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Gracilaria parva sp. nov. (Gracilariales, Rhodophyta) a Diminutive Species from the Tropical Eastern Pacific

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Abstract: DNA sequencing of the plastid encoded *rbc*L gene supported by morpho-anatomical features reveals *Gracilaria parva* sp. nov. from Panama and Ecuador in the tropical eastern Pacific Ocean. In the *rbc*L phylogram, *G. parva* occurs in a clade sister to the western Atlantic species *G. galatensis*. Morphologically and anatomically, *G. parva* is distinguished from two similar, described tropical eastern Pacific species, *G. brevis* and *G. veleroae* by its small size, to 2.5 cm tall with branch widths mostly <2 mm occasionally to 4 mm, and by its two to three cell layered cortex. *Gracilaria brevis* and *G. veleroae* are taller, have wider branches, and a one cell layered cortex. DNA sequencing is needed to resolve the many diminutive species in the tropical eastern Pacific, particularly those occurring in turf communities. DNA sequencing of historical type specimens from the 19th and 20th centuries is also needed to correctly apply names in this region.

Keywords: DNA sequencing; Ecuador; Gracilariaceae; morpho-anatomy; Panama; *rbc*L

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1. Introduction

Gracilaria Greville is an economically important genus of red algae as a producer of agarans [1]. It is a speciose genus, currently with 193 species recognized primarily from tropical and subtropical waters worldwide [2]. Distinguishing species of *Gracilaria* has been vexing [3–5] due to their simple morphology primarily as flat, dichotomously to irregularly branched blades, or as terete, irregularly branched, thalli [6,7]. About 40% of the species of *Gracilaria* were named in the 19th century; the rest more recently, particularly starting in the first decade of the 21st century with the use of DNA sequences to distinguish species. The epicentre of this recent work has been the tropical western Atlantic where 11 new blade-forming species of *Gracilaria* have been named [5,8–13].

The adjacent tropical eastern Pacific, in contrast, has received little attention. Historically, Taylor [14] named several species of *Gracilaria* while en route to the Galapagos Archipelago on the *Valero III*, and Dawson [15–18] described and listed numerous species in the northeast Pacific. Eleven species of *Gracilaria* have been reported from the tropical eastern Pacific mainland coasts of Mexico south through Ecuador (Table 1). Some of these are likely temperate species reaching their southern limits on the Mexican coast, or incorrect identifications of species with Atlantic Ocean type localities.

Table 1. Distribution of species of *Gracilaria* reported from the mainland coasts of tropical eastern Pacific countries. Reports from Guatemala and Nicaragua were not found. Numbers are reference citations.

Species	Mexico	El Salvador	Costa Rica	Panama	Colombia	Ecuador
G. brevis W.R. Taylor				[19]		[14]
G. crispata Setchell and N.L. Gardner	[17]	[18]	[16]			
G. domingensis (Kützing) Sonder						[20], p. 187
G. ecuadoreana (W.R. Taylor) E.Y. Dawson		[18]				
G. johnstonii Setchell and N.L. Gardner	[14]					
G. linearis Kylin						[14]
G. pinnata Setchell and N.L. Gardner	[14]					
G. spinigera E.Y. Dawson			[21]			
G. symmetrica E.Y. Dawson	[17]				[22]	
G. tepocensis (E.Y. Dawson) E.Y. Dawson		[18]	[16] [a]			
G. veleroae E.Y. Dawson	[17]					

[a] reported as *G. crockeri* E.Y. Dawson.

DNA sequence data have never been applied to studies of tropical eastern Pacific species of *Gracilaria*. Only one floristics paper utilized molecular assisted identification, reporting three unnamed species of *Gracilaria* from a single locality in Panama [23]. Subsequent study has revealed that one of these unidentified species was morphologically distinct from the eleven species of *Gracilaria* previously reported in the region. Herein we describe a new tropical eastern Pacific species of *Gracilaria* based on DNA sequences and the morpho-anatomy of specimens collected at sites along the coasts of Panama and mainland Ecuador.

