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NEW ULVACEAE (ULVOPHYCEAE, CHLOROPHYTA) FROM MESOPHOTIC ECOSYSTEMS ACROSS THE HAWAIIAN ARCHIPELAGO[1]

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Ulvalean algae (Chlorophyta) are most commonly described from intertidal and shallow subtidal marine environments worldwide, but are less well known from mesophotic environments. Their morphological simplicity and phenotypic plasticity make accurate species determinations difficult, even at the generic level. Here, we describe the mesophotic Ulvales species composition from 13 locations across 2,300 km of the Hawaiian Archipelago. Twenty-eight representative Ulvales specimens from 64 to 125 m depths were collected using technical diving, submersibles, and remotely operated vehicles. Morphological and molecular characters suggest that mesophotic Ulvales in Hawaiian waters form unique communities comprising four species within the genera *Ulva* and *Umbraulva*, each with discrete geographic and/or depth-related distributional patterns. Three genetically distinct taxa are supported by both plastid (*rbc*L and *tuf*A) and nuclear (ITS1) markers, and are presented here as new species: *Umbraulva kaloakulau*, *Ulva ohiohilulu*, and *Ulva iliohaha*. We also propose a new *Umbraulva* species (*Umbraulva kuaweuweu*), which is closely related to subtidal records from New Zealand and Australia, but not formally described. To our knowledge, these are the first marine species descriptions from Hawai'i resulting from the collaboration of traditional Hawaiian nomenclature specialists, cultural practitioners and scientists. The difficulty of finding reliable diagnostic morphological characters for these species reflects a common problem worldwide of achieving accurate identification of ulvalean taxa using solely morphological criteria. Mesophotic Ulvales appear to be distinct from shallow-water populations in Hawai'i, but their degree of similarity to mesophotic floras in other locations in the Pacific remains unknown.

Key index words: **Hawai'i; ITS; mesophotic coral ecosystem; molecular species concept; *rbc*L; sea lettuce; *tuf*A; *Ulva*; Ulvales; *Umbraulva***

***Abbreviations*: BI, Bayesian inference; ITS, Internal Transcribed Spacer; ML, maximum likelihood; *rbc*L, large subunit ribulose bis-phosphate carboxylase/oxygenase; *tuf*A, elongation factor *tuf*A**

Mesophotic coral ecosystems (MCEs) are characterized by communities of light-dependent corals, sponges, algae, and other organisms that are typically found at depths from 30 to over 150 m in tropical and subtropical regions (Hinderstein et al. 2010). Although MCEs often occur close to well-studied shallow coral reefs, an understanding of their biodiversity and connectivity is limited by the technical and logistical constraints of accessing these deeper depths. Despite these challenges, mesophotic surveys to date have suggested that MCEs in the Main Hawaiian Islands (MHI) contain significant and diverse cover of macroalgae (Doty et al. 1974, Agegian and Abbott 1985). Non-coralline, foliose macroalgae have been found at maximum depths ranging from 130 m (Kahng and Kelley 2007, Rooney et al. 2010) to 212 m (Spalding 2012). MCEs in the Northwestern Hawaiian Islands (NWHI) contain unique macroalgal assembalges (McDermid and Abbott 2006), dominated by species such as *Microdictyon umbilicatum* (Velley) Zanardini (Huisman et al. 2007) that form significant habitat for mesophotic reef fish (Kane et al. 2014).

Sea lettuces in the family Ulvaceae (e.g., tubular and blade-like *Ulva*, *Umbraulva*, and *Ulvaria*) are most commonly described from intertidal and shallow subtidal marine or freshwater environments worldwide (Guiry and Guiry 2015). Morphologically, this group of green algae is composed of simple, bistromatic blades (*Ulva*, *Umbraulva*), monostromatic, hollow tubes (*Ulva*, formerly *Enteromorpha*), or more rarely, monostromatic blades (*Ulvaria* and some expanded tubes such as *U. compressa*). Physiologically, sea

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lettuces are typically characterized by rapid growth and nutrient acquisition in high light, eutrophic environments (Nelson et al. 2008), and would not be expected to be significant components of low-light, oligotrophic mesophotic environments. Nevertheless, sea lettuces have been reported from MCEs and other deep-water marine habitats. For example, in the eastern Gulf of Mexico, Leichter et al. (2008) recorded a sea lettuce, assigned to *Ulva lactuca* L., as a dominant species at up to 50 m depth, and showed photographic evidence of ulvoid recruitment at 70 m. In the MHI, Abbott and Huisman (2004) reported sea lettuces dredged from mesophotic depths (up to 200 m); they assigned the specimens to *Ulva expansa* (Setchell) Setchell & N.L. Gardner, *U. fasciata* Delile, and *U. reticulata* Forsskål. In her investigations of MCEs in the MHI, Spalding (2012) found that ulvalean algae grew epiphytically (i.e., on rhodoliths, *Halimeda distorta* (Yamada) Hillis-Colinvaux and *H. kanaloana* Vroom), attached to hard substrate, or as small single blades attached to pieces of shell in soft sediment areas, and were often in high abundance with up to 75% cover in localized patches (~1–5 m^2). Individual specimens were perforate or non-perforate, and up to 80 cm in length.

An accurate description of sea lettuce biodiversity in any habitat is challenging. Since the discovery that morphological characters poorly reflect species diversity among populations of Ulvaceae based on comparisons of DNA sequence data with morphological measurements (Tan et al. 1999, Hayden et al. 2003, Hayden and Waaland 2004), numerous studies have recorded the extent to which floras have been mis- or under-reported (Shimada et al. 2003, 2008, Jiang et al. 2007, Loughnane et al. 2008, Heesch et al. 2009, Hofmann et al. 2010, Kraft et al. 2010, O'Kelly et al. 2010, Mareš et al. 2011, Duan et al. 2012, Kirkendale et al. 2012, Wolf et al. 2012, Guidone et al. 2013, Guoying et al. 2014). In the Hawaiian Islands, nearly all the names used historically for the Ulvaceae (Abbott and Huisman 2004) have been misapplied (O'Kelly et al. 2010). With the exception of the tropical Hawaiian study by O'Kelly et al. (2010), most work to date on this family has been done on temperate shallow-water biotas. In addition, understudied floras have consistently revealed novel species diversity in a genus (*Ulva*) that has typically been viewed as cosmopolitan (Heesch et al. 2009, Kraft et al. 2010, O'Kelly et al. 2010). This further compels the need for a study of sea lettuce richness from rarely studied deep-water, or mesophotic, habitats. Consequently, it remains unknown whether mesophotic sea lettuces in Hawai'i occur elsewhere, represent a subset of shallow-water species, or form a new and distinctive flora.

As part of several larger studies on mesophotic macroalgal floras in the MHI and NWHI, we used submersibles, remotely operated vehicles (ROVs), and technical divers to investigate the habitat and distribution of mesophotic sea lettuces. Molecular and morphological data were obtained and analyzed from collected ulvalean specimens to elucidate the taxon identities. Our goals were to (i) determine the species composition of mesophotic Ulvales in Hawai'i, (ii) resolve any overlap between shallow and mesophotic populations in Hawai'i, and (iii) discern if Hawaiian mesophotic specimens belong to species previously characterized from other areas of the world.

MATERIALS AND METHODS

Specimen collection and preservation. Ulvales were collected with the Hawai'i Undersea Research Laboratory (HURL) submersibles *Pisces IV* and *Pisces V*, HURL ROV *RCV-150*, and by technical divers using closed circuit rebreathers from 2004 to 2013 (Table S1 in the Supporting Information). Samples were collected from 40 to 125 m depths at seven locations in the MHI and from 30 to 85 m depths at six locations in the NWHI (Table S1, Fig. 1). The GPS coordinates for each location were reported as the starting point of the submersible dive. All green thalli visible to the naked eye were targeted for collection. Algae were photographed in situ with the GPS coordinates, depth, and habitat noted for each specimen. Collected macroalgae were saved in triplicate as herbarium presses for voucher specimens, in silica gel for molecular analyses, and in a 4% buffered formaldehyde solution in seawater. Each specimen was given an "HS" collection number in the field and an "ARS" accession number in the laboratory for molecular analyses. Algae were tentatively identified using the systematic keys and taxonomic information provided in Abbott and Huisman (2004) and Huisman et al. (2007). Vouchers were deposited at the Herbarium Pacificum, B. P. Bishop Museum, Honolulu, Hawai'i (BISH).

