REVEALING HIDDEN DIVERSITY IN A UNIQUE ISLAND ECOSYSTEM: THE EVOLUTION, ECOLOGY AND CONSERVATION OF ANCHIALINE SHRIMP IN THE PACIFIC BASIN

by

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Abstract

Due to varying anthropogenic pressures, there is an urgency to accelerate research into unique island environments and their biota before they are lost forever. For many islands, one of the most threatened ecosystems may be the anchialine ecosystem. Anchialine habitats are land-locked bodies of water which have no surface connection to the ocean yet contain salt or brackish water that fluctuate with the tides due to subterranean connections to the sea. Unfortunately, these unique habitats have received little attention while experiencing significant negative impacts from anthropogenic activities such as urbanization, groundwater extraction/contamination, and the introduction of invasive species. Given these threats, we risk losing these ecosystems without knowing the nature and extent of the biodiversity contained within them. In this regard, understanding the genetic structure and relationships of taxa endemic to these habitats may help illuminate the processes driving the evolution of anchialine organisms. Such knowledge has important implications in developing sound conservation strategies for these organisms and their imperiled ecosystem.

In this context, the research presented here examined the genetic variation, population structure and phylogeographic patterns within and between five species of anchialine Carideans (*Halocaridina rubra, Caridina rubella*, *Antecaridina lauensis*, *Metabetaeus minutus* and *Halocaridinides trigonophthalma*) endemic to the rapidly vanishing anchialine ecosystems of the Pacific Basin. Overall, analysis of intraspecific mitochondrial sequence data (*i.e.,* cytochrome oxidase subunit I [COI] and large subunit ribosomal [16S-rDNA] genes) revealed

two main patterns. First, the contrasting patterns of population structure and connectivity exhibited by each 'species' appear to result from complex interactions between intrinsic (*i.e.*, life history traits) and extrinsic (*i.e.*, historical geologic and oceanographic) processes. Second, the diversity of taxa inhabiting the anchialine habitats of the Pacific may be vastly underestimated with many of the species previously described representing cryptic species complexes.

This research not only provides additional understanding into the ecology and evolution of anchialine organisms in general, but has application in areas of conservation management; such as identifying source populations of invertebrate species in the wildlife trade as well as populations/habitats warranting conservation management and demonstrating that levels of diversity in subterranean crustaceans are in many cases vastly underestimated.

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CHAPTER 1. Introduction to Dissertation

1.1 GENERAL INTRODUCTION

Beginning over a century ago with Alfred Wallace and Charles Darwin, the study of island biogeography has contributed greatly to our understanding of fundamental evolutionary processes. It was during their travels among the world's islands that these young naturalists began developing their theories of evolution by natural selection through their observation of how closely related populations on nearby island varied in appearance. Since these times islands have long provided model systems in which theories of evolution have been developed, tested and redefined. In this context, island systems have been considered excellent "natural laboratories" for studying the fundamental processes of evolution for a variety of reasons (reviewed in Emerson, 2002): (i) they are typically of small size, (ii) are of discrete geographical nature (*i.e.*, isolated), (iii) contain a simplified biota, (iv) despite being geographically small, contain a large diversity of habitats, (v) often have dynamic geologic histories, and (vi) often occur in island groups which can function as experimental replicates. Over the past 150 years these systems have served as templates for studying biogeographic phenomena and it has become obvious that islands harbor a great diversity of organisms. However, understanding the origins of this diversity has not always been so clear (Emerson, 2002).

In this context, the examination of phylogenetic relationships and genetic structure between and within island species provides a framework in which to examine the origin of island taxa (Avise, 2004). The ability to easily acquire DNA sequence data and reconstruct the phylogenetic and demographic histories of almost any taxa has revolutionized the field of biogeography and it has now become commonplace to use phylogenetic reconstructions of lineages to interpret the evolutionary histories of islands and their biotas (Brown & Lomolino,

2000; Emerson, 2002). In this sense, the examination of phylogenetic relationships and genetic structure between and within island taxa provides a framework to examine the origin of island taxa, determine the processes that have led to species richness within island ecosystems and enable testing of general biogeographic and ecological hypotheses (Avise, 2004).

Much like isolated oceanic islands, subterranean ecosystems have been considered natural ecological and evolutionary laboratories (Poulson and White, 1969; Juan *et al.,* 2010). Similar to island systems, subterranean habitats are characterized as having discrete geographical boundaries, dynamic geological histories, simplified communities and temporally and spatially isolated biota. Given these similarities, the study of subterranean organisms can help elucidate the evolutionary processes and historical factors related to biogeography and speciation similar to what has been demonstrated for isolated oceanic islands (Cooper et al., 2007; Page et al., 2008; Juan 2010). However, diversification and speciation processes in these underground worlds are largely influenced by habitat fragmentation and are often times masked by morphological convergence, confounding interpretations leaving many aspects regarding the evolution of subterranean taxa under considerable debate (Lefebure et al., 2006; Cooper et al., 2007). To gain a better understanding of the evolution of subterranean organisms, additional systems with novel attributes are needed (Cooper et al., 2007).

Anchialine environments act as an interface between the subterranean freshwater aquifer and the open ocean and are unique from ecological and evolutionary perspectives due to the fact that they represent a habitat where salinity, water levels, and temperature fluctuate daily (Craft et al., 2008). These habitats are land-locked bodies of mixohaline water occupying basins of varying geologic origins (*i.e.*, karst caves, cenotes, fossilized coral reefs or coastal lava fields) with no surface connection to the open ocean yet contain salt or brackish water that fluctuate

with the tides due to simultaneous subterranean connections with the ocean and freshwater aquifer (Holthuis, 1973; Maciolek, 1983; Sket, 1996). In general, anchialine habitats can be classified as being either karst or pseudokarst (Mylroie & Mylroie, 2011). The latter can occur in almost any rock material by a variety of mechanisms, with the most significant being those formed by volcanic activities (*i.e.*, lava tubes). Karst habitats, on the other hand, are formed by dissolutional processes and are classified as either stream caves formed by traditional karst processes that are now interacting with the coastal environment (Mylroie & Mylroie, 2011) or coastal caves and shallow basins linked to the marine environment. While, under the right conditions, dissolution can create karst forms in a variety of rock types, anchialine habitats typically form in carbonate rocks (*i.e.*, limestone and dolomite). Given this, it is not surprising that despite being found along coastlines throughout the world from high to low latitudes, anchialine habitats dominate coastlines of tropical and subtropical islands due to historically warm seas and shallow conditions favoring coral reef development. Such habitats (karst or pseudokarst) have been reported from around the world, including the Philippines, Cuba, the South Pacific, the Sinai Peninsula, the Bahamas, Bermuda, Australia, the Yucatan Peninsula, the Canary Islands, the Caribbean, the Ryukyus and Hawai'i (Fig. 1, Holthuis 1973; Maciolek 1983; Maciolek 1986; Iliffe 2000; Becking et al., 2011).

Unfortunately, one of the most threatened habitats on many tropical islands may be these anchialine habitats (Sket, 1996; Iliffe, 2002; Santos, 2006). Despite a worldwide distribution (Fig. 1), only ~1,000 habitats fitting this ecosystem definition have been reported globally (Maciolek, 1986). Historically, these extraordinary and vulnerable environments have experienced significant negative impacts from anthropogenic activities such as coastal development, tourism, vandalism, groundwater extraction and contamination, and the

introduction of invasive species (Brock et al., 1987; Iliffe, 2002, 2003; Santos, 2006). These problems are exemplified in Hawaiian Islands, which have the single largest concentration (~600; Brock, 1985) of them in the world and is home to the only natural representatives in the United States, where >90% of Hawai'i's anchialine ecosystems have been degraded or lost by anthropogenic activities in the last 50 years and it is predicted that they will likely disappear altogether in the near future (Maciolek and Brock 1974; Brock & Bailey-Brock 1998; Santos 2006). The destruction of habitats by limestone quarries or construction activities represents another serious environmental concern for anchialine habitats as a number of habitats have been destroyed in Bermuda and the Yucatan Peninsula by quarries producing crushed aggregate for construction purposes (Iliffe & Kornicker, 2009). Given these concerns, we risk losing these unique ecosystems without knowing the nature and extent of the biodiversity contained within them. Thus, there is currently an urgency to accelerate research into these unique island environments and their biota before they are lost forever.

In order to manage anchialine habitats and their fauna, it is important to develop an understanding of the biodiversity, ecology, and evolution of organisms from this ecosystem since such knowledge will play a critical role in establishing conservation strategies around the world. To date, a large number of fascinating and unique organisms, including members of the Porifera, Cnidaria, Annelida, Mollusca, Arthropoda, Echinodermata, Pices and Chordata, are known to inhabit anchialine environments (Holthuis 1963; Maciolek & Brock 1974; Iliffe et al., 1984; Iliffe 2002; Becking et al., 2011). However, it appears that the true diversity of these habitats has only recently begun to be recognized as more than 450 new species of anchialine organisms have been discovered and described over the past 25 years (Iliffe & Kornicker, 2009; ISI Web of Science search, June 2012). Given the many threats that anchialine organisms and their habitats

face today, coupled with the fact we are only starting to scratch the surface regarding the level of diversity contained in these rare habitats, there is a timely need to determine the spatial scale at which anchialine organisms are potentially isolated since such information is critical in establishing plans to preserve these unique environments.

Interestingly, despite the geographic isolation of these habitats, many anchialine taxa show geographically widespread and disjunct distributions (Smith and Williams 1981; Maciolek, 1983; Iliffe, 2000). Given these unexpected distributions, it has been hypothesized that as a result of subterranean connections and marine dispersal, populations of anchialine organisms may experience high levels of gene flow, but low levels of genetic differentiation (Cabezas et al., 2012). However, so far, the few studies exploring the genetic connectivity of anchialine species have yielded sharply different patterns of genetic structure on the basis of mitochondrial DNA variation, from panmixia among populations hundreds of kilometers apart, suggesting frequent dispersal over long distances (Kano & Kase, 2004; Hunter et al., 2008; Page et al., 2008; Russ et al., 2010), to exceptional levels of endemism on the scale of a few kilometers due to low dispersal abilities and/or significant barriers to dispersal (Santos, 2006; Hunter et al., 2008; Craft et al., 2008).

Much of the work presented below builds on the previous work of Santos and colleagues regarding the genetic connectivity, phylogeography, and evolution of anchialine organisms in the Hawaiian Archipelago (Santos, 2006; Ivey & Santos, 2007; Craft et al., 2008; Russ et al., 2012). The anchialine biota of these islands are dominated by two species of Caridean shrimp, *Halocaridina rubra* Holthuis, 1963 (Crustacea: Decapoda: Atyidae) and *Metabetaeus lohena* Banner and Banner, 1960 (Crustacea: Decapoda: Alpheidea). Despite possessing life history traits thought to be conducive to high colonization and dispersal ability among anchialine

habitats (*i.e.*, lecithotrophic larvae; reviewed in Santos, 2006), genetic analysis of mitochondrial (mtDNA) cytochrome oxidase I (COI) gene sequences suggested that *H. rubra* actually represents a "cryptic species complex" composed of at least 13 distinct genetic groups belonging to eight discrete lineages, all exhibiting significant levels of genetic structure and restricted levels of gene flow over limited geographic scales across the islands (Santos, 2006; Craft et al., 2008). These results suggested that population differentiation and evolutionary diversification in the anchialine environments of the Hawaiian archipelago may result from extrinsic obstacles to gene flow in the form hydrological barriers imposed by the subterranean aquifers and the open ocean. However, a later study of *M. lohena*, which produces planktotrophic larvae, revealed little to no population structure among populations sampled from the same anchialine habitats as *Halocaridina* (Russ et al., 2011). This total lack of population structure at this scale is similar to what has been reported for other anchialine organisms possessing planktotrophic development, such as the neritiliid snail *Neritilia*, which covers a similar geographic range across the Philippines (Kano & Kase, 2004).

From the above-mentioned studies, several hypotheses regarding the evolutionary processes operating on endemic anchialine organisms have been developed. These include: (1) the open ocean can represent a significant isolating barrier, limiting dispersal on and between islands; (2) the compartmentalization of the hypogeal water system of an island acts as barriers to gene flow and restricts dispersal across an island; (3) life history characteristics (*i.e.*, egg size, larval stages, and larval habitat) strongly influence population structure; and (4) larval feeding mode (i.e., lecithotrophy versus planktotrophy) may influence dispersal potential and population structure. The general aim of this thesis is to further test these hypotheses using analyses of mitochondrial DNA while investigating the biogeography and evolutionary relationships within

and between anchialine Carideans of the Pacific Basin. Specifically, I am interested in determining how intrinsic (*i.e.,* life history traits) and extrinsic (*i.e.,* geology) factors influence the evolution and ecology of anchialine organisms. Hopefully, this research will not only provide additional understanding into the ecology and evolution of anchialine organisms in general, but will have applied utility in developing management strategies for these organisms and their unique environments for the future. Below, I provide a brief description of each chapter and discuss their insights into the evolution, ecology and conservation of anchialine organisms in the Pacific Basin.

The focus of my dissertation research begins with the atyid *Halocaridina rubra*, which as mentioned above, dominates the anchialine habitats of the Hawaiian Archipelago (Maciolek, 1983). Historically, *H. rubra* has been considered a single species, but recent studies by Santos (2006) and Craft et al., (2008) suggest that *Halocaridina* actually represents a "cryptic species complex" comprise of at least eight potential species. Furthermore, molecular clock estimates calibrated to the geologic age of Kilauea volcano on the island of Hawai'i have suggested that the COI gene of the *Halocaridina* complex is apparently evolving at an exceptional rate of 20% per million years (Craft et al., 2008). In Chapter 2, the validity of this rapid evolutionary rate is tested by applying it to levels of divergence measured from geographically close, yet genetically distinct, *Halocaridina* populations on the islands of Hawai'i, Maui and O'ahu. Application of the *Halocaridina* molecular clock identified a strong correlation between levels of genetic divergence and the geologic age of the region inhabited by those populations on the younger islands of Hawaii and Maui. However, this relationship weakened when applied to *Halocaridina* populations on the older island of Oahu. Given this, it appears that island age is an important factor that should be taken into consideration when conducting molecular clock analyses on

island populations. This chapter has been published as an invited contribution as: Santos, S.R., D.A. Weese (2011) Rocks and Clocks: Linking geologic history and rates of genetic differentiation in anchialine organisms. *Hydrobiologia*. 677(1):53-64.

The strong population structure observed for *Halocaridina* is utilized in Chapter 3 to examine the utility of molecular techniques for identifying source populations of ornamental invertebrates in the aquarium trade. *Halocaridina* are popular in the aquarium trade due to their ability to survive in hermetically sealed containers for extended time periods. However, commercial harvesting, coupled with habitat destruction and the strong regional endemism exhibited by these shrimp (Craft *et al.,* 2008), may lead to the depletion/extinction of unique populations. To identify the source populations of *Halocaridina* being sold in the aquarium trade, mitochondrial gene sequences from commercially purchased individuals were compared to a large database of homologous sequences from previous studies (Santos, 2006; Craft *et al.,* 2008). Commercial specimens were identified as originating from the Kona, Kaʻū (west and south coasts, respectively, Hawaiʻi), or Kinaʻu (south coast, Maui) genetic groups of these shrimp. Although the majority of individuals originated from the Kona land district as hypothesized, the finding that commercially available *Halocaridina* originate from three genetic groups spanning two islands suggest that other population also warrant management consideration. This chapter has been published as: Weese, D.A., S.R. Santos (2009) Genetic identification of source populations for an aquarium-traded invertebrate. *Animal Conservation*. 12:13-19.

In the later half of this dissertation, my research focus shifts west to the anchialine organisms of the Ryukyus Archipelago, a continuous chain of islands situated between Japan's Kyushu Island and Taiwan. The Southern Ryukyus consists of five main islands, Okinawa,

Tarama, Miyako, Ishigaki, and Iriomote. These islands, spanning a geographic distance similar to the high islands of the Hawaiian Archipelago (~200 km), offer an excellent opportunity to further test and develop hypotheses regarding the evolutionary diversification and phylogeography of anchialine taxa. This begins with an investigation of the genetic variation and population structure of the anchialine atyid, *Caridina rubella*, restricted to the island of Miyako-jima in Chapter 4. Having a planktotrophic larval stage and a potentially amphidromous life cycle, populations of *C. rubella* on the island of Miyako-jima were hypothesized to exhibit little to no structure across the island. Surprisingly, significant genetic structure was exhibited across distances ranging from < 20 m to > 10 km. Additionally, deep genetic divergence correlating with distinct morphological variation was found between closely situated, but apparently isolated, populations, suggesting that "*C. rubella*" may actually represent two distinct species on Miyako-jima. Given that this atyid is already listed as a threatened species by the Japanese government, the results presented in this chapter may have significant implications for the formulation and implementation of future conservation plans for *C. rubella* populations on Miyako-jima. This chapter has been published as Weese, D.A., Y. Fujita, M. Hidaka, S.R. Santos. (2012) The long and short of it: Genetic variation and population structure of the anchialine atyid shrimp *Caridina rubella* on Miyako-jima, Japan. *Journal of Crustacean Biology*. 32(1):109-117.

Chapter 5 takes a comparative phylogeographic approach to determine the origins of anchialine Caridean diversity in the Ryukyu Islands by investigating the population structure and evolutionary history of three Caridean shrimp from the anchialine niche. Given the geologic history of the islands, it was hypothesized that diversity across the Ryukyus originated within the archipelago and that the phylogenetic history of the three species examined (*Antecaridina*

lauensis, *Metabetaeus minutus* and *Halocaridinides trigonophthalma*) would be correlated with the sequential separation of the Ryukyu island groups as sea-levels fluctuated during the Pleistocene. However, given the phylogenetic relationships and geographic distributions of the multiple lineages found for each species coupled with the strong regional oceanographic currents of the Indo-West Pacific, the diversity of anchialine shrimp found in the Ryukyus is likely the result of an accumulation of species dispersing into the Ryukyus from independent source populations. Furthermore, the contrasting patterns of population structure and connectivity exhibited by each 'species' within the Ryukyus appear to result from complex interactions between intrinsic (*i.e.*, life history traits) and extrinsic (*i.e.*, historical geology and oceanography) processes. This chapter will be submitted to *The Biological Bulletin* as Weese, D.A.,Y. Fujita, S.R. Santos. "Multiple colonizations lead to hidden diversity in a unique island ecosystem: Comparative phylogeography and conservation of anchialine shrimp in the Ryukyu Archipelago, Japan".

Drawing from the previous four chapters, Chapter 6 offers my concluding thoughts regarding the ecology, evolution and conservation of anchialine Carideans in the Pacific Basin. I present a brief summary of the preceeding chapters and how these studies can bring novel insight into the natural history of anchialine species and how these findings can have important implications for the future management of these species and their habitats.

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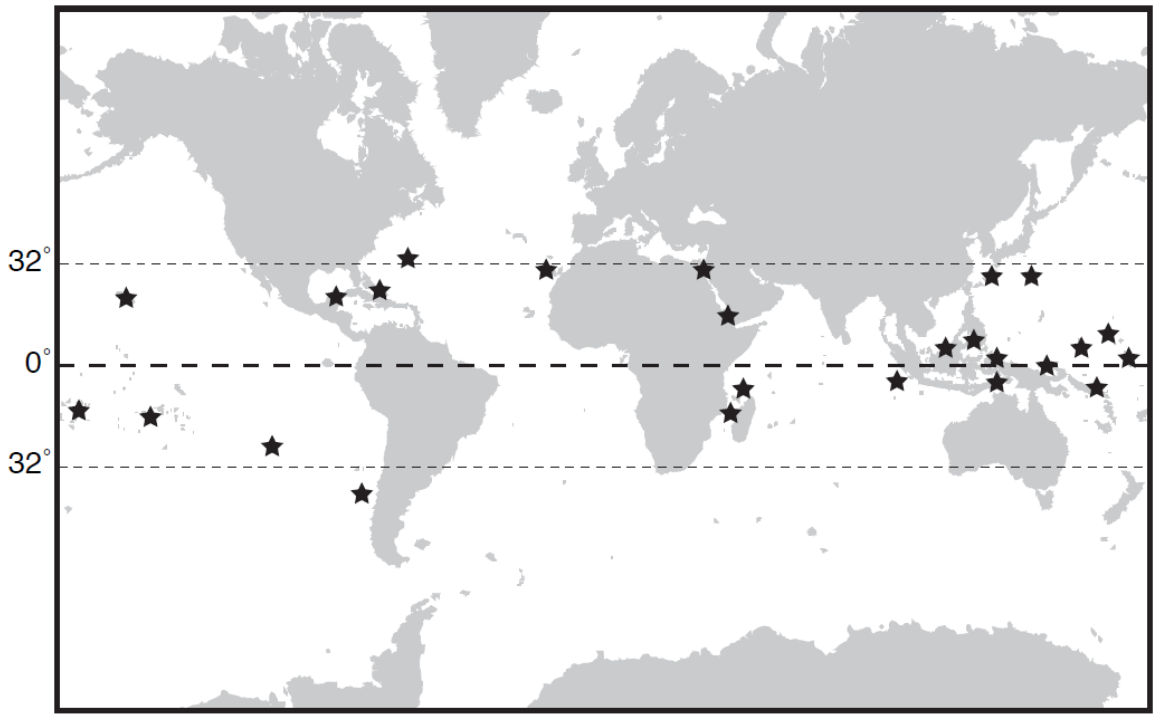

Figure 1. Global distribution of anchialine habitats.

CHAPTER 2. Rocks and Clocks: Linking Geologic History and Rates of Genetic Differentiation in Anchialine Organisms

2.1 ABSTRACT

The geologic history of a region can significantly impact the development of its flora and fauna, with past events shaping community patterns and evolutionary trajectories of species. In this context, islands are excellent "natural laboratories" for studying the fundamental processes of evolution due to their discrete geographical nature and dynamic geologic histories. An island system meeting these criteria is the Hawaiian Archipelago, which is ideal for testing how island geologic history influences the processes leading to population genetic variation and differentiation. One Hawaiian endemic whose evolutionary history is closely tied to the geology of the islands is the anchialine atyid shrimp *Halocaridina*, whose mitochondrial cytochrome oxidase I (COI) gene is hypothesized to be evolving at the rate of 20% per million years. To validate this rapid evolutionary rate, time since divergence estimates between geographically close, yet genetically distinct, populations were calculated for *Halocaridina* from anchialine habitats on the islands of Hawai‘i, Maui, and O‘ahu. On the younger (i.e. <1.5 million years) islands of Hawai‘i and Maui, where all anchialine habitats occur in basalt, application of the *Halocaridina* molecular clock identified a strong correlation between levels of genetic divergence and the geologic age of the region inhabited by those populations. In contrast, this relationship weakened when similar analyses were conducted for *Halocaridina* from limestone anchialine habitats on the older (i.e. >2.75 million years) island of O‘ahu. These results suggest geologic age, basin origin and/or composition are important factors that should be taken into consideration when conducting molecular clock analyses on anchialine flora and fauna as well as island populations in general.

2.2 INTRODUCTION

Islands serve as excellent "natural laboratories" for understanding the fundamental processes of evolution (reviewed in Emerson, 2002). For example, they have historically been templates for studying biogeographic phenomena, such as the classic hypotheses of colonization and biotic exchange proposed by MacArthur & Wilson (1967). Furthermore, the discrete and active geologic nature of many islands can regionally and significantly impact their flora and fauna, consequently altering the evolutionary trajectory of populations as well as overall community patterns. In this context, examining the genetic structure and phylogenetic relationships of island species, with reference to their specific biology and physical habitat, can provide a framework towards elucidating the forces driving the origin of novel lineages and taxa (i.e., speciation) along with the rate of their genesis (Avise, 2004).

One island system with a dynamic geology is the Hawaiian Archipelago. Situated in the central Pacific Ocean, it is an ideal location for testing how the geologic history of islands can influence the demographics and genetic structure of resident populations (e.g., Vandergast *et al.*, 2004). Additionally, the Hawaiian Islands are home to the only natural anchialine habitats in the US as well as having the single largest concentration of them (~600; Brock, 1985) in the world. Globally, such habitats are physically defined as land-locked bodies of mixohaline water with simultaneous subterranean connections to the ocean and groundwater aquifer and occupying basins of varying geologic origins (i.e., karst caves, cenotes, fossilized coral reefs or coastal lava fields) (Holthuis, 1973; Maciolek, 1983; Sket, 1996). Unfortunately, it is estimated that >90% of Hawaii's anchialine habitats have been historically and contemporarily lost or degraded by anthropogenic activities like coastal development and the spread of exotic species (Maciolek & Brock, 1974; Bailey-Brock & Brock, 1993; Brock & Bailey-Brock, 1998). For those that still

remain, it is predicted that they will also succumb to these pressures in the near future if appropriate conservation plans and management actions are not taken (Brock & Kam, 1997). Thus, anchialine habitats potentially represent one of the most threatened ecosystems in the Hawaiian Archipelago.

The Hawaiian anchialine ecosystem is home to a limited assemblage of macrofauna dominated by endemic gastropod mollusks and crustaceans (Maciolek & Brock, 1974; Maciolek, 1983). Of these, *Halocaridina rubra* Holthius, 1963 (Decapoda, Atyidae) is a small (i.e., ~12 mm) microphagous shrimp particularly characteristic of this assembly due to its near ubiquitous distribution in anchialine habitats of the islands (Maciolek, 1983). Based on this, Santos (2006) tested the hypothesis that *H. rubra* exhibits high levels of gene flow by first sampling populations from the island of Hawai‘i. Analyses of mitochondrial (mtDNA) cytochrome oxidase I (COI) gene sequences instead revealed strong subdivision between populations separated by as little as 30 km. A subsequent survey of *Halocaridina* from 34 populations on Hawai‘i, Maui, and O‘ahu identified 13 genetic groups belonging to eight (8) lineages qualifying as potential species (Craft *et al.*, 2008). In general, these genetic groups and lineages are restricted to anchialine habitats from a particular region of a single Hawaiian Island, with no individuals being exchanged between them. This phylogeographic pattern is attributed to the low dispersal potential of these atyids due to their life history traits in combination with marine and geologic barriers that compartmentalize the aquifers of each island (Santos, 2006; Craft *et al.*, 2008). Thus, evolution in *Halocaridina* is driven by population fragmentation, isolation, and subsequent diversification in anchialine habitats of discrete aquifers across Hawai‘i.

