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Analysis of Freshwater and Marine *Ulva flexuosa* in Southern California

Abstract

Mareš et al. (2011) proposed a novel approach to identifying three different subspecies within European waters for *Ulva flexuosa* (subspecies *pilifera, paradoxa,* and *flexuosa*). Their use of morphological and molecular techniques to separate each subspecies was used as a guide for this study to determine if these techniques would work for *U. flexuosa* within California waters. Eleven freshwater, five brackish water, and two seawater samples of *Ulva* were collected and were assessed using classical morphological methods and molecular techniques. The molecular methods examined the nuclear DNA region ITS1-5.8S-ITS2 (ITS) and the chloroplast RUBISCO large subunit (*rbc*L) genes. All the samples collected except one were morphologically determined to be within the species criteria for *Ulva flexuosa*, however, none of the samples could be separated down to the subspecies level based on morphology. Molecular analysis suggested that all of the freshwater samples were indeed *U.* flexuosa but only half of those could be aligned with the subspecies *paradoxa.* The other half did not align themselves with any recognized subspecies and none were aligned with the subspecies *pilifera*. Samples that were collected in the brackish or seawater habitats were not confirmed as *U. flexuosa* by molecular analysis but instead two different species, *Ulva torta* and *Ulva linza.*

The findings of this study suggest that *Ulva flexuosa* could only be found within freshwater sources in Southern California. Previous records of U. flexuosa in the region are most likely due to the morphological plasticity of the genus *Ulva* causing mistakes in the historical record, as has been seen in Europe. Future studies are needed to either confirm or correct the record in California using a combination of molecular and more classical morphological techniques.

Introduction

The genus *Ulva* was first described by Linnaeus (1753), but was later split in two genera by Heninrich Friedrich Link in 1820. The first genus, *Ulva,* consists of bright green seaweed with distromatic (two cell thick) blades and the second, *Enteromorpha* Link, is a monostromatic tubular form. The separation of these two genera based solely on morphology was not perfect because some species can display both tubular and blade-like forms, as is the case with *U. linza* (Abbot and Hollenberg 1992), *U. flexuosa* (Bliding 1963), *and U. lactuca* (Bonneau 1977). Hayden et al. (2003) used molecular analysis of two different sections of DNA, the first was the nuclear ribosomal internal transcribed spacer DNA (ITS) and the second was the chloroplast RUBISCO large subunit gene (*rbc*L), to demonstrate that *Enteromorpha* and *Ulva* should be reunited back into the same genus as Linnaeus first described, *Ulva* (Hayden et al. 2003).

Ulva is one of the most common benthic macroalgae genera in the world, with the genus occupying habitats from marine to freshwater and tolerating a wide range of salinity, temperature, and water quality (Poole and Raven, 1997). *Ulva* sp. are typically among the first weedy macroalgae to colonize open substrata and are considered to be troublesome fouling organisms (Beach et al. 1995) that can be indicators of poor (eutrophic) water quality conditions (Wallentiuns 1979). For example, *Ulva sp.* are predominately distributed in marine areas where

freshwater runoff occurs (Prakash and Jokhan 2012) and in coastal zones affected by pollution from municipal or industrial discharge (Littler and Littler 1984).

Ulva can also occur as "green tides", defined as the accumulation of unattached green algae in most polluted and eutrophic marine environments (Hernández et al. 1997, Back et al. 2000, Blomster et al. 2002, Nelson et al. , Ye et al. 2011). This phenomenon attracted the attention of the news media during the 2008 summer Olympic games in China and threatened to interfere with the sailing events (Leliaert et al. 2009, Pang et al. 2010). *Ulva* can also be considered an invasive alga colonizing habitats where disturbance is present or conditions of water quality change from agriculture runoff, wastewater, or salt runoff from roads (Lougheed and Jan Stevenson 2004). An example of *Ulva's* significant impact and invasion capabilities is in Lake Michigan in 2013 that turned green due to an *Ulva* bloom. The main component of the bloom was *U. flexuosa*, which covered the shores (Lougheed and Jan Stevenson 2004).

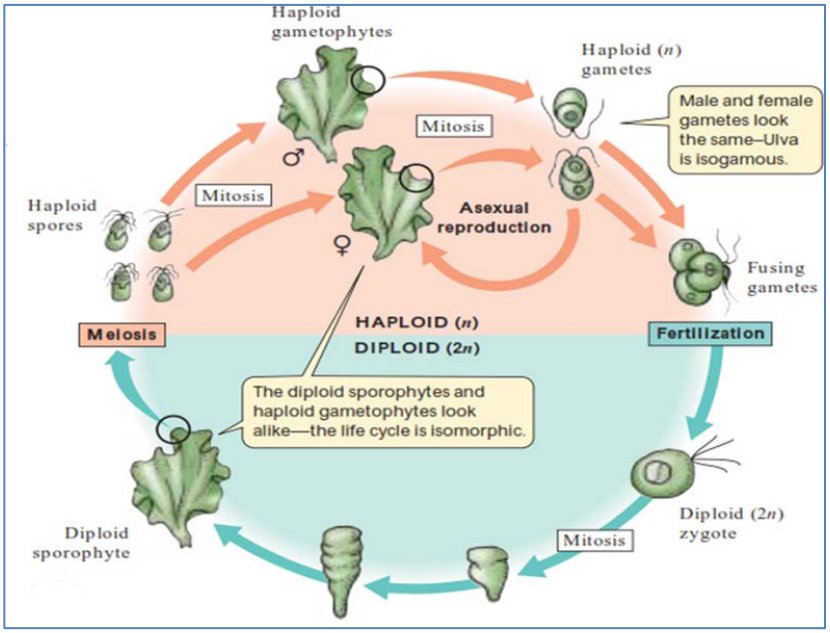

Figure 1: The life cycle of *Ulva f*lexuosa is an isomorphic alternation of generations (Plant Science 4 U 2016).

Ulva flexusoa (Chlorophyta, Ulvophyceae) thalli can be unbranched or branched, and in rare occasions have secondary branching. They can grow to 30 cm in length, but are generally smaller in size. It grows at the water body surface but can be found at depths up to approximately 5 meters (Guiry and Guiry 2016). *U. flexuosa* propagates by vegetative reproduction, using either fragmentation, growth of new upright thalli from basal cells and/or from persistent holdfasts (Beach et al. 1995). Its life history (Figure 1) is an alternation of isomorphic, unisexual haploid gametophytes and diploid sporophytes and are morphologically indistinguishable. Meiosis occurs during sporogenesis, where most cells are capable of becoming reproductive with the exception of rhizoidal cells and some basal cells. Asexual reproduction is most common, typically by quadriflagellated zoospores (swarmer) which are highly mobile and live longer due to the swarmer's ability to photosynthesize (Beach et al. 1995). Sexual reproduction is uncommon but can happen by biflagellated anisogamous or rarely isogamous gametes (Guiry and Guiry 2016).

Mareš et al. (2011) looked at morphologically similar samples of *Ulva flexuosa* from inland and coastal water throughout Europe to assess their taxonomic identity (Table 1). In addition, they used molecular techniques in combination with morphological observations to verify differences at the subspecies level for *U. flexuosa*. The subspecies of *U.* flexuosa that they describe are *U. flexuosa* subspecies *flexuosa*, *U. flexuosa* subspecies *pilifera*, and *U. flexuosa* subspecies *paradoxa* (Bliding 1963). These subspecies occupy different habitats according to this study; *pilifera* is dominant in freshwater, *flexuosa* is in marine environments, and *paradoxa* is in both marine and freshwater habitats. Their findings also suggested that the historical collection records have misidentified *U. flexuosa* as *U. intestinalis* primarily from freshwater sources, indicating those samples are most likely *U. flexuosa* subsp. *pilifera*. No other studies have been performed to determine the habitat associations of *U. flexuosa* subspecies, either in Europe or in other areas that *U. flexuosa* occurs.

Table 1: Morphological and anatomical characters useful to identify *Ulva flexuosa* subspecies from European water (Mareš et al. 2011)

	U. flexuosa* subsp. *pilifera	***U. flexuosa* subsp. *paradoxa***	***U. flexuosa* subsp.*flexuosa***
Habitat	Enriched freshwaters (ponds, pools, small streams)	Sea coast, minerally rich inland waters	Sea coast
Mode of life	Attached or free floating, occasionally in masses	Attached or free floating	Attached
General morphology	Tube-like or leaf-like	Tube-like	Tube-like
Branching	Abundant to almost absent	Frequent	Sparse
Uniseriate branches	Frequent to rare, obtusely rounded ends	Present, attenuated ends	Rare, attenuated ends
Cell shape	Rounded polygonal to quadrangular	Rectangular, quadrangular to polygonal	Rectangular, quadrangular
Cell size [µm]	7–18 (–25)	10–22 × 7–18	18–30 × 12–20
Cell arrangement	Disordered, in small groups or short rows	In long rows, sometimes less regular	In long rows
Number of pyrenoids	2–4	1–3	1–2

Mareš et al. (2011) results show the mistakes that can be made in identifying *Ulva* species. *Ulva* has proven difficult to identify morphologically due to its extensive variability of morphological traits (Brodie et al. 2007). A number of papers have also noted phenotypic plasticity of *Ulva spp*. due to salinity variations (Koeman and Van den Hoek 1984, Young et al. 1987) and/or seasonal changes (Hull 1987). Some *Ulva* species may even switch between foliose and tubular forms during their life cycle (Tan et al. 1999, Rybak et al. 2011, Rybak et al. 2014). These factors create further difficulty in identifying *Ulva* species and subspecies morphologically.

Understanding the problems associated in identifying species with the genus *Ulva*, studies have used molecular analysis to help clarify the taxonomy of this group. The most common molecular markers are the ITS region of the rRNA cstron (ITS1-5.8sITS2) and chloroplast RUBISCO large subunit (rbcL) genes (Hayden 2003, Rybak 2014). These two neutral markers have been used successfully to determine the species of algae down to the subspecies level as shown with *U. flexuosa* (Mareš et al. 2011).