Morphological assessment. In the laboratory, the morphology of each specimen was examined, and the thallus shape, size, texture, and color were recorded. Preserved slides were made for microscopic analyses according to Tsuda and Abbott (1985). Morphological and anatomical observations were made using a Zeiss Stereo Discovery V12 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) with a Canon PowerShot A640 digital camera attachment (Canon U.S.A., Inc., Lake Success, NY, U.S.A.), and an Olympus BX41 compound light microscope (Olympus America Inc., Center Valley, PA, U.S.A.) with an Olympus DP12 digital camera attachment. Cellular features were examined using wet-preserved material and herbarium samples that were rehydrated for at least 30 min. Digital images of whole-mounted and cross-sectioned basal, medial, and distal thallus regions were used to examine cell shape, cell size (maximum length and perpendicular width), thallus width, typical chloroplast positions, and the number of pyrenoids per cell. Twenty measurements were made in each thallus region for each character per specimen. Five representative specimens encompassing a range of thallus sizes, morphologies, and depths collected were analyzed for each species (as delineated by molecular analyses, described below). A balanced, nested ANOVA was used to test for significant differences in morphometrics between species. All data were examined for normality and heteroscedasticity, and Log10 transformed, if needed, to meet the assumptions of ANOVA. Only three specimens of one species contained visible holdfasts, so the average measurement for the basal cross-section width of each specimen was compared using a one-way ANOVA.

Genomic extraction and PCR. DNA from field-collected samples were extracted using a Qiagen DNeasy Plant Mini Kit

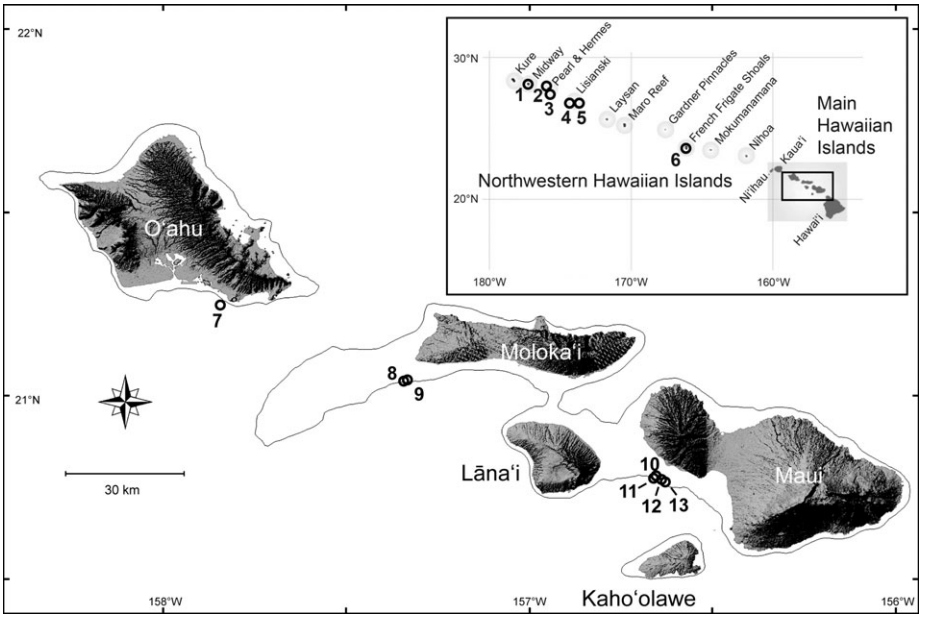

Fig. 1. Map of collection sites in the Northwestern and Main Hawaiian Islands (gray area in inset map). Site descriptions in Table S1. Gray contour lines at 100 m depth. Inset map adapted from the Papahānaumokuākea Marine National Monument.

(Valencia, CA, USA) following the manufacturer's instructions. PCR experiments (25.0 μL total) consisted of 5.0 μL of 5× MangoTaq reaction buffer (Bioline, Tauton, MA, USA), 1.5 μL of $MgCl_2$ (50 mM), 1.5 μL of 1% bovine serum albumin solution, 1.0 μL of each primer (10 mM), 1.0 μL of each dNTP (20 mM), 0.2 μL of MangoTaq DNA polymerase (Bioline; 5 mM), 9.8 μL of PCR water, and 1.0 μL genomic DNA. PCR amplification conditions for ITS1 and *rbc*L followed those given in O'Kelly et al. (2010). PCR amplification conditions for *tuf*A followed Famà et al. (2002). Successful PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol and were sequenced in both directions by the Center for Advanced Studies of Genomics, Proteomics and Bioinformatics (University of Hawai'i at Mānoa, Honolulu, HI, USA).

Alignments and phylogenetic inferences. Appropriate models of sequence evolution were assessed for the *rbc*L and *tuf*A data sets. The *rbc*L and *tuf*A data sets consisted of both newly generated sequences and additional Ulvales sequences from the National Center for Biotechnology Information (NCBI) GenBank database. Sequences were aligned using ClustalW (Thompson et al. 1994) implemented in CIPRES (http://www.phylo.org/, Miller et al. 2010), and further corrected by eye and trimmed to a similar length. Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were conducted for both data sets. The most appropriate parameter values and substitution models for the ML analyses were determined under the Bayesian Information Criterion (BIC) using jModelTest 2 (Darriba et al. 2012), also implemented in CIPRES. The optimal molecular phylogenetic tree and bootstrap values were heuristically searched by the ML method (initial tree – BioNJ [variant of NJ], nearest-neighbor interchange branch swapping, 1,000 replicates) using PhyML (Guidon and Gascuel 2003) implemented in Geneious version 5.3.6 (http://www.geneious.com, Kearse et al. 2012). The BI analyses (four chains of Markov Chain Monte Carlo, temp = 0.2, sampling every 100 generations, convergence diagnostic calculated every 1,000 generations, stop value set at 0.01) were conducted using MrBayes version 3.2.2 (Ronquist et al. 2012), and both run for 4,000,000 generations with the first 10,000 trees discarded as burn-in.

The final *rbc*L data set consisted of a total of 84 sequences and included 1,201 base-pair (bp) positions. The optimal substitution model was identified as TPM3uf+I+G (two transversion-parameters model 3 unequal frequencies with invariable sites and gamma distribution), and the parameters used were as follows: assumed nucleotide frequencies A = 0.2704, C = 0.1644, G = 0.2098, T = 0.3554; substitution rate matrix A-C substitutions = 0.3977, A-G = 7.9770, A-T = 1.0000, C-G = 0.3977, C-T = 7.9770, G-T = 1.000; proportion of invariable sites = 0.7470; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.7700. The final *tuf*A data set consisted of a total of 57 sequences and included 771 bp positions. The optimal substitution model was identified as TIM3 + I+G (transitional model with invariable sites and gamma distribution), and the parameters used were as follows: assumed nucleotide frequencies A = 0.3613, C = 0.1291, G = 0.2036, T = 0.3061; substitution rate matrix A-C substitutions = 3.4423, A-G = 5.0129, A-T = 1.0000, C-G = 3.4423, C-T = 9.4527, G-T = 1.000; proportion of invariable sites = 0.4290; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.5380.

The ITS1 region has proven useful for characterizing Ulvales OTUs (Kraft et al. 2010, Guidone et al. 2013), and was previously used to sort Hawaiian *Ulva* into 11 groups (O'Kelly et al. 2010). Yet issues with the region remain – specifically, the region's variability, which makes alignment of species from different genera difficult to nearly impossible (Saunders and Kucera 2010). Thus, the ITS1 sequence data set was compared with sequences using the Basic Local Alignment Search Tool (BLAST) from the NCBI GenBank database. A table of BLAST results was generated for each specimen with ITS sequence data (n = 22).