Identifying the widespread “*Halocaridina rubra*” as representing a “species complex” with strong regional endemism highlights the ecological significance of conserving anchialine

habitats in the Hawaiian Archipelago. Moreover, these atyids have provided unexpected perspective into the tempo of evolution. Specifically, molecular clock estimates calibrated to the geologic age of Kilauea volcano on the island of Hawai‘i lead to the revelation that the COI gene of *Halocaridina* is apparently evolving at an exceptional rate of 20% per million years (My^{-1}) (Craft *et al.*, 2008), in sharp contrast to the range of 1.7% My^{-1} (Williams & Knowlton, 2001) to 2.3% My^{-1} (Brower, 1994) commonly utilized or reported for arthropods or other atyids (e.g., Page & Hughes, 2007; Page *et al.*, 2008). In this contribution, the validity of this rapid evolutionary rate is tested by 1) applying it to levels of divergence measured from geographically close, yet genetically distinct, *Halocaridina* populations on the islands of Hawai‘i and Maui and 2) comparing the resulting time since diversification estimates to the geologic ages of their respective anchialine habitats.

2.3 Materials and Methods

2.3.1 Biological Materials

In July 2008, *Halocaridina* were sampled from previously uncharacterized populations on the northeastern and southeastern coasts of Maui and Hawai‘i, respectively (Figure 1). The anchialine habitats at Waikoloa, Maui (WKA; Figure 1A) and Wai‘ohinu, Hawai‘i (WP; Figure 1B) consist of clusters of 2-3 shallow (i.e., <0.5 m) pools whose basins are comprised of basalt. Temperatures (i.e., 25 °C), salinities (i.e., 8.0 ppt) and daily tidal fluctuations at these locations were consistent with those from other anchialine habitats in the islands (Craft *et al.*, 2008). At each site, a net was utilized to collect specimens of *Halocaridina* that were immediately preserved in 100% acetone (Fukatsu, 1999) for genetic analyses.

2.3.2 DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING

For individual *Halocaridina*, total nucleic acid extraction and polymerase chain reaction (PCR) of a partial COI gene fragment followed procedures outlined in Santos (2006). Briefly, reactions were conducted in 25 μL volumes containing ~10-30 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.0 mM $MgCl_2$, 200 μM dNTPs, 1 U *Taq* DNA polymerase and 0.4 μM each of primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) using a PTC-100 thermocycler (MJ Research, USA). Purification of amplified products prior to sequencing was done with Montage PCR Filter Units (Millipore, USA) according to the supplier's protocol. Products were cycle-sequenced in both directions using Big-Dye Terminators v.3.1 and analyzed on a PRISM 3100 (Applied Biosystems, USA). Ambiguities in the chromatograms were corrected by comparison to the complement DNA strand in Sequencher v4.8 (Gene Codes Corporation, USA) and finished sequences of 630 bp manually aligned with SE-AL version v2.0a11 (available at http://tree.bio.ed.ac.uk/software/seal/). Novel COI sequences were deposited into GenBank under accession numbers HM137541-HM137556.

2.3.3 DEMOGRAPHIC AND GENETIC STRUCTURE ANALYSES

Demographic measures, such as estimates of genetic diversity (i.e., haplotype (*h*) and nucleotide (π); [Nei, 1987]) and tests of neutrality (i.e., Tajima's *D* [Tajima, 1989]; Fu's F_S [Fu, 1997]), were done for each population using DnaSP v4.10.9 (Rozas *et al.*, 2003) and Arlequin v3.11 (Excoffier *et al.*, 2005). To test for genetic structure between these newly sampled populations and ones previously reported in Craft et al. (2008) that are in close (i.e., ~3.5-10 km) proximity, the nearest-neighbor statistic (S_{nn}; Hudson, 2000) was calculated using DnaSP. Specifically, this test was conducted between *Halocaridina* populations WKA (this study) and

WC (i.e, Waianapanapa Cave, Craft *et al.*, (2008); Figure 1A) and WP (this study) and PU (i.e, Puhi Ula Cave, Craft *et al.*, (2008); Figure 1B) on Maui and Hawai‘i (Figure 1), respectively. Additional tests for genetic structure involved calculation of pairwise Φ_{ST} statistics in Arlequin under Tamura & Nei's (1993) model of DNA evolution. Significance of the neutrality tests as well as S_{nn} and Φ_{ST} statistics was assessed with 10,000 permutations. Lastly, networks were constructed with TCS v1.21 (Clement *et al.*, 2000) in order to visualize relationships among the COI haplotypes within and between the populations under comparison. Default settings in TCS were utilized for network construction, which provides the 95% parsimoniously plausible branch connections between haplotypes.

2.3.4 Divergence Time Estimation and Geologic Age

Two different approaches were employed in estimating between-population times since divergence. Firstly, uncorrected (*p*) genetic distances of COI sequence divergence between population pairs were generated using MEGA v3.1 (Kumar *et al.*, 2004), with values calculated using the "net between-group means" option so as to correct for within-group polymorphism. These *p* genetic distances were then subjected to the 20% My^{-1} diversification rate previously hypothesized for *Halocaridina* (Craft *et al.*, 2008) to infer a time since divergence between population pairs. Secondly, theta ($\theta = 4N_e\mu$, where N_e is the effective population size and μ is the per-locus mutation rate) and divergence time ($T = t/2N_e$, where t is generation time) between populations pairs were simultaneously estimated via the Markov Chain Monte Carlo (MCMC) method described by Nielsen & Wakeley (2001) and implemented with the web-based version of the program MDIV (available at http://cbsuapps.tc.cornell.edu/). Analyses were conducted under the finite-site mutation model (i.e., HKY85 [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. A minimum of three independent runs using identical starting conditions (M_{max} = 10, T_{max} = 1.0 (e.g., for the WC and WKA populations of Maui) or 5.0 (e.g., for the PU and WP populations of Hawai‘i), length of Markov chain = 2 X 10^6 cycles, burn-in time = 5 X 10^5 cycles) but different random seeds was done to check for consistency in the estimates. Values of θ and *T* with the highest posterior probabilities were accepted as the best estimates of these parameters and utilized in generating converted estimates of the "actual years since divergence" (AYSD) with the equation $T\theta/2\mu$ (see Nielsen & Wakeley (2001) for details) and a μ of 1.26×10^{-4} substitutions per sequence per year, which is based on a COI gene fragment length of 630 bp (see above) and the 20% My^{-1} diversification rate of Craft *et al.,* (2008). Resulting point estimates of divergence time from both of these approaches were subsequently compared to the geologic age of the basalt in which that particular pair of anchialine habitats (and their resident *Halocaridina* populations) occur. Geologic ages were acquired from the U.S. Geological Survey via geographic information system (GIS) map layer files for the main Hawaiian Islands (Sherrod *et al.,* 2007; available at http://pubs.usgs.gov/of/2007/1089/) that were rendered in ArcGIS v9.3 (ESRI, USA).

2.4 RESULTS

2.4.1 *HALOCARIDINA* FROM THE NORTHEASTERN COAST OF MAUI

Two lineages and genetic groups of *Halocaridina* occur on Maui, with the Hana genetic group within the East Maui lineage localized to the northeastern coastline of the island (Craft *et al.,* 2008). Genetic analyses found that *Halocaridina* from the anchialine habitats at Waikoloa (WKA; Figure 1A) also belong to this genetic group and lineage. The only other known

population of *Halocaridina* within this lineage and genetic group inhabits Waianapanapa Cave (WC), approximately 3.5 km northwest of WKA (Figure 1A). Comparisons between the WC and WKA populations revealed similar levels of recovered haplotypes, haplotype (*h*) and nucleotide (π) diversities (Table 1). In contrast, the WC population possessed significant negative Tajima's *D* and Fu's F_S values while these same measures for the WKA population were not significant (Table 1). This reflects that an excess of haplotypes with rare polymorphisms were present at the WC site and is often a signature of recent population expansion (Tajima, 1989; Fu, 1997). Given this, the *Halocaridina* populations at WC and WKA have distinct demographic histories.

Evidence for genetic structure between the WC and WKA populations was absent with either the S_{nn} (0.563; $P = 0.096$) or pairwise Φ_{ST} (0.015; $P = 0.199$) statistics. Although genetic structure was not evident, only two haplotypes (e.g., the haplotype with the highest outgroup (i.e., ancestral) probability as well as one other) were shared between the populations, with the remaining being both private and typically recovered as a singleton in the network analysis (Figure 2A). This latter pattern suggests additional COI genetic diversity remains to be documented from these *Halocaridina* populations.

Genetic distance between the WC and WKA populations in the form of the "net between-group mean" yielded an uncorrected *p* value of 0.0001. In the context of the 20% My^{-1} diversification rate, this implies that they have diverged in the last ~500 yrs. For the MDIV analyses, the highest posterior probability of θ and *T* occurred at 4.3 and 0.07, respectively (Figure 3); application of these values to calculate the AYSD (see Materials & Methods) yielded an estimate of 1,194 yrs. Interestingly, the anchialine pools inhabited by *Halocaridina* at WC and WKA both occur in basalt with a geologic age of 750-1,500 yrs and are intersected by basalt of 200-750 yrs in age (insert in Figure 2A). Thus, the geologic ages of the most recent basalts

(i.e., lava flows) in the area strongly coincide with the estimated times since diversification (i.e., ~500/1,194 yrs) of the WC and WKA populations of *Halocaridina*.

2.4.2 *HALOCARIDINA* FROM THE SOUTHEASTERN COAST OF HAWAIʻI

The island of Hawaiʻi is home to three lineages and five genetic groups of *Halocaridina* (Santos, 2006; Craft *et al.,* 2008). Of these, the Naʻalehu genetic group within the Kaʻū lineage is the most restricted in distribution, having only been previously found on the southeastern coast of the island at Puhi Ula Cave (PU). Here, the *Halocaridina* population of anchialine habitats at Waiʻohinu (WP) was identified as members of the Kaʻū lineage, extending the lineage's range ~10 km to the southwest (Figure 1B).

Although the Kaʻū lineage is present at both PU and WP, the populations at each site are unique. For example, nearly twice as many haplotypes and 2X more nucleotide (π) diversity were recovered from the WP population relative to that at PU (Table 1). Furthermore, only the population at PU possessed a significant negative Tajima's *D* value (Table 1), implying a distinctive demography to the *Halocaridina* at this site. Lastly, significant genetic structure was detected between the WP and PU populations with both the S_{nn} (0.826; $P < 0.001$) and pairwise Φ_{ST} (0.198; $P < 0.001$) statistics. The distinct genetics of the populations are also evident in the network analysis, where of the 14 unique COI haplotypes currently identified within the lineage, each population is numerically dominated by private haplotypes while only three are shared (at low frequencies) between the sites (Figure 2B). Thus, the PU and WP populations should be considered distinct genetic groups within the Kaʻū lineage of *Halocaridina*.

An uncorrected *p* value of 0.0008 was calculated for the "net between-group mean" genetic distance between the WP and PU *Halocaridina* populations. In this case, application of

the 20% My^{-1} diversification rate resulted in an estimated time since divergence of ~4,000 yrs. Analyses with MDIV identified the highest posterior probability of θ and *T* as 2.6 and 0.5, respectively (Figure 3), with a resulting AYSD estimate of 5,158 yrs. Similar to the previous example on Maui, this strongly correlates with the geologic age of the area inhabited by these populations. Specifically, the anchialine habitats at WP and PU occupy a contiguous basalt flow whose age ranges between 3,000-5,000 yrs (Figure 2B), which is consistent with the divergence time estimates (i.e., ~4,000/5,158 yrs) for these *Halocaridina* populations.

2.5 DISCUSSION

2.5.1 VALIDATION OF THE *HALOCARIDINA* MOLECULAR CLOCK

Previously, levels of mtDNA COI gene sequence divergence calibrated to the known geologic age of Kilauea volcano on Hawai‘i suggested members of the anchialine atyid genus *Halocaridina* are diversifying at the rate of 20% My^{-1} (Craft *et al.*, 2008). This is, to the best of our knowledge, the highest mitochondrial molecular clock estimate currently reported for an invertebrate (see Burridge *et al.*, 2006, 2008; Waters *et al.*, 2007 for comparable estimates from vertebrates). One hypothesis for this is that the mitochondrial genome of *Halocaridina* is “unusual” relative to other crustaceans. However, gene order and orientation in the *Halocaridina* mitochondrial genome is syntenic with nearly all malacostracans examined to date and phylogenetic analyses encompassing the organelle’s 13 protein-coding genes found no “long-branch” (i.e., potential accelerated evolutionary rate) associated with the genus (Ivey & Santos, 2007). Thus, an alternative interpretation is required towards explaining this extraordinary divergence rate.

While the taxonomic status of the eight lineages within *Halocaridina* currently remains unresolved, numerous lines of evidence imply that they represent "cryptic species" (Santos, 2006; Craft *et al.,* 2008). Furthermore, many populations within the *Halocaridina* lineages are on trajectories towards becoming new lineages themselves, such as the South Hilo/Puna and Kalaeloa/Waiʻanae genetic groups of Hawaiʻi and Oʻahu, respectively (Santos, 2006; Craft *et al.,* 2008). Thus, the inferred rate of diversification for *Halocaridina* is an estimate at the interface between populations and "species". In this context, there is growing evidence that the derivation and application of molecular clocks at this scale represents special circumstances. Specifically, it has been advocated that molecular clocks exhibit variability in the form of an overall lower, long-term mutation rate preceded by a higher short-term (<1-2 My) substitution rate at the population-species boundary (reviewed by Ho & Larson, 2006). This apparent higher rate is thought to originate from a combination of temporal differences in the population-level retention of mutations based on their "deleteriousness" and the relative time of their observation (i.e., sampling) in the population under study (reviewed by Penny, 2005). The evolution of *Halocaridina* across the anchialine habitats of the Hawaiian Islands appears to epitomize this phenomenon, particularly when placed into the perspective of the geologic histories and ages of the islands (see below), and adds to the growing list of examples where failure to recognize this potential would have significantly altered the perceived time-scales at which ecological and evolutionary processes are occurring (see Ho *et al.,* 2008 for case studies).

The analyses of previously uncharacterized *Halocaridina* populations presented here strongly supports the 20% My^{-1} COI molecular clock first proposed by Craft *et al.* (2008) for these atyid shrimp. Specifically, divergence time estimates based on this rate for populations in two distinct lineages on two islands were highly consistent with the geologic age of the basalt

basins of their anchialine habitats (see Results). This illustrates how the demographics, population structure and evolutionary history of endemic anchialine organisms can be intimately coupled to the particular geologic history of their physical habitat. For *Halocaridina*, this coupling is due to life history traits such as large egg size, abbreviated development, restricted larval habitat and larval feeding mode effectively isolating individuals on new "islands" (i.e., distinct aquifers and their anchialine habitats) following successful dispersal and/or an allopatric fragmentation event (Santos, 2006; Craft *et al.,* 2008). Thus, it would not be surprising to find that this scenario, accompanied by exceptional rates of divergence, extends to other anchialine species with similar life history traits or belonging to groups with notoriously poor dispersal abilities (e.g., amphipods; Finston *et al.,* 2007) and for which (most importantly) well-established geologic ages of their habitats are available for calibration.

2.5.2 MAUI AND HAWAIʻI: GEOLOGIC HISTORY AND *HALOCARIDINA*

Maui is the second youngest (i.e., <1.5 My) of the current high islands in the Hawaiian Archipelago and is composed of two distinct volcanoes, West Maui and Haleakala. As discussed earlier, the Hana genetic group within the East Maui lineage of *Halocaridina* is restricted to the northeastern coast of the island, along the flank of Haleakala. In the past 10,000 years, Haleakala volcano has erupted on numerous occasions, including at least ten major eruptions in the last 1,000 years (http://hvo.wr.usgs.gov/volcanoes/haleakala/). Much of this activity was localized along two interconnecting rift zones: the southwest rift zone, which passes from La Perouse Bay on the southwest flank of Haleakala to the summit, and the east rift zone, radiating from the summit along the volcano's eastern flank to Hana. In fact, the east rift zone of Haleakala is located ~5.5 km south of the anchialine habitats at WC and WKA and is responsible for the

relatively young basalts in the area. These include lava flows ranging in age from 750-1,500 yrs, which is the basalt the WC and WKA anchialine habitats occur in, and 200-750 yrs, which traverses the two sites (Figure 1A; Sherrod *et al.*, 2007). Given this geologic history and the divergence time estimates of ~500 or 1,194 yrs, this would imply that the anchialine habitats at these sites are <1,500 yrs in age and, in the interim, that the area's recent lava flows have potentially impacted the demographics of their local *Halocaridina* by leading to cycles of contraction and expansion in some populations (i.e., WC) but not in others (i.e., WKA). Such a situation has also been reported for at least one *Halocaridina* population on the island of Hawai'i, in which the signature of a recent and strong contraction and expansion similarly correlated with a contemporary and localized volcanic episode (Santos, 2006).

The youngest (i.e., ~430,000 yrs) island in the Hawaiian Archipelago is Hawai'i, being comprised of five separate volcanoes. Of these, the Ka'ū lineage of *Halocaridina* is restricted to the southeastern flank of Mauna Loa. This particular volcano has erupted 33 times since 1843, making it one of the most active (as well as the largest) on Earth (http://hvo.wr.usgs.gov/maunaloa/). While the oldest dated basalts attributable to Mauna Loa range from 100,000-200,000 yrs, >98% of the volcano's surface is composed of flows younger than 10,000 yrs (http://hvo.wr.usgs.gov/maunaloa/history/main.html). Similar to Haleakala, these lava flows predominately originate from two rift zones radiating from the summit and towards the northeast and southwest, respectively. The southwest rift zone of Mauna Loa is the more active of the two (Macdonald *et al.*, 1983) and the source of basalt for the anchialine habitats at PU and WP. As mentioned previously, both sites occur within a contiguous flow whose age is estimated to range between 3,000-5,000 yrs (Figure 1A; Sherrod *et al.*, 2007), which coincides nearly exactly with the ~4,000 or 5,158 yrs time since divergence estimates for this pair of

Halocaridina populations (see Results). In this case, we hypothesize these anchialine habitats (i.e., PU and WP) were colonized by *Halocaridina* soon after their creation from populations in "refugia" offered by the 10,000-30,000 yr basalts immediately adjacent to this younger flow (Figure 1A). Given the ~10 km distance between the sites and the fact that peripheral populations of *Halocaridina* across a range generally exhibit some level of differentiation due to restricted gene flow (Santos, 2006; Craft *et al.*, 2008), this founder effect, reinforced via isolation by distance, then lead to the significant population structure currently evident between the Waiʻohinu and Naʻalehu genetic groups of *Halocaridina*.

2.5.3 REEVALUATING *HALOCARIDINA* EVOLUTION IN THE CONTEXT OF ISLAND GEOLOGY

Validating the 20% My^{-1} molecular clock for *Halocaridina* prompts us here to revisit evolution within the genus in the specific context of the geologic histories and ages of the Hawaiian Islands. While a well-resolved phylogeny for the *Halocaridina* "species complex" remains to be elucidated (Craft *et al.*, 2008), thus limiting our ability to fully address this topic, seven comparisons either between populations, genetic groups and/or lineages across three islands can be brought into consideration (Table 2; Figure 4). For these, estimated times since divergence were also calculated using the 1.7% My^{-1} and 2.3% My^{-1} molecular clocks of Williams & Knowlton (2001) and Brower (1994), respectively (Table 2). In all cases, utilization of these "generic" clocks resulted in divergence time estimates considerably older than the "true" age based on geologic evidence (Table 2). For example, the sister lineages of East Hawaiʻi and Kaʻū serve as the basis for the 20% My^{-1} *Halocaridina* molecular clock due to their 2% genetic distance average being calibrated to the oldest estimated atmospheric eruption of Kilauea volcano at 100,000 yrs (http://hvo.wr.usgs.gov/kilauea/; dotted line in Figure 4). In contrast, the

generic clocks suggest this divergence event occurred somewhere between 0.87-1.2 My ago (#1; Table 2), which is ~2-3X older than the island of Hawai‘i itself (~430,000 yrs, Carson & Clague, 1995). For this reason, generic molecular clocks such as these should be applied with caution since they have the potential to greatly mislead divergence time estimates for anchialine organisms or taxa from other ecosystems in general.

Within the East Hawai‘i lineage of *Halocaridina*, diversification of the Puna and South Hilo genetic groups was apparently precipitated by an allopatric fragmentation event associated with the East Rift Zone of Kilauea volcano (Santos, 2006). Here, the occurrence of that event is dated at ~60,000 yrs (#2; Table 2; Figure 4). Kilauea is the youngest and most southeastern volcano on Hawai‘i and has been in continuous eruption since January 1983. While impressive in itself, this volcano is thought to have undergone no prolonged periods of inactivity (http://hvo.wr.usgs.gov/kilauea/history/), and been in uninterrupted growth, since its emergence from the sea ~100,000 yrs ago. Thus, we hypothesize that 1) the East Hawai‘i lineage of *Halocaridina* colonized the flank of Kilauea at or prior to 60,000 yrs ago; 2) at ~60,000 yrs, establishment of, or successful dispersal around, the East Rift Zone lead to the inception of the Puna and South Hilo genetic groups that have been diverging in allopatry since the event; and 3) their present ranges and anchialine habitats are most likely not those in which the event took place due to Kilauea’s continuous activity and expansion reshaping the coastlines over its subsequent history.

O‘ahu possesses the greatest known genetic diversity of *Halocaridina* in the Hawaiian Archipelago, with four lineages and six genetic groups reported from the island (Craft *et al.*, 2008). It is also distinctive in that, unlike Maui and Hawai‘i, most of its anchialine habitats occur as a single pool per site and all have basins of fossilized, but porous, limestone materials. This

limestone is due to the development of expansive coral reefs along the island's coasts over its >2.75 My existence (Lau & Mink, 2006). Approximately 131,000 yrs ago, sea levels on Oʻahu rose ~2 m at the beginning of the last interglacial period (Szabo *et al.,* 1994), submerging low-lying areas and transforming them into near shore environments conducive to coral reef growth. Overall, this interglacial period lasted for ~17,000 yrs before sea level returned to near-present conditions around 114,000 yrs ago (Szabo *et al.,* 1994), "stranding" in a terrestrial setting any coral reef environments that had flourished during this interval. Thus, the current anchialine habitats of Oʻahu trace their origins back to this particular interglacial event and time period.

In three of the lineages on Oʻahu, the diversification of *Halocaridina* genetic groups occurred between 45,000-65,000 yrs ago (#5, #6, #7; Table 2; Figure 4), well after the last interglacial period ended ~114,000 yrs ago. Along with this, the two volcanoes on the island, Waiʻanae and Koʻolau, are considered extinct since they have not erupted in the past 500,000 yrs (Macdonald *et al.,* 1983). For these reasons, we cannot confidently identify a specific geologic event of definable age associated with the divergence times of the Kahuku/Lanikai, Waiʻanae/Kalaeloa and Kapapa/Kona genetic groups of *Halocaridina* (Table 2; Figure 4). Instead, the most parsimonious explanation is that dispersal and successful colonization at these estimated times were the events leading to their divergence since they reside allopatrically either in well-compartmentalized, but adjacent, aquifers of the same island or on different islands (Craft *et al.,* 2008). Of these, the Kapapa and Kona/Kaʻū genetic groups (#7; Table 2) within the West Hawai'i lineage are particularly interesting. For example, this lineage is the only one to date recorded from more than a single island, being found on Oʻahu and Hawai'i (Figure 4). Under the assumption of the "Progression Rule", in which populations from older islands act as founders for ones on younger islands (Funk & Wagner 1995), this implies that 1) the arrival of

the West Hawai'i lineage on the western coast of Hawai'i is a relatively recent (i.e., ~45,000 yrs ago; Table 2) event; 2) it was the second of the two lineages to colonize that island since divergence in the East Hawai‘i lineage predates this arrival time estimate (see above), and; 3) the lineage has been expanding its range along the entire western coast of Hawai‘i up to the present while diversifying into the Kona and Ka‘ū genetic groups (Santos, 2006; Craft *et al.*, 2008), which are the primary source populations of *Halocaridina* in the aquarium trade (Weese & Santos, 2009). In contrast, the evolutionary history of the Kapapa genetic group within the West Hawai'i lineage is less clear. The reason for this is that Kapapa Island, which this genetic group is restricted to, is a small (i.e., 0.012 km^2) limestone islet located ~3.2 km off the eastern coastline and is comparatively young (i.e., 80,000-143,000 yrs; Table 2) in relation to the rest of O‘ahu. Thus, from where did the migrants who colonized Kapapa Island originate? Possibilities include an undocumented *Halocaridina* population from the numerous aquifers that remain to be sampled on O‘ahu (or the other islands) or diversification from one of the previously recognized lineages. In the interim, and as answers to such questions are pursued, the genus *Halocaridina* from the anchialine habitats of the Hawaiian Archipelago has offered exciting insight into the processes and tempo of evolution in this ecosystem and it will be interesting to see if similar patterns are reported from other geographic regions and anchialine taxa in the future.

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Table 1: Genetic diversity measures and results of neutrality tests for *Halocaridina* populations from the northeastern and southeastern coasts of Maui and Hawai‘i, respectively, examined in this study. *n* = number of sampled individuals; *nh* = number of recovered haplotypes; π = nucleotide diversity; *h* = haplotype diversity. * = $p < 0.05$.

				Diversity indices		Neutrality tests	
Island Region	Population	*n*	*nh*	π	*h*	Tajima's *D*	Fu's F_s
Northeastern Maui	WC	29	13	0.004	0.702 ± 0.096	-1.64*	-5.03*
	WKA	21	9	0.005	0.795 ± 0.077	-0.72	-1.23
Southeastern Hawai‘i	PU	30	6	0.002	0.667± 0.055	-1.62*	-1.03
	WP	30	11	0.004	0.745± 0.082	-1.06	-2.63

Table 2: Summary of molecular clock comparisons for *Halocaridina*. Estimated times since divergence for each comparison were calculated using rates of 1.7% My^{-1} (Williams & Knowlton 2001), 2.3% My^{-1} (Brower 1994) and 20% My^{-1} (Craft *et al.*, 2008).

