CONSERVATION GENETICS OF RARE TREE SNAILS FROM THE HAWAIIAN AND MARIANA ISLANDS: DISTRIBUTION OF GENETIC DIVERSITY AT VARYING SCALES OF POPULATION FRAGMENTATION AND ISOLATION

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I dedicate this dissertation to my parents Shelly and Richard Sischo, and grandparents David and Patricia Sischo for instilling in me a fascination of the natural world and for always encouraging, with love and support, my pursuit of higher education, even if that meant moving to a far away island. I also dedicate this dissertation to my niece Athena Sischo, whom at only four years of age has a love of nature that I recognize in myself. Athena, may your curiosity lead you to many fascinating discoveries. Last but not least, I dedicate this dissertation to my partner Mathew Lam who has put up with me during this process, and who has provided countless hours of moral support and grammar checks. I bet you never thought you'd know so much about snails…

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ABSTRACT

Island archipelagos across the Pacific have hosted spectacular land-snail radiations. Unfortunately, due primarily to habitat fragmentation and loss and the introduction of invasive snail predators, these spectacular island radiations are now facing rapid extinctions. Although much effort is focused on tree-snail conservation, little is understood regarding how fragmentation and isolation impact the dynamics and distribution of genetic diversity in remaining populations. This dissertation consists of three separate studies using conservation genetics to broadly investigate how fragmentation and isolation of populations, at varying scales, impacts populations of tree snails, and what can be done to better manage genetic diversity to prevent extinction. First, a phylogenetic approach was used to assess the distribution of extant tree snails in the family Partulidae of the Mariana Islands. This study focused on *Partula gibba*, a species that has an unusually large and fragmented distribution across nine islands. A cryptic species endemic to the island of Rota was found, as well as two distinct clades of *P. gibba* distributed across islands. Intra-island genetic diversity was low providing further evidence that *P. gibba* self-fertilizes and highlights the need to conserve populations across the range of *P. gibba* to prevent drastic losses of genetic diversity. Next, I assessed the population structure of *Partulina redfieldi,* a tree snail once widespread on the Hawaiian island of Moloka‘i, that now exits only in a small fragmented portion of its former range. The distribution of genetic diversity across habitat patches was assessed using 11 microsatellite loci. Evidence of reduced gene flow and significant isolation-by-distance effects between habitat patches over very short distances (>20 meters) was observed. Lastly, demographic and genetic methods were used to assess a drastic population decline occurring over 20 years and four generations in an *ex situ* population of *Achatinella fuscobasis*, a tree snail endemic to the Hawaiian Island of O‘ahu. While there is evidence that the founding individuals came from an already bottlenecked population, there was no additional change in genetic diversity measures such as allelic richness, gene diversity, and observed heterozygosity among all generations. Due to the signal of a selection event in the F_3 generation, we now suspect

the population decline may be stochastic in nature, with bottleneck effects that occurred before the founding of the ex situ population, acting synergistically with other factors.

TABLE OF CONTENTS

Page

DEDICATION.....ii

ACKNOWLEDGEMENTS.....iii

ABSTRACT.....iv

LIST OF TABLES.....viii

LIST OF FIGURES.....ix

CHAPTER 1: Introduction.....1

Background…….....1

Fragmentation, isolation and small population size.....5

Purpose and Content of Chapters.....6

References……7

CHAPTER 2: PHYLOGEOGRAPHIC RELATIONSHIPS BETWEEN MULTI-ISLAND POPULATIONS OF *PARTULA GIBBA* IN THE MARIANA ISLANDS; CRYPTIC SPECIES AND LONG DISTANCE DISPERSAL **……**…….…...12

Abstract……..12

Introduction……12

Materials and methods…..……..16

Results……20

Discussion……..23

References……..30

CHAPTER 3: CONSERVATION GENETICS OF THE HAWAIIAN TREE SNAIL *PARTULINA REDFIELDI*: DISTRIBUTION OF GENETIC DIVERSITY IN A FRAGMENTED LANDSCAPE…..…….…….....36

Abstract……..36

Introduction……36

Materials and Methods……..41

Results……46

Discussion……..52

References……..55

CHAPTER 4: *EX SITU* POPULATION GENETICS OF THE ENDANGERED HAWAIIAN TREE SNAIL ACHATI*NELLA FUSCOBASIS* (ACHATINELLINAE): DEMOGRAPHIC AND GENETIC INSIGHTS INTO A CAPTIVE POPULATION DECLINE……..……..……...61

Abstract……..61

Introduction……61

Materials and methods…..……..63

Results……67

Discussion……..71

References……..75

LIST OF TABLES

Page

2.1 Island source, sampling localities and number of species and tissue samples collected ……17
2.2 Average CO1 and 16S sequence divergence…….....22
3.1 Loci summery table……..... 42
3.2 Genetic measures calculated globally and for each subpopulation…….48
3.3 Effective population size calculated globally and for each subpopulation……..49
3.4 F_{ST} Between all subpopulations……...49
3.5 Analysis of molecular variance (AMOVA)…….50
4.1 Primer information……...64
4.2 Loci summery table……..65
4.3 Genetic measures by generation……..70

LIST OF FIGURES

Page

2.1 Map of the Mariana Islands……15
2.2 *Partula gibba* and *Partula langfordi* shell comparisons.....20
2.3 Phylogenetic reconstruction for the Mariana Island Partulidae…...……...21
2.4 Scattergram of shell measurements.....…….23
2.5 *Partula gibba* clades……24
3.1A Adult *Partulina redfieldi*…..……..... 39
3.1B Fragmented patch of habitat……..39
3.2 Map of sample collection localities……40
3.3 Map of subpopulation locations…….……..44
3.4A and B Isolation by distance regression analyses…….……..47
3.5 Deviance information criterion from TESS analysis.…..…..……...50
3.6 Cluster analysis results……...……..51
4.1 *Ex situ* population trajectory….……...67
4.2 Juvenile survival……..68
4.3 Adult survival…….……69
4.4 Genetic diversity measures compared to demographic trends……73

CHAPTER 1
INTRODUCTION

BACKGROUND

Nowhere are the eccentricities of evolution more evident than on islands. In fact, much of what we know about the mechanisms of evolution was first discovered on them. Isolated from the pressures associated with large landmasses, organisms evolve in much simpler ecosystems, often with reduced predation and competition (Darwin 1845, Darwin 1909, MacArthur and Wilson 1967,Wallace 1876, Wagner and Funk 1995). This, combined with geological variation within and between isolated island archipelagos, produces the most spectacular species radiations known to science (Grant 1997, Ridley 2004, Emerson 2008, Gillespie et al. 2008, Parent et al. 2008). This is nowhere more brilliantly evidenced than with the terrestrial snail fauna of Pacific Islands (Pilsbry and Cooke 1912 – 1914, Crampton 1916, 1925, 1932, Cowie et al. 1995, Cowie 1996). Besides insects, land snails constitute the majority of terrestrial animals on many islands.

Snails are excellent passive dispersers, as evidenced by their distribution on isolated landmasses. Transoceanic dispersal events occur via several mechanisms, including birds transporting minute snails inside their guts (Kawakami et al. 2008), or attached to their bodies and or feathers (Maciorowski et al. 2012, Shikov and Vinogradov 2013). Very minute land snails may be transported by wind (Vagvolgyi 1975), as well as rafting to islands attached to vegetation washed out to sea during tsunamis, hurricanes, or other stochastic weather events (Thiel and Haye 2006, Solem and Bruggen 1984). Though excellent at passive dispersal, snails' active dispersal abilities are limited. It is this lack of vagility and the gradual fragmentation and isolation of snail populations through time (via slow geologic processes like lava flows, or the erosion of mountains into distinct valleys and ridges), that have facilitated speciation of land snails on islands at incredible scales (Wagner and Funk 1995, Cook 2008, Rundell and Price 2009). Unfortunately, these island hotbeds of evolution are more recently sites of excessive extinctions. The same processes of fragmentation and isolation that facilitate the speciation of snails on millennial timescales, when at a faster pace drives species to extinction. Unchecked habitat fragmentation and isolation combined with predation by invasive species is causing land

snails to quickly disappear from oceanic islands (Hadfield 1986, Solem 1990, Cowie 1992, Hadfield et al 1993, Régnier 2009).

Three thousand eight hundred kilometers away from the nearest significant landmass, the Hawaiian archipelago is geographically the most isolated island chain in the world. This isolation, coupled with great habitat heterogeneity has given rise to one of the most stunning examples of land snail species radiations in the world with over 750 described species (Cowie et al. 1995). Unfortunately, what remains today of this once remarkable radiation is a mere shadow of its former diversity. It is estimated that 60% to 90% of Hawaiian land snail species are now extinct (Cowie et al. 1995, Solem 1990). Most of those that remain are threatened or endangered and isolated to small patches of native vegetation high on mountain ridges (Hadfield et al. 1993).

Known for their beautiful shells, Hawai‘i’s arboreal achatinelline snail species (tree snails) are the most studied of the native snail fauna (Gulick 1905, Hadfield and Mountain 1980, Hadfield and Miller 1989, Hadfield et al 1993, Kobayashi and Hadfield 1996, Hadway and Hadfield 1999, Holland and Hadfield 2002, Erickson and Hadfield 2014, Price and Hadfield 2014). Fine scale endemism and subsequent radiations have occurred on every island they inhabit (Holland and Hadfield, 2004). The decline of achatinelline tree-snail species began when early Polynesians settlers introduced a suite of alien species and cleared lowland forests (Kirch, 1982). This pattern of anthropogenic disturbance only increased with successive waves of settlers, furthering the habitat fragmentation and introducing new alien species.

Early Hawaiians crafted lei from the beautiful shells of the tree snails, (Bryan, 1935). Later, western naturalists and shell fanciers collected hundreds of thousands of shells in the late 1800s to early 1900s, undoubtedly extirpating populations and pushing some populations and species to extinction (Hadfield, 1986). Introduced predators continue to decimate populations. Several rat species introduced by both Polynesian and western sailors feed on native snails (Hadfield 1986, Hadfield et al. 1993, Hadfield and Saufler 2009). Snails of the predatory species, *Euglandina rosea,* introduced as a biocontrol in the 1950s to reduce numbers of the invasive African snail *Achatina fulica*, devour native snails (Schalie, 1969, Hadfield, 1986). Most recently, Jackson’s chameleon, *Chamaeleo jacksonii,* introduced from Kenya by the pet trade, has expanded its

range into snail habitat. Gut-content analysis has revealed a diet inclusive of native gastropods (Holland et al, 2009). Anthropogenic disturbance and introduced ungulates, including pigs, goats, cattle, and axis deer continue to degrade native forest and further fragment tree snail populations (Hadfield, 1986).

Achatinelline species have extremely long life spans and reach sexual maturity at approximately five to seven years of age. Once mature, adult snails give live birth to four to seven offspring a year (Hadfield 1986, Hadfield et al. 1993, Hadfield & Miller 1989). Compared to other terrestrial molluscs, such as the common garden snail *Helix aspersa*, which becomes reproductive in less than a year and lays up to 100 eggs at one time (Koene 1998), Hawaiian tree snails have extremely low fecundity and are maladapted to high levels of depredation.

Similar to the achatinelline radiations that occurred in the Hawaiian Islands, land snails of the South Pacific in the family Partulidae have radiated only on a much larger geographic scale, encompassing most of Polynesia and Micronesia (Crampton 1916, 1925, 1932, Cowie 1992). The most northwestern range of the family Partulidae is the Mariana Archipelago, which hosted five recorded species (Pilsbry 1909, Crampton 1925, Kondo 1970). Quite different from the achatinelline snails in Hawaii, partulids of the Mariana Islands have been known to inhabit more than one island, most notably the endemic snail *Partula gibba*, which occurs on multiple islands from Guam to Pagan Island 485 km to the north (Kondo 1970, Hadfield 2015).

The life history characteristics of *Partula gibba* and other partulid snails of the Mariana Islands have not been well studied. However, partulid species from other islands in the Pacific have been studied in detail, and their traits are most likely comparable. In general partulid species become sexually mature at one year and give live birth of up to 18 juveniles per year (Cowie 1992). All Partulidae are hermaphroditic, and laboratory reared *P. gibba* have been known to produce offspring through self-fertilization (Cowie 1992, Johnson et al. 1977).

While not as documented as the declines of achatinelline species in Hawai‘i, partulid species in the Marina Islands are undergoing similar reductions (Hopper and Smith 1992, Bauman 1996, Hadfield 2015). Since World War II anthropogenic disturbance in the Mariana Islands has been

great, particularly on Guam, Tinian, and Saipan (Morison 2007). Introduced ungulates including pigs, goats, cattle and deer destroy native vegetation and disrupt the natural moisture regimes of microclimates necessary for snail survival (Hopper and Smith 1992). In addition, molluscan predators, including the flat worm *Platydemus manokwari,* and the predatory snails *Gonaxis kibweziensis, G. quadrilateralis, and Euglandina rosea,* have been introduced to the islands, some of them on purpose to control outbreaks of the invasive Giant African snail *Acatina fulica* (Hopper and Smith 1992, Bauman 1996). Rats have been found to depredate Hawaiian tree snails (Hadfield and Saufler, 2009). Whileno comparable research has been conducted to evaluate this possibility in the Mariana Islands, it is suspected that rats also devour partulids (Hopper and Smith 1992).

Anthropogenic disturbance, invasive predators, habitat fragmentation, and a previous existence devoid of predators, have created a 'perfect storm' for the land snail fauna of Pacific Islands. However, the depressing reports and statistics heralding the decline of tree-snail species have not gone unnoticed. In 1981 the United States Fish and Wildlife Service officially included the entire genus *Achatinella*, endemic to the Hawaiian Island of O'ahu, on the Federal Endangered Species List (U.S. Fish and Wildlife Service 1981). The University of Hawaii's Tree Snail Captive Rearing Facility was subsequently founded with the immediate goal of preserving, in vitro, genetic diversity of endangered achatinelline species, and with the ultimate goal of reintroduction to the wild (Hadfield et al 2004). Recently, three more achatinelline species in two genera from the islands of Lana'i and Maui have been federally listed as Endangered (U.S. Fish and Wildlife Service 2013). Other efforts in Hawai'i include: intensive monitoring of existing populations, predator abatement, and the construction of several predator exclusion structures around select populations of *Achatinella* species in the Wai'anae and Ko'olau mountains of O'ahu.

In the Mariana Islands, *Partula gibba* is on the Guam Endangered Species List, and in 2013 all three extant Mariana partulid species were petitioned for listing on the U.S. Endangered Species List, including *Partula gibba, Partula radiolata*, and *Saomoana fragilis* (U.S. Fish and Wildlife Service, 2013). In addition, the London Zoo's *Partula* breeding program keeps a captive population of *P. gibba* from Guam, for possible reintroductions (Johnson et al, 1977).

FRAGMENTATION, ISOLATION AND SMALL POPULATION SIZE

Fragmentation of habitat and isolation of populations is one of the main drivers of biodiversity loss across the globe, and contributes significantly to the local extirpation and extinction of vulnerable species (Henle et al. 2004, Fischer and Lindenmayer 2007). The negative effects of habitat fragmentation are a result of the altered spatial distribution of available habitat. Once large continuous areas are broken into smaller patches, habitat area and quality are reduced (Fahrig 2003).

Small fragmented habitat patches support small populations, and in general small populations are vulnerable to losses of genetic diversity over time because the fates of alleles in small populations are subject to genetic drift rather than natural selection. Genetic drift occurs due to the random sampling of genes from one generation to the next. In small populations just by chance alone deleterious alleles may become fixed, while other alleles are lost forever (Freeland and Peterson 2011, Lacy 1987). In theory, as genetic drift lowers genetic diversity and increases homozygosity, the adaptive potential of a population decreases as well. This leaves small populations and species with limited numbers of individuals without the evolutionary flexibility to adapt to change, and makes them especially vulnerable to environmental perturbation, disease, excessive predation, competition, and long-term climate change (Frankham et al. 2002, Lacy 1987).

As population size decreases, the probability of mating between related individuals increases, even if random mating occurs (Keller 2002). Inbreeding, like genetic drift, leads to a loss of heterozygosity and lowers the genetic diversity of the afflicted population. Deleterious mutations occur frequently in large populations, however, because of higher heterozygosity, deleterious mutations can go unexpressed. Individuals homozygous for a lethal allele die, while heterozygous individuals mask the deleterious allele. In large populations the chance of mating occurring between individuals that carry the same deleterious allele is small. Increased homozygosity, as a result of reduced numbers and inbreeding, increases phenotypic expression of these deleterious traits. Offspring resulting from inbred matings often experience lower

fitness compared to outcrossed offspring, i.e. inbreeding depression (Hedrick and Kalinowski 2000, Frankham 1995). Inbreeding depression has been noted in laboratory populations (Saccheri et al. 1996, Keller, 2002) and population declines and extinction in small wild populations is linked to inbreeding depression (Saccheri et al. 1998).

