Research Article

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Chondrus retortus (Gigartinales, Rhodophyta) in Hawai‘i: a taxonomic and biogeographic puzzle

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Abstract: Members of the genus *Chondrus* are well-known from temperate and cold waters. *Chondrus ocellatus* Holmes was reported from Hawai‘i Island (19° N latitude) in 1999 as a new record based on vegetative and tetrasporangial characteristics. The first specimens were collected by Setchell in 1900 in Hilo, HI. The presence of a *Chondrus* species in the subtropics has been a phycological enigma for over 100 years. We addressed the question of species identity and biogeographic affinities of the Hawaiian *Chondrus* with fresh cystocarpic material, DNA samples, and phylogenetic analyses. Analysis and comparison of five genes (nuclear: EF2; plastid: *psb*A, *rbc*L, and 23S/UPA; mitochondrial: COI) from Hawaiian *Chondrus* and holotype and topotype material of 10 of the 11 accepted *Chondrus* species indicate that Hawaiian specimens are *C. retortus* Matsumoto *et* Shimada. However, unlike type material, the Hawaiian specimens are commonly pinnulate, vary significantly in secondary medullary filament density, and have mature cystocarps filling the entire medullary space. This study shows the value of using multi-gene loci and comparing multiple sequences of several species to confirm taxonomic conclusions. Our findings suggest that *C. retortus* may have immigrated via rafting on natural floating material or on ships' hulls. Solving this old puzzle adds new insight into Hawaiian phytogeography.

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1 Introduction

The 11 currently accepted species of *Chondrus* (Guiry and Guiry 2023) are found primarily in temperate, colder regions in the intertidal to 24 m deep. *Chondrus* is a distinct genus in the Gigartinaceae delineated by reproductive morphology (Stackhouse 1797). The genus is characterized by functional generative auxiliary cells that form a few, inwardly directed protrusions bearing gonimoblast initials that grow into diffuse, filiform gonimoblast filaments that infiltrate between the medullary and secondary filaments, and often link to the medullary and secondary filaments by secondary pit connections (Fredericq et al. 1992; Hommersand et al. 1993). Inner gonimoblast cells remain narrow. Functional auxiliary cells are surrounded by an envelope composed of secondary filaments (Fredericq et al. 1992; Hommersand et al. 1993). Tetrasporangia are borne in secondary filaments formed entirely within the medulla (Fredericq et al. 1992; Hommersand et al. 1993).

Species of *Chondrus* in the northwest region of the Pacific Ocean have a rich taxonomic and nomenclatural history. *Chondrus ocellatus* Holmes is distributed along the exposed shores of China, Japan, and Korea (Taylor and Chen 1994) and exhibits habitat preferences for lower intertidal rock, reef, or pebble substrata. Okamura (1909, 1932, 1936) defined this species as comprising five forms based on morphological characters: f. *ocellatus* (as 'f. *typicus*'), f. *canaliculatus*, f. *giganteus*, f. *crispus*, and f. *nipponicus*. Mikami (1965) raised f. *canaliculatus* and f. *giganteus* to the rank of species: *C. verrucosus* Mikami and *C. giganteus* Yendo, respectively, and proposed three additional formae for *C. ocellatus*: f. *parvus*, f. *aequalis* and f. *crispoides*. Brodie et al. (1991) placed the formae f. *crispus* and *nipponicus* into the species *C. nipponicus* Yendo based on life history and morphological evidence. Later, Brodie et al. (1993) proposed to transfer f. *crispoides* to *C. nipponicus* based on morphology and culture experiments. Hommersand et al. (1994, 1999) clarified many members of the order

Gigartinales using the *rbc*L gene, including five species of *Chondrus*. Most recently, Matsumoto and Shimada (2013) separated *C. verrucosus* "small type" from *C. verrucosus* and described the new species as *Chondrus retortus* Matsumoto *et* Shimada based on its small size, canaliculated blades that are crispate (=curled), rounded medullary cells, and its *rbc*L and EF2 gene sequences.

The biogeographic history of *C. ocellatus* in the northwest Pacific Ocean was investigated by Hu et al. (2015) using DNA sequence analysis of two molecular markers, e.g. mitochondrial cytochrome oxidase subunit I (mtDNA COI) and the nuclear ribosomal internal transcribed spacer (nrDNA ITS). Hu et al. (2015) found that Hawaiian samples of *Chondrus* grouped together as "Lineage C" with samples from Amatsukominato, Chiba, Japan, whereas Lineage "A" consisted primarily of Japanese, Chinese, and a few Korean samples. Lineage "B" consisted of eight samples from China and most of the samples from Korea. Analysis using mtDNA COI indicated that the divergence time between *Chondrus ocellatus* Lineages "A" and "B" (5.96 Ma) was similar to that between lineages "A" and "C" (6.07 Ma), but that Lineage "B" may have diverged from Lineage "C" earlier, at about 7.73 Ma. The authors noted that it is possible that reproductive barriers have led to genetic isolation among the three lineages of *C. ocellatus*, and that additional cryptic formae or species may exist, but kept all specimens lumped as *C. ocellatus*, including the enigmatic Hawaiian *Chondrus.*

Yang and Kim (2022) investigated the phylogeography of three species: *C. ocellatus*, *C. nipponicus*, and *C. giganteus*, using *rbc*L and COI5-P molecular markers and found that these species exactly fit into the lineages "A", "B", and "C" defined by Hu et al. (2015) for *Chondrus ocellatus*. Thus, Hu et al. (2015) had mistakenly grouped three species as one single species, *Chondrus ocellatus*. Yang and Kim (2022) concluded that there were 11 haplotypes of *C. ocellatus* (of which two comprised 80 % of sequences: C01 and C10); *C. nipponicus* had 14 haplotypes (of which two comprised 89 % of sequences: CN1 and CN10); and *C. giganteus* could be separated into two haplotypes (CG1 and CG2) that differed by two mutations.

Abbott reported *Chondrus* from Hawai'i Island (19° N latitude) in 1999 as a new record, although specimens had "been collected many times over many years." Abbott (1999) identified specimens as *C. ocellatus* based on vegetative and tetrasporangial characteristics, and warned that lack of reproductive material made identification "hazardous" for this member of the Gigartinaceae. The earliest known specimen (UC622609) was collected by W. J. Setchell in 1900 in Hilo, HI. Most of the many herbarium specimens at Bishop Museum that are labeled as *Chondrus*, were collected on the east side of Hawai'i Island in areas where submarine groundwater discharge occurs. The occurrence of a *Chondrus* in a warm, subtropical climate, like Hawai'i, is very unusual. Fertile material and DNA have never been critically studied for specimens of Hawaiian *Chondrus*, until this study.

The objective of our research was to determine whether the Hawaiian *Chondrus* is *Chondrus ocellatus*, another species, or a new species. Our goal was to solve this long-standing, 123-year long, taxonomic and biogeographic mystery using thorough molecular, morphological, and anatomical methods including comparisons of type material or specimens from type locations.

2 Materials and methods

2.1 Field sampling

Individual thalli of *Chondrus* (Figure S1) were collected in 2014–2022 at three locations along the east Hawai'i Island shoreline (Figure 1) at or near the collection sites of specimens previously collected between 1900 and 2003 by Setchell, Doty, Abbott, Fortner, Magruder, McDermid, and Okano. Additional thalli were sampled from cultures grown at the Pacific Aquaculture and Coastal Resources Center in Hilo, Hawai'i.

Thalli were immediately preserved in 5 % formalin/seawater for morphological study, or directly placed into silica gel desiccant, or preserved in DNA/RNA Shield (Zymo Research, Cat. No. R1100, Irvine, CA, USA) for molecular work. Samples were deposited in the University of Louisiana at Lafayette Herbarium (LAF) and Bernice Pauahi Bishop Museum Herbarium in Honolulu, HI (BISH). *Chondrus* specimens from National Museum of Nature and Science (TNS) Tsukuba, Japan were used for subsequent analyses.

2.2 Morphology and anatomy

Longitudinal, transverse, and periclinal sections were stained with 1 % aniline blue in 1:1 distilled water: KARO® corn syrup or aceto-iron-haematoxylin-chloral hydrate for periods of 30 min to 4 h and mounted in 1:1 Hoyer's medium:distilled water following methods from Fredericq et al. (1992).