Comparison in Figure 4	Island: Coastal region	Population/Genetic Group/Lineage Comparison	Genetic Distance (% difference)†	Estimated Time since Divergence			
				1.7% My^{-1}*	2.3% My^{-1}**	20% My^{-1}***	"True" Geologic Age
#1	Hawai'i:Eastern coast	East Hawai'i vs. Ka'ū	2.0	1,180,000	870,000	100,000	100,000[a]
#2	Hawai'i:Eastern coast	Puna vs. South Hilo	1.2	706,000	520,000	60,000	100,000[a]
#3	Hawai'i:Southeastern coast	PU vs. WP	0.08	47,000	35,000	4,000-5,158	3,000-5,000[b]
#4	Maui:Eastern coast	WC vs. WKA	0.01	5,900	4,300	500-1,194	750-1,500[b]
#5	O'ahu: Northern coast	Kahuku vs. Lanikai	1.0	590,000	435,000	50,000	114,000-131,000[c]
#6	O'ahu: Western coast	Wai'anae vs. Kalaeloa	1.3	765,000	565,000	65,000	114,000-131,000[c]
#7	O'ahu/Hawai'i:Northern coast/Western coast	Kapapa vs. Kona/Ka'ū	0.9	530,000	390,000	45,000	80,000-143,000[d]

† calculated based on "net between-group mean" of uncorrected *p* distance. See text for additional details.
* Williams & Knowlton, (2001)
** Brower, (1994)
*** Craft *et al.*, (2008) and this paper (see Results)
[a] Carson & Clague, (1995); http://hvo.wr.usgs.gov/kilauea/
[b] Sherrod *et al.*, (2007)
[c] Szabo *et al.*, (1994)
[d] Grossman & Fletcher, (2004); Fletcher *et al.*, (2006)

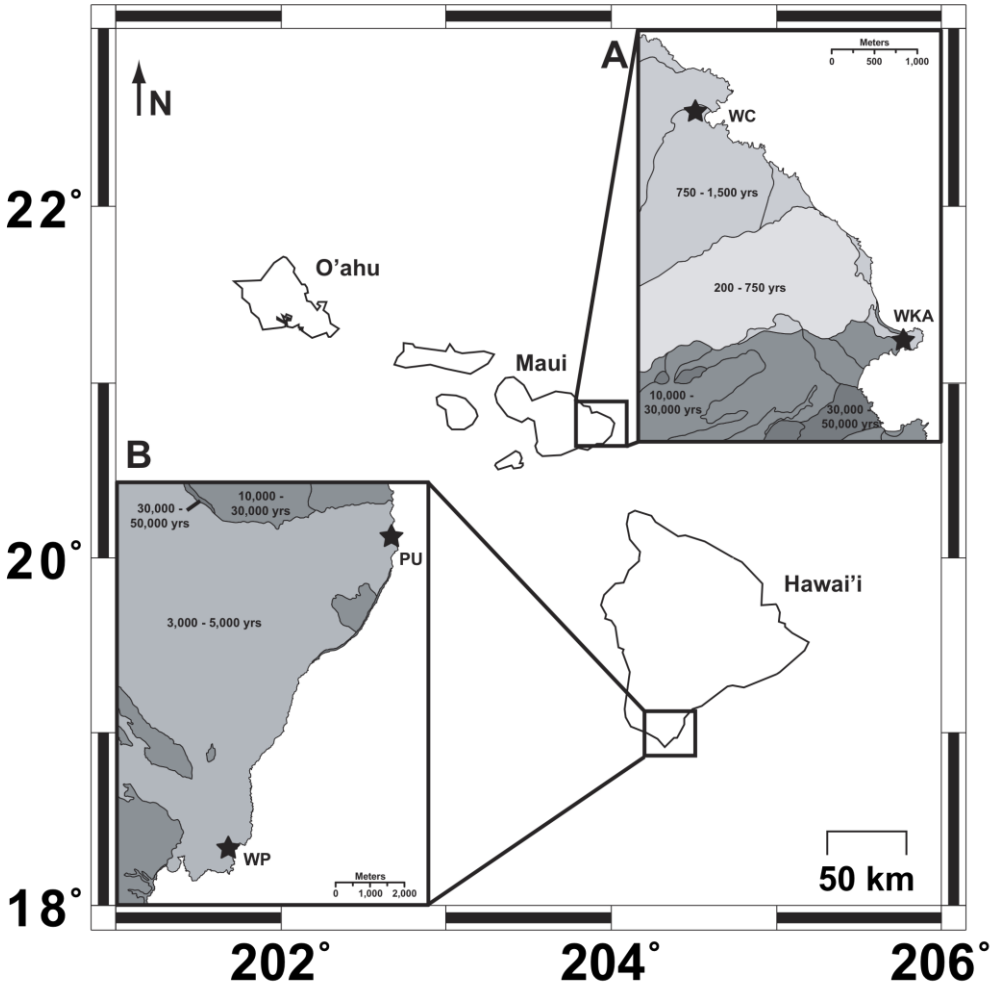

Figure 1: Map of the high Hawaiian Islands depicting anchialine pools where *Halocaridina* were sampled for this study. Inserts: Geologic maps, including ages of local basalt (i.e., lava) flows, for the northeastern coast of Maui (A) and the southeastern coast of the island of Hawai‘i (B). Site codes: Waianapanapa Cave (WC), Waikoloa (WKA), Puhi Ula Cave (PU) and Wai‘ohinu (WP).

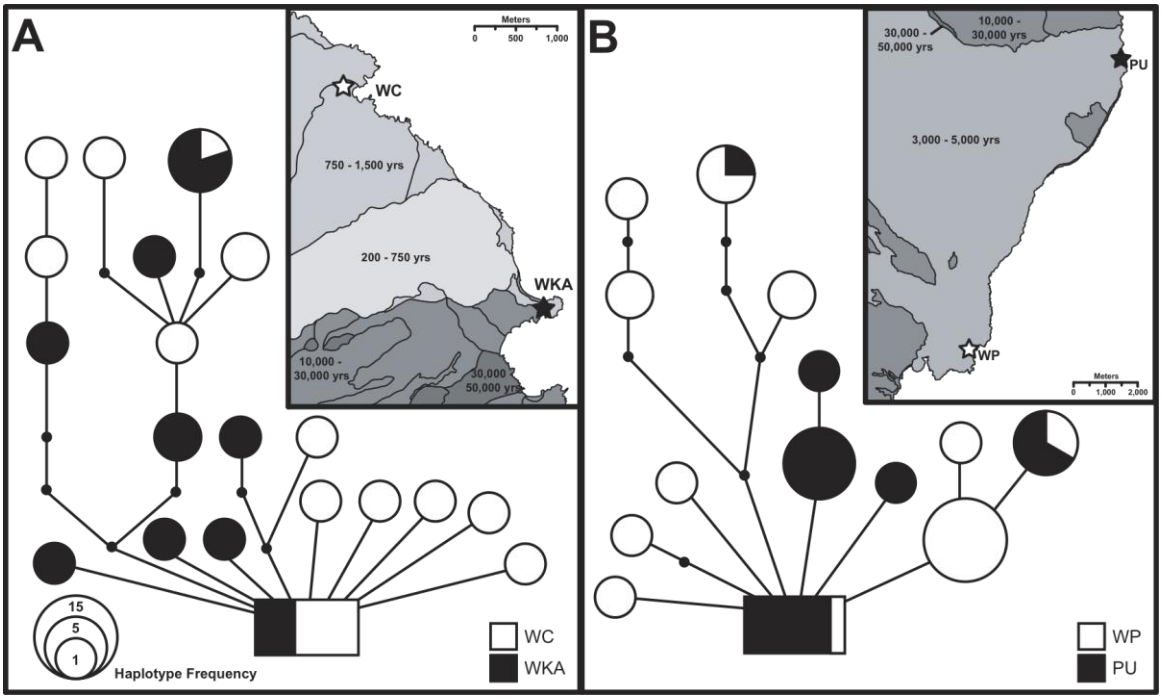

Figure 2: Network analyses for the East Maui (A) and Kaʻū (B) lineages of *Halocaridina* from northeastern Maui and southeastern Hawaiʻi, respectively. Networks depict relationships between cytochrome oxidase subunit I (COI) haplotypes within each lineage. Black dots represent DNA types that might exist in the population but remain unsampled (i.e., missing haplotypes). Rectangles represent DNA types with the highest outgroup probability according to the analysis. The size of circles and rectangles are proportional to the frequency at which a DNA type was recovered. Despite variable lengths, each branch implies a single mutational difference between haplotypes.

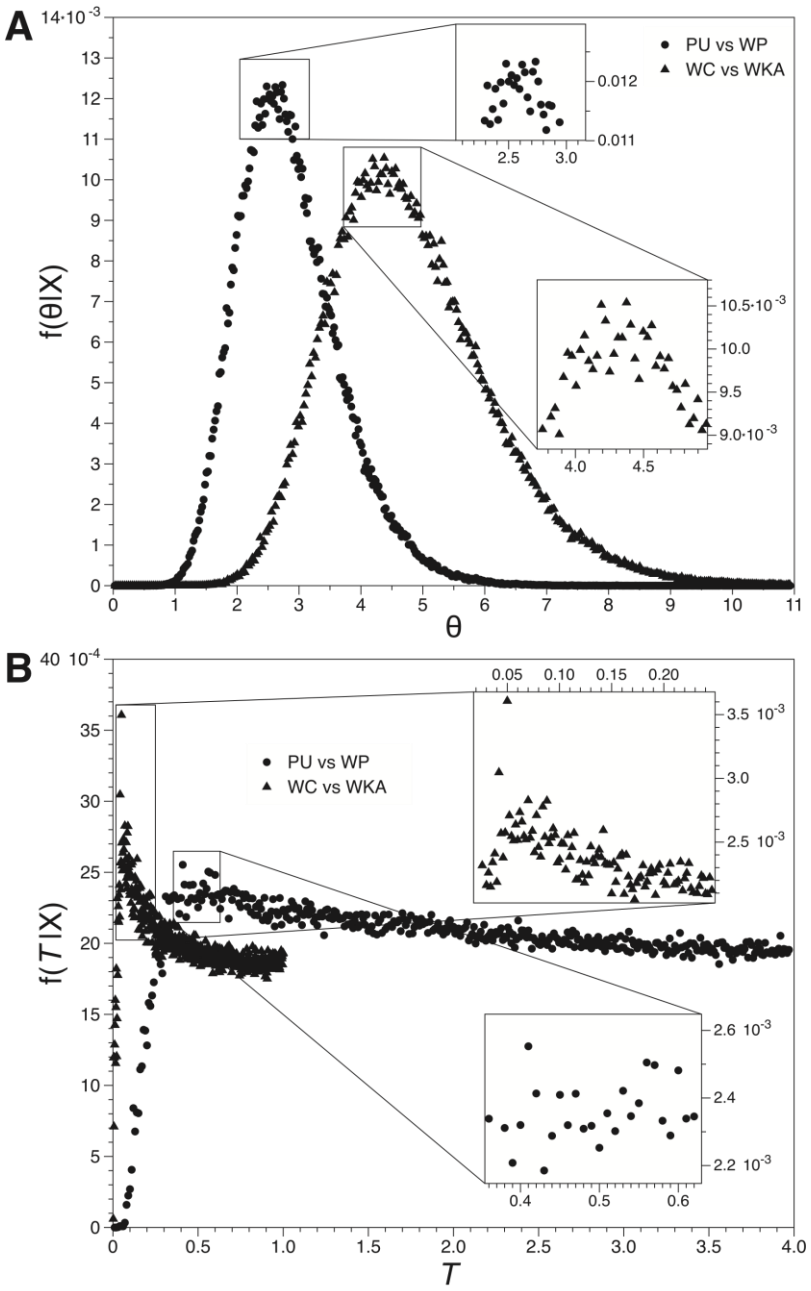

Figure 3: Posterior distributions of theta (θ; A) and divergence time (*T*; B) of *Halocaridina* population pairs from northeastern Maui and southeastern Hawai‘i. The presented posterior distributions each represent an average of three independent runs from the program MDIV (Nielsen & Wakeley 2001) utilizing identical starting conditions but different random seeds. See text for additional details.

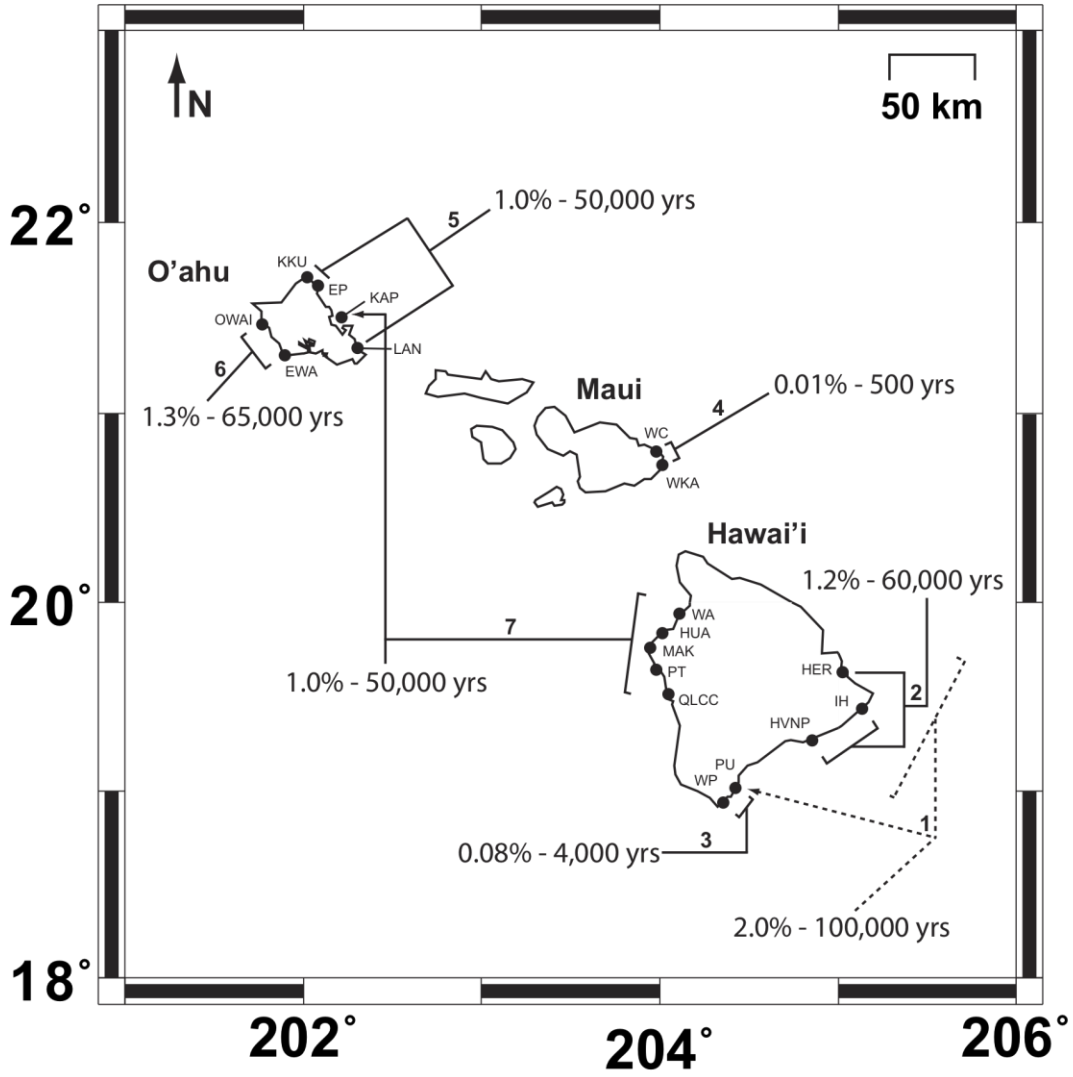

Figure 4: Genetic distances and corresponding estimated times since divergence for seven comparisons between populations, genetic groups and/or lineages of *Halocaridina* across the islands of Hawai‘i, Maui, and O‘ahu. Genetic distance were calculated as “net between-group mean” and estimated times since divergence were calculated using the 20% My^{-1} molecular clock of Craft *et al.*, 2008 calibrated from comparison # 1 (dashed line) to the geologic age of the Kilauea volcano on the island of Hawai‘i. Numbers correspond to comparisons in Table 2; see Craft *et al.*, 2008 for site codes.

CHAPTER 3. Genetic identification of source populations for an aquarium traded invertebrate

3.1 ABSTRACT

Increasingly, wildlife managers are turning to molecular genetics to aid in conservation efforts. While such approaches have been applied to large terrestrial and aquatic vertebrate species, their application to other traded organisms has not been extensively explored. Here, we examined the utility of these techniques for identifying source populations of aquarium ornamental invertebrates, using members of the Hawaiian atyid genus *Halocaridina* as a study system. These shrimp, restricted to anchialine habitats of the Hawaiian Islands, are popular in the aquarium trade due to their ability to survive in hermetically sealed containers for extended periods of time. However, commercial harvesting, coupled with habitat destruction and strong regional endemism, could lead to the depletion/extinction of unique populations. Because the land district of Kona, along the west coast of the island of Hawai‘i, has the state’s highest concentration of anchialine habitats, we hypothesized that commercially available *Halocaridina* originated from this region. To test this, mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences from 96 individuals, obtained from six vendors, were compared to 580 homologous sequences from previous studies covering the known distribution range of *Halocaridina.* Recovery of identical, regional-specific haplotypes, network analyses, and statistical assignment tests identified these commercially acquired specimens as belonging to either the Kona, Ka‘ū, (western and southern coasts, respectively, island of Hawai‘i), or Kina‘u (southern coast, island of Maui) genetic groups of these shrimp. Although 39 of the 96 individuals originated from the Kona genetic group as hypothesized, our finding that commercially available *Halocaridina* are from three genetic groups spanning two islands

suggests that other populations also warrant potential management consideration. While this study represents the first application of molecular genetics in identifying source populations of aquarium ornamental species, we feel that these techniques are amenable more broadly since they are dependent on only a few caveats.

3.2 Introduction

The annual trade in wildlife and related products, both legal and illegal, involves hundreds of millions of plant and animal specimens with an estimated value of billions of US dollars (CITES, 2008). This monetary figure includes proceeds from the stocking of public and private farms, hunting ranches, zoo displays and safari parks as well as organisms for biomedical research and teaching, consumption, and exotic pets. Given the varying demands, laws at the international, national, and local levels have been established to help preserve these resources and wildlife managers have turned to molecular genetics as an aid in these pursuits (Millions & Swanson, 2006; Kyle & Wilson, 2007; Russello *et al.*, 2007). In this context, molecular genetics has addressed issues ranging from taxonomic identification (Palumbi & Cipriano, 1998; Hoelzel, 2001; Clapham & Van Waerebeek, 2007) to determining source populations for specific individuals (Frantz *et al.*, 2006) of traded species.

While such "wildlife forensic" approaches have been primarily applied to large terrestrial (*e.g.*, Guglich, Wilson & White, 1994; Frantz *et al.*, 2006; Wasser *et al.*, 2007) and aquatic vertebrate species such as sharks, whales, and seals (*e.g.*, Malik *et al.*, 1997; Cipriano & Palumbi, 1999; Chapman *et al.*, 2003), only a few studies have investigated their utility for other traded organisms, like those popular as aquarium ornamentals (*e.g.*, Stam *et al.*, 2006). This is particularly relevant since it is conservatively estimated that 1,471 fish, 140 stony (i.e.,

scleractinian) coral, and over 500 other invertebrate species are traded as marine ornamentals on a yearly basis (Wabnitz *el al.*, 2003), with most being stocked from wild caught specimens (Andrews, 1990). Because commerce in aquatic ornamental species is a growing industry (Heerbrandt & Lin, 2006), worth US $200 - $300 million annually (Wabnitz *el al.*, 2003), effective management strategies will need to be implemented to ensure sustainability of these resources into the future.

In recent years, shrimps from the family Atyidae have grown in popularity as aquatic ornamentals (Heerdebrant & Lin, 2006). One species, *Halocaridina rubra* (Holthuis, 1963) (Crustacea: Decapoda: Atyidae), commonly referred to as ‘*ōpae* ‘*ula* (*lit*. tiny red shrimp) in the Hawaiian language, has garnered particular attention among hobbyists. This interest stems from the ability of *H. rubra* to survive in hermetically sealed containers for extended (i.e., > 10 yr) periods of time (Maciolek, 1983) and has lead to an industry where enclosed ecosystems containing these small (i.e., < 12 mm) shrimp are sold around the world as novelty “self-sustaining and perfect” pets. Additionally, *H. rubra* is marketed in both pet stores and via the World Wide Web as a live food for the rearing and maintenance of ornamental fish species. These practices raise concerns for two reasons. Firstly, *H. rubra* is restricted to anchialine habitats of the Hawaiian Islands. Anchialine habitats are land-locked bodies of brackish to marine water with no surface connection to the ocean but whose waters fluctuate with the tides due to subterranean connections (Holthuis, 1973; Maciolek, 1983; 1986). Historically, these habitats have experienced significant negative impacts from a variety of anthropogenic sources (reviewed by Santos, 2006). Secondly, mitochondrial DNA (mtDNA) analyses have identified at least 13 genetic groups of *H. rubra* across the Hawaiian Archipelago, each of which is confined to a single island or a particular subregion of an island (Craft *et al.*, 2008; Fig. 1). Thus,

commercial harvesting, coupled with habitat destruction as well as strong regional endemism, could lead to the depletion and/or extinction of unique *Halocaridina* populations or genetic groups.

An important step towards developing sound conservation strategies for aquarium ornamental species like *Halocaridina* is to first identify populations warranting management due to potential overexploitation. Here, we examine the effectiveness of "wildlife forensics" for addressing such a situation. We hypothesized that commercially available *Halocaridina* originate from the Kona genetic group of these shrimp, which is distributed on the western coast of the island of Hawai‘i (Fig. 1). Along with having the highest concentration of anchialine habitats in the Hawaiian Archipelago (Maciolek & Brock, 1974), this area is easily accessible by ground vehicles and in close proximity to an international airport, both of which would facilitate the rapid transport of live animals. To test our hypothesis, animals were purchased from local stores in Hawai‘i as well as vendors on the World Wide Web and sequence data from the mtDNA cytochrome *c* oxidase subunit I (COI) gene of these individuals were compared to a large database of homologous sequences from previous ecological and evolutionary studies covering the known distribution range of the genus (Santos, 2006; Craft *et al.*, 2008). This approach allowed us to assign these "unknown" individuals to specific *Halocaridina* genetic groups from particular geographic regions of the Hawaiian Islands and highlights the utility of molecular genetics in identifying source populations of commercially traded aquarium ornamental species.

3.3 Materials and Methods

3.3.1 Biological Materials

Commercial vendors marketing *Halocaridina* were identified through searches of the World Wide Web. From these, we purchased specimens from five stores on two Hawaiian Islands, Hawai‘i (3) and O‘ahu (2) (Table 1). Along with this, three enclosed ecosystems originating from a single distributor were acquired from retail stores, or by mail order, within the continental United States (Table 1). Each group of commercially acquired *Halocaridina* was represented by between 3 and 31 individuals, for a total sample size of 96 animals (Table 1). Cost per individual shrimp ranged from US $0.1, when sold as live fish food in Hawai‘i, to ~$22, when acquired as part of an enclosed ecosystem.

3.3.2 DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

Total nucleic acid was extracted from each *Halocaridina* individual as described by Santos (2006). Following extraction, ~10 – 30 ng of DNA was utilized as template to amplify an ~700 bp fragment of the COI gene. Polymerase chain reactions (PCRs) 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, 1 U *Taq* DNA polymerase and 0.4 μM each of primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) following the conditions outlined by Santos (2006). Amplifications were confirmed by electrophoresing an aliquot of the amplified product in a 1% agarose gel, followed by staining and viewing under UV light.

Amplified products were purified with Montage PCR Filter Units (Millipore, Billerica, MA, USA) following the supplier’s protocol, cycle-sequenced in both directions using Big-Dye

Terminators, and read on a PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Ambiguities in the chromatograms were corrected by comparison to the complementary DNA strand in Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Completed sequences of 630 bp were aligned manually using SE-AL version v2.0a11 (available at http://tree.bio.ed.ac.uk/software/seal/).

3.3.3 Genetic Analyses

In order to assign individuals of the commercially acquired *Halocaridina* to previously identified genetic groups, their sequences were compared to an existing database of homologous *Halocaridina* COI sequences (Santos, 2006; Craft *et al.*, 2008). The database contained 580 individuals possessing 221 unique mtDNA COI sequence haplotypes that have been previously identified as clustering into 13 distinct genetic groups (Craft *et al.*, 2008; Table 2). Sample sizes for each of the genetic groups were 6 – 162 individuals and 2 – 67 unique mtDNA COI haplotypes, respectively (Table 2). Each genetic group is characterized as having near or complete genetic isolation as measured via pairwise Φ_{ST} statistics and distributions restricted to ~30 km on an island (Craft *et al.*, 2008; Fig. 1).

Commercially acquired individuals of *Halocaridina* were assigned to the previously identified genetic groups via three approaches. Firstly, the total dataset of 676 sequences was analyzed with Collapse v1.2 (available from http://darwin.uvigo.es). This allowed binning of the commercially acquired *Halocaridina* individuals into two categories: region-specific haplotypes that had been sampled in previous studies and unique haplotypes recovered here. Secondly, haplotype networks were constructed with the program TCS v1.21 (Clement *et al.*, 2000) using the total dataset to determine the relationship among all haplotypes, including novel ones that

had not been sampled previously. Thirdly, statistical assignment of the commercially acquired individuals to known genetic groups was conducted with the Segregating Sites Assigner, which utilizes a model based, decision theoretic assignment approach, based on the theory of segregating sites probability, to assign newly sequenced individuals to predefined groups and calculates posterior probability and risk values for each assignment (Abdo & Golding, 2007). In cases where approximately equal posterior probabilities were obtained between two groups, such as the Kona and Kaʻū genetic groups due to their shallow genetic divergence, assignment of that individual was based on the lower risk value, which represents a smaller probability the assignment to that particular group is incorrect (Abdo & Golding, 2007).

3.4 RESULTS

The number of COI haplotypes recovered per group of commercially acquired *Halocaridina* ranged from 2 – 18 (Table 1). In total, 51 haplotypes were identified from the 96 individuals examined here (Table 1). Of these, 17 were identical across 630 bp of sequence to haplotypes previously recovered from the Kona, Kaʻū (the western and southern coasts, respectively, of the island of Hawaiʻi), or Kinaʻu (the southern coast of the island of Maui) genetic groups of *Halocaridina* (Supplementary Table S1; Craft *et al.,* 2008). The remaining 35 novel haplotypes also belonged to one of these three genetic groups, with network analyses nesting each within one of them (Fig. 2). In all cases, novel haplotypes differed by no more than 4 bp from haplotypes previously recovered in field studies (Fig. 2; Santos, 2006; Craft *et al.,* 2008). Representative sequences of these unique *Halocaridina* COI haplotypes were deposited into GenBank under Accession Numbers EU784700 – EU784734.

