Research Article

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Taxonomic assessment of blade-forming *Ulva* species (Ulvales, Chlorophyta) in the Galápagos Archipelago, Ecuador using DNA sequencing

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Abstract: DNA sequences were obtained from 32 blade-forming *Ulva* specimens collected in 2018 and 2019 from four islands in the Galápagos Archipelago: Fernandina, Floreana, Isabela and San Cristóbal. The loci sequenced were nuclear encoded ITS and plastid encoded *rbc*L and *tuf*A, all recognized as barcode markers for green algae. Four species were found, *Ulva adhaerens*, *U. lactuca*, *U. ohnoi* and *U. tanneri*, all of which have had their type specimens sequenced, ensuring the correct application of these names. Only one of these, *U. lactuca*, was reported historically from the archipelago. *Ulva adhaerens* was the species most commonly collected and widely distributed, occurring on all four islands. Previously known only from Japan and Korea, this is the first report of *U. adhaerens* from the southeast Pacific Ocean. *Ulva ohnoi* was collected on three islands, Isabela, Floreana, and San Cristóbal, and *U. lactuca* only on the last two. *Ulva tanneri* is a diminutive, 1–2 cm tall, high intertidal species that is easily overlooked, but likely far more common than the one specimen that was collected. This study of blade-forming *Ulva* species confirms that a concerted effort, using DNA sequencing, is needed to document the seaweed flora of the Galápagos Archipelago.

Keywords: DNA barcoding; *tuf*A; *Ulva adhaerens*; *U. lactuca*; *U. ohnoi*; *U. tanneri*

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1 Introduction

Our knowledge of the benthic marine algal flora of the Galápagos Archipelago is based primarily on one late 19th century collection and three early to mid-20th century collections, all published in the 20th century (Dawson 1963; Farlow 1902; Lemoine 1929; Taylor 1945). These published reports were compiled by Silva (1966) in a checklist of 311 species. Subsequent to Silva (1966) the main sources of information on the benthic marine algae, including the updating of name changes, have been the checklists, now online, assembled by the Charles Darwin Foundation, viz. Cyanobacteria (Chiriboga et al. 2014, last updated), Chlorophyta (Ruiz and Ziemmeck 2014, last updated), Phaeophyceae (Ruiz and Ziemmeck 2016a, last updated) and Rhodophyta (Ruiz and Ziemmeck 2016b, last updated). Only one genus of benthic marine algae from the Galápagos Archipelago has been treated in the 21st century, the siphonous green alga, *Codium* (Chacana et al. 2016), and likewise only one species of green algae, *Caulerpa chemnitzia* (Keith et al. 2022). All of these studies were based on the morpho-anatomical examinations of the algae.

Only two studies (Anslan et al. 2021; Boo et al. 2016) used DNA sequencing to identify algae from the Galápagos. Boo et al. (2016) sequenced the mitogenomes of two herbarium specimens of the red alga genus *Gelidium* (Gelidiales), the holotypes of *G. isabelae* and *G. galapagense*, both collected by Taylor (1945) and housed in UC (herbarium acronyms follow Thiers 2024). Anslan et al. (2021) performed a metabarcode analysis of algal DNA in fecal samples from two subspecies of the endemic Galápagos marine iguana, *Amblyrhynchus cristatus* subsp. *mertensi* and *A. cristatus* subsp. *godzilla* on Isla San Cristóbal. They also sequenced a portion of the *rbc*L gene from field-collected marine macroalgal specimens on

Isla San Cristóbal from the same sites sampled for *A. cristatus* feces. The algal species were identified mainly by comparing BLASTn analyses of publicly available sequences in NCBI and BOLD.

Many species of marine macroalgae lack diagnostic morpho-anatomical characters (Gabrielson et al. 2018; Hind et al. 2015; Vieira et al. 2014) and/or are morphologically plastic (Gao et al. 2016; Hind et al. 2014; Lewis et al. 1987), so that accurate species identifications are fraught. Since the mid-2000s DNA barcoding has become the preferred method worldwide to identify marine macroalgae (Bartolo et al. 2020; McDevit and Saunders 2009; Saunders and Kucera 2010; Torrano-Silva et al. 2018; Vieira et al. 2021). DNA based species identifications have illuminated just how incorrect many of our identifications have been in the past and revealed a striking amount of cryptic diversity in all benthic marine algae, greens, browns and reds alike, from the tropics to the polar regions. The green macroalgal genus *Ulva* is an exemplar of these problems.

A major advance in the systematics of *Ulva* in recent decades has been the application of DNA sequencing to recent field collected specimens, first by Leskinen and Pamilo (1997, as *Enteromorpha*) and subsequently by many others (e.g., Coat et al. 1998, France (Atlantic); Hayden and Waaland 2004, Northeast Pacific; Heesch et al. 2009, New Zealand; Kirkendale et al. 2013, Kraft et al. 2010, Eastern Australia; O'Kelly et al. 2010; Hawai'i; Krupnik et al. 2018, Eastern Mediterranean; Chávez-Sánchez et al. 2019, Mexico (Gulf of California); Kang et al. 2019; Korea; Steinhagen et al. 2019, North and Baltic Seas; Xie et al. 2020, China; Dartois et al. 2021, Atlantic, France; Melton et al. 2021, Northeast Atlantic/Gulf of Mexico; Lagourgue et al. 2022, New Caledonia; Tran et al. 2023, Vietnam). While this approach has been very important to explore the diversity of *Ulva* species, it has not necessarily resulted in the correct application of names for these species. The reason for this is that specimens are placed into species based on DNA sequences, but the historical names applied to these species are based on morpho-anatomical characters. We have known for decades (Papenfuss 1960) that these morpho-anatomical characters are difficult to apply to *Ulva* species, as the characters are few and the species themselves are morphologically highly variable (Gao et al. 2016).

