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Authors: Sherwood, Alison R., Paiano, Monica O., Wade, Rachael M.,
Cabrera, Feresa C., Spalding, Heather L., et al.

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Biodiversity of Hawaiian Peyssonneliales (Rhodophyta).

1. Two New Species in the Genus *Ramicrusta* from Lehua Island¹

Alison R. Sherwood,^{2,6} Monica O. Paiano,² Rachael M. Wade,^{2,3}
Feresia C. Cabrera,² Heather L. Spalding,^{2,4} and Randall K. Kosaki⁵

Abstract: Two specimens of the crustose red algal genus *Ramicrusta* were collected off Lehua Island, Hawai‘i, at 11 m and 49 m depth in September 2018, and are here described as new species on the basis of both molecular and morphological comparisons. *Ramicrusta hawaiiensis* sp. nov. is distinguished from the 12 other known species in the genus by the combination of its gross habit and coloration, pronounced perithallial zonation, frequent and robust rhizoids, presence of hair cells, and by distinctive COI and *rbcL* marker sequences, and represents a mesophotic record of the genus *Ramicrusta*. Similarly, *Ramicrusta lehuensis* sp. nov. is distinctive in the combination of its gross habit and coloration, less-pronounced perithallial zonation, presence of frequent and robust rhizoids as well as hair cells, and is also distinct in COI and *rbcL* marker sequences. These records constitute the first for the genus *Ramicrusta* in Hawai‘i, and highlight the cryptic diversity of peyssonnelioid red algae in the Hawaiian Islands. This study also adds to the growing list of novel algal species being characterized from mesophotic depths in Hawai‘i—a habitat which is being shown to contain numerous undescribed taxa and records of species and genera previously unknown to the Islands. This initial record of *Ramicrusta* in Hawaiian waters is noteworthy given the recent reports of members of the genus overgrowing and killing corals in the Caribbean and South China Sea.

Keywords: algae, biodiversity, COI, Hawai‘i, mesophotic, Peyssonneliales, *Ramicrusta*, *rbcL*, Rhodophyta

IN RECENT YEARS, the ordinal-level systematics of the peyssonnelioid red algae have become

clarified in small steps through phylogenetic analyses of diversity beyond the genus *Peyssonnelia* (Kato et al. 2006), including the recognition of the Order Peyssonneliales (Krayesky et al. 2009), and further phylogenetic characterization of additional genera within the order (e.g., Dixon and Saunders 2013). The genus *Ramicrusta* was described by Zhang and Zhou (1981) for peyssonnelioid algae possessing secondary pit connections—a feature that, to that point, had been largely absent in those fleshy, crustose red algae. The generitype, *R. nanhaiensis* Zhang and Zhou, remained the sole member of this obscure genus for several decades, until Pueschel and Saunders (2009) described *R. textilis* Pueschel & G. W. Saunders as the second species for specimens that were found to be overgrowing corals in the Caribbean. Dixon and Saunders (2013) recognized five new species from Vanuatu, the Philippines, and southern

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²School of Life Sciences, 3190 Maile Way, University of Hawai‘i, Honolulu, HI 96822, USA.

³Present address: Department of Biological Sciences, 3209 N. Maryland Avenue, University of Wisconsin Milwaukee, Milwaukee, WI 53212, USA.

⁴Present address: Department of Biology, College of Charleston, 66 George St, Charleston, SC 29424, USA.

⁵NOAA, Papahānaumokuākea Marine National Monument, 1845 Wasp Boulevard, Building 176, Honolulu, HI 96818, USA.

⁶Corresponding author (e-mail: asherwoo@hawaii.edu).

Australia. One of the 11 currently recognized species, *R. calcea* (Heydrich) K. R. Dixon, was transferred from *Peyssonnelia* on the basis of the presence of secondary pit connections in that same study (Dixon and Saunders 2013). Presently, 11 species are recognized in *Ramicrusta*, and the known distribution of the genus includes the warm waters of the South China Sea, Vanuatu, the Philippines, southern Australia, New Guinea, Tunisia, and the Caribbean (Pueschel and Saunders 2009, Dixon and Saunders 2013, Ballantine et al. 2016, Dixon 2018, Manghisi et al. 2019).

Given that *Ramicrusta* has only been recognized as a distinctive genus from *Peyssonnelia* for the last several decades, reports of its ecological role are relatively few. Nevertheless, it has recently come to the attention of scientists studying the ecology of coral reefs where it is reported as overgrowing native corals in both the Caribbean (Pueschel and Saunders 2009, Eckrich et al. 2011, Ballantine and Ruiz 2013, Eckrich and Engel 2013, K. Hollister, pers. comm.), and more recently, the South China Sea (Nieder et al. 2019). *Ramicrusta* has been described as growing upward from the base of apparently healthy corals, eventually completely covering the organisms and causing their death due to shading (Eckrich et al. 2011). Given the potential ecological impacts of this crustose red alga in coral dominated systems, further studies on the biodiversity of the genus are warranted.