Statistical assignment of the commercially acquired *Halocaridina* specimens further supported their origin from the Kona, Kaʻū, or Kinaʻu genetic groups of these shrimp since all 96 animals were placed within one of these three groups. Overall, the majority of individuals (90 of 96; ~94%) were assigned either to the Kona or Kaʻū genetic groups with mean risk values of 0.004 and 0.002, respectively (Table 3). Of these 90 individuals, 39 and 51 belonged to the Kona and Kaʻū genetic groups, respectively (Table 3). The remaining six individuals, originating from two of the three enclosed ecosystems, were assigned to the Kinaʻu genetic group with mean risk values of 0.001 (Table 3). With the exception of two cases, each batch of commercially acquired *Halocaridina* appeared to be a mixture of haplotypes from two of the three genetic groups, with the majority of individuals coming from a single genetic group (Table 3). Congruent results to the above were obtained from statistical assignment tests conducted with the program BPSI2.0 (Zhang & Savolainen 2008).

3.5 Discussion

The data presented here demonstrate the utility of molecular genetics as a tool for identifying source populations of a commercially traded aquarium ornamental species. To the best of our knowledge, this study constitutes the first such application of "wildlife forensics" to an invertebrate popular in the aquarium trade. Although we focused on *Halocaridina* from the Hawaiian Archipelago, these techniques can be applied to other organisms, either aquarium ornamental species or broader ranging, since they are dependent on only a few caveats. Firstly, markers possessing the desired level of genetic resolution (i.e., species, population and/or individual) are necessary for the organism of interest. Potential loci for taxonomic classification include genes and spacers of the nuclear rDNA operon (reviewed by Hillis & Dixon, 1991;

Coleman, 2003) or organellar "barcoding" genes such as mitochondrial COI (e.g., Herbert *et al.,* 2003) and chloroplastic *matk* (Dawnay *et al.,* 2007). The identification of populations and/or individuals, on the other hand, can also be accomplished with organellar data (as done in this study) or via high-resolution fragment-based (e.g., microsatellite loci, amplified fragment length polymorphisms ((AFLP; Vos *et al.,* 1995), etc) methods. Secondly, a database of "references" is required to facilitate assignment of "unknown" individuals (i.e., Dawnay *et al.,* 2007) and this may be generated from direct studies and/or obtained from electronic resources such as GenBank. Lastly, knowledge of the spatial distribution of genetic variation for the targeted group is needed for assignments to be done in a statistical framework. In our case, the high genetic diversity and strong population structure previously found in *Halocaridina* across the Hawaiian Islands (Santos, 2006; Craft *et al.,* 2008) allowed assignment of each individual examined here to a previously recognized genetic group. Thus, similar results should be expected from any species whose populations exhibit some level of genetic structuring between them.

In this study, the bulk of commercially acquired *Halocaridina* specimens originated from genetic groups endemic to the island of Hawai‘i. Since ~300 of the > 520 anchialine habitats in the Hawaiian Archipelago occur on this island (Maciolek & Brock, 1974; Brock *et al.,* 1987), we anticipated Hawai‘i to be the primary source of *Halocaridina* in the aquarium trade. However, while 39 specimens belonged to the Kona genetic group, which was consistent with our hypothesis, a nearly equivalent number (51 of 90; ~57%) were assigned to the Ka‘ū genetic group of these shrimp (Table 3). Likewise, when placed in the context of vendors, *Halocaridina* from three of the six originate from this genetic group (Table 3). This was unexpected because in contrast to anchialine habitats along the Kona coast (see Introduction), those inhabited by the

Kaʻū genetic group are located 10 – 15 km from the nearest major road, typically accessible only by foot or all-terrain vehicle, and > 100 km from an airport. Thus, significant investments of time and resources are being made in acquiring animals from this region of the island of Hawaiʻi.

Although the majority of *Halocaridina* are being supplied from populations on the island of Hawaiʻi, individuals from two of the three enclosed ecosystems were assigned to the Kinaʻu genetic group from the island of Maui. This was surprising since relative to Hawaiʻi, the number of anchialine habitats on Maui and Oʻahu are much fewer and restricted to small areas on these islands' coasts (Fig. 1). For these reasons, the opportunity to harvest *Halocaridina* on Maui and Oʻahu is more limited. Furthermore, most of Maui's anchialine habitats reside within the boundaries of either parks or natural area reserves administered by the State of Hawaiʻi. For example, the Kinaʻu genetic group of *Halocaridina* has only been surveyed from the Ahihi-Kinaʻu Natural Area Reserve (Maciolek, 1986) and the immediate area around it (Craft *et al.*, 2008). This suggests that some commercially available specimens of *Halocaridina* may be originating from habitats designated for conservation and protected by state laws. Though our data implies a potential case of poaching, a cautionary interpretation is warranted due to the fact that this genetic group is also found on properties adjacent to the reserve and collections may be taking place there. Thus, to confirm whether poaching is occurring within the Ahihi-Kinaʻu Natural Area Reserve will require monitoring of the area for illegal activities. In either case, however, identifying the Kinaʻu genetic group as a source of *Halocaridina* in the aquarium trade indicates that commercial harvesting of these shrimp is not restricted to only the island of Hawaiʻi.

Our finding that commercially available *Halocaridina* are from genetic groups spanning three geographic regions on two islands implies that multiple collectors may be involved in

harvesting for the aquarium trade. Multiple collectors also appear to be supplying some vendors, as is the case with animals being marketed as part of the enclosed ecosystems, where a single unit can contain individuals belonging to genetic groups from different islands (Table 3). Although our data suggest that the other vendors are also acquiring *Halocaridina* from multiple genetic groups (Table 3), this may not be the situation since low levels of gene flow have been measured between the Kona and Kaʻū genetic groups in the form of the sporadic recovery of haplotypes outside their respective regions (Santos, 2006; Fig. 2). For this reason, these outliers within a group of animals from a single vendor may represent migrants between the genetic groups rather than collections from multiple source populations.

Although overexploitation is considered one of the greatest threats to the survival of vulnerable populations (e.g., Manel, Berthier & Luikart, 2002), the negative impacts of other anthropogenic activities also need to be taken into consideration. For anchialine habitats around the world, these include habitat destruction, vandalism, and groundwater contamination (reviewed by Iliffe, 2002; 2003). Issues such as these are exemplified in the Hawaiian Archipelago, where numerous anchialine habitats have been lost or modified due to urbanization as well as by the introduction of exotic species (Maciolek & Brock, 1974; Bailey-Brock & Brock, 1993; Brock & Bailey-Brock, 1998). To mitigate this, the State of Hawai'i and private entities have established reserves in order to protect anchialine habitats and their endemic biota (reviewed by Santos, 2006). This study provides an additional resource in these efforts by identifying the geographic regions serving as sources of *Halocaridina* in the aquarium trade. However, while such information can help to focus conservation planning, it must be remembered that regulating the collection and trade of species presents great challenges (e.g., Wasser *et al.*, 2004) and other alternatives may be preferable. While *Halocaridina* is currently

being acquired from wild populations, as evident by the yearly issuing of commercial harvesting permits (State of Hawai‘i Division of Aquatic Resources, personal communication), these shrimp can be readily propagated in captivity (Couret & Wong, 1978; Bailey-Brock & Brock, 1993; personal observation). This offers the opportunity for aquarium-traded specimens to one day be solely supplied at sustainable yields via captive husbandry. For this reason, development of commercially viable aquaculture techniques should be pursued for *Halocaridina* since such a practice would satisfy growing demands and promote commerce while simultaneously alleviating pressures on natural populations and their anchialine habitats in the future.

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3.7 References

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Table 1. Numbers of individuals and unique haplotypes recovered for the commercially acquired *Halocaridina* specimens examined in this study.

	Location	***n***	***nh***
Eco_1	Georgia	4	4
Eco_2	Alabama	3	2
Eco_3	Arizona	4	4
FUKU	Hawai‘i, Hawai‘i	13	11
MP	O‘ahu , Hawai‘i	14	10
SK	O‘ahu , Hawai‘i	10	6
STPT	Hawai‘i, Hawai‘i	31	18
STY	Hawai‘i, Hawai‘i	17	13
Total		96	52

n = number of sampled individuals; *nh* = number of recovered haplotypes.

Table 2. Numbers of individuals and unique haplotypes for each of the known genetic groups of *Halocaridina* (modified from Craft *et al.* (2008)).

Island	**Genetic Group**	***n***	***nh***
Hawai‘i	Puna	61	33
	South Hilo	32	11
	Na‘alehu	30	5
	Kona	162	67
	Ka‘ū	50	26
Maui	Kinau	56	24
	Hana	29	13
Oahu	Ewa	33	13
	Kalaeloa	33	5
	Waianae	28	7
	Kahuku	30	8
	Lanikai	6	2
	Kapapa	30	7
Total		580	221

n = number of sampled individuals; *nh* = number of recovered haplotypes.

Table 3. Genetic group identification of commercially acquired *Halocaridina* specimens examined in this study based on haplotype network analysis and the Segregating Sites Assigner (Abdo and Golding, 2007).

		Kona			**Ka‘ū**			**Kina‘u**		
	n	TCS	*pp*	Risk	TCS	*pp*	Risk	TCS	*pp*	Risk
Eco_1	4	1	0.47175	0.00337	-	-	-	3	0.71945	0.00116
Eco_2	3	-	-	-	-	-	-	3	0.71643	0.00120
Eco_3	4	4	0.47504	0.00355	-	-	-	-	-	-
FUKU	13	2	0.41735	0.00374	11	0.47741	0.00243	-	-	-
MP	14	13	0.47788	0.00331	1	0.47338	0.00252	-	-	-
SK	10	2	0.47243	0.00421	8	0.47189	0.00126	-	-	-
STPT	31	2	0.47844	0.00375	29	0.47666	0.00204	-	-	-
STY	17	15	0.48512	0.00334	2	0.47524	0.00295	-	-	-
Total	96	39	0.47972	0.00375	51	0.47595	0.00200	6	0.71794	0.00118

n = number of sampled individuals; *pp* = posterior probability of assignment

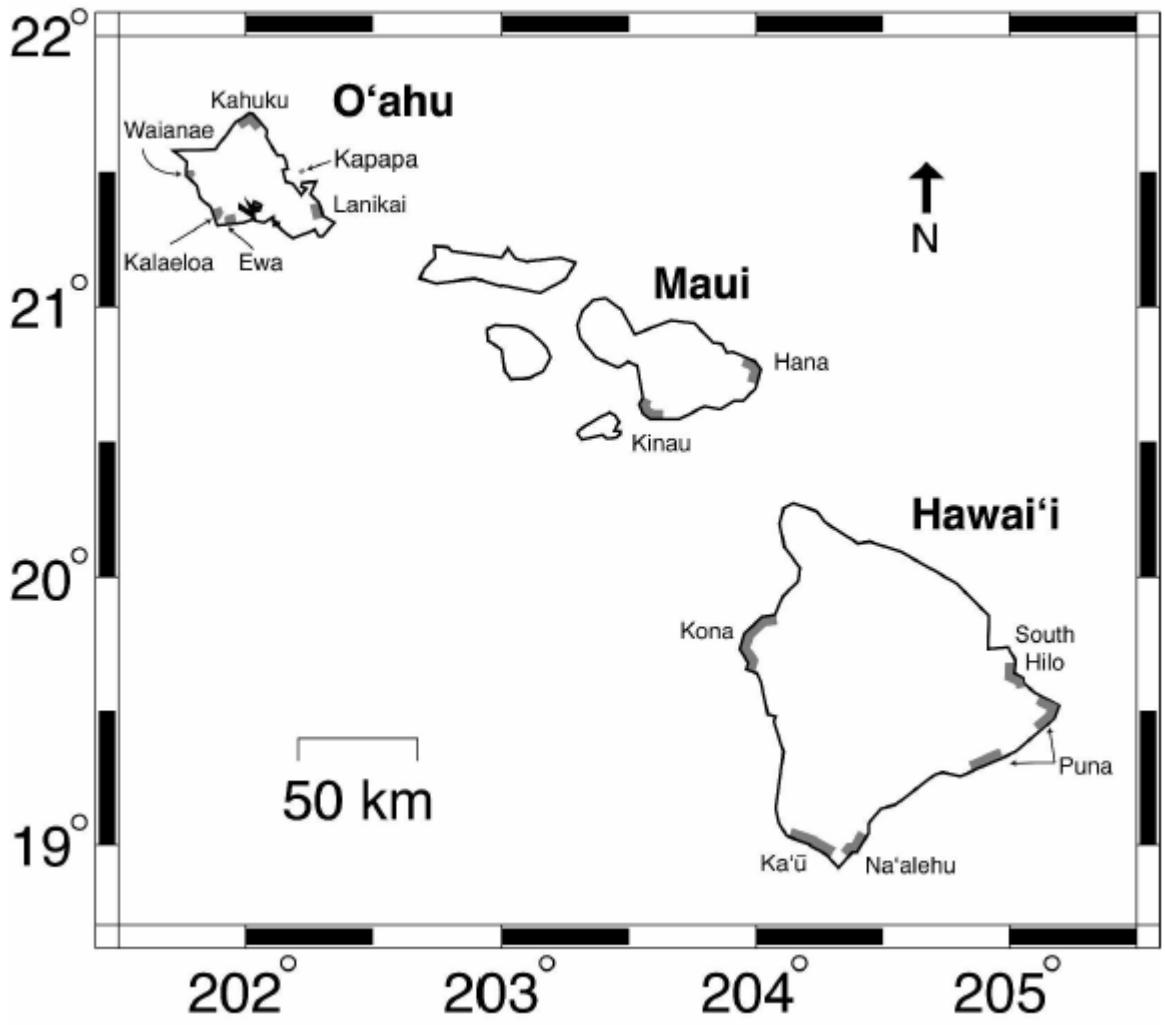

Figure 1. Localities of anchialine habitats (shaded gray) on the Hawaiian islands of O‘ahu, Maui and Hawai‘i and the range distributions of the 13 genetic groups of *Halocaridina*. See text for additional details and references.

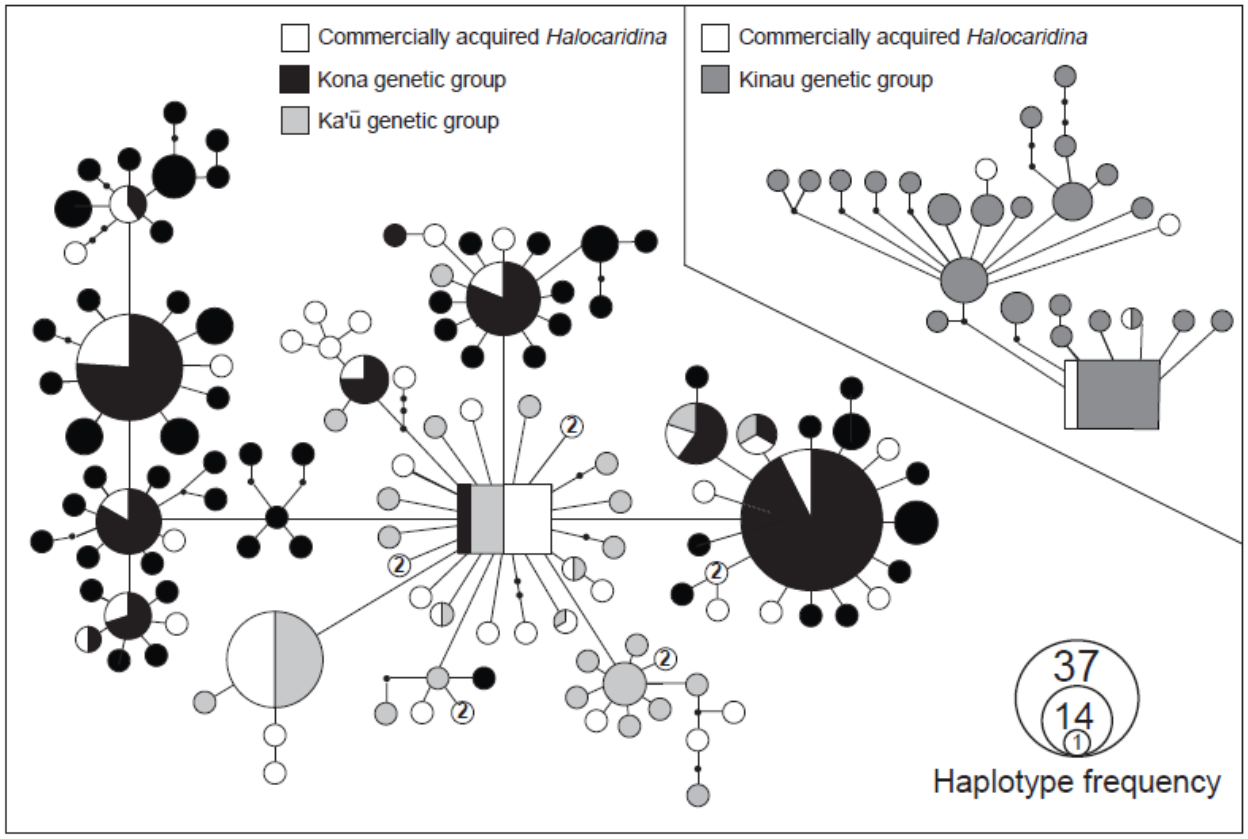

Figure 2. Haplotype network depicting the relationships between cytochrome oxidase subunit I (COI) haplotypes previously recovered from the Kona, Kaʻū, and Kinaʻu genetic groups of *Halocaridina* on Hawaiʻi and Maui (see Santos 2006 and Craft *et al.* 2008) and haplotypes recovered from commercially acquired *Halocaridina* specimens examined in this study (see Supplementary Table S1 for exact haplotype identifications). Numbers within circles represent the number of commercial specimens from which that haplotype was recovered if greater than a single individual. Rectangles represent the haplotype with the highest outgroup probability in each network and the size of circles and rectangles is proportional to the frequency at which a haplotype was recovered. Black dots in a network represent unsampled (i.e., missing) haplotypes. Despite variable lengths, each branch implies a single mutational difference between haplotypes. See Santos (2006) and Craft *et al.* (2008) for additional details on the population structure and evolution of *Halocaridina* in the Hawaiian Archipelago.

Supplementary Table S1. Posterior probability and risk for the assignment of each commercially acquired *Halocaridina* specimen to the Kona, Kaʻū (islands of Hawai'i) or Kinaʻu (islands of Maui) genetic groups of these shrimp. Previously recovered haplotypes and novel haplotypes are indicated, along with their corresponding Genbank accession numbers; *pp* = posterior probability.

	Kona		Kaʻū		Kinaʻu					
Sample	***pp***	**Risk**	***pp***	**Risk**	***pp***	**Risk**	**Identical to field collected haplotype:**	**Acession Number:**	**New haplotype:**	**New Acession Number:**
ECO1_1	0.47175	0.00337	0.47338	0.00168	0.05487	0.04650	K2	DQ399170	-	-
ECO1_2	0.21305	0.04005	0.04826	0.04682	0.73869	0.00167	HM20	EF173774	-	-
ECO1_3	0.23984	0.03740	0.06015	0.04465	0.70001	0.00048	-	-	HM25	EU784700
ECO1_4	0.22640	0.03806	0.05394	0.04494	0.71966	0.00134	HM5	EF173759	-	-
ECO2_1	0.23422	0.03897	0.05580	0.00425	0.70998	0.00092	-	-	HM26	EU784701
ECO2_2	0.22640	0.03806	0.05394	0.04494	0.71966	0.00134	HM5	EF173759	-	-
ECO2_3	0.22640	0.03806	0.05394	0.04494	0.71966	0.00134	HM5	EF173759	-	-
ECO3_1	0.47772	0.00417	0.47202	0.00252	0.05026	0.04834	-	-	K91	EU784702
ECO3_2	0.47175	0.00421	0.47338	0.00252	0.04587	0.04810	K21	DQ399189	-	-
ECO3_3	0.46812	0.00169	0.46974	0.00169	0.06213	0.04553	K7	DQ399175	-	-
ECO3_4	0.48255	0.00413	0.46132	0.00257	0.05612	0.04804	-	-	K92	EU784703
FUKU1	0.47175	0.00412	0.47338	0.00252	0.05487	0.04490	K24	DQ399192	-	-
FUKU2	0.47432	0.00335	0.46866	0.00169	0.05702	0.04639	-	-	K93	EU784704
FUKU3	0.46678	0.00425	0.48411	0.00246	0.04902	0.04840	-	-	K94	EU784705
FUKU4	0.47175	0.00421	0.47338	0.00252	0.05487	0.04810	-	-	K95	EU784706
FUKU5	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
FUKU6	0.45992	0.00344	0.47699	0.00166	0.06309	0.04451	-	-	K96	EU784707
FUKU7	0.48123	0.00497	0.46618	0.00340	0.05259	0.04822	-	-	K97	EU784708
FUKU8	0.47396	0.00589	0.48648	0.00410	0.03956	0.05215	-	-	K98	EU784709
FUKU9	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
FUKU10	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
FUKU12	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
FUKU13	0.46812	0.00339	0.46974	0.00169	0.06213	0.04297	K83	DQ399251	-	-
FUKU14	0.47126	0.00592	0.48371	0.00412	0.04503	0.05185	-	-	K99	EU784710

MP1	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
MP2	0.48255	0.00247	0.46132	0.00257	0.05612	0.04644	-	-	K100	EU784711
MP3	0.47175	0.00337	0.47338	0.00168	0.05487	0.04650	K2	DQ399170	-	-
MP4	0.48255	0.00413	0.46123	0.00257	0.05612	0.04804	-	-	K92	EU784703
MP5	0.46812	0.00169	0.46974	0.00169	0.06213	0.04553	K7	DQ399175	-	-
MP6	0.47454	0.00251	0.45367	0.00261	0.07179	0.04253	-	DQ399217	-	-
MP7	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
MP8	0.51004	0.00548	0.43064	0.00454	0.05932	0.04788	-	DQ399235	-	-
MP9	0.46812	0.00169	0.46974	0.00169	0.06213	0.04553	K7	DQ399175	-	-
MP10	0.47175	0.00421	0.47338	0.00252	0.05487	0.04810	-	-	K95	EU784706
MP11	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
MP12	0.48600	0.00410	0.46458	0.00256	0.04947	0.04838	-	-	K101	EU784712
MP13	0.47053	0.00507	0.46492	0.00341	0.06454	0.04602	-	-	K102	EU784713
MP14	0.47454	0.00251	0.45367	0.00261	0.07179	0.04253	K49	DQ399217	-	-
SK1	0.47053	0.00422	0.46492	0.00256	0.06455	0.04602	-	-	K103	EU784714
SK2	0.47432	0.00419	0.46866	0.00254	0.05702	0.04799	-	-	K104	EU784715
SK3	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
SK4	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
SK5	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
SK6	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
SK7	0.47053	0.00376	0.46492	0.00170	0.06455	0.04444	-	-	K105	EU784716
SK8	0.46812	0.00391	0.46974	0.00169	0.06213	0.04614	K85	DQ399253	-	-
SK9	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
SK10	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STPT1	0.46354	0.00342	0.48074	0.00165	0.04657	0.04657	K90	DQ399258	-	-
STPT2	0.47432	0.00352	0.46866	0.00169	0.05702	0.04639	-	-	K93	EU784704
STPT3	0.47126	0.00507	0.48371	0.00329	0.04503	0.05227	-	-	K106	EU784717
STPT4	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STPT5	0.47772	0.00417	0.47202	0.00252	0.05260	0.04834	-	-	K107	EU784718
STPT7	0.48443	0.00492	0.46928	0.00384	0.04629	0.05016	-	-	K108	EU784719
STPT8	0.48255	0.00329	0.46132	0.00171	0.05612	0.04644	-	-	K109	EU784720
STPT9	0.47999	0.00582	0.47614	0.00418	0.04385	0.05192	-	-	K110	EU784721
STPT10	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STPT11	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STPT12	0.48255	0.00330	0.46132	0.00171	0.05612	0.04644	-	-	K109	EU784720
STPT13	0.46812	0.00254	0.46974	0.00084	0.06213	0.04553	K13	DQ399181	-	-

STPT14	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
STPT22	0.47432	0.00419	0.46866	0.00254	0.05702	0.04799	-	-	K104	EU784715
STPT23	0.46678	0.00425	0.48411	0.00246	0.49110	0.04840	K70	DQ399238	-	-
STPT24	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STPT25	0.46812	0.00391	0.46974	0.00169	0.06213	0.04614	K85	DQ399253	-	-
STPT26	0.46354	0.00342	0.48074	0.00165	0.05572	0.04657	K69	DQ399237	-	-
STPT27	0.48255	0.00330	0.46132	0.00171	0.05612	0.04644	-	-	K111	EU784722
STPT28	0.46354	0.00342	0.48074	0.00165	0.04646	0.04646	K69	DQ399237	-	-
STPT29	0.45215	0.00613	0.50846	0.00392	0.03934	0.05216	-	-	K112	EU784723
STPT30	0.45590	0.00347	0.47282	0.00168	0.07128	0.04255	-	-	K113	EU784724
STPT31	0.46954	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
STPT32	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
STPT33	0.47658	0.00586	0.48189	0.00413	0.41523	0.05205	-	-	K114	EU784725
STPT34	0.46354	0.00342	0.48074	0.00165	0.05572	0.04657	K69	DQ399237	-	-
STPT35	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
STPT36	0.46812	0.00254	0.46974	0.00084	0.06213	0.04553	K13	DQ399181	-	-
STPT37	0.47772	0.00417	0.47202	0.00252	0.05026	0.04834	-	-	K115	EU784726
STPT38	0.46354	0.00342	0.48074	0.00165	0.05572	0.04646	K69	DQ399237	-	-
STPT39	0.46329	0.00601	0.49635	0.00402	0.05211	0.05211	-	-	K116	EU784727
STY1	0.47454	0.00251	0.45367	0.00261	0.07179	0.04253	K49	DQ399217	-	-
STY2	0.46812	0.00254	0.46974	0.00084	0.06213	0.04455	K13	DQ399181	-	-
STY3	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
STY4	0.49540	0.00403	0.44699	0.00353	0.05762	0.04957	-	-	K117	EU784728
STY5	0.46354	0.00256	0.48073	0.00248	0.05572	0.04646	-	-	K118	EU784729
STY6	0.49140	0.00406	0.44338	0.00444	0.06522	0.04599	-	-	K119	EU784730
STY7	0.48596	0.00410	0.46458	0.00255	0.04947	0.04838	-	-	K120	EU784731
STY8	0.47454	0.00419	0.45367	0.00261	0.07179	0.04409	K8	DQ399176	-	-
STY9	0.47454	0.00419	0.45367	0.00261	0.07179	0.04409	K8	DQ399176	-	-
STY10	0.47175	0.00337	0.47338	0.00168	0.05487	0.04650	K2	DQ399170	-	-
STY11	0.50184	0.00477	0.44091	0.00536	0.05724	0.04798	-	-	K121	EU784732
STY13	0.47772	0.00417	0.47202	0.00252	0.05026	0.04834	-	-	K122	EU784733
STY14	0.49140	0.00487	0.44338	0.00355	0.06522	0.05989	K18	DQ399186	-	-
STY17	0.47454	0.00335	0.45367	0.00348	0.07179	0.04409	K12	DQ399180	-	-
STY18	0.49140	0.00487	0.44338	0.00355	0.06522	0.05989	K18	DQ399186	-	-
STY19	0.49140	0.00487	0.44338	0.00355	0.06522	0.05989	K18	DQ399186	-	-
STY20	0.50580	0.00553	0.42706	0.00457	0.06714	0.04432	-	-	K123	EU784734

CHAPTER 4. The long and short of it: Genetic variation and population structure of the anchialine atyid shrimp *Caridina rubella* on Miyako-jima, Japan

4.1 ABSTRACT

One of the most threatened ecosystems on many islands may be anchialine habitats, or coastal land-locked water bodies with no surface connection to the sea yet containing brackish water that fluctuates with the tides. To better manage these habitats, it is important to develop a broader understanding of the biodiversity within them since such knowledge plays critical roles in establishing conservation strategies. In this study, the genetic variation and population structure of an anchialine atyid shrimp, *Caridina rubella* Fujino and Shokita, 1975, was investigated in the Southern Ryukyu Archipelago, Japan. Given a planktotrophic larval stage and its potential amphidromous life cycle, populations of *C. rubella* on the island of Miyako-jima (to which it is apparently restricted) were hypothesized to have little to no structure across the island. To test this, 61 individuals were collected from four anchialine caves and sequence variation examined at the cytochrome *c* oxidase subunit I (COI) region of the mitochondrial DNA (mtDNA). Surprisingly, significant genetic structure was exhibited across distances ranging from < 20 m to > 10 km. Additionally, deep (~17% *p*-distance) genetic divergence correlating with distinct variation in rostrum lengths was found between closely situated, but more-or-less completely isolated, populations. This implies "*C. rubella*" may actually represent two distinct species on Miyako-jima. Given that this atyid is already listed as a threatened species by the Japanese government, the results presented here are useful in the formulation and implementation of future conservation plans for *C. rubella* populations on Miyako-jima.

