Multiple Colonizations Lead to Cryptic Biodiversity in an Island Ecosystem: Comparative Phylogeography of Anchialine Shrimp Species in the Ryukyu Archipelago, Japan

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Abstract. Archipelagos of the Indo-West Pacific are considered to be among the richest in the world in biodiversity, and phylogeographic studies generally support either the *center of origin* or the *center of accumulation* hypothesis to explain this pattern. To differentiate between these competing hypotheses for organisms from the Indo-West Pacific anchialine ecosystem, defined as coastal bodies of mixohaline water fluctuating with the tides but having no direct oceanic connections, we investigated the genetic variation, population structure, and evolutionary history of three caridean shrimp species (*Antecaridina lauensis*, *Halocaridinides trigonophthalma,* and *Metabetaeus minutus*) in the Ryukyu Archipelago, Japan. We used two mitochondrial genes—cytochrome *c* oxidase subunit I (*COI*) and large ribosomal subunit (*16S-rDNA*)—complemented with genetic examination of available specimens from the same or closely related species from the Indian and Pacific Oceans. In the Ryukyus, each species encompassed 2–3 divergent (9.52%–19.2% *COI* p-distance) lineages, each having significant population structure and varying geographic distributions. Phylogenetically, the *A. lauensis* and *M. minutus* lineages in the Ryukyus were more closely related to ones from outside the archipelago than to one another. These results, when interpreted in the context of Pacific oceanographic currents and geologic history of the Ryukyus, imply multiple colonizations of the archipelago by the three species, consistent with the *center of accumulation* hypothesis. While this study contributes toward understanding the biodiversity, ecology, and evolution of organisms in the Ryukyus and the Indo-West Pacific, it also has potential utility in establishing conservation strategies for anchialine fauna of the Pacific Basin in general.

Introduction

Geological events (tectonics, volcanism, sea-level changes, *etc.*) can significantly impact the flora and fauna of an area, 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 geologic history and generally high levels of endemism, are ideal systems for studying how such events influence organismal diversification and community composition. Archipelagos of the Indo-West Pacific have received considerable interest in this regard (Liu, X. *et al.,* 2008) since the species diversity of many of these island groups is among the richest in the world (Myers *et al.,* 2000; Meijaard, 2003). Although a number of hypotheses have been proposed to explain the high species diversity of these island groups (reviewed by Carpenter *et al.,* 2011), phylogeographic 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 into surrounding areas (Benzie, 1998; 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, or Australasian biotas; Murphy and Austin, 2005; Page *et al.,* 2007; De Bruyn and Mather, 2007). However, with a few exceptions (see Bird *et al.,* 2007, 2011), many studies conducted to date have focused on single species, often leading to an inability to differentiate between these two possibilities (see De Bruyn and 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 this island chain formed during the late Miocene (~10 MYA) after subduction of the Philippine Plate by the Eurasian Plate (Konishi and Sudo, 1972; Koba, 1980; Lee *et al.*, 1980). During low sea-level stands associated with glacial periods of the late Pliocene (~2.5–3 MYA), a landbridge is thought to have extended to southeastern China, connecting the central Ryukyus with Taiwan and continental Asia (Kimura, 2000). Furthermore, this landbridge was submerged during the interglacial periods of the early Pleistocene (~2–2.5 MYA), creating islands corresponding to the present island groups (Hikida and Motokawa, 1999); these experienced sequential periods of connection and isolation due to fluctuating sea levels throughout the Pleistocene (Lin *et al.,* 2002). These latter events are believed to have significantly influenced the distributions and diversification of fauna in the Ryukyus, and the phylogenetic relationships of several terrestrial taxa reflect these paleogeographic patterns (Suzuki, 2001). 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, X. *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 is organisms from the anchialine niche. This ecosystem comprises land-locked bodies of salt or brackish water that fluctuate with the tides due to subterranean connections to both the open ocean and the freshwater aquifer systems (Holthuis, 1973; Maciolek, 1983). Such habitats have been reported from the Sinai Peninsula, Bermuda, the Caribbean, the Hawaiian Islands, the South Pacific, and the Philippines, as well as the Ryukyus (Maciolek, 1986). While exceptions exist (Kano and Kase, 2004; Russ *et al.,* 2010; Cabezas *et al*., 2012), numerous genetic studies have revealed cryptic species complexes with exceptional levels of endemism and

