AN ABSTRACT OF THE DISSERTATION OF

G Renee Albertson for the degree of Doctor of Philosophy in Wildlife Science presented on December 8, 2014.

Title: Worldwide Phylogeography and Local Population Structure of the Rough-Toothed Dolphin *(Steno bredanensis)*

Abstract approved:

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C. Scott Baker

Several dolphin species have global distributions. The extent of their radiation and limits to gene flow are presumably a product of oceanographic features both recent and historical, behavioral specializations and social organization. Rough-toothed dolphins *(Steno bredanensis)* are globally distributed in tropical and subtropical waters and are generally found in depths greater than 1,500 meters making them challenging to comprehensively sample. Although it has been assumed that pelagic dolphins range widely due to the lack of apparent barriers and unpredictable prey distribution, recent evidence suggests rough-toothed dolphins exhibit fidelity to some oceanic islands. A small number of photo-identification and genetic studies conducted to date on rough-toothed dolphins show regional population structure and stable associations in groups, with some individuals observed repeatedly in the same groups over several years. The aim of this dissertation is to describe patterns of phylogeography over evolutionary time on a global scale and expand studies of population and social structure on a regional level. The dataset contains 351 rough-toothed dolphin biopsies, tissue and teeth samples

collected from the Pacific and Atlantic Oceans and limited samples from the Indian Ocean.

To evaluate the phylogeography and test for possible species or subspecies level delineation between oceans, I used mitochondrial DNA sequences from the control region (350 bp) and 12 concatenated protein-coding genes from the whole mitogenome, as well as six nuclear introns. Although I found support for two Pacific clades and a private North Atlantic clade in the whole mitogenome, there were no genealogical patterns consistent across multiple loci, allowing me to reject species level delineation. To further evaluate the amount of gene flow and test for divisions below the species level, I used population level indices and found significant genetic differentiation for rough-toothed dolphins between the Atlantic Ocean with both the Indian/Western Pacific and Central/Eastern Pacific for both the mitochondrial datasets and the intron dataset. Significant differentiation between the Indian/Western Pacific Ocean and Eastern Pacific Ocean was found for the mitochondrial but not nuclear datasets. From these results I recommended the Atlantic Ocean basin be considered a separate evolutionary significant unit. This reflects that these populations are on independent evolutionary trajectories, but are not diagnosable species or subspecies.

To further evaluate population structure on a regional scale, I used a subset of these samples from three archipelagos in the Central Pacific Ocean including the Hawaiian Islands, the Society Island of French Polynesia and the Samoan Islands. Using a 450bp portion of the mtDNA control region and 15 microsatellite loci, an overall AMOVA

indicated strong genetic differentiation among islands within the main Hawaiian Islands (mtDNA F_{ST}=0.165; p<0.001; microsatellite F_{ST}=0.043 p<0.001) and the Society Islands of French Polynesia (F_{ST}=0.499; p<0.001; microsatellite F_{ST}=0.079 p<0.001) as well as among the three archipelagos (mtDNA F_{ST}=0.299; p<0.001; microsatellite F_{ST}=0.055 p<0.001). My results corroborate the photo-identification and the genetic studies for three archipelagos, confirming population structure on the regional level. Lastly, to test the hypothesis that social structure observed in rough-toothed dolphins is kinship based, as in other delphinid species such as killer whales, I used a subset of the main dataset from groups of living and mass stranded dolphins. I found multiple matrilines in more than half the groups, allowing me to reject a strictly matrilineal group structure, such as that observed in some killer whales. Instead I found rough-toothed dolphin groups showed weak matrilineality, where some groups are more matrilineal than expected by chance. Although group structure is stable, is not determined primarily by kin-based relationships. These analyses provide new insights into a little studied species. The use of worldwide datasets allowed me to evaluate population structure on different temporal, spatial and regional scales and delineate populations for future conservation and management.

Worldwide Phylogeography and Local Population Structure of the Rough-Toothed dolphin *(Steno bredanensis)*

by
G. Renee Albertson

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APPROVED:

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Head of the Department of Fisheries and Wildlife

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request

G. Renee Albertson, Author

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Special thanks to my committee members, Karen Martien, Kelly Benoit-Bird, Brian Sidlauskas, and Brian Haley for providing continued support and encouraging me to think like a scientist. Each member took the time to meet with me individually, read manuscripts and help shape me as a scientist, and I am very grateful for that.

Thank you to all my support system of family and friends, who helped keep me afloat. Special thanks to my wonderful, supportive and loving family, especially my mother. She always picked up the phone, and was willing to listen and/or provide advice, whatever the current crisis required. To Dave Campbell, my amazing and loving partner. You have been an absolute rock in my otherwise unstable PhD world. Thank you for your unconditional love, patience and understanding. My dear friend Trent Seager, who taught me to laugh, even in the most difficult of PhD times. My lab mates, for their support and sound advice, especially Alana Alexander and Rebecca Hamner, who were always willing to discuss analyses, even into the wee hours of the night. To Debbie Steel, for her wise words and interpretations of various analyses always brought my panic down a notch. And a huge thanks to the "extended Baker Lab" that reaches every corner of the globe, including Emma Carroll, Gaby Tezanos-Pinto, Susana Caballero, Carols Olavarria, and Jennifer Jackson, your survival tactics helped me survive!

CONTRIBUTION OF AUTHORS

C. Scott Baker: involved in discussions of chapter design, facilitating lab work, analyses, and editing of all chapters.

Marc Oremus collected a majority of the biopsy samples in the Society Islands and assisted in analyses for all chapters, and provided comments on drafts of all three chapters.

Robin W. Baird collected samples in the main Hawaiian Islands, providing a large dataset. He also read over two of the three manuscripts for this dissertation.

Alana M. Alexander assisted in laboratory analyses, and revised earlier drafts of each manuscript.

Karen K. Martien arranged for the extraction and shipping of the SWFSC rough-toothed dolphin database. She provided comments on drafts of Chapter 3.

Michael Poole arranged all the permits in French Polynesia and assisted in fieldwork.

Robert L. Brownell Jr. arranged for teeth samples to be shipped from the Smithsonian Institution.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

LIST OF TABLES (Continued)

Table Page

LIST OF APPENDIX FIGURES

LIST OF APPENDIX TABLES

1. General Introduction

1.1. Conservation Biology

Conservation biology is a field that emerged in the 1980's encompassing science and policy with the objective of conserving natural systems. It unites both scientists and nonscientists. Its practitioners use scientific tools to determine what biological flora and fauna should be considered for conservation status by the International Union for Conservation of Nature (IUCN), the Endangered Species Act (ESA) and other entities concerned with the vulnerability of natural and anthropogenic processes. Conservation biology attempts to maintain evolutionary processes within a functioning ecological setting to maintain the diversity of genes, populations and species (Groom *et al.* 2006). For animal populations, information on life history characteristics including reproduction, migration and population structure are useful features to study and monitor for protection of species.

1.2. What is a species?

If we are to conserve a species, we must first define it. Although scientists disagree on the characteristics needed to define a species, most agree that species are considered independent evolutionary units. Ayala (1976) discussed this in the context of adaptive changes. These changes can occur in an individual, a population or be extended to all members of the species. They cannot, however, be passed on to different species because species are on separate evolutionary trajectories unique to their environment (Ayala 1976). Once defined, the criteria to delimit species must also be addressed. The Unified Species Concept (De Queiroz 2007) discusses species delimitation as a continuous

process where populations are on separate paths of divergence, or considered separately evolving lineages. He highlights the importance of using geographic information for this purpose. This is important in relation to oceanic species with a global distribution since oceanographic barriers can influence gene flow (Figure 1.1)

Although the Biological Species Concept (BSC) idea of reproductive isolation (Mayr 1942) has merit, the challenges of studying cetaceans with this objective make it impractical for application. Moreover, introgression can be a confounding factor in species such as oceanic dolphins. The Phylogenetic Species Concept (PSC) improved upon this with a lineage based species concept (Cracraft 1983), but the use of character evolution as criteria creates a heavy reliance on monophyly, and it is found to be too insensitive to resolve recent speciation events for families like Delphinidae (Caballero *et al.* 2008). Similar to the Unified Species Concept of De Quierozo (2007), the Genealogical/Lineage Concordance Species Concept (GCC) (Avise & Ball 1990) reconcile elements from both the BSC and PSC, stating that phylogenetic diagnoses should be based on multiple independent genetic traits, or information contained in different types of loci (i.e. mitochondrial and nuclear). It was decided in The Workshop on Cetacean Taxonomy that at least two lines of evidence are needed to define a species, while only one line of evidence is required to define a subspecies (Reeves *et al.* 2004). In cetaceans these lines of evidence are generally a combination of genetic and morphometric comparisons.

Although not appropriate in many taxonomic designations, subspecies classifications are important in cetaceans. Due to long generation times and slow reproductive rates, there may not be sufficient evidence for species designation. Moreover, many cetaceans are widely distributed, thus requiring large amounts of time and resources to adequately sample.

1.3. Rough-toothed dolphins (Steno bredanensis)

Within the family Delphinidae, the species *Steno bredanensis*, the rough-toothed dolphin (Gray 1846), is so named for the vertical ridges on its teeth (Figure 1.2). Although (Peters 1877) originally described two species in the genus *Steno* (*rostratus* and *perspicillatus*) it was later determined that *S. perspillatus* was a *Delphinus. delphis* and there was only one species in the genus *Steno. Rostratus* was later changed to *bredanensis* (Norris 1966). Current taxonomic classification treats *Steno bredanensis* as a single species, and it is the only species belonging to the genus *Steno.* Unique morphology, including a smoothly sloping melon, makes this species quite distinct. Coloration generally consists of black, white and gray, with the lower sides and mouth dotted with white patches (Figure 1.3; (Lodi & Hetzel 1999, Jefferson 2008). Skull features distinguish them well from all but humpback dolphins (*Sousa* spp.) where tooth counts and the distinct ridges on their teeth differ (Jefferson 2008).

1.4. The impact of rapid radiation events in Delphinidae and phylogenetic discrepancies in Steno bredanensis

Paleontologists have dated the earliest delphinids to 10-12 Ma (Fordyce & de Muizon 2001). Speciation events over the next 3 Ma established all modern subfamilies

(McGowen *et al.* 2009). Rapid speciation in dolphins is intriguing, considering their relatively large size, mobility, long generation time, and sometimes sympatric associations (LeDuc & Dizon 2002). As with many other marine species, competition and oceanographic barriers most likely played a role in this radiation. Rapid radiation events can increase the likelihood of incomplete lineage sorting, where the phylogeny is different than the gene genealogy, blurring ancestral events (Frankham 2010). Therefore, incomplete lineage sorting can cause difficulty when constructing phylogenies because different genes can yield different phylogenies. This phenomenon has made resolving evolutionary patterns in the family *Delphinidae* challenging.

The placement of the genus *Steno* in phylogenetic relationships has long been disputed. An early study based on morphological characters (Perrin 1989) placed *Steno* in a clade with species in the genera *Sousa*, the Pacific humpback dolphin and *Sotalia spp.*, two species of coastal South American dolphin. In one of the first genetic studies of Delphinidae, (LeDuc *et al.* 1999) reached similar conclusions using the cytochrome *b* (cyt *b*) gene, placing *Steno* in a clade with *Sousa* and *Sotalia*. However, recent studies based on a combination of multiple genes (mtDNA+nuDNA) placed *Steno* in a clade as sister taxa to *Orcaella*, the Irrawaddy River dolphin (Caballero *et al.* 2007, Caballero *et al.* 2008, McGowen 2011). These studies robustly supported *Steno* in the *Globicephalinae* clade (Figure 1.4), also favored by separate analyses of some nuclear genes and concatenated mtDNA + nuDNA (Caballero *et al.* 2008). A mitogenome only study placed *Steno* as sister taxon to the rest of *Delphininae* subfamily, rather than within the *Globicephalinae* subfamily with a Bayesian posterior probability value of 1 (Vilstrup

et al. 2011). These discrepancies most likely represent incomplete lineage sorting of the nuclear genes. Using the support from both mtDNA and nuDNA datasets, I used the phylogenetic information derived from McGowen (2009, 2011) and Caballero *et al.* (2008) where *Steno* is most recently diverged from the genus *Orcaella*.

1.5. Consequences of worldwide phylogeography and global distribution

Phylogeography combines geographic distributions and the genealogical lineages of inter and intraspecific relationships of species (Avise 2000). Although oceanographic barriers to dispersal are not as conspicuous as land barriers, they can be just as influential to species. Biogeographic barriers identified throughout the oceans (Figure 1.1) include currents, salinity and temperature variation (Briggs & Bowen 2012). In Figure 1.1, Briggs & Bowen (2012) evaluate locations in tropical and subtropical areas that may affect gene flow of both predators and their prey due to a combination of these barriers. Thus, oceanographic features can influence population structure of marine species with even the highest dispersal capabilities including barracudas, sharks and dolphins (Daly-Engel *et al.* 2012a), forming phylogeographic structure. On a regional scale however, less productive areas of the ocean can have the opposite effect where prey are found in lower densities. This can force larger home ranges for highly mobile species like dolphins (Louis *et al.* 2014), and thus little phylogeographic structure will be apparent in these areas.

1.6. Distribution and life history parameters of the rough-toothed dolphin

Rough-toothed dolphins are considered a pelagic species with a worldwide tropical and subtropical distribution. In the Atlantic Ocean they appear to be restricted by water

temperature around the South American continent with the highest latitude on the west coast of 23^{o}S and Patagonia in Argentina on the western side of the South Atlantic (West *et al.* 2011). Stranding samples on the Virginia coastline mark the northern most latitude, 37^{o}N in the Atlantic (Miyazaki & Perrin 1994). In the Pacific Ocean, stranding samples in Oregon and Washington mark the northern extent and stranding samples from the north island of New Zealand mark the southern extent of their range. It should be noted that stranding samples may drift slightly.

Nearer the equator around oceanic islands, these dolphins are generally found in water depths between 1,000 and 2,000 meters and an average of 1.5 - 4.5 km from shore (Gannier & West 2005). Specific to the main Hawaiian Islands, rough-toothed dolphins are one of the most sighted odontocete species in depths greater than 3,000 meters (Baird *et al.* 2013). They have also been observed throughout the Eastern Tropical Pacific (ETP). However, they also have been found in depths of only 5 meters feeding off the continental shelf of Brazil (Lodi & Hetzel 1999), as well as shallow waters off Honduras (Kuczaj & Yeater 2007) and the Canary Islands (Tobeña *et al.* 2014). Therefore, rough-toothed dolphins inhabit the tropical/subtropical biogeographic regions identified by Briggs and Bowen (2012, Figure 1.1). Determining to what extent these biogeographic regions influence the pattern of dispersal in this species is to date unknown.

The few life history studies of rough-toothed dolphins indicate that males reach sexual maturity at about 14 years, and females at 10 years of age (Miyazaki 1983, Miyazaki & Perrin 1994). However, a large, pregnant female, aged at six years old was reported off

the coast of Brazil (Siciliano 2007). These dolphins are sexually dimorphic, with males (mean length = 225 cm) larger than females (215 cm), and have a life span of about 32-36 years. General population estimates conducted during large vessel nondedicated surveys estimated 145,000 individuals (CV = 0.28) in the eastern Tropical Pacific (Wade & Gerrodette 1993). Another study of the Hawaiian Islands Exclusive Economic Zone (EEZ) estimated the population in that area to be about 20,000 (CV=0.52) (Barlow 2006).

Rough-toothed dolphins feed on a variety of cephalopods and large predatory fish including mahi-mahi (*Coryphaena hippurus)* (Pitman & Stinchcomb 2002). Examination of stomach contents also yielded small fish and squid. $\delta^{15}N$ levels indicate feeding at an intermediate trophic level (Kiszka *et al.* 2010). Rough-toothed dolphins have been observed foraging on surface species such as needlefish and flying fish, occasionally in mixed species aggregations. Cooperative foraging on large predatory fish such as mahi-mahi has been recorded off the west coast of Costa Rica and Mexico as well as in the main Hawaiian Islands and French Polynesia in the South Pacific (Pitman & Stinchcomb 2002).

1.7. Conservation genetics in the context of population structure

The amount of gene flow, or movement of individuals, determines the amount of isolation and interchange between populations. Cetaceans are relatively long-lived with high survival rates and low reproductive rates. Females produce few offspring, therefore investment in maternal care may extend over several years and include learned behaviors of migratory routes and feeding strategies (Taylor *et al.* 2010). Mitochondrial DNA (mtDNA), passed down directly from mother to offspring, is useful in identifying patterns

in population structure for this reason. Nuclear DNA provides data on male and female gene flow, and indicates the structure of the breeding population. Therefore, mitochondrial and nuclear genetic markers are used to identify appropriate units to conserve for populations (Taylor *et al.* 2010).

1.8. Are rough-toothed dolphins pelagic or insular?

Rough-toothed dolphins have been described as a pelagic species (Wade & Gerrodette 1993, Perrin *et al.* 1994). In the past, pelagic species were considered to have panmictic population structure with unrestricted gene flow. Short-beaked common dolphins, for example, illustrate low genetic differentiation between populations around South Africa reflecting high mobility, fluid social structure and gene flow (Natoli *et al.* 2005, Natoli *et al.* 2006). However, to date, photo-identification and genetic studies of population structure of rough-toothed dolphins indicate populations may form small, insular communities around oceanic islands in both the Atlantic and Pacific Oceans (Baird *et al.* 2008; Mayr and Ritter 2005; Oremus *et al.* 2012) similar to spinner dolphins (Oremus *et al.* 2007). The samples used in these studies were collected from "nearshore" individuals (designated <than 40 km from shore) so offshore limits, or range of these individuals could not be determined. However, rough-toothed dolphins have also been observed in the open ocean. Wade and Gerrodette (1993) reported sightings of rough-toothed dolphins in the Eastern Tropical Pacific between the Hawaiian Islands and North America. This suggests rough-toothed dolphin populations exist in an insular framework, perhaps due to habitat specialization from nutrient-rich upwelling from the Island Mass Effect (Doty & Oguri 1956), and also as pelagic offshore populations.

1.9. Monitoring dolphin populations

Several types of conservation units exist below the species level to monitor dolphin populations, and each is applicable depending on the scale of the question (e.g. subspecies, population, etc.). Laws and policies developed by the International Union for the Conservation of Nature (IUCN) and the Marine Mammal Protection Act (MMPA) use the term evolutionary significant units (ESU) with the intent to protect genetic diversity. An ESU is defined as a population that (1) is substantially "reproductively isolated" from other populations of the species and (2) represents an "important component of the evolutionary legacy of the species" (NMFS & USFWS 1990). Taylor *et al.* (2010) elaborates on this by stating ESUs broadly represent units needed to conserve the essential genetic variability for future evolutionary potential, i.e. species can respond to environmental challenges through adaptation. ESUs and subspecies differ by time. For example, subspecies have been separated long enough that they may differ in morphological characteristics, and are therefore diagnosable. However, ESUs experience sufficiently low gene flow such that local adaptation may occur, e.g. gene flow of less than two migrants per generation has been suggested (Gardenfors 2001) .

Demographically independent populations (DIPs) focus on shorter temporal scales (human lifetime), and are typified by differences in allele frequencies (Taylor *et al.* 2010). These are particularly important for oceanic dolphins that form insular populations. Scientists can evaluate genetic differentiation between these island groups using various F_{ST} indices to answer population level questions. These indices have been used to differentiate populations in the Hawaiian and Society Islands for several dolphin

species (Oremus *et al.* 2007, Andrews *et al.* 2010, Martien *et al.* 2012, Oremus *et al.* 2012, Courbis *et al.* 2014, Martien *et al.* 2014).

1.10. Dolphin societies and social organization in the rough-toothed dolphin

Cooperative social systems are common in many mammal societies (Norris & Schilt 1988). Many social societies in mammals are characterized by stable group composition, male-biased dispersal and males forming permanent breeding associations with one or a few social groups (Archie *et al.* 2008). In many social groups females show philopatry, perhaps because known foraging areas may help them better care for their young (Moller 2012). In pelagic environments dolphin groups tend to be larger for greater protection against predators and cooperation in foraging (Norris & Schilt 1988). Around oceanic islands where prey is more predictable, dolphin groups are generally less than 10 individuals, and female philopatry is common. Although individual dolphins associate with certain other individuals, group composition may change continually. Spinner dolphins live in fission-fusion societies where observed groups are temporary associations between more stable social units (Oremus *et al.* 2007, Andrews *et al.* 2010).

The level of haplotype diversity found for rough-toothed dolphins in the Oremus *et al.* study (2012) was surprisingly low (h= 0.167-0.457). They suggest this could be explained by a highly stable social structure, similar to matrilineal species like long-finned pilot whales (De Stefanis *et al.* 2008, Oremus *et al.* 2009) and sperm whales (Christal & Whitehead 2001). Low genetic diversity of rough-toothed dolphins may be a product of social bonds, although perhaps not as homogeneous as the sperm whale. Although long-term observation of the movement within and between groups of rough-

toothed dolphins is described as “fluid”, observation of tight group formation and resighting the same individuals within the same groups suggests strong social bonds exist between individuals (Mayr & Ritter 2005, Baird *et al.* 2008). There are instances of individuals photographed within a group, and up to 12 years later photographed again with the same individuals in a group (Mayr & Ritter 2005, Baird *et al.* 2008). In the Gulf of Mexico, two rough-toothed dolphins were tagged and released after rehabilitation from a mass stranding. They were observed together 157 days after the release (Wells & Gannon 2005). In another study, three rough-toothed dolphins released together near Ft. Pierce, Florida in 2005 after a mass stranding had frequent social interactions “including food sharing and care-giving behavior” (NMFS 2008). Similar behaviors were observed off the Canary Islands (Ritter 2002, Ritter 2007), Brazil (Lodi 1992, Lodi & Hetzel 1999) and Honduras (Kuczaj & Yeater 2007), suggesting that rough-toothed dolphins have strong social bonds. It is still unknown, however, if these bonds are kinship based.

1.11. Dissertation scope and structure

For a little studied species with a global distribution like the rough-toothed dolphin, a phylogeographic description can illuminate the extent of gene flow on a worldwide scale in relation to biogeographic barriers and time, and show if species delineation is appropriate. A population level study will provide evidence of gene flow and habitat use on a smaller temporal and spatial scale. Finally, a closer look at the groups that inhabit these populations will illustrate if group structure is strictly matrilineal or kinship based. This is informative for management for Potential Biological Removal estimates and diversity issues, metrics that are critical for all species.

Here I use a worldwide database of genetic samples (Figure 1.5) of rough-toothed dolphins, and regional subsets of this database to address a number of hypotheses proposed to describe patterns of diversity and differentiation in rough-toothed dolphins. The questions, like the chapters, are listed in hierarchical geographical order beginning with the worldwide phylogegoraphy followed by identification of populations and lastly, group structure within those populations. Specifically I ask:

1) What is the pattern of genetic differentiation and gene flow among oceanic regions of rough-toothed dolphins?
2) Have rough-toothed dolphins in the Atlantic Ocean been separated long enough from the Indian and Pacific Ocean basins to be considered separate taxonomic units?
3) Do rough-toothed dolphins exist in demographically independent insular communities around oceanic islands?
4) Is this differentiation driven by increasing geographic distance or more episodic colonization and local drift?
5) Are rough-toothed dolphins with the same mtDNA haplotype more likely to associate in groups than expected by chance?
6) Is kinship a primary determinant of social groups?

For each data chapter (2-4) my contribution included assistance in sample collection in the Society and Hawaiian Islands and extraction of samples I collected as well as samples

collected after 2010 in the Hawaiian Islands. In addition I amplified, genotyped, and sequenced all samples before performing all downstream analyses.

Data files are discussed in Appendix I and are found in an electronic Appendix file. Requests for access to this file must be made in writing to G.R. Albertson and C.S. Baker and include a proposal.

*Chapter 2: Worldwide phylogeography description of the rough-toothed dolphin (*Steno bredanensis*) using the mitochondrial genome and nuclear introns.* In this chapter I use a large sample of mtDNA control region sequences and a smaller sample of protein-coding genes from the mitochondrial genome and seven nuclear introns to investigate the worldwide phylogeographic structure and phylogenetic relationship, as well as the degree and timing of divergence of rough-toothed dolphins between each ocean basin. Using a clock model and substitution rates specific to the family Delphinidae, I date the time of divergence between Atlantic and Pacific rough-toothed dolphin lineages. Since species or subspecies status requires multiple lines of evidence, I test for fixed differences and reciprocal monophyly of both mtDNA and nuclear introns. In addition, I examine divergence of both nuclear and mtDNA to determine gene flow between oceans in the context of the criteria currently recognized as defining subspecies for cetaceans.
This chapter will be submitted for publication to
The Biological Journal of the Linnean Society with the following authors: Albertson, G. Renee, Robin W. Baird, Marc Oremus, M. Michael Poole, Karen K. Martien, Alana M. Alexander, Robert L. Brownell Jr., Deborah A. Duffield, and C. Scott Baker

Chapter 3: *Staying close to home? Genetic analyses reveal insular population structure throughout the Pacific Ocean basin for a pelagic dolphin,* Steno bredanensis.

This chapter uses apriori information that islands are geographic regions for dolphin population structure expanding on previous studies that focused on photo-identification and genetics. Using microsatellites and mtDNA, I consider oceanographic and social factors to determine the amount gene flow between islands and archipelagos in the central Pacific Ocean.

Chapter 3 is reformatted from the manuscript submitted to Marine Ecology Progress Series: Albertson, G.Renee, Robin W. Baird, Marc Oremus, M.Michael Poole, Karen K. Martien, Robert L. Brownell, Jr. and C. Scott Baker.

Chapter 4: *"Are you my mother? A test of matrilineal social organization in living groups and mass strandings of rough-toothed dolphins.* After describing geographic population structure I evaluated the amount of relatedness within and among groups of dolphins in several areas of the Pacific and Western Atlantic Oceans. This included the synthesis of an index based on dyad haplotype comparisons within groups to quantify the amount of matrilineality within a group for comparison to other groups within a geographically defined population.

This chapter is intended to be submitted for publication to *Marine Mammal Science* with the following authors: Albertson, G. Renee, Marc Oremus, Robin W. Baird, M. Michael Poole, Karen K. Martien, Robert L. Brownell Jr. and C. Scott Baker.

Chapter 5, the “General Discussion” synthesizes the findings and conservation of the implications of the previous chapters. This chapter includes the proposal to management based on my work, describes the baseline for this species my work provides and concludes with suggested directions for future research that will be important for rough-toothed dolphin conservation.

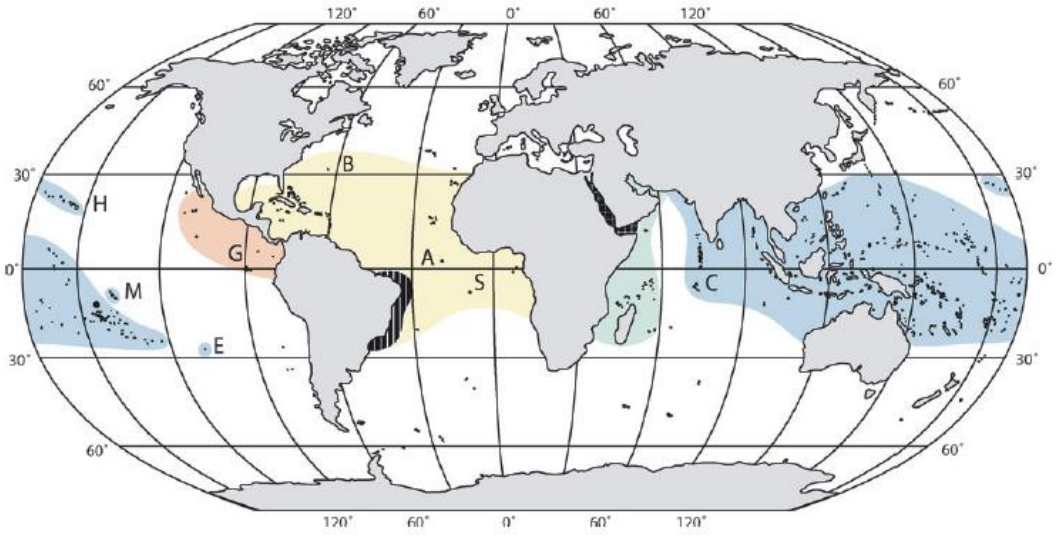

Figure 1.1: Biogeographic provinces adopted from Briggs and Bowen (2012). Tropical regions and provinces listed are all areas inhabited by rough-toothed dolphins. They include the Indo-Polynesian, Hawaiian, Marquesas and Easter Island (blue, C, H, M, E), Eastern Tropical Pacific (orange, G), the East West Atlantic regions (yellow, B, A, S) and the Western Indian Ocean Province (Green).

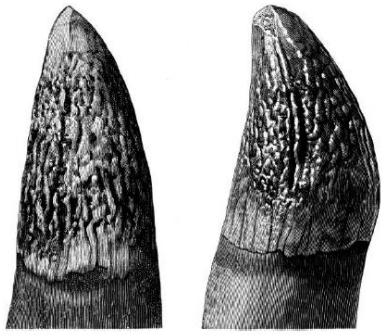

Figure 1.2: Magnified diagram of teeth ridges of rough-toothed dolphins illustrating the fine tooth ridges that are characteristic of this species. The diagram is reprinted from (Neuville 1928)and adapted from West, Mead and White (2011).

Figure 1.3: Rough-toothed dolphin off the island of Ni'ihau in the Hawaiian archipelago illustrating the unique coloration patterns and gently sloping melon of this species. Photo courtesy of Robin Baird.