RESULTS

Habitat and distribution. Four species of ulvalean sea lettuces (Figs. 2–6) were delineated based on molecular analyses, each with different distribution patterns and depth ranges (Table 1). *Umbraulva kuaweuweu* was found in the shallow mesophotic zone from 65 to 84 m depths from Moloka'i and two islands in the NWHI on carbonate reef,

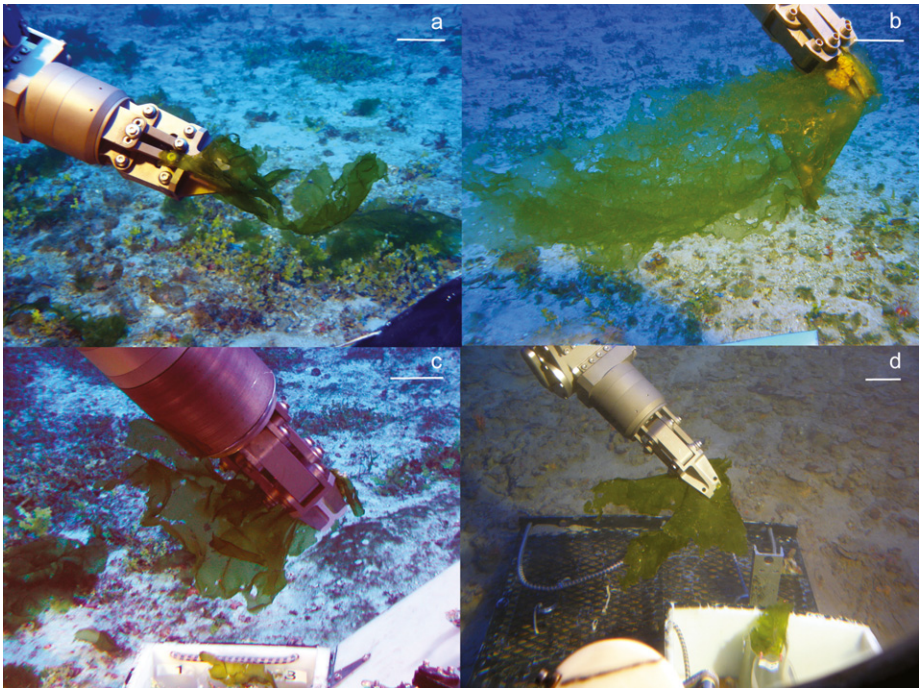

FIG. 2. Specimen collections in situ with the *Pisces IV* and *Pisces V* submersibles. White scale bar is 10 cm. Site locations in Fig. 1. The mean irradiance (PAR) levels from 80 to 125 m depths are 36 to 5 μmol photons · m^{-2} · s^{-1}, respectively (Spalding 2012). (a) *Ulva ohiohilulu* at 85 m depth (Site 7), (b) *U. ohiohilulu* at 93 m (Site 9), (c) *Umbraulva kuaweuweu* at 80 m (Site 4), (d) *Umbraulva kaloakulau* at 125 m (Site 10).

H. distorta, or attached to rhodoliths (Fig. 3, a–f; Table 1), and was often in high abundance. This species tended to be highly perforate (Fig. 3) with a dense basal rhizoidal zone in cross-section (Fig. 3d). *Umbraulva kaloakulau* (Fig. 4, a–e; Table 1) was commonly encountered in the lower mesophotic zone from 85 to 125 m depths, either within or along the edge of sparse to dense *Leptoseris* spp. coral reefs in the ʻAuʻau Channel off Olowalu, west Maui (Fig. 4, Table 1) or as isolated individuals at Pearl and Hermes, NWHI. This species had a very small holdfast, which was difficult to discern and was not readily removed from the substrate, resulting in few specimens with intact or obvious holdfasts. *Ulva ohiohilulu* (Fig. 5, a–f; Table 1) was the most commonly encountered (15 of 28 specimens) and abundant ulvalean sea lettuce species, typically occurring from 69 to 93 m depths off west Maui, south Oʻahu, west Molokaʻi (Fig. 5; Table 1), and with one specimen collected at 85 m depth from Lisiankski, NWHI. This species was found growing on pieces of shell, carbonate reefs, dead coral (*Leptoseris* spp.) skeletons, or in association with *H. distorta* or *H. kanaloana* meadows, and varied in length from 5 to 80 cm. This species was found as single individuals, or as several large individuals clumped together in 1–2 m^2 patches. In contrast, only one specimen of *Ulva iliohaha* was found at 64 m depth at French Frigate Shoals, NWHI in a mixed reef and macroalgal community (Fig. 6, a–e; Table 1).

Morphology. All thalli were foliose and distromatic. Each cell examined in all specimens contained a single parietal chloroplast with 1–2 pyrenoids. There were no significant differences in anatomical measurements between *U. ohiohilulu*, *Um. kuaweuweu*, and *Um. kulaokulau* (Table S2 in the Supporting Information), except in basal cross-section widths ($F_{2,10} = 7.02$, $P < 0.012$). *U. iliohaha* was not used for statistical comparisons given the low sample size ($n = 1$). Mean maximum cell lengths, measured in surface view of thalli for 16 specimens, ranged from 24 to 38 μm (Fig. S1 in the Supporting Information). Ratios between maximum cell length and maximum cell width in cross-section ranged from 0.26 to 2.43. Mean medial thalli cross-section widths ranged from 31 to 88 μm (Fig. S1). The largest differences found between species were in coloration, texture, and the presence of rhizoids in the basal thallus region (Table 1; Figs. 3–6). *Ulva* species were more grass green in color with membranous textures, while *Umbraulva* species had a darker, olive green coloration, and a stiffer texture. Generally, *Um. kuaweuweu* had distinctive, longitudinal rows of densely packed rhizoids in the basal thallus-to-holdfast transition zone (Fig. 3d), *U. ohiohilulu* and *U. iliohaha* possessed a thinner zone of loosely packed rhizoids (Fig. 6b), while basal rhizoids were not observed in *Um. kaloakulau*. There was also great variation between individuals in the characteristics of the basal rhizoidal zone depending on the location of the cross-section and presumed age of the thallus (as approximated based on the quantity of encrusting invertebrates and size of thallus); the width of the basal rhizoidal zone varied greatly within an individual. Thus, this feature may not be useful for delineating these particular species, especially given the diminutive size and cryptic nature of the holdfast in *Um. kaloakulau*.

Molecular analyses. Molecular analyses (Table S3 in the Supporting Information) of the *rbc*L (Fig. 7)