4.2 INTRODUCTION

Global climate change, the introduction of invasive species, and habitat destruction have become significant threats to biodiversity around the world. This loss of biodiversity is occurring at such a rate that there is the potential risk of losing numerous ecosystems without knowing the full nature and extent of the biodiversity contained within them. One of the clearest demonstrations of ecosystem degradation by human impact can be seen on island systems, which intrinsically have much higher extinction rates than continental areas (Gerlach, 2008; Gillespie *et al.*, 2008). Given this, it is not surprising that nine of the top 25 biodiversity 'hotspots' for conservation priorities are predominately, or completely, made up of islands (Myers *et al.*, 2000).

One of most threatened habitats on many tropical islands may be the anchialine ecosystem (Sket, 1996; Iliffe, 2002; 2003; Santos, 2006). Anchialine habitats are coastal land-locked bodies of water with no surface connection to the sea yet contain salt or brackish water that fluctuate with the tides due to subterranean connections to the ocean (Holthuis, 1973; Maciolek, 1983). While such environments occur in the Sinai Peninsula, Bermuda, the Caribbean, the Hawaiian Islands, the South Pacific, the Philippines, and the Ryukyus, only ~1,000 habitats fitting this ecosystem definition have been reported globally (Maciolek, 1986). Unfortunately, many of these habitats have experienced significant negative impacts worldwide from a variety of anthropogenic sources such as habitat destruction, vandalism, and groundwater contamination (Iliffe, 2002, 2003; Santos, 2006). This is alarming since despite the large number of organisms inhabiting anchialine environments, such as mollusks and crustaceans (Brock & Kam, 1987), only a few of the ~560 currently described species endemic to this ecosystem (Iliffe, 2002; ISI Web of Science search, January 2011) have been examined to date from a population genetic perspective. For some of these cases, little to no genetic structure was

identified among populations separated by ~200 km (Kano & Kase, 2004; Hunter *et al.*, 2008; Russ *et al.*, 2010), implying frequent dispersal over long distances. In contrast, a number of other anchialine species have been found to possess exceptional levels of endemism on the scale of a few km (Santos, 2006; Craft *et al.*, 2008; Hunter *et al.*, 2008; Page *et al.*, 2008), presumably due to low dispersal abilities and/or significant barriers to dispersal. Thus, given the potential for strong regional endemism along with ongoing modification and destruction of anchialine habitats around the world, there is now an urgency to accelerate research into these unique island environments and their biota before they are possibly lost forever.

The islands of East Asia are among the richest in the world in terms of biodiversity (Meijaard, 2003). Pertinent among the region's archipelagos, the Ryukyus are a continuous chain of islands situated between Japan's Kyushu Island and Taiwan. Decapod crustaceans dominate the anchialine biota of the Ryukyus, with at least eleven shrimp and crab species having been documented or formally described (Komai & Fujita, 2005; Cai & Shokita, 2006; Naruse & Tamura, 2006; Fujita, 2007; Fujita & Sunagawa, 2008). Of these, the atyid shrimp, *Caridina rubella* Fujino & Shokita, 1975 (Decapoda: Atyidae), is thought to have a disjunct distribution throughout the Indo-West Pacific, inhabiting anchialine pools and caves in the Philippines (Cai & Anker, 2004), the Cook Islands (T.J. Page personal communication, 2010), Java in Indonesia (T. von Rintelen personal communication, 2010) as well as the Ryukyus (Cai & Shokita, 2006). In the Ryukyus, however, *C. rubella* has been reported from just four of the ~55 islands in the archipelago: Okinoerabu-jima, Okinawa-jima, Minamidaito-jima, and Miyako-jima (Fujino & Shokita, 1975; Shokita & Nishijima, 1976). This restricted distribution along with continued habitat loss has led to the listing of *C. rubella* as a threatened species by the Japanese Ministry of the Environment and Okinawa Prefectural Government (Fujita & Shokita

2005; Shokita, 2006). Given this situation, it is important to develop a better biological understanding of *C. rubella* in the Ryukyus since such information is vital for developing management plans to preserve the species and, by extension, the anchialine ecosystem of these islands. Here, the genetic variation, population structure and demography of *C. rubella* from Miyako-jima in the Southern Ryukyus were investigated via sequence analyses of mitochondrial DNA (mtDNA). Specifically, given that its larvae are planktotrophic and possessing a possible amphidromous life cycle (Shokita, 1979; Fujita, unpublished data), both life history traits thought to be significant in facilitating population connectivity of anchialine taxa (Kano & Kase, 2004; Craft *et al.*, 2008; Russ *et al.*, 2010; Cook *et al.*, 2006, 2008), it is hypothesized that *C. rubella* populations are homogenized among anchialine habitats on the island due to marine and/or groundwater dispersal of larvae and/or adults.

4.3 MATERIAL AND METHODS

4.3.1 BIOLOGICAL MATERIALS

Between July and August 2009, ~42 anchialine habitats were surveyed from nine islands (including Okinawa-jima, Minamidaito-jima, and Miyako-jima) belonging to the Okinawa, Miyako, and Yaeyama island groups of the Ryukyus (Fig. 1). From these surveys, adult specimens of *C. rubella* were only found to inhabit anchialine habitats on Miyako-jima. All sites surveyed were classic examples of anchialine habitats occurring within porous limestone basins, being located < 0.95 km from the coast and showing tidal fluctuations with water depths, salinities, and surface areas ranging between 0.001-2 m, 1-15‰, and ~3-> 50 m^3, respectively. Of these, the species was found at five of seven sites. Due to the close proximity (~25 m) of two of these five habitats, only four were sampled for this study (Fig. 1). However, at one locality

(Ana Ga; ANA), samples were obtained from two ponds separated by < 20 m (ANA1 and ANA2). Notably, individuals of *C. rubella* from ANA2 possessed noticeably longer rostrums relative to individuals from the other populations sampled on the island (see below). Given this, rostrum length, as measured from the end of the eye stock to the end of the rostrum, was recorded for all collected individuals. Unfortunately, due to damage following preservation and transport, only five out of the eight individuals collected from the ANA2 population could be measured for rostrum length. Between eight (8) and 16 individuals of *C. rubella* were collected from each population (Table 1) using baited traps or small aquarium nets and immediately preserved in 99% ethanol for genetic analyses.

4.3.2 DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

Total genomic DNA was extracted from each individual following procedures outlined in Santos (2006) and utilized as template to amplify a ~670 base pair (bp) fragment of the mtDNA cytochrome *c* oxidase subunit I (COI) gene via the polymerase chain reaction (PCR). Reactions were carried out in 25 μL volumes containing ~10-30 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.0 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (*i.e.*, dATP, dCTP, dGTP, and dTTP), 1 U *Taq* polymerase, and 0.4 μM each of primers LCO1490 and HCO2198 (Folmer *et al*., 1994). Reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) following Santos (2006) and amplicons purified with Montage PCR Filter Units (Millipore, Billerica, MA, USA) according to the supplier's protocol. Purified amplicons were then cycle-sequenced in both directions using Big-Dye Terminators, and read on a PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Ambiguities in the chromatograms were corrected by comparison to the

complementary DNA strand in Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Completed sequences of 630 bp were aligned manually using SE-AL version v2.0a11 (available at http://tree.bio.ed.ac.uk/software/seal/).

4.3.3 Genetic Diversity and Parsimony Network Analyses

To assess the genetic diversity within each population of *C. rubella*, nucleotide (π) and haplotype (*h*) diversity indices were calculated according to the methods of Nei (1987) using DnaSP v5.10.00 (Rozas *et al.*, 2009). The relationships among COI haplotypes of *C. rubella* were visualized via statistical parsimony networks constructed with TCS v1.21 (Clement, Posada & Crandall, 2000). This program utilizes the cladogram estimation algorithm of Templeton et al. (1992) and was conducted using the default settings, which provide the 95% parsimoniously plausible branch connections between haplotypes. Reticulations in the network, representing ambiguous connections, were resolved using the criteria outlined in Crandall et al. (1994).

4.3.4 Population Structure, Migration, and Demographic Analyses

To test for genetic differentiation between our populations, pairwise Φ_{ST} statistics (based on haplotype frequency and molecular divergence) were conducted with Arlequin v3.11 (Excoffier & Schneider, 2005). Hudson's (2000) nearest-neighbor statistic (S_{nn}) was also calculated to test for genetic differentiation between populations using DnaSP. This statistic is a measure of how often the 'nearest neighbors' (in sequence space) are from the same locality and performs well in cases of low sample size (Hudson, 2000). Statistical significance of the pairwise Φ_{ST} and S_{nn} analyses were assessed with 10,000 and 1,000 permutations in Arlequin and DnaSP, respectively. Arlequin was also used to perform an analysis of molecular variance

(AMOVA; Excoffier et al. 1992) to determine the manner in which genetic variation was partitioned within *C. rubella* of Miyako-jima. For the AMOVA, Φ-statistics were used to estimate the relative contribution of molecular variance at three hierarchical levels: among geographic regions (Φ_{CT}), among populations within a region (Φ_{SC}) and within populations (Φ_{ST}) and significance assessed by 10,000 permutations. For the pairwise Φ_{ST} statistics and AMOVA, the Tamura & Nei (1993) model of evolution [selected by the Akaike information criterion (AIC) using the FindModel web version of Modeltest (Posada & Crandall, 1998; available at http://darwin.uvigo.es/)] was utilized.

To estimate potential migration between populations of *C. rubella*, the web version of MDIV (Nielsen & Wakeley, 2001; available at http://cbsuapps.tc.cornell.edu) was utilized under a finite-site mutation model, which accounts for multiple mutations per site, nucleotide frequencies differences and transition/transversion biases potentially present in sequence data. For these calculations, 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) were done with different random start seeds 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, as per Nielsen & Wakeley (2001). Lastly, Mantel tests (Mantel, 1967), implemented in Alleles In Space (AIS; Miller 2005), were performed to test for correlations between geographic and genetic distance (isolation by distance) and significance assessed with 10,000 permutations.

Tajima's *D* (Tajima, 1989) and Fu's F_s (Fu, 1997) tests were also conducted in DnaSP to determine whether patterns of mitochondrial COI sequence variation in *C. rubella* were consistent with predictions under the neutral model of evolution. In the absence of selection,

both methods can provide potential information on the recent demographic forces affecting a population (bottlenecks or expansions: Tajima, 1989; Fu, 1997, Akey *et al*. 2004).

4.4 RESULTS

4.4.1 GENETIC DIVERSITY AND PARSIMONY NETWORK ANALYSES OF *C. RUBELLA* ON MIYAKO-JIMA

Direct sequencing of mtDNA COI amplicons yielded a 630 bp fragment from each of the 61 *C. rubella* individuals included in this study. From these, 24 unique haplotypes were identified (Table 1) and deposited into GenBank under accession #'s JF926126-JF926149. Of the 24 haplotypes recovered, 11 occurred as singletons while the remaining 13 were sampled more than once. One hundred and twenty-one (19.2%) of the 630 bp analyzed were found to be variable across haplotypes and 117 (18.6%) of these were parsimoniously informative. Translation of the nucleotide sequences into amino acids found no stop codons and while the majority of substitutions were 'silent', one was found to be non-synonymous, with the resulting change to an amino acid with similar biochemical properties (data not shown). This implies that all sequences were derived from mitochondrial copies of COI and not nuclear copies of mitochondrial derived genes (numts; Lopez *et al.*, 1994), which can be common in arthropods (Buhay, 2009). Overall nucleotide and haplotype diversity indices were similar for all populations (Table 1).

Parsimony network analysis of the complete COI sequence data set produced two discrete networks (Fig. 2). One corresponded to 'long rostrum' (5.06 ± 2.4 mm, $n = 5$) *C. rubella* and consisted of four haplotypes and nine individuals. Eight of these nine individuals were collected from ANA2, while one individual was obtained from ANA1. The second network represented 'short rostrum' (2.55 ± 1.19 mm, $n = 53$) *C. rubella* and encompassed 20 haplotypes recovered

from 52 individuals collected at the ANA1, BUT, ZUZ, and TAG sites (Fig. 1). Uncorrected (*p*) distances among haplotypes within each network ranged from 0.20-1.7% while the *p*-distances between haplotypes in the two networks ranged from 16.3-17.5%.

4.4.2 Population Structure, Migration, and Demographic Analyses of *C. rubella* on Miyako-jima

Eight of the possible ten pairwise comparisons revealed significant genetic differentiation between the examined populations of *C. rubella*. For example, the two close proximity populations of ANA1 and ANA2 exhibited structure approaching the maximum limit of 1.0 (Table 1), implying that nearly no individuals are exchanged between these caves despite being separated by < 20 m (the exception being an apparent dispersal event by a single individual from the ANA2 to the ANA1 site; see above). Along with this, the ANA2 ('long rostrum') population exhibited similar and significant genetic structure relative to all other populations of *C. rubella* on Miyako-jima (Table 2). Due to the large genetic divergence of the ANA2 population as well as its occurrence at only a single site, ANA2 was excluded from subsequent comparative analyses. For the remaining populations of 'short rostrum' *C. rubella*, significant genetic differentiation was observed between sites separated by > 10 km (northern sites compared to southern sites, Table 2) while no significant genetic differentiation was found between populations separated by < 5 km (among northern or southern sites, Table 2). For the AMOVA, populations were grouped by geographic region (northern and southern) based on their proximity to one another. In this context, the AMOVA identified a significant amount of genetic variation as partitioned within individual populations (73%, $\Phi_{ST} = 0.273$, $P < 0.001$) while a large, but non-significant component of genetic variation occurred between geographic regions (25%, $\Phi_{CT} =$

0.250, $P = 0.319$). Likewise, the partition of genetic variation among populations within each region was not significant (2.24%, $\Phi_{SC} = 0.030$, $P = 0.169$).

To assess potential migration (*M*) between populations, estimates were generated via MDIV in a pairwise fashion for 'short rostrum' *C. rubella* (Fig. 3). For populations in close proximity to one another (< 5 km; ZUZ-BUT and ANA1-TAG; Fig. 3A and B), plateaus in the posterior probability distributions of *M* were readily apparent, implying high migration between these sites and consistent with the lack of genetic structure between them (see above). In contrast, populations separated by geographic distances of ~11-13 km possessed discernable peaks in their posterior probability distributions of *M*, indicative of lower levels of migration. For example, estimates of $M = 0.9$ and $M = 0.6$ were recovered for comparisons of ZUZ/ANA1 and ZUZ/TAG (Fig. 1, Fig. 3A), respectively. A similar result ($M = 0.7$) was obtained for BUT and TAG (Fig. 3B) while the estimate for BUT and ANA1 (~11 km) was higher ($M = 2.5$; Fig. 3B). Additionally, the Mantel test identified a significant correlation between the genetic and geographic distances of populations ($r = 0.255$; $P < 0.001$), further supporting the conclusions derived from MDIV. Taken together, these results suggest that isolation by distance drives the genetic structure of 'short rostrum' *C. rubella* on Miyako-jima, with dispersal occurring over short (< 5 km) geographic distances but decreasing with increasing distance between populations. Lastly, both Tajima's *D* and Fu's *F* were not significant for any of the *C. rubella* populations examined in this study (Table 1).

4.5 Discussion

Contrary to the proposed hypothesis, most populations of *C. rubella* of Miyako-jima exhibit significant genetic structure across scales ranging from < 20 m to > 10 km. Along with this, deep (~17% *p*-distance) genetic divergence correlating with distinct variation in rostrum lengths was identified between closely situated, but more-or-less completely isolated, populations of this anchialine atyid "species." The significance and implications of these results are discussed below.

4.5.1 Morphological and Genetic Differentiation in *C. rubella* of Miyako-jima

The relatively high (~17% *p*-distance) genetic divergence, along with the ease of visually distinguishing 'long rostrum' and 'short rostrum' morphotypes, implies "*C. rubella*" of Miyako-jima is actually comprised of two species. Here, the 'short rostrum' morphotype aligns with the original description of the species established by Fujino & Shokita (1975) and suggests that the 'long rostrum' morphotype represents either an undescribed species of *Caridina* or an already described *Caridina* sp. not previously recorded from the anchialine habitats of Miyako-jima. While additional morphological studies are required to distinguish between these possibilities, the restricted distribution of the 'long rostrum' morphotype to just a single site on one island should be taken into consideration when developing conservation and management strategies for "*C. rubella*" and anchialine habitats on Miyako-Jima and the Ryukyus in general.

Although the exact factors leading to the spatial segregation of 'long rostrum' and 'short rostrum' morphotypes have yet to be determined, several hypotheses can be proposed that might explain such a pattern. First, despite being found in close proximity to 'short rostrum' *C. rubella*, the 'long rostrum' morphotype may be physically restricted to the ANA2 pond due to

the geologic characteristics of its particular habitat. Compartmentalization of hypogeal water systems by underground barriers has been shown to be a strong isolating mechanism over short distances for anchialine fauna in the Hawaiian Islands (Santos, 2006; Craft *et al.*, 2008) and may play a similar role on Miyako-jima. If this is the case, studies incorporating isotopic tracer methods (Scholl *et al.*, 1996) could help determine the extent to which the anchialine habitat at ANA2 may be physically disconnected from the rest of the subterranean water system of the island. Second, the nearly complete isolation of the 'long rostrum' morphotype to ANA2 could be due to slight, but significant, differences in reproductive traits. For example, small variations in egg size have been shown to strongly correlate with distinct levels of population structure in other *Caridina* spp., with large-egged species having more pronounced structure (Page & Hughes, 2007). Thirdly, the two morphotypes could be segregating due to one or more ecological factors, such as dietary differences, other resource partitioning and/or competition. Thus, additional studies designed to specifically test these (or alternative) hypotheses are needed as they may help clarify the biology and taxonomic status of "*C. rubella*" on Miyako-Jima.

4.5.2 Population Structure Among 'Short Rostrum' *C. rubella* of Miyako-jima

The isolation by distance revealed here for 'short rostrum' *C. rubella* was unexpected given the fact that this atyid possesses life history traits conducive to dispersal (see Introduction). For example, *C. rubella* produces planktotrophic larvae and this reproductive mode is typically thought to contribute to higher connectivity and less structured populations in marine environments due to the ability of larvae to feed and delay metamorphosis while dispersing as plankton (reviewed by Palumbi 1994). Such a scenario apparently holds true for other anchialine species like the neritiliid snail *Neritilia cavernicola* and alpheid shrimp *Metabetaeus lohena*

Banner & Banner, 1960, both of which are planktotrophic and exhibit little to no genetic differentiation, presumably due to high gene flow, over ranges of ~200 km in the Philippines and Hawaiian Islands, respectively (Kano & Kase, 2004; Russ *et al.*, 2010). Likewise, amphidromy, defined as the active or passive movement of larvae from freshwater taxa to estuarine or marine areas for development before migrating back to adult freshwater habitats, is also thought to facilitate long distance dispersal for a number of organisms, including many atyids (Page *et al.*, 2005, 2008; Cook *et al.*, 2006, 2008, 2009). Taken together, the basic predictions of these life history traits, particularly in their most general forms, are violated by the strong population structure of 'short rostrum' *C. rubella* from Miyako-jima.

Although amphidromous species are thought to possess high dispersal potential, local selection for larval retention could be strong for such species if suitable adult habitat (*i.e.*, anchialine habitats) is rare (Sponaugle *et al.*, 2002; Strathmann *et al.*, 2002). Cases of local retention have been suggested for a number of amphidromous taxa, including gobies (Sorensen & Hobson, 2005) and snails (Haynes, 2000) as well as for the larvae of an amphidromous shrimp (Benstead *et al.*, 2000). In the case of 'short rostrum' *C. rubella*, retention of larvae in the aquifer system immediately below a particular anchialine habitat and/or in nearshore coastal waters may limit gene flow to local scales, ultimately leading to patterns of isolation by distance. Additionally, the strong population structure seen for 'short rostrum' *C. rubella* may again be a consequence of egg size (see above). Despite 'short rostrum' *C. rubella* possessing relatively small eggs (~0.52 mm; Shokita, 1979), this value falls within the range of other *Caridina* spp. with significant genetic structure between populations in relatively close proximity (Page & Hughes, 2007). Taken together, the local retention of larvae coupled with relative egg size may

limit dispersal and contribute to the patterns reported here for Miyako-jima's 'short rostrum' *C. rubella* populations.

Another potential factor influencing the population structure of 'short rostrum' *C. rubella* may be the strong oceanic currents circulating past the island. Specifically, Miyako-jima separates two western boundary currents of the North Pacific Subtropical Gyre: the Kuroshio Current of the East China Sea to the west and the Ryukyu Current to the east (Jin *et al.*, 2010). In this context, mesoscale hydrographic features of these currents such as fronts and eddies may act as mechanisms for larval retention, thereby limiting dispersal of species with otherwise high dispersal potential (Sabate`s & Olivar, 1996). For example, dynamic interactions between the Kuroshio Current and shelf waters of the East China Sea have been shown to prevent offshore larval fish dispersal (Okazaki & Nakata, 2007). Alternatively, the strong Kuroshio and Ryukyu Currents may quickly sweep larvae offshore and away from the island, in turn limiting successful dispersal of 'short rostrum' *C. rubella* around Miyako-jima and producing a similar pattern of isolation by distance.

4.5.3 Underground Dams and Conservation Aspects

Approximately 57% of the total land area of Miyako-jima is utilized for agriculture (field crops and animal husbandry). Thus, like most oceanic islands, freshwater is a valuable resource and commodity. The annual rainfall for the island is ~2,300 mm, but in the past the majority of this drained through the porous limestone of the island and either contributed to groundwater resting on the impermeable layer of clay that makes up the structural foundation of Miyako-jima or flowed out to sea (Wisniewski, 2004). To further develop groundwater resources for agriculture, the Japanese government implemented construction of two major subsurface dams

(Ishida *et al.,* 2003) in 1987 to simultaneously prevent groundwater from flowing into the sea while raising levels of the underground aquifers to facilitate water extraction by pumping (Fig. 1B). This project was completed in 2001, with a total capacity of ~20,000,000 m^3 and providing up to 50,000 m^3 of freshwater per day, making it one of the largest subsurface dam systems in the world (Ishida *et al.,* 2003).

Given the novelty of subsurface dams, little is known regarding the biological impacts such structures may have on aquifer-associated organisms like those endemic to the anchialine ecosystem. However, the ecological consequences of surface dams have been well documented, including dramatic changes in sedimentation, channelization, flooding and temperature regimes (Proff & Hart, 2002; Hall *et al.,* 2011). Similar effects have already been reported for the subterranean dams on Miyako-jima in the context of overall water quality (increased temperature, depositional rates and dissolved inorganic carbon; Kano *et al.,* 2007; Ishida *et al.,* 2011) due to significant alterations in both groundwater flow and levels. Thus, such alterations could have negative impacts on the already threatened *C. rubella* of Miyako since increased sedimentation and flow rates have been shown to reduce habitat suitability and alter community assemblages in other carideans (Iwata *et al.,* 2003). However, one direct effect of dams and other impoundments is habitat fragmentation via the erection of artificial barriers. For aquatic organisms in general, such fragmentation can decrease access to suitable habitat, restrict long distance dispersal events and impede seasonal and/or reproductive related migrations, all of which (among others) can contribute to the extirpation of populations (Dehais *et al.,* 2010). For amphidromous species like *C. rubella,* habitat fragmentation due to dam construction and water withdraw is of particular concern as it can result in a reduction or loss of connectivity between larval (marine) and adult (anchialine) habitats (Cook *et al.,* 2009). In this respect, these

underground dams should be recognized as possible (and formidable) barriers to the amphidromous life cycle of *C. rubella* that may impact the long-term viability of the "species" on Miyako-jima in multiple ways.

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Table 1. Indices of genetic diversity and tests of neutrality for each population of *Caridina rubella* on Miyako-jima, Ryukyus, Japan. *n*, number of sampled individuals; *nh*, number of unique haplotypes; π, nucleotide diversity; *h*, haplotype diversity. Values in parentheses in *n* and *nh* columns represent the actual number of individuals or recovered haplotypes, respectively, from that site. Differences in values represent individuals within a population belonging to a different lineage then what is endemic to that site. Outliers were excluded from the diversity measures and neutrality tests of that particular site. See text for additional details.