In theory, the extent of inbreeding depression depends on the abundance of deleterious recessive alleles present in a population, termed genetic load. A species or population where inbreeding has occurred throughout its history often has less of a genetic load, because historic inbreeding events may have subjected deleterious alleles to selection, purging them from the gene pool. Such species may show high levels of inbreeding without the deleterious effects of inbreeding depression (Frankham, 1995). Theoretical and empirical studies suggest that for purging to successfully remove harmful alleles from a population, the deleterious effects of a given mutation must be strong in relation to the effective population size in question (Keller, 2002). These studies have questioned the capacity of selection to purge mildly deleterious alleles, or in other words, alleles that lower fitness but are not lethal. It is generally accepted that a mutation must be lethal for purging to occur. Mildly deleterious alleles may persist in a population and contribute to inbreeding depression by reducing fitness but not causing lethality (Hedrick, 1994, Frankham 1995, Keller, 2002).

PURPOSE AND CONTENT OF CHAPTERS

Much effort is currently underway to conserve the remaining imperiled tree snail species in the subfamily Achatinellinae and the family Partulidae from the Hawaiian and Mariana Islands, respectively. Unfortunately, despite all the effort, we still know very little about how best to conserve the genetic diversity of these species in light of habitat fragmentation, isolation and small population sizes. This dissertation consists of three separate studies, each focused on a different tree snail species and aimed at assessing population structure at varying degrees of habitat fragmentation and isolation in order to provide management recommendations. In Chapter 2 I employ phylogentic approach to assess the population structure of extant tree snails in the family Partulidae from the Mariana Islands. This study focuses primarily on the species *Partula gibba,* which has an unusually large and fragmented distribution spanning nine islands within the archipelago. In Chapter 3, I present the results of a study on *Partulina redfieldi,* an

achatinelline tree snail endemic and once widespread on the island of Moloka‘i, that now only exists in a small fragmented portion of its former range. I assess the distribution of genetic diversity across habitat patches using 11 microsatellite loci and quantify isolation by distance and bottleneck effects that have occurred as a result of fine-scale habitat fragmentation. Finally, in Chapter 4, I assess the demographic changes that have occurred from 1991 – 2014 in an isolated *ex situ* captive population of *A. fuscobasis*, an achatinelline tree snail endemic to the island of O‘ahu. Demographic data from this isolated population were compared to genetic data over three generations (F_1–F_3 spanning the years 1991–2010) collected from 11 microsatellite loci to investigate the role bottleneck effects, such as inbreeding and loss of genetic diversity, may have had on this isolated snail population through time.

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CHAPTER 2
PHYLOGEOGRAPHIC RELATIONSHIPS BETWEEN MULTI-ISLAND POPULATIONS OF *PARTULA GIBBA* IN THE MARIANA ISLANDS; CRYPTIC SPECIES AND LONG DISTANCE DISPERSAL

ABSTRACT

The tree snail family Partulidae is widely distributed throughout the islands of Micronesia, Melanesia and Polynesia, and most of the 120 described species are single island endemics. In this study, we investigated the evolutionary relationships among populations of *Partula gibba*, a species having an unusually large, multi-island distribution within the Mariana Archipelago. Understanding the evolutionary relationships between these multi-island populations has become imperative, because all species of partulid tree snails in the Mariana Islands are rapidly disappearing, due mostly to introduced predators. Here we analyze two mitochondrial genes, cytochrome oxidase subunit 1 (CO1) and 16s, and the nuclear gene fragment ITS2. The results of our analyses provide two conservation imperatives. (1) Populations of *P. gibba* on islands other than Rota, including Guam, Tinian, Saipan, Sarigan, and Pagan Islands form two well supported sister clades corresponding to the most southern and most northern islands. Very low intra-island diversity supports other evidence that *P. gibba* readily self-fertilizes and may explain why *P. gibba* has colonized nine islands within the Mariana Archipelago while other congeneric species, perhaps with less tendency for self-fertilization, are restricted to the three older southernmost islands of Guam, Rota and Agiguan. (2) All genetic evidence indicates that the species formally identified as *P. gibba* on the island of Rota is, in fact, not *P. gibba,* but rather a misidentified cryptic species.

INTRODUCTION

Tree snails in the family Partulidae are widespread throughout the high islands of Oceania. Spanning some 10,000 km, their historical distribution encompasses islands throughout Polynesia, Melanesia and Micronesia (Cowie, 1992). Although they managed to populate some

of the most remote archipelagos in the world, most of the 120 described species in three genera are single island endemics, with distributions restricted to island mountain ranges or even single valleys (Pilsbry 1909-1910, Cowie 1992). While a high degree of endemism is typical for partulids, exceptions occur. A few noteworthy species such as *Partula hyalina* from the Austral, Cook and Society Archipelagos, *Partula carteriensis* from the Bismark Archipelago, and *Partula gibba* from the Mariana Archipelago, all have multi-island distributions (Pilsbry 1909-10, Kondo 1955, Lee et al. 2007, Foighil et al. 2011).

The Mariana Archipelago lies along the subduction zone of the Philippine and Pacific tectonic plates, and consists of an arc of 15 islands ranging in age from one to five million years old (figure 2.1). The older islands in the south are composed mainly of uplifted coral limestone that sits atop the summits of subterranean volcanoes, while the younger islands in the north are completely volcanic, some of which are still active (Russel 1998). The partulids of the Mariana Islands occur in the northwestern-most extent of the family's range, and formerly included six described species. Guam, the southernmost and oldest island, had four species in two genera including *Saomoana fragilis, Partula radiolata, Partula salifana*, and *Partula gibba* (Crampton 1925, Kondo 1970). Of these species, only *Saomoana fragilis* and *Partula gibba* were documented on Rota, the next island north of Guam, as well as *Partula desolata* recently described from sub-fossil shells (Bauman and Kerr, 2013). On Agiguan, the next island north of Rota, *Partula langfordi* coexisted with *Partula gibba*. On islands north of Agiguan, including Tinian, Saipan, Anatahan, Sarigan, Alamagan, and Pagan, only *Partula gibba* is known to have occurred (Kondo 1970, Kurozumi 1994, Smith 2008, Hadfield 2014). *Partula gibba*, the subject of this investigation, has a known range spanning nine Mariana Islands, with a combined distance of approximately 485 km (Kondo 1970, Hadfield 2014).

Historical monographs and other descriptions of the partulid snail species of the Mariana Archipelago make no sub-species-level distinctions between populations of *P. gibba* from different islands. Diversity in shell color and morphometrics were considered within the normal range for the species (Crampton 1925, Kondo 1970). In general, early malacologists based taxonomic assignments of land snails solely on phenotypic characters, which was often limited to shell morphology and internal anatomy. It happens that, many of these distinctions were prone to

misinterpretations due to both cryptic as well as highly polymorphic snail species. Many recent re-evaluations of molluscan species radiations using molecular techniques have found that historical species delineations do not reflect true evolutionary patterns, the results of which are often polyphyly (Kruckenhauser et al. 2014, Holland and Hadfield 2007, Kameda et al. 2007, Goodacre and Wade 2001, Kohler and Johnson 2012).

Molecular phylogenetic analyses have placed *P. gibba* from Saipan*, P. langfordi* from Agiguan and *P. radiolata* from Guam, within the clade of *Partula* from the Western Pacific region (Goodacre and Wade 2001). However, no genetic work has been conducted specifically looking at populations of *Partula gibba* across its multi-island range. Kondo (1970), in his short collection of essays entitled "*Some Aspects of Mariana Islands Partulidae (Mollusca, Pulonata),*" noted that *P. gibba* appeared to be "endowed with certain characteristics which indicate a species about to undergo diversification in several directions." He was referring mainly to the abundance and universal distribution of *P. gibba* across many islands, and the species' great morphological variation from place to place. Unfortunately, today *P. gibba* appears to be a species on the verge of extinction, with local population extirpation on all islands where it occurs. This is consistent with the status of partulid species across the Pacific, which have experienced unprecedented levels of local extirpations and extinctions due to a variety of factors, primarily including introduced predators (Bauman 1996, Clarke 1984, Hopper and Smith 1992, Murry et al. 1988, Pelep and Hadfield 2011, Regnier et al 2009). Understanding the relationships among populations of *P. gibba* throughout the Mariana Islands has now become a conservation priority as all *Partula* spp. from these US administered islands are candidate species for listing under the US Endangered Species Act (ESA) (U.S. Fish and Wildlife Service, 2013).

Legislative and management decisions pertaining to conservation, particularly for species listed under the ESA, rely on the accurate identification of biological entities. In this study, we analyze the phylogeography of Mariana Island Partulidae using two mitochondrial genes, cytochrome oxidase subunit 1 (CO1) and 16S genes, and the nuclear ribosomal internal transcribed spacer 2 (ITS2) gene. The objectives of this study include (1) examination of the diversity of *P. gibba*

within and between islands; and (2) preliminary identification of cryptic species or sub-species level distinctions.

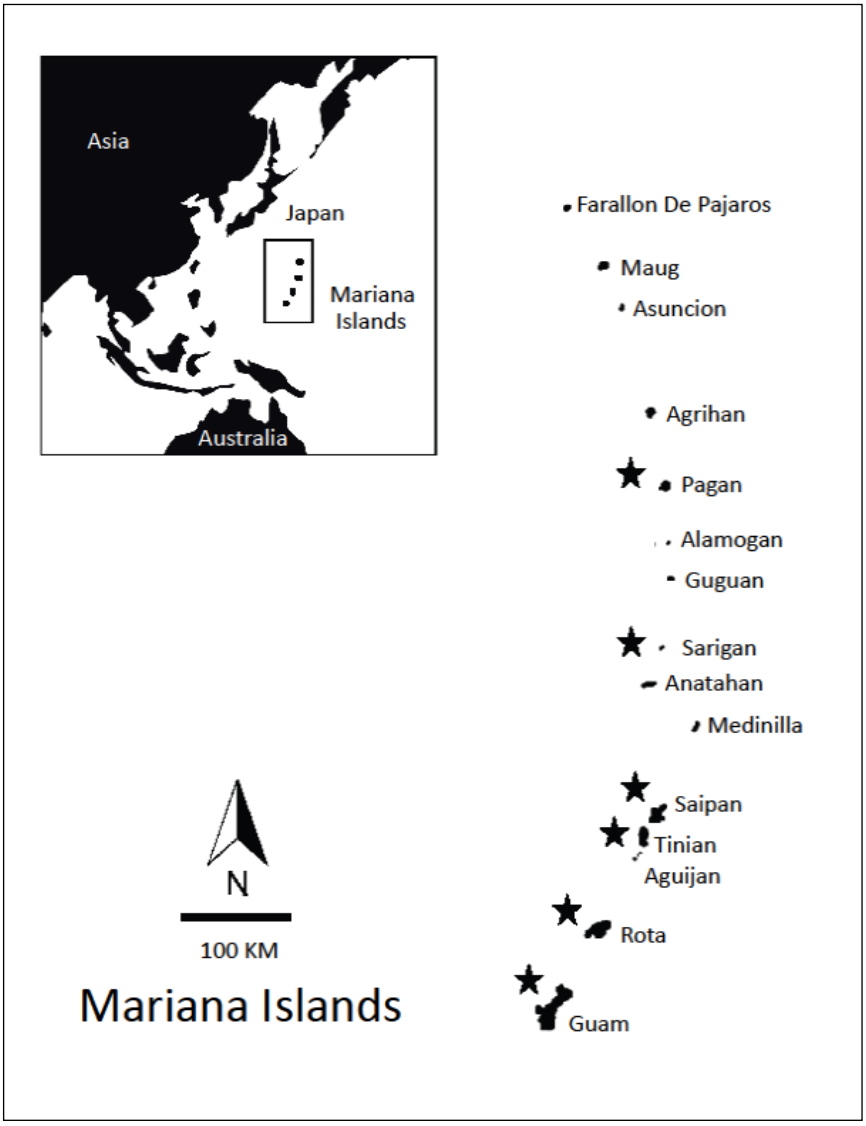

Figure 1. Map of the Mariana Islands. Islands where partulids were sampled are indicated by stars.

MATERIALS AND METHODS

Study area and sampling

We collected tissue samples from *Partula gibba* in fourteen discrete populations spanning six islands in the Mariana Archipelago. We were also given specimens from the Zoological Society of London's captive population, originally collected from Saipan. In addition, two populations of *Partula radiolata,* and one population of *Saomoana fragilis* were sampled on Guam to be used as outgroups in sequence analyses (table 2.1). Seven to twelve snails were sampled in each population. The number of tissue specimens depended on how difficult it was to find individuals and the amount of time we could devote to searching. Samples were collected from adult snails using a method originally developed for Hawaiian tree snails. This technique is non-lethal and involves slicing a very small piece of tissue, (10-20 mg), from the most posterior portion of the extended foot of an active adult snail as it crawls across a clean Petri dish (Thacker and Hadfield, 2000). Tissue samples were placed in 95% ethanol for temporary storage and transportation back to the laboratory.

Total cell DNA (tDNA) was extracted from all 172 specimens using a QIAGAN DNeasy blood and tissue kit, following the manufacture's protocol for purification of tDNA from animal tissues (QIAGEN Inc., Valencia CA, USA). tDNA was eluted in QIAGAN's elution buffer, and stored at -20°C in preparation for amplification using Polymerase Chain Reaction (PCR).

Table 2.1. Species collected, island source, number of distinct sampling localities per island, and number of tissue samples collected per island.

Taxon (species)	Island	No. of sampling locations	No. of tissue specimens
Partula gibba	Guam	1	11
	Rota	3	37
	Tinian	2	20
	Saipan	1	7
	Sarigan	2	13
	Pagan	5	61
	London Captive Population (Saipan)	1	5
Partula radiolata	Guam	2	13
Saomoana fragilis	Guam	1	5

Genetic analysis

We sequenced a partial region of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene from all tissue samples collected. PCR was conducted using the primer pair LCO1490 and HCO2198 (Folmer et al. 1994). Because initial analysis of the CO1 gene fragments indicated genetic relationships different from conventional taxonomic assignments, two more slowly evolving genes were selected to resolve deeper evolutionary relationships. We sequenced approximately 500 base-pairs of mitochondrial 16S ribosomal RNA using the primer pair 16S1: 5'CGCCTGTTTATCAAAAACAT-3' and 16S2: 5'-CTCCGGTTTGAACTCAGA-3'(Thomaz et al. 1996). In addition we sequenced approximately 700 base-pairs of the nuclear RNA internal transcribed spacer 2 gene (ITS2), between the 5.8s and 28s genes. Primer pair for ITS2 included: ITS2F 5'-GGGTCGATGAAGAACGCAG-3', ITS2R 5'-GCTCTTCCCGCTTCACTCG-3' (Yongping Wang, 2008).

All amplifications were carried out in 25ul reactions under the following PCR conditions: CO1, initial denaturation for 2 min at 92° C, followed by 35 cycles of 30s at 94° C, 45s at 40° C, and 1.0 min at 72° C, with a final extension of 7 min at 72° C; 16s: initial denaturation for 10 min at 94° C, followed by 35 cycles of 30s at 94° C, 30s at 57° C, and 45 s at 72° C, with a final

extension of 7 min at 72° C; ITS2: initial denaturation for 10 min at 95° C, followed by 35 cycles of 20s at 94° C, 30s at 50° C, and 20 s at 72° C, with a final extension of 7 min at 72° C.

Amplifications were verified using agarose gel electrophoresis. Single product samples were purified with ExoSAP-IT® (USB, Cleveland, OH) following manufacture's protocol. Samples were sequenced in both directions at the University of Hawaii's ASGPB Core Genomics Laboratory (Honolulu, USA).

Data analysis

Electropherograms were viewed and checked for errors in Geneious version 7.0.4 (Biomatters: http://www.geneious.com/). Sequences for each gene were unambiguously aligned using the ClustalW alignment tool located within the Geneious platform (Larkin et al. 2007). Unique haplotypes for each gene were identified using DNASP 5.10.1 (Librado & Rozas, 2009). Duplicate sequences were removed from alignments to prevent redundancy and save computational time during analyses. Alignments were adjusted by eye and all sequences were trimmed to the same length for analyses resulting in alignments with sequence lengths of 631 bp for CO1, 462 bp for 16s, and 588 bp for ITS2, with a concatenated total of 1683 bp.

Exploratory Bayesian and Maximum Likelihood phylogenetic analyses were conducted for the following data sets: CO1 haplotype sequences; 16s haplotype sequences; ITS2 haplotype sequences; and a concatenated analysis made up of individuals with unique combinations of haplotypes from all three genes. Individuals with poorly amplified sequences were excluded from analyses. Gaps were treated as missing data. All phylogeny reconstructions were rooted with the outgroup *Saomoana fragilis*. The genus *Saomoana* has been previously shown to occur basal to the genus *Partula* (Goodacre and Wade 2001).

