise genetic identification of these morphotypes can improve accuracy and interpretation of ecological studies as well as clarify evolutionary history and population connectivity. For 139 field samples collected from the subtropical and tropical North Atlantic, three mitochondrial genes (*cox3*, *nad6*, and *mt16S rRNA*) were used to examine genetic divergence among the three common pelagic *Sargassum* morphotypes. These gene sequences successfully differentiated among morphotypes regardless of geographic origin, confirming *in situ* morphology-based identifications. *Sargassum natans I* and *S. natans VIII* exhibited divergence consistent with that between the *S. natans*-complex and *S. fluitans III*. Phylogenetic analysis of these samples also indicated evolutionary divergence between *Sargassum* morphologies. The genetic divergence among morphotypes, compared with benthic *Sargassum* species, suggested that taxonomic reclassification of the three most common pelagic morphotypes may be warranted.

KEYWORDS

genetic diversity, pelagic *Sargassum*, phylogeny, *Sargassum fluitans III*, *Sargassum natans I*, *Sargassum natans VIII*

1 | INTRODUCTION

Sargassum (class: Phaeophyceae, order: Fucales) is a complex group of marine brown macroalgae. There are 361 accepted species, mostly benthic, distributed globally in temperate and tropical waters (Guiry & Guiry, 2021). Among these, however, the world's only holopelagic macroalgae are as follows: *Sargassum fluitans* and *S. natans*.

These drifters are suspected to reproduce asexually, maintain buoyancy via gas-filled floats, and establish a surface ocean habitat in the subtropical North Atlantic, equatorial Atlantic, Caribbean Sea, and Gulf of Mexico that is unparalleled in the open ocean. Individual, radial branching clumps are dispersed across the sea surface, aggregated by helical Langmuir circulation into parallel windrows, or concentrated into dense mats measuring 10s of meters across

(Butler et al., 1983; Ody et al., 2019; Parr, 1939; Winge, 1923). The pelagic *Sargassum* ecosystem hosts diverse organisms, from sessile and clinging motile fauna that require the algal substrate for survival (Butler et al., 1983; Coston-Clements et al., 1991; Huffard et al., 2014) to transient large fish, sea turtles, and seabirds that use pelagic *Sargassum* aggregations as nursery or foraging habitat (Moser & Lee, 2012; Trott et al., 2010).

Pelagic *Sargassum* in the Atlantic was first described by Børgesen (1914) and further examined by Winge (1923), who identified 10 distinct morphotypes. Over numerous cruises in the western North Atlantic, Caribbean Sea, and Gulf of Mexico, Parr (1939) comprehensively studied the distribution of morphotypes and refined pelagic *Sargassum* taxonomy by assigning most of Winge's morphotypes to the two pelagic species, *S. fluitans* (morphotypes III and X) and *S. natans* (I, II, VIII, IX). Presence (*S. fluitans*), or absence (*S. natans*) of thorns on the stipes was key to visual differentiation of species. The width of blades varied among morphotypes and species; for example, *S. natans* I had narrow blades while *S. natans* VIII's broad blades could, to the untrained eye, have led to misidentification as *S. fluitans* III (Parr, 1939; Schell et al., 2015). In 1934–1935, pelagic *Sargassum* density in the Sargasso Sea was four times greater than in the Gulf of Mexico and 40 times greater than in the Caribbean Sea (Parr, 1939). Morphotype distributions also differed across regions: *S. natans* I dominated the Sargasso Sea (50%–90%) and Gulf of Mexico (86%) with *S. fluitans* III accounting for the remainder, while in contrast, the western Caribbean hosted 50% *S. fluitans* III, 30% *S. natans* I, and 20% *S. natans* VIII. All other morphotypes were rare (Parr, 1939). Sampling in the Sargasso Sea (reviewed in Butler et al., 1983) and Caribbean Sea (Stoner, 1983) during the 1970s and 1980s confirmed regional pelagic *Sargassum* densities consistent with observations from the 1930s.

Since 2011, pelagic *Sargassum* distribution and diversity have changed, resulting in unprecedented strandings and management uncertainties along Caribbean reefs and beaches (Cabanillas-Terán et al., 2019; Cruz-Rivera et al., 2015; Maurer et al., 2015), as well as West African coastlines from Morocco to the Gulf of Guinea (UNEP, 2015). Shipboard observations indicated that while pelagic *Sargassum* concentrations in the Sargasso Sea remained relatively constant from 1995–2015, quantities in the western tropical Atlantic (east of the Caribbean Windward Islands) during 2011 increased to 25 times the long-term average and those in 2014 were 300 times greater than the historical record (Schell et al., 2015). *S. natans* VIII, which had not been observed since the 1930s, dominated surface net tow biomass in the tropical Atlantic (87% by wet weight) and eastern Caribbean (95% by wet weight) during the late 2014 inundation episode (Schell et al., 2015). In a recent satellite retrospective (2011–2018), pelagic *Sargassum* was annually detected in the tropics, stretching across the Great Atlantic *Sargassum* Belt from West Africa through the Caribbean to the Gulf of Mexico (Wang et al., 2019). To complicate the narrative, pelagic *Sargassum* rafts observed in the open tropics during a 2017 field expedition (Thibaut, 2018) and on Mexican Caribbean coastlines in 2018 (Garcia-Sanchez et al., 2020; Monroy-Velázquez

et al., 2019) were comprised of all three morphotypes but dominated by *S. fluitans* III. Substantial changes in density and range of pelagic *Sargassum* morphotypes imply a large-scale shift in the dynamics of the North Atlantic (Djakouré et al., 2017; Johns et al., 2020). If each morphotype hosts a different faunal community and possibly represents different levels of ecological value (Calder, 1995; Govindarajan et al., 2019; Martin et al., 2021), accurate identification of pelagic *Sargassum* is essential and can be facilitated by genetic differentiation of morphotypes.