2. Materials and Methods

Samples were collected by hand using snorkel or SCUBA. Voucher specimens were pressed in the field and a fragment was removed, patted dry and placed into a silica gel desiccant, unless the specimens were small and then all material was desiccant-dried in the field and vouchers taken in the lab. Duplicate Ecuadoran voucher specimens from the same individual were deposited in NCU and at USFQ and Panamanian specimens were deposited at WNC and PMA. Herbarium acronyms follow Index Herbariorum online [24]. See Table 2 for collection data and GenBank accession numbers. Additional information for some specimens is available through the BOLD system public database (doi: 10.5883/DS-RAPBPAN).

Table 2. *Gracilaria parva* sp. nov., specimen collection information and GenBank Accession numbers.

Collection No.	Voucher	Collection Information	GenBank Accession No.		
			*rbc*L	COI-5P	UPA
PHYKOS-4630	WNC-34055	Southwest side of Isla Burica, Chiriqui, Panama, 08.01865° N 082.88390° W, ca. 6 m depth, 10.i.11, collected by D.W. Freshwater.	KY573993	KY656553	KY573953
PHYKOS-4630	NCU-675206	Southwest side of Isla Burica, Chiriqui, Panama, 08.01865° N 082.88390° W, ca. 6 m depth, 10.i.11, collected by D.W. Freshwater.	-	-	-
PHYKOS-4541	WNC-34186	Near Mono Feliz, Punta Burica, Chiriqui, Panama, 08.03042° N 082.87574° W, 3 m depth, 08.i.11, collected by B.L. Wysor and D.W. Freshwater.	KY573973	KY656537	KY573932
PHYKOS-4519	WNC-34181	Near Mono Feliz, Punta Burica, Chiriqui, Panama, 08.03042° N 082.87574° W, 3 m depth, 08.i.11, collected by B.L. Wysor and D.W. Freshwater.	-	KY656532	KY573927
PHYKOS-6833	WNC-34185	Northwest side of Sombrero Rock Cove, Isla Cebaco, Veraguas, Panama, 07.48229° N 081.25705° W, 17 m depth, 20.i.12, collected by D.W. Freshwater.	OL690493	OL690495	OL690494
PHYKOS-6870	WNC-34184	Cebaco Bay rock pile, Isla Cebaco, Veraguas, Panama, 07.49173° N 081.22262° W, 14 m depth, 20.i.12, collected by D.W. Freshwater.	OL690496	OL690498	OL690497
E438	NCU-675207	La Chocolatera, Santa Elena Province, Ecuador, 02.18667° S 081.00583° W, 21.i.2021, epilithic low intertidal, collected by A. Eguiguren and M. Brandt.	OL690499	-	-
E438	USFQ #438	La Chocolatera, Santa Elena Province, Ecuador, 02.18667° S 081.00583° W, 21.i.2021, epilithic low intertidal, collected by A. Eguiguren and M. Brandt.	-	-	-

Whole mounts and slides of sectioned material were stained and mounted with aniline blue as described in Millar and Wynne [25]. Sections were made by hand using a razor blade while viewing through a stereomicroscope. Images were captured using a Zeiss Axio

Imager Z1 compound microscope fitted with an AxioCam MRc 5 camera system (Carl Zeiss Microimaging Inc., Thornwood, NY, USA).

DNA sequence data were generated from the studied specimens in two different laboratories and at various times from 2011 to 2021. Total genomic DNA was extracted and amplified from specimens at the University of North Carolina, Chapel Hill using the method in Hughey et al. [26]. Plastid encoded *rbc*L was amplified in two sections using the primer pairs F57/R1150 and F753/RrbcSstart [27], with the final sequences trimmed at the 3′ terminus of the gene. PCR products were cleaned with the Qiagen PCR Purification Kit, cycle sequenced, and sent to the DNA Analysis Core Facility at the Center for Marine Science, University of North Carolina at Wilmington for final sequencing. Sequences were manually aligned and compiled using Sequencher 5.4.6 (Gene Codes Corp., Ann Arbor, MI, USA).