2.3 DNA extraction and PCR amplification

In preliminary studies in 2014, 300 bp fragments of *rbc*L and ITS1 (internal transcribed spacer) were sequenced. However, the length of these sequences was not informative enough to make a confident taxonomic decision, and in April 2020, total DNA was extracted from silica gel-dried samples, and samples from Hawai'i preserved in DNA/RNA Shield (Zymo Research, Cat. No. R1100, Irvine, CA, USA) using the Zymo Quick Plant/Seed DNA Extraction kit (Zymo Research, Cat. No. D6020, Irvine, CA, USA) following the manufacturer's instructions. Total DNA was extracted from Japanese herbarium samples (National Museum of Nature and Science, Japan) of *C. ocellatus*, *C. retortus*, and *C. verrucosus* using a modified herbarium extraction protocol (Cullings 1992; Doyle and Dickson 1987; Doyle and Doyle 1987; Hughey et al. 2001)

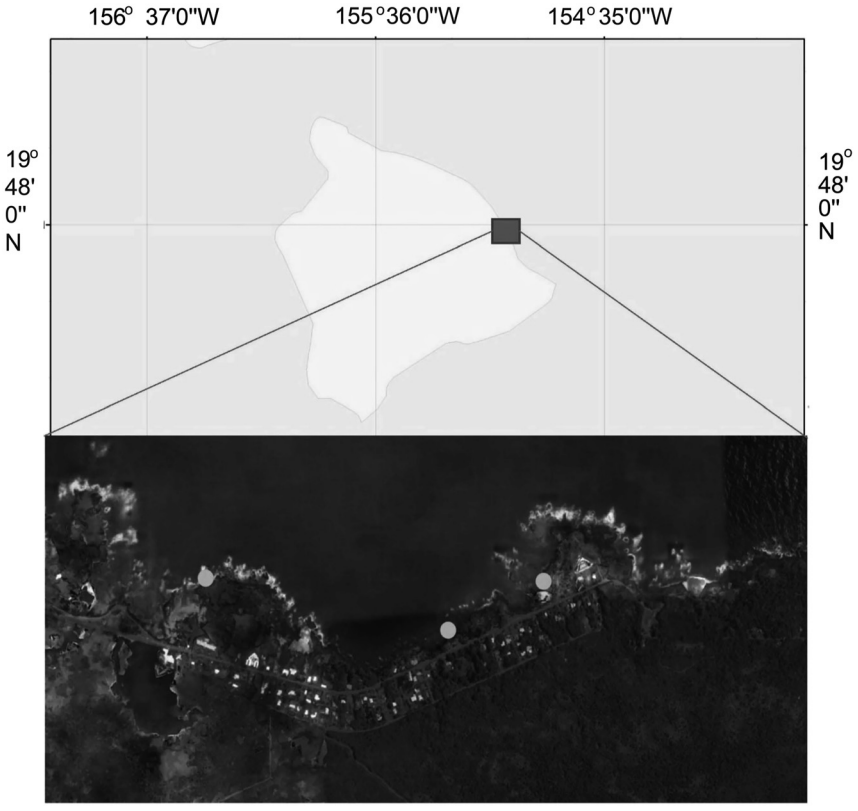

Figure 1: Collection sites of *Chondrus retortus* (light gray circles) around the Keaukaha area, east side of Hawai'i Island.

(Table S1). Additional, selected DNA extractions were chosen from material used by Hommersand et al. (1994, 1999) in the genus *Chondrus*, including *C. armatus*, *C. crispus*, *C. elatus*, *C. giganteus*, *C. nipponicus*, *C. ocellatus*, *C. pinnulatus*, *C. verrucosus*, and *C. yendoi*, as well as two other members of the family Gigartinaceae housed in UL Lafayette (Table S1). Those extractions were performed using a modified CTAB protocol with a final cesium chloride-ethidium-bromide purification step (see Hommersand et al. 1994 for more details).

Preliminary sequencing using *rbc*L and UPA determined that there were no base pair differences among four individuals from three distinct *Chondrus* populations collected in east Hawai'i Island. Therefore, only one representative (LAF 7580) was used for the multi-marker analyses using five conserved genes for PCR: the chloroplast gene *psb*A which encodes the photosystem II reaction center protein D1; the chloroplast gene *rbc*L which encodes the large subunit of the marker ribulose-l, 5-bisphosphate carboxylase/oxygenase (RuBisCO); the mitochondrial gene COI which encodes the cytochrome oxidase subunit I; a portion of the nuclear elongation factor 2 gene (EF2); and the plastid ribosomal 23S rDNA gene, also known as the universal plastid amplicon (UPA). Amplification of DNA was done through polymerase chain reaction (PCR) using a T100 Thermal Cycler (Bio-Rad).

PCR for *psb*A were performed using the primers referenced in Yoon et al. (2002) and included an initial denaturation at 94 °C for 3 min followed by 39 cycles at 94 °C for 30 s (denaturation), 52 °C for 50 s (primer annealing), and 72 °C for 1 min (extension), followed by a final extension at 72 °C for 5 min. PCR for *rbc*L were performed using the primers referenced in Freshwater and Rueness (1994), the primer F645 referenced in Lin et al. (2001), and the primer F7 referenced in Gavio and Fredericq (2002), and included an initial denaturation of 94 °C for 4 min followed by 2 cycles at 94 °C for 1 min (denaturation), 40 °C for 1 min (primer annealing), and 72 °C for 2 min (extension), then 40 cycles at 94 °C for 1 min (denaturation), 42 °C for 1 min (primer annealing), and 72 °C for 2 min (extension), followed by a final extension at 72 °C for 5 min. Multiple overlapping reads were generated for *rbc*L to ensure quality chromatograms were obtained for the full length of the consensus read. PCR for COI were conducted using the primers referenced in Saunders (2005) and Saunders and Moore (2013) with an initial denaturation at 94 °C for 2 min followed by 40 cycles at 94 °C for 1 min (denaturation), 45 °C for 1 min (primer annealing), and 72 °C for 1 min (extension), followed by a final extension at 72 °C for 5 min. PCR for EF2 were performed using the primers referenced in Le Gall and Saunders (2007) with initial denaturation at 94 °C for 4 min, followed by 38 cycles at 94 °C for 1 min (denaturation), 50 °C for 1 min (primer annealing), 72 °C for 2 min (extension), and followed by a final extension at 72 °C for 7 min. Lastly, we used primers for 23S/UPA referenced in Sherwood and Presting (2007) with an initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 20 s (denaturation), 55 °C for 30 s (primer annealing), and 72 °C for 30 s (extension), and followed by a final extension at 72 °C for 10 min.

2.4 Purification of amplification products

The PCR products were visualized through 10 mm of acrylic shielding, under low UV transillumination on a DyNA Light Dual Intensity UV Transilluminator (LabNet) in a 1.1 % agarose – 1X TAE gel containing 500 ng of ethidium bromide per ml. Single stranded primers and unincorporated dNTPs were removed from PCR products by the addition of 2 µl of ExoSAP-IT™ (USB, Cleveland, Ohio) per 5 µl of amplified DNA product. Reactions were incubated at 37 °C for 15 min, followed by inactivation of ExoSAP-IT™ at 80 °C for 15 min.

2.5 Cycle sequencing

ABI Prism® BigDye™ Terminator sequencing kits were used (Applied Biosystems). Cycle sequencing reactions using Purified PCR products were subsequently cycle sequenced using the BrightDye® Terminator Cycle Sequencing Kit (Molecular Cloning Laboratories [MCLAB], South San Francisco, CA, USA) using 20 µl volumes, 4 µl of Ready Reaction terminator mix, 2 µl of 5X Bright Dye™ sequencing buffer, 1 µl appropriate primer, and 1.5 µl of purified PCR product. Cycle sequencing was performed as follows: 96 °C for 1 min, followed by 25 cycles of 96 °C for 15 s, 50 °C for 5 s, and 60 °C for 4 min. Both strands of each PCR product were sequenced in separate sequencing reactions using primers with sequences identical to those used for amplification. Resulting PCR products were purified with ETOH/EDTA precipitation and sequenced in-house at the UL Lafayette campus on an ABI Model 3130xl Genetic Analyzer. Chromatograms were assembled using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA). *Rbc*L sequences from archived *Chondrus* extractions previously used in Hommersand et al. (1994, 1999) were re-generated in this study using the above methods to confirm the accuracy of sequences found in the NCBI GenBank database. We did not submit the re-generated *rbc*L sequences since they proved to be identical to the original sequences.

Species identification, specimen collection information, and GenBank accession numbers generated are listed in Table S1.

