Molecular Ecology (2006) **15**, 2699–2718 doi: 10.1111/j.1365-294X.2006.02965.x

Patterns of genetic connectivity among anchialine habitats: a case study of the endemic Hawaiian shrimp *Halocaridina rubra* on the island of Hawaii

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Abstract

Anchialine habitats, landlocked bodies of mixohaline water that fluctuate with the tides but have no surface connection to the sea, are known from around the world. Many anchialine organisms have widespread distributions and it has been hypothesized that high levels of gene flow and low levels of genetic differentiation are characteristic of populations from these habitats. However, the generality of this hypothesis requires further assessment, particularly in light of the significant negative impact these habitats and their biota have experienced from anthropogenic causes. This study investigated the population structure and demography of an endemic Hawaiian anchialine species, the atyid shrimp *Halocaridina rubra*, using mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences. A survey of 305 individuals from 16 populations collected on the island of Hawaii revealed 135 haplotypes. These haplotypes belonged to one of two divergent (2.7–4.9%) lineages; notably, no haplotypes were shared between the two coasts of the island. Along each coast, strong subdivision and little to no gene flow occurs between populations separated by > 30 km. The population structure and demography of *H. rubra* on Hawaii are influenced by regional hydrology, geology, volcanism and two distinct colonization events of the island. Thus, *H. rubra* on Hawaii demonstrates that populations of endemic anchialine organisms may exhibit significant levels of genetic structure and restricted levels of gene flow over limited geographic scales. This report brings novel insight into the biology of anchialine organisms and has important implications for the future management of these habitats and their biota.

Keywords: anchialine, COI, crustacean, *Halocaridina*, Hawaii, hydrology, hypogeal, volcanism

Received 7 January 2006; revision accepted 2 March 2006

Introduction

Anchialine habitats are coastal features defined as 'pools with no surface connection to the sea, containing salt or brackish water, which fluctuates with the tides' (Holthuis 1973). Such habitats have been reported from around the world, including the Philippines, the Ryukyus, the South Pacific, the Sinai Peninsula, Bermuda, the Caribbean and Hawaii (Holthuis 1973; Maciolek 1983; Thomas *et al*. 1992; Bishop *et al*. 2004). These habitats are typically landlocked open pools, pools in caves or submerged cave passages (Sket 1996; Iliffe 2000) and the observed tidal rhythms result from seawater moving between them and the sea via subterranean connections. In most cases, the infiltrating seawater is diluted by water from the underlying ground water system, resulting in a mixohaline (i.e. brackish water with salinities of 0.5‰ to 30‰) environment. Members of the Porifera, Cnidaria, Annelida, Mollusca, Arthropoda and Chordata are known to exploit anchialine environments (Maciolek & Brock 1974; Iliffe *et al*. 1984), with many being endemic to these habitats (Holthuis 1963, 1973; Maciolek & Brock 1974; Yager 1981; Kano & Kase 2004).

Interestingly, in spite of occupying such a unique niche, many anchialine organisms have geographically widespread distributions. The recovery of the crustacean class

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Remipedia from anchialine caves on both sides of the Atlantic Ocean (Iliffe *et al*. 1984) and the presence of the anchialine shrimp *Antecaridina lauensis* in the Red Sea, the Mozambique Channel, the Solomon Islands, Japan, Fiji, and Hawaii (Maciolek 1983) are just two specific examples of this phenomenon. Since the 1980s, a number of hypotheses have been developed to explain the anomalous distributions of anchialine organisms, including anthropogenic transport, vicariance events, and the regression of cosmopolitan littoral species to these habitats following a lowering of sea level (reviewed in Maciolek 1983; Iliffe 2000; Kano & Kase 2004). Although these hypotheses account for the distribution of particular anchialine taxa, Smith & Williams (1981) argued that the distribution of *A. lauensis* was best explained by passive oceanic dispersal of larval and/or postlarval stages and it has been proposed that this mode of dispersal extends to many other anchialine organisms due to similarities in life history (i.e. production of planktonic larvae) and broad physiological (i.e. salinity) tolerances (Kano & Kase 2004). Along with oceanic dispersal, the subterranean connections that supply water to these habitats are also potential corridors for individuals to disperse among seemingly disjunct populations and habitats on the same landmass. Thus, it has been hypothesized that high levels of gene flow and low levels of genetic differentiation may be the norm for populations of anchialine organisms (Kano & Kase 2004), except where strong barriers to dispersal exist. Such a pattern has been reported from the sole genetic study of an anchialine species, the gastropod *Neritilia cavernicola*: no genetic differentiation, presumably due to high gene flow, was observed for populations on two islands in the Philippines separated by ~200 km (Kano & Kase 2004). Studies of additional anchialine organisms, however, are needed to assess the generality of this hypothesis. This is particularly relevant in light of the significant, and principally negative, impact anchialine habitats have experienced from anthropogenic causes. For example, in the Hawaiian Islands, which has the single largest concentration of these habitats (~520; Brock *et al*. 1987) in the world, numerous anchialine pools have been modified or destroyed in the last 50 years either in the process of coastal development or by the human-mediated introduction and spread of exotic species (Maciolek & Brock 1974; Brock & Bailey-Brock 1998). Understanding patterns of genetic connectivity among populations inhabiting anchialine habitats is paramount to developing sound management and conservation plans for these ecosystems and their endemic biota.

The shrimp *Halocaridina rubra* (Decapoda, Atyidae), described by Holthuis (1963), is a Hawaiian endemic typically associated with anchialine habitats in the archipelago. This small (~10 mm in length) atyid, called *'öpae 'ula* (*lit*. tiny red shrimp) by the native Hawaiian people, has been reported from anchialine habitats on Hawaii (Maciolek & Brock 1974), Kahoolawe (Brock & Bailey-Brock 1998), Maui (Holthuis 1973; Maciolek 1986), Molokai and Oahu (Maciolek 1983), making the species particularly characteristic of the Hawaiian anchialine fauna. *H. rubra* is predicted to have high colonization and dispersal ability among anchialine habitats due to: (i) unusual longevity (> 10 years; Maciolek 1983; personal observation); (ii) physiological tolerance to an extensive range of salinity (0–50‰, Holthuis 1973); (iii) larvae hatching as free-swimming lecithotrophic zoeae that can remain in the plankton for 24–37 days before reaching the first juvenile stage (Couret & Wong 1978; T. Iwai, personal communication); (iv) its distribution across most of the Hawaiian Islands, coupled with the fact that each island must be colonized *de novo* following its creation, implies adults and/or larvae can enter the marine environment and cross deep (> 2000 m) oceanic channels; and (v) active migration through the ground (i.e. hypogeal) water system of an island, as evident by the rapid colonization of man-made anchialine habitats on Hawaii (Brock *et al*. 1987), Kahoolawe (Brock & Bailey-Brock 1998), and Oahu (Maciolek 1983; M. N. Yamamoto, personal communication).

The present study examined *H. rubra* populations from the island of Hawaii to determine whether high levels of gene flow and low levels of genetic differentiation typify populations of anchialine organisms. Hawaii, which is home to the majority (> 300; Maciolek & Brock 1974; personal observation) of anchialine habitats in the state, is the largest and youngest island in the archipelago, and is no older than ~430 000 years before the present (Carson & Clague 1995). Assuming that island emergence and the presence of a hypogeal water system are prerequisites for colonization, this estimate imposes a maximum age on the *H. rubra* populations inhabiting Hawaii. Sequence data from the cytochrome *c* oxidase subunit I (COI) region of the mitochondrial DNA (mtDNA) were utilized since they allow the detection of both historical and contemporary gene flow (Avise *et al*. 1987) and have proven to be informative for other atyid species (Hurwood & Hughes 2001; Chenoweth & Hughes 2003; Hurwood *et al*. 2003; Baker *et al*. 2004). Using this approach, this report provides novel insight into the processes influencing the genetic connectivity of an endemic anchialine species.