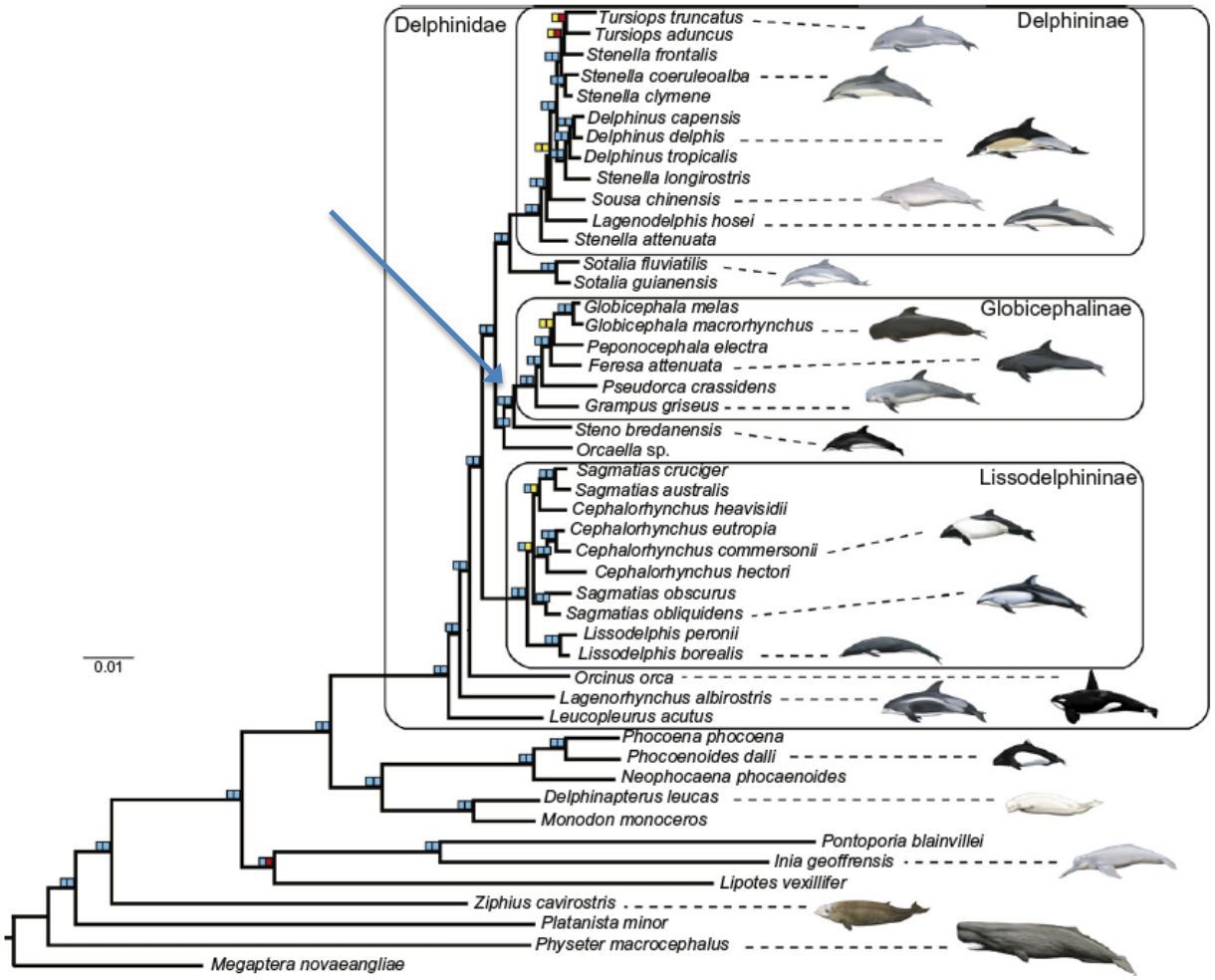

Figure 1.4. Bayesian majority consensus phylogram of cetacea generated from concatenated mtDNA+nuDNA data with 58 partitions, from (McGowen 2011). The arrow points to the well supported (Bayesian > 0.95 posterior probability) divergence of *Steno bredanensis* from *Orcaella sp.* as well as the rest of the subfamily Globicephalinae.

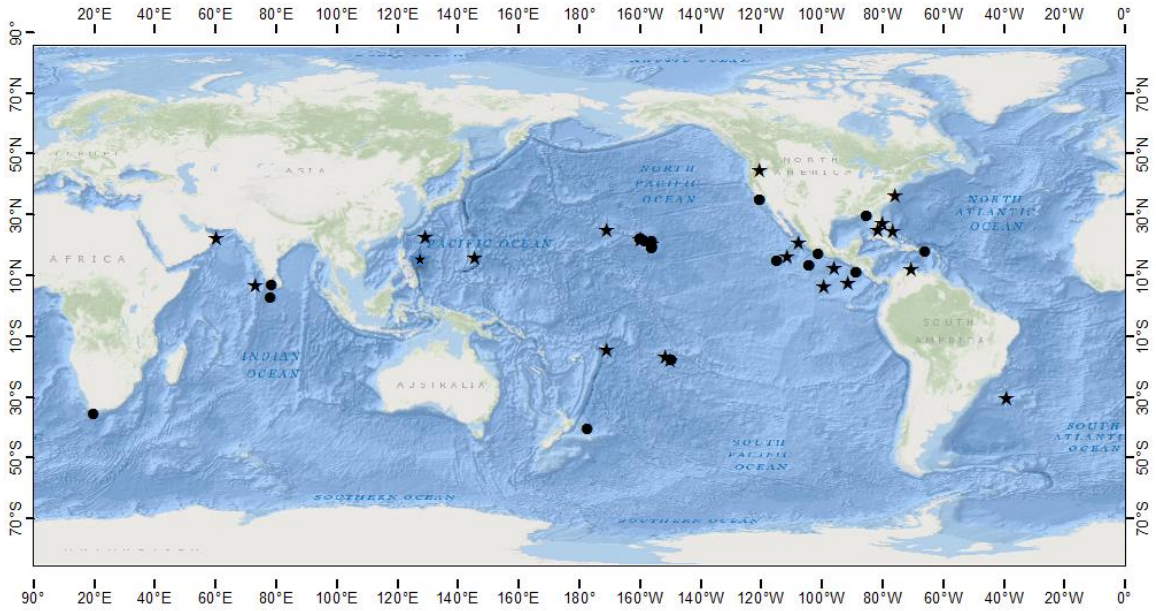

Figure 1.5. Locations of rough-toothed dolphin sample collection. Black dots represent locations of samples used (teeth and tissue) for the control region only (350 bp). Black stars represent locations of samples used in the mitogenome/intron study. These samples were collected beginning in 1976 through 2012. However, samples were not collected in all years.

2. A worldwide phylogeography description of the rough-toothed dolphin *(Steno bredanensis)* using the mitochondrial genome and nuclear introns

2.1 Abstract

Rough-toothed dolphins have a global tropical and subtropical distribution in the Pacific, Indian and Atlantic Oceans. Despite the large expanse of ocean inhabited by this species, the global phylogeography of these pelagic dolphins has not been evaluated among oceans. In this study we use the mtDNA control region (n=351), a selected representation of mitogenomes (n=19) and six nuclear introns (n=35) in order to assess multiple lines of evidence critical to the process of taxonomic evaluation. We examine the null hypothesis that rough-toothed dolphins are one panmictic worldwide species using samples from the Pacific, Indian and Atlantic Oceans. We estimated the inter-ocean divergence time using a species-specific substitution rate derived from the family Delphinidae, investigated the potential for monophyly relative to ocean basin and evaluated population level indices. MtDNA phylogenetic analyses of the mitogenome indicated a private Atlantic clade sister to a larger cosmopolitan clade containing individuals from all three oceans, and indicated three migration events. Using the mitogenome, we dated the split between the private Atlantic clade and the cosmopolitan clade to one million years ago. The population level analyses revealed significant genetic differentiation among five biogeographic regions (global $\Phi_{ST} = 0.368$, $p<0.001$), while the nuclear introns detected significant differentiation between only the Atlantic and other oceanic regions, but not within the Pacific and Indian Oceans. These results imply rough-toothed dolphins in the Atlantic have been isolated sufficiently from the other regions to be on an independent evolutionary trajectory.

2.2. Introduction

Among cetaceans with worldwide distributions, limits to gene flow are complex and considered to result from behavioral specializations, historical environmental changes and biogeographic barriers (Hoelzel *et al.* 1998, Steeman *et al.* 2009). Phylogeography identifies genetic patterns in geography where genetic markers are used to evaluate the level of gene flow between populations in regions, thus providing the opportunity to test the hypothesis of genetic pattern concordance with geography (Avise 2000). Phylogeographic patterns investigated for three of the five dolphin species with worldwide distributions found significant differentiation among populations, illustrating restricted gene flow on a regional scale, or monophyletic clades relative to geographic regions, illustrating subspecies or species designation on a worldwide scale (Natoli *et al.* 2004, Natoli *et al.* 2006, Morin *et al.* 2010a).

Rough-toothed dolphins have a worldwide tropical and subtropical distribution in the Atlantic, Pacific and Indian Oceans, as well as the Mediterranean Sea and the Gulf of Oman (Watkins *et al.* 1987, Jefferson 2008). Although they have been considered a pelagic species where they have been observed from large ship surveys in the Eastern Tropical Pacific and northwestern Hawaiian Islands, they are also observed around oceanic islands in both the Atlantic and Pacific Ocean basins, and in depths of less than 20 meters off the coast of Japan, Brazil, Mauritania and the Canary Islands (Ritter 2002, Baird *et al.* 2008, Jefferson 2008). There are currently no recognized population divisions by the International Union for Conservation of Nature (IUCN), which lists rough-toothed dolphins as least concern. In US waters the National Marine Fisheries Service (NMFS)

recognizes three stocks. These include North Atlantic and Northern Gulf of Mexico stocks in the Atlantic Ocean basin and the Hawaiian stock including American Samoa in the Pacific Ocean basin (NOAA 2008, 2011, 2014). However, these stocks are based on geography only.

Having a tropical and subtropical distribution, the equator is not a barrier for rough-toothed dolphins. However, it is clear that South America represents a thermal barrier for this species where thermoregulation restricts them to ocean temperatures greater than 21^{o}C, limiting their latitude range to about 37^{o} both north and south (West *et al.* 2011). Therefore, gene flow of rough-toothed dolphins between the Atlantic and the Pacific Oceans has been limited to going around Cape Agulhas at the southern tip of Africa and traversing the Indian Ocean since the closure of the Isthmus of Panama around four million years ago (Steeman *et al.* 2009). The southern tip of Africa is considered to be a biogeographic barrier for many tropical marine animals, including dolphins, and in several instances dolphin populations from multiple species have evolved independently on either side of Cape Agulhas in the two regions of the Atlantic and Indian Oceans (Perrin 2007).

Rough-toothed dolphins are the only delphinid with a worldwide distribution where taxonomy has not been comprehensively evaluated, either by genetic analyses or morphology. In the absence of diagnosable morphological characteristics (e.g. when skeletons are not available), taxonomic designations for cetaceans have been based on genetic evidence (Dalebout *et al.* 2002, Dalebout *et al.* 2007, Jackson *et al.* 2014).

Taxonomic delimitation recognizes that time and space can set populations on different evolutionary paths due to local adaptation (Ayala 1976, De Queiroz 2007). Considering the large geographic distance and the possible biogeographic barriers of ocean temperatures and currents around the southern tip of Africa separating the Atlantic Ocean from the Indian and the Pacific Ocean, we evaluate phylogeography, testing concordance between geography and gene flow of rough-toothed dolphins. We identify the patterns of phylogeography and evaluate the criteria for three possible delimitations used previously for dolphins with a worldwide distribution including species, subspecies and Evolutionary Significant Units (ESUs).

The Genealogical/Lineage Concordance Species Concept (GCC) states that phylogenetic diagnoses should be based on multiple independent genetic traits such as information contained in multiple loci (Avise & Ball 1990). A specialized workshop on cetacean taxonomy determined new definitions and criteria for species and subspecies, and found this species concept to be the most relevant for cetaceans because it provides evidence of irreversible divergence (Reeves *et al.* 2004). The criteria that came out of this workshop have been used for diagnosis of several cetacean species (Dalebout *et al.* 2002, Caballero *et al.* 2007) and subspecies (Morin *et al.* 2010a, Archer *et al.* 2013, Jackson *et al.* 2014), and we use it here in our definitions of species and subspecies. Species are generally defined as a collection of populations on distinct evolutionary trajectories that are not likely to recombine with any other such lineages (De Queiroz 2007). Subspecies are collections of populations that are currently on distinct evolutionary trajectories, but it is not certain they have diverged irreversibly. This could be due to being separated a short

time, and/or occasional gene flow (Taylor *et al.* 2010). Evolutionary Significant Units (ESUs) are on separate evolutionary trajectories, but experience greater gene flow than subspecies, and therefore do not exhibit diagnosable traits. Although ESUs are a human construct, they are meaningful for management purposes. ESUs represent an important component in the evolutionary legacy of the species (Waples 1991). They experience sufficiently low gene flow such that local adaptation may occur (Gardenfors 2001).

Since species are considered to be on separate evolutionary trajectories and exhibit evidence of irreversible divergence, we would expect monophyly between species (Ayala 1976, De Queiroz 2007). Due to biogeographic barriers specific to rough-toothed dolphins of South America and possibly South Africa, we would expect concordance of geography and monophyly, e.g. the Atlantic Ocean individuals to form a monophyletic clade, in order to conclude species level designation similar to the pattern found in other dolphins. Due to some degree of gene flow, we would not expect subspecies or ESUs to show monophyly. Instead, for subspecies, we would expect genetic differentiation in both the nuclear and mtDNA as well as fixed differences in either nuclear or mtDNA for diagnosability (Archer *et al.* 2013). It is this diagnosability that separates subspecies from ESU's. In addition to fixed differences in genetic diagnosability, subspecies diagnosability has been described as differences in color pattern, behavior or ecology, i.e. characters that can be correlated with evolutionary independence (Baird 2000, Foote *et al.* 2009, Braby *et al.* 2012). For ESU's, diagnosability would not be distinguishable because there is still sufficient gene flow. In ESU's we would expect genetic differentiation in mtDNA and less than two migrants per generation (Gardenfors 2001).

Here we present the first study of the phylogeographic relationship of rough-toothed dolphins. Our study included the use of both mitochondrial and nuclear markers across a comprehensive geographic area spanning three ocean basins, and analyses from phylogenetic reconstructions, divergence dating and genetic differentiation. The scope of this study allowed us to assess phylogeography on two levels: 1) Broad and shallow: many samples across the globe with one marker (350 bp of the mtDNA control region) and 2) Narrow and deep: on a subset of these samples using concatenated protein-coding genes of the mitogenome and six nuclear introns. Our dataset includes samples collected around islands, pelagic samples collected from large ship surveys, as well as bycatch and beachcast dolphins. Specifically, we test the hypothesis of species-level differentiation, using monophyly of mtDNA lineages as an indicator, and quantify the genetic diversity and differentiation of rough-toothed dolphins. We evaluate the phylogeographic patterns and divergence in light of species, subspecies and ESUs based on genetic diagnostic characters consistent with the criterion of "irreversible divergence" (Reeves *et al.* 2004). In the absence of monophyly, we evaluate conventional frequency and population structure indices to describe the pattern and magnitude of gene flow between oceans. If separation between the Atlantic and the Indo-Pacific Ocean basins has been sufficient for species status we expect supported monophyletic clades between the Atlantic and the Pacific Ocean basins. Alternatively, we evaluate genetic differentiation in both nuclear and mtDNA to assess phylogeographic patterns. For subspecies we expect fixed differences as evidence of diagnosable characteristics, whereas for ESU's we expect less than two migrants per generation.

2.3. Material and Methods

2.3.1. Ethics Statement

Procedures for ensuring animal welfare during biopsy sampling were approved as part of the scientific research permits issued by the National Marine Fisheries Service (NMFS) issued to Southwest Fisheries Science Center (permit 774), Robin W. Baird (permit 731) and C. Scott Baker (permit 13583-01) under the authority of the Marine Mammal Protection Act of 1972. Oregon State University's Institutional Animal Care and Use Committee approved a protocol for the collection of biopsy samples (permit 4285). The samples originating from outside the US jurisdiction were imported under the Convention on International Trade in Endangered Species (CITES) import permit numbers US774223, US689420 and US799055. The Southwest Fisheries Science Center and Oregon State University are Registered Scientific Institutions under CITES.

2.3.2. Sample collection

The rough-toothed dolphin tissue samples used in the mitogenome (n=19) and the worldwide study (n=360, Figure 2.1) were collected from several regional collaborators. Samples from the Society Islands of French Polynesia and Samoa (near the island of Savaii) were obtained using a modified veterinary capture rifle and biopsy dart (Krützen *et al.* 2002) and stored in 70% ethanol. All other biopsy skin samples from living dolphins were obtained using a crossbow and arrow system. Skin samples collected from fishery bycatch individuals around American Samoa (island of Tutuila) were stored in DMSO. Skin samples collected from fishery bycatch individuals around American

Samoa and mass strandings around the Gulf Coast of Florida and the northeastern Atlantic Ocean were stored in DMSO. All samples were stored at -80^{o}C.

Teeth samples used in the worldwide study (n=34, Figure 2.1) were obtained in collaboration with The Smithsonian Institution, Bayworld Port Elizabeth Museum and Oceanarium in South Africa, and the Museum of New Zealand Te Papa Tongarewa. The Smithsonian samples were collected from mass strandings in the Pacific Ocean around Maui in the main Hawaiian Islands, and the northwestern Atlantic Ocean, as well as from fishery bycatch individuals in the Eastern Tropical Pacific (ETP) and Isla Gorgona, Colombia. Bayworld samples were collected on the southwestern coast of South Africa and the New Zealand samples were collected on the eastern side of the North Island.

2.3.3. Sample location delineations for each dataset

For the 350 bp dataset, we divided our sample locations into five biogeographic regions (Figure 2.1). The delineation of these regions considered biogeographic barriers (Rocha *et al.* 2007, Briggs *et al.* 2013) as well as the constraints from the number of samples and sample locations. The Pacific Ocean was further divided into Western, Central and Eastern Pacific. The ‘western’ division represented individuals sampled near Japan, Taiwan and the Marianas Islands, ‘central’ represented individuals sampled in the Pacific Island regions including the Hawaiian, Society and Samoan Islands (see Chapter 3, Figures 3.1b-d for details) and ‘eastern’ represented individuals sampled in the traditional Eastern Tropical Pacific exclusive of the Hawaiian Islands. For the intron and mitogenome dataset, the Western Pacific and Indian Ocean regions were collapsed into

an Indian/Western Pacific region and the Central Pacific and the Eastern Pacific were collapsed into a Central/Eastern Pacific region.

2.3.4. DNA Extraction, sequencing and molecular sexing of tissue samples

Total DNA was extracted either using either a Qiagen DNeasy Blood and Tissue Kit or a standard phenol:chloroform extraction protocol (Sambrook *et al.* 1989), modified for small samples (Baker *et al.* 1994). DNA was quantified with pico-green fluorescence and normalized to 15 ng μl^{-1}. An 800 bp fragment of the 5' end of the mtDNA control region was amplified and sequenced using the primers Dlp1.5 and Dlp8 as described in Oremus *et al.* (2007).

The DNA extraction from teeth followed standard protocols for 'ancient DNA extraction'(Pimper *et al.* 2009). Total DNA was extracted from teeth samples in a lab separate from modern cetacean DNA. A laminar flow chamber and the use of UV radiation were used to provide sterile surface conditions and minimize the risk of contamination. Reagents were made up in a "DNA-free" positive pressure room separate from other laboratories. Teeth were submerged in liquid nitrogen for 20 seconds and then crushed. The resulting powder was subsampled and stored in a -20^{o}C freezer. DNA was extracted from 0.1g of tooth powder beginning with a protein digestion with 200ul of 10%SDS, 100 ul DTT (10mg/ml) and 100ul Proteinase K (20 mg/ml) and incubated at 37^{o}C overnight, and then by one hour at 50^{o}C. Samples were then centrifuged, and the rest of the extraction procedure followed Pimper *et al.* (2009), including silica suspension

(Boom *et al.* 1990). A negative control, or blank, was run as every 5th sample, and a maximum of 8 samples and two blanks were extracted at one time.

A 450 bp region of the mitochondrial control region was amplified via Polymerase Chain Reaction (PCR) in a 25ul reaction using primers M13Dlp1.5 and Dlp5 (Dalebout *et al.* 1998), 1mg/ml Bovine Serum Albumin (BSA) and 5ul of DNA template as described in Pimper *et al.* (2009). This was followed by a nested amplification using 3 ul of a 1:10 dilution of the first reaction using the primers M13Dlp1.5 and Dlp4 under the same conditions except no BSA was added. PCR products were purified for sequencing with SAPEX (Amersham). The sequencing reaction was carried out with *BigDye Vs 3.1* (Applied Biosystems, Inc.) with post-sequencing clean-up using Agencourt CleanSEQ Kit (Beckman Coulter). Products were then run on an ABI 3730 Genetic Analyzer (Applied Biosystems, Inc.).

Sequences were aligned and edited using Sequencher 4.6 (Gene Codes Corporation). Sequences were required to have a minimum average *Phred* score of >30 (e.g. 30 is a 1 in 1,000 error rate and 40 is a 1 in 10,000 error rate); (Ewing *et al.* 1998). Any variable sites with Phred<40 were visually confirmed. If sequences fell below this Phred <30 threshold, they were resequenced. If they failed again they were removed from the dataset. If a haplotype was represented by only one sample the identity of the haplotype was confirmed by re-sequencing in both directions. Variable sites and unique haplotypes were identified using Sequencher 4.6 and then MacClade, Version 4.0 (Maddison &

Maddison 2000). Samples were then trimmed to a length of 350 bp of the control region so that all skin and tooth samples represented the same subset of the control region.

Molecular sex identification was carried out using a region of the male-specific *Sry* gene along with regions of the *ZFX* genes as positive controls (Gilson *et al.* 1998). For teeth samples, two rounds of PCR were carried out using 5ul of DNA for the first round of PCR, and 3ul of a 1:10 dilution of the first PCR as input for the second round. The reactions were performed in a total volume of 25ul with the following conditions: 2.5ul Taq Gold buffer, 5ul of 4mM $MgCl_2$, 1ul of 0.4uM each primer, Y53-3C, Y53-3D, P23EZ and P15EZ, 0.25ul of 0.2mM of dNTPs and 0.25ul of *Taq* Gold. The thermocycle profile began with an initial denaturation step of 94^{o}C for 10 minutes, followed by 35 cycles of 94^{o}C for 45 seconds, 60^{o}C for 45 seconds, 72^{o}C for 60 seconds and a final extension at 72^{o}C for 10 minutes. Male and female positive controls were added to each reaction.

2.3.5. Nuclear intron amplification and sequencing

Six nuclear short-range (<1,500 bp) introns (Actin-1, CAT, CHRNA, GBA, IFN and sex marker DBY7) were amplified using PCR conditions modified from Caballero *et al.* (2008). Each reaction consisted of 15-20 ng of DNA, and a final concentration of 1x Platinum *Taq* buffer (Invitrogen), 0.4uM each primer, 20mM dNTPs, 1U/ul Platinum *Taq* polymerase and 1ul of BSA (Bovine Serum Albumin) to decrease inhibition of PCR. For Actin-1, 1.5mM $MgCl_2$ was used. For all other introns, 2.0 mM of $MgCl_2$ was used. For Actin-1, CAT, GBA and IFN temperature profile consisted of an initial denaturing step of

3 min at 94^0C, followed by 35 cycles of 94^0C for 30 s, 55^0C for 45 s and 72^0C for 30s followed by an extension at 72^0C for 10 minutes. For CHRNA1 and DBY7 touchdown temperature protocols were used. CHRNA1 consisted of an initial denaturation at 94^0C for 2 min, followed by 10 cycles at 94^0C for 20s, 64-55^0C (decrease by 1^0C per cycle) for 20s, 72^0C for 40s. This touchdown was followed by 30 cycles at 94^0C for 20s, 55^0C for 20s and 72^0C for 40s. DBY7 started with a denaturation at 94^0C for 2 min, followed by 20 cycles at 94^0C for 30s, 60-50^0C (decrease of 0.5^0C per cycle) for 1 min and 72^0C for 1.5min. This was followed by 10 cycles at 94^0C for 30s, 55^0C for 1min and 72^0C for 1.5min. A final extension at 72^0C for 10 minutes was performed for both touchdown reactions. Two female samples were used as a negative control for DBY7. A 1.6% agarose gel showed no amplification of female samples when compared to male samples.

2.3.6. Long-range amplification and Illumina MiSeq sequencing of mitogenomes

Mitogenome sequence was generated for a total of 19 rough-toothed dolphins in the Pacific (n=11), Atlantic (n=7) and Indian (n=1) Ocean basins using seven overlapping long-range fragments. The fragments ranged in size from 1,473-3,874 bp (Appendix Table II.1). The reactions, adopted from Alexander *et al.* (2013), used 2U/uL high fidelity Phusion® Polymerase (New England Biolabs, USA), 5X buffer high fidelity. Reactions conditions included 1× Phusion® HF Buffer (NEB, USA), 1 μM each primer; 2% DMSO (NEB, USA); 15-30ng of template DNA, dNTP (Promega, USA) and BSA. Thermocycle profiles began with an initial denaturation of 98^oC for 30s, followed by 35 cycles of 98 ^{o}C 8s, T_A for 30s and 72^oC for 1min 15s, followed by a final extension of 72 ^{o}C for 10 min. Several primers were redesigned to ensure amplification in the rough-toothed dolphin.

Primers, fragment length, specific location in the rough-toothed dolphin, and annealing temperatures (T_A) for each fragment are given in Appendix II, Table II.1.

Gel electrophoresis was used to quantify the PCR product from each fragment, and then fragments were combined for each individual, cleaned of excess primers and nucleotides using a Qiagen PCR purification kit (Qiagen) and individually barcoded and prepared for sequencing using a Nextera XT DNA Sample Preparation Kit (Illumina). Individuals were run on three Illumina Miseq runs (two at 250 bp paired end, one at 75bp paired end). Reads were trimmed to remove poor quality sequence and adaptor sequence using default settings in *Trim Galore! v0.2.8* (Babranham Bioinformatics 2013), and then assembled to a *Steno bredanensis* mitogenome reference (GenBank Accession no. JF339982.1) using *BWA v0.7.4* (Li & Durbin 2009). The consensus sequence from the BWA assembly was calculated using *Samtools v0.1.19* (Li *et al.* 2009). For quality control purposes, any site with a read depth <10 was resequenced using Sanger sequencing and verified for the correct base. In addition, base calls supported by fewer than 70% of reads were reviewed for possible heteroplasmy/indels/pseudogene incorporation following Alexander *et al.* (2013).

In a few samples, long-range amplification failed. For these, new primers were designed for shorter regions followed by conventional sequencing on the ABI. The primers, bp length and location of these secondary amplicons are outlined for these fragments in Appendix II, Table II.1.

Each assembled mitogenome was examined for gaps indicative of the incorporation of nuclear mitochondrial DNA (numt) pseudogenes. The mitogenome was represented by concatenated protein-coding regions (Figure 2.2) excluding ND6 due to its location on the opposing strand and distinct patterns of evolution in cetaceans (Ho & Lanfear 2010, Alexander *et al.* 2013). Consensus sequences generated final concatenation of protein-coding genes in *GENEIOUS* (Biomatters LTD) where overlap in genes was represented only once. The start of the first codon position for each gene was identified in *GENEIOUS* and then verified in *MEGA v6.0* (Tamura *et al.* 2013). Saturation of the third codon position was evaluated with *DAMBE* (Xia 2013) in order to assess accurate estimate sequence divergence.

2.3.7. Sanger sequencing

Sequencing for 350 bp of the mtDNA control region (mtDNA CR), protein-coding genes (PC mitogenome) where amplifications had failed, and all introns was done using Sanger sequencing. Free nucleotides and primers were removed from PCR products using SAP and Exo1 (shrimp alkaline phosphatase and exonuclease 1). Products were sequenced using standard protocols of BigDye (Applied Biosystems Inc.), and a final cleaning step performed using the standard CleanSEQ (Agencourt) protocol. Products were run on an ABI3730x1 DNA Analyzer. For all introns and individuals with unique mtDNA CR haplotypes, each individual was sequenced forward and reverse to ensure the sequencing of the entire fragment.

Nuclear introns were evaluated for heterozygote sites using a 25% secondary peak threshold in Sequencher. After identifying heterozygote sites, introns were phased using *Phase v2.1.1.* (Stephens *et al.* 2001), and resulting variable site alleles were concatenated for each individual.

2.3.8. Mitochondrial DNA phylogenies and divergence time

A phylogeny of the 350 bp mtDNA control region dataset was reconstructed in RAxML (Stamatakis 2014) using Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal Gateway (Miller *et al.* 2010). We used the nucleotide substitution model HKY+I+G selected by jModeltest2 (Darriba *et al.* 2012). The heuristic search conditions for ML used starting trees obtained by step-wise addition with ten random sequence addition replicates and tree-bisection-reconnection branch swapping. We used rapid bootstrapping and 1,000 iterations.

For the PC mitogenomes, the phylogeny was reconstructed using *BEAST v1.7* (Drummond *et al.* 2012). We used the nucleotide substitution model GTR, as supported by jModeltest2 (Darriba *et al.* 2012). In order to determine the correct substitution rate for rough-toothed dolphins, we first reconstructed the phylogenetic relationship for 46 cetacean species, including a randomly chosen rough-toothed dolphin sequence from this study, using the fossil calibration given in Ho and Lanfear (2010) with a minimum age constraint for Delphinoidea as discussed in Steeman *et al.* (2009). The second phylogenetic reconstruction used the substitution rate derived in the first analysis specific for rough-toothed dolphins of 0.009776 substitutions per site per Myr.

Each analysis had a burn-in period of 100,000 and 90,000,000 MCMC steps were used to generate two independent chains in *BEAST v1.7*. The multispecies analysis used the parameters, including different site models for each of the three partitions, discussed in Alexander *et al.* (2013) Supplementary Material and an uncorrelated lognormal relaxed clock and a linked Yule tree prior across the PC mitogenome partitions. In the second analysis specific to rough-toothed dolphins, the three codons were partitioned for different site models, but had the same clock model and tree. The site models are discussed in Alexander *et al.* (2013) Supplementary Material and A. Alexander (Pers Comm). A strict molecular clock was used, since lineages within a species are not expected to show rate variation (Ho & Lanfear 2010).

For each analysis, tree files generated from each of the two runs were then combined in *LogCombiner* and evaluated for convergence using *Tracer v1.6* (Rambaut *et al.* 2014), having an Effective Sample Size (ESS) of >500. The maximum clade credibility tree was produced in *TreeAnnotator* (the tree from the MCMC sampler with the highest product of all the posterior probabilities for all clades in the tree), and visualized with *Figtree v1.3.1* (Rambaut 2009).

The biogeographic sample location was traced upon the PC mitogenome phylogeny as an ancestral history using *Mesquite v3.01* (Maddison & Maddison 2014). There were three regions representing each of the oceans, Atlantic, Indian and Pacific. We used a likelihood calculation and a likelihood reconstruction.

2.3.9. Diversity and differentiation

Haplotype diversity, number of alleles, proportion of variable sites, nucleotide diversity and theta were calculated in *Arlequin v3.5* (Excoffier & Lischer 2010). F_{ST} and Φ_{ST} estimates of differentiation between regions were measured with 50,000 permutations in *Arlequin v3.5*. The average net distance between groups ($d_A = d_{XY} – ((d_X + d_Y)/2)$ where d_{XY} is the average distance between groups X and Y and d_X and d_Y are the mean within-group distances, was calculated as a metric for subspecies status in *MEGA v6.0* for the five sampling regions using the mtDNA CR dataset as well as the three region PC mitogenome dataset.