2.6 Phylogenetic, sequence divergence, and species delimitation analyses

Additional sequences were exported from the public NCBI database GenBank and added to the dataset (Table S1). Sequences for each gene were aligned separately in MEGA v. 5.2.2 (Tamura et al. 2011) using the CLUSTALW or MUSCLE algorithms, and then analyzed downstream. The DNA matrices were exported onto the CIPRES server, namely PartitionFinder 2 (Lanfear et al. 2017) to determine the best fitting model of evolution and data partition. The single gene alignments were 1446 bp for *rbc*L, 1515 bp for EF2, 947 bp for *psb*A, 369 for UPA, and 616 bp for COI.

For each of the single gene alignments, protein-coding genes were partitioned by codon position and the 23S rDNA gene was kept unpartitioned. This resulted in the GTR+I model to be used for each gene based on the AICc (corrected Akaike information criterion) and AIC (Akaike information criterion) scores. The alignment with these models and partitioning scheme was then analyzed for maximum likelihood (ML) with RAxML-HPC2 on XSEDE v. 8.2.12 (Stamatakis 2014) via the CIPRES gateway (Miller et al. 2010) with 1000 bootstrap replicates.

The *rbc*L, EF2, *psb*A, 23S, and COI alignments included 44, 14, 9, 9 and 135 sequences of sequences of *Chondrus*, respectively. One to four sequences of *Mazzaella* were used as the outgroup, except in the *psb*A alignment that included *Chondracanthus* as the outgroup since *Mazzaella* sequences are unavailable. The newick file was imported into FigTree 1.4.2 (Rambaut and Drummond 2016) as a starting point for further editing in Microsoft Publisher.

Single loci alignments of five genes were constructed for *Chondrus*. Alignments were cropped at the 5′ and 3′ ends to minimize missing data. Sequences were aligned with the CLUSTAL W (Thompson et al. 1994) algorithm in the MEGA X software (Kumar et al. 2018). For *Chondrus*, 1233 bp, 776 bp, 643 bp, 369 bp, and 616 bp alignments were constructed for *rbc*L, EF2, *psb*A, UPA, and COI, respectively. Sequence divergence values were calculated as the number of pairwise base pair differences in MEGA X (Kumar et al. 2018) and presented as a proportion (the number of base pair differences divided by the alignment length) (Tables S2–S5).

Species delimitation analysis for each gene was performed separately using assemble species by automatic partitioning (ASAP) (Puillandre et al. 2021) using the Jukes-Cantor substitution model for the following parameters: split groups below 0.01 probability, highlighting genetic distances between 0.005 and 0.05.

3 Results

3.1 Morphology and anatomy

Thalli of the Hawaiian species are up to 6.0 cm tall and up to 5 mm at the base and are commonly dichotomously branched with marginal pinnules approximately 2.5–3.3 cm long and ~1.67 mm wide (Figure 2). Female gametophytes commonly bear old cystocarps (Figure 2). Pinnules and the roundish-flattened stalk (Figure 3A) lack a constriction at the point of insertion. Thalli vary significantly in the density of secondary medullary filaments (sparse to compact) within a specimen (Figure 3B). Main branches show dense aggregations of medullary filaments (Figure 3C–E) with few enlarged stellate cells (Figure 3F) or with sparse filiform medullary filaments in the center of the blade. Pinnules show a regular

Figure 2: *Chondrus retortus.* Dichotomously branched gametophytes bearing old cystocarps. Marginal pinnules are common. Black arrows indicate cystocarps. Scale bar = 1 cm.

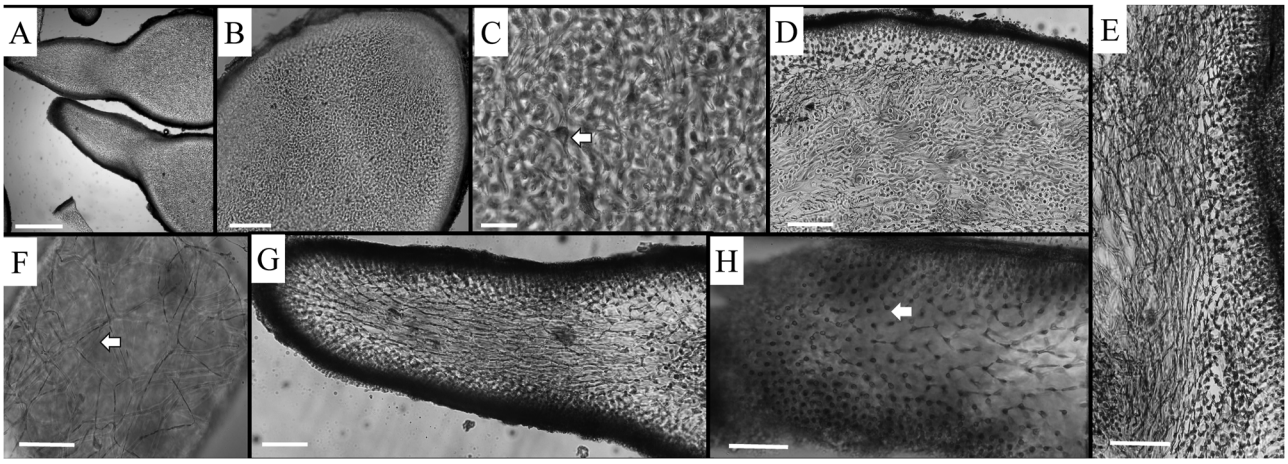

Figure 3: Structure of *Chondrus retortus*. (A) Longitudinal sections through pinnule (at left) and transverse sections through roundish-flattened stalk (at right). Note lack of constriction at point of insertion. Scale bar = 500 µm. (B) Cross section through main branch showing dense aggregation of medullary filaments. Scale bar = 200 µm. (C). Periclinal section showing dense aggregation of medullary filaments with a few enlarged stellate cells (arrow). Scale bar = 50 µm. (D) Cross section through blade showing cortex and dense medulla. Scale bar = 100 µm. (E) Longitudinal section though region shown in (D). Scale bar = 100 µm. (F) Periclinal section showing sparse filiform medullary filaments (arrow) in center of blade. Scale bar = 20 µm. (G) Longitudinal section through pinnule at left showing regular arrangement of medullary filaments, and scarcity of secondary filaments. Scale bar = 100 µm. (H) Oblique periclinal section at apical region of pinnule showing abundance of conjunctor cells issued from intercalary cells forming secondary pit connections (arrow) just below thallus surface. Scale bar = 100 µm.

arrangement of medullary filaments, and a scarcity of secondary filaments (Figure 3G and H). The apical regions of pinnules show an abundance of conjunctor cells issued from intercalary cells forming secondary pit connections just below the thallus surface (Figure 3I).

Abortive procarps scattered in the cortex are common (Figure 4A) and easily recognized because an envelope around abortive auxiliary cells is not present (Figure 4B). Abortive auxiliary cells may generate non-functional gonimoblast cells (Figure 4C). Mature cystocarps are located in the center of compressed axes, showing a central area with empty filiform networks (Figure 4D). Mature cystocarps contain carposporangia and secondary gonimoblast filaments that grow among them and reach the outer cortex (Figure 4E). A stretched network of medullary cells and filiform gonimoblast cells bearing carposporangial chains are located in the center (Figure 4F) or at the periphery of mature cystocarps (Figure 4G and H). Putative spermatangia (Figure 4I) are cut off from terminal cortical cells at the tip of female gametophytes bearing cystocarps. Tetrasporangial thalli were not observed.

Table 1 provides a comparison of morphological characters among closely related species (*Chondrus verrucosus*, *C. retortus*, *C. elatus*) and the original descriptions of *C. ocellatus* (Holmes 1895) and *C.* "*ocellatus*" in Hawai'i (Abbott 1999), *C. verrucosus* (Mikami 1965), *C. retortus* (Matsumoto and Shimada 2013) and *C. elatus* (Holmes 1895).

3.2 Phylogenetic, sequence divergence, and species delimitation analyses

The *rbc*L, EF2, and *psb*A gene sequences of Hawaiian specimens correspond to sequences of type specimens of *Chondrus retortus* (Figures 5, 6, and 7). For UPA and COI (Figures 8 and 9), sequences of Hawaiian *Chondrus* cluster in a clade with previously mislabeled *C.* "*ocellatus*" (=*C. retortus*). Earlier in 2014, our comparisons of sequences of short fragments of *rbc*L and ITS1 of the Hawaiian *Chondrus* with sequences on NCBI GenBank database, were inconclusive because of inadequate sequence length and low variability within the region.

