The Evolutionary Ecology of a Bioluminescent Vertebrate-Microbe Symbiosis

by

Alison L. Gould

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Doctoral Committee:

Professor Paul V. Dunlap, Chair
Professor Christopher William Dick
Assistant Professor Vincent J. Denef
Professor James Stephen Diana

“The best way to observe a fish is to become a fish”

– Jacques Yves Cousteau –

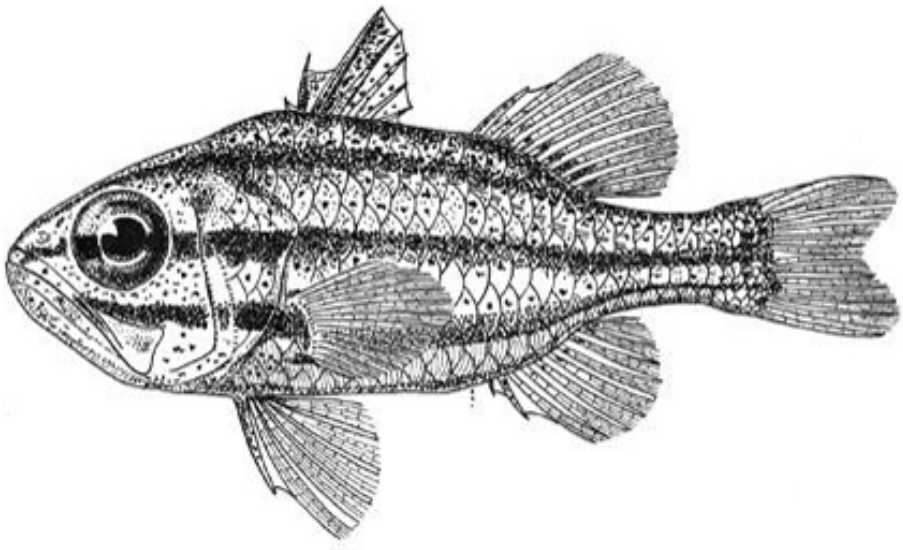

Illustration by Violet Dandridge
Smithsonian Institute

DEDICATION

To my father, the original Dr. Gould,
and to my mother, the source of my creativity

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ABSTRACT
The Evolutionary Ecology of a Bioluminescent Vertebrate-Microbe Symbiosis

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Alison L. Gould

Chair: Paul V. Dunlap

The evolution of a symbiosis requires the maintenance of an intimate host-symbiont association over ecological time. My dissertation research investigates the ecological mechanisms that help to maintain and promote specificity of a vertebrate-microbe symbiosis involving the cardinalfish, *Siphamia tubifer* (Perciformes: Apogonidae), and the luminous bacterium, *Photobacterium mandapamensis*, over host generations. I integrated field studies that describe aspects of the life history and behavioral ecology of the host in Okinawa, Japan, with newly developed genomic methods to test the hypothesis that the host's ecology genetically structures natural populations of the symbiont over time and space, consequently driving specificity of the symbiosis.

Life history of *Siphamia tubifer.* The sea urchin cardinalfish, *S. tubifer*, inhabits shallow coral reefs in the Indo-Pacific. Mouthbrooding males release their larvae into the plankton, and early in development the larvae initiate a symbiosis with the luminous bacterium, *P. mandapamensis,* which they acquire from the environment. The timing and location of symbiont acquisition in the wild was previously unknown as was the fish's pelagic larval duration (PLD) and other aspects of the host's life history, which might also shape the symbiosis. To build a foundation for understanding the host's biology relevant to the maintenance of the symbiosis, I described the growth and reproductive rates of *S. tubifer* as well as symbiont population growth in host light organs. Results indicated that the PLD of *S. tubifer* is approximately one month, during which symbiont acquisition occurs, and once settled,

the fish grows quickly, reproduces early, and typically survives much less than a year. Additionally, light organs house an average of 10^7 luminous bacterial cells, with the symbiont population continuing to grow throughout the host's lifespan. These characteristics suggest that high mortality might shape the cryptic nature of *S. tubifer* as a luminous reef fish.

Behavioral ecology of *Siphamia tubifer*. Cardinalfishes are known to express fidelity to daytime resting sites and for their ability to return to a home reef from relatively large distances, thereby affecting both nutrient cycling and the community assemblage at a reef. To determine whether such behaviors, which might also influence the symbiosis, are exhibited by *S. tubifer*, I used mark-recapture methods to define the fish's fidelity to a particular home site, its ability to return home after displacement, and its preference for relevant olfactory cues. The results revealed that the fish exhibits daily fidelity to a home site and significant homing abilities from up to two kilometers. These behaviors result in the local enrichment of a home reef with luminous symbionts released daily by the resident *S. tubifer* population and consequently, might help to maintain the specificity of the symbiosis between host generations. Additionally, in a two-channel choice flume, *S. tubifer* preferred the chemical cues of its home site water and luminous bacterial symbiont, and juvenile fish also preferred the olfactory cues of conspecifics to unconditioned seawater. These results indicate that *S. tubifer* can use site-specific chemical cues, including that of their symbiont, to home and potentially for self-recruitment and symbiont acquisition.

Population genomics of the symbiosis. To examine the possible relationship between adult homing behavior, larval recruitment patterns and symbiont acquisition, I applied double-digest, restriction site associated sequencing (ddRAD-seq) to define the fine-scale population genomic structure of *S. tubifer* and *P. mandapamensis* in the Okinawa Islands, Japan. Analysis of over 10,000 single nucleotide polymorphisms (SNPs) across nearly 300 individuals from 11 locations and over three years revealed low F_{ST} values between host fish populations and no evidence of genetic structure at spatial scales ranging from a few to over 100 kilometers. The lack of population structure in the host fish suggests that, despite adult homing, *S. tubifer* larvae disperse substantial distances and apparently do not recruit to natal reefs. However, an analysis of the presence or absence of haplotypes at 607 variable loci throughout the *P. mandapamensis* genome indicated that light organ symbiont populations are

genetically differentiated between the same reefs approximately 100 kilometers apart. Therefore, the daily site fidelity and symbiont release exhibited by *S. tubifer* apparently promotes the specificity of the symbiosis between host generations by locally enriching the symbiont pool in the seawater surrounding a reef site. This result provides evidence of host-mediated diversification of a marine bacterium and suggests symbiont acquisition by larval fish occurs near the locally enriched symbiont pool at a settlement site.

CHAPTER I
Introduction

Symbiotic associations are an integral part of life; virtually all organisms, including humans, depend on symbioses with bacteria for their success. The integration of a host organism and its microbial symbiont enables them to collectively occupy a broader ecological niche than either organism could occupy independently (Saffo 1992). Examples of such "evolutionary innovations" (Margulis 1989) driven by symbiotic interactions are well established, including nitrogen-fixing bacteria in legumes (Wilkinson and Parker 1996), endosymbiotic bacteria within various insect hosts (e.g. Smith and Douglas 1987, Nardon and Grenier 1991, Moran and Telang 1998), and chemoautotrophic bacteria in the tissues of invertebrates inhabiting hydrothermal vents (Cavanaugh 1994). However, how such intimate associations become established and evolve specificity remains poorly understood.

The evolution of symbioses between host organisms and microbial symbionts is first dependent on ecological interactions that allow for the formation and maintenance of the particular association over host generations. Such specificity is maintained in vertically transmitted symbioses through the direct transfer of symbiont from parent to offspring and commonly leads to co-speciation (e.g., Chen *et al.* 1999, Clark *et al.* 2000, Moran *et al.* 2003, Hosokawa *et al.* 2006). In contrast, the establishment of most horizontally transmitted symbioses requires both the initiation of the association with a specific symbiont from the environmental pool and the maintenance of that specificity over evolutionary time; therefore, a lower level of host-symbiont specificity is expected in horizontally transmitted symbioses. This is particularly true for horizontal symbioses established in highly-connected ocean environments, in which the microbial community is assumed to be well mixed (Mullins *et al.* 1995, Darling *et al.* 2000, Massana *et al.* 2000, Morris *et al.* 2002, Finlay 2002, Fenchel and Finlay 2004, Finlay and Fenchel 2004, Baldwin *et al.* 2005).

Nevertheless, many horizontally acquired symbioses, including those in marine environments, exhibit a surprising degree of specificity (e.g., Cavanaugh 1994, Nishiguchi *et al.* 1998, Nishiguchi 2001, Kaeding *et al.* 2007), indicating the establishment of the association between the host and a particular symbiont type from the environment is maintained by some mechanism over time. Such mechanisms might include environmental factors, genetic recognition, and aspects of the ecology, behavior, and physiology of the host organism, and are not mutually exclusive. My dissertation research investigates whether aspects of the distinct life history and behavioral ecology of a host fish facilitates the establishment and maintenance of its horizontally transmitted, bioluminescent symbiosis.

The underlying hypothesis regarding most bioluminescent symbioses suggests that the environment influences which symbiont type is acquired by a host as determined by the proportion of symbiont genotypes present in the water at the time of acquisition (Nealson and Hastings 1991). However, bioluminescent symbioses, especially those involving fish hosts, display a higher degree of specificity than what would be expected due to random within-species symbiont acquisition from the marine environment (e.g. Dunlap *et al.* 2007, Kaeding *et al.* 2007). How this specificity is achieved and maintained remains poorly understood, and may in part be due to the ecology of the host, as determined for the symbiosis involving the squid, *Euprymna scolopes* and the luminous bacterium, *Aliivibrio (Vibrio) fischeri* (Lee and Ruby 1994, Nyholm *et al.* 2000, Nyholm and McFall-Ngai 2004, Visick and Ruby 2006, Wang *et al.* 2010). In contrast, there is little direct evidence of a host's role in establishing specificity for bioluminescent vertebrate-microbe symbioses, largely due to a lack of understanding of the biology and ecology of symbiotically luminous fishes.

There are over 450 species of symbiotically luminous fishes, yet details of their ecology, especially with respect to symbiont acquisition, remain undescribed because they are challenging to study in their natural environments; many bioluminescent fish inhabit deep or open waters and are nocturnally active. In addition, the critical window of time during which fish acquire a symbiont generally occurs during larval development, and larval fish can be even more challenging to study than adults due to their small size and planktonic state. These factors make bioluminescent fish hosts and the process of symbiont acquisition by larvae extremely difficult to study in the wild. The focal host fish of my dissertation research, however, is experimentally

tractable, inhabiting shallow coral reefs; therefore, I integrate experimental field studies with population genomic studies of the host and symbiont to determine the extent to which the host's life history and behavioral ecology influences the establishment and maintenance of specificity of its bioluminescent symbiosis.

The Symbiosis

The sea urchin cardinalfish, *Siphamia tubifer,* (formerly referred to as *Siphamia versicolor* (Smith and Radcliffe 1911), now considered a junior synonym of *S. tubifer* (Gon and Allen 2012)), resides on coral reefs throughout the Indo-Pacific and is easily observed and collected in the wild. Therefore, *S. tubifer* provides a unique opportunity to describe aspects of a bioluminescent host fish's ecology and their effects on symbiont acquisition. Distinct from most other symbiotically luminous fishes, *S. tubifer* aggregates in groups among the long spines of the sea urchins *Diadema setosum* and *Echinothrix calamaris* in coral reef habitats during the day. This behavior facilitates the direct observation of fish in its natural habitat and the collection of specimens. Additionally, *S. tubifer* can be raised in culture, making the symbiosis experimentally tractable in the lab (Dunlap *et al.* 2012) and rendering it an excellent model association that can be extrapolated to other vertebrate-microbe symbioses.

Furthermore, the *S. tubifer-P. mandapamensis* symbiosis is apparently more specific than other bioluminescent fish-bacteria associations (Dunlap *et al.* 2007, Kaeding *et al.* 2007). To date, all light organ symbionts of *S. tubifer* examined are closely related, genetically clustering within the *P. mandapamensis* phylogeny (Kaeding *et al.* 2007, Dunlap *et al.* unpublished). *Siphamia tubifer* harbors a dense population of *P. mandapamensis* in a specialized, abdominal light organ attached to the fish's gut (Dunlap and Nakamura 2011, Dunlap *et al.* 2009). Initially, the light organ is unreceptive to colonization by a luminous symbiont, but after eight days of larval development in the plankton, the symbiosis with *P. mandapamensis* can become established (Dunlap *et al.* 2012). It has yet to be determined how long a light organ remains receptive to colonization, whether the timing of symbiont acquisition is the same in the wild, and where in the environment *S. tubifer* larvae are during symbiont acquisition.

Siphamia tubifer utilizes the light produced by its symbiotic bacteria to illuminate its ventral surface while foraging over the reef at night. Previous work established that *S. tubifer* releases excess symbiont cells into the intestine via a duct from the light organ such that the fish's feces are rich in the symbiotic bacteria (Dunlap and Nakamura 2011). This regular release of luminous symbiont cells into the environment can enhance the environmental concentration of strains of *P. mandapamensis* symbiotic with *S. tubifer* populations at reefs with a high density of fish hosts and consequently, might play a role in maintaining the specificity of the symbiosis. The enrichment of seawater with luminous symbionts by a host has previously been reported for other luminous fishes (Haygood *et al.* 1984, Nealson *et al.* 1984) as well as for the squid host, *Euprymna scolopes* (Lee and Ruby 1994) and was later linked to population genetic structure in the symbiont of the squid (Wollenberg and Ruby 2009). However, a better understanding of the life history and ecology of *S. tubifer* is necessary to investigate host enrichment as a potential mechanism for maintaining specificity of the *S. tubifer-P. mandapamensis* association over time.

Like other cardinalfishes (Perciformes: Apogonidae), *S. tubifer* is a paternal mouthbrooder; male parents carry their fertilized eggs in their mouth through hatching (Breder and Rosen 1966). A few days after *S. tubifer* larvae hatch in the mouth they are released into the plankton to complete larval development, after which they navigate to a suitable reef habitat for settlement. Other cardinalfishes are capable of returning to a home reef from significant distances after displacement (Marnane 2000, Døving *et al.* 2006), and some cardinalfish larvae can also navigate back to their natal reef for settlement (Gerlach *et al.* 2007). This larval homing behavior can result in significant levels of population genetic differentiation among populations of cardinalfishes (Gerlach *et al.* 2007, Kolm et al, 2005). In fact, Gerlach *et al.* (2007) provided evidence of population genetic structure of the cardinalfish, *Ostorhinchus doederleini,* at the scale of only a few kilometers. Therefore, the potential for *S. tubifer* larvae to also return to a natal reef exists and could have a direct influence on symbiont acquisition and specificity of the association.

Preview of Chapters

In my dissertation, I characterize previously undefined life history and behavioral traits of *S. tubifer*, a symbiotically luminous, coral reef fish host and use a population genomics approach to determine the role of these traits in establishing and maintaining the high degree of specificity of its symbiosis with *P. mandapamensis* over host generations. In Chapter II, I define life history characteristics of *S. tubifer*, including diet, reproduction, and growth rates, as well as the growth of the light organ bacterial population relative to the host. Results of this study provide much-needed perspective on the biology of the host relevant for symbiont acquisition. Aspects of the behavioral ecology of *S. tubifer*, also pertinent to the symbiosis, are defined in Chapters III and IV. Specifically, in Chapter III, I examine the nocturnal foraging and site fidelity behaviors of *S. tubifer* and test fish's ability to return to a home reef after displacement. Such behaviors have the potential to influence the distribution and abundance of *P. mandapamensis* in the environment and can therefore affect symbiont acquisition by larval fish. In Chapter IV, I determine whether olfaction can play a role in navigation to a settlement site, or perhaps in symbiont recognition and acquisition. To do so, I test the olfactory preferences of the *S. tubifer* for the chemical cues of its home reef and luminous bacterial symbiont over unfamiliar reef waters. In the final two chapters, I identify patterns of genomic structure in both the host fish and the luminous bacterial symbiont to test the hypothesis that the ecology of the host helps to maintain the high degree of specificity of the symbiosis observed. I apply a recently developed molecular method to test for fine scale patterns of structure between host populations across a range of spatial and temporal scales in Chapter V and then compare these patterns to those observed for the bacterial symbiont at the same scales in Chapter VI (Figure 1.1). In doing so, I indirectly determine the influence of the host's behavioral ecology on symbiont acquisition by the next generation of *S. tubifer* larvae. The combined chapters of my dissertation highlight the potential of the *S. tubifer-P. mandapamensis* symbiosis as an effective model association to investigate future questions of the evolution of symbiotic interactions, including other vertebrate-microbe associations, as well as the role of host animals in bacterial biogeography, diversification and speciation.

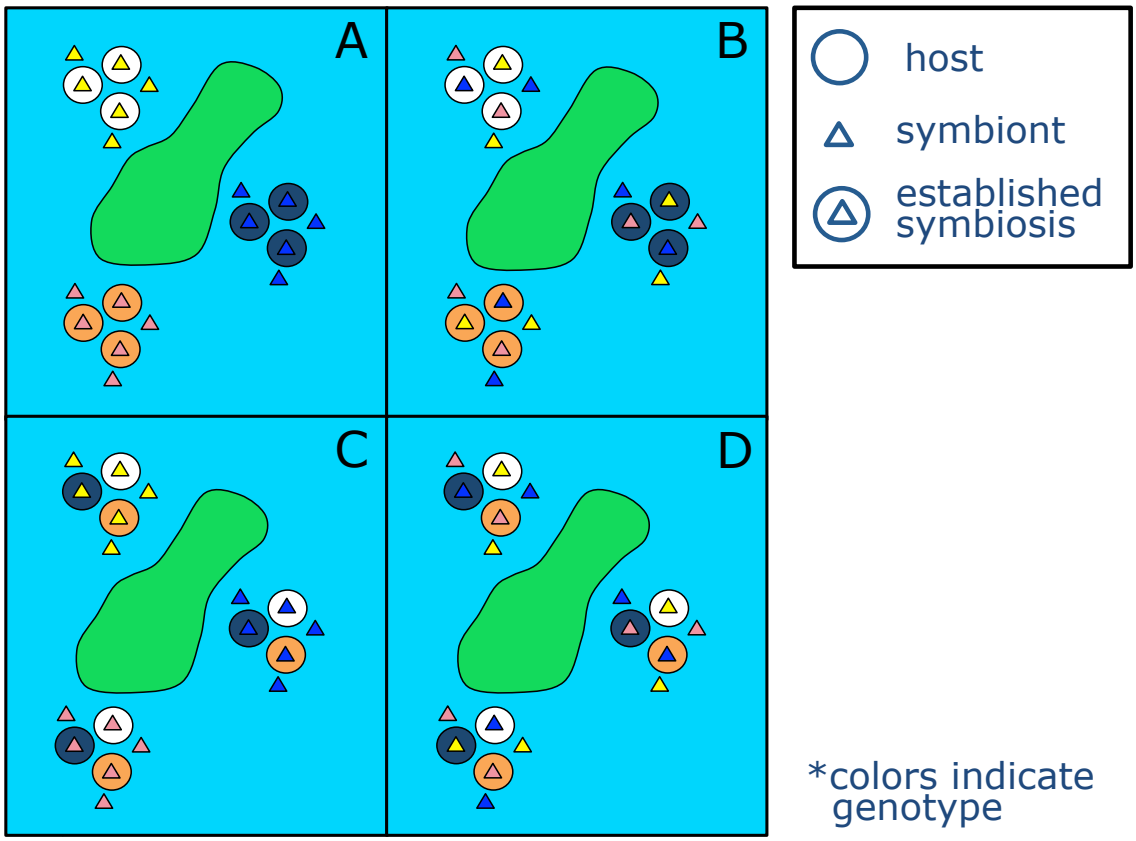

Figure 1.1 The four most extreme possible outcomes from the genomic analyses of host and symbiont populations in Chapters V and VI. Panel A represents a "pseudo-vertical" mode of symbiont transmission, where host larvae self-recruit to their natal reef and acquire a symbiont near their parents. Panel D depicts the opposite extreme outcome, where no genetic structure exists among populations of the host or symbiont. Panel B represents the scenario where host populations are genetically structured but the symbiont is genetically admixed, and panel C depicts the inverse scenario where the symbiont populations are genetically structured but the host populations are not.

CHAPTER II
Life history of *Siphamia tubifer* [1]

Abstract

Characteristics of the life history of the coral reef-dwelling cardinalfish *Siphamia tubifer,* from Okinawa, Japan, were defined. A paternal mouthbrooder, *S. tubifer* is unusual in forming a bioluminescent symbiosis with *Photobacterium mandapamensis*. The examined fish (n = 1,273) ranged in size from 9.5 mm to 43.5 standard length (L_S) and the minimum size at sexual maturity was 22 mm L_S. The number of fish associated during the day among the spines of host urchins was 22.9 ± 16.1 (*Diadema setosum*) and 3.6 ± 3.2 (*Echinothrix calamaris*). Diet consisted primarily of crustacean zooplankton. Batch fecundity (number of eggs) was related to L_S by the equations: males (fertilized eggs) = 27.5(L_S) - 189.46; females (eggs) = 31.3(L_S) – 392.63. Individual mass as a function of L_S was described by the equation: mass (g) = $9.74 \times 10^{-5}(L_S)^{2.68}$. Growth, determined from otolith microstructure analysis, was described with the von Bertalanffy growth function with the following parameters: L_∞ = 40.8 mm L_S, K = 0.026 d^{-1}, and t_0 = 23.25 d. Planktonic larval duration was estimated to be 30 days. The age of the oldest examined individual was 240 days. The light organ of *S. tubifer*, which harbours the symbiotic population of *P. mandapamensis*, increased linearly in diameter as fish L_S increased, and the bacterial population increased logarithmically with fish L_S. These characteristics indicate that once settled, *S. tubifer* grows quickly, reproduces early, and typically survives much less than a year in Okinawa. These characteristics are generally similar to other small reef fishes, but they indicate that *S. tubifer* experiences higher mortality.

[1]Published as: Gould, AL, KE Dougan, ST Koenigbauer, PV Dunlap (2016) Life history of the symbiotically luminous cardinal fish *Siphamia tubifer* (Perciformes: Apogonidae). *Journal of Fish Biology* doi:10.1111/jfb.13063

Introduction

Cardinalfishes (Perciformes: Apogonidae) are common on coral reefs worldwide and are often among the most abundant family of fish present in a reef community (Bellwood, 1996). Cardinalfishes are paternal mouthbrooders (Vagelli 2011) that actively forage at night (Marnane and Bellwood 2002) and seek shelter during the daytime, often in high densities among reef structures, including reef-dwelling invertebrates (Gardiner and Jones 2005, 2010). As highly abundant, small-bodied fish that often exhibit fidelity to a reef site, cardinalfishes play an important role as prey for larger, predatory fish and as planktivores that help recycle nutrients within the reef community (Marnane 2000, Marnane and Bellwood 2002).

Despite the diversity and ecological importance of cardinalfishes, comprehensive knowledge of the life history of individual species remains limited, and key life history traits critical for population success, such as growth and reproductive rates, are not widely documented. Nearly 350 species of cardinalfishes have been identified (Eschmeyer and Fong 2015), yet the growth rates of only six species have been described in detail (Kume *et al.* 1998, Okuda *et al.* 1998, Kume *et al.*, 2003, Longenecker and Langston 2006, Raventos 2007, Wu 2009, Ndobe *et al.* 2013, Kingsford *et al.* 2014). Many studies to date have emphasized the reproductive biology of cardinalfishes (e.g. Kuwamura 1985, Vagelli 1999, Kume *et al.* 2000b, Okuda 2001, Fishelson and Gon 2008), whereas a few studies have described larval growth and the pelagic larval duration (PLD) of some cardinalfish species (Brothers 1983, Ishihara and Tachihara 2011, Kingsford *et al.* 2014, Leis *et al.* 2015) as well as the diets and feeding ecologies of others (Chave 1978, Marnane and Bellwood 2002, Barnett *et al.* 2006). Additionally, certain aspects of the behavioural ecology, e.g., sociality, site fidelity, and homing behaviour, of a few species have been recently described (Marnane 2000, Gardiner and Jones 2005, Kolm *et al.* 2005, Døving *et al.* 2006, Gardiner and Jones 2010, Gould *et al.* 2014, Rueger *et al.* 2014).

Life history information is particularly sparse for bioluminescent cardinalfishes. There are three genera with autogenously luminous species, *Archamia*, *Jaydia*, and *Rhabdamia*, and one bacterially luminous genus, *Siphamia* (Thacker and Roje 2009). The sea urchin cardinalfish *Siphamia tubifer* Weber 1909 (Gon and Allen 2012), formerly classified as *Siphamia versicolor* (Smith and Radcliffe, in Radcliffe 1911;

Tominaga 1964), is possibly the most widespread species of luminous cardinalfish. Some characteristics of the ecology and behaviour of *S. tubifer* have been documented recently (Gould *et al.* 2014, 2015), and the fish's symbiosis with the luminous bacterium, *Photobacterium mandapamensis*, has been the subject of several studies (Leis and Bullock 1986, Wada *et al.* 2006, Kaeding *et al.* 2007, Dunlap and Nakamura 2009, 2011, Dunlap *et al.* 2012). However, key aspects of the fish's life history, including diet, reproduction, and growth, remain largely undescribed, and there is a general lack of detailed life history knowledge for the more than 450 species of bacterially luminous fishes found worldwide (Nelson 2006, Froese and Pauly 2015). Therefore, the general goal of this study was to describe in detail aspects of the life history of *S. tubifer* in order to provide a foundation of understanding of the fish's biology for future research and to provide additional perspective on the biology of both reef-dwelling cardinalfishes and bacterially luminous fishes. The specific aims were to define the fish's body size, length to weight ratio, size distribution, aggregation size, diversity of diet, reproduction, growth, and symbiont population growth in host light organs.

Materials and Methods

Field collection

Juvenile and adult *Siphamia tubifer* were collected from reefs at various locations in Okinawa, Japan (26.5° N, 128° E), during summer months (June-August) of 2011 through 2014. Fish were collected with their host sea urchin on SCUBA using a gaff hook and a 20 L bucket; the resident group of *S. tubifer* remained with their host urchin as it was gently guided into a bucket using the hook. After collection, urchins were returned to their site of capture. The protocols used here for capture, care, and handling of fish were approved by the University of Michigan's University Committee on Use and Care of Animals, and they conform to the University of the Ryukyus' Guide for Care and Use of Laboratory Animals (Dobutsu Jikken Kisoku, version 19.6.26).

The standard length (L_S) of each fish was measured to the nearest 0.5 mm using calipers and the total wet mass of several individual fish was measured to the nearest

0.001 g. The length to mass relationship was estimated using the function: $M = a(L_S)^b$, where M is total mass in g, a is a constant, and b is the growth exponent (LeCren 1951). In 2013, the numbers of fish associated with an individual host sea urchin, either *Diadema setosum* or *Echinothrix calamaris*, were recorded for analysis of aggregation size of *S. tubifer* in association with each urchin species at two reefs with a high abundance of both urchins near Sesoko Station, the University of the Ryukyu's Tropical Biosphere Research Center (26˚38'N, 127˚52'E).

Diet

The nocturnal diet of *S. tubifer* was characterised for 27 individuals captured together at dawn immediately upon their return to their host urchin (*Diadema setosum*) after nocturnal foraging (Gould *et al.* 2014). Fish stomachs were removed and preserved in 10% buffered-formalin in seawater for gut content analysis. The prey items present in each stomach were examined using a steromicroscope (Leica MZ 12.5) and identified to the best possible taxonomic classification level. The percent of empty stomachs examined, or vacuity index (VI), was calculated as well as the percent composition of each prey category for each fish with stomach contents. The mean percent composition of each prey type was compared between small, reproductively immature fish and large, reproductively mature fish. The percent occurrence (presence or absence) of each prey type among all individuals in each size class was also determined.

Reproduction

To describe the reproductive potential of *S. tubifer*, the sex of mature fish was determined by gonad examination, and the total number of fertilized eggs in the mouths of brooding males and mature eggs within the body of gravid females were counted using a steromicroscope (Leica MZ 12.5). Only the eggs from females with highly developed oocytes were counted, as they were easily separated from one another and individually sorted. The relationship between fish L_S and batch fecundity, the number of eggs and fertilized eggs per clutch for gravid females and brooding males, respectively, was fit with a linear regression To test for a difference between

batch fecundity of females and males, an analysis of covariance (ANCOVA) was performed in R version 3.1.1 (R Core Team 2014) with sex as the factor and fish L_S as the covariate. Five fertilized eggs per brood were also selected at random from 35 male fish and the egg diameters along two perpendicular axes (one slightly longer than the other) were measured to the nearest 0.05 mm using a stage micrometer and steromicroscope (Leica MZ 12.5); the mean diameter of each perpendicular axis was calculated for each of the 35 broods examined.

Growth

Pairs of sagittal otoliths from individual *S. tubifer* were removed, cleaned, and stored dry for microstructure analysis. The diameters of the right sagittae from several specimens were measured using a dissecting microscope (Zeiss SteREO Discovery.V8) equipped with a digital camera (Zeiss AxioCam MRc). Images were taken of whole otoliths (Figure 2.1a), and the longest axis through the primordium from one margin edge to the other was measured to the nearest 0.01 mm using Axio Vision 4 software. The percentage of fish L_S was also calculated for each otolith diameter.

To estimate fish age, individual otoliths were mounted and adhered onto glass slides with KrazyGlue (Elmer's Products, Inc.) and ground to their transverse mid-plane using 2000-grit wet/dry polishing paper until daily growth bands became visible. Both sides of larger otoliths were ground to clearly expose bands; however, smaller otoliths were ground only on one side if growth bands became clearly visible. Images of each cross-section were taken with transmitted light under a compound microscope (Nikon Eclipse E600) equipped with a SPOT 2 Slider (1.4.0) digital camera, and the total number of daily growth bands along a continuous radial transect, if possible, was counted twice, each time by a different observer for each image, using Adobe Photoshop (CS6 Extended). First daily increment formation typically occurs at or close to hatching in reef fishes (Thorrold and Hare 2002); therefore growth bands were counted from the first visible band after the hatch mark, a distinct, dark circle surrounding the primordium, to the outer margin of each otolith. The increments between growth bands were also measured for several individuals (n = 10) to look for a change in increment width as an indication of the timing of settlement out of the plankton (Victor 1986, Kingsford *et al.* 2011).

The average number of bands counted for each otolith by both observers was used as the final value for the age of the fish. If the two counts differed by more than 10%, a third count was made and the average of the closest two counts was used as the fish's age. If the third count was not within 10% of the first two counts, the otolith was not included in the analysis. Growth was described by the von Bertalanffy growth function: $L_t = L_\infty[1 - e^{-K(t-t_0)}]$ where L_t is the L_S at time t (days), L_∞ is the L_S at which mean asymptotic growth is reached, K is the growth coefficient ($days^{-1}$), and t_0 is the theoretical age (days) at which fish length is zero. The function was fitted to individual length-at-age data for *S. tubifer* using the package 'fishmethods' version 1.7-0 (Nelson 2014) in R version 3.1.1 (R Core Team 2014). Akaike information criterion (AIC) scores were determined and used for selection of the von Bertalanffy growth model over a linear growth model.

Daily growth bands were verified for sagittal otoliths of *S. tubifer* using a tetracycline immersion method (Kingsford *et al.* 2014) at Sesoko Station in June of 2013. Adult fish (n = 50, ranging in size from 23 to 41.5 mm L_S) were immersed in a 0.25 g/L tetracycline solution in buffered seawater with aeration for 18 hours and maintained in aquaria under natural light conditions with flowing seawater for an additional 5, 10, or 15 days after immersion. While in aquaria, fish were fed nightly with an excess of wild-caught live zooplankton, collected with a 53 mm zooplankton net using a spotlight at dusk from the pier adjacent to their home reef, to simulate the natural diet and timing of foraging. Following each post-immersion period, the sagittae of the randomly selected and sacrificed individuals were immediately removed and stored dry in the dark until processing. Otolith cross-sections were examined under a compound microscope (Nikon Eclipse E600) with fluorescent light for tetracycline marks (Odense and Logan 1974); if a fluorescent band was visible, the otolith was photographed in the same position under both transmitted and fluorescent light (Figure 2.1b). The number of bands present between the fluorescent mark and the otolith margin for each otolith with a visible tetracycline mark was counted without knowledge of the number of days post-immersion that the fish was sacrificed. The band counts were then compared to the number of days post-treatment for each fish and averaged within each treatment group.

Light organ and symbiont population growth

Light organs of *S. tubifer* were dissected from the fish and measured on the longer, anterior to posterior, axis to the nearest 0.1 mm using a stereomicroscope (Leica MZ 12.5) fitted with an ocular micrometer. To quantify bacterial population sizes, light organs from fish of different L_S were aseptically dissected from the fish and individually homogenized in 0.5 ml of buffered 70% seawater (25 mM HEPES, pH 7.25, filter-sterilized) (BSW-70) in sterile, hand-held tissue grinders. The homogenates were then serially diluted 1:100 and 1:100 in BSW-70, and 25 ml aliquots of the second dilution were spread onto plates of LSW-70 agar medium (Kaeding *et al.* 2007), which contained per litre: 10 g tryptone, 5 g yeast extract, 700 ml seawater, 300 ml de-ionized water, and 15 g of agar. The plates were then incubated at room temperature (25˚C – 29˚C) for 12 to 18 hours to allow the formation of bacterial colonies. The bacterial colonies were counted in the light, to quantify the number of colonies, and in the dark in a photographic darkroom, to confirm that all colonies were luminous and had the characteristic appearance of the symbiont, *P. mandapamensis*. Light organ population sizes were calculated from colony counts times the dilution factor used. Population sizes were $\log_{10}$-transformed, and the relationship with fish L_S was fitted linearly.

Results

Size

The body size of *S. tubifer* collected from reefs in Okinawa over the four-year study period (n = 1,273) ranged in length from 9.5 mm to 43.5 mm L_S; 12% of the fish observed were greater than 32 mm L_S (Figure 2.2). Brooding males (n = 95) ranged in size from 22 to 43.5 mm L_S (Figure 2.2). The mass of *S. tubifer* increased as a curvilinear function with fish L_S (Figure 2.3). The mean mass of all *S. tubifer* weighed was 0.780 ± 0.540 g, with a minimum and maximum mass of 0.043 and 2.300 g, corresponding to fish that were 11 mm and 42.5 mm L_S, respectively.

The numbers of *S. tubifer* associated in groups with individual host urchins varied between the two urchin species; the mean number of fish associated with *D.*

setosum was 22.9 ± 16.1, whereas the mean number associated with *E. calamaris* was 3.6 ± 3.2 (Figure 3.4). Approximately 35% of the *E. calamaris* examined (n = 69) were occupied by one *S. tubifer*, whereas only 6% of *D. setosum* (n = 36) were occupied by a single fish (Figure 3.4). Moreover, the largest group of *S. tubifer* observed with an *E. calamaris* urchin contained 15 fish, less than the mean group size associated with *D. setosum*. The largest group of *S. tubifer* associated with a *D. setosum* urchin consisted of 75 fish.

Diet

The general nocturnal diet of *S. tubifer* was diverse, consisting primarily of a variety of crustaceans and other zooplankton, and gut contents varied among individuals collected together from the same urchin (Table 2.1). The only empty stomachs observed were from the two brooding males collected (vacuity index, VI = 7.4%), confirming that *S. tubifer* does not forage while brooding (Gould *et al.* 2014). Brooding males were excluded from the diet analysis. On one occasion, 31 eggs (0.5 mm in diameter) of an unknown fish species were present in the stomach of a large, non-brooding fish (26 mm L_S) in addition to other prey items. The diets of small and large *S. tubifer* differed somewhat, although both amphipods and small decapod shrimp were common prey items for both size classes; amphipods made up approximately 18% and 10% of the diets of small and large fish, whereas decapod shrimps composed a mean of over 30% of the diet of all fish examined (Table 2.1). Several prey items were present in over half of the small individuals examined, including amphipods, decapod crab zooea, and decapod shrimps. Over half of the large fish also consumed decapod shrimps, however nearly half of this size class also consumed mysid shrimps and small teleost larvae, which were unidentifiable due to digestive state. Copepods were observed in the stomachs of both size classes but made up a larger percent of the diets of smaller fish (Table 2.1).