strong population structure on the scale of only a few kilometers for a number of anchialine organisms (Santos, 2006; Craft *et al.,* 2008; Hunter *et al.,* 2008; Page *et al.,* 2008; Santos and Weese, 2011; Weese *et al.*, 2012). For example, populations of the anchialine shrimp *Caridina rubella* Fujino and Shokita, 1975 (Decapoda: Atyidae) on the island of Miyako in the Ryukyus likely belong to two distinct species that are significantly structured across distances ranging from less than 20 m to more than 10 km (Weese *et al.,* 2012). Additionally, anchialine habitats have generally been heavily influenced by sea-level changes during the Quaternary (Mylroie and Mylroie, 2011); for anchialine organisms in the Ryukyus, such migrating coastlines may have led to multiple cycles of contraction and expansion of suitable habitats (*i.e.,* the subterranean aquifer system) in the archipelago. In this context, comparing the phylogeography of different taxa from this ecosystem provides an opportunity to understand how past environmental changes 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 Ryukyus, with at least 11 shrimp and crab species having been recorded (Komai and Fujita, 2005; Cai and Shokita, 2006; Naruse and Tamura, 2006; Fujita, 2007; Fujita and Sunagawa, 2008). Of these, three species of caridean shrimp—*Antecaridina lauensis* Edmondson, 1935 (Decapoda: Atyidae), *Halocaridinides trigonophthalma* Fujino and Shokita, 1975 (Decapoda: Atyidae), and *Metabetaeus minutus* Whitelegge, 1897 (Decapoda: Alpheidae)—have disjunct distributions in the Ryukyus as well as across the Pacific Basin (Cai and Shokita, 2006; Anker, 2010). If, as suggested by the *center of origin* hypothesis, the dynamic geologic history of the Ryukyus contributed to the distributions and diversification of anchialine diversity on these islands, it is hypothesized that the phylogeographic patterns and evolutionary relationships of *A. lauensis*, *H. trigonophthalma*, and *M. minutus* in the archipelago would reflect these events. On the other hand, if the phylogeographic patterns and evolutionary relationships of these species are not correlated with the sequential separation of island groups in the Ryukyus, this would imply that the anchialine diversity of the archipelago is due to the accumulation of distinct lineages from elsewhere *via* dispersal (*i.e.,* the *center of accumulation* hypothesis). To differentiate between these two alternatives, the genetic diversity, phylogeography, and evolutionary history of the three species were investigated across the Ryukyus. Specifically, sequence analyses of the mitochondrial (mtDNA) cytochrome *c* oxidase subunit I [*COI*] gene were employed to measure genetic variation and estimate population structure. Sequences from the mtDNA large ribosomal subunit (*16S-rDNA*) were then utilized to infer phylogenetic relationships among *A. lauensis*, *H. trigonophthalma,* and *M. minutus*
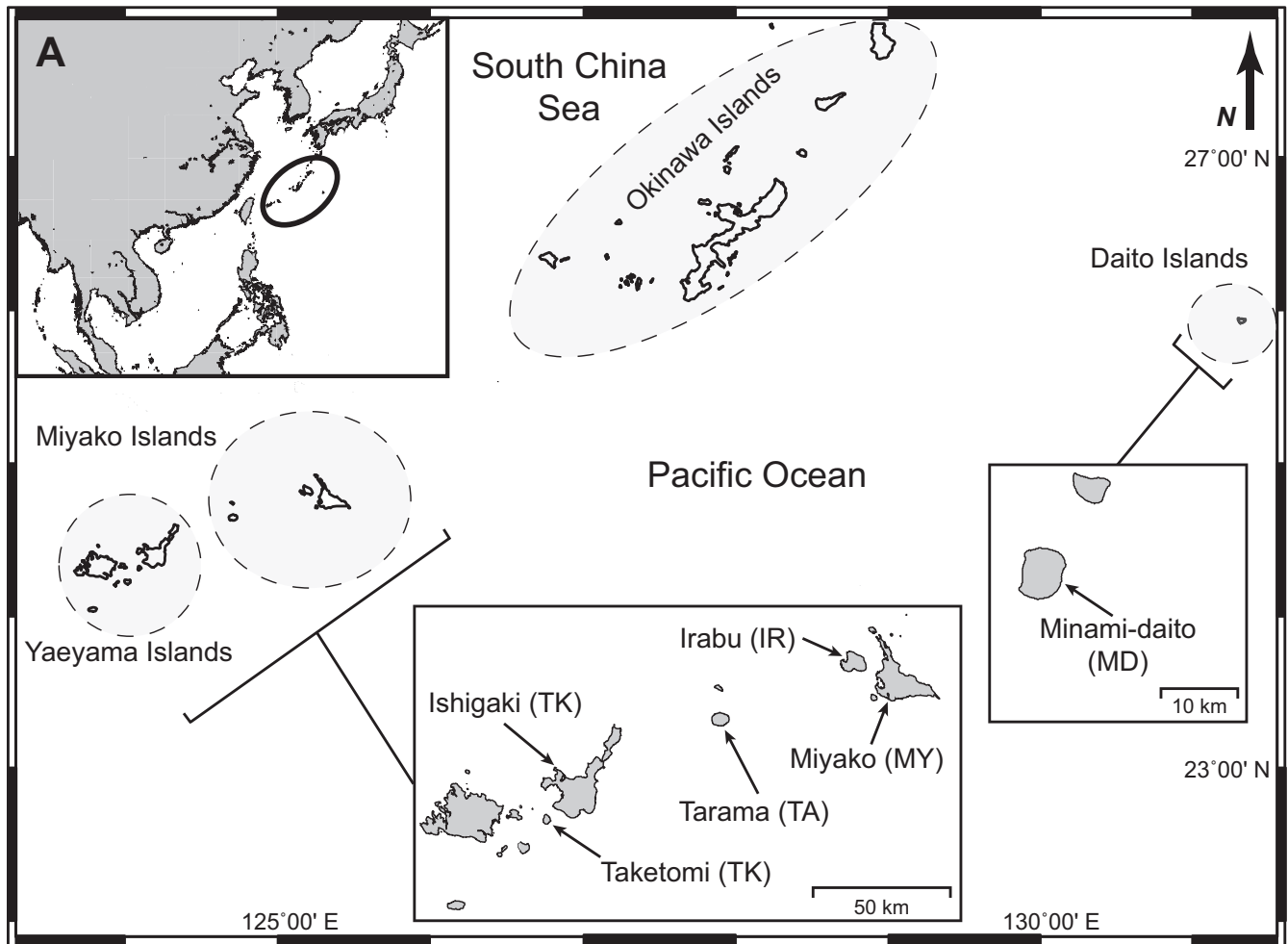
Figure 1. Map of the Southern Ryukyus Archipelago, Japan, indicating the location of the Ryukyu Archipelago (A), and enlarged maps of the Yaeyama, Miyako, and Daito island groups from which individuals of *Antecaridina lauensis, Halocaridinides trigonophthalma,* and *Metabetaeus minutus* were sampled for this study. Individual sampling sites are listed in Table 1, and geographical coordinates are available from the corresponding author upon request.

from within and outside the Ryukyus. Using this combined approach, this study illuminates the complex biogeography of these three endemic anchialine caridean shrimp species in the Indo-West Pacific.

Materials and Methods

Taxon sampling and DNA extraction and sequencing

About 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 spanning the six islands of Ishigaki, Taketomi, Tarama, Miyako, Irabu, and Minami-daito (Fig. 1). From each hab-

itat, 6–17 individuals (Table 1) were collected by using baited traps or small aquarium nets and immediately preserved in >90% ethanol for genetic analyses. For phylogenetic analyses (see below), an individual representing "short rostrum" *Caridina rubella* (see Weese *et al.,* 2012) was collected from Miyako, and additional *Antecaridina* and *Metabetaeus* specimens from outside the Ryukyus were supplied by Dr. Arthur Anker (Florida Museum of Natural History) as materials preserved in 75% ethanol.

Total genomic DNA was extracted from each individual using $2\times$ cetyltrimethyl ammonium bromide (CTAB)/chloroform according to procedures in Santos (2006). Between 10 and 30 ng of DNA was utilized as template to amplify an ~670 base pair (bp) fragment of the mitochondrial (mtDNA) cytochrome *c* oxidase subunit I (*COI*) gene *via* polymerase chain reaction (PCR). Reactions were con-

Table 1

Sample sizes and indices of genetic diversity for populations of Antecaridina lauensis, Halocaridinides trigonophthalma, *and* Metabetaeus minutus *from the Ryukyu Archipelago, Japan, based on cytochrome* c *oxidase subunit I* (COI) *sequences*

Island (code)	Site Name (code)	*Antecaridina lauensis*				*Halocaridinides trigonophthalma*				*Metabetaeus minutus*			
		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)	16	7	0.065	0.775	6	3	0.001	0.600	6	6	0.006	1.000
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)	15	9	0.004	0.876					6	5	0.006	0.933
	Shintou Cave (SHIN)	10	7	0.004	0.876								
	Miyahira Cave (MIY)									6	6	0.006	1.00
	Total	58	27	0.040	0.868	61	33	0.094	0.878	33	23	0.051	0.966

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

ducted in 25-μl volumes containing 10 mmol l^{-1} Tris–HCl (pH 8.3), 50 mmol l^{-1} KCl, 0.001% gelatin, 2.0 mmol l^{-1} MgCl$_2$, 200 μmol l^{-1} dNTPs, 0.4 μmol l^{-1} each of primers LCO1490 and HCO2198 (Folmer *et al.*, 1994), and 1 U *Taq* polymerase. Reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) using the cycling profile from Santos (2006). Additionally, sequence data from the mtDNA large ribosomal subunit (*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 μmol l^{-1} each of primers CRUST16SF and CRUST16SR (Ivey and Santos, 2007) along with the reaction constituents outlined above.

Amplicons were purified with Montage PCR filter units (Millipore, Billerica, MA) according to the supplier's directions, cycle-sequenced in both directions using Big-Dye Terminators ver. 3.1, and read on a PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Ambiguities in chromatograms were corrected by comparison with the complementary DNA strand in SEQUENCHER ver. 4.7 (Gene Codes Corporation, Ann Arbor, MI). Finished *COI* and *16S-rDNA* sequences were aligned manually using SE-AL ver. 2.0a11 (Rambaut, 2008).