For the intron dataset using the three regions (n=35), F_{ST} and G''_{ST} were calculated for phased alleles in Genodive (Meirmans & Van Tienderen 2004). Observed heterozygosity was calculated on a per-locus basis, dividing the total number of sampled heterozygotes by the total number of individuals sequenced. This differs from Jackson *et al.* (2014) who calculated heterozygosity of each single nucleotide polymorphism, rather than the phased alleles. Migration rate (Nm) was calculated with the resulting F_{ST} value between regions using the equation $Nm = ¼((1/F_{ST})-1)$ (Wright 1969, Hedrick *et al.* 2013)

2.4 RESULTS

We generated phylogeographic and population level statistics for the most comprehensive dataset of rough-toothed dolphins to date. The mtDNA CR sequences (length 350 bp) were compiled for a total of 351 individuals (the removal of replicate samples is

discussed in Chapter 3). The six nuclear loci (length 2,910 bp) were sequenced from a subset of these individuals (n=35; 10 from the Atlantic, 7 from the Indian and Western Pacific Ocean, 18 from the Central and Eastern Pacific Ocean, Appendix II Table II.3) and samples represented each of three oceanic regions, Atlantic, Indian/Western Pacific and Central/Western Pacific (Table 2.1). The protein-coding regions of the mitochondrial genome (length 10,810 bp) were concatenated for a subset of the individuals used for the intron dataset (n=19) representing three oceanic regions.

2.4.2. Mitochondrial DNA phylogenies and divergence time

Within the mtDNA CR dataset, we identified 49 haplotypes and 73 variable sites from individuals in the Pacific (n=310), Indian (n=7) and Atlantic (n=34) Oceans. Although the Pacific and Indian Oceans shared mtDNA haplotypes (n=7) there were no haplotypes shared with either of these areas and the Atlantic Ocean (Figure 2.4). There were no fixed diagnostic substitutions unique to the Atlantic Ocean or any other region. Although there was a private clade (Clade D) supported by three fixed differences in the mtDNA CR dataset, this phylogeny was not supported for any clade.

Each individual in the PC mitogenome phylogeny had a unique haplotype represented from 186 variable sites. There were four main clades, one from the Western Pacific (labeled A in Figure 2.4), one from the Central Pacific (B), and one from the North Atlantic (D). The other cosmopolitan clade included individuals from the Indian Ocean, the Central Pacific, Eastern Pacific, the South Atlantic and North Atlantic (C). The Bayesian phylogeny of the PC mitogenome dataset had posterior probabilities above 0.95

for all clades with the exception of the cosmopolitan clade (C) where the posterior probability was 0.79.

The private North Atlantic clade was reconstructed in both phylogenies (mtDNA CR and PC mitogenome). Posterior probabilities for the PC mitogenome tree were highly supported for this clade, however, the mtDNA CR clade bootstrap values illustrated no support (bootstrap value <50%). Three Atlantic haplotypes clustered with Pacific haplotypes in both reconstructions, although the clade in both phylogenies did not receive significant support. Thus, despite the support for the North Atlantic clade (D) in the PC mitogenome phylogeny, there was no evidence of overall monophyly for individuals from the Atlantic Ocean. The resolution provided by the PC mitogenome phylogeny of the other two clades (Figure 2.4 clades A and B) was not present in the 350 mtDNA CR tree. In the PC mitogenome phylogeny, two haplotypes in the central Pacific that were collapsed into one at 350 bp mtDNA (this chapter) and 450 bp (dataset in Chapter 3) represented unique haplotypes in the concatenated protein-coding regions (individuals SbrAS12588 and Sbr03FP120).

The substitutions per site per Myr calculated for the rough-toothed dolphin was 0.00976 (Figure 2.4), 95% highest posterior density (HPD) = 0.0073-0.012. Appendix Table II.3 shows rough-toothed dolphins exhibit a slightly higher substitution rate than average when compared to the other delphinids in the multispecies table. This value is well within the range of estimates from McGowen (2011) and Steeman *et al.* (2009), as well as

Alexander *et al.* (2013), for delphinid substitution rates using the PC mitogenome (median value 0.0059-0.0123, 95% HPD = 0.0039-0.0199).

The ancestral state reconstruction (Figure 2.5) suggests that rough-toothed dolphins originated in the Pacific Ocean. However, equivocal nodes after this event illustrate it is not possible to determine which direction the next migration events occurred, Pacific into the Atlantic or Atlantic into the Pacific. There could have been three migration events into the Atlantic from the Indo/Pacific Ocean. Equally likely from the PC mitogenome phylogeny and ancestral state, there could be a migration into the Atlantic and two returns to the Pacific, or two Atlantic migrations and one return to the Pacific.

2.4.3. mtDNA genetic diversity and differentiation

Genetic differentiation using the mtDNA CR dataset was highly significant among the five regions (Table 2.2) as well as highly significant differentiation for the PC mitogenomes among the three regions (Table 2.3). For both datasets, the greatest differentiation was between the Atlantic Ocean and all other regions, as expected, given the phylogenetic reconstruction. The average net divergence (d_A) ranged from 1.11% to 1.78% for the mtDNA CR (Table 2.2) and from 0.11% to 0.20% for the PC mitogenome (Table 2.3) with the largest values between the Atlantic Ocean and other regions in both datasets.

2.4.4. Nuclear diversity and differentiation

From the total of 2,910 bp of six concatenated introns there were 16 variable sites in 35 individuals. These were based on homozygotes and calling secondary peaks to identify heterozygotes. Phasing indicated 1 – 8 alleles for each autosomal intron (Table 2.1). However, the Y-linked DBY7 was invariant and naturally did not occur in female samples (n=19 of the 35 samples). Four private alleles were found in the Atlantic Ocean, but there were no fixed differences between oceanic regions for any intron. Nucleotide diversity (π) ranged across loci 0.01% (CHRNA-1) to 1.02% (Actin-1), while the average worldwide genomic (π) was 0.08% for the Atlantic, 0.10% for the Indian/Western Pacific, 0.12% for the Central/Eastern Pacific (Table 2.1).

Significant genetic differentiation was found between the Atlantic and the other two oceanic regions, but not between Indian/Western Pacific and Central/Eastern Pacific (Table 2.4).

Intron F_{ST} values yielded migration rates (Nm) for rough-toothed dolphins of 1.6 migrants/generation and 1.4 migrants/generation between the Atlantic and the Indian/Western Pacific region and the Atlantic and Central/Eastern Pacific regions respectively. An Nm of 14 migrants/generation was found between the Indian/Western Pacific and Central/Eastern Pacific regions. Nm values for mtDNA were slightly lower.

2.5. DISCUSSION

Rough-toothed dolphins are currently recognized as one species worldwide, and no taxonomic designation has been examined previously on a worldwide scale for this species. Our study evaluated the concordance between genetic isolation and oceanographic regions. We did not find monophyly of oceanic regions in either the mtDNA or nuclear intron datasets, and therefore our data do not support the designation of multiple species for the rough-toothed dolphin. Our population level analyses revealed significant differentiation and less than two migrants per generation between the Atlantic and both Indian/Western Pacific and Central/Eastern Pacific, suggesting the Atlantic ocean basin rough-toothed dolphins be recognized as a separate ESU.

2.5.1. Evidence of subspecies or ESU's for the Atlantic region?

We found evidence of genetic isolation and limited gene flow of rough-toothed dolphins in the Atlantic Ocean from the Indian and Pacific Oceans with significant F_{ST} values between the regions for both mtDNA and the nuclear introns. Significant differentiation of nuclear introns and mtDNA between regions has been sufficient to identify separate subspecies for cetaceans with worldwide distributions as it reflects low levels of gene flow (Jackson et al. 2014). Subspecies should also have a diagnosable component associated with fixed differences in either nuclear or mtDNA (Archer *et al.* 2013). We found no evidence of fixed differences in our nuclear intron or mtDNA dataset. Therefore, we do not currently have a useful measure to gauge subspecies status in our study, and we conclude from no fixed differences between oceanic regions, that subspecies status is not appropriate for the rough-toothed dolphin. The migration rate

using the nuclear introns revealed less than two migrants per generation. This has been used as a guideline in determining ESU status, as it shows that local adaptation may occur in populations that experience this low level of gene flow (Gardenfors 2001). Therefore, separate ESU's should be recognized for rough-toothed dolphins between the Atlantic region and Indian/Western Pacific and the Central/Eastern Pacific.

Between the Indian/Western Pacific and the Central/Eastern Pacific regions shared mtDNA CR haplotypes and no significant differentiation in the nuclear dataset indicate recent divergence or low levels of continued gene flow. The five regions identified in the mtDNA CR dataset should be recognized as separate populations. However, within these populations, smaller populations may exist, as shown in Oremus *et al.* (2012), and fine scale analyses including the use of microsatellites will be needed to determine the number of populations within each of these regions (see Chapter 3).

Although it is possible that rough-toothed dolphins in the Atlantic Ocean may warrant subspecies status, our study is limited in scope by 1) the use of only genetic data and 2) limited sampling in some areas. Where Jackson *et al.* (2014) and Archer *et al.* (2013) had comprehensive sampling from each ocean basin, our sampling in the Atlantic was limited to the Western Atlantic and one sample from the South Atlantic. Moreover, the few samples in the Indian Ocean may not fully represent that region. Although Morin *et al.* (2010) identified subspecies status for the killer whale using only mitochondrial DNA, they had several morphological and ecological studies that corroborated their findings. This is the first worldwide study on rough-toothed dolphins, and no morphological or

ecological based studies have been done to our knowledge. Although we found no shared haplotypes between the Atlantic and the other regions, which may be evidence of subspecies status, it may also be due to our limited sampling.

2.5.2. Colonization into or out of the Atlantic Ocean?

The PC mitogenome phylogeny implies that rough-toothed dolphins have inhabited both the Atlantic and the Pacific Ocean basins for nearly one million years. It does appear that the species originally inhabited the Pacific Ocean and there were additional radiation events that spread into both oceans, either from the Atlantic into the Indian/Pacific Ocean, or from the Indian/Pacific into the Atlantic. Ancestral state reconstruction considered alone suggests that either direction of migration is plausible (Figure 2.4). However, the oceanography of the region suggests that Pacific to Atlantic dispersal would be an easier direction of travel. This makes the option of three separate migrations into the Atlantic more likely than either two Atlantic migrations and one return to the Pacific or one migration into the Atlantic followed by two returns to the Pacific.

The Agulhas current and the fluctuating temperature around Cape Agulhas at the southern tip of Africa is referred to as the South Africa species gate (Perrin 2007). It is considered a biogeographic barrier for many species, and has affected the dispersal of many fauna with worldwide distributions (Perrin 2007, Rocha *et al.* 2007, Daly-Engel *et al.* 2012a). On several occasions in the Pleistocene, subtropical to tropical water extends around the Cape and up into the Atlantic. In colder periods this connection is interrupted, with a 20^{o} isotherm hitting the continent. Therefore, during this time, the tropical Atlantic

was completely cut off from the rest of the tropics. For species with tropical and subtropical distributions their distribution pattern may therefore be related to the South Africa species gate. For example, spotted dolphins have two recognized species (*Stenella attenuata* and *Stenella frontalis*). Although *S. attenuata* is found in both the Atlantic and Indian Oceans, *S. frontalis* is only found in the Atlantic Ocean. Moreover, the humpback dolphin on the South Atlantic Ocean coast (*Sousa tesuszii*) is a separate species than that found on the Indian Ocean coast of South Africa (*Sousa chinensis*). This pattern suggests that the occasional open gate may have been effectively a one-way filter, enabling greater movement from east to west than the reverse because when the corridor is open, the fast Agulhas current runs from northeast to southwest and the Benguela current along the west coast of Africa flows from south to north. So a tropical dolphin moving from the Atlantic into the Indian Ocean would have to travel against the currents for several thousand kilometers, while the other direction would provide an "easy ride" (Perrin 2007).

The timing of the second migration event coincides with a oceanographic event that affected several marine fauna in the South Africa region. According to the PC mitogenome phylogeny, second migration event occurred around 130 Kyr (Figure 2.4). Around the same time as this clade diverged, a vigorous exchange of fauna occurred between the Indo-Pacific and the Southwest Atlantic during the inter-glacials of the late Pleistocene promoted an enhanced Agulhas current around the southern tip of Africa (Peeters *et al.* 2004). Therefore, it is plausible that this migration event from the east occurred into the Atlantic during this time. This provides a stronger argument for the

migration event to have occurred into the Atlantic instead of into the Pacific. Although the Pacific into the Atlantic migration direction appears a more plausible option biologically for many fauna, the scope of our study is limited, and additional samples for the PC mitogenome are needed, especially samples from the eastern Atlantic, to determine which direction the rough-toothed dolphin oceanic migration events occurred.

2.5.3. Conclusion

The results of this study are the first to explicitly examine the phylogeography of the rough-toothed dolphin. The phylogeographic pattern we identified in both nuclear and mtDNA show larger F_{ST} values between the Atlantic and the other regions. This is not surprising given biogeographic barriers can be influential to pelagic species (Rocha *et al.* 2007). The South Africa species gate presents temperature fluctuations and currents that have shown it to be an influential biogeographic barrier for many fauna. Although our original metric was genetic isolation and geographic distance, the biological significance of this genetic differentiation may be more relevant to the biogeographic barrier presented by the South Africa species gate for this tropical and subtropical species.

2.6. Acknowledgements

We thank Cascadia Research Collective and many regional collaborators for access to DNA samples. Special thanks to Kelly Robertson for archiving the entire rough-toothed dolphin database outside of the Society Islands and Samoa (island of Savaii) samples. The samples were collected in collaboration with Oregon State University, the University of Auckland, Southwest Fisheries Science Center, Portland State University and the

Environment Society of Oman. We thank Ellen Garland, Ursula Gonzalez, Daniel Webster, Jessica Aschettino, Greg Schorr, James Sumich, and Pamela Carzon for field assistance in the Hawaiian and Society Islands. We thank Susana Caballero for assistance in long-range fragment primer design specific to *Steno bredanensis*, Debbie Steel for assistance in laboratory analyses and Dori Dick for ArcGIS assistance. We thank Southwest Fisheries Science Center Marine Mammal Genetics Group and the Pacific Islands Grant who provided funding for the archiving and extracting of samples from the Hawaiian Islands and American Samoa. National Marine Fisheries Service, U.S. Navy (N45, Office of Naval Research, Pacific Fleet), and Wild Whale Research Foundation provided funding for the main Hawaiian Islands fieldwork. New Zealand Marsden Fund, Pew Environmental Trust and the Mamie Markham Research Award provided funding for Society Islands and Samoa fieldwork; with additional funding provided by the Ministry of the Environment of French Polynesia and Dolphin and Whale Watching Expeditions. C.S. Baker was supported by a Pew Marine Conservation Fellowship for a study of "A Pattern of Dolphins" (aPOD) in the Pacific.

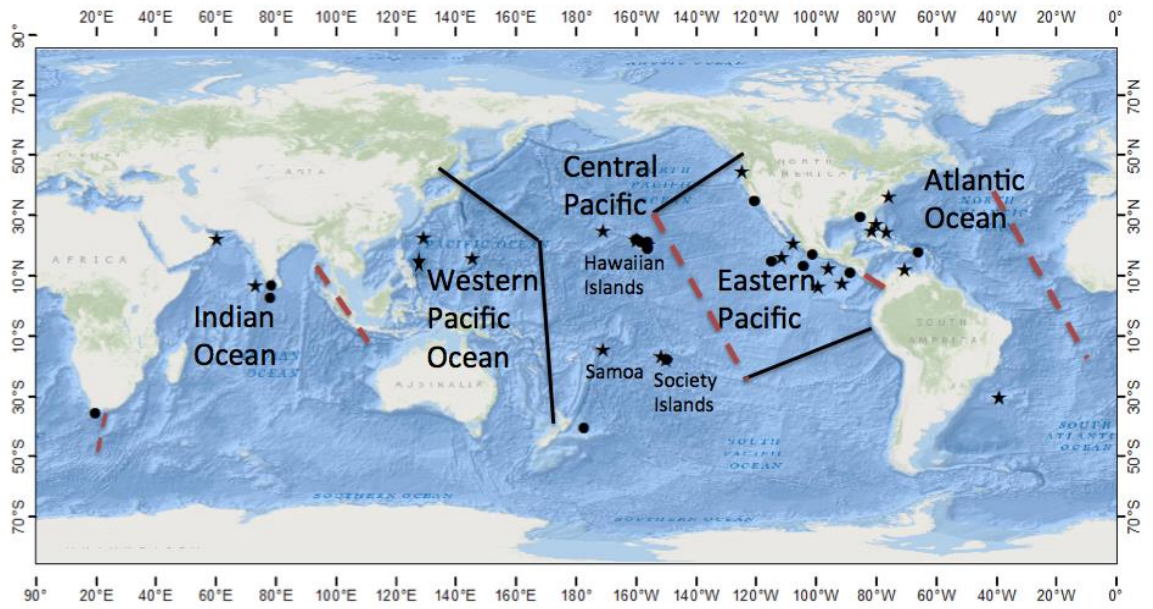

Figure 2.1. Boundaries represented by black lines show sampling regions for the worldwide mtDNA dataset. Our boundaries were designed relative to the seven biogeographic barriers described by Rocha *et al.* 2007. Boundaries from that study are shown in dashed lines. For the intron and mitogenome dataset the Western Pacific and Indian Ocean regions were collapsed into one region (Western Pacific/Indian Ocean) and the Central Pacific and the Eastern Pacific were collapsed (Central/Eastern Pacific). Circles represent locations of samples used (teeth and tissue) for the control region only (350 bp) and stars represent locations of samples used in the mitogenome/intron study. For geographic context, the Esri Ocean Basemap (http://esriurl.com/obm, courtesy of Esri and its partners) is also added.

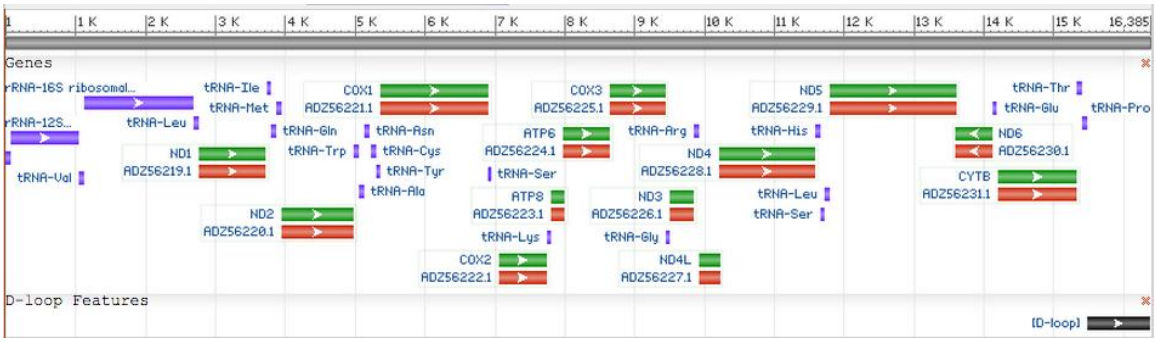

Figure 2.2. The protein-coding genes (highlighted in green) and D-loop (in black) for the sample rough-toothed dolphin sample Sbr11638. The concatenated protein-coding mitogenome dataset included all of the protein-coding genes here except ND6 (12 total genes). Reasons for the exclusion of ND6 are discussed in the Methods section.

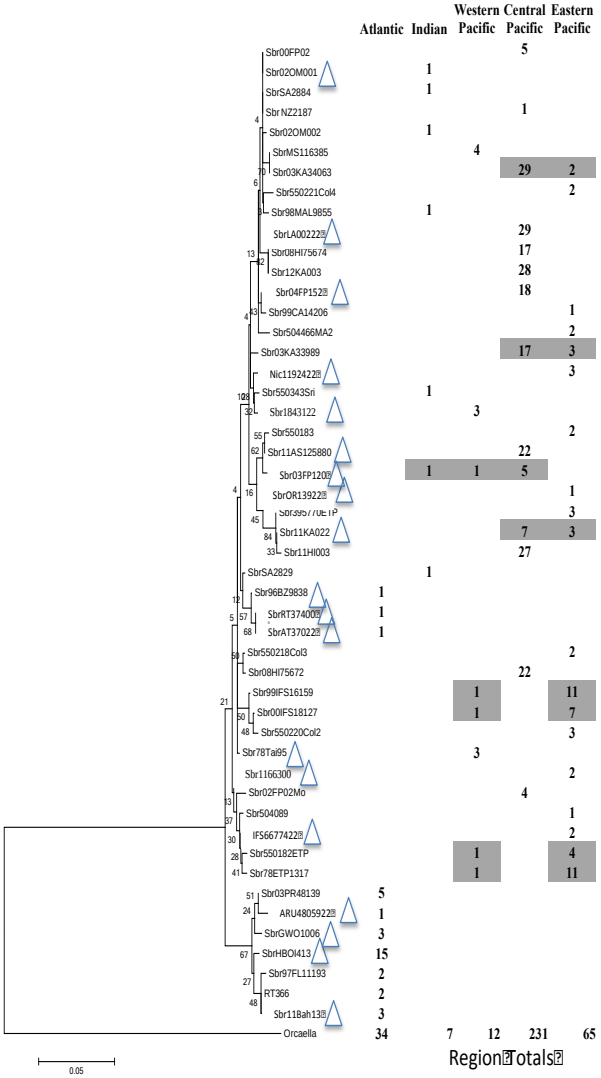

Figure 2.3. A maximum-likelihood phylogenetic reconstruction of rough-toothed dolphin mtDNA CR haplotypes (350 bp) with bootstrap values shown and rooted to the Irrawaddy dolphin (*Orcaella brevirostris*). Haplotype frequencies are shown in the table to the right, and shared haplotypes between regions are shaded. Note that some haplotypes that are separate at 450 basepairs (in Ch 3) are collapsed at 350 basepairs (n=9 that collapsed). Triangles represent samples used in the mitogenome dataset.

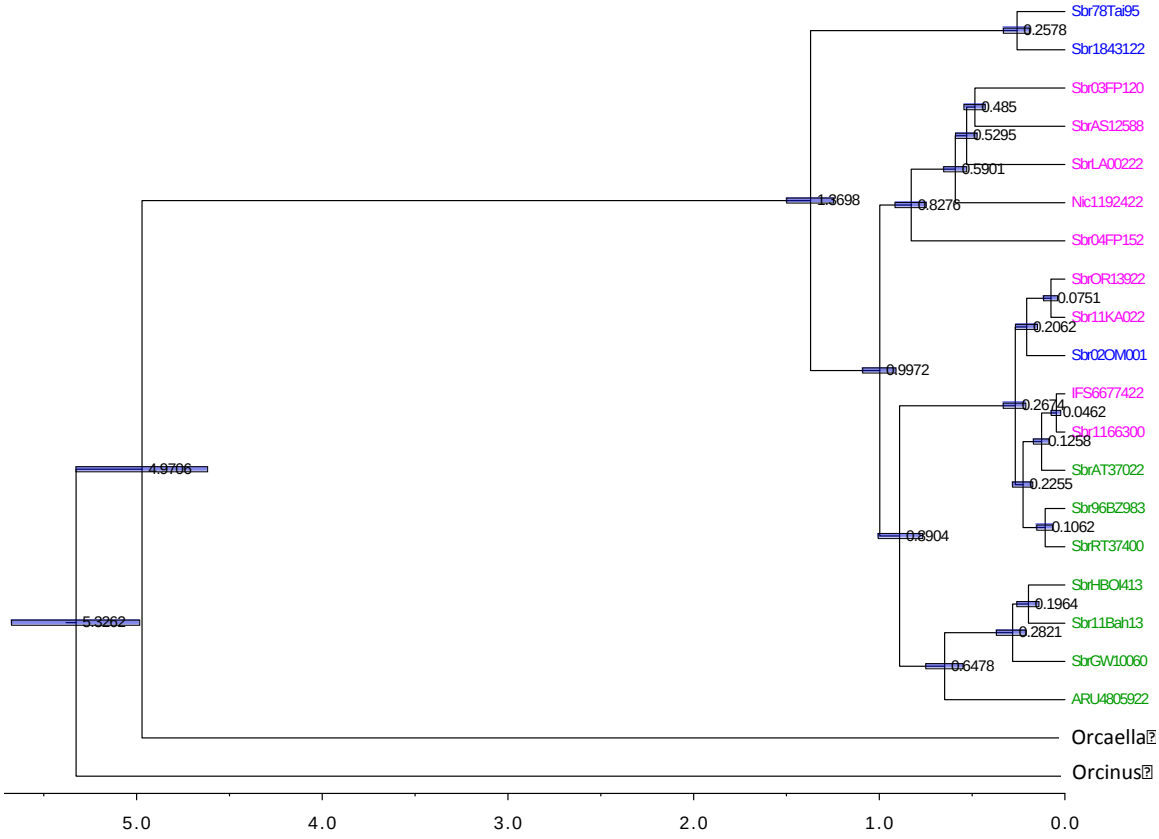

Figure 2.4. Bayesian phylogenetic reconstruction of rough-toothed dolphins based on concatenated protein-coding genes in the mitogenome rooted to *Orcaella breverostris* and *Orcinus orca* shown in black. Bayesian posterior probabilities were all above 0.98 unless indicated with an + where the posterior probability is 0.79. Individuals are color coded according to the region where they were sampled. Blue represents the Indian and Western Pacific Ocean, pink represents the Central and Eastern Pacific and green represents the Atlantic Ocean. Each letter designates a main clade. Area where samples were collected is to the right of the sample. The time scale is in millions of years, and the error bars on the nodes indicate uncertainty around divergence time estimates.

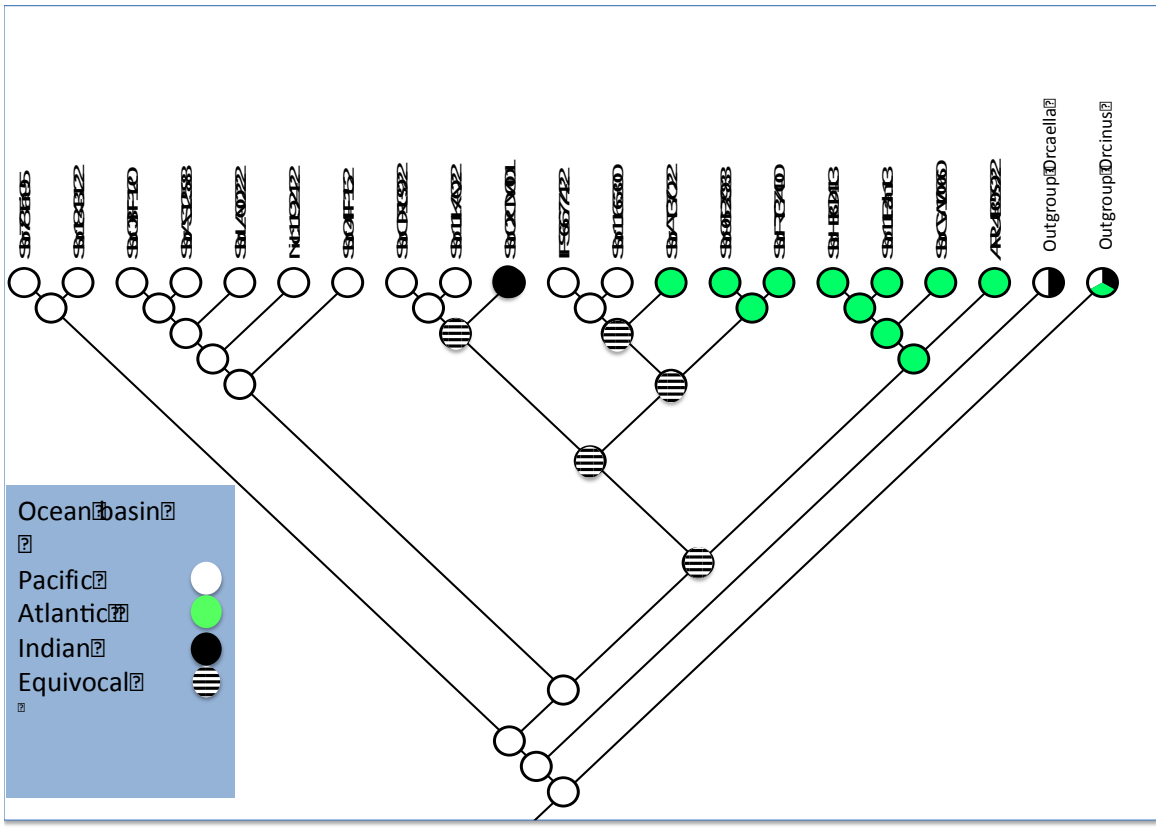

Figure 2.5. Likelihood ancestral character state reconstruction of concatenated protein-coding genes in the rough-toothed dolphin mitogenome. This was traced onto the PC mitogenome tree, so is rooted to *Orcaella breverostris* and *Orcinus orca*.

Table 2.1. Basic diversity estimates of nuclear genomic and mtDNA control region sequences. DBY-7 is not shown due to no variation across samples. 'Introns' shows statistics summed over all introns, parentheses represent private alleles to each ocean basin and n equals the number of individuals in that region. MtDNA PC refers to the concatenated mtDNA protein-coding gene sequences (10,810 bp), and mtDNA 350 bp refers to the mtDNA control region sequence that is (350 bp) in length and includes both teeth and skin samples. [a] Haplotype diversity is reported for the 350 bp mtDNA CR and mtDNA PC dataset.

	ACT	CAT	GBA	CHRNA1	IFN	Introns	mtDNA PC[a]	mtDNA 350 bp [a]
Length (bp)	980	520	310	360	340	2510	10,810	350
No. of individuals	32	35	34	35	35	35	19	351
No. of variable sites	8	2	1	1	4	16	186	76
Alleles: Atlantic n=10	5(4)	1(0)	1(0)	1(0)	5(0)	13	7(7)	10(10)
Alleles: Indian and Western Pacific n=7	4(1)	3(0)	1(0)	1(0)	5(0)	12	3(3)	15(9)
Alleles: Central and Eastern Pacific n=18	7(2)	3(0)	1(1)	2(1)	8(0)	21	9(9)	33(21)
Total no. of alleles	10	3	2	2	8	24	19	49
Observed Heterozygosity	0.400	0.200	0.028	0.057	0.911	0.155	1[a]	0.912[a]
s.d.	0.082	0.072	0.033	0.133	0.147	0.148	0.0006	0.0006
π	0.0041	0.0028	0.0002	0.0001	0.0022	0.0036	0.0126	0.0165
s.d.	0.0019	0.0016	0.0001	0.0001	0.0009	0.0017	0.0094	0.0107

Table 2.2. Inter-ocean genetic differentiation of rough-toothed dolphins at the 350 bp mtDNA control region above diagonal F_{ST} and ϕ_{ST} in parantheses above diagonal as measured in *Arlequin*. Significance is shown in bold and italics. Sample totals for each region are given in parentheses (n). Significance is shown in bold and italics with significance level *p<0.05 and ** p<0.001. dA net distance values generated in *MEGA 6.0* are shown below the diagonal.