Materials and methods

Biological materials

Specimens of *Halocaridina rubra* were collected from 10 localities in four land districts (i.e. traditional land subdivisions established by the Hawaiian royalty) on the island of Hawaii (Fig. 1 and Table 1) between December 2004 and June 2005. Localities were chosen as to survey all areas of the island with known anchialine habitats (Fig. 1)

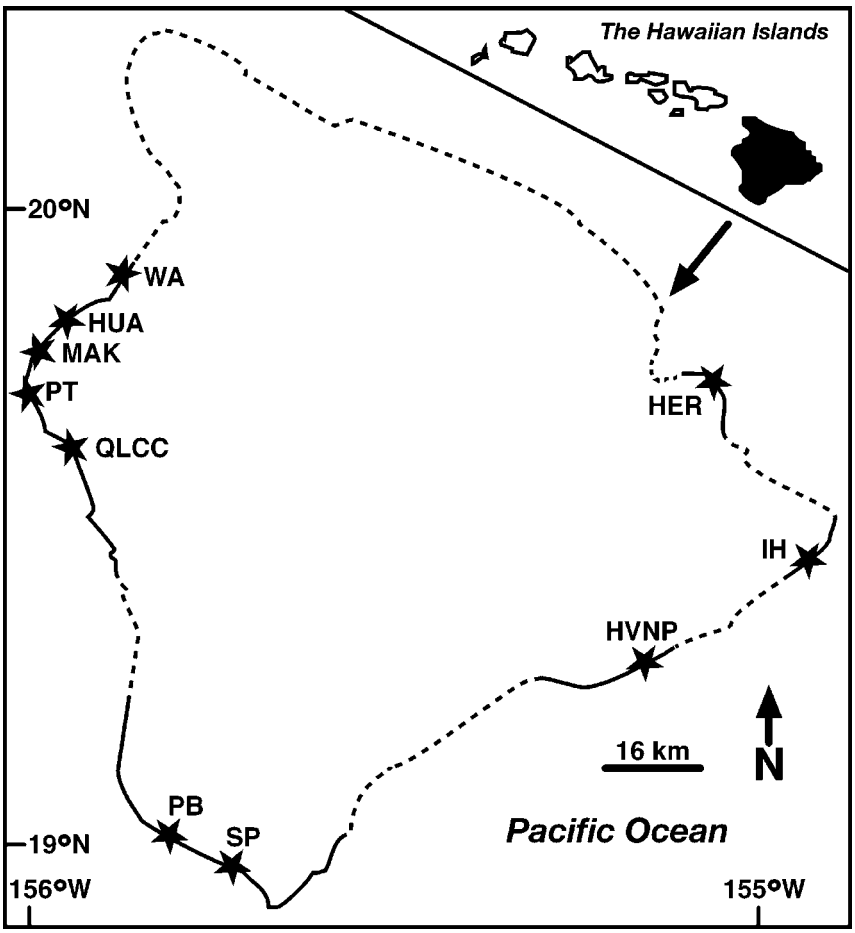

Fig. 1 Map of the island of Hawaii depicting the locations of anchialine habitats where *Halocaridina rubra* were sampled for this study. The seven localities along the western (Kona) coast are Waikaloa (WA: 19.92°N, 155.89°W); Hualālai (HUA: 19.82°N, 156.00°W); Makalawena (MAK: 19.79°N, 156.03°W); Pine Trees (PT: 19.69°N, 156.04°W); Queen Liliokalani Children's Center (QLCC: 19.65°N, 156.02°W); Pōhue Bay (PB: 19.01°N, 155.81°W) and Wai'ahukini (SP: 18.95°N, 155.70°W). The three localities along the eastern (Hilo) coast of the island are Herman's House (HER: 19.72°N, 154.99°W); Isaac Hale (IH: 19.46°N, 154.84°W) and within the boundaries of Hawaii Volcano National Park (HVNP: 19.27°N, 155.26°W). Regions of the coastline where anchialine habitats are rare or absent (Maciolek & Brock 1974; S. R. Santos, personal observation) are represented by a dashed line.

and were *H. rubra* could be collected with reasonable effort. At six (e.g. WA, HUA, QLCC, PB, HER and HVNP) localities, samples were obtained from two ponds located within 100 m of each other to allow for within-locality comparisons. Thus, *H. rubra* from 16 discrete anchialine ponds are included in this study. All sites were classic examples of anchialine habitats on the island: basins comprised of porous basalt, water depth < 2 m, temperatures and salinities of ~25 °C and 2–15‰, respectively (Maciolek & Brock 1974). Between 8 and 32 *H. rubra* were sampled from each pond (Table 1) using a small hand net and preserved in 100% acetone (Fukatsu 1999) for molecular analyses.

DNA extraction, polymerase chain reaction and sequencing

Total genomic DNA was extracted from each *H. rubra* individual according to the methods of Coffroth *et al*. (1992) and utilized as template (~10–30 ng/reaction) to amplify a ~670-bp fragment of the mitochondrial (mtDNA) COI gene. Polymerase chain reactions (PCR) were conducted in 25-μL volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.0 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM each of primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTG↑G-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994), and 1 U *Taq* DNA polymerase. Reactions were carried out in a PTC-100™ thermocycler (MJ Research) under the following profile: initial denaturing step of 94 °C for 5 min, 15 cycles of 94 °C for 45 s, 40 °C for 45 s, 72 °C for 60 s; 25 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s, and a final extension of 72 °C for 5 min. The success of amplifications were confirmed by electrophoresing 3 μL from each reaction in a 1% agarose gel, followed by staining and viewing with ethidium bromide and shortwave (265 nm) UV, respectively.

Amplified products were purified with Montage™ PCR Filter Units (Millipore) according to the supplier's recommendations, cycle-sequenced in both directions using BigDye Terminators and read on a PRISM 3100 Genetic Analyser (Applied Biosystems). Ambiguities in the chromatograms were corrected by comparison to the complement DNA strand in SEQUENCHER version 4.2 (Gene Codes Corporation). Finished sequences were aligned manually using SE-AL version 2.0a11 (available at http://evolve.zoo.ox.ac.uk/).

Table 1 Diversity measures and results of neutrality tests and mismatch distributions for populations of *Halocaridina rubra* on the island of Hawaii

				Diversity measures			Neutrality tests		
Geographic region	Land district	Population†	n↑	*nh*	π	*h*	Tajima's *D*	Fu's F_S	*Hri*
West Hawaii	Kona-	WAa	31	17	0.005 (0.0005)	0.828 (0.069)	−0.969	−9.93*	0.045
(Kona)	Kohala	WAc	16	12	0.005 (0.0007)	0.941 (0.048)	−0.854	−6.42*	0.029
		HUAa	16	11	0.005 (0.0006)	0.941 (0.041)	−0.404	−4.85*	0.06
		HUAb	14	10	0.004 (0.0005)	0.934 (0.051)	0.116	−5.28*	0.048
		MAK	16	10	0.005 (0.0005)	0.891 (0.063)	−0.454	−3.9*	0.097
		PT	22	18	0.005 (0.0005)	0.982 (0.018)	−1.101	−14.5*	0.033
		QLCCa	31	18	0.005 (0.0004)	0.907 (0.037)	−1.165	−10.67*	0.044
		QLCCb	16	12	0.005 (0.0005)	0.941 (0.048)	−0.772	−6.71*	0.05
	Ka'ū	PBa	23	13	0.003 (0.0004)	0.845 (0.071)	−2.034*	−9.05*	0.09
		PBb	17	12	0.005 (0.001)	0.933 (0.045)	−1.687*	−6.73*	0.036
		SP	10	8	0.003 (0.0005)	0.955 (0.059)	−1.127	−4.9*	0.098
	Total	11	212	90	0.005 (0.0002)	0.949 (0.009)	−2.28*	−139.42*	0.03
East Hawaii	South	HERa	16	8	0.005 (0.0009)	0.825 (0.076)	−0.607	−1.44	0.044
(Hilo)	Hilo	HERb	16	8	0.003 (0.0008)	0.758 (0.11)	−1.892*	−3.2*	0.022
	Puna	IH	32	21	0.005 (0.0005)	0.949 (0.025)	−2.095*	−17.27*	0.036
		HVNPa	21	13	0.006 (0.0011)	0.861 (0.073)	−1.502	−4.81*	0.026
		HVNPb	8	6	0.005 (0.0007)	0.928 (0.084)	0.34	−1.69	0.151
	Total	5	93	45	0.01 (0.0004)	0.954 (0.011)	−1.03	−26.16*	0.02

n, number of sampled individuals; *nh*, number of recovered haplotypes; π, nucleotide diversity; *h*, haplotype diversity; *Hri*, Harpending's raggedness index. Standard errors in parentheses.
†Lower-case letters designate discrete anchialine ponds at a site.
*$P < 0.05$.

Haplotype diversity, population structure and migration

Nucleotide (π) and haplotype (*h*) diversity estimates for the *H. rubra* populations were calculated according to the methods of Nei (1987) using the program DNASP version 4.06 (Rozas *et al*. 2003). To test for genetic differentiation between populations, the nearest-neighbour statistic, S*nn* (Hudson 2000), was employed and significance assessed by 1000 permutations in DNASP 4.06. S*nn* is a measure of how often the 'nearest neighbours' (in sequence space) of sequences are from the same locality in geographical space and is particularly suitable when haplotype diversity is large and sample sizes are small (Hudson 2000). Pairwise Φ_{ST} statistics (based on haplotype frequency and molecular divergence), obtained with the program ARLEQUIN version 2.0 (Schneider *et al*. 2000), served as an additional test of genetic differentiation between populations. To quantify the spatial distribution of genetic variation within *H. rubra*, an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) was also conducted in ARLEQUIN 2.0. For the AMOVA, Φ-statistics were used to estimate the relative contribution of molecular variance at three levels: (i) among land districts in a geographic region (Φ_{CT}); (ii) among populations within a land district (Φ_{SC}), and; (iii) within populations (Φ_{ST}). Tamura & Nei's (1993) model of evolution with rate variation among sites [TN + Γ (α = 0.76); as selected by the Akaike information criterion (AIC) in MODELTEST version 3.6 (Posada & Crandall 1998)] was utilized for the pairwise Φ_{ST} statistics and AMOVAs and their significance assessed by 10 000 permutations. Lastly, Mantel tests (Mantel 1967), implemented in the program AIS (Miller 2005) with 1000 permutations, were performed to test for correlations between geographic distance and genetic [calculated as uncorrected (*p*) distances between sequences] distance.