Genetic diversity and population genetic analyses

Population-level analyses utilized mtDNA *COI* sequence data since this gene has proven informative for other atyid and alpheid 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 ver. 5.10.00 (Rozas *et al.,* 2003). To assess potential genetic differentiation between populations, pairwise Φ_{ST} statistics (which incorporate information from both haplotype frequencies and molecular divergence) were calculated with Arlequin ver. 3.11 (Excoffier *et al.*, 2005). For these comparisons, the Tamura and Nei (1993) model of DNA evolution, as selected by the Akaike Information Criterion (AIC) with the FindModel web version of Modeltest (Posada and Crandall, 1998; Posada, 2006), was employed. As another measure of potential 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 ver. 1.21 (Clement *et al.*, 2000), which utilizes the cladogram estimation algorithm of Templeton *et al.* (1992). These analyses were conducted under default settings, providing 95% parsimony plausible branch connections between haplotypes. Reticulations in networks, representing ambiguous connections, were resolved using the criteria outlined in Crandall *et al.* (1994). In cases where multiple networks were recovered per species, the historical demography of populations in each network was inferred using Tajima's *D* (Tajima, 1989) and Fu's F_s (Fu, 1997) neutrality tests conducted in Arlequin. Both methods provide information on demographic history in the absence of selection, with significant negative or positive values generally suggesting population expansions

Table 2

Results of neutrality tests for genetic lineages of Antecaridina lauensis, Halocaridinides trigonophthalma, *and* Metabetaeus minutus *from the Ryukyu Archipelago, Japan, based on cytochrome* c *oxidase subunit I* (COI) *sequences*

Species	Lineage	*n*	*nh*	Fu's F_s	Tajima's *D*
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
Metabetaeus minutus	Minami-daito	12	9	−2.650	0.412
	Miyako/Tarama/Ishigaki	21	14	−7.328**	−1.842*

n, number of sampled individuals; *nh,* number of unique haplotypes; NA, not attempted.
* $P < 0.05$, ** $P < 0.001$.

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, as well as the S_{nn} in DnaSP, by 1000 permutations.

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 phylogenetic analyses, two datasets were assembled and analyzed in the same manner. The first consisted of atyid *16S-rDNA* sequences, including representatives of the *A. lauensis, H. trigonophthalma*, and "short rostrum" *Caridina rubella* sequences generated here along with additional *Antecaridina* spp. and *Halocaridina rubra* Holthuis, 1973 (Decapoda: Atyidae) sequences from GenBank (Table 2). The second dataset comprised alpheid *16S-rDNA* sequences, including representatives of *M. minutus, M. lohena* Banner and Banner, 1960 (Decapoda: Alpheidae), and *M. mcphersonae* Anker, 2010 (Decapoda: Alpheidae) from this study and additional *Metabetaeus* sp. and *Betaeus harrimani* Rathbun, 1904 (Decapoda: Alpheidae) sequences acquired from GenBank (Table 2). Both datasets utilized *Macrobrachium japonicum* De Haan, 1849 (Decapoda: Palaemonidae) as the outgroup. The *16S-rDNA* sequences were aligned using ClustalX (Thompson, 1997) and adjusted manually using SE-AL. For each dataset, evolutionary relationships were inferred *via* Maximum Likelihood (ML) analyses with PHYML ver. 3.0 (Guindon and Gascuel, 2003) under the appropriate model of evolution chosen by the AIC in Modeltest ver. 3.7 (Posada and 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 1000 bootstrap replicates and resulting phylogenetic trees visualized with FigTree ver. 1.3.1 (Rambaut, 2009).

Results

Genetic diversity

Direct sequencing of mtDNA *COI* amplicons yielded a 630-bp gene fragment from each of the 33, 61, and 58 individuals of *Antecaridina lauensis, Halocaridinides trigonophthalma*, and *Metabetaeus minutus* analyzed in this study. From these, 27 *A. lauensis*, 33 *H. trigonophthalma*, and 23 *M. minutus,* unique *COI* haplotypes were identified (Table 1, Table A1 [see appendix]) and deposited into GenBank under accession numbers KC879738–KC879820. Although nuclear copies of mitochondrial derived genes (numts: Lopez *et al.,* 1994) can be common and potentially problematic in arthropods (Buhay, 2009), translation of these *COI* nucleotide sequences into amino acids found no stop codons, and any identified non-synonymous changes were to amino acid residues with similar biochemical properties (data not shown). This finding implies that sequences analyzed here were derived from mitochondrial copies, rather than numts, of *COI*.

Population genetic analyses

Antecaridina lauensis. Estimates of *h* (0.775) and π (0.065) were mostly consistent across populations of *A. lauensis*, with the sole exception being Yoshino Cave (YOSH) on Ishigaki (Fig. 2A, Table 1). While no significant genetic structure was detected *via* pairwise Φ_{ST} or S_{nn} analyses between *A. lauensis* populations in the southern Ryukyus and Minami-daito, the YOSH population was significantly different from all others in the Ryukyus (Table 3). Parsimony (TCS) analysis of the *COI* sequences produced two discrete networks, with individuals from the YOSH population occurring in both (Fig. 2C). One network consisted of 23 haplotypes and 48 individuals from the islands of Ishigaki, Tarama, and Minami-daito (Fig. 2B), whereas the other included four haplotypes from 10 individuals specific to the YOSH population on Ishigaki (Fig. 2C). Uncorrected *p*-distances among *COI* haplotypes were 0.16%–1.4%
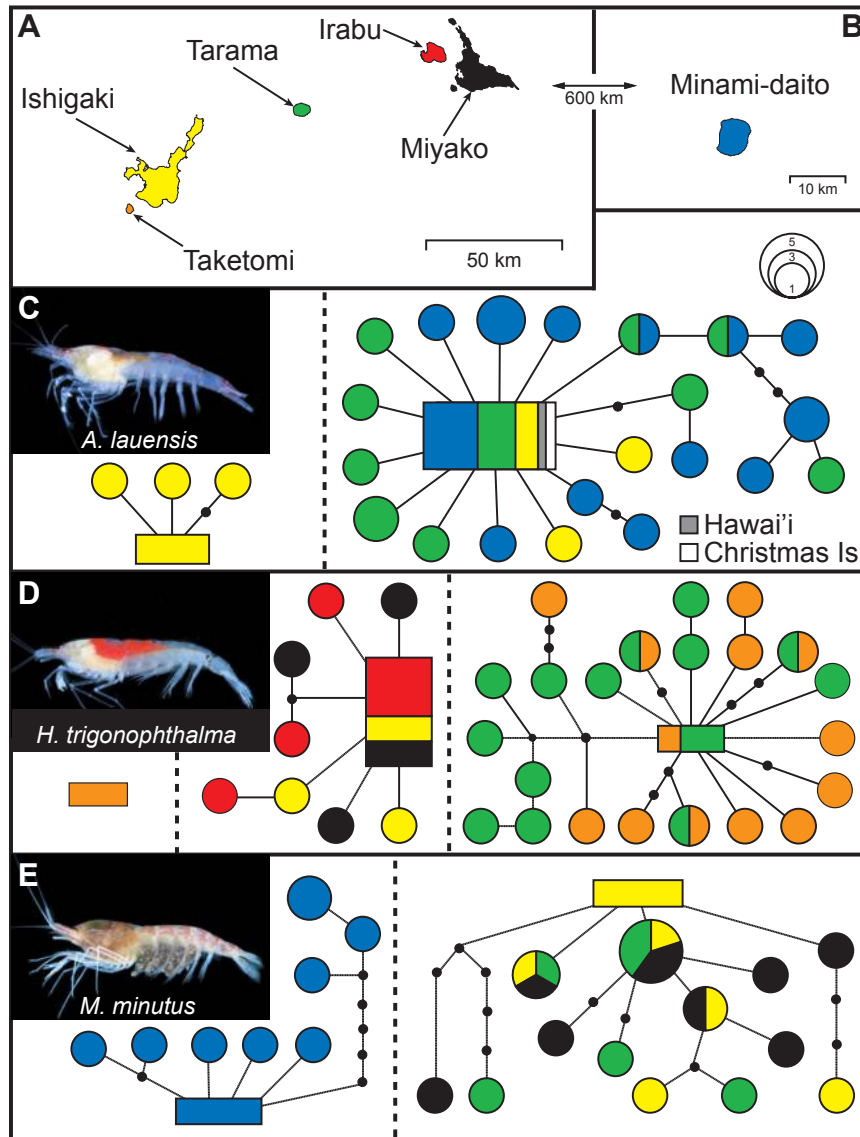
Figure 2. Networks depicting relationships among cytochrome *c* oxidase subunit I (*COI*) haplotypes of *Antecaridina lauensis, Halocaridinides trigonophthalma,* and *Metabetaeus minutus* from the Ryukyus Archipelago, Japan. Map of sampled islands: (A) Southern Ryukyus (TK—Taketomi [orange], IS—Ishigaki [yellow], TA—Tarama [green], IR—Irabu [red], and MY—Miyako [black]); and (B) Minami-daito (MD—Minami-daito [blue]). Specific networks for the genetic lineages of *A. lauensis* (C), *H. trigonophthalma* (D), and *M. minutus* (E) identified in this study, with each haplotype color-coded (as in panels A and B) by the island(s) it was sampled from. For each network, filled black dots represent unsampled (*i.e.*, missing) haplotypes, and rectangles represent the haplotype with the highest outgroup probability according to the analysis. The size of circles and rectangles is proportional to the frequency at which a specific haplotype was recovered. Despite variable lengths, each branch implies a single mutational difference between haplotypes. Dashed lines separate networks for the two or three divergent genetic lineages within each species.

