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To cite this article: Feresa P. Cabrera, John M. Huisman, Heather L. Spalding, Randall K. Kosaki, Celia M. Smith & Alison R. Sherwood (2022): Cryptic diversity in the genus *Croisettea* (Kallymeniaceae, Rhodophyta) from Hawaiian mesophotic reefs, *Phycologia*, DOI: 10.1080/00318884.2022.2096823

To link to this article: <https://doi.org/10.1080/00318884.2022.2096823>



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Published online: 03 Aug 2022.



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## Cryptic diversity in the genus *Croisettea* (Kallymeniaceae, Rhodophyta) from Hawaiian mesophotic reefs

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### ABSTRACT

Recent investigations into the species diversity of red blades in Hawai'i have yielded several specimens of Kallymeniaceae from Hawaiian Mesophotic Coral Ecosystems. Our combined morphological and mitochondrial COI-5P and plastid *rbcl* phylogenetic analyses indicated widespread cryptic diversity among those specimens commonly identified as *Kallymenia sensu lato* based on morphology. These analyses resolved four unique genetic lineages of Hawaiian taxa in the genus *Croisettea*, which are all restricted to the lower mesophotic depths (c. 60–150 m). *Croisettea* currently includes three described species distributed in the North Atlantic, Indian and South Pacific Oceans, and the Mediterranean Sea. *Croisettea* is a new genus record for the Hawaiian Islands, expanding its biogeographic range to the North Pacific. The genus has now been enlarged to include seven species comprising previously described taxa as well as four new Hawaiian taxa (*C. kalaukapuae* sp. nov., *C. haukoaweo* sp. nov., *C. ohelouliuli* sp. nov. and *C. pakualapa* sp. nov.). The known distributions of the Hawaiian *Croisettea* species are restricted to areas around their type localities. Although this pattern hints at a remarkable degree of endemism, both across depth gradients in a reef area and among islands, it is also linked to a limited sampling of the group, suggesting that additional species, and more accurate distributional ranges, remain to be detected not only in Hawai'i but also worldwide.

### ARTICLE HISTORY

Received 21 September 2021  
Accepted 29 June 2022  
Published online 03 August 2022

### KEYWORDS

COI-5P; Deepwater; Endemism; Foliose; Molecular-assisted alpha taxonomy; *rbcl*; Red algae; Red blades; Systematics; Taxonomy

## INTRODUCTION

In the last several years, Hawaiian mesophotic algal collections have been a large source of new species. Recent and ongoing floristic surveys of Hawaiian marine habitats, including Mesophotic Coral Ecosystems (MCEs) – extending from 30 to at least 150 m depths (Hinderstein *et al.* 2010), are leading to a more accurate recognition of the diversity, especially among Rhodophyta. An astounding array of new generic (i.e. *Ethelia*, *Halopeltis*, *Haraldiophyllum*, *Incendia*, *Leptofauchea*, *Meredithia*, *Psaromenia*, *Ramicrusta*, *Seiria*, *Sonderophycus*, *Umbraulva*) and species records in the Hawaiian Archipelago have resulted from Hawaiian mesophotic collections (Spalding *et al.* 2016; Paiano *et al.* 2020; Sherwood *et al.* 2020a, b, c; Alvarado 2021; Cabrera *et al.* 2021).

Despite increased research efforts, MCEs remain largely unexplored, and the taxonomy of many MCE-associated species requires clarification. Notably, many rarely recorded shallow reef members of the red blades, which often harbour cryptic species, are frequently observed and collected in Hawaiian MCEs (Spalding *et al.* 2019). Here, we follow Bickford *et al.* (2007) in considering species to be cryptic when morphologically indistinguishable taxa representing distinct entities are classified under one taxonomic name.

Phylogenetic revisions of the long-established 'red blade' genus *Kallymenia* J. Agardh (D'Archino *et al.* 2010, 2011, 2012, 2016, 2017, 2018) led to the reinstatement of the genus *Euhymenia* Kützinger, *nom. illeg.* (Saunders *et al.* 2017); however, *Euhymenia* is regarded as a superfluous name originally intended to replace *Kallymenia*, and the new genus *Croisettea* M.J. Wynne was proposed to accommodate the species in question (Wynne 2018). The emended description of the genus *Croisettea* comprises: expanded to lobed membranous red blades with 2–3-celled carpogonial branches (Norris & Womersley 1971; Wynne 2018). As currently circumscribed *Croisettea* includes three species: *C. requienii* (J. Agardh) M.J. Wynne (the generitype), *C. australis* (Womersley & R.E. Norris) M.J. Wynne and *C. tasmanica* (Harvey) M.J. Wynne. The genus has a wide and disjunct distribution in the North Atlantic, Pacific and Indian Oceans, as well as the Mediterranean (Guiry & Guiry 2021). The Southern Hemisphere has been hypothesized to be the centre of *Croisettea* diversity, with several *Croisettea* cryptic species complexes detected and yet to be described (Saunders *et al.* 2017). Additionally, the three recognized species of *Croisettea* occur both in the shallow and mesophotic – with *C. requienii* documented as low as 95 m depth (Agardh 1847),

*C. australis* at 50 m and *C. tasmanica* at 40 m (Womersley 1994). Hence, the full extent of the diversity of the genus *Croisetia* remains incompletely known without inclusion of additional specimens from unrepresented geographical areas and depth ranges.

Through combined morphological and multi-gene molecular analyses, we characterized four novel species of *Croisetia* documented exclusively in the lower mesophotic (c. 60–150 m), adding to the long list of newly recorded genera in the Hawaiian Islands. It is essential to bring attention to such rarely seen mesophotic species and to provide a taxonomic (and especially, molecular) framework for future researchers to perform more extensive fieldwork, obtain more collections or describe further new taxa.

## MATERIAL AND METHODS

Specimens were sampled during mesophotic surveys from 2006 to 2019 in the Papahānaumokuākea Marine National Monument (PMNM) (also referred to as the Northwestern Hawaiian Islands, NWHI) by National Oceanic and Atmospheric Administration (NOAA) divers using mixed gas closed-circuit rebreathers, and in the Main Hawaiian Islands (MHI) in the ‘Au‘Au Channel between the islands of Lāna‘i and Maui using the manned submersibles *Pisces IV* and *Pisces V*. The locations of the sampling sites are shown in Fig. S1 and the specimen collection details are presented in Table S1.

### Morphological characterization

Anatomical and reproductive features were observed in material that was hand-sectioned with a razor blade. Sections were rehydrated in modified Pohl’s solution (Pohl 1965) for approximately 5 min, stained with 0.5% aniline blue for approximately 5 min, and then mounted in 30% Karo™. Sections of stipe and basal regions, which were generally thicker than apical cross sections, were rehydrated from herbarium sheets and stained for at least 10 min. Note that rehydration and staining longer than 20 min will cause the blades to disintegrate into a dense mass of cells. To illustrate the full view of the sections, several successive images from individual sections were combined using Autostitch free software (Ma *et al.* 2007).