Peyssonnelioid algae have received comparatively little attention within the broader context of the Hawaiian marine algal flora, which is not surprising given the uncharismatic, crustose morphologies characteristic of the common taxa in nearshore and shallow coastal habitats in Hawai'i. Abbott (1999) reported three species of *Peyssonnelia* in the Hawaiian Islands based on morphology (*P. conchicola* Piccone & Grunow, *P. inamoena* Pilger, and *P. rubra* (Greville) J. Agardh), which were all described from elsewhere. Kato et al. (2009) added a report of *P. japonica* (Segawa) Yoneshigue to the Hawaiian flora, bringing the total number of reported species in the Order Peyssonneliales in the Hawaiian Islands to date to four.

Numerous specimens of peyssonnelioid algae have been collected as part of an effort to characterize the algal diversity of mesophotic coral ecosystems (MCEs) of the Hawaiian Islands over the past 15 years. Initial characterization of these specimens based on COI and *rbcL* DNA barcode sequencing has indicated that two of these specimens are representatives of the genus *Ramicrusta*, and that neither corresponds to known species within the genus. Both are described here as new species and are, to the best of our knowledge, endemic to the Hawaiian Islands.

MATERIALS AND METHODS

Two specimens consisting of entire pieces of coral rubble with attached crustose algae were collected in September 2018 by SCUBA as part of a marine survey expedition to Lehua and Ni'ihau, main Hawaiian Islands (Table 1). Entire specimens were preserved in silica gel desiccant. Collectors were unable to preserve specimens in formalin during the expedition, and so both molecular and morphological analyses were conducted on the desiccated material. Small pieces of peyssonnelioid algae were removed from the coral rubble under a Zeiss SteREO Discovery v12 dissecting microscope (Pleasanton, CA) and placed in 1.5 mL Eppendorf tubes for both morphological and molecular analyses. Morphological and anatomical investigations of the samples were conducted by gently rehydrating small pieces of the specimens in a detergent (Modified Pohl's Solution, Clark, unpubl.: https://www.eeob.iastate.edu/research/bamboo/pdf/anatomy_protocols.pdf) for 30 min, decalcifying in 5% HCl for 15 min to 2 h, hand sectioning with a double-edged razor blade, staining with 1% aniline blue, and mounting in 30% 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).

Specimens were extracted for genomic DNA using an OMEGA E.Z.N.A.® Plant DNA DS Kit (OMEGA Biotek, Norcross,

TABLE 1
Specimens of *Ramicrusta* from the Hawaiian Islands Characterized as Part of the Current Study

Species	Sherwood Lab Collection	BISH Accession	Collection Information (Latitude/Longitude in Decimal Degrees)	GenBank Accession (COI)	GenBank Accession (<i>rbcL</i>)
<i>Ramicrusta hawaiiensis</i> sp. nov.	ARS 09600	BISH 776043	Lehua Island, Hawai'i (22.02103, -160.10246), 49 m depth, 14.IX.2018, leg. S. Matadobra (MHI-42)	MN623629	MN623630
<i>Ramicrusta lebuensis</i> sp. nov.	ARS 09609	BISH 776044	Lehua Island, Hawai'i (22.01461, -160.10036), 11 m depth, 14.IX.2018, leg. H. Spalding (MHI-54)	MN623631	MN623632

GA, USA). A portion of the COI DNA barcode marker (cytochrome oxidase subunit I, 658 bp) was amplified using the GazF1 and GazR1 primers (Saunders 2005) or the GazF2 and Gaz R2 primers (Saunders 2005, Lane et al. 2007). The *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, 1,442 bp) marker was amplified as two overlapping fragments using the primer pairs *rbcLF7* and *rbcLJNR1* (Gavio and Fredericq 2002, Kang and Kim 2013) and *rbcLF762* and *rbcLR1442* (Kim et al. 2010). Successful PCR products were submitted for sequencing to the University of Hawai'i at Mānoa Advanced Studies for Genomics, Proteomics, and Bioinformatics (ASGPB) core facility. Raw sequence reads for each gene were assembled, edited, and aligned using the MUSCLE v. 3.8.425 plug-in (Edgar 2004) in Geneious Prime 2019.1.3 (<http://www.geneious.com>) with other available sequences for *Ramicrusta* and related genera from GenBank and BOLD (Table S1). DNA barcode analysis of the COI sequences was performed by constructing a neighbor-joining framework based on Kimura-2-parameter distances using Geneious Tree Builder in Geneious Prime 2019.1.3 (<http://www.geneious.com>). For the *rbcL*+COI concatenated phylogenetic analyses, contiguous sequences were aligned with reference sequences and analyzed with PartitionFinder v. 1.1.1 (Lanfear et al. 2012). Maximum likelihood (ML) analyses were performed on all alignments using RAxML-HPC2 on XSEDE v. 8.2.10 (Stamatakis 2014) via the CIPRES gateway (Miller et al. 2010)