Macroalgal species share common characteristics that challenge systematics based solely on morphological taxonomy, including high phenotypic plasticity, large gaps in the understanding of complex life histories, and high incidence of cryptic genetic diversity. Genetic barcoding techniques, coupled with morphological identification, have demonstrated promise in resolving taxonomic uncertainties for some macroalgal complexes (e.g. Du et al., 2014; McCoy et al., 2020; Saunders, 2005). Until the last decade, little was known about *Sargassum* genetic diversity. Most molecular taxonomy work to date focused on benthic *Sargassum* species in the Pacific, using multiple markers (Mattio & Payri, 2011) and, more recently, whole mitochondrial genomes (Liu et al., 2017). Phillips and Fredericq (2000), the first to evaluate genetic relationships among Gulf of Mexico and Caribbean species (including one sample each of *S. natans* and *S. fluitans*), found that pelagic *Sargassum* sequences of a chloroplast encoded ribulose-1, 5-biphosphate carboxylase (rbcLS) spacer region clustered with different benthic species and thus warranted continued species distinction. Camacho et al. (2015), using three molecular markers, described one new benthic species and revised the Caribbean-Columbia regional taxonomy. In their analysis, the four pelagic *Sargassum* specimens, two of each presently recognized species, grouped with most other Caribbean samples in subgenus *Sargassum* section *Sargassum* that exhibited low intraspecific divergence. A study that included nine stranded pelagic *Sargassum* samples collected from beaches in Brazil determined that a commonly used marker in such investigations, the nuclear Internal Transcribed Spacer 2 (ITS-2), could not distinguish among closely related *Sargassum* species (Sissini et al., 2017). Most recently, Amaral-Zettler et al. (2016) sequenced entire mitochondrial genomes for *S. natans* I, *S. natans* VIII, and *S. fluitans* III, revealing low divergence among the morphotypes; further analyses with two novel primer sets confirmed this low but consistent divergence. As marker selection strongly impacts the results of molecular taxonomy, a more robust assessment of genetic divergence among pelagic *Sargassum* morphotypes sampled across broader geographic ranges, was due.

This study assessed genetic differentiation among *S. natans* I, *S. natans* VIII, and *S. fluitans* III morphotypes, providing a baseline for understanding the genetic underpinnings of these morphological forms. Molecular analyses of three mitochondrial genes were conducted for a large sample archive spanning five years and temperate to tropical western North Atlantic. Given the difficulty of distinguishing between pelagic *Sargassum* morphotypes with nuanced and fine-scale phenotypic variability, genetic identification

may improve accuracy and interpretation of results and further clarify evolutionary history as well as more recent population genetic biology and connectivity. More broadly, a precise identification of the pelagic *Sargassum* morphotypes strengthens the understanding of ecological impacts following recent basin-wide shifts in pelagic *Sargassum* distribution and diversity, and informs development and implementation of effective management strategies for the now expected annual coastal inundations throughout the Tropical Atlantic.

2 | METHODS

2.1 | Sample collection

From 2015 through 2019, pelagic *Sargassum* samples were collected via dip net or neuston tow and archived by Sea Education Association (Woods Hole, MA, USA). Fresh *Sargassum* sprigs, each measuring approximately 10 cm and composed of stipe, blades, and floats were scraped to remove epiphytes and epifauna and rinsed with deionized water before transfer to granulated silica gel for desiccation and storage. A total of 135 samples were selected from the archive for molecular analysis, representing regions distributed across biogeographic provinces (Spalding et al., 2012) and defined by surface current patterns: Sargasso Sea (18 *S. fluitans III*, 40 *S. natans I*, and 8 *S. natans VIII*), Tropical/Equatorial Atlantic (10 *S. natans VIII*), Antilles Current (12 *S. fluitans III*, 7 *S. natans I*, and 14 *S. natans VIII*), Caribbean Sea (10 *S. fluitans III* and 10 *S. natans VIII*), and Gulf Stream (6 *S. natans VIII*). In most regions, sets of up to 10 samples of each morphotype derived from separate stations. For the Sargasso Sea and Antilles Current regions, additional samples from multiple stations with fewer replicates were included in order to balance the number of samples across morphotypes in the entire dataset, as all morphotypes were not present in all regions. To expand the geographic coverage of the dataset, additional specimens were collected from two stations in the Gulf of Mexico (10 *S. fluitans III* and 10 *S. natans VIII*) and a single station in the equatorial Atlantic (8 *S. fluitans III* and 8 *S. natans VIII*) by the authors in 2015 and 2016, respectively. It is important to note that due to the sample selection strategy, morphotype proportions from any given region or station in the dataset did not reflect the in situ pelagic *Sargassum* community composition.

2.2 | DNA extraction

DNA was extracted from the samples at Sea Education Association using MoBio PowerPlant Pro DNA Isolation kits (Carlsbad, CA, USA), followed by MoBio PowerClean Pro DNA purifying kits. Protocols were optimized as follows: (1) Silica-dried samples were manually ground to a fine powder using a micropestle. (2) To better homogenize the tissue, a 24-h incubation in kit buffer at 65°C with periodic vortexing was added. (3) The final volume (100 µl) of DNA was eluted a second time to maximize yield.