### DNA sequencing and phylogenetic reconstruction

Total genomic DNA was extracted from silica gel-preserved material or herbarium specimens using the OMEGA E.Z.N.A. Plant DNA Kit (OMEGA Biotek, Norcross, Georgia, USA) following the manufacturer’s protocol. The mitochondrial COI-5P region was amplified using the primers GazF1 and GazR1 and the recommended PCR profile (Saunders 2005), while the plastid rubisco large subunit (*rbcL*) gene was amplified as described in Xuan-Nguyen *et al.* (2019). Successful PCR products were sequenced by Genewiz Inc. (South Plainfield, New Jersey, USA). Sequence data were edited and aligned with additional sequences downloaded from GenBank

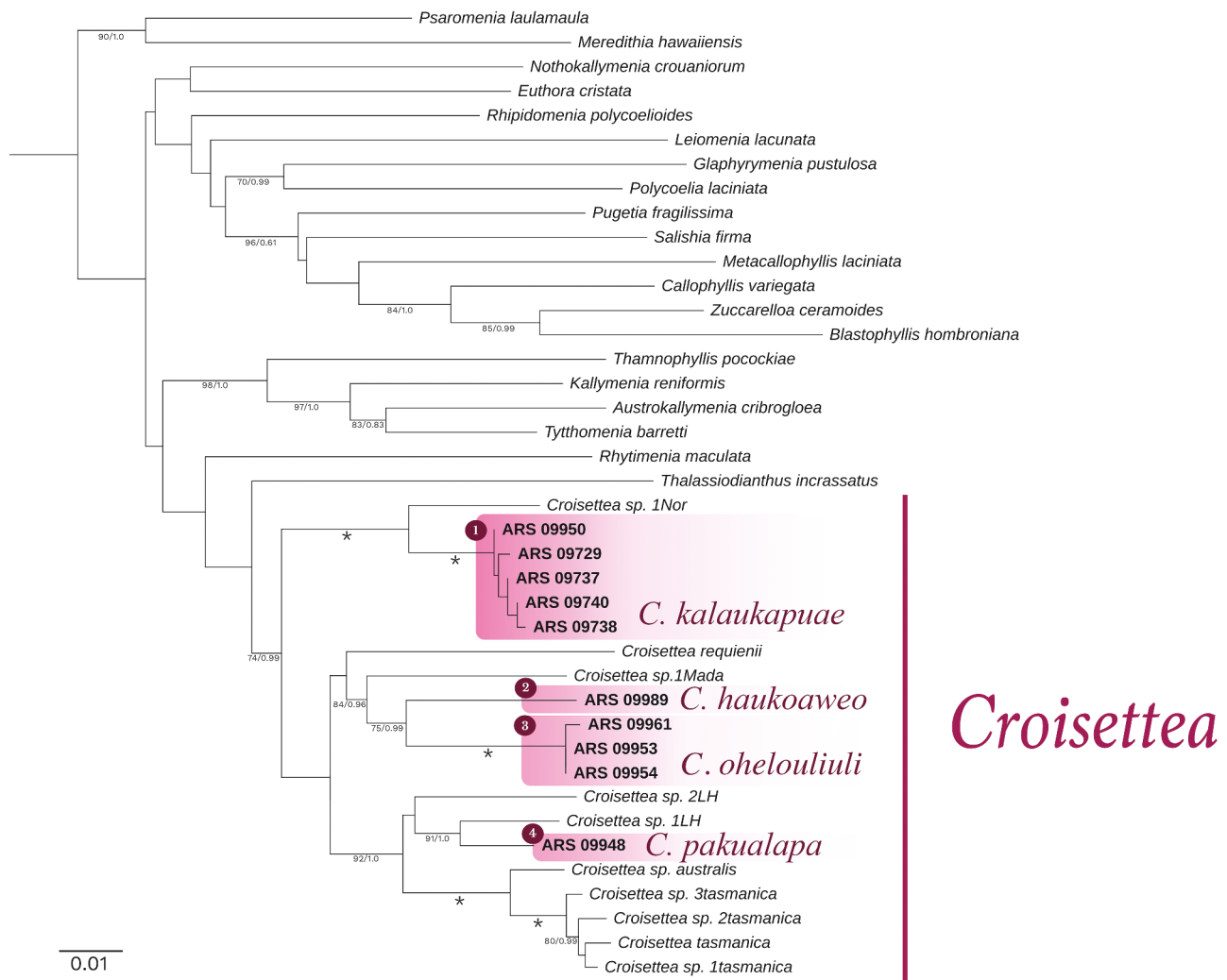
(Table S2) in Geneious Prime 2019.1.3 (<http://www.genious.com>).

Sequence alignment was performed using MUSCLE plugin (Edgar 2004) with default settings in Geneious Prime to construct alignments for each gene: COI-5P with 25 sequences of 664 base pairs (bp), and *rbcL* with 26 sequences of 1300 bp. These alignments used *Dumontia simplex* Cotton as the outgroup (Saunders *et al.* 2017). We analysed the *rbcL* and COI datasets individually and concatenated the congruent datasets (Figs S3, S4). PartitionFinder v1.1.1 analyses suggested the General Time Reversible model with gamma distributed rate variation among sites and a proportion of invariant sites for the concatenated data set (Lanfear *et al.* 2012). The concatenated dataset was used in phylogenetic reconstruction with Maximum Likelihood (ML) using RAxML (Stamatakis 2014) with 1,000 bootstrap replicates, and Bayesian Inference (BI) using MrBayes v3.2.6 (Ronquist *et al.* 2012) based on the nucleotide substitution models determined by the Akaike Information Criteria (AIC) in MrModeltest 2.3 (Nylander *et al.* 2008) through tree builder plugins in Geneious Prime. The Bayesian analysis was run with 2,000,000 generations of Markov Chain Monte Carlo iterations until the standard deviation of split frequencies was below 0.01. The first 10% of trees of each run were discarded as burn-in. Visualization of the trees was performed via the interactive Tree of Life (<https://itol.embl.de/>; Letunic & Bork 2019). All new sequences were submitted to GenBank (accession numbers: COI, OM509717–OM5097124; *rbcL*, OM621854–OM621863).

## RESULTS

### Phylogenetic analyses

Ten *rbcL* and nine COI-5P sequences were newly generated in this study (Table S1). These sequences were compared to GenBank sequences (80 for *rbcL* and 74 for COI-5P) representing all available genera in the family Kallymeniaceae (Tables S1, S2; Figs S3, S4). The BA and ML analyses of the concatenated alignment resulted in the same tree topology, and only the ML tree is shown (Fig. 1). Phylogenetic analyses confirmed the placement of four lineages of Hawaiian specimens in three moderately- to well-supported *Croisetia* subclades. The first subclade with moderate support was composed of *C. ohelouliuli* as sister to an undescribed *Croisetia* from Madagascar (sp. 1 Mada). The second subclade with good support contained *C. pakualapa* and *C. haukoawe*, as well as *C. australis* and *C. tasmanica* and undescribed *Croisetia* specimens from Lord Howe Island (LH), Australia. The third subclade with full support included *C. kalaikapuae* and an undescribed *Croisetia* from Norfolk Island, Australia. The concatenated COI+*rbcL* analyses demonstrated the distinctiveness of Hawaiian *Croisetia* from the other three recognized species in the genus. The four Hawaiian species exhibited some phenotypic variation (detailed in Table 1), mostly with differences in blade thickness and sizes of vegetative characters; however, the current evidence for their recognition as distinct species lies



**Fig. 1.** RAxML phylogenies inferred from the combined alignment of COI and *rbcl*. Outgroup (*Dumontia simplex*) pruned to facilitate presentation. Support values at nodes >70% (ML bootstrap, first value) and >0.9 (Bayesian posterior probability, second value) are shown. Asterisk on branches indicates full support; asterisk on species names indicates the generitype (\*). Scale bar = substitutions per site.

overwhelmingly in their genetic distinctiveness. They are proposed below as new species.

***Croisettea kalaukapuae* F.P. Cabrera & A.R. Sherwood  
sp. nov.  
Figs 2–13**

**DESCRIPTION:** Blades flat, thin, delicate, with smooth, pleated or undulate margins, blush to rose pink in colour, and with a soft, slippery consistency. Blades ranging from 1.5–35 cm long by 1.5–48 cm wide and 150–230 µm thick. Young blades vary in shape but typically slightly wider than high. Mature blades orbicular in shape and forming deep lobes. Blades single, erupting abruptly from a short, stiff stipe arising from a small discoidal holdfast that is usually attached to rhodoliths. Carposporophytes 600–900 µm in diameter, scattered over the blade. Tetrasporophytes and gametophytes isomorphic. Tetrasporangia scattered throughout the cortex, terminal, cruciately divided, 10–14 × 10–24 µm.