	Atlantic (n=34)	Indian (n=7)	Western Pacific (n=14)	Central Pacific (n=231)	Eastern Pacific (n=65)
Atlantic		***0.223*** ***(0.562**)***	***0.168*** ***(0.435**)***	***0.432*** ***(0.516**)***	***0.397*** ***(0.511**)***
Indian	0.0159		***0.133*** ***(0.251**)***	***0.147*** ***(0.286**)***	***0.156*** ***(0.299**)***
Western Pacific	0.0155	0.0111		***0.164*** ***(0.277**)***	***0.199*** ***(0.279**)***
Central Pacific	0.0179	0.0131	0.0124		***0.201*** ***(0.205**)***
Eastern Pacific	0.0178	0.0112	0.0119	0.0117	

Figure 2.3. Inter-ocean genetic differentiation of rough-toothed dolphins using PC mitogenome sequences ϕ_{ST} as measured in *Arlequin* (above diagonal) and dA values calculated in *MEGA 6.0* (below diagonal). Significance is shown in bold and italics. Significance is shown in bold and italics with significance level *p<0.05 and ** p<0.001.

	Atlantic	Indian and Western Pacific	Central and Eastern Pacific
Atlantic		***0.284****	***0.201****
Indian and Western Pacific	0.0020		***0.163****
Central and Eastern Pacific	0.0017	0.0011	

Table 2.4. Inter-ocean genetic differentiation of rough-toothed dolphins using nuclear intron alleles as calculated in *Genodive* for F_{ST} (below diagonal) and G''_{ST} (above diagonal). Significance is shown in bold and italics with significance level *p<0.05 and ** p<0.001.

	Atlantic	Indian and Western Pacific	Central and Eastern Pacific
Atlantic		***0.177***	***0146***
Indian and Western Pacific	***0.150***		0.0091
Central and Eastern Pacific	***0.135***	0.018	

3. Staying close to home? Genetic differentiation of rough-toothed dolphins near oceanic islands in the central Pacific Ocean

3.1. Abstract

Rough-toothed dolphins have a worldwide tropical and subtropical distribution, yet little is known about the population structure and social organization of this typically open-ocean species. Although it has been assumed that pelagic dolphins range widely due to the lack of apparent barriers and unpredictable prey distribution, recent evidence suggests rough-toothed dolphins exhibit fidelity to some oceanic islands. Here we assess the isolation and interchange of rough-toothed dolphins at the regional and trans-equatorial scale within the central Pacific Ocean using the most geographically extensive dataset for this species to date. Samples of insular communities from the Hawaiian (n=181), French Polynesian (n=75) and Samoan (n=18) archipelagos and pelagic samples off the Northwestern Hawaiian Islands (NWHI) (n=18) were analyzed using mtDNA sequencing and microsatellite genotyping of 15 loci. An overall AMOVA indicated strong genetic differentiation among islands within the main Hawaiian Islands (mtDNA F_{ST}=0.165; p<0.001; microsatellite F_{ST}=0.043 p<0.001) and the Society Islands of French Polynesia (F_{ST}=0.499; p<0.001; microsatellite F_{ST}=0.079 p<0.001) as well as between archipelagos (mtDNA F_{ST}=0.299; p<0.001; microsatellite F_{ST}=0.055 p<0.001). Our results confirm a pattern of island-specific genetic isolation among populations attached to some islands in each archipelago. These insular populations are most prevalent where there is local productivity. The shared haplotypes (n=4) between the archipelagos may be a product of a relatively recent divergence and/or periodic exchange from poorly understood pelagic

populations. Our findings have important implications for a little studied species that faces increasing anthropogenic threats around oceanic islands.

3.2. Introduction

The lack of geographic barriers in the open ocean has long been assumed to pose few restrictions to gene flow for pelagic species predicting little phylogeographic structure at the oceanic scale and negligible population structure at the local scale. However, numerous recent studies show that even species with high dispersal capabilities such as barracuda, sharks and dolphins can show strong population structure (Fontaine *et al.* 2007, Tezanos-Pinto *et al.* 2008, Daly-Engel *et al.* 2012a, Daly-Engel *et al.* 2012b, Whitney *et al.* 2012). Although biogeographic barriers are thought to be one of the forces influencing this structure on a large scale (Briggs & Bowen 2012, Daly-Engel *et al.* 2012a), population structure on a regional scale is often shaped by life history strategies, social structure and variation in oceanographic resources (Moller 2012).

The interplay of social structure, habitat specialization, and dispersal patterns has particularly important implications on the genetic structure of populations in dolphin communities. Coastal dolphins generally show patterns of strong genetic differentiation, low genetic diversity between these populations, and isolation by distance (Wiszniewski *et al.* 2010, Mirimin *et al.* 2011, Hamner *et al.* 2012, Richards *et al.* 2013), while pelagic dolphins seem to show lower, but significant, levels of genetic differentiation, and no particular pattern of isolation by distance (Gaspari *et al.* 2007, Louis *et al.* 2014). Genetic structure can also be influenced by highly organized social structure in both coastal (Moller 2012) and pelagic dolphins (Gaspari *et al.* 2007, Foote *et al.* 2011b, Baird *et al.*

2012). When social structure is the main driver in population structure, random colonization and local drift are observed (Storz 1999). Such organization may restrict genetic differentiation and reduce diversity, limiting the resilience of populations to recover from natural catastrophes or anthropogenic impacts.

In the open ocean of the tropics, where prey resources are widely scattered and less predictable, dolphins are thought to show little population structure. However, primary productivity often increases near oceanic islands due to the "Island mass effect" where nutrients from the island and sufficient light at the ocean's surface initiate photosynthesis (Doty & Oguri 1956, Longhurst 1999, Martinez 2004, Woodworth *et al.* 2012). Island topographies, and their interactions with wind and currents, create a complex system of offshore eddies that also concentrate stable and abundant prey for many mesopelagic predators (Seki *et al.* 2002, Woodworth *et al.* 2012). Surveys have shown cetacean densities are greater closer to shore around the Hawaiian Islands and French Polynesia Islands (Barlow 2006, Lambert *et al.* 2014). Several recent studies have shown dolphin species exhibit population structure and site fidelity due to resource specialization around oceanic islands that experience the island mass effect (Oremus *et al.* 2007, Andrews *et al.* 2010, Martien *et al.* 2012, Oremus *et al.* 2012).

The Hawaiian Islands in the North Pacific, and the Society and Samoan Islands in the South Pacific represent some of the most isolated archipelagos in the world (Figure 1a). Due to the island mass effect (Doty & Oguri 1956, Longhurst 1999), they also represent high levels of diversity and endemism for marine species (Bowen *et al.* 2013). The

Hawaiian Islands (Figure 1b) provide habitat to about 1/3 of the recognized cetacean species (Barlow 2006, Baird *et al.* 2013, Bowen *et al.* 2013), while the five archipelagos of French Polynesia (Figure 1c), including the Society Islands, also provide habitat to 1/3 of the world's cetacean species. The steep volcanic slope of these islands equates to deep-water species in close proximity to shore, making these islands a convenient site (Poole 1995) to study otherwise pelagic cetaceans in the wild. Within the last ten years, an increasing number of cetacean studies have provided baseline data on movement and habitat use for both nearshore and offshore species around oceanic islands. Many of these studies have found fidelity to islands for small odontocetes (Poole 1995, Oremus *et al.* 2007, Baird *et al.* 2008, Aschettino *et al.* 2012, Martien *et al.* 2012, Oremus *et al.* 2012, Baird *et al.* 2013).

Rough-toothed dolphins (*Steno bredanensis*) have a worldwide tropical and subtropical distribution (Jefferson 2008). They are a medium-sized dolphin generally found in depths greater than 1,000 meters. Although considered a pelagic species, they are also found relatively close to shore where volcanic islands provide steep bathymetric slopes (Gannier & West 2005, Baird *et al.* 2008, Oremus *et al.* 2012). Around the main Hawaiian Islands (MHI) the highest concentration of encounters of rough-toothed dolphins is at depths around 3,000 meters (Baird *et al.* 2013). A previous photo-identification (photo-ID) study found evidence of local populations around the MHI (Kauaʻi/Niʻihau and the Island of Hawaiʻi) with low levels of interchange between populations (Baird *et al.* 2008). Differences in behavior and habitat use suggested movement between the islands was infrequent. Moreover, frequent within- and between-

year re-sightings around the Big Island of Hawai‘i (from this point forward referred to as Hawai‘i) indicated high site fidelity and low abundance (N<200; (Baird *et al.* 2008). Oremus *et al.* (2012) combined molecular markers and photo-ID to describe island communities of rough-toothed dolphins in the Leeward (Mo‘orea/Tahiti) and Windward Islands (Ra‘iatea/Huahine) in the Society Islands. They found high genetic differentiation between these islands and low haplotype diversity. Additional photo-ID studies of rough-toothed dolphins have been conducted in both the Pacific and Atlantic oceans, and indicate patterns of island fidelity. In Tutuila, American Samoa, a quarter of the individuals identified were sighted in multiple years (Johnston *et al.* 2008); around Utila, Honduras, in the Atlantic Ocean, over half of the individuals identified were sighted over multiple years (Kuczaj & Yeater 2007), and around the Canary Islands of Spain, high site fidelity and association patterns suggested a small resident population, with strong social bonds between individuals of different age classes and between mother/calf/juveniles (Mayr & Ritter 2005).

Rough-toothed dolphins exhibit some behaviors associated with highly organized social structure including care-giving behaviors, synchronized travel in close formations, cooperative foraging, provisioning of large prey to calves, and mass stranding events (Nitta 1991, Ferrero & Hodder 1993, Poole 1993, Nekoba-Dutertre *et al.* 1999, Pitman & Stinchcomb 2002, Gotz *et al.* 2006, NMFS 2008, Fulgencio de Moura *et al.* 2009). Recently, Oremus *et al.* (2012) found low genetic diversity and genetic differentiation within the Society Islands where these behaviors have been observed; suggesting social organization may play a role in rough-toothed dolphin population structure.

Here we present a comparative analysis of population structure in three tropical archipelagos of the Pacific. We employ a suite of genetic markers to investigate the population structure of rough-toothed dolphins among the Hawaiian Islands, French Polynesia's Society Islands, and the Samoan Islands (Upolo and Savai'i in Samoa and Tutuila in American Samoa). We test the hypothesis suggested by Baird *et al.* (2008) and Oremus *et al.* (2012) that rough-toothed dolphins exist in demographically independent, insular populations around oceanic islands with limited interchange or gene flow between islands. We interpret the observed genetic structure in relation to two hypotheses: if isolation by distance is the primary driver, we expect that populations within archipelagos will show significant genetic differentiation, and differentiation will increase with increasing geographic distance among islands within archipelagos and between archipelagos. If social structure is the primary driver, we expect differentiation could be driven by more episodic colonization and local genetic drift.

3.3. MATERIALS AND METHODS

3.3.1. Tissue sample collection

Skin biopsy samples were collected from adult or subadult dolphins for genetic analyses during dedicated small boat surveys or from beach-cast individuals in the MHI (Kaua'i, O'ahu, Hawai'i, Figure 3.1b), the Northwestern Hawaiian Islands NWHI, Figure 3.1a large rectangle), the Society Islands (Mo'orea/Tahiti and Ra'iatea/Hu'ahine, from here forward referred to as Mo'orea and Ra'iatea respectively, Figure 3.1c), and the Samoan Islands (Figure 3.1d) including Samoa (Upolo and Savai'i) and American Samoa

(Tutuila). Biopsy samples were either collected with a crossbow or a modified veterinary capture rifle (Krützen *et al.* 2002). Samples were stored first in 70% ethanol, followed by an -80^{o}C freezer or stored directly in the freezer. Skin samples collected from individuals that stranded around the Hawaiian Islands and bycaught individuals around American Samoa were stored in DMSO at -20^{o}C. Photo-identification was collected during biopsy sampling in the Society Islands and the Hawaiian Islands. For complete methodology and analysis of photo-identification see Baird *et al.* (2008) and Oremus *et al.* (2012).

3.3.2. DNA extraction, quantification and sex identification

Total DNA was extracted using either a Qiagen DNeasy Blood and Tissue Kit or a standard phenol:chloroform extraction protocol (Sambrook *et al.* 1989), modified for small samples (Baker *et al.* 1994). DNA was quantified with pico-green fluorescence and normalized to 15 ng μl^{-1}. Sex was identified for individual dolphins by the amplification of the male-specific Sry gene multiplexed with the ZFX gene (a positive control) as described in (Gilson *et al.* 1998).

3.3.3. mtDNA sequencing

An approximately 800 bp fragment of the mtDNA control region of the 5' end was amplified via polymerase chain reaction (PCR) with primers Dlp 1.5 and Dlp 8 (Dalebout *et al.* 1998, Dalebout *et al.* 2005). The reaction was carried out in a 10μl final volume using the protocol described in Oremus *et al.* (2007). Unincorporated primers were removed from the PCR product using shrimp alkaline phosphatase (SAP) and

exonuclease I (Ex). Products were sequenced using the standard BigDye vs3.1 and CleanSeq was used to purify products before they were run on an ABI 3730 (Applied Biosystems) DNA automated sequencer. The sequences were aligned, manually edited, and haplotypes were identified using *Sequencher 5.0* (Gene Codes Co.). Following quality control guidelines of Morin *et al.* (2010), sequences with a *Phred* score of <30 were repeated (Ewing *et al.* 1998). Variable sites with a *Phred* score of <40 were visually confirmed. If a haplotype was represented by only one sample the identity of the haplotype was confirmed by re-sequencing in both directions. Three individuals from Kaua‘i showed apparent heteroplasmy at the same variable site. These sites were treated as missing values for these samples in subsequent analyses.

3.3.4. Microsatellite genotyping

Samples were genotyped at 15 previously published microsatellite loci (Appendix Table III.1). Amplifications were carried out in a 10μl final volume reaction containing 1x Perkin-Elmer reaction buffer, 1.5-4.0 mM $MgCl_2$, 0.4 μM of each primer (when fluorescent labels were pre-labeled), 0.2mM deoxynucleotide triphosphate (dNTPs), 0.25 to 0.5 U Platinum *Taq* DNA Polymerase and approximately 5ng of DNA template. Fluorescent labels were attached when loci were not labeled via an M13 tail during amplification, changing the forward primer concentration to 0.04 μM, keeping the reverse primer concentration of 0.4 μM and adding an M-13 label concentration of 0.4 μM (Schuelke 2000). The PCR thermocycle profile was 93^{o}C for 2 minutes followed by 15 cycles of 92^{o}C for 30 seconds, annealing temperature (T_A) for 45 seconds and 72^{o}C for

50 seconds. This was followed by an additional 20 cycles of 92^{o}C for 30 seconds, T_A for 45 seconds and 72^{o}C for 50 seconds and completed with a 3 minute extension at 72^{o}C for all loci except Sgui3, Sgui17 and GT6, which followed the protocol described in the original paper, and KW12, which used the profile described in Martien *et al.* (2012). Differences in annealing temperatures and concentrations of $MgCl_2$, and *Taq*, for each locus are outlined in Appendix Table III.1.

PCR products were coloaded in sets of non-overlapping loci and analyzed on a 3730 sequencer (Applied Biosystems) with formamide and 500 LIZ size standard ladder (Applied Biosystems). Following quality control guidelines (Morin *et al.* 2010b), four internal control samples were run to compare sizes across trays, a subset of randomly selected samples (5%) were repeated for all loci. Allele binning was manually checked before scoring loci. Samples with fewer than 10 of 15 loci were excluded from further analysis. An independent, experienced genotyper within the lab reviewed a subset of samples and loci. *MICROCHECKER* was used to investigate the presence of null alleles, dropout and stutter that may result from errors in allele binning (Van Oosterhout *et al.* 2006). Null alleles and dropout were confirmed using the program Dropout (McKelvey & Schwartz 2005). The program *GENEPOP v.3.4* (Raymond & Rousset 1995) was used to evaluate heterozygote deficiency, expected heterozygosity, the significance of deviation from Hardy-Weinberg Equilibrium (HWE) expectations, and test the independence of loci using linkage-disequilibrium analysis.

The program *CERVUS v3.0* (Kalinowski *et al.* 2007) was used to identify replicate samples and calculate the probability of identity (P_{ID}), or chance that a pair of randomly selected individuals will have matching genotypes. To avoid false exclusion due to potential dropout and genotyping errors (Waits *et al.* 2001), we initially used a relaxed criterion that allowed for mismatches at up to four loci. The electropherograms of the mismatching loci were then reviewed and either corrected based on visual inspection or repeated for confirmation. We required a minimum overlap of 11 matching loci to accept samples as being replicates from the same individual. A per-allele error rate was calculated from replicate genotypes identified with the initial relaxed matching and subsequent review of near matches (Pompanon *et al.* 2005), as well as the random samples that were rerun in the quality control process. Identified replicate samples were validated with photo-ID, mtDNA haplotype assignment and sex identification whenever possible.

3.3.5. mtDNA diversity and differentiation

Haplotype (h) and nucleotide (π) diversity for each island group and for all samples grouped by archipelago were calculated in *Arlequin v3.5* (Excoffier & Lischer 2010). A median-joining network of the unique haplotypes was constructed using *HapStar* (Teacher & Griffiths 2011). This construction begins by combining the minimum-spanning tree results available from the output file in *Arlequin* to construct a network relating the haplotypes (Figure 3.2).

Population differentiation was assessed in *Arlequin* with global tests followed by pairwise comparisons between ocean basins and between all pairs of island populations. The F_{ST} analogue Φ_{ST}, which accounts for the mutation process, was also calculated to determine the nucleotide diversity. *PERMUT* was used to test for the contribution of mutational divergence of haplotypes by comparing the difference between F_{ST} and Φ_{ST} (Petit 2010). To test for significant differences in haplotype diversity between geographic areas in the North Pacific (NWHI, Kauaʻi, Oʻahu, Hawaiʻi) and the South Pacific (Moʻorea, Raʻiatea and Samoan Islands), we used a permutation procedure scripted in *R*, (script available from A. Alexander GitHub). For this, random differences in haplotype diversity were calculated from 1,000 permutations and compared to the observed difference.

A hierarchical AMOVA procedure calculated standard variance components, and several haplotypic correlation measures were tested using a random permutation procedure in Arlequin (Excoffier *et al.* 1992). To conduct the hierarchical analyses, the seven populations (Figure 3.1, Table 3.1) were nested within two ocean basins, i.e. Hawaiian Islands populations in the North Pacific and Society and Samoan Islands populations in the South Pacific. Some levels could not be tested due to limited degrees of freedom.

3.3.6. Microsatellite diversity and differentiation

Observed and expected heterozygosities were calculated with *CERVUS v3.0*, and allelic richness with *FSTAT v2.9.3* (Goudet 2001). Regional differentiation in microsatellite

allele frequencies and the microsatellite specific analogue, F_{ST}, was estimated using *GenAlEx v6.5* (Peakall & Smouse 2012) with 10,000 permutations to assess significance. G''_{ST}, an analogue of F_{ST} that adjusts for the diversity of microsatellites, small sample size and small numbers of populations (Meirmans & Hedrick 2011), was calculated in *Genodive v2* (Meirmans & Van Tienderen 2004). An hierarchical AMOVA analysis between ocean basins and among populations within ocean basins described above was calculated using microsatellites (F_{ST} only) in HierFSTAT implemented through *R* (Goudet 2005) to examine the distribution of variation and differential connectivity between ocean basins. To identify possible kin sampled together we used the program *ML-RELATE* (Kalinowski *et al.* 2006) and tested each population separately. This program calculates the log-likelihood of four possible relations between individuals (unrelated, half-sibling, full-sibling and parent/offspring). We considered close kin to be three of the four (i.e. all but 'unrelated').

Population structure was also evaluated with a Bayesian clustering approach implemented in *STRUCTURE v2.3.1*. (Pritchard *et al.* 2000). For all *STRUCTURE* analyses six independent runs (iterations) were performed for each k (number of clusters). We used a burn-in of 100,000 and a run length of 1,000,000 with all other parameters left as program defaults. We used *STRUCTURE* to cluster the dataset into K=1-7 populations, with no prior information on sampling location, using an admixture model and correlated allele frequencies. We compared estimates of likelihood across runs to confirm convergence. The results were processed in *STRUCTURE HARVESTER v0.6.93* (Earl & vonHoldt 2012), and evaluated for different values of K. To determine

the most robust delineation of populations we evaluated where the ad hoc statistic, ΔK (Evanno *et al.* 2005), which estimates the rate of change in the log probability of data between successive K values. For comparison, this was then evaluated against the rate of change of the likelihood distribution (mean) calculated as L'(K). The value of K is detected when there is a sharp drop in the likelihood value or a peak in ΔK.

3.3.7. Sex-biased dispersal

Sex-biased dispersal was assessed between each island as well as among islands in an archipelago using the microsatellite dataset in *FSTAT v2.9.3* (Goudet *et al.* 2002, Goudet 2005). Sex-specific F_{ST}, inbreeding coefficient, $F_{IS,}$ mean assignment index (mAIc) and variance of mean corrected assignment index (vAIc) based on microsatellite genotypes were performed using two-tailed tests and 10,000 permutations of the resampling procedure. The dispersing sex is expected for have a lower F_{ST} value, but higher variance. For mtDNA, sex-specific F_{ST} values were calculated by coding the haplotypes as homozygotes and comparing the sexes for significant differences using the resampling procedure in *FSTAT* see Oremus *et al.* (2007).

3.3.8. Isolation by distance

Mantel tests were implemented in *GenAlEx* to assess the relationship between genetic differentiation and geographic distance. To generate the matrix for geographic distance we used the geographic coordinates of sample locations to measure the distance between

islands and archipelagos. To generate the matrix for genetic differentiation, pairwise F_{ST} values were used for both mtDNA and microsatellite data (Table 3.2).

3.4. RESULTS

3.4.1. Genotype matching and sex ratios

Skin samples of rough-toothed dolphins were collected between 2000-2012 in the main Hawaiian Islands, the NWHI, the Society Islands and the Samoan Islands. Of the 296 total samples chosen for the initial analysis, 271 met the quality control threshold by genotyping at a minimum of 10 loci of the 15 attempted (Table 1). The microsatellite loci were found to be moderately variable ranging from 3 to 14 alleles per locus (Appendix Table III.1; Appendix Table III.1). The program *MICRO-CHECKER* (Van Oosterhout *et al.* 2004) found little evidence of large-allele dropout or error due to stutter. We calculated an error rate of 2.5% per allele, most of which was due to a sizing adjustment for primers with and without an M-13 extensions for labeling (Pompanon *et al.* 2005). The relaxed matching allowed us to detect and correct much of this error. No loci showed evidence of linkage disequilibrium. There were three instances where loci deviated significantly from HWE; two for Kauaʻi (GT39 and MK9) and one for Hawaiʻi (MK9). Results were not sensitive to the inclusion of these loci, so they were retained. Using a minimum of 10 loci for each sample, the probability that two unrelated individuals share a similar genotype was very low ($P_{ID} = 1.4x10^{-15}$). From the initial relaxed matching and review of near-matches, four samples were found to have identical genotype profiles, and

also matched at haplotype and sex. All of these matches were within-island samples. These within-island matches were considered to be replicates and were removed from the dataset.

3.4.2. mtDNA diversity and differentiation

After removing four replicates, there were 274 individuals with high quality sequences for mtDNA analysis. Using 450 base pairs of the mtDNA control region we identified 30 variable sites to resolve 23 haplotypes. Of the 23 haplotypes, six were previously described from the Society Islands or Samoa (five by Oremus *et al.* 2012 and one by (Olavarria *et al.* 2004). The 17 new haplotypes were submitted to GenBank (Accession numbers xx-xxx), and regional haplotype frequencies were submitted to Dryad Digital Repository (http:xxxx). Six of the 17 new haplotypes (N, A, J, N, S, T, W) were unique to one island (Figure 3.2). Three of the four most common haplotypes (E, H and U) were shared with at least two other islands and between archipelagos in low frequencies (Figure 3.2). Interestingly, the most common haplotype at Mo‘orea (haplotype O) was not found in nearby Ra‘iatea. In fact, there were no shared haplotypes between Mo‘orea and Ra‘iatea (a distance of 190 km) compared to four shared haplotypes between Kaua‘i and Hawai‘i (a distance of 470 km). However, it should be noted that the sampling in the Hawaiian Islands has been much more extensive. Haplotype and nucleotide diversity were greatest in the Hawaiian archipelago followed by the Society Islands, with the lowest in the Samoan archipelago (Table 1). When the populations were compared separately, NWHI had the highest haplotype diversity, followed by Kaua‘i, O’ahu,

Hawai‘i, Mo‘orea, Ra‘iatea and Samoa (Table 1). Using the permutation test in R we found haplotype diversity differed significantly between the North Pacific (Hawaiian Islands) and the South Pacific (Society Islands and Samoan Islands), as well as between all populations except NWHI and O‘ahu (P values given in Appendix Table III.3 and III.4). Haplotype diversity was higher in the Hawaiian Islands than the other archipelagos.

The haplotype network showed no obvious phylogeographic structure for the central North and South Pacific Oceans (Figure 3.2). There is little geographic concordance for any archipelago. The NWHI haplotypes are widely distributed over the network. The network results agree with Oremus *et al.* (2012) where Society Island haplotypes were grouped in two different areas when compared to other haplotypes of the Pacific Ocean. The Society Island haplotypes have several steps between them with one exception (haplotype O and P), which represent a large portion of the sampled individuals in Mo'orea. Although these haplotypes represent a high frequency for Mo'orea, they were absent from other islands in the South Pacific, yet haplotype P was found in low frequencies for the NWHI and O'ahu. A haplotype found in the Society Islands and Samoa (haplotype V) is found throughout the Pacific (i.e. Japan, Taiwan, Eastern Tropical Pacific, see Chapter 2). The Samoan Islands shared haplotypes with both the Hawaiian and Society Islands, but did not have any private haplotypes. There were several private haplotypes in other areas, four in Hawai‘i and four in Mo‘orea. However, only one of these haplotypes (haplotype O at Mo‘orea) was found in high frequencies (71%).

As reflected in the network, there were marked differences in haplotype frequencies among the three archipelagos (global F_{ST} = 0.334, p-value < 0.001). Pairwise tests of differentiation showed highly significant pairwise differences between all islands except among NWHI, Oʻahu and Kauaʻi (Table 3.2). Significant differentiation was also found between archipelagos. The differences, however, were not necessarily greater between islands in different archipelagos than between islands within an archipelago, despite the large geographic distance between archipelagos. F_{ST} and Φ_{ST} values were similar and no significant differences between the two indices were found using *PERMUT* (Table 3.2). When the populations were pooled into the archipelagos, significant genetic differentiation was found for all pairwise comparisons for both mtDNA and microsatellites (Table 3.3).

The hierarchical AMOVA showed only 5.2% of the haplotype diversity was explained by the ocean basin level while 29.4% was explained by populations within ocean basins (Table 3.4). The permutation procedure showed the partitioning of variance among populations (F_{ST} = 0.346) and among populations within ocean basins (F_{CT} = 0.310) were highly significant. Limitations on the degrees of freedom at the ocean basin level of our analysis prevented a test of this variance, but a non-hierarchical analysis where populations were pooled by archipelago and ocean basin showed this was significant (F_{ST} = 0.299, $p<0.001$).

3.4.3. Microsatellite diversity and differentiation

For microsatellite data, levels of diversity were similar for each island with an average observed heterozygosity of 0.651 (Table 3.1). Summaries for diversity for each marker are presented in Appendix Table III.1. Significant genetic differentiation was found globally (F_{ST} = 0.043, p-value < 0.01) and between all island pairs (albeit lower overall values than mtDNA data) with the exception of the NWHI, Kauaʻi and Oʻahu (Table 3.2), as well as between archipelagos when populations were pooled (Table 3.3). In the hierarchical AMOVA, the total explained differentiation was 7.6% of which 2.6% was due to between-ocean basin differentiation and 5.0% was due to among-populations differentiation (Table 3.5).

Shared alleles between some individuals suggested close kinship in the sample. The program *ML-RELATE* identified possible kin (nine in Society Islands and four in the Hawaiian Islands). Although we consider kin sampled in the same group a reflection of the true population structure of dolphins (see Chapter 4), we wanted to confirm that sampling was unbiased (i.e. we didn't specifically sample related individuals). Consequently, we excluded these individuals and reran the population level analyses. The exclusion of these individuals did not change the results, and all individuals were retained in the dataset.

When all samples were included with no prior information on sampling location in the *STRUCTURE* analysis, the model with the highest log-likelihood was K=2. A graphical

representation of membership showed clear delineation for the North Pacific's Hawaiian Islands, and the South Pacific's Society Islands (Figure 3.3). However, the Samoan Islands had five individuals that clustered with North Pacific samples (Figure 3.3a). These individuals were sampled during the same encounter about 10km offshore from the island of Tutuila, American Samoa. Two of those individuals have haplotypes that are found in high frequencies in Hawai‘i and low frequencies in Kaua‘i and NWHI (haplotype H). One individual has haplotype M, found in low frequencies only in Hawai‘i. The other two have a haplotype found in all archipelagos (haplotype E), but most common in the South Pacific. All other Samoa samples clustered with South Pacific individuals, possessed haplotype E, and were collected within 2km of shore.

When only Kaua‘i and Hawai‘i were assessed in *STRUCTURE* without prior information of sampling location, and excluding the NWHI and O‘ahu, the highest likelihood was K = 2. Individuals were occasionally counter-assigned (Figure 3.3b). The cluster within Kaua‘i that looked to have ancestry or identity from Hawai‘i (illustrated with the arrows, Figure 3.3b) was made up of dolphins that were sampled together in one encounter. However, these individuals had been sighted in Kaua‘i previously, and did assign back to the main cluster in Kaua‘i in the photo-ID social network (R. Baird unpublished).

3.4.4. Sex-biased dispersal

There was no pattern of sex-biased dispersal identified among rough-toothed dolphins. Although mtDNA and microsatellite F_{ST} values were slightly higher for females, these differences were not significant between sexes (Table 3.6).

3.4.5. Isolation by distance

Mantel tests of isolation by distance conducted using pairwise comparisons of islands showed no correlation between pairwise F_{ST} values (Appendix Table III.2) and increasing geographic distance between islands. Isolation by distance was not significant for either Φ_{ST} or F_{ST} in the mtDNA data (R^2=0.004, p=0.13; R^2=0.018, p=0.08 respectively), or for the microsatellite data (R^2=0.007, p=0.16). These results allowed us to reject Wright's stepping stone model as the best explanation of the genetic differentiation (i.e. genetic differentiation increases with increasing geographic distance).

