Molecular phylogeny of pelagic Sargassum

Ethan Alley\textsuperscript{1,2}, Alesia Hunter\textsuperscript{1,3}, Walter Hutcheson\textsuperscript{1,4}, Kate Petersen\textsuperscript{1,5}, Katherine Running\textsuperscript{1,6}

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\textsuperscript{1-} Sea Education Association, Woods Hole, Massachusetts, \textsuperscript{2-} Harvard College, Cambridge, Massachusetts, \textsuperscript{3-} Beloit College, Beloit, Wisconsin, \textsuperscript{4-} New York University, New York, New York, \textsuperscript{5-} The Evergreen State College, Olympia, Washington, \textsuperscript{6-} American University, Washington, District of Columbia
Acknowledgements

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Abstract

Pelagic *Sargassum* specimens were collected and preserved at eight stations on four separate Sea Education Association (Woods Hole, Massachusetts, USA) research cruises undertaken between April 2015 and April 2016 in the tropical Atlantic Ocean, Caribbean, and Sargasso Sea. Collected specimens included *Sargassum natans I, S. natans VIII, S. fluitans III,* and two morphologically distinct samples. *Sargassum* sp. Deoxyribonucleic acid (DNA) was extracted from the specimens and the mtsp and rbcL-S genetic marker regions were amplified and sequenced. *S. fluitans III* and *S. natans (I and VIII)* are shown to be 1.5% genetically distant at the concatenated sequence of these loci. Mtsp had 10 single nucleotide polymorphisms (SNPs) (2.4%) and rbcL-S had 2 SNPs and a 9 basepair insertion (1.6%). *S. fluitans III* and *S. natans (I and VIII)* showed consistent allelic differences at both loci. No variability was found between *S. natans I* and *S. natans VIII.* *Sargassum* sp. was found to be molecularly distinguished from both *S. fluitans III* and *S. natans (I and VIII)* at the mtsp locus. The phylogeny showed that *S. natans I* and *S. natans VIII* are more closely related to each other than they are to *S. fluitans III,* with *Sargassum* sp. as an intermediate taxa. Our results do not support species delineation between *S. natans I* and *S. natans VIII.* These results support but do not confirm *S. natans* and *S. fluitans* species delineations. The analysis of *Sargassum* sp. suggests the possibility of cryptic diversity in pelagic *Sargassum.*
Introduction

As a foundation taxon, *Sargassum* provides otherwise rare deep ocean surface substrate to complex localized communities of epibiont and motile fauna (Stoner and Greening 1984; Casazza and Ross 2008). Over 200 marine species associate with pelagic *Sargassum* including endemic species such as the *Sargassum* frogfish (*Histrio histrio*), *Sargassum* crab (*Planes* spp.), and Slender *Sargassum* shrimp (*Latreutes fucorum*), which exhibit color matching and plant mimicry adaptive strategies (Coston-Clements 1991; Hacker and Madin 1991; Trott et al. 2011). These pelagic communities provide food, shelter and nursery grounds for a variety of threatened and economically important taxa such as sea turtles (Carr and Meylan 1980; Carr 1987; Luschi et al. 2003), tuna (Coston-Clements 1991), and dolphinfish (Oxenford and Hunte 1999).

Due to its primary and significant ecosystem functions and vulnerability to damage from pollution (Powers et al. 2013) and commercial extraction (Laffoley et al. 2011), pelagic *Sargassum* is of conservation concern (South Atlantic Fishery Management Council 2002; United Nations Environment Programme 2012; Sargasso Sea Commission 2014). Further, a small study suggested that the species composition of *Sargassum* associated communities may have changed over the last 40 years (Huffard 2014), with unknown ramifications for pelagic ecology. The authors pointed out that ocean temperature and acidity increased concurrently and suggested more research to investigate this correlation. Additional research is required to verify *Sargassum* associated community changes.

Øjvind Winge (1923) and Albert Parr (1939) developed the taxonomic classification system currently used to describe pelagic *Sargassum* (Genus *Sargassum*, subgenus *Sargassum*, section *Sargassum*), which is based on morphological characters such as the presence or absence of stem thorns and the size and shape of bladders and leaf blades. Parr (1939) described two
species, *Sargassum natans* and *Sargassum fluitans*, which have 6 morphological forms: *S. natans I*, *S. natans II*, *S. natans VIII*, *S. natans IX*, *S. fluitans III*, and *S. fluitans X*. These form variations are not commonly addressed in modern field guides (Schneider and Searles 1991; Littler and Littler 2000; Dawes and Mathieson 2008) or scientific literature (Stoner and Greening 1984; Calder 1995) which tend to only refer to *S. natans* or *S. fluitans* (most likely referring to *S. natans I* and *S. fluitans III*). This is probably due to the seemingly ubiquitous nature of *S. natans I* and *S. fluitans III* throughout their range (A. N. S. Siuda pers. comm.).