**HOLOTYPE:** ARS 09739/BISH 780911, Kapou (Lisianski), Hawai'i, USA (25°52.94'N, 173°57.73'W, 84 m depth, collected 15 September 2014 by R. Pyle and D. Wagner). GenBank accessions: *rbcl*, OM621858; COI, OM509720.

**ISOTYPES:** BISH 780912 and BISH 780913, collection details as for the holotype.

**ETYMOLOGY:** The species epithet *kalaukapuae* honours Laura Kalaukapu Low Lucas Thompson (1925–2020) for her advocacy for Hawai'i's cultural and natural resources, especially her significant contributions to the creation of the Papahānaumokuākea Marine National Monument, including her role as a founding member of the NWHI Coral Reef Ecosystem Reserve (see Table S3 for more information on how specific nomenclature was developed using traditional Hawaiian naming practices in collaboration with the Papahānaumokuākea Native Hawaiian Cultural Working Group, CWG).

**DISTRIBUTION:** Throughout the Papahānaumokuākea Marine National Monument including Manawai (Pearl and Hermes Atoll), Kapou (Lisianski Island) and Lalo (French Frigate Shoals), and exclusively collected from mesophotic depths, at 83–85 m.

**Morphology and ecology**

Thalli are foliose, consisting of a single blade, 1.5–35 cm long and 1.5–48 cm wide, arising from a short, stiff, cartilaginous stipe, abruptly expanding into a broad, gelatinous blade (Figs 2–6). Thalli ranging from blush to rose pink, sometimes tending to



**Table 1.** Comparison of morphological and anatomical characters among *Croisettea* species.

Characters	<i>C. kalaukapuae</i>	<i>C. haukoaweo</i>	<i>C. ohelouliuli</i>	<i>C. pakualapa</i>	<i>C. requienii</i> <sup>1</sup>	<i>C. australis</i> <sup>2</sup>	<i>C. tasmanica</i> <sup>3</sup>
Habit	Single thalli, mostly undulating or pleated margins, enlarging abruptly from the stipe	Thalli with one or more blades develop and produce marginal subdichotomously lobed blades	Single thalli, composed of procumbent perforate blades that are irregularly lobed and smooth-margined	Single thalli, composed of non-perforate blades with smooth to minutely dentate margins	Thalli lobed, typically erect or decumbent	Thallus flattened, irregularly alternately to subdichotomously branched	Foliose, often with numerous large, marginal lobes
Blade shape	Orbicular	Irregular to flabellate	Irregular in outline	Orbicular	Irregular	Irregular	Irregular to flabellate
Blade margin	Smooth undulate margins	Broadly crenate	Irregularly lobed to smooth margins	Smooth margins	Smooth	Smooth to slightly irregular margins	Smooth margins
Blade texture	Gelatinous	Gelatinous	Gelatinous	Gelatinous	Gelatinous	Cartilaginous	Gelatinous
Blade height (cm)	1.5–35	0.5–6	1.5–9	1.0	-	5–10	5–30
Blade width (cm)	1.5–48	1–8	0.5–7	0.6	-	3	0.2–5
Blade thickness (µm)	150–230	40–80	40–80	50–100	-	220–450	250–500
Stipe (cm)	0.1–0.3 × 0.1–0.6	Not observed	0.2–0.3 × 0.3–0.6	Not observed	-	0.2–0.6 × 0.2–0.6	0.2–1 × 0.1–0.33
Cortex (layers)	1–2	1–2	1–2	1–2		4–6	3–4
Outer cortical cell dimensions (µm)	1–3 × 5–10	1–3 × 4–10	3–7 × 5–12	2–7 × 5–10	1.0–1.5 (diameter)	2–5 × 2–5	5–10 × 5–10
Inner cortical cell dimensions (µm)	3–6 × 6–11	1–2 × 4–9	2–5 × 5–12	2–5 (diameter)	1.0–1.5 (diameter)	-	-
Medullary stellate cells (central bodies) (µm)	4–14 × 7–9	2–3 × 7–9	5–9 × 7–13	20–40 × 30–50	1.0–1.5 (diameter)	-	-
Medullary stellate cells (µm; number arms)	2–4 × 30–100; 6–12 arms	2–3 × 15–50; 4–6 arms	2–4 × 30–100; 4–6 arms	2–4 × 50–100; 4–6 arms	-	2–6 (diameter)	3–8 (diameter)
Carposporophyte	Slightly protruding	Not observed	Not observed	Not observed	Protruding	Largely protruding	Largely protruding
Tetrasporangial dimensions (µm) (division pattern)	10–14 × 10–24 (cruciate)	Not observed	Not observed	14–18 × 14–18 (cruciate)	-	25–38 × 10–15 (cruciate)	18–35 × 11–16 (cruciate)
Gametophytes	Dioecious	Unknown	Unknown	Unknown		Unknown	Unknown
Distribution	Manawai, Kapou and Lalo, Hawai'i, USA	Maui, Hawai'i, USA	Maui, Hawai'i, USA	Manawai, Hawai'i, USA	Atlantic and Mediterranean	Australia	Tasmania, Australia
Depth range (m)	83–85	104	94–113	85	0–95	0–50	5–40
Type locality	Kapou, Hawai'i, USA	'Au'au Channel, Maui, Hawai'i, USA	'Au'au Channel, Maui, Hawai'i, USA	Manawai, Hawai'i, USA	Cap Croisette, Marseille, France	Port Phillip Heads, Victoria, Australia	Georgetown, Tasmania