U. flexuosa is considered common within California inland and coastal water (Abbott and Hollenberg 1992, Stancheva et al. 2016, Setchell and Gardner 1919). This species within

California has never been molecularly or morphologically examined. The objective of this paper is to 1) better understand if the same subspecies of *U. flexuosa* described in Europe are present in California, based on morphology and molecular markers (ITS and *rbc*L), and 2) whether those subspecies occupy similar habitats as described in Europe.

Materials and Methods

Samples and localities

This study includes 18 samples of *U. flexuosa* collected around California by either the author or Surface Water Ambient Monitoring Program (SWAMP), this program is apart of the California Water Board, which is tasked with assessing the water quality in all of California's surface waters. Seventeen samples were collected in the Southern California region (4 from SWAMP) with only one sample coming from north of San Francisco, California (SWAMP). Thirteen of the samples were collected from freshwater sources, 5 were collected from brackish habitats, and

Figure 2: Southern California Collection Locations. Freshwater locations are in green, brackish water locations are in orange and marine locations are in blue. All locations were determined to have *U. flexuosa* based on morphological analysis.

2 were collected in Marine habitats (Figure 2). Additionally, 28 other locations (15 marine, 8 brackish and 5 freshwater) were examined for *Ulva flexuosa* in Southern California, but were not found in those areas even though *U. flexuosa was* historically present in those areas (Setchell and Gardner 1919, Abbott and Hollenberg 1992, Stancheva et al. 2014). If Samples were collected

in these areas they were identified as *Ulva intestinalis*, a alga with a similar macorscopic appearance but a different dramatically when viewed under microscope, with cup shaped chloroplasts.

Each sampling location was recorded via Garmin Differential GPS and water quality parameters were measured with a Hydrolab Quanta water quality meter recording pH, temperature, and conductivity (which measures salinity, Table 2). In addition, field notes and pictures were taken at each site. Samples obtained from SWAMP only included water quality and location data, pictures and field notes were not provided.

Morphology and anatomy of the samples were assessed as soon as possible for the field collection (within 2 days of collection or sooner) and for the SWAMP specimens the samples were process within 10 day of collection. The SWAMP specimens showed some signs of degradation making it difficult to collect some of the thallus morphology, but cellular information could still be recorded. Specimens were photographed fresh, if possible, and pictures were taken microscopically for all specimens to show cell arrangement, cell size, and branching patterns. Part of the material from a single individual algal sample was cleaned and preserved in silica gel for molecular analysis. A representative piece of material from each specimen was also fixed in 2.5% histological grade glutaraldehyde as a historical collection record.

Table 2: Water quality parameters collected. *The Bouton locations were collected during low tide and appear to be in freshwater based on conductivity, however this is a tidally influenced channel and should be considered a brackish environment as noted in the table.

Location ID	pH	Conductivity (mS/cm)	Temperature (°C)	Habitat
Bouton1*	7.94	1.02	16.50	Brackish
Bouton2*	7.94	1.02	16.50	Brackish
Doheny	9.69	7.82	24.90	Brackish
Bouton3*	7.94	1.02	16.50	Brackish
La Costa	7.47	28.35	23.60	Brackish
Garcia (SWAMP)	7.89	0.20	19.66	Fresh
403 (SWAMP)	7.76	1.30	20.23	Fresh
SMC-36 (SWAMP)	7.87	2.05	21.10	Fresh
Oceanside	8.40	1.25	18.50	Fresh
Pico	7.84	5.26	21.30	Fresh
Harmony	8.06	2.29	18.90	Fresh
Aliso	7.97	2.91	19.30	Fresh
SMC-15 (SWAMP)	8.97	2.52	33.20	Fresh
San Juan	7.84	3.64	21.60	Fresh
801 (SWAMP)	8.58	1.96	30.34	Fresh
Escondido	7.81	2.04	18.90	Fresh
Las Olas	8.15	48.82	31.90	Marine
Kraken	7.80	31.65	18.00	Marine

Morphology and anatomy

Samples were determined to be *U.* flexuosa if the following criteria was met: a) thalli was slender with a soft fragile consistency without proliferations, b) thallus can be either tubular or blade like, c) branching can be present but rarely has secondary branching, d) cells arranged in

longitudinal and transverse rows, and e) chloroplast almost fills the cell with more than one pyrenoid (Mareš et al. 2011, Bliding 1963 Abbot and Hollenberg 1992). The macroscopic morphology of the specimen was documented from photographs of both the fresh and SWAMP samples. All of the specimens were examined with an Olympus BX41 compound light microscope (LM) equipped with a camera (Insight Firewire Spot 2 with 2 MP resolution) to document the size and the arrangement of the cells within the thallus and the microscopic branching. Cell size measurements (lengths and widths) were performed by computer-aided planimetry with Spot 5.2 imaging software. Due to the varying shape of the cells length and width were measured with the axes passing through the middle of the cell to the midpoint of the edge of the cell. At least 200 cells were measured for each specimen from different parts of the middle region of the thallus. Special attention was paid to the presence of microscopic brachlets and the number of pyrenoids within the chloroplast, which are regarded as the main anatomical features with taxonomic value (Mareš et al. 2011). Each specimen was morphologically similar, except the Garcia sample (visibly smaller cells) and all were assumed to *be U. flexuosa*. There were no zoospores or gametes observed within any of the specimens.

An ANOVA would have been performed to assess if there was any significant differences between any of the morphological features collected and post-hoc analysis using Hotelling's T2 would also be performed.

Molecular Analysis

DNA Extraction:

DNA extraction was performed from silica-gel-preserved specimens. The Invisorb Spin Plant Mini Kit (Invitek Inc., Hayward, CA, USA) was used to extract DNA from each of the preserved samples following the manufacturer's instructions with two modifications for better DNA extraction. First, the initial incubation step was extended to at least 30 minutes, and second, the last incubation step was extended to 30 minutes, with a temperature of 30 degrees Celsius. These two modifications, learned in the molecular lab at CSU San Marcos, can increase the DNA yield by 10 fold in addition to following manufacturing recommendations.

PCR Setup and DNA sequencing:

Total genomic DNA between 100 to 200 ng with 150 ng being optimal was added to 50 µl PCR reaction containing final concentrations of 25 µl of Qiagen HotStarTaq Master Mix, 5 µl of 0.2uM of each primer then the rest of the volume was made up of RNase-free water. For the ITS1-5.8s-ITS2 gene the PCR reaction also contained a 4% DMSO. PCR amplification was either carried out on CSU Programmable Thermal Controller or performed by Retrogen. The Primers that were used to amplify and sequence ITS and *rbc*L gene are listed in Table 3.

Table 3: Primers for DNA amplification and sequencing (Mareš et al. 2011)

Primer	Sequence	Target	Direction
ITS1[1]	5′ TCCGTAGGTGAACCTGCGG 3′	ITS	Forward
ITS4[1]	5′ TCCTCCGCTTATTGATATGC 3′	ITS	Reverse
RH1[1]	5′ ATGTCACCACAAACAGAAACTAAAGC 3′	rbcL	Forward
1385r[1]	5′ AATTCAAATTTAATTTCTTTCC 3′	rbcL	Reverse
rbc571[2]	5′ TGTTTACGAGGTGGTCTTGA 3′	rbcL	Forward
rbc590[2]	5′ TCAAGACCACCTCGTAAACA 3′	rbcL	Reverse

1 – amplification and sequencing; 2 – only for sequencing.

The ITS1-5.8s-ITS2 ribosomal subunit was amplified in a single reaction using the primers ITS1 and ITS4. The reaction profile consisted of an initial denaturation step (15 min at 95◦C as per manufacture recommendations), followed by 35 cycles of 1 min at 94◦C, 2 mins at 45◦C, and 3 mins at 65◦C, with a final extension step at 65◦C for 10 mins.

The *rbc*L gene was amplified with RH1 and 1385r primers (Manhart 1994). These primers amplified the first 1,357 bp (95%) of the gene (without primers), excluding the variable 3' terminus (Mareš et al. 2011). The reaction profile included an initial denaturation step (15 min at 95◦C as per manufacture recommendations), followed by 35 cycles of 1 min at 94◦C, 1 min at 52◦C, and 1 min at 72◦C, with a final extension step at 72◦C for 10 mins. PCR products were then tested on 1% agarose gel with 15 µl of product and the rest was sent to Retrogen (San Diego, California) to be purified and sequenced. PCR-amplified products were directly sequenced using the primers listed in Table 3. Retrogen performed the sequencing. All the sequences were rerun multiple times to ensure that the sequences were correct.

Phylogenetic analysis

Raw data from the molecular sequences were assembled into the final nucleotide sequences using CodonCode Aligner (CodonCode Corp.) computer software application. Sequences that could not be aligned with confidence were removed before additional analysis. For comparison additional *U. flexuosa* sequences for phylogenetic analysis were downloaded from GenBank. The original sequences from this study were compared to the GenBank database using the BLAST algorithm and suitable sequences from other species were chosen as well as from previous papers (Table 4) (Hayden et al. 2003, Mareš et al. 2011, Rybak et al. 2014). The additional sequences obtained from both sources were used to create the phylogenetic trees.

Once all of the sequences were obtained for each of the loci they were aligned with ClustalW within the Mega7 computer application. Both alignments contained 61 total taxa (57 ingroup taxa plus 4 outgroups), of which 18 were original sequences. These alignments were then used to create ITS, *rbc*L, and concatenated ITS-*rbc*L phylogenetic tress. Before the ITS-*rbc*L can be concatenated to form a phylogenetic tree, an incongruence length difference (ILD) test (Paup4.0a150, Swofford 2016) was performed to assess whether or not it was appropriate to combine the DNA into a single database. This test assesses heterogeneity among user-designed partitions, e.g. genes of codons positions. A non-signification result indicates that user-designation data partitions are not significantly different from random partitions of the combined data set. The data may then be combined and used in a single-phylogenetic analysis (de Queiroz et al. 1995).