3.5. DISCUSSION

Our results demonstrate a high degree of insular population structure for three archipelagos in the Central Pacific for rough-toothed dolphins despite the typically oceanic deep-water habitat of this species. These results support a growing body of evidence that otherwise pelagic species form isolated insular populations in areas with increased local productivity. Significant hierarchical partitioning of genetic variation was found between the North Pacific and South Pacific Ocean basins. We found significant genetic differentiation in both mtDNA and microsatellite analyses between all pairs of islands with the exception of the NWHI, Kauaʻi and Oʻahu. Our findings extend and support conclusions from less extensive previous genetic and photo-ID studies on rough-toothed dolphins around oceanic islands in both the Pacific (Baird *et al.* 2008, Johnston *et*

al. 2008, Oremus *et al.* 2012) and the Atlantic Oceans (Mayr & Ritter 2005, Kuczaj & Yeater 2007). Together with other studies, the pattern we observe is consistent with island colonization and occasional ongoing gene flow with pelagic populations.

3.5.1. Gene flow throughout the Central Pacific

The significant genetic differentiation between islands within archipelagos and the low genetic diversity are similar to other dolphin populations that are considered insular (Oremus *et al.* 2007, Andrews *et al.* 2010, Martien *et al.* 2012, Courbis *et al.* 2014). However, our results suggest that the genetic differentiation is most likely evidence of genetic drift. Although the genetic and photo-ID data support the existence of demographically independent populations around Kauaʻi and Hawaiʻi, between Moʻorea and Raʻiatea in the Society Islands, and the Samoan Islands, these populations could still experience low levels of gene flow from conspecifics throughout the Pacific Ocean. We found no evidence of isolation by distance, with F_{ST} values between archipelagos similar to values within archipelagos.

The pattern we observed with rough-toothed dolphins among the archipelagos in this study is consistent with a larger-scale study of common bottlenose dolphin *(Tursiops truncatus)* population structure in the Central Pacific. For this species populations were also connected by low levels of gene flow across large distances, either through occasional long-distance dispersal or gene flow with pelagic populations (Tezanos-Pinto *et al.* 2008). The shared haplotypes in our study among all three archipelagos (n=1) and

between ocean basins (n=4) indicate a genetic connection between the northern and southern hemispheres or a recent isolation. Moreover, the haplotypes that are found in both hemispheres were also found in Japan, Taiwan and the Eastern Tropical Pacific. However, it is not clear if this represents current or historic gene flow. As suggested by Andrews *et al.* (2010) for spinner dolphins *Stenella longirostris*, such shared haplotypes may be remnant haplotypes from an ancestral population that colonized all three archipelagos, or they may have originated after colonization from a pelagic population that has occasional gene flow with the insular populations. Similarly, the large number of undetected intermediate haplotypes shown in the network between Kauaʻi and Hawaiʻi could be the result of unsampled "offshore" pelagic populations that have occasional influxes of gene flow into the insular populations or the result of an occasional long distance migration from another insular population.

3.5.2. Local productivity and population structure

In the MHI the island mass effect is responsible for the highly productive areas directly surrounding each island where runoff provides enhanced productivity nearshore, but then drops off sharply. This phenomenon creates a pattern of strong population differentiation between islands for many insular species spanning trophic levels from limpets to dolphins (Polovina *et al.* 1994, Bird *et al.* 2007, Martien *et al.* 2012). The island of Hawaiʻi has additional oceanographic mechanisms of isolation. The tall mountains on Maui (Haleakalā) and Hawaiʻi (Mauna Kea) funnel the wind between them creating an exceptionally strong current and one of the most treacherous channels in the world.

Moreover, the area around 4-islands area of Maui, Molokai, Lanea and Kahoʻolawe (the neighboring islands to Hawaiʻi) is surrounded by relatively shallow water, which could act as a barrier for deep-water species. Indeed, Baird *et al.* (2008) noted sightings of rough-toothed dolphins in this area have been rare (Baird *et al.* 2008, Baird *et al.* 2013). In addition, the leeward side is an active area of eddy generation, and these productive areas have been linked to the frequent occurrence of large mesopelagic predators (Holland & Mitchum 2001, Seki *et al.* 2001) making the need to travel to other islands for foraging unnecessary.

In contrast, the NWHI experience a mixing zone known as the Transition Zone Chlorophyll Front resulting in much higher average productivity in offshore waters (Schmelzer 2000). This reduces the habitat differences between nearshore and offshore waters in that part of the archipelago. Although there are several islands in the NWHI, each island is much smaller than the islands in the MHI. Lacking the large mass and tall mountains, the island mass effect and offshore eddies are not as prevalent in the NWHI, and the productivity here is substantially less (Bowen *et al.* 2013). It has been hypothesized that less productive areas require larger habitat ranges for efficient foraging due to unpredictability of prey (Silva *et al.* 2008). Spinner dolphins in the NWHI were found to have lower levels of genetic differentiation and higher group stability, which the authors attributed to a lower density of prey (Andrews *et al.* 2010). False killer whales in the NWHI showed lower levels of population structure than in the MHI, and this was also attributed to lower productivity in the NWHI (Martien *et al.* 2014). No significant differentiation among Kauaʻi, Oʻahu and the NWHI in our study suggests some level of

gene flow between these islands. Telemetry data of NWHI false killer whales indicates the population ranges from the NWHI to Kauaʻi (Baird *et al.* 2013). For rough-toothed dolphins, Baird *et al.* (2008) found lower site fidelity around Kauaʻi and suggested a larger home range as an explanation. Rough-toothed dolphins in the NWHI may be required to travel longer distances for sufficient foraging, thus causing their home range to overlap periodically with those in the MHI, and subsequently, occasional gene flow would occur between these areas. However, more samples from the NWHI as well as information on habitat use are needed to confirm this.

In the Society Islands and the MHI, rough-toothed dolphins are most commonly found in depths greater than 1,000 meters (Gannier 2000, Gannier & West 2005, Baird *et al.* 2008, Oremus *et al.* 2012, Baird *et al.* 2013). Due to the steep slope of these oceanic islands, the distance from shore at these depths is generally within a few kilometers. Similar to the MHI, the Society Islands experience increased productivity nearshore and a drop in productivity as the distance from shore increases (Longhurst 1999, Mannocci *et al.* 2014). So it is not surprising that our study and Oremus *et al.* (2012) found genetic differentiation between the islands of Moʼorea and Raʻiatea in the Society Islands. However, rough-toothed dolphins have also been observed tens of kilometers offshore from the Society Islands (Nekoba-Dutertre *et al.* 1999, Gannier 2000, Mannocci *et al.* 2014). It is possible that the individuals observed were part of a larger, pelagic population. If so, and these individuals provided occasional gene flow between the Leeward (Raiatea) and Windward islands (Moʼorea /Tahiti) we would expect the levels of differentiation between the two islands would be lower. Additional sampling

throughout the Society Islands would help to determine if there are separate offshore populations, or if those dolphins sighted were part of a nomadic population. The possibility of offshore populations was also apparent with Samoas samples. The *STRUCTURE* analysis identified a few individuals from Samoa as 'Hawaiian'. The field notes showed these individuals were sampled further offshore as part of a large vessel survey (SWFSC Pers. Comm.). This could be a difference in nearshore vs offshore dolphins around Samoa or evidence of long-distance dispersal.

3.5.3. Social organization and foraging differences

Genetic drift, and not isolation by distance, appears to be the mechanism driving the insular communities of rough-toothed dolphins. Our results showed no clear correlation between geographic distance and genetic differentiation among the islands or archipelagos. This suggests independent colonization events, followed by rapid drift in small populations. Prey specialization and social organization would presumably only enhance this through cultural adaptation supported by fidelity to social groups. Socially-defined population structure has the general effect of increasing the importance of genetic drift relative to other evolutionary forces (Storz 1999). Differences in specializations such as foraging strategies and habitat use have been suggested as causes for genetic differentiation of populations observed in coastal bottlenose dolphins (Moller 2012), as well as pelagic species such as killer whales *Orcinus orca,* (Hoelzel *et al.* 2007, Pilot *et al.* 2010), false killer whales *Pseudorca crassidens* (Martien *et al.* 2014) and striped dolphins *Stenella coeruleoalba,* (Gaspari *et al.* 2007). Rough-toothed dolphins exhibit

characteristics such as care giving and cooperative foraging common in other delphinids with a highly organized social structure and cultural specialization (Lodi 1992, Pitman & Stinchcomb 2002, Fulgencio de Moura *et al.* 2009). To date, however, there is no evidence available to assess whether there are differences in foraging specialization at any of the islands. Further studies on foraging, diving patterns and habitat use could help determine the extent of social organization drives specialization and influences genetic isolation in rough-toothed dolphins.

3.5.4. Conservation Implications

This comparative study of rough-toothed dolphins in the central Pacific provides evidence of insular populations of dolphins in three archipelagos. The presence of island-associated populations in pelagic species has important conservation implications due to concentrated human impacts near shore. Fishing depredation by rough-toothed dolphins is increasing in the main Hawaiian Islands and the Society Islands (Poole 1993, Baird *et al.* 2008). Around Hawai‘i, rough-toothed dolphins steal fish off lines in local fisheries and it has been reported that fishermen shoot dolphins as a method of deterrent (Kuljis 1983, Nitta & Henderson 1993). Although there has been no confirmed documentation of shootings by fishermen in the Society Islands, reports from local fishermen at Moorea and Tahiti indicate negative interactions are increasing between rough-toothed dolphins and local fisheries and that some fishermen have resorted to shooting dolphins (Poole unpublished). In the Samoan archipelago there were two bycatch incidences (2011 and 2012), and evidence of entanglement from a beach cast animal in 2010 (J. Ward pers.

comm). These examples provide evidence that insular rough-toothed dolphins are likely to be more vulnerable to anthropogenic impacts than their pelagic conspecifics.

3.6. Acknowledgements

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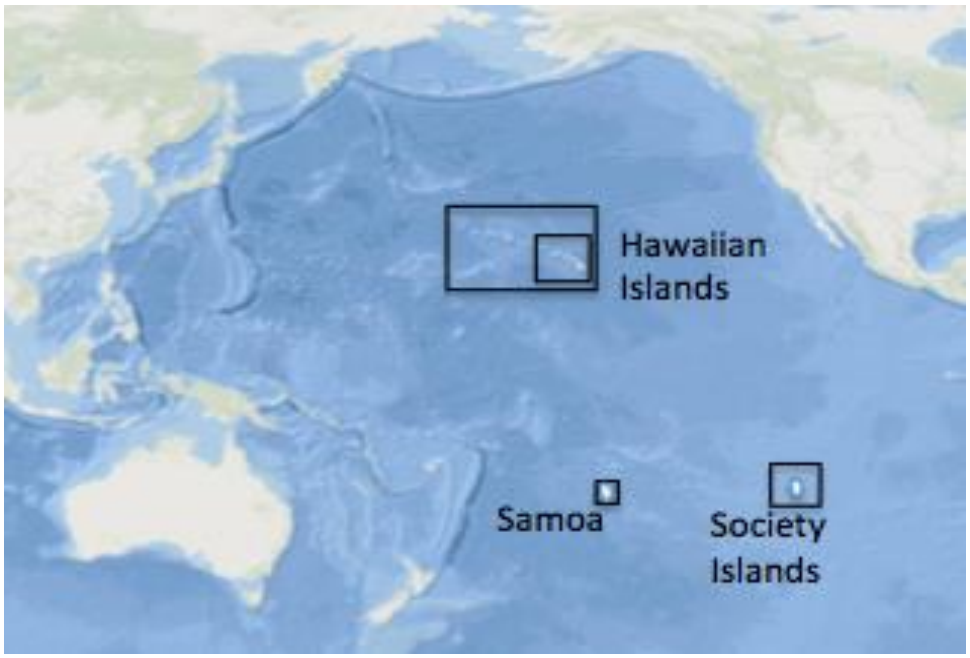
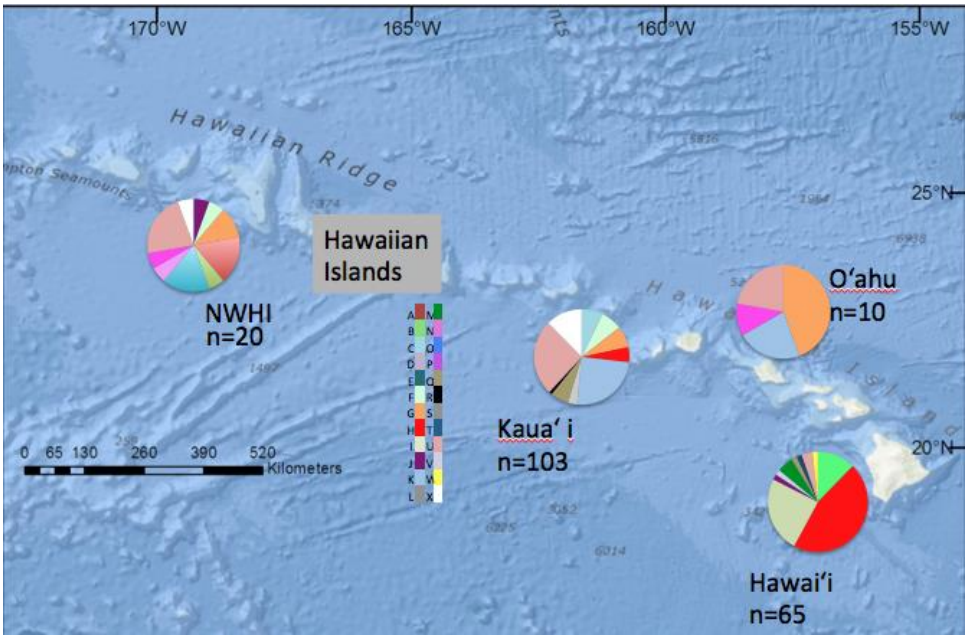
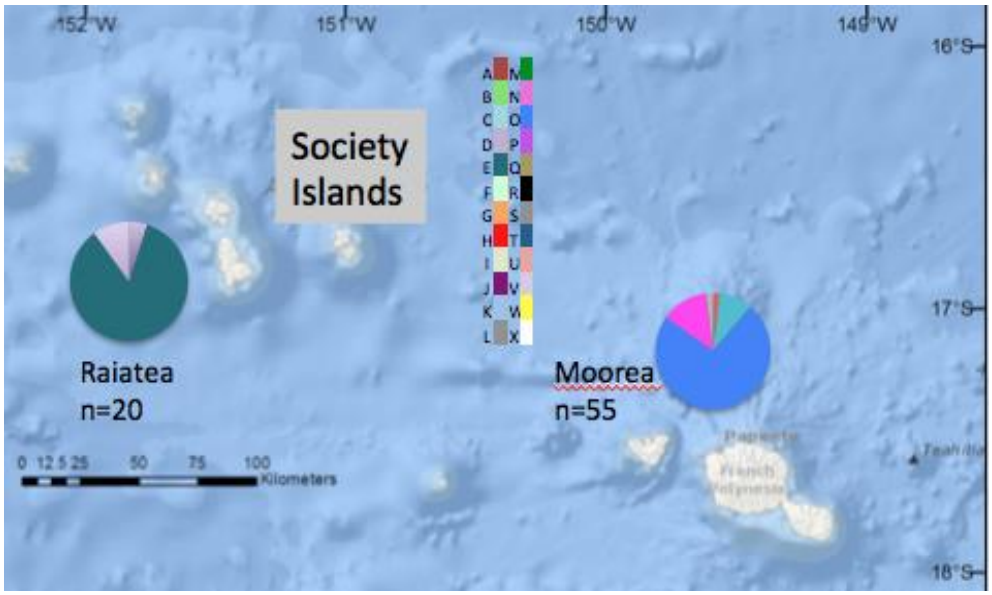


a) Pacific Ocean

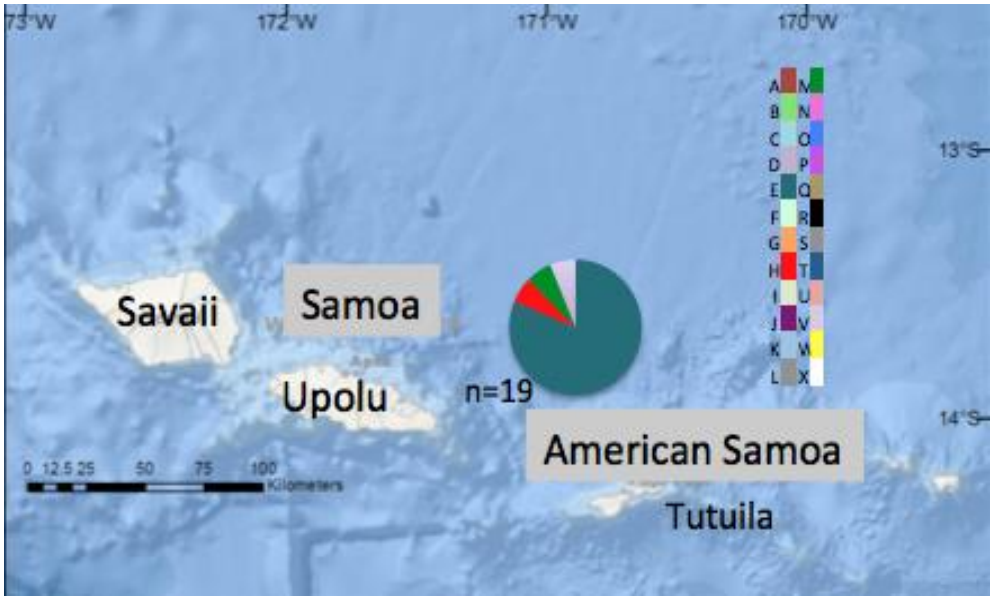


b) Hawaiian Islands

Figure. 3.1: Sampling locations (with enlargements) for rough-toothed dolphins, showing frequencies of mtDNA haplotypes. Pie charts reflect mtDNA haplotype frequencies for each island(s). (a) Pacific Ocean map showing locations where samples were collected. The large box for the Hawaiian Islands includes the Northwest Hawaiian Islands. The smaller box includes the main Hawaiian Islands only (b) Hawaiian Islands (c) Society Islands (d) Samoan archipelago.



c) Society Islands of French Polynesia



d) Samoan Islands

Figure. 3.1: Sampling locations (with enlargements) for rough-toothed dolphins, showing frequencies of mtDNA haplotypes. Pie charts reflect mtDNA haplotype frequencies for each island(s). (a) Pacific Ocean map showing locations where samples were collected. The large box for the Hawaiian Islands includes the Northwest Hawaiian Islands. The smaller box includes the main Hawaiian Islands only (b) Hawaiian Islands (c) Society Islands (d) Samoan archipelago.

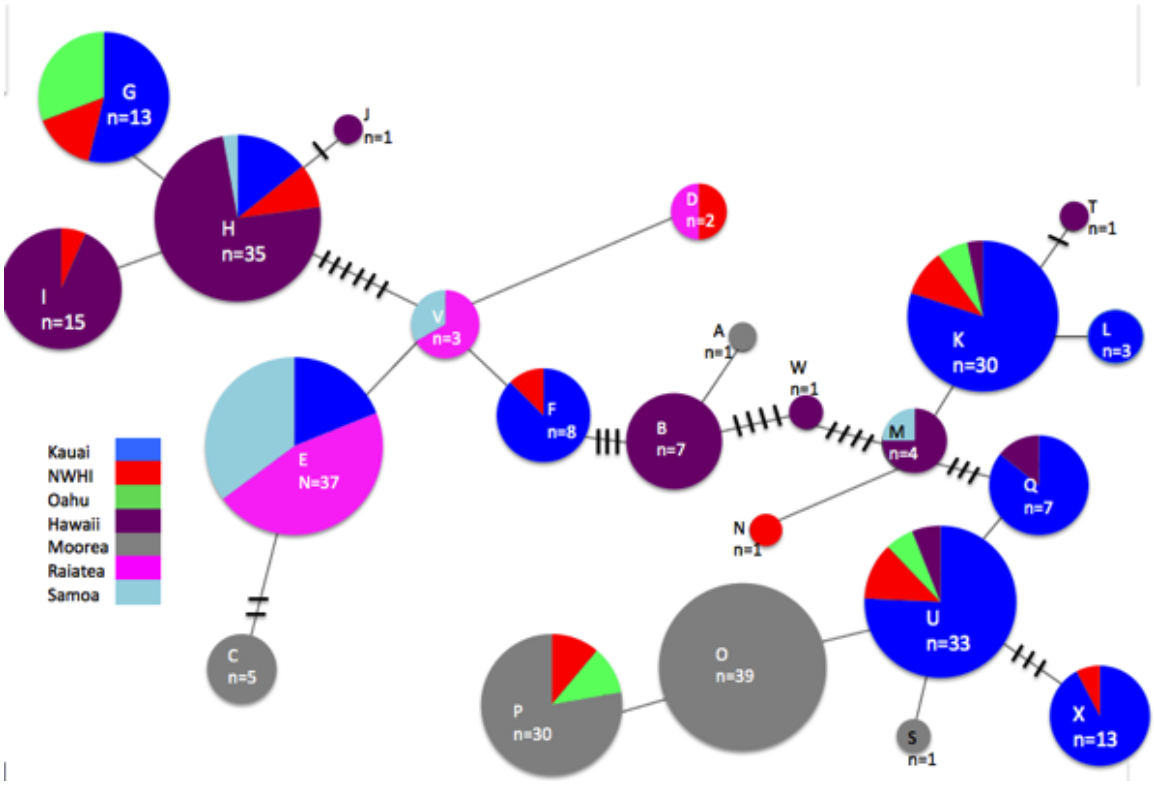
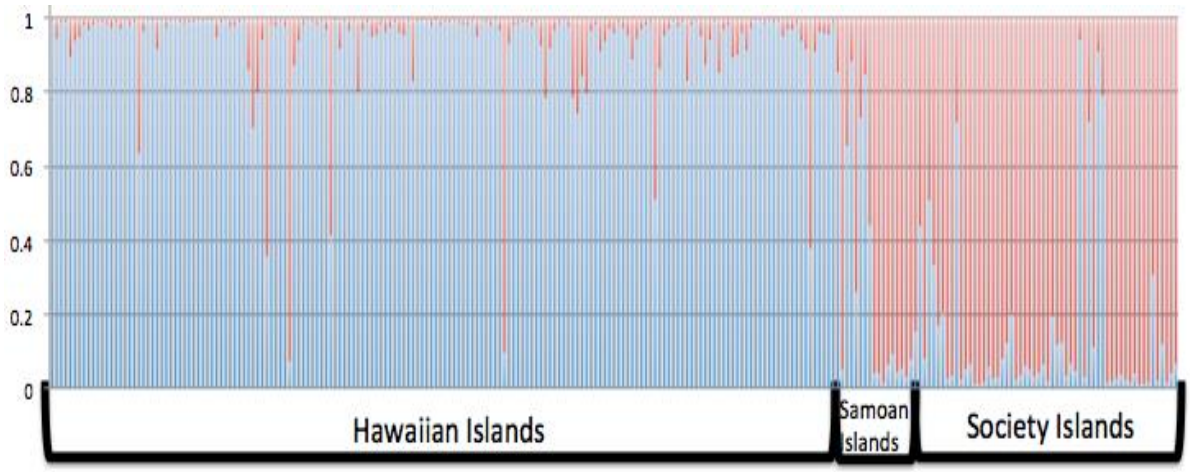
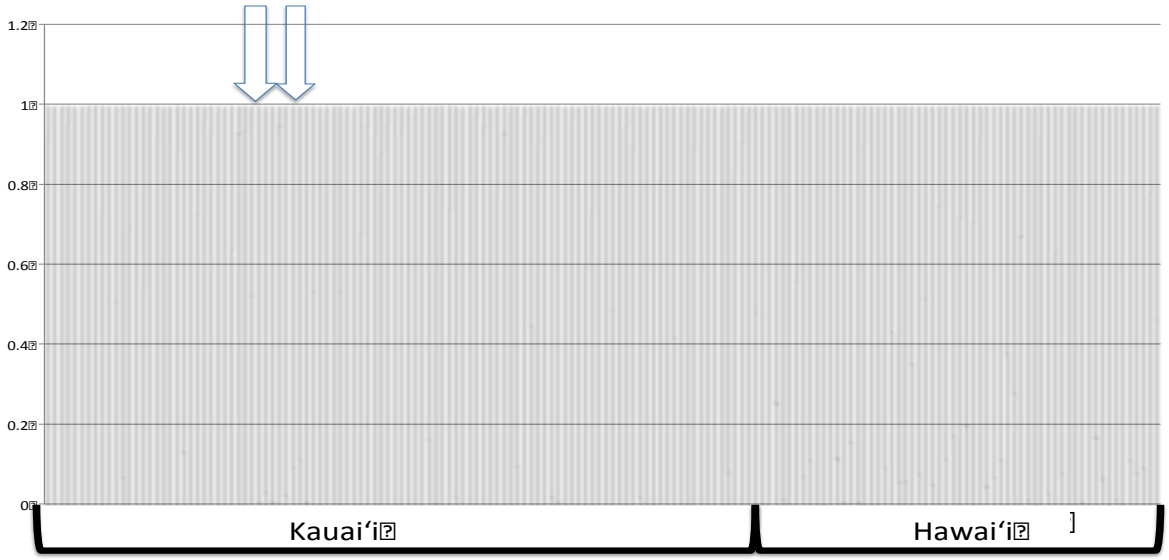


Figure. 3.2: Median-joining network of mtDNA control region 450 bp haplotypes found in rough-toothed dolphins from the Pacific Ocean. Each circle represents a unique haplotype and each line connecting the haplotypes indicates a single base pair change. Small black dashed lines represent an additional substitution. The size of each circle is proportional to the sample size, and each is colored according to the region(s) where the haplotype was sampled.



a)



b)

Figure. 3.3: Graphical representation of the results of the STRUCTURE analysis for the a) archipelagos where the most probable model was k=2 and a prior was used for sampling location. b) Island populations of Hawaiʻi and Kauaʻi where the most probable model was k=2 and no prior was used for sampling location. Each vertical bar represents an individual. Bars are shaded as to the proportion of the individual's ancestry that is attributable to groups one (blue) and two (red) as defined by STRUCTURE. Arrows point to individuals who did not assign correctly that were sampled together during an encounter.

Table 3.1: Oceanic and island samples sizes (n) for mtDNA in the Hawaiian, Society and Samoan Islands. For mtDNA, number of haplotypes (*k*), haplotype diversity (*h*) and nucleotide diversity (in %, π) presented, with standard deviations calculated in ***Arlequin***. For microsatellites numbers of individuals are given by sex (*F, M*) and total sample size (*n)*, K is the mean number of alleles per locus across 15 loci.

		mtDNA				Microsatellites						
	Geographic area	*n*	k	*h (s.d.)*	π % (*s.d.*)	F	M	*n*	K	Allelic Richness	H_O	F_{IS}
North Pacific	NWHI	18	10	0.912 (0.059)	1.64(0.93)	8	6	14	12.5	4.3	0.836	0.010
	Kauaʻi	96	9	0.798 (0.0168)	1.39(0.75)	58	43	101	8.75	3.7	0.855	-0.068
	Oʻahu	10	4	0.711 (0.086)	1.38(0.81)	7	3	10	7.3	4.4	0.735	0.079
	Hawaiʻi	57	9	0.623 (0.046)	1.13(0.62)	36	19	55	7.61	4.6	0.802	0.096
	Hawaiian Islands Total	**181**	**18**	**0.880 (0.0093)**	**1.61(0.85)**	**109**	**71**	**180**	**9.04**	**4.3**	0.810	0.065
South Pacific	Moʻorea	55	5	0.409 (0.081)	0.501(0.31)	29	24	55	7.65	4.9	0.691	0.045
	Raʻiatea	20	3	0.189 (0.108)	0.42(0.06)	14	3	17	4.37	3.9	0.723	-0.004
	Society Islands Total	**75**	**8**	**0.477 (0.093)**	**0.510(0.19)**	**43**	**27**	**72**	**6.01**	**4.5**	0.711	0.012
	Samoan Islands Total	**18**	**4**	**0.350 (0.148)**	**0.451(0.30)**	**12**	**5**	**18**	**4.31**	**3.8**	0.678	-0.001
	Total all Islands	**274**	**23**	**0.665**	**1.12(0.43)**	**166**	**105**	**271**	**5.81**	**4.01**	**0.685**	**0.057**

Table 3.2: Pairwise F_{ST} values for mtDNA and microsatellites between seven populations in the Hawaiian, Society and Samoan Islands for 15 microsatellite loci above diagonal and mtDNA control region below diagonal. Φ_{ST} and G''_{ST} values in Appendix Table III.2. Statistically significant F_{ST} values are bolded and italicized, with * significant at $p < 0.05$; ** significant at $p < 0.001$.

	***n* mtDNA**	**Kaua'i**	**NWHI**	**O'ahu**	**Hawai'i**	**Mo'orea**	**Ra'iatea**	**Samoan Islands**
***n* microsatellite**		101	14	10	55	55	17	18
Kaua'i	96		0.023	0.013	***0.035*** **	***0.039*** **	***0.077*** **	***0.057*** **
NWHI	18	0.002		0.015	***0.047*** **	***0.062*** **	***0.073*** **	***0.041*** **
O'ahu	10	0.044	0.012		***0.094*** **	***0.109*** **	***0.100*** **	***0.102*** **
Hawai'i	57	***0.186*** **	***0.142*** **	***0.168*** **		***0.058*** **	***0.047*** *	***0.021*** **
Mo'orea	55	***0.350*** **	***0.307*** **	***0.498*** **	***0.429*** **		***0.079*** **	***0.048*** **
Ra'iatea	20	***0.393*** **	***0.404*** **	***0.511*** **	***0.441*** **	***0.499*** **		***0.091*** **
Samoan Islands	18	***0.301*** **	***0.315*** **	***0.495*** **	***0.396*** **	***0.410*** **	***0.334*** **	

Table 3.3: Pairwise F_{ST} differentiation among the three archipelagos with islands pooled into the corresponding archipelago for 15 microsatellite loci above diagonal and mtDNA control region below diagonal with corresponding (Φ_{ST} and G''_{ST} values). Statistically significant F_{ST}, Φ_{ST} and G''_{ST} values are bolded and italicized, with * significant at $p < 0.05$; ** significant at $p < 0.001$.