Distinguishing between *Sargassum* forms in scientific research is necessary because *Sargassum* species and form identity appears to be a driver of species abundance and diversity in *Sargassum*-associated pelagic ecosystems. For example, a study of *S. natans* and *S. fluitans* found that these species support distinct hydroid species assemblages (Calder 1995). In another study, *Litiopa melanostoma*, a common snail, was found to be more abundant on *S. natans* than on *S. fluitans* (Stoner and Greening 1984). No form distinctions were made in either study. Winge (1923) also observed prolific hydroid colonization on *S. fluitans III* and very limited colonization on *S. natans II*. The only ecological study addressing *S. natans I*, *S. natans VIII*, and *S. fluitans III* found species diversity differences between the three forms, with *S. natans VIII* having the lowest diversity (L. M. Martin unpubl.).

Unprecedented and economically disruptive *Sargassum* beach inundation events were reported in 2011, 2012, 2014, and 2015 in the western Caribbean and Gulf of Mexico (Higgins 2011; MercoPress 2015). Some news outlets suggested that the inundations came from the Sargasso Sea (Boodram 2015; Miller 2015), an area of the north Atlantic Ocean named for its *Sargassum* population. However satellite data and backtracking analysis suggested a previously unknown equatorial Atlantic source (Johnson et al. 2012; Gower et al. 2013). Both *S. natans* and
S. fluitans (without form descriptions) were implicated in the inundations by various sources (Johnson et al. 2012; Economist 2015; Fardin et al. 2015). However, verified morphological identifications and voucher specimen collections were sparse and not always accurate. For instance, Szechy et al. (2012) reported an equatorial Atlantic S. natans bloom in 2011, but voucher photographs clearly show S. fluitans specimens, which can be conclusively identified by the presence of thorns on the stem (Parr 1939). Further complicating the investigation of the beach inundations was the identification of huge quantities of floating S. natans VIII in 2014 (Schell et al. 2015), a form that had not been treated in the literature since Parr (1939). Schell et al. (2015) subsequently documented S. natans VIII throughout the tropical Atlantic Ocean, Caribbean, Antilles Current, and southern Sargasso Sea, a phenomena not previously observed during their 20 years of shipboard research.

The confusion surrounding the Sargassum beach inundation events underlines the need to clarify ambiguities in species and form definitions, which complicate Sargassum research at a time when form distribution and abundance may be changing and original baseline data is limited. The lack of widespread treatment of the different morphological forms in the literature challenges the development of functional ecological narratives regarding pelagic Sargassum. The degree to which existing ecological data for pelagic Sargassum could be confounded by incorrect or incomplete species or form identification is unknown.

As a complex and morphologically plastic taxa (Kilar 1992), molecular techniques are necessary to corroborate and refine morphological species and form delineations in Sargassum. Genetic markers used previously to delineate taxa in Sargassum and other brown algae groups include the chloroplastic Ribulose bisphosphate carboxylase large chain + spacer + partial Ribulose bisphosphate carboxylase small chain (rbcL-S) (N. E. Phillips unpubl.; Phillips et al.
2005; Dixon et al. 2014), the nuclear Internal Transcribed Spacer 2 (ITS-2) (Stiger et al. 2000, 2003; Mattio et al. 2010), and the mitochondrial cytochrome oxidase 3 subunit (cox3) (Mattio et al. 2008, 2009; Dixon et al. 2014). In these studies, the rbcL-S, ITS-2, and cox3 markers were successfully used to obtain consistent phylogenetic resolution at the subsections level or higher, but resulted in shallow unresolved trees below the subsection level.

An additional marker, the mitochondrial intergenic spacer region (mtsp) between the mitochondrial 23S gene and transcription ribonucleic acid Valine (tRNA-Val), was proposed for use within Sargassum and other Sargassaceae by Draisma et al. (2010) after development for use in the Fucus genus by Coyer et al. (2006). This non-coding region was shown to be the most variable of those studied, but could not be aligned for a multi-genus data set (Draisma et al. 2010). A subsequent marker assessment analysis of ITS-2, the chloroplast partial RubisCO operon (rbcL-S), cox3, mtsp and the mitochondrial cytochrome oxidase unit 1 gene (Co1) by Mattio and Payri (2010) identified mtsp as a potentially useful barcoding marker, but also found difficulties in alignment.