TABLE 1 List of GenBank Accession numbers for mitochondrial genomes used both for gene segment selection and benthic outgroup comparisons

Species/ Morphotype	Accession #	Citation
<i>S. natans I</i>	KY084907	Amaral-Zettler et al. (2016)
<i>S. natans VIII</i>	KY084908	Amaral-Zettler et al. (2016)
<i>S. fluitans III</i>	KY084909	Amaral-Zettler et al. (2016)
<i>S. thunbergii</i>	KP280065	Liu and Pang (2015)
<i>S. muticum</i>	KJ938301	Liu and Pang (2014)
<i>S. muticum</i>	NC_0264614	Liu and Pang (2014)
<i>S. polycystum</i>	KT280278	Liu et al. (2017)
<i>S. spinuligerum</i>	KT276514	Liu et al. (2017)
<i>S. ilicifolium</i>	KT272403	Liu et al. (2017)
<i>S. aquifolium</i>	KT266809	Liu et al. (2017)
<i>S. vachellianum</i>	KR132242	Bi and Zhou (2016)

2.3 | Gene selection and primer design

Whole mitochondrial genomes for *S. fluitans III*, *S. natans I*, and *S. natans VIII* were retrieved from GenBank (Table 1). From these, gene segments exhibiting multiple polymorphisms among pelagic *Sargassum* morphotypes were targeted using Geneious Prime (Biomatters Ltd., Version 2020.0.5), including mitochondrial 16S rRNA (mt16S), mitochondrial 23S rRNA (mt23S), 40S ribosomal protein S13 (rps13), cytochrome III oxidase (cox3), and NADH dehydrogenase subunit 6 (nad6). Primers of optimal length, melting temperature, and PCR product size (Roux, 2009) were designed for each region (Table 2) using Primer3 in Geneious Prime and were confirmed to have no hairpins or primer dimers using OligoCalc (Kibbe, 2007). Existing primer sets for *Sargassum* (referenced in Camacho et al., 2015 and designed by Amaral-Zettler et al., 2016) did not capture the most variable segments of the mitochondrial genome in pelagic species. The mt23S rRNA and rps13 regions proved difficult to sequence and thus were excluded from this study. Sequencing of nad6 required two sets of primers: External primers were used to amplify the gene, whereas internal primers ensured successful sequencing across the short repetitive T region in the middle of the gene.

2.4 | DNA amplification

A step-down thermocycler protocol was used to denature, anneal, and extend all samples. This protocol effectively amplified all three genes of interest by having an initial annealing temperature that was higher than the optimal melting temperature (T_m) of the primers, thereby reducing the temperature over a number of cycles to increase PCR specificity. PCR amplification consisted of an initial denaturation temperature of 94°C for 4 min followed by 5 cycles at 94°C for 60 s, 50°C for 30 s, and 68°C for 60 s, 5 cycles at 94°C for 60 s, 48°C for 30 s, and 68°C for 60 s, 10 cycles

Gene name and read direction	Nucleotide sequence	Length	T _m (°C)	%GC
<i>Sarg nad6 F (External)</i>	TATGATTCTTGGGGCTGGT	19	55.6	47.4
<i>Sarg nad6 R (External)</i>	GGGATCATTCAAAGCAGAAGA	21	55.9	42.9
<i>Sarg nad6 R (Internal)</i>	CTGTTTTTGCCAGAAGACCA	21	52.4	48
<i>Sarg nad6 F (Internal)</i>	TACGGTTTTTATAGGAASTTCCTATG	26	54.5	35
<i>Sarg cox3 F</i>	GTTCAATCTATCCCCTTCTTAA	24	57.8	41.7
<i>Sarg cox3 R</i>	GGCCAAACCCCTCCAATATTA	21	57.7	47.6
<i>Sarg mt16S F</i>	GTAGTCGGTTGGGTTAGGCC	20	60.1	60
<i>Sarg mt16S R</i>	GTTGAACCCCGCCAATTC	20	60	55

TABLE 2 List of primers, with their gene names, nucleotide sequences, lengths, melting temperatures, and guanine–cytosine contents (%)

at 94°C for 60 s, 46°C for 30 s, and 68°C for 60 s, 10 cycles at 94°C for 60 s, 44°C for 30 s, and 68°C for 60 s, 10 cycles at 94°C for 60 s, 42°C for 30 s, and 68°C for 60 s, and a final extension at 68°C for 7 min.

Amplification was confirmed using standard gel electrophoresis. Before sequencing, PCR products were purified using Qiagen QIAquick PCR Purification Kit (Germantown, MD, USA) and concentrated when necessary. Quantity and quality of the purified products were determined using a Nanodrop 8000 (Thermo Fisher Scientific).

2.5 | Sequencing, assembly, and alignment

Purified PCR products generated at Eckerd College were sent to Eurofin Genomics (Louisville, KY, USA) and those generated at Sea Education Association to the DNA Analysis Facility at Yale University (New Haven, CT, USA) for bi-directional Sanger sequencing. A BLAST search was performed in Geneious Prime for all sequences to confirm identification as a member of Phaeophyceae. Forward and reverse sequences for *cox3* and *mt16S* were assembled via De Novo Assembly in Geneious Prime. Sequences were assembled at the highest sensitivity and were not trimmed before assembly. *Nad6* sequences were assembled by mapping the two forward and two reverse sections to the original gene segment for each morphotype. Any ambiguous calls (i.e., if the forward and reverse complement sequences did not match) were resolved via examination of the original chromatograms. None of the ambiguities coincided with the polymorphic sites. Assembled sequences were then aligned via MUSCLE alignment using a ClustalW sequence weighting scheme. The ends of each alignment were trimmed and regions containing gaps that could not be confidently aligned were removed. Every polymorphism was confirmed by examination of the original forward and reverse chromatograms. All new sequences are available in GenBank under accession numbers MT813198–MT813425.

2.6 | Phylogenetic analysis

Alignments were exported as fasta files and converted to nexus files. Nexus files were uploaded to TCS (Version 1.21; Clement et al.,

2000), which was used to generate a haplotype network for each gene. When the haplotype assignment did not match the original morphological identification, voucher samples and photographs were reexamined; this verification process uncovered eight obviously misidentified or mislabeled samples. The final networks were stylized in TCS beautifier (dos Santos et al., 2016) to indicate the proportionate morphotype composition for each haplotype. Another network was stylized to show geographic distribution of samples for each *cox3* haplotype.

