South(east) by Southwest: Identification of a New *Halocaridina rubra* Holthuis, 1963 (Decapoda: Atyidae) Genetic Group From Oʻahu, Hawaiʻi[1]

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Abstract: The anchialine ecosystem, comprised of coastal landlocked habitats containing brackish water, experiences tidal fluctuations due to simultaneous underground connections with the sea and terrestrial aquifer system. Anthropogenic impacts have led to substantial habitat degradation and loss, potentially making the anchialine ecosystem and its biota one of Hawaiʻi's most threatened. Prior analyses of mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) sequences from the Hawaiian anchialine atyid shrimp *Halocaridina rubra* revealed a potential cryptic species complex with strong regional endemism. This study hypothesized that a *Halocaridina* population discovered in 2018 in southeast Oʻahu, an area with no historically documented anchialine habitats but where a specimen was collected in 1998, represents a unique lineage or genetic group endemic to the region. Comparison of newly generated mtDNA COI sequences to previous ones identified the population as belonging to the South Oʻahu lineage of *Halocaridina*. However, fixed mutations and significant genetic differentiation distinguish it from the closely related ʻEwa genetic group of southwest Oʻahu. Given this, we propose the new Maunalua genetic group for *Halocaridina* from southeast Oʻahu, with an apparent split between groups occurring at approximately the Holocene-Pleistocene Epoch boundary, corresponding well to the geological age of where the new population was discovered. Notably, the 1998 specimen shared the same distinctive coloration as the Maunalua and ʻEwa genetic groups, implying inclusion within the South Oʻahu lineage. Conservation efforts should consider the currently recognized 15 *Halocaridina* genetic groups since they represent unique units from all others in both their genetics and geographic distribution.

Keywords: anchialine, crustacean, evolution, Hawaiʻi, *Halocaridina*, invertebrate, phylogeography

The anchialine ecosystem is defined as "bodies of haline waters, usually with restricted exposure to air, always with extensive subterranean connections to the sea, and showing noticeable marine and terrestrial influences" (Stock 1986). While they have been described from primarily tropical locations worldwide (reviewed by Holthuis 1973, Maciolek 1983, Thomas et al. 1992, Bishop et al. 2004, Shishido et al. 2021), the Hawaiian Archipelago possesses the only natural anchialine habitats in the US while having the single largest concentration of them (~600; Brock 1985) globally. In the Hawaiian Islands, anchialine habitats are typically characterized

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as land-locked open pools or ponds occurring in either fossilized limestone materials (e.g., Oʻahu) or basalt (e.g., Maui and Hawaiʻi Island) but can also encompass holes and other depressions of anthropogenic origin (Holthuis 1973, Maciolek 1983, Shishido et al. 2021). In all cases, seawater (i.e., ~35‰ salinity) infiltrating the habitats from the sea via subterranean connections generate the observed tidal fluctuations with dilution from the aquifer groundwater system producing their mixohaline (i.e., brackish water of 0.5‰ to 30‰ salinity) environments. Regrettably, anthropogenic impacts like coastal development, nonpoint source pollution and the spread of exotic species have resulted in ~90% of the islands' anchialine habitats being lost or degraded (Maciolek and Brock 1974, Bailey-Brock and Brock 1993, reviewed by Shishido et al. 2021), potentially making this ecosystem one of the most threatened in the Hawaiian Archipelago.