The phylogenic tree construction used in this study, were Maximum-Parsimony (MP), Maximum-Likelihood (ML) and Bayesian Inference (BI) Trees were constructed for MP and ML using Mega7 and the BI tree was constructed using MrBayes 3.2.6 software (Ronquist and Huelsenbeck 2003) through the CIPRES online super computer (Miller et al. 2010).

MP trees were constructed using the tree bisection-reconnection (TBR) branch-swapping algorithm. One thousand nonparametric bootstrap replications were performed with the default settings for branch support. All bases and base changes were weighted equally and the gaps coded as missing data. The ML analyses were produced using the generalized time-reversible (GTR) substitution model with the discrete gamma distribution in six categories (one for each pair of nucleotides). The gamma shape parameter α as well as the proportion of the variable sites

were estimated from the data set (GRT+I+G model) and 1,000 bootstrap replications were used to evaluate the relative support of the branches (Mareš et al. 2011).

Table 4 : Sources of taxa used to create the phylogenic trees

Taxon	Source	GenBank accession	
Outgroup taxa from *Percursaria, Ulvaria* and *Umbraulva* genus		**ITS**	***rbc*L**
Percursaria percursa[1]	Hayden et al. 2003, Hayden & Waaland 2004	**AY260570**	**AF499674**
Ulvaria obscura var. *blyttii*[1]	Hayden *et al.* 2003	AY260571	AF499673
Umbraulva olivascens[1]	Hayden *et al.* 2003	**AY260564**	**AY255876**
Blidingia minima	Tan *et al.* 1999	AJ000206	AF8387109
Ingroup taxa from *Ulva* genus			
Ulva australis[1]	Kraft *et al.* 2010	EU933985	EU933957
Ulva californica[1]	Hayden *et al.* 2003	**AY260560**	**AY255866**
Ulva californica[1]	Hayden & Waaland 2004	AY422515	AY422558
Ulva clathrata[1]	Blomster *et al.* 1999,	**AF127170**	**AY422563**
Ulva compressa[1]	Hayden et al. 2003	AF035350	AY255859
Ulva fasciata[1]	Hayden & Waaland 2004	**AY422524**	**AY422565**
Ulva flexuosa subsp. *Flexuosa*[2]	Mareš *et al.* 2011	HM447564	HM447574
Ulva flexuosa* subsp. *Flexuosa	Ogawa et al. 2013	**AB830510**	**AB830522**
Ulva flexuosa subsp.*linzaformis*	Taku *et al.* 2013	AB830511	AB830522
Ulva flexuosa* subsp. *Paradoxa[2]	Tan *et al.* 1999	**AJ234306**	**HM447575**
Ulva flexuosa subsp. *Paradoxa*[2]	Mareš *et al.* 2011	HM447561	HM447565
Ulva flexuosa* subsp. *Pilifera[1,2]	Mareš *et al.* 2011	**HM447579**	**HM447566**
Ulva flexuosa subsp. *Pilifera*[1,2]	Mareš *et al.* 2011	HM447584	HM447568
Ulva flexuosa* subsp. *Pilifera[1,2]	Mareš *et al.* 2011	**HM447583**	**HM447567**
Ulva flexuosa subsp. *Pilifera*[1,2]	Mareš *et al.* 2011	HM481175	HM447569
Ulva flexuosa* subsp. *Pilifera[1,2]	Mareš *et al.* 2011	**HM447580**	**HM447576**
Ulva flexuosa subsp. *Pilifera*[1,2]	Mareš *et al.* 2011	HM447582	HM447577
Ulva flexuosa* subsp. *Pilifera[1,2]	Mareš *et al.* 2011	**HM447581**	**HM447578**
Ulva flexuosa subsp. *Pilifera*[1,2]	Mareš *et al.* 2011	HM481176	HM447570
Ulva flexuosa* subsp. *Pilifera[1,2]	Mareš *et al.* 2011	**HM481171**	**HM447572**
Ulva flexuosa subsp. *Pilifera*[1,2]	Mareš *et al.* 2011	HM481173	HM447573
Ulva intestinalis[1]	Kraft *et al.* unpublished	**EU933966**	**EU933939**
Ulva intestinalis[1]	Hayden & Waaland 2004	AY422508	AY422552
Ulva lactuca[1]	Hayden & Waaland 2004	**AY422499**	**AY422543**
Ulva lactuca[1]	Tan *et al.* 1999, Hayden & Waaland 2002	AJ234310	AF499669
Ulva linza[1]	Hayden *et al.* 2003	**AY260557**	**AY255861**
Ulva pertusa[1]	Hayden & Waaland 2004	AY422504	AY422549
Ulva procera (linza)[1]	Hayden & Waaland 2004	**AY422521**	**AY422562**
Ulva procera (linza)[1]	Hayden *et al.* 2003	AY260558	AY255863
Ulva prolifera[1]	Tan *et al.* 1999, Hayden et al. 2003	**AJ234304**	**AY255864**
Ulva prolifera[1]	Hayden *et al.* 2003	AY260559	AY255865
Ulva pseudocurvata[1]	Tan *et al.* 1999, Hayden et al. 2003	**AJ234312**	**AY255869**
Ulva rigida[1]	Hayden & Waaland 2004	AY422522	AY422564
Ulva scandinavica[1]	Tan *et al.* 1999, Hayden et al. 2003	**AJ234317**	**AY255870**
Ulva stenophylla[1]	Hayden *et al.* 2003	AY260569	AY255874
Ulva taeniata[1]	Hayden & Waaland 2004	**AY422525**	**AY422566**
Ulva tanneri[1]	Hayden & Waaland 2004	AY422519	AF499672
Ulva torta	Ogawa et al. 2013	**AB830502**	**AB830519**

BI analysis was run using a likelihood model. The Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analysis with three heated chains and one cold chain per independent runs were processed for 5 million generations, until the split frequencies standard deviation were lower than 0.01 and the potential scale reduction factor (PSRF) of all the parameters reached a value between 1.00 and 1.01. A 50 % majority-rule consensus tree with the posterior

probabilities of branches was constructed with a 10% generation burn-in for the likelihood value. All trees were drawn and edited in TreeGraph2 (Stöver and Müller 2010).

Results

Morphology and anatomy

General morphology of 17 of 18 samples was consistent with the description of the species *U. flexuosa* (Bliding 1963, 1968, Abbott and Hollenberg 1992, Kaštovský et al. 2010, Mareš et al. 2011) using the following site and morphological criteria: a) location collected (Figure 4, a, b, and c) b) size and shape of thallus(Figure 4, d, e, g, and h), c) arrangement of cell in longitudinal and transverse rows (Figure 4: f, i, j, and k), d) cell size (Figure 4: j and k), e) chloroplast filled the cell with more than one pyrenoid present (Figure 4: J and k), and f) branching characteristics (Figure 4: d, e, g, h, I, and l) seen in Table 5. Due to morphologic plasticity of this species and the different habitats in which they were collected, none of the morphological information could be used to identify *U. flexuosa* samples to the subspecies level (Table 1) following either Mareš et.al. (2011) or Bliding (1963) descriptions of *U. flexuosa* subspecies. This was the results of single organisms having multiple criteria present representing all subspecies.

Due to the overlap of subspecies characteristics, cell size was used to see if there was any differences between collection locations. Figure 3 shows the distribution of cell length and width demonstrating the considerable overlap between 17 of the collection locations. This figure also show the Garcia location separated form the other locations almost completely with only one or two cells overlapping.

The Garcia location was originally identified by algae laboratory at California State University, San Marcos (CSUSM) as *U. flexuosa*, but classification was changed to *Blidingia minima* , based on the defining characteristic of this species is the cell size being less than 10 µm in both length and width. All other morphological observations are similar to *U. flexuosa*. Even though this location proved to be a different species it was kept in the study to confirm the identification with molecular techniques (Figures 6, 7, & 8).

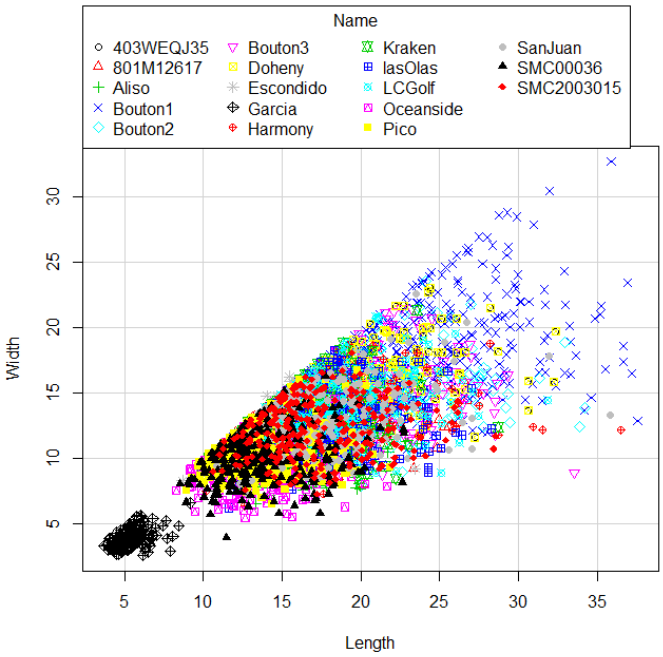

Figure 3: Graph of cell length versus width of specimens collected. The lower left corner show a distinct separation between the collection at Garcia River and the rest of the collected samples. This collection location had a specimen with cells under 10um in both length and width. As a result the specimen was identified as *Blidingia minima*.