			Diversity indices			Neutrality tests	
Population	**Site Code**	***n***	***nh***	***π***	***h***	**Fu's F_s**	**Tajima's *D***
Ana Ga 1	ANA1	15 (16)	8 (9)	0.006	0.867	-1.134	-0.877
Ana Ga 2	ANA2	8	4	0.002	0.750	-0.903	-1.030
Tomori Ama Ga	TAG	12	9	0.008	0.939	-2.368	-0.672
Butura Ga	BUT	12	6	0.005	0.803	0.027	-0.881
Zuza Ga	ZUZ	13	6	0.005	0.782	0.083	0.942
	Total	60 (61)	24	0.045	0.945		

Table 2. Pairwise Φ_{ST} (above diagonal) and S_{nn} (below diagonal) estimates as measures of genetic differentiation between populations of *Caridina rubella* on Miyako-jima, Ryukyus, Japan. *$P < 0.05$, ** $P < 0.001$

	ANA 1	ANA 2	TAG	BUT	ZUZ
ANA 1	-	0.980**	0.056	0.204**	0.224*
ANA 2	1.000**	-	0.976**	0.982**	0.982**
TAG	0.465	1.000**	-	0.337**	0.317**
BUT	0.745**	1.000**	0.833**	-	-0.006
ZUZ	0.850**	1.000**	0.859**	0.666*	-

Table 3. Hierarchical analysis of molecular variance (AMOVA) for northern and southern populations of *Caridina rubella* on Miyako-jima, Ryukyus, Japan. $^{**}P < 0.001$

Source	d.f.	Sum of squares	Variance component	% Variation	Φ statistic
Among geographic regions	1	19.566	0.651 V_a	25.03	0.250 Φ_{CT}
Among populations within regions	2	5.292	0.058 V_b	2.24	0.030 Φ_{SC}
Within populations	48	90.857	1.893 V_c	72.73	0.273** Φ_{ST}
Total	51	115.715	2.603		

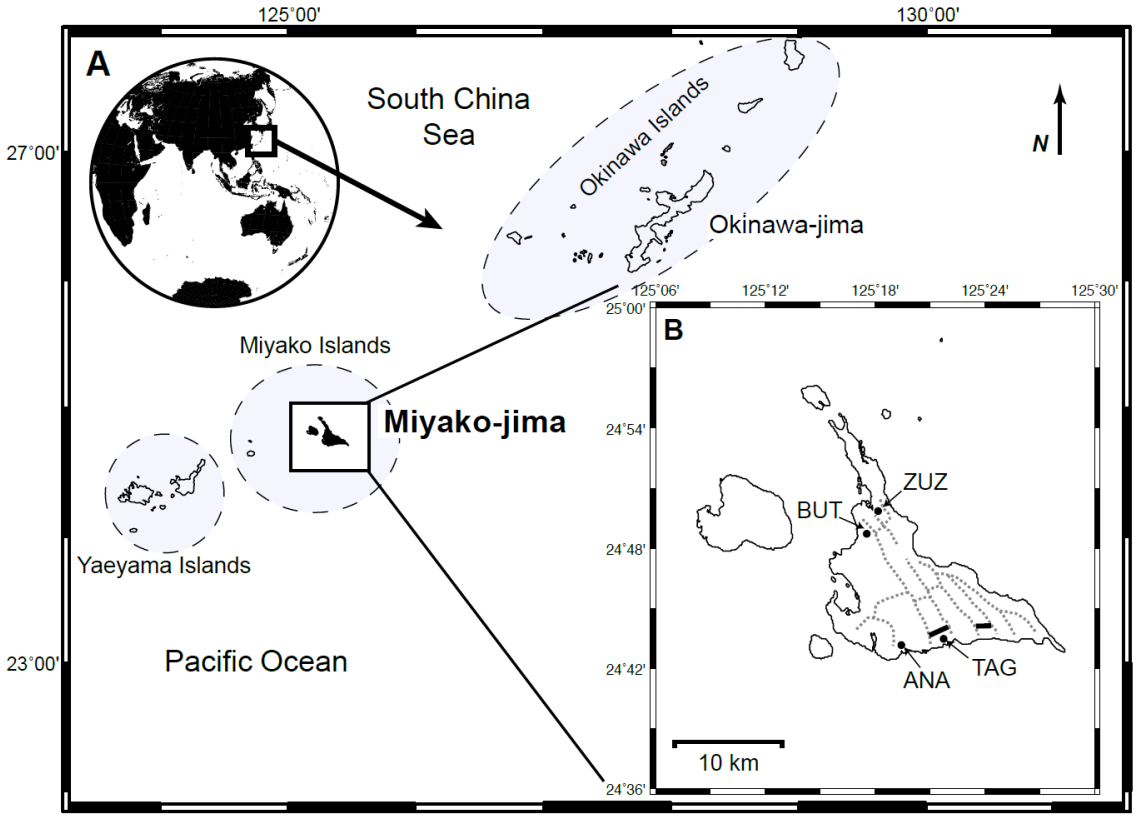

Figure 1. Map of the Southern Ryukyus Archipelago, Japan, indicating the location of Miyako-jima (A) and enlarged map of Miyako-jima depicting anchialine habitats from which *Caridina rubella* were sampled for this study (B). In panel B, grey dashed lines represent underground aquifer boundaries and thick dark lines represent the location of major subsurface dams of Miyako-jima. Site labels are listed in Table 1 and geographical coordinates of sampling sites are available from the corresponding author upon request.

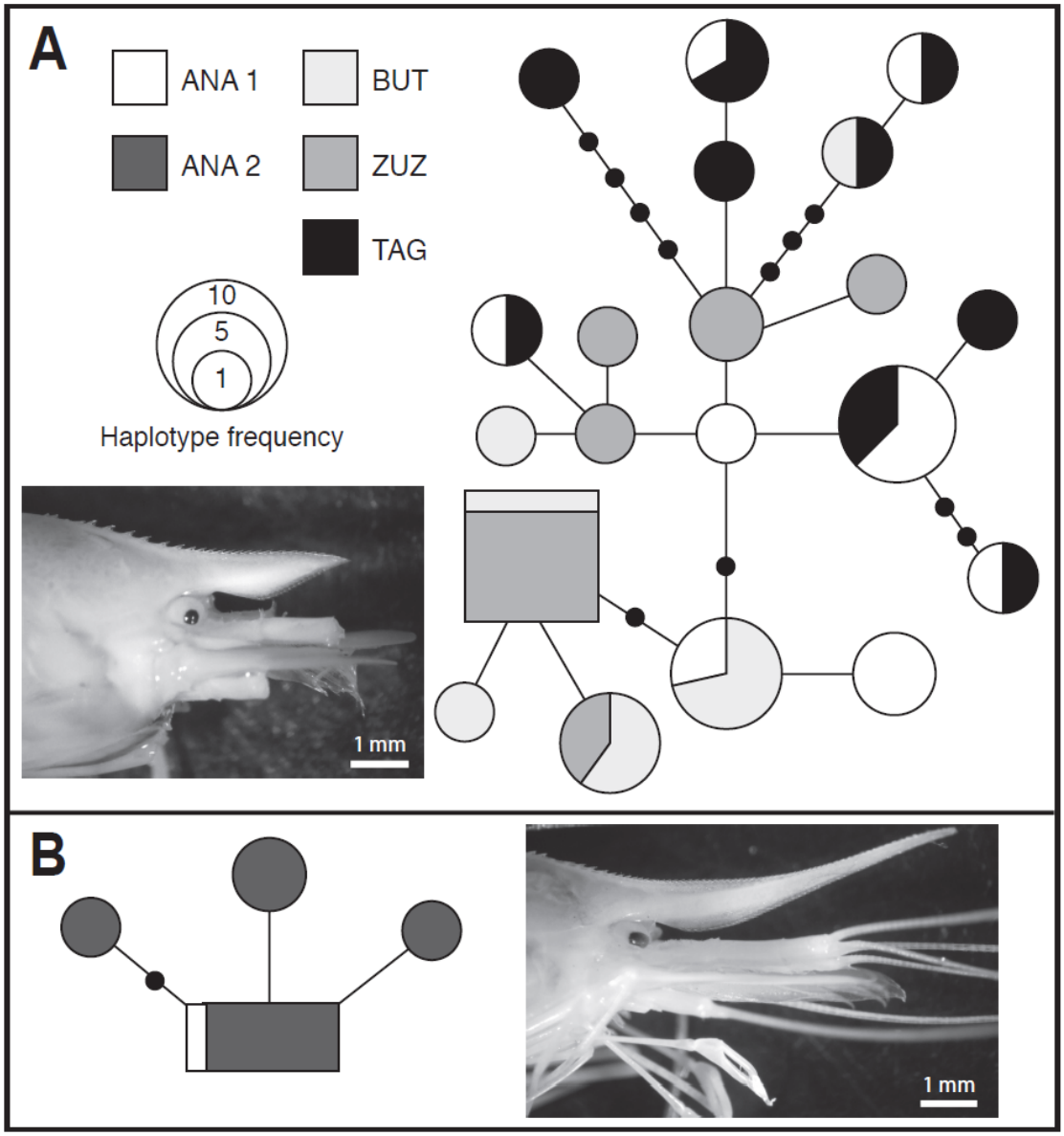

Figure 2. Networks depicting relationships among cytochrome *c* oxidase subunit I (COI) haplotypes recovered from the two *Caridina rubella* lineages on Miyako-jima, Ryukyus, Japan. (A) 'short' and (B) 'long' rostrum morphotypes of *C. rubella*. For each network, black dots represent unsampled (missing) haplotypes while a rectangle represents the haplotype with the highest outgroup probability according to the analysis. The size of circles and rectangles are proportional to the frequency at which a specific haplotype was recovered. Despite variable lengths, each branch implies a single mutational difference between haplotypes.

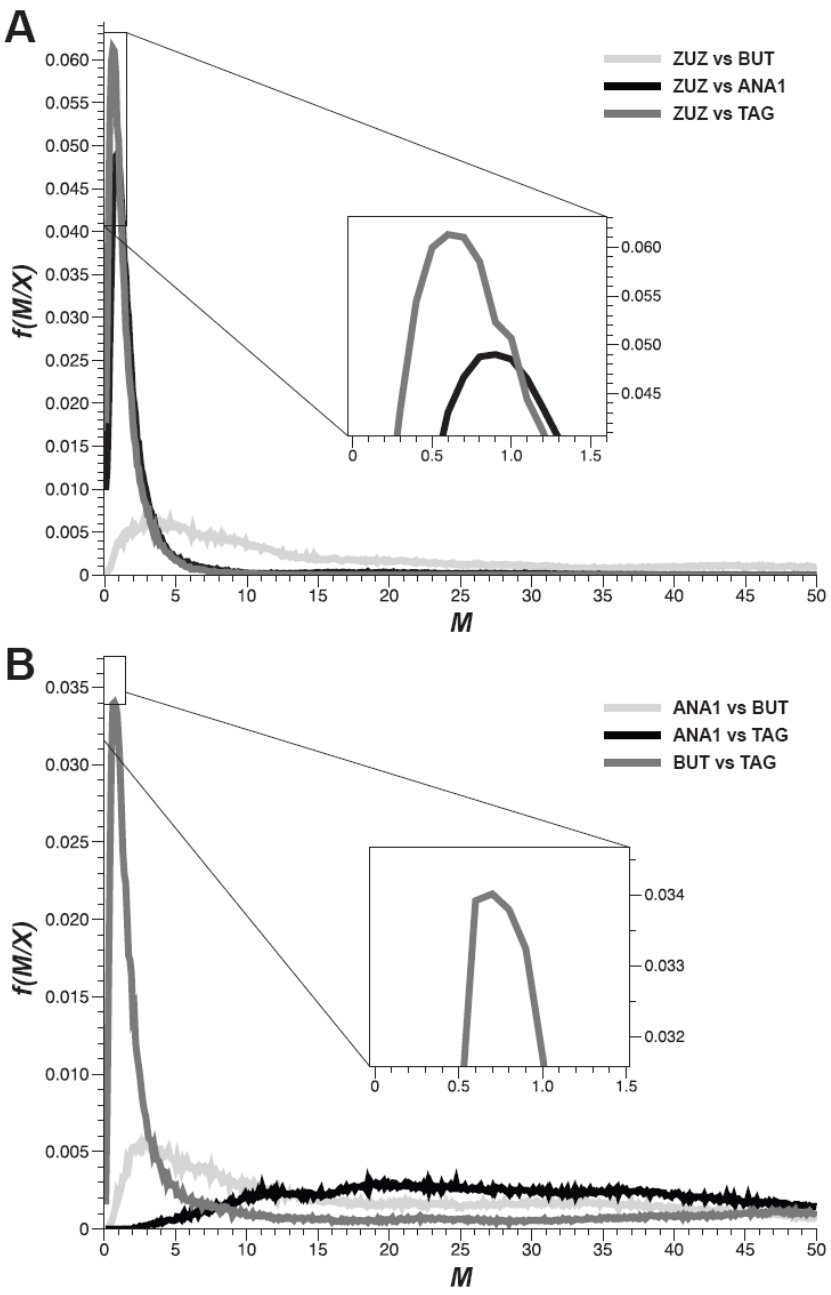

Figure. 3. Posterior probability distributions of migration rates (*M*) between populations of *Caridina rubella* on Miyako-jima, Ryukyus, Japan. Presented posterior distributions are the averages of three independent runs with MDIV (Nielsen and Wakeley 2001) (see text for additional details). Panels A and B represent all possible pairwise comparisons between populations of 'short rostrum' *C. rubella* and are presented as two graphs for readability.

CHAPTER 5. Multiple colonizations lead to hidden diversity in a unique island ecosystem: Comparative phylogeography of anchialine shrimp in the Ryukyu Archipelago, Japan

5.1 ABSTRACT

Geological events (*i.e.*, tectonics, volcanism, sea level changes) can significantly impact an area's flora and fauna, consequently shaping the structure and evolutionary trajectory of populations. In this context, the archipelagos of the Indo-West Pacific are among the richest in the world in terms of biodiversity due to the region's dynamic geological past and stable climatic conditions. To explain this high species diversity, recent molecular studies have supported one of two alternative hypotheses: the *center of origin hypothesis* or the *center of accumulation hypothesis.* To differentiate between these two hypotheses, we took a comparative phylogeographic approach to determine the origins of the anchialine diversity in the Ryukyu Islands by investigating the genetic variation, population structure and evolutionary history of three shrimp species endemic to the anchialine niche. Given the dynamic geologic history of the islands, it was hypothesized that the anchialine diversity of the Ryukyus originated within the archipelago and that the phylogenetic history of these shrimp would be correlated with the sequential separation of the Ryukyu Islands as sea-levels fluctuated during the Pleistocene (*i.e.*, Center of Origin). Accordingly, sequence analyses of two mitochondrial (*i.e.*, cytochrome oxidase subunit I [COI] and large subunit ribosomal [16S-rDNA]) genes found multiple genetic lineages with considerable genetic diversity and population structure for each species sampled. However, given the phylogenetic relationships and geographic distributions of the multiple lineages found and the strong regional oceanographic currents of the Indo-West Pacific, the diversity of the anchialine Carideans

of the Ryukyus is likely the result of an accumulation of species dispersing into the Ryukyus from independent source populations. Furthermore, the contrasting patterns of population structure and connectivity exhibited by each 'species' within the Ryukyus appear to result from complex interactions between intrinsic (*i.e.*, life history traits) and extrinsic (*i.e.*, historical geology and oceanography) processes.

5.2 INTRODUCTION

Geological events (*i.e.*, tectonics, volcanism, sea level changes) can significantly impact an area's flora and fauna, consequently shaping the structure and evolutionary trajectory of populations (*e.g.,* Vandergast *et al.,* 2004; Liggins *et al.,* 2008; Santos and Weese, 2011). In this respect, oceanic islands, with their discrete geography and generally high levels of endemism, are ideal systems for studying how such events influence organismal diversification. The archipelagos of the Indo-West Pacific have received considerable interest in this regard (Liu *et al.,* 2008) since these island groups are among the richest in the world in terms of species diversity (Myers *et al.,* 2000; Meijaard, 2003). Although a number of hypotheses have been proposed to explain this high species diversity (reviewed in Carpenter *et al.*, 2011), phylogenetic studies generally support one of two alternatives: *(i)* the *center of origin hypothesis*, in which the majority of the region's biodiversity is endemic (*i.e.,* due to repeated bouts of isolation by geologic events) and a source of species for surrounding areas (Benzie, 1989, Briggs 2000, Barber *et al.*, 2006), or *(ii)* the *center of accumulation hypothesis*, whereby this high biodiversity is due to species accumulation via dispersal from surrounding areas (*i.e.*, Indian, Pacific and Australasian biotas; Murphy & Austin, 2005, Page *et al.*, 2007, de Bruyn & Mather,

2007). However, most studies conducted to date have focused on single species; often leading to an inability to differentiate between these two possibilities (see de Bruyn & Mather, 2007).

The Ryukyu Archipelago, located in the Indo-West Pacific and stretching between Japan and Taiwan, has experienced an extensive range of geological events over its history. Specifically, the backbone of the islands formed during the late Miocene (~10 MYA) following subduction of the Philippine Plate by the Eurasian Plate (Konishi & Sudo, 1972; Koba, 1980; Lee *et al*., 1980). During low sea level stands associated with glacial periods of the late Pliocene (~2 MYA), a landbridge is thought to have extended to southeastern China, connecting the central Ryukyus with Taiwan and continental Asia (Kimura, 2000). Furthermore, this land bridge was submerged in the interglacial periods of the early Pleistocene (~1.5 MYA), creating islands corresponding to the present groups (Hikida & Motokawa, 1999) that experienced sequential periods of connections and oceanic isolation due to fluctuating sea levels throughout the Pleistocene. These latter events are believed to have significantly influenced the diversification and distributions of fauna in the Ryukyus and the phylogenetic relationships of several terrestrial taxa reflect these paleogeographic patterns. For example, Lin *et al.* (2002) found that the phylogeny of *Takydromus* grass lizards strongly correlated with the sequential separation of islands during the late Pleistocene. Similar patterns have also been reported for *Salganea* and *Panesthia* wood-feeding cockroaches (Maekawa *et al.,* 1999), *Parachauliodes* fishflies (Liu *et al.,* 2008), and *Anomala* beetles (Muraji *et al.,* 2008).

Another group whose evolutionary history is predicted to correlate with the geologic history of the Ryukyu Archipelago are organisms from the anchialine

ecosystem. This ecosystem is comprised of land-locked bodies of salt or brackish water in proximity to the sea that fluctuate with the tides due to subterranean connections to both the ocean and aquifer system (Holthuis, 1973; Maciolek, 1983). Such habitats have been reported from around the world, including the Sinai Peninsula, Bermuda, the Caribbean, Hawaii, the South Pacific, the Philippines, as well as the Ryukyus (Maciolek, 1986). With a few exceptions (Kano & Kase, 2004; Hunter *et al.*, 2008; Russ *et al.*, 2010), population genetic studies have revealed exceptional levels of endemism and strong structure on the scale of only a few km for a number of anchialine organisms (Santos, 2006; Craft *et al.*, 2008; Hunter et al., 2008; Page et al., 2008). For example, populations of the anchialine shrimp *Caridina rubella* Fujino & Shokita, 1975 (Decapoda: Atyidae) on the island of Miyako in the Ryukyus are significantly structured across distances ranging from <20 m to >10 km (Weese *et al.*, 2012). Additionally, anchialine habitats in general have been heavily influenced by sea-level changes during the Quaternary (Mylroie & Mylroie, 2011), and for anchialine organisms in the Ryukyus, such migrating coastlines may have lead to cycles of isolation and connection as suitable habitats (*i.e.*, the subterranean aquifer system) contracted and expanded with fluctuating sea-levels. In this context, comparing the phylogeography of different taxa endemic to this ecosystem provides an opportunity to understand how past environmental changes (*i.e.*, fluxes in sea levels) may have impacted patterns of anchialine biodiversity in the Ryukyus and the species diversity of Indo-West Pacific archipelagos in general.

Decapod crustaceans are the dominant macrobiota of the anchialine ecosystem in the Ryukyu Archipelago, with at least eleven species of shrimp and crabs having been recorded (Komai & Fujita, 2005; Cai & Shokita, 2006; Naruse & Tamura, 2006; Fujita,

2007; Fujita & Sunagawa, 2008). Of these, three caridean shrimp species, *Metabetaeus minutus* Whitelegge, 1897 (Decapoda: Alpheida), *Antecaridina lauensis* Edmondons, 1935 (Decapoda: Atyidae) and *Halocaridinides trigonophthalma* Fujino & Shokita, 1975 (Decapoda: Atyidae), have disjunct distributions both in the Ryukyus as well as across the Pacific Basin (Cai & Shokita, 2006; Anker, 2010). If *M. minutus, A. lauensis* and *H. trigonophthalma* exhibit genetic differentiation in the Ryukyus and this diversification was driven by geological events directly impacting the archipelago, it is hypothesized that the phylogeography of these species will reflect the history of the islands. Alternatively, it can also be hypothesized that any observed "differentiation" in these species is due to the accumulation of distinct lineages into the Ryukyu Archipelago from elsewhere via dispersal. To differentiate between these two hypotheses, the genetic variation, population structure and evolutionary history of these three shrimp species were investigated via sequence analyses of two mitochondrial (*i.e.*, cytochrome oxidase subunit I [COI] and large subunit ribosomal [16S-rDNA]) genes.

5.3 Material and Methods

5.3.1 Taxon sampling, DNA extraction and sequencing

Approximately 42 anchialine habitats were surveyed from nine islands belonging to the Daito, Miyako, and Yaeyama island groups of the Ryukyu Archipelago (Fig. 1) between January and August 2009. From these, specimens of *Antecaridina lauensis*, *Halocaridinides trigonophthalma* and *Metabetaeus minutus* were acquired from 11 anchialine habitats on the islands of Ishigaki, Taketomi, Tarama, Miyako, Irabu and Minami-daito (Fig. 1). From each habitat, 6 -17 individuals were collected (Table 1)

using baited traps or small aquarium nets and immediately preserved in 99% ethanol for genetic analyses. For phylogenetic analyses, an individual representing "short rostrum" *Caridina rubella* (Weese *et al.*, 2012) was collected from Miyako and additional *Metabetaeus* and *Antecaridina* specimens from outside the Ryukyus were supplied as 75% ethanol preserved materials by Dr. Arthur Anker (Florida Museum of Natural History).

Total genomic DNA was extracted from each individual using 2X cetyltrimethyl ammonium bromide/chloroform (CTAB) according to procedures outlined in Santos (2006). Between 10-30 ng of DNA were utilized as template to amplify an ~670 base pair (bp) fragment of the mtDNA cytochrome *c* oxidase subunit I (COI) gene via polymerase chain reaction (PCR). Reactions 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 and HCO2198 (Folmer *et al*., 1994), and 1 U *Taq* polymerase. Reactions were conducted in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) under the conditions outlined in Santos (2006). Additionally, sequence data from the mtDNA large subunit ribosomal (16S-rDNA) gene were obtained from one to two individuals for each divergent genetic lineage within the three "species" (see below) for phylogenetic analyses. The PCRs for an ~850 bp fragment of the 16S-rDNA were conducted in 25 μL volumes with the "touchdown" thermocycling profile outlined in Craft *et al.*, (2008) and containing 0.4 μM each of primers CRUST16SF and CRUST16SR (Ivey & Santos 2007) along with the reaction constituents outlined above.

Amplicons were purified with Montage PCR filter units (Millipore, Billerica, MA, USA) according to the supplier's directions, cycle-sequenced in both directions

using Big-Dye Terminators v3.1 and read on a PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Any ambiguities in the chromatograms were corrected by comparison with the complementary DNA strand in SEQUENCHER v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Finished COI and 16S-rDNA sequences were aligned manually using SE-AL v2.0a11 (available at http://tree.bio.ed.ac.uk.software/seal/).

5.3.2 Genetic diversity and population genetic analyses

Population-level analyses utilized mtDNA COI sequence data since this gene has proven to be informative for other alpheid and atyid species (Cook *et al.,* 2006; Page *et al.* 2007; Russ *et al.,* 2010). Nucleotide (π) and haplotype (h) diversity estimates were calculated according to the methods of Nei (1987) using DnaSP v5.10.00 (Rozas *et al.,* 2009). To assess genetic differentiation, pairwise Φ_{ST} statistics (which incorporates information from both haplotype frequencies and molecular divergence) were calculated with Arlequin v3.11 (Excoffier & Schneider, 2005). For these comparisons, the Tamura & Nei (1993) model of DNA evolution, selected by the Akaike Information Criterion (AIC) with the FindModel web version of Modeltest (Posada & Crandall, 1998; available at http://darwin.uvigo.es/), was utilized. As another measure of genetic differentiation, Hudson's (2000) nearest-neighbor statistic (S_{nn}; which measures how often the 'nearest neighbors' (in sequence space) are from the same locality) was calculated using DnaSP.

To visualize relationships among COI haplotypes, networks were constructed via TCS v1.21 (Clement *et al.*, 2000), which utilizing the cladogram estimation algorithm of Templeton *et al.,* (1992). The analyses were conducted under default settings, providing

95% parsimony plausible branch connections between haplotypes. Reticulations in the networks, representing ambiguous connections, were resolved using the criteria outlined in Crandall *et al.,* (1994). For each of the resulting networks, historical demography was inferred using Tajima's *D* (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests conducted in Arlequin. Both methods provide information regarding population history in the absence of selection, with significant negative or positive values generally suggesting expansions or bottlenecks (Tajima, 1989; Fu, 1997; Akey *et al.*, 2004), respectively. Statistical significance in the pairwise Φ_{ST} and neutrality tests was assessed in Arlequin by 1,000 permutations and the S_{nn} in DnaSP by 1,000 permutations.

5.3.3 PHYLOGENETIC ANALYSES

Phylogenetic analyses utilizing mtDNA 16S-rDNA sequence data were used to infer evolutionary relationships among divergent genetic lineages recovered from the three shrimp "species" (see Results) examined in this study. For these analyses, two data sets were created and analyzed in the same manner. The first consisted of alpheid 16S-rDNA sequences, including those of *Metabetaeus "minutus"* from this study and an additional *Metabetaeus* spp. and *Betaeus harrimani* sequence downloaded from GenBank (Table 2). The second dataset consisted of atyid 16S-rDNA sequences, including the *Antecaridina "lauensis", Halocaridinides "trigonophthalma"*, and *Caridina* spp. sequences generated here along with additional *Antecaridina* spp. and *Halocaridina rubra* sequences from GenBank (Table 2). Both datasets utilized *Macrobrachium japonicum* as an outgroup. The 16S-rDNA sequences were aligned using ClustalX (Thompson *et al.,* 1997) and checked manually using SE-AL. For each data set,

evolutionary relationships among species were inferred via Maximum Likelihood (ML) analyses with PHYML v3.0 (Guindon & Gascuel, 2003) under the appropriate model of evolution chosen by the AIC in Modeltest v3.7 (Posada & Crandall, 1998). For each phylogeny, the transition/transversion ratio and proportion of invariable sites were estimated, with the starting tree determined by BioNJ (default settings). Branch supports were estimated by 1,000 bootstrap replicates. The resulting phylogenetic trees were viewed with FigTree v1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree/).