The second major advance in *Ulva* systematics has been to obtain DNA sequences from *Ulva* type specimens, either by Sanger sequencing a portion of the plastid encoded *rbc*L gene or by high-throughput sequencing to obtain mitochondrial and or plastid genomes (Hanyuda and Kawai 2018; Hughey et al. 2018, 2019, 2021, 2022, 2024). This enables DNA sequences from field-collected material to be directly compared to type sequences, thus ensuring the correct application of names. Indeed, when Fort et al. (2020) compared type specimen sequences to contemporary *Ulva* DNA sequences in GenBank whose identities were determined using morpho-anatomy, many of the over 1000 named sequences of *Ulva* species in GenBank from localities around the world had been assigned incorrect names. The authors estimated that 21 % of named *Ulva* sequences in GenBank were misidentified, and this ranged up to 65 % for *U. lactuca* L., the generitype species of *Ulva*. These misidentifications have profound consequences for our understanding of the physiology, ecology, biogeography, and even commercial utilization of *Ulva* species.

To correctly identify the blade-forming species of *Ulva* in the Galápagos Archipelago, we used targeted PCR methods to characterize field-collected specimens. Four blade-forming *Ulva* species were found, *U. adhaerens* Kaoru Matsumoto *et* S.Shimada, *U. lactuca*, *U. ohnoi* M.Hiraoka *et* S.Shimada, and *U. tanneri* H.S.Hayden *et* Waaland. These species were also found by Anslan et al. (2021) in their macroalgal sampling, although *U. adhaerens* was called *Ulva* sp. None of these species were found in the fecal samples of the marine iguanas (Anslan et al. 2021)

2 Materials and methods

Samples of *Ulva* were collected from four islands in the Galápagos Archipelago. Isla San Cristóbal was the island more intensively sampled for algae at four sites: La Lobería, Playa Baquerizo, Punta Carola and Tijeretas; then Isla Isabela at Concha y Perla, Cuatro Hermanos and Tintoreras; then Isla Floreana at Sur Tres Cuevitas and La Botella; while sampling at Isla Fernandina was more opportunistic and included two sites: Punta Espinosa and Punta Mangle (Figure 1). Specimens were collected by hand via snorkel or SCUBA. Voucher specimens were pressed in the field or at the Galápagos Science Center on Isla San Cristóbal. Fragments for DNA analysis were removed, patted dry and placed into silica gel desiccant. Duplicate voucher specimens were deposited in NCU and QUSF (herbarium acronyms follow Thiers 2024). See Supplementary Table S1 for collection data and GenBank accession numbers. Throughout this paper specific epithets of green algae whose type specimens have not been sequenced are indicated by single quotation marks.

Total genomic DNA was extracted and amplified from Galápagos field-collected specimens at the University of North Carolina, Chapel Hill using the method in Hughey et al. (2001). Plastid encoded *rbc*L from field-collected specimens was amplified in two amplicons using the primer pairs *rbc*L start/R750 and F650/*rbc*Lend from Shimada et al. (2003), with the final sequences trimmed to 1355 base pairs (bp) to remove primer sequences; plastid encoded *tuf*A was amplified using the primer pair *tuf*G4/*tuf*AR (Saunders and Kucera 2010), with the final sequence trimmed to 774 bp; nuclear encoded partial SSU, complete ITS1, complete 5.8S, complete ITS2, and partial LSU were sequenced using the primers ITS1 and ITS4 (Shimada et al. 2003), with sequences trimmed to 674 bp. Amplification protocols for all PCR reactions followed Hughey et al. (2001). PCR products were cleaned with the Qiagen PCR Purification Kit,

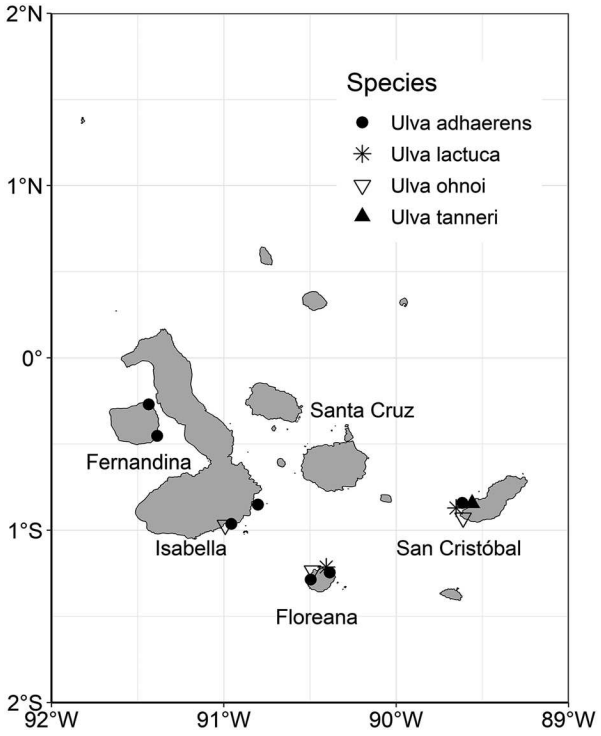

Figure 1: Map of the Galápagos Archipelago with major islands named and where *Ulva* specimens were collected.

cycle sequenced, and sent to the DNA Analysis Core Facility at the Marine Sciences Center, University of North Carolina, Wilmington for Sanger sequencing. Sequences were manually aligned and compiled using Sequencher 5.4.6 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Three datasets were prepared, one for each of the sequenced loci, and all were compiled and aligned using MUSCLE (Edgar 2004) as implemented in Geneious Prime (2020.2.4, Biomatters, Auckland, New Zealand) and checked manually. Named sequences of *Ulva* were downloaded from GenBank to compare to sequences from Galápagos specimens, 50 of ITS ranging in length from 272 bp (ITS2 was available only for *U.* '*sublitoralis*' Segawa) to 807 bp (depending what proportion of flanking SSU and LSU sequences were included); 50 of *rbc*L ranging in length from 626–1355 bp; 47 of *tuf*A ranging in length from 680 to 774 bp. Not all of the same loci have been sequenced for all of the *Ulva* species used in the phylogenetic analyses – for some species only one of the markers is present in GenBank; for others only two are present (see Supplementary Table S1). Because the datasets did not contain the same taxa, each was analyzed separately. For all datasets three taxa from the family Ulvaceae were used as outgroups, *Percursaria* '*percursa*' (C.Agardh) Rosenvinge, *Ulvaria* '*obscura* var. *blyttii*' (Areschoug) Bliding and *Umbraulva* '*japonica*' Bae *et* I.K.Lee. The application of these names to sequences in GenBank may or may not be correct.