To discriminate between the relative effects of ongoing migration ($M = 2N_{ef}m$) vs. recent divergence between particular pairs of *H. rubra* populations, the Markov chain Monte Carlo (MCMC) method described by Nielsen & Wakeley (2001) was utilized via the Web-based version of the program MDIV (available at http://cbsuapps.tc.cornell.edu/). Analyses were conducted under the finite-site mutation model, which accounts for the possibility of multiple mutations per site, differences in nucleotide frequencies and the presence of transition/transversion bias. A minimum of three independent runs using identical starting conditions ($M_{max} = 50$, $T_{max} = 10$, length of Markov chain = 2×10^6 cycles, burn-in time = 5×10^5 cycles) but different random seeds was done to check for consistency in the estimates. The *M* values with the highest posterior probabilities were accepted as the best estimates of migration rate per generation.

Haplotype network and nested clade analysis

The relationships among *H. rubra* haplotypes were visualized in a network constructed with the program TCS version 1.21 (Clement *et al*. 2000), which utilizes the cladogram estimation algorithm of Templeton *et al*. (1992). The analysis was conducted using the default settings, which provides the 95% parsimoniously plausible branch connections between haplotypes. Loops or reticulations between haplotypes, which represent ambiguous connections in the network, were resolved using the methods of Crandall *et al*. (1994). Haplotypes within the network were then nested according to the procedures outlined in Crandall (1996) prior to conducting nested clade analyses (NCA; Templeton *et al*. 1995). The NCA, which allows separation of population history from population structure (reviewed in Templeton 1998), was done with GEODIS version 2.4 (Posada *et al*. 2000) and 5000 permutations were employed in order to detect significant associations between particular clades within the nesting structure and their geographic locations. Inferences regarding the historical processes giving rise to the current genetic patterns were made using the November 2005 NCA inference key (Templeton 2004; available at http://darwin.uvigo.es/). Since *H. rubra* has the potential to migrate/disperse through either the hypogeal water table of the island or ocean, separate NCAs were conducted which utilized geographic (great circle) or coastal distances between populations, as suggested by Fetzner & Crandall (2003).

Demographic analyses

Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) tests were conducted to determine whether patterns of *H. rubra* COI sequence variation were consistent with predictions of the neutral model. Although these neutrality tests are most commonly used to detect the influence of selection on a gene, they can also be potentially informative about the demographic forces that have affected a population (Tajima 1989; Fu 1997). Significance of the neutrality tests for each of the 16 populations was assessed by 10 000 permutations using ARLEQUIN 2.0. In addition, Harpending's raggedness index (*Hri*, Harpending 1994), based on mismatch distributions (i.e. the frequency distribution of pairwise differences among all haplotypes in a sample), was estimated to test whether the sequence data from each population deviated from what is expected under a sudden expansion model. The significance of *Hri* was assessed by 10 000 permutations using ARLEQUIN 2.0. A significant *Hri* value ($P < 0.05$) is taken as evidence for rejecting the sudden population expansion model (Schneider & Excoffier 1999).

Results

Genetic diversity of Halocaridina rubra *on the island of Hawaii*

A total of 630 bp of COI was obtained from each of the 305 *Halocaridina rubra* included in the study. From these individuals, 135 distinct haplotypes were identified and deposited into GenBank under accession numbers DQ399124–DQ399258. Only 33 haplotypes were sampled more than once while the remaining 102 occurred as singletons (Appendices I and II). In all, 97 (15.4%) sites were variable, with the difference between any two haplotypes ranging from 1 (0.16%) to 31 (4.9%) substitutions. While the majority (i.e. 102) of substitutions were 'silent', 8 of the 110 were nonsynonymous in nature, with the resulting change to an amino acid with similar biochemical properties (data not shown). Nucleotide diversity (π) values were consistent for individual populations and among groups of populations (Table 1). Within a population, the number of haplotypes ranged from 6 to 21 and haplotype diversity (*h*) values were similar for each (Table 1).

Haplotype networks and NCAs for H. rubra *on the island of Hawaii*

Two discrete networks were recovered from the statistical parsimony (TCS) analysis; one encompassed the 212 individuals of *H. rubra* sampled along the western coast of Hawaii (Fig. 2) while the second was comprised of the 93 individuals from the eastern coast of the island (Fig. 3). Genetic distances between haplotypes within each network were comparable (i.e. western Hawaii = 0.16–1.7%; eastern Hawaii = 0.16–2.2%) whereas sequence divergence between haplotypes in the two networks ranged from 2.7% to 4.9% [all estimates calculated as uncorrected (*p*) distances to allow comparisons to published values; see below]. Mutation rates for COI have been reported to range from 1.7% per million years (Myr) in the snapping shrimp genus *Alpheus* (Williams & Knowlton 2001) to 2.3% per Myr for arthropods in general (Brower 1994). Based on these rate estimates, the level of divergence between western and eastern Hawaii *H. rubra* indicates the two groups have been separated for 1.17–2.13 Myr. Due to the large genetic divergence between haplotypes in the two networks and the fact that no haplotypes were shared between the western and eastern coasts of Hawaii (i.e. no apparent gene flow is occurring across the island), subsequent analyses (see below) were conducted separately for the *H. rubra* populations in each geographic region.

The NCA of the western *H. rubra* populations revealed four 1-step clades, two 2-step clades, one

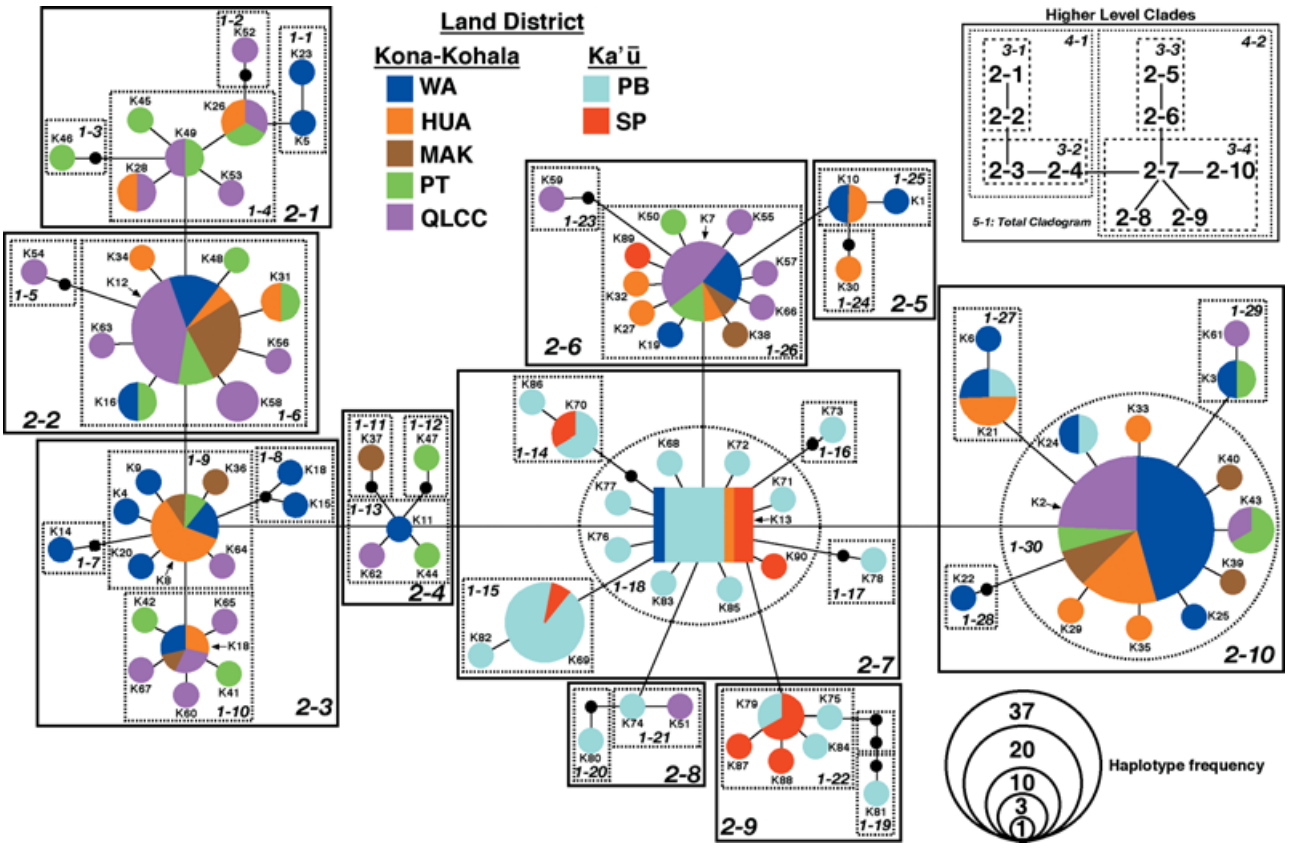

Fig. 2 Haplotype network depicting the nesting levels used to infer historical processes in *Halocaridina rubra* populations along the western (Kona) coast of the island of Hawaii. Sampled haplotypes are indicated by a name (i.e. K1–K90) while black dots represent unsampled (i.e. missing) haplotypes. The rectangle represents the haplotype with the highest outgroup probability according to the TCS (Clement *et al*. 2000) analysis. The size of ovals and the rectangle are proportional to the frequency at which a haplotype was recovered (see Appendix I for exact haplotype frequencies). Colour codes for each population or group of populations are presented in the legend. Note that in spite of variable lengths, each branch implies a single mutational difference between haplotypes.