Reproduction

Total numbers of fertilized eggs in the buccal cavity of brooding *S. tubifer* males ranged from 415 to 838 with a mean of 650 ± 146, and the numbers increased linearly

with L_S (Figure 2.5). Similarly, the total number of eggs in ovaries of gravid females increased linearly with L_S, ranging from 440 to 838 total eggs, and the mean was very similar to that for males, 678 ± 164. The relationship between batch fecundity and L_S was not significantly different between males and females (ANCOVA, F = 2.7, P = 0.11; Figure 2.5). Fertilized eggs in the mouths of male *S. tubifer* were nearly round, with one axis slightly longer than its perpendicular axis; the mean long axis diameter was 0.87 ± 0.07 mm, whereas the shorter axis mean was 0.80 ± 0.07 mm. The ranges in diameter were 0.65 - 1.00 mm and 0.70 -1.20 mm for the short and long axes, respectively.

Growth

Sagittal otolith diameter increased linearly with fish length ($L_S = 15.0(O_L) - 1.16$, $r^2 = 0.97$, where O_L is otolith diameter in mm) and ranged from 0.83 mm to 3.07 mm. The mean length of all otolith diameters as a percentage of fish L_s was 7.05 ± 0.44%. From counts of otolith daily increments, the growth of *S. tubifer* was described by the von Bertalanffy growth model (VBGM) (Figure 2.6), which indicated that asymptotic growth is reached at 40.8 mm L_S. The relationship between $\ln(L_\infty - L_t)$ and apparent age of *S. tubifer* was linear ($r^2 = 0.82$) and validated the use of the VBGM (Everhart and Youngs 1981), as did a comparison of AIC scores between a linear model and the VBGM; the AIC score for the VBGM was considerably lower (ΔAIC = 145). Based on this growth curve, the age at first reproduction of *S. tubifer* is 57.5 days at 22 mm L_S, the smallest size observed of reproductively mature fish. Furthermore, the age of the oldest individual examined was estimated to be 240 days at 43 mm L_S. Settlement marks were not evident in *S. tubifer* otoliths and there was no observable pattern in increment width between growth bands that would indicate the timing of settlement; increment widths varied overall from 4.8 – 19.5 mm (13.8 ± 3.2 mm, mean ± S.D). However, the youngest fish analyzed was 31 days old (11.5 mm L_S), which was close to the smallest size of *S. tubifer* collected with an urchin (Figure 2.2); therefore the PLD for *S. tubifer* in Okinawa is estimated to be approximately 30 days.

The tetracycline immersion method confirmed that the growth bands used for aging represented daily growth increments of *S. tubifer*. Sagittal otoliths of 22% of the chemically treated fish showed clear incorporation of tetracycline into their otolith microstructure, visible as a fluorescent band under UV light (Figure 2.1b). The number

of bands between the fluorescent mark and the otolith margin of these otoliths corresponded with the number of days post-immersion for these individuals (Table 2.2).

Light organ and symbiont population growth

Light organs of *S. tubifer* increased linearly in diameter as fish L_S increased with no sign of asymptotic growth ($r^2 = 0.82$, Figure 2.7a). The smallest light organ measured was 0.8 mm in diameter (11.0 mm L_S), and the two largest light organs were both 2.9 mm in diameter (36.0 and 37.0 mm L_S). The population sizes of *P. mandapamensis* in light organs also increased with fish L_S ($r^2 = 0.62$, Figure 2.7b), increasing from 7.0×10^6 (13.8 mm L_S) to 8.7×10^7 (38.2 mm L_S) cells.

Discussion

Among cardinalfishes, *S. tubifer* is unusual for its symbiosis with the luminous bacterium, *Photobacterium mandapamensis*. This study provides additional evidence of the fish's distinct biology and also highlights some biological similarities to other apogonids, including a diverse, carnivorous diet and large group aggregation sizes. In particular, the results presented here indicate that *S. tubifer* is the shortest-lived cardinalfish studied to date, which in addition to a high natural mortality rate, might result from high predation pressure, as direct predation on *S. tubifer* by other reef fishes has been observed at the study site (Gould *et al.* 2014). As a consequence, predation might have played a role in shaping the fish's cryptic behaviour as a small, bioluminescent coral reef fish that seeks refuge among urchin spines during the day and uses ventral luminescence, potentially for countershading, while foraging at night (Dunlap and Nakamura 2011).

Within the cardinalfish family, there is an overall positive, linear relationship between maximum species size and longevity (Marnane 2001). This relationship holds true for small species such as Doederlein's cardinalfish *Ostorhinchus doederleini* (Jordan and Snyder 1901) and the rubyspot cardinalfish *Ostorhinchus rubrimacula* (Randall and Kulbicki 1998), the life histories of which have both been recently

described (Table 2.3). Results of this study are similar to that of *O. rubrimacula*, suggesting that *S. tubifer* is also short-lived in Okinawa (<2/3 year), and support the positive relationship between maximum body size and longevity of apogonids. In addition, the size distributions reported for other *Siphamia* species are similar to that of *S. tubifer*; the siphonfishes *S. corallicola* and *S. jebbi*, range in size from 10.7-30.5 mm L_S (n = 55) and 11.7-24.8 (n = 39), respectively (Allen 1993). It should be noted, however, that a larger maximum body size (7.0 cm L_T) of *S. tubifer* was reported from Tahiti on FishBase (Froese and Pauly 2015), which indicates that the fish has the potential to live longer than observed in this study.

Aggregation sizes vary between different species of cardinalfish and can range from solitary or paired individuals to hundreds of fish. Gardiner and Jones (2010) determined that the mean group size of the five-lined cardinalfish *Cheilodipterus quinquelineatus* Cuvier 1982 was 13 with over half of the observed groups consisting of only 1-6 individuals, whereas the longspine cardinalfish *Zoramia leptacanthus* Bleeker 1856 groups were much larger, averaging 98 individuals and containing as many as 700 fish. The numbers of fish per aggregation reported for *S. jebbi* was between 20 and 40 individuals in association with pocilloporid coral heads (Allen 1993). This aggregation size is similar to the number of *S. tubifer* reported here in association with *D. setosum*, however the group size associated with *E. calamaris* was much lower, likely due to the shorter spines of *E. calamaris*, which cannot physically accommodate or protect a large number of fish. The group size of *S. tubifer* associated with *E. calamaris* was similar to the aggregation size reported for *Siphamia* sp. among the spines of the crown-of-thorns sea star *Acanthaster planci* (2–18 fish per group, mean = 6.2) (Stier *et al.* 2009), which also have shorter spines than *D. setosum*. Conversely, the numbers of silver siphonfish *Siphamia argentea* Lachner 1953 associated with the sea urchin *Astropyga radiate* in Madagascar were reported to be so large that the sea urchin could not accommodate all of the fish; the fish therefore formed a dense aggregation in the form of an urchin directly above the urchin itself (Fricke 1970).

Despite the use of ventral luminescence while foraging, the diet of *S. tubifer* was similar to that reported for non-luminous cardinalfishes. Most apogonids are nocturnal predators and feed primarily on benthic invertebrates and zooplankton; their diet is diverse, yet often dominated by crustaceans (Hiat and Strasburg 1960, Allen 1993,

Marnane and Bellwood 2002, Longenecker and Langston 2006). Similar to the findings reported here, the diet of *S. tubifer* (*S. permutata*) from the Red Sea was reported to consist of copepods, gastropod veligers, worm chaeta, stomatopod larvae, benthic amphipods and juvenile shrimps (Fishelson *et al.* 2005). All brooding males examined in this study had empty stomachs, and therefore, apparently do not forage during this incubation period. One non-brooding individual had several eggs in its stomach, but the eggs were smaller in diameter than *S. tubifer* eggs; filial cannibalism, as reported for other cardinalfishes (Okuda and Yanagisawa 1996, Okuda 1999, Kume *et al.* 2000a) was not observed in this study. Although daytime feeding was not examined, *S. tubifer* may consume small zooplankton prey throughout the day while sheltered among its host urchin's spines (Magnus 1967, Tamura 1982). Overall, *Siphamia* spp., like most cardinalfishes, have a generalist carnivore diet and forage nocturnally on a diverse array of benthic zooplankton prey, especially decapod shrimps.

Mouthbrooding is one of the most effective ways of protecting offspring under high predation pressure (Oppenheimer 1970), and is therefore a successful reproductive strategy for the relatively small-bodied family of cardinalfishes. Within the family, however, brood sizes carried by males vary widely, from as low as 40 to tens of thousands of eggs, as do egg diameters (Vagelli 2011), and there is no indication that brood or egg size varies in relation to fish body size. However, smaller cardinalfish species, including *Siphamia* spp., generally have ovaries that are relatively large compared to their body size and spawn fewer, larger eggs than do larger species (Fishelson and Gon 2008). Within *Siphamia*, a physically small cardinalfish genus, brood sizes have been reported as low as 162 eggs for *S. corallicola* (25.0 mm L_S) (Allen 1993) and up to 600 for the crown-of thorns cardinalfish *Siphamia fuscolineata* Lachner, 1953 (27.7 mm L_S) (Vagelli 2011); both instances were reported for a single brooding male. The mean number of eggs per brood for *S. tubifer* was similar to that reported for *S. fuscolineata*, however, the largest brood in this study contained over 800 eggs. The number of eggs previously reported in the ovaries of *S. tubifer* (26 mm L_S) (Fishelson and Gon 2008) corresponds with the lower range of total eggs counted in gravid *S. tubifer* females in this study. There was little to no difference between the total number of fertilized eggs in broods carried by male *S. tubifer* and eggs in female gonads, indicating that few, if any, eggs are lost in the process of fertilization and transfer to the male.

Egg diameters reported for other *Siphamia* spp. are similar to those reported here and elsewhere (Tominaga 1964); fertilized eggs of *S. corallicola* and *S. fuscolineata* were between 0.95-1.0 mm and 0.7-0.8 mm in diameter (Allen 1993, Vagelli 2011), respectively. However, egg diameters in female ovaries of *S. tubifer* (*S. permutata*) and *S. roseigaster* were 1.2 mm and 1.3 mm in diameter (Fishelson and Gon 2008), both larger than the maximum diameter of fertilized eggs observed in the mouths of *S. tubifer* males in this study. Overall, *Siphamia* spp. eggs are average in size compared to the eggs of other apogonids and correspond with the general trend that fish with larger broods have smaller eggs (Vagelli 2011).

In a survey of sagittal otolith diameter as a percentage of fish L_s across 247 species in 147 marine fish families, Paxton (2000) determined that nearly half of the species with the largest otoliths (> 7% L_s) surveyed were luminous, including one species of apogonid (*Archamia fucata*), which had a larger otolith than its non-luminous counterpart examined (*Apogon aureus*). This trend was true for most families with both luminous and non-luminous members (Paxton 2000). However, the otolith diameter of *S. tubifer* appears to be similar (~7% L_s) to that of the non-luminous apogonid species examined by Paxton (2000).

The von Bertalanffy growth parameters for *S. tubifer* are similar to those reported for another relatively small cardinalfish, *O. rubrimacula* (Longenecker and Langston 2006, Table 2.3). Both fish have similar asymptotic lengths (L_∞) and longevities less than one year, but *S. tubifer* had an initial growth rate (K) twice that of both *O. rubrimacula* and *O. doederleini* (Longenecker and Langston 2006, Kingsford *et al.* 2014). Additionally, the maximum age observed for *S. tubifer* in Okinawa was even shorter than those reported for two Ostorhinchus spp; the oldest observed *O. rubrimacula* in Fiji was 274 days (Longenecker and Langston 2006), and the oldest *O. doederleini* reported in the southern Great Barrier Reef, Australia was 368 days. Yet, much like this study, few fish examined were older than 200 days (Kingsford *et al.* 2014).

No indication of the timing of settlement was evident in the otolith microstructure of *S. tubifer*; however the youngest fish observed was 31 days old and was similar in size (11.5 mm L_s) to the settlement sizes of other apogonid species (Leis *et al.* 2015). Assuming that *S. tubifer* settle directly onto the reef and immediately take up residence among the spines of a host urchin, the PLD of *S. tubifer* could be

approximately 30 days. This result is similar to that reported for the weed cardinalfish *Foa brachygramma* (Jenkins 1903) in Okinawa with a mean PLD of 30.6 days at 11 mm L_S (Ishihara and Tachihara 2011), and it is relatively long in comparison to the PLDs reported for other species (Leis *et al.* 2015). However, some apogonids undergo a two-phase recruitment process, settling first onto sand rubble habitat before eventually taking up residence on a continuous reef with adults (Finn and Kingsford 1996). Thus far, there is no evidence to suggest that *S. tubifer* settles out of the plankton onto non-urchin habitat prior to taking residence with adults at an urchin. There is also an undefined period of time (estimated as a few days) after *S. tubifer* embryos have hatched in the male's mouth but prior to their release into the plankton as larvae (Dunlap *et al.* 2009, 2012), which could have an influence on the PLD of the fish and should be considered in future studies.

There are few studies of the life histories and ecology of other bacterially luminous fishes. Previous studies either described aspects of the life history the fish with no examination of the fish's symbiosis with luminous bacteria (Murty 1986, Okuda *et al.* 2005), or they focused primarily on the bioluminescent symbiosis (Hastings and Mitchell 1971, Haygood 1993); few studies have examined the growth of the light organ and the symbiont population relative to the growth of the host fish (Dunlap 1984, McFall-Ngai and Dunlap 1984). This study shows that light organs of *S. tubifer* continue to increase linearly in diameter with fish L_S, and that the number of luminous symbionts housed within a light organ also increases throughout the fish's life span (Figure 2.7). However, the maximum estimated symbiont population size in *S. tubifer* was lower than that reported for leiognathids, monocentrids, and anomalopids, and may be consistent with the generally smaller light organ of adult *S. tubifer* compared to light organs of adults of these other fishes (Haygood 1993).

The symbiosis with *P. mandapamensis* does not begin immediately upon hatching in *S. tubifer* (Dunlap *et al.* 2012); the light organ of *S. tubifer* becomes receptive to colonisation by the symbiotic bacteria after one week or more of development post-release from the male's mouth (Dunlap *et al.* 2012), and larvae that were 2.8 mm in length had no luminous symbionts in light organs, whereas larvae that were 3.5 and 10.4 mm L_S had symbionts (Leis and Bullock 1986). It remains unknown how many bacterial cells initially colonise a light organ and for how long initial colonisation is possible, but evidence from this study suggests that, once established,

the population size of *P. mandapamensis* within a light organ increases throughout a host fish's lifespan.

Before the current analysis of *S. tubifer* in Okinawa, *O. rubrimacula* in Fiji (Longenecker and Langston 2006) was the smallest and most short-lived cardinalfish reported. However, results of this study suggest that *S. tubifer*, despite its similar size, is even more short-lived than *O. rubrimacula*, and once settled on a reef, grows at a rate twice that of both *O. doederleini* and *O. rubrimacula* (Longenecker and Langston 2006, Kingsford *et al.* 2014, Table 2.3). Despite having similar lengths of asymptotic growth, *S. tubifer* reaches sexual maturity sooner than *O. rubrimacula* and reproduces over more of its lifespan, as reflected by the lower reproductive load in *S. tubifer* (Table 2.3); however, the mean brood size of *S. tubifer* is much lower than that of *O. rubrimacula* (Longenecker and Langston 2006). The rapid growth to maturity and short lifespan of *S. tubifer* correspond with the fish's cryptic behaviour and support the hypothesis that high mortality, possibly due to predation, has influenced the ecology of this small, bioluminescent coral reef fish.

Table 2.1 Summary of the diet of *Siphamia tubifer* (n = 25) in Okinawa, Japan. Percent gut content is the mean percent composition of each prey item of the total diet across all individuals of the size class indicated. Percent occurrence is the percentage of individuals in each size class in which that prey category was present in the diet. Rank indicates the relative importance of each prey item to the diet of each size class as a reflection of percent occurrence combined with percent content

Prey type	fish < 22 mm L_S (n = 14)			fish > 22 mm L_S (n = 11)		
	% content (± SE)	% occurrence	rank	% content (± SE)	% occurrence	rank
Amphipoda	18.1 (± 4.4)	64.3	2	10.1 (± 9.1)	18.2	6
Decapod crab megalops	2.8 (± 2.1)	14.3	8	6.0 (± 2.7)	36.4	4
Decapod crab zooea	8.9 (± 2.9)	50.0	3	5.1 (± 3.6)	18.2	8
Decapod shrimp	34.4 (± 8.0)	78.6	1	31.7 (± 9.0)	63.6	1
Chaetognatha	0.7 (± 0.7)	7.1	10	-	-	-
Copepoda	11.3 (± 5.6)	35.7	5	1.5 (± 1.0)	18.2	9
Isopoda	-	-	-	0.6 (± 0.6)	9.1	13
Mollusca	-	-	-	0.7 (± 0.7)	9.1	10
Mysidacea	6.5 (± 5.3)	21.4	6	9.4 (± 3.6)	45.5	3
Ostracoda	0.5 (± 0.5)	7.1	11	0.7 (± 0.7)	9.1	10
Polychaeta	2.2 (± 1.2)	21.4	7	10.6 (± 5.6)	27.3	5
Stomatopoda	12.5 (± 7.2)	35.7	4	3.9 (± 3.1)	18.2	7
Tanaidacea	2.3 (± 1.6)	14.3	9	0.7 (± 0.7)	9.1	10
Teleost larvae	-	-	-	19.2 (± 9.5)	45.5	2
Fish eggs	-	-	-	0.6 (± 0.6)	9.1	13

Table 2.2 Otolith growth band validation. Treatment refers to the number of days after tetracycline immersion that otoliths were sampled, n_i and n_f indicate the numbers of fish treated and the number of otoliths recovered with visible UV bands, respectively

Treatment (days)	n_i	*Ls* range (mm)	n_f	Mean count (± SE)
5	12	27.5 - 41.0	1	5
10	15	27.5 - 41.5	5	10.8 (± 1.7)
15	15	29.0 - 37.5	5	14.6 (± 2.7)

Table 2.3 The von Bertalanffy growth parameters, L_∞, K, and t_0, available for apogonid fishes. Minimum size and time at maturity, L_m and t_m, and maximum size and longevity, L_{max} and t_{max} are also listed. Reproductive load, L_m/L_∞, was calculated when possible. Lengths in bold are reported as total lengths, L_T, and times or rates in bold are in years; all other lengths are reported as standard lengths, L_S, and times or rates are in days.

Species	L_∞ (mm)	K (time^{-1})	t_0	L_m (mm)	t_m	L_{max} (mm)	t_{max}	L_m/L_∞	Location	Reference
Apogon fasciatus	**105.5**	**1.88**	**-0.04**	**46.4**	-	-	-	-	SW Taiwan	Wu (2009)
Apogon imberbis	**120.5**	**0.41**	**-0.57**	**55**	**1**	**121**	**5**	0.46	NW Mediterranean Sea	Raventos (2007)
Apogon lineatus	**86.6 (118.5)**	**1.12 (0.37)**	**-0.01 (-1.03)**	**53 (65)**	**1**	**103 (112)**	**3**	0.61 (0.55)	Tokyo Bay, Japan	Kume *et al.* (1998)
	94.7 (85.0)	**0.50 (1.23)**	**-0.88 (-0.07)**	**51.3 (65)**	**1**	**110**	**4 (5)**	0.54 (0.77)	Niigata Prefecture, Japan	Kume *et al.* (2003)
Ostorhinchus doederleini	65.04	0.01	-	-	-	74 mm	368	-	Great Barrier Reef, Australia	Kingsford *et al.* (2014)
	86.5 (88.6)	**1.56 (1.62)**	**-0.02 (-0.02)**	69 (73)	**1**	92.5 mm*	**6 (7)**	0.80 (0.82)	Shikoku Island, Japan	Okuda *et al.* (1998)
Ostorhinchus rubrimacula	40.8	0.014	22.45 d	35	162	43 mm**	274	0.86	Koro, Fiji	Longenecker and Langston (2006)
Pterapogon kauderni	71	**0.74**	**-0.11**	40	**1**	66 mm	**3-5**	0.56	Banggai Islands, Indonesia	Ndobe *et al.* (2013)
Siphamia tubifer	40.8	0.026	23.25	22	53	43.5 mm	240	0.54	Okinawa, Japan	this study

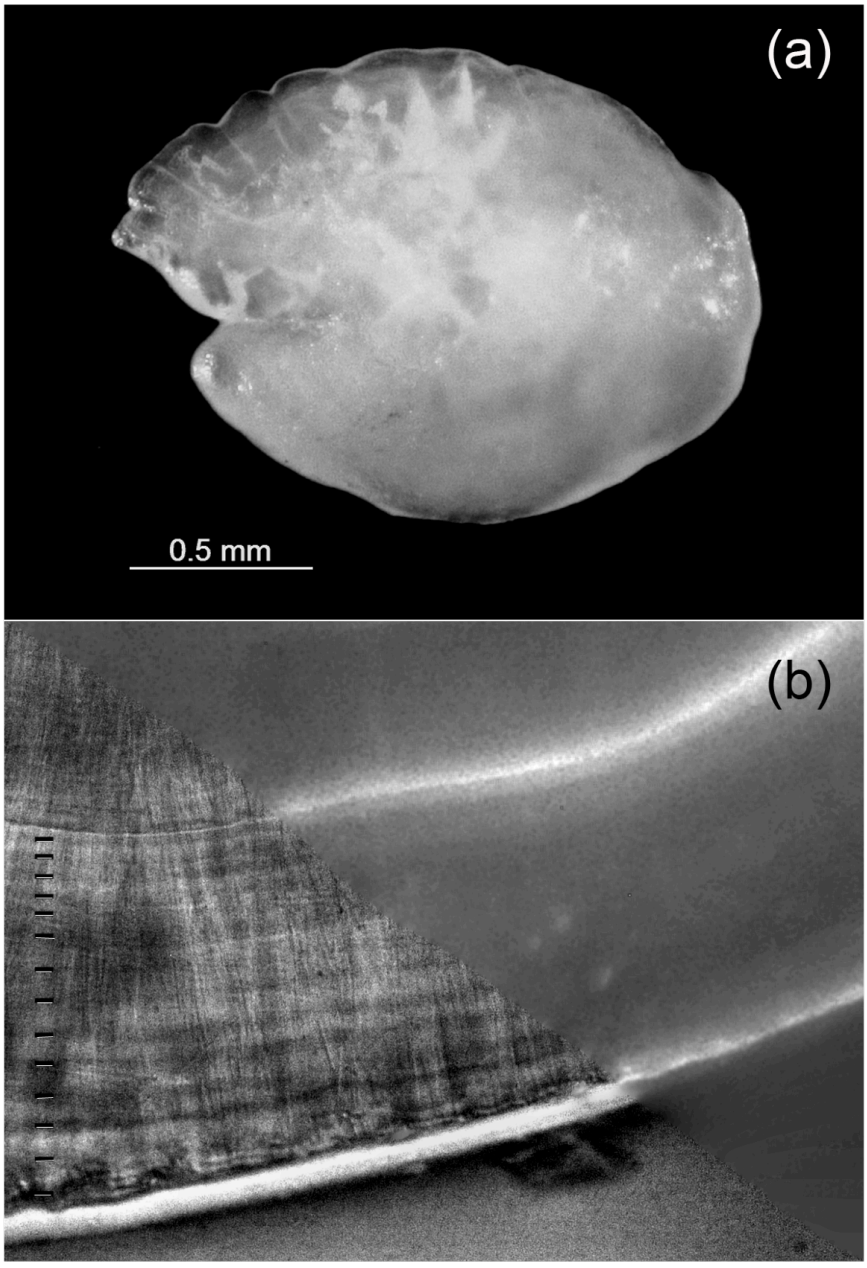

Figure 2.1 (a) Photograph of a right sagittal otolith of *Siphamia tubifer* (27 mm L_s) and (b) overlay of two images of the outer edge of a sagittal otolith cross-section from an individual taken 15 days post-immersion in a tetracycline-seawater solution. For (b), the image at the left was captured under transmitted light, and the image at the right was captured under ultraviolet light. The black tick marks indicate daily growth bands

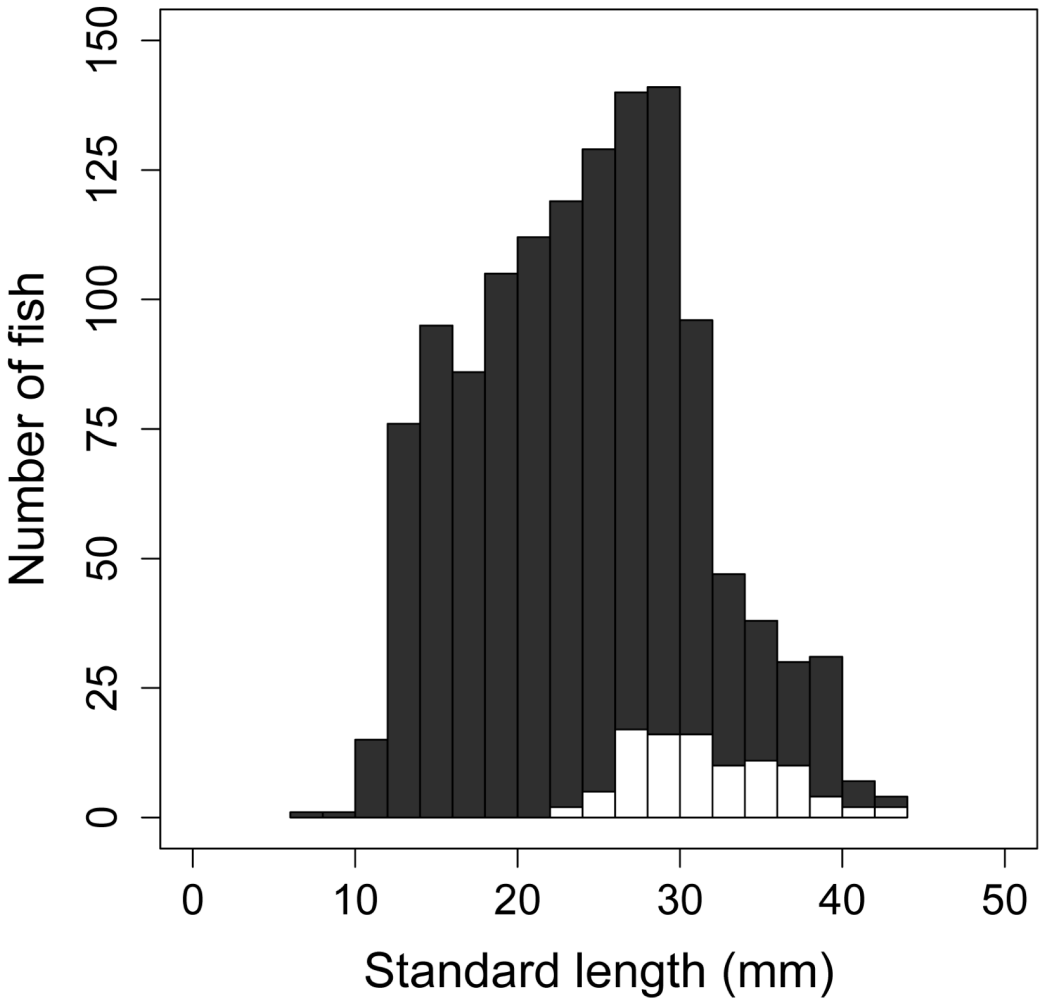

Figure 2.2 Frequency histogram of the lengths of *Siphamia tubifer* from various locations in Okinawa, Japan from 2011-2014. White bars indicate length frequencies of brooding males

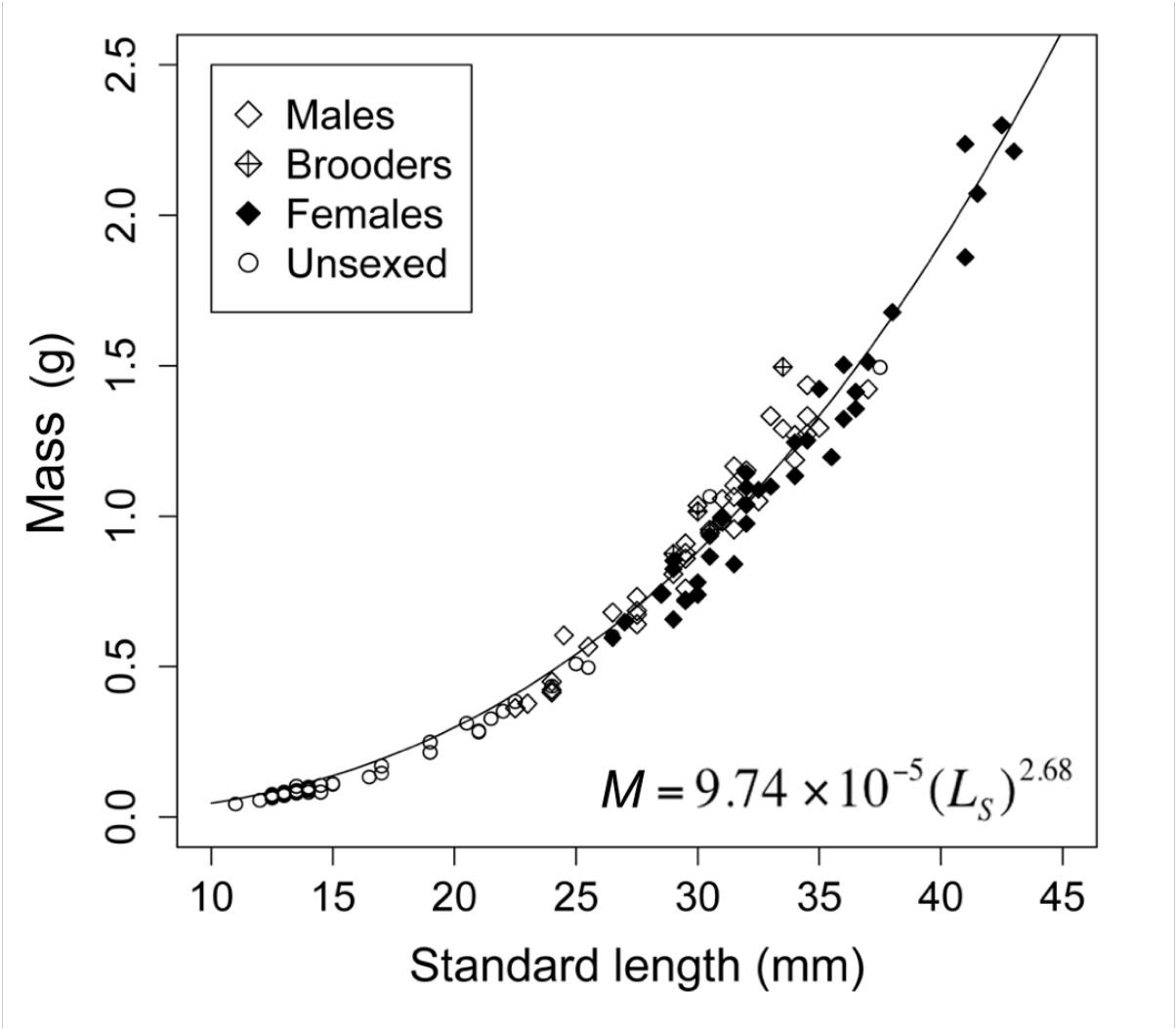

Figure 2.3 Total wet mass of *Siphamia tubifer* as a function of fish length (n = 121) fitted with the curvilinear function indicated

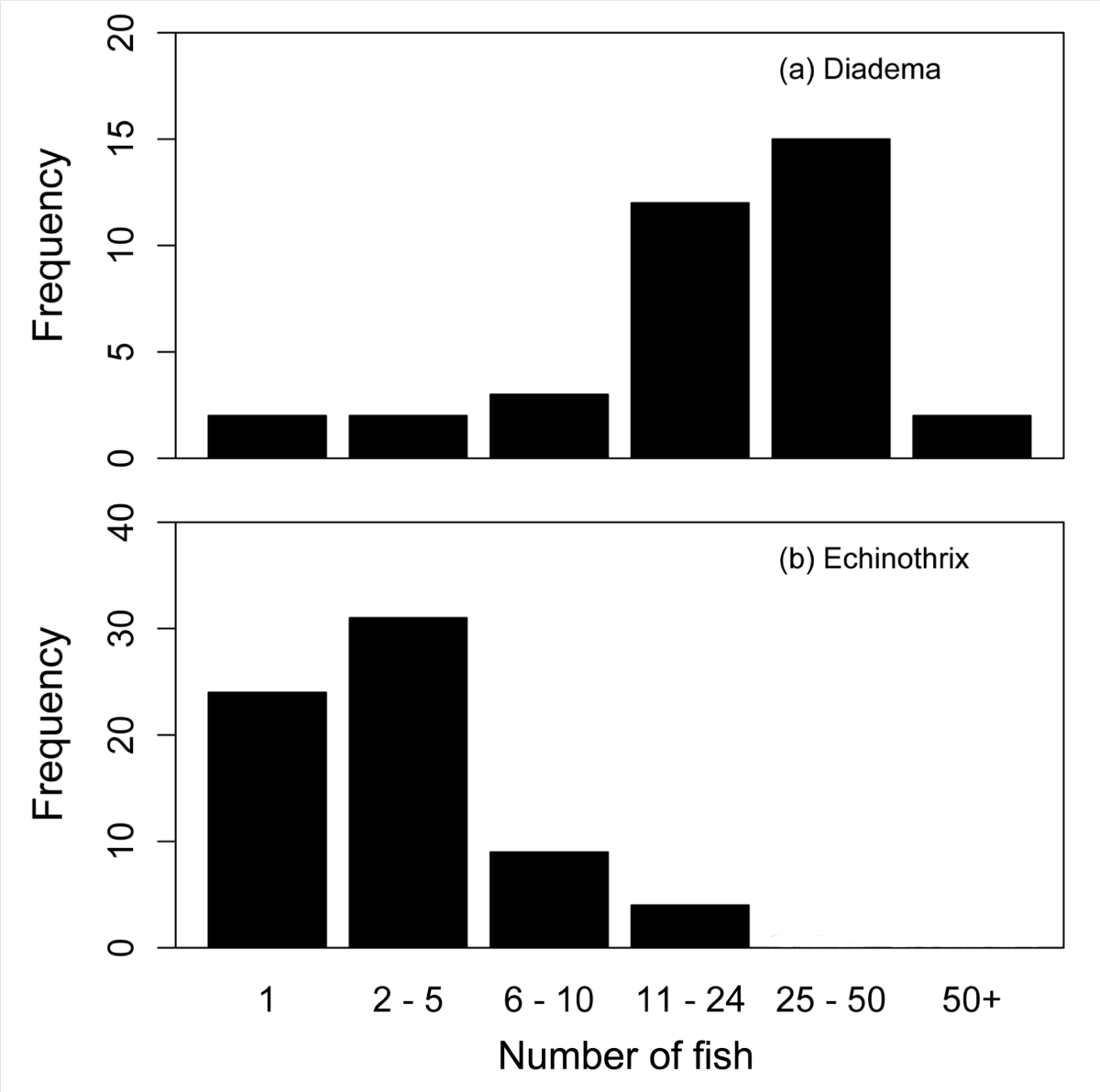

Figure 2.4 The number of *Siphamia tubifer* aggregated together among the spines of a host sea urchin: (a) *Diadema setosum* and (b) *Echinothrix calamaris*