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 sequences in the two networks (Table 2), suggesting recent population expansions in both groups. When *COI* sequences from *A. lauensis* and *Antecaridina* sp. sampled in Hawai'i, Christmas Island in the Indian Ocean (both supplied by Dr. Timothy J. Page, Grif-

fith University, Queensland, Australia; unpubl. data), and East Timor (GenBank accessions EF173843–EF173847) from Craft *et al.* (2008) are included in the parsimony analysis, those from Hawai'i (*n* = 1) and Christmas Island (*n* = 2) were identical to the inferred ancestral haplotype found in the Ishigaki, Tarama, and Minami-daito populations (Fig. 2B), while a third network, composed of five

Table 3.

Pairwise Φ_{ST} (below diagonal) and Snn (above diagonal) estimates as measures of genetic differentiation between populations of Antecaridina lauensis, Halocaridinides trigonophthalma, *and* Metabetaeus minutus *from the Ryukyu Archipelago, Japan, based on cytochrome* c *oxidase subunit I* (COI) *sequences*

	Population Site Code				
	Antecaridina lauensis				
	JR	SHIN	SHUG	YOSH	
JR	–	0.454	0.525	0.747*	
SHIN	0.000	–	0.408	0.707*	
SHUG	0.000	0.000	–	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	0.000	–	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	0.000	0.000	0.982*	0.901*	–
	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*	0.000	–	0.325
YOSH	0.951*	0.945*	0.000	0.000	–

Site code: See Table 1.

* $P < 0.05$.

haplotypes from the eight East Timor individuals (*i.e.*, an *Antecaridina* sp.), was also recovered (data not shown).

Halocaridinides trigonophthalma. For *H. trigonophthalma*, estimates of h spanned from 0.371 to 0.983, and with the exception of the one site on Taketomi, π values were consistent among populations (Table 1). Significant genetic structure was found between those populations on the islands of Taketomi and Tarama relative to ones on the islands of Miyako, Irabu, and Ishigaki (Table 3), and three discrete networks were recovered from the parsimony analysis of *H. trigonophthalma* (Fig. 2D). The first corresponded to 9 haplotypes and 29 individuals from populations on Miyako, Irabu, and Ishigaki; the second was composed of 24 haplotypes and 30 individuals from the Taketomi and Tarama populations (Fig. 3A); and the third consisted of two individuals and one haplotype exclusive to the island of Taketomi (Fig. 2D). Here, *p*-distances among haplotypes in the same network ranged from 0.16% to 1.2%, while those between the three networks were from 9.52% to 19.2%. Two of the three *H. trigonophthalma* groups displayed evidence of recent population expansions as evident from significantly negative Tajima's D and Fu's F_s values (Table 2).

Metabetaeus minutus. Estimates of haplotype diversity (h) for *M. minutus* ranged from 0.93 to 1.0, with nucleotide diversity (π) being consistent among populations (Table 1). Pairwise Φ_{ST} or S_{nn} values approaching, or at, their upper limit of 1.0 (Table 4) were estimated between the Southern Ryukyus and Minami-daito, implying strong genetic structure between populations separated by more than 600 km (Fig. 2A, B). On the other hand, no genetic structure was identified among *M. minutus* populations of the southern Ryukyus spanning a distance of about 200 km (Table 3). Two discrete networks (Fig. 2E) were recovered from the parsimony analysis: one represented *M. minutus* from the Southern Ryukyus, consisting of 14 haplotypes from 21 individuals collected on Ishigaki, Miyako, and Tarama islands; the second was from populations on Minami-daito, encompassing 9 haplotypes recovered from 12 individuals (Table 3). Uncorrected (*p*) distances among haplotypes within each network ranged from 0.16% to 1.4%, while those for haplotypes between the two networks ranged from 9.7% to 10.6%. Both Tajima's D and Fu's F values were significantly negative for *M. minutus* of the Southern Ryukyus, while those for populations on Minami-daito were not significant (Table 2).