Geographic region	Clade	Inference chain	Inferred pattern
West Hawaii (Kona)	1-9	1, 2, 11, 12, 13, Yes	PF/RE
	1-26	1, 2, 11, 17, No	Inconclusive outcome
	1-30	1, 2, 11, 12, 13, Yes	PF/RE
	2-1	1, 2, 11, 17, No	Inconclusive outcome
	2-3	1, 2, 11, 12, No	CRE
	3-4	1, 2, 11, 12, No	CRE
	4-2	1, 2, 3, 4, No	RGF/IBD
	Total	1, 2, 3, 4, No	RGF/IBD
East Hawaii (Hilo)	3-3	1, 2, 3, 5, 6, 7, 8, No	Cannot discriminate IBD vs. LDD due to sampling
	Total	1, 2, 3, 4, 9, No	AF

Table 2 Summary of the nested clade analyses (NCA) for populations of *Halocaridina rubra* on the island of Hawaii. Only clades with significance at $P < 0.05$ based on 5000 permutations in GEODIS are shown. Complete results of the NCAs are presented in Appendices III and IV

AF, allopatric fragmentation; CRE, contiguous range expansion; IBD, isolation by distance; LDD, long-distance dispersal; PF, past fragmentation; RE, range expansion; RGF, restricted gene flow.

4-step clade and the total cladogram exhibiting significant geographic structure (Table 2 and Appendix III). Although two of these clades (i.e. 1-26 and 2-1) provided inconclusive outcomes, the others suggest a range of processes have affected the genetic structure of *H. rubra* along the western coast of Hawaii. For lower level clades, past fragmentation/range expansion and contiguous range expansion were inferred as probable causes of the observed patterns

Table 3 Pairwise Φ_{ST} (lower triangle) and S*nn* (upper triangle) values as measures of genetic differentiation for populations of *Halocaridina rubra* along the western (Kona) coast of the island of Hawaii

		Population†										
Land district	Population†	WAa	WAc	HUAa	HUAb	MAK	PT	QLCCa	QLCCb	PBa	PBb	SP
Kona-Kohala	WAa	—	0.5061	0.5068	0.5112	0.5462	0.537	0.568*	0.636*	0.862*	0.91*	0.873*
	WAc	–0.025	—	0.373	0.403	0.434	0.54	0.565	0.447	0.915*	0.9*	0.917*
	HUAa	–0.004	–0.025	—	0.435	0.46	0.533	0.592	0.459	0.901*	0.907*	0.928*
	HUAb	–0.029	–0.04	–0.033	—	0.467	0.559	0.656	0.557	0.795*	0.854*	0.791*
	MAK	0.017	–0.004	–0.039	–0.032	—	0.465	0.507	0.474	0.932*	0.895*	0.879*
	PT	0.051*	0.035	–0.013	0.01	–0.026	—	0.454	0.476	0.929*	0.942*	0.909*
	QLCCa	0.041*	0.025	–0.015	0.005	–0.022	–0.013	—	0.51	0.961*	0.951*	0.939*
	QLCCb	0.069*	0.04	–0.021	0.027	–0.022	–0.025	–0.018	—	0.948*	0.943*	0.897*
Ka'ū	PBa	0.179*	0.192*	0.22*	0.218*	0.264*	0.256*	0.265*	0.271*	—	0.477	0.629
	PBb	0.148*	0.147*	0.173*	0.169*	0.21*	0.211*	0.228*	0.219*	0.005	—	0.451
	SP	0.163*	0.166*	0.178*	0.198*	0.212*	0.195*	0.209*	0.208*	0.008	–0.006	—

†Lower-case letters designate discrete anchialine ponds at a site.
*$P < 0.05$.

Table 4 Pairwise Φ_{ST} (lower triangle) and S*nn* (upper triangle) values as measures of genetic differentiation for populations of *Halocaridina rubra* along the eastern (Hilo) coast of the island of Hawaii

		Population†				
Land district	Population	HERa	HERb	IH	HVNPa	HVNPb
South Hilo	HERa	—	0.453	1***	0.959***	1***
	HERb	0.032	—	1***	0.945***	1***
Puna	IH	0.722*	0.754*	—	0.77*	0.802*
	HVNPa	0.671*	0.712*	0.195*	—	0.594
	HVNPb	0.723*	0.79*	0.166*	–0.033	—

†Lower-case letters designate discrete anchialine ponds at a site.
*$P < 0.05$; ***$P < 0.001$.

(Table 2); at higher clade levels, inferences of restricted gene flow and isolation by distance are supported by the tendency of haplotypes to cluster according to the land district from which they were collected (Fig. 2). In contrast, the NCA of eastern *H. rubra* populations found only a single higher-level clade and the total cladogram to be significant (Table 2 and Appendix IV). For clade 3-3, isolation by distance could not be distinguished from long-distance dispersal due to sampling strategy whereas allopatric fragmentation was inferred for the total cladogram (Table 2). Additional evidence for allopatric fragmentation in eastern Hawaii *H. rubra* comes from the larger than average number of steps connecting haplotypes in clades 3-1 and 3-2 (South Hilo land district) with those in 3-3 (Puna land district) (Fig. 3), which is indicative of such an event (Templeton 2004). Identical inferences were obtained using either geographic or coastal distances in the NCAs (data not shown), suggesting both measures adequately describe the distances between populations (Fetzner & Crandall 2003).

Population structure and migration in H. rubra *on the island of Hawaii*

Significant genetic differentiation was observed between many of the *H. rubra* populations on Hawaii (Tables 3 and 4). The exceptions to this were those populations in relative proximity to each other. For example, no differentiation was detected at the six localities where two ponds within a 100-m distance were sampled (Tables 3 and 4). Over larger areas, estimates of differentiation tended to increase with distance and became significant for populations separated by at least 30–45 km. This was clearly evident in *H. rubra* from the Kona-Kohala land district on the western coast of Hawaii. A Mantel test detected a weak ($r = 0.039$) but significant ($P = 0.016$) correlation between geographic and genetic distance for these populations. Although most population pairwise comparisons along this transect failed to detect differentiation, significant values were observed for *H. rubra* populations at WA and PT (~30 km geographic distance, ~36 km coastal distance) and WA and QLCC

