Molecular Systematics of the Native Seagrass, *Ruppia* cf. *maritima* (Ruppiaceae, Alismatales), on Hawai'i Island[1]

Brandie A. Colwell ,[2,5] *Ronald P. Kittle III*,[3] *Renee L. Corpuz* ,[4] *and Karla J. McDermid* [2,6]

Abstract: *Ruppia* cf. *maritima* is one of the few native Hawaiian brackish water flowering plants, but its identity has never been examined using genetic analysis. The ability of this seagrass to tolerate a wide range of salinities and temperatures is reflected in its morphological variability among locations worldwide. Three populations on the island of Hawai'i were sampled, and molecular analyses of the nuclear gene *ITS* and two chloroplast genes *trnH-psbA* and *rbcL* were used to examine the identity of Hawaiian *Ruppia*. Concatenated analyses showed that the populations contained little intra- or interpopulation variability, and indicated greatest genetic similarity to specimens from Japan, India, Vietnam, and Africa. Slight variations in tree topologies were present among the individual nuclear and two plastid markers; however, all Hawaiian specimens nested within other sequences reported as *R. maritima*. Molecular phylogenetic analyses demonstrate that there are multiple clades of samples from around the world labeled as *R. maritima*, and that the Hawaiian samples are allied with one of these clades. The geographic isolation and geologic age of each Hawaiian island, as well as the disjunct distribution of *Ruppia* populations among islands and within each island suggest a multiplex biogeography and evolutionary history of Hawaiian *Ruppia*.

Keywords: *Ruppia maritima*, Ruppiaceae, *rbcL*, *trnH-psbA*, *ITS*, Hawaii

The seagrass family Ruppiaceae consists of one globally distributed genus, *Ruppia*, comprising several species, found in diverse, brackish water habitats from subarctic to tropical zones in both hemispheres (Richardson 1980, Koch and Dawes 1991, Lazar and Dawes 1991, Dawes 1998, Den Hartog and Kuo 2006, Ito et al. 2015, Martínez-Garrido et al. 2016). The diagnostic morphological characteristics of *Ruppia*, such as leaf length, leaf tip shape, flower position in the water column, peduncle length, peduncle coiling, and fruit size, can show high phenotypic plasticity among species, among populations within a species, as well as within a population, leading to substantial taxonomic confusion. *Ruppia maritima* L., also known as widgeon grass or beaked tasselweed, can be extremely morphologically variable in response to differing environmental conditions (Graves 1908) making accurate species identification difficult (Ito et al. 2010). Several authors have stated that the uncertainty about *Ruppia* at the species and family level has a long history since it was first described by Linnaeus in 1753 probably from a trip to Westgota,

[1]Manuscript accepted 28 January 2021.
[2]Marine Science Department, University of Hawai'i at Hilo, 200 West Kawili Street, Hilo, HI 96720-4091, USA.
[3]Department of Biology, University of Louisiana at Lafayette, 410 E. St. Mary Boulevard, Billeaud Hall, Lafayette, LA, USA.
[4]Tropical Conservation Biology and Environmental Sciences Program, University of Hawai'i at Hilo, 200 West Kawili Street, Hilo, HI 96720-4091, USA.
[5]Current address: Master of Biotechnology Program, California State University, San Marcos, 333 S. Twin Oaks Valley Road, San Marcos, CA 92096, USA.
[6]Corresponding author (e-mail: mcdermid@hawaii.edu).

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Sweden (Linnaeus 1753, pp. 127–128, Setchell 1946, Jacobs and Brock 1982, Kantrud 1991, Les et al. 1993, Zhao and Wu 2008, Iles et al. 2013, Ito et al. 2017). Eleven species are currently accepted taxonomically (Guiry 2019), two of which are cosmopolitan species (*R. maritima* and *R. spiralis* Linnaeus ex Dumortier = *R. cirrhosa* (Petagna) Grande), two described from New Zealand and Australia (*R. megacarpa* R. Mason, *R. polycarpa* R. Mason), one with an Australasian distribution (*R. tuberosa* Davis & Tomlinson), one reported only from Mexico (*R. mexicana* Hartog & Van Tussenbroek), one only known from South Africa (*R. bicarpa* Ito & Muasya), two limited to East Asia (*R. brevipedunculata* Shuo Yu & Hartog and *R. sinensis* Shuo Yu & Hartog), one from the Mediterranean (*R. drepanensis* Tineo), and one known only from southern South America and the Falkland Islands (*R. filifolia* (Philippi) Skottsberg) (Zhao and Wu 2008, Ito et al. 2010, 2013, Martínez-Garrido et al. 2016).

The simple morphology of the genus and existence of polyploidy have also complicated morphology-based studies that were intended to resolve species delimitation and phylogenetic relationships (Ito et al. 2013). Molecular research to resolve phylogenetic relationships among *Ruppia* species and to answer identification questions has been conducted by Ito et al. (2010), and others (Ito et al. 2013, Triest and Sierens 2014*b*, 2015, Yu et al. 2014, Martínez-Garrido et al. 2016, 2017). Ito et al. (2010) examined the phylogenetic relationships of Mediterranean *Ruppia* species, as well as the role of hybridization and polyploidization in the evolution of the genus at a global scale. In the Mediterranean, *Ruppia* species show high genetic diversity, and Ito et al. (2013) concluded that two *Ruppia* species (*R. drepanensis* and *R. maritima*) and one species complex (*R. cirrhosa* complex) recognized from the Mediterranean by Triest and Sierens (2014*b*, 2015) should all be placed within the *R. maritima* complex. Along the coast of China, Yu et al. (2014) used molecular and morphological data to identify three distinct clades corresponding with three species: *R. cirrhosa*, *R. maritima*, and *R. megacarpa*. Martínez-Garrido et al. (2016) utilized microsatellite markers to distinguish among three distinct *Ruppia* species (*R. drepanensis*, *R. maritima*, *R. cirrhosa*) and one hybrid population (*R. maritima* × *R. cirrhosa*) on the southern Iberian peninsula (Spain). Using morphology and molecular genetics, Martínez-Garrido et al. (2017) reported *R. maritima* in West Africa, which expanded its known distribution. The DNA sequences from these Cape Verde Archipelago populations were more closely aligned with sequences from Europe and eastern North America than the Indo-Pacific. The body of literature is building toward an understanding of the genotypic diversity within and genetic connectivity among *Ruppia* species and populations; however, less attention has been paid to genetic diversity of Pacific *Ruppia*.