Phylogenetic reconstructions with Maximum Likelihood (ML) and Bayesian Inference (BI) for all data sets were carried out using the RAxML (Stamatakis 2014) and MrBayes (Huelsenbeck and Ronquist 2001) plugins in Geneious Prime (2020.2.4, Biomatters, Auckland, New Zealand), respectively. The RAxML analyses were performed using the GTR + CAT + I model and rapid hill-climbing algorithm for 20 random trees to determine the best starting tree for determining node confidence. Node confidence was then assessed by 1000 bootstrap replicates and search for best-scoring ML tree. Bayesian analyses were performed using the GTR + gamma + invariable sites model with four heated Monte-Carlo Markov Chains for 1,000,000 generations, sampling every 750 generations and with a burn-in period of 250,000 generations.

3 Results

For each of the three loci, the ML and BI phylograms were mostly congruent; only the BI phylograms (Figures 2, 3 and 4) are presented with ML bootstrap values shown at the nodes when ≥75 % and posterior probabilities when ≥0.8. The Galápagos species occurred in the same major clades within *Ulva* for all three loci (Figures 2, 3 and 4). *Ulva adhaerens* consistently occurred in a clade with *U. rigida* C.Agardh, and in *rbc*L was sister to *U. piritoka* Kuri, Heesch *et* W.A.Nelson for which only a *rbc*L sequence was generated (Figure 2). *Ulva lactuca* and *U. ohnoi* consistently occurred in a clade with *U. conglobata* Kjellman, *U. dactylifera* Setchell *et* N.L.Gardner, *U. lacinulata* (Kützing) Wittrock and *U. taeniata* (Setchell) Setchell *et* N.L.Gardner, with support ranging from moderate (ITS RAxML, Figure 3) to strong (*rbc*L RAxML, Figure 2) to full (*rbc*L BI, Figure 2 and *tuf*A RAxML and BI, Figure 4). Sister taxon relationships among these species varied depending on the locus that was sequenced. *Ulva tanneri* occurred in a clade that was more variable with various combinations of *U. californica* Wille, *U. clathratioides* L.G.Kraft, Kraft *et* R.F.Waller, *U.* '*aragoënsis*' (Bliding) Maggs and *U.* '*torta*' (Mertens) Trevisan that lacked support from RAxML and with good to strong support from BI (Figures 2, 3 and 4). Note also that the correct application of the names *U.* '*torta*' and *U.* '*aragoënsis*' is uncertain, as type material has not been sequenced. Despite its equatorial location, none of the *Ulva* species found in the Galápagos occurs in the large and predominantly tropical to warm water clade (Figures 2, 3 and 4) of 14 species (*U. arbuscula* Lagourgue *et* Payri, *U. batuffulosa* Lagourgue *et* Payri, *U. finissima* Lagourgue *et* Payri, *U. iliohaha* H.L.Spalding *et* A.R.Sherwood, *U. kraftiorum* Huisman, *U. meridionalis* R.Horimoto *et* S.Shimada, *U. pennata* Lagourgue *et* Payri, *U. planiramosa* Lagourgue *et* Payri, *U. pluriramosa* Lagourgue *et* Payri, *U. scolopendra* Lagourgue *et* Payri, *U. siganiphylla* Lagourgue *et* Payri, *U. spumosa* Lagourgue *et* Payri, *U. tentaculosa* Lagourgue *et* Payri, *U. tepida* Masakiyo *et* S.Shimada, and *U. vietnamensis* L.Tran, Leliaert *et* De Clerck).

The DNA sequencing results for all of the markers identified unequivocally the Galápagos *Ulva* specimens to be one of four species, *U. adhaerens*, *U. lactuca*, *U. ohnoi* or *U. tanneri* (Figures 2, 3 and 4). 20 specimens of *U. adhaerens* (Supplementary Figures S1 and S2) were collected from sites on all four islands, and 15 were sequenced for *rbc*L with 11

