Population Structure of Island-Associated Pantropical Spotted Dolphins (Stenella attenuata) in Hawaiian Waters

Sarah Shelby Courbis
Portland State University
Population Structure of Island-Associated Pantropical Spotted Dolphins (*Stenella attenuata*) in Hawaiian Waters

by

Sarah Shelby Courbis

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Biology

Dissertation Committee:
Deborah Duffield, Chair
Robin W. Baird
Mitch Cruzan
Suzanne Estes
Natalie Vasey

Portland State University
2011
Abstract

Understanding gene flow, diversity, and dispersal patterns is important for predicting effects of natural events and anthropogenic activities on dolphin populations. With the very recent exceptions of false killer whales (*Pseudorca crassidens*), spinner dolphins (*Stenella longirostris*), and common bottlenose dolphins (*Tursiops truncatus*), Hawaiian odontocete species are managed as single stocks within the U.S. Hawaiian Exclusive Economic Zone. These exceptions are a result of recent studies that have indicated that some species have populations that show fidelity to individual islands or groups of islands, resulting in genetic differentiation, often with management implications. The first part of my study (following the introductory chapter) focused on population structure of pantropical spotted dolphins (*Stenella attenuata*) near the Hawaiian Islands. Because of the level of human interaction, pantropical spotted dolphin populations need to be defined accurately to be managed in a way that will avoid local population losses, especially given that the commercial and recreational troll fisheries near the islands “fish on dolphins” to catch tuna. I analyzed genetic samples for mtDNA and microsatellite loci from four island regions: Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau. My results support genetic differentiation among the regions of Hawai‘i, the 4-islands area, and O‘ahu and suggest that pantropical spotted dolphins near Kaua‘i/Ni‘ihau are likely transient and in very low numbers. There was no strong evidence to support sex-biased dispersal or group fidelity. Possibly, differentiation is mediated by behavior adapted to differing habitat types. From a management perspective, spinner and bottlenose dolphin populations near the Hawaiian Islands have
been split into separate stocks for management based on levels of genetic differentiation similar to those found for pantropical spotted dolphins. These precedents suggest that comparable action should be taken to split pantropical spotted dolphin stocks near the Hawaiian Islands.

Most population studies rely heavily upon fixation indices like $F_{ST}$ to determine whether populations are genetically differentiated. When $F_{ST}$ values are low but significantly different from zero, it can be difficult to interpret the biological significance of these values. As part of my study, I suggest that one way to evaluate whether small $F_{ST}$ values indicate significant differentiation is to compare $F_{ST}$ values with other populations considered to be separate based on factors such as extreme distance or morphological differences. I examined pantropical spotted dolphins from the coastal and offshore Eastern Tropical Pacific (ETP), Hawaiian Islands, and China/Taiwan to examine the utility of comparing $F_{ST}$ values across separate populations. Among Hawaiian Island regions, $F_{ST}$ values are significantly different from zero but small. The comparison of these $F_{ST}$ values with more distant populations in the ETP and China/Taiwan indicated that differences among Hawaiian Island regions were similar in magnitude to those found between the offshore and coastal ETP sub-species, but smaller than between the Hawaiian Island regions and the other regions examined. This suggests a level of reproductive isolation among the Hawaiian Islands regions that is comparable to that of offshore and coastal ETP populations, and supports the value of fixation index comparisons in evaluating differentiation among putative populations. My results suggest that assigning specific numerical baseline $F_{ST}$ values may not always be biologically
meaningful but that determining whether related populations with geographic or other separation show a preponderance of similar, lower, or higher fixation index values can help evaluate whether genetic differences among sympatric or parapatric groups warrants designating them as separate populations for management.

Lastly, I explore whether the fast evolving mtDNA control region may be more suited to phylogenetic comparisons among the *Stenella* than slower evolving gene regions and whether the small number of haplotypes generally used in phylogenetic analyses is adequate for defining relationships among dolphins. Usually, slow evolving regions, such as gene regions, are used in phylogenetic analyses because species and genera have been isolated long enough for variation to have accumulated in such regions but not so long that many reversals (*i.e.* a mutational change in sequence that later changes back to the original sequence) have occurred. The mtDNA control region is typically used for population genetic comparisons rather than phylogenetic comparisons because it is considered to be a fast evolving region. Historically, dolphin phylogeny has been examined using gene regions, which have resulted in ambiguous and unexpected relationships. However, the lack of variation in the mtDNA control region for pantropical spotted dolphin populations and the fact that recent studies have found that the mtDNA control region in cetaceans evolves at about one quarter the rate of other mammals, raises the question as to whether this region would be better suited to phylogenetic studies for the *Stenella* (and potentially other dolphin species). In comparing 346 haplotypes from five species of *Stenella* world-wide, I found that the mtDNA control region is probably not a good region to use for phylogenetic analyses,
and that even faster evolving regions might perform better. The differences in the
mtDNA control region were not sufficient to distinguish clear relationships among the
*Stenella*. I also found that when subsets of haplotypes chosen at random were compared,
the results differed among comparisons, suggesting that there is value in using more than
the usual one or two haplotypes when making phylogenetic comparisons. Given the
recent increases in sequence availability (*e.g.* GenBank) and computing power,
researchers should strongly consider using many haplotypes from a variety of populations
in their phylogenetic comparisons.
Table of Contents

Abstract ................................................................................................................................. i
List of Tables .......................................................................................................................... vii
List of Figures ....................................................................................................................... viii
CHAPTER 1: Introduction to Population Genetics & Spotted Dolphins .................................. 1
  Pantropical Spotted Dolphins in the Eastern Tropical Pacific and China/Taiwan ............ 1
  Pantropical Spotted Dolphins and Atlantic Spotted Dolphins in the Atlantic ............... 3
  Pantropical Spotted Dolphins near the Hawaiian Islands .............................................. 4
  Genetic Studies of Other Odontocete Species .............................................................. 7
Hypotheses ............................................................................................................................ 12
Significance ......................................................................................................................... 13
Project Background .......................................................................................................... 17
Population Genetics Background .................................................................................... 19
Population Genetics Statistics ......................................................................................... 21
Phylogenetics Statistics .................................................................................................... 23

CHAPTER 2: Evidence of Multiple Populations of Pantropical Spotted Dolphins
  (Stenella attenuata) within Hawaiian Waters ................................................................. 24
  Introduction ....................................................................................................................... 24
Methods ............................................................................................................................... 29
  Study site and sample collection .................................................................................. 29
  Sexing, mtDNA, & Microsatellites ............................................................................... 30
  Statistical Analyses ........................................................................................................ 32
Results .................................................................................................................................. 39
  General results ............................................................................................................... 39
  Relationships among the Regions ................................................................................ 42
Tables & Figures ................................................................................................................. 46
Discussion ............................................................................................................................ 61

CHAPTER 3. Comparisons to explore potential baselines for biologically significant
genetic differentiation levels: Population structure of pantropical spotted dolphins in the
North Pacific Ocean as an example ...................................................................................... 72
  Introduction ....................................................................................................................... 72
Methods ............................................................................................................................... 77
  Microsatellites ................................................................................................................. 78
  MtDNA ............................................................................................................................. 81
Results .................................................................................................................................. 82
  Microsatellites ............................................................................................................... 82
  MtDNA ............................................................................................................................ 85
Tables & Figures ................................................................................................................. 87
Discussion ............................................................................................................................ 100
  General Relationships .................................................................................................. 100
  Baselines ....................................................................................................................... 103

CHAPTER 4. Are small sample sizes and use of slow evolving DNA regions hindering
resolution of the phylogenetic relationships among delphinids: Stenella as an example
genus ..................................................................................................................................... 109
Introduction ................................................................. 109
Methods ............................................................................. 113
Results .............................................................................. 115
Tables & Figures .................................................................. 118
Discussion .......................................................................... 123
CHAPTER 5: Conclusions .................................................. 127
References .......................................................................... 128
Appendices .......................................................................... 147
  Appendix A: DNA Extraction Procedures ..................... 147
  Appendix B: DNA Purification Procedures ...................... 148
  Appendix C: Primer Sequences ....................................... 149
  Appendix D: Phylogenetic Consensus Tree ..................... 150
List of Tables

Table 1: Total number of samples at each island region, the number of groups from which samples were obtained, number of males and females, group size range, depth range, and distance from shore range and observation effort near the Hawaiian Islands 2002-2008 for all observations (not just sampling events) ... p 54.

Table 2: Number of alleles, allelic diversity, and expected & observed heterozygosity at each microsatellite locus for pantropical spotted dolphin samples collected near the Hawaiian Islands 2002-2003 ... p 55.

Table 3: Assignment probability to each of three clusters from TESS analysis ... p 56.

Table 4: Mean pairwise relatedness comparisons between regions ... p 57.

Table 5: $F_{ST}$ and $R_{ST}$ for microsatellite data; $F_{ST}$ and $\Phi_{ST}$ for mtDNA sequences; and $F'_{ST}$ (standardized $F_{ST}$) ... p 58.

Table 6: Allelic diversity for microsatellites and diversity, nucleotide diversity, and mean pairwise differences among mtDNA haplotypes ... p 59.

Table 7: Migration rate ($N_{m}$) between pairs of regions in Hawaiian Islands waters ... p 59.

Table 8: Mean pairwise relatedness comparisons for dolphins sampled in the same group and in different groups for each region ... p 60.

Table 9: Assignment probability to each of five clusters from STRUCTURE analysis ... p 94.

Table 10: $F_{ST}$ and $R_{ST}$ for microsatellite data, and $F'_{ST}$ (standardized $F_{ST}$) ... p 95.

Table 11: Migration rate ($N_{m}$) between pairs of regions ... p 96.

Table 12: Number of overlapping mtDNA haplotypes among regions ... p 97.

Table 13: $F_{ST}$ and $\Phi_{ST}$ for mtDNA sequences, and $F'_{ST}$ (standardized $F_{ST}$) ... pp 98 & 99.

Table 14: Haplotypic diversity, nucleotide diversity, and mean pairwise differences among haplotypes ... p 99.

Table 15: Sequences used in this study ... pp 121 & 122.
List of Figures

Figure 1: Photo of a small vessel fishing for tuna over a group of pantropical spotted dolphins off the island of Hawai‘i May 11, 2011. Photo provided by Cascadia Research Collective...p 47.

Figure 2: Locations of samples, sightings without samples, and search effort for pantropical spotted dolphins near the main Hawaiian Islands 2002-2008...p 48.

Figure 3: Correlogram showing combined genetic correlation for microsatellite loci as a function of distance...p 49.

Figure 4: Mean DIC for each K value run in TESS...p 50.

Figure 5: Example of assignment probability bar plot for K=3 from TESS analysis...p 51.

Figure 6: Mean number of distinct alleles per locus estimated based on sample size in ADZE and mean number of private alleles per locus estimated based on sample size using ADZE...p 52.

Figure 7: Haplotype network generated in TCS...p 53.

Figure 8: Locations of sampling are shown for the Eastern Tropical Pacific, China/Taiwan, the pelagic, and Hawaiian Islands...p 88.

Figure 9: Mean posterior probability from STRUCTURE analyses...p 89.

Figure 10: STRUCTURE bar plot by region of assignment probabilities for K=5... p 90.

Figure 11: Mean number of distinct alleles per locus estimated based on sample size in ADZE, and mean number of private alleles per locus estimated based on sample size using ADZE...p 91.

Figure 12: Mean number of private alleles per locus for pairs of regions, and mean number of private alleles per locus for triads of regions...pp 92 & 93.

Figure 13: Simplified phylogenetic tree obtained with Maximum Likelihood Analysis in GARLI...p 119.

Figure 14: Phylogenetic trees obtained with Maximum Likelihood Analysis in GARLI each using 1000 bootstrap replicates comparing mtDNA control region sequences for three sets of random pairs of haplotypes of each Stenella species...p 120.
CHAPTER 1: Introduction to Population Genetics & Spotted Dolphins

My study focused on population structure of pantropical spotted dolphins 
(*Stenella attenuata*) near the Hawaiian Islands, ranging from the island of Hawai`i to the 
island of Ni‘ihau, using mitochondrial DNA (mtDNA) and microsatellites (nuclear 
DNA). I also explored genetic relationships among pantropical spotted dolphins in the 
Eastern Tropical Pacific (ETP), near the Hawaiian Islands, in pelagic waters near the 
Hawaiian Islands Exclusive Economic Zone (EEZ), and near China/Taiwan to compare 
fixation index values in an effort to establish whether this method could be used to assess 
whether low levels of genetic differentiation are likely to be biologically significant. 
Further, I used published DNA sequences and sequences from my study to investigate 
phylogenetic relationships among the *Stenella* as a genus and assess the use of the 
mtDNA control region as a phylogenetic, rather than population genetic, marker for 
*Stenella*.

**Pantropical Spotted Dolphins in the Eastern Tropical Pacific and China/Taiwan**

Pantropical spotted dolphins, as the name suggests, have a tropical, sub-tropical, 
and warm-temperate distribution, which lies between ~40°N and ~40°S latitudes (Perrin 
2001). Extensive studies have been done on the morphological differences among 
pantropical spotted dolphin populations (Douglas *et al.* 1984, Perrin *et al.* 1985, Schnell 
Schnell *et al.* (1986) and Yao *et al.* (2008) found that oceanographic conditions 
correlated with some morphological characteristics of pantropical spotted dolphins in the
offshore Eastern Tropical Pacific and near China and Taiwan respectively. For example, Schnell et al. (1986) found that cranial morphology, such as width of the temporal fossa, and environmental measures, such as sea surface temperature and thickness of the oxygen minimal layer, both tended to be strongly clinal, suggesting ocean conditions affected physical characteristics. However, they found a strong division between northern and southern offshore populations. Based on morphological differences (Perrin et al. 1985), and more recently on genetic evidence (Escorza-Treviño et al. 2005, Rosales 2005), pantropical spotted dolphins in the ETP are considered to be two sub-species: offshore (S. a. attenuata) and coastal (S. a. graffmani). The offshore sub-species have been further subdivided into northern and southern populations based on differences in morphology, differences in seasonality of calving, and a potential gap in distribution (Barlow 1984, Perrin et al. 1985, Schnell et al. 1986). Dizon et al. (1994b) defined them as a northeastern and a southern-western stock. There is geographical overlap among the morphotypes that could allow interbreeding to occur (Perrin et al. 1985). Generally, the coastal sub-species is described as occurring within 100nmi of the coast, although the offshore sub-species has been recorded within 16nmi of the coast (Perrin et al. 1985). The northeastern and southern-western offshore stocks are generally divided near the equator (Perrin et al. 1985).