Genetic divergence among pelagic *Sargassum* morphotypes and seven western Pacific benthic outgroups was calculated in Geneious Prime for each gene (*cox3*, *mt16S*, and *nad6*) alone and concatenated, using the percentage of identical bases/residues (Table 3). Outgroups were selected as examples from the 12 available mitochondrial genome sequences for benthic *Sargassum* species (Table 1), all of which were generated from samples collected in coastal China; five of these species were from *Sargassum* subgenus *Sargassum* and two fell in the section *Sargassum* with the pelagic morphotypes (Camacho et al., 2015). Concatenated (*cox3*, *nad6*, and *mt16S*) sequences were generated for 13 samples from this study representing all pelagic morphotypes as well as the seven outgroups. Concatenated sequences were used for a maximum likelihood phylogenetic inference using the phyML plugin on Geneious Prime (Guindon et al., 2010). This tree was created using the Kimura substitution model (K80) and the robustness bootstrap branch support with 1000 bootstraps. Transition and transversion ratio as well as gamma distribution parameters were estimated, and proportion of invariable sites was fixed. The number of substitution rate categories was four, and topology, length, and rate were optimized. Gene segments (*ITS-2*, *rcbLS*, and *cox3*) from *S. fluitans*, *S. natans*, and *S. thunbergii* sequenced by Camacho et al. (2015) were also concatenated and aligned to investigate the comparative level of divergence.

3 | RESULTS

Sequences were successfully generated from 139 samples (48 *S. fluitans III*, 43 *S. natans I*, and 48 *S. natans VIII*) across 38 western North Atlantic stations (Figure 1). Sequences from *S. natans VIII* samples were distributed across all regions and those from *S. fluitans III*



TABLE 3 Molecular divergence between pelagic *Sargassum* morphotypes and benthic outgroups

	<i>S. natans VIII</i>	<i>S. fluitans III</i>	<i>S. vachellianum</i>	<i>S. spinuligerum</i>	<i>S. polycystum</i>	<i>S. ilicifolium</i>	<i>S. aquifolium</i>	<i>S. thunbergii</i>	<i>S. muticum</i>
<i>S. natans I</i>	cox3 0.004	0.008	0.012	0.016	0.055	0.05	0.055	0.047	0.070
	16S 0.002	0.002	0.004	0.004	0.008	0.014	0.017	0.045	0.035
	nad6 0.003	0.006	0.025	0.019	0.048	0.063	0.066	0.169	0.162
	conc. 0.003	0.007	0.007	0.011	0.034	0.042	0.047	0.109	0.108
<i>S. natans VIII</i>	cox3 0.004	0.004	0.008	0.008	0.051	0.047	0.055	0.051	0.044
	16S 0.004	0.004	0.006	0.006	0.011	0.016	0.019	0.047	0.037
	nad6 0.009	0.009	0.025	0.016	0.051	0.066	0.069	0.173	0.165
	conc. 0.008	0.008	0.008	0.012	0.035	0.043	0.048	0.110	0.109
<i>S. fluitans III</i>	cox3 0.004		0.004	0.012	0.047	0.050	0.051	0.047	0.070
	16S 0.002		0.002	0.002	0.010	0.012	0.016	0.045	0.033
	nad6 0.032		0.032	0.019	0.048	0.057	0.069	0.169	0.168
	conc. 0.009		0.009	0.013	0.036	0.044	0.049	0.111	0.110
<i>S. vachellianum</i>	cox3 0.004			0.004	0.043	0.043	0.047	0.043	0.066
	16S 0.000			0.000	0.010	0.014	0.017	0.047	0.035
	nad6 0.035			0.035	0.059	0.079	0.085	0.186	0.164
	conc. 0.006			0.006	0.032	0.039	0.044	0.106	0.106
<i>S. spinuligerum</i>	cox3 0.043				0.043	0.047	0.051	0.047	0.070
	16S 0.010				0.010	0.014	0.017	0.047	0.035
	nad6 0.053				0.053	0.063	0.076	0.173	0.170
	conc. 0.036				0.036	0.044	0.049	0.111	0.110
<i>S. polycystum</i>	cox3 0.047				0.047	0.047	0.082	0.070	0.094
	16S 0.008				0.008	0.008	0.019	0.045	0.035
	nad6 0.047				0.047	0.047	0.079	0.173	0.170
	conc. 0.031				0.031	0.031	0.062	0.124	0.124
<i>S. ilicifolium</i>	cox3 0.074				0.074		0.074	0.070	0.094
	16S 0.041				0.041		0.041	0.041	0.029
	nad6 0.088				0.088		0.088	0.017	0.190
	conc. 0.070				0.070		0.070	0.132	0.131
<i>S. aquifolium</i>	cox3 0.063				0.063		0.063	0.063	0.094
	16S 0.049				0.049		0.049	0.049	0.033
	nad6 0.170				0.170		0.170	0.170	0.167
	conc. 0.123				0.123		0.123	0.123	0.123

(Continues)

TABLE 3 (Continued)

	<i>S. natans VIII</i>	<i>S. fluitans III</i>	<i>S. vachellianum</i>	<i>S. spinuligerum</i>	<i>S. polycystum</i>	<i>S. ilicifolium</i>	<i>S. aquifolium</i>	<i>S. thunbergii</i>	<i>S. muticum</i>
<i>S. thunbergii</i>									
cox3									0.039
16S									0.031
nad6									0.100
conc.									0.066

Numbers represent divergence among groups via aligned sequences generated in Geneious Prime. Includes divergence among concatenated sequences (conc.). The most common sequence for each morphotype of pelagic *Sargassum* was used to calculate inter-species divergence.

samples were represented in all regions, except the Gulf Stream. In contrast, *S. natans I* samples and resulting sequences were isolated to the Sargasso Sea and the Antilles Current regions.