with 1,000 bootstrap replicates, and using the GTRCAT model. Bayesian inference was performed using the MrBayes plug-in v. 3.2.6 (Huelsenbeck and Ronquist 2001) through Geneious Prime 2019.1.3 (<http://www.geneious.com>) using four chains of Metropolis-coupled Markov Chain Monte Carlo for 1,000,000 generations and sampling every 100 generations; 100,000 chains were removed as burn-in prior to determining posterior probabilities.

RESULTS

Mitochondrial COI sequences were available for 10 of the 11 previously described species of *Ramicrusta* as well as an undescribed species recently reported from Tunisia (Manghisi et al. 2019), and DNA barcoding analysis of the COI marker clearly indicated that the Hawaiian specimens were distinct from all of these as well as from each other, with specimen ARS 09600 sharing 93.15–94.13% identity with its most similar congeners (*R. aranea* and *R. trichaurea*) and specimen ARS 09609 sharing 91.94–92.85% identity with its most similar congener (*R. appressa*) (Figure 1). Phylogenetic analysis of concatenated COI+*rbcL* sequences supported the Hawaiian specimens as members of the genus *Ramicrusta* within the Peyssonneliales (Figure 2). Specimen ARS 09600 was allied with a sequence of *R. aranea* from Vanuatu with full support, and specimen ARS 09609 was basal to the clade of *R. textilis*, *R. aranea*, and specimen ARS 09600, again, with full

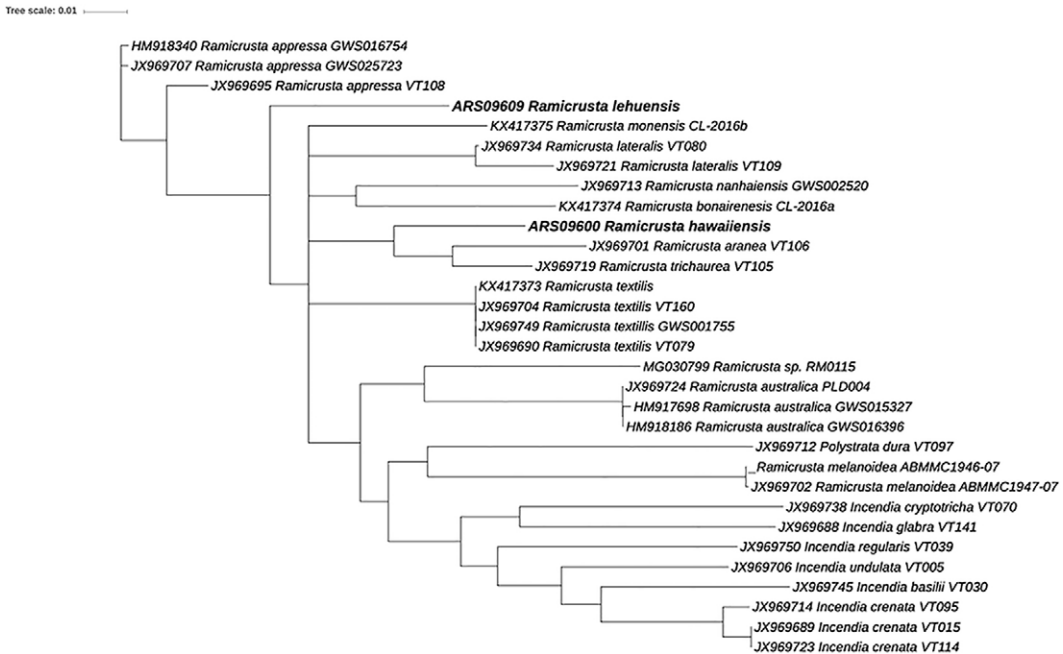


FIGURE 1. Neighbor-joining (NJ) phylogram (K2P distances) of COI sequences of *Ramicrusta* and the closely related genus, *Incendia*. Scale bar = substitutions per site.

support (Figure 2). Based on the evidence of these unique phylogenetic entities and the morphological characters outlined below, the two new specimens of *Ramicrusta* from Lehua Island, Hawai'i, are described here as new species.