Escorza-Treviño et al. (2005) investigated genetic differentiation of pantropical spotted dolphins of the coastal and offshore sub-species in the ETP near Mexico and Central America. They also investigated population structure within the coastal sub-species. Both mitochondrial and microsatellite analyses showed that genetic
differentiation exists between coastal and offshore dolphins despite overlap in their ranges. In addition, mitochondrial results indicated genetic partitioning among the coastal dolphins in six geographic locations based on genetic distances ($F_{ST}$ and $\Phi_{ST}$) among haplotypes. However, microsatellite loci revealed low levels of genetic partitioning among these coastal areas. These results suggested differing dispersal rates between sexes, with females showing higher philopatry and therefore local differentiation. Subsequent analyses with additional samples from coastal areas resulted in nine distinct populations (Rosales 2005), although Rosales (pers. comm.) reduced this to seven upon further analyses.

Pantropical spotted dolphins near eastern Taiwan, in the Taiwan Strait, and in the South China Sea showed significant differentiation in mtDNA between the South China Sea and the other two locations based on $F_{ST}$ analysis, but the difference was not significant based upon $\Phi_{ST}$ analysis (Yao et al. 2004).

Pantropical Spotted Dolphins and Atlantic Spotted Dolphins in the Atlantic

In the Atlantic, two species of spotted dolphins are recognized, pantropical spotted dolphin and Atlantic spotted dolphin ($S. frontalis$). There is no published information regarding the population genetics of pantropical spotted dolphins in the U.S. waters of the Western North Atlantic (Waring et al. 2011). However, some samples from pantropical spotted dolphins in the western Atlantic have been collected, and mtDNA sequences are available on GenBank (Bero 2001, Kingston et al. 2009). Pantropical spotted dolphins in the U.S. Atlantic are divided into two stocks for management:
Northern Gulf of Mexico and Western North Atlantic. The separation of these stocks is provisional, and there is no genetic or morphological evidence available that differentiates these stocks (Waring et al. 2011).

The Atlantic spotted dolphin is also separated into a Western North Atlantic stock and a Northern Gulf of Mexico stock in U.S. waters. This stock delimitation is supported by genetic studies showing significant differences in mitochondrial and nuclear DNA between the two areas (Bero 2001, Adams & Rosel 2006). Adams and Rosel (2006) also reported that mtDNA population differentiation (based upon $F_{ST}$ and $\Phi_{ST}$) was twice as large for females than males, whereas microsatellite population differentiation (based upon $F_{ST}$ and $R_{ST}$) was comparable between the two sexes. This supported female philopatry as a driving force in genetic differentiation among these populations. Martinez-Vergara et al. (2004) reported significant differences in Atlantic spotted dolphin mitochondrial DNA from three geographic locations: North Western Atlantic, Gulf of Mexico, and West Africa. The authors concluded that there has been recent gene mixing and/or moderate to high levels of historical gene flow and suggested that microsatellite markers may be informative in future studies. Currently, a study is underway to assess population genetic structuring of Atlantic spotted dolphins near the Bahamas based on fecal samples (Green et al. 2007).

**Pantropical Spotted Dolphins near the Hawaiian Islands**

Pantropical spotted dolphins are an important part of the Hawaiian ecosystem and economy, interacting with fisheries, acting as predators, prey, and competitors, and are
becoming increasingly important for ecotourism (Carretta et al. 2011). Because of human interaction associated with fishing and ecotourism, pantropical spotted dolphin stocks need to be defined accurately to be managed in a way that will avoid local population losses, especially given that the commercial and recreational troll fisheries near the islands “fish on dolphins” to catch tuna (Figure 1), causing, at least occasionally, dolphins to sustain injuries that are evident in photos (Baird 2009 unpub. data). Rizzuto (2007) reported a fisher describing the “first time” he caught a spotted dolphin on a lure and brought it up to the boat before cutting off the line with the lure still stuck in the dolphin’s mouth. Two decades before Rizzuto, Shallenberger (1981) also reported that trollers fished through groups of pantropical spotted dolphins to catch tuna. Pantropical spotted dolphins near the Hawaiian Islands are managed as a separate stock from ETP pantropical spotted dolphins that are impacted by the tuna purse seine fishery (Carretta et al. 2011). ETP populations have not shown recovery from declines brought on by the fisheries interactions of the 1960s and early 1970s, despite conservation efforts (Gerrodette & Forcada 2005, Cramer et al. 2008).

Dizon et al. (1994b) reported that the Hawaiian stock of pantropical spotted dolphins is discriminated from the other Pacific stocks based on its proximity to the Hawaiian Islands rather than on any genetic, behavioral, or major morphological differences. The stock boundary is the U.S. Exclusive Economic Zone surrounding the Hawaiian Islands (Carretta et al. 2011). Pantropical spotted dolphins near the Hawaiian Islands tend to have less spotting than and differing cranial morphology from northern offshore pantropical spotted dolphins, but similar coloration and cranial morphology to
southern offshore pantropical spotted dolphins (Dizon et al. 1994b). Reeves et al. (2004) stated that numerous additional sub-species of Pacific pantropical spotted dolphins may be added in the future as further studies reveal more information on levels of differentiation. Population size estimates for the Hawaiian Islands regions included in our study area range from approximately 2,928 (CV=0.45) (Mobley et al. 2000) to 4,283 (Barlow 2006), although the area included by Barlow et al. (2006) (212,892 km$^2$) was larger than that of Mobley et al. (2000) (71,954 km$^2$), which may explain the difference in estimates. For the Barlow (2006) estimate an approximate CV of 0.55 was estimated by assuming that the variance in each stratum is proportional to the number of sightings (Barlow, pers. comm.). Within the entire Hawaiian EEZ, population size is estimated to be approximately 8,978 (CV=0.48) (Barlow 2006). CV is the coefficient of variation and measures the ratio of standard deviation to the mean.

Baird et al. (2001) suggested that movements among the islands may be limited based on differences in scar pattern among islands; however, it is unknown whether there are genetically separate stocks of pantropical spotted dolphins near these islands. For my study, four regions were defined, based on distance among islands and depths of the channels between them per Baird et al. (2008b): the island of Hawai‘i; the “4-islands area” including Maui, Lana‘i, Kaho‘olawe, and Moloka‘i; the island of O‘ahu; and the islands of Kaua‘i and Ni‘ihau (Figure 2). In an earlier study, Baird et al. (2001) reported that pantropical spotted dolphins near the 4-islands area were generally found at least 3-5km from shore between the islands in waters 70-300m deep. They concluded that in comparison with other dolphin species, pantropical spotted dolphins near the 4-islands
area have distinct habitat use patterns. Pantropical spotted dolphins showed a tendency to move into deeper water as the day progressed and preferred deeper portions of the study area than spinner and common bottlenose dolphins (Baird et al. 2001). Deeper and longer dives occurred at night, suggesting possible feeding at night (Baird et al. 2001).

If pantropical spotted dolphins near the Hawaiian Islands consist of multiple populations or sub-populations, two possible driving forces for this are female philopatry, as has been seen in pantropical spotted dolphins in the ETP (Escorza-Treviño et al. 2005), and niche specializations. Hoelzel (1998) reviewed cetacean genetics studies and discussed potential reasons for sympatric populations to genetically diverge. He proposed niche specialization as the main force behind sympatric speciation. This kind of specialization can lead to behavioral changes that affect sexual selection. Ultimately, mating location preferences that differ among members of a sympatric species can lead to genetically distinct races that may eventually evolve into separate species (Bush 1994, Hoelzel 1998). As we begin to learn where there are genetic subdivisions in populations, we can build on that foundation to look for causes, such as behavioral isolation created by differential prey preferences, hunting pressures, mating behaviors, etc. and then begin to explore genetic correlates for behaviors. Hoelzel (1998) proposed that learning could be important in maintenance of such specializations.

**Genetic Studies of Other Odontocete Species**

Studies of other dolphin species have shown genetic differentiation of geographically contiguous or overlapping populations. The hypothesis that pantropical
spotted dolphins near the Hawaiian Islands would exhibit site fidelity that would drive genetic differentiation among regions was based on the results of such studies. Among *Stenella*, a recent study of Hawaiian spinner dolphins found significant genetic differentiation among populations near most of the Hawaiian Islands regions (Andrews et al. 2010). Garrison *et al.* (1999) reported that spinner dolphin genetic differentiation was correlated with habitat preference. Spinner dolphins near French Polynesian islands have been found to have small populations, relatively isolated by distance (Oremus *et al.* 2007). Females showed more site fidelity than males (Oremus *et al.* 2007). García-Martínez *et al.* (1999) reported two genetically distinct populations of striped dolphins (*S. coeruleoalba*) in the Mediterranean and Atlantic. They stated that dolphins preferring shallow water or island habitats become isolated. Gaspari *et al.* (2007) found genetic differentiation between striped dolphin populations in the Adriatic and Tyrrhenian Seas and between inshore and offshore populations within the Tyrrhenian Sea.

Among other genera, two previously unrecognized species of sympatric common bottlenose dolphins found in Chinese waters were found to be genetically distinct and are now considered separate species, Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) and common bottlenose dolphins (Wang *et al.* 1999, Yang *et al.* 2005). Hoelzel *et al.* (1998b) found that offshore and nearshore Atlantic common bottlenose dolphin populations were genetically distinct despite geographical overlap; Sellas *et al.* (2005) found that common bottlenose dolphins in the Gulf of Mexico showed population subdivisions indicative of philopatry of both sexes; Parsons *et al.* (2002) determined that common bottlenose dolphins near the United Kingdom had local genetic populations,
some of which were more similar to distant neighboring populations than to nearer neighboring populations; and Dowling and Brown (1993) found that common bottlenose dolphins along the western Atlantic coast showed regional differentiation. Studies of a resident common bottlenose dolphin population on the west coast of Florida have demonstrated a high degree of female philopatry, coupled with a significant level of male-based gene flow in this local population (Duffield & Wells 1991, 2002). Common bottlenose dolphins near New Zealand showed considerable genetic differentiation indicating little gene exchange among Northland, Marlborough Sounds, and Fiordland (Tezanos-Pinto et al. 2009). Common bottlenose dolphins in the Black Sea have been shown to be genetically distinct from those in the Mediterranean (Viaud-Martinez et al. 2008). Parsons et al. (2006) found that there was genetic differentiation between common bottlenose dolphins found near East Abaco, South Abaco, and White Sand Ridge in the Bahamas Islands. Further, Martien et al. (2011) found genetic differentiation among common bottlenose dolphin populations near regions of the Hawaiian Islands. Genetic analyses by Möller and Beheregaray (2004) revealed that Indo-Pacific bottlenose dolphins near Australia showed female philopatry and much less male philopatry than previously suspected. Genetic analyses of Indo-Pacific bottlenose dolphins in Australia have also shown differentiation based on habitat type (Möller et al. 2007, Wiszniewski et al. 2010). Some genetic differentiation has also been found among putative coastal and migratory populations of Indo-Pacific bottlenose dolphins caught in the KwaZulu-Natal shark fishery near South Africa (Natoli et al. 2008). Bottlenose dolphins sampled near Victoria Australia were found to be divergent from both
recognized species of bottlenose dolphins, suggesting the possibility of a third species
(Charlton et al. 2006). What were considered sympatric morphotypes of common
dolphins (Delphinus) in the Northeast Pacific have been shown to be genetically
separated species (Rosel et al. 1994) and have been split into short-beaked common
dolphins (D. delphis) and long-beaked common dolphins (D. capensis) (Heyning &
Perrin 1994). Short-beaked common dolphins have been reported to have genetically
distinct populations within the California/Oregon/Washington stock (Chivers et al. 2003).
Natoli et al. (2006, 2008) have reported genetic differentiation among short-beaked
common dolphin sub-species in the Mediterranean and Black Sea areas. Short-beaked
common dolphins have also been found to have unexpected levels of genetic
differentiation across a distance of ~1500km near Australia, with marked differentiation
between Southern Australia and Southeastern Tasmania (Bilgmann et al. 2008). The
authors suggest differences in temperature, habitat, and fish abundance may contribute to
this genetic isolation (Bilgmann et al. 2008). Short-beaked common dolphins also form
at least two genetically distinct populations in the western and eastern Atlantic Ocean
(Mirimin et al. 2009). Genetic analyses of Hector’s dolphins (Cephalorhynchus hectori)
from three areas of New Zealand indicated female philopatry and lower levels of gene
flow than expected (Pichler et al. 1998). Baker et al. (2002) reported four regional
populations of Hector’s dolphins in New Zealand, including a distinct sub-species, C. h.
maui, near the North Island. Differentiation has been reported among Commerson’s
dolphins (Cephalorhynchus commersonii) in five regions of Tierra del Fuego (Pimper et
al. 2010). Coastal and offshore Pacific white-sided dolphins (Lagenorhynchus
obliquidens) near Japan exhibited severely restricted gene flow between these two parapatric groups (Hayano et al. 2004). However, Cassens et al. (2005) found that dusky dolphins (Lagenorhynchus obscurus) near Argentina and Peru showed male specific gene flow between the populations. Fish-eating and mammal-eating killer whales (Orcinus orca) have been shown to have restricted gene flow between populations despite considerable geographic overlap (Hoelzel 1991, Hoelzel & Dover 1991, Hoelzel et al. 1998a). More recently, analysis of the entire mitochondrial genome suggests that North Pacific mammal-eating killer whales should be considered a separate species (Morin et al. 2010). False killer whales near the Hawaiian Islands are genetically distinct from offshore populations (Chivers et al. 2007, 2010). Frère et al. (2008) found that humpback dolphins (Sousa chinensis) near China and Australia are more genetically diverged than humpback dolphins (S. plumbea) near China and South Africa, calling their current taxonomy into question. Unique haplotypes of humpback dolphins have also been found near India (Jayasankar et al. 2008).