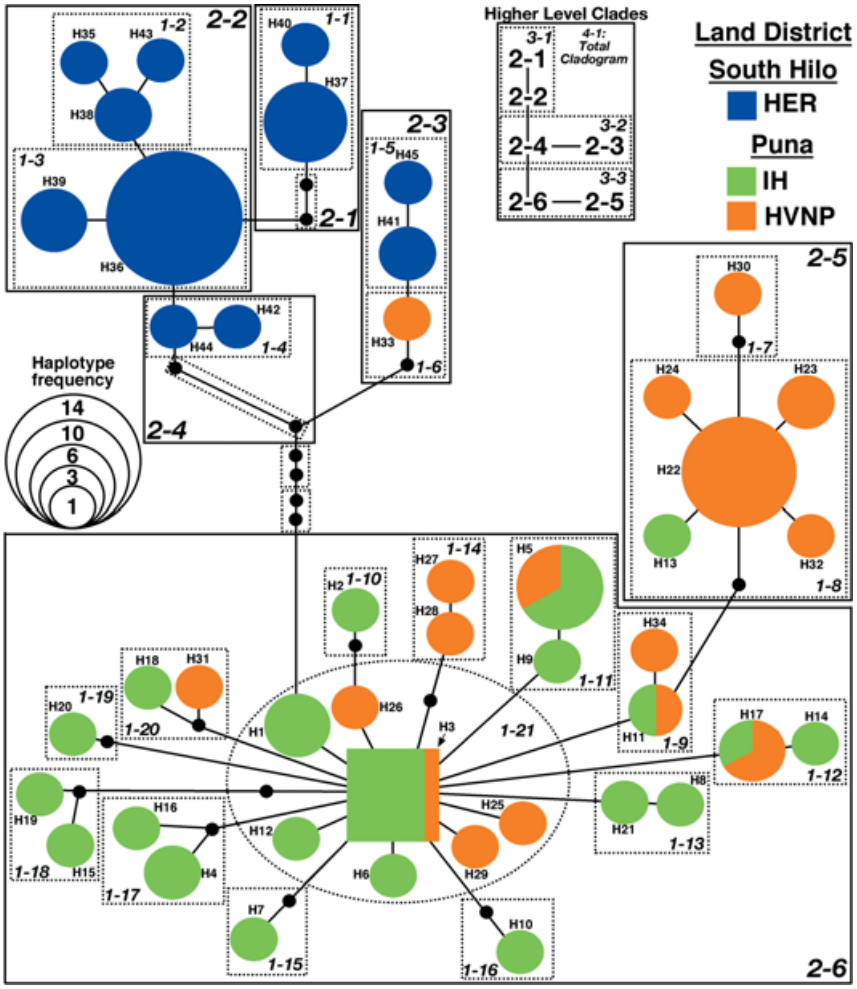

Fig. 3 Haplotype network depicting the nesting levels used to infer historical processes in *Halocaridina rubra* populations along the eastern (Hilo) coast of the island of Hawaii. Sampled haplotypes are indicated by a name (i.e. H1–H45) while black dots represent unsampled (i.e. missing) haplotypes. The rectangle represents the haplotype with the highest outgroup probability according to the TCS (Clement *et al.* 2000) analysis. The size of ovals and the rectangle are proportional to the frequency at which a haplotype was recovered (see Appendix II for exact haplotype frequencies). Colour codes for each population or group of populations are presented in the legend. Note that in spite of variable lengths, each branch implies a single mutational difference between haplotypes.

(~33 km geographic distance, ~43 km coastal distance) with the Φ_{ST} statistics whereas only the WA and QLCC comparison was significant with the S*nn* statistic (Table 3). It should be noted that these cases of significant differentiation were confined to the *H. rubra* population from WAa (Table 3), implying the larger sample size from this pond (Table 1) is responsible for the observed patterns. In all other instances, the significance of pairwise Φ_{ST} and S*nn* values were congruent (Tables 3 and 4).

The analysis of molecular variance of *H. rubra* populations from the western coast of Hawaii found a significant proportion of the observed genetic variation occurring within individual populations (~80%; $\Phi_{ST} = 0.197$; $P < 0.001$), and to a lesser extent, between the Kona-Kohala and Ka'ū land districts (~19%; $\Phi_{CT} = 0.192$; $P < 0.01$) (Table 5). For eastern Hawaii *H. rubra*, the AMOVA found most variation to be between the South Hilo and Puna land districts (~68%; $\Phi_{CT} = 0.679$), while variation within populations and among populations within the land districts explained ~28% and ~4%, respectively, of the total variance. In this case, all variance components were significant ($P < 0.001$; Table 5).

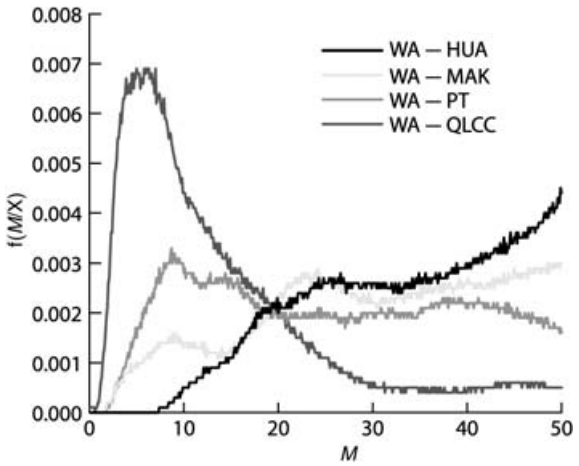

Fig. 4 The posterior distributions of ongoing migration rate (*M*) for *Halocaridina rubra* populations in the Kona-Kohala land district on the western coast of the island of Hawaii. The presented posterior distributions each represent an average of three independent runs from the program MDIV (Nielsen & Wakeley 2001) utilizing identical starting conditions but different random seeds. See text for additional details.

Table 5 Analysis of molecular variance (AMOVA) for the cytochrome *c* oxidase subunit I (COI) sequences of *Halocaridina rubra* on the island of Hawaii

Geographic region	Source of variation	d.f.	Sum of squares	Variance component	% variation	Φ statistic
West Hawaii (Kona)	Among land districts	1	28 289	0.349 V_a	19.19	Φ_{CT} = 0.192**
	Among populations within land districts	9	14 728	0.009 V_b	0.51	Φ_{SC} = 0.006
	Within populations	201	293 406	1.459 V_c	80.30	Φ_{ST} = 0.197***
	Total	211	336 423	1.818		
East Hawaii (Hilo)	Among land districts	1	157 740	3.621 V_a	67.97	Φ_{CT} = 0.679***
	Among populations within land districts	3	15 978	0.222 V_b	4.16	Φ_{SC} = 0.13***
	Within populations	88	130 648	1.485 V_c	27.87	Φ_{ST} = 0.721***
	Total	92	304 366	5.327		

V_a, V_b and V_c are the associated covariance components.
** = $P < 0.01$; *** = $P < 0.001$.

Restricted gene flow and isolation by distance characterizes Hawaii *H. rubra* populations separated by 30–45 km (see above). For this reason, ongoing migration rates (*M*) were estimated for *H. rubra* within the Kona-Kohala (western Hawaii) and Puna (eastern Hawaii) land districts, where populations are maximally separated by ~45 km (Fig. 1) but possess common haplotypes (Figs 3 and 4). For each, the posterior distributions of divergences were almost uniform, suggesting there is little evidence for large (i.e. evolutionary) divergences between populations within these land districts and is consistent with previous analyses (see above). In the Kona-Kohala land district, the data were insufficient to estimate *M* between most population pairs due to a continue rise or plateau in the estimate (Fig. 4). Hence, for these comparisons, migration between populations appears to be high (i.e. greater than 50, the limit at which the analyses were performed). The one exception involved the most distal populations, WA and QLCC, where a maximum in the distribution of the posterior probability occurred at $M = 6$ (Fig. 4). Within the Puna land district, $M = 4$ was estimated between populations at IH and HVNP (data not shown). Taken together, it appears an average of five individuals (females, in this case, since mtDNA is maternally transmitted) per generation are exchanged between these populations. Data from laboratory cultures suggest that *H. rubra* attains full maturity and begins reproducing in a period of *c.* 2 years (T. Iwai, personal communication). Using this value as an approximation of generation time for these shrimp, *M* translates to 2–3 females migrating per year between localities separated by ~45 km.

Demography of H. rubra *on the island of Hawaii*

Tests of neutrality using Tajima's *D* were negative, but not significant ($P > 0.1$), for the majority of western Hawaii *H. rubra* populations; significant negative values of *D* were only recovered for the two populations from PB and when the 11 populations were treated as a single group (Table 1). In contrast, significant negative values of Fu's F_S were obtained from each western population and for the entire group (Table 1). For *H. rubra* on the eastern coast, two populations (i.e. HERb and IH) possessed significant negative values of *D* and F_S whereas one additional population (i.e. HVNPa) and the pooled group were significant for F_S only (Table 1). Significant negative *D* or F_S values were also attained when populations were grouped and analysed according to land district (data not shown). Significant negative values in neutrality tests reflect an excess of rare polymorphisms in the population, and although such values may be indicative of positive selection, they can also be obtained from populations that have experienced a recent expansion (Tajima 1989; Fu 1997). Furthermore, Tajima's *D* statistic has been found to be less sensitive than Fu's F_S in detecting events such as this (Ray *et al*. 2003). Taken together, it appears that nearly all of the *H. rubra* populations have undergone recent population expansions. Additional support for this conclusion comes from Harpending's raggedness index (*Hri*) of the mismatch distributions; an absence of significant *Hri* values (Table 1) implies that the sudden population expansion model cannot be rejected for the populations in this study.

Discussion

Diversity and population structure

Substantial genetic diversity exists within *Halocaridina rubra* on the island of Hawaii. Most mutational differences between haplotypes were synonymous substitutions that had no effect on the sequence or reading frame of the mitochondrial COI protein. This nonrandom pattern suggests that the observed variation is not due to the inadvertent sampling of COI nuclear pseudogenes, a

phenomenon that has complicated some studies (e.g. Williams & Knowlton 2001). Although the genetic diversity within *H. rubra* appears high (i.e. 44% of the individuals represent unique haplotypes), it does fall within a range defined by other Atyidae. For instance, a sampling of *Paratya australiensis* across New South Wales, Australia, yielded 6% unique haplotypes (30 COI haplotypes/518 individuals) (Baker *et al*. 2004) whereas a study of the *Caridina indistincta* species complex from Queensland, Australia, found 57% unique haplotypes (30 COI haplotypes/53 individuals) (Chenoweth & Hughes 2003).