Ramicrusta hawaiiensis A. R. Sherwood, *sp. nov.* (Figure 3A–F)

Description: Thallus surface smooth and lacking protuberances. Thallus tightly adherent and closely appressed, dark dusky rose color when dried, 210–250 μm thick, moderately calcified. Hypothallus as for the genus. Hypobasal cells globose, 28–30 μm diameter by 23–25 μm in height. Perithallus with irregularly shaped cells in lower regions, 15–20 μm diameter \times 25 μm height. Perithallial cells gradually decreasing in size toward epithallus, becoming more regular in shape. Epithallus composed of several rows of small, regularly shaped cells, 8–9 μm diameter by 5–7 μm in height in the uppermost row. Hair

cells present. Hypobasal cuticle moderately thick, 8–11 μm . Rhizoids common, 60–120 μm long, 10–15 μm diameter. Gametangia and tetrasporangia unknown.

Holotype: BISH 776043 (ARS 09600, Lehua Island, Hawai'i, 22.02103, –160.10246, 49 m depth, 14.IX.2018, leg. S. Matadobra (MHI-42)).

Etymology: Named for the Hawaiian Islands, the only known location (thus far) of this species.

Distribution: Lehua Island, Hawai'i, USA, 49 m depth.

Specimens examined: ARS 09600 (BISH 776043).

DNA sequence data: GenBank accessions MN623629 (COI) and MN623630 (*rbcL*).

Habit and morphology: Growing on coral rubble with sponges and crustose coralline algae (Figure 3A). Thallus relatively thin for the genus. Perithallus composed of distinct upper and lower regions, with zonation evident (Figure 3B,C). Shape of cells in lower perithallus differs strongly from those in epithallus, with the former being larger,

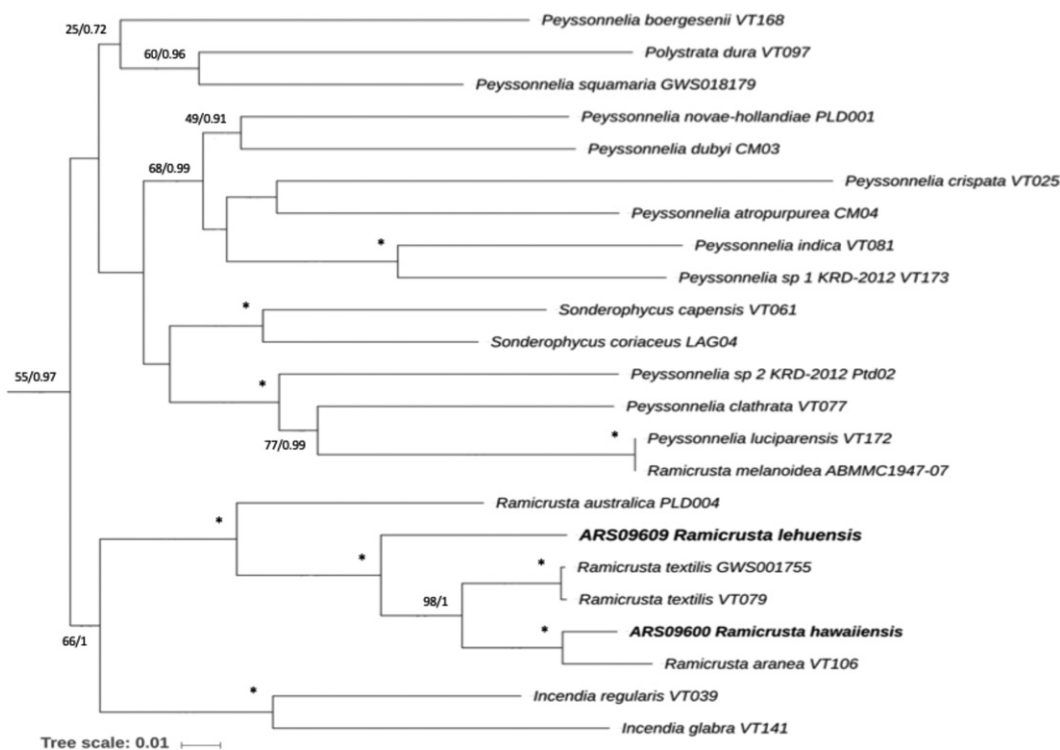


FIGURE 2. Maximum likelihood phylogenetic tree of concatenated COI + *rbcL* sequences for *Ramicrosta* and closely related genera in the red algal order Peyssonneliales. Numbers along branches indicate nodal support (first value = bootstrap support, second value = Bayesian posterior probabilities). Nodes with full support are indicated with an asterisk. Scale bar = substitutions per site.

globose to oval-shaped, and the latter being smaller, very regular in size and shape, and rectangular, with a larger diameter than height (Figure 3D). Thallus lightly calcified, and reacting only moderately with 5% HCl during decalcification. A slight mottling pattern is evident on the dorsal surface of the thallus, but no striations or other patterns are visible. A dorsal view of the thallus demonstrates the presence of hair cells in addition to the smaller perithallial cells (Figure 3E). Rhizoids are abundant, extending to 120 μm in length, and are broad (Figure 3F). Only a single specimen collected thus far, which was not reproductive.