	Hawaiian Islands	**Society Islands**	**Samoan Islands**
Hawaiian Islands		***0.035*** ** ***(0.112**)***	***0.043*** ** ***(0.159**)***
Society Islands	***0.232*** ** ***(0.182**)***		***0.087*** ** ***(0.114**)***
Samoan Islands	***0.258*** ** ***(0.282**)***	***0.418*** ** ***(0.338**)***	

Table 3.4: Hierarchical AMOVA based on 450 bp of the mtDNA control region and microsatellites in the Pacific Ocean. Ocean basins delineates North Pacific and South Pacific; Populations represents the 7 populations from Fig. 1/Table 1. P-value estimates are based on 10,000 permutations across the full data set. df = Degrees of Freedom.

mtDNA hierarchical AMOVA Analysis	df	% of total variation	F-statistic	P-value
Among Ocean basins	1	5.2%	CT=0.052	n.a.
AmongPopulations/ Ocean basins	5	29.4%	SC= 0.310	0.001
Within Populations	271	65.4%	ST=0.346	0.001

Table 3.5: Hierarchical AMOVA based on 15 microsatellite loci in the Pacific Ocean. Ocean basins delineates North Pacific and South Pacific; Populations represents the 7 populations from Fig. 1/Table 1. df = Degrees of Freedom. P-value estimates are based on 10,000 permutations across the full data set.

Microsatellite loci AMOVA analysis	df	% of total variation	F-statistic	P-value
Among Ocean basins	1	2.6%	CT= 0.028	n.a.
Among populations/ Ocean basins	5	5.0%	SC =0.052	0.001
Within populations	268	92.4%	ST= 0.076	0.001

Table 3.6: Two-tailed tests of sex-biased dispersal based on the comparison of sex-specific microsatellite (msat) F_{ST}, mtDNA F_{ST}, inbreeding coefficient (F_{IS}), mean corrected assignment index (mAIc), and variance of corrected assignment index (vAIc). The mtDNA sample size (n) is indicated in parentheses if it differed from the microsatellite sample size.

	Female	**Male**	**P**
North Pacific (4 populations)			
n	111	73 (70)	
F_{ST} mstat	0.027	0.016	0.420
F_{ST} mtDNA	0.137	0.109	0.222
F_{IS}	0.051	0.090	0.240
mAIc	-0.088	0.057	0.320
vAIc	14.54	22.94	0.110
South Pacific (3 populations)			
n	55 (58)	32 (35)	
F_{ST} mstat	0.084	0.070	0.434
F_{ST} mtDNA	0.152	0.123	0.255
F_{IS}	0.002	0.001	0.752
mAIc	0.498	-0.287	0.100
vAIc	17.03	10.32	0.192
Overall (7 populations)			
n	166 (170)	105(104)	
F_{ST} mstat	0.050	0.044	0.615
F_{ST} mtDNA	0.144	0.116	0.512
F_{IS}	0.042	0.061	0.441
mAIc	0.103	-0.064	0.773
vAIc	15.42	18.53	0.412

4.Are you my mother? A measure of matrilineal social organization in wild-ranging groups and mass strandings of rough-toothed dolphins (*Steno bredanensis*)

4.1. Abstract

Group structure has potential consequences for both evolutionary processes and conservation genetics as it can influence the rate at which genetic diversity is lost. Rough-toothed dolphins, *Steno bredanensis*, are reported to form stable social groups within isolated populations around some oceanic islands. Within these groups, rough-toothed dolphins exhibit cooperative foraging and care-giving behavior, including the sharing of large prey. Similar social characteristics are found in other delphinid species (e.g. killer whales), where social groups are comprised of maternally-related individuals forming "strict matrilines" as reflected by a single mitochondrial (mt) DNA haplotype. Here, we test the hypothesis that rough-toothed dolphins form "strict matrilines", by identifying maternal lineages in groups of rough-toothed dolphins in both the Pacific and Atlantic Oceans. We used mtDNA control region haplotypes as markers for maternal lineages and infer kinship within groups using 15 microsatellite markers. The collection includes 186 biopsy samples from 47 groups at sea and two fisheries bycatch groups, as well as 26 teeth samples taken from six groups from either mass strandings or bycatch. The number of samples per group ranged from 2 to 90. Our results revealed multiple matrilines in 33 of the 55 groups, illustrated by multiple mtDNA haplotypes, including 29 from groups at sea, two from mass strandings and two from bycatch groups. Following the rejection of the hypothesis of 'strict matrilineal structure', we assessed how rough-toothed dolphins partition genetic diversity by assessing the level of matrilineality. We calculated a matrilineal index based on dyad relationships within groups. Our index indicated that rough-toothed dolphin groups display "weak matrilineality", with most

groups slightly more matrilineal than expected by chance, and some significant differences overall, when accounting for regional differences in haplotype frequencies. Fine-scale analyses of kinship of wild-ranging groups revealed relatedness on the order of no relation to first cousins (R = 0.007-0.146) with four groups showing significantly greater relatedness than expected by chance. Our results suggest stable groups of rough-toothed dolphins are not necessarily kin based or matrilineal. This study provides a unique insight into group structure and social kin associations of a little studied, pelagic species.

4.2 Introduction

Dolphins (Family Delphinidae) are one of the most highly social taxa among all mammals (Mann *et al.* 2000, Moller 2012). However, the extent of relatedness and stability of groups varies. In coastal waters, bottlenose dolphins *Tursiops truncatus*, show philopatry where females develop moderate social bonds with related females in small groups. In comparison, males form alliances that are generally not related, but are stable and cooperate in gaining access to females for mating (Moller *et al.* 2001, Krützen *et al.* 2004, Moller 2012). In general, pelagic dolphins are found in larger and more fluid groups than coastal dolphins. Oceanic bottlenose dolphins in the Northeast Atlantic maintain high levels of gene flow (Quérouil *et al.* 2007). This is thought to be related to resources being relatively sparse over a large area, forcing dolphins into larger home ranges and less stable associations (Gowans *et al.* 2007). In the Mediterranean however, striped dolphins *Stenella coeruleoalba*, display genetic structure and relatedness in groups, as shown by higher than average kinship for females within groups, and higher than average kinship for smaller rather than larger groups (Gaspari *et al.* 2007).

Moreover, pelagic dolphins around oceanic islands are known to form small, but stable groups, with high site fidelity (Chivers *et al.* 2007, Baird *et al.* 2012). Larger delphinids, such as long finned pilot whales *Globicephala melas*, exhibit strong social bonds, but can be found in groups made up of multiple matrilines (Amos *et al.* 1993, Pilot *et al.* 2010, Oremus *et al.* 2013), while killer whales *Orcinus orca*, show philopatry of both sexes and live in strictly matrilineal societies their entire lives (Pilot *et al.* 2010, Foote *et al.* 2011a, Oremus *et al.* 2013).

Social structure has many important implications for both evolutionary processes and conservation genetics. Fine-scale population structure can be shaped by patterns of oceanographic processes, prey availability and social structure, including nonrandom mating and group associations. Social structure within populations suggests that mating is non- random, and therefore social organization can influence the rate at which genetic diversity is lost from natural populations (Archie *et al.* 2008) as well as impact processes of local adaptation (Storz 1999). The degree to which matrilineality or relatedness affects population structure can have important conservation implications. Species such as killer whales, sperm whales and elephants have stable groups that are maternally related. These groups are afforded more protection in many cases since social structure can restrict diversity, (Archie *et al.* 2008, Morin *et al.* 2010a) possibly affecting a population's ability to adapt to anthropogenic pressures. However, groups can also be stable without being related. Chimpanzee populations, for example, are composed of stable, cooperative groups that participate in similar behaviors, but these groups are not strictly matrilineal

(Goldberg & Wrangham 1997). Therefore, determining the degree of relatedness in populations can aid in prioritizing conservation measures.

Rough-toothed dolphins are a pelagic delphinid known to form small groups within insular populations around oceanic islands (Mayr & Ritter 2005, Baird *et al.* 2008, Oremus *et al.* 2012) and are the third most likely dolphin species to strand in the Atlantic Ocean (NOAA 2014). Rough-toothed dolphins show genetic differentiation between islands in the main Hawaiian Islands and the Society Islands of French Polynesia (Albertson *et al.* 2011, Oremus *et al.* 2012) suggesting habitat specialization. Within some of these island populations as well as others in the Caribbean and Canary Islands, photo-identification studies (photo-ID) have shown evidence of long-term associations (several years) between individuals (Ritter 2002, Kuczaj & Yeater 2007, Baird *et al.* 2008, Oremus *et al.* 2012). In the Caribbean, 12 individuals were resighted together on four different occasions (Kuczaj & Yeater 2007), suggesting stable group associations. According to previous studies in the Pacific Ocean, mean group size of rough-toothed dolphins generally ranges from 6-15 individuals (Oremus *et al.* 2012, Baird *et al.* 2008). A mean group size of 10 has been found in the Atlantic Ocean (West *et al.* 2011).

However, all groups are not stable. Although uncommon, "super groups" have been observed in the Pacific Ocean where groups contain at least 40, and up to 105 individuals (Baird *et al.* 2008). A maximum group size of 50 and 30 individuals has been reported in the Canary Islands (Ritter 2002) and the Caribbean (Kuczaj & Yeater 2007) respectively. The largest group recorded for rough-toothed dolphins of 160 was observed in the

Mediterranean Sea (Watkins *et al.* 1987). This "super group" consisted of eight subgroups of about 20 dolphins each and included all age categories. Super groups have also been observed in the Pacific Ocean. Baird *et al.* (2008) reported super groups ranging in size from 65 to 90 individuals around the island of Kauaʻi, Oʻahu and the island of Hawaiʻi. On every occasion each super group was composed of smaller subgroups. This same super group/small subgroup pattern was also observed in large groups of long-finned pilot whales (Oremus *et al.* 2013).

Stability of groups is evident in strictly matrilineal species (e.g. killer whales). Rough-toothed dolphins also exhibit social characteristics similar to species that form long-term bonds with matrilineal social structure such as killer whales and pilot whales (Jefferson 2008, Foote *et al.* 2011a, Oremus *et al.* 2013). These characteristics include cooperative foraging, food provisioning, mass strandings and care-giving (epimeletic) behavior (Lodi 1992, Pitman & Stinchcomb 2002, Wells & Gannon 2005). Although mass strandings have been recorded for rough-toothed dolphins in the Pacific (Ferrero & Hodder 1993, Miyazaki & Perrin 1994, Wells & Gannon 2005, NMFS 2008), a majority of documented mass strandings have been in the North Atlantic (NMFS 2008, Wells & Gannon 2005). Although the causes of mass strandings are unknown, these events also provide evidence of strong social bonds among member of the groups. The degree of relatedness in rough-toothed dolphin mass strandings has not been investigated. However, three dolphins rescued from a mass stranding in Florida were observed to participate in food sharing and "intense communication". When released back into the wild, the dolphins stayed together for the duration of the satellite tags, 23 days; (Wells & Gannon 2005).

Here we evaluate the matrilineality and relatedness within rough-toothed dolphin groups to assess if stable group structure has a genetic component in this species. We use a collection of genetic samples from the Pacific and Atlantic Ocean basins representing 55 groups of biopsy samples from wild-ranging dolphins, tissue samples from bycatch and teeth samples from mass strandings and bycatch. The biopsy samples represent groups of dolphins that are a subset of samples in Chapter 3 where genetic diversity was partitioned geographically by islands, and groups are also delineated by island here. We consider that individuals with different haplotypes cannot be maternal relatives, while individuals with a shared haplotype will represent a common maternal lineage, including both close and distant maternal relatives (Weinrich *et al.* 2006). Due to the similarities in social characteristics between rough-toothed dolphins and strictly matrilineal species such as killer whales, we predict rough-toothed dolphins with the same mtDNA haplotype are more likely to associate in groups than expected by chance. Alternatively, we quantify the degree of matrilineality of groups with multiple matrilines.

We acknowledge our scope is limited by two main challenges associated with sampling pelagic dolphin species (1) accounting for regional differentiation of mtDNA haplotypes and (2) accounting for incomplete sampling of most groups. However, we address these challenges by using dyad combinations of the number of haplotypes within groups to create a matrilineal index. This matrilineal index is then tested using a Monte Carlo simulation based on the observed haplotype frequencies of the island population where the groups were sampled. This helps to prevent confounding social structure of groups

with the regional population structure. Finally, we tested the hypothesis that adult group members are more related (kinship) than expected. Similar to the study on striped dolphins by Gaspari *et al.* (2007), we predict greater levels of relatedness in smaller groups than in larger groups, and that larger groups are made up of smaller subgroups of related individuals, similar to killer whale and pilot whale groups (Pilot *et al.* 2010, Foote *et al.* 2011a).

4.3 Methods

4.3.1. Tissue sample collection, region delineation and definition of groups

Skin samples were collected from adult or sub-adult rough-toothed dolphins at sea (n=214) in 47 different groups in the main Hawaiian Islands (Kaua‘i, O‘ahu, and the Big Island of Hawai`i, termed Hawai‘i from this point forward), the Society Islands of French Polynesia (Mo’orea and Raiatea) and Samoan archipelago (Figure 4.1). In the Hawaiian Islands and American Samoa biopsy samples were collected using a crossbow and biopsy arrow (Lambertsen 1987) and stored either as tissue at -80^{o}C or first place in 70% ethanol in the field and then stored at -80^{o}C. In the Society Islands and Samoa (Island of Savaii), biopsy samples were collected using a modified veterinary capture rifle and biopsy dart (Krützen *et al.* 2002) and stored in 70% ethanol at -80^{o}C .

Samples were also collected from groups in either fisheries related mortality (‘bycatch’) or mass strandings (8 groups, Figure 4.1a and 4.1d). Skin samples collected from fishery bycatch individuals around American Samoa and a stranding around the Gulf Coast of Florida, USA were stored in DMSO at -80^{o}C. Teeth samples (courtesy of the

Smithsonian Institution) were collected from individuals that mass stranded near Maui in the main Hawaiian Islands, and off the central US coast of the Atlantic Ocean. Teeth samples were also collected from bycatch groups in the Eastern Pacific near Mexico and Isla Gorgona, Colombia (Figure 4a).

Groups at sea were identified as assembled individuals that appear to be involved in a similar activity, for example foraging, socializing, resting or travelling, (Shane *et al.* 1986). Here, the spatial scale used to define groups had a mean area of 500m x 800m, however two groups in the Hawaiian Islands occupied a larger area, with a group envelope of up to 1,000m x 1,200m and another group occupying 1,500m x 2,000m during the encounter. Group size was estimated by two or more researchers in the field, and the average estimate of the two was recorded. Therefore group size was considered an absolute value with no variance. Events where dolphins were caught in the same net/fishing operation or identified in a mass strandings were considered to be a part of the same group for analyses.

4.3.2. DNA Extraction and molecular sexing of tissue samples

Total DNA was extracted either using a Qiagen DNeasy Blood and Tissue Kit or a standard phenol:chloroform extraction protocol (Sambrook *et al.* 1989), modified for small samples (Baker *et al.* 1994). DNA was quantified with pico-green fluorescence and normalized to 15 ng μl^{-1}. An 800 bp fragment of the 5' end of the mtDNA control region was amplified and sequenced as described in Oremus *et al.* (2007). Variable sites and unique haplotypes were identified as described above. Sex was identified by

coamplification of the *Sry* gene, and the *ZFX* gene was used for a positive control (Gilson *et al.* 1998) following the protocol described in Oremus *et al.* (2007).

4.3.3. DNA Extraction and molecular sexing of teeth samples

The DNA extraction from teeth followed standard protocols for 'ancient DNA extraction'(Pimper *et al.* 2009). Total DNA was extracted from teeth samples in a lab separate from modern cetacean DNA. A laminar flow chamber and the use of UV radiation were used to provide sterile surface conditions and minimize the risk of contamination. Reagents were made up in a "DNA-free" positive pressure room separate from other laboratories. Teeth were submerged in liquid nitrogen for 20 seconds and then crushed. The resulting powder was subsampled and stored in a -20^{o}C freezer. DNA was extracted from 0.1g of tooth powder beginning with a protein digestion with 200ul of 10%SDS, 100 µl DTT (10mg/ml) and 100ul Proteinase K (20 mg/ml) and incubated at 37^{o}C overnight, and then for one hour at 50^{o}C. Samples were then centrifuged, and the rest of the extraction procedure followed Pimper *et al.* (2009), including silica suspension from (Boom *et al.* 1990). A negative control, or blank, was run as every fifth sample, and a maximum of eight samples and two blanks were extracted at one time.

A 450 bp region of the mitochondrial control region was amplified via Polymerase Chain Reaction (PCR) in a 25µl reaction using primers M13Dlp1.5 and Dlp5 (Dalebout *et al.* 1998), 1mg/ml Bovine Serum Albumin (BSA) and 5µl of DNA template as described in Pimper *et al.* (2009). This was followed by a nested amplification using 3 µl of a 1:10 dilution of the first reaction using the primers M13Dlp1.5 and Dlp4 under the same

conditions except no BSA was added. PCR products were purified for sequencing with SAPEX (Amersham). The sequencing reaction was carried out with BigDye Version 3.1 (Applied Biosystems, Inc.) with post-sequencing clean-up using Agencourt CleanSEQ Kit (Beckman Coulter). Products were then run on an ABI 3730 Genetic Analyzer (Applied Biosystems, Inc.). Variable sites and unique haplotypes were identified as described for tissue samples. Teeth samples were trimmed to 350 bp of the control region.

Molecular sex identification was carried out using a region of the male-specific *Sry* gene along with regions of the *ZFX* genes as positive controls (Gilson *et al.* 1998). Two rounds of PCR were carried out using 5µl of DNA for the first round of PCR, and 3ul of a 1:10 dilution of the first PCR as input for the second round. The reactions were performed in a total volume of 25µl with the following conditions: 2.5µl Taq Gold buffer, 5ul of 4mM $MgCl_2$, 1ul of 0.4µM each primer, Y53-3C, Y53-3D, P23EZ and P15EZ, 0.25ul of 0.2mM of dNTPs and 0.25µl of *Taq* Gold. The thermocycle profile began with an initial denaturation step of 94^{o}C for 10 minutes, followed by 35 cycles of 94^{o}C for 45 seconds, 60^{o}C for 45 seconds, 72^{o}C for 60 seconds and a final extension at 72^{o}C for 10 minutes. Male and female positive controls were added to each reaction.

4.3.4. mtDNA sequencing

Sequences were aligned and edited using *Sequencher v4.6* (Gene Codes Corporation). Sequences were required to have a minimum average Phred score of >30 (e.g. 30 is a 1 in 1,000 error rate and 40 is a 1 in 10,000 error rate). Any variable sites with Phred <40

were visually confirmed. If sequences fell below this Phred<30 threshold, they were resequenced. If they failed a second time they were removed from the dataset. If a haplotype was represented by only one sample, the identity of the haplotype was confirmed by re-sequencing in both directions. Variable sites and unique haplotypes were identified using *Sequencher v4.6* and then *MacClade v4.0*.

4.3.5. Delineation of regions

The regions of Kaua‘i, Hawai`i, O‘ahu, Mo’orea, Raiatea and Samoa were delineated based on previous studies of genetic analysis and photo-ID that illustrated regional differentiation (see Chapter 3 in addition to (Baird *et al.* 2008, Albertson *et al.* 2011, Oremus *et al.* 2012). The Atlantic region samples were considered one region and separate from the Pacific Ocean samples based on phylogenetic analysis (see Chapter 2) that showed the two ocean basins are separate populations. The Eastern Tropical Pacific (ETP) region included groups from the ETP (defined as the triangle of ocean extending from the Hawaiian Islands to California and Peru) and included groups with similar haplotype frequencies from within this area.

4.3.6. Matrilineal index for groups

To quantify matrilineality of groups, and compare these values across regions, we developed a Standardized Matrilineality Index (SMI). This index accounts for unequal and incomplete sampling in groups by calculating shared and unshared haplotypes among dyads. This method is similar to Weinrich *et al.* (2006) who used dyads of mtDNA haplotypes of associated humpback whales *Megaptera noveangliae*, to quantify matrilineality. However, unlike Weinrich *et al.* (2006), our matrilineality index only

considers the sharing of identical haplotypes between all pairs of individuals in a group (i.e. 1 or 0). For each group, the observed proportion of haplotype identity (Hi_{obs}) is calculated based on the number of dyad combinations of haplotypes in that group. These calculations are shown with an example dataset in Appendix Table VI.1. To generate a null distribution of the region where the sample was collected and determine the expected haplotype identity (Hi_{exp}), a null distribution for the matrilineal index was generated by Monte Carlo sampling of background haplotype frequencies of each island or region was performed with 1,000 total replicates carried out in *R v3.0.2* (R Core Team 2013; script available from A. Alexander, or see Appendix Figure IV.2). The Standard Matrilineal Index (SMI) is the difference between the observed and expected haplotype identities (e.g. subtracting the Hi_{exp} from Hi_{obs}). The formula is then:

$$\text{Standard Matrilineal Index (SMI)} = Hi_{obs} - Hi_{exp}$$

This index describes the degree of matrilineality and ranges from -1 to 1, where positive values indicate groups are more matrilineal than expected given the baseline region haplotype frequencies, and negative values indicate groups are less matrilineal than expected.

The SMI can be calculated at the group level as well as at the regional level by summing across the total observed number of within-group dyads, and the number of same-haplotype within-group dyads. It is important to note that the SMI is not informative if a regional sample consists of only one haplotype because a null distribution cannot be generated. Likewise, the background haplotype frequencies should be representative of the entire region to have enough power to detect a deviation from Hi_{exp}. Therefore, for our study, due to the limited number of samples in groups, we generated the Hi_{exp} for the

entire region and used this value to calculate the SMI for each group and for the region. Hi_{obs} was calculated for each group and for each region, and the SMI was calculated for each group and each region, but significance was only tested for the sum of the dyads in each region.

The baseline frequencies used to generate the null distribution were a subset of the data in Chapter 2 (Atlantic Ocean and Eastern Tropical Pacific haplotype frequencies) and Chapter 3 (Central Pacific Ocean haplotypes) for each region and are shown in Appendix IV (Table IV.2.). The subset included only haplotypes found in groups from that specific region (e.g. Kauaʻi haplotypes that were identified in Chapter 3 were used in the baseline here if they are found in Kauaʻi groups listed in Table 4.1). The Maui stranding (n=8) contained two haplotypes that are found in high frequency in the Eastern Tropical Pacific (ETP) and one new haplotype. Although this group was geographically located in the Hawaiian Islands, the haplotype frequencies were better represented by the ETP haplotype baseline, allowing for a more conservative test. Therefore the null distribution of the Maui samples was generated using the ETP haplotypes.

4.3.7. Microsatellite genotyping

All biopsy samples from Kauaʻi, Hawaiʻi, Oʻahu, Moʼorea, Raiatea and Samoa were genotyped using up to 15 previously published microsatellite loci developed from different cetacean species in order to estimate relatedness (Table 3.2, Chapter 3). PCR reactions were performed in 10ul volumes with varying annealing temperatures (Table 3.2, Chapter 3) using the reaction protocols discussed in Chapter 3. PCR products were

co-loaded in sets of non-overlapping loci and analyzed on a 3730 sequencer (Applied Biosystems). The protocol for quality control, testing for genotyping errors, deviation from Hardy-Weinberg equilibrium, the identification of replicates and the probability of identity are discussed in detail in Chapter 3. When replicates were identified, if an individual was sampled on one occasion in one group, and on a different day in another group, the individual was retained both times. This occurred on a single occasion in Kauaʻi.

4.3.8. Measuring relatedness

Levels of relatedness (estimated as R) were calculated with microsatellite data in *GenAlEx v6.5* (Peakall & Smouse 2012) using the Queller and Goodnight (1989) estimator. R ranges from 0-0.5 in wild populations, where 0 is unrelated, 0.25 is half siblings and 0.5 is parent-offspring or full sibs (Queller & Goodnight 1989, Blouin 2003). To evaluate whether individuals within groups were more related than expected by chance we used the Excel macro *GroupRelate* (Valsecchi *et al.* 2002). The program uses the pairwise relatedness values generated in *GenAlEx* to determine significance. The significance of any individual comparison is tested by drawing alleles from one region to create genotypes of unrelated individuals. This is repeated 1,000 times, and the number of times the observed relatedness value exceeds the randomized value provides the approximate probability of observing the data under the null hypothesis of no relatedness. Relatedness could be calculated in groups where only two individuals were sampled. To examine any possible correlation between group size and relatedness, we used Pearson's correlation coefficient.

4.4. Results

A total of 55 groups were sampled in seven regions in the Pacific Ocean basin and one region in the Atlantic Ocean basin. Variation in the mtDNA sequences revealed 28 haplotypes (Appendix Table IV.1). Where sex could be determined for individuals within groups, there were six groups where all females were sampled, 16 groups where all males were sampled and 33 groups where both females and males were sampled (Table 4.1). Sex ratio differences could only be tested for Kauaʻi, the Big Island of Hawaiʻi and Mo'orea due to the number of individuals sampled at each island. There was a male sex bias in sampling for Hawaiʻi (p=0.03) and Mo'orea (p=0.01), but no sex bias observed in Kauaʻi (p=0.13).

4.4.1. Group sizes and haplotype diversity

The 55 rough-toothed dolphin groups in this study contained at least two sampled individuals that passed quality control (n= 214 individuals total, Table 4.1). Of the 55 groups, 26 had two sampled individuals, 15 had three or four sampled individuals, and 14 had five or more sampled individuals in a group. Estimated group sizes ranged from 2-90 dolphins (Table 4.1). Kauaʻi had the largest observed group (90 individuals). Estimated group size was not statistically different between regions in most cases (Figure 4.2). The number of haplotypes present in each region ranged from 2 to 9, with Kauaʻi and Hawaiʻi containing the highest (9,6 respectively).

4.4.2. The degree of matrilineality in groups

Of the 55 groups sampled in eight regions, 33 (60%) contained multiple haplotypes, allowing us to reject the strict matriline hypothesis. In the strictly matrilineal groups, the

largest estimated group size was 28 (2 samples) in Hawai‘i and 27 (2 samples) in Mo’orea. The largest strictly matrilineal group with the largest number of samples collected was a mass stranding off the Virginia coast. Eight samples were collected from this group of 26.

We then considered the degree of matrilineality for each group using the standard matrilineal index based on the observed haplotype identity for the group and the expected haplotype identity for region. The values of the index for all groups ranged from -0.791 (less matrilineal than expected based on regional haplotype frequencies) to 0.827 (more matrilineal than expected, Table 4.1). Of the 33 groups that had multiple haplotypes, the index showed 17 groups were more matrilineal than expected by chance.

The standardized matrilineal index was also calculated summing across each region, and the Monte Carlo test was used to evaluate significance. With this, the total dyads for Hawaii, North Pacific bycatch events and the Northwest Atlantic were significantly more matrilineal than expected by chance, while Kaua‘i, Hawai‘i, O‘ahu, Mo‘orea, Raiatea and Samoa were not significant. However, Kaua‘i contained the largest group size of 90 where eight haplotypes were found from 24 samples collected. No other groups contained more than four sampled haplotypes. Therefore we also calculated the matrilineal index for Kaua‘i excluding this super group (Table 4.1). When this group was excluded Kaua‘i as a region had an SMI of 0.118 and was significantly more matrilineal than expected by chance. When the group was included, Kaua‘i as a region had an SMI of 0.027 and was not significantly more matrilineal than expected by chance. Differences in the index and

significance when the group is included and excluded are shown by the histograms in Appendix Figure IV.1.

4.4.3. Relatedness within groups

The relatedness values for the 47 groups with microsatellite data ranged from 0.005 (no relation) to 0.142 (on the order of first cousins; Table 4.1). Four groups were significantly more related than expected by chance (one in Kaua‘i and three in Mo‘orea). Three of the four groups were strictly matrilineal for the mtDNA dataset and were composed of both sexes. These groups included Kaua‘i, an estimated group size of 14, with two males and two females sampled, Mo‘orea, a group of seven, with two females and three males sampled, and Mo‘orea, a group of 11, with two females and one male sampled. The fourth group was a group in Mo‘orea, a group of six, containing all males. Mo‘orea had several groups that contained only one haplotype. However, the overall SMI was not significant because of low mtDNA diversity.

In general, it appeared as group size increased, relatedness values decreased, and the larger groups were less likely to be related. The R-value from the Pearson’s correlation for this relationship was highly negative, the corresponding p-value was only of marginal significance ($r = -0.6661$; $P=0.054$).

4.5 Discussion

The results of this study provide new insights into the social organization and molecular ecology of rough-toothed dolphins in two ocean basins. We found that a majority (33 out of 55) of rough-toothed dolphin groups are composed of multiple matrilines and relatedness values are low in most groups. From this we can reject that rough-toothed dolphins form strict matrilines. Instead, we found that rough-toothed dolphin groups show a weak but significant tendency towards matrilineality and some kinship. Our study adds a genetic component to previous photo-ID studies of association that found rough-toothed dolphins assemble in stable groups. In addition, our study corroborates other studies on pelagic dolphin group structure that there is relatedness in some groups, and large groups may be the combination of smaller related subgroups (Gaspari *et al.* 2007, De Stefanis *et al.* 2008).

4.5.1. Weak matrilineality and stable groups

Rough-toothed dolphin groups show weakly matrilineality where some groups have one haplotype and other groups have multiple haplotypes. Despite the evidence that rough-toothed dolphins are thought to have a complex social system, exhibiting behaviors like food sharing, cooperative foraging, mass stranding events and reciprocal altruism (Lodi 1992, Pitman & Stinchcomb 2002, Wells & Gannon 2005) they did not exhibit the typical genetic pattern observed in other strictly kinship based social mammals such as killer whales and elephants (Archie *et al.* 2008, Johnston *et al.* 2008, Pilot *et al.* 2010). However, it has been shown from previous photo-ID studies throughout regions in both the Atlantic and Pacific that they do form stable groups where they associate with the same individuals repeatedly (Mayr & Ritter 2005, Baird *et al.* 2008). Moreover, in

several locations of their range it has been documented that rough-toothed dolphins 'participate in complex social interaction' where they exhibit care-giving behavior among group members (Lodi 1992, Wells & Gannon 2005, Fulgencio de Moura *et al.* 2009) and participate in cooperative foraging (Pitman & Stinchcomb 2002). Therefore, rough-toothed dolphin group structure may be more similar to chimpanzees where stable groups are formed, but are not directly motivated by kinship (Goldberg & Wrangham 1997). Stable, but not related groups are also observed in other cetaceans including humpbacks. Individuals are found in the same foraging groups year after year, yet these groups are not necessarily related (Weinrich *et al.* 2006). Spinner dolphins exhibit stable group behavior and cooperative foraging, yet members of these groups are not necessarily related (Karczmarski 2005, Olavarría *et al.* 2007, Oremus *et al.* 2007, Andrews *et al.* 2010).