The Hawaiian Archipelago is a remote location for *Ruppia*, geographically isolated from any surrounding freshwater or brackish water habitats. Ancestors of this native indigenous aquatic plant traveled great distances to the middle of the Pacific Ocean, perhaps as fruits ingested by water birds or stuck to birds' feet (Carlquist 1982, Ziegler 2002, Ito et al. 2010, Triest and Sierens 2014*a*, Yu et al. 2014) and successfully established populations in the Hawaiian Islands. The population genetics of *Ruppia* in the Hawaiian Islands has not been studied; plants morphologically identified as *R. maritima* may be misidentified, and the genetic variability within and among populations is currently unknown. The oldest collection of *R. maritima* at the Bishop Museum was made by Forbes on Moloka'i in 1912 (BISH sheet number 47371, BISH barcode 1049016, http://nsdb.bishopmuseum.org/). Subsequent collections have been made on all the other main Hawaiian Islands, except Kaho'olawe. A new variety *R. maritima* var. *pacifica* was reported by St. John and Fosberg (1939) from Kailua Beach, O'ahu. On Hawai'i Island, *Ruppia* populations are usually found in anchialine pools (brackish water coastal ponds that rise and fall with tidal cycles, but without a surface connection to the sea) which are specialized habitats at risk because of coastal development. The objectives of this research were (1) to compare *Ruppia* DNA sequences

within and among populations collected in anchialine pools on Hawai'i Island, and (2) to compare DNA sequences of *Ruppia* found on Hawai'i Island with those reported from other parts of the world. The aquatic habitats of *Ruppia* species are threatened by anthropogenic and abiotic disturbance (Short and Neckles 1999, Peyton 2009, Unsworth et al. 2014). In addition, although *Ruppia maritima* and four other species are considered to have stable global populations (Short et al. 2010), their current conservation status can be difficult to assess because the species are difficult to distinguish. Accurate information on the identity and distribution of *Ruppia* species is critical to conservation and management, especially in the Hawaiian Islands.

MATERIALS AND METHODS

Specimen Collections

Three populations were sampled from the island of Hawai'i: two from the west or leeward coast and one from the east or windward coast of the island (Figure 1). Plants were putatively identified as *Ruppia maritima* based on the distinct traits of *R. maritima*: plants submerged; stems slender, terete, branched, arising from creeping rhizome; leaves long, narrow, ribbon-like (up to 10 cm long, up to 1 mm wide), in clusters; leaf bases enclosed in sheaths; fruits small, pyriform on a peduncle (less than 2 cm long), in clusters; and one root at each node on rhizome (Wagner et al. 1999). The collection sites were

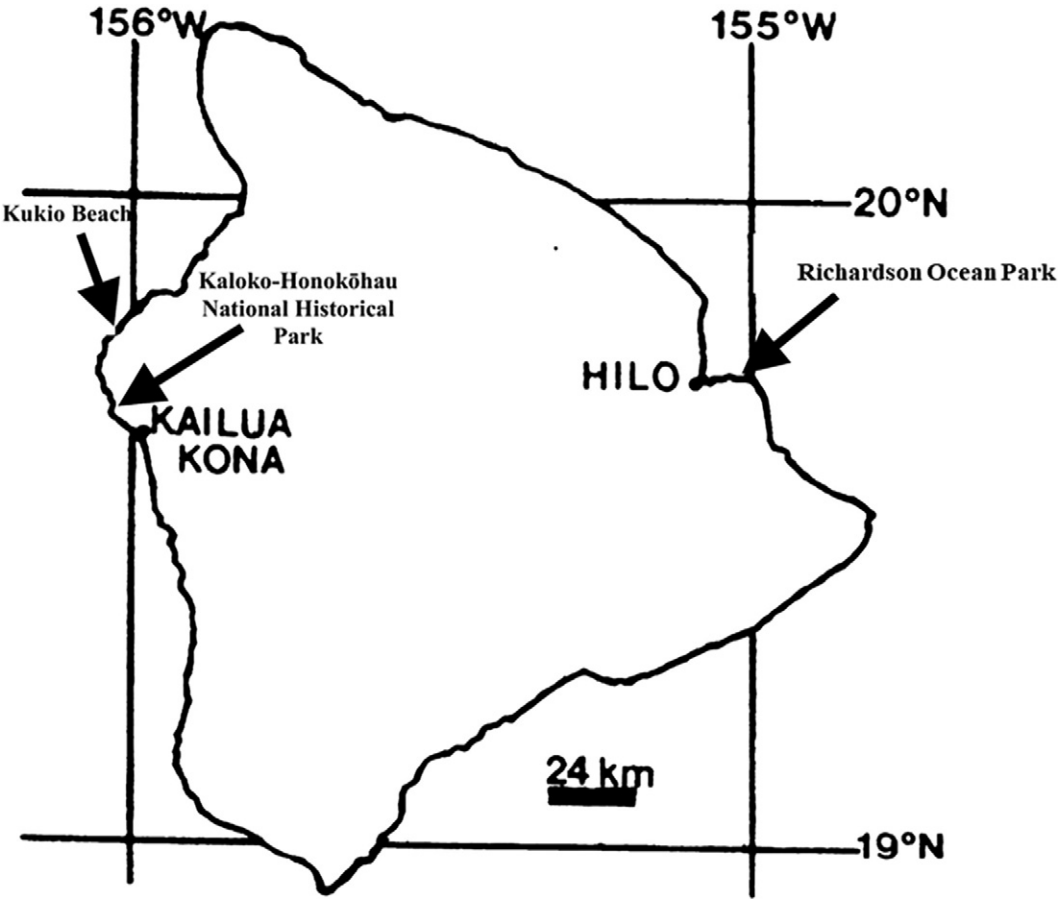

FIGURE 1. Map of *Ruppia* collection sites on Hawai'i Island: two on the leeward side (Kukio Beach and Kaloko-Honokōhau National Historic Park) and one on the windward side (Richardson Ocean Park).