Phylogenetic analyses

Analyses of *A. lauensis*, *H. trigonophthalma,* and *M. minutus* from the Ryukyus at the population level identified each as being composed of two to three distinct and diver-
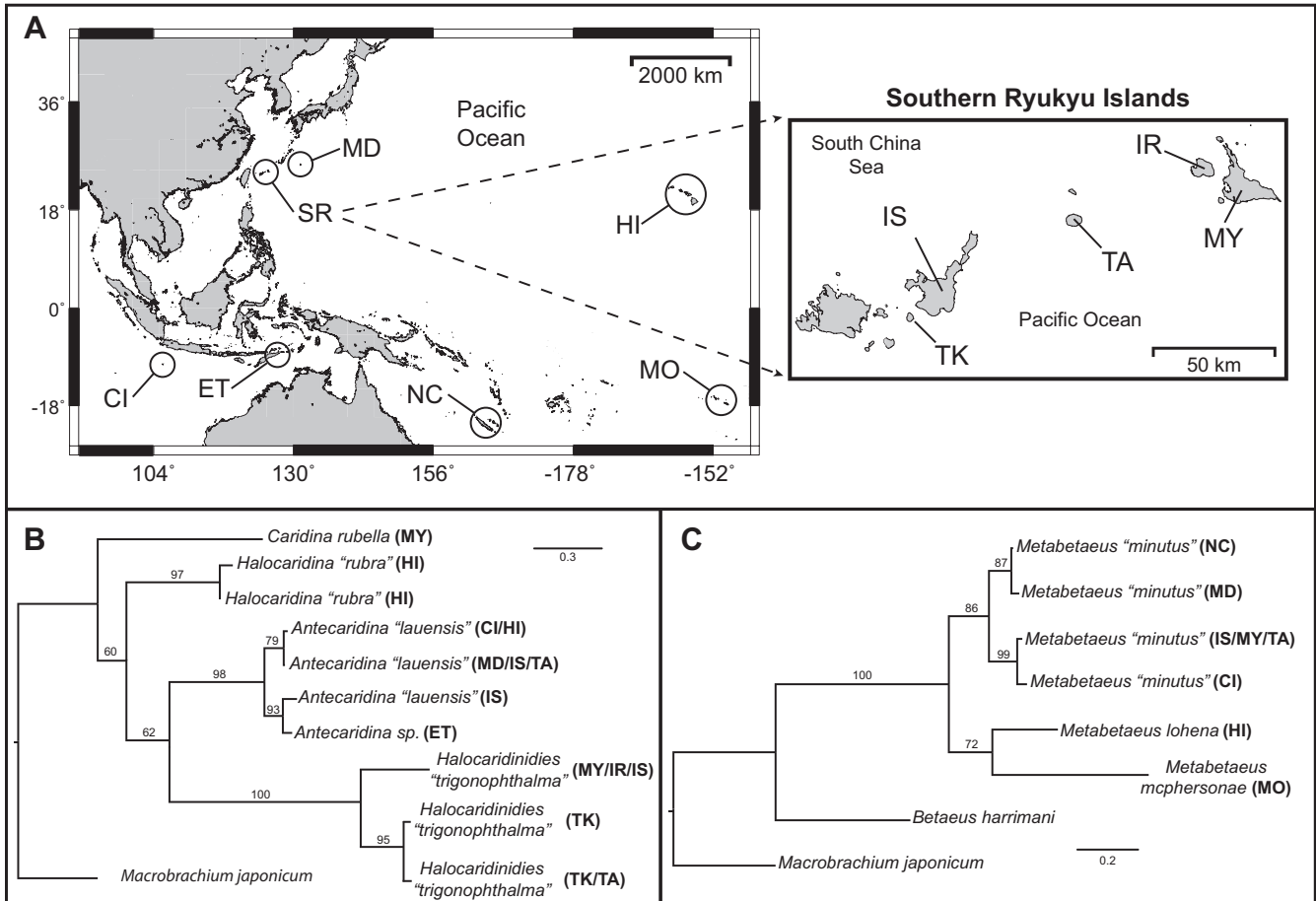
Figure 3. Phylogenetic relationships inferred for selected anchialine endemic caridean shrimp species of the Pacific Basin. (A) Map of sampling localities in the Pacific Basin and the Southern Ryukyus (zoomed in). CI—Christmas Island, ET—East Timor, HI—Hawai'i, IS—Ishigaki, MD—Minami-daito, MO—Moorea, MY—Myiako, NC—New Caledonia, TA—Tarama, TK—Taketomi. Maximum likelihood (ML) trees of atyid (ln likelihood $= -4958.78$; B) and alpheid (ln likelihood $= -3493.34$; C) 16S-rDNA datasets. Values above tree branches represent bootstrap support as percentages of 1000 re-samplings.

gent genetic lineages. To test whether each "species" was monophyletic as well as to infer the evolutionary relationships among lineages, *16S-rDNA* sequences were generated for one to two individuals per lineage, as well as from specimens of the same or closely related species from outside the Ryukyus (Fig. 3A, Table 4), for phylogenetic analyses (Table 4). Maximum likelihood (ML) trees, inferred under the GTR model of evolution (as chosen by the AIC), for both datasets were well resolved with moderate-to-strong bootstrap support among branches (Figs. 3B, C).

In the analysis of the atyid dataset, both *Antecaridina* and *Halocaridinides* were monophyletic at the level of genera with strong (*i.e.*, 98%–100% bootstrap) support (Fig. 3B). For *Antecaridina,* the lineage found on Ishigaki and Tarama in the southern Ryukyus as well as on Minami-daito was sister with moderate (*i.e.*, 79% bootstrap) support to *A. lauensis* from Christmas Island in the Indian Ocean and Hawai'i in the central Pacific Ocean; the sequence diver-

gence in *16S-rDNA* was less than 1% across this geographic range (Fig. 3A). The second lineage, which was confined to the YOSH population on Ishigaki, was sister with strong (*i.e.*, 93% bootstrap) support to an *Antecaridina* sp. from East Timor, which lies between the South China Sea and Indian Ocean (Fig. 3A). Notably, both groups of *A. lauensis* in the Ryukyus were more closely related to lineages from outside the archipelago then to one another. For the three genetic lineages of *H. trigonophthalma*, the two lineages restricted to Tarama and Taketomi formed a monophyletic group sister to the third lineage, which was found on Ishigaki, Miyako, and Irabu (Fig. 3B), with strong (*i.e.*, 95%–100% bootstrap) support. Unfortunately, no specimens or genetic data for *H. trigonophthalma* from outside the Ryukyus could be obtained for this study.

Similar patterns were recovered from the phylogenetic analysis of the alpheid dataset. Specifically, *Metabetaeus* was monophyletic with strong (*i.e.*, 100% bootstrap) sup-

Table 4. *Additional caridean specimens and sequences included in* 16S-rDNA *phylogenetic 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
	Hawai'i[†]	–	Unpublished
	Ishigaki	KC879824	This study
	Minami-daito	KC879825	This study
Caridina rubella	Miyako	KC879826	This study
Halocaridina rubra	Hawai'i	EF173734	Craft *et al.,* 2008
	Hawai'i	EF173734	Craft *et al.,* 2008
Halocaridinides trigonophthalma	Miyako	KC879823	This study
	Taketomi	KC879821	This study
	Taketomi	KC879822	This study
Metabetaeus minutus	Christmas Island[††]	KC879829	This study
	Ishigaki	KC879828	This study
	Minami-daito	KC879827	This study
Metabetaeus mcphersonae	Moorea[††]	KC879831	This study
Metabetaeus lohena	Hawai'i	KC879830	This study
Metabetaeus sp.	New Caledonia	FJ943435	Bracken *et al.,* 2009
Betaeus harrimani		FJ943434	Bracken *et al.,* 2009
Macrobrachium japonicum	Okinawa	DQ194935	Liu, M., *et al.,* 2007

[†] Sequence provided by Timothy Page, Griffith University, Queensland Australia.
[††] Samples provided by Arthur Anker, Florida Museum of Natural History.

port (Fig. 3C). Within *Metabetaeus,* all four *M. minutus* lineages were also monophyletic with relatively strong (*i.e.*, 86% bootstrap) support to the exclusion of *M. lohena* (Hawai'i) and *M. mcphersonae* (Moorea). For the two *M. minutus* lineages of the Ryukyus, the one from islands in the southern Ryukyus (*i.e.*, Ishigaki, Miyako, and Tarama) was sister with strong (*i.e.*, 99% bootstrap) support to *M. minutus* from Christmas Island in the Indian Ocean (Fig. 3A, C). The second lineage, apparently confined to the island of Minami-daito more than 600 km east of the southern Ryukyus, was sister with relatively strong (*i.e.*, 87% bootstrap) support to a *Metabetaeus* sp. from New Caledonia in the southwest Pacific Ocean (Fig. 3A, B). As with the case for *A. lauensis*, both groups of *M. minutus* in the Ryukyus were more closely related to lineages from outside the archipelago then to one another. Furthermore, previously recognized morphological variation and character states also support the phylogenetic relationships inferred here. In this context, Anker (2010) noted differences in rostrum length and the angle of orbital teeth between *M. minutus* of Minami-daito and Christmas Island. In this study, specimens from the Minami-daito lineage conformed to the description of materials from the same locality, while those from the southern Ryukyus (*i.e.*, Ishigaki, Miyako, and Tarama) lineage resemble the description of the Christmas Island specimens (Fig. A1, see Appenix) presented in Anker (2010).