Diverse assemblages of microorganisms, along with a macrofauna of gastropod mollusks and crustaceans, inhabit the Hawaiian anchialine ecosystem (Maciolek and Brock 1974, Maciolek 1983, Bailey-Brock and Brock 1993, Hoffman et al. 2018). Of these, the endemic and small (i.e., ~10 mm in length) microphagous grazer shrimp *Halocaridina rubra* Holthuis, 1963 (Decapoda: Atyidae), commonly called *ʻōpae ʻula* (*lit.* tiny red shrimp), is considered the signature species given its ubiquitous distribution in anchialine habitats spanning the islands. Such a distribution, along with a surprising long life span (i.e., >10+ years) (Maciolek 1983), wide salinity tolerance (i.e., 0–50‰) (Holthuis 1973, Havird et al. 2014) and the ability to migrate actively through the aquifer groundwater system (Brock et al. 1987), led to the initial hypothesis that *H. rubra* exhibits high levels of gene flow and subsequent low genetic differentiation on Hawaiʻi Island (Santos 2006). Instead, analyses of mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) DNA sequences demonstrated strong genetic subdivision between populations separated by as little as 2 km (Santos 2006, Craft et al. 2008). To date, 14 genetic groups, defined as one or more populations exhibiting significant genetic differentiation from all others, belonging to eight divergent (i.e., ~5% uncorrected *p* genetic distance) lineages, have been identified from Oʻahu, Maui and Hawaiʻi Island (Craft et al. 2008, Santos and Weese 2011). Notably, these *Halocaridina* genetic groups and lineages are typically restricted to anchialine habitats from a particular region of a single island, with no individuals being exchanged between them, and this pattern is attributed to intrinsic organismal properties like having large (i.e., ~1.0 mm) eggs that produce energy-constrained lecithotrophic (i.e., yolk-bearing) larvae with abbreviated development as well as extrinsic barriers in the form of regional hydrology driving their diversification in aquifer "islands" under the terrestrial islands they occupy (Craft et al. 2008).

Compared to Maui and Hawaiʻi Island, many of Oʻahu's anchialine habitats occur as a singleton per site or are anthropogenic in origin (reviewed by Yamamoto et al. 2015, Shishido et al. 2021). Oʻahu also possesses the highest genetic diversity of *Halocaridina* in the islands, with six genetic groups belonging to four lineages (Craft et al. 2008). Interestingly, a sole shrimp was collected in December 1998 from near a freshwater seep in the marine environment at Kawaikuʻi Beach Park on southeast Oʻahu (Bailey-Brock et al. 1999), an area with no historically documented natural or anthropogenic anchialine habitats. While several follow-up collecting efforts were unsuccessful in securing more specimens (Bailey-Brock et al. 1999), this single instance implies *Halocaridina* resides in the aquifers of southeast Oʻahu. Here, we address this 20+ year mystery by describing and genetically characterizing a population of *Halocaridina* discovered from a remediated well shaft located <5 km northeast of Kawaikuʻi Beach Park. Given the strong genetic subdivision typical of the genus (see above), we analyzed newly generated mtDNA COI DNA sequences relative to those previously collected from Oʻahu, Maui and Hawaiʻi Island to test the hypothesis that this newly discovered population represents either a novel lineage or genetic group of *Halocaridina* endemic to southeast Oʻahu.

MATERIALS AND METHODS

Habitat Characteristics and Biological Materials

Keawāwa (KWWL, Figure 1, panel *A*) is a spring-fed estuarine wetland in close proximity to Maunalua Fishpond, now commonly referred to as Hawai‘i Kai Marina, which was once the largest (~2 km^2) *loko kuapā* (i.e., a type of reef-walled fishpond) on O‘ahu. The wetland provides habitat for indigenous *‘auku‘u* (black-crowned night heron, *Nycticorax nycticorax*), endemic *pinao* (giant green darner dragonfly, *Anax strenuus*), various species of native damselflies, and the critically endangered *‘alae‘ula* (Hawaiian moorhen, *Gallinula galeata sandvicensis*). In 2014, KWWL and the adjacent Hāwea Heiau (i. e., sacred structure of native Hawaiian religious ceremonial and spiritual significance) complex were incorporated into a community-owned and managed Hawaiian cultural heritage preserve. Subsequently, an ancient well on the property, approximately 1 m in diameter, 7 m deep and lined with