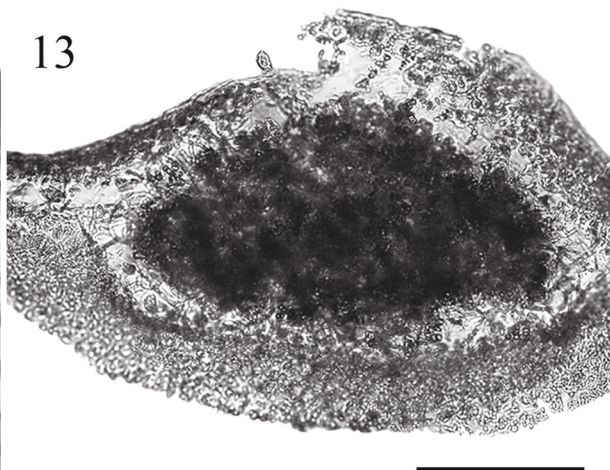
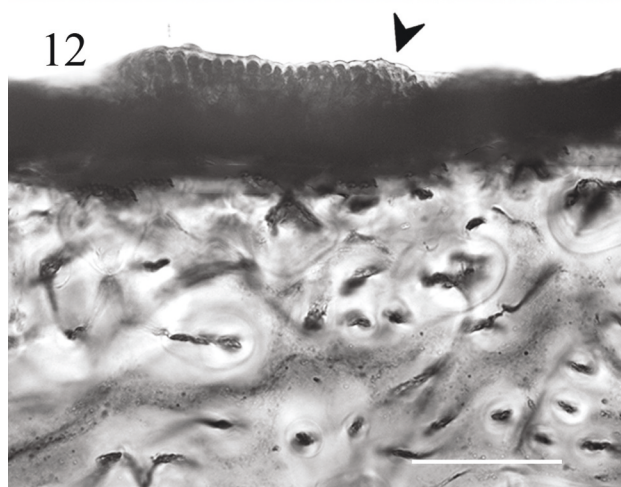
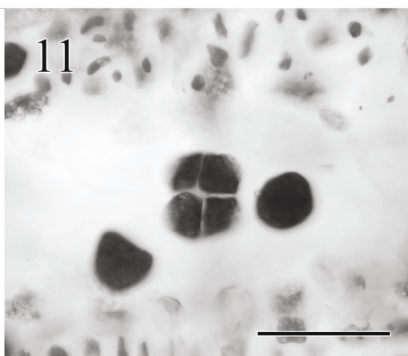
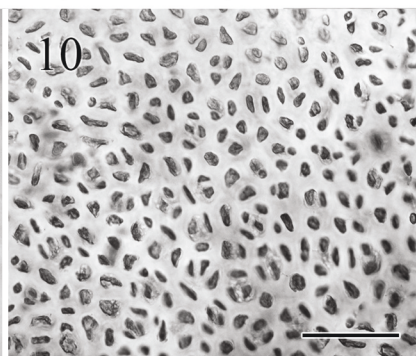
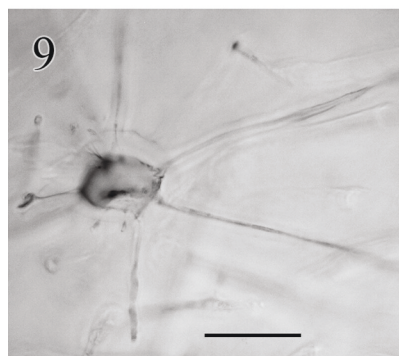
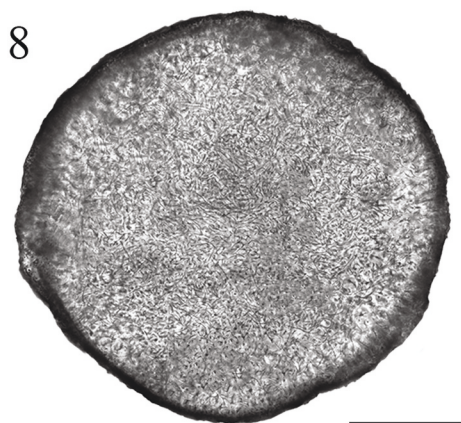
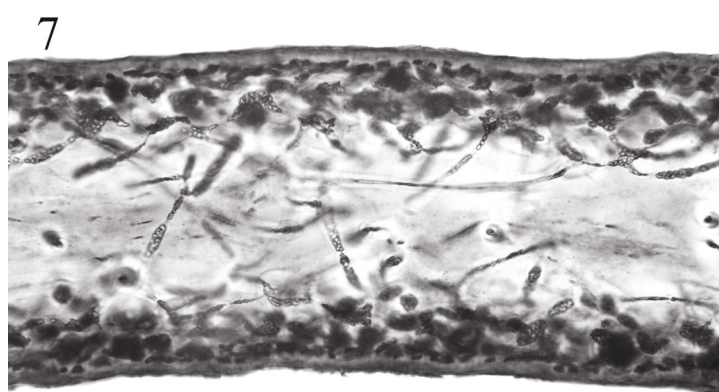
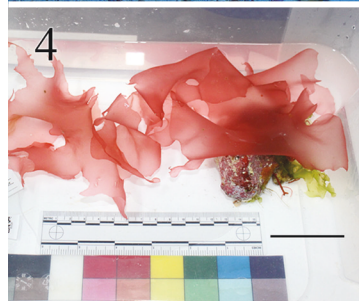
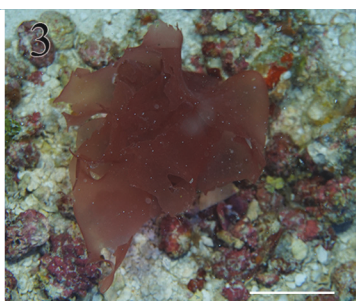
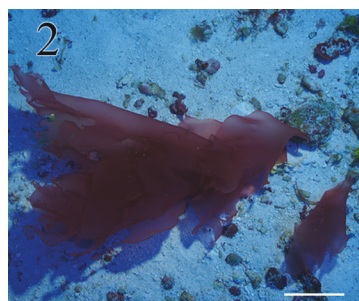
## References:

<sup>1</sup>Agardh (1847) and Rodríguez-Prieto & Hommersand (2009).<sup>2</sup>Womersley (1994, pp 241, 242).<sup>3</sup>Womersley (1994, pp 235, 236).

a pinkish brown colour. Blade margins are mostly pleated to undulate. Blades 150–230 µm thick in section (Fig. 7) with peripheral cells ultimately bearing cortex of one or two layers of periclinally compressed inner cortical cells, 3–6 × 6–11 µm, and a cartilaginous stipe <0.6 cm in length and 1.0–1.5 mm in diameter (Fig. 8). Medulla lax with an interconnected network of darkly staining stellate cells, typically with central bodies 7–9 µm in diameter and long, thin arms 2–4 µm wide by 30–100 µm long

(Fig. 9), extending parallel to the blade surface. Stellate cells connected to the cortex of one or two layers of small isodiametric outer cortical cells 1–3 µm wide by 5–10 µm long (Fig. 10).

Tetrasporangia scattered in the cortex and cruciately divided (Fig. 11), 5–7 × 5–12 µm, on both surfaces and terminal in blade. Thalli are dioecious. Spermatangia are formed in nemathecium, scattered across median parts of the thallus; nemathecium develop on both sides of the blade, are





darkly staining, and are elongate, with irregular margins (Fig. 12). Spermatangia (3–6  $\mu\text{m}$  in diameter) borne singly on spermatangial mother cells (10–30  $\mu\text{m}$  long) in the outer cortex. Cystocarps are approximately 600–900  $\mu\text{m}$  in diameter, slightly protruding from the thallus surface, and are distributed across the blade surfaces except in the basal region. Carpospores 10–15  $\mu\text{m}$  in diameter, forming a singular dense mass (Fig. 13).

These blades are relatively abundant on the mesophotic reefs in the PMNM (Manawai, Kapou and Lalo). They have been so far only documented from mesophotic depths (83–85 m). Blades are typically attached at a single point to coral rubble on a sandy bottom and are often observed to have a sprawling habit.

***Croissetea haukoaweo* F.P. Cabrera & A.R. Sherwood**  
***sp. nov.***

Figs 14–16

**DESCRIPTION:** Blades typically flabellate, single or clustered, 1–6 cm long by 1–8 cm wide and 40–80  $\mu\text{m}$  thick, lobed with broadly crenate margin, magenta pink to rose red, with a soft, slippery consistency. One or more blades developing and producing in turn several to many marginal, subdichotomously highly lobed blades, often overtopping one another. Medulla uniform throughout with a sparse arrangement of elongated filamentous stellate cells with 4–6 arms; cortex 1–2 layers of ovoid cells, 1–3  $\mu\text{m}$  wide by 5–10  $\mu\text{m}$  high.

**HOLOTYPE:** ARS 09989/BISH 780919, ‘Au‘Au Channel, Maui, Hawai‘i, USA (104 m depth), collected 29 September 2006 by H. Spalding and T. Kerby. GenBank accession: *rbcl*, OM621863.

**ETYMOLOGY:** The species epithet *haukoaweo* refers to “the vibrant limu entwined with pūko‘ako‘a (*Halimeda* sp.) found in the cool deep waters”. The term ‘hau’ in the name also honours Mr. Skippy Hau, conservationist and retired State of Hawai‘i Division of Aquatic Resources staff on Maui, for his lifetime dedication to the ocean and his community (see Table S3 for more details on how the CWG developed the species name).