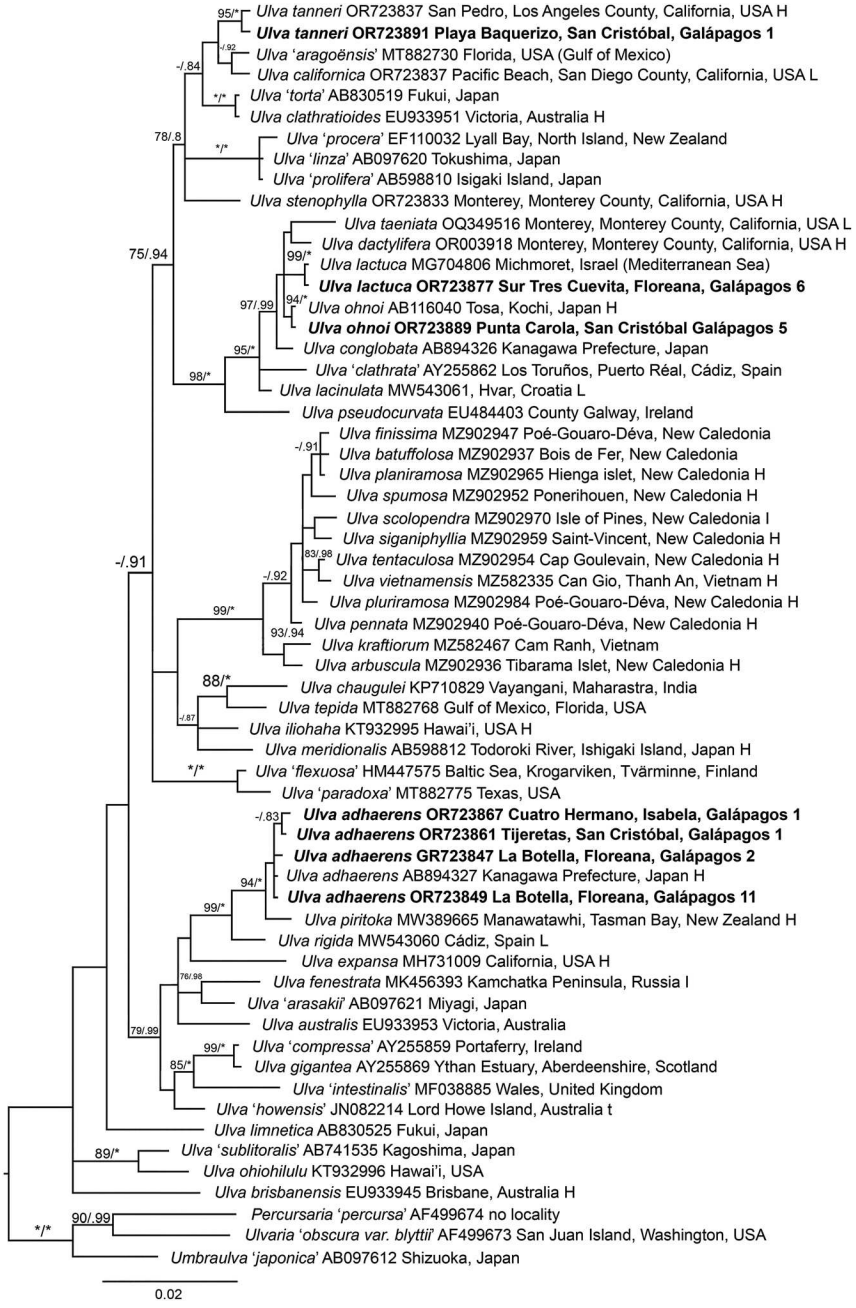

Figure 2: Phylogram of *Ulva* species based on *rbc*L sequences. RAxML and Bayesian analyses yielded the same topology. Branch lengths are from ML analysis. The topology is rooted with outgroups *Percursaria* '*percursa*', *Ulvaria* '*obscura* var. *blyttii*' and *Umbraulva* '*japonica*' (family Ulvaceae). Galápagos sequences in bold; species names in single quotation marks have not had their type specimens sequenced. Support values at each node are shown as bootstrap percentage/Bayesian posterior probability. Bootstrap percentages (nreps = 1000) are shown when ≥75 %; Bayesian posterior probabilities are shown when ≥0.8, and *indicates full support. The single letters at ends of some localities indicate: H, holotype; I, isotype; L, lectotype; t, topotype. Numbers at end of Galápagos sequences indicate total number of identical sequences.

identical to the holotype sequence from Japan and four differing by 1–2 bp; 15 were sequenced for *tuf*A, eight were identical and seven differed by 1–2 bp; 10 were sequenced for ITS and varied by 2–5 bp from the holotype sequences from Japan. For *U. lactuca* six specimens (Supplementary Figure S3) were collected from three sites on Isla San Cristóbal and one site on Isla Floreana. All six *rbc*L sequences were identical to each other and to a sequence from the Mediterranean Sea that is linked to the lectotype sequence of *U. lactuca*, and likewise for *tuf*A, except for two ambiguous bp in one Galápagos sequence. Three ITS sequences were obtained and differed from each other by 1–3 bp with one sequence identical to a *U. lactuca* sequence from Chile. Five specimens of *U. ohnoi* (Supplementary Figure S4) were collected from three sites on Isla San Cristóbal and one each from Isla Isabela and Isla Floreana, and the *rbc*L sequences from these were identical to the *rbc*L holotype sequence from Japan. Likewise, the five *tuf*A sequences were identical to a *U. ohnoi tuf*A sequence from a specimen from Queensland Australia that can be linked to the *U. ohnoi* holotype specimen. The three Galápagos ITS sequences were identical to the ITS sequence from the holotype specimen. Only one

