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Confirming a new species of *Amalthea* D'Archino & W.A.Nelson (Halymeniales, Rhodophyta) from the mesophotic zone of Hawai'i through taxogenomics

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Biodiversity,
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ABSTRACT

The order Halymeniales represents a significant number of species in the mesophotic red algal flora of Hawai'i, with numerous undescribed taxa. The cultivation and restoration of species in the Halymeniales in Hawai'i, particularly members of *Grateloupia* C.Agardh and *Halymenia* C.Agardh, have focused substantial attention on these taxa, highlighting uncertainty in the genetic diversity within this group. In this study, we adopt a taxogenomic approach integrating taxonomic, phylogenetic and phylogenomic analyses. We present the first report of the genus *Amalthea* D'Archino & W.A.Nelson in Hawai'i, focusing on the species description of *Amalthea mahilanii* F.P.Cabrera &

A.R.Sherwood, sp. nov. Our initial *rbc*L analysis suggested the presence of two lineages within Hawaiian *Amalthea*, but robust support from plastome-wide phylogenies contradicted that analysis, emphasizing the value of taxogenomics in clarifying cryptic diversity. Morphologically distinct from congeners *Amalthea freemaniae* D'Archino & W.A.Nelson and *A. rubida* H.W.Lee & M.S.Kim, *A. mahilanii* F.P. Cabrera & A.R.Sherwood, sp. nov. features rough, leathery blades with a unique cortical and medullary cell arrangement, including 9-10 layers of ovoid cortical cells and a dense arrangement of medullary filaments. Its blades maintain uniform thickness. The presence of larger ostiolate cystocarps (300-500 µm diameter) further distinguishes *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. from its Pacific conspecifics. Syntenic comparisons of published plastomes within the Halymeniales revealed remarkable similarities in gene content, with no detected rearrangements, indicating conserved plastidial evolution within the order. Overall, this study significantly advances our understanding of Halymeniales diversity in mesophotic ecosystems, shedding light on the remarkable biodiversity of macroalgae in Hawaiian mesophotic reefs. We emphasize the need for further study of Halymeniales and propose ongoing efforts in taxogenomics to enhance taxonomic resolution, particularly in mesophotic habitats.

RÉSUMÉ

*Confirmation d'une nouvelle espèce d'*Amalthea *D'Archino & W.A.Nelson (Halymeniales, Rhodophyta) de la zone mésophotique d'Hawai'i par la taxogénomique.*

L'ordre d'Halymeniales représente un nombre important d'espèces dans la flore d'algues rouges mésophotiques d'Hawaï, avec de nombreux taxons non décrits. La culture et la restauration d'espèces d'Halymeniales à Hawaï, en particulier des membres de *Grateloupia* C.Agardh et *Halymenia* C.Agardh, ont attiré une attention considérable sur ces taxons, soulignant l'incertitude quant à la diversité génétique au sein de ce groupe. Dans cette étude, nous adoptons une approche taxogénomique intégrant des analyses taxonomiques, phylogénétiques et phylogénomiques. Nous présentons le premier rapport du genre *Amalthea* D'Archino & W.A.Nelson à Hawai'i, en nous concentrant sur la description de l'espèce *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. Notre analyse *rbc*L initiale suggérait la présence de deux lignées au sein des spécimens hawaïens d'*Amalthea*, mais le solide soutien des phylogénies à l'échelle du plastome contredisait cette analyse, soulignant la valeur de la taxogénomique pour clarifier la diversité cryptique. Morphologiquement distinct des congénères *Amalthea freemaniae* D'Archino & W.A.Nelson et *A. rubida* H.W.Lee & M.S.Kim, *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. présente des lames rugueuses et coriaces avec un arrangement unique de cellules corticales et médullaires, comprenant 9 à 10 couches de cellules corticales ovoïdes et un arrangement dense de filaments médullaires. Ses lames maintiennent une épaisseur uniforme. La présence de cystocarpes ostiolés plus grands (300 à 500 µm de diamètre) distingue encore *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. de ses congénères du Pacifique. Les comparaisons synténiques des plastomes publiés au sein des Halyméniales ont révélé des similitudes remarquables dans le contenu génétique, sans réarrangements détectés, indiquant une évolution plastidienne conservée au sein de l'ordre. Dans l'ensemble, cette étude fait progresser considérablement notre compréhension de la diversité d'Halymeniales dans les écosystèmes mésophotiques, mettant en lumière la remarquable biodiversité des macroalgues dans les récifs mésophotiques hawaïens. Nous soulignons la nécessité d'une étude plus approfondie d'Halymeniales et proposons des efforts continus en taxogénomique pour améliorer la résolution taxonomique, en particulier dans les habitats mésophotiques.

MOTS CLÉS
Biodiversité,
chloroplaste,
génomes,
phylogénomique,
plastidien,
*rbc*L,
lame rouge,
espèces nouvelles.

INTRODUCTION

In recent years, the deeper waters of Hawaiian Archipelago has been the focus of intensive marine biodiversity studies (Pyle & Copus 2019). The mesophotic reefs surrounding these islands, which are found at depths of 30 to >150 m, have received particular attention because these reefs have unique ecological and biogeographic characteristics (Asher *et al.* 2017; Copus *et al.* 2022). These reefs are known to support a high diversity of marine organisms, including many endemic (Kosaki *et al.* 2017) and rare species of Hawaiian marine fauna (Rooney *et al.* 2010). Recent studies have revealed that these reefs also harbor a high diversity of red macroalgae, many of which are undescribed and poorly understood (Spalding *et al.* 2019; Sherwood & Guiry 2023). The order Halymeniales is a major component of the mesophotic red algal flora in Hawai'i, with numerous undescribed species (Cabrera 2020). The restoration and cultural significance of Halymeniales in Hawai'i (Abbott 1996), particularly through local efforts organized by Limu Hui (https://kuahawaii.org/limu-hui/), cultivation and reef plantings have brought renewed attention to beloved taxa such as *Grateloupia* C.Agardh and *Halymenia* C.Agardh. However, the extent of the genetic diversity among the Halymeniales is likely still dramatically under-characterized.

Halymeniales species that have been described in Hawai‘i are not yet adequately represented in global phylogenetic studies, leaving a significant gap in our understanding of the genetic landscape of these taxa.

The taxonomic classification of the family Halymeniaceae established by Bory in 1828 has undergone notable transformations. Initially categorized within the Cryptonemiales (Kylin 1956), the family Halymeniaceae underwent a first taxonomic shift to the Gigartinales (Kraft & Robins 1985) before attaining recognition as an independent order based on molecular characterization (Saunders & Kraft 1996). The order Halymeniales is characterized by a diverse array of thallus forms, ranging from erect or decumbent, simple to richly branched, blade-like, foliose, or terete thalli (Saunders & Kraft 1996; Kim *et al.* 2021). The internal structure is multiaxial, featuring a pseudoparenchymatous cortex with numerous secondary pit connections in the filamentous medulla, or lacking secondary pit connections (Saunders & Kraft 2002). Halymenialean genera were classically set apart by the reproductive anatomy of the female plants; specifically, the separate ampullae for the formation of 2-4-celled carpogonial branches and auxiliary cells (Chiang 1970). Tetrasporangia are distinctively decussately or cruciately divided, and the life history is triphasic, involving isomorphic gametophytes and tetrasporophytes. The Halymeniales includes the families Grateloupiaceae, Halymeniaceae, and Tsengiaceae (Guiry & Guiry 2023). In Hawai‘i, the Halymeniales is currently represented by 15 species belonging to six genera *Dermocorynus* P.Crouan, *Grateloupia* C.Agardh and H.Crouan, *Carpopeltis* F.Schmitz, *Cryptonemia* J.Agardh, *Halymenia* C.Agardh, and *Polyopes* J.Agardh (Abbott 1967; Sherwood & Guiry 2023). Six of these species are believed to be endemic to the Hawaiian archipelago, with all distributed exclusively across the Main Hawaiian Islands. These species include *Grateloupia corymbifera* (I.A.Abbott) S.Kawaguchi & A.W.Wang, *G. hawaiiana* E.Y.Dawson, *Halymenia chiangiana* I.A.Abbott & Kraft, *H. cromwellii* I.A.Abbott, *H. hawaiiana* J.J.Hernández-Kantún & A.R.Sherwood (Tsuda 2013), and *Polyopes hakalauensis* (Tilden) I.A.Abbott.