Ramicrosta lehuensis A. R. Sherwood, sp. nov. (Figure 4A–F)

Description: Thallus surface smooth and lacking protuberances. Thallus tightly adherent

and closely appressed, except margins are occasionally free, dark rosy pink when living and drying to a brownish red to caramel color, 210–240 μm thick, heavily calcified. Hypothallus as for the genus. Rhizoids common, 120–170 μm long, 11–13 μm diameter. Hypobasal cells irregular in shape, 20–28 μm diameter \times 25–30 μm height. Perithallus thick in lower regions, lacking in obvious zonation, composed of irregularly shaped cells. Epithallus composed of a single row of small and flattened cells, 15–17 μm diameter by 7–10 μm in height. Hair cells present. Hypobasal cuticle thin, 5–8 μm thick. Gametangia and tetrasporangia unknown.

Holotype: BISH 776044 (ARS 09609, Lehua Island, Hawai'i, 22.01461, -160.10036, 11 m depth, 14.IX.2018, leg. H. Spalding (MHI-54)).

Etymology: Named for Lehua Island, the only known location (thus far) of this species.

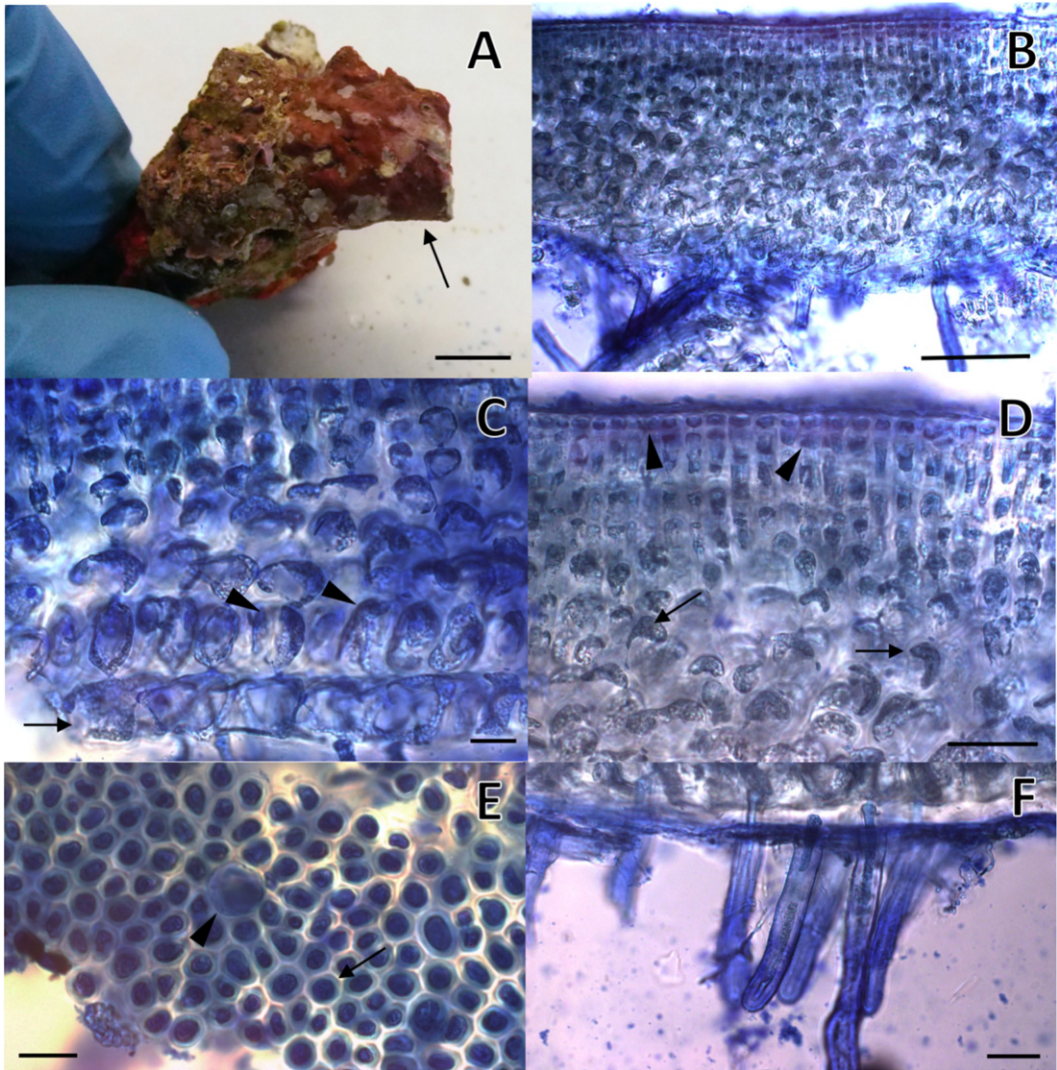


FIGURE 3. Morphology of *Ramicrusta hawaiiensis* sp. nov. (A) The holotype specimen (BISH 776043) overgrowing coral rubble (arrow). Scale bar = 1 cm. (B) Radial vertical section through thallus. Scale bar = 100 μ m. (C) Hypobasal layer (arrow) and lower perithallial cell arrangement (arrowheads). Scale bar = 20 μ m. (D) Mid-perithallus cells (arrows) and epithallial cells (arrowheads). Scale bar = 50 μ m. (E) Dorsal surface of crust showing small perithallial cells (arrow) and larger hair cells (arrowhead). Scale bar = 20 μ m. (F) Rhizoids extending from the hypobasal cuticle region of the thallus. Scale bar = 20 μ m.

Distribution: Lehua Island, Hawai'i, USA, 11 m depth.

Specimens examined: ARS 09609 (BISH 776044).

DNA sequence data: GenBank accessions MN623631 (COI) and MN623632 (*rbcL*).

Habit and morphology: Growing on coral rubble with other crustose red and brown algae (Figure 4A,B). Thallus relatively thin for the genus. Upper and lower regions of the perithallus not as clearly zoned as some other species of *Ramicrusta* (Figure 4C), although