Discussion

Here, we applied a comparative phylogeographic approach to three caridean shrimp species toward differentiating the potential origins of anchialine crustacean biodiversity in the Ryukyu Archipelago. In this context, *Antecaridina lauensis*, *Halocaridinides trigonophthalma,* and *Metabetaeus minutus* were identified as comprising two to three lineages with considerable population structure and genetic divergence. If diversification within these three species followed the *center of origin* hypothesis and occurred within the Ryukyus, one would expect the evolutionary history of the *A. lauensis*, *M. minutus,* and *H. trigonophthalma* lineages to be correlated with the sequential separation of the island groups in the archipelago as sea levels fluctuated during the Pleistocene. While phylogenetic patterns supporting the *center of origin* hypothesis of Indo-West Pacific fauna have been reported for a number of marine species in this geographic area (reviewed in Benzie, 1998; Carpenter *et al.,* 2011), this does not appear to be the case for the anchialine species examined here. Instead, the phylogenetic relationships of the multiple lineages within each of these three anchialine carideans, coupled with regional oceanographic current patterns and geographic distributions, suggest the Ryukyus has been a sink to divergent lineages coming from independent source populations, consistent with the *center of accumulation* hypothesis.

Evidence for multiple colonizations of the Ryukyus by anchialine carideans

Whether the occurrence of related taxa across an island archipelago reflects diversification *in situ* or multiple colonizations can potentially be reconciled through phylogenetic reconstruction. For example, if island taxa are found to be monophyletic, a single colonization is favored and the speciose *Drosophila* (Grimaldi *et al.,* 1990) and silverswords (Baldwin *et al.,* 1991) of the Hawaiian Islands are exemplars of such a situation. On the other hand, paraphyly typically implies that multiple colonizations have occurred (Emerson, 2002). For *A. lauensis* and *M. minutus* in the Ryukyus, the two lineages within each species were not recovered as sister taxa in our phylogenetic analyses (Fig. 3). Rather, they exhibit paraphyletic relationships to *A. lauensis* and *M. minutus* lineages from outside (*i.e.*, Christmas Island, East Timor, or New Caledonia) the Ryukyu Archipelago. Given these patterns, the three lineages of *H. trigonophthalma* found in the Ryukyus are likely the result of multiple colonizations from sources outside the archipelago as well. Similar phylogenetic patterns attributed to multiple colonizations have also 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) island taxa throughout the Pacific, including the Ryukyus (see Introduction).

Could the diversification of *A. lauensis*, *M. minutus,* and possibly *H. trigonophthalma* into multiple genetic lineages have occurred within the Ryukyus, with subsequent dispersal and colonization to regions outside the archipelago? Although phylogenetic patterns similar to those reported here would be recovered in such a scenario, this seems unlikely given the general oceanographic patterns of the region. Specifically, the Indo-West Pacific is dominated by the historically stable Kuroshio Current, which diverges from the North Equatorial Current east of the Philippine Islands, flowing northward into the Okinawa Trough (past the Ryukyus) and eventually out into the Pacific Ocean through the Tokara Strait (Ujiie *et al.,* 2003). As the world's second-largest oceanic current (Shen *et al.,* 2011), it is thought to have 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.,* 2012). For example, the North Equatorial and Kuroshio Currents contribute measurably to the dispersal of marine fishes from the central and southern Indo-West Pacific to Japan and the Ryukyus (*e.g.,* Tsukamoto, 2006; Mukai *et al.,* 2009). Similarly, the Kuroshio Current (along with the South China and North China Coastal Currents) apparently influenced the re-colonization of the Ryukyus by the flathead mullet, *Mugil cephalus,* from southern refugia after Plio-Pleistocene sea-level changes (Shen *et al.,* 2011). Given these recurring patterns, one could hypothesize that these strong northward currents aided in the dispersal and multiple colonizations of the Ryukyus by caridean species and lineages originating from anchialine habitats elsewhere in the Pacific Basin, most likely in the southern Indo-West Pacific.

As mentioned in the Introduction, climatic oscillations during the Pleistocene globally drove large fluctuations in sea-level stands, dramatically impacting the diversification and distributions 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 levels likely influenced the availability of island, habitat, or both, as multiple "waves" of colonizers encountered the Ryukyu Archipelago. Assuming that higher and larger islands have been above sea level for longer time periods than low-lying and smaller ones, a strong correlation is apparent between current distributions of the anchialine caridean lineages examined here and island elevation and area in the Ryukyus. For example and consistent with this idea, the islands of Ishigaki, Miyako, and Irabu, with elevations of more than 100 m and areas greater than 200 km^2, appear to have been colonized by one of the *H. trigonophthalma* lineages at a time when sea levels were higher than present and smaller islands would have been submerged. As sea levels receded to present day conditions (or lower), a second (and third) *H. trigonophthalma* lineage swept through the Ryukyus, colonizing the previously submerged islands of Taketomi and Tarama, both of which have much lower (*i.e.,* <30 m) elevations and smaller (*i.e.,* <15 km^2) areas than Ishigaki, Miyako, or Irabu. The hypothesis of a recent colonization for these lower and smaller islands is supported by the fact that the *H. trigonophthalma* lineage found on both Taketomi and Tarama possesses the signal of a strong population expansion, as evident by the largest negative Tajima's D and Fu's F_S values encountered in the study (Table 3), indicative of a potentially recent founder event. Furthermore, the only anchialine caridean lineage that exhibits no evidence of a recent population expansion (Table 3) is *M. minutus* found in the Daito Islands (*i.e.,* Minami-daito and Kita-daito), which are thought to have migrated to their current location *via* plate tectonics and be considerably older (*i.e.,* ~50 MYA; Kawana and Ohde, 1993; Ohde, 2007) than the islands in the Southern Ryukyus (*i.e.,* ~10 MYA; see Introduction). Given this as well as the evolutionary relationship between the Minami-daito lineage and the *M. minutus* found on New Caledonia (Fig. 3), the anchialine habitats of Minami-daito may have been colonized by this alpheid when the islands were volcanically formed in the southern Indo-West Pacific near present-day New Guinea (Kawana and Ohde, 1993; Ohde, 2007) and prior to the emergence of (and habitat availability on) the southern islands in the Ryukyus. A similar pattern can be seen for *A. lauensis*, where one lineage is restricted to the

island of Ishigaki and may represent an older colonization event, while the second lineage has an exceptionally impressive range of about 11,000 km, with an identical haplotype being shared among populations on Christmas Island (Indian Ocean), three islands in the Ryukyus (*e.g.*, Ishigaki, Tarama, and Minami-daito), and Hawai'i (Central Pacific), suggesting either a recent colonization or ongoing dispersal and gene flow across the Indian and Pacific Basins.