Although analyses of these loci have produced important taxonomic insights across the genus in benthic species, molecular phylogeny of pelagic Sargassum is highly understudied. A 2015 study of S. fluitans III, S. natans I, and S. natans VIII at the CO1 marker found a single nucleotide difference between S. fluitans III and S. natans (I and VIII), but no variation between S. natans I and S. natans VIII at this locus (A.N.S. Suida pers.comm.). Camacho et al. (2015) included two samples identified as S. natans and two identified as S. fluitans in a molecular phylogeny of Sargassum subgenus Sargassum from the Caribbean and western tropical Atlantic using ITS-2, rbcL-S, and cox3. Form distinctions were not made in this study. As a whole, the phylogeny constructed from these markers did not have sufficient resolution within Sargassum.
sect. *Sargassum*. When the pelagic species’ sequences were retrieved from GenBank and examined, only rbcL-S appeared to be variable between *S. natans* and *S. fluitans*. Camacho et al. (2015) also sequenced mtsp but did not include these sequences in the phylogeny due to alignment difficulty. However, mtsp was one of the few markers to show any variability within *Sargassum* sect. *Sargassum* (O. Camacho pers.comm.).

In this study, we amplify and sequence rbcL-S and mtsp loci of pelagic *Sargassum* which represent the predominant morphological forms collected in the Sargasso Sea, western tropical Atlantic and Caribbean, *S. natans I*, *S. natans VIII*, and *S. fluitans III*, as well as two specimens of *Sargassum* sp. (provisionally identified as *S. natans II*) collected in the south Sargasso Sea. We then construct a molecular phylogeny based on these markers.

**Methods**

Pelagic *Sargassum* specimens were collected and preserved at eight stations on four separate Sea Education Association (Woods Hole, Massachusetts, USA) research cruises (C259, C263, C264, and C266) undertaken between April 2015 and April 2016 in the tropical Atlantic Ocean, Caribbean, and Sargasso Sea (Table 1). Sampling from a broad geographic area was necessary due to the minimally overlapping ranges of pelagic *Sargassum* forms (Schell et al. 2015). Every 12 hours along these cruise tracks, at 0000 h and 1200 h, a one-meter wide, half-meter high Neuston net with 333-micrometer mesh was deployed. The net was towed at a speed of two to three knots for 30 minutes, for a total approximate sampling distance of one nautical mile. Concurrent sampling using a dip net with 333-micrometer mesh occurred at morning stations. *Sargassum* was preserved in silica for molecular analysis when at least seven individuals of a particular morphological form were collected at a single station. All specimens
were identified using morphological characters described by Parr (1939). Morphological vouchers for all samples were collected and are kept at Sea Education Association.

A 5-10 cm sample was clipped from the apical end of each Sargassum replicate and persistent epiphytes were removed with a razor blade. The samples were then rinsed in freshwater and dried in silica desiccant for at least 48 hours before extraction. Two to four leaf blades or an approximately equivalent volume of floats and stems from the dried samples were ground in 1.5 ml microcentrifuge tubes using disposable pellet pestles.

DNA was extracted using MoBio Power Plant Pro Kit (Carlsbad, California, USA) according to manufacturer protocols as modified by Wilson et al. (2016) to replace the bead beating step with 24hrs of heating at 65°C with periodic vortexing. Extractions were cleaned using MoBio Power Clean Pro Kit according to manufacturer protocols. The clean genomic DNA was profiled using a Nanodrop ND-1000 Spectrophotometer.

A mitochondrial spacer (mtsp), and the chloroplast-encoded partial Ribulose bisphosphate carboxylase large chain + spacer + partial Ribulose bisphosphate carboxylase small chain (rbcL-S) regions were amplified using Polymerase Chain Reaction (PCR). PCR products were amplified using site-specific primers (Table 2) and reaction protocols were optimized from those developed by Coyer et al. (2006) for mtsp and Mattio et al. (2008) for rbcL-S. The cycling conditions included: (i) a 4 minute initial denaturation at 95°C, (ii) 40 cycles of a 40 second denaturation at 95°C, (iii) a 30 second annealing at 50°C (mtsp), 45°C (rbcL-S), (iv) a 45 second extension at 68°C, and (v) a 7 minute final extension at 68°C. New England Biolabs HotTaq 2x Master Mix with Standard Buffer (Ipswich, Massachusetts, USA) was used for all reactions.