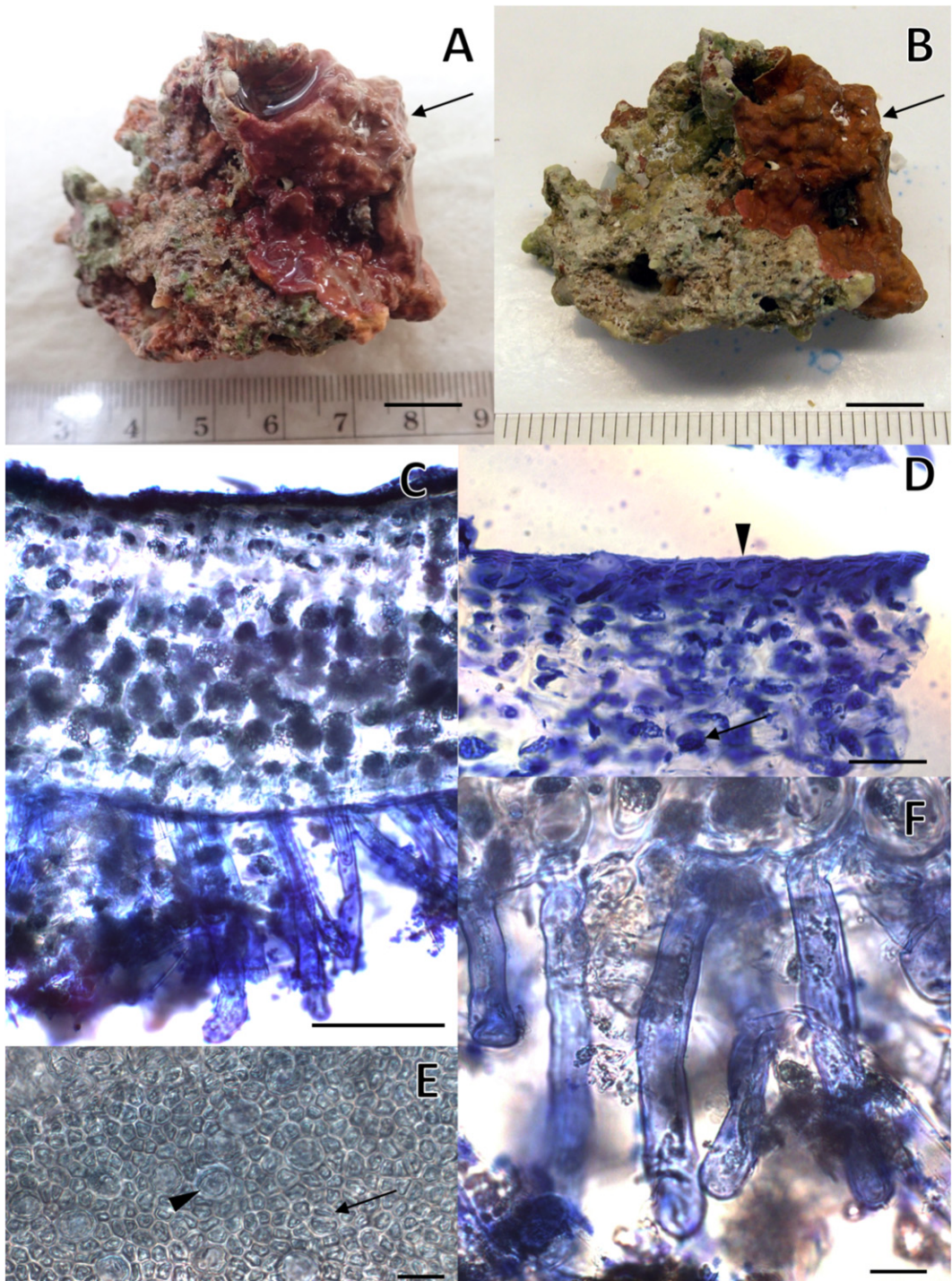


FIGURE 4. Morphology of *Ramicrusta lebuensis* sp. nov. (A) The holotype specimen (BISH 776044) overgrowing coral rubble (arrow); photograph of newly collected, living specimen. Scale bar = 1 cm. (B) The holotype specimen (BISH 776044) overgrowing coral rubble (arrow); photograph of dried specimen. Scale bar = 1 cm. (C) Radial vertical section through thallus. Scale bar = 100 μ m. (D) Mid-perithallus cells (arrow) and epithallial cells (arrowhead). Scale bar = 100 μ m. (E) Dorsal surface of crust showing small perithallial cells (arrow) and larger hair cells (arrowhead). Scale bar = 20 μ m. (F) Rhizoids extending from the hypobasal cuticle region of the thallus. Scale bar = 20 μ m.

lower perithallial cells are larger than those in the epithallus (Figure 4C,D). Thallus heavily calcified and reacting strongly with 5% HCl during decalcification process. No striations or other patterns visible on the surface of the thallus. Hair cells are evident from the dorsal surface (Figure 4E). Rhizoids are unicellular, abundant, long (to 170 μm) and broad (Figure 4F). Only a single specimen collected thus far, which was not reproductive.

DISCUSSION

The two new species of *Ramicrosta* described here represent further examples of the undescribed algal biodiversity being characterized from the shallow and mesophotic depths of the Hawaiian Islands. The current emphasis on understanding the diversity of Hawaiian mesophotic algae has led to the recent description of groups of species within the green algae (Ulvaceae—four new species; Spalding et al. 2016) and red algae (*Martensia*—four new species; Sherwood et al. 2019), with a number of others in preparation. The Peyssonneliales, in particular, represent a remarkable opportunity for study of cryptic diversity in Hawaiian red algae given their simple, crustose morphology, which is often not characterized in detail at the species level, resulting in these species typically being grouped together at the functional form level in ecological and diversity studies (e.g., Spalding 2012).