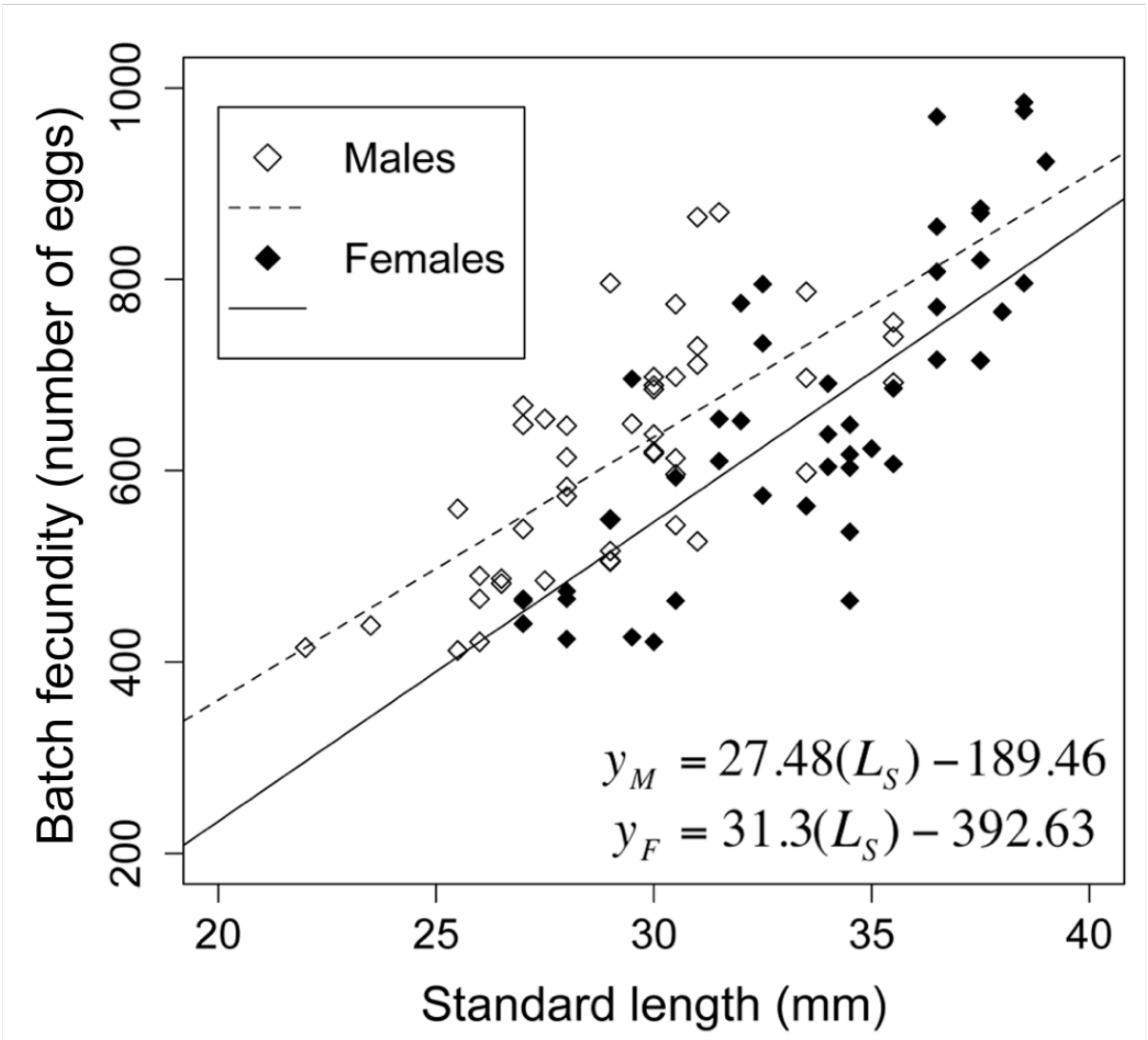

Figure 2.5 Batch fecundity of *Siphamia tubifer* represented as the number of fertilized eggs in the mouths of brooding males (n = 46, r^2 = 0.46, F = 39.47, P < 0.001) and eggs in the ovaries of females (n = 49, r^2 = 0.63, F = 82.59, P < 0.001) as a function of fish length

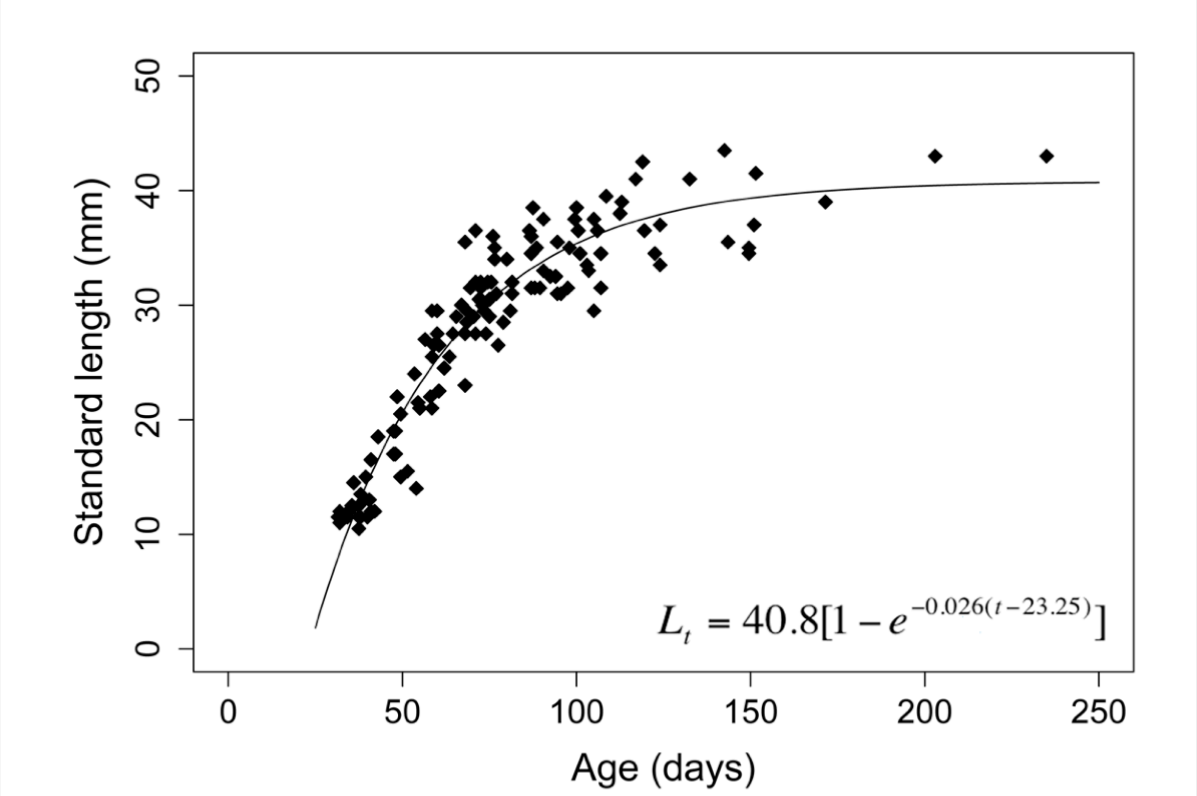

Figure 2.6 Length of *Siphamia tubifer* by age as determined from counts of daily growth bands in sagittal otoliths fitted to the von Bertalanffy growth function indicated

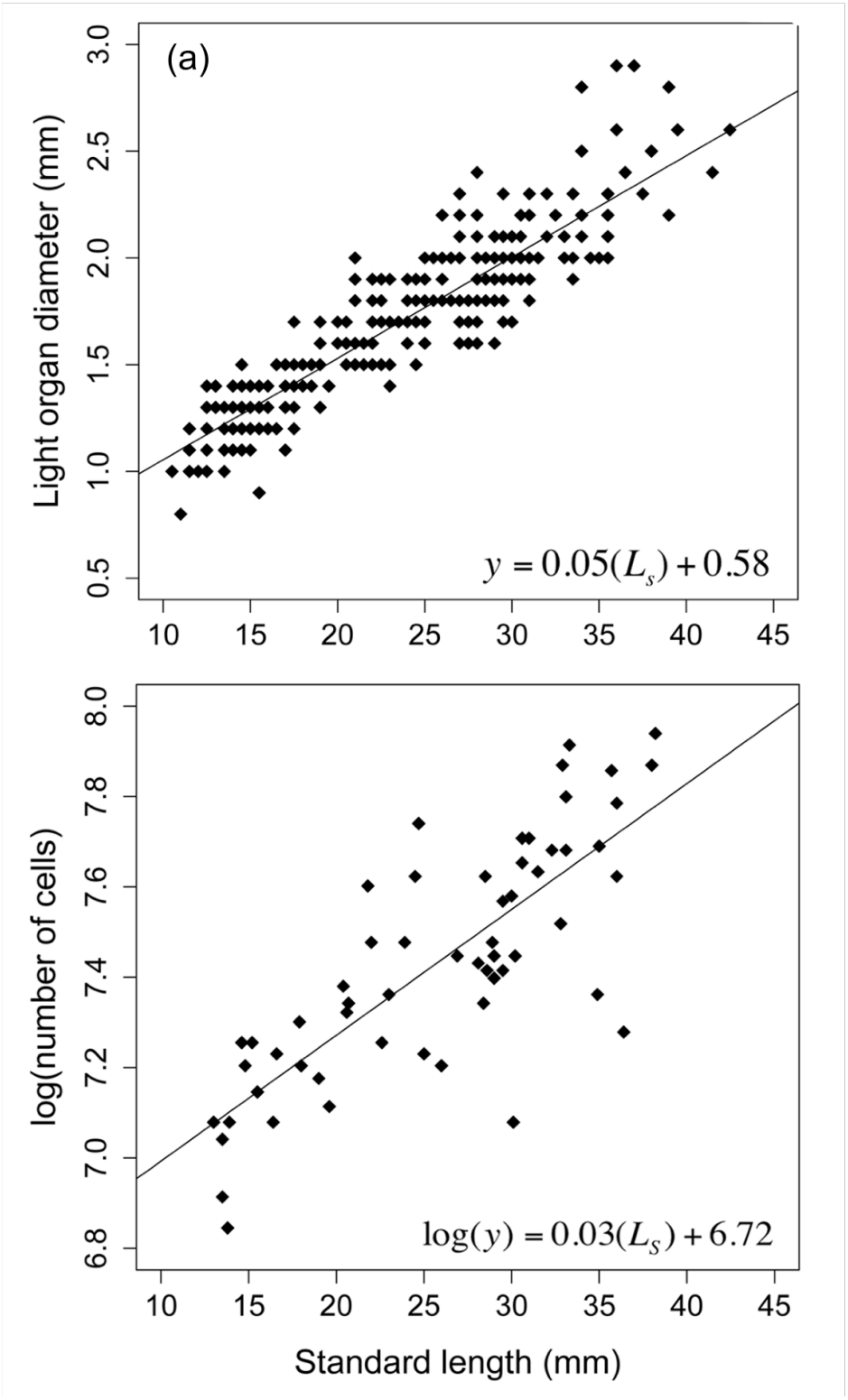

Figure 2.7 Light organ growth of *Siphamia tubifer*. (a) The diameter of a *Siphamia tubifer* light organ (n = 299, r^2 = 0.82, F = 1,345, P < 0.001) and (b) the number of luminous bacteria present in a light organ (n = 58, r^2 = 0.62, F = 92.5, P < 0.001) as a function of fish length

CHAPTER III
Homing and site fidelity of *Siphamia tubifer* [2]

Abstract

The sea urchin cardinalfish, *Siphamia tubifer* (Perciformes: Apogonidae), is unusual among coral reef fishes for its use of bioluminescence, produced by symbiotic bacteria, while foraging at night. As a foundation for understanding the relationship between the symbiosis and the ecology of the fish, this study examined the diel behavior, host urchin preference, site fidelity, and homing of *S. tubifer* in June and July of 2012 and 2013 at reefs near Sesoko Island, Okinawa, Japan (26º38'N, 127º52'E). After foraging, *S. tubifer* aggregated in groups among the spines of the longspine sea urchin, *Diadema setosum*, and the banded sea urchin, *Echinothrix calamaris*. A preference for *D. setosum* was evident ($P < 0.001$), especially by larger individuals (> 25 mm standard length, $P < 0.01$), and choice experiments demonstrated the ability of *S. tubifer* to recognize and orient to a host urchin and to conspecifics. Tagging studies revealed that *S. tubifer* exhibits daily fidelity to a host urchin; 43-50% and 26-37% of tagged individuals were associated with the same urchin after three and seven days. Tagged fish also returned to their site of origin after displacement; by day two, 23-43% and 27-33% of tagged individuals returned from displacement distances of one and two kilometers. These results suggest that *S. tubifer* uses various environmental cues for homing and site fidelity; similar behaviors and cues might be used by larvae for recruitment to settlement sites and for the acquisition of luminous symbiotic bacteria.

[2] Published as: Gould, AL, S Harii, and PV Dunlap (2014) Host preference, site fidelity and homing behavior of the symbiotically luminous cardinalfish, *Siphamia tubifer* (Perciformes: Apogonidae). *Marine Biology* 161(12):2897-2907

Introduction

Many coral reef fishes have restricted home ranges and return to home sites daily after foraging and after displacement (Sale 1978a). Having a home site can enhance an individual fish's fitness through benefits associated with familiarity of local resources and the location of competitors, predators, and mates (Shapiro 1986, Noda *et al.* 1994, Brown and Dreier 2002). Furthermore, the diel homing behavior of fishes can directly affect nutrient transfer within a reef environment (e.g. Meyer *et al.* 1983, Bellwood 1995) as well as processes that influence population dynamics, such as mortality and recruitment (Sale 1978b). Among reef fishes, the cardinalfishes (Perciformes: Apogonidae) are one of the most abundant and species-rich groups in the Indo-Pacific (Allen 1993, Bellwood 1996). Cardinalfishes typically forage at night and form aggregations during the day around reef structures, such as branching corals (Greenfield and Johnson 1990, Gardiner and Jones 2005, 2010). Some cardinalfishes exhibit fidelity to their daytime home sites over the course of months (Kuwamura 1985, Okuda andYanagisawa 1996, Marnane 2000), and few species are known to return to home sites when displaced substantial distances (Marnane 2000, Kolm *et al.* 2005). However, despite their abundance in reef communities, cardinalfishes remain one of the least studied families of reef fishes (Bellwood 1996). In particular, little is known of the behavioral ecology of members of the symbiotically luminous genus of cardinalfish, *Siphamia*.

Siphamia tubifer may be the most widespread *Siphamia* species; a recent taxonomic revision reclassified *Siphamia versicolor* (Smith and Radcliffe, in Radcliffe 1911; Tominaga 1964), reported from many locations throughout the Indo-West Pacific region, as a junior synonym of *S. tubifer* Weber, 1909 (Gon and Allen 2012). Like other cardinalfishes, *S. tubifer* is a paternal mouth brooder; the adult male orally broods his fertilized clutch of eggs (Breder and Rosen 1966, Thresher 1984, Dunlap *et al.* 2012) and releases pre-flexion larvae into the plankton (Dunlap *et al.* 2009). Unusual for most cardinalfishes and other coral reef fishes, however, bioluminescence apparently plays a major role in the biology of *S. tubifer*. The abdominal light organ of *S. tubifer*, which is connected to the intestine by a duct, begins to develop in larvae after their release into the plankton and remains free of bacteria for at least seven days of post-release development (Leis and Bullock 1986; Dunlap *et al.* 2009). The luminous bacteria,

identified as members of clade II of *Photobacterium mandapamensis* (Kaeding *et al.* 2007, Urbanczyk *et al.* 2011), are then taken up from the environment and colonize the fish's light organ (Dunlap and Nakamura 2011, Dunlap *et al.* 2012). The fish carries a large population of the symbiotic bacteria in the light organ and emits the bacterial light as an even glow over its ventrum while it forages at night (Dunlap and Nakamura 2011). After returning to an urchin from foraging, the fish release fecal material containing large numbers of the symbiotic bacteria (Dunlap and Nakamura 2011).

Despite progress in understanding the symbiosis of *S. tubifer* and *P. mandapamensis*, the behavioral ecology of the fish and the functional role of the symbiosis in its daily life remain largely unknown. During the day, *S. tubifer* associates in small to large groups with the longspine sea urchin, *Diadema setosum*, or the banded sea urchin, *Echinothrix calamaris*, remaining quiescent among the urchin's spines (Lachner 1955, Eibl-Eibesfeldt 1961, Tamura 1982). A preference for a host urchin species would indicate which reef sites are suitable for incoming recruits, and predictable home sites could influence the distribution of competitors and predators at that reef. However, whether the fish exhibits the homing behavior and site fidelity seen other cardinalfishes and whether the symbiosis is influenced by or contributes to these activities are not known. Therefore, to begin building a foundation for understanding the ecology of this group of apogonids with respect to the bioluminescent symbiosis, I examined the diel behavior, host urchin preference, site fidelity, and homing of *S. tubifer* at reefs in Okinawa, Japan.

Materials and Methods

Study sites

This study was carried out at shallow coral reefs at Sesoko Island, Okinawa, Japan (26º38'N, 127º52'E) and at nearby reefs on Motobu Peninsula (Figure 3.1) during June and July of 2012 and 2013. Observations of diel behavior of *Siphamia tubifer* were made at reefs fronting Sesoko Station (Tropical Biosphere Research Center, University of the Ryukyus) on Sesoko Island, as were site fidelity experiments. Transects and homing experiments were carried out at a site in the vicinity of Motobu town, across

the channel from Sesoko Island (Figure 3.1). The protocols used here for the capture, care, and handling of *S. tubifer* were approved by the University of Michigan's Institutional Animal Care and Use Committee, and they accord with animal handling guidelines of the University of the Ryukyus Guide for Care and Use of Laboratory Animals.

Diel behavior

Observations of groups of *S. tubifer* associated with *Diadema setosum* were made using SCUBA to determine the timing of departure from and return to a host urchin. On July 1, 2012, the group of fish at an urchin was monitored from approximately fifteen minutes before sunset until no other fish left the urchin. An additional observation of the urchin was made at midnight to determine whether any fish had returned from foraging by this time. On July 4, 2012, the same urchin was monitored beginning at one hour before sunrise until the time after which no additional fish returned to the urchin. One group of fish (n = 26) was collected immediately after their return to an urchin and examined for stomach fullness and contents.

Host preference

To determine the natural preference for *S. tubifer* to associate with *D. setosum* or *Echinothrix calamaris* (Figure 3.2), surveys along randomly placed transects were carried out at a site approximately 40 m offshore where both species of urchin were abundant (Figure 3.1). A total of six independent 50 m transects were surveyed using SCUBA for the number of urchins and associated *S. tubifer* along the backside of the reef and the adjacent sand flat. Transects were randomly placed, regardless of substrate (reef or sand), at least 20 m apart, and each urchin within two m of either side of the transect tape was examined by divers. The urchin species and number of *S. tubifer* associated with each urchin were recorded along with the substrate type. The size of each *S. tubifer* observed was also estimated and recorded as either "small" (< 25 mm standard length, SL) or "large" (> 25 mm SL) for three of the transects, as a difference in size of fish associated with each host urchin species became evident during the first three transects.

For choice experiments, six groups of *S. tubifer*, which varied in standard lengths and number of fish, were collected with their urchins from reefs fronting Sesoko Station (Figure 3.1) and maintained in aerated aquaria with flowing natural seawater. Individual fish were placed in the middle area of a large aquarium (two m x one m x one m) that contained approximately 1,200 L of natural seawater. The tank was partitioned into three equal sections with square plastic mesh (20 x 20 mm^2) through which the fish could swim. Different combinations of choices were presented to each fish in the two opposing sections of the aquarium, and the sides in which the stimuli were presented were randomly and periodically switched between fishes to ensure no side bias existed in the tank. For each trial, the side that an individual fish swam to and remained settled at for at least 30 seconds was recorded. Fish were allowed up to two minutes to choose a side, and any individual that did not choose a side within the two minutes was not included in the analysis. The aquarium was flushed with flowing seawater after each trial, and each fish was tested only once. The combination of choices presented and the number of fish tested for each combination were: a *D. setosum* urchin from a different patch of reef at least 20 m away from the collected fish (unfamiliar urchin) or no urchin (only seawater), n = 38; the *D. setosum* urchin collected with the fish (familiar urchin) or an unfamiliar *D. setosum* (unfamiliar urchin), n = 87; a group of ten *S. tubifer* collected from an urchin > 20 m away (unfamiliar fish) or no fish (only seawater), n = 28; a group of ten *S. tubifer* collected from the same urchin (familiar fish) or an unfamiliar group of ten *S. tubifer* collected from a different urchin > 20 m away, n = 57; a familiar *D. setosum* or a familiar group of ten *S. tubifer*, n = 19; and an unfamiliar *D. setosum* or an unfamiliar *E. setosum* (both collected > 20 m away), n = 35. All groups of *S. tubifer* presented as a conspecific choice were kept in place in the aquarium with the same mesh structure used to partition the tank. On no occasion did any of these fish swim away from the group of fish or the mesh structure.

Tagging

Groups of *S. tubifer*, which varied in numbers and in standard lengths of individuals (Table 3.1), were collected from the reef with their associated host urchin, taken to the laboratory, and tagged. Individual fish were lightly anesthetized with 2-phenoxyethanol (Acros Organics) (0.2 mL per L of seawater) and measured to the

nearest 0.5 mm SL prior to tagging. The standard length of all tagged fish (n = 313) ranged from 12.5 to 38.5 mm, with a mean length of 26.8 ± 5.1 (SD) mm. Brooding male fish were not included in these experiments, as they do not leave an urchin while brooding (Dunlap and Nakamura 2011, this study). Fluorescent visual implant elastomer (VIE) tags (Northwest Fisheries Supplies, Inc.) of different colors were injected subcutaneously at varying body locations to uniquely identify each group of *S. tubifer* collected with an individual urchin. After tagging, fish were given a four-hour recovery period in aquaria with aerated flowing seawater and were then released back into the field as a group with a *D. setosum* urchin. No fatalities occurred during this four-hour period in the experimental groups. To test for mortality associated with tagging, an additional group of *S. tubifer* (n = 41) was tagged and maintained in an aquarium for one week and fed daily with wild-caught zooplankton. Of this group of fish, one individual did not survive handling, and another fish was found dead in the aquarium one day after tagging. The remaining individuals (> 95%) were seemingly healthy by the end of one week after tagging, and all tags were clearly visible, indicating that mortality due to handling and tagging is less than 5% and likely occurs during handling or by day one and that the tags remain in place and visible for this period of time.

Site fidelity

Analysis of site fidelity was carried out using groups of *S. tubifer* associated with individual *D. setosum*. Observations during this study indicated that divers could recognize individual urchins by their appearance and specific locations at reef sites, to which the urchins returned daily from short nocturnal foraging distances (generally < 5 m) (Magnus 1967, this study). Three groups of *S. tubifer* (Table 3.1) were collected with their urchins from reefs fronting Sesoko Station in June 2013 and uniquely marked with VIE tags. After a four-hour recovery period, each group of fish was released with their urchin at its site of origin at least two hours before sunset. The number of tagged fish from each group that were associated with their original urchin was determined on days one, two, three, and seven after release. In addition, the surrounding 10 m-radius area was surveyed for the presence of tagged fish on other urchins.

Homing behavior

To determine the homing ability of *S. tubifer*, three replicate groups of uniquely tagged *S. tubifer* (Table 3.1) were released with an unfamiliar *D. setosum* urchin (collected from a different reef) at sites one or two kilometers from their reef of origin (Figure 3.1) and monitored for their return over one week. The original urchin with which a group of fish was collected was returned back to its capture site at its reef of origin. Three control groups of fish were released with their urchin of origin at their capture site after tagging and recovery. Additional groups (three groups per displacement distance) were released one and two kilometers from their reef of origin; the two-kilometer release site was located northeast of the site of origin, and the one-kilometer site was located southwest of the site of origin (Figure 3.1). An additional one-kilometer release site northeast of the site of origin was also tested to determine if the direction of the release site relative to the capture site influenced the homing ability of the fish. The percentage of fish that returned from this experimental group after one week (19%) was within the range of those returning from the other one-kilometer site (19-24%). Original urchins and the surrounding 10 m-radius area at the reef of origin were monitored for the presence of tagged individuals on days one, two, three, and seven after displacement.

Statistical analysis

Each transect at the study site was treated independently for the analysis of the distribution of *S. tubifer* on *D. setosum* and *E. calamaris* as host urchins. Because the data were not normally distributed, a Wilcoxson rank-sum test was performed, with correction for continuity, to test the preference of *S. tubifer* to associate with *D. setosum* or *E. calamaris*. To test whether small (≤ 25 mm SL) and large (> 25 mm SL) fish associate more frequently with an urchin species, chi-square tests of independence were preformed on the number of fish of each size category in association with *D. setosum* or *E. calamaris*. In addition, Manly's alpha scores (Manly *et al.* 1972, Chesson 1978) were calculated for all fish surveyed and converted into electivity indices (Chesson 1983, Shima 2001) to analyze the use of each urchin species as a host relative

to their abundance on both reef and sand substrate at the study site. To analyze the choice experiments, chi-square tests of independence were performed on the number of fish that chose either stimulus for each pair of choices presented.

To analyze site fidelity data, a repeated-measures ANOVA followed by pairwise t-tests between days was used to test for the effect of time on the proportion of individuals that returned to the same urchin daily. Homing data were analyzed using a generalized linear mixed model with a binomial distribution and a logit link function, with time in days, distance, and mean body length (mm, SL) of each group of fish (Table 3.1) as fixed effects and each replicate group as a random effect. The final model was chosen by stepwise selection based on lowest Akaike information criterion (AIC) scores. Individual body size was measured only during the initial tagging process, therefore the correlation between homing success and fish body size was examined using metrics of size describing an entire group of tagged fish, such as the proportion of small individuals (< 25 mm) and mean body length. All statistical analyses were performed in R, version 2.15.1 (R Development Core Team 2012).

Results

Diel behavior

Field observations of *Siphamia tubifer* associated with *Diadema setosum* revealed that the fish alternates between a non-feeding, protective association with an urchin during the day and foraging for zooplankton away from the urchin at night (Table 3.2); ambient light levels at dusk and dawn apparently cue this behavior. As dusk approached after sunset, the fish changed from a uniform nearly black, dark-brown color to a pattern of silver with three lengthwise dark stripes. At this time, the fish moved away from the urchin test toward the outer ends of the spines. The fish hovered at this position for several minutes, facing outward from the urchin. They then turned entirely silver in color and individually darted away from the urchin; approximately ten fish would leave the urchin within a few seconds of each other. All fish except brooding males had left the urchin, presumably to forage, within a few minutes (Table 3.2). Brooding males, identified by their swollen, distended jaws,

remained dark-brown in color among the urchin spines throughout the night. As dawn approached, the foraging fish returned to the urchin, arriving singly or in pairs, and were silver in color. All fish arrived within several minutes of each other and had assembled among the urchin's spines by approximately half an hour before sunrise. In one instance, a returning fish was chased by a larger, presumably predatory, fish (unknown species); the chased fish darted into a crevice of a *Porites* coral close to an urchin, remained still in this crevice for several minutes, and then darted among the spines of a nearby *D. setosum* urchin. Examination of the stomachs of the fish collected from an urchin immediately after their return at dawn revealed the stomachs to be full and to contain mostly benthic zooplankton. In contrast, the stomachs of brooding males were empty.

Host preference

The natural and apparently exclusive daytime hosts of *S. tubifer* are *D. setosum* and *Echinothrix calamaris* in the Motobu Peninsula area. During the day, I found *S. tubifer* primarily in association with the longspine urchin, *D. setosum*, but also frequently with the banded urchin *E. calamaris*, which has shorter spines (Figure 3.2). Despite extensive observations, I did not find the fish during the day in association with any other urchin species, with corals, with the crown-of-thorns seastar *Acanthaster* (Stier *et al.* 2009), or in other areas of the reef. The transect site (Figure 3.1) contained more *E. calamaris* than *D. setosum*, and both urchin species occurred on the backside of the reef as well as on adjacent sand flats; however, 85% of all urchins surveyed were located on the sand flat (Figure 3.3). Of the *D. setosum* surveyed, 65% were found on the reef, whereas only 3% of *E. calamaris* were on reef substrate (Figure 3.3). The distribution of *S. tubifer* at this site was therefore influenced by the distribution of host urchins.

Of all urchins surveyed, 41% had *S. tubifer* associated with them, but fish were found more frequently in association with *D. setosum*; 56% of all fish surveyed were associated with *D. setosum* despite its low relative abundance at the study site (Figure 3.3, Wilcoxson ranked-sum test, $T = 7911.5$, $P < 0.001$). When comparing host urchins occupied by small (< 25 mm SL) and large (> 25 mm SL) *S. tubifer*, more small fish were associated with *E. calamaris* than large fish ($X^2 = 78.7$, $df = 1$, $P < 0.0001$); 82% of fish

associated with *E. calamaris* were small (Figure 3.3). Conversely, there was little difference in the numbers of small and large fish associated with *D. setosum* ($X^2 = 0.30$, df = 1, $P < 0.58$); 53% and 47% of the fish surveyed with *D. setosum* were small and large, respectively (Figure 3.3). An electivity score (ε) of 0.68 for all fish surveyed over both substrates indicate that *S. tubifer* selectively associate with *D. setosum*, although this preference is stronger for large fish (ε = 0.37) than for small fish (ε = 0.15) (Table 3.3). In contrast, all electivity scores calculated for fish associated with *E. calamaris* were negative, which indicates a lack of preference for *E. calamaris* as a host urchin. On the reef, all *S. tubifer* surveyed appeared to avoid *E. calamaris* as a host; no fish were seen in association with *E. calamaris* on the reef and consequently the electivity scores were -1.00 for all fish, regardless of size (Table 3.3).

The results of choice experiments in the aquarium confirmed the observed preference of *S. tubifer* for *D. setosum*. Compared to an empty area with no urchin, *S. tubifer* associated more frequently with *D. setosum* ($X^2 = 13.88$, $P < 0.0001$) as well as with conspecifics ($X^2 = 4.95$, $P < 0.05$) (Figure 3.4). The fish also exhibited a preference for *D. setosum* over *E. calamaris*; 71% of *S. tubifer* tested associated with *D. setosum* ($X^2 = 2.85$, $P = 0.09$). Although not statistically significant, the preference for *D. setosum* (Figure 3.4) is consistent with the higher numbers of *S. tubifer* associated with *D. setosum* compared to *E. calamaris* in the wild. With respect to choosing between familiar and unfamiliar urchins and conspecifics, *S. tubifer* showed no obvious preference; 49% and 54% of fish tested associated with familiar urchins ($X^2 = 0.03$, $P = 0.86$) and conspecifics ($X^2 = 0.15$, $P = 0.70$), respectively (Figure 3.4).

Site fidelity

Consistent with field observations, *S. tubifer* exhibits daily fidelity to an individual urchin at a site. Tagged fish were re-sighted on their original urchin seven days after tagging, with an average of 55%, 51%, 46%, and 33% of tagged individuals re-sighted on the same urchin on days one, two, three, and seven, respectively (Figure 3.5). Time after release had a significant effect on proportion of fish found with the same urchin ($P < 0.01$); a lower proportion of fish were re-sighted at the same urchin after one week than on days one and two ($P < 0.05$). In some instances, up to 5% of tagged fish were sighted with other *D. setosum* within five meters from their original urchin.

Homing behavior

In addition to host urchin preference and site fidelity, *S. tubifer* is able to return to its home reef site from substantial distances, regardless of the direction of displacement. When fish were displaced one kilometer (southwest of their capture site) and two kilometers (northeast of capture site) (Figure 3.1), an average of 35% and 29%, respectively, were re-sighted on an urchin within a ten m radius of their original urchin at their capture site by day two, with up to 24% of individuals returning to their original urchin. On day seven, an average of 34% and 24% of fish from the one- and two-kilometer groups, respectively, were re-sighted within a ten m radius of their original urchin (Figure 3.6a). Averages of control-group fish, tagged and released at their capture site with their original urchin, re-sighted on days one, two, three, and seven after release, were 48%, 35%, 42%, and 17%, respectively (Figure 3.6a). Thus, displacement distance had a significant effect on the proportion of fish that returned to their site of origin ($P < 0.01$). There was also a strong effect of mean group standard length (Table 3.2) on homing ($P < 0.0001$); a smaller proportion of fish homed from groups with a lower mean standard length than from groups with a higher mean standard length, irrespective of release distance (Figure 2.6b). The proportion of small fish in a group, however, did not have a significant effect on the proportion of fish that homed ($P = 0.38$) and its relationship with homing was weaker ($R^2 = 0.17$, $F = 6.85$, $P = 0.01$) than that of mean body length ($R^2 = 0.45$, $F = 29.9$, $P < 0.001$) (Figure 3.6b).

Discussion

Together with the ability to emit ventral luminescence, the behaviors and preferences described here for *S. tubifer* appear to function to minimize predation. The daytime association with an urchin allows the fish, which typically is dark in coloration at that time, to be cryptic. Consistent with our field observations, Tamura (1982) observed *S. tubifer* at dusk and documented the fish's body color change, from dark brownish black to silver striped to all silver, as the fish left an urchin. The fish remained silver all night, which presumably helps *S. tubifer* avoid detection while

foraging. Ventral luminescence, which begins to be emitted at dusk (Dunlap and Nakamura 2011), might complement the silver coloration, helping the fish remain cryptic while foraging. Nonetheless, predation rates on *S. tubifer* are probably high; direct predation by lionfish has been observed (Michael 2013), and during this study, predatory fish, including larger apogonid species, were often sighted near urchins occupied by *S. tubifer* and observed preying on fish leaving and returning to an urchin.

The preference of *S. tubifer* for *Diadema setosum* as its daytime host over *Echinothrix calamaris*, a shorter-spined urchin (Figure 3.2), is consistent with the observation that the fish prefer urchins with longer spines (Tamura 1982). Longer spines presumably provide better protection from predators, especially for larger *S. tubifer*. When both *E. calamaris* and *D. setosum* are present at a reef, small fish may be able to find adequate protection from predators among the shorter spines of *E. calamaris*. It is also possible that learning occurs with age; larger fish might have learned that the longer spines of *D. setosum* provide better protection than those of *E. calamaris*. Intraspecific interference competition (e.g. Holbrook and Schmitt 2002) could also influence the distribution of small and large *S. tubifer* associated with both urchin species; larger fish may outcompete smaller fish for space among the more protective *D. setosum* spines, and consequently displace smaller individuals to take residence among the shorter spines of *E. calamaris*. Additional studies, e.g. testing different size classes of the fish, "small" and "large", with *D. setosum* versus *E. calamaris* in choice experiments, would provide further insight on host characteristics important for the fish throughout development.

This study establishes that *S. tubifer* exhibits daily site fidelity and returns to a home site after being displaced one and two kilometers. Like host preference, site fidelity and homing by *S. tubifer* are likely to be shaped by the need to avoid predators. Knowledge of the local reef structure and the location of urchins and resident predators presumably enhances survival of fish departing from and returning to a home reef site and urchin. In this study, the percentage of tagged fish re-sighted at home reef sites for the control group of the homing study was similar to the proportion of fish re-sighted in the site fidelity experiment, which suggests that the lower numbers of fish returning over time to a home site and urchin reflect losses due to predation. The natural mortality rate of *S. tubifer* might also be relatively high, as the lifespans of other apogonids are short (< 1-2 years) (Chrystal *et al.* 1985, Marnane

2000, Kingsford *et al.* 2014). Another factor that could have influenced the proportion of fish recovered during the homing study was the study site itself; the collection (control) site was selected due to the high abundance of *S. tubifer*, which correlated with a high density of host urchins. Consequently, more tagged fish may have returned to the general area but were not re-sighted in the surveyed home site radius.