Species delimitation analyses from ASAP are within the species *C. retortus* for four genes and we can infer that the Hawaiian *Chondrus* is *C. retortus* and not *C. ocellatus*. A total of 10 species were delimited using the *rbc*L marker (*C. yendoi*, *C. armatus*, *C. pinnulatus*, *C. crispus*, *C. elatus*, *C. verrucosus*, *C. retortus*, *C. giganteus*, *C. ocellatus*, *C. nipponicus*). For the EF2 gene, ASAP delimited six known species (*C. crispus*, *C. retortus*, *C. verrucosus*, *C. elatus*, *C. ocellatus*, and *C. giganteus*). No sequences are available for *C. yendoi*, *C. armatus*, *C. pinnulatus*, and *C. nipponicus* for the EF2 marker. For the *psb*A marker, ASAP delimited six species (combined *C. retortus* and *C. verrucosus*, *C. elatus*, *C. giganteus*, *C. ocellatus*, *C. yendoi*, *C. crispus*). No sequences are available for *C. nipponicus*, *C. pinnulatus*, *C. armatus* for the *psb*A marker. For UPA, ASAP delimited

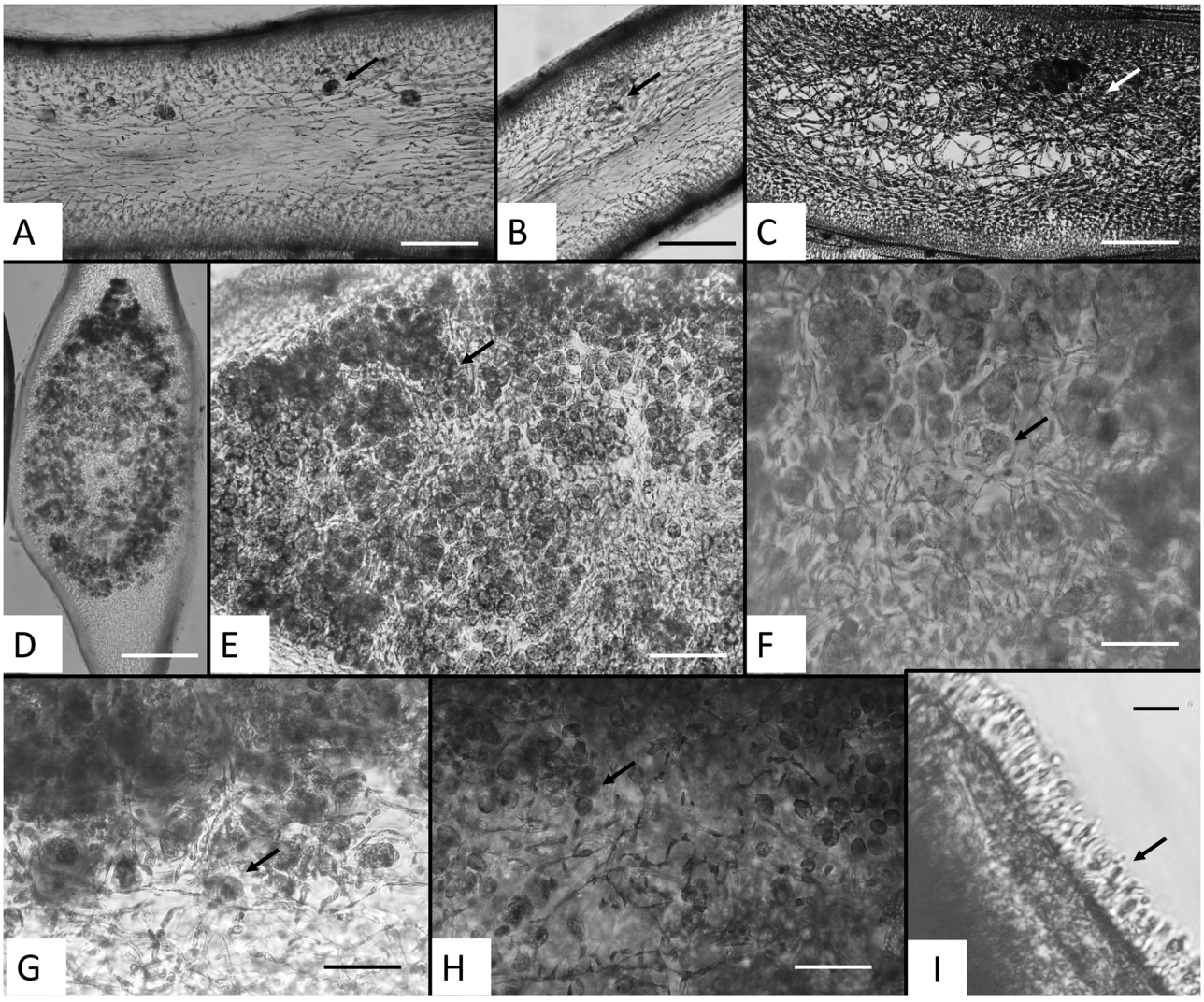

Figure 4: Structure of *Chondrus retortus*. (A) Abortive procarps scattered in cortex. Longitudinal section. Note the lack of envelope around auxiliary cells. Scale bar = 100 µm. (B) Abortive auxiliary cell with degenerating protrusions, and medulla with sparse medullary filaments. Longitudinal section. Scale bar = 100 µm. (C) Abortive auxiliary cell with non-functional gonimoblast cells, and medulla with dense aggregation of medullary filaments (arrow). Longitudinal section. Scale bar = 100 µm. (D) Mature cystocarp located in center of compressed axis, showing central area with empty filiform network. Cross section. Scale bar = 200 µm. (E) Mature cystocarp containing carposporangia and secondary gonimoblast filaments (arrow) that grow among them and reach the outer cortex. Cross section. Scale bar = 75 µm. (F) Stretched network of medullary cells and gonimoblast cells bearing carposporangial chains (arrow) in center of mature cystocarp. Periclinal section. Scale bar = 50 µm. (G) Stretched network of medullary cells and gonimoblast cells bearing carposporangial chains (arrow) at periphery of mature cystocarp. Periclinal section. Scale bar = 50 µm. (H) Mixture of medullary filaments and filiform gonimoblast filaments bearing carposporangia (arrow). Periclinal section. Scale bar = 50 µm. (I) Putative spermatangia (arrow) produced from terminal cortical cells at tip of female gametophyte bearing cystocarps. Cross section. Scale bar = 7.5 µm.

taxa into five species (the Hawaiian and Chinese "*C. ocellatus*," *C. elatus*, *C. giganteus*, *C. yendoi*, and *C. pinnulatus*). No UPA gene sequences were successfully generated for *C. ocellatus*, *C. crispus*, *C. verrucosus*, *C. armatus*, and *C. retortus*. However, we used data available on NCBI GenBank for UPA. For COI, ASAP delimited taxa into 10 taxa (*C. yendoi*, *C. pinnulatus*, *C. armatus*, *C. crispus*, *C. retortus*, *C. verrucosus*, *C. elatus*, *C. giganteus*, *C. ocellatus*, and *C. nipponicus*).

From the 23S UPA dataset, there is a 100 % match of *C. ocellatus* ARS04629 and ARS00883 (Hilo, Hawaii) with voucher LAF 7580 of *Chondrus retortus*, collected in the same locality. In the COI dataset, *C. ocellatus* ARS00883 and *C. ocellatus* haplotype 16 (both Hawaii samples from the same locality) show a 100 % match, and both exhibit a 99.8 % match with *C. ocellatus* mbccc36 (Huiquan Bay, Qingdao); therefore, this close match of Hawaiian and Chinese *Chondrus* specimens indicates that *C. retortus* also occurs in

Table 1: Comparison of morphological characters among *Chondrus* species, including the type descriptions of *C. ocellatus* and *C. retortus.*