Although the molecular analyses are clear in supporting *R. hawaiiensis* sp. nov. and *R. lebuensis* sp. nov. as distinct from other species of *Ramicrosta* (except for *R. calcea*, which does not, as of yet, have DNA sequences available), they are also distinct based on combinations of morphological characters (Pueschel and Saunders 2009, Dixon and Saunders 2013, Ballantine et al. 2016, Dixon 2018). Both species are thin for the genus (maximum 250 μm), tightly adherent and smooth-surfaced, and either completely lacking in striations and other patterns on the dorsal surface of the thallus (*R. lebuensis*) or possessing only a faint mottled appearance (*R. hawaiiensis*). These gross morphological features distinguish the two new Hawaiian

species from those with surface protuberances or bumps and/or obvious striations and patterns (*R. australica*, *R. bonairensis*, *R. calcea*, *R. melanoidea*, *R. monensis*, *R. nanhaiensis*, and sometimes *R. textilis*). The Hawaiian species are pinkish red in color, which differs from the brown or golden-brown coloration of *R. aranea*, *R. lateralis*, and *R. trichaurea*. Both *R. hawaiiensis* and *R. lebuensis* also have hair cells, which are lacking in *R. australica* and *R. bonairensis*. The frequent, robust, and long rhizoids (maximum 150–170 μm in length) of Hawaiian *Ramicrosta* distinguish these species from the short, peg-like rhizoids of *R. appressa*. Moreover, the perithallial cell arrangements in *R. hawaiiensis* and *R. lebuensis* do not resemble the distinctive cell arrangements of *R. aranea* and *R. lateralis*.

Ramicrosta represents a well-characterized genus in terms of sequence data, which allows nearly unambiguous determination of the Hawaiian specimens as new species. Almost all described species have representative COI sequences available, excepting *R. calcea*, and this taxon can be ruled out as a match to the Hawaiian specimens based on the branching protuberances produced on the thallus surface in the former taxon (Pueschel and Saunders 2009, Ballantine et al. 2016). Interestingly, Dixon (2018) presented a COI DNA barcoding analysis of *Ramicrosta* species with *R. melanoidea* K. R. Dixon, which suggested that this species may not be a member of the genus *Ramicrosta* and may be better aligned with a clade of *Peyssonnelia*. Our analyses lead to the same conclusion, and this possibility is also suggested by the presence of multiple versions of the same sequences on public databases labeled variously as *R. melanoidea* (BOLD: ABMMC1947-07) or *P. "sp.1 luciparensis"* (GenBank: JX969702.1).