**DISTRIBUTION:** A single collection, from ‘Au‘Au Channel, Maui, Hawai‘i, USA; mesophotic depth of 104 m.

### Morphology and ecology

Blades single or clustered, 1–6 cm in height, 1–8 cm wide, lobed with broadly crenate margins (Fig. 14). Blades in cross section uniformly 40–80  $\mu\text{m}$  thick. Stipe and mode of attachment to substrate not observed. The medulla consisting primarily of sparse filaments and stellate cells with central bodies ranging from 5–10  $\mu\text{m}$  wide by 1–3  $\mu\text{m}$  high, and elongate, slender arms (4–6 in

number) that are 2–3  $\mu\text{m}$  wide by 15–50  $\mu\text{m}$  long (Fig. 15). Surface view of outer cortical ovoid cells 1–3  $\mu\text{m}$  wide by 5–10  $\mu\text{m}$  high (Fig. 16). Tetrasporangial and gametangial reproduction not observed.

Although mode of attachment of blades was not identified, parts of blades were found entwined with species of mounding, prostrate species of *Halimeda* J.V. Lamouroux, which are abundant in the ‘Au‘Au Channel, Maui (see Spalding *et al.* 2019, fig. 29.1b). Blades of *C. haukoaweo* are only documented in the MHI in the ‘Au‘Au Channel, Maui at 104 m depth.

***Croissetea ohelouliuli* F.P. Cabrera & A.R. Sherwood**  
***sp. nov.***

Figs 17–21

**DESCRIPTION:** Blades foliose, magenta pink to rose red, blades sometimes wider than high, 5–9 cm in height and 0.5–7 cm wide. Blades are smooth-surfaced, irregularly lobed, membranous, and attached with a short stipe to the substratum by a small discoid holdfast. Blades uniformly 40–80  $\mu\text{m}$  thick, the medulla composed primarily of sparse filaments and stellate cells with central bodies 5–9  $\mu\text{m}$  wide by 7–13  $\mu\text{m}$  high, and elongate, slender arms (4–6 in number), 2–4  $\mu\text{m}$  wide by 30–100  $\mu\text{m}$  long. The outer cortical layer subtending 1–2 layers of refractive isodiametric cells, 3–7  $\mu\text{m}$  wide by 5–12  $\mu\text{m}$  high.

**HOLOTYPE:** ARS 09953/BISH 780920, ‘Au‘Au Channel, Maui, Hawai‘i, USA (113 m depth), collected 29 September 2006 by H. Spalding and T. Kerby. GenBank accessions: *rbcl*, OM621861; *COI*, OM509723.

**ISOTYPE:** ARS 09954/BISH 780921, ‘Au‘Au Channel, Maui, Hawai‘i, USA (94 m depth), collected 29 September 2006 by H. Spalding and T. Kerby.

**ETYMOLOGY:** The species epithet *ohelouliuli* refers to “the dark and vibrant ‘ōhelo” (or algae with no known species attached to it). ‘Ōhelo also describes the colour of the limu in its reference to a mauka (land) plant, *Vaccinium reticulatum* Smith, and its deep, red-coloured berries and endemism to Hawai‘i (see Table S3 for more details on how the species name was developed by the CWG).

**DISTRIBUTION:** Two specimens collected from the ‘Au‘Au Channel, Maui, Hawai‘i, USA; depth range of 94–113 m.

### Morphology and ecology

Thalli are bright fuchsia to dark red, soft and fleshy, irregularly lobed or with smooth margins, 1.5–9 cm in length and 0.5–7 cm in width (Figs 17, 18). Blades emerge from a short stipe with a small discoid holdfast that attaches to the substratum, beset with numerous perforations and small protuberances, and are uniformly 40–80  $\mu\text{m}$  thick (Fig. 19). The medulla is composed primarily of sparse filaments with darkly

**Figs 2–13.** Habit, general morphology and anatomy of *Croissetea kalaukapuae* sp. nov.

**Fig. 2.** Holotype specimen, male gametophyte (BISH 780911) *in situ*, collected at Papa‘āpoho (Lisianski) at 84 m. Scale bar = 5 cm.

**Fig. 3.** Paratype specimen (BISH 780917) *in situ*, collected at Lalo (French Frigate Shoals) at 83 m. Scale bar = 5 cm.

**Fig. 4.** Live holotype specimen (BISH 780911), cleaned of epiphytes. Scale bar = 5 cm.

**Fig. 5.** Live holotype specimen (BISH 780917), cleaned of epiphytes. Scale bar = 5 cm.

**Fig. 6.** Voucher for BISH 780911 (holotype, tetrasporophyte). Scale bar = 5 cm.

**Fig. 7.** Cross section through blade showing filamentous medulla and cortex, BISH 780911. Scale bar = 100  $\mu\text{m}$ .

**Fig. 8.** Cross section through stipe showing dense aggregation of narrow internal filaments, BISH 780910. Scale bar = 400  $\mu\text{m}$ .

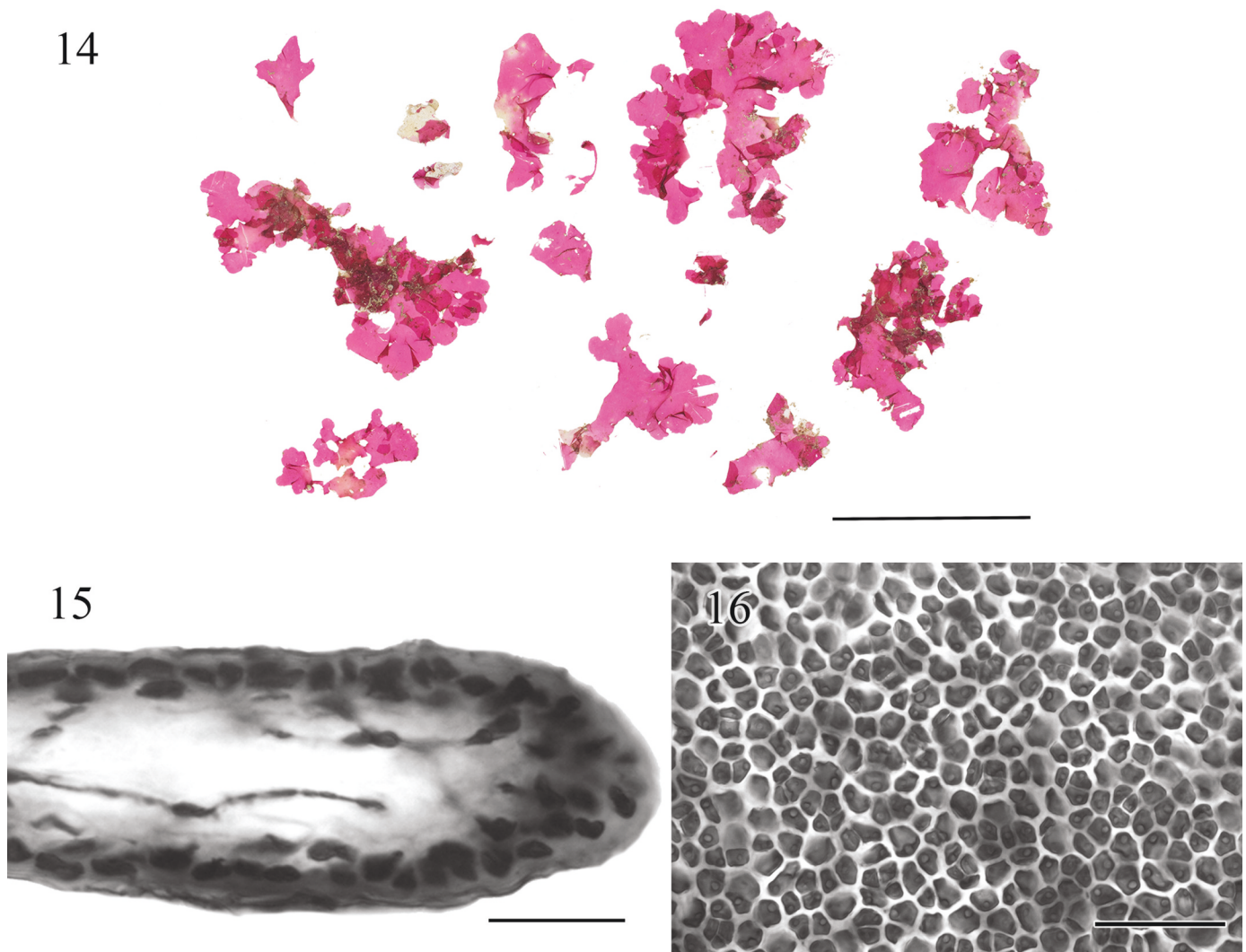
**Fig. 9.** Squash preparation showing stellate cell, BISH 780912. Scale bar = 50  $\mu\text{m}$ .