Cox3 sequences were trimmed to 256 base pairs and exhibited two single-nucleotide polymorphisms; mt16S rRNA sequences were trimmed to 516 base pairs and also exhibited two single-nucleotide polymorphisms. Aligned sequence sets for *cox3* ($n = 96$) and mt16S rRNA ($n = 65$) yielded three haplotypes each; each haplotype corresponded to a separate pelagic *Sargassum* morphotype (Figure 2). This demonstrates that, for mt16S rRNA and *cox3*, genetic divergence was consistent with morphotype identity, with no genetic variation observed within morphotype groups. Sequences for *nad6* were trimmed to 317 base pairs with six variable positions within the alignment. Aligned sequences for *nad6* ($n = 67$) consisted of three common and 12 rare haplotypes (Figure 2). All three common haplotypes were comprised of individuals sharing a consistent *Sargassum* morphotype identification. Only one rare haplotype was exhibited by both *S. natans*-complex morphotypes, with all other rare haplotypes being consistent to *Sargassum* morphotype.

For each gene, the genetic distances between pelagic *Sargassum* morphotypes and from these morphotypes to benthic outgroups were calculated (Table 3). The differences among the pelagic *Sargassum* morphotypes, ranging between 0.002 and 0.009, were generally smaller than the divergence range between pelagic and benthic species (0.002–0.173), but the two benthic morphotypes in subgenus *Sargassum* sec. *Sargassum* (*S. vachellianum* and *S. spinuligerum*) were separated by the same order of divergence from each other as pelagic morphotypes were from each other (0.000–0.035; Table 3). Furthermore, the divergence between *S. natans I* and *S. natans VIII* (0.002–0.004) was on par with divergence exhibited between the *S. natans*-complex and *S. fluitans III* (0.002–0.009).

Concatenated *cox3*, *nad6*, and 16S sequences were used in a maximum likelihood phylogenetic tree analysis, and genetic divergence was calculated among pelagic morphotypes and seven benthic *Sargassum* species (Figure 3, Table 3). The three pelagic *Sargassum* morphotypes grouped into individual clades, phylogenetically distinct from one another. The two *S. natans* morphotypes formed a single clade, separate from *S. fluitans*. The *S. natans* clade was further broken down into genetically distinct and monophyletic sub-clades consistent with *S. natans I* and *S. natans VIII* morphology (Figure 3). For this concatenated dataset, average genetic distance between *S. fluitans III* ($n = 4$) and *S. natans I* ($n = 5$) was 0.007, 0.008 between *S. fluitans III* and *S. natans VIII* ($n = 4$), and 0.003 between *S. natans* morphotypes. Greater average genetic divergence was observed between *S. natans* and *S. fluitans* lineages at the concatenated *cox3*, *nad6*, and 16S genes (0.006) than reported in Camancho et al. (2015), who found a range of 0.001–0.002 divergence between *S. natans* and *S. fluitans* at concatenated ITS-2, *rbcLS*, and *cox3* genes; this is likely due to differences in the genes analyzed and the sample size between these studies. Genetic distance among distinct clades of *S. natans* morphotypes reported here (0.002–0.004) was on par with genetic

FIGURE 1 Distribution of pelagic *Sargassum* samples, collected from 2015 to 2019 during Sea Education Association cruises, as well as from the Gulf of Mexico and equatorial Atlantic expeditions

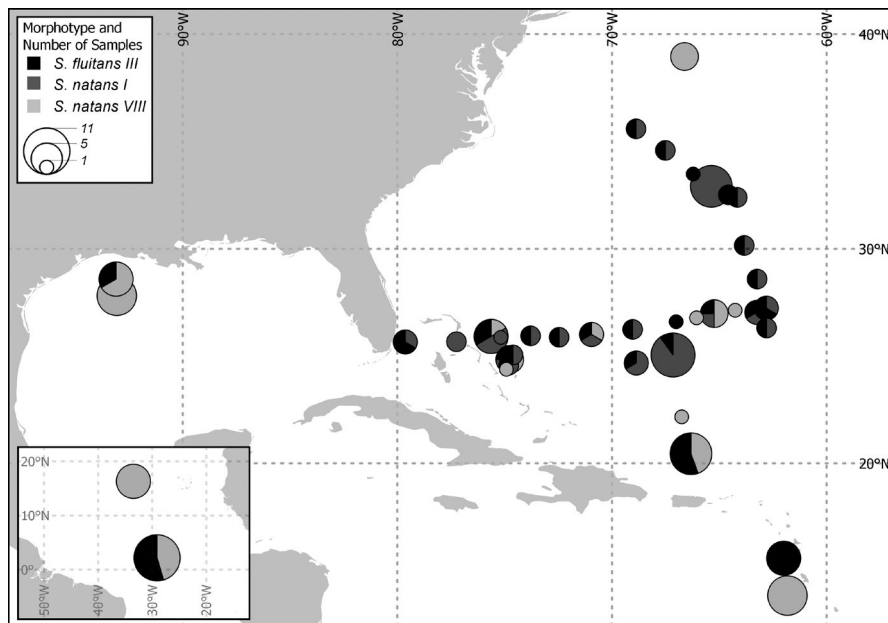
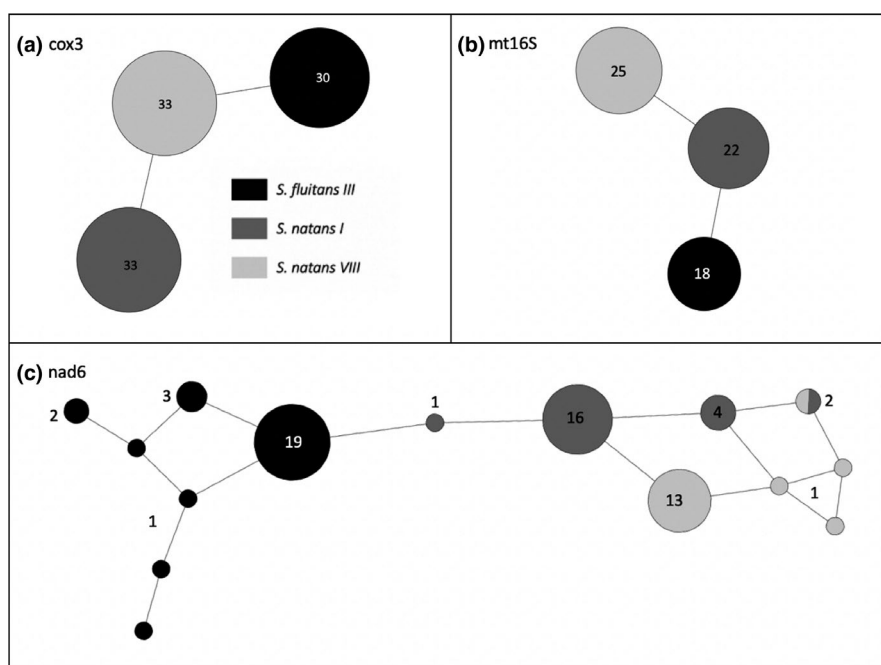