Ongoing refinements to the taxonomy of the Halymeniales has involved a progressive exploration of its phylogeny. Initially relying on SSU gene marker, the approach has evolved to include the LSU gene marker (Withall & Saunders 2006) and, more recently, multi-gene alignments incorporating *rbc*L, *cox*1, *psa*A, *psb*A, or UPA (Wynne 2011; Azevedo *et al.* 2016; Le Gall *et al.* 2018; Tan *et al.* 2018; Kim *et al.* 2021; Nguyen *et al.* 2023). Morphological features, while informative, have frequently proved to be inadequate in capturing subtle genetic variations among morphologically similar Halymenialean taxa (Kim *et al.* 2023). This limitation has persisted even as researchers have transitioned to the use of molecular markers, revealing instances of cryptic diversity that were previously undetected. More recently, Next-Generation Sequencing (NGS) technologies have revolutionized the exploration of genetic diversity in non-model organisms, including challenging-to-study mesophotic species (Gijsbers *et al.* 2022).

Taxogenomics, the integration of taxonomic and genomic approaches, is revolutionizing the field of systematics, providing a more comprehensive and accurate understanding of biological diversity, evolution, and ecological roles. Organellar genome analyses, especially plastome-wide (Park *et al.* 2023) or mitogenome-wide phylogenies (Li *et al.* 2018), have allowed researchers to address cryptic diversity challenges, overcoming limitations of using only one or a few molecular markers. Chloroplast genomes or plastomes (cpDNA), in particular, have played a key role in resolving taxonomy of Rhodophyta (Díaz-Tapia *et al.* 2023). Díaz-Tapia *et al.* (2017) showcased how cpDNA can revolutionise phylogenetic analyses. They prompted a reevaluation of the classification of the Rhodomelaceae, emphasizing the need for revisions. Similarly, Costa *et al.* (2016) strategically used cpDNA to reclassify species of the Nemaliales. Iha *et al.* (2018) leveraged cpDNA sequencing in the Gracilariaceae, revealing evolutionary relationships. These new highlights summarize the crucial role of whole-plastome cpDNA sequencing in understanding the evolution of the Rhodophyta. The advent of platforms like RealDB (Chen *et al.* 2018) and Rhodoexplorer (Lipinska *et al.* 2023) underscores the growing importance of organellar genomic data. Despite this, the limited availability of complete red algal genomes poses a significant obstacle to robust phylogenetic inference. Comprehensive genomic data not only aids in precise phylogenetic studies but also plays a pivotal role in conservation efforts, advancing our understanding of ecosystem dynamics and signatures of adaptation through genome-wide association studies (Beilsmith *et al.* 2019; Cho *et al.* 2023).

The genus *Amalthea* D'Archino & W.A.Nelson (order Halymeniales) comprises two known Pacific taxa. The genus was first described from New Zealand collections at intertidal to subtidal depths of 5-30 m, resulting in one species, *A. freemaniae* D'Archino & W.A.Nelson (D'Archino *et al.* 2014). *Amalthea rubida* H.W.Lee & M.S.Kim was discovered as a Northeastern Pacific basin species from greater subtidal depths of 15-20 m, highlighting the benefits of a global study to determine the full species diversity and phylogenetic relationships of *Amalthea* (Lee *et al.* 2016a). Prior to this study, the genus *Amalthea* has not been previously documented in Hawai‘i (Abbott 1999; Sherwood & Guiry 2023). The aim of this study was to provide a comprehensive taxonomic description of a unique species within *Amalthea*. This new species was discovered during sampling surveys targeting mid- to lower mesophotic Hawaiian ecosystems (60-150 m depths) in 2006. Through an integrated taxogenomic approach, we describe a newly discovered species within *Amalthea* and provide complete *de novo* assemblies of the cpDNA for this apparent endemic Hawaiian species.

MATERIAL AND METHODS

INSTITUTIONAL ABBREVIATIONS

BISH	Bishop Museum's *Herbarium Pacificum*, Honolulu;
HPC	University of Hawai‘i High Performance Computing cluster, Honolulu;

NCBI	National Center for Biotechnology Information, Bethesda;
PMNM	Papahānaumokuākea Marine National Monument, northwestern portion of the Hawaiian archipelago.

Others abbreviations

CTAB	Cetyltrimethylammonium bromide;
cpDNA	Chloroplast genome;
IR	inverted repeat;
LCBs	locally collinear blocks;
MCE	Mesophotic Coral Ecosystem;
MCMC	Markov Chain Monte Carlo;
MHI	Main Hawaiian Islands;
NWHI	Northwestern Hawaiian Islands;
ML	Maximum Likelihood;
ORFs	open-reading frames;
PCGs	protein-coding genes;
*rbc*L	large subunit of the ribulose-bisphosphate carboxylase oxygenase gene.

Collection details

Three specimens of red blades were sampled during lower mesophotic surveys from 2006 at Hoʻokuleana (Penguin Banks) at 106-107 m using the manned submersible *Pisces IV* (representing the Main Hawaiian Islands, or MHI), and at Hōlanikū (Kure Atoll) at 90 m, Mokupāpapa, Papahānaumokuākea Marine National Monument (PMNM) in 2016 (representing the Northwestern Hawaiian Islands, or NWHI) by National Oceanic and Atmospheric Administration (NOAA) divers using mixed gas closed-circuit rebreathers (Appendix 1 and Appendix 2 in Supporting information). Specimens were saved in silica gel for molecular analyses, and in 4% formalin in seawater solution and pressed onto herbarium paper directly after collection for morphological vouchers, or frozen and pressed at a later date. Specimen vouchers BISH 791136-791139 are deposited at the *Herbarium Pacificum*, Bernice P. Bishop Museum (BISH).

Morphological characterisation

Morphological investigations were conducted by hand sectioning with a double-edged razor blade, staining with 0.5% aniline blue, and mounting in 30-50% Karo™. Photomicrographs were taken on a Zeiss AxioImager A1 compound light microscope (Pleasanton, CA) with an Infinity2-1RC digital camera (Lumenera Corporation, Ottawa, Ontario, Canada). To illustrate the full view of the sections, several successive images from individual sections were combined using Autostitch free software (Ma *et al.* 2007). Morphological characters, measured or determined as described above, were used to compare the new specimens to all previously described taxa within the genus *Amalthea* to determine if they represented a currently recognized taxon or an undescribed species.

DNA extraction and organellar genome sequencing

Total genomic DNA was extracted using a CTAB DNA extraction protocol with minor modifications (Doyle & Doyle 1987). Library preparation and sequencing of all three collected samples (ARS 09490, ARS 10058 and ARS 10059) were conducted on an Illumina NovaSeq 6000 platform in paired-end mode at the Huntsman Cancer Institute's High-Throughput Genomics (HCI-HTG) facility at the University of Utah. FASTQ files of the raw sequence reads are deposited under the BioSample Accessions SAMN37204901, SAMN37204902, SAMN37204903.

Genome assembly and annotation

Illumina paired-end reads were trimmed for adaptors and filtered for low-quality reads using fastp v0.20.1 with default settings (Chen *et al.* 2018). Remaining high quality filtered reads were used to reconstruct organellar genomes via NOVOPlasty v4.3.4 (Dierckxsens *et al.* 2017) using default settings. The *rbc*L sequence served as the seed for cpDNA assembly. Preliminary annotations for chloroplast genomes were acquired using MFannot (Lang *et al.* 2023). GeSeq (Tillich *et al.* 2017), which also supports the use of tRNAscan-SE v2.0.7 (Chan & Lowe 2019), was utilized with default settings to corroborate the protein-coding genes, rRNAs, and tRNAs, using published Halymeniales cpDNA (OR635816, OR635817, OR635818, NC_021618, NC_037841, NC_046751, NC_066971) as its BLAT (BLAST-Like Alignment Tool) reference. Open reading frames (ORFs) were identified using the "Find ORF" function in Geneious, with translation table 11, maintaining a minimum size of 300 bp. The annotations were subsequently imported and aligned in Geneious. To validate gene predictions from GeSeq, MFannot, and Geneious ORFinder, a thorough examination of start/stop codons, as well as intron/exon boundaries, was conducted. All annotations underwent manual vetting, leading to the establishment of a comprehensive master annotation track derived from these validated annotations in Geneious 2023.2 (http://www.geneious.com). The physical circular maps were visualized using OrganellarGenomeDRAW v. 1.3.1 The final annotated cpDNA assemblies of Hawaiian *Amalthea* have been deposited under GenBank Accessions PP777198 – PP777200.