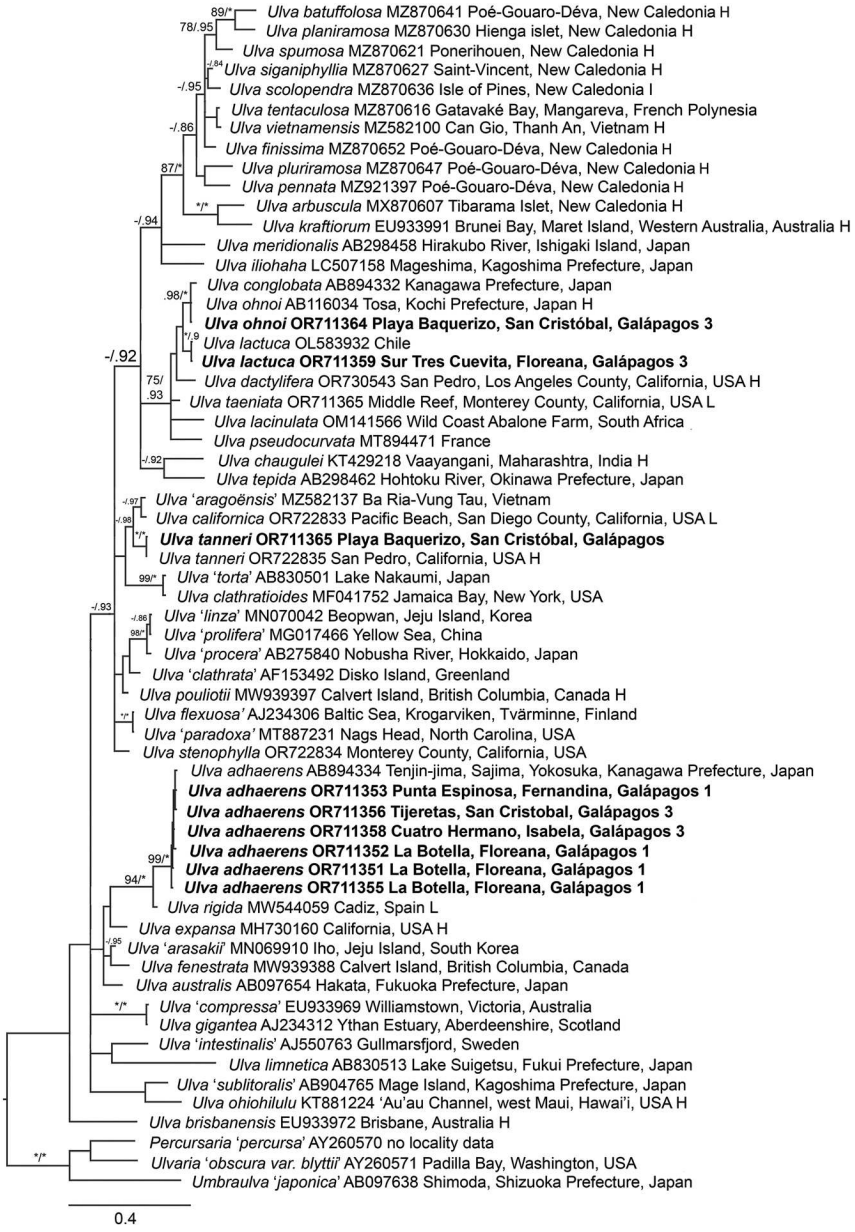

Figure 3: Phylogram of *Ulva* species based on ITS sequences. RAxML and Bayesian analyses yielded the same topology. Branch lengths are from ML analysis. The topology is rooted with outgroups *Percursaria* '*percursa*', *Ulvaria* '*obscura* var. *blyttii*' and *Umbraulva* '*japonica*' (family Ulvaceae). Galápagos sequences in bold; species names in single quotation marks have not had their type specimens sequenced. Support values at each node are shown as bootstrap percentage/Bayesian posterior probability. Bootstrap percentages (nreps = 1000) are shown when ≥75 %; Bayesian posterior probabilities are shown when ≥0.8, and *indicates full support. The single letters at ends of some localities indicate: H, holotype; I, isotype; L, lectotype. Numbers at end of Galápagos sequences indicate total number of identical sequences.

specimen of *U. tanneri* was collected from Playa Baquerizo, Isla San Cristóbal (Supplementary Figure S5), and its *rbc*L, *tuf*A and ITS sequences were identical to these same sequences from the holotype specimen of *U. tanneri* (=*Chloropelta caespitosa* Tanner).

4 Discussion

As a preface to the discussion, what is evident is that all blade-forming *Ulva* species worldwide are cryptic due to their simple construction as a two-cell layered blade and their morphological plasticity. It is very difficult to confidently identify *Ulva* species based on morpho-anatomy, either macroscopically (e.g., size and shape of blades, presence/absence of marginal teeth on blade) or microscopically (e.g., cell size, cell shape, number of pyrenoids/cell). All of these characters have been used historically to identify *Ulva* specimens to species. An example of the difficulty of identifying *Ulva* specimens using morpho-anatomy are the studies by Chávez-Sánchez et al. (2017, 2019) in the Gulf of California. In the first paper (Chávez-Sánchez et al. 2017), eight *Ulva* species were identified by morpho-anatomy: *U.* '*acanthophora*' (Kützing) H.S.Hayden, Blomster, Maggs, P.C.Silva, Stanhope *et* Waaland, *U.* '*clathrata*' (Roth) C.Agardh, *U.* '*intestinalis*' L., *U.* '*flexuosa*' Wulfen, *U. lactuca*,