FIGURE 2 Haplotype networks for (a) *cox3* gene, (b) *mt16S* rRNA gene, and (c) *nad6* gene. Each node (circle) represents a distinct haplotype (nucleotide sequence) and is scaled to the number of samples with that particular sequence. The numbers within and adjacent to each node reflect sample size. Each line represents a single-nucleotide difference between nodes. Grayscale indicates the pelagic *Sargassum* morphotype from which the sequence was generated



distance reported among taxonomically distinct *Sargassum* species reported by Camacho et al. (2015) at concatenated ITS-2, *rbclS*, and *cox3* genes (0.008–0.021). Divergence among *S. natans* and *S. fluitans* lineages (0.003–0.008) was lower than divergence between more evolutionarily distant benthic forms (Table 3).

To explore whether geography was related to pelagic *Sargassum* genotype or phenotype, sample collection region was superimposed on the *cox3* haplotypes. The *cox3* gene was chosen because the dataset was most comprehensive with haplotypes subdivided according to *Sargassum* morphology. No trend was observed linking geographic region of sample collection to genotype or phenotype (Figure 4). For example, *S. natans I* was found in the Sargasso Sea and Antilles Current, whereas *S. fluitans III* and *S. natans VIII* were observed in every sampling region.

4 | DISCUSSION

Over the past decade, unprecedented annual inundations of pelagic *Sargassum* have occurred across the Caribbean and broader tropical Atlantic (Franks et al., 2016; Wang et al., 2019). This increase in pelagic *Sargassum* biomass, outside historical areas of accumulation in the Sargasso Sea and Gulf of Mexico, was marked by a biogeographic shift that included proliferation of the previously rare *S. natans VIII* morphotype (Schell et al., 2015). As new hypotheses are proposed to explain underlying processes causing the regional biomass increase, it is increasingly critical to ensure consistent and accurate identification of pelagic *Sargassum* morphotypes in biological and ecological studies. The results presented in this study demonstrate that pelagic *Sargassum* morphotypes are genetically distinct, perhaps to a degree

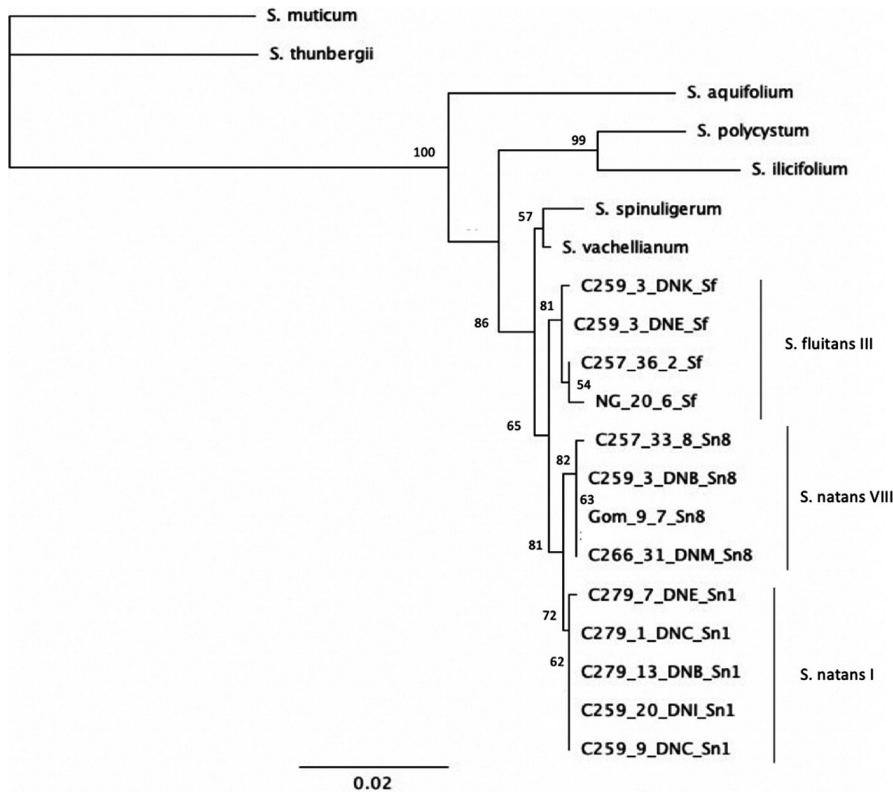


FIGURE 3 Maximum likelihood phylogenetic tree hypothesizing the relationship among concatenated (cox3+nad6+16S) pelagic *Sargassum* sequences (listed as Cruise_Station_Sample_Morphotype) and concatenated sequences of seven benthic western Pacific outgroups: *S. thunbergii*, *S. muticum*, *S. aquifolium*, *S. ilicifolium*, *S. polycystum*, *S. spinuligerum*, and *S. vachellianum*. Scale bar represents evolutionary difference, and numbers at the nodes represent bootstrap confidence values of 50 or greater