MrBayes version 3.2.0 (Ronquist et al., 2011) was used to reconstruct phylogenies using Bayesian methods. We applied models of sequence evolution based on the best-fitting model estimated by the Bayesian information criterion (BIC) implemented in the program JModeltest 2.1.4 (Darriba 2012, Guindon and Gascuel 2003). Best fit models used for each gene region

included: CO1 = HKY+G; 16S = TPM1uf+G; and ITS2 = HKY. Two runs each containing four Marcov Chain Monte Carlo chains were conducted and compared for consistency to insure analyses were not trapped on local optima. Analyses were run for one million generations with a sampling frequency of every 100 generations. The first 25 percent of trees were discarded as burn-in. 50% majority-rule consensus trees were generated for each gene and a concatenated analysis of all three genes.

Maximum likelihood (ML) phylogeny reconstruction for all individual gene trees and the concatenated alignment of all three genes was inferred with the program Raxml Version 7.4.2 (Stamatakis 2014). The general time reversible (GTR) with GAMMA rate substitution model was used. Bootstrap support was evaluated with 10,000 nonparametric pseudoreplicates.

Substitution saturation for the complete CO1 alignment and single codon positions was tested for, with DAMBE 5.2.68 (Xia and Xie 2001). No sign of saturation was detected, and all characters were used in analyses.

Intra and inter-island CO1, 16S and ITS2 variation was assessed using a distance analysis of percent sequence divergence calculated in the program MEGA version 6 (Tamura 2013).

Shell morphological assessment

No useful DNA from *P. langfordi* is known to exist. Therefore shells housed in the mollusc collection at the Bernice Pauahi Bishop Museum in Honolulu, Hawai‘i were used to compare shell metrics of *P. langfordi* (Bishop Museum lot numbers 213092, 21309, 213104, 213012, 213024) originally collected from Agiguan, with shells from *P. gibba* (Bishop Museum lot numbers, 217155, 213251, 213248, 213151, 213241) originally collected on Rota (Figure 2.2). Length and width measurements were recorded from 48 and 47 shells of each species respectively, with precision calipers to 0.01mm. Adult *Partula* stop growing and become sexually mature when they form a characteristic flare around the aperture of their shells, here referred to as a lip. All shells measured where lipped indicating they had reached terminal

growth. Mean shell length and width were compared with independent-samples, two-sided t-tests, assuming unequal variances, using Microsoft Excel (2011).

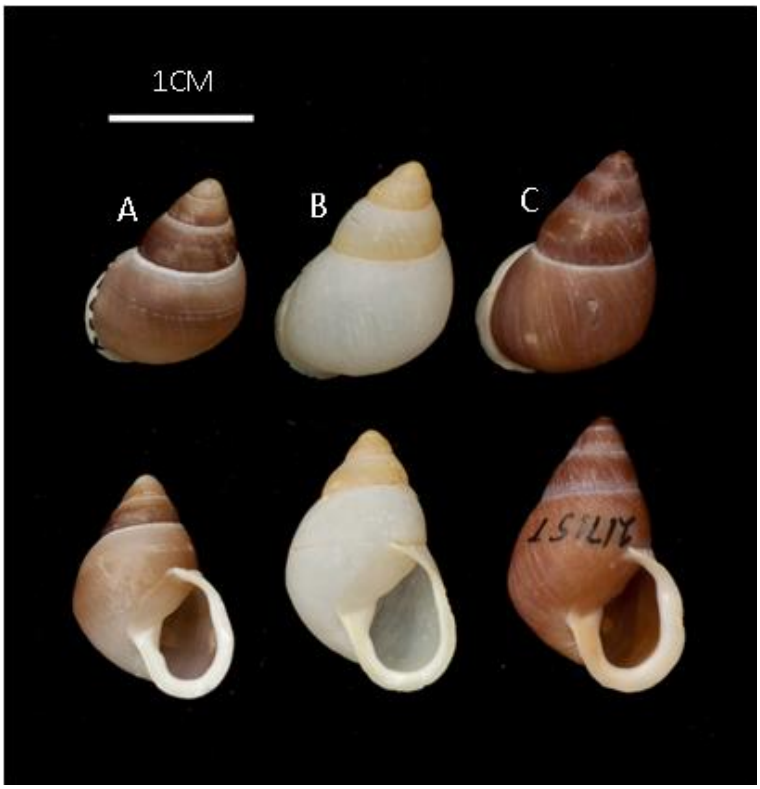

Figure 2.2. Typical shell of *P. langfordi* from Agiguan (A) compared to light and dark shell morphs of *P. gibba* from Rota (B and C). The three shells are shown in adapertural view in the upper row and apertural view below.

RESULTS

All Bayesian and Maximum Likelihood phylogenies constructed using individual gene alignments and a concatenated alignment incorporating all three genes had comparable topologies. For the sake of conciseness, we report only the phylogenetic- tree reconstructions for the concatenated alignment (Figure 2.3). The reported phylogeny contains sequences from 24 individuals from seven islands, all with unique haplotype combinations of CO1, 16S and ITS2 genes. We report Bayesian posterior probabilities and Maximum Likelihood bootstrap vales on nodes with greater than .50 or 50%, respectively (Figure 2.3).

The three extant partulid species of the Mariana Islands, including *P. gibba, P. radiolata* and *S. fragilis,* were well supported in all phylogenetic analyses. However, all individuals of *P. gibba* sampled from the island of Rota formed a clade that is polyphyletic with respect to all other *P. gibba* sampled from Guam, Tinian, Saipan, Sarigan and Pagan Islands (figure 2). *P. gibba* from Rota forms a sister clade, not to other *P. gibba*, but to *P. radiolata* from Guam.

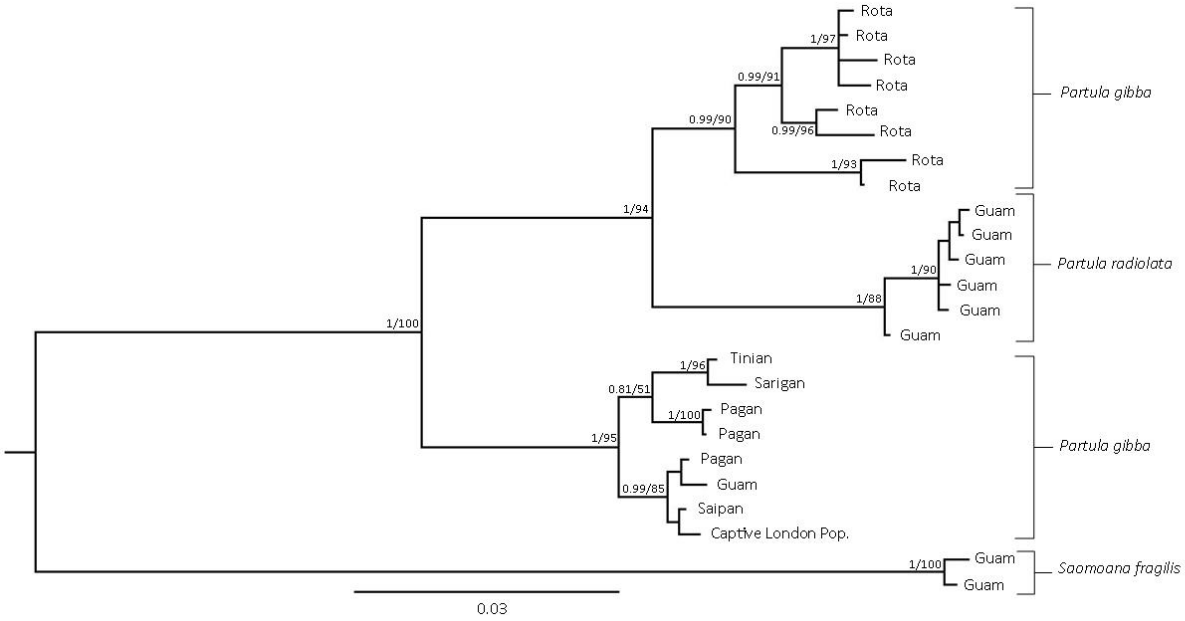

Figure 2.3. Bayesian phylogenetic tree illustrating the relationship between three extant species in the family Partulidae from the Mariana Islands. The resulting phylogeny is based on a concatenated alignment of three genes (CO1, 16s, and ITS2) with a combined sequence length of 1683 base pairs. The phylogeny contains sequences from 24 individuals with unique haplotypes from 7 islands. Maximum Likelihood analysis recovered the same topology. Therefore, Bayesian posterior probabilities and Maximum likelihood bootstrap vales are reported on all nodes with greater than .50 or 50% support, respectively. This tree is rooted with the outgroup *Saomoana fragilis*. The Genus *Saomoana* has previously been assigned as basal to the genus *Partula* (Goodacre and Wade 2001). Terminal labels indicate island of collection. Outside brackets identify species based on conventional assignments.

Average intra and inter-island percent sequence divergence (distance) is reported for the CO1 and 16S genes (Table 2.2) Percent sequence divergence within ITS2 was uninformative among *P. gibba* from different islands and is therefore not reported. All *P. gibba* individuals sampled had very low intra-island CO1 and 16s sequence divergence. We calculated Pagan Island to have

an average intra-island sequence divergence of 0.008 for CO1 and 0.00 for 16S, while *P. gibba* from Sarigan, Saipan, Tinian and Guam were found to have 0.00 intra-island sequence divergence for both CO1 and 16S. This is in stark contrast to *P. gibba* from Rota, which had an average intra-island sequence divergence of 0.030 and 0.023 for CO1 and 16S respectively, and *P. radiolata* from Guam having divergence of 0.023 and 0.001.

P. gibba from Guam, Tinian, Saipan, Sarigan and Pagan Islands had an average inter-island sequence divergence of 0.057 for CO1 and 0.019 for 16S (Table 2.2). Because *P. gibba* from Rota is polyphyletic with respect to the *P. gibba* from all other islands, we did not include it in the average inter-island sequence divergence calculation for *P. gibba*.

Table 2.2. Average CO1 and 16S percent sequence divergence within (intra) and between (inter) islands and species. Percent sequence divergence was calculated in the program MEGA (Tamura 2013). Top rows (grey) indicate CO1 percent sequence divergence; bottom rows (white) indicate 16S percent sequence divergence. Bold numbers indicate percent island-intraspecific sequence divergence.

		Pagan *P. gibba*	Sarigan *P. gibba*	Saipan *P. gibba*	Tinian *P. gibba*	Rota *P. gibba*	Guam *P. gibba*	Guam *P. radiolata*	Guam *S. fragilis*
Pagan, *P. gibba*	CO1	**0.008**							
	16S	**0.000**							
Sarigan, *P.gibba*	CO1	0.050	**0.000**						
	16S	0.022	**0.000**						
Saipan, *P. gibba*	CO1	0.019	0.052	**0.000**					
	16S	0.011	0.020	**0.000**					
Tinian, *P. gibba*	CO1	0.022	0.036	0.027	**0.000**				
	16S	0.015	0.007	0.013	**0.000**				
Rota, *P. gibba*	CO1	0.072	0.094	0.075	0.076	**0.030**			
	16S	0.061	0.064	0.059	0.062	**0.023**			
Guam, *P. gibba*	CO1	0.067	0.105	0.057	0.073	0.119	**0.000**		
	16S	0.011	0.024	0.004	0.018	0.064	**0.000**		
Guam, *P. radiolata*	CO1	0.101	0.117	0.103	0.104	0.085	0.134	**0.023**	
	16S	0.065	0.069	0.063	0.067	0.051	0.063	**0.001**	
Guam, *S. fragilis*	CO1	0.165	0.171	0.163	0.163	0.16	0.162	0.169	**0.024**
	16S	0.119	0.128	0.115	0.121	0.116	0.117	0.111	**0.000**

Analysis of shell morphologies

Shell length and Width of *P. langfordi*, from Agiguan, and *P. gibba* from Rota were compared with independent-samples, two-sided t-tests, assuming unequal variances. Shell length of *P. langfordi* (M=13.83, SD=0.37) was significantly shorter than the length of *P. gibba* (M=15.98, SD=0.75), t(82) = -13.91, P < 0.001. Similarly, shell width of *P. langfordi* (M=9.74, SD=0.36) was significantly less than that of *P. gibba* (M=10.64, SD=0.24), t(90)= -8.02, P <0.001(Figure 2.3).

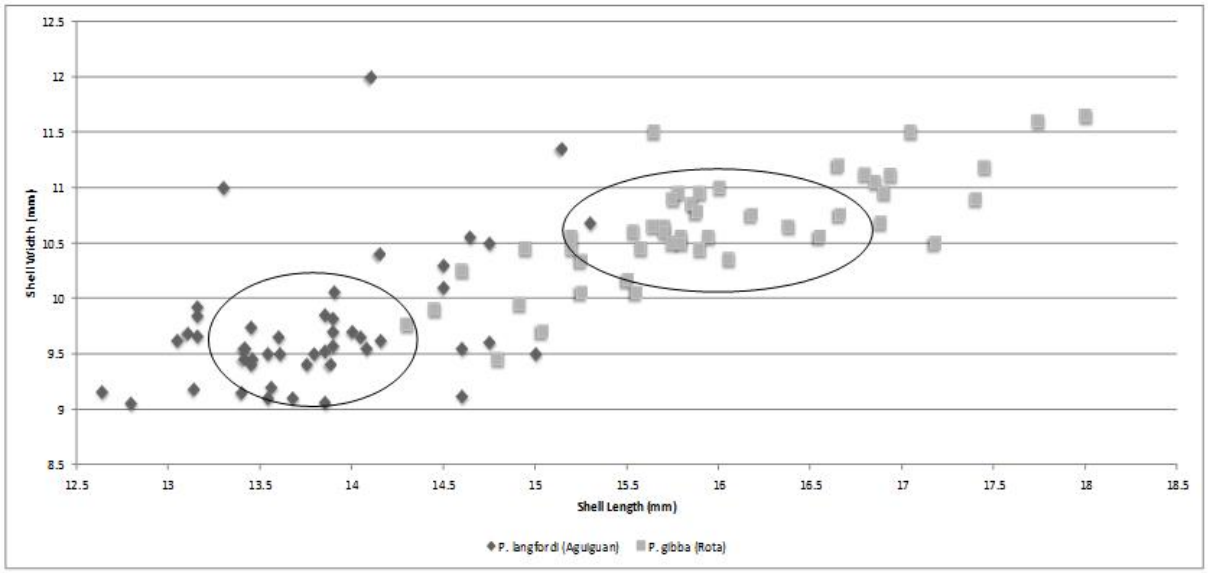

Figure 2.4. Scattergram of Bishop Museum shell measurements comparing shell width (y-axis) by shell length (x-axis) of 48 *P. langfordi* from Agiguan, and 47 *Partula* sp. from Rota. The mean shell length and width plus standard deviation of each species are encircled. All shells were lipped indicating snails were mature and had reached terminal growth.

DISCUSSION

Distribution of Mariana Island Partulidae

No haplotypes of CO1 or 16S from *P. gibba* were shared between islands. However, closely related haplotypes cluster into two sister clades (Figure 2.4). Clade I consists of individuals from Tinian, Sarigan, and Pagan Islands, and Clade II includes individuals from Guam, Saipan, and

other snails from Pagan Island. Interestingly, both clades contain individuals from the southern (Guam, Saipan, and Tinian), and the northernmost islands (Sarigan and Pagan). Approximately ten percent of snails sampled on Pagan Island, the northern limit of *P. gibba,* had a CO1 haplotype very closely related to *P. gibba* sampled from Guam (clade II), which harbors the most southerly populations (Figure 2.4).