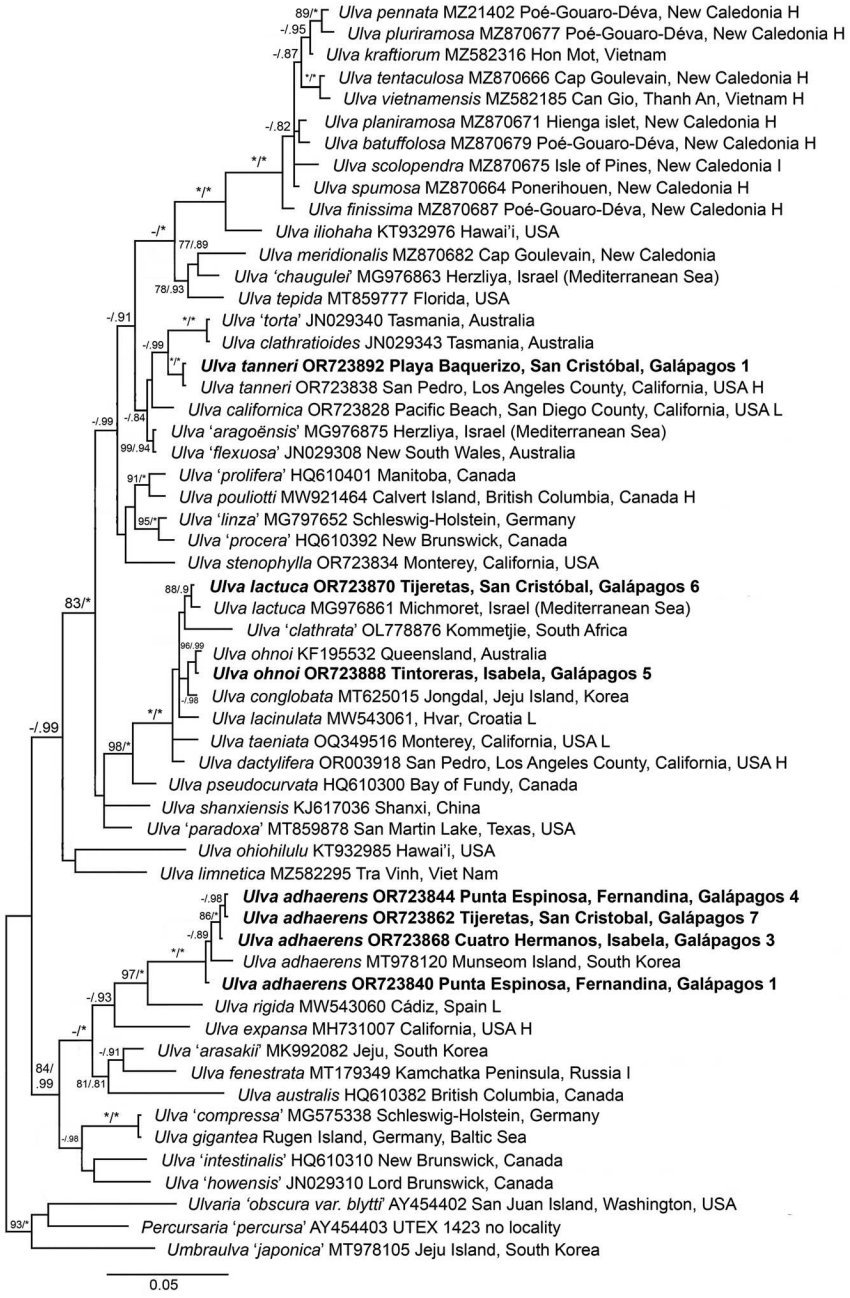

Figure 4: Phylogram of *Ulva* species based on *tuf*A sequences. RAxML and Bayesian analyses yielded the same topology. Branch lengths are from ML analysis. The topology is rooted with outgroups *Percursaria 'percursa'*, *Ulvaria* '*obscura* var. *blyttii*' and *Umbraulva 'japonica*' (family Ulvaceae). Galápagos sequences in bold; species names in single quotation marks have not had their type specimens sequenced. Other details as in Figure 3 legend.

U. lobata (Kützing) Harvey, *U. nematoidea* Bory and *U. rigida*. Subsequently these same specimens were sequenced using the barcoding markers *rbc*L, *tuf*A and ITS2 (Chávez-Sánchez et al. 2019). Specimens previously identified by morpho-anatomy as *U. lactuca* and *U. rigida* were *U. ohnoi* by DNA sequencing; *U.* '*intestinalis*' was *U.* '*tepida*'; *U.* '*clathrata*' and *U. 'flexuosa'* were *U.* '*torta*'; *U.* '*acanthophora*' matched no sequenced *Ulva* specimens in publicly available databases; and *U. lobata* and *U. nematoidea* specimens failed to amplify. Thus, none of the specimens that could be sequenced had been correctly identified by morpho-anatomical characters.

Named *Ulva* specimens in herbaria that have not been sequenced may or may not be correctly identified. Sequences in GenBank of species whose type specimens have not been sequenced to apply names correctly also may not be correctly identified. If specimens in a local flora have been sequenced, such that one understands the habitat and seasonality of each *Ulva* species present, then it may be possible to identify these species based on morpho-anatomical

characters. But, to our knowledge, this comprehensive sequencing has not been done for any local *Ulva* flora anywhere in the world. Moreover, if a species of *Ulva* is subsequently introduced to a flora, it may not be recognized.

For the Galápagos Archipelago, the green algal checklist compiled by Ruiz and Ziemmeck (2014) listed the following *Ulva* species that had been identified historically using morpho-anatomy: *U. fasciata* Delile (type locality: Alexandria, Egypt) that was first reported by Farlow (1902), *U. lactuca* (type locality: unknown, but possibly the Indo-Pacific), *U. lobata* (type locality: Chile), and *U. taeniata* (type locality: Monterey, California, USA), all reported by Taylor (1945), the last as *U. dactylifera*. However, based on DNA sequencing of type specimens, Hughey et al. (2019) showed that the names *U. fasciata* and *U. lobata* are junior, heterotypic synonyms of *U. lactuca,* thereby reducing the *Ulva* species recognized by morpho-anantomy in the Galápagos Archipelago to two species, *U. lactuca* and *U. dactylifera*.

In contrast, DNA sequences from field-collected, blade-forming, *Ulva* specimens in the Galápagos Archipelago are identical to or highly similar (>99.5 %) to sequences from the type specimens of *U. adhaerens*, *U. lactuca*, *U. ohnoi* and *U. tanneri*, conclusively documenting their presence. It is indeed fortunate that type specimens of each of these species have been sequenced, particularly for *U. lactuca* and *U. tanneri* that were described before the advent of DNA sequencing to identify seaweed species. Linnaeus (1753) described *U. lactuca*, the generitype of *Ulva*, and Tanner (1980) described *U. tanneri* (as *Chloropelta caespitosa*). The *rbc*L sequence of the lectotype of *U. lactuca* was made available in a public database (GenBank) and reported in Hughey et al. (2019), and the *rbc*L, *tuf*A and ITS sequences from the holotype of *U. tanneri* (=*C. caespitosa*) were recently published (Hughey et al. 2024). Anslan et al. (2021) found these same species, although they used the designation '*Ulva* sp.' for *U. adhaerens.*