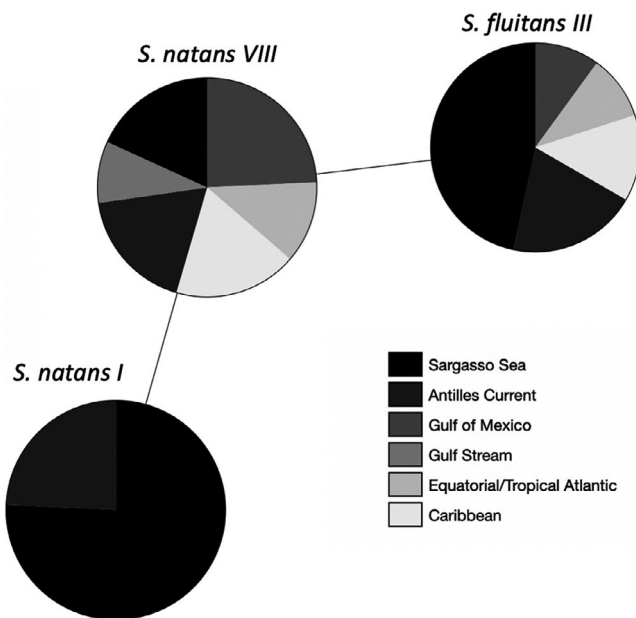


FIGURE 4 Geographic distribution of pelagic *Sargassum* cox3 haplotypes (same sample set as in Figure 2). Grayscale indicates the proportion of sequences from each oceanographic region for each haplotype

warranting updated taxonomic classification on the sub-species level. Although morphological identification of pelagic *Sargassum* morphotypes may be challenging in the field, the present research shows that reliable genetic identification is possible; here, low but consistent genetic differentiation among pelagic *Sargassum* morphotypes was observed in samples collected across the western North

Atlantic over the course of five years. The use of genetic identification of pelagic *Sargassum* morphotypes may help elucidate underlying evolutionary and ecological drivers of recent proliferation.

In this study, pelagic *Sargassum* morphotypes *S. natans I*, *S. natans VIII*, and *S. fluitans III* formed distinct taxonomic clades, and genetic divergence was on par with other species-level classifications within the *Sargassum* genus. The present results align with those reported by Amaral-Zettler et al. (2016) from an analysis of six entire mitochondrial genomes, representing the three common pelagic *Sargassum* morphotypes. For cox3, mt16S rRNA, and nad6 genes, a single polymorphic site in each gene differentiated between *S. natans I* and *S. natans VIII*; the resulting divergence between these morphotypes was comparable to species-level divergence between the *S. natans*-complex and *S. fluitans III*, as well as with that between *S. vachellianum* and *S. spinuligerum*, also within subgenus *Sargassum* section *Sargassum* (Table 3). Multiple marker phylogenetic methods were previously applied in the revision of regional subsets of benthic *Sargassum* species in the Pacific Ocean (Mattio & Payri, 2009; Mattio et al., 2008), Indian Ocean (Mattio et al., 2013), and Caribbean Sea (Camacho et al., 2015). These analyses used a common set of three markers, covering segments of the mitochondrial, plastidial, and nuclear genomes and adopted from earlier studies that were successful in resolving interspecific relationships among diverse brown algal groups. However, those markers appear suboptimal for differentiating among a large subset of species in subgenus *Sargassum* section *Sargassum*. For example, *S. hystrix* and *S. filipendula*, common Caribbean benthic species, were differentiated by a single polymorphic site in the ITS-2 region; no variable sites were found along

the targeted plastidial or mitochondrion gene segments (Camacho et al., 2015). Similarly, Phillips and Fredericq (2000) and Camacho et al. (2015) measured divergence between *S. natans* and *S. fluitans* in the plastidial *rbclS* locus, but there were no polymorphic sites in the ITS-2 or *cox3* segments targeted by Camacho et al. (2015) and Sissini et al. (2017). *Sargassum natans I* and *S. natans VIII* were identical across the *rbclS* region and only differed at one site in the ITS-2 region (Amaral-Zettler et al., 2016). Subsequent efforts to assess *Sargassum* diversity in the western North Atlantic to further understand population-level genetic relatedness among distinct pelagic *Sargassum* morphotypes may require the use of a higher number of independent genetic markers.

The three mitochondrial markers *cox3*, *nad6*, and *16S*, broadly tested here, were successful in resolving the three most common pelagic *Sargassum* morphotypes. *Sargassum* species are difficult to identify and often exhibit phenotypic plasticity (Mattio & Payri, 2011). Intraspecific morphological variation in macroalgae can be influenced by environmental factors; for example, a single Florida, USA population of *S. polyceratum* included 47 intermixed phenotypes based on blade morphology (Kilar & Hanisak, 1989). Blade length–width ratios in the same population further varied by seasonal growth stage (Kilar & Hanisak, 1989). For pelagic *Sargassum*, Parr, (1939) noted the many intergradations of *S. natans* and *S. fluitans* morphotypes both across and within species. Moreover, the description of *S. natans* was based primarily on absence of traits present on *S. fluitans* (Parr, 1939). As such, accurate identification of pelagic *Sargassum* morphotypes can be entirely dependent upon the quality of the sample. Upon initial review, haplotype results (Figure 2) for several samples conflicted with the morphological identification assigned upon collection. Careful re-examination of voucher specimens revealed that key morphological features had likely been overlooked when first examined. For example, the *S. fluitans III* samples had stipes with thorns, a defining physical characteristic for the morphotype. Accurate identification is essential for comparison of results across studies; integrated morphological and molecular methods provide a pathway to achieve this goal.