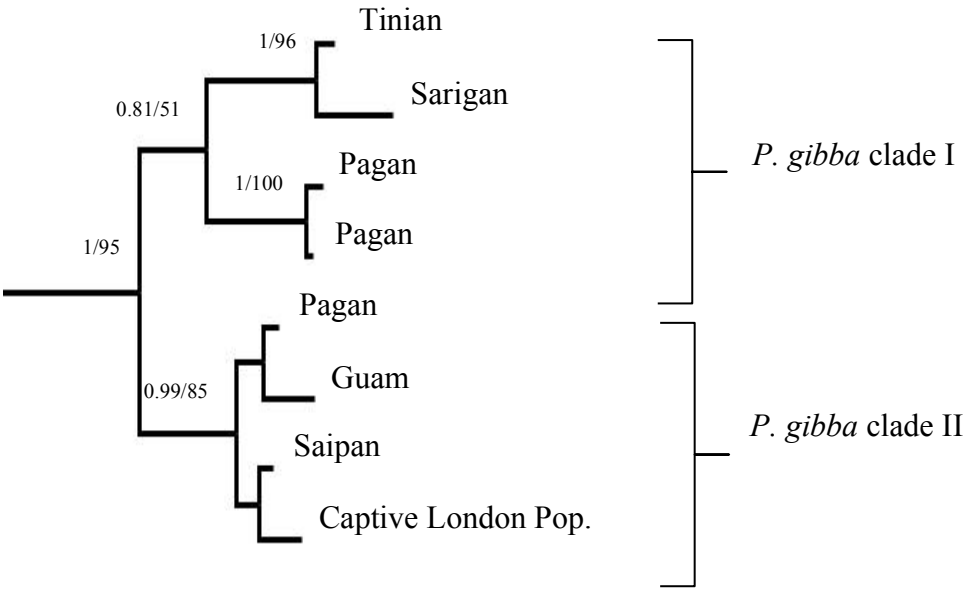

Figure 2.5. Bayesian phylogenetic tree showing *P. gibba* clades taken from Figure 2.3. The known extant of populations of *P. gibba* on five islands sort into two distinct clades made up of closely related haplotypes from both the northern and southern islands.

It is clear the vagility of *P. gibba* is remarkable. For example, our data suggest that Pagan Island had two colonization events, one of which appears to have come from Guam relatively recently (Figure 2.4). Another noteworthy relationship is between the *P. gibba* on the islands of Tinian and Sarigan, which share a recent common ancestor, and yet are on islands approximately 200 km apart, with Saipan and Anatahan in between them. If we are detecting this pattern with a species on the brink of extinction, it is likely that if we had sampled *P. gibba* prior to the introduction of predators and major habitat loss in the southern islands, the pattern might have been more complex.

Kondo (1970) attributed the multi-island distribution of *P. gibba* to typhoons that frequent the region and have a generally northerly direction. However, recent findings indicate a more likely mode of molluscan dispersal is by passive means: in the guts or on the bodies of birds, or through the accidental or purposeful introduction by humans (Kawakami et al. 2008, Maciorowski et al. 2012).

The Mariana Islands have been inhabited for over three thousand years by indigenous Chamorro people (Russell 1998). It is not farfetched to speculate that snails could have been accidentally or purposefully brought to other islands by prehistoric inhabitants who regularly sailed between them. At all of our sampling locations *P. gibba* could be found on plants closely associated with current and prehistoric cultivation including: *Cocos nucifera* (coconut), *Artocarpus altilis* (bread fruit), *Alocasia macrorrhiza* (Taro) and *Piper guahamense.* Prehistoric human-mediated dispersal of other *Partula* spp. was described by Lee et al. (2007) in the Society Islands, and by O'Foighil et al. (2011), in the Papua New Guinean Archipelago.

While it is clear that *P. gibba* is capable of long distance dispersal, it is curious that *P. radiolata*, *P. langfordi* and *Saomoana fragilis* do not also occur on the younger northern islands. Interestingly, individuals of *P. gibba* that are completely isolated in the laboratory produce offspring at the same rate as those with mates, indicating an ability to self-fertilize with no reduction in fecundity (Johnson et al. 1977). The very low intraspecific CO1 and 16S genetic distance we calculated for all islands with *P. gibba* (except Rota) appear to reinforce the laboratory findings about self-fertilization. For example, individuals occurring on Guam, Tinian, Saipan, and Sarigan had only one CO1 and one 16S haplotype recovered per island, equating to 0.000 sequence divergence. On Pagan Island, we sampled over 60 individuals from five discrete populations which had an average of 0.008 intra-island percent sequence divergence. This is in stark contrast to the *Partula* sp. we sampled on Rota, which had average CO1 and 16S percent sequence divergence of 0.030 and 0.023 respectively, with much less sampling effort. This pattern holds true for *P. radiolata* and *S. fragilis* from Guam as well. We hypothesized that the propensity of *P. gibba* for self-fertilization may allow it to survive extreme bottlenecks making the species a very successful island colonizer. Theoretically, with an ability to self-fertilize, *P. gibba* would be capable of establishing a new population with only one individual.

While *P. gibba* is the most widely distributed *Partula* species in the Mariana Archipelago, on most islands it is imperiled. This is most notable on Guam where *P. gibba*, once the most populous *Partula* species (Crampton 1925) has all but vanished from most of its former range (Hopper and Smith 1992) Conversely, *P. radiolata* is persisting in numerous locations and in very high local densities (thousands of individuals personal observation). We suspect that *P. radiolata* has some aspect of its life history or behavior that has allowed it to persist in the wake of extreme predation by introduced predators. Faster maturation, higher fecundity, or predator avoidance behavior are likely involved. While this is speculation, it might not be coincidence that the *Partula* sp. from Rota, which we now know is a sister taxon to *P. radiolata* on Guam, is also persisting at locally high densities. Perhaps this lineage shares advantageous traits. Only careful study of the life-history characteristics of these species will reveal the answer.

***Polyphyly in* Partula gibba.**

All individuals sampled from three discrete populations on Rota, previously attributed to *P. gibba* (Kondo, 1970, Bauman 1996), form a distinct genetic clade, sister to *Partula radiolata* from Guam and not to *P.* gibba as all previous work has suggested based on shell similarities. These data indicate that *P. gibba*, as it stands, is a polyphyletic group (Figure 2.3). This distinct clade from Rota was recovered in all individual analyses of CO1, 16s and ITS2 genes, and is well supported in the combined analysis including all three genes.

Goodacre and Wade (2001) reconstructed a phylogeny for the entire Pacific-wide Partulidae using part of the ribosomal RNA gene cluster. Their analyses included a sample of *P. gibba* from Saipan, a *P. radiolata* from Guam, and a supposed *P. langfordi* reportedly from Agiguan. During the exploratory stages of our data analyses we found that ITS2 sequences from *P. gibba* sampled on Rota closely matched the portion of the ribosomal RNA gene cluster of the *P. langfordi* reported by Goodacre and Wade (2001). No mitochondrial sequences were published for this *P. langfordi* sample; we therefore were unable to draw any conclusions, because ITS2 was only informative at deeper nodes. This led us to ask if the Rota snails were, in fact, *P. langfordi*.

After correspondence with colleagues in Guam and the *Partula* captive breeding program in the United Kingdom, we discovered that the *P. langfordi* sample used in the Goodacre and Wade (2001) study came from a population once reared in the Zoological Society of London's *Partula* breeding program. Furthermore, we learned from Mr. Barry Smith (pers. comm. 2014) that he originally collected this captive population of *P. langfordi* on Rota, not Agiguan. These snails were unofficially identified as *P. langfordi* based on perceived morphological similarity. Although the captive population in the *Partula* breeding program has subsequently died, we obtained tissue from frozen specimens. We extracted DNA from several specimens, sequenced a region of the CO1 gene and were able to confirm, with closely matching CO1 haplotypes, that indeed individuals from this captive population were likely collected in close proximity to, or directly from, one of the same populations of *P. gibba* we sampled on Rota. The question now remains: was the perceived identification of the *Partula* sp. on Rota as *P. langfordi* correct, or do the populations on Rota represent a misidentified cryptic species endemic to that island?

Over the past several decades, invasive goats have ravaged the native flora of Agiguan and subsequently caused the likely extinction of *P. langfordi* and the extirpation of sympatric *P. gibba* (Smith 2008, Bauman 1996, Jill Liske-Clark pers. comm. Dec. 2014). However, the Bernice Pauahi Bishop Museum in Honolulu, Hawai'i, houses preserved specimens of *P. langfordi* originally collected on Agiguan over a half century ago by Y. Kondo and others. Unfortunately, our attempts to extract DNA from these specimens all failed. Although Bishop Museum specimens are currently stored in ethanol, it is unknown how they were originally preserved. It is expected that specimens were preserved in ethanol after originally being drowned in water or fixed in formalin. Drowning mollusc specimens prior to preservation is known to damage DNA (Schander and Hagnell 2003). Likewise, preservation in formalin damages DNA by crosslinking base pairs making downstream PCR exceptionally problematic (Srinivasan et al. 2002).

P. langfordi was described as a distinct species endemic to Agiguan by Kondo (1970). Prior to Kondo's description, it was believed *P. langfordi* and larger sympatric *P. gibba* were two extreme variants of the same species. This was logical because *P. gibba* is known to be highly polymorphic, especially on Guam (Crampton 1925). However, the absence of intermediate forms

on Agiguan prompted further study. Kondo (1970) compared the length and width of specimens of sympatric *P. langfordi* and *P. gibba*, showing distinct non-overlapping shell sizes. In a photographic figure, Kondo also compared a shell of *P. langfordi* directly to a shell of what he calls a color morph of *P. gibba* from Rota. Although he did not state it, Kondo presumably chose this particular morph from Rota because of its likeness to *P. langfordi*. When comparing the two specimens Kondo explained, "*P. langfordi* is much smaller; its spire is shorter and convexity of whorls much less; the last whorl is proportionately more capacious and its gibbous outline emphatic, whereas the Rota specimen does not show this" (Kondo 1970).

P. langfordi was described as a distinct species based on its purple shell coloration, small shell size and lack of intermediate forms when compared to sympatric *P. gibba*. Without DNA to confirm whether the *Partula* sp. on Rota is or is not *P. langfordi*, we assessed dimensions of a large number of Bishop Museum shells of the *Partula* sp. from Rota and P. *langfordi* from Agiguan. Differences in shell length and width between the two species were statistically significant. On average *P. langfordi* is smaller than *P. gibba* from Rota with little overlap (figure 3). Kondo and Easly (1970) had noted the same relationship between shell metrics of sympatric *P. langfordi* and *P. gibba* from Agiguan

Based on the same methods Kondo (1970) used to describe *P. langfordi* as a distinct species, our data indicate *P. gibba* from Rota is not *P. langfordi*. Our phylogenetic reconstruction reveals that the *Partula* sp. on Rota is not *P. gibba* either. Rather, both molecular-genetic and shell morphometric data indicate a misidentified cryptic species endemic to Rota. In the interim, while we work to describe the species, we suggest it be noted as distinct from *P. gibba* and managed accordingly for conservation purposes.

Conservation Implications

Populations of *P. gibba* on all islands had low sequence divergence. Therefore local extirpation will disproportionately impact the remaining diversity of *P. gibba* compared to other *Partula* sp. with higher intra-island sequence divergence. The diversity of *P. gibba* is not captured on single

islands or in single populations but rather is spread across the entire archipelago. For example, the loss of *P. gibba* from Pagan Island would represent a 43% reduction in observed genetic diversity for the species, because Pagan Island harbors individuals from both clade I and II. This makes Pagan Island particularly important for conservation. By protecting populations on Pagan, two distinct *P. gibba* lineages will be preserved. Remaining populations on all islands must be protected to preserve the adaptive potential of *P. gibba*.

Our analysis revealing closely related mitochondrial haplotypes for *P. gibba* across multiple islands has management implications, mainly that long-distance dispersal of *P. gibba* has occurred previously (whether naturally or human-mediated), which may justify the use of inter-island translocations and reintroductions as a conservation tool. The discovery of a misidentified cryptic species of *Partula* on Rota has several additional implications. First, the range of *P. gibba* may no longer, or have never included Rota, thus the number of populations of *P. gibba* is less than was previously thought. Second, the cryptic species found on Rota appears to be an island endemic with a range that is very small, and therefore at a high risk of extinction.

It is clear there is much left to be understood regarding the life histories and phylogeograpic relationships of partulids of the Mariana Islands. This investigation has generated many new questions, which, while exciting, are overshadowed by the fact that these evolutionary lineages may be running out of time. Extirpation and extinction are imminent for many of the populations and species of tree snails from which we sampled.

It is important to remember that we sampled only from known populations and island surveys were not exhaustive. No doubt there are other undiscovered populations of *Partula* in the islands. Thorough surveys on all islands within the historical range of Mariana Island partulids are urgently needed if we are to understand the magnitude of what is disappearing and how to best maintain it. Our finding of a cryptic species on Rota should be considered a lesson: looks can be deceiving. Because certain morphological characteristics of partulids may converge, an approach scrutinizing both morphology and phylogentics should be employed.

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CHAPTER 3
CONSERVATION GENETICS OF THE HAWAIIAN TREE SNAIL *PARTULINA REDFIELDI*: DISTRIBUTION OF GENETIC DIVERSITY IN A FRAGMENTED LANDSCAPE

ABSTRACT

Small populations occurring in fragmented habitat are more vulnerable to environmental, demographic and genetic stochasticity. This is particularly true for organisms such as terrestrial molluscs, which have very limited vagility. Hawaiian tree snails in the subfamily Achatinellanae have experienced drastic range reductions and extinctions due to habitat loss and fragmentation, combined with predation by introduced species. Here we present the results of a study on *Partulina redfieldi,* a tree snail once widespread on the island of Moloka‘i, that now only exists in a small fragmented portion of its former range. We assess the distribution of genetic diversity across habitat patches using 11 microsatellite loci and 186 specimens. We found evidence of reduced gene flow and significant isolation-by-distance effects between habitat patches over very short distances (>10 meters). In addition, within patch bottleneck effects were significant in the form of reduced genetic diversity and high inbreeding. Our results highlight the importance of maintaining and restoring habitat connectivity inside protected reserves and around wild populations to prevent loss of genetic diversity. In addition, it may be necessary to include individuals from multiple habitat patches when founding *ex situ* populations, or establishing new colonies via translocation to prevent and reverse bottleneck effects.

INTRODUCTION

Fragmentation of habitat is one of the main drivers of biodiversity loss across the globe, and contributes significantly to the local extirpation of populations and extinction of vulnerable species (Fischer and Lindenmayer 2007). The negative effects of habitat fragmentation are a result of the altered spatial distribution of available habitat. Once large continuous areas are broken into smaller patches, habitat area and quality are reduced (Fahrig 2003). Small habitat fragments host small populations, and small population size increases vulnerability to extinction

or local extirpation from demographic and or environmental stochasticity (Frankham et al 2010). Similarly, small population size and limited migration (gene flow) can make populations susceptible to extinction due to bottleneck effects. Small populations are prone to lose alleles randomly as a result of genetic drift. In addition, mating that occurs between closely related individuals, or an increased rate of selfing (for organisms where this is possible), can result in a reduction of heterozygotes, inbreeding, and possibly inbreeding depression (Johansson et al. 2007, Frankham et al 2010, Leberg et al 2010). Organisms with limited dispersal capability are often disproportionally impacted by the effects of habitat fragmentation (Henle et al. 2004). This is especially true for terrestrial molluscs, which have very low vagility (Kappes et al. 2009, Schweliger et al. 2004).

The Hawaiian Islands were host to a spectacular radiation of land snails with over 750 described species in 13 families (Cowie et al. 1995). During the last two centuries the Hawaiian snail fauna has experienced drastic range reductions and extinctions as a result of historical habitat destruction and fragmentation, over-collection, and introduced predators. It is estimated that Hawaiian land snail diversity has decreased by 60 – 90%, and extant species now only occur in small fragmented patches of their former ranges (Hadfield and Mountain 1980, Hadfield 1986, Solem 1990).

The most well documented Hawaiian snail declines have occurred in the sub-family Achatinellinae. These large tree snails, famous for their beautiful shells, are endemic to the Hawaiian Islands, and are characterized by low fecundity (4-7 offspring per year), late age at maturity (3-5 years) and long lifespans (10 + years) (Hadfield and Mountain 1980, Hadfield et al 1993, Kobayashi and Hadfield 1996, Holland and Hadfield 2002). Forty-four species of Hawaiian? tree snails are included on the US Endangered Species List and most are considered critically rare by the IUCN (IUCN 2015). As a result, much effort is focused on protecting remaining species. Because invasive snail predators are ubiquitous in the islands, limited tools are available for snail conservation. Currently, captive rearing (Hadfield et al. 2004) and the construction of predator-proof fences around snail habitat are the only means to protect species from all known invasive molluscivores.

Non-native ungulates and weeds are responsible for much of the current habitat loss and fragmentation in Hawaii (Dixon 2011). Prior to humans arriving in the islands, and the subsequent introduction of these invasive species, tree snails, in most places, would have existed in continuous native forest. This is with the exception of natural isolation of forest habitats by large geological features such as lava flows, mountain ridges and rivers (Ziegler 2002). Here we present results of a study of *Partulina redfieldi* (Figure 3.1), a tree snail endemic to the island of Moloka‘i where it was formerly widespread. Currently the only known *P. redfieldi* now exist within the Nature Conservancy’s Kamakou Preserve on the summit of Molokai’s eastern mountains (Figure 3.2) (Hadfieldi and Saufler 2007). Remaining snails occupy single trees or small patches of interconnected or closely spaced trees that are separated from other suitable habitat by large tracts of invasive pants (Figure 3.1). We assess the distribution of genetic diversity of *P. redfieldi* by measuring isolation by distance, and quantifying bottleneck effects, such as the loss of genetic diversity and inbreeding within and among subpopulations isolated in habitat fragments. This pattern of habitat fragmentation is comparable to what many extant achatinelline species experience, and consequently results of this study may have implications for management across species on several Hawaiian Islands.