Consistent with our results, previous studies have shown that various apogonids can return to a home reef site when displaced substantial distances (Marnane 2000, Kolm *et al.* 2005). Marnane (2000) showed that between 33% and 63% of three apogonid species returned to their site of origin within three days when translocated two kilometers. Additional studies have shown that other apogonids, including members of *Siphamia*, exhibit site fidelity and remain at the same reef site for weeks to months (Strasburg 1966, Allen 1972, Kuwamura 1985, Okuda and Yanagisawa 1996, Marnane 2000), the consequences of which may directly affect nutrient distribution within a reef as well as the assembly of predator and prey species at that reef (Marnane 2000, Marnane and Bellwood 2002). The daily site fidelity and homing by *S. tubifer* might lead to a local enrichment of their luminous symbiont in the water at a home site because excess symbiont cells are released daily with the fish's feces (Dunlap and Nakamura 2011).

Previous studies have also shown that site fidelity and homing behavior of fishes can vary with ontogeny (Yoshiyama *et al.* 1992, Shima *et al.* 2012, White and Brown 2013); older fish are more likely to risk the return to a home site across unfamiliar waters, although this is not always the case for all fishes (White and Brown 2013). Our homing results appear to be consistent with this view, but additional studies are needed to empirically test whether larger *S. tubifer* are actually more successful at homing than smaller fish. The lower proportion of fish that homed from groups with smaller mean body size, however, may reflect a greater loss of smaller fish to predation. High predation risk could, therefore, play a critical role in shaping the highly cryptic life history of *S. tubifer* and provide incentive for the homing behaviour observed in this study; prior knowledge of the predator and urchin communities in an area could outweigh the risks of making the return trip home.

The mechanisms used by fishes to navigate daily to home sites and those used by recruitment-stage larvae to find suitable settlement sites may involve visual, olfactory, and auditory cues. From short distances, *S. tubifer* likely uses visual cues to recognize

and navigate within a familiar area to its daytime urchin host and probably has some spatial memory of a home site (e.g. White and Brown 2013), including the location of the host urchins in the area. However, to navigate back to a home site after displacement or to find a settlement site as a larva, *S. tubifer* presumably uses additional cues. Other cardinalfishes use olfaction to discriminate between familiar and unfamiliar reef waters, and settlement-stage apogonids might use chemical cues to recruit to their natal reefs (Atema *et al.* 2002, Døving *et al.* 2006, Geralch *et al.* 2007). Previous studies have also shown that apogonids are attracted to reef sounds, which could also serve as cues for larval fish to navigate to a settlement site (Leis *et al.* 2003, Simpson *et al.* 2004, 2005). Sound can propagate relatively long distances through water, regardless of the direction of current flow (Rogers and Cox 1988), and urchins produce distinct sounds at frequencies detectable by fish against the background noises of coral reef communities (Radford *et al.* 2008, 2010). Therefore, *S. tubifer* could use a combination of olfactory and auditory cues for homing, which could also convey habitat quality to incoming *S. tubifer* recruits searching for a suitable settlement site.

The homing and site fidelity behavior of *S. tubifer* described here, together with other studies that suggest settling fishes might use environmental cues to navigate to natal reefs (Atema *et al.* 2002, Leis *et al.* 2003, Simpson *et al.* 2004, 2005, Geralch *et al.* 2007), lead us to speculate that *S. tubifer* larvae use similar environmental cues to recognize and recruit to reefs inhabited by adult conspecifics. If so, the larvae might encounter higher numbers of symbiotic bacteria near the reef compared to in the plankton, due to the daily release of the bacteria by adults at their daytime home sites (Dunlap and Nakamura 2011). Depending on the developmental timing of recruitment, light organ development, and the timing of symbiont acquisition by *S. tubifer* larvae (Leis and Bullock 1986, Dunlap *et al.* 2012), this interaction might function to ensure the successful initiation of the symbiosis, by establishing a quasi-vertical, adult to larvae, form of symbiont transfer. However, the environmental cues used by *S. tubifer* larvae for settlement and the relationship between settlement and initiation of the symbiosis remain to be determined.

Table 3.1 Number and standard lengths of individuals from groups of *Siphamia tubifer* tagged for the homing and site fidelity studies

Study (treatment)	N	Mean fish length (± SD, mm)	Minimum fish length (mm)	Maximum fish length (mm)	Proportion of fish ≤ 25 mm (SL)
Homing (0 km)	11	31.5 (± 4.9)	21.0	37.5	0.09
	13	27.1 (± 3.4)	19.0	34.0	0.15
	22	29.2 (± 4.1)	22.0	36.0	0.14
Homing (1 km)	18	28.6 (± 3.6)	21.5	37.0	0.05
	23	27.9 (± 4.7)	19.5	37.0	0.22
	22	21.8 (± 5.8)	13.0	30.0	0.55
Homing (2 km)	34	28.0 (± 4.1)	21.0	38.0	0.56
	22	22.3 (± 7.6)	12.5	36.5	0.24
	25	23.7 (± 5.6)	23.5	37.0	0.59
Site fidelity	58	27.8 (± 3.6)	20.0	37.0	0.25
	30	25.7 (± 4.5)	15.5	38.5	0.33
	35	28.6 (± 3.1)	21.0	35.5	0.09

Table 3.2 Timetable of observations of *Siphamia tubifer* leaving the protection of the spines of a *D. setosum* urchin to forage at dusk, and returning to the same urchin at dawn on a reef fronting Sesoko Station, Okinawa, Japan. All observations from sunset through midnight were made on July 1, 2012. Observations in the morning hours through sunrise were made on July 4, 2012.

Time	Observation
19:26	Sunset
19:50	Color change from black to striped
19:59	Fish moved to the end of the urchin's spines and hovered, changing to silver in color
20:01	First group of approximately 10 fish left the urchin
20:03	Another group of approximately 10 fish left the urchin
20:07	Last two fish left the urchin (two brooders remained with the urchin)
00:00	Two brooders still remained with the urchin (no other fish with the urchin)
04:45	Only the two brooders with the urchin
05:00	One fish returned to the urchin
05:04	Three fish returned to the urchin
05:07	One fish returned to the urchin
05:11	One fish returned to the urchin
05:12	Two fish returned to the urchin
05:13	One fish returned to the urchin
05:14	The last fish returned to the urchin, pausing first near a coral head to avoid a predator fish
05:42	Sunrise

Table 3.3 Electivity indicies (e) for *Siphamia tubifer* calculated from Manly's alpha indicies based on the numbers of fish observed on either host urchin species on each substrate (reef or sand) relative to the abundance of each urchin on that substrate. Small fish are < 25 mm SL and large fish are > 25 mm SL. A positive index score indicates more freuqent habitat use and a negative score idicates a lack of preference for that habtiat

	Reef		Sand		Both substrates	
	D. setosum	*E. calamaris*	*D. setosum*	*E. calamaris*	*D. setosum*	*E. calamaris*
Small fish	1.00	-1.00	0.48	-0.48	0.15	-0.15
Large fish	1.00	-1.00	0.87	-0.87	0.37	-0.37
All fish	1.00	-1.00	0.83	-0.83	0.68	-0.68

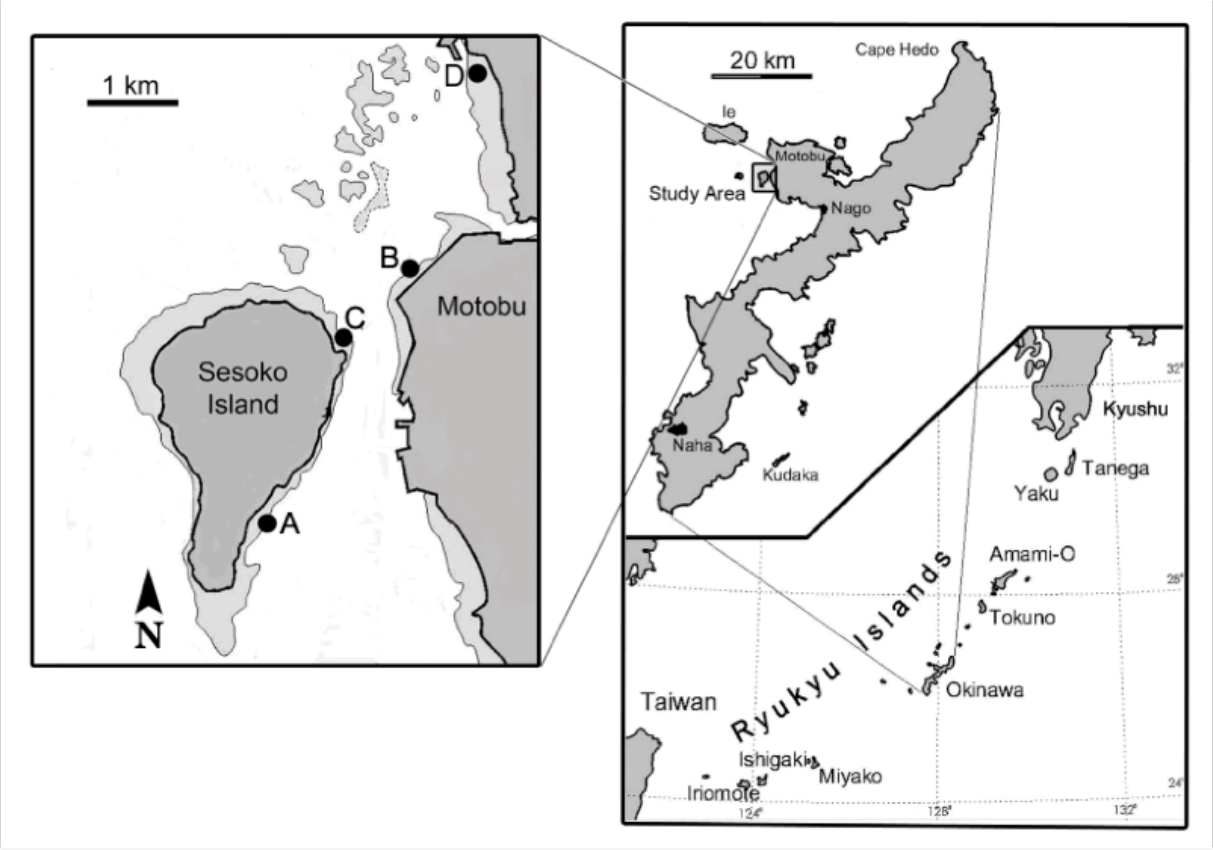

Figure 3.1 Map of the study area in Okinawa, Japan. Study sites are indicated with black circles and labeled as follows: **A**) Sesoko Station (site fidelity study); **B**) study site near Motobu, the point of origin for the homing study and site of all field transects; **C**) 1 km release site for the homing study; and **D)** 2 km release site for the homing study. Light gray shaded areas (left) indicate areas <10 m in depth. Map modified from Hohenegger *et al.* (1999)

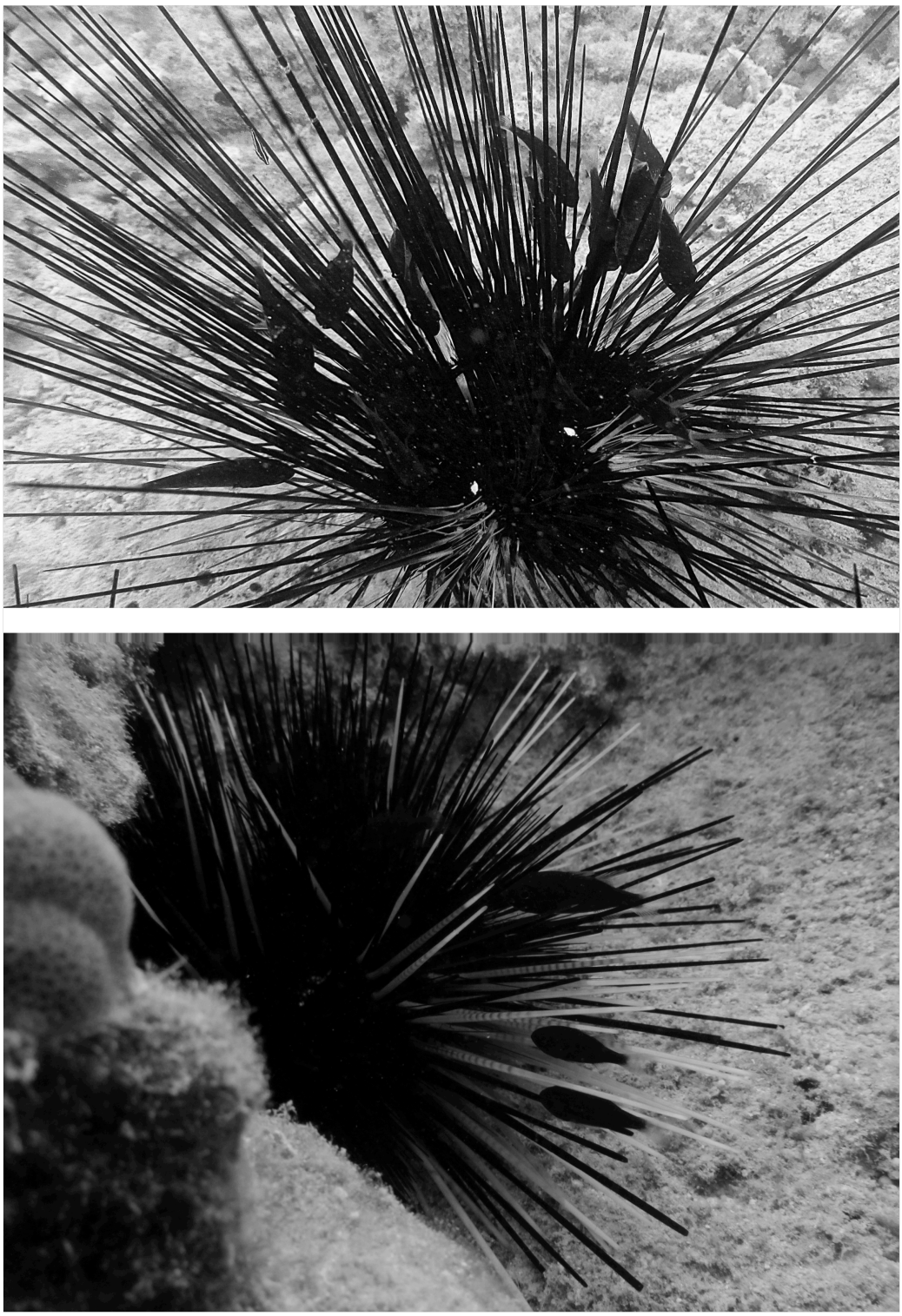

Figure 3.2 Groups of *Siphamia tubifer* associated with a longspine sea urchin, *Diadema setosum,* (top) and a banded sea urchin *Echinothrix calamaris* (bottom) on the reef fronting Sesoko Station in Okinawa, Japan

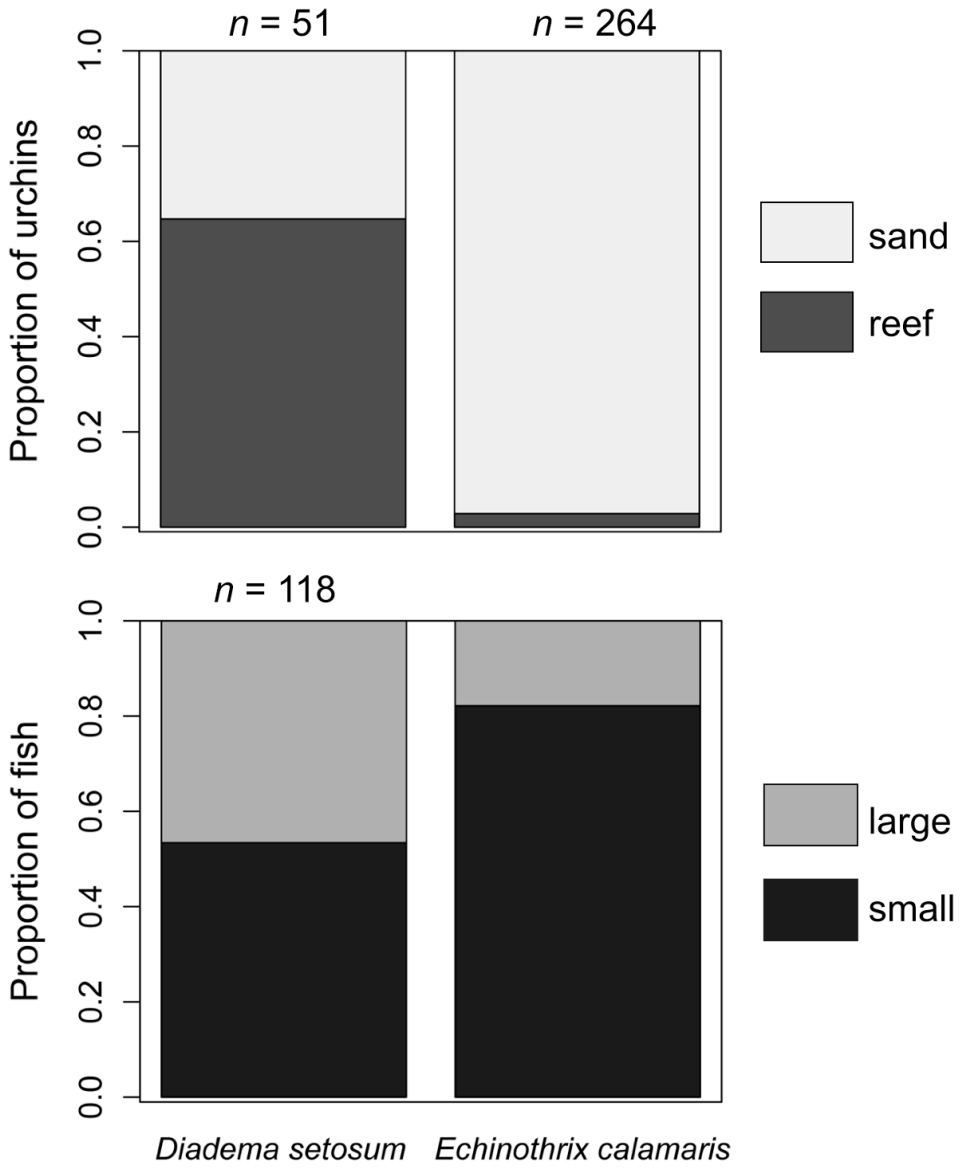

Figure 3.3 Habitat preference of host urchins and of *Siphamia tubifer***.** The proportion of host urchin species surveyed along transects that were associated with sand or reef as substrate (top). The proportion of large (> 25 mm SL) and small (< 25 mm SL) *Siphamia tubifer* surveyed that were associated with each host urchin species (bottom). Total numbers of individuals surveyed are indicated at the top of each bar

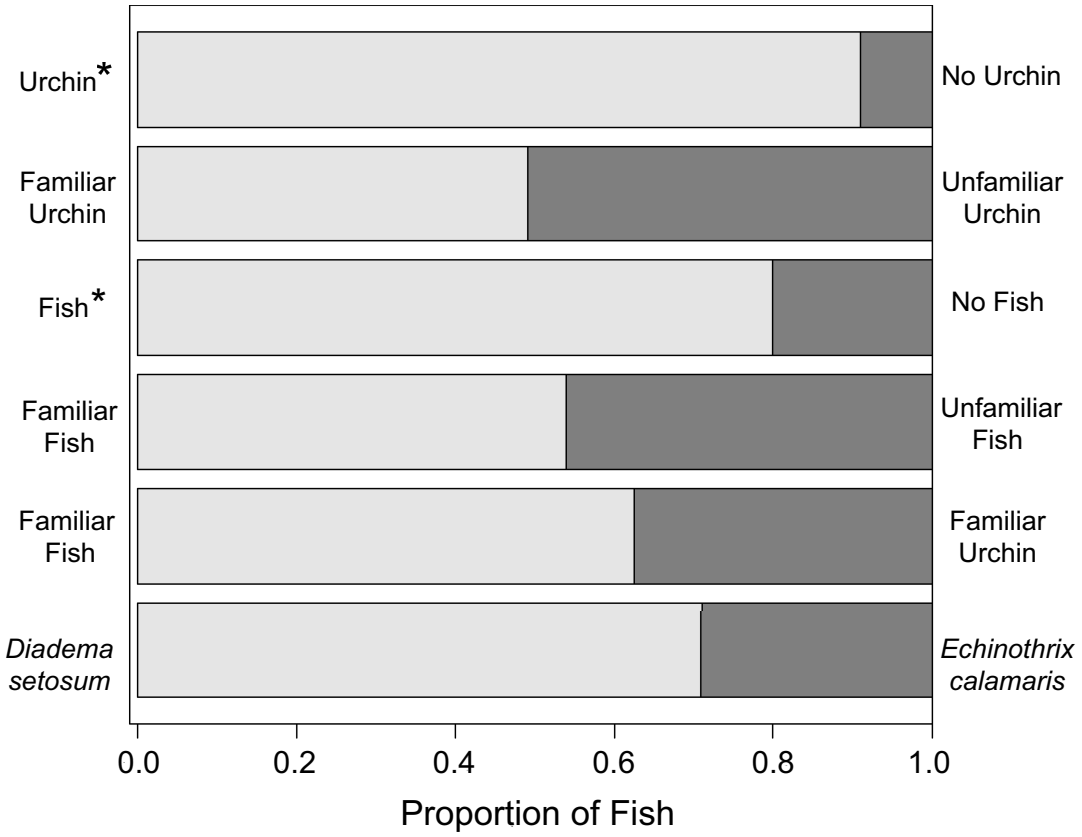

Figure 3.4 Choices made by *Siphamia tubifer* when provided stimuli on opposing sides of an aquarium. Numbers of fish tested that made a choice (and the number that did not make a choice) for each experiment, from top to bottom were: 34(4), 80(7), 25(3), 52(5), 16(3), 31(4). All urchins were *Diadema setosum* with the exception of the choice between host urchin species (bottom). Significant differences in choices made by fish ($P < 0.05$) are indicated by *

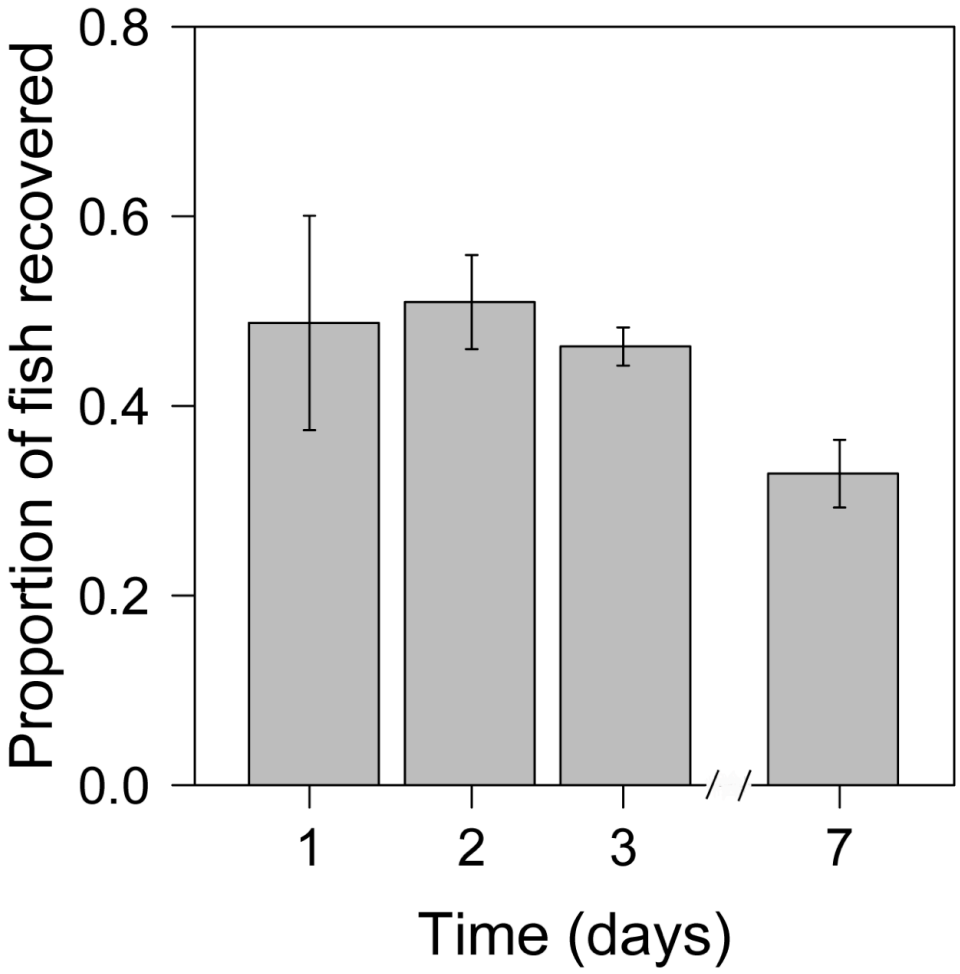

Figure 3.5 Site fidelity of *Siphamia tubifer.* The mean proportion of tagged fish per group that were observed with their original urchin on days one, two, and three, and seven. Error bars indicate standard error

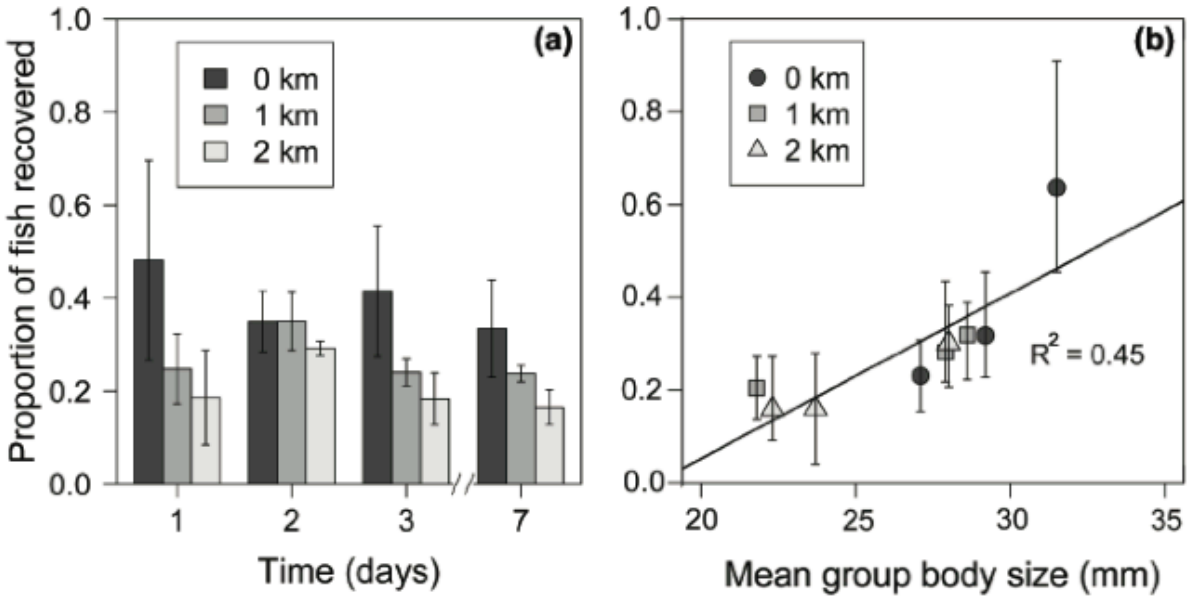

Figure 3.6 Homing behavior of *Siphamia tubifer.* Results from homing experiments in which *Siphamia tubifer* were tagged and released distances of 0 (control), 1, or 2 km. **(a)** The mean proportion of tagged fish per group that were observed at their site of origin over seven days. Error bars indicate standard error. **(b)** The mean proportion of fish from a group that were recovered at their site of origin by mean body size (standard length) of the group ($F = 29.9$, $P < 0.001$). Bars indicate the range of the mean proportion of fish recovered across all time points sampled for each group

CHAPTER IV
Olfactory preferences of *Siphamia tubifer* [3]

Abstract

The symbiotically luminous, reef-dwelling cardinalfish, *Siphamia tubifer* (Perciformes: Apogonidae), exhibits daily site fidelity, homing behavior, and a preference for the long-spined urchin, *Diadema setosum,* as its daytime host. The fish acquires its symbiont during larval development and releases large numbers of the bacteria with its feces daily at a host urchin. To examine the role of olfaction in site fidelity and homing by *S. tubifer*, juvenile and adult fish were tested in a two-channel choice flume for their olfactory preferences. Neither juveniles nor adults showed a preference for seawater conditioned by *D. setosum*. Juvenile fish, but not adults, preferred seawater conditioned by conspecific fish versus unconditioned seawater. Both juveniles and adults preferred seawater conditioned by their luminous symbiont and also preferred home site water to foreign reef water. These results suggest that *S. tubifer* uses chemical cues for homing and possibly settlement and symbiont acquisition, but not for host urchin recognition.

Introduction

Cardinalfishes (Apogonidae) display strong site fidelity and homing ability through navigation using a combination of auditory, visual, and chemical cues (Marnane 2000, Kolm *et al.* 2005, Døving *et al.* 2006, Fukumori *et al.* 2010, Gardiner and Jones 2010, Gould *et al.* 2014). Olfaction, in particular, plays an important role for

[3] Published as: Gould, AL, S Harii, and PV Dunlap (2015) Cues from the reef: olfactory preferences of a symbiotically luminous cardinalfish. *Coral Reefs 34(2):673-677*

both larval and adult fish returning to reefs, with various chemical cues known to indicate habitat quality (Atema *et al.* 2002, Dixson *et al.* 2010, 2011, 2014, Døving *et al.* 2006, Gerlach *et al.* 2007, Coppock *et al.* 2013). Adult apogonids have strong olfactory abilities, distinguishing between the odors of conspecifics from a home reef and those from a foreign reef (Døving *et al.* 2006), and larval apogonids use olfactory cues for settlement, sometimes to a reef of origin (Atema *et al.* 2002, Gerlach *et al.* 2007). Analysis of the use of olfactory cues can therefore provide insight into how fish recognize and navigate to their daytime resting sites, identify suitable settlement habitat, and return to natal reefs.

The sea urchin cardinalfish, *Siphamia tubifer*, inhabits coral reefs in the Indo-Pacific and aggregates in groups by day among the spines of sea urchins, particularly the long-spined sea urchin, *Diadema setosum*. Early in development, *S. tubifer* initiates a symbiosis with the luminous bacterium, *Photobacterium mandapamensis*, which it acquires from the environment (Dunlap *et al.* 2012). The bacteria establish a large light-producing population in an abdominal light organ that connects to the intestine; the fish uses the bacterial light to illuminate its ventrum while foraging at night (Dunlap and Nakamura 2011, Gould *et al.* 2014). Along with strong homing ability, returning to a reef of origin when displaced two kilometers, adult *S. tubifer* exhibit daily site fidelity, returning to a specific urchin each dawn after foraging (Gould *et al.* 2014). The fish's diel behavior is thought to enrich the concentration of the symbiotic bacteria near a host urchin; *S. tubifer* release feces rich in the symbiotic bacteria after foraging (Dunlap and Nakamura 2011).

To examine the possible use of olfactory cues by adult *S. tubifer* for homing after displacement and in returning daily to a home site, I used a two-channel choice flume to test the preferences of adult fish for water conditioned by the presence of the host sea urchin, conspecific fish, and their luminous symbiont, and for seawater from their home site. I also investigated the potential use of these chemical cues by recent recruits for settlement by testing the olfactory preferences of small juvenile *S. tubifer* and determined whether their olfactory preferences differ from those of adults.

Materials and Methods

Study site

This study was carried out at Sesoko Station (Tropical Biosphere Research Center, University of the Ryukyus) on Sesoko Island, Okinawa, Japan (26º38'N, 127º52'E). Test fish were collected from coral reefs nearby Sesoko Station and from two additional sites: a small harbor near Itoman in Kyan town at the southern tip of Okinawa main island (26º05'N, 127º39'E) and a reef at Ikei Island on the east side of Okinawa (26º23'N, 127º59'E) (Figure 4.1). The protocols used for the capture, care, and handling of *S. tubifer* were approved by the University of Michigan's Institutional Animal Care and Use Committee, and they accord with animal handling guidelines of the University of the Ryukyus' Guide for Care and Use of Laboratory Animals.

Olfactory choice trials

A two-channel choice flume was designed after the Atema flume (Atema *et al.* 2002, Gerlach *et al.* 2007, Dixson *et al.* 2011, 2014, Munday *et al.* 2013), with the dimensions scaled up (40 x 8 x 6 cm^3) to test both adult (≥23 mm standard length, SL) and juvenile (<20 mm SL) *S. tubifer* (Figure 4.2). The discrete size categories were defined by the size at which *S. tubifer* are reproductively mature (22 mm SL or greater), based on the presence of developed gonads. Many of the juveniles tested were 12 to 15 mm SL and had presumably recently settled on the reef. A 12 cm divider separated the two channels, and the remaining test area, partitioned by 2 mm plastic screens, measured 8 x 8 cm^2. The flume was constructed with gray, translucent acrylic to increase opacity and had a removable piece of acrylic that was placed over the test area to increase shade and minimize stress on the test fish. The fish were easily monitored through the translucent lid and from behind the test area where the lid was not covering. Water flow was collimated at the entry by a series of 1 mm mesh screens, and the gravity-driven flow rate was set at 100 ml min^{-1} for juveniles (water height: 3 cm) and at 150 ml min^{-1} for adults (water height: 4 cm). Following a three-minute acclimation period, individual fish were tested for two minutes, during which the fish's position (side A or B; Figure 4.2) was recorded every five seconds. Fish that appeared stressed, as noted by quick and erratic movements, were not tested, and fish that did

not actively swim in both water sources during both acclimation periods were excluded from the study. To eliminate possible side-bias in the flume, the two water sources were switched from one side to the other by momentarily stopping the flow, switching the hoses connected to the water sources, and turning on the valves to allowing the new flow to re-establish during a one-minute rest period; no observations of the fish were recorded during this time. The process of switching water sources took no longer than ten seconds and did not appear to affect the test fish. The three-minute acclimation and two-minute test periods were then repeated. I carried out periodic dye tests and carefully monitored the laminar flow to ensure that the two water sources remained parallel and separate throughout the test area (Figure 4.3).

Pairwise choice experiments were carried out on individual *S. tubifer* to test the fish's preference for seawater conditioned by *D. setosum* urchins, conspecifics, and the symbiotic luminous bacteria versus unconditioned seawater, and for the preference for home site water versus foreign reef water. All seawater used for conditioned and unconditioned water treatments, with the exception of home site water, was pumped from approximately 100 m offshore at the reef fronting Sesoko Station. To condition the treatment water, unoccupied sea urchins collected near Sesoko (two in 20 L) and conspecifics (15 to 20 fish from the same site as the test fish, without an urchin, in 20 L) were held, respectively, in seawater for two to four hours; the seawater was then filtered through 150-μm mesh before use in the flume to remove any particulates that accumulated during the holding period. To determine if *S. tubifer* prefers seawater conditioned by the symbiont, treatment water was prepared by incubating a dense culture of the bacteria, isolated from the light organ of *S. tubifer* and grown on agar plates of a seawater-based medium (Dunlap and Nakamura 2011), in 1 L volumes of seawater for eight hours. Each 1 L of treatment water was then filtered through several layers of cheesecloth to remove most of the bacteria, and the filtrate was added to 20 L of seawater to be used in the flume. To test the preference for home site water versus water from the reef fronting Sesoko Station, *S. tubifer* and seawater were collected from two distant sites, Ikei and Itoman (Figure 4.1). All fish were held overnight in captivity in their home site water with the host urchin from their collection site for at least twelve hours prior to testing. After testing, each fish was measured (standard length, mm) and released back at the collection site with its urchin or preserved for future analyses.