Ulva adhaerens was originally described in 2015 from Tenjin-jima, Kanagwa Prefecture, Japan (Matsumoto and Shimada 2015). The study was undertaken to examine small sized (2–4 cm tall) *Ulva* specimens that, when sequenced for ITS and *rbc*L, comprised four different species: *Ulva* sp. 1, later shown by Hughey et al. (2021) to be *U. conglobata*; *U. pertusa* Kjellman (=*U. australis* Areschoug); *U. tanneri*; and *Ulva* sp. 2 that was named *U. adhaerens* due to the presence of rhizoids linking adjacent blades. *Ulva adhaerens* was known only from its type locality until some sequences were recently deposited in GenBank from Munseom Island, Korea (*rbc*L MT978111-MT978113 and *tuf*A MT978120--MT978122). Thus, it was surprising, not only to find *U. adhaerens* in the Galápagos, but also that it was the *Ulva* species that we found most commonly (20 samples) and was the only species that occurred on all four islands where we collected, namely Isla Fernandina, Isla Floreana, Isla Isabela, and Isla San Cristóbal (Supplementary Table S1). All of the *U. adhaerens* specimens that were collected were small in size ranging from 1 to 4 cm tall (Supplementary Figures S1 and S2), although this size range was typical for nearly all of *Ulva* species collected.

The two *rbc*L sequences from the holotype collection of *U. adhaerens* both have a large (2521 bp) Group II intron within the gene. Interestingly, this intron was not found in any of the 17 *rbc*L sequences that we obtained from Galápagos specimens. We used the *rbc*L primer pairs designed by Shimada et al. (2003) to sequence this gene from all *Ulva* specimens that we collected. If this intron had been present, we would not have been able to sequence the *rbc*L gene due to the size of the intron. Thirteen of our *U. adhaerens* specimens had *rbc*L sequences identical to *U. adhaerens* from Japan when the intron is removed. Two of four specimens from one site, La Botella, on the west coast of Isla Floreana, differed by the same SNP (single nucleotide polymorphism), and the specimen from Cuatro Hermanos, on Isla Isabela differed by two other SNPs, one shared with one of the specimens from Tijeretas, on Isla San Cristóbal. All *rbc*L sequences from *U. adhaerens* in the Galápagos differed from each other at most by 2 bp.

Ulva piritoka from New Zealand was proposed by Heesch et al. (2021) based only on a *rbc*L sequence, and is sister to *U. adhaerens*. It differs over its 1355 bp *rbc*L sequence by 5 bp from *U. adhaerens*, and none of these base-pair differences are found in any of the various haplotypes of *U. adhaerens.* Given that *U. lactuca* and *U. ohnoi* typically differ by 3 bp over the same length of *rbc*L, *U. piritoka* is recognized as a distinct species from *U. adhaerens.*

Ulva lactuca, first reported for Galápagos Archipelago by Taylor (1945), is present, based on our DNA sequencing, on two of the four islands, Isla San Cristóbal and Isla Floreana (Supplementary Figure S3), where blade-forming *Ulva* species were collected. Whether historical specimens in MICH or CDS (herbarium acronyms follow Thiers 2024) are indeed *U. lactuca* needs to be confirmed by DNA sequencing. However, this may not be possible with Taylor's specimens as they were preserved in formaldehyde before being pressed. DNA from specimens preserved in this manner has been very difficult to amplify. *Ulva lactuca* occurs in Chile and Perú based on DNA sequenced specimens, so its presence in the archipelago is not surprising.

Another species that was found by both the present study and Anslan et al. (2021) was *U. ohnoi. Ulva ohnoi* (type locality: Tosa Bay, Tosa, Kochi Prefecture, Japan) was described by Hiraoka et al. (2003). Previously, Ohno (1988), while studying a "green tide" (a bloom of one or more *Ulva*

species that are free-floating) of *U. australis* (as *U. pertusa*) in southeastern Japan, proposed that another species, "*Ulva* sp.," was present based on temporal and physiological differences. Hiraoka et al. (2003) then performed crossing experiments between the two species and observed that the gametes did not cross, supporting Ohno's hypothesis. They then described *Ulva* sp. as a new species, *U. ohnoi*, based on ITS and *rbc*L sequences from field-collected and cultured specimens that were attached at Tosa Bay and unattached at Naminoue Beach (Okinawa Prefecture, Japan). Subsequently, *U. ohnoi* has been reported from the Northwest Pacific Ocean: Japan (Suzuki et al. 2018) and South Korea (Kang et al. 2019), attached and unattached; North Central Pacific Ocean: Hawai'i, USA (O'Kelly et al. 2010, attached); Northeast Pacific Ocean: Gulf of California, Mexico (Chávez-Sánchez et al. 2019, attached); Southwest Pacific Ocean: temperate Australia (Kirkendale et al. 2013, habitat not reported); Northwest Indian Ocean: Gujarat and Maharastra States, India (Kazi et al. 2016, attached) and Persian Gulf, Iran (Pirian et al. 2016, attached); Northwest Atlantic Ocean: Florida, USA (Melton et al. 2016a, attached and unattached); Gulf of Mexico: Alabama and Texas, USA (Melton et al. 2016a, attached and unattached); Caribbean Sea: Venezuela (Melton et al. 2016b, attached); Mediterranean Sea: Italy, Tunisia (Miladi et al. 2018) and Israel (Krupnik et al. 2018), all attached.

The Galápagos Archipelago *U. ohnoi* specimens were collected from the islands of Floreana, Isabela and San Cristóbal, all the islands from which samples were obtained except for Fernandina. All specimens were epilithic and found from the low intertidal to the subtidal (5 m maximum depth). These specimens were also small in size ranging up to 2–3 cm tall (Supplementary Figure S4). Based on the *tuf*A marker, Melton et al. (2016a,b) identified three haplotypes in *U. ohnoi*. All of the Galápagos specimens belong to haplotype 2, which also contains the holotype specimen from Tosa Bay, Japan whose plastid genome (Suzuki et al. 2018) is publicly available in GenBank (AP018696) and from which we extracted the *tuf*A sequence. This haplotype is also found in the Northwest Atlantic (Biscayne Bay, Florida, USA), Gulf of Mexico (Florida, Texas, USA; Yucatan, Mexico, Melton et al. 2016a,b) and the Southwest Pacific (New South Wales, Australia; GenBank JN029329, Kirkendale et al. 2013). Haplotype 1 is also found in the Gulf of Mexico and in Australia, whereas haplotype 3 is only found in Australia (Melton et al. 2016a,b).