selected based on accessibility and presence of *Ruppia*. At each collection site, five separate samples were collected by hand at depths of 0.5–1.0 m, and placed in 20 mL tubes with the surrounding brackish water; additional plants were collected as voucher specimens. At Richardson Ocean Park (19° 44′ 2.99″ N, −155° 00′ 29.40″ W), all five samples were collected within a 30 m radius within an approximately 2,000 m^2 anchialine pool on 26 September 2017. At Kukio Beach (19° 49′ 23″ N, 156° 00′ 03″ W), the five samples were collected within an 85 m radius from two separate anchialine pools (with areas of approximately 2,500 m^2 and 940 m^2) on 27 October 2017. The Kaloko-Honokōhau National Historic Park (19° 41′ 02″ N, 156° 01′ 45″ W) site sampled on 17 January 2018, included two anchialine pools, approximately 500 m apart, one with an area of 74 m^2 and the other 2 m^2, adjacent to the ʻAiʻōpio Fish Trap. The fresh samples were brought to the Marine Science Department, University of Hawaiʻi at Hilo, and rinsed with freshwater to remove any invertebrates, hand-cleansed of any epiphytes, blotted dry with paper towels, and stored in 20 mL tubes filled with silica gel to desiccate samples prior to DNA extraction.

DNA Extraction

Genomic DNA of 15 *Ruppia* specimens was extracted from 20 mg of desiccated leaves and stems using a Nucleospin Plant II Extraction Kit (Machery-Nagel) following the manufacturer's optional protocol modifications to increase the overall yield of eluted DNA. These adjustments were done to account for the loss of product when transferring from the mortar to 2 mL tubes, and included doubling the amount of PL2 Buffer, RNase PL3 Buffer, and Buffer PC. The quality and concentration of DNA extracts were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Primer Design

Three genomic regions (1 nuclear and 2 plastid) were targeted in this study: (1) the complete Internal Transcribed Spacer (*ITS*) region of the nuclear ribosomal DNA (*ITS1*, 5.8S rRNA, and *ITS4*), (2) the chloroplast *trnH-psbA* intergenic spacer region, and (3) a fragment of the ribulose-bisphosphate carboxylase (*rbcL*) gene, also found in the chloroplast genome. These genes were selected based on their use as successful barcoding regions in plants (Kress and Erickson 2007, Martínez-Garrido et al. 2016), as well as their use in previous phylogenetic reconstructions of *Ruppia* species (Ito et al. 2010, Ito et al. 2013, Triest and Sierens 2014*b*) that allowed direct comparison of the *Ruppia* samples collected from Hawaiʻi Island to those from other regions of the world.

The *ITS* region was amplified using the primers *ITS1* and *ITS4* (White et al. 1990). In this study, the two plastid primers were redesigned to target the noncoding *trnH-psbA* intergenic and the protein-coding *rbcL* regions of the chloroplast genome because of inefficient amplification of the samples using previously described primers for these regions. Primers were redesigned using sequences obtained from the National Center for Biotechnology Information's (NCBI) GenBank nucleotide sequences repository (Supplemental Table 1). These sequences were selected to target the same genetic regions of interest (*trnH-psbA* and *rbcL*) for *R. maritima*. The levels of nucleotide conservation between the sequences were assessed by alignment in MEGA7 v.7.0.26 (Kumar et al. 2016), and primers were chosen in regions showing high levels of nucleotide conservation. Primers for *rbcL* and *trnH-psbA* were redesigned manually using the generally accepted criteria (Gerischer and Dürre 2001): (1) GC content about 50% of the sequence, (2) length of about 20 nucleotides, (3) no homopolymer runs greater than five nucleotides, and (4) a GC clamp at the 3′ end. Table 1 lists primers used in this study, including the two that were manually redesigned.

PCR Conditions and DNA Sequencing

Each gene was amplified in a 30 μL reaction containing 6.0 μL of 5× GoTaq Flexi Buffer

TABLE 1

Forward and Reverse Primer Sequences Used in This Study to Amplify DNA (Based on White et al. 1990, Kress and Erickson 2007)

Primers, Forward (F) and Reverse (R)	Sequence	Annealing Temperature (°C)
ITS 1-F	5′-TCC GTA GGT GAA CCT GCG G-3′	61
ITS 4-R	5′-TCC TCC GCT TAT TGA TAT GC-3′	61
trnH-psbA-F	5′-ACT GCC TTG ATC CAC TTG GC-3′	58
trnH-psbA-R	5′-CGA AGC TCC ATC TAC AAA TGG-3′	58
rbcL-F	5′-ATG TCA CCA CAA ACA GAG ACT-3′	58
rbcL-R	5′-CCG AAT TGT AGT ACG GAA TC-3′	58

Best annealing temperatures used for each primer, are listed. A touchdown PCR for the *ITS* gene utilized an annealing temperature of 61 °C for the first 10 cycles and decreased by 5 °C to 56 °C for the following 30 cycles.

(Promega), 2.4 μL of $MgCl_2$ (25 mM) (Promega), 1.5 μL of 10× bovine serum albumin solution (10 mg/μL) (Promega), 0.6 μL of each primer (10 μM, IDT), 3.0 μL of deoxynucleotide triphosphates (1.25 mM each) (Promega), 0.15 μL *Taq* DNA polymerase (5U/μL) GoTaq Flexi DNA Polymerase, and 2.25 μL template DNA.