Analysis

Choice experiments were conducted on a minimum of 15 individuals, with the exception of fish from Ikei where only four adults were collected, and each fish was only tested once. The difference in the percent of time spent in each water source, as determined by the percent of five second observations in which an individual was in the conditioned or unconditioned seawater was calculated and two-tailed Wilcoxon signed rank tests were performed to determine whether the differences were significantly different from zero for each experimental treatment and size group. Zero difference (a random distribution) in time spent between water masses was expected if an individual did not prefer either water source. The few individuals (6% of all fish tested) that did not switch sides during the test periods, and therefore showed a zero preference for both water source, were included in the analysis; the inclusion of these fish had no significant effect on the results. Wilcoxson rank sum tests were used to determine whether there was a difference in the response of juvenile versus adult fish for each odor stimulus. All statistical analyses were carried out in R, version 2.15.1 (R Development Core Team 2012).

Results and Discussion

Although *S. tubifer* return daily after foraging to urchins at home reef sites (Gould *et al.* 2014), the fish showed no preference for seawater conditioned by *D. setosum* urchins. Both juveniles and adults spent approximately equal time in urchin-conditioned and unconditioned seawater ($p=0.41$ and $p=0.18$) (Figure 4.4). Therefore, urchin-specific odors are apparently not used by the fish to navigate to a home reef site or urchin. Spatial memory and other cues not tested here, such as visual and auditory cues, are presumably involved in urchin recognition. For example, apogonids are attracted to reef sounds (Leis *et al.* 2003, Simpson *et al.* 2004, 2005), and urchins produce distinguishable sounds above background reef noises (Radford *et al.* 2008, 2010).

Juvenile *S. tubifer*, but not adults, showed a preference for seawater conditioned by conspecific fish ($p<0.01$). Juveniles spent approximately 38% more time in

conspecific-conditioned seawater than in unconditioned seawater ($p<0.001$), whereas adults spent an average of 53% of their total time in the treatment water ($p=0.07$) (Figure 4.4). The preference exhibited by juveniles might relate to age- and size-specific use of conspecific odors for settlement or to minimize the predation risk of smaller individuals by fish, such as other apogonid species and lionfish (Gould *et al.* 2014). Døving *et al.* (2006) showed similar results for the five-lined cardinalfish, *Cheilodipterus quinquelineatus*; adults exhibited no preference for conspecific odors over background reef water. However, *C. quinquelineatus* adults did prefer conspecific fish from their home reef to those from a foreign reef, indicating that adult fish do recognize conspecific odors (Døving *et al.* 2006). It is therefore possible that adult *S. tubifer* recognize but do not exhibit a preference for conspecific odors over background reef water.

Siphamia tubifer also preferred seawater conditioned by the presence of their luminous symbionts to unconditioned seawater. Juveniles and adults spent an average of 34% and 26% more time, respectively, in seawater conditioned with their symbiont than in unconditioned seawater ($p<0.01$ and $p<0.05$) (Figure 4.4). On the reef, the symbiont odor is presumably released daily at a home urchin with the fish's feces, which is rich in luminous bacteria (Dunlap and Nakamura 2011, Gould *et al.* 2014). Other fishes respond to specific chemical cues in feces, such as conspecific alarm cues in predators' diets (Brown 2003). Symbiont odor, a possible sign of the presence of adults and juveniles, could serve as a cue for daily site fidelity and homing after displacement and possibly as an indication of good habitat quality for incoming recruits.

Responses of juveniles and adults to home site water varied between the two locations. Adult fish collected from a small harbor near Itoman (Figure 4.1) preferred home site water ($p<0.05$), whereas juveniles spent nearly equal time in home and foreign site water ($p=0.38$) (Figure 4.4); however, the difference in preference between juveniles and adults from this location was not significant ($p=0.61$). Conversely, juveniles from the reef at Ikei (Figure 4.1) preferred their home reef water to foreign reef water ($p<0.01$); too few adult fish were examined from this location to test for a significant preference. The difference in response between juveniles from the two locations might reflect a difference in the conditions between sites; water in the protected harbor at Itoman had low visibility and little current flow, whereas water at

the natural reef at Ikei was clearer and exposed to ocean currents. Similar to the percent of time that *S. tubifer* spent in their home site water, Devine *et al.* (2012) showed that adult *Cheilodipterus quinquelineatus* spent 67% of their time in a home versus foreign site conspecific odor source. Furthermore, larval apogonids appear to prefer lagoon water to open ocean water (Atema *et al.* 2002) and exhibit preferences (7.0-17.1%) for their home site water to foreign reef water, corresponding to genetic differentiation between populations a few kilometers apart (Gerlach *et al.* 2007). In our study, juveniles from the reef home site seemingly preferred their home water to unfamiliar reef water, therefore *S. tubifer* larvae might also use olfaction to recognize and remain near their natal reef.

Our results contribute to the growing body of evidence demonstrating the importance of olfaction in marine and freshwater fishes, particularly as a mechanism used by apogonids and other reef fishes for navigation (Atema *et al.* 2002, Døving *et al.* 2006, Gerlach *et al.* 2007, Dixson *et al.* 2008, 2011, 2014, Gerlach and Atema 2012). It should be noted, however, that studies using experimental choice flumes are carried out with the assumption that the behaviors observed in such artificial conditions are reflective of what occurs in nature. In addition, studies here and elsewhere have certain limitations such as small sample sizes, limited amounts of treatment water and short acclimation times. Nonetheless, this and other studies that use choice flumes demonstrate that fish exhibit distinct responses to olfactory cues and may use certain chemical cues in the environment for navigation. Specifically, I demonstrate here the potential for *S. tubifer* to use olfaction to return daily to a resting site, to a home reef when displaced and possibly for recruitment, and the novel ability of this symbiotically luminous fish to recognize and orient to the odor of its luminous bacterium.

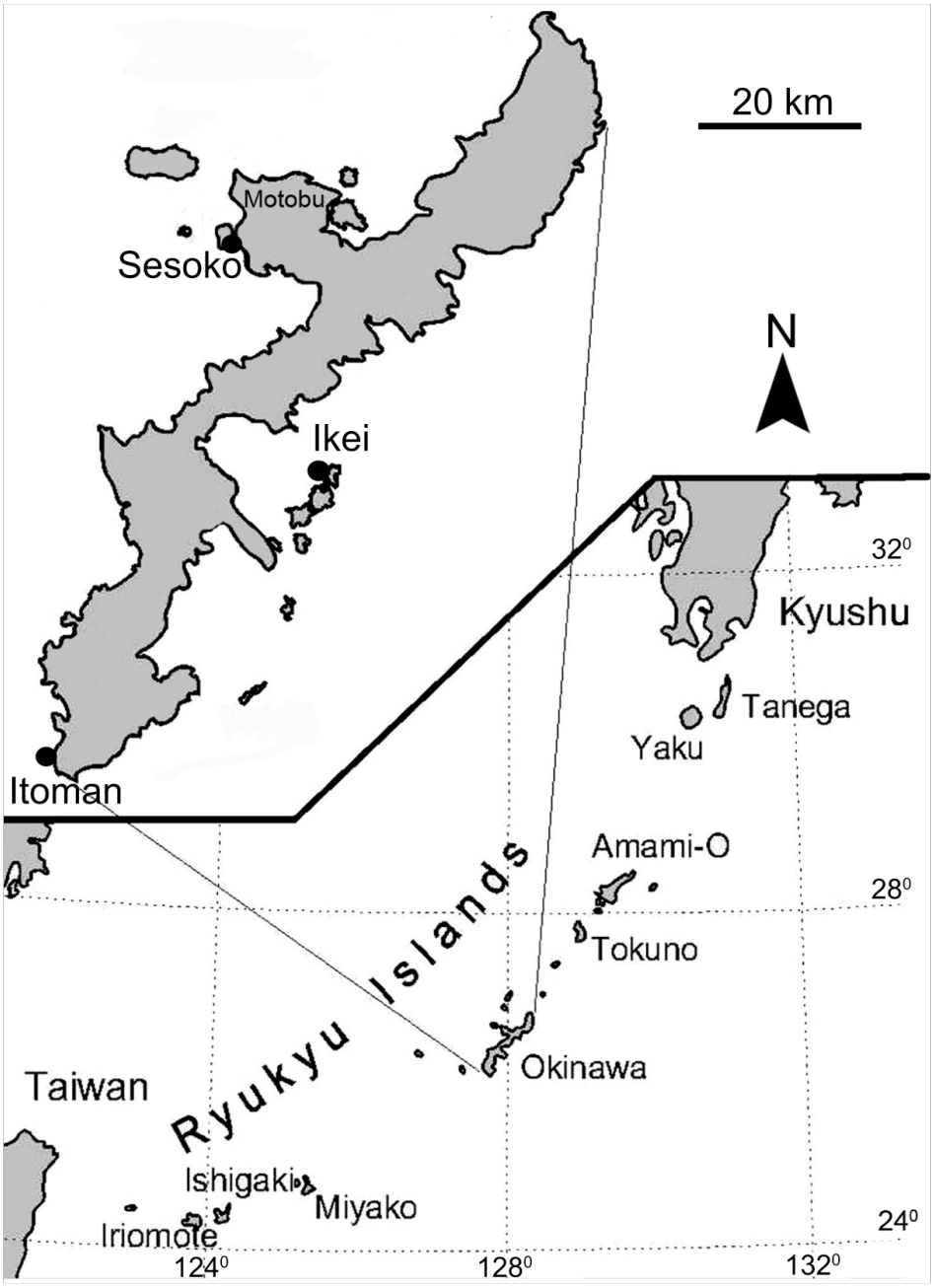

Figure 4.1 Map of the study area in Okinawa, Japan, with labels designating Sesoko Station ("Sesoko"), home site 1 - a harbor south of Itoman ("Itoman"), and home site 2 - a reef at Ikei Island ("Ikei")

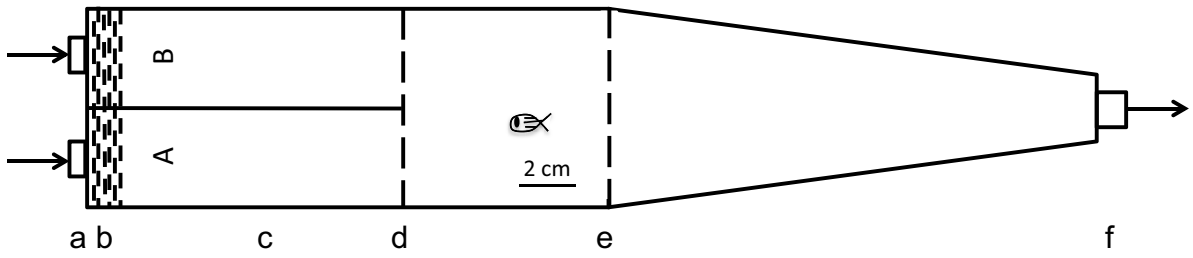

Figure 4.2 Schematic of the two-channel choice flume used to test olfactory preferences of *Siphamia tubifer*. Odor stimuli were presented on either side (A or B) of the two separated channels. a) inflow valves, b) collimator of plastic screens to evenly disperse water flow, c) channels separated by a barrier, d) upstream and e) downstream screens to contain test fish, and f) outflow valve

Figure 4.3 Photograph of a dye test highlighting the separation of the two water masses in the two-channel choice flume (left, blue dye; right, green dye)

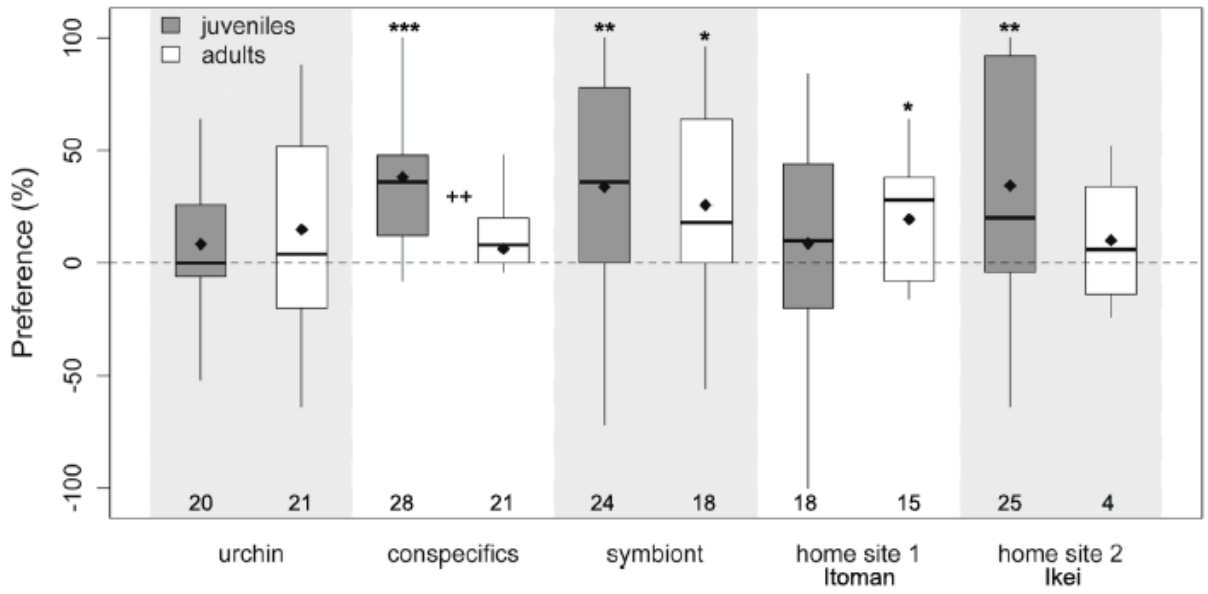

Figure 4.4 The olfactory preferences of juvenile and adult *Siphamia tubifer* for chemical cues in a two-channel flume. Preference is reported as the difference between the percent of total time fish spent in seawater conditioned by each olfactory cue and the percent of time spent in unconditioned seawater; positive values indicate preferences for conditioned water. Seawater from home sites 1 and 2 were tested on fish from those locations, respectively, against seawater from the reef fronting Sesoko Station. Box plots indicate the upper and lower quartiles (box), the median value (line), the mean value (diamond), and values within 1.5 times the interquartile range (whiskers); outlier points removed. Significant preferences are indicated by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), and a significant difference between preferences of juveniles and adults is indicated by ++ **(**$p<0.01$). Sample sizes are shown below the boxes

CHAPTER V
Population genomics of *Siphamia tubifer*[4]

Abstract

Discrepancies between potential and observed dispersal distances of reef fish indicate the need for a better understanding of the influence of larval behavior on self-recruitment and population connectivity patterns. Population genetic studies can reveal the degree to which populations are genetically connected, providing insight on larval recruitment and dispersal patterns relative to expectations based on behavior. The recent development of restriction site associated sequencing (RAD-Seq) methods has made genomic analyses of non-model organisms more accessible. In this study, I applied double digest RAD-Seq methods to test for population differentiation in the coral reef-dwelling cardinalfish, *Siphamia tubifer,* which based on behavioral studies of newly settled recruits, have the potential to use navigational cues to return to natal reefs. Analysis of 11,836 SNPs from fish collected at coral reefs in Okinawa, Japan from eleven locations over three years reveal little genetic differentiation between groups of *S. tubifer* at spatial scales from 2 to 140 kilometers and between years at one location: pairwise F_{ST} values ranged from 0.0116 to 0.0214. These results suggest *S. tubifer* exists as one panmictic population within the study region, probably due to larval dispersal influenced by the Kuroshio Current. Therefore, and in contrast to expectations based on studies of a similar cardinalfish, *S. tubifer* larvae do not self-recruit and instead retain high levels of population connectivity. These findings highlight the need for more studies of individual species within and between geographic regions to better understand the potential connections between larval behavior and population connectivity in reef fishes.

[4] *In review* in *Molecular Ecology* as: Gould, AL and PV Dunlap. Genomic analysis of a coral reef cardinalfish reveals high connectivity despite larval homing potential.

Introduction

The degree to which coral reef fish populations are connected has been debated for decades and has large consequences on their persistence and resilience to disturbances. For most reef fishes, dispersal occurs during a planktonic larval phase that lasts from a few days to months, creating the potential for large dispersal distances (Barlow 1981, Shulman and Bermingham 1995), but the challenges associated with tracking the direct movement of relatively small larvae in a dynamic, fluid environment has limited our knowledge of the connectivity patterns of most reef fishes (Jones *et al.* 2009). Recently, however, the rapid advancement of genetic tools has facilitated the study of reef fish populations, revealing the wide variation of spatial scales at which marine populations exhibit connectivity (e.g. Terry *et al.* 2000, Planes 2002, Jones *et al.* 2005, Taylor and Hellberg 2003, Purcell *et al.* 2006, Gerlach *et al.* 2007, Horne *et al.* 2008, van der Meer *et al.* 2012). Most coral reef fish metapopulations lie somewhere in the middle of the spectrum between having relatively closed (low connectivity and high self-recruitment) to open (high connectivity and no self-recruitment) populations (Cowen *et al.* 2000, Mora and Sale 2002, Jones *et al.* 2009) yet a surprising number of studies have provided evidence that reef fish populations are more closed than expected and exhibit a significant degree of local recruitment (Jones *et al.* 1999, 2005, Swearer *et al.* 1999, 2002, Cowen *et al.* 2000, 2006, Bode *et al.* 2006, Almany *et al.* 2007) or genetic differentiation at relatively small spatial scales (e.g. Planes 1993, Planes *et al.* 1998, Taylor and Hellberg 2003, Gerlach *et al.* 2007).

In response to the growing evidence of somewhat restricted gene flow among reef fish populations, the links between early life history traits, larval behavior, and patterns of larval recruitment and dispersal have been examined. Contrary to expectations, levels of genetic differentiation of marine fishes are generally not correlated with pelagic larval duration (PLD) or spawning mode (Shulman 1998, Bowen *et al.* 2006, Galarza *et al.* 2009, Jones *et al.* 2009), and the genetic structure of reef fish populations is often observed at smaller scales than predicted by larval dispersal alone (e.g. Taylor and Hellberg 2003, Rocha *et al.* 2005). These discrepancies can often be explained by larval fish behavior, through which larvae can employ some control over their dispersal trajectories (Kingsford *et al.* 2002, Leis and McCormick 2002, Sponaugle

et al. 2002, Leis *et al.* 2006, Montgomery *et al.* 2006), including larval swimming ability (Fisher *et al.* 2005), vertical migration (Paris and Cowen 2004), and the use of various navigational cues, such as chemical and acoustic cues in the environment (Kingsford *et al.* 2002, Leis *et al.* 2003, 2011, Dixson *et al.* 2008, Simpson *et al.* 2008, Atema *et al.* 2012, Paris *et al.* 2013). Studies of larval fish behavior suggest that fish larvae are not passive particles in the plankton, but instead can actively orient and navigate to settlement sites. Incorporating larval behavior into dispersal models can therefore dramatically alter projected population connectivity patterns (e.g. Paris and Cowen 2004, Cowen *et al.* 2006, Sale *et al.* 2005, Leis 2007, Paris *et al.* 2007, Staaterman *et al.* 2012). Nonetheless, few studies have linked larval traits and behaviors to gene flow and levels of population genetic differentiation, and it remains unknown how conserved such links may be across species and within regions.

Carindalfishes (family Apogonidae) are known for their homing ability from relatively large distances and for their fidelity to particular daytime resting sites (Marnane 2000, Kolm *et al.* 2005, Døving *et al.* 2006, Gould *et al.* 2014, Rueger *et al.* 2014). However, there are only a few population genetic studies of cardinalfishes, most of which examined the same focal species, the Banggai cardinalfish *Pterapogon kauderni*, which lacks a planktonic larval stage, and all have indicated genetic differentiation at small spatial scales (Bernardi and Vagelli 2004, Hoffman *et al.* 2005, Gerlach *et al.* 2007, Vagelli *et al.* 2008, Gotoh *et al.* 2009). One study of the cardinalfish, *Ostorhinchus doederleini*, which has a PLD of 16-27 days, linked larval behavior to self-recruitment and genetic differentiation between populations only a few kilometers apart. Furthermore, the population genetic structure observed was linked to olfactory preferences exhibited by *O. doederleini* larvae for their home reef water over the other nearby reefs examined (Gerlach *et al.* 2007). Due to the lack of population genetic studies of other cardinalfishes, it remains unknown whether the results of the *O. doederleini* study were specific to the particular study system and whether other cardinalfishes with planktonic larvae also exhibit the same degree of fine-scale genetic structure attributed to larval homing.

To further investigate the link between larval behavior and recruitment in cardinalfishes, I examined the population genetics of the sea urchin cardinalfish *Siphamia tubifer*, for which homing and olfaction preferences have been described (Gould *et al.* 2014, 2015). Similar to other cardinalfishes, *S. tubifer* adults and juveniles

exhibit fidelity to a home site and return to a home reef from displacement distances of at least two kilometers (Gould *et al.* 2014). As described for *O. doederleini* (Gerlach *et al.* 2007), recently settled *S. tubifer* also exhibit a preference for the olfactory cues of their home reef to that of a foreign reef (Gould *et al.* 2015). These findings suggest the possibility that *S. tubifer* larvae also use olfaction to recognize and return to their natal reef. Additionally, *S. tubifer* are short-lived (typically <200 days) like *O. doederleini*, with a slightly longer PLD of up to 30 days (Kingsford *et al.* 2014, Gould *et al.* 2016). Despite these similarities, *S. tubifer* is distinct among cardinalfishes in that it is symbiotically bioluminescent, hosting in an abdominal light organ a dense population of the luminous bacterium *Photobacterium mandapamensis*, which it acquires from the environment during larval development, and uses the bacterially-emitted light while foraging at night (Iwai 1958, Dunlap and Nakamura 2011, Dunlap *et al.* 2012). Therefore, describing the dispersal distances and connectivity patterns of *S. tubifer* may also provide insight on the location of symbiont acquisition by developing larvae.

To test for population genetic differentiation in *S. tubifer*, I applied double digest restriction site-associated sequencing (ddRAD-Seq) methods to identify fine-scale patterns of genetic divergence among groups of *S. tubifer* collected over a three-year period from various locations in Okinawa, Japan. I used ddRAD-Seq methods as they do not require an extensive marker discovery process and enable the development of thousands of genomic markers without any prior genetic data for the focal species (Davey and Blaxter 2010, Seeb *et al.* 2011). Our specific aims were to test for patterns of population genetic differentiation between groups of *S. tubifer* sampled at various spatial scales and to test the stability of *S. tubifer* populations by examining temporal patterns of genetic divergence at certain reef sites. If the link between larval olfactory preferences, homing potential, and self-recruitment are conserved across similar cardinalfish species, I predicted that populations of *S. tubifer* would have significant levels of genetic differentiation at small spatial scales but no differentiation at the same reef over time. However, if the observed behaviors do not indicate larval homing, I expected to detect little or no genetic differentiation between groups of *S. tubifer* sampled from different reefs and between years at the same reef.

Materials and Methods

Sampling

A total of 300 *Siphamia tubifer* were collected from different locations (approximately 20 individuals per location) over three years in the Okinawa Islands, Japan. Ten of these locations were sampled during the summer of 2013, three of which were again sampled in 2014, and one location was sampled in three consecutive years, 2012, 2013, and 2014 (Figure 5.1). Fish of various sizes associated with several different host sea urchins and from a broad sampling area were collected at each sampling location (Table 5.1). Upon collection, fish were immediately euthanized and placed on ice. The intact light organ of each fish was then aseptically dissected and individually preserved in RNAlater®, and the remainder of the fish specimen was stored in 98% ethanol at -20˚C.

DNA extraction and library preparation

Genomic DNA was extracted from the intact, preserved light organ using QIAGEN DNeasy Blood and Tissue Kits and following the manufacturer's protocol from whole, preserved light organs, which are comprised of fish tissue and contain the symbiotic population of luminous bacteria. A total of six ddRAD-Seq DNA libraries were constructed, each from the genomic DNA of 50 *S. tubifer* light organs, for a total of 300 samples, following a modified combination of the methods described in Parchman *et al.* (2012) and Peterson *et al.* (2012). For each library, approximately 200 ng of genomic DNA from each light organ was digested with the high-fidelity restriction enzymes *MseI* and *EcoRI* at 37˚C for three hours. A standardized concentration per library of each digestion product was then ligated to a uniquely barcoded Illumina adaptor at the *EcoRI* cut site and an Illumina adaptor at the *MseI* cut site. The ligation products were individually amplified with the Illumina Illpcr1 and Illpcr2 primers in two 20 ml PCR reactions per sample with a 98˚C start, 12 cycles of 98˚C for 20 seconds, 65˚C for 30 seconds, 72˚C for 40 seconds, followed by 10 minutes at 72˚C, after which, the PCR products from all samples within a library were pooled and concentrated to an approximate volume of 150 ml. Samples were purified with

Agencourt AMPure XP magnetic beads following standard protocols after the digestion, ligation, and PCR steps. The pooled, purified PCR products were then size-selected between 300-400 bp on a Pippin Prep (Sage Science) machine, and the size-selected DNA libraries were each sequenced in one lane on the Illumina HiSeq2000 platform (San Diego, CA) at the Center for Applied Genomics, Toronto, ON, Canada, to generate 100 bp, single-end sequence reads.

Sequence analysis and processing

Raw sequence reads were quality filtered and processed primarily using the program *Stacks* v. 1.35 (Catchen *et al.* 2011, 2013). Raw reads were demultiplexed, trimmed to 90 bp, and quality filtered for a Phred score of 33 or higher using the *process_radtags* command in *Stacks*. To distinguish sequence reads that belonged to the host fish from those of the bacterial symbiont, I used the '*very_sensitive*' command in *Bowtie2* v. 2.2.0 (Langmead and Salzberg 2012) to filter all reads against the reference genome of *Photobacterium mandapamensis* (Urbanczyk *et al.* 2011). To ensure that all bacterial reads were removed, I also filtered all reads against the genomes of *Escherichia coli* K12 (Durfee *et al.* 2008) and *Vibrio campbellii* (Lin *et al.* 2010) in the same manner. Sequence reads that did not align to the bacterial genomes were assigned as fish (*S. tubifer*) sequences.

Fish sequence reads were processed and assembled *de novo* to call single nucleotide polymorphisms (SNPs) using the *Stacks* pipeline with the *rxstacks* correction step. I first ran the *denovo_map* program with the parameters -m 3, -M 2, and -n 3, optimized to prevent over- and under- merging of homologous loci. These parameters were previously recommended to increase the number of loci but minimize genotyping error for the *de_novo* assembly of other RAD datasets (Mastrett-Yanes *et al.* 2015). I then implemented the *rxstacks* correction step using a bounded model (--bound_high 0.1) in which excess haplotypes were pruned, and loci for which 25% of individuals had a confounded match in the catalog (--conf_lim 0.25) or an average log likelihood less than (-10.0) were removed. After running this correction step, the *cstacks* (-n 3) and *sstacks* programs were re-applied to produce the final set of RAD tags across all individuals in the study.

Population analysis

The mean depth of coverage per locus across all individuals was determined from the *Stacks* output files, and individuals with a mean coverage less than 10 across all loci were excluded from the analyses. Population summary statistics were computed with the *populations* program in *Stacks* for sites present in at least 10 populations and in 70% of individuals per population. Population differentiation was evaluated with pairwise F_{ST} values calculated in the *populations* program in *Stacks* for loci found in all populations and in 70% of individuals per population with a minor allele frequency greater that 5%. Pairwise AMOVA F_{ST} values were also computed in *GenoDive* (Meirmans and Van Tienderen 2004) with 1,000 bootstrap resampling steps, producing *P*-values for all pairwise population comparisons. Isolation by distance (IBD) was assessed by conducting Mantel tests with the Isolation by Distance Web Service (Jensen *et al.* 2005) on pairwise F_{ST} values and the natural logarithm of the shortest distances over water between sites; one-sided *P*-values were calculated by randomizing the data 30,000 times. Using the *hierfstat* program (Goudet 2005) in R v. 3.1.1 (R Core Team 2014) the per locus F_{ST} values across all individuals for each dataset were calculated to compare and examine their distributions.

To visualize genetic structure, principal components analyses (PCAs) were implemented on the same dataset used to calculate F_{ST} values in R with the *dudi.pca* function in the *adegenet* v. 1.4.2 package (Jombart 2008, Jombart and Ahmed 2011). Missing data values were replaced with the mean value across the entire dataset at that locus. Analyses of molecular variance (AMOVA) (Excoffier *et al.* 1992) were carried out in *GenoDive* to test for genetic differences between populations and region (populations on the east and west coast of Okinawa). Both PCAs and AMOVAs were performed separately on the 2013 and 2014 populations as well as on the three-year dataset from the Sesoko site (S) (Figure 5.1).

Complimentary clustering analyses were also performed with the program *STRUCTURE* v. 2.3.4 (Pritchard *et al.* 2000) using the output data files from *Stacks* comprised of only the first SNP per locus (to eliminate any SNPs that are linked within the same RAD site from the analysis) for loci present in at least 70% of all individuals and in at least 10 populations for each dataset. Group assignments in *STRUCTURE* were made using the admixture model with 10,000 burn-in steps and 10,000 MCMC

iterations for each number of pre-determined genotypic groups (K). Analyses were repeated ten times for each value of K. For the 2013 and 2014 datasets, K was set from 1 to the total number of sites sampled (10 and 4, respectively). Probable K values were inferred by examining the change in the posterior probability of the log likelihood across all K values (ln P(X|K)) and by applying the Evanno D*K* method (Evanno *et al.* 2005) with *STRUCTURE HARVESTER* (Earl 2012). All *STRUCTURE* results were visualized using the program *DISTRUCT* (Rosenberg 2004).

Ethics statement

The protocols used here for the capture and handling of fish specimens were approved by the University of Michigan's University Committee for the Use and Care of Animals (PRO00004825), and they followed the requirements outlined in the University of the Ryukyus' Guide for Care and Use of Laboratory Animals (Dobutsu Jikken Kisoku, version 19.6.26).

Results

Sequence analysis and processing

The six ddRAD libraries each produced high quality sequence data with sufficient depth of coverage across most individuals for population-level genetic analyses. Thirteen individuals that had fewer than 800,000 remaining reads after quality filtering (Figure 5.2) were discarded from the analysis. On average, 87.05 ± 2.58% of all reads were retained from each library, with an average of 9.72 ± 2.29% and 3.23 ± 2.51% of reads discarded for having ambiguous barcodes or RADtags or for low quality, respectively (Table 5.2).

Prior to genetic analysis, sequence reads that aligned to the genome of the light organ symbiont, *P. mandapamensis*, or to the other examined bacterial genomes were removed from the dataset. The average percent of quality-filtered reads per individual that aligned to the *P. mandapamensis* genome was 26.9 ± 9.1% (Figure 5.2), and of the reads that did not align to *P. mandapamensis,* the average percent per individual that

aligned to the *V. campbelli* or the *E. coli* genomes were 0.293 ± 0.155% and 0.003 ± 0.155%, respectively. These bacterial sequence reads were removed from the dataset, and the remaining reads were assigned as *S. tubifer* sequences, resulting in an average of 72.7 ± 9.3% of total reads per individual assigned as *S. tubifer* (Figure 5.2). After *de novo* assembly of the *S. tubifer* sequence reads across all remaining individuals, the mean depth of coverage per locus was 21.6 ± 8.3. One individual with mean sequence coverage per locus below 10 was also removed from further analyses (Figure 5.2), resulting in a total of 280 individuals in the dataset (Table 5.1).

Population summary statistics

For RAD sites that were polymorphic in at least one population (Table 5.3), the average major allele frequency (P) and observed heterozygosity (H_{obs}) across all populations ranged from 0.9593 to 0.9607 and 0.0485 to 0.0519, respectively. These values changed as expected when also including sites that are fixed across all populations (Table 5.4); P increased up to 0.9983 and H_{obs} values all decreased to 0.0022. Across variant sites, the percentages of polymorphic loci in populations sampled in 2013 were between 30.92% and 36.09%, whereas the percentages of polymorphic loci in populations sampled in 2014 were slightly higher, ranging from 36.79% to 41.75% (Table 5.3). Levels of overall nucleotide diversity (p) across all sites, fixed and variant, were similar for all groups of fish sampled, varying between 0.0026 and 0.0028. Average measures of Wright's inbreeding coefficient (F_{IS}) calculated for all variant sites ranged from 0.0407 to 0.0567 across all populations (Table 5.3). These F_{IS} values are all relatively low, indicating a lack of cryptic population structure or assortative mating within populations.

Analysis of genetic differentiation

Population genetic analyses of the 11,836 SNPs identified revealed no significant genetic structure over the sampling range or over time. In 2013, pairwise F_{ST} values were consistently low between sites, ranging between 0.0157 and 0.0214, with only a few significant values (Table 5.5), most of which were for comparisons with fish sampled at the Motobu (M) site (Figure 5.1). Pairwise F_{ST} values for the 2014 samples

were similarly low and non-significant, ranging from 0.0116 to 0.0139 (Table 5.6). An analysis of temporal differentiation at the Sesoko (S) site (Figure 5.1) over three years also revealed no significant differentiation between years (F_{ST} values ranged from 0.0158 to 0.0177, Table 5.7). Similarly the additional two sites that were sampled in consecutive years, Itoman (It) and Ikei (Ik) (Figure 5.1), had low and non-significant F_{ST} values (It: F_{ST} = 0.0151, Ik: F_{ST} = 0.0165) between years. Per locus F_{ST} values for each dataset were all low with seemingly normal distributions around zero. The maximum F_{ST} value calculated across all datasets was 0.166 for a locus in the Sesoko dataset, whereas the maximum values in the 2013 and 2014 datasets were 0.0954 and 0.148, respectively (Figure 5.3).

Isolation by distance analyses revealed no significant relationship between the low levels of population differentiation observed and geographic distance between locations within either sampling year (2013: $F_{1,43}$ = 0.369, R^2 = 0.0085, P = 0.547; 2014: $F_{1,4}$ = 3.372, R^2 = 0.457 P = 0.140; Figure 5.4). Similarly, results from the AMOVA indicate that all of the observed genetic variation is attributed to variation within (F_{IT}) and among (F_{IS}) individuals and none is attributed to differences between populations or between the east and west coast regions of Okinawa (Table 5.8).

Principal components analyses confirmed the lack of genetic structure, with no apparent clustering of individuals by sampling location (Figure 5.5). The first two PC axes for the 2013 analysis each described less than 1% of the variation in the data. These values rose slightly for the 2014 data, accounting for a combined (PC1 and PC2) total of 2.57% of the total genetic variation (Figure 5.5). The PCA carried out on the temporal dataset from the Sesoko site also indicates a lack of structure over time (Figure 5.6) and is consistent with the low, non-significant pairwise F_{ST} values between groups of fish collected at that site in subsequent years (Table 5.5). Similarly, clustering analyses in *STRUCTURE* revealed that a K = 1 is most likely for both the 2013 and 2014 datasets (Figure 5.7). In the case where K = 1, the Evanno ΔK method of detecting the true value of K is ineffective (Evanno *et al.* 2005). I therefore examined the mean posterior probabilities for each K value in both datasets; the log likelihood was highest for K = 1 in both years (Figure 5.8). These results provide further evidence that one panmictic population of *S. tubifer* was sampled in Okinawa, spanning the area around Okinawa Island and including Kume Island (K), one hundred kilometers to the west (Figure 5.1).