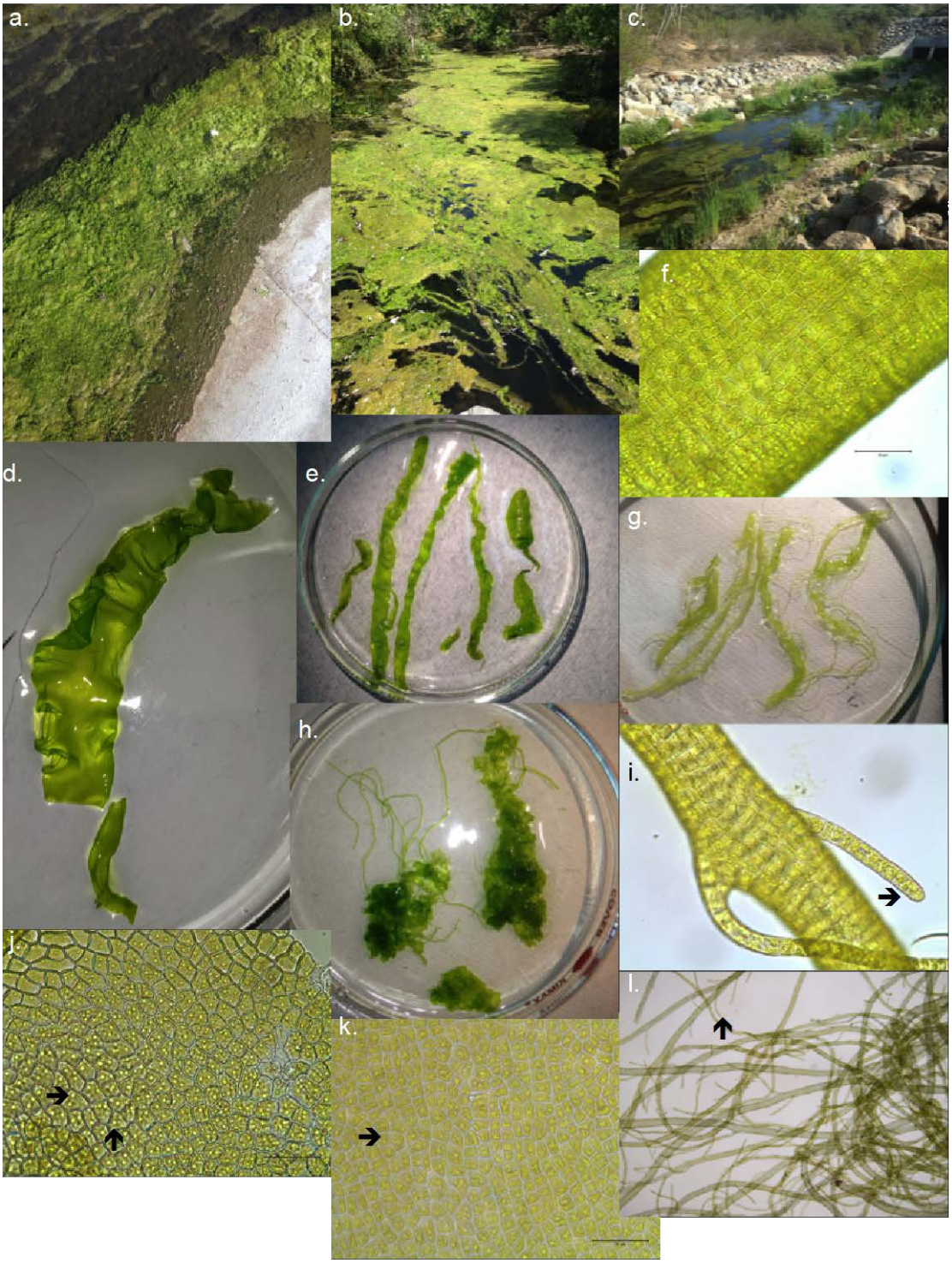

Figure 4: General morphology of collected material. Algae collected from concrete channels, (a.) occur in floating mats (b.), and attached to creek bottoms (c). Thalli occurred in blade-like forms (d.), tube-like forms with no branching (e.), tube-like with branching (f)), and combination of blade-like form with tube-like branches (h.). Branching had attenuated ends (i & l arrows). Cellular arrangement was typically in long rows (i-k) with rectangular to quadrangular cell shapes (j & k). Chloroplast with visible pyrenoids (j & k arrows).

Table 5: General morphology of the U. flexuosa from each collection location.

Location ID	Habitat	Range of Pyrenoids	Range of Cell Size (Width X Length) (µm)	General morphology	Branching	Uniseriate branching	Cell shape	Cell arrangement
Garcia (SWAMP)	Fresh	1-2 (3)	3-7 X 4-9	Tube-like	Frequent	None	Angular to polygonal	In long Rows, sometimes less regular
403 (SWAMP)	Fresh	1-3 (4)	10-23 X 13-32	Tube-like	Frequent	Present with attenuated ends	Rectangular	In long rows
801 (SWAMP)	Fresh	2-4 (5)	8-18 X 11-27	Tube-like	Sparse	Present with attenuated ends	Rectangular to Quadrangular	In long Rows
Aliso	Fresh	1-5 (6)	7-17 X 10-24	Tube-like	None	None	Rectangular to Quadrangular	In Long Rows
Escondido	Fresh	(1) 2-3 (4)	8-18 X 9-23	Blade to Tube-like	Present	Present with attenuated ends	Rectangular to Quadrangular	In long rows
Harmony	Fresh	1-3	6-19 X 10-37	Tube-like	Present	Present with attenuated ends	Rectangular to Quadrangular	In long rows
Oceanside	Fresh	1-4	5-15 X 8-22	Tube-like	None	None	Rectangular to Quadrangular	In long rows
Pico	Fresh	(1) 2-3 (5)	6-17 X 9-22	Tube-like	None	None	Rectangular to Quadrangular	In long Rows
San Juan	Fresh	1-3 (5)	7-23 X 11-36	Tube-like	None	None	Rounded Polygonal	In Long rows, sometimes less regular
SMC-15 (SWAMP)	Fresh	2-4	7-18 X 11-29	Tube-like	Present	Present with attenuated ends	Rounded Polygonal	In long Rows
SMC-36 (SWAMP)	Fresh	1-4	4-16 X 9-23	Tube-like	Present	Present with attenuated ends	Rounded Polygonal	In long rows sometimes less regular
La Costa	Brackish	2-4 (6)	9-21 X 12-32	Tube-like	Sparse	Present with attenuated ends	Polygonal	In Long rows, sometimes less regular
Bouton1	Brackish	1 (2)	11-33 X 13-38	Tube-like	Frequent	Present with attenuated ends	Quadrangular to Polygonal	In long rows
Bouton2	Brackish	(1) 2-3 (4)	8-24 X 14-34	Blade-like	None	Present with attenuated ends	Quadrangular to Polygonal	In long rows
Bouton3	Brackish	1-4 (5)	9-21 X 12-34	Tube-like	Present	Present with attenuated ends	Quadrangular to Polygonal	In Long rows, sometimes less regular
Doheny	Brackish	NA	10-23 X 13-32	Tube-like	Frequent	Present with attenuated ends	Rounded Polygonal	In long rows
Kraken	Marine	(1) 2-4 (5)	8-21 X 11-29	Blade to Tube-like	Sparse	None	Quadrangular	In Long rows
Las Olas	Marine	1-4	6-18 X 11-28	Tube-like	Present	Present with attenuated ends	Rectangular to Quadrangular	In long rows

Molecular analysis

In total, 17 of the 18 samples that were determined morphologically to be U. flexuosa, along with the *B. minima* sample from the Garcia location were sequenced for the ITS and *rbc*L regions. ITS sequences were between 510 and 560 bp long and the *rbc*L sequence were between 1291 and 1309 bp long. The length of each sequence is shown in Table 6. Sequence identity results for all the sequences can be found in the supplemental material Tables S1, S2 and S3. All referenced sequences used for comparisons are in Table 4.

All of the 18 samples were compared against the GenBank database using the Basic Local Alignment Search Tool (BLAST) distance tree results to give a starting point for each of the sequences. The initial findings of these comparisons were that all of the freshwater collections, except Garcia location, were placed into the species of *Ulva flexuosa.* The Garcia location was paired with *Blidingia minima* species. The two marine locations (Kraken and Las Olas) and two of the brackish water collections (Doheny & Bouton 3) were placed in the species *Ulva torta.* Two of the other brackish water locations (Bouton 1 & 2) were placed in the species *Ulva linza.* For the actual molecular analysis a Bayesian, MP and ML trees were constructed for each gene and then genes were combined together with the results below.

Table 6: Lengths of base pairs (bp) by sequences location for ITS region, *rbc*L, and the concatenated ITS/*rbc*L.

Location	ITS Region	rbcL Region	Concatenated
403	560	1307	1867
801	560	1307	1867
Aliso	514	1308	1822
Bouton 1	540	1306	1846
Bouton 2	538	1308	1848
Bouton 3	536	1307	1845
Doheny	558	1308	1844
Escondido	560	1308	1866
Garcia	550	1307	1867
Harmony	555	1307	1857
Kraken	558	1308	1863
La Costa	527	1291	1849
Los Olas	545	1308	1835
Oceanside	560	1308	1853
Pico	548	1308	1868
San Jose	560	1308	1856
SMC-36	541	1309	1857
SMC-15	510	1308	1868

Due the molecular analysis revealing that the samples collected represent three different species. An MANOVA was performed comparing the comparing species by cell lengths and widths, water types, and collection locations, there was significant difference (degrees of freedom (DF) = 7662, p-value<2.2e-16). Performing an ANOVA continue to show significant differences between all species cells lengths and widths (DF = 17, p-values < 2.2e-16), and all water types (DF= 17, p-values < 2.2e-16). Post-hoc analysis with Hotelling's T^2 test showed that differences between species and water type was also significant.