A touchdown PCR was performed for *ITS* following Martínez-Garrido et al. (2016) using the following reaction conditions: 95 °C for 6 min, followed by 10 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s (decreasing 0.5 °C/cycle), extension at 72 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and a final elongation at 72 °C for 5 min, and a 4 °C hold.

Additionally, the two chloroplast primers (*trnH-psbA* and *rbcL*) were included in reactions following a slightly modified PCR protocol from Martínez-Garrido et al. (2016), with changes made only to the optimal annealing temperatures for each set of primers. Reactions were incubated for 2 min at 94 °C, followed by 32 cycles of denaturation at 92 °C for 45 s, annealing at 57 °C for *trnH-psbA* and 58 °C for *rbcL* for 1 min, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min and a 4 °C hold. All PCRs were performed using an Applied Biosystems ProFlex thermocycler and products were visualized using a 1.5% agarose gel and Gel Red Nucleic Acid Gel Stain (Biotium).

Following amplification, PCR products were purified using 2% Size-Select E-gel stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen) following the manufacturer's protocol. However, rather than using nuclease-free water as directed in the manufacturer's protocol, all recovery wells were filled with 50 μL of TE buffer (10 mM Tris and 0.1 mM EDTA). This modification was done to allow for better storage and preservation of the PCR product upon collection. A volume of 7.5 μL consisting of approximately 200 ng of purified PCR product and 5.0 pmol of either forward or reverse primer for each sample was added to a 96-well plate and submitted for Sanger sequencing. Samples were sequenced in both directions using an Applied Biosystems 3500 Genetic Analyzer at the University of Hawai'i at Hilo's Evolutionary Genomics Core Facility. Sequences were viewed and edited using Sequencher v. 5.2.4 (Gene Codes Corporation). Chromatograms of 10 of the 15 samples showed evidence of overlapping double peaks at consistent locations on the *ITS* gene. These ambiguous loci suggested areas of Single Nucleotide Polymorphisms (SNPs), which suggest two or more possible alleles for a given locus.

A cloning method was used in order to distinguish the possible allelic diversity that seemed to be present on the *ITS* gene of the Hawaiian *Ruppia* samples. The PCR products of these samples were cloned using a TOPO-TA Cloning kit with One Shot

Chemically Competent TOP 10 *E. coli* cells (Invitrogen). At least five transformed clones were selected for each specimen. Plasmids were isolated from transformed bacterial colonies using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Isolated plasmids (10 μL) were then sent to the Advanced Studies in Genomics, Proteomics and Bioinformatics lab (ASGPB) at the University of Hawai'i at Mānoa. A volume of 6.0 μL consisting of approximately 200 ng of plasmid DNA and 3.2 pmol of M13 Forward (-20) primer 5′-GTAAAACGACGGCCAG-3′ for each sample was prepared for Sanger sequencing. Sequencing of the PCR inserts was carried out in one direction using an Applied Biosystems 3730XL DNA Analyzer at the University of Hawai'i at Mānoa's ASGPB lab.

Phylogenetic Analyses

Sequences for *trnH-psbA*, *rbcL*, and *ITS* were aligned separately using MUSCLE v. 3.8.4 (Edgar 2004), concatenated using Sequence Matrix v. 1.8 (Vaidya et al. 2011), and imported into CIPRES for phylogenetic analysis. The subsequent alignments were analyzed in PartitionFinder2 on XSEDE (Lanfear et al. 2016) in CIPRES to determine the best fitting model of evolution and data partition. The concatenated alignments of *ITS*, *trnH-psbA*, and *rbcL* resulted in the selection of the General Time Reversible model plus gamma (GTR+G) with three partitions (the three codon positions of *ITS*, the three codon positions of *trnH-psbA*, and the three codon positions of *rbcL*) based on AICc and AIC scores. Concatenated alignments *of ITS*, *trnH-psbA*, and *rbcL* in total 1,984 bp in length and consisted of sequence data from 47 specimens (15 specimens sequenced from Hawaii, 29 belonging to the genus *Ruppia* downloaded from GenBank, and three outgroup taxa: *Potamogeton wrightii* and two specimens of *Posidonia oceanica*) (Aires et al. 2011; Supplemental Table 2). No DNA sequences were available from type material or Swedish locales.

Sequence divergence analyses was also calculated for inter- and intraspecies taxa using MEGAX using the Pairwise Distance algorithm using the p-distance model for each gene individually. The model does not make any correction for multiple substitutions at the same site, substitution rate biases, or differences in evolutionary rates among sites (Supplemental Tables 3–5).

Phylogenetic reconstructions were generated using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches for each gene individually and concatenated. An ML analysis on alignments exported in PHYLIP format was performed in RAxML v. 8.2 (Stamatakis 2014), and a BI analysis on alignments exported in NEXUS format was run in MrBayes v. 3.2.7 (Ronquist and Huelsenbeck 2003). Both ML and BI analyses were run using the best-fitting model of evolution and partitioning scheme determined by PartitionFinder2 above. Maximum likelihood analyses were run using 1,000 restarts to find the tree with the lowest likelihood score and 1,000 Bootstrap (BS) replications. MrBayes was run using Markov Chain Monte Carlo (MCMC) searches that consisted of two independent runs of four chains with three heated chains and two cold chain for 20,000,000 generations, sampled every 1,000 generations. Convergence was assessed by comparing the bipartition posterior probability estimates derived from the post-likelihood stabilization phase of each Markov chain for each temperature setting until the values were under 0.01. Likelihood values were plotted against generation number to determine the number of trees that were to be discarded as burn-in (25%) and a majority rule consensus tree was constructed from the remaining trees. Bayesian Inference and Maximum-Likelihood trees were exported using the "phangorn" package v. 2.5.5 (Schliep et al. 2017) in R statistical software v. 3.6.1 (R Core Team 2019, https://www.R-project.org/) and then visualized in FigTree v. 1.4.4 (Rambaut 2012).