Total genomic DNA was extracted from specimens at the Center for Marine Science, University of North Carolina at Wilmington using methods described in Hughey et al. [26], Freshwater et al. [23] or Taylor et al. [28]. Amplification and sequencing of loci followed the methods of Taylor et al. [28] and Freshwater et al. [23]. Primer pairs for *rbc*L were F57/R1144 or R1150 and F753/RrbcSstart [23,27,29]; for COI-5P were GWSFn/GWSRx [30,31]; for UPA were p23r-V-f1/p23r-V-r1 [32].

The *rbc*L tree included sequences of species of *Gracilaria* recovered in a BLAST search of GenBank that had a >95% identity to the studied tropical eastern Pacific specimens. Sequences from GenBank were trimmed to 1416 bp in length. *Gracilariopsis carolinensis* L.M. Liao and Hommersand was used as the outgroup. The dataset was compiled and aligned using MUSCLE [33] as implemented in Geneious Prime (2020.2.4, Biomatters, Auckland, New Zealand).

Phylogenetic reconstructions with maximum likelihood (ML) and Bayesian inference (BI) were carried out using the RAxML [34] and Mr Bayes [35] Geneious Prime plugins, 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 replications and search for best-scoring ML tree. Bayesian analyses were performed using the GTR + gamma + invariable sites model with 4 heated Monte-Carlo Markov Chains for 1,000,000 generations, sampling every 750 generations and with a burn-in length value of 250,000 generations.

3. Results

3.1. Molecular Analyses

Specimens from Panama and Ecuador of the studied tropical eastern Pacific species of *Gracilaria* were resolved in a clade with two western Atlantic species, *G. gurgelii* Freshwater from the northwest Gulf of Mexico and *G. galatensis* Gurgel, Fredericq and J.N. Norris from the Caribbean coast of Panama. The clade had full support in the Bayesian analysis but was unsupported in the RAxML analysis (Figure 1). This clade was sister to a clade of mostly tropical to subtropical western Atlantic and eastern Pacific species, also with full support in the Bayesian analysis, but was unsupported in the RAxML analysis (Figure 1).

Blast results from GenBank showed that the closest taxon match to the tropical eastern Pacific species was 97.76% for a sequence of *G. galatensis* from the Caribbean coast of Panama. Although they occurred on two different coasts, the Caribbean and the Pacific coasts of Panama, these species were sister taxa in both analyses, with full support in the Bayesian analysis, but lacking support in the RAxML analysis. The genetic distance of over 2% in *rbc*L sequence from all other species of *Gracilaria*, indicated that the Pacific entity is a distinct species that is described below.