On the other hand, Cassens et al. (2003) reported a matrilineal genealogy of dusky dolphins that showed no obvious geographical partitioning between the Eastern and Western Atlantic, indicating that the separation of the populations was very recent or some animals occasionally cross the ocean. Also, Harlin et al. (2003) found that dusky dolphins along the New Zealand coast did not show genetic subdivisions among regions, and Jansen van Vuuren et al. (2002) did not find significant genetic differences between populations of Heaviside’s dolphin (Cephalorhynchus heavisidii) from South Africa and Nambia, although they did find some variation within the two geographic regions. Also,
no evidence of significant mtDNA differentiation was found between northern right whale dolphins (*Lissodelphis borealis*) in the offshore Pacific and along the U.S. west coast (Dizon et al. 1994a). Further, Atlantic white-sided dolphins (*Lagenorhynchus acutus*) near Ireland were found to be a large, single population along the coast (Mirimin et al. 2011). In addition, when Quérouil et al. (2007) compared common bottlenose dolphins among two archipelagos (Azores and Madeira) and the Atlantic Basin, they did not find significant differentiation within or among these three locations, concluding that pelagic bottlenose dolphins in the eastern temperate North Atlantic belong to one oceanic population. Quérouil et al. (2010) reported similar results for short-beaked common dolphins and Atlantic spotted dolphins in the same regions.

**Hypotheses**

I hypothesize that island-associated populations of pantropical spotted dolphins will exhibit female philopatry and/or niche specializations resulting in genetic differentiation among island regions. This hypothesis is based on previous studies that suggest female site fidelity among some dolphins, for example, spinner dolphins near French Polynesia (Oremus et al. 2007), and on other studies that have shown that dolphin habitat use patterns can correlate to genetic differentiation (e.g. Möller et al. 2006, Bilgmann et al. 2008). I expect to find evidence of significant differentiation among microsatellites and mtDNA sequences from different island regions in the Hawaiian Islands, as has been found for spinner dolphins (Andrews et al. 2010) and common bottlenose dolphins (Martien et al. 2011) near these islands. If female philopatry is
occurring, mtDNA population differentiation will be higher for females than corresponding males while microsatellite population differentiation will be similar when analyzed separately by sex, as was found for pantropical spotted dolphins in the ETP (Escorza-Treviño et al. 2005). I expect intra-group relatedness to be higher than inter-group relatedness if sex-biased dispersal is occurring because it is likely females would stay with natal groups. For many mammal species, females stay in family groups throughout their lives (Greenwood 1980). Female group fidelity occurs in at least some dolphin populations (Möller et al. 2006).

**Significance**

Understanding dispersal and gene flow among populations is important for predicting effects of human activities (e.g. fishing, introduction of exotics, pollution) and natural events (e.g. hurricanes) on populations (Kokko & López-Sepulcre 2006). Intrinsic species properties can have profound effects on the ability of populations to disperse to new habitats and maintain patterns of dispersal and gene flow, regardless of geographic features of the environment (Kokko & López-Sepulcre 2006). My study aims to elucidate patterns of gene flow and dispersal for an island-associated dolphin species, thereby providing data for design of models to predict these parameters in other island-associated dolphin populations and to predict how populations may react to anthropogenic activity (e.g. predict if disturbed dolphins near one island would disperse to a different island). Models of this nature are particularly important for conservation and management of elusive, endangered, and/or otherwise strategic populations for which
data cannot be or has not been collected. These data can also be more broadly applied through comparisons with other vertebrate species, such as island-associated marine turtles and fish, to explore more general barriers to gene flow in island ecosystems.

Known differences or similarities in dispersal and gene flow patterns among island-associated species can be used in conjunction with differences and similarities in habitat and prey preferences and other niche specializations. These will help us begin to determine what environmental and life history factors will allow us to predict patterns of dispersal and gene flow in other island-associated dolphin species. For example, Möller et al. (2007) found that habitat type affected gene flow among Indo-Pacific bottlenose dolphins near Port Stephens, Australia. Similar habitat differences elsewhere, in this case open coast versus enclosed embayment, could be used as a predictor of lack of gene flow.

Intra-group structure is also important. Association patterns may vary along a continuum from random associations in groups to closely related individuals in groups. Management is affected by these relationships because if groups consist of closely related individuals, entire family groups may be adversely affected by disturbance at the same time. This raises questions, for example, of how long a vessel should be allowed to stay with one group or how many animals can be approached in one group.

Informed management is particularly important for this species given that the commercial and recreational troll fisheries near the islands “fish on dolphins” to catch tuna. In this practice, fishers drive their boats through groups of dolphins pulling lines behind them (Figure 1). Off the island of Hawaiʻi from November, 2006 to May, 2011 ~29% of groups of pantropical spotted dolphins encountered had fishing vessels fishing
through or in the middle of groups (# vessels range 1-8, mean = 2.4), in an attempt to
catch tuna that may be associated with the dolphins (R.W. Baird unpub. data). NOAA
Fisheries has proposed to elevate the Hawai‘i charter vessel and trolling, rod and reel
fisheries from Category III to Category II based on these fishing techniques, the large
number of vessels (the List of Fisheries notes the Hawaiian troll fishery includes 2,210
participant vessels/persons), and anecdotal reports of hookings of this species
(Department of Commerce 2011). Categories are defined as follows: I frequent
incidental mortality or serious injury of marine mammals; II occasional incidental
mortality or serious injury of marine mammals; III remote likelihood of/no known
incidental mortality or serious injury of marine mammals (Department of Commerce
2011). It is possible that if pantropical spotted dolphins are split into multiple stocks near
the Hawaiian Islands, the troll fishery will exceed 1% of Potential Biological Removal
for the island of Hawai‘i, which would legally require the reclassification of the fishery to
a Category II from a Category III. Further, the Hawaiian-based longline fishery does
cause some mortality of pantropical spotted dolphins, with one death reported between
1994 and 2005 in this fishery, or 0.04 pantropical spotted dolphins killed per 1000 sets
(Forney & Kobayashi 2007).

Also, there have been no studies comparing genetic data from the Hawaiian stock
of pantropical spotted dolphins to the ETP stocks that are heavily impacted by the tuna
purse seine fishery, or between these stocks and other Pacific populations. There has also
been little attempt to determine the biological significance of levels of genetic
differentiation among sympatric or parapatric odontocete populations. One way to do
this is to establish baseline values using populations that are not sympatric or parapatric and, therefore, not likely to have significant gene flow. My study examines this question using pantropical spotted dolphins from the North Pacific. On a finer scale, this study also clarifies genetic relationships and inter-island movement patterns that need to be known in order to properly designate management stocks of pantropical spotted dolphins. Because most Hawaiian populations of dolphins are currently managed as single stocks (Carretta et al. 2011), a lack of gene flow among islands would necessitate the restructuring of stock boundaries in order to appropriately manage and conserve these species. The information from my study will be used by the NOAA Fisheries to readdress stock structure of pantropical spotted dolphins. The results will allow managers to incorporate genetic relationships into their management schemes.

Hoelzel (1992) stated that to ensure long term survival of whale and dolphin populations, it is necessary to preserve genetic diversity by identifying and protecting populations with restricted gene flow, assessing variation in local populations, and gaining understanding of reproductive and dispersal behavior. He points out that neither geographic boundaries nor morphological differences and/or similarities always correlate with genetic distance between populations. Therefore, the genetic relationships of pantropical spotted dolphins near the Hawaiian Islands and other areas needs to be explored, particularly because there are currently no genetic data from the Hawaiian Islands on which to base management of this species.
Project Background

Population genetics models have been widely applied to a variety of taxa to describe gene flow and diversity within populations, as well as to define stocks (units for management) and geographical population borders. Applications of such studies range from protists (Kusch 1998) to trees (Boshier et al. 1995) to turtles (Bowen et al. 1992). Because of the low cost of locomotion (Williams 1999) and the documentation of long distance movements (>1,000km) for some species (Wells & Gannon 2005), dolphins are typically considered capable of wide dispersal among islands and island groups. However, recent studies have indicated that several species have populations that show fidelity to individual Hawaiian island regions, resulting in potential genetic differentiation with management implications. For example, photo-identification (Baird et al. 2009) and population genetics studies (Martien et al. 2011) of common bottlenose dolphins were used to create new stock boundaries in 2010 (Carretta et al. 2011). Likewise, new stock boundaries have been created for spinner dolphins based on genetic differentiation among several regions of the Hawaiian Islands (Andrews et al. 2010). As a result of genetic (Chivers et al. 2007, 2010), photo-identification (Baird et al. 2008a), and tagging (Baird et al. 2010) studies on false killer whales, NOAA Fisheries has divided false killer whales into three Pacific Islands Region management stocks, including insular and offshore stocks (Carretta et al. 2011). These stocks are not separated by the island regions that define bottlenose and spinner dolphin stocks. Further, photo-identification studies indicate that rough-toothed dolphins (Steno bredanensis) (Baird et al. 2008b) show some site fidelity to individual Hawaiian Islands. Photo-
identification study of melon-headed whales (*Peponocephala electra*) near the Hawaiian Islands has shown that these dolphins move among islands and that there is evidence of a small, demographically independent population found mainly in comparatively shallow waters near the northwestern side of the island of Hawai‘i (Aschettino 2010, Aschettino et al. 2011). There have been re-sightings of individual Cuvier’s beaked whales (*Ziphius cavirostris*) and Blainville’s beaked whales (*Mesoplodon densirostris*) near the island of Hawai‘i over a 21yr period of photographing these whales, suggesting long-term site fidelity to the area (McSweeney et al. 2007). Overall, the studies that explore dispersal and gene flow of odontocetes associated with island habitats yield varied results with respect to patterns of site fidelity. Additional studies of such species are needed to resolve questions of site fidelity and the effects this site fidelity may have on the distribution of genetic diversity and population structure and ecology.

Spinner and common bottlenose dolphins are primarily found in shallow water near the Hawaiian Islands (Norris et al. 1994), suggesting they may not use deep water channels to the extent that other odontocetes do. Understanding dispersal and barriers to gene flow for island-associated marine species will allow us to begin to design studies to address the proximate (mechanistic) and ultimate (evolutionary) causes of reproductive isolation and the effects that isolation has on genetic diversity of island-associated marine populations. My study is designed to inform pantropical spotted dolphin management by revealing population sub-structure of this species among the Hawaiian Islands.

Intra-group structure is important as well. It is common in mammals for females to stay in family groups (Greenwood 1980). Group fidelity by females has been
documented in some dolphin populations, for example, Indo-Pacific bottlenose dolphins near Australia (Möller et al. 2006) and common bottlenose dolphins near Sarasota, Florida (Duffield & Wells 1991). This female fidelity to the group may occur whether dolphins are dispersing among islands within an archipelago or not. My study represents a preliminary look at intra-group relationships among pantropical spotted dolphins near the Hawaiian Islands help to address the question of female fidelity.

Further, genetic data from previous studies of other populations of *Stenella* across oceans were examined to explore the phylogenetic relationships among *Stenella* species and sub-species and assess whether mtDNA control region sequences are useful for phylogenetic analysis among *Stenella*.

**Population Genetics Background**

The following general description of population genetics theory can be found in most textbooks. Halliburton (2004) was used as a general reference. DNA sequencing, particularly of the mtDNA control region, has been used to assess variation and relatedness within and among populations since the 1980’s. Sequencing DNA allows the researcher to assess how many distinct haplotypes (unique sequences of DNA) are found within populations and in what proportions they are found. MtDNA is inherited only from the mother and does not undergo recombination, making it a good indicator of female gene flow and female philopatry. Sequences chosen for genetic analysis need to have sufficient variation to allow comparison, but not so much that there is significant homoplasy (*i.e.* similarities for reasons other than common ancestry, such as a changing
to a new sequence then back to the original sequence—this cannot be detected and appears as if no change occurred) (Hillis et al. 1996). When focusing on population genetics, a fast evolving region is preferable because there will be few differences in slower evolving regions among individuals upon which to base comparisons. This is in contrast to higher level phylogenetic studies in which slower evolving sequences are generally preferred because the likelihood of homoplasy in fast evolving sequences is higher among genera and higher taxonomic levels. Also, if all sequences are the same or there are not at least two different nucleotides with each variant occurring at least twice, there are no phylogenetically informative characters with which to create possible trees.

In animals, mtDNA has some of the highest rates of nucleotide substitution, particularly in the non-coding control region (Dowling et al. 1996). The most common variation is due to base substitutions. The use of mtDNA in association with geographic information creates a means for determining genetic structure of populations (Hillis et al. 1996). Using DNA that is uni-parentally inherited (e.g. mtDNA) also allows comparison with bi-parentally inherited sequences (e.g. microsatellites) to detect differences between the two sexes (Dowling et al. 1996).