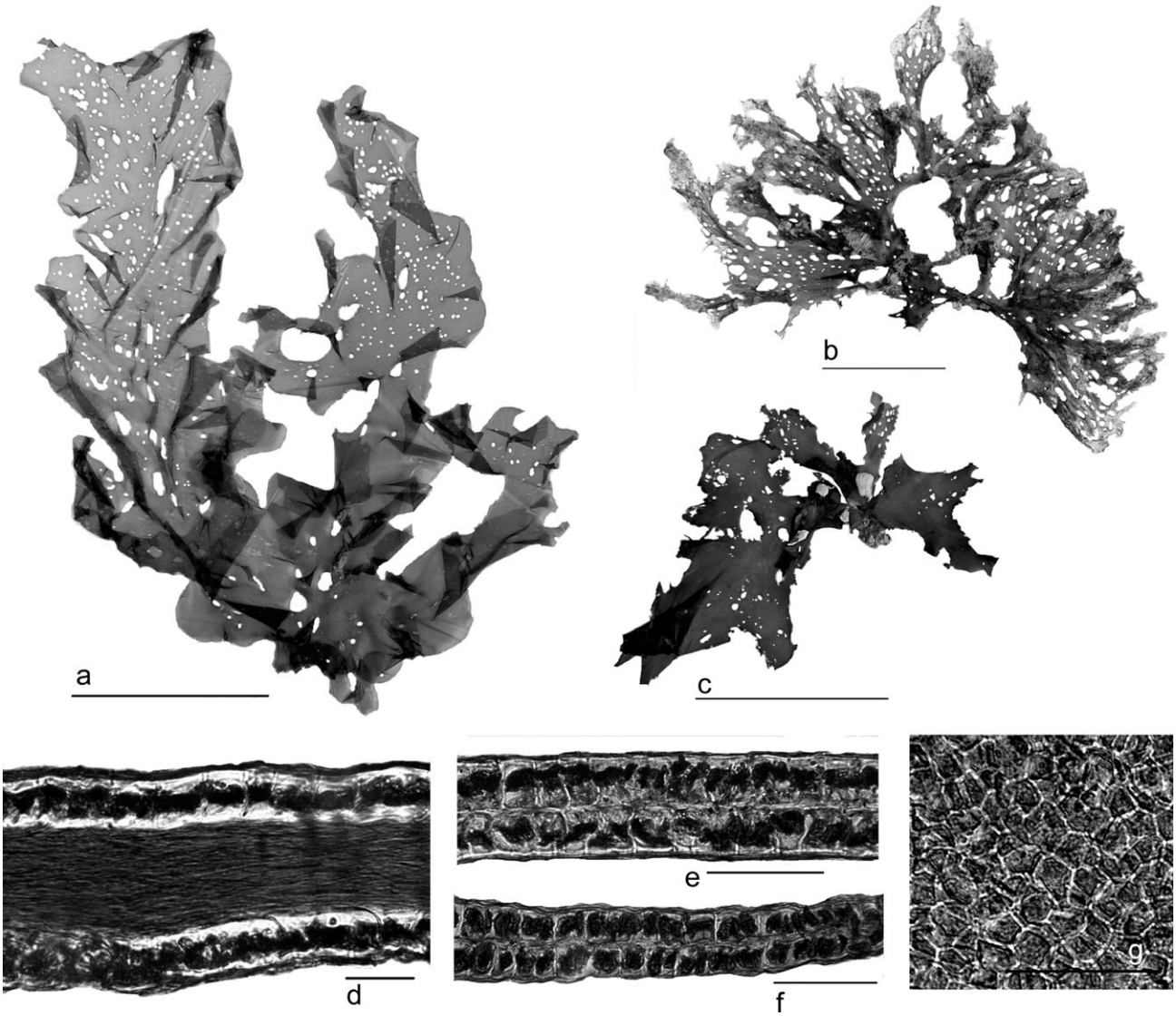

FIG. 3. Morphology of *Umbraulva kuaweuweu*. Macroscopic and microscopic scale bars are 5 cm and 100 μm, respectively. (a) ARS 08671. (b) ARS 08676. (c) Holotype BISH 759425 (ARS 03961). (d) Cross-section immediately above the basal rhizoid zone. (e) Cross-section mid-blade (f) Cross-section near blade tip. (g) Surface view mid-blade.

*tuf*A (Fig. 8) and ITS (Table S4 in the Supporting Information) regions confirmed the presence of four mesophotic species belonging to the genera *Umbraulva* and *Ulva*. Sequence divergence among the four species clades was well-supported in *tuf*A (Fig. 8), which was shown to have high resolution power at the species level for Ulvales (Saunders and Kucera 2010). The mesophotic species appeared distinct from the shallow-water flora in Hawai'i that was characterized by molecular analyses (O'Kelly et al. 2010), although one species (*Um. kuaweuweu*) clustered with sequences obtained from an undescribed *Umbraulva* species in the Kermadec Islands (Heesch et al. 2007, 2009, Fig. 7) and Australia (Kirkendale et al. 2012, Fig. 8). However, relationships among species of *Umbraulva*, aside from *Um. kuaweuweu* from Hawai'i, New Zealand, Australia, were largely unresolved. The two new species of *Ulva* clustered strongly within *Ulva* (Figs. 7 and 8; Table S4) and were in distinctly different clusters within the genus.

The final ITS1 data set consisted of 22 sequences ranging in length from 213 to 315 bp (Table S4). ITS1 sequences from all three currently described species of *Umbraulva* were compared with the mesophotic *Umbraulva* species, without any exact matches. *Um. kuaweuweu* was most similar to *Um. dangeardii* M. J. Wynne & G. Furnari (listed in GenBank, and consequently in Figs. 7, 8 and Table S4, as *Um. olivascens* (P.J.L. Dangeard) G. Furnari, *nom. inval.*; Wynne and Furnari 2014), with bp differences ranging from 15 to 16. *Um. kaloakulau* grouped with *Um. amamiensis* (Tanaka) Bae & I.K. Lee, with 22–29 bp differences between species.

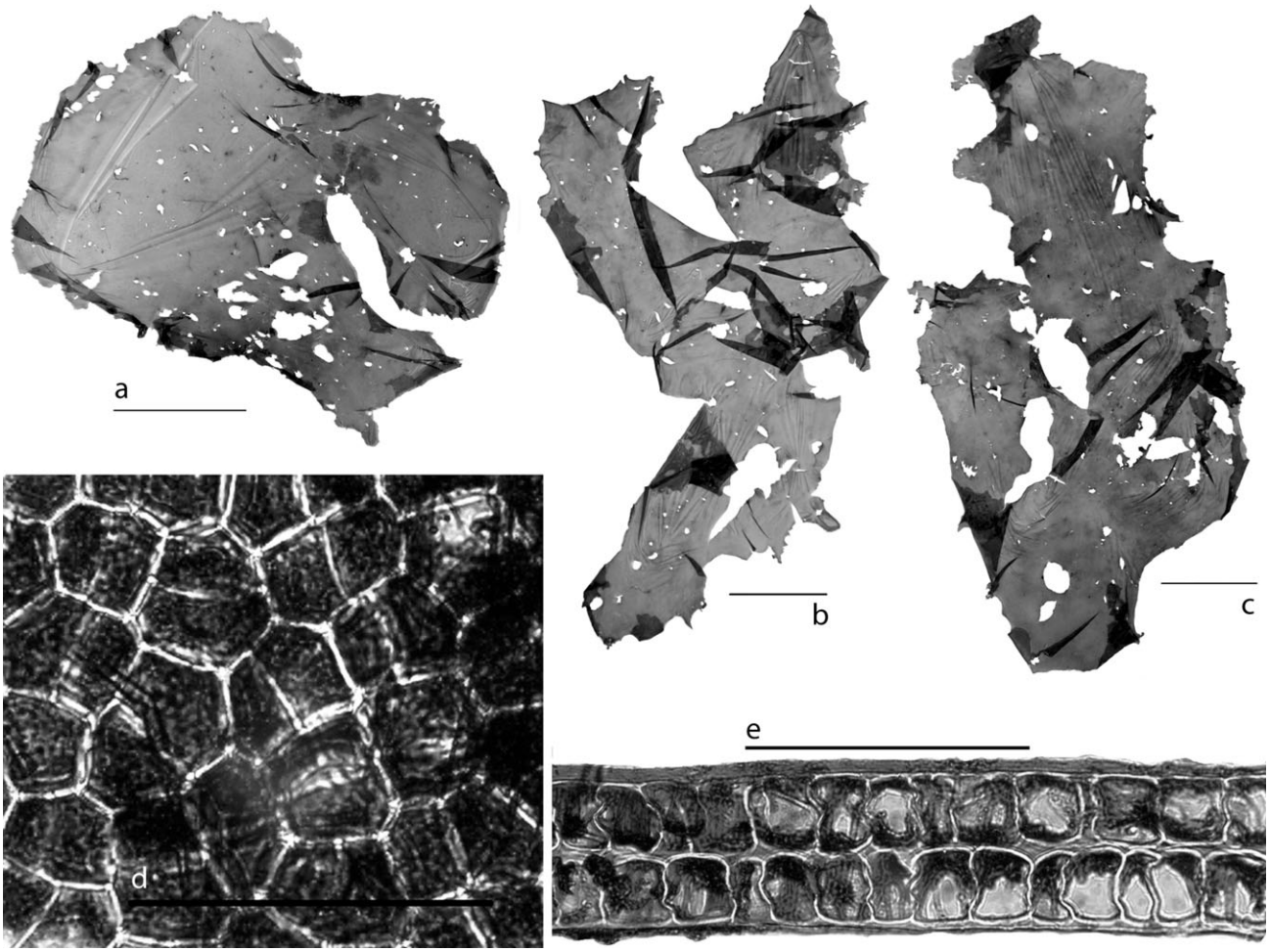

FIG. 4. Morphology of *Umbraulva kaloakulau*. Macroscopic and microscopic scale bars are 5 cm and 100 μm, respectively. (a) ARS 08675. (b) Isotype BISH 759427 (ARS 07553). (c) Holotype BISH 759426 (ARS 07552). (d) Surface view mid-blade. (e) Cross-section mid-blade.