5. 4 RESULTS

5.4.1 GENETIC DIVERSITY

Direct sequencing of mtDNA COI amplicons yielded a 630 bp fragment from each of the 33, 61, and 58 individuals of *Antecaridina lauensis, Halocaridinides trigonophthalma*, and *Metabetaeus minutus* analyzed in this study. From these, 23 *A. lauensis*, 33 *H. trigonophthalma*, and 27 *M. minutus* unique COI sequences were identified and deposited into GenBank under assession #'s ###-###. While nuclear copies of mitochondrial derived genes (numts: Lopez *et al.*, 1994) can be common among arthropods (Buhay, 2009), translation of these COI nucleotide sequences into amino acids found no stop codons and any non-synonymous changes identified were to residues with similar biochemical properties (data not shown). This implies that sequences analyzed here were derived from mitochondrial copies of COI. Specific sample sizes and number of haplotypes recovered for each species are summarized in Table 1.

5.4.2 PHYLOGENETIC ANALYSES

From inspection of the COI sequence alignments, it was evident that *A. "lauensis"*, *H. "trigonophthalma"* and *M. "minutus"* from the Ryukyus were each potentially comprised of 2-3 divergent genetic lineages. To test whether each "species" was monophyletic as well as to infer relationships among lineages, 16S-rDNA sequences were generated for one to two individuals per lineage, as well as from specimens of the same and/or closely related species from outside the Ryukyus (Table 2), for analyses in a phylogenetic context. Maximum likelihood (ML) trees, inferred under the GTR model of evolution (as chosen by the AIC), for both datasets were well resolved and are presented in Figs. 2B and 2C.

The ML tree for the alpheid data set found *Metabetaeus* to be monophyletic with strong (100%) bootstrap support (Fig. 2B). Within *Metabetaeus*, *M. "minutus"* was also monophyletic to the exclusion of *M. lohena* (Hawai'i) and *M. mcphersonae* (Moorea). For *M. "minutus"* of the Ryukyus, two lineages were recovered. One of these, collected exclusively from the southern Ryukyu Islands of Ishigaki (IS), Miyako (MY), and Tarama (TA), was sister to *M. "minutus"* from Christmas Island (CI) in the Indian Ocean (Fig. 2B). The second lineage of *M. "minutus"* is apparently confined to the island of Minami-daito (MD), > 600 km east of the southern Ryukyus. The phylogenetic analysis placed this lineage with relatively strong (*i.e.*, 87%) bootstrap support as sister to a *Metabetaeus sp*. (a potential member of *M. "minutus"*) from New Caledonia (NC) in the southwest Pacific Ocean (Figs. 2A and 2B). Thus, both groups of *M. "minutus"* in the Ryukyus are more closely related to lineages collected from other geographic regions then they are to each other. Furthermore, morphological variation also supports the

inferred phylogenetic relationships. Specifically, Anker (2010) noted differences in rostrum length and the angle of orbital teeth between *M. "minutus"* collected from Minami-daito and Christmas Island. Here, specimens from the Minami-daito lineage conformed to the description of materials from the same locality, while specimens from the southern Ryukyus lineage closely resemble the description of the Christmas Island specimen (Supplementary Figure 1) presented in Anker (2010).

Similar phylogenetic patterns were recovered from the atyid ML analysis. In this case, *Antecaridina* and *Halocaridinides* were each monophyletic with strong (98% and 100%, respectively) bootstrap support (Fig. 2C). For *Antecaridina*, one of the two genetic lineages, localized to Ishigaki (IS) and Tarama (TA) in the southern Ryukyus and Minami-daito (MD), was sister to *A. "lauensis"* from Christmas Island (CI) and Hawai'i (HI) in the central Pacific Ocean, with < 1% sequence divergence in 16S-rDNA across this geographic range (Figs. 2C). The second lineage in the Ryukyus also occurs on Ishigaki (IS) but, unlike the other (and more widespread) *A. "lauensis"* lineage, appears restricted to one anchialine habitat on this single island. Phylogenetically, this lineage is sister to an *Antecaridina* sp. from East Timor (ET), which lies between the South China Sea and Indian Ocean (Fig. 2A). Just as in the case of *M. "minutus"*, both groups of *A. "lauensis"* are more closely related to lineages from outside the Ryukyus then to one another. Lastly, three genetic lineages were recovered from *H. "trigonophthalma"*, with two of the lineages restricted to Taketomi (TK) and Tarama (TA) forming a monophyletic group sister to a third lineage exclusive to the islands of Ishigaki (IS), Miyako (MY), and Irabu (IR) (Fig. 2C). Unfortunately, no specimens or genetic data for *H. "trigonophthalma"* from outside the Ryukyus could be obtained for this study.

5.4.3 POPULATION GENETIC ANALYSES

Metabetaeus "minutus": Estimates of haplotype diversity (*h*) for *M. "minutus"* ranged from 0.93-1.0, with nucleotide diversity (π) being consistent among populations (Table 1). Significant genetic structure, in the form of pairwise Φ_{ST} and/or S_{nn} values approaching, or at, their upper limits of 1.0 (Table 4), was observed between the Southern Ryukyus and Minami-daito, implying no individuals are exchanged between these populations and distinct genetic lineages separated by > 600 km (Figs. 3A and 3B). On the other hand, an absent of structure was evident among *M. "minutus"* populations of the southern Ryukyus lineage spanning a distance of ~ 200 km (Table 4). This homogenization of *M. minutus* inhabiting anchialine habitats on islands, isolated by expanses of ocean of ~50 km, implies an absence of isolating barriers at this scale, thus leading to panmixia between populations on the three islands. The total lack of population structure at this scale is similar to what has been reported for *M. lohena* in the Hawaiian Islands (Russ *et al.* 2011), which covers a similar (~ 260 km) geographic range across the three main Hawaiian islands. Two discrete networks (Fig. 3C), corresponding to the two lineages identified in the phylogeny (Fig. 2B), were recovered from parsimony analysis of the COI sequences. One represented *M. "minutus"* from the Southern Ryukyus, consisting of 14 haplotypes from 21 individuals collected on Ishigaki (IS), Miyako (MY) and Tarama (TA) islands (Table 3). The second network was of *M. "minutus"* from Minami-daito (MD), encompassing nine haplotypes recovered from 12 individuals (Table 3). Uncorrected (*p*) distances among haplotypes within each network ranged from 0.16-1.4% while those for haplotypes between the two networks ranged from 9.7-10.6%. Both Tajima's *D* and Fu's *F* values were significantly negative for *M.*

"minutus" populations in the Southern Ryukyu (Table 3), implying recent population expansion for this lineage.

Antecaridina "lauensis": With the exception of one outlier (i.e., Yoshino Cave (YOSH) on Ishigaki [see below]), estimates of *h* and π were consistent across populations of *A. "lauensis"* (Table 1). Unlike *M. "minutus"*, no significant genetic structure was detected between *A. "lauensis"* populations in the southern Ryukyus and Minami-daito (Table 4). However, the *A. "lauensis"* population at YOSH was significantly different from all others in the Ryukyus (Table 4), due to being the only site having both lineages of *A. "lauensis"* (see above). Parsimony analysis of the COI sequences produced two discrete networks, representing the two lineages of *A. "lauensis"* in the Ryukyus, with individuals from the YOSH population occurring in both (Fig. 3D). One network consisted of 23 haplotypes and 48 individuals from the islands of Ishigaki (IS), Tarama (TA) and Minami-daito (MD) whereas the other included four haplotypes from 10 individuals specific to the island of Ishigaki (IS) (Fig. 3D). Uncorrected *p*-distances among COI haplotypes were 0.16-1.4% within a network and 12.7-13.5% between the two networks. Tajima's *D* and Fu's *F* values were found to be significant and negative for the two lineages (Table 3), suggesting recent population expansion in both. If COI sequences from *A. "lauensis"* and *Antecaridina* sp. sampled in Hawai'i, Christmas Island (both supplied by Dr. Timothy J. Page, unpublished), and East Timor (EF173843 - EF173847) are included in the parsimony analysis, those from Hawai'i (1) and Christmas Island (2) are identical to the ancestral haplotype of the Ishigaki (IS), Tarama (TA) and Minami-daito (MD) lineage while a third network, comprised of five haplotypes from

eight East Timor individuals (i.e., the *Antecaridina* sp.), is also recovered (data not shown).

Halocaridinides "trigonophthalma": For *H. "trigonophthalma"*, estimates of *h* spanned 0.371-0.983 and, with the exception of the one site on Taketomi (TK) (π = 0.028), π values were consistent for all populations (Table 1). Significant genetic differentiation and population structure was found between populations on the islands of Tarama (TA) and Taketomi (TK) relative to those on the islands of Miyako (MY), Irabu (IR) and Ishigaki (IS) (Table 4). Three discrete networks were identified in the parsimony analysis of COI sequences for *H. "trigonophthalma"* (Fig. 3E). The first corresponded to 9 haplotypes and 29 individuals from Ishigaki (IS) and Miyako (MY) islands while the second was composed of 24 haplotypes and 30 individuals from Tarama (TA) and Taketomi (TK) (Fig. 3A). The third network, consisting of two individuals and one haplotype, was exclusive to the single Taketomi (TK) population (Fig. 3E). Here, *p*-distances among haplotypes in the same network ranged from 0.16-1.2% while those between the three networks were 17.1-19.2%. Two of the three *H. "trigonophthalma"* lineages exhibited recent population expansion as evident from significantly negative Tajima's *D* and Fu's F_s values (Table 3).

5.5 Discussion

Here, we took a comparative phylogeographic approach to determine the origins of the anchialine diversity in the Ryukyu Islands by investigating the population structure and evolutionary history of three Caridean shrimp from the anchialine niche. Following the Center of Origin hypothesis (see Introduction), it was hypothesized that the anchialine

diversity of the Ryukyus originated within the archipelago and that the phylogenetic history of *A. lauensis*, *M. minutus* and *H. trigonophthalma* would be correlated with the sequential separation of the Ryukyu island groups as sea-levels fluctuated during the Pleistocene. Accordingly, multiple genetic lineages with considerable genetic diversity and population structure were found to occur within the archipelago for each of the three "species" investigated. Similar patterns have been seen for a number of marine species in the Coral Triangle supporting the center of origin hypothesis for the Indo-West Pacific (reviewed in Benzie, 1989; Carpenter *et al.*, 2012). However, taken together, the phylogenetic relationships and geographic distributions of the multiple lineages found for each species coupled with the strong regional oceanographic currents of the Indo-West Pacific suggest that the diversity of anchialine shrimp found in the Ryukyus is the result of an accumulation of species dispersing into the Ryukyus from independent source populations followed by subsequent diversification within the archipelago.

5.5.1 Evidence for multiple colonization of the Ryukyus by anchialine Carideans

Whether the occurrence of related taxa on an island/archipelago reflects in situ diversification or multiple colonizations can be reconciled through the reconstruction of phylogenetic relationships; if island taxa are monophyletic, a single colonization is favored, whereas paraphyly suggests multiple colonizations (Emerson, 2002). For example, in the Hawaiian Islands, phylogenetic monophyly of the specieous Hawaiian *Drosophila* (Grimaldi *et al.*, 1990) and silverswords (Baldwin *et al.*, 1991) revealed that these spectacular radiations were each the result of a single colonization event. Here, the

occurrence of multiple lineages of each species and their similar phylogenetic relationships to populations outside the archipelago (Fig. 2), suggests that the anchialine fauna of the Ryukyus is the result of an accumulation of species dispersing into the archipelago from multiple source populations around the Indo-West Pacific. For *Metabetaeus and Antecaridina*, the sympatric lineages of each species occurring in the Ryukyus are not sister taxa, but rather exhibit paraphyletic relationships to *Metabetaeus*/*Antecaridina* lineages from outside the archipelago (Christmas Island, East Timor or New Caledonia). Furthermore, given these patterns, it is likely that the multiple lineages of *Halocaridinides* found in the Ryukyus are also the result of multiple colonizations from sources outside the archipelago. Similar phylogentic patterns attributed to multiple colonizations have recently been elucidated for a number of terrestrial (Harbaugh and Baldwin, 2007; Swenson et al., 2007; Nattier *et al.*, 2011) and marine (Holland et al., 2004 Burridge et al., 2006) taxa.

While similar phylogenetic patterns could arise from anchialine species diversifying in the Ryukyus and subsequently dispersing out of the archipelago, this seems unlikely given the phylogenetic relationships of the multiple lineages recovered here to Carideans sampled from islands of the south Indo-West Pacific (*i.e.*,, Christmas Island, East Timor and New Caledonia) and the oceanographic patterns of the Indo-West Pacific. The currents of the area are dominated by the, historically stable, Kuroshio Current which diverges from the North Equatorial Current in the area east of the Philippine Islands and flows northward into the Okinawa Trough, past the Ryukyu Islands and out into the Pacific Ocean through the Tokara Strait (Ujiie *et al.* 2003). As the worlds second-largest ocean current (Shen *et al.*, 2011), the Kuroshio Current has

played a major role in generating the species diversity of the Indo-West Pacific seen today (*e.g.*, Kojima *et al.*, 1997; Mukai *et al.*, 2009; Soeparno *et al.*, 2011). For example, the North Equatorial and Kuroshio Currents have been shown to contribute to the dispersal and migration of marine taxa from the Central/Southern Indo Pacific to the Japan and Ryukyu Islands (*e.g.*, Tsukamoto, 2006; Mukai *et al.*, 2009). Furthermore, the Kuroshio Current (along with the South China and North China Coastal Currents) is thought to have influenced the recolonization of the Ryukyus by the flathead mullet, *Mugil cephalus*, from southern refugia following Plio-Pleistocen sea level changes (Shen *et al.*, 2011). For anchialine taxa, the strong northward currents of the Indo-West Pacific may have resulted in the colonization of the anchialine habitats of the Ryukyus from the southern Indo-West Pacific into the Ryukyus

As mentioned above (see Introduction), climatic oscillations during the Pleistocene caused large fluctuations in sea levels globally, dramatically impacting the diversification and distribution of many species throughout the Indo-West Pacific (*e.g.*, Barber *et al.*, 2006; De Bruyn and Mather, 2007; Crandall *et al.*, 2008; Fitzpatrick *et al.*, 2011). For anchialine Carideans, these cyclical fluctuations in sea-level may have lead to multiple "waves" of colonizations into the Ryukyus associated with periods of increased dispersal followed by subsequent divergence in isolation. Assuming larger/higher islands have been above sea level for longer periods of time then smaller, lower-lying islands, there appears to be a strong correlation between the distribution of anchialine lineages and island age. For example, one could hypothesize that, given their current elevation (> 100 m) and size (> 200 km^2), the islands of Ishigaki, Miyako and Irabu may have been colonized by *Halocaridinides* at a time in the past when sea levels were higher then

present and many of the Ryukyu Islands were submerged. Then later, as sea levels receded to present day conditions or lower, as second (and maybe third) wave of *Halocaridinides* may have swept through the islands colonizing the once submerged islands of Taketomi and Tarama (elevation <30 m; area <15 km^2). The more recent colonization of these smaller/younger islands is supported by the fact that the Taketomi/Tarama lineage possesses the signal of a recent strong bottleneck and subsequent expansion indicative of a recent colonization/founder event, as evident by the largest negative Tajima's *D* and Fu's F_S values encountered in the study (Table 3). Furthermore, the only lineage that shows no evidence of a recent population expansion (Table 3) is lineage *M. minutus* endemic Minami-daito, which is thought to be considerably older then the islands of the Southern Ryukyus (Ota and Omura, 1992) suggesting it may have been colonized prior to the emergence and colonization of the southern islands of Ishigaki, Miyako and Tarama. For *Antecaridina,* one lineage has an impressive distributional range with haplotypes being shared among Christmas Island, Hawai'i, and three islands in the Ryukyus (Ishigaki, Tarama and Minami-daito) suggesting a recent colonization or ongoing dispersal/gene flow throughout the Pacific while the other lineage is restricted to the island of Ishigaki and may represent an older colonization. While the timing of specific colonization events cannot be determined with the current sampling, all three species appear to share a history of recurring periods of increased dispersal associated with sea-level fluctuations.

5.5.2 Population Structure of anchialine Carideans in the Ryukyus Archipelago

Despite having similar ecologies and broadly sympatric distributions within the Ryukyu Archipelago, lineages *Metabetaeus*, *Antecaridina* and *Halocaridinides* exhibit radically different scales of geographic distributions, intraspecific diversity, and population structuring. While the similar phylogenetic patterns throughout the Indo-West Pacific suggest similar evolutionary histories for all three species, subtle differences in intrinsic life-history characteristics may have played a role in creating this phylogeographical discordance within the Ryukyus. For Caridean shrimp, differences in life history characteristics (*i.e.*, egg size, larval stages, and larval habitat) and larval feeding mode (*i.e.*, lecithotrophy versus planktotrophy) have been shown to strongly influence population structure and dispersal potential (Shokita, 1979, Page and Hughes, 2007; Craft *et al.*, 2008; Russ *et al.*, 2010).

Very little is known about the life history of *Metabetaeus minutus*, but recent field and laboratory observations suggests that *Metabetaeus* typically produce 20-29 eggs that develop as planktotrophic larvae (Fujita, unpublished). As planktotrophic larvae are typically associated with wide dispersal abilities and low genetic differentiation, it is not suprising that populations of *Metabetaeus* from the Southern Ryukyu genetic lineage are homogenized across the three southern islands of Miyako, Tarama and Ishigaki, which span a distance of ~150 km (Fig. 3). This homogenization of *M. minutus* inhabiting anchialine habitats on islands, isolated by expanses of ocean of ~50 km, implies an absence of isolating barriers at this scale, thus leading to panmixia between populations on the three islands. This total lack of population structure at this scale is similar to what has been reported for other anchialine species like the neritiliid snail *Neritilia cavernicola* and alpheid shrimp *Metabetaeus lohena*, both of which possess planktotrophic larvae and

exhibit little to no genetic differentiation over similar ranges of ~200 km in the Philippines and Hawaiian Islands, respectively (Kano and Kase, 2004; Russ *et al.*, 2010). Additionally, the two populations of *M. minutus* sampled from the Minami-daito lineage show no population structure across this island, again similar to populations of *M. lohena* occurring on any single Hawaiian Island. However, as evident from the deep (~10% COI *p*-distance) genetic divergence found between the two lineages separated by > 600 km, *M. minutus* appear to be isolated over greater geographic distances. Collectively, this suggests that *Metabetaeus* in general (*minutus* or *lohena*) may be a "good" disperser in "ecological" time when islands are in close (*i.e.*, < 200 km) proximity. However, once islands get further apart (*i.e.*, > 600 km), dispersal and colonization becomes an "evolutionary sweepstake" event, with an apparent absence of gene flow following colonization.

For atyids, such as *Antecaridina* and *Halocaridinides*, egg size is thought to be an effective predictor of dispersal ability and population structure (Craft *et al.*, 2008). For example, *Caridina* species possessing large geographic ranges and low levels of genetic structure generally have relatively small eggs (~0.4 mm), whereas those with large eggs (~1.6 mm) are more restricted in their distributions and exhibit higher levels of genetic structure (Page and Hughes, 2007). However, despite producing relatively small (~0.35 mm eggs; Fujita, personal observation) with planktotrophic development, *Halocaridinides* exhibits surprisingly strong population structure in the Ryukyus with strong differentiation found between islands separated by > 5 km of shallow ocean (*i.e.*, Ishigaki *vs.* Taketomi). While local retention of larvae within nearshore coastal waters or the strong regional oceanic currents might explain the unexpected levels of genetic

structure (Weese *et al.*, 2012), given their distributions the multiple lineages of *Halocaridinides* are more likely the result of independent colonizations from outside the archipelago (see above) that have remained genetically isolated due to subtle difference in reproductive traits, ecological factors, such as dietary differences, resource partitioning or competition.

While *Antecaridina "lauensis"* has historically been thought to have an "extremely disjunctive" distribution due to high dispersal abilities (Smith and Williams, 1981), the two lineages of *Antecaridina* in the Ryukyus represent two opposite ends of the dispersal spectrum. One lineage appears to have an impressive distributional range occurring on Christmas Island, the Hawaiian Islands, and at least three islands in the Ryukyus (Ishigaki, Tarama and Minami-daito), with minimal divergence found across this range. Given the correlations seen between reproductive traits (*e.g.*, egg size and larval feeding mode) and dispersal ability, it could be predicted that this lineage would produce relatively small eggs developing as planktonic larvae. However, the second lineage is restricted to only the island of Ishigaki, suggesting this lineage may possess larger eggs; reducing dispersal abilities. Such intraspecific variation in egg size has been seen between cryptic lineages and species for Australian atyids (Paige *et al.*, 2005; Cook *et al.*, 2006; Paige and Hughes, 2007). In studying the Caridean shrimp of the Ryukyus, Shokita (1979) suggested that such an increase in egg size occurs as species transition from an amphidromous to a totally landlocked lifecycle; which may explain the difference in rage size of the two lineages. Alternatively, *Antecaridina* as a genus may produce medium size eggs undergoing planktonic development with moderate dispersal abilities, limiting long distance marine dispersal to evolutionary sweepstake events as

discussed above. In this context, the widespread Ryukyu/Hawai'i/Christmas Island lineage may be a recent dispersal event with insufficient time for divergence between populations.

5.5.3 Distribution and hidden diversity of anchialine shrimp of the Indo-West Pacific

The anchialine fauna of the Indo-West Pacific is dominated by Caridean shrimp, with 11 species of shrimp being distributed throughout Pacific (de Grave and Sakihara, 2011). The majority of these anchialine Carideans, such as *A. lauensis, M. minutus, Calliasmata pholidota* Holthuis, 1973, *Ligur uveae* Borradaile, 1899 and *Periclimenes pholeter* Holthuis, 1973, are thought to have extremely widespread and disjunct distributions occurring throughout the Indo-West Pacific (see Maciolek, 1983). However, a number of shrimp species, such as *Halocaridina rubra, M. lohena* and *Procaris hawaiana* from the Hawaiian Archipelago (Maciolek, 1983), are characterized as being endemic to single islands or archipelagos in the Pacific with the open ocean hypothesized as a strong barrier to dispersal (Craft *et al.,* 2008; Weese *et al.,* 2012). Given the apparent multiple colonizations of the Ryukyus by *Metabetaeus, Antecaridina* and *Halocaridinides* and the presence of anchialine shrimp on isolated archipelagos such as the Hawaiian Islands, passive marine dispersal (*i.g.,* Smith and Williams,1981), by either adult or larval stages, may be likely for these species as well and may be a common characteristic of anchialine Carideans in general. However, the varying levels of genetic structure and distributions revealed here for the multiple lineages of *Metabetaeus, Antecaridina* and *Halocaridinides* with in the Ryukyus, coupled with the restricted

distributions and populations structure of other anchialine Carideans such as *H. rubra, M. lohena, Procaris hawaiana* and *C. rubella* (Maciolek, 1983; Santos, 2006; Craft *et al.,* 2008; Russ *et al.,* 2011; Weese *et al.,* 2012) suggest that the open ocean acts as a strong barrier to dispersal and long distance oceanic dispersal likely occurs on evolutionary, rather than ecological, timescales.

Given the patterns that are beginning to be emerge for anchialine shrimp in the IWP, the diversity of taxa inhabiting the anchialine habitats of the Pacific may be vastly underestimated with many of the species previously described representing cryptic species complexes. In this study for example, both *M. minutus* and *A. lauensis* appear to represent possible species complexes comprised of at least, given the current sampling, two possible divergent species occurring in the Ryukyus. Furthermore, the population structure and genetic divergence found within *H. trigonophthalma* suggests a potential species complex of at least three *Halocaridinides* species occurring in the Ryukyu Islands. Additionally, cryptic species complexes have recently been revealed for the two anchialine atyids *Caridina rubella* (two potential species) on Miyako (Weese *et al.,* 2012) and *Halocaridina rubra* (eight potential species) in the Hawaiian Archipelago (Santos 2006; Craft *et al.,* 2008). In fact, of the six Pacific anchialine species studied from a genetic prospective to date, only *M. lohena* has failed to reveal a species complex (Russ *et al.,* 2010). Given these results, additional genetic analyses of other widely distributed Pacific Carideans, such as *C. pholidota, P. pholeter* and *L. uveae,* are likely to reveal additional cryptic taxa. Given this, what has traditionally been thought to be 11 species of anchialine Carideans distributed throughout the Pacific (de Grave and Sakihara, 2011) may actually represent over 24 genetically distinct cryptic species, all more restricted in

their distributions then previously thought. While additional morphological studies will be required to resolve the taxonomic status of each complex, the restricted distribution of these ‘potential species’ should be taken into consideration when developing conservation and management strategies for anchialine Carideans and their habitats throughout the Indo-West Pacific.

5.6 REFERENCES

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Table 1. Measures of genetic diversity and tests of neutrality for populations of *Metabetaeus minutus*, *Antecaridina lauensis* and *Halocaridinides trigonophthalma* from the Ryukyu Archipelago, Japan.

		Metabetaeus minutus				*Antecaridina lauensis*				*Halocaridinides trigonophthalma*			
Island (code)	**Site Name (code)**	***n***	***nh***	***π***	***h***	***n***	***nh***	***π***	***h***	***n***	***nh***	***π***	***h***
Taketomi (TK)	Mi-na-ga (MIN)									16	14	0.028	0.983
Ishigaki (IS)	Yoshino Cave (YOSH)	6	6	0.006	1.000	16	7	0.065	0.775	6	3	0.001	0.600
Tarama (TA)	Shuga-ga (SHUG)					17	10	0.003	0.838				
	Futatsu-ga (FUT)	6	5	0.007	0.933								
	Fushato-ga (FUSH)									16	14	0.005	0.975
Miyako (MY)	Kikya-ga (KIK)	9	8	0.005	0.972								
	Tomori Ama-ga (TAG)									8	4	0.002	0.643
Irabu (IR)	Sabaoki Well (SW)									15	4	0.001	0.371
Minami-daito (MD)	Jr. High Cave (JR)	6	5	0.006	0.933	15	9	0.004	0.876				

Shintou Cave (SHIN)					10	7	0.004	0.876				
Miyahira Cave (MIY)	6	6	0.006	1.000								
Total	33	23	0.051	0.966	58	27	0.040	0.868	61	33	0.094	0.878

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

Table 2. Additional specimens and sequences included in 16S-rDNA analyses.