Non-protein-coding regions of the nuclear DNA, such as microsatellites, are also fast evolving sequences used for population genetics study (Dowling et al. 1996). Microsatellites are sequences made up of two to five repeating nucleotide units. Microsatellite loci tend to be highly variable, and are generally considered to be neutrally evolving. Microsatellites are inherited from both parents, so unlike mtDNA, microsatellite loci can be used to examine male-mediated gene flow.
Population Genetics Statistics

Common calculations used in population genetics to detect sub-populations or separate populations are fixation indices such as $F_{ST}$, $R_{ST}$, and $\Phi_{ST}$. $F_{ST}$ can be calculated for individual loci and averaged across all loci. $F_{ST}$ is a calculation that determines the proportion of total heterozygosity that is due to allele frequency differences among sub-populations. The calculation is $(\text{Total Heterozygosity Expected in Population} - \text{Mean of Expected Heterozygosities Over All Sub-populations}) / (\text{Total Heterozygosity Expected in Population})$ (Wright 1951). $R_{ST}$ was developed for use specifically with microsatellite data (Slatkin 1995). $R_{ST}$ removes the assumption of low mutation rates and the assumption that size of new mutant alleles does not depend on size of the allele that mutated (Slatkin 1995). It uses a step-wise mutation model in which mutations are accrued in steps (such as increasing microsatellite size by one repeated element at a time). However, it should be noted that microsatellite mutations are not necessarily limited to step-wise and can occur in large jumps (Duffield, pers. comm.) $\Phi_{ST}$ is an $F_{ST}$ analog that uses Analysis of Molecular Variance (equivalent to ANOVA) (Excoffier et al. 1992). $F_{ST}$ and $R_{ST}$ calculations use departure of allele frequencies from panmictic expectations (Excoffier et al. 1992), while $\Phi_{ST}$ evaluates the correlation of random haplotype sequences within populations relative to random pairs drawn from the whole species (Excoffier et al. 1992). For $\Phi_{ST}$, a hierarchical Analyses of Molecular Variance is constructed directly from the matrix of squared-distances between all pairs of haplotypes (Excoffier et al. 1992). These statistics can be calculated using a variety of
computer programs and compared for levels of similarity in results to look for how changes in assumptions affect the results.

$F_{ST}$ based calculations require the researcher to define potential sub-populations ahead of time. This makes it possible to miss cryptic sub-structure not based on obvious barriers or long distances. Another approach to evaluating population structure is to use Bayesian statistics that test different models of population structure to determine if the data fit the models, rather than building a model based on the data, as is done in $F_{ST}$, $R_{ST}$, and $\Phi_{ST}$ calculations. TESS 2.3 (Chen et al. 2007, Durand et al. 2009) is an example of a program that uses Bayesian model-based clustering methods.

Mantel tests can be used to assess significance of correlation between geographic and genetic distances (Mantel 1967, Smouse et al. 1986). Wright (1943) first described isolation by distance. If a population has short range dispersal, individuals are more likely to breed with others that live close, causing nearby neighbors to be more genetically similar than distant neighbors (Wright 1943). This situation is not indicative of separate populations, but can appear to be so if sampling is discontinuous across a population isolated by distance (Wright 1943).

Genetic relatedness among individuals within and among populations can be estimated using programs like COANCESTRY 1.0 (Wang 2011). This program can also be used to compare relatedness within and among a variety of types of groups designated by the user.
Phylogenetics Statistics

Often, PAUP*4.0b10 (Swofford 2003) is used for phylogenetic analyses, but large sample size and some close similarities in haplotypes in my dataset precluded the use of this program because PAUP* did not have the computational power necessary. For large datasets or very similar haplotypes, other programs, such as GARLI (www.bio.utexas.edu/faculty/antisense/garli/Garli.html) can be used. Originally, such analyses were based on a model of evolution in which random evolutionary changes occur at a stochastically constant rate (Felsenstein 1988). Current software programs allow the user to alter the model of evolution and weigh characters differently if desired. Best models of evolution based on the data can be determined using programs such as ModelTest 3.7 (Posada & Crandall 1998, Posada & Buckley 2004, Posada 2006). This program chooses among 56 models, and implements three different model selection frameworks: hierarchical likelihood ratio tests (hLRTs), Akaike information criterion (AIC), and Bayesian information criterion (BIC) to choose the best of the 56 models (Posada & Crandall 1998, Posada & Buckley 2004, Posada 2006). I used bootstrapping in GARLI, in which the data are re-sampled with replacement multiple times, which allows for calculation of the probability of a tree given the level of variation in the estimator (Felsenstein 1988).
CHAPTER 2: Evidence of Multiple Populations of Pantropical Spotted Dolphins

(*Stenella attenuata*) within Hawaiian Waters

Introduction

Population genetics has been widely applied to a variety of taxa to describe gene flow and diversity among populations and to define stocks and geographical population boundaries. Recently, population genetics studies have begun to be used to examine island-associated dolphin populations near the Hawaiian Islands (*e.g.* Andrews *et al.* 2010, Martien *et al.* 2011). Because of the relatively low cost of locomotion (Williams 1999) and the documentation of long distance movements (>1,000km) for some species (Wells & Gannon 2005), dolphins are typically considered capable of wide dispersal among islands and island groups. Therefore, it is often assumed that populations of dolphins near archipelagos are panmictic among the regions of archipelagos. However, studies of island-associated populations, such as spinner dolphins (*Stenella longirostris*) near the Hawaiian Islands (Andrews *et al.* 2010) and French Polynesia (Oremus *et al.* 2007), common bottlenose dolphins (*Tursiops truncatus*) (Baird *et al.* 2009, Martien *et al.* 2011) near the Hawaiian Islands and the Bahamas (Parsons *et al.* 2006), and melon-headed whales (*Peponocephala electra*) near the island of Hawai‘i (Aschettino *et al.* 2011) have shown that dolphins may exhibit fidelity to individual island regions within archipelagos. As a result of genetic (*Chivers et al.* 2007, Chivers *et al.* 2010) and photo-identification studies (Baird *et al.* 2008a), NOAA Fisheries has divided false killer whales (*Psuedorca crassidens*) into three Pacific Islands Region management stocks,
including insular and offshore stocks within the Hawaiian Islands Exclusive Economic Zone (EEZ) (Carretta et al. 2011). NOAA Fisheries has also recently split the Hawaiian stocks of common bottlenose dolphins and spinner dolphins into multiple stocks based on recent genetic and photo-identification studies (Baird et al. 2009, Andrews et al. 2010, Carretta et al. 2011, Martien et al. 2011).

In contrast, when Quérouil et al. (2007) compared common bottlenose dolphins among two archipelagos (Azores and Madeira) and the Atlantic Basin, they did not find significant differentiation within or among these three locations, concluding that pelagic common bottlenose dolphins in the North Atlantic belong to one oceanic population. Likewise, Quérouil et al. (2010) found that short-beaked common dolphins (Delphinus delphis) and Atlantic spotted dolphins (Stenella frontalis) likely belong to single populations that include the Azores and Madeira archipelagos. Overall, the studies that have explored dispersal and gene flow of dolphins associated with island habitats have had varied results, likely reflecting varied ecological circumstances around different islands. Additional studies of island-associated dolphins are needed to resolve questions of site fidelity for specific species and to investigate the effect this fidelity may have on genetic diversity and population ecology.

My study focused on population structure of pantropical spotted dolphins (Stenella attenuata) near the Hawaiian Islands from the island of Hawai‘i to Ni‘ihau. There has been little study of this species near the Hawaiian Islands (e.g. Baird et al. 2001). More extensive study has been done on their Eastern Tropical Pacific counterparts that are impacted by the tuna purse seine fishery (e.g. Scott & Chivers 2009)
and on pantropical spotted dolphins near China and Taiwan (Wang et al. 2003, Yao et al. 2004, 2008). Population size estimates for the Hawaiian Islands included in our study area range from approximately 2,928 (CV=0.45) (Mobley et al. 2000) to 4,283 (Barlow 2006), although the area included by Barlow et al. (2006) (212,892 km$^2$) was larger than that of Mobley et al. (2000) (71,954 km$^2$), which may explain the difference in estimates. For the Barlow (2006) estimate an approximate CV of 0.55 was estimated by assuming that the variance in each stratum is proportional to the number of sightings (Barlow, pers. comm.). Within the entire Hawaiian EEZ, population size is estimated to be approximately 8,978 (CV=0.48) (Barlow 2006).

The goal of assessing population structure and defining populations is to manage populations in such a way that they continue to be functioning elements of their ecosystems. Genetics is one avenue to learning about demographic isolation of populations, but I agree with Palsbøll et al. (2007) that simply finding evidence against panmixia is not necessarily sufficient to indicate that populations are isolated enough to justify re-defining stocks for management. Palsbøll et al. (2007) suggest pre-defining a threshold value for genetic divergence, although this can be difficult to do. In the case of pantropical spotted dolphins, I used several approaches to decide if populations should be defined as separate. First, I compared my genetic differentiation values to those of other dolphin populations near the Hawaiian Islands and examined management actions taken. Second, I considered the potential for population impacts caused by fisheries if potentially separate populations continue to be combined as one stock near the Hawaiian Islands. Further, because population differentiation measures require pre-defined
populations, I also used individual assignment testing to evaluate whether there are genetic differences among island regions. It should be noted that “population” and “stock” are not necessarily the same. For the purposes of this paper, “populations” are biologically differentiated by a lack of gene flow, although some gene flow may occur at low levels. “Stocks” are groups of marine mammals of the same species or smaller taxa in a common spatial arrangement that interbreed when mature (Marine Mammal Protection Act, 1972 as amended 2007). The NOAA Guidelines for the Assessment of Marine Mammal Stocks (Wade & Angliss 1997) has interpreted this definition to include both demographically and genetically distinct groups. Genetics is only one line of evidence by which a “stock” is defined.

Because of the threats posed by human interaction, pantropical spotted dolphin populations need to be defined accurately to be managed in a way that will avoid local population depletion. Informed management is particularly important for this species given that the commercial and recreational troll fisheries near the islands “fish on dolphins” to catch tuna. In this practice, fishers drive their boats through groups of dolphins pulling lines behind them (Figure 1). Off the island of Hawai‘i from November, 2006 to May, 2011 ~29% of groups of this species encountered had fishing vessels fishing through or in the middle of groups (# vessels range 1-8, mean = 2.4), in an attempt to catch tuna that may be associated with the dolphins (R.W. Baird, unpub data). Injury and mortality to pantropical spotted dolphins from this fishing technique has not been quantified, but fishers asked about the practice admit that occasionally, hooking of pantropical spotted dolphins occurs (R.W. Baird unpub data). Rizzuto (2007) reported a
fisher describing the “first time” he caught a spotted dolphin on a lure and brought it up to the boat before cutting off the line with the lure still stuck in the dolphin’s mouth. Several individual pantropical spotted dolphins that have marks associated with fishing (e.g. a straight cut across the dorsal fin sometimes bending the fin) or vessel interactions (e.g. multiple straight cuts from a propeller across the side) have been photographed in the region (R.W. Baird unpub. data). NOAA Fisheries has proposed to elevate the Hawai‘i charter vessel and trolling, rod and reel fisheries from Category III to Category II based on these fishing techniques, the large number of vessels (the List of Fisheries notes the Hawaiian troll fishery includes 2,210 participant vessels/persons), and anecdotal reports of hookings of this species (Department of Commerce 2011). It is possible that if pantropical spotted dolphins are split into multiple stocks near the Hawaiian Islands, the troll fishery will exceed 1% of Potential Biological Removal for the island of Hawai‘i, which would legally require the reclassification of the fishery to a Category II from a Category III (Department of Commerce 2011). Further, the Hawaiian-based longline fishery does cause some mortality of pantropical spotted dolphins, with one death reported between 1994 and 2005 in this fishery, or 0.04 pantropical spotted dolphins killed per 1000 sets (Forney & Kobayashi 2007).

Near the Hawaiian Islands, pantropical spotted dolphins are managed as a single stock (Carretta et al. 2011); however, Baird et al. (2001), suggested that movements among the islands may be limited based on differences in scar pattern among islands. Here, I address the question of gene flow in pantropical spotted dolphins near the Hawaiian Islands using analysis of mitochondrial DNA (mtDNA) sequences and
microsatellite profiles. Further, I examine intra-group relatedness as a way to evaluate if group fidelity may drive genetic differentiation.

Methods

Study site and sample collection

Survey effort attempted to cover a wide survey area (Baird et al. 2008a, 2008b). While all groups (individuals found swimming together) of pantropical spotted dolphins were approached for species identification and group size estimation, not all were sampled for genetic analyses (Figure 2; Table 1). For a thorough description of survey techniques see Baird et al. (2008a, 2008b).

Four regions were defined, based on distance among islands and depths of the channels between them per Baird et al. (2008b): the island of Hawai‘i; the “4-islands area” including Maui, Lana‘i, Kaho‘olawe, and Moloka‘i; the island of O‘ahu; and the islands of Kaua‘i and Ni‘ihau (Figure 2). Samples were collected up to 40m from shore, and depths were shallower near the 4-islands area (Table 1). Depth was determined by taking GPS locations for each sample/sighting and overlaying the point locations on a bathymetric raster using ArcGIS 9.1 (ESRI, Redlands, CA). Distance from shore was determined using GPS locations and distance measures in ArcGIS.

Genetic samples were collected as skin biopsies from live animals encountered during surveys. Biopsy samples were taken using either a pole spear or a Barnett RX-150 crossbow (Lambertsen 1987). Biopsy tips were 25mm in length and 8mm in
diameter with a collar to limit penetration to approximately 18mm. Samples were preserved in a DMSO/saturated salt solution (Milligan 1998).