In all, there are six recognized pelagic *Sargassum* morphotypes (Parr, 1939). *Sargassum natans II*, *S. natans IX*, and *S. fluitans X* are rare but present in the field (unpublished data). As suggested by the *S. natans VIII* biomass surge in the Tropical Atlantic, each morphotype may require different nutrient concentrations and environmental conditions for optimal growth; however, such basic information is limited; existing studies on growth rates and nutrient composition do not consider all common morphotypes (Lapointe et al., 2014). Given continued oceanic transformation induced by climate change and land-use modifications, it is possible that pelagic *Sargassum* diversity could shift again and other rare morphotypes could proliferate in the future. Further testing is necessary to determine the suitability of these or other molecular markers for differentiation among all pelagic *Sargassum* morphotypes. The ability to accurately assess the relative proportions of morphotypes in the environment could provide insight into the multiple and competing factors contributing to the unprecedented Tropical Atlantic blooms. Changes to

these proportions have already been recorded over the last decade. Although early shipboard observations of Caribbean inundations found a dominance of *S. natans VIII* in late 2014 (Schell et al., 2015), recently, *S. fluitans III* was noted as most abundant during beaching events in 2018 (García-Sánchez et al., 2020).

This study was the first to examine molecular diversity among three distinct morphotypes of pelagic *Sargassum* species using extensive specimens collected across broad temporal and geographic range throughout the western North Atlantic. Despite the geographic co-existence of pelagic *Sargassum* morphotypes, their genetic differentiation appeared stable. The same pattern of stable, albeit low, genetic divergence has been reported in benthic *Sargassum* species as well; for example, *S. muticum* has low divergence across the entire genome yet exhibits strong invasive behavior (Le Cam et al., 2019). Genetic divergence among studied pelagic *Sargassum* morphotypes was lower compared with benthic species, suggesting more recent evolutionary divergence (Figure 3). Genetic clades aligned with *S. natans I*, *S. natans VIII*, and *S. fluitans III* morphologies despite all three morphotypes co-existing and being sampled from subtropical to temperate western North Atlantic waters across five years. For example, *Sargassum* identified morphologically as *S. natans VIII* was genetically identical, regardless of collection region. This suggests either sympatric speciation from other pelagic *Sargassum* morphotypes or, following the law of parsimony, allopatric speciation with dispersal from an inconspicuous source region. A combination of ecological and evolutionary factors, including environmental tolerances, life history, or dispersal pathways, may drive genetic differentiation among pelagic *Sargassum* morphotypes. Genetic divergence of *Sargassum* morphotypes may be based on differences in source regions and seed populations that are likely associated with different environmental conditions. We lack the data to identify the location of potential source regions of *Sargassum* morphotypes. However, future studies and publications related to *Sargassum* distribution will elucidate these dynamics. In particular, exploring the environmental drivers of *Sargassum* growth, reproduction, and senescence will shed light on the evolutionary mechanisms responsible for the divergence of unique *Sargassum* morphotypes. While we observe that known *Sargassum* morphotypes exhibit distinct and consistent genetic signatures, our sampling scheme is too limited to elucidate the potential environmental or evolutionary drivers of observed genetic divergence, and yet, our study offers a foundation for future work exploring the drivers of genetic divergence and distributional patterns of genetically distinct *Sargassum* morphotypes. Future investigation of gene flow among pelagic *Sargassum* morphotypes may also offer a clearer understanding of both the genetic mixing potential and whether these genetically and morphologically distinct lineages should be re-classified as separate species.

Future work to resolve pelagic *Sargassum* diversity and its connection to morphotype identity would benefit from high-resolution population genetic markers. For example, single-nucleotide polymorphisms (SNPs) across the genome could provide population-level perspective on the possibility of gene flow among pelagic *Sargassum* morphotypes (Schreiber et al., 2019). Previous benthic

studies undertaking *Sargassum* reclassification and taxonomic revision have accomplished this via whole-genome comparisons, utilizing mitochondrial and chloroplast genomes and extracting regions of interest (Liu et al., 2017). Utilizing the whole-genome sequencing for SNPs, without selective focus on highly conserved genes, could provide a broader understanding of genetic differences among morphotypes and has been proven a high-resolution taxonomic tool (Larraín et al., 2018). Moving forward, molecular and phylogenetic tools will guide our understanding of the evolutionary history of the pelagic *Sargassum* morphotypes and shed light on evolutionary and ecologically relevant patterns of gene flow. That said, full taxonomic reclassification may require both complementary analyses of morphology and higher-resolution molecular markers.

5 | CONCLUSIONS

The three common pelagic *Sargassum* morphotypes (*S. natans I*, *S. natans VIII*, and *S. fluitans III*) were genetically distinct. Low but consistent divergence was observed in all three mitochondrial genes examined, and coding regions were recognized as highly conserved. A large sample size lends confidence to these distinctions. Still unknown, however, remains the association of each morphotype with physiological characters, growth rates, or processes of senescence. Recognizing that the three common pelagic morphotypes are genetically distinct and likely source from different geographic regions (Govindarajan et al., 2019; Schell et al., 2015), it is integral that research focus shifts to factors that limit or enhance pelagic *Sargassum* growth. Controlled laboratory investigations on environmental conditions may offer insight into where, why, and how each individual morphotype grows and ultimately declines, and will perhaps explain why pelagic *Sargassum* distributions overlap but are not well-mixed across their range. Understanding the causes of recent large-scale pelagic blooms that are increasing in frequency and severity is imperative. The integration of complementary *in situ* and satellite work may allow a better picture of changing accumulation patterns between morphotypes. This molecular genetics work allows future studies to consider distinct morphotypes in their exploration of pelagic *Sargassum* ecology. As evaluation of *Sargassum*'s ecological services and possible environmental causes of tropical Atlantic blooms continues, each morphotype of pelagic *Sargassum* must be kept distinct. An understanding of this ecologically significant organism's deeper evolutionary history in tandem with recognition of genetic distinctions among morphotypes may allow for future taxonomic reclassification of *S. natans I* and *S. natans VIII* as distinct species or sub-species.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All new sequences are available in Genbank under accession numbers MT813198-MT813425.

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