	Chondrus retortus **Hawaii**	***Chondrus* "*ocellatus*"**	***Chondrus ocellatus***	***Chondrus verrucosus***	***Chondrus retortus***	***Chondrus elatus***
Reference(s)	This study	Abbott (1999)	Holmes (1895), Mikami (1965), Brodie et al. (1993)	Mikami (1965)	Matsumoto and Shimada (2013)	Holmes (1895), Mikami (1965)
Plant length (cm)	Up to 6.0	To 5.0	5.0–7.62 (reported as 2–3 in. tall)	8–17	4–18	15.0–20.32 (reported as 6–8 in.)
Branching	Subdichotomously to irregularly, branching 1 or 3 times	Subdichotomously to irregularly	Twice to thrice forked from base	1–4 times dichotomously branched, very regularly to irregularly divided	Branching 1–3 times dichotomously, with round axils	Rarely branching below middle, arcuate and divaricate
Cortex	Consisting of roundish cells that become stellate or irregularly shaped in inner cortical region	~ 100 µm thick, narrow subcortex of rounded cells	Thick composed of 6–8 small oblong to elongated cells	5–8 rows (or more) of small, elongated or ellipsoidal cells	Composed of roundish cells, which become stellate to irregularly shaped in inner cortical region	6–8 rows of small oblong or elongated cells; subcortical cells 3–5 star-shaped cells arranged in anticlinal order
Medullary cells	Very dense, with sparse filiform medullary filaments in the center of the blade	Mesh-like at lateral margins, becoming elongate centrally and periclinally (to 400 µm thick)	Slender rhizoidal cells, arranged parallel to the frond; 2.5–12 µm in diameter and 75–100 µm in length	Very thick, composed of slender cell rows	5.6–11 µm in diameter	Consisting of somewhat swollen, mostly rhizoidal cells mostly arranged to the horizon to the surface of the frond in longitudinal section
Tetrasporangia	Not observed	Abundant in midsections of blade, 1–2 mm diameter	Network among medullary layer, except central medulla	Sori scattered on upper part of frond, as elliptical or irregular spots on surface view	Formed in shallow medullae. Sori scattered on both sides of the middle to upper portion of fronds	Densely scattered on upper branches
Cystocarps	Located in center of compressed axes, commonly filling-up the entire medullary space	2–4 mm wide, irregularly present, occupying up to 80 % of section	Surrounded by a ring, giving ocellated appearance	Very large and numerous. Wart-like, strongly on one side, 1–4 mm wide	Formed on outer side of canaliculation, scattered over middle to upper fronts	Slightly prominent on one side, elliptical to ocellated, scattered over upper branches, 1–2 mm in diameter
Gonimoblasts	Filiform and abundant	Size not reported	Size not reported	Abundant gonimoblast threads connecting to plentiful swollen medullary cells	Size not reported	Gonimoblasts abundant composed of extremely slender cells that directly connect to medullary nutritive cells
Spermatangia	Size not reported	Size not reported	Size not reported	2.8–3.3 µm in diameter and 5.2–5.9 µm in length	2.9–3.6 µm in diameter and 3.9–5.5 µm in length	Size not reported
Reported distribution	Richardson Ocean Park, Hilo, HI; Leileiwi Beach Park, Hilo, HI	Hawaii: Richardson Ocean Park, Hilo, HI and Shipman Estates, Kea'au, HI	Type: Shimoda, Izu Peninsula, Shizuoka Prefecture, Japan	Type: Inubozaki, Chiba Prefecture, Japan	Type: Enoshima, Kanagawa Prefecture, Japan; Honghyeon-ri, Namhae, Korea	Type: Enoshima, Kanagawa Prefecture, Japan

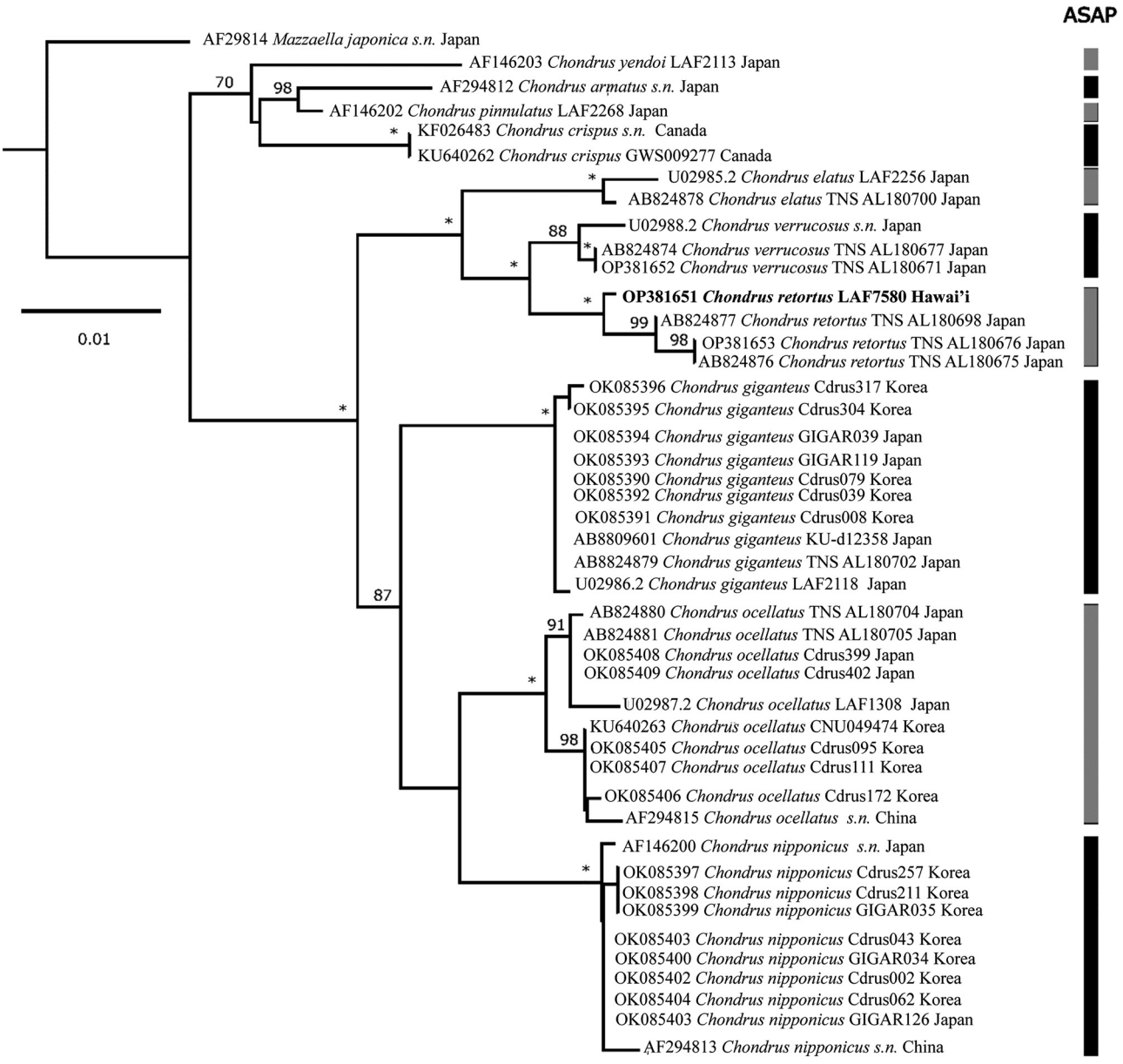

Figure 5: Maximum likelihood analyses of *rbc*L sequences (1446 bp). Numbers at branches indicate bootstrap values out of 1000 replicates. *Denotes full support. The results of assemble species by automatic partitioning (ASAP) species delimitation are indicated by vertical bars.

Huiquan Bay. The sequence divergence analyses comparing Hawaiian *Chondrus retortus* LAF 7580 for *rbc*L shows that there is a 99.984 % similarity with the *Chondrus retortus* voucher specimen TNS:AL:180698 (Kisami, Shimoda, Japan). Thus, the Hawaiian "*Chondrus ocellatus*" samples should be treated as *C. retortus,* as well as the voucher mbccc36 "*C. ocellatus*" samples from China.

Sequence divergence analyses comparing Hawaiian *Chondrus* LAF 7580 with other *C. retortus* specimens were <0.4 % for *rbc*L, <0.6 % for EF2, <0.1 % for *psb*A, <0.8 % for UPA, <0.2 % for COI (Tables S2–S6). For these same gene sequences, interspecific divergences between Hawaiian *Chondrus* LAF 7589 and its closest relative, *C. verrucosus*, were greater than intraspecific divergences and ranged from 0.56 % (*psb*A) to 2.4 % (COI). Sequence divergence between Hawaiian *Chondrus* LAF 7580 and all other *Chondrus* species was greater than these intraspecific and interspecific divergences (Tables S2–S6).