From 2002-2003, a total of 101 pantropical spotted dolphin samples were collected from the four defined regions as part of a long-term study of dolphins and beaked whales. Samples with all microsatellite alleles, sex, and mtDNA haplotype the same were considered to be suspected duplicates. One sample was removed from the study as a result of suspected duplication based on these criteria, making the total samples 100 (Table 1). There were 76 additional samples collected near the island of Hawai‘i from 2005-2008 (Table 1). For this study, these 76 additional samples were used in analysis to investigate whether individuals within the same group were more genetically similar than individuals from different groups to examine whether group fidelity may affect gene flow. These samples were used to increase sample size of number of groups with multiple samples from the same group. These samples were also used for estimation of effective population size near the island of Hawai‘i.

**Sexing, mtDNA, & Microsatellites**

DNA was isolated from tissue samples using the DNeasy extraction kit (QIAGEN, Valencia, CA) (Appendix A). The following microsatellite loci were used: EV14, EV37, EV94, (Valsecchi & Amos 1996), SL8-49, SL9-69, SD8 (Galver 2002, Escorza-Treviño et al. 2005), MK5, MK6, MK8 (Krützen et al. 2001), KWM2A, and KWM12A (Hoelzel et al. 1998a). The PCR cycling profile for EV14, EV37, EV94, SL8-49, SL9-69, SD8, MK5, MK6, and MK8 was 5min at 95°C; then 10 cycles of 1min at
93°C, 1min at 52°C, and 50sec at 72°C; then 45sec at 90°C; then 25 cycles of 1min at 90°C, 1min at 73°C; then 5min at 72°C. The PCR cycling profile for KWM2A and KWM12A was 5min at 95°C; then 10 cycles of 1min at 93°C, 1min at 48°C, and 50sec at 72°C; then 45sec at 90°C; then 25 cycles of 1min at 56°C and 1min at 73°C. Allele sizes were determined on an ABI 3100 genetic analyzer using the standard GS500 ROX and scored with GENESCAN 2.11 (Applied Biosystems, Foster City, CA). EV37 and EV14 were multiplexed for amplification and run jointly on the ABI 3100. All other loci were amplified and run separately from each other. Amplification and analysis of mitochondrial control region sequences were done according to procedures used by Escorza-Treviño et al. (2005), modified to amplify the proline transfer RNA gene and hypervariable region I of the control region with primers H00034 (Rosel et al. 1994) and L15824 (Rosel et al. 1999) to obtain ~650bp. For PCR amplification, 1uL of 10uM L15824, 1uL of 10uM H00034, and 22uL of water were combined with PuReTaq™ Ready-To-Go PCR beads (GE Healthcare UK Limited, Piscataway, NJ). A PTC-100 Peltier thermal cycler (MJ Research, Ramsey, MN) was used for all reactions. The cycling profile for control region amplifications was 5min at 95°C; then 10 cycles of 1min at 93°C, 1min at 52°C, 50sec at 72°C; 45sec at 90°C; then 25 cycles of 1min at 60°C and 1min at 73°C; followed by a final extension period of 5min at 72°C. Amplified DNA was purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) (Appendix B) and sequenced on an AB 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Chromatograms were visualized using LASERGENE 6 (DNASTAR 2004).
Sex of the individuals sampled was determined by amplification of the zinc finger gene specific for the X and Y chromosomes of cetaceans using primers ZFXY, ZFX, and ZFY following the procedures of Bérubé and Palsbøll (1996). DNA was visualized on an agarose E-gel (Invitrogen, Carlsbad, CA). Females had one distinct band, whereas males exhibited three bands. Primer sequences are shown in Appendix C.

Statistical Analyses

All basic statistics, such as Kruskal-Wallis, Anderson-Darling, and ANOVA analyses, were performed using MINITAB 13 (MINITAB, Inc., State College, PA). Kruskal-Wallis tests were used for depth, distance from shore, and group size comparisons among regions because data were not normally distributed based on Anderson-Darling tests and sample sizes were low in all regions except Hawai‘i. H-values were adjusted for ties. Nemenyi tests and Tukey tests for multiple range analyses on ANOVA results were performed using critical Q-values and q-values from Zar (1999).

ARLEQUIN 3.0.1.2 (Schneider et al. 2000) was used to check microsatellite loci for pairwise linkage disequilibrium for each population with 50,000 Markov steps and a 50,000 step burnin and for deviations from Hardy-Weinberg equilibrium for each locus for each population with 1,000,000 Markov steps and a 100,000 step burnin. LOSITAN (Antao et al. 2008) was used to evaluate neutrality of microsatellite loci. Neutral Mean $F_{ST}$ and Force Mean $F_{ST}$ were chosen and run for 50,000 simulations with a stepwise mutation model. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to test for null alleles and allelic dropout using 10,000 permutations at 95% confidence for each
island region. BOTTLENECK 1.2.02 (Piry et al. 1999), based on methods described in Cornuet & Luikart (1996) and Luikart & Cornuet (1998), was used to determine if there were deviations from mutation-drift equilibrium indicating possible population bottlenecks. The TPM model with 95% single-step mutations and 5% multiple-step mutations with a variance of 12 among multiple steps was used. These parameters are recommended for microsatellites (Piry et al. 1999). BOTTLENECK was run for 1,000 replications, and both the sign test and Wilcoxon’s test were used. Piry et al. (1999) report that for fewer than 20 loci, the Wilcoxon’s test is the most appropriate and powerful but that results of this test can be more difficult to interpret than the sign test.

TESS 2.3 (Chen et al. 2007, Durand et al. 2009) was used to analyze microsatellite data to estimate the number of populations near the Hawaiian Islands and calculate assignment probability of each individual to each population cluster. Latitude and longitude in the WGS1984 geographic coordinate system were used. TESS was run using the admixture model and the linear trend model. I used a run length of 500,000 sweeps with a burn-in of 100,000 sweeps. Log likelihood and regression coefficient graphs did not indicate a problem with convergence. The TESS manual recommends a burn-in of only 10,000 and run length of 50,000 sweeps. TESS was run with both the CAR and BYM models. Results did not differ significantly, so only the results for the BYM model are reported here. K values were set from 2 to 6 and the algorithm was run 100 times for each K. For each K, the ten runs with the lowest DIC values were kept for further analysis. The mean DIC for each K was calculated using the ten runs for each and plotted against K. Assignment probabilities for each of the ten runs for each K were
exported from TESS into CLUMPP (Jakobsson & Rosenberg 2007), which calculated mean assignment probabilities for each individual to each cluster for each K value. In CLUMPP, the Greedy Option was used with all possible input orders repeated 1000 times. Mean assignment to cluster for each region was calculated by averaging the mean individual assignment probabilities for each region. FSTAT 2.9.3.2 (Goudet et al. 2002) was used to investigate the possibility of sex-biased dispersal. Permutations were set to 10,000, and two-tailed tests were used to compare $F_{ST}$ values; $F_{IS}$, $mAIC$, and $\Delta AIC$ values are also reported.

ARLEQUIN was used to calculate pairwise population $F_{ST}$ and $R_{ST}$ for microsatellite data. $F_{ST}$ and $R_{ST}$ of microsatellites reported from ARLEQUIN are equivalent to $\Theta_{ST}$ of Weir and Cockerham (1984) based on a pairwise distance matrix using number of different alleles for $F_{ST}$ and sum of squared differences for $R_{ST}$. $R_{ST}$ differs from $F_{ST}$ in that it uses a step-wise mutation model rather than an infinite alleles model, which may be more appropriate for the evolution of microsatellites.

To test for isolation by distance using microsatellites, IBD 1.52 (Bohonak 2002) was used to run Mantel tests to determine whether there were correlations between population level geographic and genetic distances. Number of randomizations was set to 50,000. The genetic distances used in this analysis were $F_{ST}$ and $R_{ST}$ values. Geographic distances among islands were calculated by taking the mean of the latitude and longitude for one sample from each encounter for each region and using the posdist function in Excel (Microsoft, Redmond, WA) to calculate the distances among regions from the means. Mean distance from Hawai‘i ($n=11$) to the 4-islands area ($n=9$) was 144.2km.
from Hawai‘i to O‘ahu \((n=8)\) 308.8km, from Hawai‘i to Kaua‘i/Ni‘ihau \((n=1)\) 393.9km, from the 4-islands area to O‘ahu 176.5km, from the 4-islands area to Kaua‘i/Ni‘ihau 267.9km, and from O‘ahu to Kaua‘i/Ni‘ihau 92.9km. To test for isolation by distance at an individual level, GENALEX 6.4.1 (Peakall & Smouse 2006) was used following the procedures of Peakall et al. (2003) to create a correlogram to compare combined genetic correlation for microsatellite loci as function of distance. First, GENALEX was used to create a distance matrix in kilometers using latitude and longitude coordinates for each sample. GENALEX was also used to create a genetic distance matrix combined across microsatellite loci. Because the question is whether there is one population that is isolated by distance, the autocorrelation analysis (see Peakall et al. 2003, Peakall & Smouse 2006 for details) was run for all samples as one population. Distance classes were set with an effort to have similar pairwise sample size in each class. This resulted in nine distance classes. The analysis was run using the spatial function in GENALEX, with 9,999 permutations for assessing 95% CI around the null hypothesis of random distribution and with 10,000 bootstraps for determining the 95% CL around the calculated correlation \((r)\). The autocorrelation coefficient produced in this analysis is bounded by -1 and 1 and is closely related to Moran’s I (Peakall et al. 2003).

KININFOR 1.0 (Wang 2006) was used to assess the informativeness of the microsatellite loci for five relatedness estimators—namely those of Lynch (1988), Queller & Goodnight (1989), Li et al. (1993), Ritland (1996), Lynch & Ritland (1999), Wang (2002), and Milligan (2003). Genotype error rate was set to 0.03 for all loci based on allele size call error rates reported by Chivers et al. (2010). Primary and null
hypotheses were full siblings and parent–offspring and the significance level was set at 5%.

COANCESTRY 1.0 was used to calculate relatedness among pairs of individuals. Genotype error rate was set to 0.03 for all loci (as in KININFOR). I did not check the “account for inbreeding” box, as there were no indications that inbreeding was an issue with these populations (see BOTTLENECK results). Number of reference individuals was set to 100 (as suggested by the program author), and bootstraps were set to 10,000. Pairwise relatedness was compared among populations for the 100 samples used in this study. Comparisons among populations were made using four different estimators (TrioML, Wang, LynchLi, and QuellerGt) to assess whether the outcome was consistent with different estimators. The Wang, LynchLi, and QuellerGt estimators were chosen based on KININFOR results, and TrioML was included as a likelihood estimator. Bootstraps were set to 100,000 for each comparison. Pairwise relatedness was also compared among pairs of individuals found in the same groups (encounters) and in different groups near each region for an extended dataset of 166 samples (Kaua‘i/Ni‘ihau excluded) to determine if group fidelity might affect genetic relatedness in the regions. Comparisons were made using the TrioML and LynchLi estimators with 100,000 bootstraps. This allowed for comparison between a moment estimator and a likelihood estimator. Likelihood estimators are generally better than moment estimators when there are a large number of polymorphic markers (Wang 2011). There were no samples available from different groups near Kaua‘i/Ni‘ihau, so no inter- and intra-group comparison could be made for this region. An overall comparison of intra- and inter-
group relatedness across the pooled dataset of all four regions ($n=173$) was also made with TrioML and LynchLi estimators.

In order to estimate effective population size, ONeSAMP 1.2 (Beaumont et al. 2002, Tallmon et al. 2004, 2008) and LDNe 1.31 (Waples & Do 2010) were used. These programs allow estimation of effective population size with microsatellite data collected during one sampling period rather than over multiple generations. LDNe uses linkage disequilibrium (Waples & Do 2010) and OneSamp uses a Bayesian approach (Tallmon et al. 2008) to estimate effective population size. In OneSamp, minimum effective population size was set to 100 and maximum effective population size was set to 10,000. In LDNe, $p_{\text{crit}}$ of 0.05 and random mating were selected. Only the extended dataset from the island of Hawai‘i ($n=113$) was used for this analysis. The other island regions did not have sufficient sample size.

Mean number of distinct alleles per locus and mean number of private alleles per locus, accounting for sample size, was calculated using AZDE 1.0 (Szpiech et al. 2008) to determine if number of private alleles was sufficient to estimate migration rates. AZDE was also used to determine mean number of private alleles per locus for pairs of putative populations to examine whether any pair of populations shared enough alleles only with each other to indicate more recent migration or founder events. Kaua‘i/Ni‘ihau was not included in the pairwise analysis because the program can only calculate means up to the smallest population sample size, which would limit the comparisons to sample size of eight. To estimate migration rates ($N_m$) the private alleles method of Barton & Slatkin (1986) was applied using GENEPOP 4.0.1.0 (Raymond & Rousset 1995, Rousset 2008).
MtDNA sequence neutrality was tested in ARLEQUIN using Tajima’s $D$ at 50,000 simulated samples. To test for isolation by distance using mtDNA, IBD was used to run Mantel tests as described above for microsatellites but using $F_{ST}$ and $\Phi_{ST}$ as genetic distances. ARLEQUIN was used to calculate $F_{ST}$ and $\Phi_{ST}$ for the mtDNA sequence data. The number of permutations in a randomization test was set to 50,000, and $\Phi_{ST}$ was calculated using a basic pairwise comparison model without weighting mutation type because haplotype differences consisted only of transitions. TCS (Clement et al. 2000) was used to create a haplotype network to examine the relationships among mtDNA haplotypes. A 95% connection limit was chosen in TCS, and gaps were set to a 5th state, though as there were no gaps in the aligned sequences, this did not affect the network.