In contrast when comparing the differences between the collection locations and cell size data, the ANOVA did not show any significant differences revealing that there was considerable overlap in cell length and width between species and water types. For example, cell lengths and widths did not differ between *U. torta* and *U. flexuosa* collected from marine locations (Las Olas and Kraken) and a handful of freshwater locations (Harmony, 801, San Juan, SMC15 and

Table 7: Hotelling's T^2 analysis comparing cell width and cell length between pairs of all of sample locations. The selected comparisons below did not show significant differences between cell length and width at these locations and those locations represent a overlap between water type and species. An alpha level of 0.025 was used for all comparisons. (F)=freshwater, (B)=brackish, and (M)=marine

Comparison	P-value	Comparison	P-value
403(F)-Doheny(B)	1.00	Pico(F)-SMC-36(F)	0.05
Harmony(F)-Kraken(M)	0.93	Harmony(F)-San Juan(F)	0.05
Las Olas(M)-San Juan(F)	0.72	Aliso(F)-Escondido(F)	0.05
801(F)- San Juan(F)	0.67	Harmony(F)-SMC-15(F)	0.04
801(F)-lasOlas(M)	0.25	801(F)-SMC-15(F)	0.03
801(F)-Harmony(F)	0.21	801(F)-Kraken(M)	0.03
Bouton 2(B)-Bouton 3(B)	0.12		

Escondido), with p-values greater than 0.05. In addition, Doheny (brackish, *U. torta)* overlapped with the freshwater *U. flexuosa* location 403 (p-value= 1.00), and Bouton 2 (*U. linza)* and Bouton 3 (*U. torta)* also overlapped in both cell width and length (p-value = 0.12). These analyses were performed using a post-hoc Hoteling's T^2, p-values greater that 0.025 would indicate that the cells would not have differed significantly in both width or length (Table 7).

These analyses demonstrate that cell length and width is not useful for determining the differences between these species except for *Blidingia minima*. To better illustrate the point, a Principle Component Analysis (PCA) was performed comparing cell lengths and widths size by species and water type. PC1 explained more than 85% of the variance for the means of length and width of the cell. Figure 5 shows the overlapping relationship between the cell size vs. water type and species.

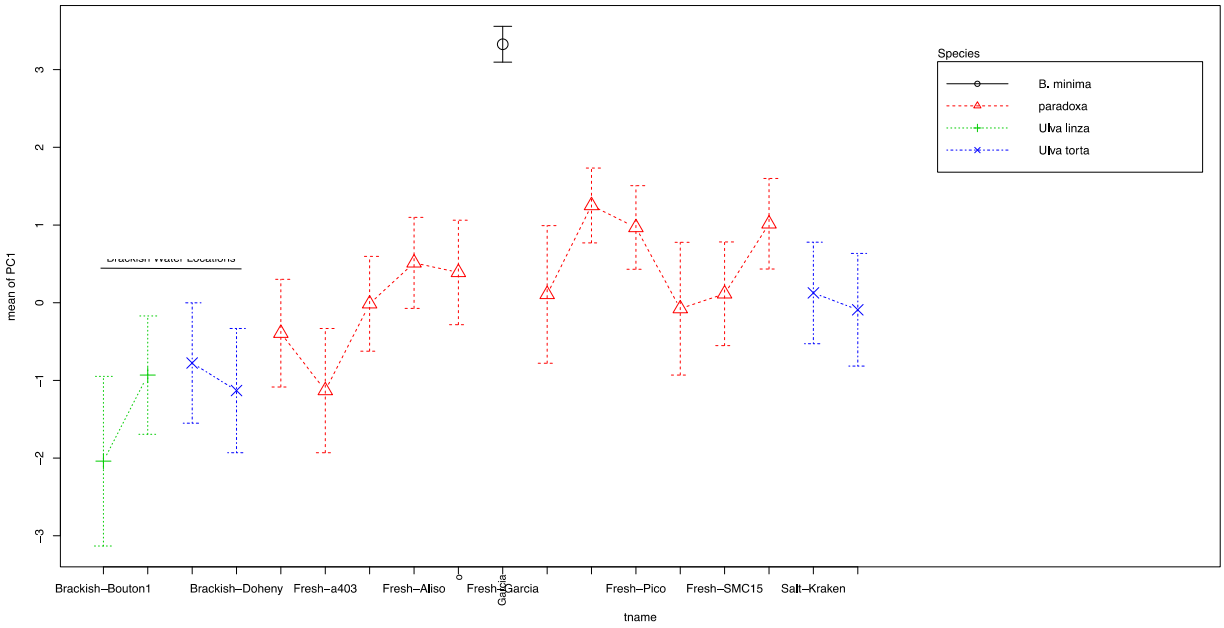

Figure 5: Plot of cell width and length means with error bars of 1 standard deviation. Data were produced by principle component analysis (PCA) in which more than 85% of the variance can be explained by PC1. This graph demonstrates that both width and length of cells overlap substantially between species and water types.

ITS region phylogenetic tree

The analysis for the ITS data set results for MP were a 50% majority-rule consensus tree (length = 437), consistency index (CI) = 0.584, retention index (RI) = 0.757 and rescaled consistency index (RCI) = 0.442. The ML Analysis produced a tree with a log maximum likelihood value of -2334.68, and the Bayesian analysis produced a 50% majority consensus of two trees from independent runs. Each of the phylogenetic analysis produced similar results as shown in Figure 5 where the Bayesian tree is mapped with consensual branches from the ML and MP Trees. The Trees were rooted using the outgroup taxa *Percursaria percursa,* with three additional non- *Ulva* taxa, *Umbraulva olivascens, Ulvaria obscura var. blyttii, and Blidingia minima*. These taxa have been commonly used as outgroups for similar studies (Hayden et al. 2003, Mareš et al. 2011, Rybak et al. 2014). *Blidingia minima* was used as an additional outgroup to show a relationship with the Garcia location.

In the Bayesian and MP trees, all of the freshwater samples consistently fell into one clade containing three different subspecies of *Ulva flexuosa* (*linziformis, flexuosa*, and *paradoxa),* with 77% MP bootstrap support and 1.0 Bayesian Inference Posterior Probability (BP), none of these samples were associated with the subspecies *pilifera.* The Garcia River and the La Costa

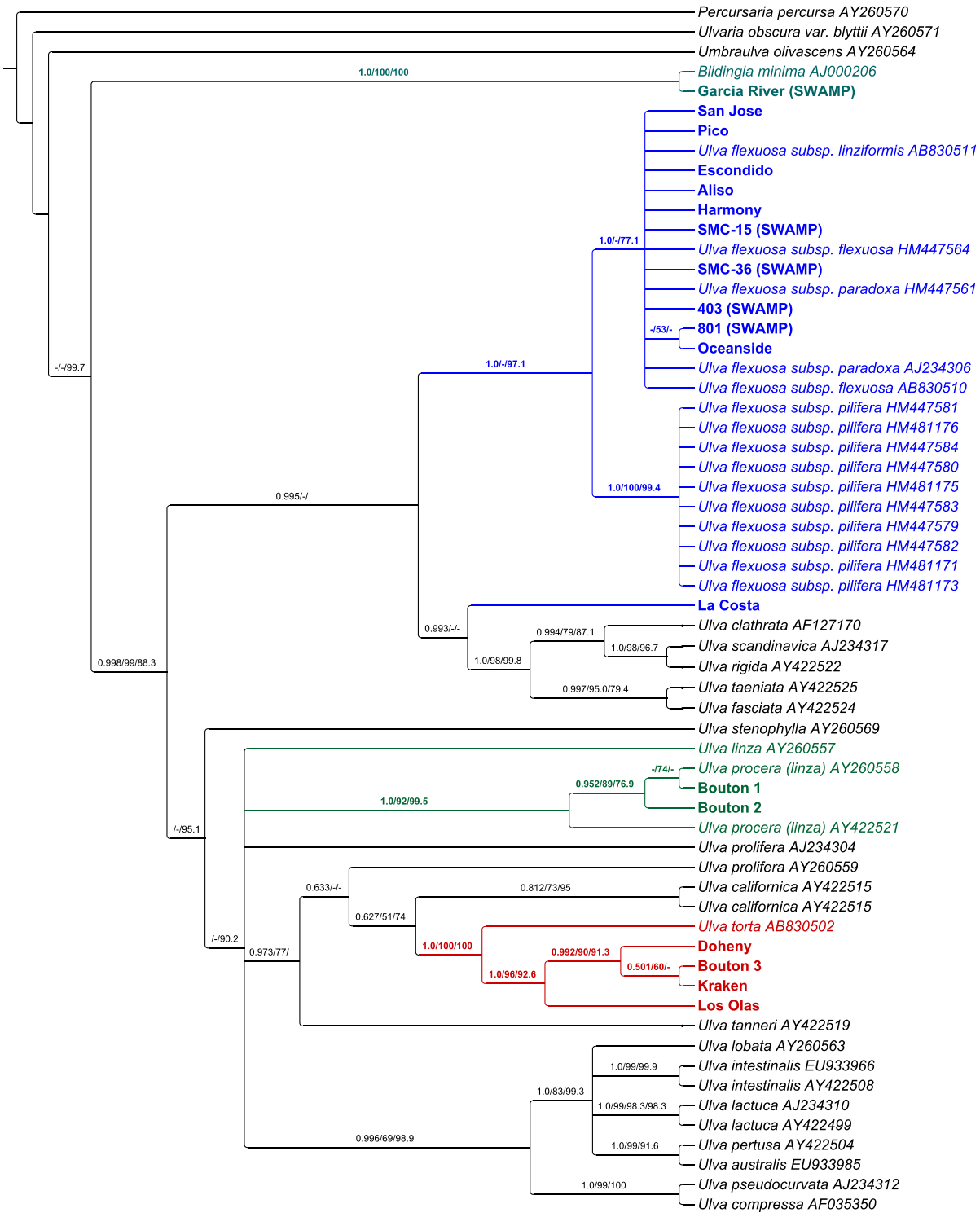

Figure 6: ITS Bayesian Tree with support from Bayesian probabilities, MP and ML bootstrap supports values (1,000 replications) are given at the nodes (BP/MP/ML) (0.1 substitution per site). Dashes (-) represent less than 0.50 (BP) or 50% (MP and ML) support. Sequences produced in this study are bolded. All other sequences are labeled with taxon name and GenBank accession number. Species are grouped by color within the tree, the *Ulva flexuosa* is in blue, *Ulva linza* is in green, *Ulva torta* is in red and Garcia River is in the dark green. The La Costa location was included in blue as it is associated this species once the sequences are concatenated (Figure 8).