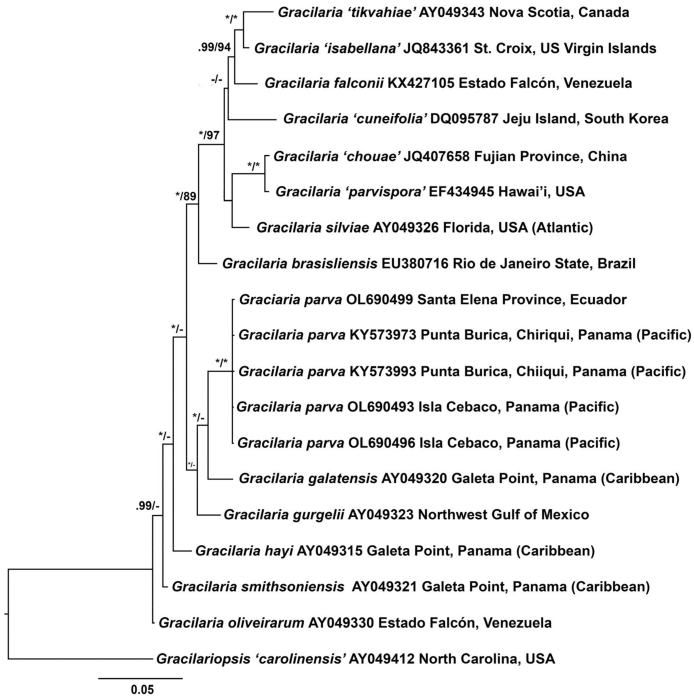

Figure 1. Bayesian phylogram of *rbc*L sequences showing placement of *Gracilaria parva* in a clade with tropical western Atlantic species. The species of *Gracilaria* in single quotation marks (' ') have not had their type specimens sequenced. Support values at phylogram nodes are posterior probabilities (PP) followed by bootstrap percentages (BP): * at nodes indicate full support; - at nodes indicate support values below 0.8 PP and 80% BP that are not shown.

3.2. New Species Description

Gracilaria parva Freshwater, B. Williamson, P.W. Gabrielson and Margarita Brandt sp. nov. Figures 2–4.

DESCRIPTION: Thalli were coriaceus, rose to dark or brownish red in colour, and fixed to hard substratum by small discoidal holdfasts that gave rise to one or several generally recumbent axes (Figure 2). Axes were basally cylindrical, but quickly transitioned to compressed or flattened strap like blades with smooth margins that were 0.5–2.0 (4.0) mm wide and mostly less than 1.5 cm, but up to 2.5 cm long (Figure 2). Axis branching was dichotomous, or cervicorn, up to four times. Branches arising through normal axis development were not constricted at the base, but those developing as new axes from wound sites were constricted (Figure 2B). Apices were obtuse to truncate and had inconspicuous apical cells. Blades were composed of a three to six layered medulla of 70–160 µm diameter globose to longitudinally ovoid cells, and a 2–3 layered cortex of pigmented, 4–10 µm diameter polyhedral cells (Figure 3). There was an abrupt transition between the medulla and cortex.

Spermatangial sori were scattered, developed on both blade surfaces and had shallow textori-type conceptacles (Figure 4A,B). Individual conceptacles were loosely surrounded by anticlinally elongated cells that extended into the thick overlaying cuticle. Mature cystocarps were hemispherical and only slightly to not constricted at the base (Figure 4C). The outer pericarp was composed of 11–17 layers of small cells and an inner pericarp of 3–5 layers of small darkly staining cells covered the cystocarp floor. A conspicuous fusion cell was present (Figure 4D) and small tubular nutritive filaments connected the gonimoblasts to both the outer and inner pericarp. Tetrasporangia developed on both blade surfaces, were cruciately or decussately divided and surrounded by radially elongated, flanking cortical cells (Figure 4E). DNA sequences of *rbc*L, GenBank no. KY573993; COI-5P, GenBank no. KY656553; UPA, GenBank no. KY573953.

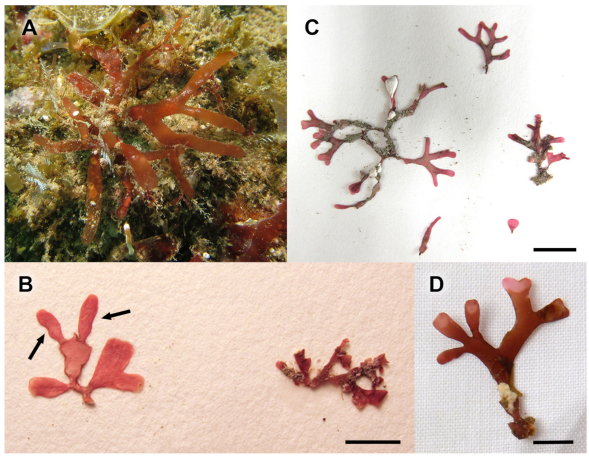

Figure 2. *Gracilaria parva* sp. nov. (**A**) In situ image of specimen PHYKOS-6870, Isla Cebaco, Panama. (**B**) Pressed holotype, WNC-34055, Isla Burica, Panama. Arrows indicate new branches with constricted bases developing from a wound site. Scale = 0.5 cm. (**C**) PHYKOS-6833 herbarium specimen WNC-34185, Isla Cebaco, Panama. Scale = 1.0 cm. (**D**) Specimen from La Chocolatera, Ecuador before splitting and pressing as NCU-675207 and USFQ #438. Scale = 0.5 cm.