**Fig. 10.** Cortical cells in surface view, BISH 780909. Scale bar = 50  $\mu\text{m}$ .

**Fig. 11.** Detail of cruciate tetrasporangia, BISH 780910. Scale bar = 50  $\mu\text{m}$ .

**Fig. 12.** Cross section through blade with arrows showing male nemathecium, BISH 780918. Scale bar = 100  $\mu\text{m}$ .

**Fig. 13.** Cross section through mature female cystocarp, BISH 780909. Scale bar = 200  $\mu\text{m}$ .



**Figs 14–16.** Morphology and anatomy of *Croissetea haukoaweo* sp. nov.

**Fig. 14.** Pressed voucher for BISH 780919 (holotype, vegetative). Scale bar = 5 cm.

**Fig. 15.** Cross section through apical portion of the blade showing inner cortical cells and medullary stellate cells, BISH 780919. Scale bar = 25  $\mu$ m.

**Fig. 16.** Cortical cells in surface view, BISH 780919. Scale bar = 50  $\mu$ m.

staining stellate cells with large central bodies 5–9  $\mu$ m wide by 7–13  $\mu$ m, bearing high and elongate, slender, radiating arms (4–6 in number), 2–4  $\mu$ m wide by 30–100  $\mu$ m long (Fig. 20). Cortex compact with 1–2 layers of isodiametric cells, 3–7  $\mu$ m wide by 5–12  $\mu$ m high (Fig. 21). Tetrasporangial and gametangial reproduction not observed.

Blades found growing attached either to dense assemblages of *Halimeda* spp or coral rubble. Numerous perforations and small protuberances on blades either ontogenetic or marks of grazing pressure from mesophotic herbivores. So far, this species has only been documented in the MHI, ‘Au‘Au Channel, Maui at a depth range of 94–113 m.

***Croissetea pakualapa* F.P. Cabrera & A.R. Sherwood  
sp. nov.**

**Figs 22–26**

**DESCRIPTION:** Thalli single, composed of non-perforate blades with smooth to minutely dentate margins, rose pink in colour. Thalli with a soft, slippery consistency, 1.0 cm long by 0.6 cm wide and 50–100  $\mu$ m thick, and orbicular in shape. Crucially divided tetrasporangia scattered

in the cortex of both blade surfaces, typically spherical and regularly cruciate, 14–18  $\times$  14–18  $\mu$ m. Medulla with a loose arrangement of elongate stellate cells 30–50  $\mu$ m in diameter, bearing 6–8 radiating arms 2–4  $\times$  50–100  $\mu$ m; cortex of 1–2 layers of small isodiametric cells, 2–5  $\mu$ m in diameter.

**HOLOTYPE:** ARS 09948/ BISH 780907, Manawai (Pearl and Hermes Atoll), Hawai‘i, USA (27°44.48’N, 175°57.50’W, 84 m depth, collected 15 September 2014 by B. Hauk). GenBank accessions: *rbcL*, OM621860; *COI*, OM509722.

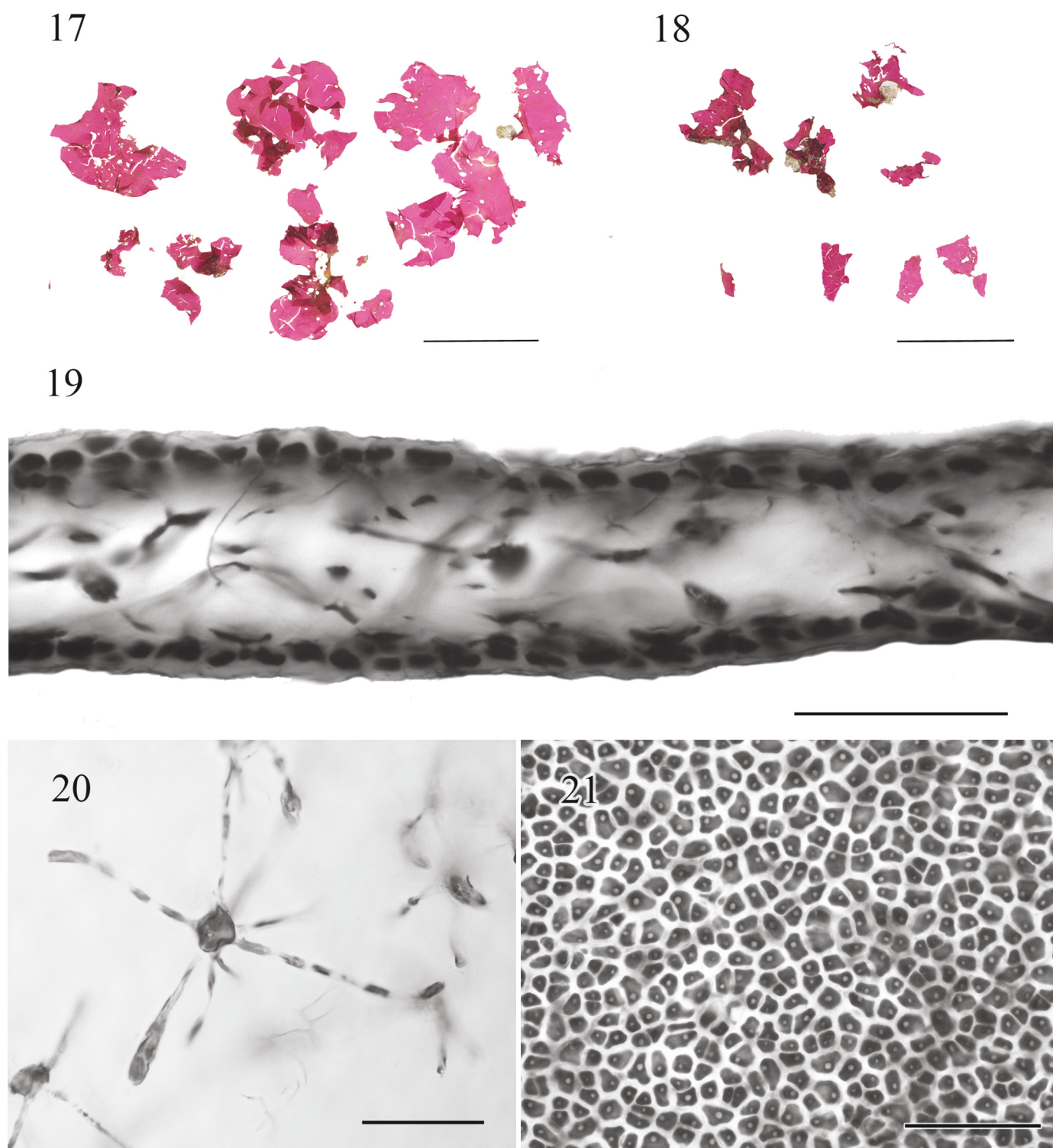
**ETYMOLOGY:** The species epithet *pakualapa* refers to “a sprawling, tapered limu found on the ridge” (see Table S3 for more details on how the species name was developed by the CWG).