Results

DNA with little impurity (Nanodrop values close to 1.8) was successfully extracted from all samples (Table 2). Very little sequence variation for any of the three markers was

TABLE 2

Nanodrop Results from DNA Extractions Prior to Submitting Samples for Sequencing

Voucher Number	Concentration (ng/μL)	A 260/280
BC2018R1	20.89	1.79
BC2018R2	24.63	2.14
BC2018R3	41.22	2.00
BC2018R4	41.90	1.93
BC2018R5	44.93	1.54
BC2018K1	19.93	1.51
BC2018K2	31.35	1.51
BC2018K3	85.74	1.56
BC2018K4	51.07	1.51
BC2018K5	28.70	1.66
BC2018KH1	40.88	1.81
BC2018KH2	42.58	1.79
BC2018KH3	85.98	1.89
BC2018KH4	55.23	1.84
BC2018KH5	28.75	1.79

found among samples from the three populations. Cloning was performed on 10 of the *ITS* gene samples based on two ambiguous nucleotide locations visualized on chromatograms from samples taken from Richardson Ocean Park and Kaloko-Honokōhau National Historic Park. This variation found at these specific loci seemed to be indicative of the location and habitat of the *Ruppia*. This possibility of a greater allelic diversity was found in Richardson and Kaloko-Honokōhau samples that were located in the larger anchialine pools, while the samples that were extracted from the small 2 m^2 pool at Kaloko-Honokōhau exhibited little to no allelic diversity based on the final cloned sequences.

Using the *rbcL*, *ITS*, and *trnH-psbA* genes, individually and concatenated, the samples from Hawai'i Island populations indicated a strong match with *Ruppia maritima* sequences recorded in the National Center for Biotechnology Information (NCBI) database.

The *ITS* genes for all Hawaiian samples showed 100% identity to *Ruppia maritima* voucher specimens YI01491 (Malta) and YI01552 (Spain). Hawaiian samples had ≤1.25% difference compared to other *R. maritim*a voucher specimens: SJ9694 (Australia), YI01575 (Italy), YI01233 (Mississippi, USA), YI000958 (Maryland, USA), YI00743 (Japan), and YI01209 (India), which all clustered in the same clade as the Hawaiian samples. A second clade consisting of *R. cirrhosa* YI01299 and a few *R. maritima* sequences was apparent with a 5.97% sequence difference compared to the first clade, plus *R. drepanensis* BRVULTR91 (Spain) with a 7.0% difference in sequences. A third clade of *R. megacarpa* SJ9681 (Australia) showed a 16% difference. Outgroups composed of the seagrass *Posidonia oceanica* (Linnaeus) Delile and the freshwater aquatic plant, *Potamogeton wrightii* (Morong) showed ≥28% sequence differences (Supplemental Table 3, Figure 2).

Comparison of sequences of the plastid gene, *trnH-psbA*, showed 100% similarity among all Hawaiian sequences. The Hawaiian samples nested closely within a clade of other *R. maritima* specimens, *R. mexicana* and *R. brevipedunculata* (Supplemental Table 4, Figure 3). *Ruppia megacarpa* showed a 6% difference compared to the Hawaiian samples. Outgroups showed ~18% sequence difference compared to the Hawaiian specimens. Analysis using the other plastid gene, *rbcL*, displayed little to no divergence among all sequences within the genus *Ruppia* (≤1.48% difference), and they are nested in a single clade that includes the outgroup *Potamogeton wrightii* isolate TC from China (7% difference) (Supplemental Table 5, Figure 4).

The concatenated tree (Figure 5) separated Hawaiian *Ruppia* into a distinct clade closely related to other *R. maritima* sequences found globally, with tree topology similar to that shown in the *trnH-psbA* tree. Short branch lengths, or lack thereof, in the tree indicated a lack of sequence variation among Hawaiian samples. Little to no divergence was present among different species of *Ruppia* (*R. brevipedunculata*, *R. mexicana*, *R. tuberosa*) compared to other *R. maritima* specimens (Figure 5).

DISCUSSION

The objectives of this research were achieved: *Ruppia* DNA sequences within and among