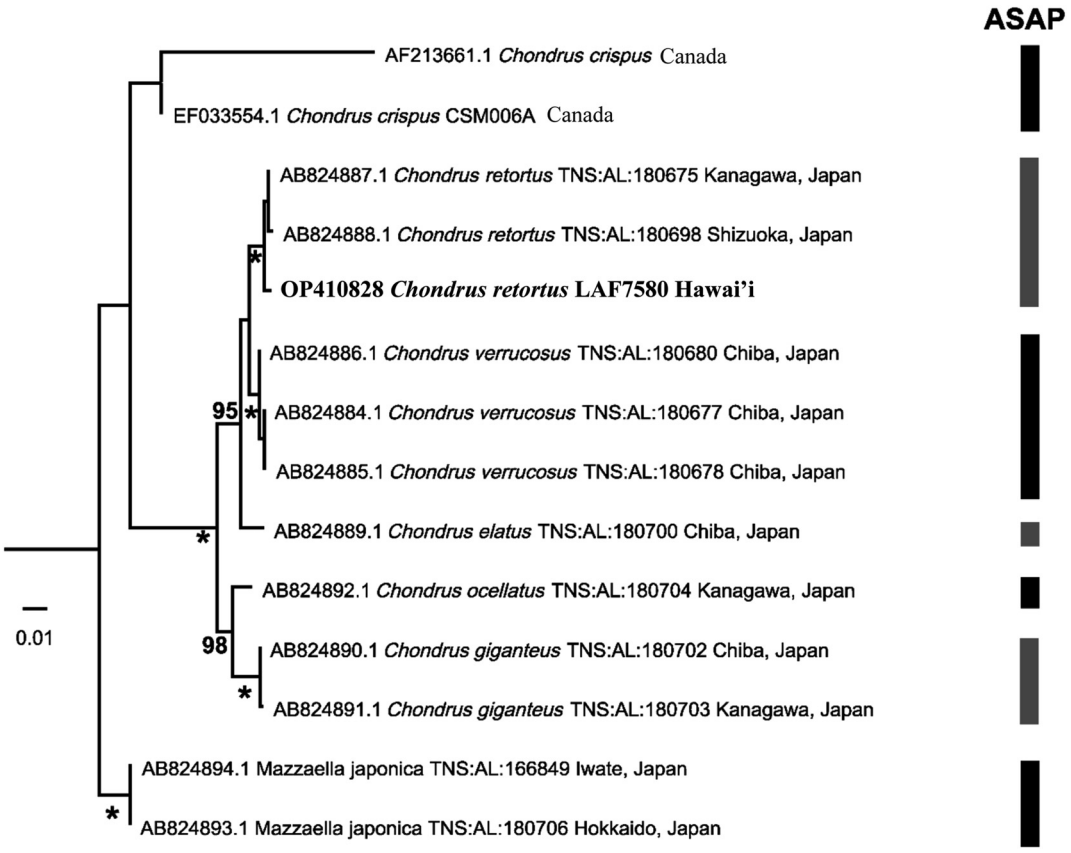

Figure 6: Maximum likelihood analyses of EF2 sequences (1515 bp). Other details as in Figure 5.

4 Discussion

Biogeographical, taxonomic, and ecological studies have treated the *Chondrus* in Hawai'i as *C. ocellatus* (Abbott 1999; Hu et al. 2015; McDermid and Stuercke 2003; Sherwood et al. 2010; Sherwood and Guiry 2023). However, our study shows that after comparing the anatomy, morphology, and five gene sequences from type material (isotypes) or thalli from type habitats (topotypes) in Japan, the Hawaiian species is not *Chondrus ocellatus*, but *C. retortus*. Unlike the descriptions of type material of *C. retortus*, the Hawaiian *C. retortus* is commonly pinnulate, varies significantly in density of secondary medullary filaments (sparse to compact) within a specimen, and has mature cystocarps filling up the entire medullary space. However, there are many overlapping morphological characters among closely related species (Table 1), so caution must be used when interpreting morphology alone for species identification. Fredericq et al. (1992) used developmental reproductive traits to distinguish species with overlapping vegetative traits. Future studies involving morphological comparisons within the genus *Chondrus* must include longitudinal and periclinal sections to better understand the developmental morphology.

There are currently 11 taxonomically accepted species within *Chondrus* (Guiry and Guiry 2023). Ten of the species have at least two molecular markers available on NCBI GenBank. Many NCBI GenBank sequences labeled "*Chondrus ocellatus*" have been historically misidentified and lumped together, without proper comparisons with the type specimens, leading to paraphyly in gene trees or misinterpretations in phylogeographic analyses. Our study demonstrates how DNA sequences can clarify the identity of misidentified samples. Kang et al. (2020) reported *C. retortus* in Honghyeon-ri, Namhae, Korea based on the *rbc*L gene. It is possible that this species has been established in other localities around the Pacific region, but historically misidentified and labeled as *C. ocellatus*.

Chondrus retortus is known from Japan, South Korea, and perhaps China (Guiry and Guiry 2023; Kang et al. 2020; Matsumoto and Shimada 2013; Yoshida et al. 2015), and has been established in east Hawai'i Island since at least 1900 (as *Chondrus ocellatus*; Abbott 1999). The earliest collection of *Chondrus* in Hawai'i was made by William Setchell in 1900 at Seaconnet Point, now known as Kēōkēa in Hilo, Hawai'i Island (UC622609), and the dried herbarium specimens are recognizable as *C. retortus* (Figure S2). Since then, many

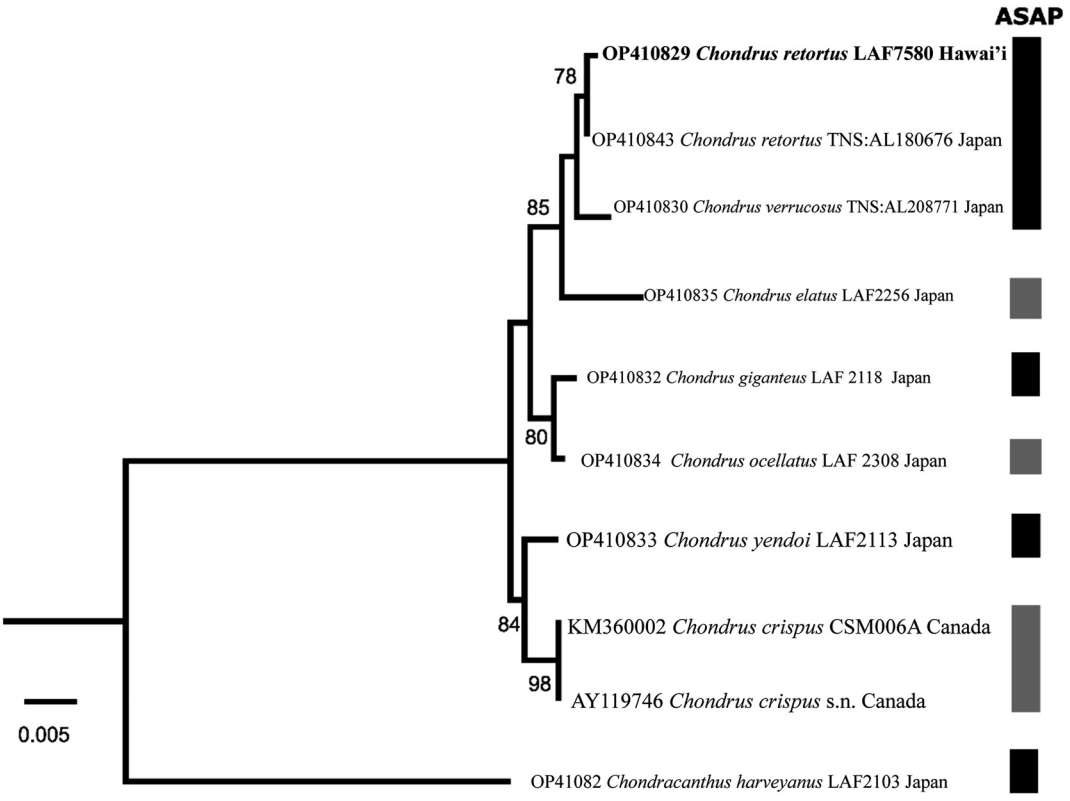

Figure 7: Maximum likelihood analyses of *psb*A sequences (947 bp). Other details as in Figure 5.