samples were the only freshwater specimens that fell outside of the *U. flexuosa* clade. La Costa was associated with neighboring species to *U. flexuosa* and Garcia River showed strong support (1.0 BP, 100% ML, and 100% MP) associating with *Blidingia minima.* The other samples collected in both saltwater and brackish environments separated out into two well-supported species with 1) *Ulva torta* (Doheny, Bouton 3, Kraken, and Las Olas, 1.0 BP, 96% ML, and 92.6% MP) and 2) *Ulva linza* (Bouton 1 and 2, 1.0 BP, 92% ML, and 99.5% MP). The *Ulva flexuosa subsp. pilifera* separated out form the rest of the subspecies within the species with 1.0 BP, 0.97 ML Bootstrap, and 0.96 MP Bootstrap supports (Figure 5).

*rbc*L Region

The *rbc*L analysis for the MP produced one most parsimonious tree with a 50% majority-rule consensus tree (l=639, CI=0.754, RI=0.795, RCI=0.600). ML analyses produce a single tree (-lnL=-3436.54), and two trees from Bayesian independent runs were combined to produce a similar structured tree as the ITS gene. The Bayesian tree was mapped with the bootstrapped braches of the MP and ML analysis (Figure 6). The tree is rooted as described before.

The *U. flexuosa* group separated out as before except that the subspecies tended to be separate and not lumped together. As with ITS, all the of the freshwater samples, except Garcia River and La Costa, were associated as a subspecies of *U. flexuosa*; however, this time they were strongly associated with the subspecies *paradoxa* (0.916 BP, 63% ML, and 79.6% MP). The exceptions were again Garcia River, which was strongly associated to *Blidingia minima* (1.0 BP, 96% ML, and 91.2% MP), and La Costa fell outside of *U. flexuosa* clade. As with the previous trees, the brackish and fresh water samples were placed in the same well-supported species, *Ulva torta* (Bouton 3, Doheny, Las Olas, and Kraken, 0.998 BP and 95% ML) and *Ulva linza* (Bouton 1, 0.875 BP, 63% ML, and 80.1% MP). Bouton 2 fell was not related to any species on the tree and was on par with the La Costa location. Again the *U. flexuosa* subsp. *pilifera* separated out within the species *U. flexuosa* from the other subspecies with strong support (1.0 BP, and 99% ML) (Figure 6).

Combining of Both Regions

Prior to combining the two genes an ILD test was performed, which was non-significant (P=0.76), thus allowing for the combination of the data sets into a single analysis. The same analyses as described before were performed on the combined data matrix. The MP analysis of the combined data produced a most parsimonious tree that was collapsed into a 50% majority-rule consensus tree (l=1726, CI=0.671, RI=0.685, RCI=0.460). The ML tree (lnL=-9987.13) and the 50% majority-rule consensus Bayesian tree were obtained as in previous analysis (Figure 5). The general appearance of the tree is similar to the ITS and the rbcL trees with separation and support for the flexuosa clade (0.557 BP and 99.9% MP). However, this time not only all of the freshwater locations, except for Garcia River, but the La Costa location was included as well. The collection locations of SMC-36, SMC-15, 403, Pico, and San Jose sites tended to be aligned with the subspecies *paradoxa* with only ML Support (76.4%). The subspecies *pilifera* were again separated from the other subspecies with *U. flexuosa* with strong support (1.0 BP, 90% ML and 100%MP). Oceanside, Aliso, and Escondido were also not associated with any of the other the subspecies groups' *paradoxa, linziformis, and flexuosa,* but instead they were shown to be strongly associated with each other (1.0 BP, 90.6% ML, 98.4% MP). La Costa and Harmony

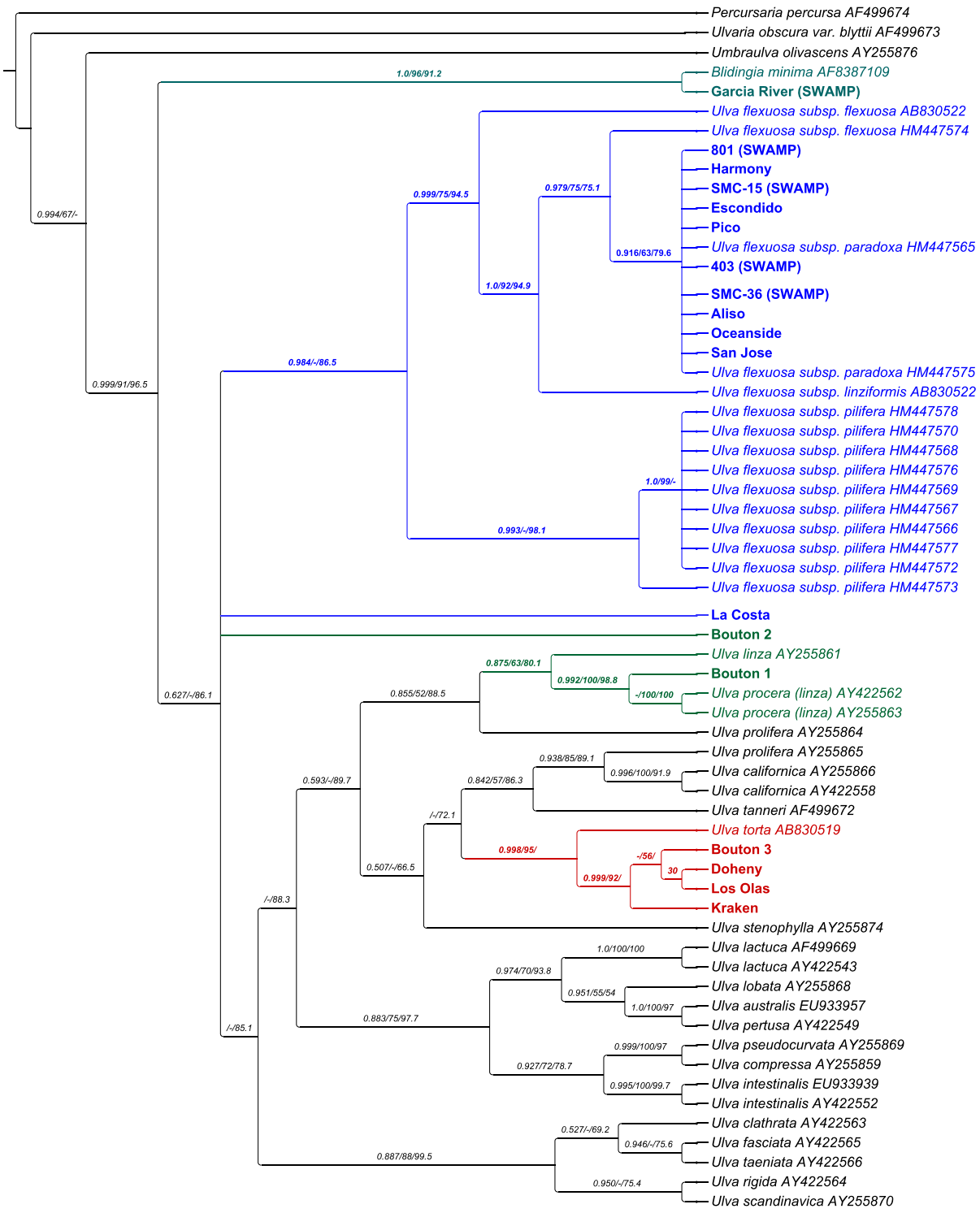

Figure 7: The *rbc*L Bayesian Tree with support from Bayesian probabilities, MP and ML bootstrap supports values (1,000 replications) are given at the nodes (BP/MP/ML) (0.1 substitution per site). Dashes (-) represent less than 0.50 (BP) or 50% (MP and ML) support. Sequences produced in this study are bolded. All other sequences are labeled with taxon name and GenBank accession number. Species are grouped by color within the tree, the *Ulva flexuosa* (blue), *Ulva linza* (green), *Ulva torta* (red) and Garcia River (dark green). La Costa and Bouton 2 are colored to demonstrate that they will be included with those species once the two sequences are concatenated (Figure 8).