Discussion

The observed discrepancies between potential and actual dispersal distances of reef fish larvae (e.g. Taylor and Hellberg 2003, Rocha *et al.* 2005, Bowen *et al.* 2006, Galarza *et al.* 2009, Jones *et al.* 2009) highlight the need for a better understanding of the influence of larval fish behavior on connectivity patterns of reef fish populations. To narrow this gap, general links between larval behavior across groups of fishes and species' patterns of population connectivity are needed. I applied RAD-Seq methods to look for evidence of larval homing in *S. tubifer*, which had been observed for another cardinalfish species with similar life history traits and behavior (Gerlach *et al.* 2007). Despite the potential of RAD-Seq methods to detect fine-scale genetic structure, and in contrast to other genetic studies of cardinalfishes, I found no evidence of genetic differentiation between groups of *S. tubifer* at spatial scales ranging up to 140 kilometers. The observed genetic admixture of *S. tubifer* within Okinawa indicates a high level of connectivity in the region. Presumably, this connectivity results from larval dispersal and mixing by ocean currents.

Evidence suggests that the typical dispersal distances of reef fish larvae may be on the order of 50 to 100 kilometers, with some local retention, and that populations of fishes with high mortality rates tend to be subsidized with larvae from greater distances (Cowen *et al.* 2006). Consistent with this model, *S. tubifer* is apparently subject to high mortality rates (Gould *et al.* 2014, 2016), and may therefore depend on larval subsidies for population persistence. *Siphamia tubifer* also has highly specialized habitat requirements; groups of *S. tubifer* closely associate with the sea urchins *Diadema setosum* and *Echinothrix calamaris* during the daytime, seeking shelter among the urchins' long spines (Lachner 1955, Eibl-Eibesfeldt 1961, Tamura 1982, Gould *et al.* 2014). The distribution of diademed urchins in reef habitats can be patchy, driven partially by variation in sediment size (Nishihira *et al.* 1991, Dumas *et al.* 2007). Therefore, the distribution of suitable settlement habitat available for dispersing *S. tubifer* larvae may be highly variable between reefs. In addition to specific habitat requirements, larval *S. tubifer* must acquire the appropriate luminous bacterium from the environment during development to establish its bioluminescent symbiosis. Both of these factors may limit the recruitment success of *S. tubifer* and

could largely contribute to the admixture observed, as the amount of gene flow required to maintain genetic connectivity over a large scale is on the order of only a few individuals per generation (Shulman and Bermingham 1995, Shulman 1998, Leis 2002). If few, far-dispersing *S. tubifer* larvae settle sporadically on reefs, there is little potential for genetic divergence to accumulate between populations over time and in the absence of local recruitment.

Corresponding with high genetic connectivity in the region, the islands in Japan's Ryukyu Archipelago receive larval supply from other reef habitats in the south. In particular, small reef fish from the Philippines have an ecologically significant linkage potential to the Ryukyu Islands (Treml *et al.* 2015). The connectivity potential from the northern Phillipines is driven by the strong ocean currents in the region; the Kuroshio Current originates off the coast of the Philippines and flows northward, passing through the Ryukyu Archipelago (Figure 5.1). A study of the crown-of-thorns sea star *Acanthaster planci*, which has a PLD of over 14 days (Lucas 1973), established evidence of genetic homogeneity among the Philippine Islands and across the Ryukyu Islands following the Kuroshio Current. There was also no signature of IBD within the region, consistent with substantial larval dispersal and connectivity (Yasuda *et al.* 2009). Similarly, there is high genetic connectivity for the broadcast-spawning coral *Acropora digitifera* throughout the Ryukyu Island chain (Nakajima *et al.* 2010), and within Okinawa there is evidence of gene flow for several coral species (Nishikawa *et al.* 2003, Nishikawa 2008). However, two distinct genetic clusters, between the North and South regions of Okinawa Island, exist for the scleractinian coral *Goniastrea aspera*, indicating a substantial number of locally-produced recruits and the potential for self-recruitment in the region despite the strong influence of currents in the region (Nishikawa and Sakai 2005).

Connectivity between reef fish populations is dependent on the certain larval characteristics, such as dispersal duration and swimming behavior. However, the degree to which these behaviors influence a species' dispersal potential and self-recruitment is disputed and is most likely species-specific (Cowen *et al.* 2000, Mora and Sale 2002, Warner and Cowen 2002, Sale 2004). Furthermore, abiotic factors, including the direction and speed of ocean currents and the availability and distribution of downstream settlement habitat, have large effects on connectivity patterns of marine populations (Treml *et al.* 2008). Our study indicates that one

panmictic population of *S. tubifer* exists in Okinawa; these results suggest that larval dispersal maintains genetic connectivity over substantial distances. For similarly "open" populations of reef fishes, a large scale for management would be necessary to maintain the appropriate levels of gene flow. However, inferences regarding ecologically important levels of connectivity are limited when there is panmixia, as it is difficult to directly measure connectivity patterns under high-gene-flow conditions (Hedgecock *et al.* 2007, Jones *et al.* 2009).

In addition to spatial differentiation, I examined temporal genetic divergence in *S. tubifer* at one of our study sites by sampling over three consecutive years. The dynamics of temporal genetic structure may be even more informative than spatial dynamics in marine systems, but this issue has been largely overlooked for most marine populations (Hellberg *et al.* 2002, Hedgecock *et al.* 2007). Of the studies that have examined temporal structure, instances of temporal stability have been rare (Bernal-Ramírez *et al.* 2003, Larsson *et al.* 2010), whereas temporal genetic differentiation has been reported for several marine fishes (e.g. Planes and Lenfant 2002, Maes *et al.* 2006, Selkoe *et al.* 2006, Klanten *et al.* 2007). Genetic differentiation over time at a location can result from selection, random genetic drift, or from variable larval supply from different source populations (Hedgecock *et al.* 2007). Between groups of *S. tubifer* sampled at the same site over three years, I observed low levels of differentiation similar to those observed between sampling locations within the same year. *Siphamia tubifer* is short-lived, with an expected longevity of less than 200 days (Gould *et al.* 2016); therefore some degree of genetic turnover within a location is reasonable to expect, particularly when considering the potential variability in larval supply consistent with the predicted dispersal distances in this study. However, to further understand the importance of genetic variation over time in predicting patterns of marine connectivity and population genetic structure more studies are needed that investigate the temporal aspect of population genetic differentiation.

Overall our study supports the need to identify general patterns of connectivity within specific geographic regions across diverse fish species, as well as species-specific patterns of genetic differentiation across groups of fishes, to adequately define the relationships between life history traits, larval behavior, and gene flow. I highlight the effectiveness of RAD-Seq methods, which have been used very recently to examine the genomics of other coral reef fishes (Puebla *et al.* 2014, Gaither *et al.* 2015,

Saenz-Agudelo *et al.* 2015, Stockwell *et al.* 2016, Picq *et al.* 2016). RAD-Seq methods have the ability to continue to narrow our gap in knowledge of the patterns of connectivity, speciation, and adaptation for an array of species, as no extensive marker discovery process and no prior genetic information are required (Davey and Blaxter 2010), and they can be used to infer genetic differentiation with finer precision, even with small sample sizes (Luikart 2003, Coates *et al.* 2009, Willing *et al.* 2012, Bradbury *et al.* 2015).

In this study, I show that in contrast to the cardinalfish *O. doederleini* in the Great Barrier Reef (Gerlach *et al.* 2007), *S. tubifer* in Okinawa lacks population genetic structure, indicating that larvae disperse significant distances during their planktonic phase, despite the species' homing potential and olfactory preferences for a home reef. These findings suggest that larval settlement behavior does not promote genetic divergence in *S. tubifer*. Instead, strong ocean currents combined with a month-long pelagic larval phase promote dispersal and gene flow in the region. Future investigations of *S. tubifer* at varying locations across this species' broad, Indo-Pacific distribution will provide insight on whether the scale of genetic admixture observed here is region-specific or if there are consistent patterns of larval dispersal maintained at the species level.

Table 5.1 Sampling locations and years of *Siphamia tubifer* in Okinawa, Japan. The range and mean standard lengths (SL) of fish specimens collected at each sampling site are listed as well as the numbers of individuals collected (N_i) and included in the final genomic dataset after quality filtering (N_f)

ID	Site	Latitude	Longitude	Year	SL (mean); mm	N_i	N_f
S	Sesoko	26.6354	127.8658	2012	15.0 - 31.0 (22.9)	17	17
				2013	22.0 - 38.5 (28.5)	18	18
				2014	15.0 - 38.0 (28.4)	22	21
M	Motobu	26.6558	127.8803	2013	21.0 - 35.5 (29.5)	26	20
N	Nago	26.6037	127.9324	2013	18.5 - 42.5 (29.5)	24	21
Hd	Hedo	26.8488	128.2525	2013	17.5 - 37.5 (26.3)	17	17
It	Itoman	26.0952	127.6585	2013	23.0 - 36.5 (27.9)	15	14
				2014	13.5 - 20.0 (16.8)	27	27
O	Ou	26.1280	127.7690	2013	16.5 - 25.0 (20.1)	16	16
Y	Yonabaru	26.2030	127.7712	2013	21.0 - 38.5 (28.7)	16	16
Ik	Ikei	26.3935	127.9886	2013	11.5 - 31.0 (17.3)	16	15
				2014	13.0 - 30.5 (21.5)	22	22
Hk	Henoko	26.5346	128.0461	2013	14.5 - 27.5 (19.6)	17	17
A	Ada	26.7420	128.3211	2013	23.0 - 34.5 (28.5)	16	15
K	Kume	26.3516	126.8201	2014	15.5 - 41.5 (27.9)	26	24

Table 5.2 Details for each of the six ddRAD-Seq libraries. Numbers of total reads from each library that did not pass the filters set in *Stacks* are listed as ambiguous or low quality reads. The total number and percent of total reads retained for further processing and analysis are also listed

Library	Total reads	Ambiguous reads		Low quality reads		Retained reads	
		total	%	total	%	total	%
1	115,966,572	16,262,201	14.02	2,100,938	1.81	97,603,433	84.17
2	124,642,686	10,945,491	8.78	9,698,364	7.78	103,998,831	83.44
3	129,099,530	9,280,426	7.19	5,504,011	4.26	114,315,093	88.55
4	145,903,444	13,321,386	9.13	3,482,628	2.39	129,099,430	88.48
5	142,669,390	13,708,785	9.61	3,409,093	2.39	125,551,512	88.00
6	154,050,631	14,812,053	9.62	1,126,946	0.73	138,111,632	89.65

Table 5.3 Population genetic summary statistics calculated for each group of *Siphamia tubifer* sampled using only nucleotide positions that are polymorphic in at least one population. Statistics listed are the average number of individuals analyzed at each locus (*N*), the total number of nucleotide positions in the dataset (Sites), the number of unique variable sites in each population (Private), the percent of polymorphic sites (% Poly), the average frequency of the major allele (P), the average per locus observed heterozygosity (H_{obs}), the average nucleotide diversity (π), and Wright's average inbreeding coefficient (F_{IS}). All statistics were calculated in *Stacks*

Population	*N*	Sites	Private	% Poly	P	*Hobs*	π	*FIS*
A-13	14.3	109,236	2,474	32.38	0.9600	0.0517	0.0631	0.0416
Hd-13	16.0	109,504	2,663	33.96	0.9598	0.0512	0.0630	0.0452
Hk-13	16.0	107,956	2,627	34.03	0.9597	0.0514	0.0632	0.0455
Ik-13	14.1	108,401	2,284	31.71	0.9602	0.0509	0.0625	0.0419
Ik-14	20.7	106,997	3,274	37.55	0.9601	0.0505	0.0623	0.0502
It-13	13.1	109,264	2,120	30.92	0.9598	0.0516	0.0632	0.0407
It-14	25.6	110,648	4,581	41.75	0.9593	0.0519	0.0635	0.0545
K-14	22.4	98,587	3,210	38.43	0.9595	0.0501	0.0631	0.0567
M-13	18.5	82,512	2,166	34.92	0.9607	0.0485	0.0615	0.0534
N-13	19.6	98,854	2,692	36.09	0.9601	0.0492	0.0624	0.0550
O-13	15.0	106,753	2,382	32.67	0.9603	0.0507	0.0624	0.0439
S-12	15.5	97,232	2,070	32.56	0.9602	0.0495	0.0622	0.0473
S-13	16.8	108,356	2,846	34.69	0.9598	0.0510	0.0630	0.0473
S-14	19.7	107,381	3,086	36.79	0.9599	0.0505	0.0627	0.0511
Y-13	15.1	108,186	2,539	33.07	0.9603	0.0514	0.0624	0.0418

Table 5.4 Population genetic summary statistics calculated for each group of *Siphamia tubifer* sampled across all nucleotide positions (variant and fixed). Statistics listed are the average number of individuals analyzed at each locus (*N*), the total number of nucleotide positions in the dataset (Sites), the number of unique variable sites in each population (Private), the percent of polymorphic sites (% Poly), the average frequency of the major allele (P), the average per locus observed heterozygosity (H_{obs}), the average nucleotide diversity (π), and Wright's average inbreeding coefficient (F_{IS}). All statistics were calculated in *Stacks*

Population	*N*	Sites	Private	% Poly	P	*Hobs*	π	*FIS*
Ad-13	14.3	2,570,792	2,474	1.38	0.9983	0.0022	0.0027	0.0018
H-13	16.0	2,573,525	2,663	1.44	0.9983	0.0022	0.0027	0.0019
Hk-13	16.0	2,529,561	2,627	1.45	0.9983	0.0022	0.0027	0.0019
Ik-13	14.0	2,546,147	2,284	1.35	0.9983	0.0022	0.0027	0.0018
Ik-14	20.7	2,511,160	3,274	1.60	0.9983	0.0022	0.0027	0.0021
ItH-13	13.1	2,569,206	2,120	1.32	0.9983	0.0022	0.0027	0.0017
ItH-14	25.7	2,601,977	4,581	1.78	0.9983	0.0022	0.0027	0.0023
Ku-14	22.4	2,241,314	3,210	1.69	0.9982	0.0022	0.0028	0.0025
M-13	18.5	1,851,345	2,166	1.56	0.9982	0.0022	0.0027	0.0024
N-13	19.6	2,251,233	2,692	1.58	0.9982	0.0022	0.0027	0.0024
Ou-13	15.0	2,501,900	2,382	1.39	0.9983	0.0022	0.0027	0.0019
S-12	15.5	2,209,429	2,070	1.43	0.9983	0.0022	0.0027	0.0021
S-13	16.8	2,535,756	2,846	1.48	0.9983	0.0022	0.0027	0.0020
S-14	19.7	2,503,012	3,086	1.58	0.9983	0.0022	0.0027	0.0022
Y-13	15.1	2,549,868	2,539	1.40	0.9983	0.0022	0.0026	0.0018

Table 5.5 Pairwise F_{ST} values (top diagonal) and the shortest distance (km) through water (bottom diagonal) between groups of *Siphamia tubifer* sampled in 2013. F_{ST} values in bold have a *P*-value < 0.05

2013	A	Hd	Hk	Ik	It	M	N	O	S	Y
A	-	0.0187	0.0191	0.0197	0.0207	**0.0182**	0.0169	0.0192	0.0183	0.0194
Hd	22.4	-	0.0179	0.0191	0.0198	**0.0172**	0.0161	0.0184	0.0170	0.0183
Hk	38.2	59.7	-	0.0192	0.0198	**0.0173**	0.0163	0.0184	0.0176	0.0184
Ik	51.4	73.3	17.7	-	0.0214	0.0183	0.0172	0.0193	0.0185	0.0194
It	104.5	117.3	71.5	54.8	-	**0.0189**	0.0174	0.0205	0.0192	0.0199
M	68.0	47.8	105.3	119.5	72.4	-	**0.0157**	0.0176	0.0169	0.0176
N	78.2	58.0	115.5	129.3	80.8	10.4	-	0.0167	0.0157	**0.0168**
O	89.0	111.3	58.6	38.1	15.8	84.4	83.5	-	0.0181	0.0186
S	70.4	50.1	107.7	121.9	68.2	2.8	8.6	82.1	-	0.0175
Y	83.6	105.0	51.8	33.4	32.1	100.5	99.8	16.8	98.4	-

Table 5.6 Pairwise F_{ST} values (top diagonal) and the shortest distance (km) through water (bottom diagonal) between groups of *Siphamia tubifer* sampled in 2014

2014	Ik	It	K	S
Ik	-	0.0120	0.0130	0.0139
It	54.8	-	0.0116	0.0127
K	141.8	80.0	-	0.0135
S	121.9	68.2	100.0	-

Table 5.7 Pairwise F_{ST} values between groups of *Siphamia tubifer* sampled at the Sesoko site (see Figure 5.1) over three consecutive years

Sesoko	2012	2013	2014
2012	-	0.0177	0.0161
2013		-	0.0158

Table 5.8 Analysis of molecular variance (AMOVA) of groups of *Siphamia tubifer* sampled in 2013. Populations were grouped into regions determined by collection sites on the east or west coast of Okinawa (see Figure 5.1)

Source of variation	Nested in	% Variance	*F*-statistic	*P*-value
Within individual	--	86.4	F_{IT}	--
Among individual	Population	13.6	F_{IS}	0.001
Among population	Region	0.0	F_{SC}	0.245
Among region	--	0.0	F_{CT}	0.012

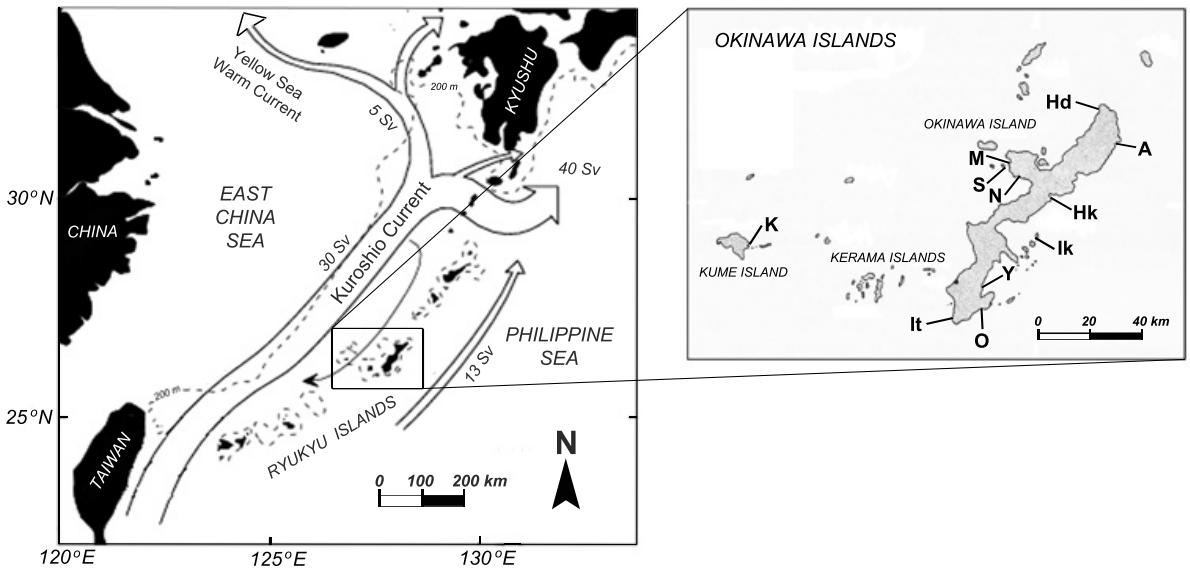

Figure 5.1 Sampling locations of *Siphamia tubifer* in Okinawa, Japan from 2012 to 2014, and the general current patterns in the region

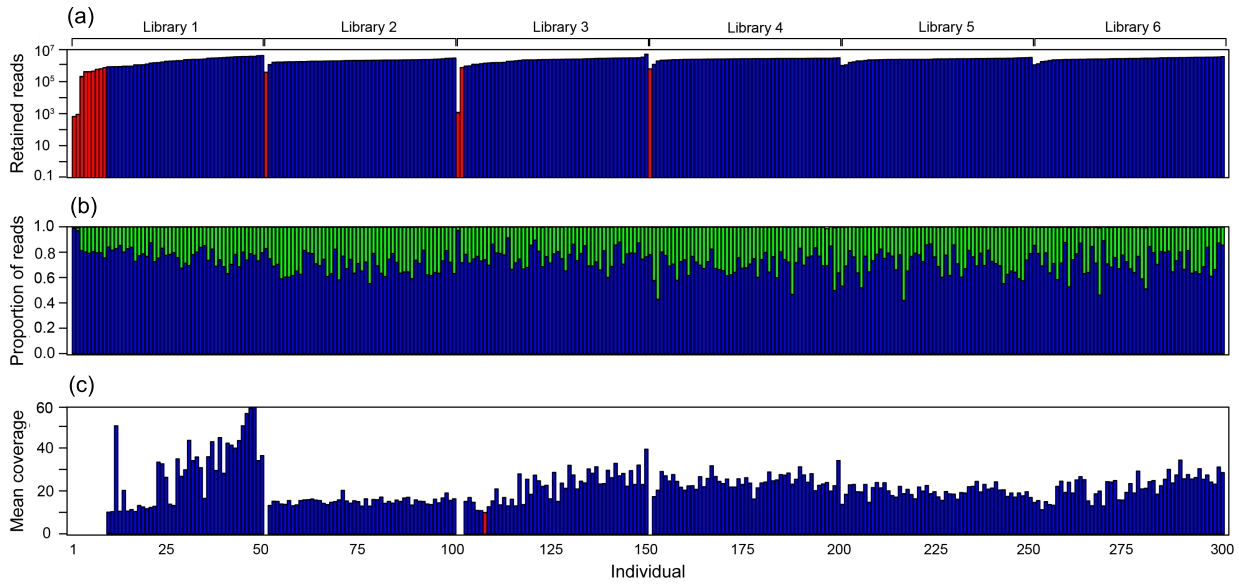

Figure 5.2 Summary of sequence reads produced from each RAD library. (a) The number of retained reads after quality filtering in *Stacks* for each individual sequenced. Individuals that were discarded due to a low number of sequence reads are in red. (b) The proportion of retained reads that aligned to the bacterial genome sequences and subsequently discarded (green) or that were assigned as *Siphamia tubifer* sequences (blue) for the remaining analyses. (c) Mean sequence coverage per locus across all loci for quality-filtered individuals. Individuals that were discarded from the analysis due to low mean coverage are in red

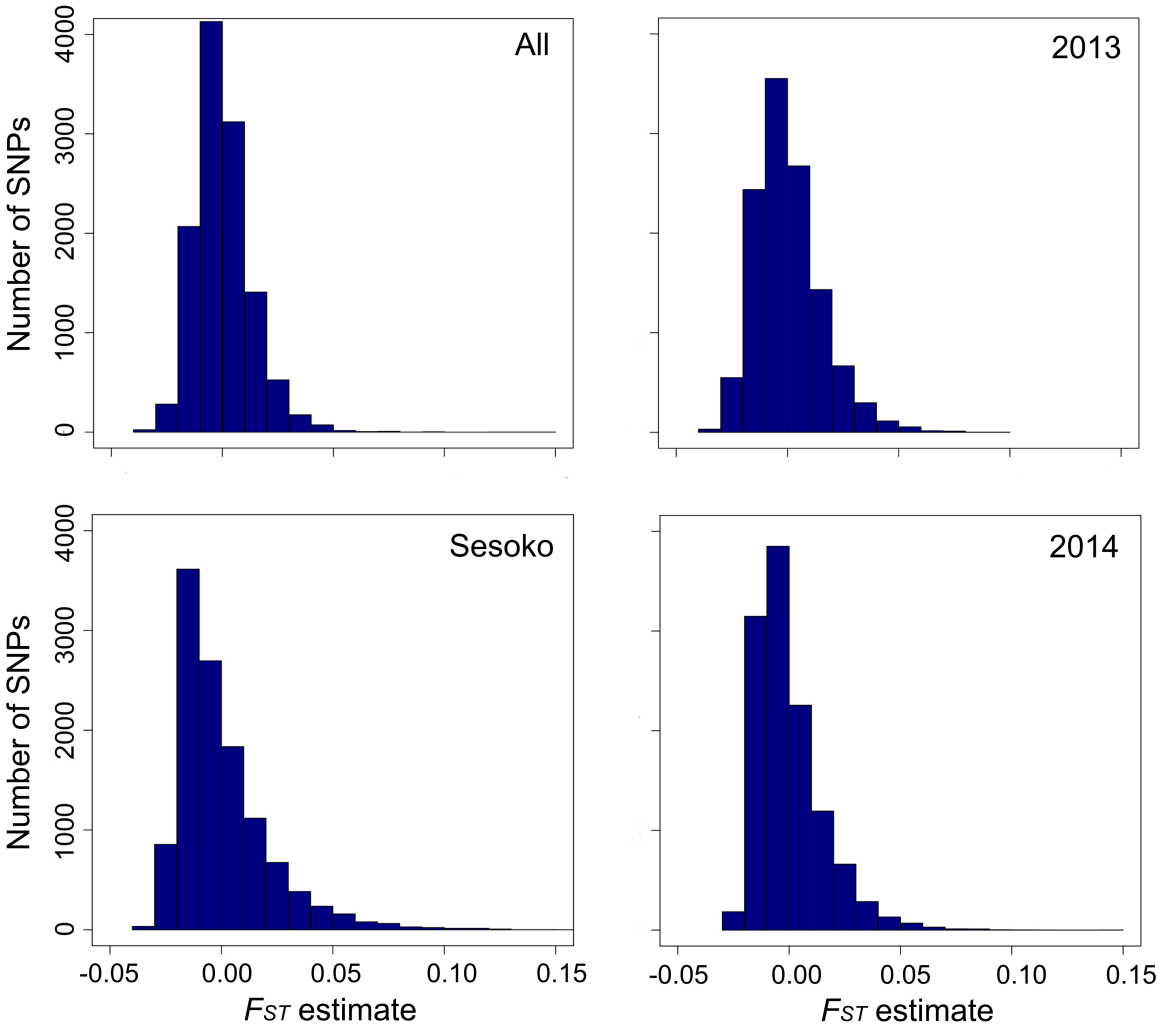

Figure 5.3 Per locus F_{ST} estimates for 11, 836 SNPs identified for *Siphamia tubifer* calculated across all individuals, individuals sampled in 2013 and 2014, and individuals sampled consecutively over three years (2012 – 2014) at the Sesoko site

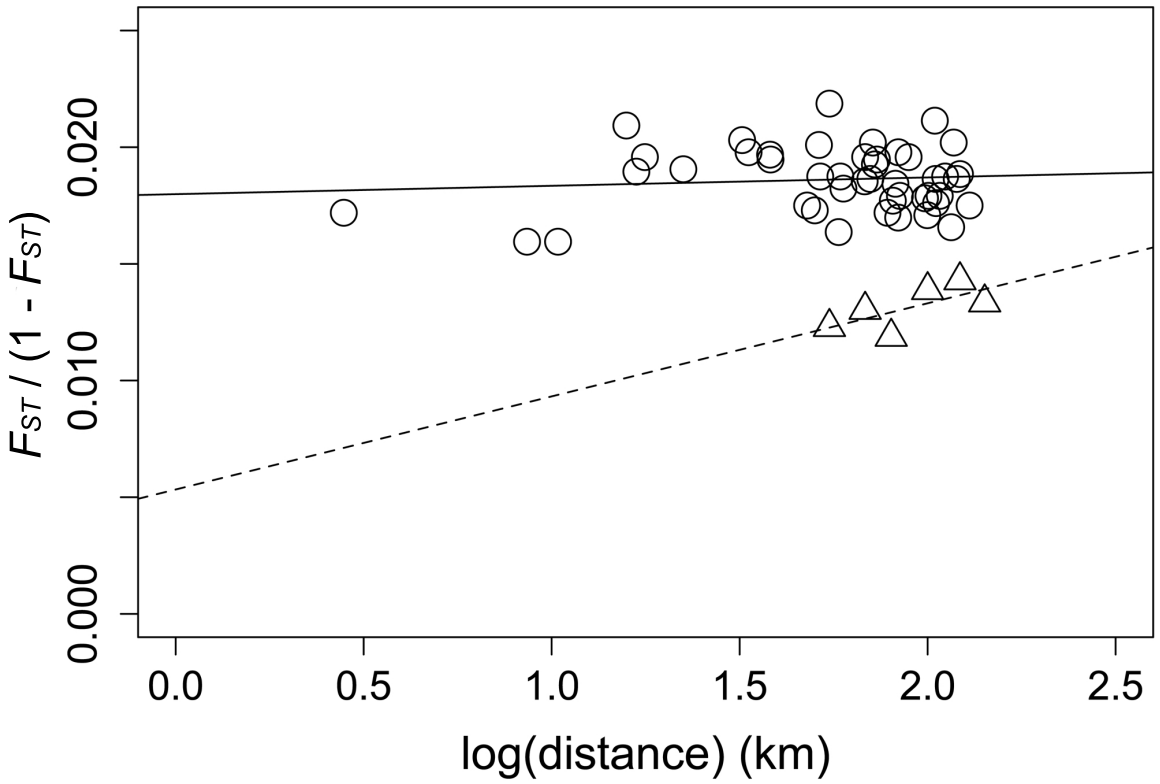

Figure 5.4 Analysis of isolation by distance based on pairwise differentiation calculated between groups of *Siphamia tubifer* collected in 2013 (circles) and 2014 (triangles). Distances were measured as the shortest straight-line distance across water between each pair of sites

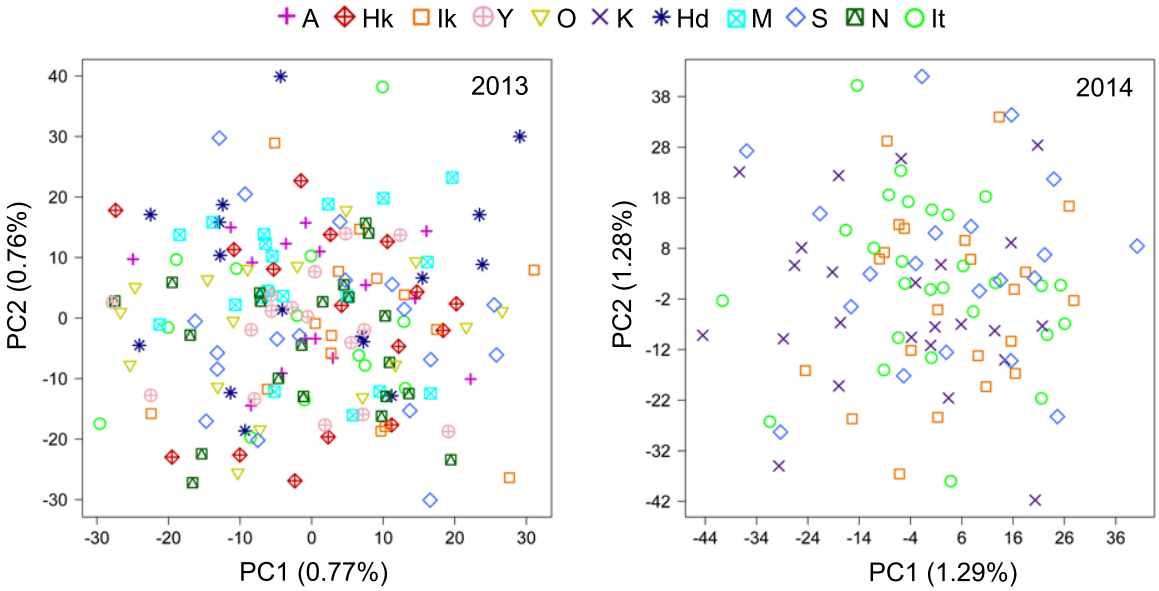

Figure 5.5 Principal components analyses (PCA) of genetic differentiation among the 280 genotyped *Siphamia tubifer* in 2013 and 2014. Points represent individuals along the PC1 and PC2 axes of genetic variation with the amount of variation explained by each axis in parentheses. Different colors and shapes indicate the sampling locations (see Figure 5.1)

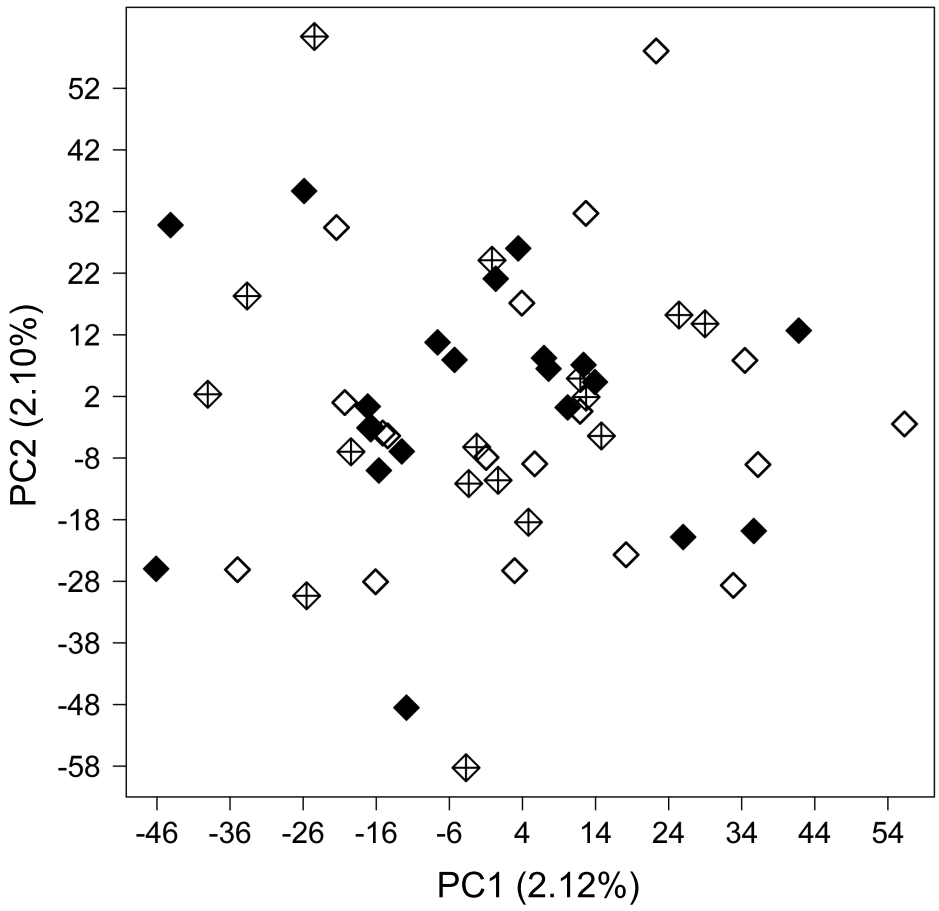

Figure 5.6 Principal components analyses (PCA) of genetic differentiation among the genotyped *Siphamia tubifer* sampled from the Sesoko site (see Figure 1) in three consecutive years, 2012 (crossed), 2013 (open), and 2014 (closed). Points represent individuals along the PC1 and PC2 axes of genetic variation with the amount of variation explained by each axis in parentheses

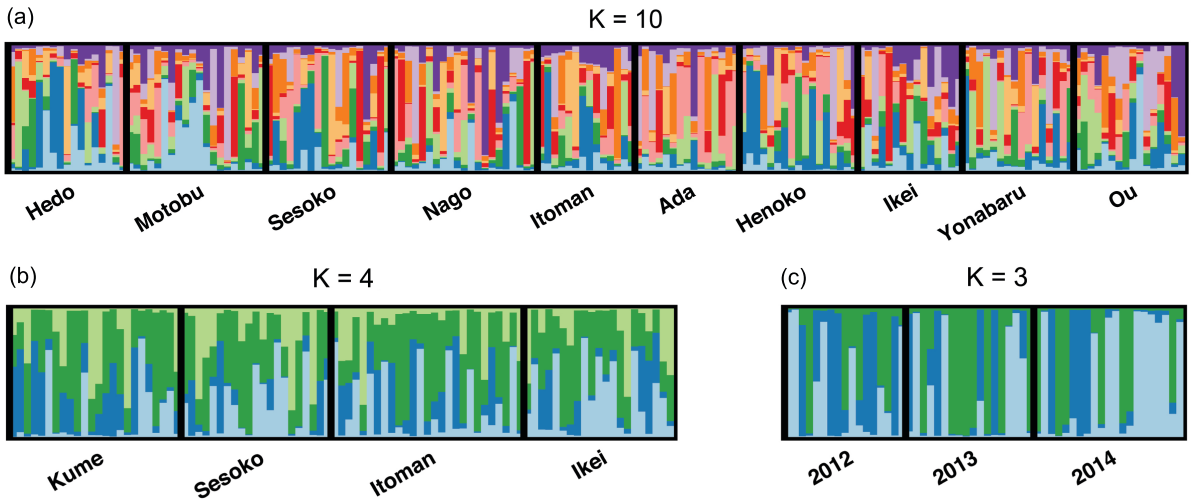

Figure 5.7 Posterior probabilities from the *STRUCTURE* analyses for individual *Siphamia tubifer* that were assigned to the number of groups (K) indicated. Analysis of (a) individuals collected in 2013 (b) individuals collected in 2014 and (c) individuals collected from the Sesoko site over three consecutive years: 2012-2014. The K values depicted represent the number of groups sampled for that dataset. Each bar represents an individual fish and the colors within each bar correspond with the probability of membership in a genotypic group. Individuals are grouped by sampling locations, which are divided by black lines

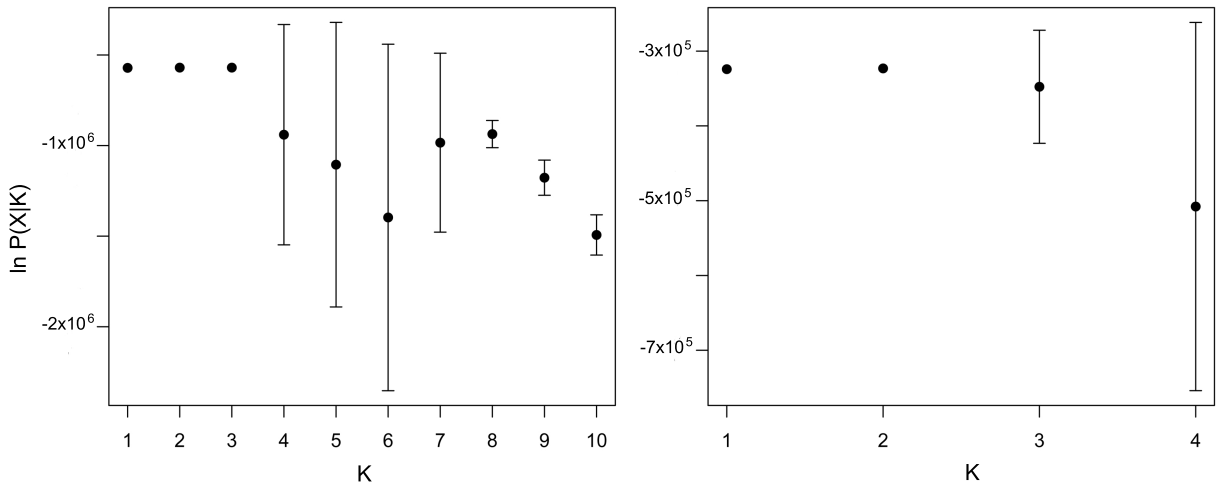

Figure 5.8 Mean estimates (±SD) of the posterior probability of each K value from the *STRUCTURE* analysis (Pritchard 2000) of *Siphamia tubifer* for the 2013 (left) and 2014 (right) datasets

CHAPTER VI

Population genomics of *Photobacterium mandapamensis*[5]

Abstract

The study of biogeography provides insights into the mechanisms that promote the evolutionary processes of diversification, speciation, and extinction. Until recently, this field has been dominated by studies of macroorgansims, limiting our understanding of the processes that have produced the immense diversity of bacteria described to date. Additionally, the classic view in microbiology is that "everything is everywhere", especially in highly connected marine environments. In this study, I introduce the application of a recently developed reduced representation genomic method to define and compare patterns of the population genomic structure of both a host fish and its bacterial symbiont in a specific, pairwise bioluminescent symbiosis. I show significant genetic differentiation between light organ symbiont populations 100 km apart, despite genetic admixture in the host fish at this scale. I also demonstrate temporal stability of the luminous symbiont populations over a three-year period. Collectively, our results provide evidence of fine-scale biogeographic patterns of a facultative marine symbiont and suggest a host-mediated mechanism for promoting genetic divergence of bacterial symbiont populations. In doing so, I highlight the effectiveness of restriction site associated sequencing as a tool to investigate the biogeographic patterns of symbiotic associations and of natural bacterial populations.