A

B

Figure 3.1. A) Adult *Partulina redfieldi* on an O‘hia lehua (*Metrosideros polymorpha)*. Note the large distance (>20 meters) to next host tree. B) Typical fragmented patch of O‘hia lehua (*Metrosideros polymorpha)* surrounded by non-native grasses unusable by tree snails.

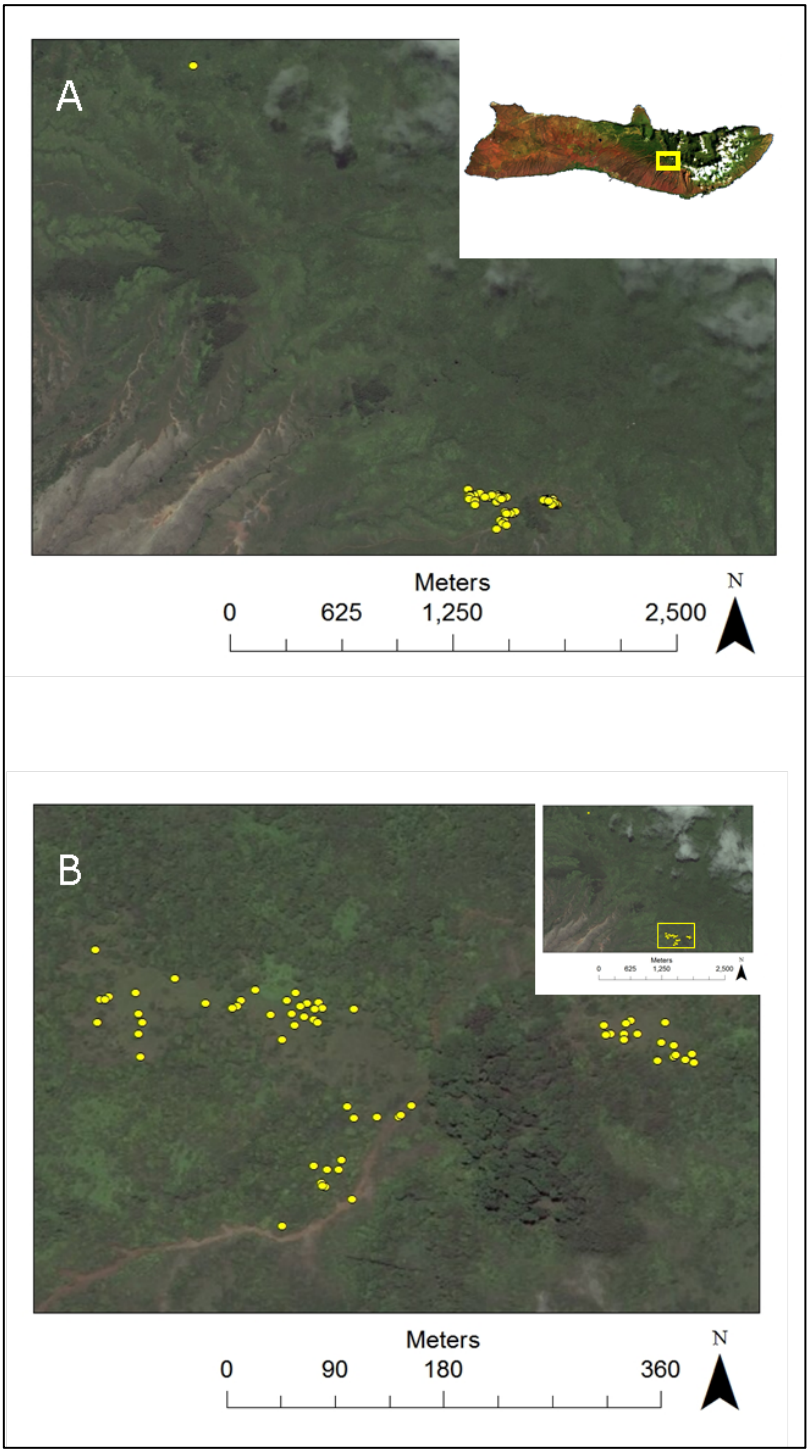

Figure 3.2. A) Location of the 65 trees we collected genetic samples of *P. redfieldi* from. Note two trees occurred approximately three kilometers northwest from the rest of the trees, which were all within 400 meters of each other. B) Higher resolution of all the sampling trees except for the two occurring approximately three kilometers to the northwest as displayed above.

MATERIALS AND METHODS

Sampling

186 adult and large sub-adult (>12.5 mm) *Partulina redfieldi* were sampled from 65 trees near the summit of the eastern Molokaʻi mountains (Figure 3.2). Population extent was determined by searching transects through habitat until snails were no longer at detectable densities. All trees where snails were detected were carefully searched until no more snails could be found. Tissue samples were collected from *P. redfieldi* using a non-lethal technique, whereby a 10-20 mg piece of tissue was cut from the extended foot of an adult or large sub-adult (>12.5 mm) snail as it crawled across a clean petri dish (Thacker and Hadfield 2000). Tissue samples were placed in individual vials of 95% ethanol for temporary storage and transportation back to the laboratory.

Laboratory methods

Total cell DNA (tDNA) was extracted from tissue samples using a QIAGEN DNeasy blood and tissue kit and following the manufacture's protocol for purification of tDNA from animal tissue (QIAGEN Inc., Valencia CA, USA). Eluted samples were stored at -20 ^{o}C until amplification by the Polymerase Chain Reaction (PCR).

All individuals were genotyped at eleven microsatellite loci (Table 3.1). Eight loci were previously identified and published by Erickson and Hadfield (2008), and for these eight loci, we followed the authors' recommended PCR conditions. The remaining three loci had been previously identified by Erickson and Hadfield but never optimized or published. We amplified the three new primers using the same reaction conditions described in Erickson and Hadfield (2008), with the exception of annealing temperature, which we optimized for each of the new loci. Genotyping was conducted at the Center for Genomic, Proteomic, and Bioinformatic Research (CGPBR) at the University of Hawaiʻi at Mānoa. The software Peakscanner 1.0 (Applied Biosystems 2006) was used to interpret chromatograms and manually score alleles.

Table 3.1. Loci identification and Genebank accession number followed by number of alleles (N_a), size range of alleles per loci, and null allele frequencies estimated by the program INEst.

Loci Identification (GenBank Assession No.)	N_a	Size range (bp)	INEst null allele freq.
AS812 (EU119381)	2	235-237	0
AS32 (EU119382)	3	195-201	0.032
AS46 (EU119383)	3	213-222	0
AS53 (EU119384)	11	240-280	0
AS61 (EU119385)	7	184-211	0
AS62 (EU119386)	3	206-212	0
AS82 (EU119387)	2	156-159	0
AS110 (EU119388)	9	229-268	0.005
AS50 (KR872615)	4	360-387	0
AS96 (KR872616)	5	191-212	0
AS100 (KR872617)	8	251-305	0

Data analysis

Hall and Hadfield (2009) conducted a dispersal study on several achatinelline species using harmonic radar technology. During their study, snails did not disperse between discrete habitat patches except during high wind events. However, all detectable snail dispersal was less than 10 meters from original host trees. Based on this finding, we identified subpopulations of *P. redfiedli* based on host tree proximity (< 10 meters apart and or interconnected trees) and sample size (more than 10 snails, a sample size large enough to analyze) (Figure 3.3). Out of the 65 trees we sampled, we found seven distinct habitat patches with large enough snail numbers for genetic analysis. These seven distinct habitat patches are here henceforth referred to as subpopulations. There were conspecific snails that occurred solitarily in trees greater than 10 meters from any other trees with snails. These samples were analyzed in the global calculations including all samples, but were not assigned to any of the seven subpopulations.

We used the program CONVERT version 1.31 (Glaubitz 2004) to format data appropriately for different population-genetics software programs. It was expected that *P. redfieldi* existing in fragmented habitat would have high rates of selfing and or inbreeding (Kobayashi & Hadfield 1996). Because homozygote excess can arise from null alleles (allele scoring, amplification problems, or alleles identical in size but not by decent) and inbreeding (alleles identical by

decent), the program INEst version 2.0 was used to estimate null allele frequencies. INEst, through an iterative process, simultaneously measures inbreeding and estimates null allele frequencies providing a more accurate estimate when inbreeding is possible (Chybicki and Burczyk 2009). No estimated null allele frequencies were higher than 0.032, therefore null alleles were not a concern for our analyses (Table 3.1). We checked for linkage disequilibrium and Hardy-Weinberg equilibrium globally (all 186 specimens) and within all seven subpopulations with the program Genepop 4.0 (Raymond and Rousset 1995, Rousset 2008).

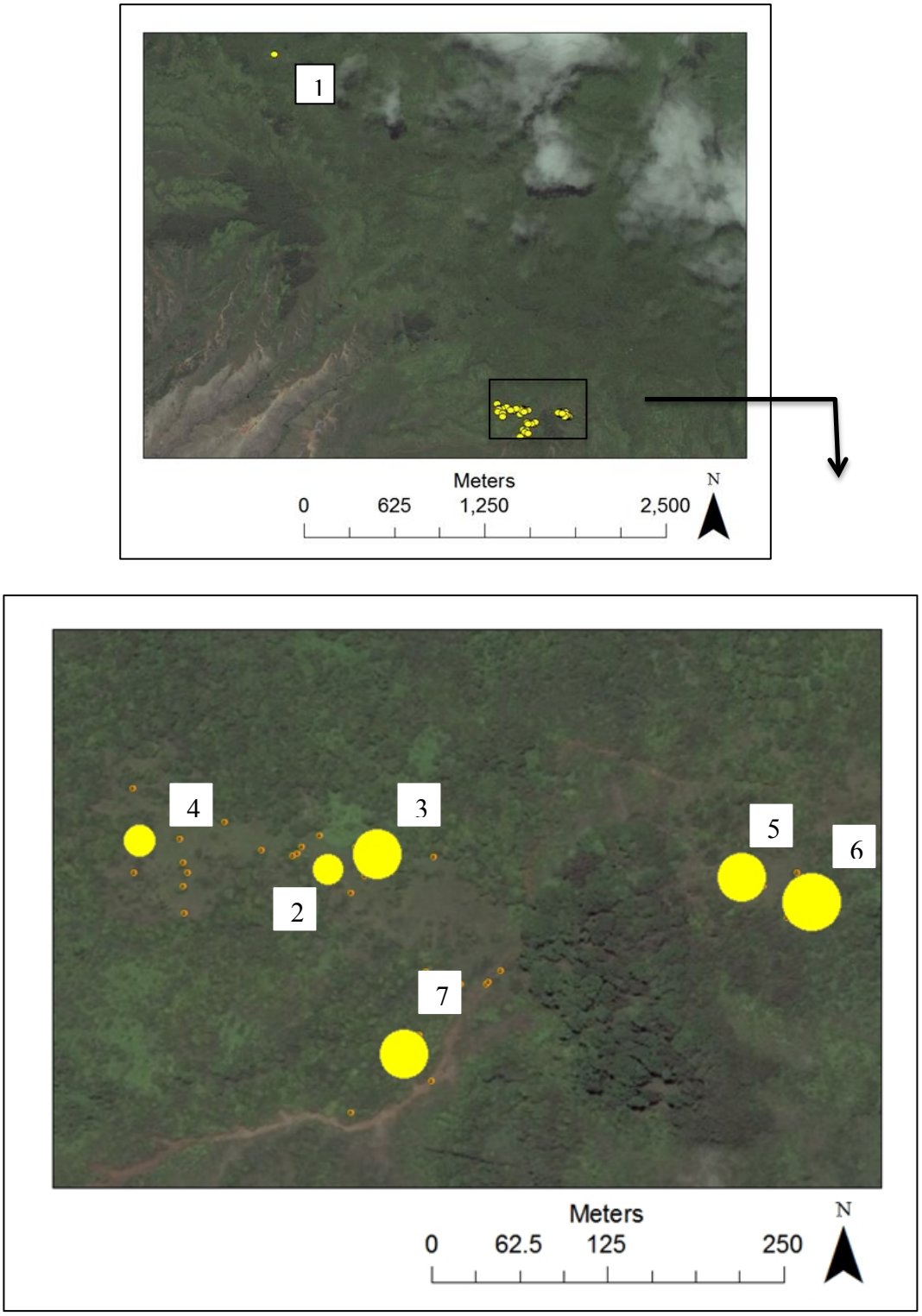

Figure 3.3. Yellow circles are placed on the seven subpopulations composed of closely spaced (<10 M) and or interconnected trees with ten or more snails. Small orange circles represent trees farther than 10 meters from any other snail tree and have less than 10 snails per tree.

Allelic richness was calculated with the program HP Rare version 1.0 with a minimum size of n = 20 gene copies (10 diploid snails, the smallest sample size from any of the subpopulations). HP Rare uses rarefaction methods to control for unequal sample sizes (Kalinowski 2004, Kalinowski 2005).

Observed heterozygosity (H_o), expected heterozygosity (H_E), gene diversity and inbreeding (F_{is}) were calculated globally and for all subpopulations with the program Arlequin (Excoffier et al. 2005). Also using the program Arlequin, F_{ST} was calculated between all subpopulations using 10,000 permutations (Weir and Cockerham 1984), and an analysis of molecular variance (AMOVA) was run. AMOVA was used to determine the proportion of genetic variation partitioned among subpopulations, within subpopulations, and within individuals.

We measured the effective population size (N_e) globally (all individuals sampled) and within each subpopulation using the program LDNE version 1.31 (Waples and Do 2008). LDNE does not require temporally spaced samples and instead calculates N_e with only one sample. We used the random mating model with the lowest allele frequency cutoff at 0.02. Ninety-five percent confidence intervals were calculated with the jackknife method implemented within the program. Self-fertilization has been recorded in *P. redfieldi* (Kobayashi and Hadfield 1996). For organisms that self, effective population size is generally interpreted as the number of breeders (Waples and Do 2008).

We evaluated the relationship between geographic distance (in meters) and genetic distance (F_{ST}) between the seven subpopulations with a mantel test (10,000 permutations) and reduced major axis (RMA) regression analysis implemented in IBDWS version 3.23 (Jensen et al. 2005). To ensure that the subpopulation farthest away from the others was not biasing the results, the analysis was rerun excluding this cluster.

A cluster analysis was conducted to empirically estimate the population structure of *P. redfieldi*, independent of our population groupings based on geographic proximity. Because of the detection of significant isolation-by-distance effects, the Bayesian-clustering program TESS 2.1 (Chen et al. 2007) was used instead of other programs such as Structure (Prichard et al. 2000).

TESS implements an allele-frequency algorithm based on Hardy-Weinberg equilibrium that includes an interaction parameter with geographic distance via GIS data. TESS therefore performs better than other programs when isolation by distance effects are significant (Chen et al. 2007). TESS works by grouping individuals into a user-specified number of clusters ("k"). A higher probability of assignment to a specific cluster is given to individuals within closer geographic proximity to one another. Ten runs were conducted for each value of k from 2 – 10 using the BYM admixture model (Durand et al. 2009). An initial burn-in of 10,000 steps followed by 40,000 steps was conducted for each run. The true number of clusters ("k") was determined by Q-matrix stabilization as well as interpreting when the deviance information criterion (DIC) reached a plateau.

RESULTS

Every subpopulation except Subpopulation 1 showed significant heterozygosity deficit ($P = < 0.000$). There was no significant heterozygosity excess globally or in the subpopulations. In addition, there was no significant (alpha = 0.05) linkage disequilibrium observed, after Bonferroni correction, within subpopulations. However significant linkage disequilibrium was observed between 10 pairs of loci when tested globally. Heterozygosity deficit and global linkage disequilibrium are typical of populations split into isolated subpopulations, also known as a Wahlund effect (Frankham et al. 2010).

The effect of isolation-by-distance was significant between all seven subpopulations ($r = 0.79$, $P = < 0.001$ figure 3.4A). Furthermore, the effect of isolation-by-distance remained significant even when the subpopulation farthest away from the others was removed ($r = 0.65$, $P = 0.03$, figure 3.4B).