Melton et al. (2016a,b) hypothesized that *U. ohnoi* was non-native to Atlantic Florida and the Gulf of Mexico due to the low genetic diversity found in the *tuf*A and ITS1 markers compared to the global genetic diversity. We observed a similar low genetic diversity for the Galápagos specimens of *U. ohnoi*, but collecting was limited to four islands, and a sample size of five is far too low to support any hypothesis about the origins of this species in the archipelago. What is needed, and would be helpful in understanding how long this species has been in the Galápagos in recent historical time, is to sequence *Ulva* herbarium specimens collected in the 20th Century to see if any of those specimens is *U. ohnoi*, as well as a greater sampling effort throughout the archipelago.

All specimens of *U. ohnoi* in the Galápagos were attached. This species has been reported to cause green tides (blooms of unattached specimens) in bays in Japan, South Korea and the USA, but in all other countries the species grows attached. No green tides of *U. ohnoi*, nor of any other species of *Ulva*, have been reported from the Galápagos Archipelago.

Ulva tanneri was originally described as *Chloropelta caespitosa* Tanner (1980) from epilithic specimens in the upper littoral zone from San Pedro, Los Angeles County, California, USA. It was established as a monotypic genus based on a different developmental pattern from other *Ulva* species to form the characteristic distromatic blade. Tanner (1980) characterized *C. caespitosa* as forming dense tufts with orbicular, peltate or split thalli ranging in size from a few mm to 60 mm. He also cited specimens from Los Angeles, Laguna, La Jolla and Pacific Beach, California. Stewart (1991) included *C. caespitosa* in her survey of the marine algae and seagrasses of San Diego County, California based on morpho-anatomy. Joska and Bolton (1992) examined and cultured thalli from Dalebrook, South Africa, and concluded they represented *C. caespitosa*. Using morphology and culture studies, Lima and Fukusumi (1996) reported *C. caespitosa* from Japan.

Based on phylogenetic analyses using ITS sequences and *rbc*L gene sequences of *C. caespitosa* from Kobe, Japan, Hayden et al. (2003) concluded that *Chloropelta* should not be recognized as a separate genus. They proposed the new combination, *Ulva tanneri* H.S.Hayden *et* Waaland, the currently accepted name. Additional molecular studies have supported the transfer, including the analysis of specimens from Monterey, California, USA (Hayden and Waaland 2004), Brisbane, Australia (Kraft et al. 2010) and North Island, New Zealand (Nelson et al. 2021). Based on morpho-anatomy, Wysor (2004) included *U. tanneri* (as *C. caespitosa*) in his annotated list of marine Chlorophyta from Panamá, and Huisman et al. (2007) also recorded *U. tanneri* from Hawai'i, USA. Fernández-García et al. (2011) also reported *U. tanneri* from Costa Rica.

DNA sequences generated from the holotype specimen of *U. tanneri* (Hughey et al. 2024) are the same as those named *U. tanneri* in GenBank, showing that they were

correctly identified. The phylogenetic analyses published by Hayden et al. (2003) using ITS and *rbc*L DNA sequences placed *U. tanneri* sister in position to *U. californica* and *Enteromorpha* sp. 1. A following study (Hayden and Waaland 2004), using the same two genetic markers, resolved *U. tanneri* in a clade sister to *U. californica* and *U.* '*prolifera*' O.F.Müller. Kraft et al. (2010), in an investigation of *Ulva* from southern Australia also using ITS and *rbc*L sequences, found that their new species *U. clathratioides* and *U. tanneri* were closely related, and sister to *U. californica*. More recent analyses using *rbc*L sequences (Hughey et al. 2019, 2021, 2022) are consistent with these previous results as well as the findings reported herein. They show that *U. tanneri* occupies a strong (BI) to unsupported (RAxML) sister taxon relationship to *U. clathratioides* in the *rbc*L and *tuf*A phylograms, also unsupported in ITS.

In addition to previously published DNA sequences of *U. tanneri* from California, USA, Japan, and eastern Australia and New Zealand, the data herein confirm that *U. tanneri* is present in the Galápagos Archipelago. The specimens are small (<1 cm tall, Supplementary Figure S5), but this is typical for species found in high intertidal habitats. This and Anslan's et al. (2021) finding of *U. tanneri* on Isla San Cristóbal should not be used to infer that *U. tanneri* is a recent introduction to the Galápagos Archipelago. Rather, it is likely that this species has been present historically, but has been misidentified in collections by using morpho-anatomy or, more likely, is not represented in historical collections. High intertidal macroalgae frequently are overlooked entirely or not collected in surveys as they are assumed to be depauperate specimens of species found lower in the intertidal. Still, historical voucher specimens of *Ulva* species in herbaria should be sequenced to determine their correct identities and to test hypotheses about how long *U. adhaerens, U. ohnoi* and *U. tanneri* have been present in the Galápagos Archipelago.

Despite its historical biological importance and the uniqueness of the terrestrial flora and fauna of the Galápagos Archipelago, the seaweed flora is very poorly known. Only one of the blade-forming *Ulva* species, *U. lactuca*, previously reported for the archipelago based on morpho-anatomical characters, is confirmed with DNA sequencing. This study and Anslan et al. (2021) have demonstrated for the first time the presence of *U. adhaerens*, *U. ohnoi* and *U. tanneri* not only from the Galápagos Archipelago, but from the entire southeast Pacific Ocean. A concerted effort is needed to document, with DNA sequencing, the seaweed flora of this treasured and unique archipelago.

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Research ethics: Procedures are/were in accordance with national laws.

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Data availability: The raw data can be obtained on request from the corresponding author.

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Bionotes

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