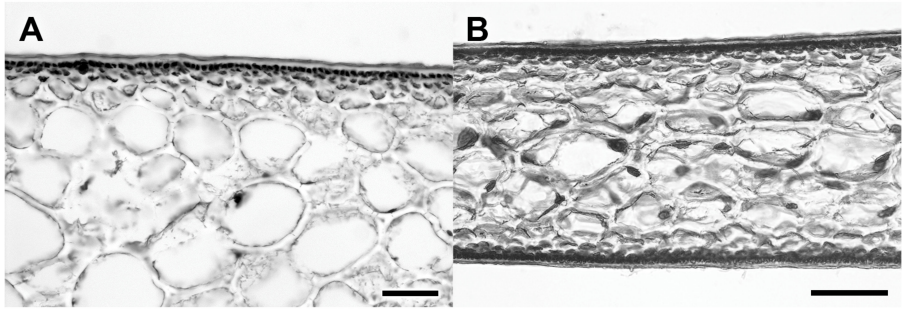

Figure 3. *Gracilaria parva* sp. nov., internal vegetative anatomy. (**A**) Transverse section, PHYKOS-4541, specimen WNC-34186. Scale = 50 µm. (**B**) Longitudinal section, PHYKOS-4541, specimen WNC-34186. Scale = 100 µm.

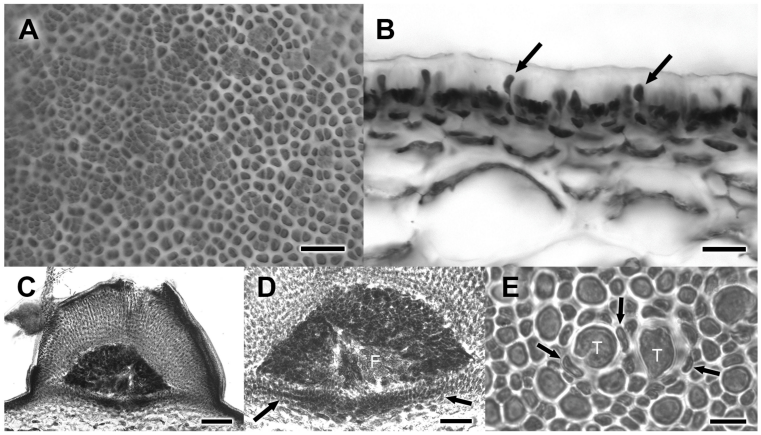

Figure 4. *Gracilaria parva* sp. nov., reproductive structures. (**A**) Surface view of spermatangial sorus, WNC-34055 slide A. Scale = 20 µm. (**B**) Transverse section of spermatangial sorus showing shallow, textori-type conceptacles and surrounding anticlinally elongated cells (arrows), WNC-34055 slide B. Scale = 20 µm. (**C**) Transverse section through mature cystocarp, WNC-34186 slide A. Scale = 100 µm.

(**D**) Transverse section through mature cystocarp showing conspicuous fusion cell ("F") and layers of small, darkly staining cells covering cystocarp floor (arrows), WNC-34186 slide A. Scale = 50 μm. (**E**) Surface view of developing tetrasporangia ("T") surrounded by radially elongated, flanking cortical cells (arrows), WNC-34055 slide A. Scale = 10 μm.

HOLOTYPE: WNC-34055, collection number PHYKOS-4630, growing on rock, southwest side of Isla Burica, Chiriqui, Republic of Panama, 08.01865° N 082.88390° W, ca. 6 m depth, 10 January 2011, collected by D.W. Freshwater.

ISOTYPE: NCU-675206.