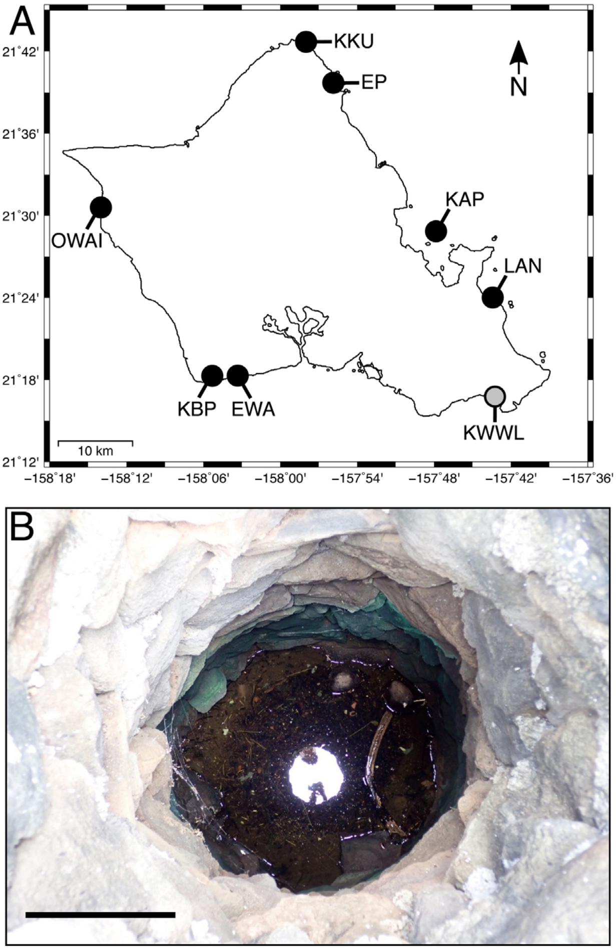

Figure 1. (*A*) Map of the island of O‘ahu, Hawai‘i, depicting sites where the endemic Hawaiian anchialine atyid genus *Halocaridina* was previously sampled by Craft et al. (2008) (black circles) as well as the location of the newly discovered shrimp population at Keawawa Wetland (KWWL; gray circle). (*B*) Vertical view into the remediated well shaft at KWWL. Note pattern of algae growth on its stone/rock lining between 10 and 2 o’clock position relative to water surface at a low tide being indicative of tidal influences on the well. Scale bar in lower left of (*B*) represents 0.5 m.

stones/rocks all the way to the bottom (Figure 1, panel *B*), was remediated by removal of litter from the shaft as well as vegetation from the general area. Initially, some water wetted the bottom of the shaft before eventually rising to a depth of 18–23 cm. While fed by Kanehala Spring, seawater intrusion into, and tidal influences on, the well are apparent by a salinity of ~3.5‰ and the pattern of algae growth relative to the water surface on its stone/rock lining during a low tide (Figure 1, panel *B*), respectively.

In Summer 2018, a GoPro® camera was lowered to the well's bottom to investigate the source of movement in its waters and video footage revealed a few thiarid snails together with a population of *Halocaridina* (Supplementary Data 1). Individual shrimp were observed to actively transverse the water column and graze on and among organic materials like plant leaves and wood covering the benthos (Supplementary Data 1). While shrimp population size was not formally quantified, it is apparently large based on the video footage along with the fact that 30–40 individuals were captured each time a baited inverted-bottle trap was deployed for ~5 min intervals. From the trapping efforts, ~150 individuals were shipped to Auburn University, Auburn, AL, USA within ~2 days of collection and housed in a 38 L aquarium under laboratory conditions described in Vaught et al. (2014).

DNA Extraction, Polymerase Chain Reaction, and Sequencing

Total genomic DNA was extracted in Fall 2018 from individual *Halocaridina* (n = 12) of the KWWL laboratory colony using a DNeasy® Tissue Kit (Qiagen; Valencia, CA, USA) according to the manufacturer's protocol. Approximately 10–30 ng of extracted DNA was employed as template to amplify an ~700 base pair (bp) fragment of the mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) gene via the polymerase chain reaction (PCR). PCR constituents and thermocycling conditions followed those outlined by Santos (2006). Amplified COI products were shipped to the University of Washington Genomics Unit, Seattle, WA, USA for purification and sequencing in both directions on an ABI3100 Genetic Analyzer (Applied Biosystems; Waltham, MA, USA). Sequences were complemented for correction of ambiguities before being trimmed to 630 bp using Sequencher v. 4.7 (Gene Codes, Ann Arbor, MI, USA). Novel mtDNA COI sequences were deposited into GenBank under accession numbers ON153177–ON153179.