4.5.2. Insights into mass strandings

Our study presents the first documentation of the mtDNA haplotypes of rough-toothed dolphins from mass strandings. Similar to our results of wild-ranging groups of dolphins, four of the six mass strandings in this study consisted of multiple matrilines. Unfortunately, the limitation of using teeth prevented the use of microsatellites to infer kinship. There is evidence of rough-toothed dolphins that were rehabilitated together after a mass stranding showing care-giving behavior during rehabilitation, and when released remained together for the duration of the satellite location tags (Wells & Gannon 2005). From these observations the authors inferred the dolphins form stable bonds between individuals. However, since not genetic samples were taken it is not known if there was kinship or maternal relatedness between the individuals. In pilot whales it has been

shown that groups are cohesive, exhibiting herding behavior during drive kills (Fielding 2007), and often strand in large groups. However, unexpectedly, a study of long finned pilot whale mass strandings found multiple matrilines were present in 75% of the mass strandings (Oremus *et al.* 2013).

4.5.3. The composition of "super groups"

Long-finned pilot whales are sometimes seen in groups of 500 or more individuals, and it has been shown these groups are made up of smaller more stable subgroups (Ottensmeyer & Whitehead 2003). Gasari *et al.* (2007) found large groups of striped dolphins were often made up of smaller subgroups, and the smaller subgroups were more related than the larger groups. Rough-toothed dolphin groups have been documented in several locations where large groups appeared to be made up of smaller subgroups (Watkins *et al.* 1987, Ritter 2002, Baird *et al.* 2008). In our study, we tested to see if there was a correlation between group size and relatedness. This negative correlation ($r = -0.661$) was marginally significant in our data. It is possible then that the larger groups encountered in our study were made up of smaller subgroups, similar to pilot whales or striped dolphins. However our relatedness values were low in general, and there may not be a clear distinction in relatedness with large vs. small groups.

One such instance of a super group was around Kauai where the group size was estimated to be 90 individuals and these individuals were spread over an area of 1.5 km by 2 km. The 24 samples collected in this group contained eight different haplotypes, more than any other group in our study. This group influenced the results of the Kauai dataset, such

that when it was removed the region changed to being significantly more related by chance (Table 4.1 and Appendix IV.3). This was not a typical group in our study, in the size or the number of haplotypes represented, and this group most likely reflects a "super-group" where several subgroups make up a large group. More comprehensive sampling of groups could help determine if this is a pattern in rough-toothed dolphin groups as seen in long finned pilot whales and striped dolphins.

4.5.4. Conservation concerns about local behavior

Despite their preference for deep water, rough-toothed dolphins are affected by increasing anthropogenic impacts around oceanic islands. There is documentation in every area where they have been studied of stable social groups. The behaviors associated with these groups may present other conservation issues if there are learned behaviors that include negative anthropogenic interactions. For example, around the Society Islands in French Polynesia and the Big Island of Hawaii, rough-toothed dolphins are increasingly involved in fisheries depredation with individuals occasionally shot by fishermen as a way to discourage them from taking bait and hooked fish (Kuljis 1983, Nitta & Henderson 1993, Poole 1993). The absence of this behavior in other locations suggests that, similar to other cetacean species, this may be a learned behavior in a specific isolated, population. Sperm whales in the Gulf of Alaska, for example, remove sablefish from longline fishing gear, and there is evidence of 'repeat offenders' in multiple years (Straley *et al.* 2014). In bottlenose dolphin communities where shrimp trawling is used, one population of bottlenose dolphins participates in the foraging behind shrimp trawlers, while another sympatric population does not (Chilvers & Corkeron

2001). In the cases of depredation, if this behavior is either vertically transmitted, passed down from mother to offspring, or horizontally transmitted within social groups, the numbers of dolphins that are participating in this behavior could increase in the future as more dolphins learn the behavior (Cantor & Whitehead 2013). This will increase the conflict between dolphins and fishermen. The outcome of such an interaction will require innovative management and potentially technological solutions to discourage this undesirable behavior.

4.6 Acknowledgements

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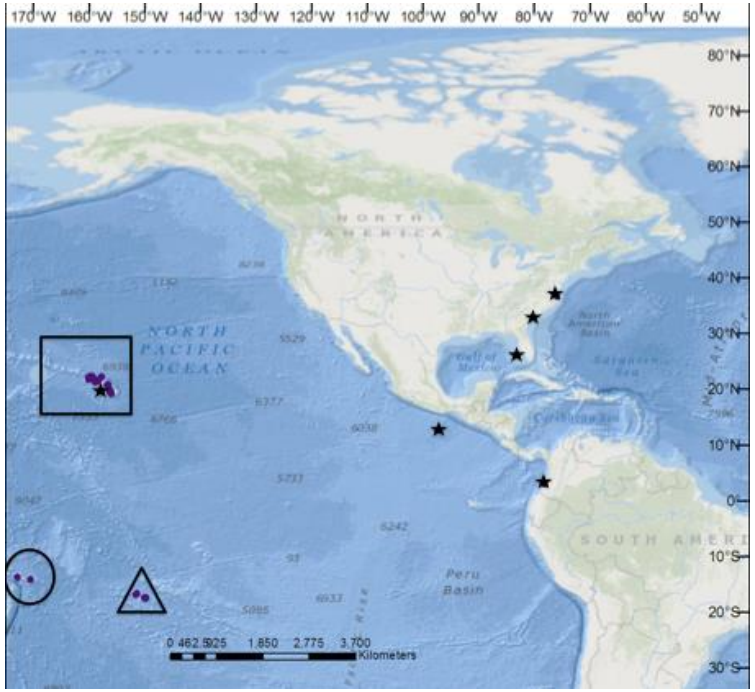
for providing funding for archiving and extracting of all biopsy samples outside of French Polynesia.

a) 

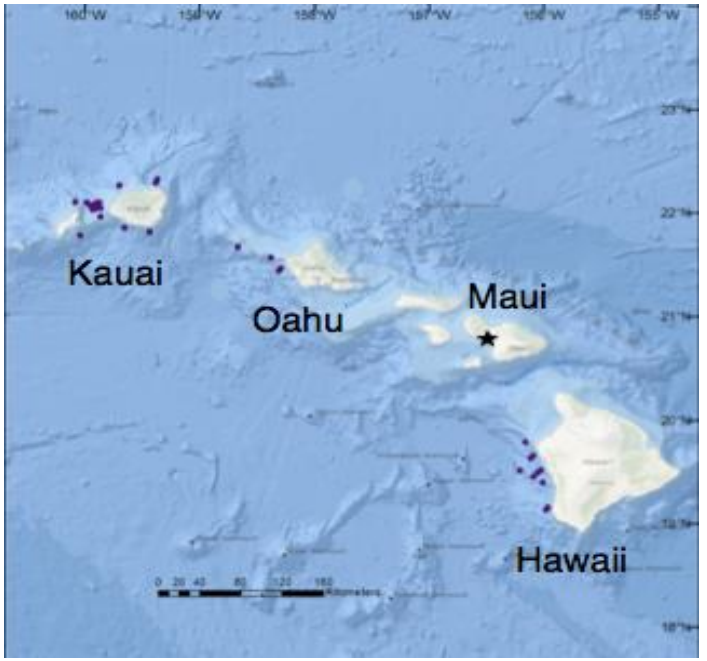
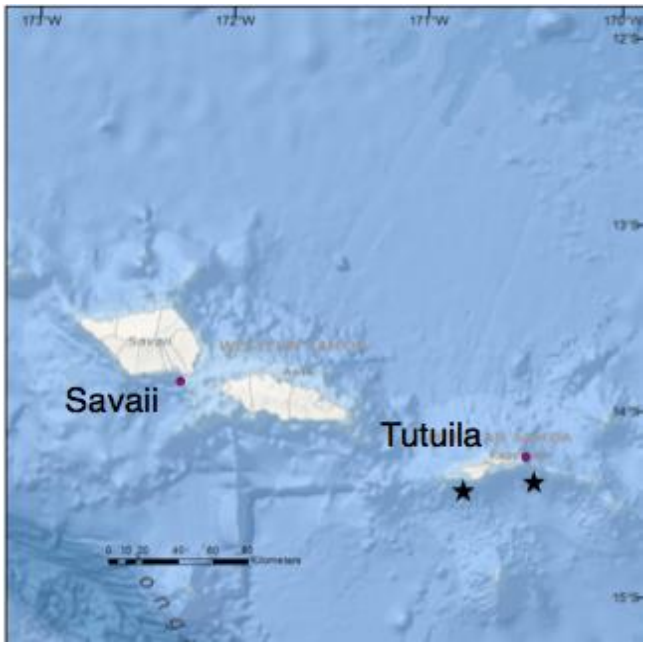
b) 

Figure 4.1. Location of data collection for groups of rough-toothed dolphins for (a) all samples for the study (sample locations shown with a star for mass strandings and bycatch events and circles for biopsies. The outline rectangle is the Hawaiian Islands, the circle is the Samoan archipelago and the triangle is the Society Islands of French Polynesia (b) the Hawaiian Islands group sample locations, circle for each group biopsy and star for each mass stranding (c) Samoan archipelago group locations, circle for each group biopsy and star for each bycatch events (d) Society Islands of French Polynesia group sample locations, circle for each group biopsy.

c) 

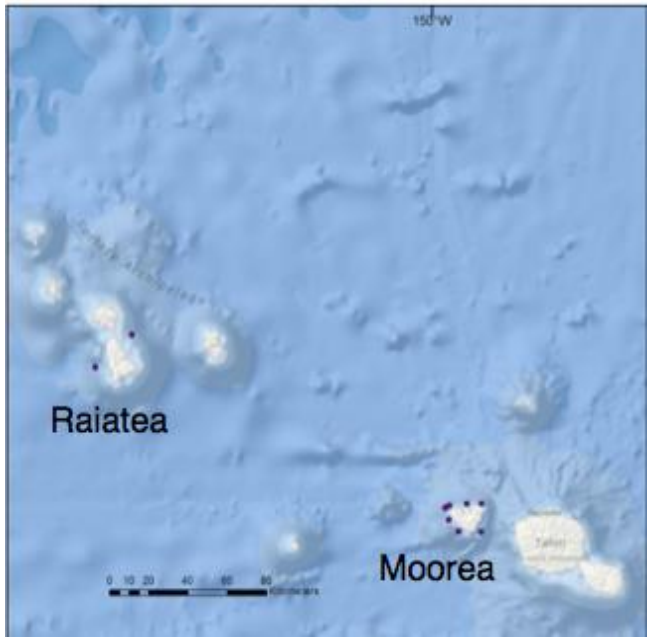
d) 

Figure 4.1. Location of data collection for groups of rough-toothed dolphins for (a) all samples for the study (sample locations shown with a star for mass strandings and bycatch events and circles for biopsies. The outline rectangle is the Hawaiian Islands, the circle is the Samoan archipelago and the triangle is the Society Islands of French Polynesia (b) the Hawaiian Islands group sample locations, circle for each group biopsy and star for each mass stranding (c) Samoan archipelago group locations, circle for each group biopsy and star for each bycatch events (d) Society Islands of French Polynesia group sample locations, circle for each group biopsy.

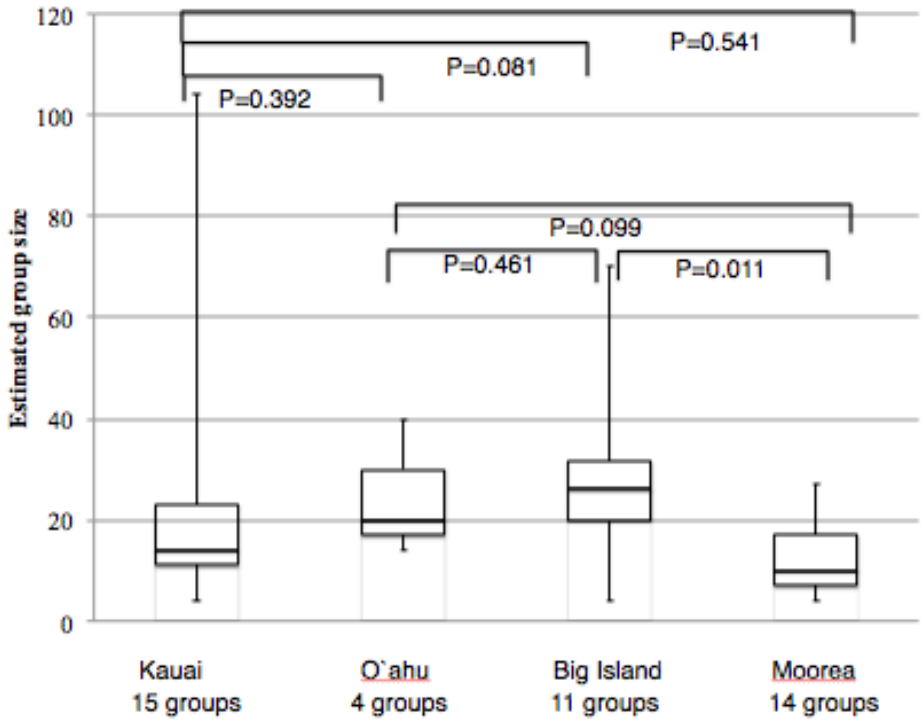


Figure 4.2. An assessment of estimated group sizes in rough-toothed dolphin in the Pacific Ocean basin where more than three groups in a region were available for comparison. P-values were adjusted for multiple comparisons using the false discovery rate function implemented in *R*. Minimum, lower quartile, median, upper quartile and maximum estimated group size are given for each region.

Table 4.1: Summary of genetic samples for groups of rough-toothed dolphins from islands in the main Hawaiian Islands (North Pacific), Society Islands and Samoa (South Pacific), the Eastern Tropical Pacific (ETP), and the east coast of the US and the Gulf of Mexico. Regions are separated in the table and sample collection years for each region is given. Estimated group size is the total number of dolphins estimated to be in the group, n QC samples are the number of dolphins that were biopsied/sampled, genotyped and sequenced successfully during the group encounter for *n* Females and *n* Males (male and female total may not add up to the *n* samples, as some samples did not amplify for sex). K is the total number of haplotypes in a group, dyads is the total observed same haplotype dyad combinations and Relatedness (R) is the within group relatedness value measured by GenAlEx. Hi_{obs} is the observed haplotype identity calculated from the number of same haplotype dyads. Hi_{exp} is not listed, but examples of Kauaʻi and the North Atlantic are shown in Appendix IV Figure IV.1. The Standard Matrilineality Index (SMI) values are presented by island (all groups together). NA identifies where calculations were not possible either due to low sample size or where microsatellite data was not available. The highlighted row identifies the largest group for Kauaʻi. The total for Kauaʻi includes an additional row where the region values are calculated excluding this group. R and SMI are bolded and italicized with an asterisk if significant at *p <0.05; ** p< 0.001.

Date of group encounter	Estimated group size	n QC	n Females	n Males	*K*	Total dyads within each group	R	H_{obs}	SMI (Hi_{obs}-Hi_{exp})
Kauaʻi, North Pacific, 2003-2012									
02 Jun 03	11	3	1	2	2	3	0.058	0.30	0.127
03 Jun 03	4	2	2	0	1	1	0.082	1	0.827
04 Jun 03	25	9	3	6	4	36	0.018	0.22	0.027
07 Jun 03	12	7	1	6	4	21	0.064	0.19	0.017
07 Jun 03	15	3	2	1	2	3	0.031	0.30	0.127
08 Jun 03	11	4	1	3	3	6	0.044	0.20	0.027
09 Jun 03	14	4	2	2	1	6	***0.120****	1	0.827
30 Oct 05	42	9	2	7	4	36	0.015	0.30	0.127
14 Nov 05	90	24	13	11	8	253	0.005	0.06	-0.113
17 Feb 11	12	2	1	1	2	1	0.047	0	-0.173

Date of group encounter	Estimated group size	n QC	n Females	n Males	*K*	Total dyads within each group	R	H_{obs}	SMI (Hi_{obs}-Hi_{exp})
18 Feb 11	4	2	2	0	2	1	0.028	0	-0.173
21 Jul 11	65	2	0	2	2	1	0.024	0	-0.173
31 Jul 11	25	3	1	2	3	3	0.012	0	-0.173
08 Aug 11	14	2	2	0	1	1	0.066	1	0.827
14 Jan 12	14	2	0	2	1	1	0.078	1	0.827
	Kauaʻi Total	78	33	45	9	372		0.20	0.027
	Kauaʻi Total Excluding 14Nov 05	54	20	34	8	119		0.29	***0.118****
Oʻahu, North Pacific, 2003, 2010									
24 May 03	40	2	0	2	2	1	0.003	0	-0.291
10 Oct 10	14	2	1	1	2	1	0.001	0	-0.291
10 Oct 10	16	2	1	1	2	1	0.003	0	-0.291
11 Oct 10	24	4	1	3	2	6	0.027	0.5	0.210
	Oʻahu Total	10	3	7	4	9		0.33	0.039
Hawaiʻi, North Pacific, 2004-2012									
04 Oct 04	4	2	0	2	2	1	0.086	0	-0.246
21 Nov 04	10	2	1	1	1	1	0.091	1	0.625
22 Nov 04	28	2	0	2	1	1	0.044	1	0.625
30 Nov 04	24	2	1	1	2	1	0.038	0	-0.246
25 Jul 06	28	2	1	1	1	1	0.020	1	0.625
20 Jul 08	70	3	0	3	2	3	0.015	0.33	0.04

Date of group encounter	Estimated group size	n QC	n Females	n Males	*K*	Total dyads within each group	R	H_{obs}	SMI (Hi_{obs}-Hi_{exp})
21Jul 08	35	2	0	2	2	1	0.013	0	-0.246
01 May 09	20	2	0	2	2	1	0.011	0	-0.246
29 Oct 09	26	5	4	1	3	10	0.052	0.2	0.175
22 Aug 11	35	3	1	2	1	3	0.028	1	0.625
23 Aug 12	25	2	0	2	2	1	0.008	0	-0.246
	Hawai`i Total	27	8	19	6	24		0.375	***0.129****
Mo‘orea, South Pacific, 2002-2004									
01 Sept 02	9	2	0	2	2	1	0.099	0	-0.588
02 Sept 02	7	2	0	2	2	1	0.071	0	-0.588
27 July 03	22	5	2	2	2	10	0.007	0.6	0.012
08 Aug 03	4	2	2	0	1	1	0.100	1	0.412
07 Oct 03	17	2	0	2	1	1	0.032	1	0.412
22 Nov 03	15	7	3	4	3	21	0.013	0.33	-0.258
19 Aug 04	7	5	3	2	1	10	***0.134****	1	0.412
26 Aug 04	11	3	2	1	1	3	***0.242****	1	0.412
12 Sept 04	13	6	0	6	2	15	***0.125****	0.67	0.082
20 Sept 04	9	3	3	0	1	3	0.035	1	0.412
21 Sept 04	7	4	1	2	2	6	0.062	0.5	-0.088
24 Sept 04	6	2	1	1	1	1	0.044	1	0.412
12 Oct 04	22	3	2	1	1	3	0.019	1	0.412

Date of group encounter	Estimated group size	n QC	n Females	n Males	*K*	Total dyads within each group	R	H_{obs}	SMI (Hi_{obs}-Hi_{exp})
16 Oct 04	27	2	0	2	1	1	0.032	1	0.412
	Mo‘orea Total	48	19	29	4	75		0.636	0.048
Raiatea, South Pacific, 2004, 2011									
07 Nov 04	12	6	1	5	2	15	0.064	0.467	-0.270
13 Nov 04	8	7	1	6	1	21	0.092	1	0.263
	Raiatea Total	13	2	11	2	36		0.78	0.042
Samoan archipelago, South Pacific 2003, 2006 biopsy and 2010, 2011 bycatch events									
2003	NA	3	0	3	1	3	0.103	1	0.299
20 Feb 06	NA	5	1	4	3	3	0.009	0.33	-0.371
2010	2	2	2	0	1	1	0.111	1	0.299
2011	2	2	1	1	1	1	0.086	1	0.299
	Samoa Totals	12	4	8	3	8		0.53	-0.171
Mass stranding and bycatch events, North Pacific									
Maui 1976									
28 Jun 76	17	8	3	1	3	28	NA	0.393	0.163
Colombia 1982									
16 Mar 81	4	4	2	1	3	1	NA	0.167	-0.066
East Pacific 1981									
16 Oct 82	4	4	1	3	2	6	NA	0.5	0.267
	ETP Total	16	6	5	8	15	NA	0.375	***0.142****

Date of group encounter	Estimated group size	n QC	n Females	n Males	*K*	Total dyads within each group	R	H_{obs}	SMI (Hi_{obs}-Hi_{exp})
Mass stranding events, Northwest Atlantic									
Virginia 1976									
12 Oct 76	26	8	6	0	1	28	NA	1	0.290
North Carolina									
15 Oct 76	2	2	0	1	1	1	NA	1	0.290
Gulf of Mexico									
14 Dec 97	2	2	1	1	2	1	NA	0	-0.791
	Northwest Atlantic Total	12	7	2	3	30	NA	0.967	***0.257****

5. General discussion

5.1. Objectives and major findings

In this dissertation my main objective was to describe patterns of diversity and differentiation in rough-toothed dolphins using a worldwide dataset. Both mitochondrial and nuclear markers proved useful to evaluate gene flow for rough-toothed dolphins on different spatial scales. I began with a worldwide dataset and ended with assessing genetic structure within groups in specific regions.

Chapter two was the first time a worldwide dataset was used to evaluate the phylogeography of rough-toothed dolphins. The five regions I describe were based on biogeographic barriers discussed in Rocha (2007), Bowen (2013) and Briggs and Bowen (2012). The limitations of the dataset meant that I had a shallow but wide description (many samples with one limited marker) and a narrow but deep description (fewer samples, but both nuclear introns and mitogenomes). I did not find a pattern of monophyly with mitochondrial or nuclear introns, and therefore explored an alternative hypothesis to evaluate genetic differentiation and gene flow using population level indices. I determined evolutionary significant units, a taxonomic level below subspecies status, was a meaningful way to delineate rough-toothed dolphins in the Atlantic and the Indian/Pacific Oceans and acknowledge the two ocean basin populations are on separate evolutionary trajectories. Although many samples were not available from the Indian Ocean the shared haplotypes with the Pacific Ocean illustrate there may be occasional long distance dispersal. Differentiation was significant between regions in the

Central/Eastern Pacific and the Indian/Western Pacific for mitochondrial DNA, but not for the nuclear intron dataset.

One of the most surprising findings of this study was the Atlantic clade nested with the Pacific and Indian lineages. This was apparent in both the control region and the mitogenome phylogenies. The mitogenome phylogeny showed there were two or three migration events of the Atlantic to the Indo-Pacific or Indo-Pacific to the Atlantic. Due to the timing of this second invasion, the dolphins would have come from the Indian Ocean and around the southern tip of Africa. During this time period (late Pleistocene, 130 Kyr) there was an enhanced Agulhas current around the southern tip of Africa and several megafauna were known to migrate into the Atlantic from the Indo-Pacific at this time (Peeters *et al.* 2004). A figure diagramming the ancestral state color-coding the oceans would have been useful to give a more robust estimate of timing of the invasions.

In Chapter 3 I confirmed the presence of insular populations in three archipelagos in the Central Pacific. Previous studies had demonstrated insular structure using photo-identification for samples in the Main Hawaiian Islands, but I expanded the dataset, and included 'offshore' samples from the Northwest Hawaiian Islands as a comparison. The offshore waters surrounding the Northwest Hawaiian Islands are more productive than the nearshore waters, perhaps reducing the habitat differences between nearshore and offshore waters. Similar to other pelagic species in areas with low productivity (Chivers *et al.* 2007, Louis *et al.* 2014), rough-toothed dolphins in the Northwest Hawaiian Islands showed higher genetic diversity and lower genetic differentiation with the neighboring

islands of Kauai and Oahu. In contrast to the Northwest Hawaiian Islands, I found the island of Hawaii was isolated compared to the islands of Kauai and Oahu. This corroborates previous interpretations that offshore eddies, steep bathymetric shelves off the island (which concentrates plankton) and the island mass effect provide local productivity supporting island communities.

In the South Pacific my findings in Chapter three confirmed what Oremus *et al.* (2012) had found using both photo-identification and genetics; that strong differentiation existed between the Leeward and Windward islands in the Society Islands. There were shared haplotypes between the North and South Pacific, but it could not be determined if this was due to occasional influxes from pelagic populations, as suggested for bottlenose dolphins (Tezanos-Pinto *et al.* 2008) or remnant haplotypes from an ancestral population. The genetic differentiation among the three archipelagos was similar to the differentiation found within an archipelago, illustrating it was not driven by isolation by distance, and was most likely due to strong genetic drift within the isolated communities.

A few genotypes in the Chapter three dataset revealed possible kin relations in some groups. Groups of rough-toothed dolphins from the regions defined in Chapter 3 were evaluated for the degree of relatedness in Chapter four. These pairs of individuals that were identified as kin in Chapter three were in groups that were more related than expected by chance when evaluated for within group relatedness in Chapter four. I found rough-toothed dolphins may associate with kin occasionally, but sampled groups do not

appear to be made up of only close kin. This supports our findings in Chapter four that rough-toothed dolphin groups are not exclusively composed of close kin.

In Chapter four I used the geographic regions I defined in chapter three to evaluate genetic structure within groups. Specifically I tested if rough-toothed dolphins in the same groups were more likely to share mtDNA haplotypes than expected by chance. Alternatively, I tested the degree of matrilineality by evaluating within group dyad combinations of haplotypes. I found some groups were strictly matrilineal, but most groups showed weak matrilineality. Only four groups showed relatedness on the order of half siblings illustrating that, although other studies had shown rough-toothed dolphin groups to be stable, groups are not necessarily related. This social structure is more similar to chimpanzees than strictly matrilineal species like elephants and killer whales.

Although we found a negative correlation between group size and relatedness, it was only marginally significant. A definitive answer to this may not be possible considering how low our relatedness values were in general. Although this study did identify a ‘super group’ in Kauai it was not clear if other large groups were true ‘super groups’. In order to determine this we would need sampling of the dolphins in each the small subgroups (e.g. these samples came from a specific subgroup and other samples in the encounter came from another subgroup). Lastly, the information I provide on mass strandings was the first genetic information for rough-toothed dolphin mass strandings. However, due to using teeth samples I was not able to infer kinship from microsatellites. It would be useful to have access to skin samples from mass strandings in order to determine kinship,

and also determine whether or not close kin (i.e. mother/offspring pairs) strand near each other, similar to the study done on long-finned pilot whales (Oremus *et al.* 2013)

5.2. Implications and future research

In almost any study of cetaceans it goes without saying that 'more concerted sampling should be done', but it is worth saying what the priorities of future sampling should be. Indeed, the information presented here is some of the first of this species, and answering these questions brings about more questions. In order to answer additional questions, it would be useful to collect samples in specific areas. The haplotypes from the Indian Ocean were also found in the Pacific Ocean, but the power to detect differentiation between the Indian Ocean and other regions was limited by the low number of samples. Additional sampling in the Indian Ocean could be used to assess a more robust estimate of gene flow between the Indian Ocean and the Pacific Ocean regions (western, central and eastern). These samples could also be compared to the Atlantic, which appears from this study to present more of a barrier than the Pacific Ocean. In addition, surveys around South Africa would be useful to determine if this area is current habitat for rough-toothed dolphins since our South Africa samples represented single stranded animals, and surveys of small cetaceans around Mayotte in the Indian Ocean yielded no sightings of rough-toothed dolphins (Gross *et al.* 2009).

It has been shown that rough-toothed dolphins inhabit the waters around Brazil and are not just occasional visitors (Lodi & Hetzel 1999, Lailson-Brito *et al.* 2012). Therefore a concerted sampling effort around Brazil would establish the local population structure in

the Western South Atlantic. In addition, those samples could be used to evaluate the amount of exchange from the Indian Ocean (which seems to be very limited in our study) and the North Atlantic. In this study, most samples from the North Atlantic were in a private clade with the Brazil sample. Therefore, additional samples in both locations could be used to evaluate differentiation between these regions since the equator is not a barrier for this species.

Another important sampling consideration would be offshore sampling in areas of the North and South Pacific. In Chapter 3, the STRUCTURE plot showed differences between dolphins sampled in Samoa, where individuals that were more similar to Hawaiian Islands individuals were sampled tens of kilometers offshore, and individuals sampled within two kilometers of shore were more similar to South Pacific individuals. This was confirmed with the haplotypes of these individuals. However, our sample size was not large enough to have power to test whether an 'offshore' populations exists. In areas around French Polynesia, where rough-toothed dolphins have been observed 'offshore' they haven't been sampled so it could not be determined if these dolphins represent part of a separate population, or if they were simply part of the sampled population nearshore that happened to be farther offshore at that particular time. The samples that were collected around the northwest Hawaiian Islands did show characteristics similar to other populations of species considered to be 'offshore' with high haplotype diversity and low genetic differentiation to surrounding areas (Louis *et al.* 2014). Therefore, this may represent the first 'offshore' population of rough-toothed dolphins. Large ship surveys (albeit expensive) may be the best method for offshore

sampling as they can cover a large area, deploying small boats for sampling only when animals are present and weather is agreeable.

The use of several methods in concert to infer habitat use is becoming more prevalent in cetacean studies. In the main Hawaiian Islands, satellite tags have been deployed on rough-toothed dolphins since 2011, and photo-identification has been collected since 2000. The use of these methods with genetic data could tell a robust story of habitat use of this species. In French Polynesia aerial surveys were used to record identity and location of species. These data were then modeled with oceanographic productivity to predict cetacean habitat use (Mannocci *et al.* 2013).

Appendix I
Supplementary material for entire document

The files listed below are archived with the author, as well as the Cetacean Conservation and Genetics Laboratory at Oregon State University's Hatfield Marine Science Center. Requests for data access can be submitted to Renee Albertson (renee.albertson@oregonstate.edu) and C. Scott Baker (scott.baker@oregonstate.edu), and should be accompanied by a proposal describing how the data would be used. A two-year embargo applies to the data, however, requests made before two years will be considered on a case-by-case basis, depending upon the proposed use and potential for collaboration.

Nuclear genotypes for individuals sequenced in this study

File name: Nuclear_genotypes_Sbr_24Nov2014.xlsx

Individual code and allele calls for the nuclear DNA sequence generated in this study. Sequence definition for allele calls given in 'Nuclear allele definitions' below.

Nuclear alleles

Folder name: Nuclear_allele_definitions_Sbr_25Sept2014.xlsx

Fasta files with the DNA sequence for the rough-toothed dolphins defined for each nuclear locus sequenced in this study.

mtDNA sequences

Folder name: mtDNA_Sbr_25Aug2014

A CAF file with the mtDNA control region sequences for this study.