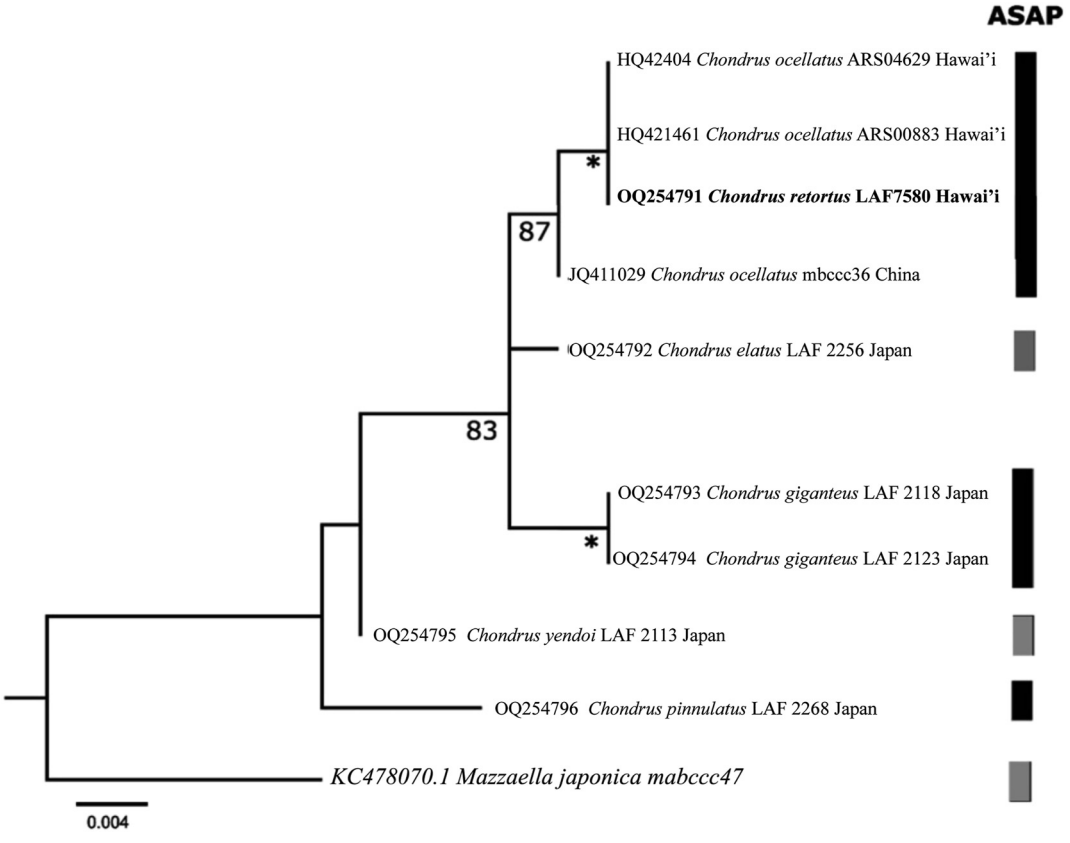

Figure 8: Maximum likelihood analyses of 23S (UPA) sequences (369 bp). Other details as in Figure 5.

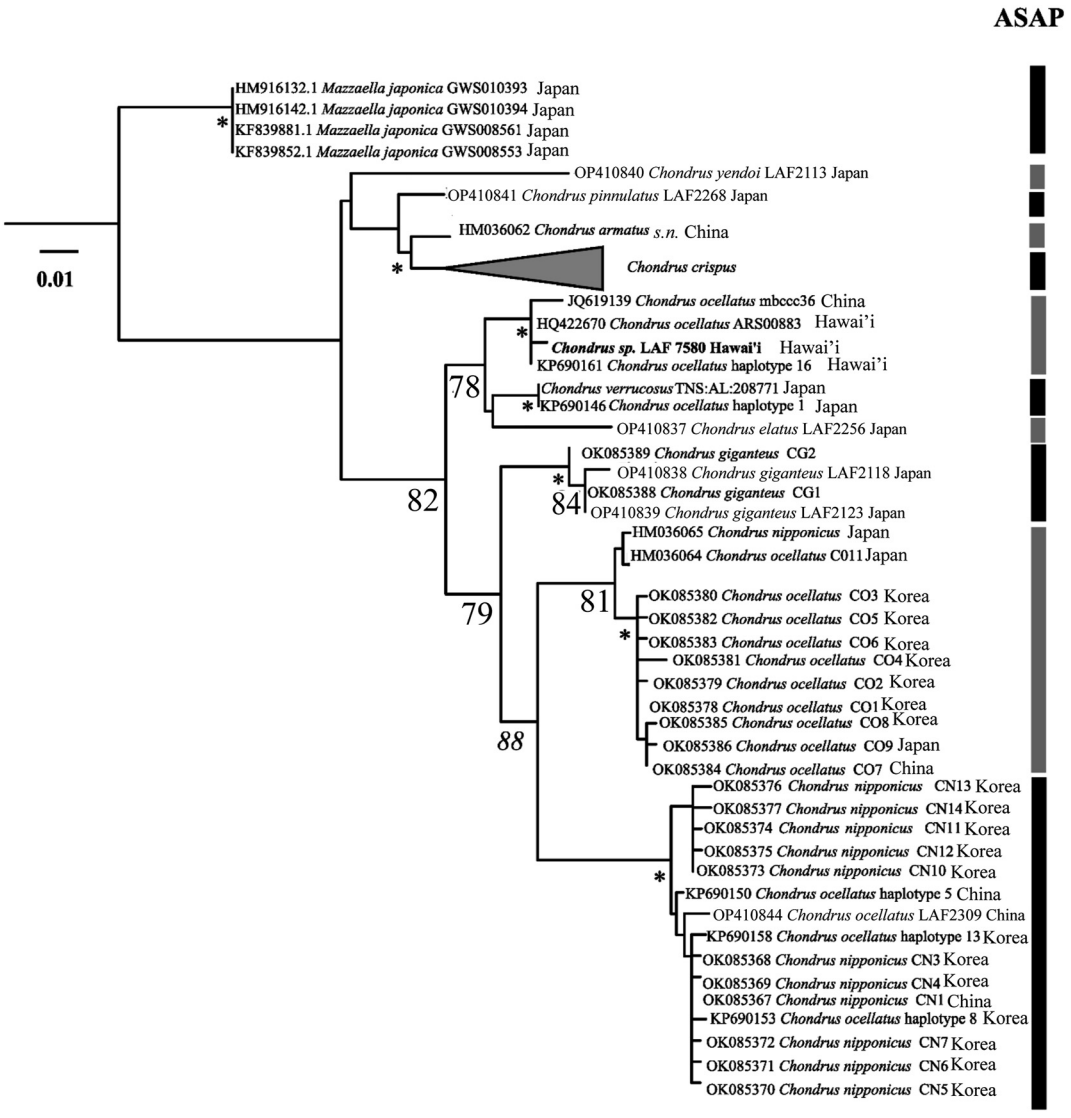

Figure 9: Maximum likelihood analyses of COI sequences (616 bp). Other details as in Figure 5.

phycologists have collected *Chondrus* in the same vicinity along the rocky basalt shoreline (Figure S3). A few Bishop Museum (BISH) herbarium specimens from O'ahu and Maui collected by non-phycologists are labeled *Chondrus* (Figure S4, Table S7), but the thallus morphologies do not seem to correspond to this genus and their gene sequences have not been analyzed.

The Hawaiian Islands, because of their geographic isolation but rich marine flora of over 660 species (Sherwood and Guiry 2023), present a biogeographical enigma. How and when *C. retortus* made the trek from its native population range in the northwest Pacific to the Hawaiian Islands is unknown. Other indigenous Hawaiian macroalgae with disjunct distributions and cold-temperate water affinities have been reported from the Northwestern Hawaiian Islands in the subtidal zone (10 m and deeper) where water temperatures are cooler than in the Main Hawaiian Islands (McDermid and Abbott 2006), i.e. red algae: *Crouania magesshimensis* (Abbott 1989) and *Kallymenia sessilis* (Abbott and McDermid 2002), and several browns: *Desmarestia ligulata*, *Nereia intricata*, and *Sporochnus moorei* (Abbott and Huisman 2003). In contrast, most non-native macroalgal species in the Main Hawaiian Islands are tropical species that were intentionally introduced for commercial cultivation in the 1970–1980s, e.g. *Kappaphycus alvarezii*, *Hypnea musciformis*, or unintentional hitchhikers on the hulls of ships from Guam in the 1950s, e.g. *Acanthophora spicifera* (Coles et al. 1999; Conklin and Smith 2005; Eldredge and Smith 2001; Padilla and Williams 2004). However, *Chondrus retortus* is not a tropical species and probably was not intentionally introduced. The small genetic divergence between Hawaiian and Japanese specimens suggests that