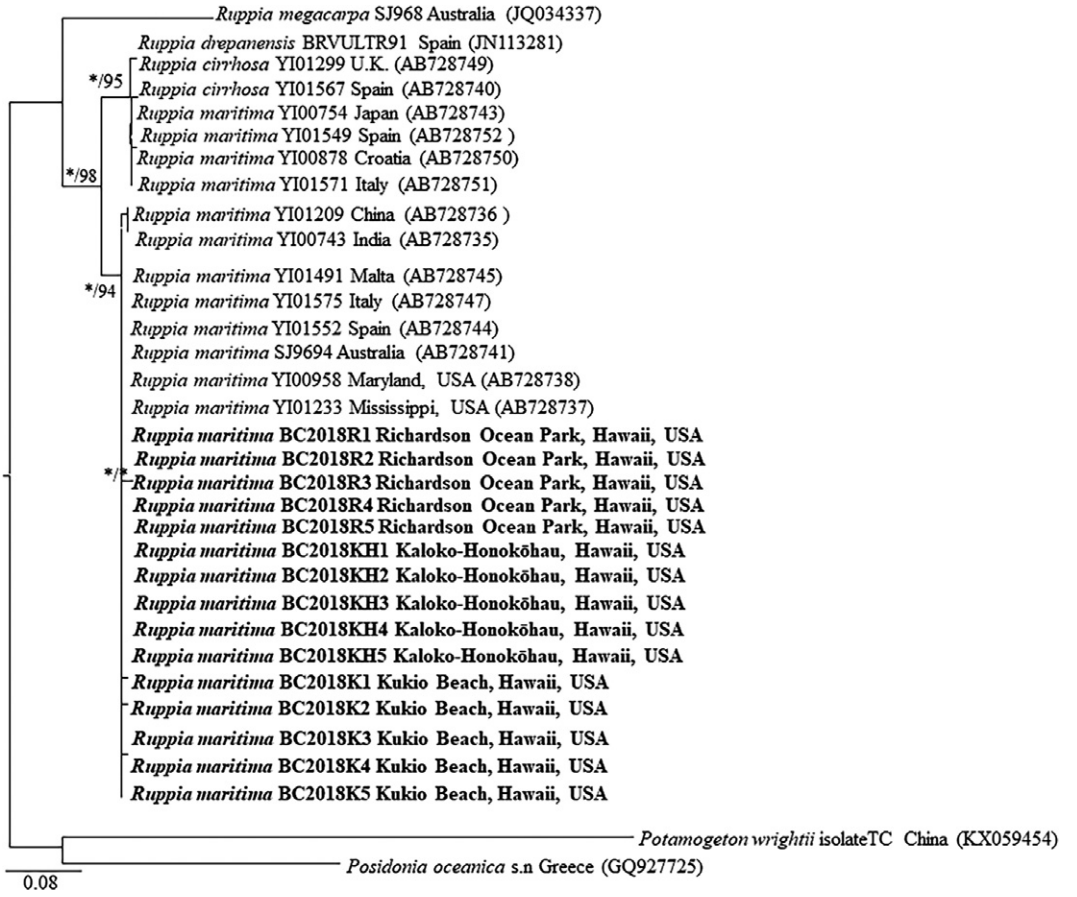

FIGURE 2. *ITS* combined Bayesian Inference and Maximum Likelihood tree with posterior probabilities values on the left and bootstrap values on the right (* denotes full support). Each tip represents one specimen: voucher specimen collection numbers are given after the species names, followed by locality, and accession number in parentheses. Bold denotes newly generated sequences from this study.

populations collected in anchialine pools on Hawai'i Island were compared; and DNA sequences of *Ruppia* found on Hawai'i Island were compared with *Ruppia* reported from other parts of the world. There was a lack of variation in the amplified sequences of all Hawai'i Island *Ruppia* individuals and populations. These results could have a number of possible explanations. Given the history of other taxa in the Hawaiian archipelago, it is possible that *Ruppia* is a recent arrival, likely from southeast Asia, where some of the *Ruppia* specimens with greatest genetic similarity to Hawaiian samples occurred. In this case, *Ruppia* on Hawai'i Island could be the product of one ancestral colonization event leading to limited genetic diversity, an example of the Founder Effect (Mayr 1942). Low genetic variability within and among populations on Hawai'i Island may also indicate strong selection on phenotypes as noted in the seagrass, *Zostera capensis*, along southern African coastlines (Phair et al. 2019). In this case, any new variants arising by mutation would be selected against, thus maintaining the original limited genetic variation present in the founder. Additionally, it is possible that two of the genes selected for sequencing in this study, for example, plastid DNA, do not evolve rapidly enough for change to be