Phylogenetic analyses

All phylogenetic analyses, including determination of partitioning schemes and models of molecular evolution using the greedy algorithm with unlinked branch lengths, were conducted uniformly across datasets using PartitionFinder v.2.1.1 (Lanfear *et al.* 2017). Subsequent analyses encompassed Bayesian Inference (BI) in MrBayes v3.1.2 (Ronquist *et al.* 2012) and Maximum Likelihood (ML) in RAxML-NG v1.0.1 (Kozlov *et al.* 2019) all of which were run on the MANA University of Hawaiʻi High Performance Compute (HPC) cluster. Visualization of the trees was performed via the interactive Tree of Life v5 (Letunic & Bork 2021).

For the initial *rbc*L-based phylogenetic analyses, three newly generated *rbc*L sequences, extracted from their respective cpDNA assemblies, were combined with selected sequences from the family Halymeniaceae obtained from GenBank, including *Aeodes nitidissima* J.Agardh (EU497914) and *Pachymenia laciniata* J.Agardh (AY583301) as outgroups.

The alignment, conducted using the ClustalW plugin (Larkin *et al.* 2007) with default settings in Geneious Prime 2023.2, underwent removal of ambiguous regions, resulting in a 42-sequence (1259 bp) *rbc*L alignment. This alignment was further partitioned by codon position, employing the gamma-distributed rate variation among sites and a proportion of invariable sites (GTR+I+G) model, which was consistently applied in subsequent BI and ML analyses. Bootstrap values for ML analysis were calculated based on 1,000 replicates. BI involved Metropolis-coupled Markov Chain Monte Carlo runs of five million generations, with two independent runs, four chains, and tree sampling every 100 generations. The burn-in period was determined graphically by tracking likelihoods, removing 25% of saved trees, and using the remaining trees to calculate Bayesian posterior probabilities (BPPs).

To elucidate the phylogenetic relationships among our sequences and other available plastomes within Halymeniales, we retrieved GenBank sequences of *Grateloupia asiatica* S.Kawaguchi & H.W.Wang (OR635816), *Polyopes affinis* (Harvey) Kawaguchi & Wang (OR635817), *Pachymeniopsis lanceolata* (Okamura) Yamada ex Kawabata (OR635818), *Grateloupia taiwanensis* S.-M.Lin & H.Y.Liang (NC_021618), *Grateloupia subpectinata* Holmes (NC_037841), *Halymenia maculata* J.Agardh (NC_046751), *Grateloupia turuturu* Y.Yamada (NC_066971), with sequences of *Rhodymenia pseudopalmata* (J.V.Lamouroux) P.C.Silva included as the outgroup (KX284709). Initially, a whole cpDNA alignment approach was employed for phylogenetic tree reconstruction. Standardization of the start position of all cpDNA allowed the assessment of synteny at the large subunit of the *rbc*L gene. Halymeniales cpDNA were aligned using the progressive MAUVE plug-in (Darling *et al.* 2010) in Geneious Prime 2023.2, applying the full alignment option, with automatically calculated seed weights and minimum locally collinear blocks (LCB) scored and compared for genetic synteny. The resulting file was subjected to HomBlocks (Bi *et al.* 2018) to extract syntenic alignments. Orthologous sequences at the genome scale generated by HomBlocks were used for constructing phylogenetic trees. The HomBlocks alignment was partitioned based on the identified conserved locally collinear blocks (LCBs), using the (GTR+I+G+X) model that incorporates features to handle non-uniform substitution rates, invariant sites, rate heterogeneity, and a mixed model approach. This partitioning scheme was consistently applied in subsequent Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. The ML analysis included 10,000 bootstrap replicates, and BI followed the same procedure as described above.

RESULTS

PHYLOGENETIC ANALYSES

rbc*L phylogenetic analyses*

A total of 42 *rbc*L sequences were selected for analysis, representing key taxa within the order Halymeniales. Sequences from *Aeodes nitidissima* J.Agardh (EU497914) and *Pachymenia laciniata* J.Agardh (AY583301) were used as outgroups. The alignment included 1259 sites, omitting 104 and 103 bp from the 5' and 3' end of the *rbc*L (1467 bp) gene, respectively, due to missing or ambiguous regions. Topologies for ML and BI analyses were congruent and support values for both are displayed on a single *rbc*L tree figure (Fig. 1). Molecular phylogenetic analyses of *rbc*L gene sequences provided robust support for the *Amalthea* clade, comprising two lineages: Lineage 1 with ARS 09490 and Lineage 2 with ARS 10058 and ARS 10059 found in the Hawaiian Islands, *A. freemaniae*, *A. rubida*, and *Halymenia abyssicola* E.Y.Dawson. While Hawaiian *Amalthea* specimens are separated into two distinct lineages, they exhibit an astonishing degree of similarity, with a genetic correspondence of 99.60% for *rbc*L (5 differences out of 1259 bp). Interestingly, this clade exhibits significant genetic divergence from other Halymenialean genera, specifically the clade which includes the type species *H. floresii*, as well as Galene, and the *Cryptonemia* clade (D'Archino *et al.* 2014; Lee *et al.* 2016 a).

Plastidial genome features of Hawaiian Amalthea

This study successfully *de novo* assembled three complete plastomes for Hawaiian *Amalthea*, with lengths ranging from 186,321 to 186,357 bp. These genomes were characterized by a wide range of depth coverage, from 462-1364X. Hawaiian *Amalthea* CP genomes shared the same set of genes and GC content (29.7%). In total, the cpDNA of Hawaiian *Amalthea* harbored 238 genes, including 200 PCGs (Table 1), inclusive of 31 transfer RNA (tRNA) genes and 3 ribosomal RNA (rRNA) genes. To elucidate the genomic landscape and diversity among the Hawaiian *Amalthea* cpDNA, we visualized the genome map of *Amalthea* sp. ARS 09490, which has the highest depth coverage, at 1364X. (Fig. 2). The overall base composition of the provided sequence is as follows: 35.65% for A (66,406 bp), 15.15% for C (28,201 bp), 14.56% for G (27,136 bp), and 34.64% for T (64,614 bp). *Amalthea* cpDNA lacked the large rRNA operon-encoding inverted repeat (IR). Instead, *Amalthea* cpDNA sequences showcased an exceptionally compact and circular structure. *Amalthea* shared nearly all of its genes with one or more Halymenialean taxa, namely *Halymenia maculata*, *Grateloupia asiatica*, *G. subpectinata*, *G. taiwanensis*, *G. turuturu*, *Polyopes affinis*, and *Pachymeniopsis lanceolata*. Notably, one tRNA gene (trnM-CAU) is tripled and scattered singly in the plastome, with one trnM-CAU copy interrupted by a group II intron.

Plastome-wide phylogenetic analyses

In December 2023, plastidial genomes of nine Halymeniales species were published in NCBI. Seven were selected for our analysis, while *Grateloupia livida* (Harvey) Yamada (NC_058752) and *G. lanceola* (J.Agardh) J.Agardh (HM767098 and HM767138) were excluded due to incongruence, specifically having a significantly shorter length at 50,000-120,000 bp. Additionally, although *G. lanceola* was published with genome characterization by Janouškovec *et al.* (2013), their submission to NCBI consists of two different contigs without annotations, which reduces our

Table 1. — Functional classification of genes in the *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. chloroplast genome. Categories for classification follow Ohta *et al.* (2003).