C. retortus arrived in Hawai'i relatively recently – in the last few hundred years, not millions of years ago. Carlton (1987) discussed major transoceanic marine biological invasions around the Pacific Ocean, and stated that "major *donor* areas are the northwest Pacific Ocean and the Australasian-southern Asia-southwest Pacific region," whereas "major *receiver* areas for introduced [organisms] around the Pacific Ocean are the Hawaiian Islands, the Pacific coast of North America, and Australasia." Long distance dispersal of marine species, such as macroalgae and invertebrates via rafting on floating logs, pumice stones, and coral rubble in the Pacific, has been reported, but the survival of these organisms was hypothesized to be low (Jokiel 1990). However, Haram et al. (2023) documented diverse, living, coastal marine invertebrates on 70.5 % of the floating debris sampled in the eastern North Pacific Subtropical Gyre. If shallow-water, benthic invertebrates can float, persist, reproduce, and develop dynamic populations and communities in pelagic waters far from their native shores, then coastal macroalgae might similarly disperse and survive when suitable substrata are present. In March 2011, the tsunami that hit the Tōhoku region of eastern Japan, carried countless coastal objects into the North Pacific Ocean, and by 2012, living, coastal Japanese marine organisms, including macroalgae, were washing ashore in the Hawaiian Islands and North America (Carlton et al. 2017, 2018; Haram et al. 2023). On the tsunami debris that stranded along the northwest coast of North America, Hanyuda et al. (2018) found at least 49 macroalgal species that were genetically identical to members of populations of the Tōhoku region of Japan. In fact, living *Chondrus giganteus* thalli were collected attached to the transom (back wall at the stern of a boat) of the Japanese tsunami-wrecked vessel, *Sou-you,* on the coast of Washington, USA (Carlton et al. 2013). Modern dispersal patterns of tsunami debris (Maximenko et al. 2018) show that the natural processes for transport, e.g. tsunamis, storms, buoyant objects, and currents continue to occur today as they might have in the past.

Since red algal spores do not have long competency periods (Norton 1992) and probably no ability for independent long-distance dispersal across the Pacific, *Chondrus retortus* thalli may have migrated, via rafting on natural floating material in the Kuroshio Current and the Kuroshio Extension (Qiu 2002) to the North Pacific, into the North Pacific Convergence Zone and its eastern tip called the "Great Pacific Garbage Patch" (Moore and Phillips 2011), which periodically ejects flotsam towards the Hawaiian Islands (Berg et al. 2024). Evidence for this route is currently visible in the plastic debris and derelict fishing nets that accumulate on windward reefs in the Hawaiian Archipelago, as well as leeward shores subject to the islands' wake effect (De Falco et al. 2022; Xie et al. 2001).

Another means for *Chondrus retortus* to immigrate to east Hawai'i Island could have been on boat hulls (Godwin 2003). Prior to Captain Cook's arrival in the Hawaiian Islands in 1778, Japanese boats caught in typhoons or storms may have drifted to the Hawaiian Islands (Braden 1976). Whaling vessels that docked in Hilo, Hawai'i over 200 years ago traveled to and from Japan primarily in early January to late April, with less frequent visits between mid-October and the end of December (Lebo 2010). Even before whaling vessels, dozens of successful voyages to Hawai'i from Japan were made between 1600 and 1778 based on shogunate shipping records (Braden 1976). Many ships carrying Japanese immigrants sailed from Japan to Hawai'i in the 1800s and early 1900s. The first official group of Japanese immigrants sailed from Yokohama, the capital of Kanagawa Prefecture, to Honolulu in 1868. The type locality of *C. retortus* is Enoshima, Fuijisawa, Kanagawa Prefecture. The trans-Pacific trip in the 1800s would have taken two months from Japan to Hawai'i and, although thalli or spores would probably not have survived in the ballast water, they might have persisted in crannies in the wooden hulls. Similarly, Carlton and Eldredge (2009) proposed that Japanese transport ships in the late 1800s introduced the Japanese littleneck clam, *Venerupis philippinarum*, which was first documented in the Hawaiian Islands in the early 1910s.

Upon arrival on Hawaiian shores, *Chondrus retortus* survival might have been influenced by light, temperature, salinity, and nutrients. Cold seeps created by submarine groundwater discharge occur in some coastal areas on the Hawaiian Islands and may have helped the cold-temperate immigrant to persist in a tropical environment, although in spatially restricted populations. Submarine groundwater discharge on the east coast of Hawai'i Island creates localized plumes of colder, brackish water ranging from 20.5 to 25.26 °C that can be nutrient-rich (Hart 2016; M & E Pacific 1980; Nakoa III 2022; Waiki 2022).

Chondrus retortus on Hawai'i Island may have been the product of one introduction event leading to low genetic diversity – an example of the Founder Effect (Mayr 1942). Low genetic variability within and among populations on east Hawai'i Island may also indicate strong selection on phenotypes. Japanese *Chondrus* species exhibit a wide variety of morphological characters which may be genetically based and/or environmentally induced (Figures S5–S7). Environmental differences between Hawaiian and Asian habitats may explain the slight morphological differences between Hawaiian specimens and *Chondrus retortus* specimens from Japan (Figure S5).

Ancestral range reconstruction, as well as comparison of different models of range evolution and habitat distribution

models, should be examined to better understand the biogeography of populations of *Chondrus retortus*, especially in unusual habitats like Hawai'i, and the genus *Chondrus* as a whole. Although several studies on *C. verrucosus* have explored the subtle, but significant, differences in spore ecophysiology, thallus biomechanics, and phenology which may contribute to the distribution and abundance of isomorphic 1N and 2N life-history stages (Bellgrove and Aoki 2006, 2008, 2020; Bellgrove et al. 2019), information on these traits in *C. retortus* in Hawai'i is lacking. Future studies should also investigate the environmental tolerances, including nutrient loading simulations, of *C. retortus* to predict how this species may, or may not adapt, to future climatic and anthropogenic changes around the Hawaiian Islands.

This study emphasizes the value of using multi-gene loci comparisons and the necessity of comparing results with known type material or specimens from type localities. The 300 bp fragments of *rbc*L and ITS1 of the Hawaiian *Chondrus* sequenced in 2014 compared with sequences on NCBI GenBank database, erroneously suggested that our specimens were *C. crispus* from the Atlantic (ITS) or *C. ocellatus* (*rbc*L), but sequence length and lack of variability within the region did not allow any phylogenetic conclusions with known type specimens. Previous taxonomic and phylogeographical *Chondrus* studies used only one or two molecular markers that were not always the same, which made inter-specific comparisons difficult (Table S8), thus we re-extracted DNA from holotypes, isotypes, and topotypes to compare five gene sequences to the Hawaiian specimens. In our study of Hawaiian *Chondrus*, we combined classical morphological comparisons of specimens, especially type material, with exhaustive use of modern molecular data, all enhanced by the cooperation of phycologists around the Pacific to solve a long-standing taxonomic puzzle. Molecular methods in combination with phylogenetic systematics and paleo- and modern oceanographic data, may help define ancestral populations and dispersal pathways and decipher the biogeographic mystery of many species in the Hawaiian Islands.

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Research ethics: All activities and procedures involved in this research have been in accordance with national laws.
Author contributions: Ronald P. Kittle III conducted the multi-gene sequencing of Hawaiian and Japanese specimens of *Chondrus* spp., completed the species delineation analyses, prepared phylogenetic trees, prepared all figures and tables, and interpreted the results. Anne Veillet performed initial sequencing of Hawaiian *Chondrus*, supervised UH Hilo Core Genetics Lab students, and analyzed results. William E. Schmidt carried out PCR and sequencing of *Chondrus*, and assisted with the interpretation of the data. Suzanne Fredericq prepared specimens for morphological analyses, collected, extracted, and sequenced initial *Chondrus* specimens used for comparison, and interpreted the data. Karla J. McDermid conceived the idea of solving this long-standing taxonomic mystery, collected many specimens, tracked down the location of the oldest known scientific collection of *Chondrus* in Hawai'i, contacted fellow phycologists throughout the Pacific for advice and specimens, and interpreted the results. All authors have had a role in writing the manuscript and have read and approved the entire manuscript.
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Data availability: All sequence data generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) database. Detailed information, including sample metadata and associated sequences, can be found in Supplementary Table 1 accompanying this manuscript.

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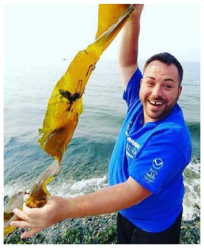
Bionotes



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