Identities among the mesophotic *Umbraulva* species ranged from 90% to 92% similarity with *Um. japonica* (Holmes) Bae & I.K. Lee specimens. *U. ohiohilulu* specimens were most similar to specimens from deep-water sites in India that have been described as *U. gigantea* (Kützing) Bliding, with 4–36 bp differences between species, and identity similarities ranging from 89% to 98%. *U. iliohaha* was most similar to sequences assigned to *U. fasciata* Delile (= *U. lactuca* Linnaeus; O'Kelly et al. 2010), but with 41 bp differences.

Taxonomic results. In consideration of the morphological and molecular analyses provided here, two new species of *Ulva* and two new species of *Umbraulva* are hereby described from the mesophotic zone in the Hawaiian Archipelago. Traditional Hawaiian naming practices were used to develop each species names (Appendix S1 in the Supporting Information).

Umbraulva kuaweuweu H. L. Spalding & A.R. Sherwood **sp. nov.** (Figs. 2c and 3; Table 1; and Audio S1 in the Supporting Information)

Description. Plants foliose and distromatic, elliptical to flabellately expanded with cuneate lobes, 7–21 cm long by 7–17 cm wide, holdfast attached to carbonate surfaces, such as rhodoliths or carbonate reef. Thalli slightly to extensively perforate with a light to dark olive green coloration and a slightly lubricous and stiff texture. Cells in surface view polygonal and angular, arranged irregularly, 17–49 (-53) μm long by 8–25 (-39) μm wide, with 1–2 pyrenoids per cell. Distal thallus 29–69 (-83) μm thick, cells in x.s. cuboidal; median thallus 24–98 μm thick with cuboidal to rectilinear cells in x.s.; proximal x.s. at start of rhizoidal zone to 267 μm with densely packed longitudinal rhizoids. Cells in x.s. 9–49 μm tall by 10–50 μm wide.

Holotype: ARS 03961 (Fig. 3c) collected on November 28, 2006 at Penguin Bank, west Moloka'i, Hawai'i (21° 02.727′ N, 157° 21.312′ W) by M. Cremer, K. Peyton, and A. Faucci with the *Pisces IV* submersible (dive 189) at 80 m depth on carbonate reef with *H. distorta* (Yamada) Hillis-Colinvaux and rhodoliths. Deposited at BISH (BISH 759425).

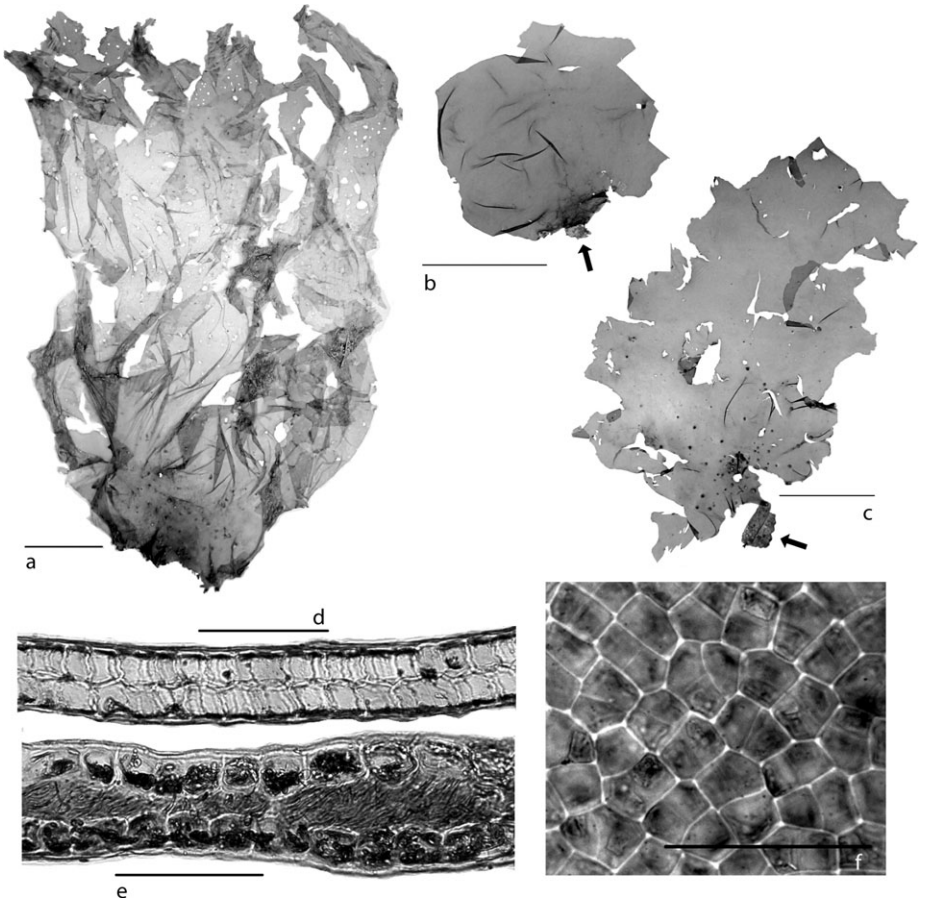

Fig. 5. Morphology of *Ulva ohiohilulu*. Macroscopic and microscopic scale bars are 5 cm and 100 μm, respectively. Arrows indicate a shell fragment attached to a holdfast. (a) Holotype BISH 759429 (ARS 07531). (b) ARS 08539. (c) ARS 08537. (d) Cross-section mid-blade. (e) Cross-section immediately above the basal rhizoid zone. (f) Surface view mid-blade.

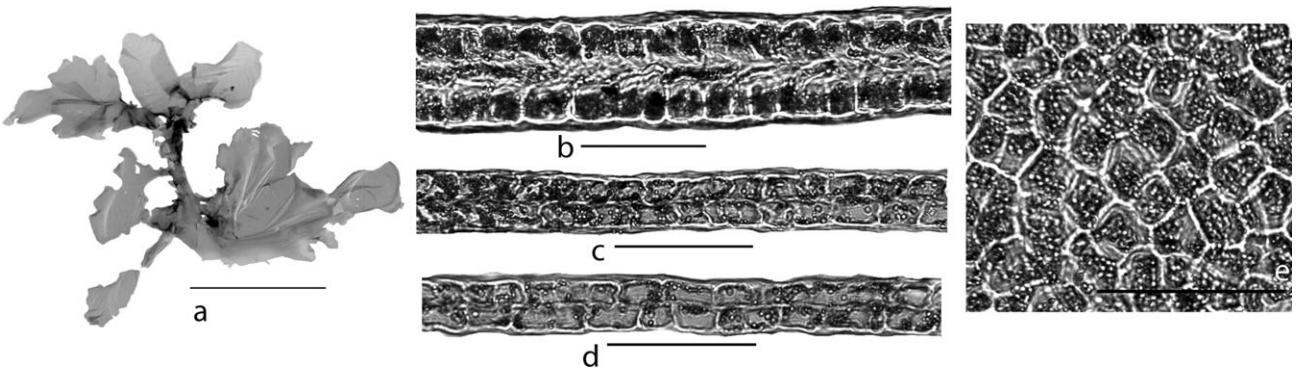

Fig. 6. Morphology of *Ulva iliohaha*. Macroscopic and microscopic scale bars are 5 cm and 100 μm, respectively. (a) Holotype BISH 759430 (ARS 08535). (b) Cross-section immediately above the basal rhizoid zone. (c) Cross-section mid-blade. (d) Cross-section near tip. (e) Surface view mid-blade.

Isotype: None.

Etymology: The specific nomenclature is in the Hawaiian language and was developed using traditional Hawaiian naming practices by M. K. Souza, with critical feedback from P. Nogelmeier and K. Kuoha (Appendix S1). The name was inspired by the Hawaiian god of prosperity (Kū) and the area north of Maui, and is intended to refer to "grass of Kū" (M. K. Souza, pers. comm.). The proper spelling and pronunciation from a Hawaiian cultural context is *kū'āweuweu* (Audio S1).