Population Genetic and Demographic Analyses

DNA sequences generated from the KWWL shrimp were manually aligned using SE-AL v. 2.0a11 (available at http://tree.bio.ed.ac.uk/software/seal/) to a database of 624 mtDNA COI sequences previously collected from *Halocaridina* on Oʻahu (n = 160), Maui (n = 106) and Hawaiʻi Island (n = 358) by Santos (2006), Craft et al. (2008) and Santos and Weese (2011). Networks were then constructed with the program TCS v. 1.21 (Clement et al. 2000) using default settings to determine the 95% parsimoniously plausible branch connections between the KWWL mtDNA COI sequences (hereafter referred to as haplotypes) to any of those in the database. To generate uncorrected (p) genetic distances between haplotypes from KWWL and any closely related populations, values were calculated using the "net between-group means" option in MEGA v. 11 (Tamura et al. 2021) to account for within-group polymorphism. Tests for genetic differentiation were then conducted with the nearest-neighbor statistic, S_{nn} (Hudson 2000), and significance assessed by 10,000 permutations. The S_{nn} was specifically selected for the genetic differentiation analysis since it is well-suited for small sample sizes (Hudson 2000). Diversity indices were estimated as the number of unique COI haplotypes (nh) as well as nucleotide (π) and haplotype (h) diversities (Nei 1987). Lastly, the historical demography of the KWWL and any closely related populations were inferred using Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) neutrality tests, with significance assessed by 10,000 permutations. In the absence of selection, these tests provide potential information on population demography, revealing possible expansions or bottlenecks. All population genetic and demographic analyses were conducted with DnaSP v. 6.12.03 (Rozas et al. 2017).

Divergence Time Estimation

To estimate divergence time between populations, time in years (t) was estimated via the Markov Chain Monte Carlo (MCMC) method described by Hey and Nielsen (2004) and implemented in the program IM (available from https://bio.cst.temple.edu/~tuf29449/software). The analysis was conducted under the finite-site mutation model (i.e., HKY; Hasegawa et al. 1985), which accounts for the possibility of multiple mutations per site, differences in nucleotide frequencies and the presence of transition/transversion bias. Three independent runs using identical starting conditions (length of Markov chains = 5×10^8 cycles, burn-in time = 2×10^6 cycles) but different random seeds were done to check for consistency between estimates. To estimate t on a demographic scale, a generation time for *Halocaridina* of 2 yrs (Iwai 2005) was employed along with a mutation rate (μ) of 1.26×10^{-4} substitutions per sequence per year, which is based on a mtDNA COI gene fragment length of 630 bp and the 20% My^{-1} diversification rate for the genus presented in Craft et al. (2008) and substantiated by Santos and Weese (2011). The value of t with the highest posterior probability was accepted as the best estimate of time in years since divergence.

RESULTS

Laboratory Colony From KWWL

As in the wild (Supplementary Data 1), individuals from KWWL are highly active in the laboratory, constantly grazing on the volcanic rock substrate in the aquarium or filter-feeding via back swimming at the surface. Reproduction, as apparent by the presence of positively phototaxic larvae, was first observed ~6 month following colony establishment and has been routine over the last >2 yrs. However, longer-term monitoring is required to determine specific reproductive patterns relative to other laboratory-maintained *Halocaridina* colonies (Havird et al. 2015). While individual shrimp from KWWL varied in coloration, ranging from lightly-tinged reddish pink to nearly translucent (Supplementary Data 1), they are highly reminiscent in overall phenotype to those from the ʻEwa genetic group (Vaught et al. 2014) (Figure 1, panel *A* site EWA) (Figure 2, panel *A*) of the South Oʻahu lineage of *Halocaridina* (Craft et al. 2008).

Population Genetic and Demographic Analyses

Parsimony network analysis of the combined *Halocaridina* mtDNA COI haplotype database identified the KWWL population from southeast Oʻahu as belonging to the South Oʻahu lineage, but clustering to the exclusion of those at EWA from southwest Oʻahu (Figure 3). Specifically, the two clusters have an uncorrected p genetic distance of 0.315%, with three positions in the DNA alignment possessing fixed, transition mutations exclusive to KWWL relative to EWA (i.e., 120: fixed A in KWWL but either A/G in EWA; 159: fixed T in KWWL but fixed C in EWA; 483: fixed G in KWWL but either A/G in EWA). Significant genetic differentiation occurs between KWWL and EWA given that the S_{nn} statistic reached the maximum limit of 1.0 ($P < 0.001$) due to a lack of shared mtDNA COI haplotypes between populations. Compared to EWA, KWWL uniformly possessed lower diversity indices as measured by the number of unique haplotypes (nh) as well as nucleotide (π) and haplotype (h) diversities (Table 1). Lastly, while significance was absent from the neutrality tests (Table 1), the recovery of positive (i.e., population bottleneck for KWWL) and negative (i.e., population expansion for EWA) values implies subtly differing demographics between the two.