PCR products were verified with agarose gel electrophoresis. PCR products were purified with Qiagen Qiaquick PCR Cleanup Kit (Germantown, Maryland, USA). The DNA
concentration of purified PCR products was quantified with a Nanodrop ND-1000 Spectrophotometer. Purified PCR products were sequenced by Eurofins MWG Operon (Huntsville, Alabama, USA) in both directions with primers from the amplification step (Table 2) using the Sanger sequencing method. In total, this analysis included 13 replicates of *Sargassum natans VIII*, 15 replicates of *Sargassum natans I*, 14 replicates of *Sargassum fluitans III*, and two replicates of *Sargassum* sp., which were morphologically distinct specimens suggestive of *Sargassum natans II*.

Results

This study produced 65 sequences for mtsp and 49 for rbcL-S which are available on Genbank (Table 3). Sequences were aligned with ClustalW (Larkin et al. 2007) in Geneious 9.1.7 software (Biomatters Ltd.) to an appropriate outgroup obtained from Genbank via the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (NCBI BLAST). The mtsp alignment was 195 base pairs (bp) long including a two and three bp gap introduced by the outgroup comparison. This is significantly shorter than previously reported (Coyer et al. 2006, Draisma et al. 2010). The rbcL-S alignment was longer, at 687 bp, including a 9 bp gap. Both alignments were examined and it was determined that all sequences from *Sargassum natans I* and *Sargassum natans VIII* were identical to one another at both the mtsp and rbcL-S loci. All *S. fluitans III* sequences were also identical to one another at each loci. Lastly, the sequences obtained from *Sargassum* sp. were found to be identical to one another at both loci.

We discovered 10 polymorphic loci (5.2%) in mtsp at which *S. fluitans III* and *S. natans I* and *VIII* showed a consistent allelic pattern, which corresponded exactly to the species delineation (*S. natans* or *S. fluitans*). We found 2 polymorphic loci and a 9 bp indel in the rbcL-S
sequences, which also corresponded to the *S. natans*/*S. fluitans* delineation and did not vary between the morphological forms of *S. natans* (0.2%).

Sequences from samples with both rbcL-S and mtsp represented in the alignments were concatenated for *S. natans* I (n=15), *S. natans* VIII (n=13), *S. fluitans* III (n=14), and *Sargassum* sp. (n=2) resulting in a combined alignment of 882 bp. Identical sequences for each form were removed. Gaps in both sequences were treated according to the simple indel coding method of Simmons and Ochoterena (2000) to inform the phylogeny.

A consensus Neighbor Joining Tree was constructed from the concatenated, gap-coded sequences in Geneious 9.1.7 software (Biomatters Ltd.) using the Tamura-Nei genetic distance model with 10,000 bootstrap replications and a support threshold of 95% (Figure 1). The concatenated consensus tree showed genetic distance of 1.5% between *S. natans* (I and VIII) and *S. fluitans* III, 0.07% between *S. natans* (I and VIII) and *Sargassum* sp., and 0.08% between *Sargassum* sp. and *S. fluitans* III. Genetic distances were also calculated for each locus individually (not concatenated) using the same parameters above. This showed rbcL-S was less variable, with 0.4% genetic distance between *S. natans* (I and VIII) and *S. fluitans* III, 0.4% genetic distance between *Sargassum* sp. and *S. fluitans* III, and no distance between *S. natans* (I and VIII) and *Sargassum* sp. Contrarily, mtsp alone produced genetic distances of 5.5% between *S. natans* (I and VIII) and *S. fluitans* III, 5.6% between *S. natans* (I and VIII) and *Sargassum* sp., and 2.2% between *Sargassum* sp. and *S. fluitans* III.

Discussion

We found genetic distance between *S. fluitans* III and *S. natans* (I and VIII) at both the mtsp (5.5%) and rbcL-S (0.4%) loci. This finding corroborates the morphologically based species delineations proposed by Parr (1939) as well as the 1 SNP difference observed in Co1 between
these taxa (A.N.S. Siuda pers. comm.). Our observed variation does not meet the criteria for species delineations proposed by Mattio and Payri (2010) who conducted the only study of the effectiveness of various genetic markers as barcodes for *Sargassum* subgenus *Sargassum*. This study included the section *Sargassum*, to which pelagic *Sargassum* belongs, along with a number of additional sections, but did not include any samples from pelagic *Sargassum*. The authors reported a range of interspecific genetic distances for mtsp (16-19.5%) and rbcL-S (1-6%) (Mattio and Payri 2010), much higher than the genetic distances found between our *S. natans* group and *S. fluitans III* at these loci.