Population structure of anchialine carideans in the Ryukyus Archipelago

Despite having similar ecologies and broadly sympatric distributions within the Ryukyus, each of the *A. lauensis*, *H. trigonophthalma,* and *M. minutus* lineages exhibits radically different levels and scales of population structure and geographic range. One potential driver for this phylogeographical discordance among lineages and populations is subtle differences in intrinsic life-history characteristics. Such differences, particularly in life-history traits (*i.e.,* egg size and larval stages), larval feeding mode (*i.e.,* lecithotrophy *vs.* planktotrophy), and larval habitat have been implicated in influencing the dispersal potential and genetic structure of a number of caridean species from anchialine as well as stream habitats (Shokita, 1979; Page and Hughes, 2007; Craft *et al.,* 2008; Russ *et al.,* 2010). The significance of this is discussed in the following paragraphs.

For atyids such as *A. lauensis* and *H. trigonophthalma*, egg size is thought to be an effective and significant predictor of both dispersal ability and level of genetic structure (Shokita, 1979; Page and Hughes, 2007; Craft *et al.,* 2008). For example, *Caridina* spp. from Australian streams possessing more restricted distributions and exhibiting higher levels of genetic structure typically have large (~1.6 mm) eggs, whereas those with extensive geographic ranges and low levels of genetic structure generally have relatively small (~0.4 mm) eggs (Page and Hughes, 2007). However, *Halocaridinides,* which produces relatively small eggs (~0.35 mm) with planktotrophic development (Fujita, unpublished), exhibits surprisingly strong genetic structure (*i.e.*, approaching, or at, the maximum limit of 1.0) between islands (*i.e., Ishigaki vs. Taketomi*) separated by less than 5 km of shallow ocean. This is similar to 'short rostrum' *Caridina rubella,* another atyid possessing life-history traits conducive to dispersal (*i.e.,* small egg size and planktotrophic larvae), from anchialine habitats on the island of Miyako (Weese *et al.,* 2012), suggesting that the correlation between egg size and genetic structure in the Atyidae may not be as generally applicable as previously thought. As for *A. lauensis,* the species has historically been reported to have an "extremely disjunctive" distribution due to high dispersal abilities (Smith and Williams, 1981). The two *A. lauensis* lineages sampled to date represent opposite ends of the spectrum of range distributions, with one being confined

to a single anchialine habitat (*i.e.*, YOSH) of a single island (*i.e.*, Ishigaki) and the other inhabiting multiple anchialine habitats in both the Indian and Pacific Oceans. Unfortunately, no information is currently available on egg size, larval feeding mode, or larval habitat for *A. lauensis*. However, it can be hypothesized that the lineage with a highly restricted range differs significantly in these traits from the other lineage that has a much broader distribution, and laboratory reproductive studies of these two *A. lauensis* lineages would directly address this possibility. In any case, one vexing question is this: How did multiple *A. lauensis* and *H. trigonophthalma* lineages successfully colonize the Ryukyus on several occasions from likely distant sources when current populations remain in isolation over small spatial scales (*i.e.*, an apparent absence of short-distance dispersal)? Local retention of larvae within nearshore waters might help explain this level and pattern of genetic structure (Weese *et al.,* 2012). It remains to be determined, however, to what extent additional biotic (*i.e.*, differences in other reproductive or ecological/physiological traits (*e.g.,* salinity tolerance, dietary differences, resource partitioning, competition) or abiotic (*i.e.*, local oceanic currents, geography, and/or geology) factors contribute to this situation as well.

Although little is known about the life history of the alpheid *M. minutus*, field and laboratory observations suggest that the species produces many (*i.e.,* 70–170) small (0.72–0.52 mm) eggs that develop as planktotrophic larvae (Fujita, unpublished). In contrast to the lecithotrophic (*i.e.*, yolk-bearing) larvae produced by atyids like *Halocaridina rubra* (Craft *et al.,* 2008), planktotrophic larvae are considered to be less energy constrained because of the ability to feed during planktonic dispersal, typically leading to low genetic structure between populations (reviewed by Palumbi, 1994). Given this, it is not surprising that populations of the *M. minutus* lineage from the Southern Ryukyus are homogenized across the islands of Miyako, Tarama, and Ishigaki, which span a distance of about 200 km (Fig. 2). The lack of population structure at this scale is similar to that in a few other anchialine species like the neritiliid snail *Neritilia cavernicola* (Kano and Kase, 2004) in the Philippines and *Metabetaeus lohena* (Russ *et al.,* 2010) in the Hawaiian Islands, where both species also possess planktotrophic larvae and exhibit little-to-no genetic differentiation over ranges of about 200 km. Additionally, the two populations of *M. minutus* sampled from the lineage confined to Minami-daito exhibited no genetic structure across the island, which is again similar to *M. lohena* populations occurring on any single island in Hawai'i (Russ *et al.,* 2010). However, the fact that no *M. minutus* haplotypes are shared between populations in the Southern Ryukyus and Minami-daito implies isolation by distance over greater (*i.e.*, >600 km) geographic scales. Collectively, this suggests that while some caridean species such as *Metabetaeus*

spp. (*i.e.*, *minutus* or *lohena*) may be "good" dispersers in "ecological" timescales when habitats or islands are in close (*i.e.,* ~200 km) proximity, dispersal and potential colonization for the three anchialine shrimp examined here becomes more of an "evolutionary sweepstake" event as habitats or islands become further apart (*i.e.*, from <5 km to >600 km), with subsequent isolation following successful colonization.

Distribution and cryptic diversity of anchialine shrimp of the Indo-West Pacific

The anchialine fauna of the Indo-West Pacific is dominated by caridean species, with 11 being recorded throughout the Pacific Basin (de Grave and Sakihara, 2011). While some, such as *H. rubra*, *M. lohena,* and *Procaris hawaiana* Holthuis, 1973 (Decapoda: Procarididae), are characterized as endemic to single archipelagos (*e.g.*, the Hawaiian Islands), many others, such as *A. lauensis, H. trigonophthalma, M. minutus, Calliasmata pholidota* Holthuis, 1973 (Decapoda: Hippolytidae)*, Parhippolyte uveae* Borradaile, 1899 (Decapoda: Barbouriidae), and *Periclimenes pholeter* Holthuis, 1973 (Decapoda: Palaemonidae), are thought to have extremely widespread and disjunct distributions throughout the Indo-West Pacific (reviewed by Maciolek, 1983). However, the emerging patterns of multiple genetic lineages with strong population structure (Santos, 2006; Craft *et al.,* 2008; Santos and Weese, 2011; Weese *et al.,* 2012; this study) imply that the caridean biodiversity of anchialine habitats in the Pacific Basin may be vastly underestimated, with many previously described species actually representing cryptic species complexes. For example, both *A. lauensis* and *M. minutus* in this study appear to represent complexes composed of at least (given the current sampling) two potentially divergent species in the Ryukyus alone. Furthermore, the population structure and genetic divergence found within *H. trigonophthalma* of the Ryukyus suggest a complex of at least three species. Likewise, cryptic species complexes have previously been described for the anchialine atyids *Caridina rubella* (two potential species) on Miyako in the Ryukyus (Weese *et al.,* 2012) and *Halocaridina rubra* (eight potential species) in the Hawaiian Islands (Santos 2006; Craft *et al.,* 2008). In fact, of the six Pacific anchialine caridean species studied to date from a population genetic perspective, 18 distinct and divergent lineages have been identified, with only *M. lohena* failing to be a cryptic species complex (Russ *et al.,* 2010). Given this, it would not be surprising if genetic analyses of other widely distributed Pacific anchialine crustaceans, such as *C. pholidota, P. pholeter,* and *P. uveae,* reveal cryptic species complexes as well. Although additional morphological studies will be required to resolve the taxonomic status of the lineages within each complex, the restricted distributions of these potential "species" should be taken into consid-

eration when developing conservation and management strategies for anchialine carideans and their habitats throughout the Indo-West Pacific.