Appendix II.
Worldwide phylogeography description of the rough-toothed dolphin (Steno bredanensis) using the mitochondrial genome and nuclear introns

Electronic files available for Chapter 2
mtDNA sequences (PC dataset)
Folder name: mtDNA_ PC_Ch2_Sbr_24Nov14
There is one excel file for each sample

mtDNA haplotypes (350 dataset) with sex and GPS data
File name: mtDNA_350_Ch2_Sbr_24Nov14.xlsx

mtDNA sequences (350 dataset)
A CAF file of sequences that will open in Sequencher or Geneious

Intron data sequences
File name: Introns_Ch2_Sbr_24Nov14.xlsx
A CAF file of sequences that will open in Sequencher or Geneious

Appendix Table II.1. Long-range PCR fragments used to sequence the mitogenome identified by fragment number. Origin and Terminal End specify where the primer started and ended specific to *Steno bredanensis* when aligned with published *Steno bredanensis* from Vilstrup *et al.* (2012) sequence of 16,385bp (GenBank Accession no. JF339982.1). T_A refers to the annealing temperature used in the thermocycle protocol.

Fragment	Primer Pair	Fragment length	Origin *S.bredanensis*	Terminal End *S. bredanensis*	T_A
1	1.4UPF DelND2R	1,952	2,536	4,488	62
2	Pma6800CO1F DelHDND4R	3,610	6,779	10,389	59
3	Mys10000ND4LF Mys13000ND5R	3,024	9,986	13,011	64
4	PmaHS13660F Pma12sRNAR	3,834	12,741	190	62.5
5A	Ejm13tPheF 1.4UPR	2,502	55	2,557	60
5B	Eu1635F DelHD3106R	1,452	1,654	3,106	63
6	DelND2F Pma6916tSerR	2,465	4,463	6,928	62.5
4**	Mys13000NDH5F CytB2	1,624	12,996	15,220	62.5
Control Region	Dlp1.5 tPhe	1,235	15,150	16,385	55

Appendix Table II.2. Species-specific substation rates (per site per Myr) for the family Delphindae generated in the multispecies *BEAST* phylogenetic reconstruction using the PC mitogenome. The *BEAST* xml file was that used in Alexander et al. (2013) with the addition of a *Steno bredanensis* sequence from this study.

Species	lower HPD	higher HPD	median	mean
Lagenorhynchus obscurus	0.0041	0.008	0.0059	0.0073
Grampus griseus	0.0052	0.0113	0.0078	0.0105
Stenella attenuata	0.0056	0.0107	0.008	0.0097
Stenella coeruleoalba	0.0053	0.0113	0.008	0.0096
Lagenorhynchus albirostris	0.0084	0.0159	0.0119	0.0156
Cephalorhynchus heavisidii	0.0068	0.0131	0.0097	0.0117
Orcinus orca	0.0088	0.016	0.0122	0.016
Tursiops truncatus	0.0069	0.0199	0.0123	0.0158
Delphinus delphis	0.0041	0.0115	0.0072	0.0093
Globicephala melas	0.0067	0.0146	0.0101	0.0137
Sousa chinensis	0.0055	0.0103	0.0078	0.0093
Cephalorhynchus commersonii	0.0054	0.0139	0.0091	0.0114
Tursiops aduncus	0.0064	0.014	0.0098	0.0117
Cephalorhynchus eutropia	0.0047	0.0123	0.0081	0.0101
Lagenorhynchus cruciger	0.0039	0.0107	0.0068	0.0083
Delphinus capensis	0.0059	0.0167	0.0104	0.0135
Cephalorhynchus hectori maui	0.0053	0.0145	0.0092	0.0125
Lagenorhynchus australis	0.0039	0.0108	0.0069	0.0085
Steno bredanensis	0.0073	0.012	0.00978	0.0115

Appendix Table II.3. Alleles from the six introns used in this study. *Sotalia*, one proposed sister taxa of *Steno*, is shown in the last row for comparison purposes (name includes Genbank Accession number). DBY7 is not shown since no variable sites were identified. The green sequence is representative of sample Sbr07Sa03.

		IFN	IFN	IFN	IFN	IFN	CHRNA1	CHRNA1	CHRNA1	CHRNA1	GBA	GBA	CAT	CAT	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1	Act-1
		216	221	235	259	285	94	135	253	294	198	222	184	447	105	153	179	234	239	289	297	306	332	349	545	590	665	690	695		
		G	G	G	G	G	C	T	A	T	A	C	G	A	T	T	T	G	T	A	A	C	C	A	A	C	C	A	T		
Atlantic Ocean	Sbr96BZ9838	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.		
	Sbr03PR48139	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrARU48059	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.		
	SbrAT370	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.		
	SbrGWO1006	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrHBOI0413	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrOW1021921	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr11Bah134000	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrRT900	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	.	.		
	SbrRT374red	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	.	.	.		
Indian and Western Pacific Ocean	Sbr98Mal9855	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr02OM001	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr13MS116865	S	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr78ETP1316	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.		
	Sbr78Tai9535	S	S	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.		
	Sbr03IFS18431	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.		
	Sbr99IFS16159	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
Central and Eastern Pacific Ocean	SbrHICE123348	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr00HI70966	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	Y	.	.	.	.	.	.		
	Sbr12LA002	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr03KA34040	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr12KA003	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr12KA002	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.		
	Sbr05KA51113	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr12HI001	.	S	.	.	.	.	.	.	.	.	S	.	R	.	.	.	.	Y	.	.	Y	.	.	.	.	.	.	.		
	Sbr04FP15	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr03FP12	S	S	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr11RA03	.	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	.		
	Sbr07SA03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrAS125880	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr11KA022	S	S	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	Sbr06IFS66774	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
	SbrXX3816	.	S	.	.	.	.	.	.	.	.	.	S	R	.	.	.	.	.	.	.	.	.	.	.	Y	T	.	.		
	SbrNic11924	.	S	.	.	R	.	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.		
	Sbr91OR139	S	S	R	.	.	.	W	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	Y	.	.	.	.	.	.		
	Sotalia EF027041	C	C	.	C	.	T	C	G	C	G	.	C	.	C	G	G	.	.	.	T	.	.	G	G	.	.	.	C		

Appendix III
Staying close to home? Genetic differentiation of rough-toothed dolphins near oceanic islands in the central Pacific Ocean

Electronic files available for Chapter 3

Genotypes for individuals
A file with the individual and genotype
File name: Nuclear_genotypes_Sbr_24Nov14.xlsx

mtDNA haplotypes
A file with the individual, sex and haplotype
File name: mtDNA_Sbr_24Nov14.xlsx

mtDNA sequences (450 dataset)
A CAF file of sequences that will open in Sequencher or Geneious

Appendix Figure III.1: R code for carrying out permutations of haplotype diversity based on islands

```
MM <- rep.int(1,1)
BB <- rep.int(2,2)
N <- rep.int(3,8)
Y <- rep.int(4,19)
B <- rep.int(5,55)
A <- rep.int(6,69)
C <- rep.int(7,128)
X <- rep.int(8,157)
# Number of each haplotype in the background haplotype frequency to be
# sampled R needs these to be coded as #'numerical' so haplotypes MM-X
have been recoded here as 1-8.

Pop_1 <- 40
Pop_2 <- 14
Pop_3 <- 87
Pop_4 <- 230
Pop_5 <- 0
Pop_6 <- 0
# Sample size for each population that haplotype diversity will be
#estimated for

haplotypes <- c(MM,BB,N,Y,B,A,C,X)
# Combining the haplotypes defined above in one array of haplotypes

no_haps <- 8
# Total number of haplotypes (not sequences) in the 'haplotypes' array

i <- NULL
j <- NULL
# Parameterizing i and j for use in loops below

diff_Pop_12 <- c("diff_Pop_12")
diff_Pop_13 <- c("diff_Pop_13")
diff_Pop_14 <- c("diff_Pop_14")
diff_Pop_15 <- c("diff_Pop_15")
diff_Pop_16 <- c("diff_Pop_16")
diff_Pop_23 <- c("diff_Pop_23")
diff_Pop_24 <- c("diff_Pop_24")
diff_Pop_25 <- c("diff_Pop_25")
diff_Pop_26 <- c("diff_Pop_26")
diff_Pop_34 <- c("diff_Pop_34")
diff_Pop_35 <- c("diff_Pop_35")
diff_Pop_36 <- c("diff_Pop_36")
diff_Pop_45 <- c("diff_Pop_45")
diff_Pop_46 <- c("diff_Pop_46")
```

Figure III.1 (Continued)

```
diff_Pop_56 <- c("diff_Pop_56")
# Naming the array for each population comparison

for (i in 1:1000) {
# This sampling process will be completed 1000 times

a <- sample(haplotypes, Pop_1, replace=TRUE, prob=NULL)
b <- sample(haplotypes, Pop_2, replace=TRUE, prob=NULL)
cc <- sample(haplotypes, Pop_3, replace=TRUE, prob=NULL)
d <- sample(haplotypes, Pop_4, replace=TRUE, prob=NULL)
e <- sample(haplotypes, Pop_5, replace=TRUE, prob=NULL)
f <- sample(haplotypes, Pop_6, replace=TRUE, prob=NULL)
# For each replicate, take a sample from the background haplotype
# frequencies equal to the observed sample size for each population

pi_a <- 0
pi_b <- 0
pi_c <- 0
pi_d <- 0
pi_e <- 0
pi_f <- 0
# Initializing the 'pi' values for each replicate

j <- 1
while (j < no_haps+1) {
# While j is less than or equal to the total number of haplotypes in
#the population

pi_a <- pi_a + ((sum(a==j))/Pop_1)^2
pi_b <- pi_b + ((sum(b==j))/Pop_2)^2
pi_c <- pi_c + ((sum(cc==j))/Pop_3)^2
pi_d <- pi_d + ((sum(d==j))/Pop_4)^2
pi_e <- pi_e + ((sum(e==j))/Pop_5)^2
pi_f <- pi_f + ((sum(f==j))/Pop_6)^2

# Count the number of haplotypes in each population that equals
# haplotype code 'j'. Divide them by the total sample size (to get the
# frequency of that haplotype in the sample) and square this
# difference. Add this to the value calculated for other haplotypes.

j <- j+1
# The counter for the while loop goes up one (we move on and do these
# calculations for the next haplotype)
}

Pop_1H <- Pop_1/(Pop_1 - 1)*(1-pi_a)
Pop_2H <- Pop_2/(Pop_2 - 1)*(1-pi_b)
Pop_3H <- Pop_3/(Pop_3 - 1)*(1-pi_c)
```

Figure III.1 (Continued)

```
Pop_4H <- Pop_4/(Pop_4 - 1)*(1-pi_d)
Pop_5H <- Pop_5/(Pop_5 - 1)*(1-pi_e)
Pop_6H <- Pop_6/(Pop_6 - 1)*(1-pi_f)
# Using the values calculated in the while loop above, we calculate
# haplotype diversity for each of our samples

diff_Pop_12 <- rbind(diff_Pop_12, abs(Pop_1H - Pop_2H))
diff_Pop_13 <- rbind(diff_Pop_13, abs(Pop_1H - Pop_3H))
diff_Pop_14 <- rbind(diff_Pop_14, abs(Pop_1H - Pop_4H))
diff_Pop_15 <- rbind(diff_Pop_15, abs(Pop_1H - Pop_5H))
diff_Pop_16 <- rbind(diff_Pop_16, abs(Pop_1H - Pop_6H))
diff_Pop_23 <- rbind(diff_Pop_23, abs(Pop_2H - Pop_3H))
diff_Pop_24 <- rbind(diff_Pop_24, abs(Pop_2H - Pop_4H))
diff_Pop_25 <- rbind(diff_Pop_25, abs(Pop_2H - Pop_5H))
diff_Pop_26 <- rbind(diff_Pop_26, abs(Pop_2H - Pop_6H))
diff_Pop_34 <- rbind(diff_Pop_34, abs(Pop_3H - Pop_4H))
diff_Pop_35 <- rbind(diff_Pop_35, abs(Pop_3H - Pop_5H))
diff_Pop_36 <- rbind(diff_Pop_36, abs(Pop_3H - Pop_6H))
diff_Pop_45 <- rbind(diff_Pop_45, abs(Pop_4H - Pop_5H))
diff_Pop_46 <- rbind(diff_Pop_46, abs(Pop_4H - Pop_6H))
diff_Pop_56 <- rbind(diff_Pop_56, abs(Pop_5H - Pop_6H))
# We then record the differences in haplotype diversity between
# populations based on this random sampling procedure. The for (i)
# loop then resets and we do this for the number of permutations (1000)
# that we defined
}

result_table <- cbind(diff_Pop_12, diff_Pop_13, diff_Pop_14,
diff_Pop_15, diff_Pop_16, diff_Pop_23, diff_Pop_24, diff_Pop_25,
diff_Pop_26, diff_Pop_34, diff_Pop_35, diff_Pop_36, diff_Pop_45,
diff_Pop_46, diff_Pop_56)
# This summarizes the differences in haplotype diversity between each
# of our populations, for each of our 1,000 permutations

write.table(result_table, "haplotype_diversity_differences.txt",
sep="\t")
# Using this table of haplotype diversity differences based on the
# random sampling procedure, we assess the significance of our observed
# differences in haplotype diversity between populations
```

Appendix Figure III.2: R code for measuring the nearest geographic distance between samples located in different geographic areas

```
#Make sure geosphere and aspaces packages are loaded and your input
# file has no headers
rm(list=ls()) # This just clears off variables that were already in
# your R space

temp_file <- read.csv(file="Input.csv",sep=",",
stringsAsFactors=FALSE,header=FALSE) #read in your csv file. The
#following column format is expected (but include no headers):
#Ocean    Region    Sample_name    Lat(decimal degrees)    Long(decimal
#degrees)
#Each row should have a separate sample listed. This script is designed
#to find the nearest distance between different regions located in the
#same ocean
maxcolumn <- dim(temp_file)[1]  #just getting some dimensions for the
#for loop below
maxrow <- dim(temp_file)[2]     #just getting some dimensions for the
# for loop below
output <- c("Sample_1", "Lat1", "Long1", "Sample_2", "Lat2", "Long2",
"Dist", "Area1", "Area2", "Comparison") #defining
#how many fields are in our output table "output"

for (i in 1:maxcolumn) { #for each sample
for (w in 1:maxcolumn) { #compare it to every other sample that...
if ((temp_file[i,2])!=(temp_file[w,2])) {  #is NOT from the same region
if ((temp_file[i,1])==(temp_file[w,1])) {  #but IS from the same ocean
#(because the nearest distance between oceans is over land, we manually
#calculate the nearest distance between oceans to ensure a non-
# terrestrial path)
if (i < w) {
distance <- acos(cos(as_radians(90-temp_file[w,4]))*cos(as_radians(90-
temp_file[i,4]))+sin(as_radians(90-temp_file[w,4]))*sin(as_radians(90-
temp_file[i,4]))*cos(as_radians(temp_file[w,5]-temp_file[i,5])))*6371
#calculating the distance between samples in km
temp <-
cbind(temp_file[i,3],temp_file[i,4],temp_file[i,5],temp_file[w,3],temp_
file[w,4],temp_file[w,5], distance, temp_file[i,2],temp_file[w,2],
temp_file[i,1]) #for each sample, binding together the fields we
# mentioned in output above
output <- rbind(output, temp) } } } } }  #binding all the rows together
#to make a full table

write.csv(output,file="output.csv") #writing results out
```

Appendix Table III.1: Summary of microsatellite loci over 278 individuals genotyped in this study. *N* gives the number of individuals successfully typed at each locus. K is the number of alleles per locus. $\mathbf{T^{o}_{A}}$ is the annealing temperature used for each locus. H_o is the observed heterozygosity and H_e is the expected heterozygosity calculated for each locus.

Locus	**N**	**K**	Mg^{+2} (mM)	T^{o}_{A}	Ho	He	Label	Reference
DlrFCB1	302	11	2.5	45	0.925	0.743	VIC	**(Buchanan *et al.* 1996**
GT6	261	10	1.5	61	0.804	0.858	VIC	**(Caldwell *et al.* 2002)**
GT39	308	13	2.5	62	0.766	0.790	VIC	**(Caldwell *et al.* 2002)**
Ppho110	289	3	1.5	60	0.365	0.359	FAM	**(Rosel *et al.* 1999)**
415/416	301	9	2.5	45	0.700	0.726	NED	**(Amos *et al.* 1993)**
MK5	291	14	1.5	55	0.907	0.807	VIC	**(Krützen *et al.* 2001)**
MK6	297	9	1.5	50	0.558	0.534	NED	**(Krützen *et al.* 2001)**
MK8	282	10	1.5	50	0.755	0.838	NED	**(Krützen *et al.* 2001)**
MK9	278	8	1.5	50	0.818	0.815	VIC	**(Krützen *et al.* 2001)**
Sgui 3	307	6	2.5	57	0.624	0.702	FAM	**(Cunha & Watts 2007**
Sgui 17	299	6	2.5	57	0.487	0.511	NED	**(Cunha & Watts 2007**
KWM12	302	10	1.5	45	0.741	0.699	VIC	**(Hoelzel *et al.* 1998)**
TexVet5	261	5	1.5	50	0.633	0.683	FAM	**(Rooney *et al.* 1999)**
SloSl-9	269	7	2.5	55; 53.5	0.465	0.494	NED	**(Galver 2002)**
SloSl-4	270	12	2.5	50.5; 50	0.677	0.701	NED	**(Galver 2002)**

Appendix Table III.2: Pairwise Φ_{ST} differentiation below diagonal and G''_{ST} above the diagonal among populations in the Hawaiian, Society and Samoan Islands mtDNA control region Statistically significant Φ_{ST} values are bolded and italicized, with * significant at $p < 0.05$; ** significant at $p < 0.001$.

	n mtDNA	Kaua`i	NWHI	O‘ahu	Hawai‘i	Mo‘orea/ Tahiti	Ra‘iatea/ Huahine	Samoan Islands
Kaua‘i	97		0.079	0.065	***0.064***	***0.143***	***0.220***	***0.161***
NWHI	18	0.004		0.076	***0.047***	***0.126***	***0.294***	***0.213***
O‘ahu	9	0.021	0.008		***0.100***	***0.115***	***0.312***	***0.146***
Hawai‘i	56	0.235**	0.092*	0.216**		***0.149***	***0.281***	***0.148***
Mo‘orea/ Tahiti	54	0.239**	0.387**	0.423**	0.378**		***0.248***	***0.234***
Ra‘iatea/ Huahine	20	0.342**	0.219**	0.598**	0.571**	0.591**		***0.207***
Samoan Islands	16	0.356**	0.237**	0.513**	0.377**	0.394**	0.222**	

Appendix Table III.3: P values generated for population comparisons of haplotype diversity derived from mtDNA control region haplotype frequencies for rough-toothed dolphins in the Hawaiian, Society and Samoan Islands. Significant values are bolded and italicized.

	Kaua‘i	NWHI	O‘ahu	Hawai‘i	Mo‘orea/ Tahiti	Ra‘iatea/ Huahine	Samoan Islands
Kaua‘i							
NWHI	***0.013***						
O`ahu	***0.046***	0.06					
Hawai‘i	***0.006***	***0.019***	***0.041***				
Mo‘orea/ Tahiti	***0.008***	***0.006***	***0.045***	***0.013***			
Ra‘iatea/ Huahine	***0.008***	***0.006***	***0.047***	***0.013***	***<0.01***		
Samoan Islands	***<0.001***	***0.013***	***0.047***	***0.007***	***0.007***	***0.007***	

Appendix Table III.4: P values generated for archipelago comparisons of haplotype diversity derived from mtDNA control region haplotype frequencies for rough-toothed dolphins in the Hawaiian, Society and Samoan Islands. Significant values are bolded and italicized.

	Hawaiian Islands	Society Islands	Samoan Islands
Hawaiian Islands			
Society Islands	***0.019***		
Samoan Islands	***0.001***	***0.009***	

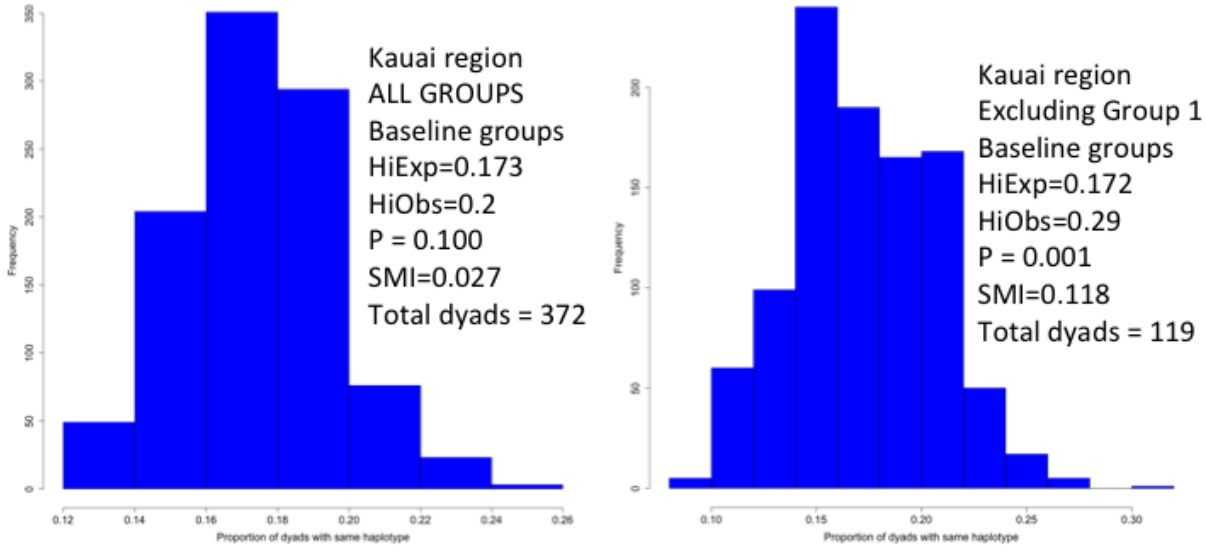
APPENDIX IV

Are you my mother? A measure of matrilineal social organization in social groups and mass strandings of rough-toothed dolphins (*Steno bredanensis*)

Electronic files available for Chapter 4
Genotypes for individuals sequenced in groups in this chapter
File name: Nuclear_genotypes_Groups_Ch4_Sbr_24Nov14.xlsx

mtDNA haplotypes for groups including sex and GPS location
File name: mtDNA_Groups_Sbr_24Nov14.xlsx

Appendix Figure IV.1: Histograms for the region of Kaua‘i with (a) all groups included and (b) when the largest group (estimated group size of 90 individuals) is removed. Hi_{Exp}, Hi_{Obs} and SMI are derived as explained in Appendix Table III.1 above.



Appendix Figure IV.2.
R script for generating the null distribution of expected same-haplotype dyads, based on mtDNA haplotype frequencies for each island population used to calculate the expected haplotype identity for the Standard Matrilineality Index.

```
A <- rep.int (1,  4)
B <- rep.int (2,  36)
C <- rep.int (3,  15)
X <- rep.int (4,  156)
Y <- rep.int (5,  19)

x <- c(A,B,C,X,Y)

# x is an array of your regional haplotypes. These need to be coded as numbers

ndyads <-268
# This is the number of within-group dyads you calculate directly from your group data

reps <- 1000
# This is the number of times you want to simulate drawing the same number of within-group dyad pairs (ndyads) from your total regional haplotypes

result_table <- NULL
# This will be the table results are ultimately written to

j <- NULL

for (j in 1:reps) {
e <- 1
# e is used as the counter to get ndyad draws from the regional haplotype definitions
matrl_count <- 0
# This will be a count for the number of pairs over each ndyad draw that have the same haplotype
```

```
while (e <= ndyads) {
# while e is less than ndyads: this just means that we are doing ndyad draws

g <- sample(x, 2, replace=TRUE, prob=NULL);
# Sampling 2 individuals, i.e. a dyad from our data
if(g[1]==g[2]) {matrl_count <- matrl_count + 1 }
e <- e+1
}
rep_result <- matrl_count/ndyads
result_table <- rbind(result_table, rep_result)
}

write.table(result_table, "null_matrilineality_index.txt", sep="\t"
```

Appendix Figure III.3.
R script for generating the histogram

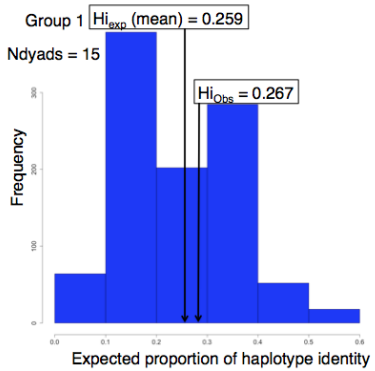
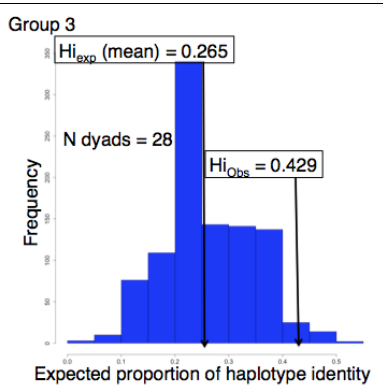
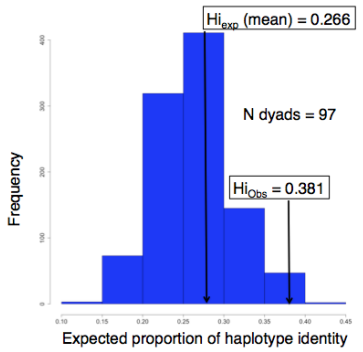
```
file.data <- read.csv(file =" file.csv", header = TRUE, check.names=TRUE)
file.breaks <- read.csv(file=" file.csv", header = TRUE, check.names=TRUE)

png(filename=" file -Histogram.png", width = 1500, height = 1200)
hist(x= file.data$x, breaks = file.breaks$bin, xlab = "X LABEL HERE", ylab = "Y LABEL HERE", main = "TITLE HERE OR
EMPTY IF NONE", cex.lab = 1, cex.axis = 1.5, cex.main = 3, col = 'blue')
dev.off()

#cex.lab - % font size for labels
#cex.axis - % font size for axis tick marks
```

Appendix Table IV.1. An example of the calculation of the SMI with four groups and four haplotypes present in an island population (A, B, C, D). The number of same-haplotype dyads within a group are calculated by haplotype, and then summed to give the total number of same-haplotype dyads within a group. The proportion relative to the total number of within group dyads is then calculated (Hi_{obs}). Using the baseline island community haplotype frequencies (shown here as the 'regional totals'), Monte Carlo sampling of the expected proportion of same-haplotype frequencies is carried out (code in Appendix I. The mean of this distribution (Hi_{exp}) can be used to calculate the SMI ($Hi_{obs} - Hi_{exp}$). Significance can be assessed as the number of permutations that exceed Hi_{obs} divided by the total number of permutations. As different sizes of groups (n = 6, 15 dyads; and n = 7, 21 dyads) were present in this example, four separate Monte Carlo simulations were carried out. The same calculations can also be carried out at the island level by summing over the same-haplotype within-group dyads and total within group dyads calculated on a per-group basis (shown in right column of table). The observed haplotype identify proportion was calculated for each group in an island community by combining the number of possible dyad combinations for each haplotype present within a group (x*(x-1)/2) where x is the number of individuals with that haplotype within that group.

Haplotype	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Regional totals**
A	2	2	0	4	8
B	3	2	0	0	5
C	1	0	4	5	10
D	0	3	4	0	7
Total group size	6	7	8	9	
Observed haplotype A-A dyads	= (2*(2-1))/2 = 1	1	0	6	= 1 + 0 + 0 + 6 = 7
Observed haplotype B-B dyads	= (3*(3-1))/2 = 3	1	0	0	= 3 + 1 + 0 + 0 = 2
Observed haplotype C-C dyads	= (1*(1-1))/2 = 0	0	6	10	= 0 + 0 + 6 +10 = 16
Observed haplotype D-D dyads	= (0*(0-1))/2 = 0	3	6	0	= 0 + 3 + 6 + 0 = 12
Total observed same haplotype dyads	= 1 + 3 + 0 + 0 = 4	5	12	16	= 7 + 2 + 16 + 12 = 37
Total within group dyads	= (6*(6-1))/2 = 15	21	28	36	= 15 + 21 + 28 + 36 = 97
Observed proportion of haplotype identity (Hi_{obs})	= 4/15 = 0.267	0.190	0.429	0.444	= 37/97 = 0.381

			
Expected mean prop. of haplotype identity (Hi_{exp}) Standardized matrilineality index = Hi_{obs} - Hi_{exp} Significance = $\frac{\text{no. of permutations} \geq Hi_{obs}}{\text{total no. of permutations}}$	= 0.259 (from graph for number 1) = (0.267 - 0.259) = 0.008 = 356/1000 = 0.356	0.265 (from graph for number 3) 0.164 0.016	0.266 for regional total 0.115 0.009

Appendix Table IV.2. Background haplotype frequencies used to generate the null distribution for each region. Not all rough-toothed dolphin haplotypes are represented in groups, and frequencies shown here are restricted to groups. A list of haplotype frequencies from the entire rough-toothed dolphin dataset can be found in electronic Appendix I.

Haplotype ID	Atlantic	ETP	Kauai	Oahu	Hawaii	Moorea	Raiatea	Samoa	Total
A	0	0	0	0	0	1	0	0	1
B	0	0	0	0	4	0	0	0	4
C	0	0	0	0	0	7	0	0	7
D	0	2	0	0	0	0	1	0	3
E	0	0	0	0	0	0	11	13	24
F	0	0	7	0	0	0	0	0	7
G	0	0	5	4	0	0	0	0	9
H	0	0	4	0	11	0	0	1	16
I	0	0	0	0	9	0	0	0	9
K	0	0	20	2	1	0	0	0	23
L	0	0	3	0	0	0	0	0	3
M	0	0	0	0	3	0	0	1	1
O	0	0	0	0	0	36	0	0	36
P	0	5	0	1	0	4	0	0	10
T	0	0	0	0	1	0	0	0	1
U	0	0	18	3	0	0	0	0	21
V	0	0	0	0	0	0	2	1	3
X	0	0	6	0	0	0	0	0	6
AA	0	2	0	0	0	0	0	0	2
BB	0	1	0	0	0	0	0	0	1
CC	0	1	0	0	0	0	0	0	1
DD	0	1	0	0	0	0	0	0	2
EE	0	1	0	0	0	0	0	0	1
FF	0	5	0	0	0	0	0	0	5
HH	0	3	0	0	0	0	0	0	6
MM	1	0	0	0	0	0	0	0	1
NN	1	0	0	0	0	0	0	0	1
SS	10	0	0	0	0	0	0	0	10
Total	12	21	63	10	29	48	14	16	214

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