**DISTRIBUTION:** A single specimen collected from Manawai (Pearl and Hermes Atoll), Hawai‘i, USA; at a depth of 85 m.

**Morphology and ecology**

Only a single blade was collected, non-stipitate, 1.0 cm in height, 0.6 cm wide, with smooth to minutely dentate margins (Figs 22–23). Blade cross section 50–100  $\mu$ m thick (Fig. 24). Tetrasporangia





**Figs 17–21.** Morphology and anatomy of *Croisetia ohelouliuli* sp. nov.

**Fig. 17.** Pressed voucher for BISH 780920 (holotype, vegetative). Scale bar = 5 cm.

**Fig. 18.** Pressed voucher for BISH 780921 (paratype, vegetative). Scale bar = 5 cm.

**Fig. 19.** Cross section through blade, BISH 780920. Scale bar = 50 μm.

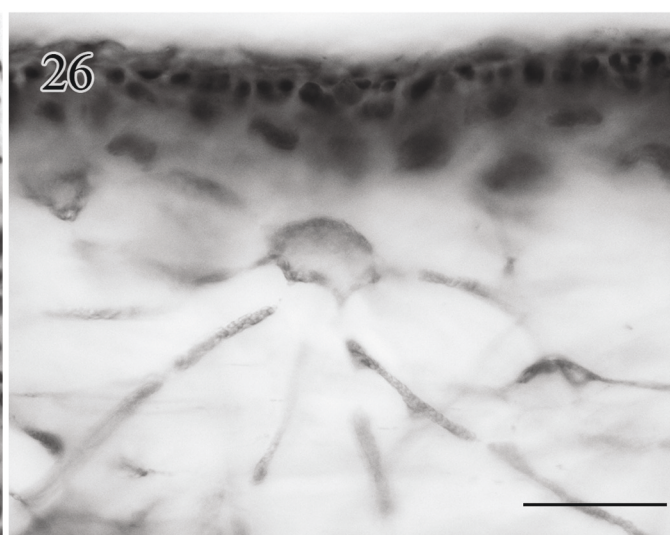
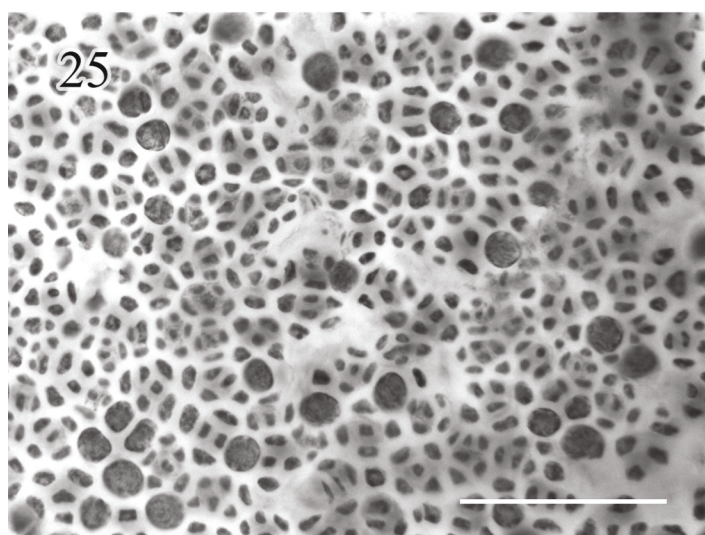
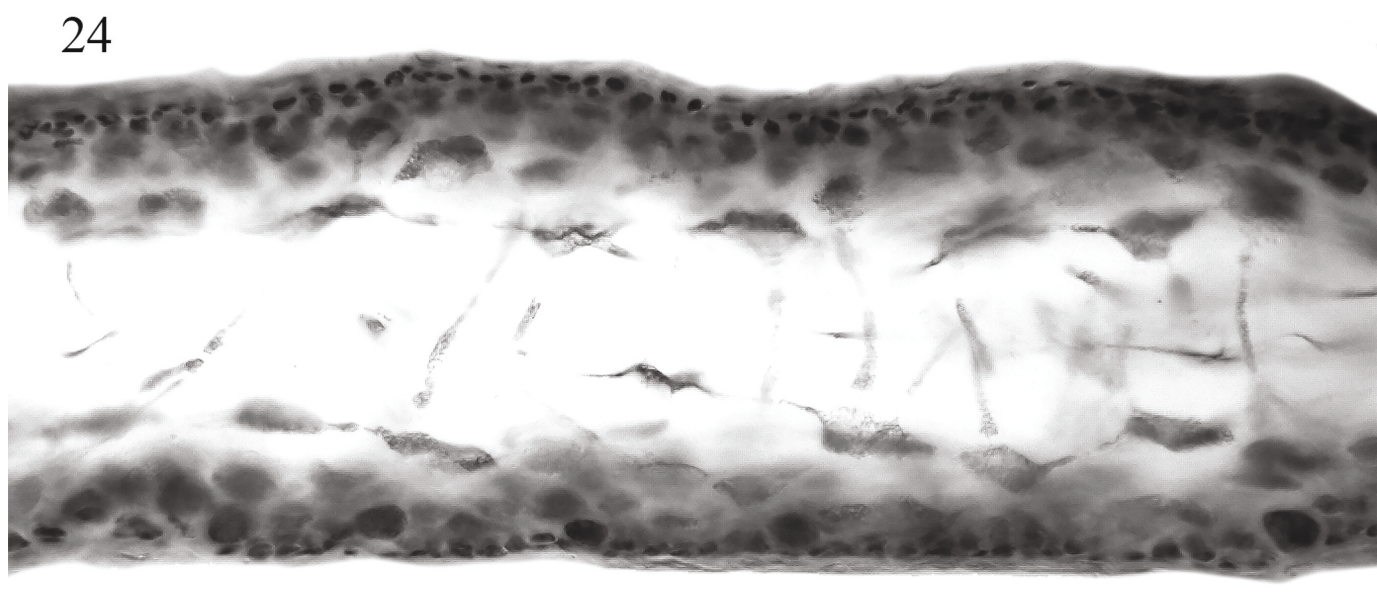
**Fig. 20.** Detail of a stellate cells in a squash preparation. BISH 780920. Scale bar = 50 μm.

**Fig. 21.** Cortical cells in surface view, BISH 780921. Scale bar = 50 μm.

scattered in the cortex of both blade surfaces, typically spherical and regularly cruciate,  $14 \times 14$  to  $18 \times 18$  μm (Fig. 25). The inner cortex is composed of 1–2 layers of small, isodiametric cells, 2–5 μm in diameter, which subtends one or two layers of periclinally

elongated outer cortical cells covering the subsurface cells (Fig. 25). Medullary stellate cells 30–50 μm in diameter, bearing 6–8 radiating arms  $2\text{--}4 \times 50\text{--}100$  μm (Fig. 26). Gametangial reproduction not observed.





**Figs 22–26.** Morphology and anatomy of *Croisettea pakualapa* sp. nov.

**Fig. 22.** Live holotype specimen, BISH 780907. Scale bar = 1 cm.

**Fig. 23.** Pressed voucher (visible cut from obtaining tissue for DNA extraction, BISH 780907 (holotype, tetrasporophyte). Scale bar = 1 cm.

**Fig. 24.** Cross section through blade showing medullary filaments, BISH 780907. Scale bar = 50  $\mu$ m.

**Fig. 25.** Cortical cells and tetrasporangia in surface view, BISH 780907. Scale bar = 100  $\mu$ m.

**Fig. 26.** Detail of a stellate cell, BISH 780907. Scale bar = 50  $\mu$ m.

The single blade was collected at Manawai (Pearl and Hermes Atoll, PMNM) at a depth of 85 m. Although mode of attachment was not identified, this small blade was collected on a flat sandy surface, adjacent to a ridge.