The known global distribution of *Ramicrosta* includes the South China Sea, Vanuatu, the Philippines, southern Australia, New Guinea, Tunisia, and the Caribbean (Zhang and Zhou 1981, Pueschel and Saunders 2009, Dixon and Saunders 2013, Ballantine et al. 2016, Dixon 2018, Manghisi et al. 2019), with the southern Australian and Tunisian locations representing the only distribution points beyond the tropics for this relatively

understudied genus. We add Lehua Island of Hawai'i to this distributional range, and reiterate the likelihood of this range being expanded with detailed study of peyssonnelioid specimens from other tropical locations, as was noted by [Dixon and Saunders \(2013\)](#). In terms of the known depth range for the genus, previous taxonomic reports have recorded it to depths of 21 m, with most records extending from shallow depths to approximately 8 m. However, two additional *rbcL* sequences on GenBank (EU349195—Western Florida Shelf, 41 m depth, and EU349196—Dry Tortugas, FL, 90 m depth), labeled as species of *Peyssonnelia*, yet clearly allied with *Ramicrosta* based on phylogenetic analyses ([Figure 2](#)), illustrate that the depth distribution of the genus can extend as deep as 90 m, which is in the middle range of the mesophotic in the tropical Hawaiian Islands ([Hinderstein et al. 2010](#), [Spalding et al. 2019](#)).

Several species of *Ramicrosta* have been recorded as overgrowing live corals, causing their eventual death (*R. textilis* in the Caribbean and South China Sea: [Pueschel and Saunders 2009](#), [Ballantine et al. 2011](#), [Ballantine and Ruíz 2013](#), [Ballantine et al. 2016](#), [Nieder et al. 2019](#); *R. bonairensis* in the Caribbean: [Eckrich et al. 2011](#), [Eckrich and Engel 2013](#), [Ballantine et al. 2016](#)). Other *Ramicrosta* species, including *R. lateralis*, *R. trichaurea*, and the two new taxa described here, are known to grow on coral rubble but are not reported to cause coral death ([Dixon and Saunders 2013](#), [Ballantine et al. 2016](#)). However, given the potential ecological impacts of some members of *Ramicrosta*, this first record of the genus in Hawaiian waters should be noted as additional justification for continued monitoring of reefs in the state. In addition, Lehua Island (the location of the species in Hawai'i) is positioned at the northwestern end of the main Hawaiian Islands, and its proximity to the Pāhānaumokuākea Marine National Monument adds cause for concern if *Ramicrosta* is found in the coral reefs of the Northwestern Hawaiian Islands. [Ballantine et al. \(2016\)](#) not only speculated that the appearance of *Ramicrosta* in the Caribbean may have been a recent phenomenon related to the mass die off

of the long spine sea urchin or general coral reef health decline, but also noted that a recent focus on the systematics of tropical crustose red algae may have led to the recognition of the alga that had long been present but had only recently become a problem. If either of their first two hypotheses are correct, however, there is indication that changes in coral reef health (e.g., from climate change-related impacts) may facilitate spread of invasive species or other negative ecological impacts by previously unnoticed species in the marine environment.

Additional work is needed due to the cryptic morphology and unknown distribution of these algae, including in the Hawaiian flora. A DNA barcoding-based biodiversity survey of the Hawaiian red algae focused on the shallow water flora, reported between six (LSU) and nine (UPA) species-level groupings of peyssonnelioid algae (with very little sequencing success for the COI marker at that time) ([Sherwood et al. 2010a, b](#)). Moreover, specimens that were morphologically identified as one of the three previously reported species of *Peyssonnelia* for the Hawaiian Islands (*P. conchicola*, *P. inamoena*, and *P. rubra*) often clustered together with identical sequences, casting doubt on the straightforward identification of these algae using only microscopy. The phylogenetic clarification of the Peyssonneliales since that time ([Krayesky et al. 2009](#)) and recognition of additional genera that can be distinguished using molecular as well as morphological characters (e.g., *Incardia* and *Ramicrosta*; [Dixon and Saunders 2013](#)), have allowed the fleshy crustose red algae of the Hawaiian Islands to be investigated in greater detail than was previously possible. This study represents the first of several to unravel the peyssonnelioid diversity of the Hawaiian Islands, and to place these species in the context of what is known elsewhere, with comparisons between the known shallow and mesophotic flora of Hawai'i. This initial analysis describes two new species within a genus of red algae previously unrecorded in the islands, hinting at the diversity that likely remains to be discovered among the crustose red algae of the Hawaiian Islands and in warm water (or tropical) mesophotic ecosystems.

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