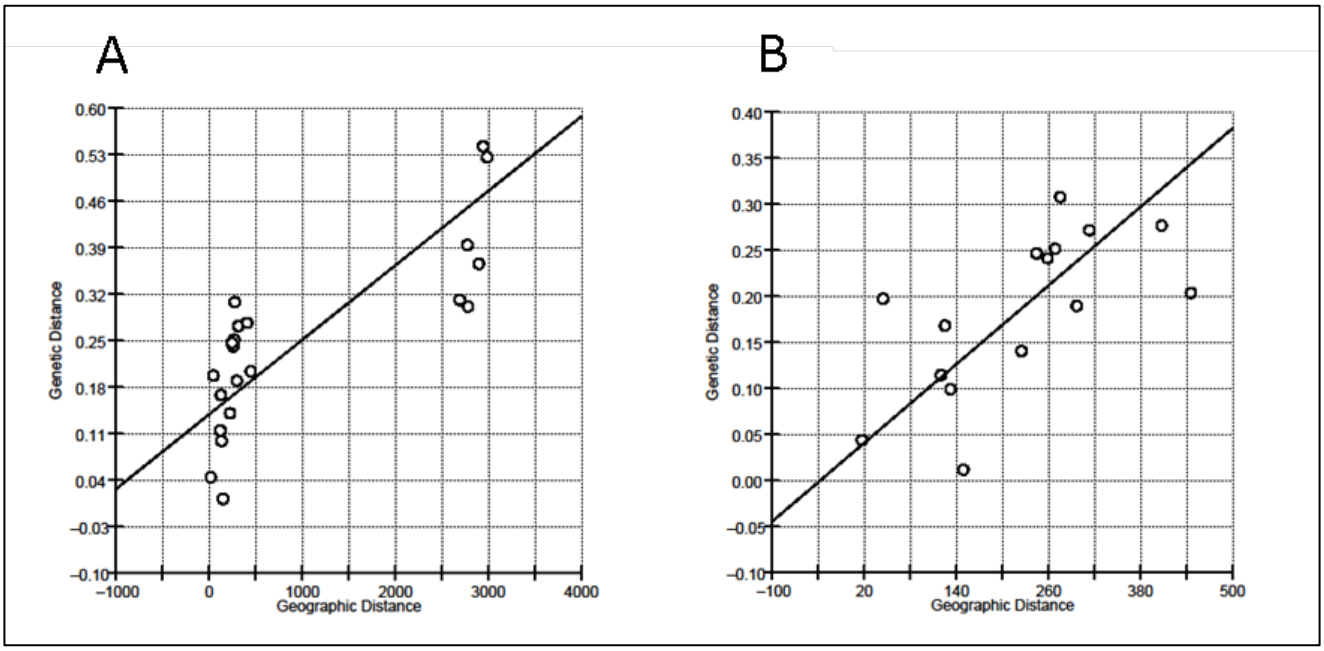

Figure 3.4 A) Reduced major axis regression showing clear linear relationship between geographic distance (x-axis) and genetic distance (F_{ST}) (y-axis) between all seven population patches. B) Reduced major axis regression analysis showing clear linear relationship between geographic distance (x-axis) and genetic distance (F_{ST}) (y-axis) with the outlier population farthest away from the others removed.

Mean numbers of alleles within the subpopulations ranged from 1.72 to 3.27, and globally the mean number of alleles was 5.18 (Table 3.2). Inbreeding (F_{is}) was high (> 0.200) globally and within all subpopulations with significant (alpha = 0.05) departures from Hardy-Weinberg (HW) expectations, except for Subpopulation 1 (Table 3.2). Subpopulation 1 had the lowest F_{is} (0.086), with no significant departures from HW expectations (Table 3.2). Observed heterozygosity was less than expected when measured globally and for most subpopulations except again for Subpopulation 1. Allelic richness (A_R) ranged from 1.73 to 2.53 within the subpopulations and was 3.14 when measured globally (Table 3.2).

Table 3.2. Genetic measures of all individuals pooled (186 snails) and of each geographically assigned population (containing 10-24 snails) including number of individuals (N), mean number of alleles, inbreeding coefficient (F_{is}), observed heterozygosity (H_O), expected heterozygosity (H_E), gene diversity and rarified allelic richness (A_R).

Population	N	Mean # of alleles ± s.d.	F_{is}	H_O ± s.d.	H_E ± s.d.	Gene Diversity	A_R
All	186	5.182±3.093	0.386*	0.282±0.172**	0.459±0.240	0.753±0.226	3.14±1.545
1	10	1.727±0.905	0.086	0.182±0.244	0.198±0.217	0.491±0.151	1.73±0.905
2	19	2.818±1.779	0.201*	0.306±0.247**	0.381±0.282	0.552±0.242	2.53±1.482
3	22	3.273±2.005	0.251*	0.314±0.203**	0.417±0.271	0.605±0.232	2.73±1.483
4	11	2.272±1.954	0.264*	0.331±0.241**	0.488±0.284	0.63±0.283	2.71±1.924
5	12	2.545±1.293	0.349*	0.235±0.235**	0.355±0.261	0.687±0.229	2.51±1.262
6	24	2.091±0.944	0.231*	0.231±0.245**	0.299±0.233	0.61±0.204	1.92±0.730
7	12	2.455±1.368	0.277*	0.288±0.231**	0.394±0.249	0.675±0.244	2.40±1.307

* F_{is} values with significant ($P< 0.001$) departure from Hardy-Weinberg expectations
** Significant heterozygote deficit

Effective population size (N_E), interpreted as the number of breeders for species that can self-fertilize, was calculated globally at 27.9 individuals. Results for subpopulations ranged from 1.6 to 8.5 except for four of the populations, which had negative or extremely high estimates (Table 3.3). These erroneous results are likely due to the model's difficulty measuring extremely small effective population sizes, or non-adherence to model assumptions (Waples and Do 2008). For subpopulations with erroneous results we interpret them to mean very small effective population sizes (likely less than five individuals).

Table 3.3. Effective population size (N_e), calculated globally and for each of the subpopulations, using the LDNE method. The jackknife method implemented within the LDNE software was used to calculate confidence intervals. Note N_e is considered number of breeders for species that can self-fertilize.

Population	N_e	95% confidence limits
All	27.9	19.7 – 39.1
1	1.6	0.6 - 24.4
2	-30.9	471.7 - infinite
3	43.5	14.9 - infinite
4	-39.1	11.4 - infinite
5	6.4	2.6 - 16.6
6	8.5	2.2 - 45.1
7	110.2	4.4 - infinite

In general, F_{ST} between 0 and 0.04 is an indication of low genetic differentiation, while 0.05 – 0.25 indicates moderate genetic differentiation, and F_{ST} greater than 0.25 indicate high levels of genetic differentiation (Freeland and Peterson 2011). Pairwise F_{ST} between all subpopulations was generally high (0.09 – 0.54) and significant ($P < 0.001$). However, pairwise F_{ST} between Population 3 and 4 was low and non-significant, likely indicating dispersal occurring between the two patches (Table 3.4).

Table 3.4. F_{ST} between all pairs of subpopulations

Population	1	2	3	4	5	6	7
1	0						
2	0.394*	0					
3	0.301*	0.044*	0				
4	0.311*	0.099*	0.011	0			
5	0.526*	0.271*	0.189*	0.203*	0		
6	0.542*	0.307*	0.241*	0.276*	0.197*	0	
7	0.365*	0.168*	0.114*	0.140*	0.251*	0.2468*	0

* Indicates significant F_{ST} (Alpha = 0.05)

Analysis of molecular variance (AMOVA) indicates that genetic variation is highest within individuals (68.89%), followed by among subpopulations (21.51%), and finally among individuals within subpopulations (9.60%; Table 3.5). This result is consistent with other

diversity measures, which suggest low diversity and high inbreeding within subpopulations. High variation within individuals and among subpopulations indicates restricted gene-flow between discrete habitat patches.

Table 3.5. Analysis of molecular variance (AMOVA) of 150 *P. redfieldi* in seven subpopulations.

Source of variation	df	Sum of squares	Variance components	Percentage of variation (%)
Among populations	6	1277.212	6.09684 va	21.51
Among individuals within populations	103	2572.438	2.72166 vb	9.60
Within individuals	110	2148.5000	19.53182	68.89
Total	219	5998.150	28.35031	

Results from the Bayesian clustering analysis conducted with TESS showed distinct clustering corresponding roughly to the populations we defined based on geographic proximity. The Q-matrix stabilized around K=5 to K=6, and the deviance information criterion (DIC) plateaued around K=6 (Figure 3.5). Therefore the likely number of genetically defined population clusters is approximately six.

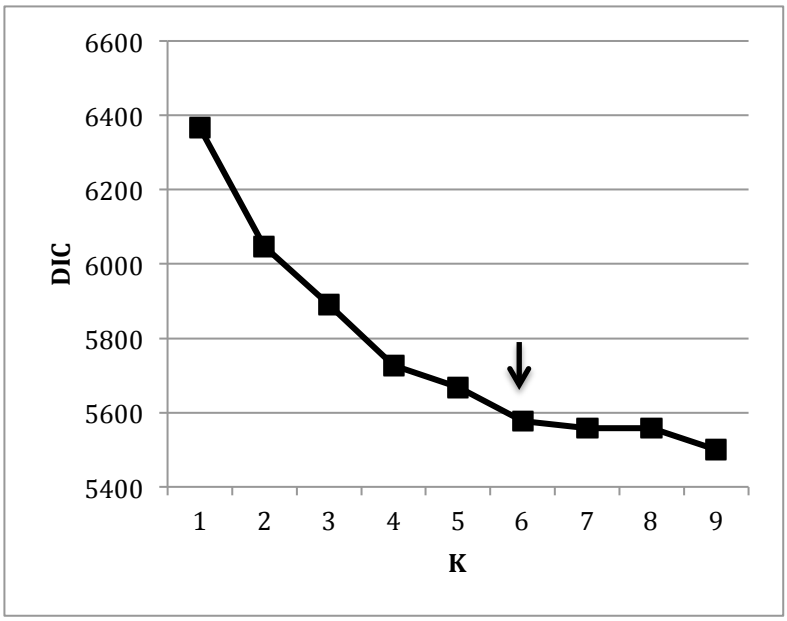

Figure 3.5. Deviance information criterion (DIC) from TESS output plotted over number of clusters (K). Note plateau reached at approximately K = 6. A Plateau of the DIC is an indication of the model fit.

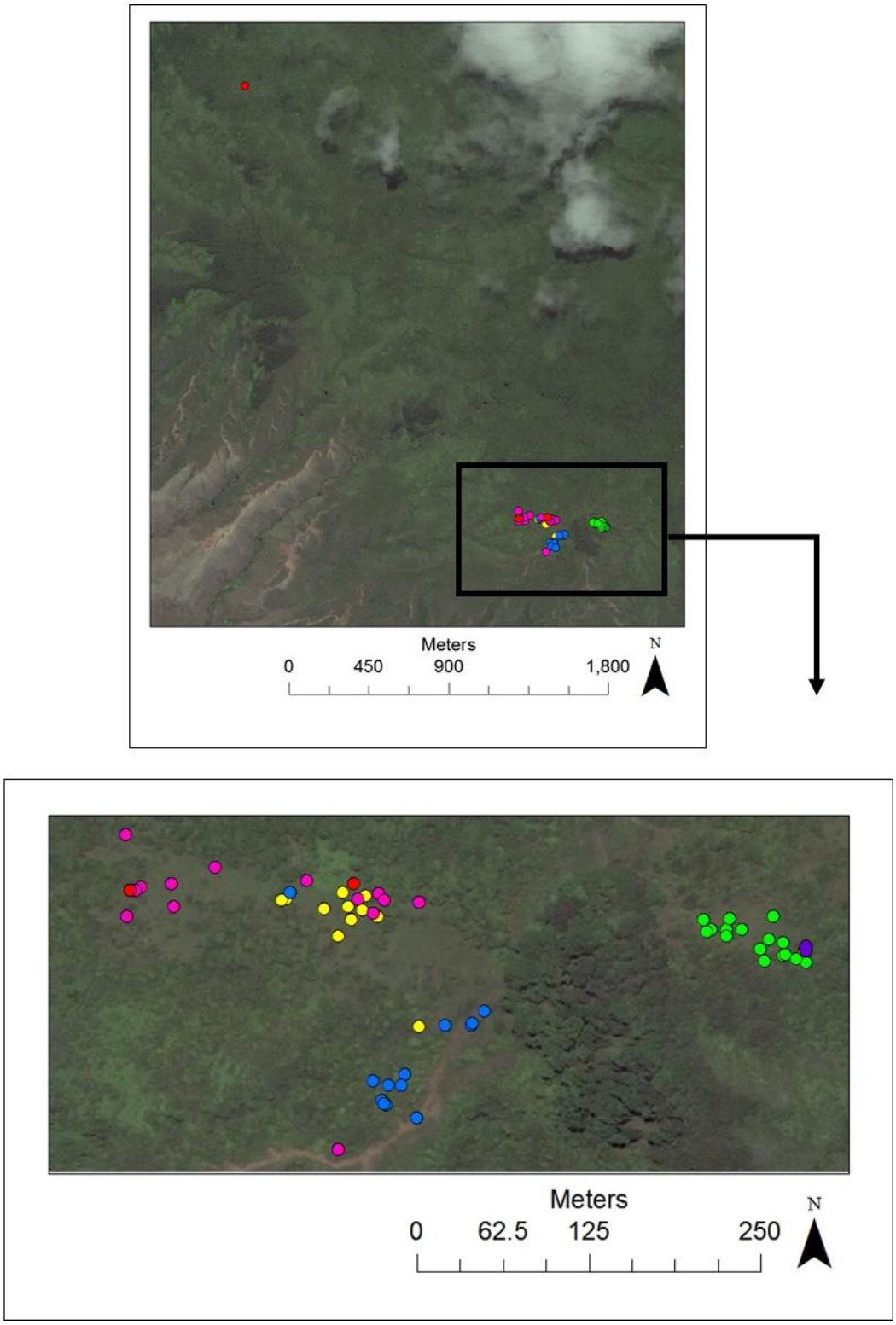

Figure 3.6. Six genetic populations were identified with the Bayesian clustering program TESS. Here we display tree coordinates color coded by cluster. For this figure individuals were assigned to a cluster if they had admixture proportions greater than or equal to 50% for a given cluster.

DISCUSSION

This study is the first to assess fine scale population structure across a fragmented landscape that once likely hosted a continuous population of Hawaiian tree snails. Based on our results, *P. redfieldi* exist in a highly structured state with meta-population dynamics occurring between subpopulations isolated in habitat fragments. Our data show these subpopulations are experiencing significant isolation-by-distance effects over very short distances (≥10 meters). We found significant F_{ST} between most subpopulations in discrete habitat patches, and reduced genetic diversity (H_O and A_R) within patches compared to global calculations. Wahlund effects were observed in the form of heterozygosity deficits in all but one subpopulation, and linkage disequilibrium was found between 10 pairs of loci when measured globally. These Wahlund effects are typical symptoms of population subdivision (Frankham et al. 2010, Garnier-Géré and Chikhi 2013). In addition, results of the analysis of molecular variance suggested reduced gene-flow, and very low variance within subpopulations.

The cluster analysis conducted with TESS empirically assigned individuals to six clusters, matching almost exactly the populations we assigned based on geographic proximity. This result confirms that discrete habitat patches separated by invasive vegetation at distances greater than 20 meters may represent a significant barrier to gene-flow in Hawaiian tree-snail populations.

Subpopulation 1 (Fig. 3.3), separated from the rest of the known *P. redfieldi* subpopulations by approximately three kilometers, was in Hardy-Weinberg equilibrium, and had a relatively low inbreeding coefficient when compared to the rest of the subpopulations. These results indicate that individuals within this subpopulation are able to mix freely. Interestingly, this subpopulation was the only one that was sampled from habitat not fragmented by invasive vegetation. Instead individual trees within the immediate habitat were connected either directly, with interlocking branches, or with other native shrubs and ferns. Although this population had low genetic diversity (observed H_O and A_R), likely a result of small population size, it did not display the same patterns as those snails in fragmented habitat, which included high inbreeding (F_{is}) and significant heterozygote deficit. While we can only speculate, it is possible that the connected habitat at this site has maintained gene flow.

Stoll et al (2009), through experimental fragmentation of grassland harboring six species of ground snail, found that small-scale habitat fragmentation increased the local extirpation of snails. Though taking place in different ecosystems, our results highlight the genetic effects that may contribute to such declines in fragmented habitat. Genetic diversity measures of *P. redfieldi*, including observed heterozygosity (H_O) and allelic richness (A_R), are some of the lowest reported for Hawaiian tree snails and other species of rare terrestrial molluscs, using microsatellite markers. (Buckley et al. 2014, Mejia et al. 2012, Erickson and Hadfield 2014, Price and Hadfield 2014, Price et al. in review, Sischo et al. in review,). Such low diversity within habitat patches indicates that *P. redfieldi* may have experienced sustained and or reoccurring population bottlenecks.