Genetic system	**Number**	**Genes**							
Maintenance	2	dnaB	rne						
RNA Polymerase	4	rpoA	rpoB	rpoC1	rpoC2	rpoZ			
Transcription factors	4	ntcA	ompR	rbcR	ycf29				
Translation	4	infB	infC	tsf	tufA				
Ribosomal proteins									
Large subunit	28	rpl1	rpl2	rpl3	rpl4	rpl5	rpl6	rpl9	rpl11
		rpl12	rpl13	rpl14	rpl16	rpl18	rpl19	rpl20	rpl21
		rpl22	rpl23	rpl24	rpl27	rpl28	rpl29	rpl311	rpl32
		rpl33	rpl34	rpl35	rpl36				
Small subunit	18	rps1	rps2	rps3	rps4	rps5	rps6	rps7	rps8
		rps9	rps10	rps11	rps12	rps13	rps14	rps16	rps17
		rps18	rps19						
TRNA processing	1	tilS							
Protein quality control	4	clpc	dnak	ftsH	groEL				
Photosystems									
Phycobilisomes	11	apcA	apcB	apcD	apcE	apcF	cpcA	cpcB	cpcG
		cpeA	cpeB	nblA					
Photosystem I	13	psaA	psaB	psaC	psaD	psaE	psaF	psaI	psaJ
		psaK	psaL	psaM	ycf3	ycf4			
Photosystem II	19	psbA	psbB	psbC	psbD	psbE	psbF	psbH	psbI
		psbJ	psbK	psbL	psbN	psbT	psbV	psbW	psbX
		psbY	psbZ	Psb30					
Cytochrome complex	11	ccs1	ccsA	petA	petB	petD	petF	petG	petJ
		petL	petM	petN					
Redox system	7	acsF	bas1	dsbD	ftrB	grx	pbsA	trxA	
ATP synthesis									
ATP synthase	9	atpA	atpB	atpD	atpE	atpF	atpG	atpH	atpI
		clpC	sufB	sufC					
Metabolism									
Carbohydrates	5	odpA	odpB	pgmA	rbcL	rbcS	cbbx		
Lipids	5	accA	accB	accD	acpP	fabH			
Nucleotides	2	carA	upp						
Amino acids	8	argB	gltB	ilvB	ilvH	syfB	syh	trpA	trpG
Cofactors	6	chlI	moeB	preA	thiG	thiS	dfr		
Transport									
Transport	7	cemA	secA	secG	secY	ycf38	ycf63	tatC	
Unknown									
Conserved ORFs	31	ORF58	ORF83	ORF745	ORF101	ORF152	ycf17	ycf19	ycf20
		ycf21	ycf22	ycf23	ycf33	ycf34	ycf35	ycf36	ycf37
		ycf39	ycf41	ycf45	ycf46	ycf52	ycf53	ycf54	ycf55
		ycf58	ycf60	ycf62	ycf65	ycf80	ycf86	ycf92	
RNA genes									
rRNAs	3	rrn5	rrn23	rrn16					
tRNAs	29	trnA-UGC	trnC-GCA	trnD-GUC	trnE-UUC	trnF-GAA	trnG-GCC	trnG-UCC	trnH-GUG
		trnI-GAU	trnK-UUU	trnL-CAA	trnL-UAA	trnL-UAG	trnM-CAU	trnM-CAU	trnM-CAU
		trnN-GUU	trnP-UGG	trnQ-UUG	trnR-ACG	trnR-CCG	trnR-UCU	trnS-GCU	trnS-UGA
		trnT-GGU	trnT-UGU	trnV-GAC	trnW-CCA	trnY-GUA			
Miscellaneous RNAs	1	rnpB							

confidence in using these sequences. The Rhodymeniales, represented by *Rhodymenia pseudopalmata* (KC284709), is recognized as sister to the Halymeniales (Lee *et al.* 2016b), and served as the outgroup. MAUVE alignment analysis identified 5 LCBs consistently shared across all four species, demonstrating no evidence of rearrangements. HomBlocks then selected and trimmed these 5 LCBs using GBlocks, covering LCB1 = 1–57 080; LCB2 = 57 081–113 118; LCB3 = 113 119–148 549; LCB4 = 148 550–153 749; LCB5 = 153 750–156 209, resulting in an alignment spanning 156 209 bp. Topologies for ML and BI analyses were congruent, and their support values are presented on a unified plastome tree figure (Fig. 3A). Every lineage in this tree is robustly supported, including the Hawaiian *Amalthea* specimens identified as a distinct and unique lineage. The plastome-wide phylogeny recovered a close relationship between *Polyopes affinis* (OR635817) with full support. Additionally, syntenic comparison revealed a consistent

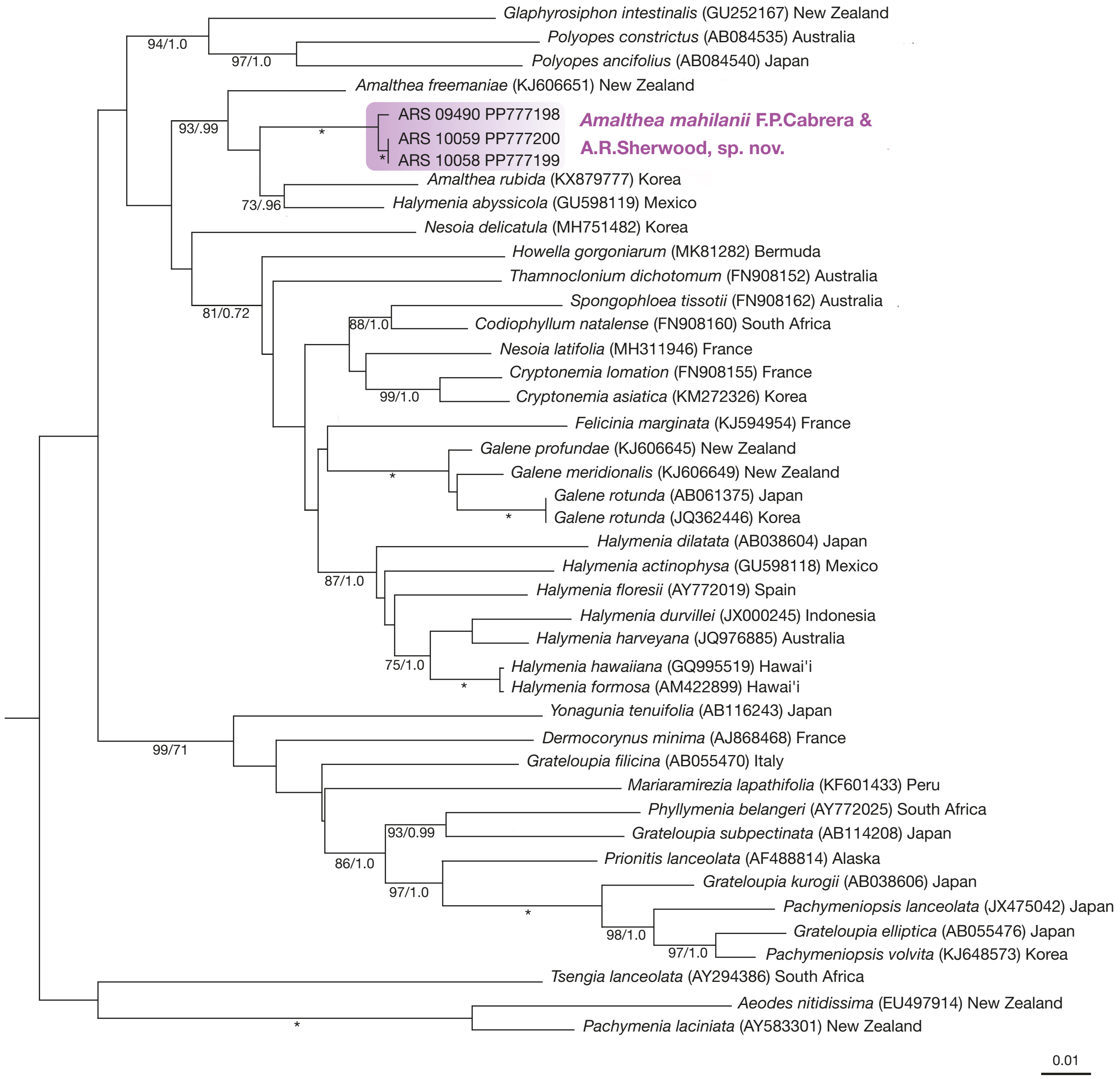

FIG. 1. — Maximum Likelihood phylogenetic tree inferred in RAxML with GTR + I + G nucleotide substitution model from 44 *rbc*L sequences of representative Halymeniaceae. Support (ML/PP: ML ≥ 70% and PP ≥ 0.95) are given below the branches. Full support indicated by *. Scale bar: substitutions per site.

collinear structure and nearly identical gene content within the order Halymeniales (Fig. 3B). Notably, there were two minor regions where connecting LCB lines were removed, potentially representing alignment artifacts and unlikely to represent homology across different genes. After excluding this incongruent segment, the MAUVE-HomBlock analysis identified five LCBs shared consistently across all 11 taxa in the Halymeniales (including the outgroup in the order Rhodymeniales), with no evidence of rearrangements. Additionally, syntenic comparison revealed a consistent collinear structure and nearly identical gene content within the order Halymeniales (Fig. 3B).

The results from our phylogenetic and genomic analyses are unequivocal in supporting the samples from Hawai'i as a distinct and undescribed lineage. We described these specimens as a new species of *Amalthea*:

Family HALYMENIACEAE Bory
Genus *Amalthea* D'Archino & W.A.Nelson

Amalthea mahilanii
F.P.Cabrera & A.R.Sherwood, sp. nov. (Fig. 4)

EXAMINED MATERIAL. — **United States** • Hawai'i, Ho'okuleana (Penguin Banks); 106 m depth; 17.XI.2006; collected by Terry Kerby; *ARS 10059*; holotype, BISH 791136; female gametophyte (Fig. 4A, C) • same collection data as for the holotype; *ARS 10059*; isotype, BISH 791137; female gametophyte (Fig. 4D).