Standardizations of microsatellite and mtDNA $F_{ST}$ values were calculated using ARLEQUIN based on Meirmans & Van Tienderen (2004), Hedrick (2005), and Meirmans (2006). These standardizations account for high within-population variation, differences in effective population size, and markers with different mutation rates (Meirmans 2006). To standardize $F_{ST}$, microsatellite data were re-coded such that all populations contained unique alleles without changing the within-population variation (maximizing among-population variation). Similarly, all mtDNA sequences were re-coded such that all populations contained unique haplotypes without changing the within-population variation of haplotypes. These new values generate an $F_{ST \text{ max}}$ (Meirmans 2006). $F'_{ST} = F_{ST}/F_{ST \text{ max}}$ (Hedrick 2005, Meirmans 2006). It is inappropriate to do significance testing on standardized measures because the calculation of the maximum
value uses a hypothetical dataset (Meirmans 2006), so \( p \)-values are not reported for standardized values. \( R_{ST \text{ max}} \) values were \( \sim 1 \), so no standardizations were necessary. \( \Phi_{ST} \) takes mutational distances into account and so is independent of mutation rate; its performance is related to the accuracy of the mutation model (Kronholm et al. 2010), so standardizations are not necessary. Standardized \( F_{ST} \) values have been reported for spinner dolphins (Andrews 2009, Andrews et al. 2010) and common bottlenose dolphins (Martien et al. 2011) near the Hawaiian Islands, so these values are useful for comparisons among Hawaiian species. Further, standardized \( F_{ST} \) values better represent the actual magnitude of differentiation. Bonferroni corrections were not applied to the significance level of \( F \)-statistics. Bonferroni corrections tend to increase the likelihood of type II errors. In the case of determining population structure for conservation purposes, there is greater concern about type II error than type I error.

**Results**

**General results**

Survey effort from 2002 to 2008 near the Hawaiian Islands regions considered in this study suggests that pantropical spotted dolphins are rarely found near Kaua‘i/Ni‘ihau and commonly found near the island of Hawai‘i (Figure 2; Table 1). Kruskal-Wallis tests were used to compare median depths, distances from shore, and group sizes (Table 1) of pantropical spotted dolphins near Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau for all encounters and for encounters during which a biopsy occurred. For encounters with biopsies, Kaua‘i/Ni‘ihau could not be statistically compared to other regions because
only one encounter occurred. This analysis was done to investigate bias in sampling and potential differences in distribution among regions. Kruskal-Wallis tests indicated significant differences in median depth for all encounters ($H=60.47$, $p<0.001$) and for encounters resulting in biopsies ($H=16.39$, $p<0.001$). Nemenyi tests for unequal sample sizes indicated that there were significant differences ($p<0.05$) for all encounters in median depth between Hawai‘i (1781m, Mean Rank (R) =120.4) and the 4-islands area (177m, R=18.4) between Hawai‘i and O‘ahu (977m, R=62.6), between the 4-islands area and Kaua‘i/Ni‘ihau (3477m, R=160.2), and between O‘ahu and Kaua‘i/Ni‘ihau reflecting, at least in part, differences in depth of survey effort in these areas. Nemenyi tests also indicated that there were significant differences ($p<0.05$) for biopsy encounters in depth between Hawai‘i (1666m, R=20.5) and the 4-islands area (192m, R=5.7) and between the 4-islands area and O‘ahu (955m, R=17.3). Kruskal-Wallis tests also indicated significant differences for median distance from shore ($H=11.07$, $p=0.004$) and median group size ($H=8.61$, $p=0.013$) for encounters during which biopsies occurred, but no significant differences in distance from shore ($H=5.19$, $p=0.158$) and group size ($H=6.39$, $p=0.094$) for all encounters. Nemenyi tests indicated that there were significant differences for biopsy encounters in median distance from shore ($p<0.05$) between Hawai‘i (12.3m, R=20.8) and O‘ahu (8.2m, R=13.2) and significant differences ($p<0.05$) in median group size between Hawai‘i (72.5, R=19.6) and the 4-islands area (35.0, R=87.0) and between the 4-islands area and O‘ahu (65.0, R=15.2).
Microsatellites were successfully amplified for all samples except one sample each from Hawai‘i \((n-1=37)\), the 4-islands area \((n-1=26)\), and O‘ahu \((n-1=26)\). Sex determination and mtDNA sequencing were successful for all samples.

For mtDNA analyses, 571 base pairs of control region sequence from each individual were compared. For samples from 2002-2003, ten haplotypes were found (GenBank accession numbers GQ852567-GQ852573, GQ852575, GQ852577, GQ852578). Seven haplotypes were unique (found in only one individual), and 77% of all samples were haplotype 3, which was found in all four regions. Four different haplotypes, two of which were unique, were found in eight dolphins sampled from one group of 35 individuals near Kaua‘i/Ni‘ihau. Overall, five out of the seven dolphins with unique haplotypes were female. All differences among mtDNA sequences were transition mutations. For the complete island of Hawai‘i dataset from 2002-2008 \((n=113)\), ten haplotypes were found, three of which were not found in the other three regions (GenBank accession numbers GQ852574, GQ852576, GQ852579). Of these samples, 80% were haplotype 3. Four out of the five dolphins near Hawai‘i with unique haplotypes were female. Only samples collected from 2002-2003 were used in comparisons among the four island regions. The microsatellite profiles from 2002-2008 were used in group (encounter) intra- and inter-relatedness comparisons and for estimating effective population size.
Relationships among the Regions

There was no indication of linkage disequilibrium or deviations from Hardy-Weinberg Equilibrium ($p$-values<0.05) for the microsatellite loci. MICRO-CHECKER indicated no significant excess of homozygotes for the combined probability test for any of the island regions, suggesting the loci did not have null alleles or allelic dropout. Allelic diversity and heterozygosity by locus are shown in Table 2. LOSITAN analyses indicated that all microsatellite loci were neutrally evolving with 95% confidence. Both the sign test and Wilcoxon’s test in BOTTLENECK indicated that no populations were likely to have experienced a recent bottleneck ($p$-values for heterozygosity excess were all >0.05). However, BOTTLENECK indicated a heterozygosity deficiency for O‘ahu (sign test $p=0.007$, Wilcoxon’s test $p=0.001$ for one-tailed test and 0.002 for two-tailed test).

The analysis in IBD indicated that microsatellite $F_{ST}$ and $R_{ST}$, as measures of genetic relatedness, did not significantly correlate with geographic distance ($Z=43.352$, $r=-0.206$, $p=0.750$ for $F_{ST}$; $Z=37.203$, $r=-0.445$, $p=0.790$ for $R_{ST}$). In individual comparisons, the correlogram of distance classes compared with the autocorrelation coefficient ($r$) as defined by Peakall et al. (2003) indicated that there was some positive spatial autocorrelation for individuals up to approximately 130km, although there was no significant correlation from approximately 35km to 90km (Figure 3). For pairs more than 130km apart, correlation is not significant up to approximately 195km, at which point the correlation becomes significantly negative and then returns to insignificant in the final distance bin (Figure 3).
A plot of K values versus DIC values from TESS indicated DIC leveled off at three populations (Figure 4). Bar plots of the probability of assignment to each cluster for K=3 showed clustering of assignments for Hawai‘i, the 4-islands area, and O‘ahu as three clusters, with Kaua‘i/Ni‘ihau clustering equally with Hawai‘i and O‘ahu (Figure 5). Mean probability of assignment to clusters one, two, or three differed within regions (Figure 5; Table 3). Based on Tukey tests, all cluster assignment probabilities for three clusters were significantly different except Kaua‘i/Ni‘ihau clusters one and two (Table 3).

KININFOR indicated that the LynchLi (Lynch 1988, Li et al. 1993), QuellerGt (Queller & Goodnight 1989), Wang (Wang 2002), and LynchRd (Lynch & Ritland 1999) estimators all had similar values for multilocus reciprocal of the mean squared deviations of relatedness estimates (RMSD; 48.966, 49.281, 49.541, and 48.319 respectively), suggesting that the loci used in this study have similar levels of informativeness for each of these estimators. Multilocus RMSD for the Ritland estimator was lower (8.760).

Mean relatedness among regions (based on 100,000 bootstraps in COANCESTRY) was calculated using the TrioML triad likelihood estimator (described in Wang 2007) and using the LynchLi, QuellerGt, and Wang moment estimators, as the loci were equally informative for these moment measures (see KININFOR results). The difference in observed mean pairwise relatedness between regions was outside of the 95% bootstrap confidence interval for mean pairwise relatedness for O‘ahu compared to Hawai‘i and the 4-islands area for all four estimators and for O‘ahu compared to Kaua‘i/Ni‘ihau for the Wang and LynchLi estimators (Table 4). This indicates that
individuals sampled near O‘ahu were more closely related to each other than those sampled at other island regions.

Most microsatellite $F_{ST}$ and $R_{ST}$ values were significantly different from zero (Table 5). Overall $F_{ST}$, 0.033 (95% CL 0.024-0.042) was significantly different from zero ($p=0.001$), as was overall $R_{ST}$, 0.035 (95% CL 0.005-0.074, $p=0.001$) (Table 5).

Kaua‘i/Ni‘ihau was included in these analyses as a separate region based on the unusual situation in which few dolphins were seen despite high effort, the high diversity in mtDNA, and the ambiguity of assignment to a specific cluster in TESS; although, it should be noted that sample size was very small for this region ($n=8$). An AMOVA based on microsatellites showed that 97% of the variation was within populations, with 3% among populations. Allelic diversity was similar for all regions (Table 6). With such high within-population variation, standardized $F_{ST}$ values can be better indicators of actual genetic differentiation because maximum value of $F_{ST}$ is less than one. In this case, standardized $F_{ST}$ values were larger in magnitude than non-standardized values (Table 5). FSTAT results indicated no significant difference between female and male $F_{ST}$ values (females $n=39$, $F_{ST}=0.021$; males $n=50$, $F_{ST}=0.039$; $p=0.151$). $F_{IS}$ and $vAIC$ values were slightly higher for females, while $mAIC$ values were lower (females $F_{IS}=0.083$, $H_o=0.765$, $mAIC=-0.941$, $mAIC=11.142$; males $F_{IS}=0.040$, $H_o=0.778$, $mAIC=0.734$, $mAIC=10.570$).

Two estimates of effective population size near the island of Hawai‘i were generated using an extended microsatellite data set of 113 individuals from that region: 156 individuals (95% CI 132-261, Bayesian OneSamp method), and 373 individuals
(95% CI 209-1248, LD method of LDNe), with the CI’s of the two methods overlapping from 209 to 261 individuals. Sample sizes were too small for other regions to estimate effective population size.

Mean number of distinct alleles per locus and mean number of private alleles per locus for Hawai‘i (0.858 SE±0.71, 1.61 SE±0.31 respectively), the 4-islands area (8.31 SE±0.64, 1.40 SE±0.27), and O‘ahu (7.90 SE±0.62, 1.12 SE±0.18) for sample size 26 were within the values successfully tested by Barton & Slatkin (Barton & Slatkin 1986) for their private alleles method of estimating migration rate (Nm) (Figure 6). Mean number of distinct alleles per locus and mean number of private alleles per locus for Kaua‘i/Ni‘ihau for sample size of eight were 5.17 SE±0.37 and 1.31 SE±0.24 respectively. Standard errors of mean numbers of private alleles for a sample size of 26 overlapped for pairs of regions, indicating no differences (Hawai‘i-4-islands area 1.29 SE±0.21, Hawai‘i-O‘ahu 1.15 SE±0.23, 4-islands area-O‘ahu 1.10 SE±0.20). As another indicator of gene flow, using the Barton & Slatkin (Barton & Slatkin 1986) method, Nm was calculated to estimate pairwise migration rates for all four island regions (Table 7).

Tajima’s D results were neutral for the mtDNA control region (D=-1.191, \(p=0.212\)). The analysis in IBD indicated that mtDNA \(F_{ST}\) and \(\Phi_{ST}\), as measures of genetic relatedness, did not significantly correlate with geographic distance (\(Z=91.584, r=-0.507, p=0.915\) for \(F_{ST}\); \(Z=52.641, r=-0.671, p=0.959\) for \(\Phi_{ST}\)).

For mtDNA, most \(F_{ST}\) and \(\Phi_{ST}\) values were not significantly different from zero except for O‘ahu and the 4-islands area (Table 5). Overall \(F_{ST}\) (0.055) was significantly different from zero \((p=0.023)\), as was overall \(\Phi_{ST}\) (0.039, \(p=0.044\)) (Table 5).
Confidence intervals were not calculated because those intervals are calculated over loci and the control region is only one locus. An AMOVA based on mtDNA showed that 94% of the variation was within populations, with 6% among populations. Standardizations of $F_{ST}$ for mtDNA resulted in values larger in magnitude than unstandardized $F_{ST}$ (Table 5). Haplotypic diversity, nucleotide diversity, and mean pairwise differences among mtDNA haplotypes were highest for Kaua‘i/Ni‘ihau and lowest for O‘ahu (Table 6), but in general, mtDNA haplotypic diversity among the four regions studied was low (0.450 SD±0.255), with 77 out of 100 samples representing a single haplotype. Two additional haplotypes were seen in nine and seven samples respectively, and seven individuals had unique haplotypes. Unique haplotypes were found in all four island regions. A haplotype network indicated that minor haplotypes have branched off the major haplotype several times (Figure 7).

*Intra-group Structure*

Mean pairwise relatedness comparisons for microsatellites for samples taken from the same group in comparison with samples taken from different groups indicated no significant differences in relatedness for any region or for samples pooled across regions (Table 8).