While there is no documentation regarding the land use history of this particular part of Molokaʻi, more specifically how it became fragmented, it is likely that non-native deer and cattle are to blame. These ungulates were particularly abundant on Molokaʻi prior to island-wide eradication beginning in 1967 as a result of an outbreak of bovine tuberculosis on the island (Hadfield and Saufler 2007). *P. redfiedli* mature in approximately five years (Kobayashi and Hadfield 1986), therefore if we assume the habitat in this part of Molokaʻi became fragmented between the years 1900 and 1950, then it is possible that approximately 10 to 20 generations of *P. redfieldi* have existed in this fragmented state.

A portion of the subpopulations of *P. redfieldi* that we sampled from in this investigation was part of a long-term study (>20 years) monitoring the demographic trends of *P. redfieldi* (Hadfield and Miller 1989, Kobayashi and Hadfield 1996, Hadfield and Saufler 2007). During the span of this study, the population sizes within individual habitat patches (mostly individual trees in a wide meadow) varied considerably. Initially, 1983-1995, individual populations grew 100-900%. However, after 1995 all populations declined precipitously (>85%), the result of intense rat predation (Hadfield and Saufler 2007). It is not surprising that we see evidence of this drastic population bottleneck in our genetic data, including low genetic diversity and high inbreeding estimates within subpopulations occupying discrete habitat patches.

Land snails in general have low vagility, and it is this characteristic that has likely contributed greatly to the impressive snail-species radiations seen on islands. In Hawai‘i, and other island archipelagos, the slow volcanic processes that form land, and the gradual geologic processes that lead to its weathering, are the same vicariant forces that have gradually fragmented snail populations over time and have resulted in these spectacular radiations (Gulick 1872, 1887, Crampton 1916, 1925, 1932, Zimmerman 1948, Solom 1990, Holland and Hadfield 2004).

While population isolation, as a result of habitat fragmentation, may be a driver of terrestrial mollusc evolution, these natural processes over millennial time scales should not be confused with the fragmentation and subsequent bottlenecks experienced by contemporary populations in Hawai‘i. The rate and scale of habitat fragmentation occurring in recent time, paired with extreme predation from invasive molluscivores, are certainly drivers of extinction.

Based on our data, we recommend that when starting an *ex situ* population or when selecting individuals for translocation into protected habitat, individuals should be from multiple patches across what would have been a continuous habitat and or population. This is particularly important when snails occur in heavily fragmented habitat, or when small population patches are separated from other small patches by distances greater than 10 – 20 meters. There may be concern for outbreeding depression, which might occur when mixing individuals from divergent lineages that may have local adaptations to the specific microclimates in which they occur (Frankham et al 2010). Therefore, we caution that our recommendations should not be extrapolated to distances greater than what would have likely been connected populations prior to the fragmentation in question occurring.

In addition, when designing fenced, predator-proof snail reserves, or restoring habitat around wild populations, managers should aim to make the habitat as connected as possible to ensure gene-flow. As noted here, habitat patches fragmented by distances greater than ten meters represent significant barriers to dispersal for achatinelline snails. Thus, even in protected habitat, loss of genetic diversity can occur, undermining conservation efforts, due to Whalund effects over very short distances.

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CHAPTER 4

EX SITU POPULATION GENETICS OF THE ENDANGERED HAWAIIAN TREE SNAIL ACHATI*NELLA FUSCOBASIS* (ACHATINELLINAE): DEMOGRAPHIC AND GENETIC INSIGHTS INTO A CAPTIVE POPULATION DECLINE

ABSTRACT

Hawaiian tree snails in the sub-family Achatinellinae have experienced drastic population declines and range reductions as a result of habitat destruction, over-collection and introduced predators. In 1991, 11 individuals from one of the last remaining populations of *Achatinella fuscobasis*, a federally listed species, were brought to the University of Hawaii's tree-snail captive-rearing facility. After increasing in population size to 441 snails in 2005, the population subsequently declined to only 120 snails by 2014, a 73% reduction. In this study we assessed demographic and genetic changes that occurred in the captive-bred population of *A. fuscobasis*. Demographic data were compared to genetic data collected from seven microsatellite markers for the $F_1 – F_3$ generations to investigate the role bottleneck effects such as loss of genetic diversity and inbreeding may have had on the population decline. Demographic measures such as juvenile survival and survival to maturity remained constant for the F_1 and F_2 generations, and significantly decreased in the F_3 generation. There was no difference in genetic diversity measures such as allelic richness, gene diversity, and observed heterozygosity among generations, but all generations were characterized by low genetic diversity and high inbreeding. The drastic population decline appears to have been due to stochastic demographic dynamics characteristic of small populations, with bottleneck effects that occurred before the founding of the *ex situ* population acting in synergy with the genetic factors.

INTRODUCTION

Island endemic species, isolated from the pressures of continental ecosystems, often evolve life history traits, behavior, and immune systems naïve to the presence of mainland predators and diseases (Blackburn et al. 2004, Fordham and Brook 2008, Paulay 1994). Achatinelline tree snails, endemic to the Hawaiian Islands are one such group. Late maturity (5–6 years), low

fecundity (1–7 offspring per year) and long lifespans (>10 years) are characteristics of snails within this sub-family (Hadfield 1986, Hadfield et al. 1993, Hadfield and Miller 1989). Unfortunately, achatinelline tree snails have experienced rapid declines and massive range reductions over the last century due to historical habitat loss, predation and over-collection. Currently the more urgent and insidious threat comes from introductions of predators including rats, predatory snails, and chameleons (Hadfield 1986, Solem 1990, Hadfield et al.1993, Hadfield and Saufler 2009, Holland et al. 2010). In light of these declines, in 1981, the entire genus *Achatinella*, endemic to the island of O‘ahu, was listed as Endangered under the US Endangered Species Act (US Fish and Wildlife Service 1993). The formal recognition of these declines elicited emergency conservation efforts that included the establishment of an *ex situ* captive rearing facility at the University of Hawai‘i at Mānoa (UH) (Hadfield et al. 2004). Since 1986 this facility has reared 20 species of Hawaiian tree snails with the ultimate goal of augmenting or reestablishing wild populations. However, heavy predation in remaining wild populations has prevented the release of captive-bred snails, and populations have remained in captivity for multiple generations. Despite initial success and for unknown reasons, most *ex situ* populations at the captive rearing facility have experienced drastic declines in recent years.

In 1991 a population of *Achatinella fuscobasis*, was visited in the summit region of Mt. Konahuanui in the southern Ko‘olau Mountains on O‘ahu. This small remnant population was under enormous pressure from introduced predators, so to safeguard it against extirpation a sample of the population was brought to the lab for captive rearing. The captive population was founded with eleven snails and subsequently increased to 441 snails by 2004. Since 2005 the population has experienced a 73% reduction in size. This decline corresponded with declines in other species reared at the facility (Price and Hadfield 2014).

In this study we investigated potential contributions of demographic and genetic factors to the drastic population decline observed in *A. fuscobasis* between 2005 and 2014. Demographic trends in juvenile survival, survival to maturity, and fecundity were compared among four generations (F_1, F_2, F_3,F_4) for snails born in the captive population. Using 11 microsatellite markers, demographic trends for the first three generations ($F_1 – F_3$) were compared with genetic measures such as heterozygosity, inbreeding coefficient, and genetic diversity.

MATERIALS AND METHODS

Captive propagation methods

Eleven *A. fuscobasis* (10 adults, 1 sub-adult) were brought to the Hawaiian tree snail captive-rearing facility at the University of Hawai‘i at Mānoa in 1991 under USFWS permit PRT-826600. In the captive-rearing facility all snails were kept in small plastic cages inside environmental chambers that mimicked environmental conditions experienced by snails in the wild (Hadfield et al. 2004). Cages were cleaned every other week, at which time freshly cut leafy branches from native snail host plants were added to provide grazing opportunities. In addition, snail diets were supplemented with a common native fungus (*Cladosporium* sp.) isolated from the leaves of wild host trees and cultured on calcium-supplemented agar. Through the years, as the abundance of *A. fuscobasis* individuals increased in the laboratory, juvenile snails were separated from adults and moved to separate cages.

During every cage cleaning, demographic data were recorded, including births and deaths, and the total numbers of juveniles (shell length < 9.5 mm), sub-adults (9.5 mm - lipped shell) and adults. Adults stop growing when they form a characteristic thickening around the shell aperture, here referred to as the lip. Lip formation is correlated with sexual maturity in achatinelline snails (Pilsbry and Cooke 1912–1914). Every snail that died in the lab was placed in a vial of 95% ETOH for long-term tissue preservation and storage. The species identity, date of death, laboratory population and shell length and width were recorded on each vial. Demographic data obtained from preserved snails were cross-checked with logbook data.

Tissue sample collection, DNA extraction and amplification

A small piece of tissue (10–20 mg) was cut from the foot of preserved, deceased snails with a sterile blade and placed in 100% ETOH in preparation for DNA extraction. Total cell DNA was extracted from all samples using a QIAGEN DNeasy blood and tissue kit, following manufacture’s protocols for animal tissues (QIAGEN Inc., Valencia CA, USA). DNA was eluted in 200 µl of QIAGEN’s proprietary elution buffer and kept at -20^{o} C until amplification using Polymerase Chain Reaction (PCR).

All samples were genotyped at eleven microsatellite loci. For eight previously-published loci (Erickson and Hadfield 2008), we followed the recommended PCR conditions with the addition of 10 μm bovine serum albumin (BSA) to each reaction to bind inhibitor molecules (Kreader 1996, Woide et al. 2010). We additionally amplified three novel primers using the same amplification protocol described in Erickson and Hadfield (2008) with optimized annealing temperatures (Table 4.1, Table 4.2). Genotyping was conducted at the Center for Genomic, Proteomic, and Bioinformatic Research (CGPBR) at the University of Hawai‘i at Mānoa. The software Peakscanner version 1.0 (Applied Biosystems 2006) was used to score alleles.

Table 4.1. Primer information for the three new loci originally identified by Erickson and Hadfield and optimized in this study. Locus name and Genbank accession number are followed by primer sequence, repeat motif, and annealing temperature (T_a).

Loci identification (GenBank Accession no.)	Primer sequence (5' - 3')	Repeat motif	T_a
AS50 (KR872615)	F: CGCGAGCTACGGATATAGGA R: GCGGTTTTGTTGAGATGTCTT	$(AGT)_6$…$(AGT)_3AGA(AGT)_3TGC(AGT)_6ATT(AGT)_6$	54
AS96 (KR872616)	F:CAACAACAATAACAACAACAGCA R: TCCAGGTTGAGACCAACAGA	$(ACT)_3GCTACTACC(ACT)_2CCT(ACT)_{10}$	58
AS100 (KR872617)	F: GAGGTTGTTAGGGTATGGAGATT R: GTTTAGCGCCGGAAAGTTGT	$(ACAT)_{32}AAAT(ACAT)_3AAAT(ACAT)_7ATAT(ACAT)_{10}$	59

Table 4.2. Locus identification and GeneBank accession number, followed by number of alleles (N_A), size range of alleles, and null allele frequencies estimated by Microchecker and INEST for *Aachatinella fuscobasis*.

Loci Identification, Genbank accession No.)	N_A	Size range (bp)	Microchecker null allele freq.	INEST null allele freq.
AS812 (EU119381)	13	219–337	0.204	0.003
AS32 (EU119382) *	10	165–210	0.374	0.416
AS46 (EU119383)	13	219–270	0.286	0.075
AS53 (EU119384)	20	176–296	0.107	0.054
AS61 (EU119385)	20	157–229	0.196	0.03
AS62 (EU119386)	8	206–228	0.179	0.054
AS82 (EU119387) *	31	129–273	0.208	0.284
AS110 (EU119388)*	11	208–283	0.222	0.353
AS50 (KR872615)	9	315–417	–0.506	0.057
AS96 (KR872616)	26	208–403	0.198	0.156
AS100 (KR872617)*	49	217–466	0.251	0.246

* Indicates loci removed from all further analyses due to high null allele frequencies.

Demographic analysis

Demographic data were collected for 1,390 *A. fuscobasis* that died in the laboratory between 1991 and 2014. In addition, demographic data were collected for 119 live snails at the captive-rearing facility in 2014. Growth curves were used to estimate birth dates for living and deceased snails (Severns 1981, Price and Hadfield 2014). Birth dates were then used to calculate juvenile survival (individuals that lived to at least one year of age), and survival-to-maturity (proportion of individuals that reached sexual maturity). To assess trends in juvenile survival and survival-to-maturity we used the statistical package JMP version 10.0 (2012). Linear regression analysis was used to test for changes in juvenile and adult survival over time. Chi-square analysis was used to test for changes in survival to maturity among generations.

Genetic analysis

Microsatellite genotypes were collected for 342 snails (27% of the deceased collection) that died in the years 1991 to 2010 ($F_1 – F_3$ generations). The program Microchecker version 2.2.3 was used to assess scoring problems and to estimate the frequency of null alleles (Van Oosterhout et al. 2004). Because homozygote excess can arise from null alleles (allele scoring, amplification problems, or alleles identical in size but not by decent) and inbreeding (alleles identical by decent), the program INEst version 2.0 was used in addition to Microchecker to independently estimate null allele frequencies. INEst, through an iterative process, simultaneously measures inbreeding, and estimates null-allele frequencies providing a more accurate estimate when inbreeding is possible (Chybicki and Burczyk 2009). Using the combined null-allele estimates from both programs, we eliminated four loci from all down stream analyses that had null-allele estimates greater than 25%. The remaining seven alleles had lower estimated null-allele frequencies, were polymorphic, and were therefore used in all further analyses.

Microsatellite data were divided into generations spanning 5.59 years using the equation $M – 1 + (1/(1 – v)) = T$ (M = maturation time, v = mean adult survival and T= generation time) (Nunney and Elam 1994). Within each generation the data were further divided into individuals that survived to maturity and those that did not. We were not able to recover DNA from the majority of individuals comprising the founding generation; therefore we did not include founders in this study.

Within each generation, we calculated the inbreeding coefficient (F_{is}), observed heterozygosity (H_O), expected heterozygosity (H_E), and genetic diversity using the program Arlequin (Excoffier et al. 2005). Because data divisions had uneven sample sizes, rarified allelic richness (A_R) was calculated using the program HP Rare (Kalinowski 2004, Kalinowski 2005). Individual heterozygosity was calculated for each snail by tabulating the proportion of heterozygous loci. Individual heterozygosity data was then arcsine transformed. Allelic richness and individual heterozygosity were compared across generations with an analysis of variance. Within-generation allelic richness and individual heterozygosity of those that survived to maturity was

compared to those that did not with t-tests, using the statistical software JMP version 10.0 (2012).

RESULTS

Demographics

In 1991 the captive population of *A. fuscobasis* was founded with 11 individuals and grew at an average rate of 29% per year between 1991 and 2004. The population reached a peak of 441 individuals in 2004 and began a decline in 2005 that continued through the completion of this study in 2014. After 2005 the population declined 73%, resulting in 117 individuals at the end of 2014 (Figure 4.1).

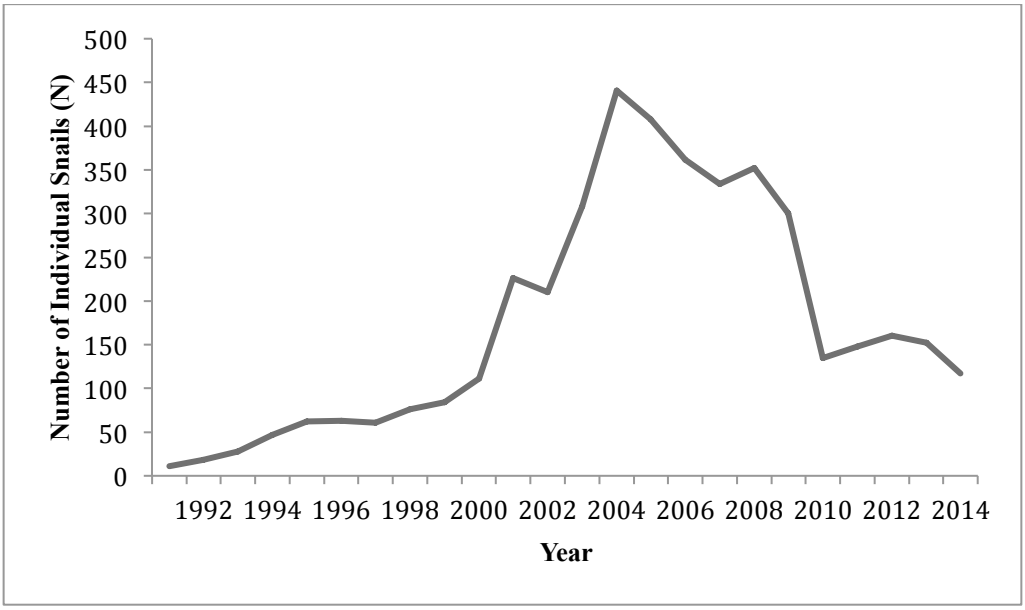

Figure 4.1. Population trajectory of captive *Achatinella fuscobasis* beginning in 1991 with 11 founding individuals. The population peaked in 2005 with 441 individuals and has since declined to 117 as of December 2014.