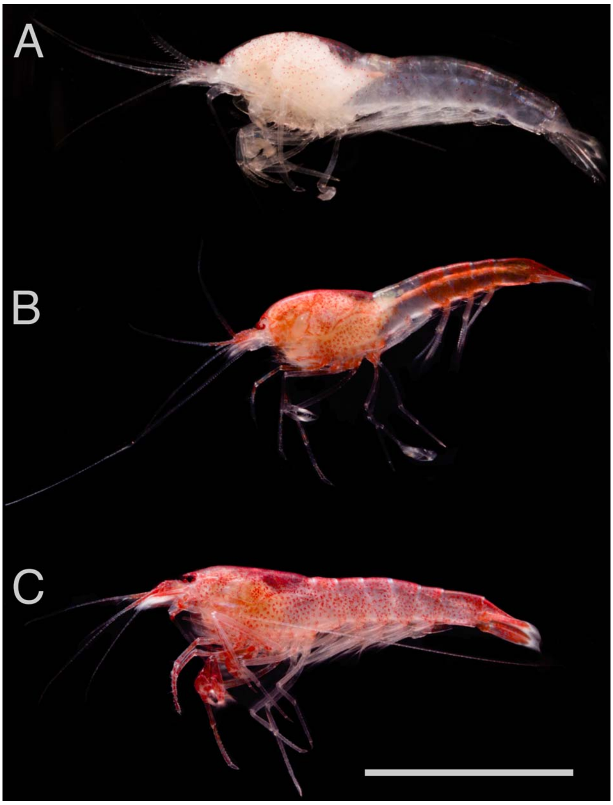

Figure 2. Examples of phenotypic variation in coloration among lineages of *Halocaridina* from the island of O‘ahu, Hawai‘i. Individual shrimp represent the typical coloration observed for members of the (*A*) South, (*B*) West, and (*C*) Windward O‘ahu lineages. Scale bar in lower right represents 5.0 mm. Photographs credit: Ryan J. Weaver (Iowa State University).

Divergence Time Estimation

All three independent runs of the IM analysis produced tight overlaps in their distributions of posterior probability (Figure 4). A clear maximum in the distribution for *t* occurred at ~13,320 years (insert of Figure 4). This implies that KWWL and EWA split from an ancestral population at approximately this timepoint.

DISCUSSION

Once hypothesized to possess high levels of gene flow and subsequent low genetic differentiation because of biological traits conducive to dispersal along with a ubiquitous distribution across multiple islands (Santos 2006), the Hawaiian anchialine atyid shrimp genus *Halocaridina* is now recognized as a potential "cryptic species complex" with strong regional endemism (Craft et al. 2008, Santos and Weese 2011). The present study further supports this latter point by testing the hypothesis that a newly discovered *Halocaridina* population from a remediated well shaft at KWWL and located on the southeast O‘ahu coast (Figure 1, panel *A*) represents either a novel lineage or genetic group of these shrimp. While our genetic analyses identified the KWWL population as belonging to the South O‘ahu lineage of *Halocaridina*, several fixed mutations in their mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) gene differentiate them from the closely related ‘Ewa genetic group (Figure 3) from southwest O‘ahu (Figure 1,