Representative specimens examined: ARS 03959, ARS 03960, ARS 03961, ARS 08671a, ARS 08671b, ARS 08676a, ARS 08676b.

Habitat and geographic distribution: Blades were found attached to rhodoliths and carbonate reef in the NWHI (Midway Atoll and Lisianski) and offshore of Penguin Bank, west Moloka'i in the MHI

(Fig. 1) from 65 to 84 m depths. The *rbc*L and *tuf*A molecular data for this species are also consistent with subtidal specimens from the Kermadecs (New Zealand), Lord Howe Island, and western Australia (GenBank EF110288 in Fig. 7; GenBank JN029349 and JN029352 in Fig. 8).

Molecular sequences of type material: *rbc*L GenBank (KT932987); *tuf*A GenBank (KT932969); ITS GenBank (KT881220).

Umbraulva kaloakulau H. L. Spalding & A.R. Sherwood **sp. nov.** (Figs. 2d and 4; Table 1; and Audio S2 in the Supporting Information)

Description: Plants foliose and distromatic, elliptical to cuneate, 12–37 cm long by 4–25 cm wide, with a very small holdfast that is difficult to discern. Thalli irregularly perforate with a deep to dark olive green coloration and a slightly lubricous and stiff texture. Cells in surface view polygonal and angular, arranged irregularly, 14–42 (-49) μm long by 11–36 μm wide, with 1–2 pyrenoids per cell. Distal thallus 27–70 μm thick with cuboidal to rectilinear cells in x.s.; median thallus 38–94 μm thick, and basal thallus 61–93 μm thick and lacking rhizoidal cells. Cells in x.s. 11–39 μm tall by 12–41 μm wide. Chloroplast parietal and filling most cells.

Holotype: ARS 07552 (Fig. 4c), collected on March 3, 2011 by T. Kirby, J. Rooney, and K. Binsted with the submersible *Pisces V* (dive 758) at 125 m depth from the ʻAuʻau Channel, west Maui, Hawaiʻi (20° 45.945′ N, 156° 40.200′ W). Deposited at BISH (BISH759426).

Isotype: ARS 07553 (Fig. 4b) deposited at BISH (BISH759427).

Etymology: The specific nomenclature is in the Hawaiian language and was developed using traditional Hawaiian naming practices by M. K. Souza, with critical feedback from P. Nogelmeier and K. Kuoha (Appendix S1). The name was inspired by the god Kanaloa (M. K. Souza, pers. comm.), and is in reference to the tendency of this species to be abundant and growing prostrate along the bottom. Kāloa is a variant of the name Kanaloa most commonly used in the identification of the 24th–26th days of the lunar calendar, which are linked in various ways to Kanaloa (K. Kuoha, pers. comm.). A literal translation could be "long vine [that] stands/exists in abundance," with a more figurative meaning of "Kanaloa [who] alights/exists upon the leaf" (K. Kuoha, pers. comm.). The proper spelling and pronunciation from a Hawaiian cultural context is *kāloakūlau* (Audio S2).

Representative specimens examined: ARS 07552, ARS 07553, ARS 08550, ARS 08675a, ARS 08675b, ARS 08677.

Habitat and geographic distribution: Large blades found attached to carbonate reef, shells, rhodoliths, and in areas with sparse coral (*Leptoseris* spp.) cover from 85 to 125 m depths. This was the deepest mesophotic Ulvaceae observed, and the only Ulvaceae species occupying the 125 m depth range. Specimens were collected from both the MHI (Olowalu, west Maui) and the NWHI (Pearl and Hermes; Fig. 1).

Molecular sequences of type material (ARS 07552, ARS 07553): *rbc*L GenBank (KT932990, KT932991); *tuf*A GenBank (KT932971, KT932973); ITS GenBank (KT881216).

Ulva ohiohilulu H.L. Spalding & A.R. Sherwood **sp. nov.** (Figs. 2, a and b; and 5; Table 1; and Audio S3 in the Supporting Information)

Description: Plants foliose and distromatic, orbicular to elliptical, reniform, or falcate, 6–80 cm long by 4–25 cm wide with a small holdfast penetrating into carbonate substrates. Thalli slightly perforate or complete with a grass green coloration and membranous texture that dries to a lighter yellow. Cells in surface view polygonal and angular, arranged irregularly, 19–38 (-68) μm long by 9–27 (-45) μm wide, with 1–2 pyrenoids per cell. Distal thallus 13–57 μm thick with squared to rectilinear cells in x.s.; median thallus 43–65 μm thick. Basal thallus 68–144 μm thick with a distinct longitudinal rhizoidal zone. Cells in x.s. 18–38 (-71) μm tall by 2–31 (-76) μm wide. Chloroplast thin and parietal, occasionally cup-shaped.

Holotype: ARS 07528 (Fig. 5a) collected on April 5, 2009 by R. Boland, C. Bradley, M. Cremer with the *Pisces V* submersible (dive 736) at 85 m depth in the ʻAuʻau Channel off Olowalu, west Maui, Hawaiʻi (20° 46.808′ N, 156° 40.438′ W). Specimen deposited at BISH (BISH759428).

Isotype: ARS 07531 (Fig. 5c) deposited at BISH (BISH759429).

Etymology: The specific nomenclature is in the Hawaiian language and was developed using traditional Hawaiian naming practices by K. Kuoha, with critical feedback from C. P. Pata, U. Victor, and K. Winter (Appendix S1). The name literally means "flourishing (in the) leeward calm," and is in reference to the first place this species was found, the ʻAuʻau Channel between the islands of Maui and Lānaʻi. This channel is known for its calm waters, as the name ʻAuʻau (to swim) alludes to a tranquil sea that allows one to swim from one island to the other. The ʻAuʻau Channel also contains some of the most extensive MCEs in Hawaiʻi, with luxuriant beds of this species. The sound and rhythm of this name portrays a playfulness and poetry often represented in the Hawaiian language, when referred to as *līpahapaha ohiohilulu* (Audio S4 in the Supporting Information). The name *līpahapaha* refers to the genus *Ulva*.

Representative specimens examined: ARS 07528, ARS 07531, ARS 07546, ARS 07547, ARS 07549, ARS 07550, ARS 08535, ARS 08536, ARS 08537, ARS 08538, ARS 08539, ARS 08540, ARS 08546, ARS 08548, ARS 08599, and ARS 08672.

Habitat and geographic distribution: This was the largest and most commonly encountered (15 specimens of this taxon of 28 specimens total)

TABLE 1. Summary of macroscopic features and habitat.

Species	Thallus dimensions (cm) Range of lengths (mean), range of widths (mean)	Color	Texture	Form	Habitat
Ulva ohiohihulu	6–80 (18), 4–25 (10)	Grass green	Membranous	Slightly perforate or no holes; orbicular to elliptical, reniform, or falcate	Carbonate reef, *Halimeda* spp., dead coral plates (*Leptoseris* spp.), rhodoliths, or on dead shells over sand
Ulva iliohaha	9, 12[a]	Pale green	Membranous	Slightly perforate, ruffled with cordate to falcate lobes	Mixed carbonate reef with macroalgae
Umbraulva kuaweuweu	7–21 (14), 7–17 (12)	Light to dark olive green	Slightly lubricous, stiff	Slightly to extensively perforate, elliptical to flabellately expanded with cuneate lobes	Rhodolith beds and carbonate reef
Umbraulva kalaokulau	12–37 (23), 15–20 (18)	Deep green to dark olive green	Slightly lubricous, stiff	Irregularly perforate, elliptical to cuneate	Coral (*Leptoseris* spp.), carbonate reef, shells, and rhodoliths

[a]Only one specimen collected ($n = 1$).

mesophotic Ulvaceae, found growing attached to mixed carbonate reef with a thin covering of sand, dead segments of *H. distorta*, pieces of dead coral plates (*Leptoseris* spp.), rhodoliths, or on dead pieces of shell from 69 to 93 m depths. Currently, this species has been found at multiple locations in the MHI off Olowalu (west Maui), Penguin Bank (west Moloka'i), and Waikīkī (south O'ahu), and from one location in the NWHI (Lisianski) (Fig. 1).