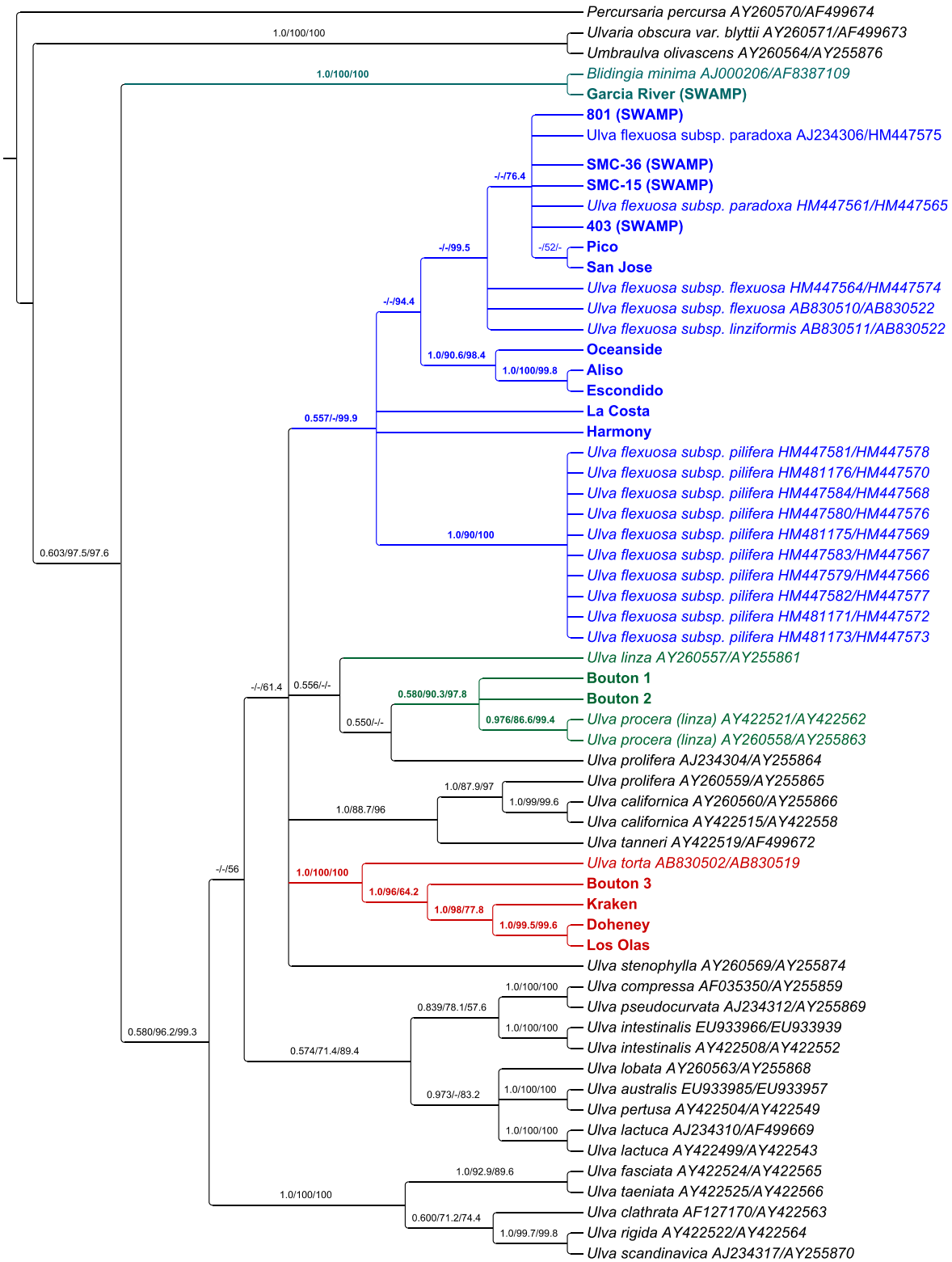

Figure 8: Concatenated ITS and *rbc*L Bayesian Tree with support from Bayesian probabilities, MP and ML bootstrap supports values (1,000 replications) are given at the nodes (BI/MP/ML) (0.1 substitution per site). Dashes (-) represent less than 0.50 (BP) or 50% (MP and ML) support. Sequences produced in this study are

bolded. All other sequences are labeled with taxon name and GenBank accession number. Species are grouped by color within the tree, the *Ulva flexuosa* (blue), *Ulva linza* (green), *Ulva torta* (red) and Garcia River (dark green).

locations were not associated with any of the subspecies but were still associated within the *U. flexuosa* clade.

The other locations were similarly aligned as in the previous trees with Garcia River strongly aligned with *Blidingia minima* (1.0 BP, 100% ML, and 100% MP), Bouton 3, Kraken, Doheny, and Las Olas were strong support as with *U. torta* (1.0 BP, 100% ML, 100% MP) and Bouton 1 and 2 were strongly associated with *U. linza* (0.580 BP, 90.3% ML, 97.8% MP) (Figure 7).

Discussion

The morphological identification of species within the genus *Ulva* has been notoriously difficult, due to the plasticity of features observed at the thallus and cellular level (Bliding 1963, Bliding 1968, John et al. 2002). The keys needed for identifying *Ulva* are the following: a) microscope morphology of the thallus (length, width, color and structure), b) branching and structure of branch types, c) number of pyrenoids per chloroplast, d) shape of chloroplast, e) cell size, f) cell configuration, g) mode of reproduction, and h) habitat (Koeman and Van den Hoek 1984, Kolwalkar et al. 2007, Kaštovský et al. 2010, Mareš et al. 2011).

Because of its phenotypic plasticity, *Ulva* species and subspecies are often incorrectly identified (Rybak et al. 2014). Through the use of molecular and classic morphological analysis, Mareš et al (2011) was able to confirm the corrected identification of historic herbarium specimens and recent collection to be *Ulva flexuosa* and its various subspecies in Europe. Hayden et al (2003) also used molecular techniques to demonstrate that the *Enteromorpha* Link should be returned to the genus *Ulva*. Each of these studies used ITS and *rbc*L regions for the molecular basis of their analysis and were the primary reason they were chosen to be use in this paper.

Currently, it is very difficult to conduct ecological and morphological studies with the genus of *Ulva* without the use of these molecular techniques (Leskinen and Pamilo 1997, Kaštovský et al. 2010, Mareš et al. 2011, Rybak et al. 2011, Rybak et al. 2014). In this study, analysis of both the ITS and *rcb*L revealed that not all the samples collected were *Ulva flexuosa,* even though their morphology suggested they were, but were four different species (*U. flexuosa*, *U. torta, U. linza, and Blidingia minima*) with only one that had distinctly different morphology that was supported statistically (Garcia – *Blidingia minima*). Water type seems to play the most important role in distinguishing between species in the study, with all of the freshwater samples, except Garcia, being *U. flexuosa* and the material collected from the more saline habitats were associated with two different species, *U. torta* or *U. linza.* The major exception was the location, La Costa (brackish water), which was loosely associated with *U. flexuosa* but only when the genes were concatenated (Figures 6, 7 & 8).

The morphological diversity of *U. flexuosa* and its subspecies has been described in great detail in a number of studies (Burrows 1959, Bliding 1963, Mareš et al. 2011), however, these details provided a taxonomic key in which morphological identification can be made within Europe. In this study, the samples collected exceeded the limitation of this key though morphological variation. In addition, the findings also suggest that the dominant subspecies occurring in California freshwaters could be U. flexuosa subsp. paradoxa, and not the more common U.

flexuosa subsp. *pilifera* collected in European waters (Mareš et al. 2011, Messyasz et al. 2013, Rybak et al. 2011). This was demonstrated in the *rbc*L tree (Figure 7) and to some extent in the concatenation of ITS and *rbc*L gene tree (Figure 8). What was most noticeable is that the subspecies *pilifera* was not associated with any of the material collected.

The literature suggests that *Ulva flexuosa* is common along the west coast of the United States, Canada, and Mexico in marine intertidal waters (Abbot and Hollenburg 1992, Setchell and Gardner 1919). However, Abbot and Hollenberg (1992) using the European morphology, stated that they did not find similar specimens that has branching, whereas Setchell and Gardner (1919) refer to an obscure collection record of one individual, Mrs. Bingham in Santa Barbara, credited by J. G. Agardh in 1883. Another publication Marine Algae and Seagrasses of San Diego County by Joan Steward (1991) refers to Dawson's check list as proof for the presence of this within Southern California, which in turns refers back to Setchell and Garners recount of 1883. This difference may suggest that *U. flexuosa* is not as common as suggested by historical records within the marine coastal waters of California. This study attempted to collect a number of samples from marine sources within Southern California (Figure 9) but only produced two samples. Marine samples were collected from boat hulls, intertidal zones, mud flats, dock and other favorable locations where *U. flexuosa* is typically situated. Samples collected from marine environment are likely to be *Ulva intestinalis*. *U. flexuosa* has been commonly mistaken for *U. intestinalis* in the past within European waters (John et al. 2002, Mares et al. 2011) and only recently has been corrected through the use of molecular sequencing (Mares et al. 2011). The results of this study suggest that U. flexuosa may also have been misidentified within California waters, and with the use of current molecular techniques these mistakes can be corrected.

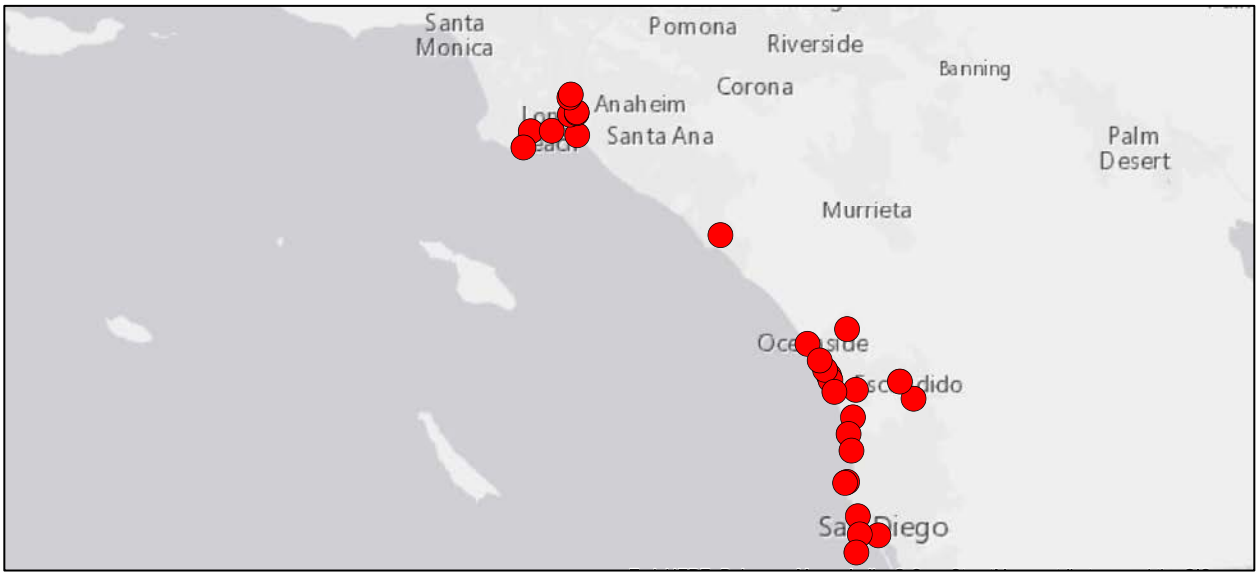

Figure 9: Collection locations in which did not produce any samples of *Ulva flexuosa*. Fifteen marine, eight brackish and five freshwater locations failed to produce a sample of *U. flexuosa*. The most common species collected, if at all, in the marine and brackish environments was *Ulva intestinalis*.