TABLE 2. — Taxonomic characteristics comparison of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. and other *Amalthea* species.

	***Amalthea freemaniae* D'Archino et W.A.Nelson**	***Amalthea rubida* H.W.Lee et M.S.Kim**	***Amalthea mahilanii* F.C.Cabrera et A.R.Sherwood, sp. nov.**
Type locality	Karikari Bay, North Island, New Zealand	Udo, Jeju, Korea	Penguin Banks, Main Hawaiian Islands
Ecology	Subtidal between 5-30 m depth, attached to small cobbles, shells, or rhodoliths	Subtidal habitats at depths of 15-20 m, attached to rhodoliths or pebbles on sandy bottoms	mesophotic 90-107 m, attached to rhodoliths or pebbles on sandy bottoms
Thallus	Foliose, usually undivided, longer than wide; 330-410 µm thick in the basal part and 230-270 µm in the apical part	Foliose, solitary, usually entire, rounded or obtusely ellipsoid in large; 350-380 µm thick in the basal part and 290-320 µm in the apical part	Foliose, solitary, usually entire, rounded or obtusely ellipsoid when mature; 280-440 µm thick
Stipe	Present, cuneate	Present (inconspicuous), short and compressed, obtusely cuneate	Present (inconspicuous), narrow-cuneate
Margin	Mostly entire or wavy when mature	Entire or slightly wavy when mature	Entire or slightly wavy when mature
Color	Pale red to light pink, lightly mottled on the surface	Pale reddish brown to red	Chestnut red to dark mauve when live and dries to a mauve to a taffy pink
Length (cm)	About 40 (up to 70)	5-15 (up to 35)	About 50
Width (cm)	25-35	5-10 (up to 20)	3-25
Texture	Soft but not slimy	Soft, slick and quite gelatinous	Firm and leathery
Surface view	Oval outline, not compacted	Roundish, not compacted	Roundish, not compacted
Vegetative structure	Different between basal and distal regions of thallus	Different between basal and distal regions of thallus	Uniform from basal to distal regions of thallus
Cortex	One-celled, ovoid	One-celled, ovoid	9-10 layers of ovoid cortical cells 1-3 µm in diameter, 4-8 fascicles of stellate subcortical cells
Subcortex	1-2 layers of subcortical cells, stellate cells forming loose network parallel to the surface	1-2 layers with stellate cells forming a network parallel to the surface	4-8 layers with stellate cells forming a network parallel to the surface
Medulla	Loosely arranged anticlinal filaments	Loosely arranged anticlinal filaments with medullary stellate cells	Densely arranged anticlinal filaments with medullary stellate cells
Cystocarp	180-250 µm diameter	Not observed	300-500 µm diameter
Tetrasporangia	Cruciately divided	Cruciately divided	Not observed
Reference	D'Archino *et al.* (2014)	Lee *et al.* (2016a)	This study

ADDITIONNAL EXAMINED MATERIAL. — **United States** • Hawai'i, Ho'okuleana (Penguin Banks); 107 m depth; 17.XI.2006; collected by Terry Kerby; *ARS 10058*; BISH 791138; female gametophyte (Fig. 4E) • Hawai'i, Hōlanikū (Kure Atoll), Mokupāpapa, PMNM; 90 m depth; VI.5.2016; collected by John Hansen & Brian Hauk; *ARS 09490*; BISH 791139; male gametophyte (Fig. 4F).

TYPE LOCALITY. — Hawai'i, Ho'okuleana (Penguin Banks); at 106 m.

ETYMOLOGY. — The epithet "mahilanii" honors Uncle Jerry Leroy Mahilani Kaluhiwa, also known as Na Koa Limu, for his significant role in conserving and preserving Hawaiian limu (algae) and traditional Hawaiian values that emphasize the importance of cultural stewardship in conserving Hawaiian biodiversity.

HABITAT. — *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. inhabits the mesophotic zone at 90-107 m depth, attaching to rhodoliths or pebbles on sandy sediments. In the PMNM, they occur at a mean temperature of 18-19°C at 90 m and currents of 10-15 cm^{-1} at 70-90 m depths. In the MHI, the mean temperature is 20-21°C at 100-120 m, with stagnant currents at 100-110 m depths.

DISTRIBUTION. — Mesophotic and Hawai'i-endemic.

SEASONALITY. — Collected from June (Kau/ Summer) to November (Hooilo/Winter) in Hawai'i.

DESCRIPTION

Thallus typically foliose, solitary, and expanding broadly, growing up to 50 cm in length and widths ranging from 3 to 25 cm, arising from a narrow-cuneate stipe (3-7 mm long, 3-4 mm wide) attached to a discoid holdfast. Female and male gametophytes are isomorphic and dioecious. Blade color transitions from chestnut red to dark mauve when living, eventually drying to a mauve to taffy pink hue. Blade surfaces incised with various-sized round perforations. Thallus margins typically eroded, torn and wavy. Texture firm, cartilaginous, and leathery, and in surface view, the thallus appears compact with 1-2 layers of isodiametric outer cortical cells. Thallus thickness remains uniform between the basal and distal portions. Elongated spermatangial parent cells occur in both sides of the thallus. Cystocarps are embedded in the medulla and cause protuberances on one side of the thallus surfaces as small hemispherical blisters measuring up to 500 µm. Carposporangia are spherical to ovoid, 6-10 µm in diameter, and are released through an ostiole. Tetrasporic blades absent.

Vegetative characteristics

Collected blades of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. were predominantly mature, characterized by a single thickened blade reaching lengths of up to 50 cm and widths ranging from 3 to 25 cm (Fig. 4A-F). The internal anatomy of younger blades was not observed. The thallus emerges from an inconspicuous narrow-cuneate tough stipe (Fig. 4D, E, arrowheads) measuring 3-7 mm in