Halocaridina rubra populations on the island of Hawaii are, in general, highly structured. While significant genetic structuring on similar spatial scales has been observed in the freshwater atyids *Caridina zebra* (Hurwood & Hughes 2001) and *P. australiensis* (Baker *et al*. 2004), this finding in *H. rubra* is surprising since the species possesses a number of traits conducive to dispersal between anchialine habitats (see Introduction). Similar dispersal abilities and physiological tolerances in the anchialine gastropod *Neritilia cavernicola* are thought to contribute to panmixia between different island populations (Kano & Kase 2004), so it is not difficult to envision that *H. rubra* populations on a single island would exhibit little to no differentiation. The genetic structure of *H. rubra* on Hawaii, however, clearly demonstrates that populations of anchialine organisms can experience restricted gene flow over limited geographic (intra-island) scales, in spite of high dispersal potential. For this reason, the hypothesis that populations of endemic anchialine species will typically exhibit high levels of gene flow and low levels of genetic differentiation is not supported and highlights the need to evaluate the population structure of organisms from these habitats on an individual species basis.

Genetic differentiation between *H. rubra* populations on Hawaii results from a general pattern of restricted gene flow and isolation by distance. Thus, there are evidently limits to the distances these shrimp can disperse, either through the hypogeal water system of the island or via oceanic routes. Although *H. rubra* has the potential to disperse through the ocean, this appears to occur infrequently, at least over long distances. This conclusion is supported by the confinement of distinct lineages to each coast of the island as well as the occasional recovery of haplotypes outside the region that they would be expected to be found in. An example of this latter point is the sampling of haplotype K51 in the Kona-Kohala land district although it is deeply nested among haplotypes specific to the district of Ka'ū (Fig. 2). Similarly, the presence of haplotype H33 in a population from the Puna land district (Fig. 3) is also best explained by a rare oceanic dispersal event. The sporadic nature of these cases suggests that the ocean represents a significant barrier and dispersal via this route is not a major contributor to gene flow in *H. rubra*. Instead, the primary mechanism of gene flow in these shrimp appears to be dispersal through the hypogeal water system of the island, as evident by no differentiation and high estimates of migration between closely situated populations. However, even in this case, dispersal through the hypogeal water system is limited by distance to a range of ~30 km since significant differentiation and low estimates of migration are characteristics of populations separated by > 30 km.

Regional hydrology and geology can also influence the population structure of anchialine organisms, as demonstrated by *H. rubra* on Hawaii. Using isotopic tracer methods, Scholl *et al*. (1996) found the hypogeal water system on the eastern coast of the island to be hydrologically compartmentalized by rift zones associated with the Kilauea volcano. One of these rift zones, the East Rift Zone (ERZ), is situated between the *H. rubra* populations in the South Hilo (e.g. HER) and the Puna (e.g. IH and HVNP) land districts. The inference of a historic allopatric fragmentation event for these populations suggests *H. rubra* colonized the hypogeal water system of eastern Hawaii prior to its subdivision by the ERZ. Thus, the genetic divergence and strong structure between populations in these land districts is due to their long-term confinement and current isolation within distinct hypogeal water systems.

Demographic history

The NCA for western Hawaii *H. rubra* suggests particular clades have expanded their geographic range while demographic tests found nearly all populations on the island undergoing expansion. Taken together, this implies *H. rubra* is continuing to actively colonize the hypogeal water systems of Hawaii. Volcanic episodes, and the impact these events have on the hypogeal environment, also appear to have influenced the demography of *H. rubra* on the island. For instance, the population from Isaac Hale (i.e. IH) possesses the signal of a strong bottleneck and subsequent expansion, as evident by the largest negative Tajima's D and Fu's F_S values encountered in the study (Table 1). Coincidently, this *H. rubra* population is from an anchialine pond ~5 km south of the primary eruption site for the 1960 Kapoho episode, which is considered the third largest eruption of Kilauea volcano in the 20th century. On the first day of the episode (13 January 1960), several anchialine ponds adjacent to the eruption site were covered by magma and more than 11 h of powerful blasts occurred as the hypogeal water system of the area flashed to steam (http://hvo.wr.usgs.gov/kilauea/history/1960Jan13/). The steam blasts ended within 24 h, suggesting the water reservoirs of the immediate hypogeal region were exhausted during that period. In areas where water was not blasted to steam, the hypogeal system would have experienced a substantial, and inhospitable, increase in temperature. As testimony to this, ocean temperature increased from 22 °C

to 39 °C at a site 300 m offshore from the Kapoho area on 15 January 1960 (http://hvo.wr.usgs.gov/kilauea/history/1960Jan13/). The 1960 Kapoho episode, which spanned 36 days and erupted a volume of magma conservatively estimated at 122 million cubic metres, represents just a single event in Kilauea's history. An additional 61 eruptions of Kilauea have been recorded in the past 200 years (http://hvo.wr.usgs.gov/kilauea/history/) while two other volcanoes on the island, Mauna Loa and Mauna Kea, have also been highly active over the last 10 000 years (Lockwood 1995). Volcanic events like those described above have probably impacted the hypogeal water systems of Hawaii (and by extension, the *H. rubra* that reside within them) on numerous occasions, leading to cycles of population contraction and expansion in these shrimp. It is proposed that such events, on a scale of tens of thousands of years, could be responsible for the observed demography of *H. rubra* on Hawaii. Given the magnitude and frequency of these types of events, it should not be surprising that volcanism can significantly impact the demographic history of an organism (*sensu* Vandergast *et al*. 2004), including those occupying anchialine habitats on geologically active landmasses.

Colonization history

An appreciable level of genetic divergence (2.7–4.9%) separates western and eastern Hawaii populations of *H. rubra*. These values are comparable to other Atyidae, with divergences of ~6% being reported within species of *Caridina* (Hurwood & Hughes 2001; Chenoweth & Hughes 2003) and *Paratya* (Hurwood *et al*. 2003). As noted above, applying a COI mutation rate of 1.7% or 2.3% per Myr (Brower 1994; Williams & Knowlton 2001) implies that the western and eastern Hawaii populations of *H. rubra* diverged from each other approximately 1.2–2.1 million years ago. However, if the age estimate of Hawaii (~430 000 years; Carson & Clague 1995) is accurate, the divergence event pre-dates the formation of the island by at least 0.8–1.7 Myr. One hypothesis that could account for the incongruence between observed genetic divergence and island age is that the COI gene of *H. rubra* is evolving at an accelerated rate. However, nonsignificant relative rate tests between *H. rubra* and other Atyidae offer no support for this idea (data not shown). Thus, a single early colonization event followed by subsequent diversification does not adequately explain the genetic divergence between the populations from the two coasts of Hawaii. Instead, a more parsimonious conclusion is that two lineages of *H. rubra*, each with a distinct evolutionary history, have independently colonized the island.

Dispersal and colonization in the Hawaiian archipelago has generally been from older islands (e.g. Kauai and Oahu in the northwest) to younger islands (e.g. Maui and Hawaii in the southeast). This pattern, known as the 'progression rule' (Funk & Wagner 1995), has been reported from other endemic Hawaiian organisms such as terrestrial arthropods (reviewed in Roderick & Gillespie 1998; Hormiga *et al*. 2003; Jordan *et al*. 2003), birds (Tarr & Fleischer 1993), tree snails (Holland & Hadfield 2004), ferns (Ranker *et al*. 2000; Schneider *et al*. 2005) and plants (Filatov & Burke 2004). It is hypothesized that *H. rubra* and other Hawaiian anchialine species also observe the progression rule, with the colonizers of Hawaii originating from populations on the older islands. Although the evolutionary history of *H. rubra* across the Hawaiian Islands remains to be elucidated, possible origins of the Hawaii founders include Oahu (2.6–3.7 Myr), Molokai (1.76–1.9 Myr), Kahoolawe (> 1.03 Myr), and Maui (0.75–1.32 Myr); alternatively, they may have come from undocumented populations that likely occur throughout the islands. While oceanic dispersal could potentially involve adults, as supported by the sighting of a single *H. rubra* individual in a reef flat (marine) habitat (Bailey-Brock *et al*. 1999), a more plausible scenario is the passive dispersal of larvae or postlarvae, as suggested for a number of other anchialine species (Smith & Williams 1981; Kano & Kase 2004). In either case, the population structure of *H. rubra* on Hawaii implies that long-distance oceanic dispersal is rare in this species (see above), suggesting inter-island dispersal and colonization probably occur on evolutionary, rather than ecological, timescales.

Conservation issues

The anchialine habitats of the Hawaiian Islands have historically experienced a negative impact from anthropogenic causes. The native Hawaiian people were the first to exploit these ecosystems by manipulating the physical structure of larger ponds for aquaculture purposes while numerous other anchialine habitats have been modified or lost more recently due to urbanization (Maciolek & Brock 1974; Brock & Bailey-Brock 1998). Along with this, the introduction and spread of exotic species, such as cichlids and the Tahitian prawn, *Macrobrachium lar*, have increased competition and predation pressures on the endemic biota in those anchialine habitats that remain (Maciolek & Brock 1974; Bailey-Brock & Brock 1993; Brock & Bailey-Brock 1998). The longevity of *H. rubra* and ability to maintain them in hermetically sealed containers (Maciolek 1983; personal observation) has also garnered the interest of aquarists and companies in the pet trade industry. Over the last few years, closed ecosystems containing these shrimp have been marketed under a variety of trademarked names in retail stores and on the World Wide Web. Although *H. rubra* will propagate in captivity (Couret & Wong 1978; Bailey-Brock & Brock 1993; T. Iwai, personal communication; personal observation), it remains unclear if commercially available specimens are solely the product

of breeding programmes (and therefore represent a sustainable resource) or are being supplemented with individuals harvested from natural populations. Thus, habitat destruction and modification, as well as the potential for over-harvesting, could ultimately lead to the extinction of some *H. rubra* populations. This is particularly troublesome given the significant levels of genetic subdivision in the populations of Hawaii reported here and elicits questions on how best to conserve the species and their habitats.