## DISCUSSION

Genus-level relationships within the family Kallymeniaceae remain equivocal because of varying degrees of phylogenetic support (Selivanova *et al.* 2020; Skriptsova 2021). The placement of *Croisettea* has not been fully resolved in our molecular phylogenetic analyses, and support is lacking for the *Croisettea* clade. Our phylogenetic analyses allied the four newly proposed species as distinct lineages within *Croisettea*. While relationships among *Croisettea* species remain largely unresolved or weakly supported in our analyses, our expanded phylogeny suggests that there are potentially many more genera in Kallymeniaceae. With the scale of phylogenetic studies continuing to grow and more taxa, particularly in the understudied MCEs, being included, additional independent lineages are likely to emerge. Further studies revisiting the morphology of this group and providing robust resolution of the molecular phylogenies will be necessary to better understand evolutionary relationships in the group and help define the taxonomic status of *Croisettea*.

There is considerable overlap in the morphological characters of *C. kalaukapuae*, *C. ohelouliuli* and *C. pakualapa*, such that it would be impossible to distinguish them in the field. Only *C. haukoaweo* has a suite of characters, particularly its deeply lobed blades, that clearly distinguishes it from the other Hawaiian *Croisettea* species. However, the young blades of *C. haukoaweo*, lacking lobes, also have overlapping morphological characters with all congeners. The potential for misidentification is incredibly high when preserved or juvenile specimens are the only material available for comparison. For example, *C. pakualapa* is easily confused with *C. kalaukapuae* or *C. ohelouliuli*. Overall, our phylogenetic and morphological analyses corroborate previous reports that cryptic speciation is rampant among red blades, which exhibit extremely high phylogenetic diversity (Rodríguez-Prieto *et al.* 2019; D'Archino & Zuccarello 2020).

Although cosmopolitan in distribution, kallymeniacean diversity is concentrated in the temperate regions of the world (Saunders *et al.* 2017). Abbott (1999) reported her earliest encounters with *Kallymenia*, which she previously identified as *Pugetia* Kylin (Abbott 1996), in the tropical Pacific to be an 'unusual occurrence'. Tropical Hawaiian *Croisettea* species are locally restricted to lower temperature and irradiance levels (Spalding *et al.* 2019), similar to temperate congeners. Yet, in contrast to its congeners distributed across shallow to mesophotic depths, Hawaiian *Croisettea* is exclusively documented in the lower mesophotic. For these reasons, we believe these new species to be endemic. Moreover, having distinct genetic differences in spite of overlapping in their geographical distribution (i.e. *C. haukoaweo* and *C. ohelouliuli* in 'Au'au Channel; and *C. kalaukapuae* and *C. pakualapa* at Manawai) hints at a remarkable degree of endemism. Thus, the Hawaiian MCEs appears to be a diversity hotspot for *Croisettea*.

Our study of Hawaiian *Croisettea*, similar to a study of Hawaiian mesophotic Ulvaceae (Spalding *et al.* 2016), does not support the Deep Reef Refuge Hypothesis, which postulates that mesophotic reefs function as refugia when there is considerable species overlap with shallow-water counterparts (Bongaerts & Smith 2019). In both of these studies, novel species were only documented at mesophotic depths. Yet, species overlap across spatial ranges differs by genus and species. For instance, among Hawaiian representatives of the genus *Martensia* K. Hering, *M. tsudae* A.R. Sherwood & Showe M. Lin and *M. hawaiiensis* A.R. Sherwood & Showe M. Lin occur both at shallow and mesophotic depths, whereas *M. abbottiae* A.R. Sherwood & Showe M. Lin and *M. lauhiekoeloa* A.R. Sherwood & Showe M. Lin are only documented in MCEs (Sherwood *et al.* 2019). At present, the Hawaiian endemic flora associated with the MCEs suggests two ecotypes: depth generalists that occur both in the shallow and mesophotic, and depth specialists usually in the lower mesophotic zone. With ample evidence for the existence of depth-specialists compared to depth-generalist algae, there is lower empirical support of the DRRH, and thus a lower likelihood of Hawaiian MCEs serving as refugia. During the course of field collections for this study, the PMNM experienced two major coral bleaching events (Couch *et al.* 2017) and a direct hit from a major hurricane (Pascoe *et al.* 2021), demonstrating that MCEs are not without vulnerabilities.

Describing new species from limited specimens cannot represent the whole picture of phenotypic diversity for a species, and this affects the completeness and utility of species descriptions. In this study, we describe *C. haukoaweo* and *C. pakualapa* from single specimens and *C. ohelouliuli* based on two specimens. Despite numerous expeditions to the 'Au'au Channel and PMNM being one of the most extensively collected MCEs globally, the current distribution ranges of these taxa cannot be determined due to lack of access to MCEs with manned ROV or submersibles and consistent funding for mesophotic expeditions. These limitations greatly hinder a comprehensive sampling of marine algae from these unique habitats and, in turn, our ability to describe comprehensively spatial distributions, diversity and endemism of the mesophotic flora. Nevertheless, expedient formal taxonomic recognition of *C. haukoaweo*, *C. pakualapa* and *C. ohelouliuli* is beneficial in providing a taxonomic (and especially, molecular) framework for future researchers to compare future collections or describe further new taxa, and ensures the diversity is recognized, as a starting point for future research. Schneider *et al.* (2019) used single mesophotic algal specimens to describe new species that corresponded morphologically to old herbarium collections, which is a good workaround for limited collections that are non-cryptic. Description of some mesophotic organisms (i.e. fishes, decapods, etc.) based on single specimens are justified by the low likelihood for timely acquiring of additional samples from the logistically challenging mesophotic reefs (Shepherd *et al.* 2018; Felder & Lemaitre 2020).

The description of *C. kalaukapuae*, *C. haukoaweo*, *C. ohelouliuli* and *C. pakualapa* raises the total number of *Croisettea* species from three to seven. Notably, these new species represent half of the currently recognized diversity of



the Hawaiian Kallymeniaceae and contribute to the broader knowledge of MCE algal biodiversity. Our present knowledge of diversity of the Hawaiian macroalgal flora is far from exhaustive, and further species are to be expected among red blades. Given the continuing interest in Kallymeniaceae as model species for palaeobiology and biogeography (Bringloe & Saunders 2018), further investigation of its evolutionary history will be critical.

## ACKNOWLEDGEMENTS

We thank the NOAA Papahānaumokuākea Native Hawaiian Cultural Working Group for their invaluable contributions in developing specific epithets for the Hawaiian species of *Croisettea*. G. McFall, J. Leonard, K. Lopes, S. Matadobra, B. Hauk, T. Efrid, A. Fukunaga, D. Wagner and H. Owen assisted with diving and small boat operations. Our gratitude goes to Terry Kirby and the Hawai'i Undersea Research Laboratory (HURL) *Pisces IV* and *V* submersible and *RCV-150* pilots and crew, as well as the crew of the R/V *Ka'imikai-o-Kanaloa*, for access to these great depths. Fieldwork and specimen collections in the Northwestern Hawaiian Islands were authorized under Papahānaumokuākea Marine National Monument research permits PMNM-2014-15, PMNM 2015-029 and PMNM-2018-029 issued to R. Kosaki.

## DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

## FUNDING

This work was supported by the U.S. National Science Foundation [DEB-1754117]; the U.S. National Fish & Wildlife Foundation [NFWF 0810.18.059023]; the National Oceanic and Atmospheric Administration (NOAA) Papahānaumokuākea Marine National Monument, NOAA Coastal Ocean Program [NA07NOS4780187 and NA07NOS478190 to the University of Hawai'i]; NOAA's Undersea Research Program and Coral Reef Conservation Program through the Hawai'i Undersea Research Laboratory [NA09OAR4300219 and NA05OAR4301108], and NOAA's Office of Ocean Exploration. The scientific views and conclusions and any views or opinions expressed herein are those of the authors and do not reflect the opinions of the above organizations.

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