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Appendix

Table A1 *Distribution of caridean cytochrome c oxidase subunit I (COI) haplotypes for populations of* Antecaridina lauensis, Halocaridinides trigonophthalma, *and* Metabetaeus minutus *from the Ryukyus Archipelago, Japan*

Haplotype	Genebank Accession	Sampling Localities											Individuals/ haplotype
		MIN	YOSH	SHUG	FUT	FUSH	KIK	TAG	SW	JR	SHIN	MIY	
					Antecaridina lauensis								
Al_IS1	KC879738		7										7
Al_IS2	KC879739		1										1
Al_IS3	KC879740		1										1
Al_IS4	KC879741		1										1
Al_IS5	KC879742		4	7						5	4		20
Al_IS6	KC879743		1										1
Al_IS7	KC879744		1										1
Al_MD1	KC879745									3			3
Al_MD2	KC879746									1			1
Al_MD3	KC879747									1			1
Al_MD4	KC879748			1						1			2
Al_MD5	KC879749									1			1
Al_MD6	KC879750									1			1
Al_MD7	KC879751									1			1
Al_MD8	KC879752									1	1		2
Al_MD9	KC879753			1							1		2
Al_MD10	KC879754										1		1
Al_MD11	KC879755										1		1
Al_MD12	KC879756										1		1
Al_MD13	KC879757										1		1
Al_TA1	KC879758			1									1
Al_TA2	KC879759			1									1
Al_TA3	KC879760			1									1
Al_TA4	KC879761			1									1
Al_TA5	KC879762			2									2
Al_TA6	KC879763			1									1
Al_TA7	KC879764			1									1
Individuals/location			16	17						15	10		
					Halocaridinides trigonophthalma								
Ht_TK1	KC879765	1											1
Ht_TK2	KC879766	1											1
Ht_TK3	KC879767	2											2
Ht_TK4	KC879768	1											1
Ht_TK5	KC879769	2				3							5
Ht_TK6	KC879770	1											1
Ht_TK7	KC879771	1											1
Ht_TK8	KC879772	1											1
Ht_TK9	KC879773	1											1
Ht_TK10	KC879774	1				1							2
Ht_TK11	KC879775	1				1							2
Ht_TK12	KC879776	1				1							2
Ht_TK13	KC879777	1											1
Ht_TK14	KC879778	1											1
Ht_TA1	KC879779					1							1
Ht_TA2	KC879780					1							1
Ht_TA3	KC879781					1							1
Ht_TA4	KC879782					1							1
Ht_TA5	KC879783					1							1
Ht_TA6	KC879784					1							1
Ht_TA7	KC879785					1							1
Ht_TA8	KC879786					1							1
													Continued

Table A1 *Continued*

Haplotype	Genebank Accession	Sampling Localities											Individuals/ haplotype
		MIN	YOSH	SHUG	FUT	FUSH	KIK	TAG	SW	JR	SHIN	MIY	
Halocaridinides trigonophthalma													
Ht_TA9	KC879787				1								1
Ht_TA10	KC879788				1								1
Ht_MY1	KC879789							1					1
Ht_MY2	KC879790		4					5	12				21
Ht_MY3	KC879791							1					1
Ht_MY4	KC879792		1										1
Ht_MY5	KC879793		1										1
Ht_MY6	KC879794								1				1
Ht_MY7	KC879795								1				1
Ht_MY8	KC879796								1				1
Ht_MY9	KC879797							1					1
Individuals/location		16	6			16		8	15				
Metabetaeus minutus													
Mm_IS1	KC879798		1		1		1						1
Mm_IS2	KC879799		1										1
Mm_IS3	KC879800		1										1
Mm_IS4	KC879801		1										1
Mm_TA1	KC879802				1								1
Mm_TA2	KC879803				1								1
Mm_TA3	KC879804				1								1
Mm_MY1	KC879805						1						1
Mm_MY2	KC879806						1						1
Mm_MY3	KC879807						1						1
Mm_MY4	KC879808						1						1
Mm_MY5	KC879809						1						1
Mm_MY6	KC879810		1				1						2
Mm_MY7	KC879811		1		2		2						5
Mm_MD1	KC879812									2		1	3
Mm_MD2	KC879813									1			1
Mm_MD3	KC879814									1			1
Mm_MD4	KC879815									1			1
Mm_MD5	KC879816									1		1	2
Mm_MD6	KC879817											1	1
Mm_MD7	KC879818											1	1
Mm_MD8	KC879819											1	1
Mm_MD9	KC879820											1	1
Individuals/location			6		6		9			6		6	

Individual sampling sites are listed in Table 1 and geographical coordinates are available from the corresponding author upon request.
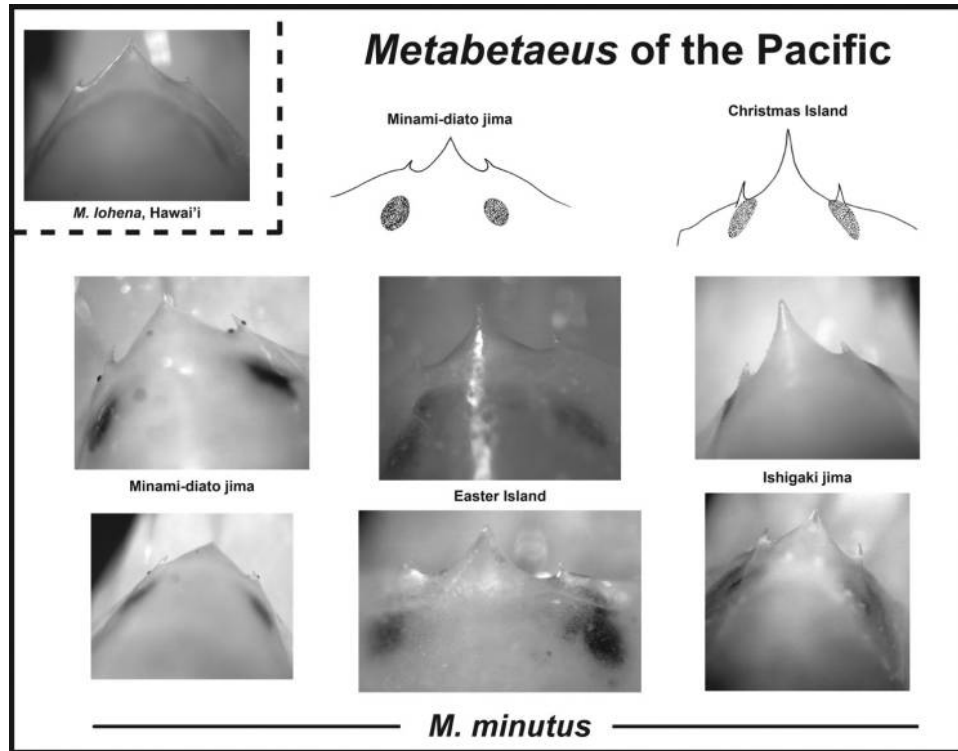
Figure A1. Morphological variation in rostrum length and orbital teeth of *Metabetaeus minutus* collected from anchialine habitats in the Pacific Basin. Illustrations for *M. minutus* (reprinted, with permission, from Anker, 2010. ©*Zootaxa*) are presented for comparison to photographs (this study) of the same species as well as *M. Iohena*.