Molecular sequences of type material (ARS 07528, ARS 07531): *tuf*A GenBank (KT932977); ITS GenBank (KT881224, KT881226).

Ulva iliohaha H.L. Spalding & A.R. Sherwood **sp. nov.** (Fig. 6; Table 1; and Audio S5 in the Supporting Information)

Description: Plants foliose and distromatic, ruffled with cordate to falcate lobes, 9 cm long by 12 cm wide and attached epiphytically to turf algae. Thalli slightly perforate with a light green coloration and membranous texture. Cells in surface view polygonal and angular, arranged irregularly, 17–34 μm long by 16–24 μm wide, with 1 pyrenoid per cell. Distal thallus 35–46 μm and median thallus 40–46 μm thick with rectangular cells in x.s.; basal thallus 80–110 μm thick with cuboidal cells and longitudinal rows of loosely packed rhizoids. Cells in x.s. 16–37 μm tall by 18–45 μm wide. Chloroplast parietal.

Holotype: ARS 08535 (Fig. 6a), collected by K. Gleason on May 20, 2013 at 64 m depth from French Frigate Shoals, NWHI, Hawai'i (23° 62.872′ N, 166° 19.598′ W), in a mixed carbonate reef and macroalgal assemblage. Specimen deposited at BISH (BISH759430).

Isotype: None.

Etymology: The specific nomenclature is in the Hawaiian language and was developed using traditional Hawaiian naming practices (Appendix S1) by K. Kuoha and C. P. Pata, with critical feedback from U. Victor and K. Winter. The name is literally translated as "dog (that) feels about in search of something," and is in reference to the Hawaiian monk seal's (*Neomonachus schaunslandi* Matschie, or '*īlio-holoikauaua* in Hawaiian) observed behavior of foraging in algal-dominated, mesophotic depths (Parrish et al. 2002, 2005, Parrish and Boland 2004). The Hawaiian word "hāhā" means "to grope, feel, as with the hands" (Appendix S1). Precedent for the application of '*īlio* to an *Ulva* species can be found in the traditional name *'īlioha'a*. Naming this algal species in reference to the critically endangered Hawaiian monk seal is believed to add a supportive element to the monk seal's environment at French Frigate Shoals, NWHI. The proper spelling and pronunciation from a Hawaiian cultural context is '*īliohāhā* (Audio S5).

Representative specimens examined: ARS 08535.

Molecular sequences of type material: *rbc*L GenBank (KT932995); *tuf*A GenBank (KT932976); ITS GenBank (KT881223).

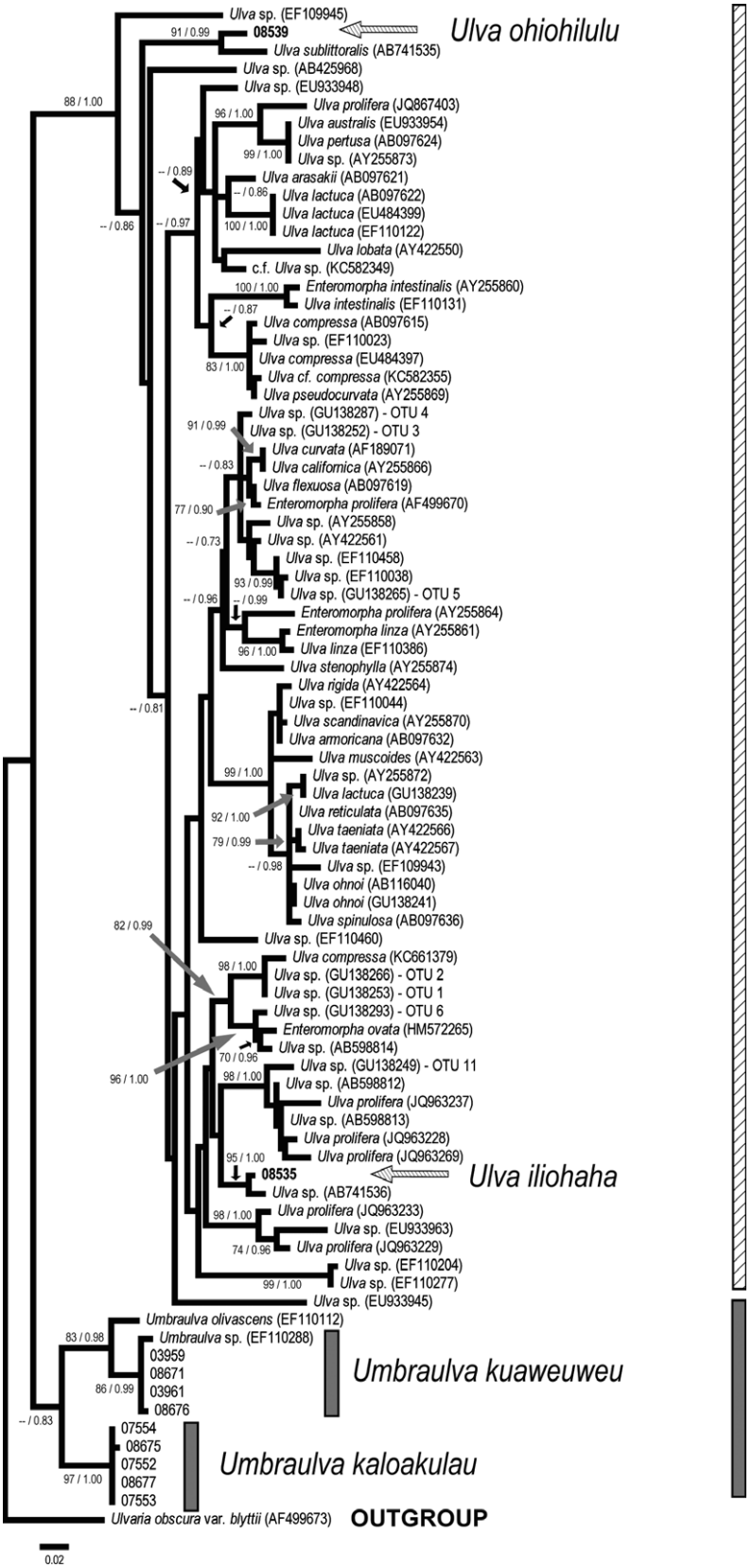

FIG. 7. Maximum likelihood phylogram inferred topology for the *rbc*L region of mesophotic Ulvales with other *Ulva* and *Umbraulva* taxa. (BIC-derived parameters, -*ln*L = 4,514.6362). Branch lengths are proportional to the amount of sequence change. ML bootstrap values (first, 1,000 replicates) and posterior probabilities from Bayesian Inference analyses (second, 4,000,000 generations) are indicated at the nodes, and support values lower than 50% are not shown. Genera are shown to the right. GenBank accessions listed, with OTUs from shallow-water Hawaiian Ulvales (O'Kelly et al. 2010). Diagonal striped bar = *Ulva* species. Solid gray bar = *Umbraulva* species. Scale bar indicates nucleotide substitutions per site.