Difficulty resolving closely related taxa at or below the section level within the *Sargassum* genus is not uncommon (Phillips 2005; Mattio et al. 2010; Dixon et al. 2014). N. E. Phillips (unpubl.) argues that there is unlikely to be a universal pattern of genetic variation for spacer regions in macroalgae, because these regions demonstrate distinct patterns of variation depending on the studied taxa. It may be that the barcode assessment of Mattio and Payri (2010) was too broad to definitively represent *Sargassum* sect. *Sargassum* and that this section has patterns of genetic variation distinct from other sections in subgenus *Sargassum*. This hypothesis is corroborated by the observation that excluding *Sargassum* sect. *Sargassum* from barcode analysis changed the identification success of all markers especially rbcL-S (Mattio and Payri 2010) and the evidence that a molecular phylogeny with the best known markers was unable to resolve between species within *Sargassum* section *Sargassum* (Camacho et al. 2015). The section has been noted as a primary target for barcode development efforts because “...no good
barcoding marker will be identified for the genus *Sargassum* before one can genetically
discriminate between the very closely related and morphologically distinct species of *S. section Sargassum*” (Mattio and Payri 2010).

We found *S. natans I* and *S. natans VIII* to be genetically identical at the mtsp and rbcL-S loci despite the fact that both morphological forms possess distinct morphological characters (Parr 1939; Schell et al. 2015) and associated ecological communities (L.M. Martin unpubl.). This data corroborates the previously mentioned Co1 marker study which also found no difference between these forms (A.N.S. Siuda pers. comm.) However, our results clearly show that *S. natans I* and *S. natans VIII*, traditionally considered to be morphological variants of the same species, are more closely related to each other than they are to either *S. fluitans III* or *Sargassum* sp. This highlights important areas of future research, both for development of new genetic markers to resolve evolutionary relationships in pelagic *Sargassum* and to investigate how distinct phenotypes and ecological characteristics can arise from yet undiscovered genetic variation.

We found that *Sargassum* sp. was .07% distant from *S. natans (I and VIII)* and .08% distant from *S. fluitans III*, suggesting the possibility of cryptic diversity in pelagic *Sargassum*. These samples had subtle, but noticeable morphological differences which resembled *S. natans II* (Parr 1939). Unfortunately, we were unable to complete our identification before the majority of our specimens were discarded. More specimens are required to determine whether this form was described by Parr (1939) and that the observed grouping is not anomalous. This finding
illustrates the importance of further integrative taxonomy research on the less common morphological forms of pelagic *Sargassum*, which could clarify evolutionary relationships and may also contribute to the understanding of inter and intraspecific genetic diversity in *Sargassum* sect. *Sargassum*.

Further research is also necessary to establish new genetic markers within *Sargassum* sect. *Sargassum* to facilitate integrative taxonomy work, with barcoding assessments on large representative samples from within the section. Lastly, research into life history and reproduction patterns of pelagic *Sargassum* could clarify morphological changes that occur during an individual’s life cycle.
References

Boodram, K. 2015. Tobago’s western coast escapes seaweed. Trinidad Express Newspaper, August 11.


Table 1: Station summary information for samples of each morphological form.

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<tr>
<th>Date</th>
<th>Position</th>
<th>Region</th>
<th># Samples</th>
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<td><strong>Sargassum natans VIII</strong></td>
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<td></td>
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<td>26 Nov 2015</td>
<td>16°20’N x 33°20’W</td>
<td>E. Tropical Atlantic</td>
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<tr>
<td>7 Dec 2015</td>
<td>14°54’N x 53°04’W</td>
<td>W. Tropical Atlantic</td>
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<td></td>
<td></td>
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<td>4</td>
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<tr>
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<td>17°56’N x 65°06’W</td>
<td>E. Caribbean</td>
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<td><strong>Sargassum natans I</strong></td>
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Table 2: Primers used to isolate the selected markers from genomic DNA. AT = PCR annealing temperature (°C).

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<td>Mattio et al. 2008</td>
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<td>S97R</td>
<td>5′-CATCTGTCCATTCWACACTAAC-3′</td>
<td>Mattio et al. 2008</td>
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Table 3: GenBank accession numbers for sequenced samples

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<td>rbcL-S</td>
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<td><em>Sargassum species</em></td>
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Figure 1: Consensus Neighbor Joining Tree of the concatenated rbcL-S and mtsp loci. Bootstrap support is indicated at nodes, with those less than 95% supported collapsed. Number of replicates of each sequence shown in parentheses. Branch length is unconstrained and corresponds to genetic distance at a scale of 0.002. Outgroup sequences obtained from GenBank using NCBI Blast.