*Tables & Figures*
Figure 1. Photo of a small vessel fishing for tuna over a group of pantropical spotted dolphins off the island of Hawai‘i May 11, 2011. Photo provided by Cascadia Research Collective.
Figure 2. Locations of samples, sightings without samples, and search effort for pantropical spotted dolphins near the main Hawaiian Islands 2002-2008.
Figure 3. Correlogram showing combined genetic correlation for microsatellite loci as a function of distance, 95% CI (dotted lines) about the null hypothesis of random distribution, and 95% CL bars around the genetic correlation as determined by bootstrapping. Distance classes were created with an effort to include approximately the same number of pairwise comparisons in each class. This resulted in nine distance classes, the mid-points of which are shown plotted against genetic correlation above. The numbers of pairwise comparisons per class are indicated as $n$ values. This analysis was run in GENALEX using the spatial function assuming one population with 9,999 permutations and 10,000 bootstraps to test whether genetic differentiation indicating separate populations could be a function of gaps in sampling and isolation by distance. There was slight positive correlation between genetic and geographic distance for pairs up to approximately 130km apart, indicating that some isolation by distance may be occurring within island regions, but mean distances among island regions were all greater than 130km, except for the mean distance between O’ahu and Kaua’i/Ni’ihau, which was 92.9km.
Figure 4. Mean DIC for each K value run in TESS. TESS was run using the admixture model and the linear trend model. I used a run length of 500,000 sweeps with a burn-in of 100,000 sweeps and the BYM model. K values were set from 2 to 6 and the algorithm was run 100 times for each K. For each K, the ten runs with the lowest DIC values were used to calculate the mean DIC. Standard error bars are shown. DIC levels off at K=3, suggesting three populations.
Figure 5. Example of assignment probability bar plot for $K=3$ from TESS analysis. Note that Hawai‘i is predominantly light gray, the 4-islands area is predominantly medium gray, O‘ahu is predominantly dark gray, and Kaua‘i/Ni‘ihau is approximately equally light gray and dark gray. These three shades of gray indicate assignment probability of each individual to each of the three clusters.
Figure 6. Top: Mean number of distinct alleles per locus estimated based on sample size in ADZE. Kaua‘i/Ni‘ihau is not included because of small sample size. Figure 6. Bottom: Mean number of private alleles per locus estimated based on sample size using ADZE. Mean number of private alleles per locus is reasonable for making estimates based on private allele method by Barton & Slatkin (1986), which is robust to mean private allele values even less than one. Standard errors are shown with error bars.
Figure 7. Haplotype network generated in TCS. A 95% correction limit was chosen and gaps were set to a 5th state. Each node represents one transition. Size of oval represents number of individuals with the haplotype. Pie charts indicate relative numbers of individuals with each haplotype from each population.
Table 1. Top: Total number of samples at each island region, the number of groups from which samples were obtained, number of males and females, group size range, depth range, and distance from shore range. Table 1. Bottom: Observation effort near the Hawaiian Islands 2002-2008 for all observations (not just sampling events).

<table>
<thead>
<tr>
<th>Location</th>
<th>Total Samples</th>
<th># of Groups</th>
<th>Males</th>
<th>Females</th>
<th>Group Size Range</th>
<th>Depth Range (m)</th>
<th>Distance from Shore Range (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002-2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i</td>
<td>38</td>
<td>11</td>
<td>15</td>
<td>23</td>
<td>40-120</td>
<td>633-2681</td>
<td>7.2-34.3</td>
</tr>
<tr>
<td>4-islands area</td>
<td>27</td>
<td>8</td>
<td>16</td>
<td>11</td>
<td>25-75</td>
<td>60-817</td>
<td>5.9-14.7</td>
</tr>
<tr>
<td>O‘ahu</td>
<td>27</td>
<td>8</td>
<td>19</td>
<td>8</td>
<td>4-100</td>
<td>839-2278</td>
<td>4.9-14.5</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>35-35</td>
<td>3477</td>
<td>33.9-33.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additional samples used for comparisons of relatedness in groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005-2008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i</td>
<td>76</td>
<td>40</td>
<td>24</td>
<td>53</td>
<td>3-194</td>
<td>85-4662</td>
<td>2.5-64.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Region</th>
<th>Days</th>
<th>Hours</th>
<th>Km</th>
<th># encounters</th>
<th>encounters/ 100 km</th>
<th>Median Group Size</th>
<th>Median Depth</th>
<th>Median Distance from Shore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i</td>
<td>270</td>
<td>1974</td>
<td>31012</td>
<td>180</td>
<td>0.58</td>
<td>60</td>
<td>1781</td>
<td>8.5</td>
</tr>
<tr>
<td>4-islands</td>
<td>97</td>
<td>657</td>
<td>8224</td>
<td>22</td>
<td>0.27</td>
<td>40</td>
<td>177</td>
<td>10.5</td>
</tr>
<tr>
<td>O‘ahu</td>
<td>22</td>
<td>168</td>
<td>2649</td>
<td>9</td>
<td>0.34</td>
<td>60</td>
<td>977</td>
<td>7.6</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau</td>
<td>55</td>
<td>394</td>
<td>6225</td>
<td>5</td>
<td>0.08</td>
<td>30</td>
<td>3477</td>
<td>28.3</td>
</tr>
<tr>
<td>Total</td>
<td>444</td>
<td>3218</td>
<td>48074</td>
<td>216</td>
<td>0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Number of alleles, allelic diversity, and expected & observed heterozygosity at each microsatellite locus for pantropical spotted dolphin samples collected near the Hawaiian Islands 2002-2003. Total samples were 97 for each locus (194 alleles). LOSITAN was used to calculate $F_{ST}$ and probability that simulated $F_{ST}$ was less than sample $F_{ST}$. Neutral Mean $F_{ST}$ and Force Mean $F_{ST}$ were chosen and run for 50,000 simulations with a stepwise mutation model. All loci were indicated to be neutrally evolving with 95% confidence.

<table>
<thead>
<tr>
<th>Locus</th>
<th>#of Alleles</th>
<th>Allelic Diversity</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$F_{ST}$</th>
<th>$p$ (Simulated $F_{ST}$ &lt; sample $F_{ST}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK8</td>
<td>9</td>
<td>0.046</td>
<td>0.779</td>
<td>0.699</td>
<td>0.055</td>
<td>0.936</td>
</tr>
<tr>
<td>MK5</td>
<td>6</td>
<td>0.031</td>
<td>0.670</td>
<td>0.668</td>
<td>0.024</td>
<td>0.571</td>
</tr>
<tr>
<td>KWM12a</td>
<td>10</td>
<td>0.052</td>
<td>0.833</td>
<td>0.796</td>
<td>0.030</td>
<td>0.742</td>
</tr>
<tr>
<td>KWM2a</td>
<td>18</td>
<td>0.093</td>
<td>0.916</td>
<td>0.850</td>
<td>0.013</td>
<td>0.625</td>
</tr>
<tr>
<td>MK6</td>
<td>17</td>
<td>0.088</td>
<td>0.867</td>
<td>0.867</td>
<td>0.010</td>
<td>0.242</td>
</tr>
<tr>
<td>EV14</td>
<td>20</td>
<td>0.103</td>
<td>0.929</td>
<td>0.903</td>
<td>0.033</td>
<td>0.967</td>
</tr>
<tr>
<td>EV37</td>
<td>17</td>
<td>0.088</td>
<td>0.848</td>
<td>0.850</td>
<td>0.049</td>
<td>0.945</td>
</tr>
<tr>
<td>SD8</td>
<td>19</td>
<td>0.098</td>
<td>0.912</td>
<td>0.810</td>
<td>0.039</td>
<td>0.988</td>
</tr>
<tr>
<td>SL849</td>
<td>14</td>
<td>0.072</td>
<td>0.853</td>
<td>0.814</td>
<td>0.022</td>
<td>0.564</td>
</tr>
<tr>
<td>SL969</td>
<td>14</td>
<td>0.072</td>
<td>0.841</td>
<td>0.761</td>
<td>0.054</td>
<td>0.967</td>
</tr>
<tr>
<td>EV94</td>
<td>20</td>
<td>0.103</td>
<td>0.916</td>
<td>0.889</td>
<td>0.024</td>
<td>0.883</td>
</tr>
</tbody>
</table>
Table 3. Assignment probability to each of three clusters from TESS analysis. One hundred iterations were performed at K=3 and the ten with the lowest DIC values were used. Means, standard errors, and significant differences among means based on ANOVA analyses are shown here. Bold values are the largest assignment probabilities for each region. Tukey tests were performed on these data using critical $q$-values from Zar (1999). These tests indicated that there were significant differences for all pairs of clusters for each region except Kaua‘i/Ni‘ihau Cluster 1 and Cluster 2.

<table>
<thead>
<tr>
<th>Region</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
<th>Cluster 3</th>
<th>$F$-value</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i ($n=37$)</td>
<td>0.27 (SE±0.02)</td>
<td><strong>0.63 (SE±0.04)</strong></td>
<td>0.10 (SE±0.02)</td>
<td>98.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4-islands Area ($n=26$)</td>
<td>0.24 (SE±0.04)</td>
<td>0.06 (SE±0.02)</td>
<td><strong>0.70 (SE±0.05)</strong></td>
<td>77.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>O‘ahu ($n=26$)</td>
<td><strong>0.72 (SE±0.01)</strong></td>
<td>0.11 (SE±0.01)</td>
<td>0.17 (SE±0.01)</td>
<td>2725.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau ($n=8$)</td>
<td><strong>0.43 (SE±0.06)</strong></td>
<td><strong>0.50 (SE±0.07)</strong></td>
<td>0.07 (SE±0.02)</td>
<td>16.97</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 4. Mean pairwise relatedness comparisons between regions. Mean pairwise relatedness for each region was found using COANCESTRY with 100,000 bootstraps. Results for four estimators are shown below. For Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau pairs were 666, 325, 325, and 28 respectively. Significant differences occur when the observed mean difference is outside of the bootstrap 95%CL indicated by the 2.5% and 97.5% quantiles. These differences are indicated in bold. Relatedness was higher using the likelihood estimator than the moment estimators. These results indicate that pairwise relatedness among individuals was higher near O‘ahu than near the other regions.

<table>
<thead>
<tr>
<th>Regions Compared (1-2)</th>
<th>Mean 1</th>
<th>Mean 2</th>
<th>Obs Mean Diff</th>
<th>2.5% Quant</th>
<th>97.5% Quant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrioML</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>0.036 (SD±0.057)</td>
<td>0.036 (SD±0.058)</td>
<td>0.000</td>
<td>-0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>0.036 (SD±0.057)</td>
<td>0.081 (SD±0.096)</td>
<td>-0.045</td>
<td>-0.100</td>
<td>0.010</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>0.036 (SD±0.057)</td>
<td>0.055 (SD±0.070)</td>
<td>-0.019</td>
<td>-0.024</td>
<td>0.019</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>0.036 (SD±0.058)</td>
<td>0.052 (SD±0.144)</td>
<td>-0.045</td>
<td>-0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>0.036 (SD±0.058)</td>
<td>0.055 (SD±0.070)</td>
<td>-0.019</td>
<td>-0.025</td>
<td>0.020</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.081 (SD±0.096)</td>
<td>0.055 (SD±0.070)</td>
<td>-0.026</td>
<td>-0.034</td>
<td>0.039</td>
</tr>
<tr>
<td>Lynch Li</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>-0.028 (SD±0.134)</td>
<td>-0.041 (SD±0.127)</td>
<td>0.013</td>
<td>-0.018</td>
<td>0.017</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>-0.028 (SD±0.134)</td>
<td>0.052 (SD±0.144)</td>
<td>-0.080</td>
<td>-0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>-0.028 (SD±0.134)</td>
<td>0.008 (SD±0.136)</td>
<td>-0.020</td>
<td>-0.051</td>
<td>0.051</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>-0.041 (SD±0.127)</td>
<td>0.052 (SD±0.144)</td>
<td>-0.093</td>
<td>-0.022</td>
<td>0.022</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>-0.041 (SD±0.127)</td>
<td>0.008 (SD±0.136)</td>
<td>-0.033</td>
<td>-0.049</td>
<td>0.050</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.052 (SD±0.144)</td>
<td>0.008 (SD±0.136)</td>
<td>-0.060</td>
<td>-0.055</td>
<td>0.057</td>
</tr>
<tr>
<td>QuellerGt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>-0.008 (SD±0.125)</td>
<td>-0.013 (SD±0.123)</td>
<td>0.006</td>
<td>-0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>-0.008 (SD±0.125)</td>
<td>0.071 (SD±0.141)</td>
<td>-0.078</td>
<td>-0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>-0.008 (SD±0.125)</td>
<td>0.030 (SD±0.128)</td>
<td>-0.037</td>
<td>-0.048</td>
<td>0.047</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>-0.013 (SD±0.123)</td>
<td>0.071 (SD±0.141)</td>
<td>-0.084</td>
<td>-0.021</td>
<td>0.021</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>-0.013 (SD±0.123)</td>
<td>0.030 (SD±0.128)</td>
<td>-0.043</td>
<td>-0.048</td>
<td>0.047</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.071 (SD±0.141)</td>
<td>0.030 (SD±0.128)</td>
<td>-0.041</td>
<td>-0.053</td>
<td>0.056</td>
</tr>
<tr>
<td>Wang</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>-0.036 (SD±0.057)</td>
<td>-0.030 (SD±0.119)</td>
<td>-0.007</td>
<td>-0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>-0.036 (SD±0.057)</td>
<td>0.056 (SD±0.138)</td>
<td>-0.092</td>
<td>-0.018</td>
<td>0.018</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>-0.036 (SD±0.057)</td>
<td>-0.008 (SD±0.127)</td>
<td>-0.028</td>
<td>-0.048</td>
<td>0.047</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>-0.030 (SD±0.119)</td>
<td>0.056 (SD±0.138)</td>
<td>-0.085</td>
<td>-0.021</td>
<td>0.021</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>-0.030 (SD±0.119)</td>
<td>-0.008 (SD±0.127)</td>
<td>-0.022</td>
<td>-0.046</td>
<td>0.046</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.056 (SD±0.138)</td>
<td>-0.008 (SD±0.127)</td>
<td>-0.064</td>
<td>-0.053</td>
<td>0.054</td>
</tr>
</tbody>
</table>
Table 5. Top: $F_{ST}$ and $R_{ST}$ for microsatellite data calculated in ARLEQUIN. 50,000 permutations were used for calculations. $R_{ST}$ is above the diagonal, and $F_{ST}$ is below the diagonal. 95% CL for values are shown in brackets. CL’s were calculated in ARLEQUIN using 20,000 bootstraps. Numbers in bold are significantly different from zero, and $p$-values are in parentheses. Table 5. Middle: $F_{ST}$ and $\Phi_{ST}$ for mtDNA sequences. $\Phi_{ST}$ was calculated using a basic pairwise comparison model without weighting mutation type because haplotype differences only consisted only of transitions. 50,000 permutations were used for calculations. $\Phi_{ST}$ is above the diagonal, and $F_{ST}$ is below the diagonal. Confidence intervals could not be calculated because those intervals are calculated over loci and the control region is only one locus. Numbers in bold are significantly different from zero, and $p$-values are shown in parentheses. Table 5. Bottom: $F'_{ST}$ (standardized $F_{ST}$) is shown: $F'_{ST} = F_{ST} / F_{ST\ max}$. These values were calculated in ARLEQUIN using 50,000 permutations. MtDNA $F_{ST}$ is above the diagonal, and microsatellite $F'_{ST}$ is below the diagonal. It is not statistically appropriate to calculate $p$-values for standardized values.