Juvenile survival significantly decreased over time ($r^2 = 0.01$, $P < 0.0001$) and differed among generations ($X^2 = 47.6$ $P < 0.0001$). Juvenile survival was comparable in the F1 and F2

generations but dropped in the F3 generation and remained low through the F4 generation (Figure 4.2)

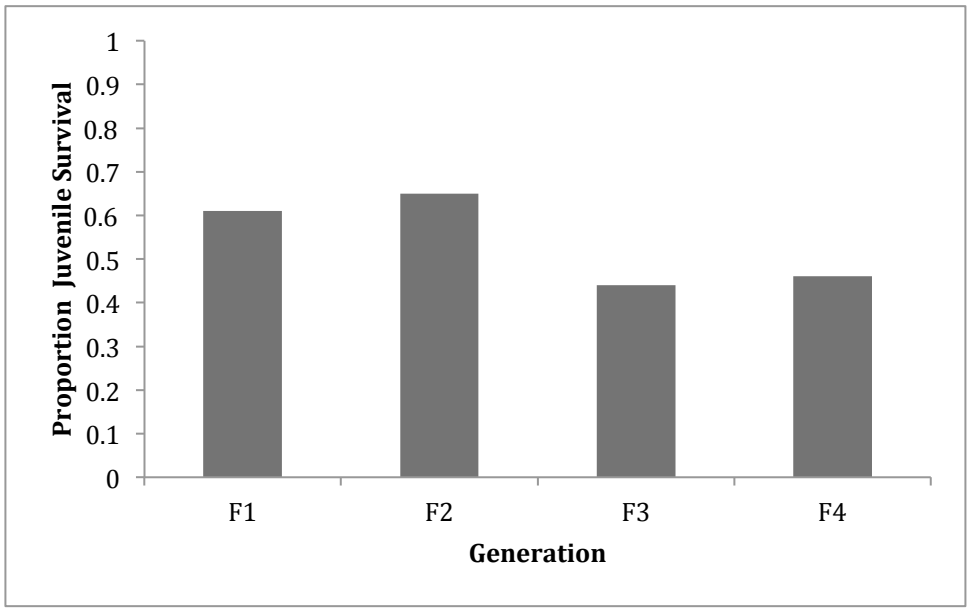

Figure 4.2. Proportion of newborn snails surviving past their first year of life per generation.

Survival to maturity significantly decreased over time ($r^2 = 0.03$ $P < .0001$) and differed among generations ($X^2 = 0.03$ $P < 0.0001$). Survival to maturity was 55% and 53% in the F_1 and F_2 generations, respectively. In the F_3 generation survival to maturity dropped to 32% and to 23% in the F_4 generation (Figure 4.3).

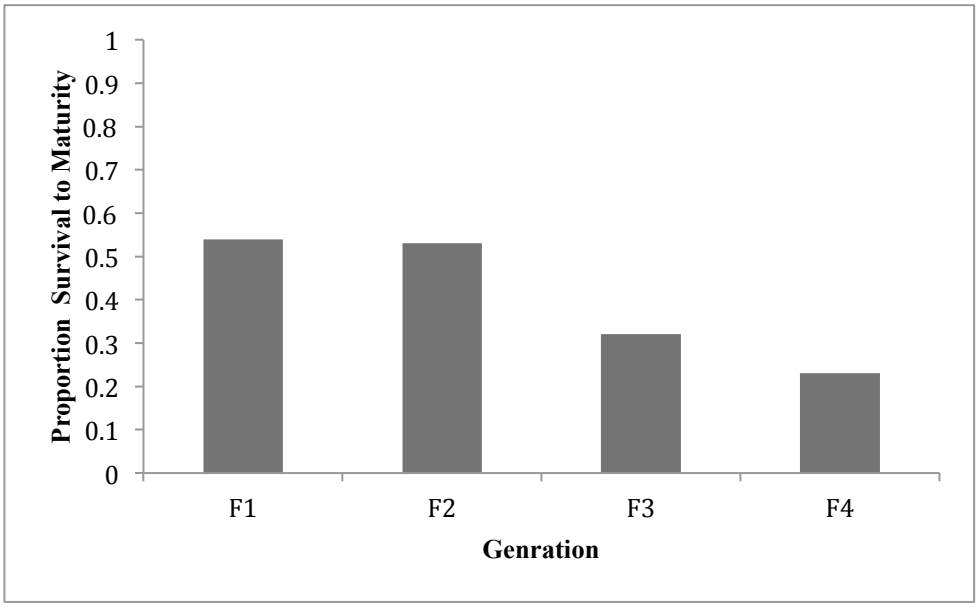

Figure 4.3. Proportion of individuals surviving to maturity per generation.

Genetics

Heterozygosity did not differ among the three generations examined in this study ($F = 0.066$, $df = 2$, $P = 0.94$). In the F_2 generation, there was no significant difference in heterozygosity ($t = 0.91$, $P = 0.37$) between those that survived to maturity, and those that did not. However, in the F_3 generation those that survived to maturity were less heterozygous than those that did not survive to maturity ($t = 2.07$, $P = 0.041$). This comparison was not conducted for the F_1 generation because all genetic samples came from individuals that survived to maturity.

In the F_2 generation, there was no statistical difference in heterozygosity between juveniles that survived past their first year of life and those that did not ($t = 1.72$, $P = 0.088$). Similarly, in the F_3 generation there was no statistical difference between juveniles that survived past their first year of life and those that did not ($t = 1.26$, $P = 0.21$). The inbreeding coefficient (F_{is}) was high in the F_1 generation (> 0.300) and remained high in all three generations, even among snails that survived to maturity (Table 4.3).

There was no significant difference in allelic richness (A_R) among the three generations ($F = 3.44$, df = 2, $P = 0.956$). In addition, there was no statistical difference between A_R of those that

survived to maturity compared to those that did not in the F_2 generation (t=1.78, P=0.212) and in the F_3 generation (t=1.81, P=0.355).

Table 4.3. Genetic measures by generation ($F_1 - F_3$) for captive *A. fuscobasis* including number of individuals (N), inbreeding coefficient (F_{is}), observed heterozygosity (H_o), expected heterozygosity (H_E), gene diversity, and allelic richness (A_R). Generations are further subdivided by those that survived to maturity and those that did not. Note, all individuals sampled in the F_1 generation survived to maturity.

Population	N	F_{is}	H_o ± s.d.	H_E ± s.d.	Gene diversity ± s.d.	A_R
F1						
Survived to Maturity	23	0.419*	0.54±0.20	0.74±0.10	0.72±0.43	6.8±2.0
Did not survive to Maturity	--					
F2						
Survived to Maturity	98	0.330*	0.54±0.22	0.69±0.11	0.65±0.37	6.6±2.4
Did not survive to Maturity	84	0.354*	0.52±0.22	0.74±0.11	0.71±0.48	6.9±3.0
F3						
Survived to Maturity	39	0.516*	0.48±0.26	0.67±0.16	0.62±0.37	6.4±3.1
Did not survive to Maturity	74	0.350*	0.54±0.19	0.73±0.10	0.69±0.39	6.9±1.9

*Indicates F_{is} values with significant ($P < 0.001$) departure from Hardy-Weinberg expectation.

DISCUSSION

The recent population declines in the tree-snail captive-rearing facility at the University of Hawaii have raised concerns regarding the future of Hawaiian tree snails and our ability to conserve the last remaining species. Declines in *ex situ* populations of any organism may stem from a variety of causes, including but not limited to disease, parasites, sanitation, diet, improper conditions and failure of mechanical life support equipment (Jacobson 1993, Snyder et al. 1996). In addition, erosion of genetic diversity and increased inbreeding are a major concern for *ex situ* populations founded with small numbers of closely related individuals (Freeland 2005). These bottleneck effects in particular, can act synergistically with the above mentioned factors (Frankham 1998, Frankham et al. 2014).

The demographic measures of population viability assessed, including juvenile survival and survival to maturity, remained relatively stable in the F_1 and F_2 generations, but significantly decreased in the F_3 and F_4 generations. We expected to observe bottleneck effects such as a loss of genetic diversity and increased inbreeding correlating to these declines, but this was not the case (Figure 4.4). All measures of genetic diversity, including allelic richness (A_R), observed heterozygosity (H_o), and gene diversity remained relatively stable over 20 years and three generations (Table 4.3).

This captive population was founded with some of the last known wild individuals of the species. Our data indicate that these founders may have already experienced severe genetic bottleneck effects. In the F_1 generation, we observed low genetic diversity measures, departures from Hardy-Weinberg equilibrium, and high levels of inbreeding. However, despite the indication of a genetic bottleneck, this population grew at a rate of 29% per year from 1991 to 2004 (F_1 – F_2 generations) with no changes in genetic trends (Table 4.3). This rapid rate of increase may have prevented further loss of genetic diversity (Freeland 2005, Kekkonen and Brommer 2015).

There was a significant difference in the observed heterozygosity of the individuals that survived to maturity in the F_3 generation compared to those that did not. Those that survived were significantly less heterozygous than those that died. This is a curious result because we expect individuals with lower heterozygosity to be more susceptible to early mortality. For example, Price and Hadfield (2014) observed that snails of the sister species *Achatinella lila* that had higher heterozygosity were more likely to survive to maturity and produce offspring than those with lower heterozygosity. However, small populations are susceptible to stochastic demographic change, and our results illustrate the dynamic nature of the interaction between genetic and demographic factors. Small populations experiencing drastic declines caused by factors such as disease or environmental fluctuation may lose genetic diversity through the death of heterozygous individuals carrying low-frequency alleles. This loss of genetic diversity in a captive population may lead in turn to further demographic decline, especially if the lost alleles were correlated with higher reproductive output or conferred a selective advantage in the natural environment.

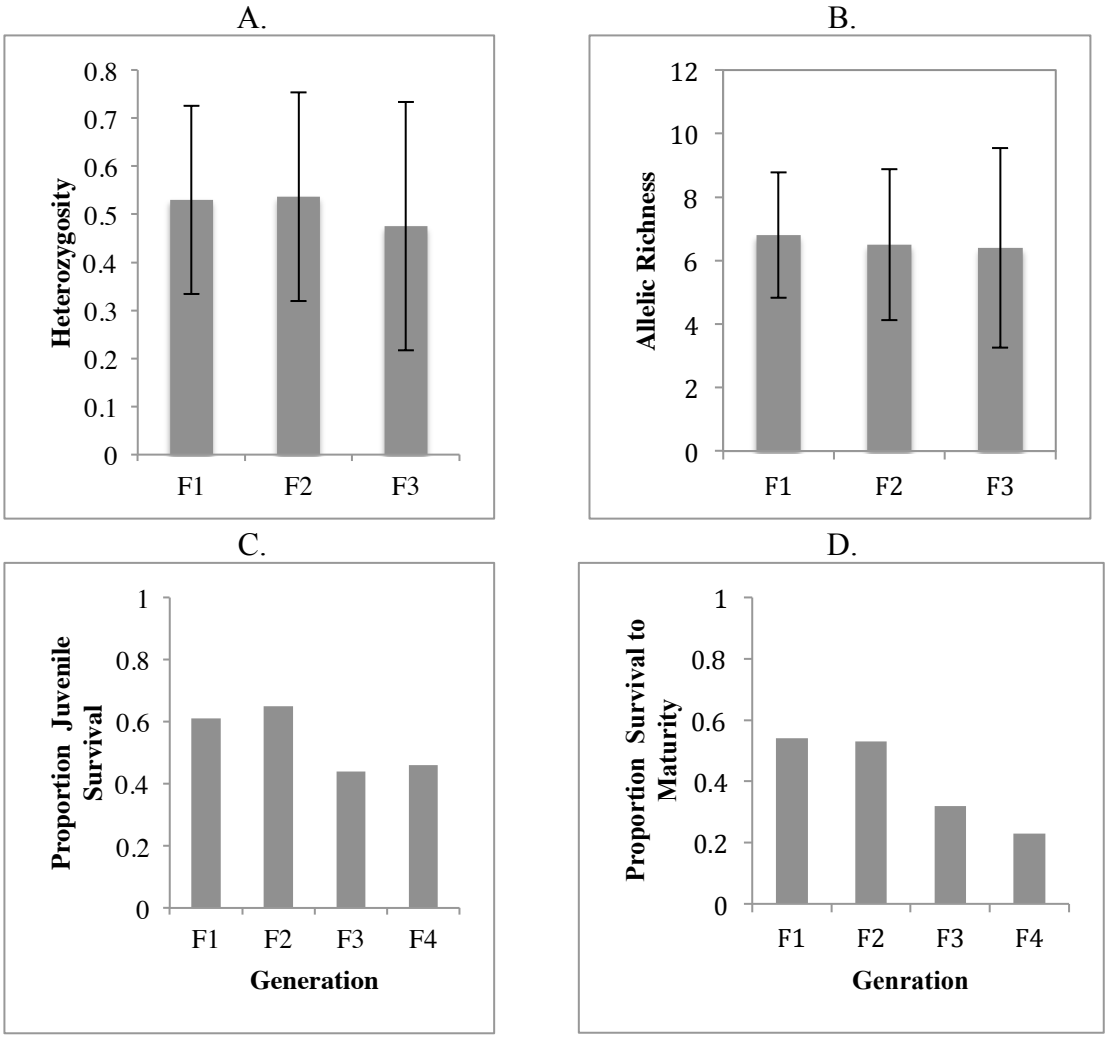

Figure 4.4. Genetic diversity measures compared to demographic trends. A) Heterozygosity by generation, B) Allelic richness by generation, C) Juvenile survival by generation, D) Survival to maturity. Note, demographic trends continue through the F_4 generation while genetic measures stop at the F_3 generation.

It is likely that the *ex situ* decline described here was not a direct result of a further loss of genetic diversity following the severe bottleneck this population experienced. Instead, bottleneck effects that occurred prior to the founding of the laboratory population may have acted in synergy with other factors, such as disease. Island species often have immune systems naïve to alien diseases and may be highly vulnerable to them (Paulay 1994, Fordham and Brook 2008). In addition, Low genetic diversity and high inbreeding likely increase vulnerability to disease (Khlat and Khoury 1991, Jacobson 1993, Ross-Gillespie et al. 2007). Furthermore, diseases affecting captive populations are often

density-dependent; large numbers of individuals kept in close proximity may allow disease to spread quickly among cages or environmental chambers (Anderson and May 1986, Arneberg et al. 1998).

The laboratory population of *Achatinella fuscobasis* grew to 441 individuals. Similarly, Price and Hadfield (2014) report a maximum population size of *A. lila* that was over 600 individuals at the captive-rearing facility. With such high numbers of inbred, immunologically-naïve island taxa, kept in close proximity, the Hawaiian tree-snail captive-rearing facility may be extremely vulnerable to outbreaks of disease and parasites. It is possible that the decline of *A. fuscobasis* was an epidemiological response typical for captive populations. Lafferty and Gerber (2002), in a meta-analysis of parasite and disease effects on populations of rare species, summarized 29 examples of disease-related decline. In all studies a parasite, or a viral, fungal or bacterial pathogen was identified and attributed to population declines of 50 -100%. The only documented extinction of a rare molluscan species in captivity is the complete collapse of a population of *Partula turgida* kept at the London Zoo, which was attributed to a microsporidian parasite (Cunningham and Daszak 1998). Little is known of molluscan diseases and detection is difficult. However, the rapid 73% reduction in population size experienced by *A. fuscobasis* is characteristic of similar disease-induced declines reported by Lafferty and Gerber (2002).

Our demographic and genetic data indicate that despite a genetic bottleneck, captive rearing of *A. fuscobasis* can be successful, as was the case for two generations of laboratory-reared snails over fifteen years. The captive population of *A. fuscobasis* was initiated with individuals from a genetically bottlenecked population but retained its founding genetic diversity, likely due to rapid growth (Kekkonen and Brommer 2015, Freeland 2005). Our results demonstrate that we need better understanding of the stochastic risks associated with keeping rare, inbred taxa in captive-propagation facilities so we can implement management strategies appropriate to the level of risk. We recommend: (1) that the Hawaiian tree-snail captive-rearing facility undergoes a thorough operational review to identify all potential routes of disease transmission, and adopt

rigorous hygiene and quarantine protocols. (2) Avoid keeping all individuals in one place by releasing excess individuals back into the wild, or establishing other captive populations at off-site locations. This prevents the proverbial "all eggs in one basket" scenario and will ensure that when a disease or other stochastic perturbation occurs at one locality, the existence of the species is not in jeopardy, as is currently the case with *A. fuscobasis.* 3) Develop a breeding plan by selecting founder snails and making appropriate pairings to maintain maximum genetic diversity.

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