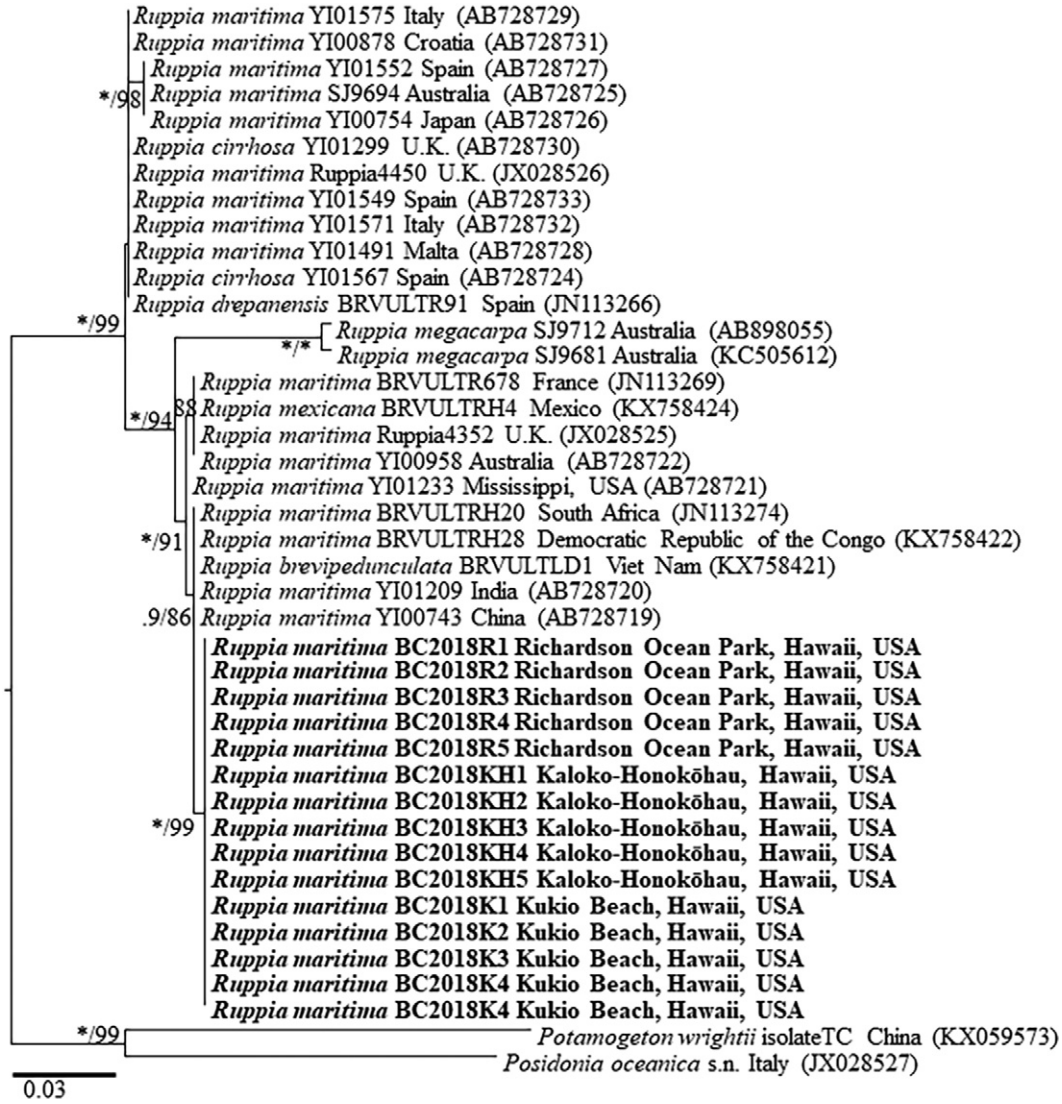

FIGURE 3. *trnH-psbA* combined Bayesian Inference and Maximum Likelihood tree with posterior probabilities values on the left and bootstrap values on the right (* denotes full support). Each tip represents one specimen: voucher specimen collection numbers are given after the species names, followed by locality, and accession number in parentheses. Bold denotes newly generated sequences from this study.

detected using methods employed here and because they do not undergo recombination. Another possible factor in the lack of allelic diversity among Hawai'i Island populations may be due to the small sample size of collecting localities and numbers of individuals per location. Additionally, genetic variability within and among populations of *Ruppia* is known to be affected by reproductive patterns, that is, self-fertilization, out-crossing, clonal growth, and vegetative propagation (Witz and Dawes 1995, Triest and Sierens 2015, Triest et al. 2018*b*). Although flowering and fruiting plants are common in Hawai'i, the reproductive ecology of *Ruppia*, *vis-a-vis* its genetic diversity in the

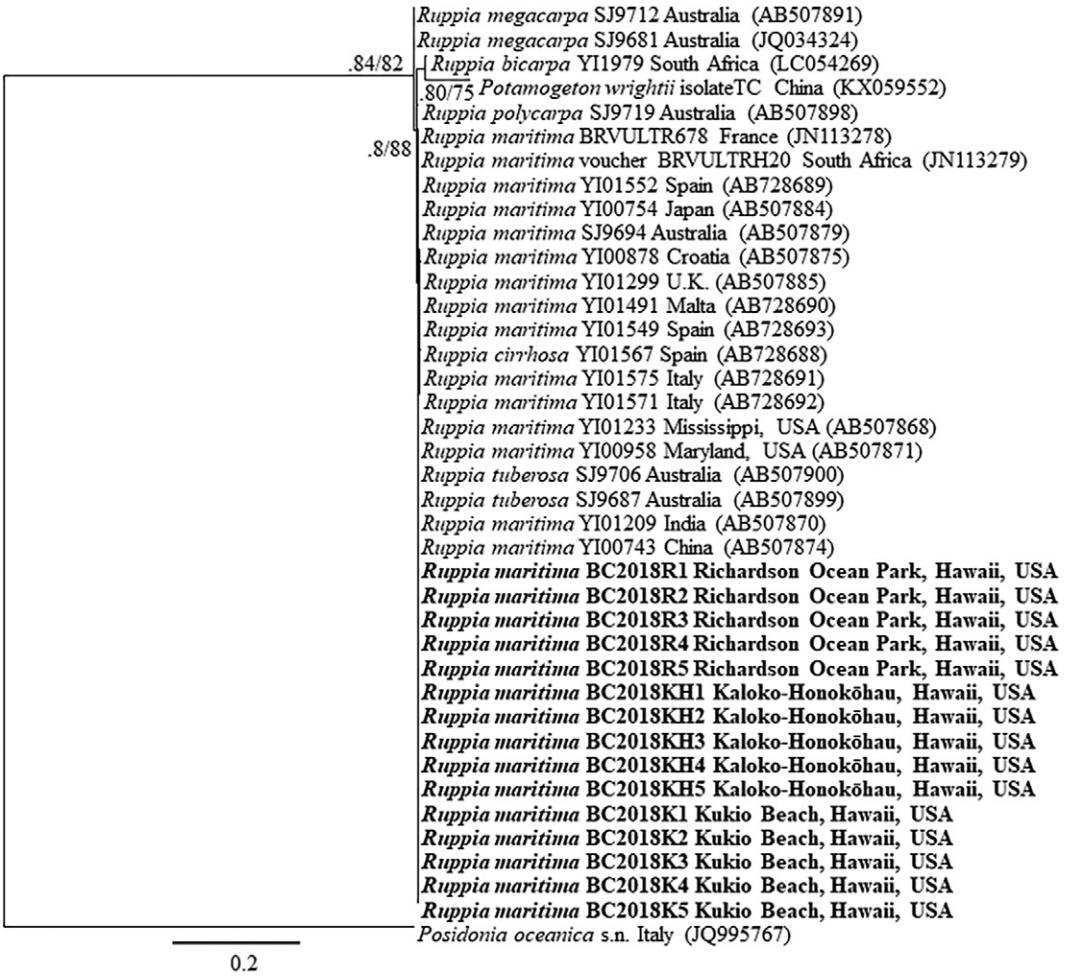

FIGURE 4. *rbcL* combined Bayesian Inference and Maximum Likelihood tree with posterior probability values on the left and bootstrap values on the right (* denotes full support). Each tip represents one specimen: voucher specimen collection numbers are given after the species names, followed by locality, and accession number in parentheses. Bold denotes newly generated sequences from this study.

Hawaiian Islands, has not previously been studied, so the role this might play remains unknown.