Efforts have been made by the State of Hawaii to protect anchialine habitats and their endemic biota. In 1973, a complex of pools on Cape Kinau, Maui, were designated as the first natural area reserve in the state (Maciolek 1986). Similarly, a 4.9-ha reserve of 66 ponds on Hawaii was recognized as the Waikoloa Anchialine Pond Preservation Area in 1986 (Brock *et al*. 1987). Along with the creation of reserves, one broadly applicable framework for recognizing populations and habitats that warrant conservation is through the identification of evolutionary significant units (ESUs) for a species. According to Ryder (1986), ESUs are comprised of one or a set of populations with a distinct and long-term evolutionary history. Using this criterion, the island of Hawaii is home to (at least) two ESUs of *H. rubra* (i.e. a western and an eastern lineage). Although further work is required to define the taxonomic status of these ESUs, the level of genetic divergence between the lineages implies isolation over significant time spans and the possibility of subsequent speciation. Furthermore, surveys of *H. rubra* populations from the other Hawaiian Islands suggest that the western and eastern Hawaii lineages of these shrimp may be endemic to the island (unpublished data), which places greater emphasis on their conservation and management. It is envisioned that the protection of ESUs and their habitats will contribute to the long-term viability of endemic anchialine species such as *H. rubra* and help to ensure that these unique organisms are not lost to future generations.

Acknowledgements

I would like to thank Dr Robert A. Kinzie III (University of Hawaii at Manoa), Dr Tonya L. Shearer (Georgia Institute of Technology), Mike N. Yamamoto, Tom Iwai (State of Hawaii Division of Aquatic Resources–Oahu) and three anonymous reviewers for valuable suggestions that improved this work. I am indebted to Alfred 'Kekoa' Santos III for first showing me the QLCC site as well as Atlantis Russ, Charles T. Chong (University of Hawaii at Hilo), and John N. Kahiapo (State of Hawaii Division of Aquatic Resources–Hawaii) for collection assistance in the field and additional *H. rubra* samples. I am extremely grateful to the following for allowing me access to anchialine ponds on lands that they administer: Vern Yamanaka (Pōhue Bay, Ka'ū), Mike Ikeda (Queen Liliokalani Children's Center, Kona-Kohala), David Chai (Hualālai, Kona-Kohala) and the US National Park Service (Hawaii Volcano National Park; permit # HAVO-2005-SCI-0030). This paper is dedicated to the memory of Charles T. Chong, a good friend who shared an interest in understanding the biology of Hawaiian anchialine organisms; he will be greatly missed. This is contribution #11 to the Auburn University (AU) Marine Biology Program.

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The laboratory of S. R. Santos focuses on questions of molecular evolution, population genetics and resource conservation in aquatic microbes and multicellular organisms. More information on Hawaiian anchialine ecosystems and their endemic biota is available on the laboratory's website: http://www.auburn.edu/~santosr

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Appendix I

Distribution of *Halocaridina rubra* haplotypes at sampling localities along the western (Kona) coast of the island of Hawaii

Haplotype ID	GenBank Accession	Localities							Individuals/haplotype
		WA	HUA	MAKA	PTA	OKA	PB	SP	
K1	DQ399169	1							1
K2	DQ399170	17	6	3	2	9			37
K3	DQ399171	1			1				2
K4	DQ399172	1							1
K5	DQ399173	1							1
K6	DQ399174	1							1
K7	DQ399175	3	1	1	2	6			13
K8	DQ399176	2	6	1	1				10
K9	DQ399177	1							1
K10	DQ399178	1	1						2
K11	DQ399179	1							1
K12	DQ399180	3	1	5	2	8			19
K13	DQ399181	1	1				7	2	11
K14	DQ399182	1							1
K15	DQ399183	1							1
K16	DQ399184	1			1				2
K17	DQ399185	1							1
K18	DQ399186	2	2	1		2			7
K19	DQ399187	1							1
K20	DQ399188	1							1
K21	DQ399189	1	2				1		4
K22	DQ399190	1							1
K23	DQ399191	1							1
K24	DQ399192	1					1		2
K25	DQ399193	1							1
K26	DQ399194		1		1	1			3
K27	DQ399195		1						1
K28	DQ399196		1			1			2
K29	DQ399197		1						1
K30	DQ399198		1						1
K31	DQ399199		1		1				2
K32	DQ399200		1						1
K33	DQ399201		1						1
K34	DQ399202		1						1
K35	DQ399203		1						1
K36	DQ399204			1					1
K37	DQ399205			1					1
K38	DQ399206			1					1
K39	DQ399207			1					1
K40	DQ399208			1					1
K41	DQ399209				1				1
K42	DQ399210				1				1
K43	DQ399211				2	1			3
K44	DQ399212				1				1
K45	DQ399213				1				1
K46	DQ399214				1				1
K47	DQ399215				1				1
K48	DQ399216				1				1
K49	DQ399217				1	1			2
K50	DQ399218				1				1
K51	DQ399219					1			1
K52	DQ399220					1			1
K53	DQ399221					1			1
K54	DQ399222					1			1

Appendix I *Continued*

Haplotype ID	GenBank Accession	Localities							Individuals/haplotype
		WA	HUA	MAKA	PTA	OKA	PB	SP	
K55	DQ399223					1			1
K56	DQ399224					1			1
K57	DQ399225					1			1
K58	DQ399226					2			2
K59	DQ399227					1			1
K60	DQ399228					1			1
K61	DQ399229					1			1
K62	DQ399230					1			1
K63	DQ399231					1			1
K64	DQ399232					1			1
K65	DQ399233					1			1
K66	DQ399234					1			1
K67	DQ399235					1			1
K68	DQ399236						1		1
K69	DQ399237						12	1	13
K70	DQ399238						2	1	3
K71	DQ399239						1		1
K72	DQ399240						1		1
K73	DQ399241						1		1
K74	DQ399242						1		1
K75	DQ399243						1		1
K76	DQ399244						1		1
K77	DQ399245						1		1
K78	DQ399246						1		1
K79	DQ399247						1	2	3
K80	DQ399248						1		1
K81	DQ399249						1		1
K82	DQ399250						1		1
K83	DQ399251						1		1
K84	DQ399252						1		1
K85	DQ399253						1		1
K86	DQ399254						1		1
K87	DQ399255							1	1
K88	DQ399256							1	1
K89	DQ399257							1	1
K90	DQ399258							1	1
Individuals/location		47	30	16	22	47	40	10	

Appendix II

Distribution of *Halocaridina rubra* haplotypes at sampling localities along the eastern (Hilo) coast of the island of Hawaii

Haplotype ID	GenBank Accession	Localities			Individuals/haplotype
		IH	HVNP	HER	
H1	DQ399124	3			3
H2	DQ399125	1			1
H3	DQ399126	6	1		7
H4	DQ399127	2			2
H5	DQ399128	4	2		6
H6	DQ399129	1			1
H7	DQ399130	1			1
H8	DQ399131	1			1
H9	DQ399132	1			1
H10	DQ399133	1			1
H11	DQ399134	1	1		2
H12	DQ399135	1			1
H13	DQ399136	1			1
H14	DQ399137	1			1
H15	DQ399138	1			1
H16	DQ399139	1			1
H17	DQ399140	1	2		3
H18	DQ399141	1			1
H19	DQ399142	1			1
H20	DQ399143	1			1
H21	DQ399144	1			1
H22	DQ399145		10		10
H23	DQ399146		2		2
H24	DQ399147		1		1
H25	DQ399148		1		1
H26	DQ399149		1		1
H27	DQ399150		1		1
H28	DQ399151		1		1
H29	DQ399152		1		1
H30	DQ399153		1		1
H31	DQ399154		1		1
H32	DQ399155		1		1
H33	DQ399156		1		1
H34	DQ399157		1		1
H35	DQ399158			1	1
H36	DQ399159			14	14
H37	DQ399160			5	5
H38	DQ399161			2	2
H39	DQ399162			3	3
H40	DQ399163			1	1
H41	DQ399164			2	2
H42	DQ399165			1	1
H43	DQ399166			1	1
H44	DQ399167			1	1
H45	DQ399168			1	1
Individuals/location		32	29	32	

Appendix III

Flow chart of the nested clade analysis (NCA) of *Halocaridina rubra* COI haplotypes from the western (Kona) coast of the island of Hawaii based on 5000 permutations in GEODIS