[5] *In preparation* as: Gould AL and PV Dunlap. Host-mediated symbiont divergence in a bioluminescent vertebrate-microbe symbiosis.

Introduction

The classic view in microbial biogeography that "everything is everywhere, but the environment selects" was proposed nearly a century ago (Baas-Becking 1934) and remains a central belief of many microbiologists today. This perspective has been particularly upheld for microbes living in highly connected ocean environments, for which numerous studies have portrayed cosmopolitan distributions (Mullins *et al.* 1995, Darling *et al.* 2000, Massana *et al.* 2000, Morris *et al.* 2002, Finlay 2002, Fenchel and Finlay 2004, Finlay and Fenchel 2004, Baldwin *et al.* 2005). However, as molecular methods advanced and genetic examinations of microorganisms accumulated, an increasing number of studies revealed unexpected patterns of geographic structure in certain marine bacteria (Staley and Gosink 1999, Cho and Tiedje 2000, Whitaker *et al.* 2003, Ivars-Martinez *et al.* 2008), although many of these patterns were observed at broad geographic or taxonomic scales (Fuhrman *et al.* 1992, Pommier *et al.* 2005, 2007, Brown *et al.* 2012) or can be attributed to environmental factors (Karner *et al.* 2001, Herndl *et al.* 2005, Martiny *et al.* 2006). Despite the increasing evidence of biogeographic patterns of microbes, few studies have examined the spatial scale of intra-specific patterns of marine bacterial populations. Using a novel application of recently developed molecular methods, I provide evidence of host-mediated population genomic structure of a symbiotically luminous bacterium within a homogeneous marine environment. Our results suggest that the distinct ecology of a host animal can help to promote the genetic divergence of a facultative bacterial symbiont through the local enrichment and isolation of symbiont populations over host generations, further challenging the conventional panmictic view of marine microorganisms and broadening our understanding of the mechanisms that can promote speciation in bacteria.

The primary mechanisms by which bacterial diversity is believed to arise are by lateral gene transfer (LGT), homologous recombination, and the accumulation of mutations that can result in a competitive advantage upon which selection can act. This view of adaptive bacterial evolution is primarily supported by the large size of most bacterial populations and by the ability of bacteria to disperse freely, thereby limiting the potential of neutral processes like genetic drift to promote divergence. Facilitating these processes, however, the physical isolation of microbial populations

can facilitate microbial diversification (Papke and Ward 2004), as is the case for certain extremophiles associated with hot springs (Papke *et al.* 2003) and hydrothermal vents (Whitaker *et al.* 2003). Additionally, host organisms can act as ecological 'islands" for microbial populations, promoting isolation over time without physical barriers to dispersal and gene flow (Papke and Ward 2004, Taylor *et al.* 2005). This has been well-documented for many vertically transmitted host-microbe associations, for which co-evolution and diversification have been documented and can even result in symbiont genome reduction over time (reviews by Baumann 2005, Dale and Moran 2006, Moran *et al.* 2008, McCutcheon and Moran 2011). However, the role of a host organism in promoting population divergence of horizontally acquired symbionts is much less understood.

Bacterial symbionts acquired from the environment can persist, although often dormant or inactive, outside of their host for variable amounts of time. Consequently, horizontally transmitted symbionts have greater dispersal potential than do their vertically transmitted counterparts. This is particularly true in the marine environment, in which ocean currents can maintain environmental homogeneity over large distances and promote dispersal and population connectivity. Therefore, in open marine environments lacking physical barriers, the role of host organisms as "islands" for their microbial symbionts may be especially important in promoting microbial diversification (Taylor *et al.* 2005). To better understand this potential, I examined a highly specific, pairwise association between the coral reef-dwelling, sea urchin cardinalfish *Siphamia tubifer* and the luminous bacterium *Photobacterium mandapamensis*, a member of the Vibrionaceae, in the Okinawan Islands of Japan.

A bioluminescent vertebrate-microbe symbiosis

The symbiosis involving *S. tubifer* and *P. mandapamensis* is a mutualistic association in which the symbiotic bacterium is provisioned in a ventral light organ by the host fish in exchange for light production. The fish host, which remains quiescent among the long spines of sea urchins during the daytime (Lachner 1955, Eibl-Eibesfeldt 1961, Tamura 1982, Gould *et al.* 2014), uses the bacterially produced light to illuminate its ventral surface while foraging at night. The light organ, containing a dense population of *P. mandapamensis* (~10^{7-8} cells, Gould *et al.* 2016), is connected by

a duct to the host's gut, and luminous bacterial cells are released periodically from the duct into the intestines and eventually, into the seawater with fecal material (Dunlap and Nakamura 2011). This purging of bacterial cells from the light organ presumably acts to promote growth and light production by the symbiont population.

The initiation of the symbiosis occurs during larval development, however, aspects of this critical process remain undefined, including the location and timing of symbiont acquisition in the wild as well as the number of bacterial cells that initially colonize a light organ. In culture, the light organs of *S. tubifer* larvae were not receptive to colonization by *P. mandapamensis* until at least eight days of having been released into the plankton; therefore, the direct transfer of symbiont from parent to offspring is not possible (Dunlap *et al.* 2012). The planktonic larval duration of *S. tubifer* is approximately 30 days (Gould *et al.* 2016), and the light organs of all juvenile fish collected from reefs have an established symbiont population, suggesting that the symbiosis becomes established between day eight in the plankton and settlement onto a reef. How long a light organ remains receptive to colonization by *P. mandapamensis* and how many symbiont cells initially infect a light organ have yet to be determined. The host fish also exhibits fidelity to a host urchin at a reef and will return to that reef if displaced distances of at least 2 km (Gould *et al.* 2014). Therefore, once settled out of the plankton, *S. tubifer* remain associated with a home reef through the rest of their lifespan, which is typically less than 200 days (Gould *et al.* 2016).

In addition to being both biologically and experimentally tractable (e.g. Dunlap *et al.* 2012, Gould *et al.* 2014, 2015), this pairwise association appears to be more specific in comparison to other bioluminescent fish symbioses (Kaeding *et al.* 2007), thereby providing an ideal binary association to investigate the ecological processes that lead to the evolution of host-bacteria associations, including other vertebrate-bacteria symbioses. To do so, I applied a recently developed molecular method that utilizes next generation sequencing technology to examine the influence of the host's ecology on the fine-scale genomic patterns of its luminous bacterial symbiont.

Application of RAD-Seq methods

Restriction site associated sequencing methods were developed within the past decade (review by Rowe *et al.* 2011) but have not been used to examine the genetic

structure of natural bacterial populations to date. This reduced representation genomics approach has the ability to produce genome-wide markers that can be used to detect fine scale patterns of population structure without any prior genetic information of the focal species. Therefore the application of RAD-Seq methods has become widespread and it has the potential to expand our understanding of the biogeographic patterns of microbial populations and the processes that shape them.

In this study, I used double digest, restriction-site associated sequencing (ddRAD-Seq) methods to define and compare the population genomic structure of both the host fish and its light organ symbionts. In doing so, I identified thousands of single nucleotide polymorphisms (SNPs) throughout the host fish's genome and hundreds of variant sites throughout the bacterial symbiont's genome (Figure 6.1) with which Idefined and compared patterns of population structure of host and symbiont. Specifically, using the restriction enzymes *EcoRI* and MseI, I identified 607 90-bp regions (RADtags) distributed randomly throughout the *P. mandapamensis* genome with sequence variability with which to examine population genomic structure of the luminous symbiont populations. Using these variable loci, I addressed the following questions: Is there population genomic structure of the light organ symbionts of *S. tubifer* at spatial scales ranging from 2 to over 100 kilometers and between years at the same location? and if symbiont population genomic structure exists, does it correlate with patterns of genomic structure of the host?

Symbiont population differentiation

I sampled the light organs of hundreds of *S. tubifer* specimens from various locations in the Okinawa Islands over a range of spatial scales and in three consecutive years (Table 6.1) to examine the effects of local symbiont enrichment on the population genomic structure of *P. mandapamensis*. Upon examination of the 607 RADtags throughout the *P. mandapamensis* genome (Figure 6.1), I detected a significant signature of population genomic structure between the light organ symbionts from Kume and Okinawa Islands, which are approximately 100 km apart (Figure 6.2). Additionally, the genetic distance between light organ symbiont populations correlated significantly with geographic distance ($r = 0.19$, $P = 0.001$), and there was a significant effect of sampling location on genetic distance (Table 6.2). In

contrast, the host fish exhibited no genomic structure at this spatial scale (Figure 6.2) and showed no significant signature of isolation by distance (Gould *et al. in review*). Consequently, there was also no significant correlation between the observed genetic distances of populations of the symbiont and that of their hosts (r = 0.59, P = 0.201).

At the finer geographic scales of tens of kilometers examined, symbiotic *P. mandapamensis* around Okinawa Island were more genetically homogeneous, as were populations of their hosts (Figure 6.3); although a significant correlation between the genetic distance of light organ symbionts and geographic distance was also evident at this scale (r = 0.052, P = 0.001). As seen at the regional scale, there is no correlation between genetic distance of the host and symbiont at these scales as well (r = 0.45, P = 0.17). The ocean currents in the study region are largely influenced by the Kuroshio Current, which provides the potential for long distance dispersal and mixing of free-living marine microbes over a broad geographic area. This mixing potential is made evident by the genetic admixture observed in the host fish at the same geographic scales examined (Figures 6.2, 6.3, Gould *et al. in review*). Future studies over broader geographic ranges and in regions with weaker currents might reveal even higher levels of genetic divergence of symbiont populations and perhaps of the host fish as well.

To test the temporal stability of the symbiont population, I examined the genomic structure of *P. mandapamensis* from the light organs of *S. tubifer* collected from one location (Sesoko, Figures 6.2, 6.3) in three consecutive years. In contrast to the regional scale differences observed, populations of symbiotic *P. mandapamensis* were not significantly different between years (Figure 6.4, Table 6.2). Therefore populations of light organ symbionts appear to be temporally stable over host generations at a particular reef.

The observed genetic differentiation between populations of symbiotic *P. mandapamensis* suggests that the luminous symbionts acquired by *S. tubifer* larvae that settle on reefs at Kume and Okinawa Islands are from genetically differentiated symbiont pools in the seawater. Moreover, the genetic panmixia observed for the host fish in the region indicates that *S. tubifer* larvae settling at both islands might have originated from the same southern source population, including the Philippines (Gould *et al. in review*). Consequently, it is unlikely that larvae remain near their natal reef during symbiont acquisition and there must be some mechanism serving to promote the isolation of *P. mandapamensis* genotypes that colonize the light organs of *S.*

tubifer between Kume and Okinawa Islands. The currents are highly mixed in the region and there are no apparent differences in environmental conditions between the two islands, therefore I propose that the distinct ecology of the host fish is facilitating the observed symbiont population differentiation.

The distinct life history and behavioral ecology of *S. tubifer* as a coral reef dwelling cardinalfish of releasing excess symbiont cells from the light organ and returning to the same home reef site after foraging can enrich the seawater at a reef with the *P. mandapamensis* genotypes present in light organs of the resident host fish population at that reef. This continual local enrichment of the seawater with symbionts can consequently be promoting the genetic isolation of *P. mandapamensis* populations over time, and is the most likely mechanism behind the observed regional pattern of genomic differentiation between symbiotic populations of *P. mandapamensis.* The local enrichment of symbiotic *P. mandapamensis* by the resident *S. tubifer* population at a reef also supports the temporal stability of light organ symbiont populations that I observed at the Sesoko site. This example of host-mediated bacterial divergence provides evidence of how patterns of biogeographic structure of marine microbes can arise in the absence of physical boundaries to dispersal and gene flow in the marine environment.

Loci correlated with population divergence

Between symbiotic populations of *P. mandapamensis* at Kume and Okinawa Islands, I examined specific loci potentially driving the divergence patterns observed. Of the 607 RADtags examined throughout the *P. mandapamensis* genome, 32 loci located at various sites throughout the genome contained haplotypes that were highly correlated (either positively or negatively) with the primary axis of genetic variance differentiating the Kume and Okinawa Island populations (Figure 6.5). Of these 32 candidate loci, only two were in non-coding regions of the genome and 18 had haplotypes that were positively correlated with the primary axis of variation driving the genetic divergence between the light organ symbionts at Kume and Okinawa Island (Table 6.3). Nucleotide substitutions within these haplotypes were primarily non-synonymous (76%) but showed no obvious pattern of functional effects (Table 6.4).

The environmental conditions within the study region are presumably uniform with respect to nutrient availability and other abiotic factors affecting *P. mandapamensis* fitness. Similarly, conditions within the host light organs between sampling locations are expected to be constant, especially considering the host fish sampled are one panmictic population (Gould and Dunlap *in review*). The patterns of genetic divergence observed for the bacterial symbionts in this study do not appear to be due to a selective advantage between the genotypes at these locations, but rather are a result of neutral processes that carried genetic novelties through the symbiont population over host generations.

Symbiont acquisition

The RAD-Seq methods applied also enabled us to infer previously undefined aspects of symbiont acquisition by developing *S. tubifer* larvae in the wild. First, the regional scale of genetic differentiation observed between symbiotic populations of *P. mandapamensis* suggests that settling larvae acquire a symbiont from the locally enriched waters near the adult host population at their settlement reef; therefore, I deduce that the window of time during which a light organ remains receptive to colonization by a luminous symbiont lasts through larval development until *S. tubifer* are near their settlement site. Second, I determine the minimum possible number of *P. mandapamensis* genotypes that initially colonized a light organ as the maximum number of haplotypes across all 607 RADtags examined within each light organ. On average, 6.0 ± 1.6 (S.D.) distinct symbiont genotypes were observed within a light organ, and the minimum and maximum numbers of strains across all 282 light organs were 2 and 9; no monocultures of light organ symbionts were evident (Figure 6.6). Furthermore, to determine whether the observed symbiont diversity was due to multiple colonization events over time or is a reflection of the number of cells that initially colonize a light organ, I tested for an increase in symbiont diversity over time, using fish standard length as a proxy for age (Gould *et al.* 2016). There was no correlation between fish length and minimum number of symbiont genotypes ($F_{1,290}$ = 1.477, R^2_{Adj} = 0.00164, P = 0.225), indicating that continual colonization by new *P. mandapamensis* cells throughout a host's lifespan is unlikely. This also corresponds with the observation of the regular release of symbiotic bacteria out of the duct, the

only pathway connecting the light organ to the rest of the host fish (Dunlap and Nakamura 2011).

The rapid accumulation of mutations giving rise to new symbiont genotypes within a light organ after colonization is also unlikely because older *S. tubifer* do not have higher light organ symbiont diversity than do younger individuals. Therefore, the genetic diversity of *P. mandapamensis* within a light organ is a direct reflection of the initial colonizing cells, and on average at least six distinct *P. mandapamensis* genotypes enter a light organ during the window of colonization. This is in contrast to the sepiolid squid symbiosis, for which light organ crypts are initially colonized by at most two luminous bacterial cells (Wollenberg and Ruby 2009). Despite the opportunity for multiple luminous bacterial cells to colonize a light organ, the *S. tubifer – P. mandapamensis* symbiosis remains highly specific (Kaeding *et al.* 2007) indicating that additional mechanisms (i.e. physiologic or genetic) function to maintain the specificity of the association. Future investigations of these mechanisms are needed and will provide further insight into the evolution and maintenance of other vertebrate-microbe symbioses.

Discussion

The concurrent genomic analyses of the host fish and light organ symbiont populations using ddRAD-Seq methods suggests that *S. tubifer* larvae disperse significant distances within the Okinawan Islands and acquire a symbiont from the enriched environment near the resident host population at their non-natal settlement site. The mechanism of bacterial symbiont enrichment by a marine host was first proposed by Ruby *et al.* (1980) to explain the unexpected, high concentrations of a luminous bacterium in seawater sampled at several hundred meters, the typical depth inhabited by midwater, luminous fishes. The release of luminous bacterial symbionts from light organs into the surrounding seawater was also demonstrated in the laboratory for other symbiotically luminous fishes (Haygood *et al.* 1984, Nealson *et al.* 1984) and observed in natural habitats in Hawaii of the squid host, *Euprymna scolopes* (Lee and Ruby 1994), which was similarly linked with local geographic symbiont population structure (Wollenberg and Ruby 2009). Furthermore, evidence of a non-

random geographic distribution of particular strains of *Photobacterium leiognathi* was observed in the light organs of other luminous fish hosts (Ast *et al.* 2007). These examples of symbiont enrichment further support our conclusion that host animals can shape the distribution patterns of their facultative marine symbionts and can subsequently promote the isolation of distinct symbiont genotypes over time.

Our study also highlights the effectiveness of the application of RAD-Seq methods to define and compare the fine-scale patterns of genomic structure of an animal host and its symbiotic bacteria. This approach can be used to examine an array of other host-microbe associations, including other bioluminescent symbioses and those involving vertebrate hosts. Such future applications will broaden our understanding of the influence host animals have on the biogeographic patterns of their microbial symbionts and the evolutionary processes that lead to host-symbiont integration and specificity. Moreover, RAD-Seq methods provide a genome-wide snapshot of nucleotide sequence information that can be applied to examine other natural populations of bacteria without any prior genetic information. The accessibility of this powerful genomic method therefore has the potential to further advance our limited view of microbial population genomics and biogeography beyond the broad taxonomic and geographic scales of most studies to date.

The study of biogeography provides insights into the mechanisms that promote the evolutionary processes of diversification, speciation, and extinction. Until recently, this field has been dominated by studies of macroorgansims, limiting our understanding of the processes that have produced the immense diversity of bacteria I see today. In this study, I examined a pairwise vertebrate-microbe symbiosis to highlight the role of a host animal as a promoter of genetic divergence of its bacterial symbiont. Furthermore, in highly connected ocean environments where the classic view of microbial biogeography suggests that “everything is everywhere” I illustrated that fine-scale patterns of genomic population structure can arise, even in a region dominated by strong ocean currents and without physical barriers to gene flow, when facilitated by a host organism. The environmental symbiont enrichment behavior of *S. tubifer* ensures that the next generation of host fish can initiate its bioluminescent symbiosis with its particular luminous bacterial partner, a critical process for the success of the host. This host facilitation functions to maintain the high degree of specificity observed of the partnership over time, and can incidentally, promote

symbiont population divergence. Overall, using a novel molecular approach, this study provides evidence of the genetic divergence of a facultative symbiotic bacterium with fine-scale resolution and reveals host-mediated microevolutionary processes that can lead to diversification and ultimately speciation in a marine bacterium.

Methods

Sampling, DNA extraction and library preparation

Siphamia tubifer specimens were collected over three years from various locations in the Okinawa Islands, Japan (Table 6.1). Ten collection sites were sampled during June and July of 2013, and in June 2014, light organs were sampled from *S. tubifer* at Kume Island as well as from fish at three sites previously sampled in 2013. One reef site (Sesoko, "S") that was sampled in 2013 and 2014 was also sampled in July of 2012, providing a three-year dataset from that location. Fish ranging in body lengths and associated with different host sea urchins were targeted for collection at each sampling location. Fish were immediately euthanized upon collection following an approved protocol by the University of Michigan's Institutional Animal Care and Use Committee and in accord with animal handling guidelines of the University of the Ryukyus Guide for Care and Use of Laboratory Animals.

The whole, intact light organ, composed of fish tissue and containing the luminous bacterial symbiont population, was aseptically removed from each fish specimen and individually preserved in RNAlater®. Genomic DNA was extracted from each light organ using a QIAGEN DNeasy Blood and Tissue Kit following the manufacturer's protocol, and a total of six double digest restriction site-associated sequencing (ddRAD-Seq) DNA libraries were built from the DNA of 50 *S. tubifer* light organs, each individually barcoded with unique 10 bp DNA sequences for downstream identification. The protocol used to construct the ddRAD-Seq libraries followed a modified combination of the methods in Parchman *et al.* (2012) and Peterson *et al.* (2012), using the restriction enzymes *MseI* and *EcoRI,* and is described in further detail in Gould and Dunlap (*in review*). All DNA libraries were sequenced at the Center for Applied Genomics, Toronto, ON, Canada, on the Illumina HiSeq2000 platform (San

Diego, CA) generating100 bp single-end reads.

Sequence processing and data analysis

Raw sequence reads were de-multiplexed and quality-filtered for a Phred score of 33 or higher with the *process_radtags* command in *Stacks* v1.35 (Catchen *et al.* 2011, 2013). After the DNA sequence barcodes were removed, the remaining 90-bp sequence reads were aligned against the ~4.5 Mb reference genome of *Photobacterium mandapamensis* (Urbanczyk *et al.* 2011) using the *very_sensitive* command in *Bowtie2* v2.2.0 (Langmead and Salzberg 2012) to remove the fish sequences from the bacterial dataset. Aligned *P. mandapamensis* sequences in *.SAM* file format were then additionally quality filtered using *SAMtools* v1.3 (Li *et al.* 2009), retaining only reads with a MAPQ score greater than 10. The quality-filtered, aligned *P. mandapamensis* sequences were then processed with the *ref_map* command in *Stacks*, requiring a minimum stack depth of three (-m 3). Individuals with fewer than 100,000 total sequence reads or a mean depth of coverage per stack less than 100x were discarded from the analysis (Figure 6.7), resulting in 282 light organs included in the genetic analysis (Table 6.1).

Loci present in at least 200 light organs with a maximum of 25 SNPs per locus across all samples were extracted from the entire catalog of loci produced by *Stacks* for the analysis. Subsequently, loci not present in at least 50% of individuals in each population were removed from the dataset. The final dataset consisted of 607 loci (RADtags), from which haplotypes with at least 5x coverage within a light organ were analyzed for patterns of genomic structure between symbiont populations of the groups of host fish. To analyze patterns of both spatial and temporal genomic structure, locations sampled in 2013 and 2014 were analyzed separately and the Sesoko site was analyzed over three consecutive years. Principal coordinates analyses (PCoA) were performed on the Bray-Curtis distance matrices calculated from the presence or absence of each symbiont haplotype across the 607 RADtags within a light organ. Calculations were carried out with the “vegan” package (Oksanen *et al.* 2016) in R v.3.1.1 (R Core Team 2014).

Analyses of variance were performed with the *adonis* function to test for the effects of various factors on the genetic distance matrices calculated across individuals

within each dataset (2013, 2014, Sesoko). Factors tested included RAD library, sampling site, region, year, as well as all possible interaction effects of these factors (Table 6.2). Additional analyses of similarity (ANOSIM) were performed on the same Bray-Curtis distance matrices to test for genetic differences between groups within each factor above. Tukey's post hoc tests were carried out on factors that had a significant effect in the ANOSIM to identify significantly different levels within that factor. To test for pearson correlations between genetic and geographic distances, Mantel tests with 1,000 permutations were performed between the Bray-Curtis distance matrix calculated for light organ genotypes and a matrix of pairwise distances, estimated as the shortest straight-line distance through water between collection sites. All analyses were performed with the "vegan" and "ecodist" (Goslee and Urban 2007) packages in R.

Patterns of genetic structure between the luminous bacterial symbiont and the host fish were also compared by first analyzing the genomic structure of the host fish at the same scales as the bacterial symbiont. Briefly, *S. tubifer* sequence reads were assembled *de novo* in *Stacks* and genotypes across a set of identified loci were assigned to all individuals (see Gould *et al. in review* for details). In this study, I analyzed a subset of the genetic data of *S. tubifer* presented in Gould and Dunlap (*in review*). Specifically, the first single nucleotide polymorphism (SNP) within each of 8,637 loci present in 70% of individuals in each population with a minor allele frequency of 5% or greater were analyzed across the host fish from the 282 light organs described previously. A principal components analysis (PCA) was performed on the *S. tubifer* SNP dataset with the "adegenet" package (Jombart 2008, Jombart and Ahmed 2011) in R. Pairwise genetic distances between each individual were calculated with the Kosman and Leonard (2005) method using the "PopGenReport" package (Adamack and Gruber 2014) in R. The genetic distance matrix of the host fish was then compared to that of its light organ symbionts with a Mantel test as described above.

To examine loci driving the patterns of genetic divergence observed between light organ symbionts at Kume and Okinawa Islands, a constrained analysis of principal coordinates (CAP) was performed on the 2014 *P. mandapamensis* genetic distance matrix with "vegan" in R. The resulting CAP scores for each light organ and for all haplotypes examined across all individuals were examined to identify haplotypes driving the observed patterns. The light organ symbionts from Kume Island

were divergent from the other populations primarily along the CAP1 axis (Figure 6.5). Haplotypes with the highest and lowest CAP1 scores were therefore identified as candidate drivers of divergence of *P. mandapamensis* populations and the loci to which they belong were identified (Table 6.3). Differences in nucleotide sequences within the outlier haplotypes were then analyzed and changes between the Kume and Okinawa populations were identified as synonymous or non-synonymous and categorized as having low, moderate, or a modifying effect on the gene products with the SnpEff (Cingolani *et al.* 2012) program (Table 6.4).

Table 6.1. Sampling locations of *Siphamia tubifer* light organs in the Okinawan Islands, Japan for the population genomic analysis

ID	Site	Latitude	Longitude	Year	N
S	Sesoko	26.635409	127.865832	2012	16
				2013	18
				2014	21
M	Motobu	26.655806	127.880286	2013	20
N	Nago	26.603673	127.932404	2013	21
Hd	Hedo	26.848756	128.252513	2013	17
It	Itoman	26.095109	127.658478	2013	14
				2014	27
O	Ou	26.127916	127.768981	2013	16
Y	Yonabaru	26.203032	127.771178	2013	16
Ik	Ikei	26.393535	127.988601	2013	15
				2014	22
Hk	Henoko	26.534554	128.046181	2013	17
A	Ada	26.741936	128.321107	2013	16
K	Kume	26.351617	126.820149	2014	26

Table 6.2. Results of the analyses of genetic variance (Adonis) and similarity (ANOSIM) across the datasets indicated. For each model, the factors' degrees of freedom (DF), sum of squares (SS), mean of squares (MS), predicted F-value (F), R^2 and P-value are shown. Library refers to which RAD-Seq library an individual was sequenced in, Region indicates whether an individual was sample from Kume Island, or the West or East Coast of Okinawa Island, and Location refers to the sampling location of an individual. Non-significant P-values are listed as NS (>0.1)

Model	Adonis test							ANOSIM		
	DF	SS	MS	F	R^2	P		F	P	
All samples										
Library	1	0.350	0.350	3.115	0.011	0.001	***	9.739	0.000	***
Region	2	0.458	0.229	2.041	0.014	0.001	***	30.387	0.000	***
Location	11	1.739	0.158	1.407	0.053	0.001	***	11.903	0.000	***
Year	1	0.112	0.112	0.996	0.003	0.488	NS	0.801	0.450	NS
Library:Region	2	0.259	0.130	1.155	0.008	0.042	*	-	-	-
Library:Location	9	0.952	0.106	0.942	0.029	0.930	NS	-	-	-
Library:Year	1	0.113	0.114	1.010	0.003	0.445	NS	-	-	-
2014										
Library	1	0.096	0.096	0.836	0.009	0.933	NS	0.083	0.775	NS
Region	2	0.546	0.273	2.377	0.049	0.001	***	23.102	0.000	***
Location	1	0.165	0.164	1.433	0.015	0.007	**	18.172	0.000	***
Library:Region	2	0.242	0.121	1.052	0.022	0.261	NS	-	-	-
Library:Location	1	0.085	0.085	0.739	0.008	0.991	Ns	-	-	-
2013										
Library	1	0.379	0.379	3.405	0.020	0.001	***	29.298	0.000	***
Region	1	0.126	0.126	1.132	0.007	0.132	NS	20.345	0.000	***
Location	8	1.133	0.142	1.272	0.059	0.001	***	10.821	0.000	***
Library:Region	1	0.105	0.105	0.945	0.005	0.667	NS	-	-	-
Library:Location	6	0.663	0.111	0.993	0.034	0.555	NS	-	-	-
2013, w/o Lib. 2										
Library	1	0.243	0.243	2.323	0.018	0.001	***	0.531	0.589	NS
Region	1	0.122	0.122	1.168	0.009	0.076	.	4.001	0.048	*
Pop	6	0.637	0.106	1.014	0.047	0.359	NS	1.561	0.153	NS
Library:Region	1	0.105	0.105	1.005	0.008	0.464	NS	-	-	-
Library:Location	6	0.663	0.111	1.056	0.049	0.116	NS	-	-	-
Sesoko										
Library	1	0.152	0.152	1.447	0.027	0.002	**	0.212	0.930	NS
Year	1	0.110	0.110	1.046	0.019	0.300	NS	0.027	0.974	NS
Library:Year	1	0.105	0.105	0.995	0.018	0.499	NS	-	-	-

Table 6.3. RADtags within the *Photobacterium mandapamensis* genome correlated with the primary axis of genetic variation driving the patterns of divergence between symbiont populations at Kume and Okinawa Islands, Japan. Listed are the RADtag ID, whether haplotypes within the locus are positively or negatively correlated with the axis of variation, the scaffold in which a locus is located, and the assigned COG group and the putative gene product of the protein coding region in which the RADtag is located

RADtag	Correlation	Scaffold	COG	Gene product
47	-	II	K	DNA-directed RNA polymerase subunit beta
92	+	II	J	RNA-binding protein Hfq
166	+	II	M	rare lipoA family protein
356	-	IV	R	smr domain protein
600	+	IV	-	non-coding
667	+/-	IV	O	molecular chaperone DnaK
712	+	IV	K	transcriptional regulator
715	-	IV	G	PTS acetylglucosamine transporter subunit IIB
145	-	IV	H	thiamine biosynthesis protein ApbE
239	-	IV	R	radical SAM protein
286	+	IV	S	hypothetical protein
355	+	IV	V	acriflavin resistance protein
375	-	IV	G	D-hexose-6-phosphate mutarotase
391	-	IV	O	clpX -ATP-dependent Clp protease ATP-binding subunit ClpX
794	+/-	V	G	phosphoglycerate kinase
811	+	V	T	hybrid sensor histidine kinase/response regulator
822	-	V	J	alanine--tRNA ligase
890	-	VI	C	glutathione-disulfide reductase
991	-	VII	C	glycerol dehydrogenase
1089	+	VIII	-	non-coding
1235	-	VIII	M	penicillin-binding protein1B; penicillin-sensitive transpeptidase
1254	+	VIII	R	phenazine biosynthesis protein PhzF
1261	-	VIII	G	chitinase
1269	+	VIII	P	cobalamin ABC transporter substrate-binding protein
1407	+	VIII	E	glycine dehydrogenase (aminomethyl-transferring)
1417	+	VIII	R	GTPase YlqF
1429	+	VIII	R	paraquat-inducible protein B (pqiB)
1098	-	VIII	T	transcriptional regulator
1160	+	VIII	R	conserved repeat domain protein
1162	+	VIII	S	hypothetical protein
1163	-	VIII	S	hypothetical protein
1195	-	VIII	M	AsmA family protein