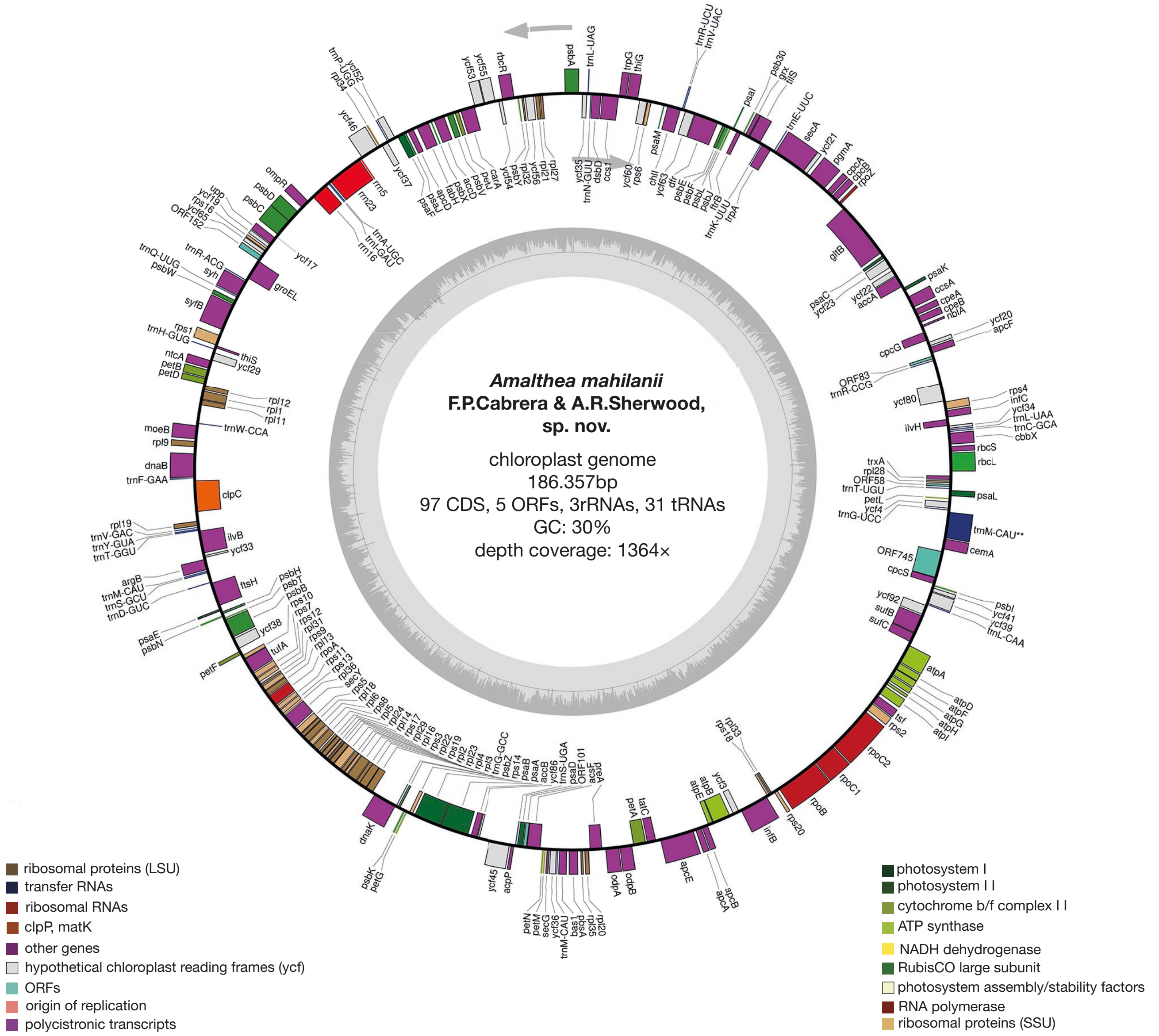

FIG. 2. — Chloroplast genome (CP) of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. (ARS 09490, GenBank PP777198, 1364x coverage). Overview of the chloroplast genome features. Genes outside the circle are transcribed clockwise, while genes inside are transcribed counter-clockwise. Functional groups are color-coded. Inner circles represent Guanine-Cytosine (GC) and Adenine-Thymine (AT) content.

length and 3-4 mm in width, firmly attached to a discoid holdfast (Fig. 4F, arrowheads). When living, the thallus exhibits a striking chestnut red to dark mauve color. Blades collected in Holaniku retained their dark mauve hue until pressed, while those collected in Hoʻokuleana dried to a taffy pink shade. The blade has various-sized round perforations, eroded margins, and a firm to rough, leathery thallus with compact outer cortical cells (Fig. 4G). The cortex consists of 9-10 layers of ovoid cortical cells (1-3 µm in diameter) and is accompanied by 4-8 fascicles of stellate subcortical cells (Fig. 4H, J). Below the cortex, the subcortex forms a layered structure with stellate cells, creating a network parallel to the surface (Fig. 4H). Within the medulla, anticlinal and intertwined filaments are densely arranged, accompanied by medullary stellate cells connecting inner cortical cells and medullary filaments. No tetrasporophytes were collected.

Reproductive features

Elongated spermatangial parent cells are found on both sides of the thallus surface (Fig. 4H, J), which undergo division to form rosettes of 8-12 parent cells (Fig. 4I). Upon maturity, each spermatangial parent cell yields a spermatangial initial, which subsequently elongates, occasionally bifurcating, to give rise to 1-2 ovoid spermatangia at the distal ends (Fig. 4J, arrowheads). Carpogonial branches and early post-fertilization stages were not observed in mature female specimens. When cystocarps are present, they are nestled within the medulla, inducing protuberances on one side of the thallus surface, manifesting as small

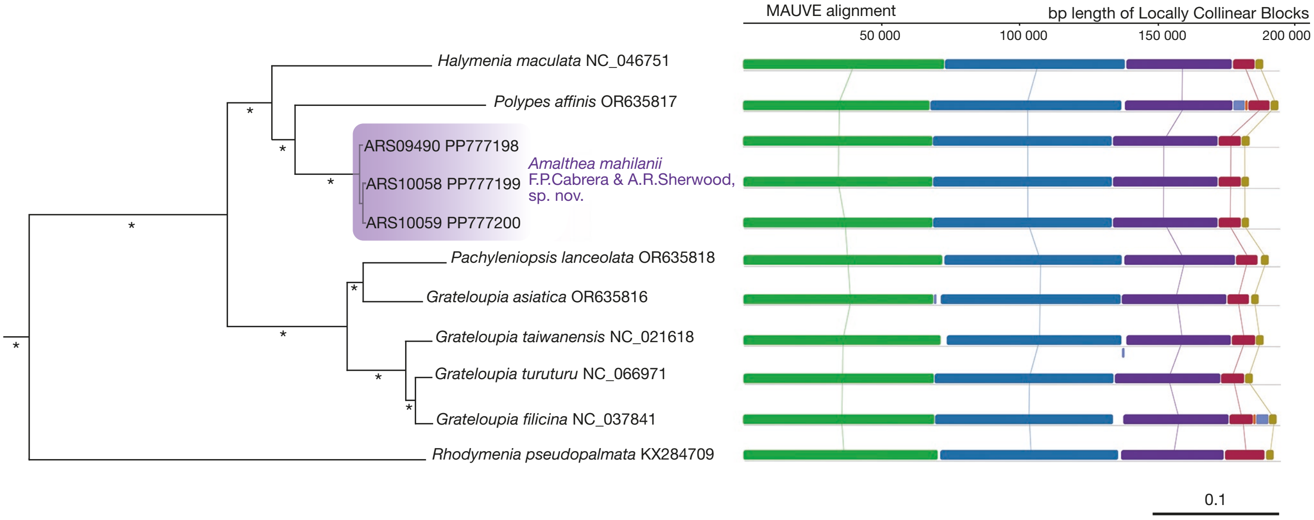

Fig. 3. — Phylogenetic analysis: **A**, phylogenetic tree (Maximum Likelihood) of the order Halymeniales based on 11 cpDNA sequences, with *Rhodymenia pseudopalmata* (J.V.Lamouroux) P.C.Silva used as outgroup (bootstrap values based on 1000 replicates). *Amalthea* chloroplast genomes are newly determined; **B**, syntenic comparison 11 chloroplast genome sequences from the order Halymeniales based on MAUVE package (Darling et al. 2010). The colored boxes represent Locally Collinear Blocks (LCBs), signifying homologous regions of sequences devoid of significant rearrangement.

hemispherical blisters, measuring up to 500 µm (Fig. 4K). Within these cystocarps, the carposporangia are spherical to ovoid, measuring 6-10 µm in diameter. Carposporangia are released through apparent ostioles (Fig. 4G, arrowhead; 4L), or distinct openings in the thallus surface (Fig. 4G, arrowhead).

DISCUSSION

This study marks the first report of the genus *Amalthea* in Hawai'i, with a particular focus on *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. Distinguished from other *Amalthea* species in several key aspects (Table 2), this mesophotic seaweed exhibits features that suggest suitability for life in the mesophotic. Firstly, its ecological niche sets it apart, as it appears to be specifically adapted to mesophotic environments, thriving at depths of 90-107 m in the Hawaiian Islands. The apparent depth specialization exhibited is in stark contrast to *A. freemaniae* and *A. rubida*, taxa predominantly found at shallower subtidal depths of 5-30 m and 15-20 m, respectively. While *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. were collected from deeper, mesophotic depths in the Hawaiian Archipelago compared to the subtidal in *A. rubida* Korea and *A. freemaniae* New Zealand, the irradiance levels at the depth of collection in these regions were likely similar. Calculations based on the attenuation coefficient (KPAR) from similar bodies of water (Idso & Gilbert 1974; Howard-Williams *et al.* 1995; Kim *et al.* 2015; Lee *et al.* 2018; Spalding *et al.* 2019) show that all collections were made just beyond the 1% optical depth, regardless of the depth of collection (Appendix 3). In Hawai'i, these depths of collection (90-107 m) correspond to irradiance levels of 26-13 µE m-2 s-1, respectively (Padilla-Gamiño *et al.* 2019; Spalding *et al.* 2019) (Appendix 3). Thus, the light environment of the region may be more pertinent than the depth of collection from bodies of water with different optical properties. Morphologically, *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. exhibits distinctive features. In contrast to the soft and gelatinous thalli of *A. freemaniae* and *A. rubida*, the blades of *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. are rough and leathery, a characteristic likely attributed to its distinct cortical and medullary cell arrangement. Furthermore, we did not encounter young specimens, so it is plausible that the blades of *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. are tough and leathery due to their maturity, as descriptions for *A. rubida* and *A. freemaniae* considered characteristics of young specimens. The cortex of *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. is characterized by 9-10 layers of ovoid cortical cells measuring 1-3 µm in diameter, accompanied by 4-8 fascicles of stellate subcortical cells. Additionally, *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. displays a very dense arrangement of medullary filaments, which significantly differs from the cortical and subcortical cell arrangements observed in *A. freemaniae* and *A. rubida*, where the medulla for both is loosely packed. *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. maintains uniform thickness across both basal and distal portions of its blade. The incorporation of dense medullary tissue, as seen in *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov., enhances flexural stiffness, limiting reconfiguration in flowing water and increasing drag (Stewart 2006). While thicker tissues may hinder flexibility (Millar *et al.* 2021), they may also provide increased strength and resistance to breakage, allowing *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. to balance both structural stability and hydrodynamic efficiency. Lastly, the presence and size of cystocarps also distinguish *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. from the other two species, as it exhibits larger cystocarps (300-500 µm), while *A. freemaniae*