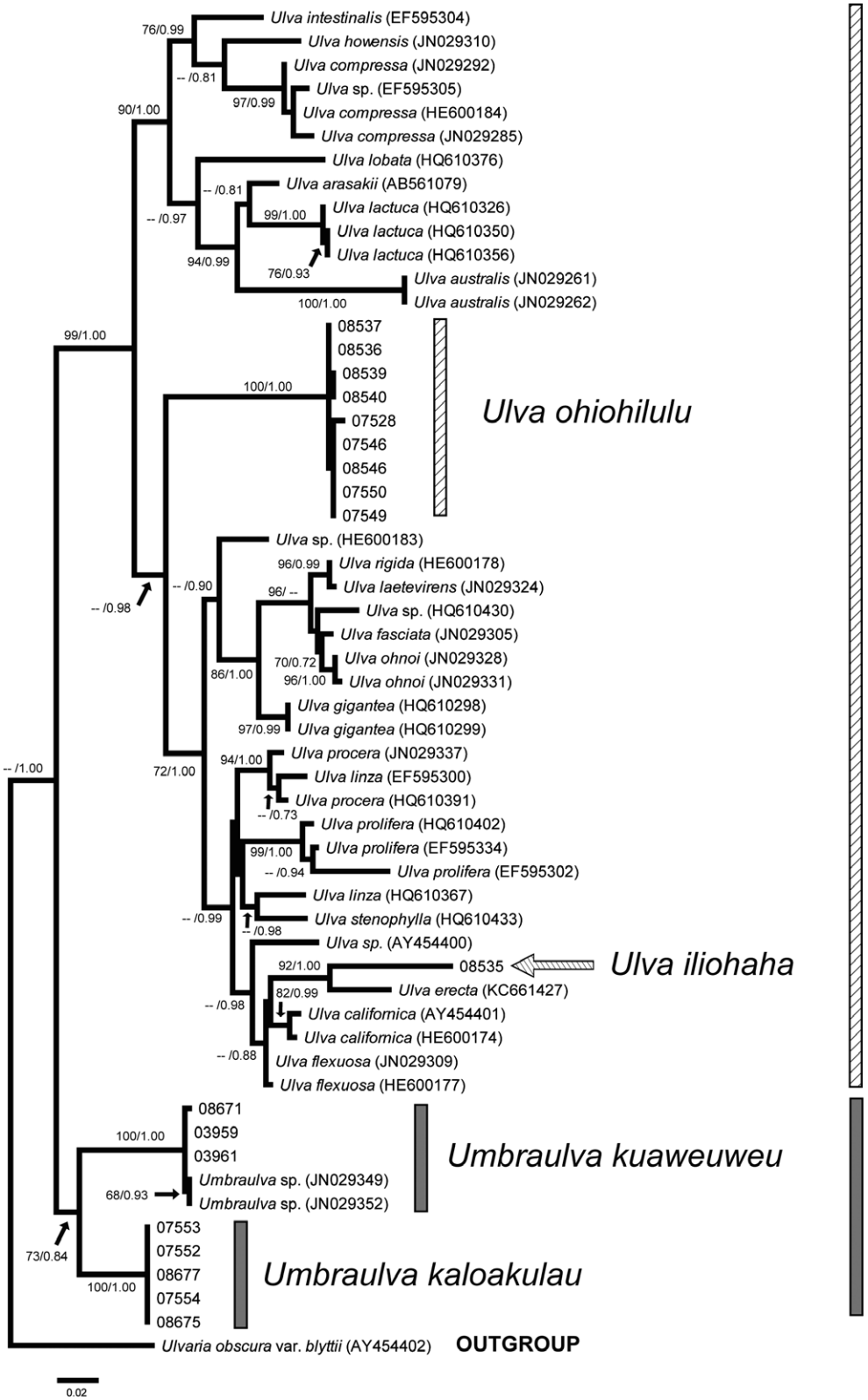

FIG. 8. Maximum likelihood phylogram inferred topology for the *tuf*A region of mesophotic Ulvales with other *Ulva* and *Umbraulva* taxa (BIC-derived parameters, -*ln*L = 3,796.2138). Branch lengths are proportional to the amount of sequence change. ML bootstrap values (first, 1,000 replicates) and posterior probabilities from Bayesian Inference analyses (second, 4,000,000 generations) are indicated at the nodes, and support values lower than 50% are not shown. Genera are shown to the right, with specimens listed by GenBank accession. Diagonal striped bar = *Ulva* species. Solid gray bar = *Umbraulva* species. Scale bar indicates nucleotide substitutions per site.

DISCUSSION

The ulvalean sea lettuces of mesophotic ecosystems in Hawaiian waters form a unique community that is distinct from the shallow-water flora. We found four genetically distinct entities in our samples, three of which are well-supported at the molecular level as previously unrecorded taxa. The absence of clear morphological markers for these species reflects the common problem worldwide of achieving proper ulvoid identifications using morphological criteria (Hayden and Waaland 2004), although further investigation into the basal rhizoidal transition zone with a larger sample size may help to delineate some species.

Moreover, two of the four entities belong to a genus, *Umbraulva*, for which there are no prior records in the Hawaiian Islands. One of these two *Umbraulva* taxa is closely related to an alga found, but not formally described, from the Kermadec Islands, New Zealand (Heesch et al. 2007, 2009), and from both Lord Howe Island and the vicinity of Perth, Australia (Kirkendale et al. 2012). Interestingly, the specimens from Lord Howe Island and western Australia were collected from 3 to 16 m depths, suggesting that this species of *Umbraulva* is not limited to mesophotic depths. *Ulva* and *Umbraulva* spp. were also recently described from the shallow subtidal to 90 m depths in New Zealand (Nelson et al. 2015). Our results coupled with Nelson et al. (2015) suggest that Ulvales possess the ability to occur over wide depth gradients, and may be more common at mesophotic depths than previously thought.

The two *Ulva* species from Hawaiian mesophotic habitats in the MHI and NWHI are molecularly distinct from all other *Ulva* species previously reported. It cannot yet be ascertained whether *U. ohiohilulu* represents *U. expansa* (Abbott and Huisman 2004), because it has not yet been possible to obtain DNA sequences from herbarium specimens assigned to *U. expansa*, and morphological characters are unlikely to be helpful. It is also doubtful that any Hawaiian specimen represents authentic *U. expansa*, for which the type locality is cold-temperate Monterey, California (Setchell 1905, as *U. fasciata* f. *expansa* Setchell). We think it best to describe this mesophotic species as new, and consider it separate from historical Hawaiian records assigned to *U. expansa* pending additional investigations. Although only one specimen of *U. iliohaha* was collected, it was distinct from *Ulva ohihilulu* and all other species based on molecular analyses and located in a very isolated region and depth, meriting its designation as a new species. None of the algae investigated here represents either true *U. lactuca* (formerly *U. fasciata*) or the temperate-zone alga formerly known under this name (O'Kelly et al. 2010). Reports of *U. lactuca* that are resident and actively growing in deep-water communities (Olson and Kellogg 2010) – as distinct from those that have descended into the depths from shallow-water populations (Britton-Simmons et al. 2012) – should be reevaluated in the light of our findings. It is, in fact, an open question whether the algae we have found are widely distributed in tropical mesophotic zones, represent a community specific to (some portion of) the Pacific basin, or are endemic to the Hawaiian Archipelago. However, the mesophotic ulvalean sea lettuces were distinct from all other shallow Ulvales studied to date in Hawai'i (O'Kelly et al. 2010), suggesting limited connectivity between deep and shallow macroalgal communities.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Mean (±SD) microscopic measurements of four proposed Ulvales species listed by specimen number.

Table S1. Collection information for samples used in this study. Collections beginning with P4- or P5- were Hawai'i Undersea Research Laboratory *Pisces IV* or *Pisces V* submersible dives, respectively.

Table S2. Results of nested or one-way (*) ANOVA analyses on morphometrics among *Ulva ohiohilulu*, *Umbraulva kuaweuweu*, and *Umbraulva kaloakulau* ($n = 20$ measurements per specimen, $n = 5$ specimens per variable). *Ulva iliohaha* was not used in analyses because of limited sample size ($n = 1$).

Table S3. GenBank accessions for sequences used in the phylogenetic analyses.

Table S4. ITS1 BLAST table for new *Umbraulva* (*Um.*) and *Ulva* (*U.*) species.

Appendix S1. Traditional Hawaiian nomenclature for macroalgae: *Inoa Limu.*

Audio S1. The Hawaiian pronunciation for the species name of *Umbraulva kuaweuweu.* The Hawaiian spelling is *kū'āweuweu.* Audio file created by K. Kuoha.

Audio S2. The Hawaiian pronunciation for the species name of *Umbraulva kaloakulau.* The Hawaiian spelling is *kāloakūlau.* Audio file created by K. Kuoha.

Audio S3. The Hawaiian pronunciation for the species name of *Ulva ohiohilulu.* Audio file created by K. Kuoha.

Audio S4. The Hawaiian pronunciation for *līpahapaha ohiohilulu*, which refers to the genus species name in Hawaiian of *Ulva ohiohilulu.* Audio file created by K. Kuoha.

Audio S5. The Hawaiian pronunciation for the species name of *Ulva iliohaha.* The Hawaiian spelling is *'īliohāhā.* Audio file created by K. Kuoha.