OTHER SPECIMENS EXAMINED: WNC-34186, coll. no. PHYKOS-4541, growing on subtidal mudstone, near Mono Feliz, Punta Burica, Chiriqui, Panama, 08.03042° N 082.87574° W, 3 m depth, 8 January 2011, *leg*. B.L. Wysor and D.W. Freshwater; WNC-34181, coll. no. PHYKOS-4519, growing on subtidal mudstone, near Mono Feliz, Punta Burica, Chiriqui, Panama, 08.03042° N 082.87574° W, 3 m depth, 8 January 2011, *leg*. B.L. Wysor and D.W. Freshwater; WNC-34185, coll. no. PHYKOS-6833, growing on rock, northwest side of Sombrero Rock Cove, Isla Cebaco, Veraguas, Panama, 07.48229° N 081.25705° W, 17 m depth, 20 January 2012, *leg*. D.W. Freshwater; WNC-34184, coll. no. PHYKOS-6870, growing on rock, Cebaco Bay rock pile, Isla Cebaco, Veraguas, Panama, 07.49173° N 081.22262° W, 14 m depth, 20 January 2012, *leg*. D.W. Freshwater; NCU 675207, coll. no. E438, La Chocolatera, Santa Elena, Province, Ecuador, 21.i.2021, epilithic low intertidal, leg. A. Eguiguren and M. Brandt

HABITAT: Epilithic from low intertidal to 17 m depth.

DISTRIBUTION: *Gracilaria parva* is currently known from the tropical eastern Pacific coasts of Panama and Ecuador. Considering Punta Burica's position at the border of Panama and Costa Rica, the species' range likely extends into the latter country as well.

ETYMOLOGY: The species epithet reflects the small size of this species.

4. Discussion

Gracilaria parva is the only described tropical eastern Pacific species of *Gracilaria* to be characterized by both DNA sequences (*rbc*L, COI and UPA) and morpho-anatomy. Previously, Freshwater et al. [23] had provided DNA sequences (*rbc*L-3P) for three undescribed species of *Gracilaria* from Punta Burica, Panama, as *Gracilaria* sp. 1, *Gracilaria* sp. 2 and *Gracilaria* sp. 3. These were not described, as it was not known whether they were small specimens of previously described species, or unknown species. Additional specimens of one of these species, *Gracilaria* sp. 2, were later found at another locality in Panama, and also in Ecuador, and were reproductive, and thus are described herein as *G. parva*.

Eleven species of *Gracilaria* have been previously reported from the mainland coasts of tropical eastern Pacific countries (Table 1), all based on only morpho-anatomical descriptions [14–22,36]. DNA sequences subsequently have not been reported for any of these species. All are recorded as being larger than and having various morpho-anatomical characteristics that distinguish them from the newly described *Gracilaria parva*. For example, *G. crispata*, reported from Mexico, El Salvador and Costa Rica (Table 1), was described as being 4–7 cm high, which is only slightly larger than *G. parva*, but with axes that branch into decreasingly smaller segments with crisped margins to the point of giving the thallus a distally fasciculate appearance [17,37], unlike the broad, truncate blades of *G. parva*. Only two of the described tropical eastern Pacific species of *Gracilaria*, *G. brevis* from Ecuador and Panama and *G. veleroae* from Mexico, might be confused with *G. parva*.

Gracilaria veleroae has a similar branching pattern to *G. parva* and mostly obtuse to truncated apices, but it is larger at 6–8 cm high, has wider axes of 5–7 mm, and a single-layered cortex of periclinally flattened cells [17,38]. The largest specimen of *G. parva* was only 2.5 cm high; axes widths were mostly <2 mm and maximally 4 mm, and it has a 2–3 layered cortex of variably polyhedral cells. *Gracilaria brevis* was described by Taylor [14] to be larger reaching 5 cm in length versus 2.5 cm in *G. parva.* Axes of *Gracilaria brevis* are also wider (3–10 mm vs. <2 (4) mm), and it has up to six orders of dichotomous branching, in

contrast to *G. parva* where the branching pattern was dichotomous to cervicorn and the maximum number of divisions observed was four. The central medullary cells of *G. brevis* are generally smaller in diameter (40–120 μm) than those of *G. parva* (70–160 μm), and *G. brevis* also has only a single cortical cell layer in contrast to the 2–3 cortical cell layers of *G. parva*. A single cortical cell layer is also shown in the images of Panama specimens identified as *G. brevis* by Littler and Littler [19].