To this author's knowledge this is the first attempt within Southern California to assess the morphological features of *U. flexuosa* and compare them using molecular techniques. This study confirmed that *U. flexuosa* is very common in freshwaters within California (Stancheva et al. 2016) and suggest that the freshwater algae are most likely the subspecies *U. flexuosa* subsp. *paradoxa*. Future studies may be able to better compare the *U. flexuosa* from different water

sources or they may be able to find out that those historical collections could be just masquerading as *Ulva flexuosa* as found within Europe (Mareš et al. 2011).

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Appendices

Supplemental material

Table S1: Sequence identity results for the ITS region for both the original sequences and the published sequences

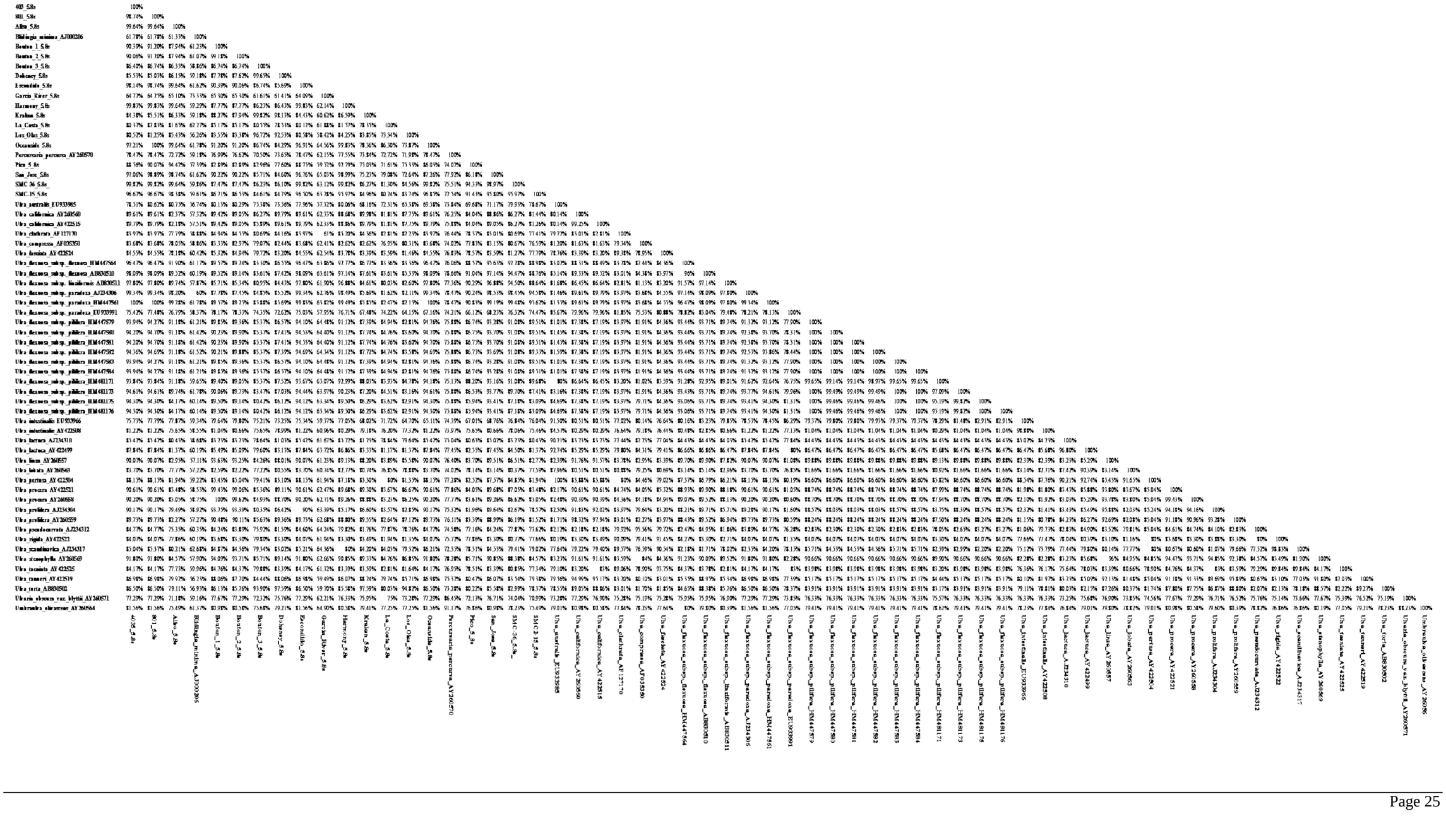

Table S2: Sequence identity results for the *rbc*L region for both the original sequences and the published sequences.

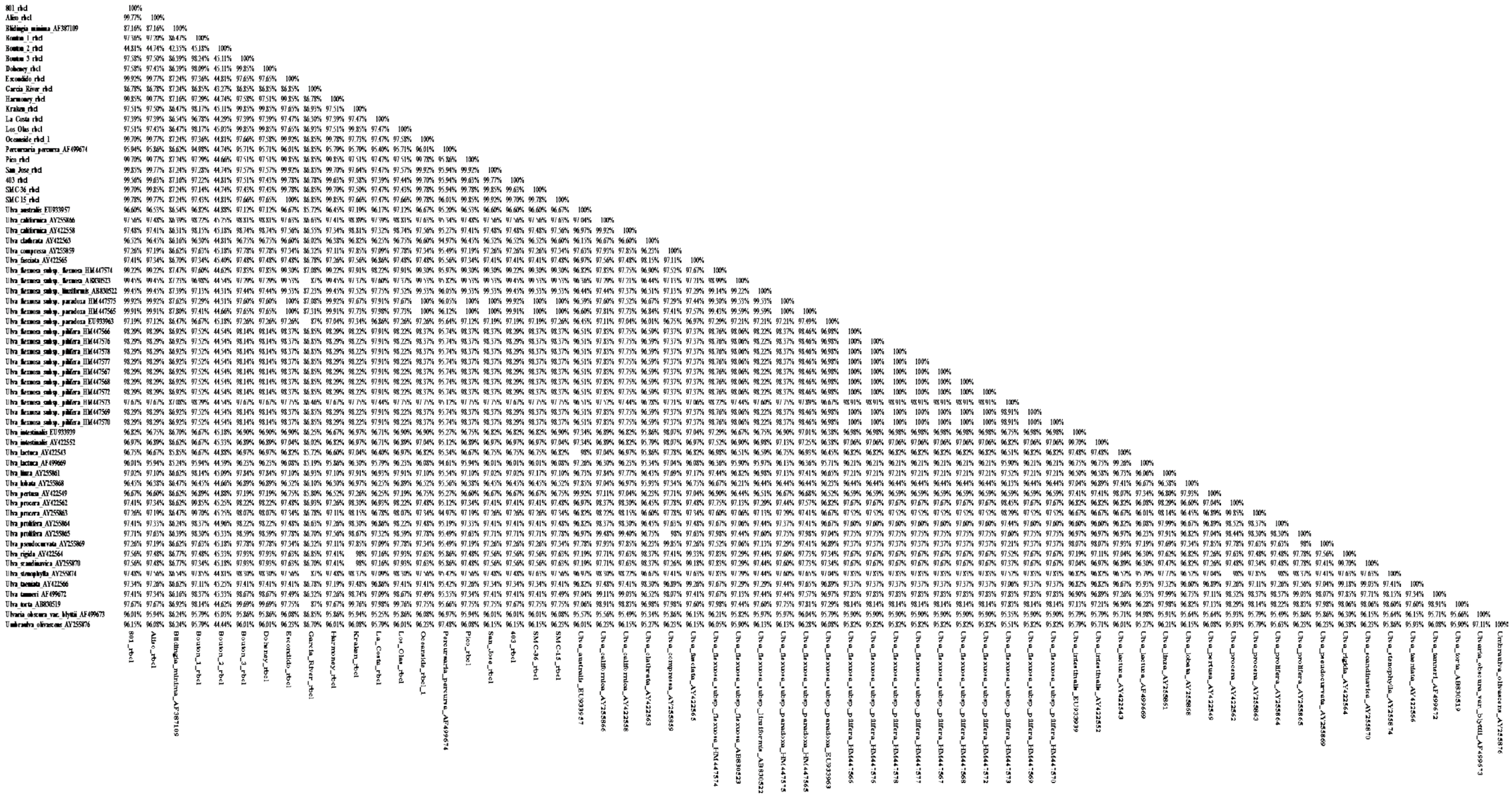

Table S3: Sequence identity results for both the ITS and rbcL regions that have been concatenated together for both the original sequences and the published sequences.