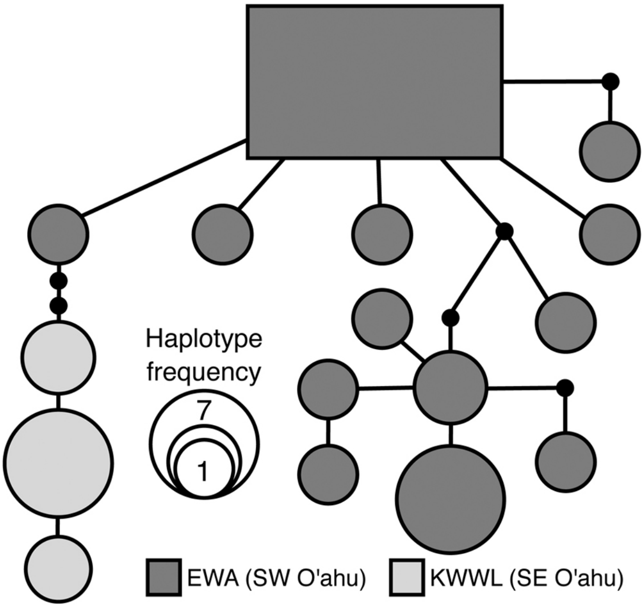

FIGURE 3. Parsimony network depicting relationships among mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) haplotypes from the South O‘ahu lineage of *Halocaridina*. Each haplotype is color-coded according to the southwestern (EWA; dark gray) or southeastern (KWWL; light gray) O‘ahu site it was sampled from. In the network, filled black dots represent unsampled (i.e., missing) haplotypes, and the rectangle represents the haplotype with the highest outgroup probability according to the analysis. The size of circles and rectangle is proportional to the frequency at which each specific haplotype was recovered. Despite variable lengths, each branch implies a single mutational difference between haplotypes.

TABLE 1

Diversity Indices and Neutrality Test for Populations from the South O‘ahu Lineage of *Halocaridina* Examined in this Study

		Diversity Indices			Neutrality Tests	
Population/Site	*n*	*nh*	π	*h* ± SD	Tajima's *D*	Fu's F_S
EWA	38	13	0.004	0.748 ± 0.004	–1.024	–4.49
KWWL	12	3	0.001	0.667 ± 0.008	0.554	0.217

n = number of sampled individuals; *nh* = number of unique haplotypes; π = nucleotide diversity; *h* = haplotype diversity.

panel *A*). Furthermore, no mtDNA COI haplotypes recovered at KWWL were shared with other *Halocaridina* populations on O‘ahu, Maui or Hawai‘i Island, leading to significant genetic differentiation from all others sampled to date. Given this, the KWWL population qualifies as a novel genetic group within the South O‘ahu lineage of *Halocaridina*. Here, we propose naming it the "Maunalua" genetic group following the "regional genetic groups nested within lineage" nomenclature convention of Craft et al. (2008). Additional sampling along both the southeastern and south coasts of O‘ahu are needed to refine the exact distributional range of this 15th recognized genetic group of *Halocaridina* as well as identify the specific barrier(s) (i.e., geohydrologic boundaries like volcanic rift zones, geographic distance, etc.) isolating it from the ‘Ewa genetic group.

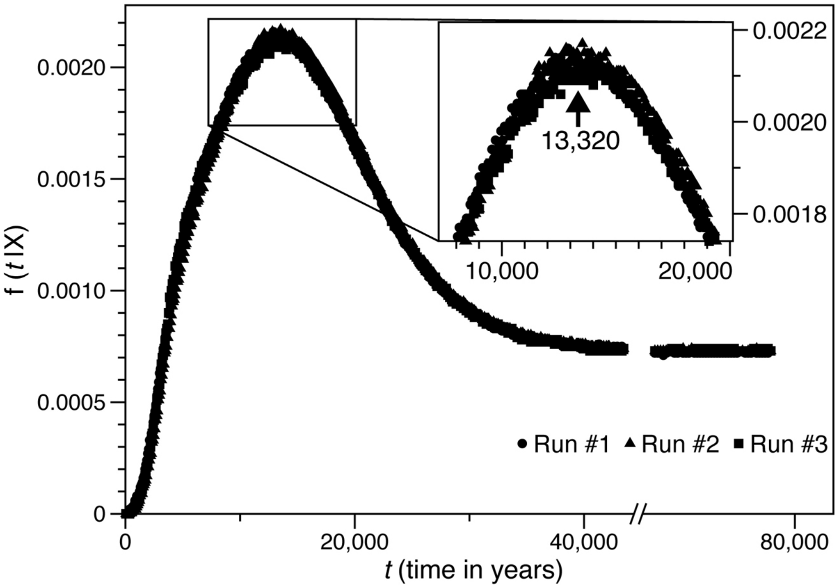

FIGURE 4. Posterior probability distributions of time in years (*t*) since divergence for populations from the South O‘ahu lineage of *Halocaridina*. The presented posterior distributions are from three independent runs of the program IM (Hey and Nielsen 2004) utilizing identical starting conditions but different random seeds. See text for additional details.