Table 6.4. Effects of the nucleotide substitutions examined within the divergent *Photobacterium mandapamensis* RADtag sequences between Kume and Okinawa Islands. The ID of each variant corresponds to each locus' RADtag ID. Multiple variant regions examined within a single RADtag are indicated as _n, where n corresponds to the order in which that variant appears in the 90 bp sequence. Also listed are the types of variants and the degree to which they affect function as determined with the SnpEff program (Cingolani *et al.* 2012)

ID	Type	Effect
92	synonymous variant	low
166_1	downstream gene variant	modifier
166_2	downstream gene variant	modifier
286	downstream gene variant	modifier
355	missense variant	moderate
600	upstream gene variant	modifier
667	upstream gene variant	modifier
712	upstream gene variant	modifier
794	missense variant	moderate
811	upstream gene variant	modifier
1089_1	upstream gene variant	modifier
1089_2	upstream gene variant	modifier
1160	synonymous variant	low
1162	synonymous variant	low
1235	missense variant	moderate
1254	upstream gene variant	modifier
1269	synonymous variant	low
1407	upstream gene variant	modifier
1417_1	upstream gene variant	modifier
1417_2	upstream gene variant	modifier
1417_3	synonymous variant	low

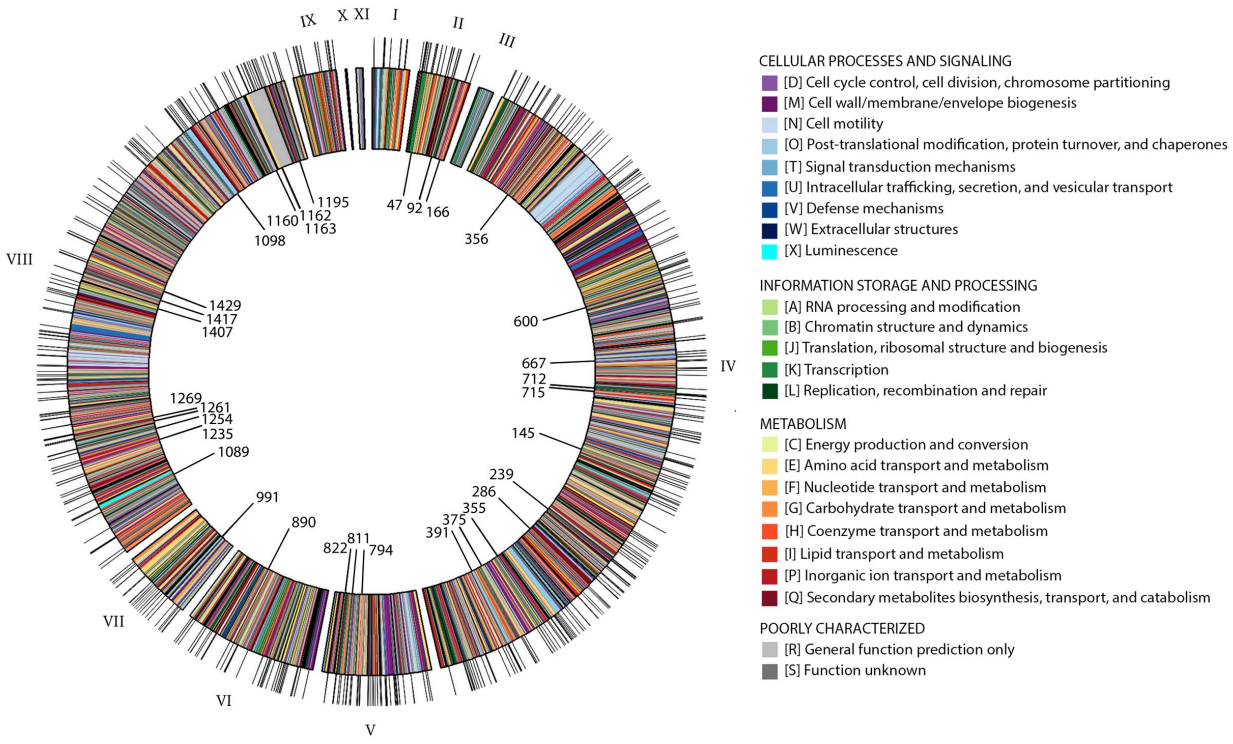

Figure 6.1. Genome map of *Photobacterium mandapamensis* (Urbanczyk *et al.* 2011), the light organ symbiont of *Siphamia tubifer*. Sequence scaffolds I - XI are indicated on the outside of the map. Protein-coding genes are color coded according to the COG categories listed. Outer tick marks represent the locations of the 607 90-bp RADtags analyzed for genomic differentiation. Candidate RADtags identified as potential drivers of population differentiation between Kume and Okinawa Islands are highlighted on the inner circle of the map

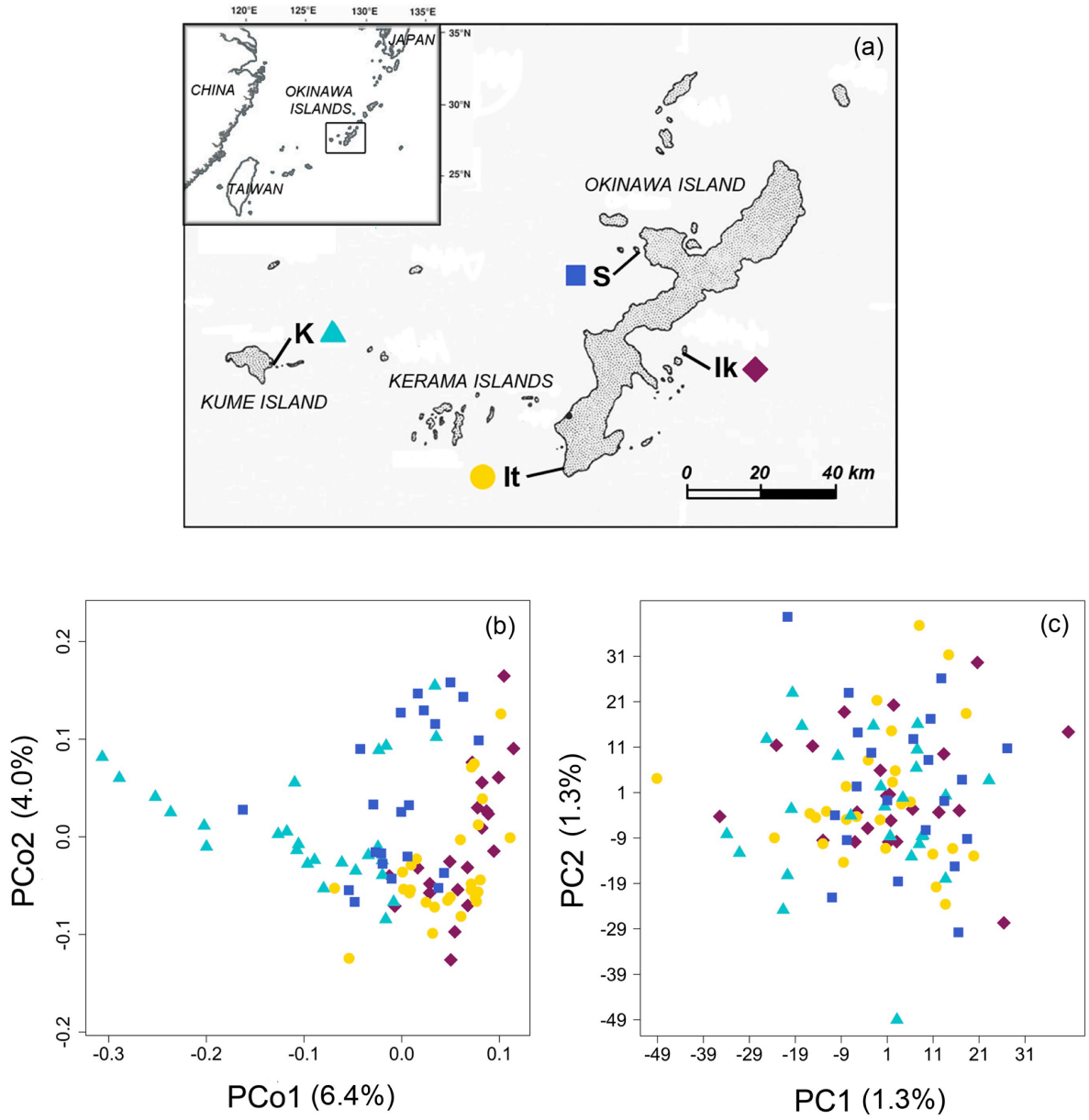

Figure 6.2. Genomic analysis of host and symbiont sampled from fish light organs in 2014. (a) Sampling locations of *S. tubifer* light organs in the Okinawan Islands, Japan. (b) Principal coordinates analysis of genetic differentiation of symbiotic *P. mandapamensis* calculated from Bray-Curtis distances of the presence or absence of haplotypes within a light organ across 607 RADtags identified throughout the bacterial genome. (c) Principal components analysis of the genetic differentiation of the corresponding *Siphamia tubifer* hosts based on 8,637 SNPs. Points in (b) and (c) represent individuals along the first and second axes of genetic variation with different colors and shapes representing the sampling locations in (a)

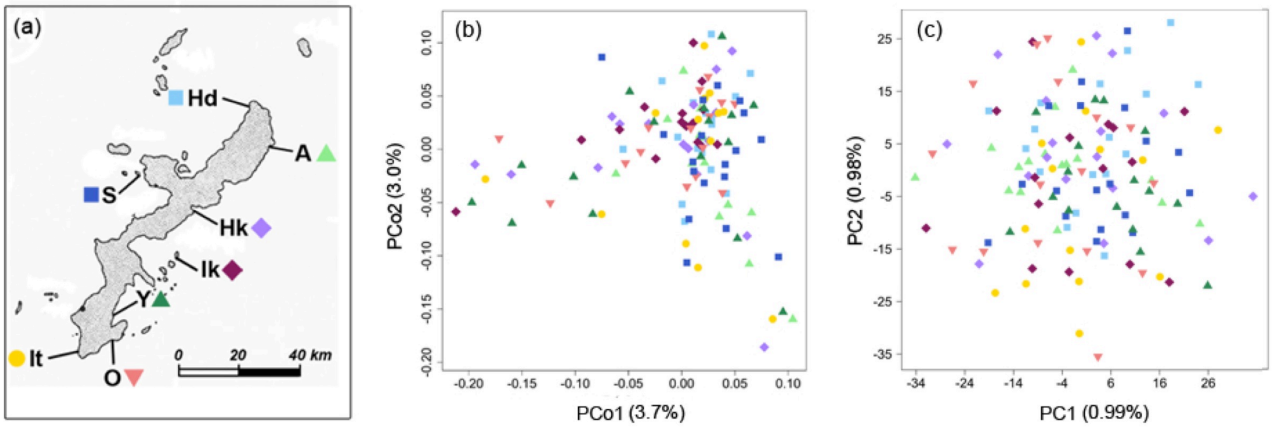

Figure 6.3. Genomic analysis of host and symbiont sampled from fish light organs in 2013. (a) Sampling locations of *S. tubifer* light organs around Okinawa Island, Japan. (b) Principal coordinates analysis of genetic differentiation of light organ symbiont population across the 607 identified RADtags for *P. mandapamensis*. (c) Principal components analysis of the genetic differentiation of the corresponding *Siphamia tubifer* hosts based on 8,637 SNPs. Points in (b) and (c) represent individuals along the first and second axes of genetic variation with different colors and shapes representing the sampling locations in (a)

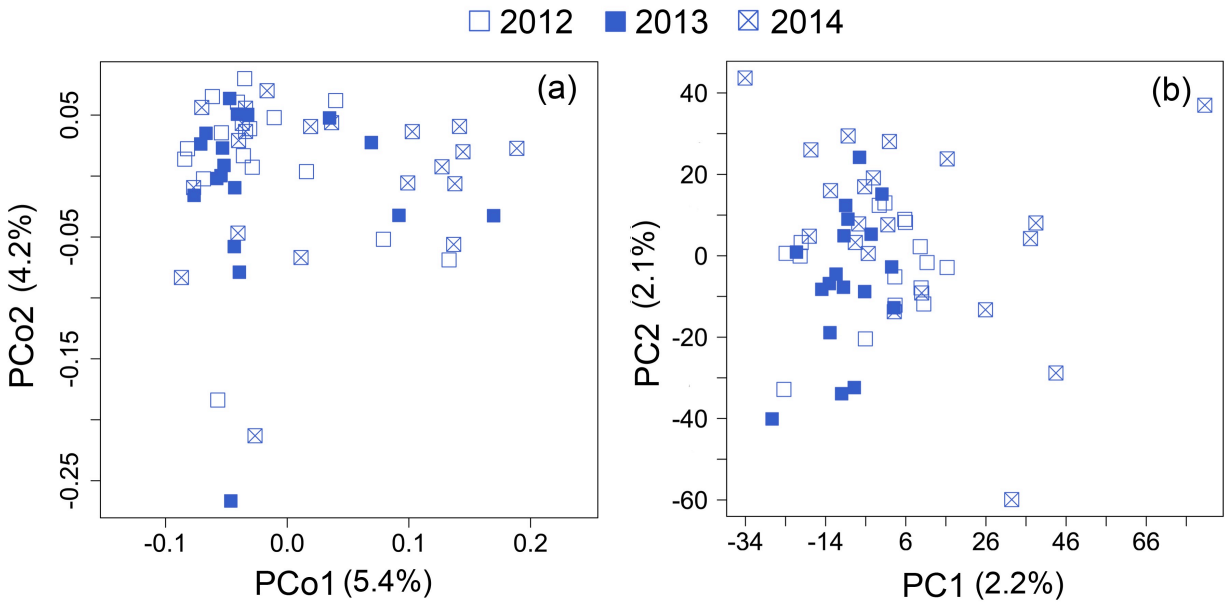

Figure 6.4. Genomic analysis of host and symbiont sampled from fish light organs at Sesoko Island in Okinawa, Japan in 2012, 2013, and 2014. (a) Principal coordinates analysis of genetic differentiation of symbiotic *P. mandapamensis* across 607 RADtags throughout the bacterial genome. (b) Principal components analysis of the genetic differentiation of the corresponding *Siphamia tubifer* hosts based on 8,637 SNPs. Points represent individuals along the first and second axes of genetic variation with different shapes representing the sampling year

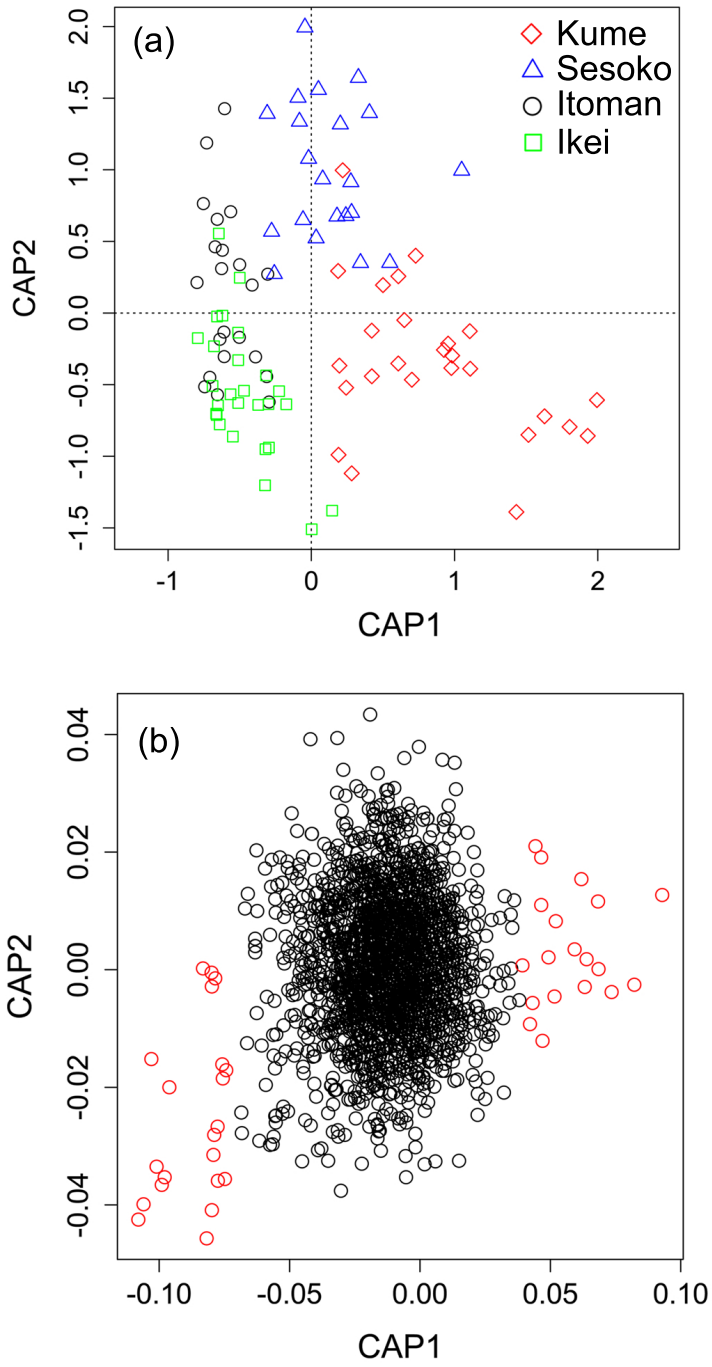

Figure 6.5. Results of the constrained analysis of principal coordinates (CAP) on the 2014 genetic distance matrix of *Photobacterium mandapamensis* haplotypes across 607 RADtags. The resulting CAP scores (a) for each light organ and (b) for all haplotypes within each RADtag. The following colors and shapes in (a) represent each sampling location: Kume Island (red diamonds), Sesoko (blue triangles), Itoman (black circles), Ikei (green squares). In (b), outlier haplotypes as putative drivers of the divergence patterns between light organ symbiont populations are highlighted in red

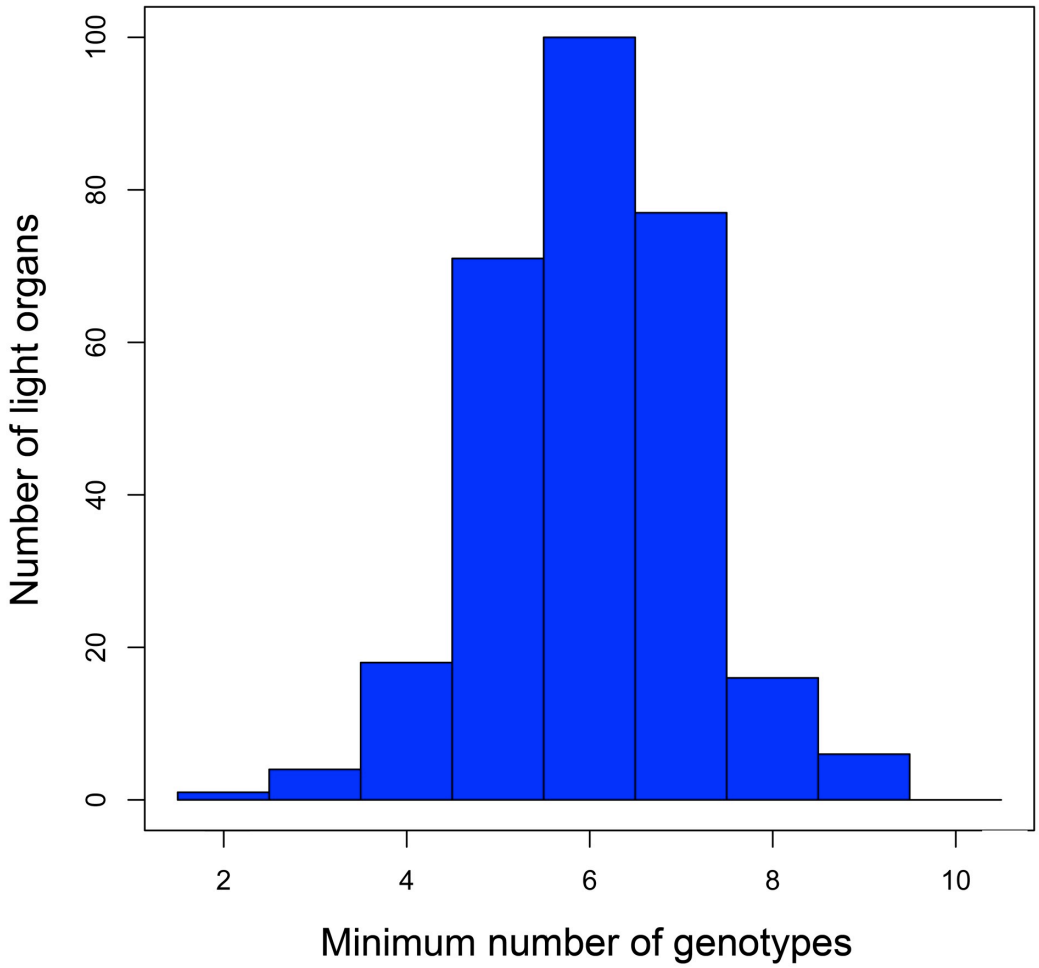

Figure 6.6. Frequency distribution of the minimum number of *Photobacterium mandapamensis* genotypes within *Siphamia tubifer* light organs (N = 282) as determined by the maximum number of haplotypes observed across all 607 RADtags within a light organ

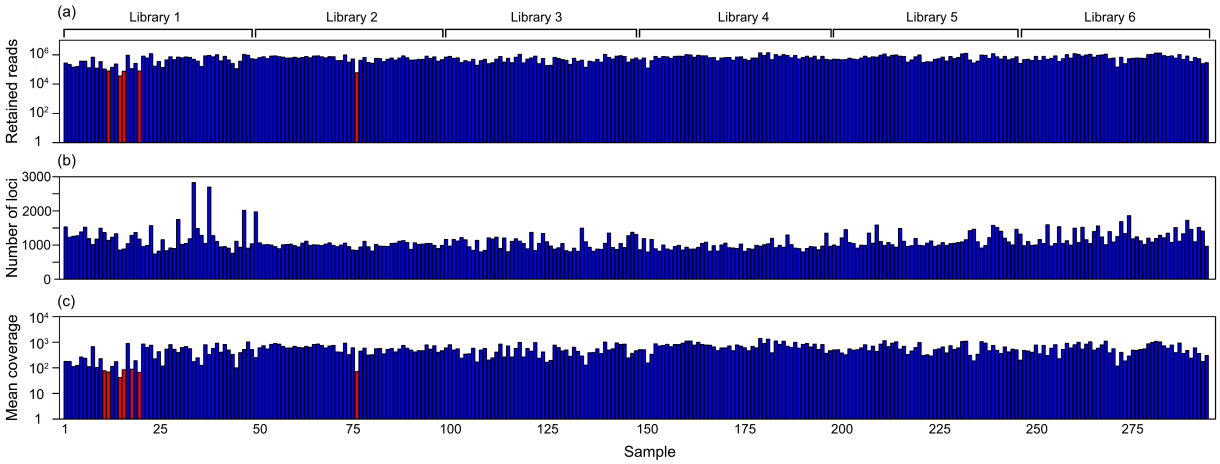

Figure 6.7. Summary of *Photobacterium mandapamensis* sequence reads produced from each RAD-Seq library. (a) The number of aligned *P. mandapamensis* reads retained after quality filtering in *Stacks* (Catchen *et al.* 2011, 2013) for each light organ. Individuals that were discarded form the analysis due to a low number of sequence reads are in red. (b) The number of sequenced loci per light organ as determined with the *ref_map* command in *Stacks* within the *P. mandapamensis* genome. (c) Mean sequence coverage per locus across all loci for quality-filtered individuals. Individuals that were discarded from the analysis due to low mean coverage are in red

CHAPTER VII
Conclusion

The primary objectives of my dissertation were to define key characteristics of the biology and behavioral ecology of a host fish that can facilitate the maintenance specificity of a horizontally acquired bioluminescent symbiosis and to test whether such characteristics influence the process of symbiont acquisition by developing larvae, and hence, the specificity of the symbiosis. To address these objectives, I examined a model vertebrate-microbe symbiosis involving the sea urchin cardinalfish, *Siphamia tubifer*, and the facultative symbiotic bacterium, *Photobacterium mandapamensis*, which is both experimentally tractable and maintains high specificity in the marine environment. I integrated life history descriptions with behavioral field experiments of the host fish and population genomic studies of both the host and bacterial symbiont to investigate potential mechanisms involved in the establishment and maintenance of specificity for a pairwise marine symbiosis over host generations. The main results of my dissertation research are as follows:

(1) *Siphamia tubifer* has similar life history attributes as other small-bodied coral reef cardinalfish (Perciformes: Apogonidae), including short longevity, a fast initial growth rate, and early age at first reproduction. However, *S. tubifer* has a slightly longer pelagic larval duration of approximately 30 days in comparison to other similar apogonid fishes.

(2) *Siphamia tubifer* exhibited daily fidelity to a particular host urchin at a reef and returned to that reef when displaced at least 2 kilometers. Both adults and juveniles exhibited homing behavior, although larger fish were more successful at returning to their home reef.

(3) Adult and juvenile *S. tubifer* recognized and preferred the chemical signature of their home reef water to that of unfamiliar reef water. Adults and juveniles also preferred seawater conditioned by their luminous bacterial symbiont but not by their host urchins over unconditioned seawater. Juvenile *S. tubifer* but not adults preferred the chemical cue of conspecifics.

(4) Within the Okinawa Islands, Japan, *S. tubifer* is genetically admixed; populations ranging from 2 to 140 kilometers apart were not genetically differentiated and showed no signature of isolation by distance. During the month-long pelagic larval duration, *S. tubifer* larvae can disperse significant distances due to the strong current patterns in the region.

(5) Despite genetic admixture of the host fish at the same geographic scale, symbiotic populations of *P. mandapamensis* from the light organs of *S. tubifer* were genetically divergent between reefs 100 kilometers apart. Light organ symbiont populations are more genetically admixed at smaller spatial scales of tens of kilometers around Okinawa Main Island, but a significant correlation still exists between genetic and geographic distances at this scale.

Overall, the combined results of my dissertation chapters provide evidence that the life history and behavioral ecology of *S. tubifer* as a coral reef cardinalfish help to maintain and promote the specificity of its pairwise symbiosis with *P. mandapamensis* over host generations. Specifically, the daily release of excess *P. mandapamensis* cells from the light organs of the resident *S. tubifer* population at a reef combined with the host fish's fidelity to that reef provides a constant source of symbiotic *P. mandapamensis* cells to the surrounding seawater. The light organs of *S. tubifer* larvae are not receptive to colonization by *P. mandapamensis* until at least eight days of having been released into the plankton (Dunlap *et al.* 2012) and larvae can travel significant distances during their 30-day larval phase before finding a suitable reef upon which to settle. Based on the olfactory preferences of newly settled *S. tubifer*, larval fish might also have the ability to recognize the chemical signature of both conspecifics and their luminous symbiont in the seawater, and can use these cues to locate and navigate to a reef with an established resident population of *S. tubifer*. The

bacterial pool surrounding a settlement site is enriched with symbiotic *P. mandapamensis* genotypes from the light organs of the host fish at that reef, therefore the larvae settling at that reef are more likely to encounter symbiotic *P. mandapamensis* genotypes that are abundant in the resident host population at that reef. This proposed mechanism of maintaining specificity of the symbiosis between host generations through the local symbiont-enrichment of the seawater can also incidentally promote genetic divergence of symbiotic *P. mandapamensis* populations by isolating the light organ symbiont populations between regions and enabling genetic differences within these populations to accumulate over time. The patterns of genetic divergence observed between light organ symbionts from Kume and Okinawa Islands support this hypothesis and provide evidence that host organisms can facilitate the diversification of marine bacteria.

Future Directions

This research further establishes the *S. tubifer-P. mandapamensis* symbiosis as a model symbiosis for future studies of bacterial associations and as a vertebrate counterpart to the bioluminescent invertebrate squid symbiosis. Yet many aspects of the *S. tubifer –P. mandapamensis* association remain undefined or poorly understood, one of which remains the specificity of the symbiosis. Future studies are warranted to address whether the specificity of the association is attributed solely to the host-mediated mechanisms described here, or whether other factors such as host physiology and genetics also help to maintain the pairwise association. For example, additional light organ colonization experiments in which the luminous symbionts of other host animals are presented to *S. tubifer* larvae in culture would reveal whether *S. tubifer* is capable of establishing a symbiotic association with any luminous bacterium other than *P. mandapamensis*. The results of such studies might also lead to future questions of the cellular processes involved in symbiont recognition and colonization of the light organ.

Additional studies expanding upon the olfactory preferences of *S. tubifer* presented here can also enhance our understanding of symbiont acquisition and larval recruitment. Specifically, the preferences of *S. tubifer* larvae for the chemical cues of

other marine bacteria, both luminous sand non-luminous, can be determined to test the robustness of the olfactory preference for *P. mandapamensis* observed here. Similarly, the density of *P. mandapamensis* required to evoke an olfactory response by *S. tubifer* can be tested, and this density threshold can then be compared to the density of *P. mandapamensis* cells in the seawater surrounding a resident *S. tubifer* population. Results of this comparison would provide a better indication as to whether *S. tubifer* does in fact use the chemical cue of its luminous symbiont to detect and navigate to a settlement reef, and perhaps, even for symbiont acquisition.

Thus far, only the luminous symbionts of *Siphamia tubifer* from the Okinawa Island region of Japan have been identified. Whether the *S. tubifer-P. mandapamensis* symbiosis maintains the same level of specificity over the fish's entire geographic range remains unknown. Similarly, it is unknown whether other *Siphamia* spp. exhibit the same degree of specificity with *P. mandapamensis* or are hosts to other luminous bacteria and whether the symbiosis has remained stable over time. Future studies identifying the light organ symbionts of *S. tubifer* across the host's entire Indo-Pacific home range, from southern Africa through the Indian Ocean and into Australia, will reveal the degree to which the specificity of the symbiosis with *P. mandapamensis* is conserved. Additionally, the stability of the symbiosis over time can be examined by comparing museum specimens collected nearly a century ago to *S. tubifer* specimens collected from the same locations today. Such an examination of the temporal stability of the symbiosis might reveal more insight into the evolutionary processes that lead to host-symbiont integration and specificity.

Future research efforts can also focus more broadly across the entire *Siphamia* genus, which in addition to being the only symbiotically luminous group of cardinalfishes, diverged approximately 50 million years ago from all other apogonids (Cowman and Bellwood 2011, Thacker 2014), is apparently monophyletic (Mabuchi *et al.* 2014), and is the third most speciose of the 40 cardinalfish genera (Froese and Pauly 2015) (Figure 7.1). The importance of microbial symbionts in providing new function to their hosts, often enabling the exploitation of new resources is highly recognized (Margulis and Fester 1991); yet there are still few examples of symbiont-assisted speciation in the literature (Brucker and Bordenstein 2012). Future investigations of the role of the symbiosis in promoting the initial divergence of *Siphamia* as well as facilitating speciation within the *Siphamia* genus can provide a

better understanding of the evolution of specialized pairwise associations between vertebrate hosts and their microbial symbionts over evolutionary time, potentially broadening our understanding of symbiont-assisted speciation.

Significance

Symbioses with microorganisms are ubiquitous in nature and can provide new functions to their hosts, yet I still know relatively little regarding the processes involved in the establishment and maintenance of symbiotic associations between host generations. This is especially true for horizontally transmitted symbioses in which symbionts are acquired from the environment. Through an integrative investigation of a highly specific, bioluminescent vertebrate-microbe symbiosis, my dissertation research exemplifies the importance of a hosts' ecology in promoting a pairwise symbiotic association over time.

Furthermore, the role of microbial symbionts in altering animal behavior has been studied in some detail (review by Shropshire and Bordenstein 2016), but there are fewer examples of the effects of animal behavior on their microbial symbionts (Ezenwa *et al.* 2012). In my dissertation, I demonstrate that the behavioral ecology of *S. tubifer* has significant effects on the biogeography of its symbiotic luminous bacterium, promoting population divergence over time. Such genetic divergence is the first step towards diversification and ultimately, speciation; therefore, my research highlights the potential for host animals to play an important role in promoting speciation in bacteria.

Host organisms might play an especially critical role in bacterial diversification in the oceans, where microorganisms have few physical boundaries to dispersal and consequently have been thought to have very cosmopolitan distributions. Yet there is a immense diversity of bacterial species in the ocean, suggesting that genetic divergence and speciation has occurred regularly through time and creating a gap in our understanding of microbial marine biogeography and diversification processes. The hypothesis of host organisms as "ecological islands" that can both alter the biogeographic patterns of bacterial populations and promote the isolation of genetic novelties between different populations is one potential mechanism that narrows this

gap, and the *S. tubifer-P. mandapamensis* association is one example, building a foundation for future research in this area.

In a broader sense, understanding the ecological processes involved in the establishment and maintenance of species-specific symbioses over time is especially important today as rapidly changing environments threaten to de-couple these associations. Yet the ecological interactions that facilitate the maintenance of most symbiotic associations remain poorly understood. My research demonstrates the importance of a host organism's daily behavior and ecology to its symbiosis with a specific bacterium. With this knowledge, improved predictions as to how the association might respond to future environmental changes can be made. For example, if olfaction is essential for *S. tubifer* recruitment and symbiont acquisition as suggested, then environmental changes, such as ocean acidification, that can affect the olfactory abilities of *S. tubifer* might have detrimental effects on the symbiosis. Therefore, with improved knowledge of the behavioral and ecological mechanisms involved in maintaining symbiotic associations, the fate of these critical partnerships can be better predicted in our changing world.

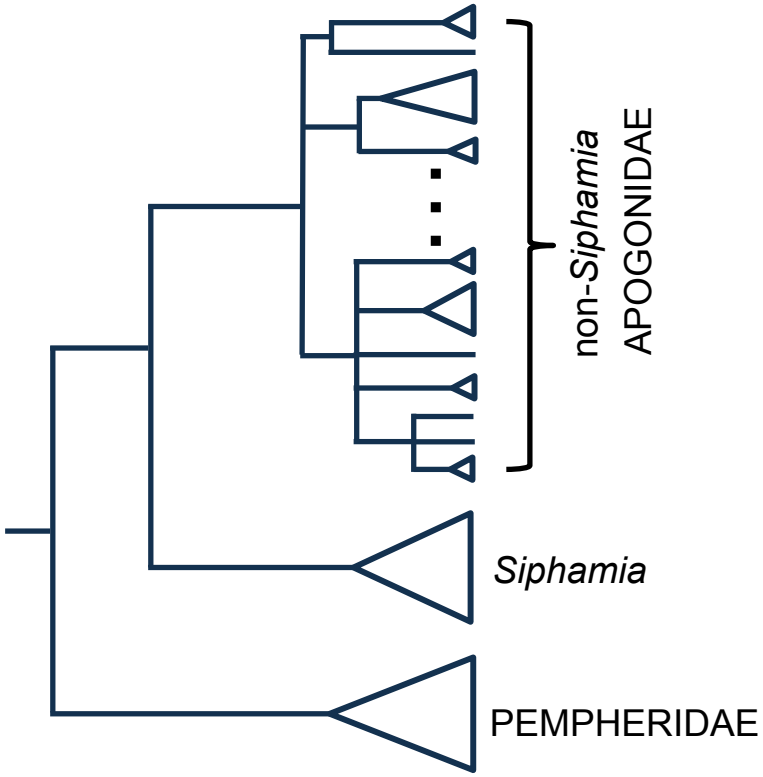

Figure 7.1. Non-representative depiction of the phylogenetic relationships among Apogonoidei fishes and the relative membership to various Apogonidae genera including *Siphamia* (simplified from Thacker 2014)

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