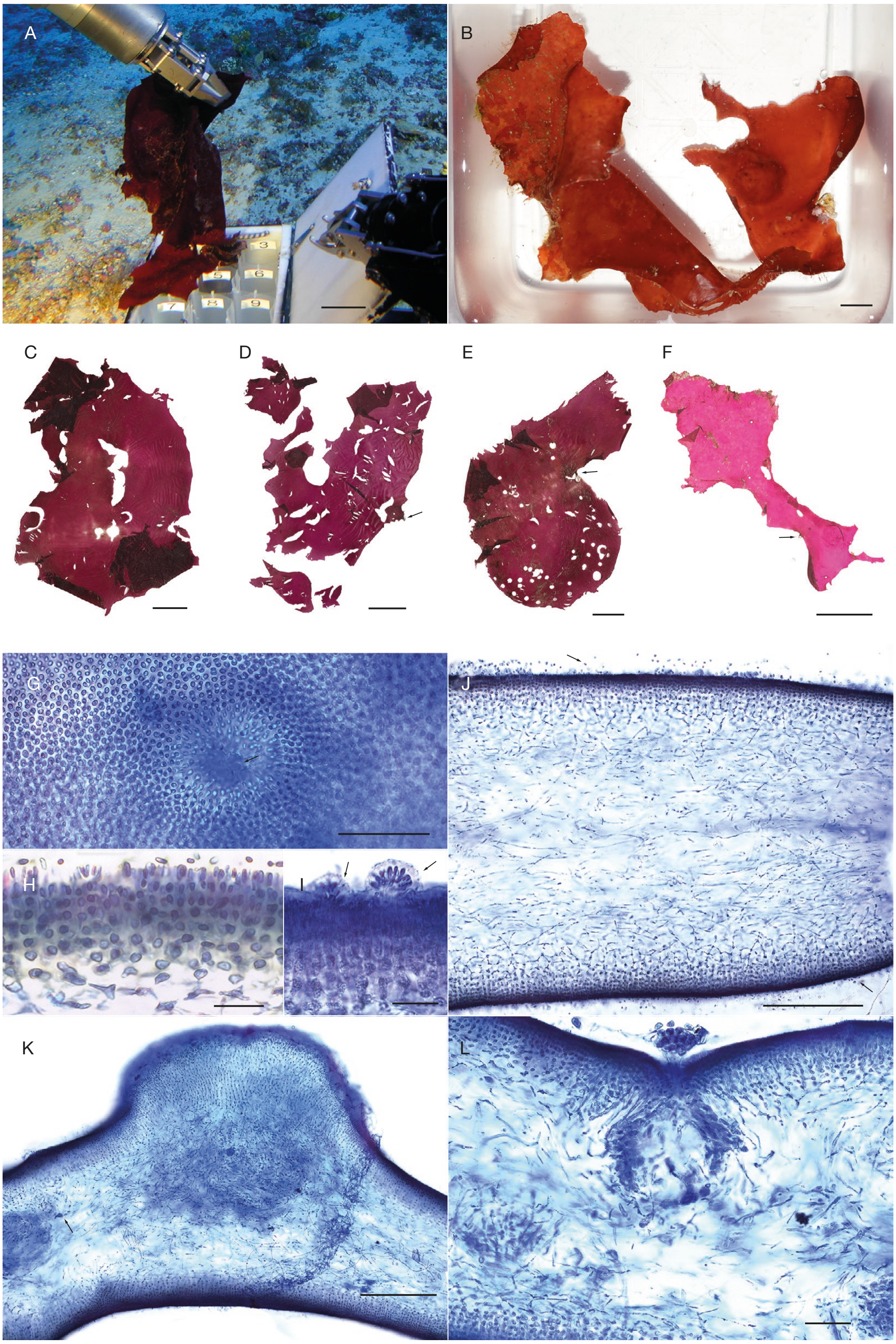

Fig. 4. — Morphology of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov.: **A**, *in situ* image of *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. *ARS 10058* being collected by the submersible robotic arm at Hoʻokuleana (Penguin Banks) at 106 m; **B**, image of live *A. mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. HI *ARS 09490* several hours after being collected at Hōlanikū (Kure Atoll) at 90 m; **C**, habit of pressed specimen, a female gametophyte (holotype: *ARS 10059*, BISH 791136); **D**, isotype specimen, a female gametophyte (*ARS 10059*, BISH 791137). The parallel rips/holes in the middle resulted from the collector arm of *Pisces IV* Deep Diving Manned Submersible gripping the alga. **Arrow** pointing at stipe; **E**, topotype specimen, a female gametophyte (*ARS 10058*, BISH 791138). **Arrow** pointing at stipe; **F**, paratype specimen, a male gametophyte (*ARS 09490*, BISH 791139). **Arrow** pointing at holdfast; **G**, upper layer surface view of the cortex showing outer cortical cells and ostiole (**arrowhead**); **H**, lower layer surface view of the cortex showing inner cortical cells and spermatangial parent cells; **I**, surface cortical cell dividing to form rosettes of spermatangial parent cells (**arrowheads**); **J**, cross-sectional view of a male gametophyte blade, revealing the release of spermatangia (**arrowheads**); **K**, cross-sectional view of female gametophyte showing cystocarp protruding on one side and a developing auxiliary cell ampulla (**arrowhead**); **L**, cross-sectional view of female gametophyte with an ostiole releasing carpospores. Scale bars: A-F, 5 cm; G, H, L, 50 µm; I, 25 µm; J, K, 100 µm.

has smaller cystocarps (180-250 µm), and cystocarps have not been observed for *A. rubida*. Moreover, cystocarps of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. are ostiolate, while *A. freemaniae* lacks this characteristic. Ostioles, present in *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov., serve as a direct pathway for spore release (Lobban & Wynne 1981; Hurd *et al.* 2014), contributing to reproductive success at mesophotic depths. Thus ostiolate cystocarps appear to serve as a distinguishing morphological factor between the two species and a trait of some ecological significance.

Amalthea mahilanii F.P.Cabrera & A.R.Sherwood, sp. nov. shares a distinctive cpDNA trait with certain other red algae in the Halymeniales, including *Halymenia maculata* (Tan *et al.* 2019), *Grateloupia turuturu* (Han & Li 2020), and *G. subpectinata* (Zhang *et al.* 2018; Sumikawa *et al.* 2020): an atypical cpDNA structure that differs from the canonical quadripartite arrangement commonly seen in land plants (Green 2011). In the typical quadripartite structure, cpDNA comprise a pair of inverted repeats separated by the small single-copy and large single-copy regions, along with multiple copies of the rRNA gene cluster, including 16S, 23S, and 5S rRNA genes (Cutolo *et al.* 2022). However, the cpDNA of *Amalthea* is exceptionally compact and streamlined; it lacks the inverted repeat region and contains only a single copy of the rRNA gene cluster, encompassing the 16S, 23S, and 5S rRNA genes. *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. displays a distinctive feature in its plastome where a single tRNA-Met gene (trnM-CAU) is tripled and dispersed individually, and one of the copies is interrupted by a group II intron. This intronic tRNA-Met has also been observed in *Grateloupia lanceola* (HM767098 and HM767138), *Calliarthron tuberculosum* (KC153978), *Chondrus crispus* (HF562234) (Janouškovec *et al.* 2013), and *Gracilaria salicornia* (NC_023785) (Campbell *et al.* 2014). This finding emphasizes that the intron within the tRNA-Met gene is a characteristic shared by some florideophytes, indicating its widespread presence across Rhodophyta. It is noteworthy that within the order Halymeniales, the presence of this unique intronic copy of the tRNA-Met gene is observed only in *Grateloupia lanceola* and *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov., suggesting a specific pattern within this taxonomic group.