In the future, if finer scale genetic analyses reveal more genetic variation in Hawaiian *Ruppia*, these findings could be used to investigate geographic and temporal patterns of colonization and recolonization within the archipelago as has been done for numerous terrestrial plants (Ranker et al. 2000, Percy et al. 2008, Knope et al. 2012, Appelhans et al. 2014, Price and Wagner 2018) and animals (Fleischer and McIntosh 2001, Shaw 2002, Jordan et al. 2003, Magnacca and Danforth 2006, Cowie and Holland 2008, Rubinoff 2008, Garb and Gillespie 2009, Bennett and O'Grady 2012). For example, does *Ruppia* follow the "progression rule" of colonization and diversify from older to younger islands or did the species island hop randomly? Gillespie (2016) emphasized the importance of islands in remote archipelagos with a known geological chronology to the study of biodiversity. The disjunct distribution of

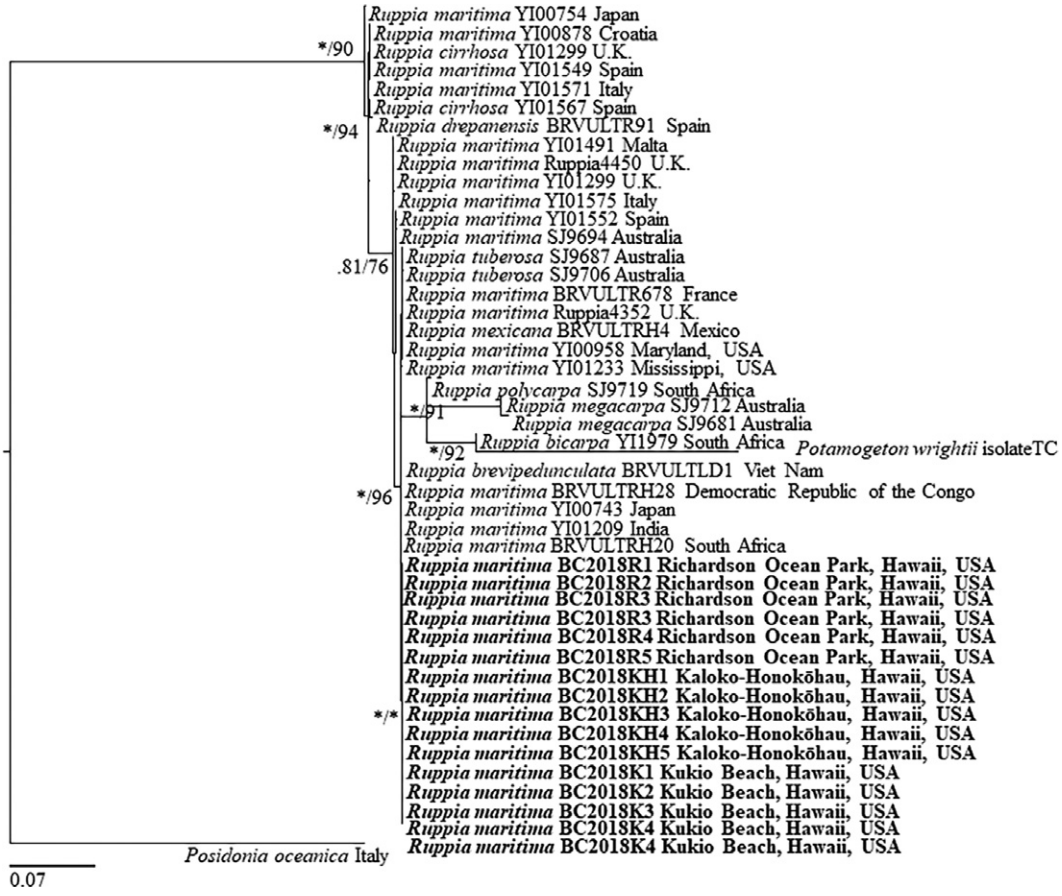

FIGURE 5. Concatenated *ITS*, *trnH-psbA*, and *rbcL* combined Bayesian Inference and Maximum Likelihood tree with posterior probabilities values on the left and bootstrap values on the right (* denotes full support). Each tip represents one specimen: voucher specimen collection numbers are given after the species names, followed by locality. Bold denotes sequences from this study.

Ruppia populations among the islands and within each island suggests that there may have been periods of population separation and reunification when diversification at the gene level could have occurred, or not, although this remains to be determined.

In order to expose more of the possible variability within Hawaiian *Ruppia*, future studies should sample from more islands, determine ploidy levels and chromosome counts of collected plants, and use introns (between gene segments of DNA, which do not code for proteins and are not under selective pressure), instead of only plastid or nuclear genes. DNA barcoding is an accurate way of determining the taxonomic identity of plant species (Kress et al. 2005, de Vere et al. 2012, Lucas et al. 2012, Kuzmina et al. 2017). However, because there is no clear consensus on genes used in molecular studies on seagrasses, and without full genome sequences, comparisons can be difficult because of the different genes chosen by various researchers. Polyploidy, hybridization, and the possibility of morphologically misidentified seagrasses may also confound molecular studies (Ito et al. 2013). In flowering plants and conifers, de Vere et al. (2012) found that the maturase K (*matK*) genes provide greater clarity on interspecific

divergence than *rbcL*, but *matK* is not used as often because of difficulty in obtaining amplification in certain orders of flowering plants. The three genes used in the current study suggested a polyphyletic relationship among *R. maritima* samples. There are no sequences available from the generitype or the type locality of *R. maritima*; however, these should be critically examined in future phylogenetic work within the genus *Ruppia*. Without the ability to compare DNA from type material, the absolute confirmation of Hawaiian *Ruppia* as *R. maritima* is not possible. The molecular phylogenetic analyses demonstrate that there are multiple clades of samples from around the world labeled as *R. maritima*, and that the Hawaiian samples are allied with one of these clades. Triest et al. (2018*a*) stressed the importance of identifying and monitoring unique or rare *Ruppia* lineages in order to understand connectivity and survival strategies of populations, as well as to determine the conservation status of coastal wetland habitats. Concordantly, the conservation of *Ruppia* and its habitats in the Hawaiian Islands depends on accurate information about distribution and genetic diversity of the species.

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