Current reports of species of *Gracilaria* in the tropical eastern Pacific suggest that their diversity is low, in contrast to the tropical western Atlantic. This reflects the traditional view that despite the presence of a wide variety of potential habitats, the tropical eastern Pacific has a depauperate macroalgal flora [16,39]. Reasons cited for the reduced diversity include the extreme tides [39,40], high water temperatures and low salinities in large gulfs [16], upwelling induced variable water temperatures [41], low nutrients [40], herbivory [42], and changes in current patterns since the close of the Central American isthmus [43]. While all these factors may be causes of a reduction in diversity as compared to the tropical western Atlantic, it is also possible that these factors have resulted in the evolution of different morphological forms and shifts in occupied habitats of the macroalgal community, and a depauperate flora is more apparent than real.

Our surveys along the Pacific coast of Panama have revealed many areas with extensive rocky intertidal zones where long exposure and high temperatures prevent the development of a macroalgal community. An exception was found on the Burica Peninsula where the particular type of intertidal rock (mudstone) allows extensive intertidal turf, and small, foliose tidal pool and subtidal macroalgal communities to flourish [23]. New explorations of little studied habitats and areas in the tropical eastern Pacific will likely increase the known diversity of this region. Similarly, the application of DNA sequence data is revealing an increased diversity of tropical eastern Pacific marine algae. Recent studies of species of *Gelidium* that grow as intertidal turfs have revealed multiple species that were not recognized with traditional morphological characters [23,44–46], and our DNA-barcode based surveys of macroalgae from unexplored subtidal habitats around Isla Cebaco, Panama have found six different small species of *Gracilaria* [47], including *G. parva*. These data, combined with that previously published for Punta Burica, indicate that the diversity of species of *Gracilaria* from just the Pacific coast of Panama is much greater than recognized.

The absence of available DNA sequence data for tropical eastern Pacific species of *Gracilaria* makes the assignment of names for these species difficult. Morphological characteristics are sometimes distinguishing, but other times subjective and prone to variable interpretations. *Gracilaria crispata* is an informative example. Setchell and Gardner's [37] and Dawson's [17] descriptions and illustrations of specimens of *G. crispata* from Pacific Mexico are very similar and reasonably represent the same species, but they differ considerably from the El Salvador specimen of *G. crispata* illustrated by Dawson [18]. As noted by Norris [7], the true range of many species of *Gracilaria* described from the Gulf of California and Pacific Baja Mexico requires study, especially those with reported distributions extending south into the tropical eastern Pacific. The sister relationship of *G. parva* from the Pacific coast and *G. galatensis* from the Caribbean coast of the Central American isthmus in the *rbc*L tree, might prove useful as a calibration point for dating phylogenies of *Gracilaria*. However, preliminary analyses of sequence data from additional specimens of *Gracilaria* indicate that there may be multiple tropical eastern Pacific species of *Gracilaria* within the clade with *G. parva* and *G. galatensis* [47]. Further study is required to determine the appropriate geminate species pair for molecular clock calibration. DNA sequence analyses will be essential for addressing these and other questions related to the biodiversity of tropical eastern Pacific marine macroalgae, and a concerted effort is underway by regional scientists to bridge this data gap [48].

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D.W.F., P.W.G. and M.B. D.W.F., B.W., P.W.G. and M.B. have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All DNA sequences used in this study are in GenBank (https://www.ncbi.nlm.nih.gov, accessed on 31 October 2021).

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