Our initial analysis focused on the *rbc*L gene, revealed the presence of two distinct lineages within the genus *Amalthea*. However, a comprehensive examination of the plastome-wide phylogeny demonstrated that the three specimens under consideration exhibit high degrees of similarity, suggesting recognition as a single species. This discrepancy between the individual-based *rbc*L phylogenetic analyses and the plastome-wide phylogeny highlights an essential point for taxonomic considerations within the order Halymeniales. In current taxonomic practices, individual-based *rbc*L phylogenetic analyses are still commonly employed for describing new species within the order. For instance, *Grateloupia oligoclora* H.W.Wang & Y.Bian was initially described as a new species from China based on *rbc*L gene sequence analysis (Duan *et al.* 2022) but was later synonymized as *Grateloupia subpectinata* (Kim *et al.* 2023) when including different data such as biogeography and genomic analyses (DePriest *et al.* 2013; DePriest 2015; Han & Li 2020; Sumikawa *et al.* 2020), and multi-gene alignment (Kim *et al.* 2021). Presently, Grateloupiaceae is recognized as a monophyletic family. Kim *et al.* (2021) emphasized the need for further study on Halymeniaceae *sensu lato*. Our study highlights the importance of robust taxogenomic support to prevent and alleviate taxonomic confusion. It represents a progressive step in addressing taxonomic challenges within the Halymeniaceae *sensu lato*, aligning with the dynamic landscape of genomic research.

In conclusion, our study has significantly expanded our understanding of red algae within the genus *Amalthea* through the description of a new species from the Hawaiian mesophotic zone. This taxonomic expansion underscores the dynamic nature of our comprehension of this group and highlights the exciting potential for further discoveries within Hawaiian mesophotic ecosystems. The presence of these unique species in lower mesophotic environments, distinct from the typical shallow reef habitats, emphasizes the remarkable biodiversity of Hawaiian mesophotic ecosystems that remain a reservoir of great diversity (Spalding *et al.* 2016; Cabrera *et al.* 2020, 2022a, 2022b; Paiano *et al.* 2020, 2022; Sherwood *et al.* 2021a, b, c, 2022; Alvarado *et al.* 2022) in a region of the world largely isolated from the impacts of invasive species overgrowth, overfishing and our changing ocean change. As we delve deeper into the taxonomy and genomics of red algae, particularly those thriving in distinctive environments such as Hawaiian mesophotic reefs, we continue to gain valuable insights into biodiversity that may have been lost elsewhere and evolutionary adaptations of the Rhodophyta. Finally, our ongoing efforts to assemble organellar genomes of red algae associated with the marine flora of the Hawaiian mesophotic reefs signify our commitment to unraveling the genetic intricacies of these fascinating organisms, and hold promise for exciting avenues of future research and discovery (Cabrera *et al.* 2023).

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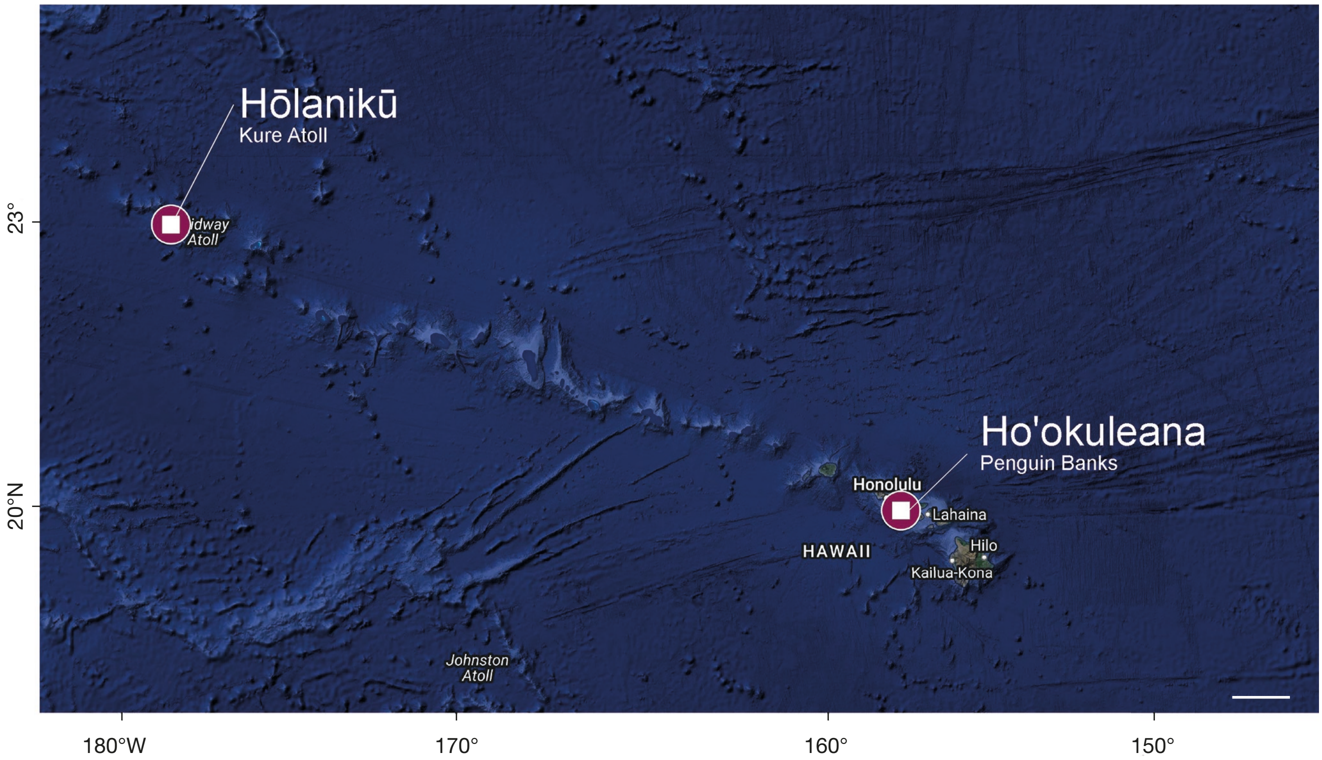
APPENDICES

Appendix 1. — Distribution of *Amalthea mahilanii* F.P.Cabrera & A.R.Sherwood, sp. nov. in the Hawaiian Archipelago. Collection locations are represented by encircled square points outlined in **white**.



Appendix 2. — Collection information for Hawaiian specimens of *Amalthea* D'Archino & W.A.Nelson used for morphological analysis and GenBank accession numbers for newly generated sequence data used in the phylogenetic analyses. Field codes with NWHI indicate rebreather dives, while field codes with P4/P5 denote submersible dives.

Species	BISH number	Specimen voucher	Field code	Collection date	Location	Collection depth (m)	Collecters	BioSample accession	CP Genome accession
Amalthea mahilanii	Holotype BISH 791136	*ARS 10059*	P4-184 103-1	11/17/2006	Ho'okuleana (Penguin Banks), Hawai'i (20.98643, – 157.45014)	106	Terry Kerby	SAMN37204903	PP777199
	Isotype BISH 791137	—	P4-184 103-2	11/17/2006	Ho'okuleana, Hawai'i (20.98643, – 157.45014)	106	Terry Kerby	—	—
	Topotype BISH 791138	*ARS 10058*	P4-184 99	11/17/2006	Ho'okuleana, Hawai'i (20.98643, 157.45014)	107	Terry Kerby	SAMN37204902	PP777200
	Paratype BISH 791139	*ARS 09490*	NWHHI – 541	06/05/2016	Hōlanikū (Kure Atoll), Hawai'i (28.49845, – 178.34172)	90	John Hansen & Brian Hauk	SAMN37204901	PP777198

APPENDIX 3. — Comparison of depth of collection and 1% optical depths in each region. The 1% optical depth is defined as the euphotic zone depth (Zeu, m), or depth where Photosynthetically Active Radiation (PAR) (Zeu) is 1% of PAR at the surface (0 m) (Lee *et al.* 2018).

Location of collection	**Depth range (m) of collection**	**Diffuse Attenuation Coefficent (KPAR, m-1)**	**1% Optical Depth (m) (4.6/KPAR, Kirk 2011)**	**Source**
Ho‘okuleana (Penguin Banks), Hawai‘i	90-107	0.041	112	Spalding *et al.* 2019
Udo, Jeju Korea	15-20	0.163	28	Kim *et al.* 2015 (calculated from 10.4 m average Secchi depth (ZSD), where K=1.7/ ZSD from Idso & Gilbert 1974)
Karikari Bay, North Island, New Zealand	5-30	0.132	35	Howard-Williams *et al.* 1995 (K for Coastal I waters)