Taxonomic Group	Location	Accession Number	Source
Antecaridina sp.	East Timor	EF173754	Craft *et al.,* 2008
Antecaridina lauensis	Christmas Island	EU123850	Page *et al.,* 2008
	East Timor	EU123853	Page *et al.,* 2008
	Hawai'i[†]	------	Unpublished
	Ishigaki	XXXXX	This study
	Minami-daito	XXXXX	This study
Caridina rubella	Miyako-jima	XXXXX	This study
Halocaridina rubra	Hawai'i	EF173734	Craft *et al.,* 2008
	Hawai'i	EF173734	Craft *et al.,* 2008
Halocaridinides trigonophthalma	Taketomi	XXXXX	This study
	Taketomi	XXXXX	This study
	Miyako-jima	XXXXX	This study
Metabetaeus minutus	Minami-daito	XXXXX	This study

	Ishigaki	XXXXX	This study
	Christmas Island[††]	XXXXX	This study
Metabetaeus mcphersonae	Moorea[††]	XXXXX	This study
Metabetaeus lohena	Hawai'i	XXXXX	This study
Metabetaeus sp.	New Caledonia	FJ943435	Bracken *et al.*, 2009
Betaeus harrimani		FJ943434	Bracken *et al.*, 2009
Macrobrachium japonicum	Okinawa	DQ194935	Liu *et al.*, 2007

[†]Sequence provided by Timothy Page, Griffith University, Queensland Australia

[††]Samples provided by Arthur Anker, Florida Museum of Natural History

Table 3. Indicators of demographic expansion events in lineages of of *Metabetaeus minutus, Antecaridina lauensis* and *Halocaridinides trigonophthalma* of The Ryukyu Archipelago, Japan. *$P < 0.05$, ** $P < 0.001$

Species	Lineage	*n*	*nh*	Fu's F_s	Tajima's *D*
Metabetaeus minutus					
	Minami-daito	12	9	-2.650	0.412
	Miyako/Tarama/Ishigaki	21	14	-7.328**	-1.842*
Antecaridina lauensis					
	Ishigaki	10	4	-1.345*	-1.667*
	Ishigaki /Tarama/Minami-daito	48	23	-19.819**	-1.951**
Halocaridinides trigonophthalma					
	Ishigaki/Miyako/Irabu	29	9	-6.021**	-2.021**
	Tarama/Taketomi	30	24	-22.994**	-2.024**
	Taketomi	2	1	NA	NA

NA, not attempted.

Table 4. Pairwise Φ_{ST} (below diagonal) and S*nn* (above diagonal) estimates as measures of genetic differentiation between populations of *Metabetaeus minutus*, *Antecaridina lauensis* and *Halocaridinides trigonophthalma* of The Ryukyu Archipelago, Japan. **P* < 0.05

Metabetaeus minutus

	JR	MIY	KIK	FUT	YOSH
JR	-	0.389	1.000*	1.000*	1.000*
MIY	0.028	-	1.000*	1.000*	1.000*
KIK	0.956*	0.951*	-	0.392	0.244
FUT	0.947*	0.940*	-0.048	-	0.325
YOSH	0.951*	0.945*	-0.054	-0.061	-

Antecaridina lauensis

	JR	SHIN	SHUG	YOSH
JR	-	0.454	0.525	0.747*
SHIN	-0.031	-	0.408	0.707*
SHUG	-0.001	-0.039	-	0.727*
YOSH	0.575*	0.597*	0.597*	-

Halocaridinides trigonophthalma

	TAG	IR	FUSH	MIN	YOSH

TAG	-	0.541	1.000*	1.000*	0.468
IR	-0.002	-	1.000*	1.000*	0.552
FUSH	0.982*	0.986*	-	0.364	1.000*
MIN	0.908*	0.929*	0.070*	-	1.000*
YOSH	-0.013	-0.026	0.982*	0.901*	-

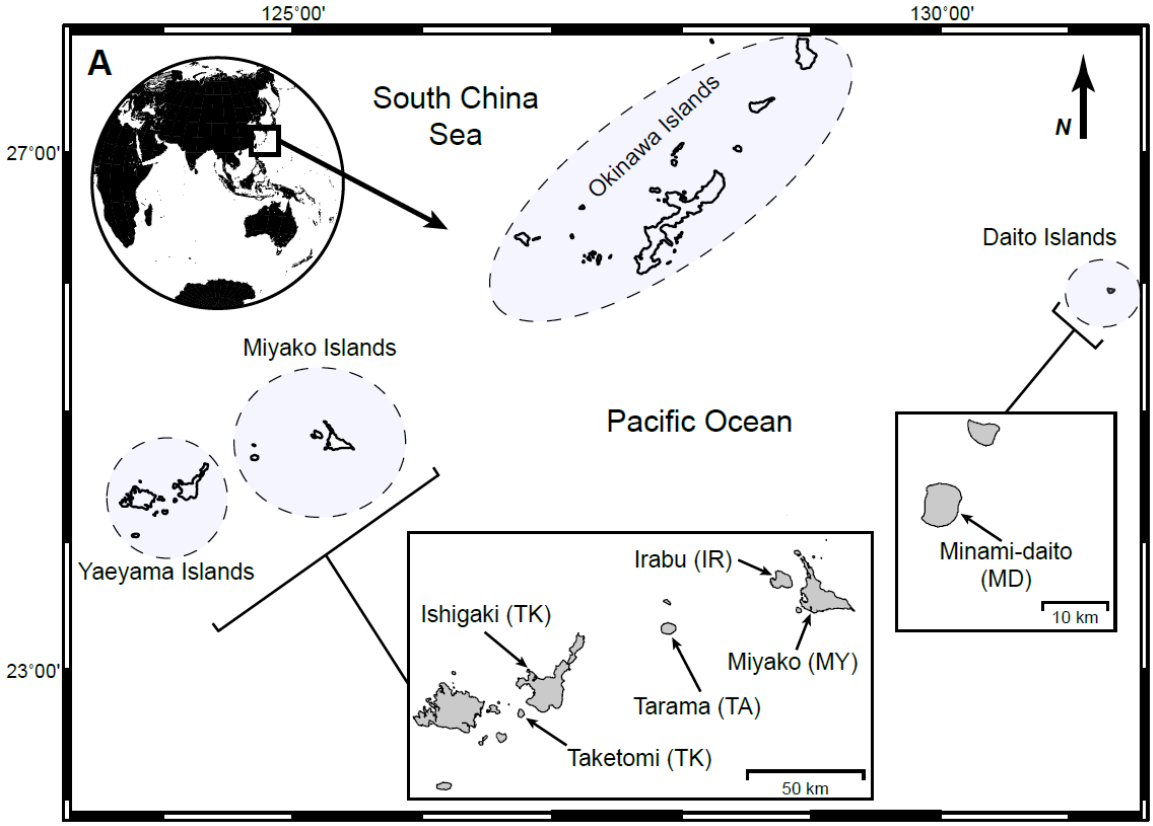

Figure 1. Map of the Southern Ryukyus Archipelago, Japan, indicating the location of the Ryukyu Archipelago (A) and enlarged maps of the Yaeyama, Miyako and Daito island groups from which individuals of the genus *Metabetaeus*, *Antecaridina*, and *Halocaridinides* were sampled for this study (B). Individual sampling site are listed in Table 1 and geographical coordinates of sampling sites are available from the corresponding author upon request.

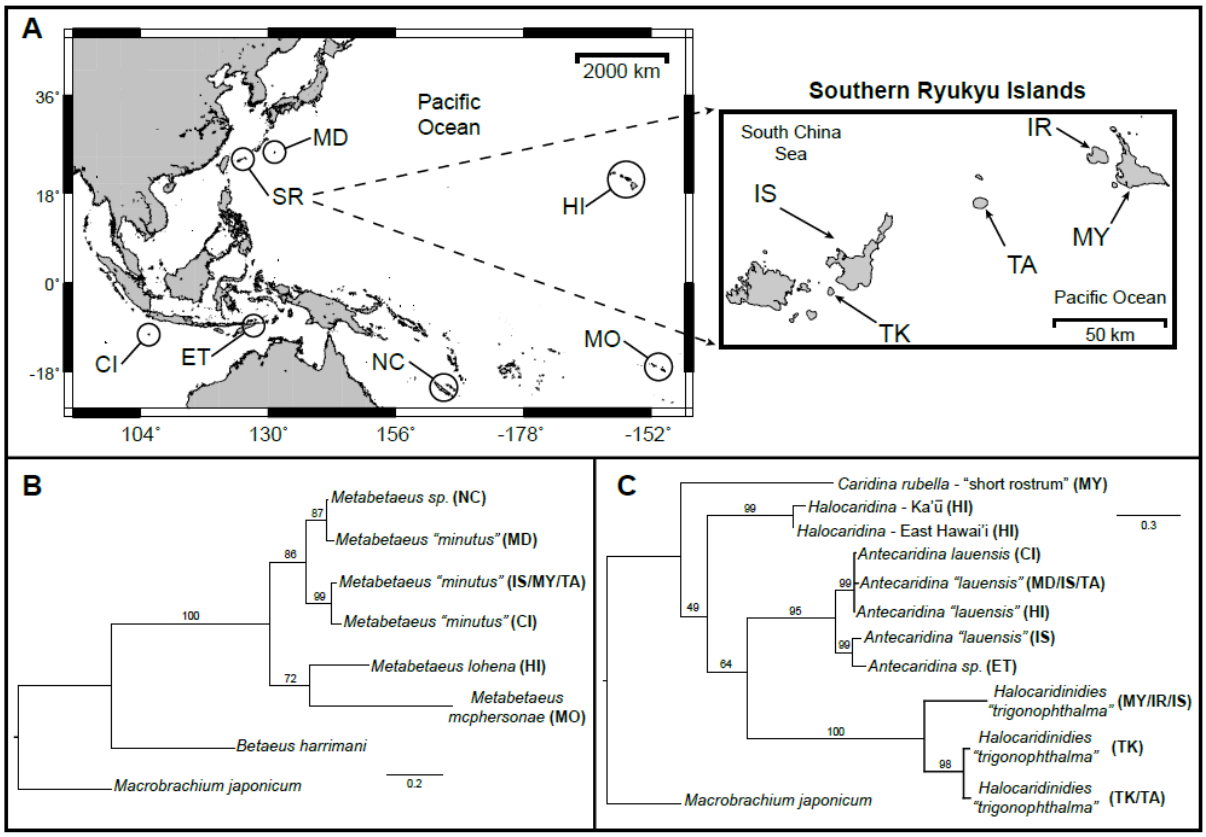

Figure 2. Phylogenetic relationships inferred for selected species of endemic Caridean shrimp of the Pacific Basin. (A) Map of sampling localities from the Pacific Basin and the Southern Ryukyus. Maximum likelihood (ML) trees for the Alpheid dataset (-lnL = -3,493.34; B) and the Atyid data set (-lnL = -4,958.78; C). Values above vertical lines represent bootstrap support as percentages of 100 re-samplings for ML analyses. CI- Christmas Island, ET- East Timor, HI- Hawai'i, IS- Ishigaki, MD- Minami-daito, MO- Moorea, MY- Myiako, NC- New Caledonia, TA- Tarama, TK- Taketomi.

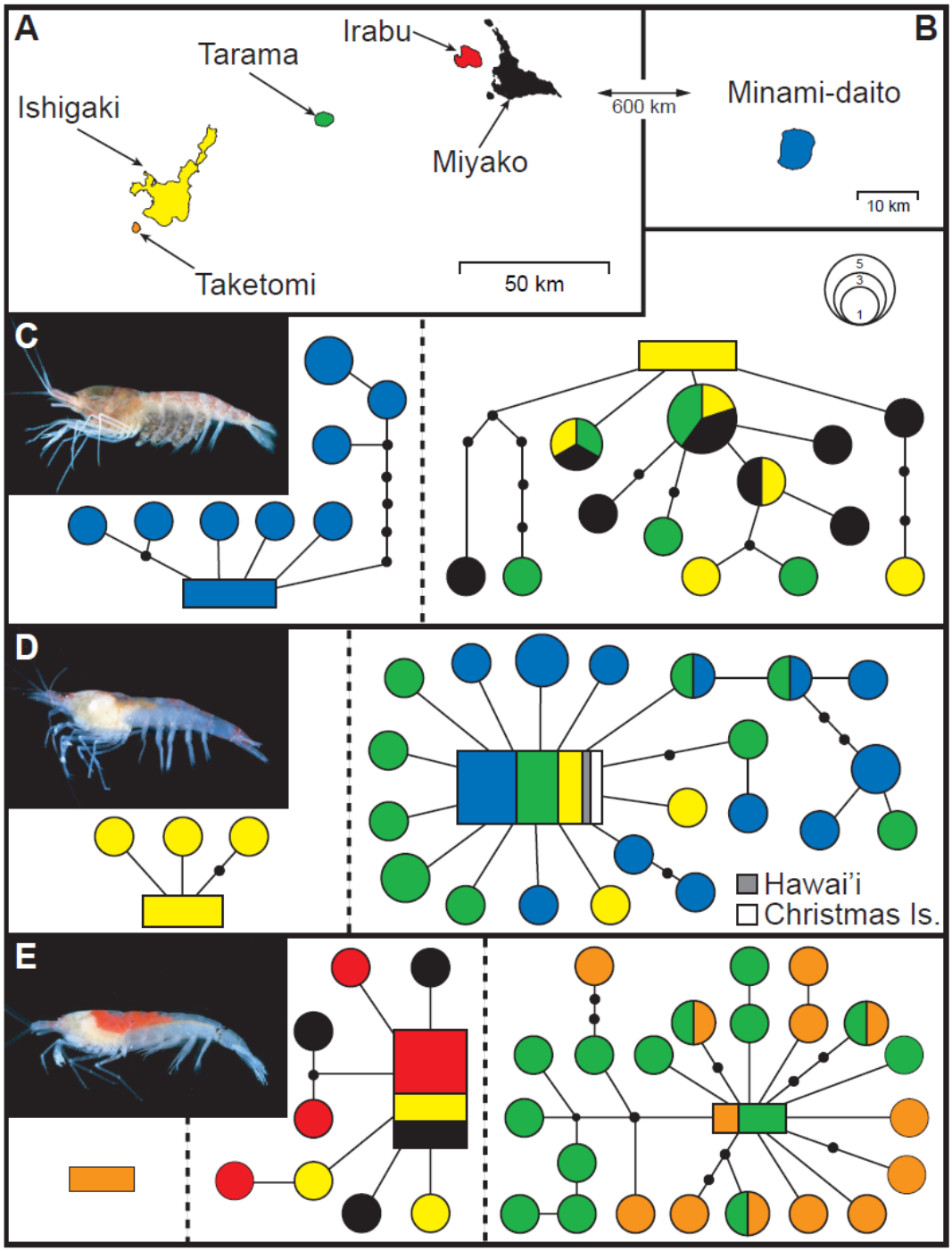

Figure 3. Networks depicting relationships among cytochrome *c* oxidase subunit I (COI) haplotypes recovered for three genera of endemic anchialine shrimp sampled from the Ryukyus Archipelago, Japan. Map of sampled islands; Southern Ryukyus (A; TK-Taketomi [orange], IS-Ishigaki [yellow], TA- Tarama [green], IR- Irabu [red], and MY- Miyako [black]) and Minami-

daito (B; MD- Minami-daito [blue]). Geographic distribution of *Metabetaeus* (C), *Antecaridina* (D) and *Halocaridinides* lineages (E). For each network, black dots represent unsampled (missing) haplotypes while rectangles represent the haplotype with the highest outgroup probability according to the analysis. The size of circles and rectangles are proportional to the frequency at which a specific haplotype was recovered. Despite variable lengths, each branch implies a single mutational difference between haplotypes. Color codes for each lineage match island colors in panel A and B. Dashed lines separate lineages.

CHAPTER 6. Conclusions

6.1 GENERAL CONCLUSIONS

We are currently witnessing the greatest loss of species since the extinction of the dinosaurs 65 million years ago (Warrick, 1998), with global climate change, the introduction of invasive species, and habitat destruction being significant threats to biodiversity around the world. Given this, there is now an urgency to accelerate research into unique island environments and their biota before they are lost forever. In this context, one of most threatened ecosystems on numerous islands may be the anchialine habitat. Historically, anchialine environments have experienced significant negative impacts from anthropogenic activities such as urbanization, groundwater extraction and contamination, and the introduction of invasive species. Thus, in order to manage these habitats and their fauna, it is important to develop an understanding of the biodiversity, ecology, and evolution of organisms from the anchialine niche since such knowledge will play a critical role in establishing conservation strategies for these environments around the world.

Subterranean environments, such as the anchialine niche, have long fascinated biologists (*e.g.*, Darwin's 'wrecks of ancient life'; 1859) and much of this fascination has centered on understanding the geographic distributions through space and time (*i.e.*, biogeography) of their inhabitants (Porter, 2007). Analyzing the population genetic and geographic structures of sympatric taxa can provide valuable information on the processes involved in generation patterns of diversity (Avise, 2000). If these processes are dominated by intrinsic factors (i.e. biological, ecological, physiological or behavioral), discordant patterns of population structure are expected to result, whereas extrinsic factors (i.e. physical, geological, environmental) are likely to result in similar patterns of population structure even among diverse taxa (McMillen-Jackson and Bert,

2003; Page and Hughes 2007). The goal of this dissertation was to examine the evolutionary history and phylogeography of organisms from the anchialine niche to gain a better understanding of the ecological and evolutionary forces influencing current biogeographic and diversity patterns in these unique habitats. Specifically, through the use of molecular markers (*i.e.*, mitochondrial cytochrome oxidase subunit I [COI] and large subunit ribosomal [16S-rDNA] genes), I investigated the population structure, phylogeography and evolution of five Caridean shrimp (*Halocaridina rubra, Caridina rubella, Antecaridina lauensis*, *Metabetaeus minutus* and *Halocaridinides trigonophthalma*) endemic to the anchialine ecosystem of the Pacific Basin to gain a better understanding of the combination of factors involved in creating current distribution patterns, including dispersal ability, potential vicariant events and rates of evolution. Furthermore, using *H. rubra* as an example, I demonstrate how such genetic information can be utilized to identify populations and habitats warranting conservation management.

Given the result presented here, it appears that many anchialine Carideans are much more restricted in their distributions then previously thought (*e.g.* Smith and Williams, 1981; Kano and Kase, 2004). In all cases, what has been previously thought to be one species distributed across an island (*e.g., C. rubella*), archipelago (*e.g., H. rubra*) or ocean (*e.g., M. minutus*, *A. lauensis* or *H. trigonophthalma*) actually consisted of multiple "cryptic species" (see below) restricted to a particular aquifer, island or island group. Following the reasoning presented above, these concordant patterns seen across multiple taxa suggest that extrinsic factors, such as geologic history and oceanographic conditions, have a large impact on the population structure and evolution of anchialine organism. For example, in Chapter 4, it was shown that divergence time estimates between populations, genetic groups and/or lineages across the islands of Maui and

Hawai'i were highly consistent with the geologic age of the basalt basins of their anchialine habitats of *H. rubra* from the Hawaiian Archipelago. Similarly, the apparent repeated colonizations of the Ryukyus by multiple lineages of *M. minutus, A. lauensis* or *H. trigonophthalma*, given their population structure and distributions across the islands, appear to be correlated with the geologic history and emergence of the islands that they inhabit (Chapter 5). Other extrinsic factors influencing the population structure of anchialine organisms appear to include regional oceanography (*i.e.*, circulation patterns, oceanographic currents and thermal/salinity gradients) and selection pressures (*i.e.*, habitat availability) (Chapter 4).

If these "regional" factors (Page and Hughes, 2007) were the sole processes influencing geographic structuring in these environments, the anchialine taxa inhabiting a particular region (*i.e.*, the Hawaiian or Ryukyu Archipelago) would exhibit similar patterns of geographic structuring. However, despite overall patterns of restricted dispersal, varying patterns of genetic differentiation and population structure was uncovered between sympatric taxa, suggesting the influence of intrinsic processes as well. For instance, while *H. rubra* exhibits extremely low levels of gene flow between populations over limited geographic scales (Santos, 2006; Craft et al., 2008) a later study of *M. lohena,* which produces planktotrophic larvae, revealed little to no population structure among populations sampled from the same anchialine habitats (Russ et al., 2011). Furthermore, if extrinsic barriers to gene flow were the sole factors influencing anchialine diversity, one would have expected similar phylogeographic patterns between *C. rubella, M. minutus, A. lauensis* and *H. trigonophthalma* distributed across Ryukyus (Chapters 4-5). However, the phylogenetic relationships and geographic distributions of these species suggest that each responded uniquely to the dynamic geologic and oceanographic histories of the Indo-West Pacific. These unique responses are likely due to slight, but significant, differences in

intrinsic life-history characteristics or ecological factors, such as dietary differences, other resource partitioning and/or competition. For example, small differences in life history characteristics (*i.e.*, egg size, larval stages, and larval habitat) and larval feeding mode (*i.e.*, lecithotrophy versus planktotrophy) have been shown to strongly influence both population structure and dispersal potential of Carideans (Shokita, 1979, Page and Hughes, 2007).

Collectively, these studies demonstrate how the ecology, population structure and evolutionary history of organisms endemic to the anchialine niche result from a complex interaction between extrinsic obstacles to gene flow (*i.e.*, geologic history and oceanography) and intrinsic limits to dispersal (*i.e.*, life history traits and developmental mode). Understanding these complex interactions is important since such information is vital for developing management plans to preserve the species and, by extension, the anchialine ecosystem in the Pacific Basin.

These studies also suggest that the diversity of taxa inhabiting the anchialine habitats of the Pacific may be vastly underestimated. The anchialine fauna of the Indo-West Pacific is dominated by Caridean shrimp, with 11 species of shrimp thought to be distributed throughout Pacific (de Grave and Sakihara, 2011). However, in Chapter 4, it was shown that *C. rubella* on the island of Miyako-jima actually consists of two morphologically and genetically distinct species. Furthermore, *M. minutus*, *A. lauensis* and *H. trigonophthalma* of the Ryukyus were all found to represent possible species complexes, each comprised of at least two possible divergent species, all more restricted then previously thought (Chapter 5). Additionally, *H. rubra* of the Hawaiian Archipelago has also recently been revealed to be a complex of at least eight potential species (Santos 2006; Craft *et al.*, 2008). In fact, of the six anchialine species from the Pacific Basin studied from a genetic prospective to date, only *M. lohena* has failed to reveal a species

complex (Russ *et al.*, 2010). Given these results, genetic analyses of additional Carideans inhabiting the anchialine niche are likely to reveal additional cryptic taxa. While further morphological studies are needed to resolve the taxonomic status of each complex and its potential species, the restricted distribution of these 'potential species' need to be taken into consideration when developing conservation and management strategies for these Carideans and the anchialine habitats of the Indo-Pacific.

6.2 Future Perspectives

While these phylogeographic and population genetic studies have contributed to our understanding of the evolutionary and biogeographic history of anchialine Carideans, many questions sill remain. As with most population genetic studies, additional sampling localities need to be visited. For example, further sampling of *M. minutus, A. lauensis* and *H. trigonophthalma* from outside the Ryukyus would better elucidate the evolutionary history of these shrimp in the Pacific Basin. Furthermore, with additional samples, a better understanding of thier phylogenetic relationships and the identification of a few geologic calibration points, it may be possible to utilize the molecular divergence between populations from the different island groups to infer the geologic history and formation of the Southern Ryukyus (the original goal of my NSF proposal) for which no hypothesis has received universal acceptance (Muraji *et al.*, 2008). Lastly, as mentioned above, cryptic species complexes were revealed for each of these three "species". With increased sampling from areas such as New Caledonia, Easter Island, Christmas Island, the Philippines, Palau, Guam and other locations where these "species" have been reported, additional morphological studies will be needed to resolve the taxonomic status of each complex and its potential species.

For *Halocaridina,* the proverbial "white whale" appears to be a well-resolved phylogeny between the eight lineages. It is hypothesized that colonization patterns of *Halocaridina* across the Hawaiian Islands would follow the "Progression Rule", in which populations from older islands act as founders for ones on younger islands (Funk & Wagner 1995). However, despite a number of attempts with a wide range of molecular markers including microsatellites, exon-priming intron-crossing markers, amplified fragment length polymorphism (AFLP) and a number of nuclear loci, the relationships between the eight *Halocaridina* lineages are largely unresolved (Craft *et al.* 2008). Hopefully, through the utilization of next-generation sequencing technologies, appropriate markers can be identified to fully resolve these relationships which might provide new insights on dispersal and colonization in the archipelago (Craft *et al.* 2008).

Leaving the anchialine system, I believe it would be interesting to investigate the population structure and phylogeographic history of some of the freshwater atyids inhabiting the streams of the Hawaiian and Ryukyu Archipelagos since such studies may shed new light on both the origin and causes of species diversity both within an archipelago and on individual islands (Emmerson, 2002). Although the more than 21 species of freshwater shrimp (Cai and Shokita, 2006) inhabiting the river and streams of the Ryukyu Islands have been relatively well studied in recent decades (Cai and Shokita, 2006), none have been examined from a genetic prospective. Such studies would offer the opportunity to (i) investigate the colonization history, evolution and origin of the Ryukyu shrimp fauna (*e.g.*, Maekawa *et al.*, 1999), (ii) test hypotheses regarding the effects of life history traits on the dispersal abilities and colonization of the "inlandwater shrimps" of the Ryukyus put forth by Shokita (1979), (iii) examine the biological impacts of the Takara and Keroma Gaps on the freshwater diversity of the Ryukyus (*e.g*. Muraji *et al.*, 2008). Furthermore, comparative phylogeographic studies between

freshwater and anchialine Carideans may provide further insight into the ecological and evolutionary forces acting on anchialine taxa in general.

Lastly, recent advances in next-generation sequencing technologies have transformed the way we think about many aspects of molecular biology and evolution (Tautz *et al.*, 2010). Utilizing these new techniques, a number studies regarding anchialine crustaceans could be envisioned, including, but not limited to: studying the genetics of phenotypic differences between subterranean and surface taxa through transcriptional profiling, examining genomic signatures of adaption to life in the anchialine environment and identifying single nucleotide polymorphisms (SNPs) to better understand diversity between closely related populations and species.

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