0-step clades			1-step clades			2-step clades			3-step clades			4-step clades		
Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n
K5	0	0												
K23	0	0	**1-1**	0	25**L**									
K53	0	0	**1-2**	0	8.46									
K46	0	0	**1-3**	0	4.94									
K26	7.32	6.77												
K28	9.04	10.27												
K45	0	2.57												
K49	2.13	3.82												
K53	0	6.49	**1-4**	6.07	7.28	**2-1**	8.98	9.48						
I-T	–3.59	–3.31	***I-T***	6.07	–8.6**S**									
			1, 2, 11, 17, No: Inconclusive outcome											
K54	0	0	**1-5**	0	10.31									
K12	8.49	8.69												
K16	13.02	11.99												
K31	7.34	7.42												
K34	0	8.5												
K48	0	6.64												
K56	0	10.54												
K58	0	10.54												
K63	0	10.54	**1-6**	8.87	8.87	**2-2**	8.9	8.9	**3-1**	9.07	9.56			
I-T	4.42	–0.92	***I-T***	8.87	–1.44	***I-T***	–0.07	–0.57						
K14	0	0	**1-7**	0	17.46									
K15	0	0												
K18	0	0	**1-8**	0	17.46									
K4	0	15.58												
K8	5.22S	5.5												
K9	0	15.58												
K20	0	15.58												
K36	0	5.13												
K64	0	18.59	**1-9**	7.7**S**	8.26									
I-T	5.22**S**	–8.6S												
1, 2, 11, 12, 13, Yes: PF/RE														
K18	8.78	10.87												
K41	0	7												
K42	0	7												
K60	0	10.67												
K65	0	10.67												
K67	0	10.67	**1-10**	9.88	11.12	**2-3**	9.81	9.89						
I-T	8.78	1.66	***I-T***	–0.14	–4.12									
			1, 2, 11, 12, No: RE											
K37	0	0	**1-11**	0	5.41									
K47	0	0	**1-12**	0	6.26									
K11	0	23.4												
K44	0	6.67												
K62	0	9.8	**1-13**	11.47	11.48	**2-4**	8.33	10.25	**3-2**	9.96	10.36	**4-1**	9.92**S**	25.9**S**
I-T	0	15.17	***I-T***	11.47	5.65	***I-T***	–1.48	0.36	***I-T***	0.89	0.8			
K30	0	0	**1-24**	0	6.23									
K1	0	7.02												
K10	7.6	8.21	**1-25**	7.87	8.21	**2-5**	7.6	22.9						
I-T	7.6	1.19	***I-T***	7.87	1.98									
K59	0	0	**1-23**	0	5.76									
K7	10.85	14.48												
K19	0	32.02												
K27	0	20.29												
K32	0	20.29												

Appendix III *Continued*

0-step clades			1-step clades			2-step clades			3-step clades			4-step clades		
Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n
K38	0	17.98												
K50	0	9.64												
K55	0	5.92												
K57	0	5.92												
K66	0	5.92												
K89	0	81.5**L**	**1-26**	23.86	23.78	**2-6**	24.63	23.45	**3-3**	22.2S	41.7**S**			
I-T	10.85	–7.69	***I-T***	23.86	18.02	***I-T***	17.03	0.54						
1, 2, 11, 17, No: Inconclusive outcome														
K70	5.93	6.35												
K86	0	7.62	**1-14**	6.53	8.1									
I-T	5.93	–1.27												
K69	5.00	4.9												
K82	0	3.14	**1-15**	4.8	5.6									
I-T	5	1.77												
K73	0	0	**1-16**	0	3.88									
K78	0	0	**1-17**	0	3.88									
K13	23.2	20.41												
K68	0	5.82												
K71	0	5.82												
K72	0	5.82												
K76	0	5.82												
K77	0	5.82												
K83	0	5.82												
K85	0	5.82												
K90	0	13.62	**1-18**	15.82	14.09	**2-7**	10.3S	44.4S						
I-T	23.2	13.62	***I-T***	11.16	8.15									
K81	0	0	**1-19**	0	10.67									
K75	0	11.23												
K79	2.64	3.12												
K84	0	11.23												
K87	0	2.11												
K88	0	2.11	**1-22**	3.55	3.93	**2-9**	4.27S	47.79						
I-T	2.64	–3.55	***I-T***	3.55	–6.74									
K80	0	0	**1-20**	0	22.22									
K51	0	40.2												
K74	0	34.22	**1-21**	36.97	36.00	**2-8**	31.17	38.8						
I-T	0	–5.98	***I-T***	36.97	13.79									
K6	0	24.7												
K21	32.98	30.8	**1-27**	29.83	24.59									
I-T	32.98	6.1												
K22	0	0	**1-28**	0	18.14									
K3	13.02	12.01												
K61	0	9.8	**1-29**	11.47	14.12									
I-T	13.02	2.21												
K2	11.74	11.96												
K24	50.4**L**	55.7**L**												
K25	0	17.63												
K29	0	4.31												
K33	0	4.31												
K35	0	4.31												
K39	0	5.22												
K40	0	5.22												
K43	1.51	12.97	**1-30**	12.42	12.7	**2-10**	13.8S	50 L	**3-4**	47.4	47.8**L**	**4-2**	46.6**L**	45.3**L**
I-T	2.17	–5.42	***I-T***	–7.99	–7.69	***I-T***	–3.2S	–4.9S	***I-T***	25.1**L**	6.1**L**	***I-T***	36.7**L**	19.4**L**
1, 2, 11, 12, 13, Yes: PF/RE						**1, 2, 11, 12, No: RE**			**1, 2, 3, 4, No: RGF/IBD**			**1, 2, 3, 4, No: RGF/IBD**		

Clade (D_c) and nested clade (D_n) distances are given. An '**S**' indicates the distance is significantly small, while an '**L**' indicates the distance is significantly large, at the 5% ($P < 0.05$) level. In clades with both tip and interior nested clades, the row '***I-T***' indicates the average distance between tip and interior clades. Shaded regions indicate interior groupings.

Appendix IV

Flow chart of the nested clade analysis (NCA) of *Halocaridina rubra* COI haplotypes from the eastern (Hilo) coast of the island of Hawaii based on 5000 permutations in GEODIS

0-step clades			1-step clades			2-step clades			3-step clades		
Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n	Name	D_c	D_n
H37	0	0									
H40	0	0	**1-1**	0	0	**2-1**	0	0			
H35	0	0									
H38	0	0									
H43	0	0	**1-2**	0	0						
H36	0	0									
H39	0	0	**1-3**	0	0	**2-2**	0	0	**3-1**	0**S**	26.62
H42	0	0									
H44	0	0	**1-4**	0	0	**2-4**	0	10.38			
H41	0	0									
H45	0	0	**1-5**	0	41.99						
H33	0	0	**1-6**	0	15.44	**2-3**	22.58	20.24	**3.2**	17.01	27.92
			I-T	0	–26.55	***I-T***	–22.58	–9.86			
H30	0	0	**1-7**	0	2.78						
H13	0	45.85									
H22	0	2.97									
H23	0	2.97									
H24	0	2.97									
H32	0	2.97	**1-8**	5.58	5.41	**2-5**	5.25**S**	24.79**L**			
I-T	0	–8.58	***I-T***	5.58	2.63						
H11	24.35	23.96									
H34	0	15.2	**1–9**	20.95	27.78						
I-T	24.35	8.73									
H2	0	0	**1–10**	0	15.44						
H5	22.37	21.67									
H9	0	14.94	**1–11**	20.74	20.93						
I-T	–22.37	–6.73									
H14	0	25.6									
H17	20.95	23.96	**1-12**	24.35	24.85						
I-T	20.95	–1.64									
H8	0	0									
H21	0	0	**1-13**	0	15.44						
H27	0	0									
H28	0	0	**1-14**	0	33.38						
H7	0	0	**1-15**	0	15.44						
H10	0	0	**1-16**	0	15.44						
H4	0	0									
H16	0	0	**1-17**	0	15.44						
H15	0	0									
H19	0	0	**1-18**	0	15.44						
H20	0	0	**1-19**	0	15.44						
H18	0	25.6									
H31	0	23.21	**1-20**	24.35	24.85						
I-T	0	0									
H1	0	13.97									
H3	12.81	17.22									
H6	0	13.97									
H12	0	13.97									
H25	0	34.84									
H26	0	34.84									
H29	0	34.84	**1-21**	19.95	20.58	**2-6**	21.11**S**	24.25S	**3.3**	24.4**S**	26.81
I-T	12.81	–4.58	***I-T***	7.74	–0.65	***I-T***	15.87**L**	–0.54**S**	***I-T***	0.18	1.17
						1, 2, 3, 5, 6, 7, 8, No: Cannot discriminate IBD vs. LDD due to sampling			**1, 2, 3, 4, 9, No: PF**		

Clade (D_c) and nested clade (D_n) distances are given. An '**S**' indicates the distance is significantly small, while an '**L**' indicates the distance is significantly large, at the 5% ($P < 0.05$) level. In clades with both tip and interior nested clades, the row '***I-T***' indicates the average distance between tip and interior clades. Shaded regions indicate interior groupings.