As the Hawaiian name for *‘ōpae ‘ula* literally translates to “tiny red shrimp”, the coloration of *Halocaridina* is considered to typically be a bright, vibrant red. However, phenotypic variation in coloration can be high within and between *Halocaridina* lineages, with individuals ranging from red to intermediates to nearly translucent. Despite this, analyses of color cell morphology and their chromatic properties found that the *Halocaridina* lineages statistically differed from one another in varying numbers of chromatosomal properties (Vaught et al. 2014) as well as in having lineage-specific amounts of the red ketocarotenoid astaxanthin (Weaver et al. 2020). Such analyses consistently identified members of the ‘Ewa genetic group in the South O‘ahu lineage as being the least colored in the genus and nearly translucent in nature (Figure 2, panel *A*). For the Maunalua genetic group from KWWL, individual shrimp fit this same phenotypic description (Supplementary Data 1) and alluded to their inclusion in the South O‘ahu lineage even before genetic identification. In fact, Bailey-Brock et al. (1999) remarked that the single specimen collected at Kawaiku‘i Beach Park was cream in color and resembled those from the ‘Ewa Beach area (i.e., in proximity to site EWA) since ones from other O‘ahu populations (and now known to represent distinct lineages) were much redder (Figure 2, panels *B*–*C*). Thus, the individual reported by Bailey-Brock et al. (1999) was likely from the South O‘ahu lineage and the Maunalua genetic group specifically given the relatively close proximity (i.e., <5 km) of KWWL to Kawaiku‘i Beach Park in southeast O‘ahu.

Overall, diversification time in *Halocaridina* according to a genus-specific 20% My^{-1} rate has shown remarkable correlations with the geologic ages of the regions inhabited by those populations under consideration (reviewed by Santos and Weese 2011). In this context, the time since divergence between the Maunalua and ‘Ewa genetic groups was estimated to be ~13,320 yrs before present, just prior to the start of the Holocene Epoch ~11,700 yrs before present (Walker et al. 2009). Notably, the area of southeast O‘ahu where KWWL is located is comprised of alluvium materials dated to the Holocene-

Pleistocene Epoch boundary (Sherrod et al. 2021*a*, 2021*b*), in good agreement with the time since divergence estimate between the genetic groups. Additionally, Maunalua Fishpond and the vicinity around it was likely submerged in the middle to late Holocene Epoch during the last highstand, which was 1.6 (±0.45) m above present sea level (Fletcher and Jones 1996). Taken together, we propose that the South Oʻahu lineage of *Halocaridina* has dispersed across the southern coast of the island since the start of the Holocene Epoch from the older Waiʻanae Range in the west to the younger Koʻolau Range in the east as suitable habitat became available. Such a scenario, where the Maunalua genetic group is more recently derived, would be consistent with its lower genetic diversity indices and positive (but nonsignificant) neutrality test values relative to the ʻEwa genetic group (Table 1).

Our study highlights how biodiversity of the anchialine ecosystem in the Pacific Basin is underestimated, particularly when genetic approaches are applied to either its microorganismal communities (e.g., Hoffman et al. 2020) or macrofaunal populations (reviewed by Weese et al. 2013). This is especially concerning given the rapid degradation and loss of habitats around the Pacific Basin in general and the Hawaiian Archipelago in particular. For *Halocaridina* in Hawaiʻi, overlapping morphological characters within, as well as between, populations from Oʻahu and Hawaiʻi Island (Bailey-Brock and Brock 1993), which represent distinct lineages and likely “cryptic species” (Craft et al. 2008), suggests morphology alone has limited use for describing potential species in the genus. While one goal of ongoing work is to generate additional data that can be applied towards formally recognize each of the eight *Halocaridina* lineages as a distinct species according to multiple species concepts, conservation efforts should actually focus on the currently recognized 15 genetic groups since they represent unique units from all others in both their genetics and geographic distribution. It is hoped that further understanding the phylogeography of endemic organisms like *Halocaridina* will contribute to developing sound, culturally sensitive management strategies for the unique biota and exceptional habitats of the Hawaiian anchialine ecosystem.

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