<table>
<thead>
<tr>
<th>$F_{ST}$ and $R_{ST}$</th>
<th>Hawai’i</th>
<th>4-islands area</th>
<th>O’ahu</th>
<th>Kaua’i/Ni’ihau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai’i ($n=37$)</td>
<td>0.044 [0.019-0.079] (0.004)</td>
<td><strong>0.055 [-0.007-0.132] (0.001)</strong></td>
<td>-0.014 [-0.036-0.003] (0.769)</td>
<td></td>
</tr>
<tr>
<td>4-islands area ($n=26$)</td>
<td><strong>0.028 [0.013-0.045] (&lt;0.001)</strong></td>
<td>0.018 [-0.002-0.042] (0.061)</td>
<td><strong>0.047 [-0.005-0.091] (0.048)</strong></td>
<td></td>
</tr>
<tr>
<td>O’ahu ($n=26$)</td>
<td><strong>0.038 [0.023-0.053] (&lt;0.001)</strong></td>
<td><strong>0.037 [0.020-0.056] (&lt;0.001)</strong></td>
<td><strong>0.039 [-0.032-0.087] (0.065)</strong></td>
<td></td>
</tr>
<tr>
<td>Kaua’i/Ni’ihau ($n=8$)</td>
<td>0.016 [-0.002-0.026] (0.057)</td>
<td><strong>0.045 [0.023-0.064] (&lt;0.001)</strong></td>
<td><strong>0.029 [0.009-0.048] (0.003)</strong></td>
<td></td>
</tr>
</tbody>
</table>

$F_{ST\ overall} = 0.033 [0.024-0.042] (<0.001)$; $R_{ST\ overall} = 0.036 [0.005-0.074] (0.001)$

<table>
<thead>
<tr>
<th>$F_{ST}$ and $\Phi_{ST}$</th>
<th>Hawai’i</th>
<th>4-islands area</th>
<th>O’ahu</th>
<th>Kaua’i/Ni’ihau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai’i ($n=38$)</td>
<td>0.017 (0.212)</td>
<td>0.005 (0.336)</td>
<td>0.028 (0.155)</td>
<td></td>
</tr>
<tr>
<td>4-islands area ($n=27$)</td>
<td>0.011 (0.229)</td>
<td><strong>0.105 (0.032)</strong></td>
<td>0.005 (0.387)</td>
<td></td>
</tr>
<tr>
<td>O’ahu ($n=27$)</td>
<td>0.016 (0.180)</td>
<td><strong>0.112 (0.010)</strong></td>
<td><strong>0.191 (0.012)</strong></td>
<td></td>
</tr>
<tr>
<td>Kaua’i/Ni’ihau ($n=8$)</td>
<td>0.087 (0.064)</td>
<td>0.018 (0.315)</td>
<td><strong>0.282 (0.013)</strong></td>
<td></td>
</tr>
</tbody>
</table>

$F_{ST}=0.055 (0.023)$; $\Phi_{ST\ overall} = 0.039 (0.044)$

<table>
<thead>
<tr>
<th>$F'_{ST}$</th>
<th>Hawai’i</th>
<th>4-islands area</th>
<th>O’ahu</th>
<th>Kaua’i/Ni’ihau</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai’i</td>
<td>0.019</td>
<td>0.021</td>
<td>0.171</td>
<td></td>
</tr>
<tr>
<td>4-islands area</td>
<td>0.165</td>
<td>0.169</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>O’ahu</td>
<td>0.205</td>
<td>0.195</td>
<td>0.415</td>
<td></td>
</tr>
<tr>
<td>Kaua’i/Ni’ihau</td>
<td>0.098</td>
<td>0.262</td>
<td>0.156</td>
<td></td>
</tr>
</tbody>
</table>

$F'_{ST\ overall}$ for microsatellites = 0.186; $F'_{ST\ overall}$ for mtDNA = 0.089
Table 6. Top: Allelic diversity for microsatellites from samples collected 2002-2003 was calculated using ARLEQUIN. Table 6. Bottom: Haplotypic diversity, nucleotide diversity, and mean pairwise differences among mtDNA haplotypes from samples collected 2002-2003 were calculated using ARLEQUIN.

<table>
<thead>
<tr>
<th>Region</th>
<th>Allelic Diversity</th>
<th>n alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i (n=37)</td>
<td>0.835 (SD±0.430)</td>
<td>74</td>
</tr>
<tr>
<td>4-islands area (n=26)</td>
<td>0.826 (SD±0.429)</td>
<td>52</td>
</tr>
<tr>
<td>O‘ahu (n=26)</td>
<td>0.794 (SD±0.413)</td>
<td>52</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau (n=8)</td>
<td>0.841 (SD±0.457)</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Region</th>
<th>Mean Haplotypic Diversity</th>
<th>Mean Nucleotide Diversity</th>
<th>Mean Pairwise Differences</th>
<th># Haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i (n=38)</td>
<td>0.376 (SD±0.098)</td>
<td>0.004 (SD±0.002)</td>
<td>2.125 (SD±1.210)</td>
<td>6</td>
</tr>
<tr>
<td>4-islands area (n=27)</td>
<td>0.527 (SD±0.097)</td>
<td>0.006 (SD±0.004)</td>
<td>3.402 (SD±1.796)</td>
<td>4</td>
</tr>
<tr>
<td>O‘ahu (n=27)</td>
<td>0.145 (SD±0.090)</td>
<td>0.001 (SD±0.001)</td>
<td>0.519 (SD±0.450)</td>
<td>3</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau (n=8)</td>
<td>0.750 (SD±0.139)</td>
<td>0.008 (SD±0.005)</td>
<td>4.321 (SD±2.390)</td>
<td>4</td>
</tr>
<tr>
<td>Overall</td>
<td>0.450 (SD±0.255)</td>
<td>0.005 (SD±0.003)</td>
<td>2.592 (SD±1.429)</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 7. Migration rate (Nm) between pairs of regions in Hawaiian Islands waters. These rates were calculated using the private alleles method of Barton & Slatkin (1986). Error cannot be estimated with this method. This method assumes no admixture and populations in equilibrium, so values likely do not reflect exact migration rates but do suggest that migration is relatively low.

<table>
<thead>
<tr>
<th>Pairwise Regions</th>
<th>Mean Frequency of Private Alleles across Both Regions</th>
<th>Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>0.03</td>
<td>3.45</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>0.04</td>
<td>2.90</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>0.05</td>
<td>2.63</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>0.04</td>
<td>2.51</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>0.07</td>
<td>1.49</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.07</td>
<td>1.69</td>
</tr>
</tbody>
</table>
Table 8. Mean pairwise relatedness comparisons for dolphins sampled in the same group and in different groups for each region. Mean pairwise relatedness was found using COANCESTRY with 100,000 bootstraps. Results for the TrioML and LynchLi estimators are shown below. Significant differences occur when the observed mean difference is outside of the bootstrap 95% CL indicated by the 2.5% and 97.5% quantiles. There were no significant differences. Relatedness was higher using the likelihood estimator than the estimator. Kaua’i/Ni’ihau was excluded from regional comparisons because all samples were from one group, but those samples were included in the All Samples comparisons.

<table>
<thead>
<tr>
<th>Region</th>
<th>TrioML Grp Mean</th>
<th>TrioML Non-Grp Mean</th>
<th>TrioML Grp n pairs</th>
<th>TrioML Non-Grp n pairs</th>
<th>Obs Mean Diff</th>
<th>2.5% Quant</th>
<th>97.5% Quant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai’i</td>
<td>0.047 (SD±0.077)</td>
<td>0.041 (SD±0.066)</td>
<td>142</td>
<td>6186</td>
<td>-0.006</td>
<td>-0.011</td>
<td>0.01</td>
</tr>
<tr>
<td>4-islands area</td>
<td>0.037 (SD±0.050)</td>
<td>0.036 (SD±0.059)</td>
<td>48</td>
<td>277</td>
<td>-0.002</td>
<td>-0.019</td>
<td>0.016</td>
</tr>
<tr>
<td>O’ahu</td>
<td>0.083 (SD±0.099)</td>
<td>0.071 (SD±0.077)</td>
<td>51</td>
<td>274</td>
<td>0.011</td>
<td>-0.029</td>
<td>0.028</td>
</tr>
<tr>
<td>All Samples</td>
<td>0.051 (SD±0.073)</td>
<td>0.043 (SD±0.068)</td>
<td>269</td>
<td>6737</td>
<td>-0.008</td>
<td>-0.009</td>
<td>0.008</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Region</th>
<th>LynchLi Grp Mean</th>
<th>LynchLi Non-Grp Mean</th>
<th>LynchLi Grp n pairs</th>
<th>LynchLi Non-Grp n pairs</th>
<th>Obs Mean Diff</th>
<th>2.5% Quant</th>
<th>97.5% Quant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai’i</td>
<td>&lt;0.000 (SD±0.143)</td>
<td>-0.008 (SD±0.138)</td>
<td>142</td>
<td>6186</td>
<td>0.008</td>
<td>-0.230</td>
<td>0.023</td>
</tr>
<tr>
<td>4-islands area</td>
<td>-0.042 (SD±0.114)</td>
<td>-0.041 (SD±0.129)</td>
<td>48</td>
<td>277</td>
<td>-0.001</td>
<td>-0.039</td>
<td>0.038</td>
</tr>
<tr>
<td>O’ahu</td>
<td>0.028 SD±0.127</td>
<td>0.056 (SD±0.147)</td>
<td>51</td>
<td>274</td>
<td>-0.029</td>
<td>-0.042</td>
<td>0.044</td>
</tr>
<tr>
<td>All Samples</td>
<td>-0.004 (SD±0.137)</td>
<td>-0.007 (SD±0.139)</td>
<td>269</td>
<td>6737</td>
<td>0.003</td>
<td>-0.017</td>
<td>0.020</td>
</tr>
</tbody>
</table>
Discussion

My results suggest that pantropical spotted dolphins are not mating randomly across the Hawaiian Islands regions considered in this study. Microsatellite analyses, including genetic cluster assignments and fixation indices, support the separation of dolphins found in the Hawai‘i, O‘ahu, and 4-islands area regions into different populations, and mtDNA analyses support splitting at least O‘ahu and the 4-islands area. There is also some support for a separate population near Kaua‘i/Ni‘ihau, but these relationships should be explored further with larger sample sizes if possible. There was no support for the hypothesis of female philopatry driving differentiation. Possibly, local behavioral adaptations to differing environmental conditions in each region may drive genetic isolation, although further research is needed to examine this hypothesis.

In support of the conclusions above, the microsatellite $F_{ST}$ and $R_{ST}$ values among Hawai‘i, the 4-islands area, and O‘ahu were generally low but significantly different from zero (Table 5), the exception being $R_{ST}$ of the 4-islands area compared to O‘ahu. These $F_{ST}$ and $R_{ST}$ values were similar to other studies of dolphins that concluded populations were differentiated (e.g. Escorza-Treviño et al. 2005, Natoli et al. 2005), including studies that found differentiation among island regions (Parsons et al. 2006, Oremus et al. 2007). NOAA Fisheries has split spinner dolphins in the Hawaiian Islands into Hawai‘i, O‘ahu/4-islands, and Kaua‘i/Ni‘ihau stocks (Carretta et al. 2011) based mainly on genetic evidence of population differentiation (Andrews 2009, Andrews et al. 2010) and split common bottlenose dolphins into Hawai‘i, the 4-islands area, O‘ahu, Kaua‘i/Ni‘ihau, and Hawai‘i Pelagic stocks based on both genetics (Martien et al. 2011) and photo-
identification evidence (Baird et al. 2009). The $F_{ST}$ values for microsatellites for spinner dolphin and common bottlenose dolphin stocks found in the Hawaiian Islands regions overlapping my study range from 0.004 to 0.096 for spinner dolphins (Andrews et al. 2010) and 0.019 to 0.050 for common bottlenose dolphins (Martien et al. 2011). These values are lower than those for pantropical spotted dolphins, which ranged from 0.098 to 0.262 (Table 5). The spinner and bottlenose dolphin precedents suggest that comparable action could be taken to split pantropical spotted dolphin populations into separate management stocks near the Hawaiian Islands. It should be noted that O‘ahu showed evidence of heterozygosity deficiency for microsatellites. This may explain the high level of relatedness within O‘ahu. Luikart & Cornuet (1998) report that heterozygosity deficiency may indicate a recent population increase or a recent influx of rare alleles from immigrants. However, they also state that loci may seldom be at mutation-drift equilibrium because of natural fluctuations in population size or natural selection.

TESS DIC and bar plot analyses suggested that three populations were present. TESS performs well at low genetic differentiation levels, with mis-assignment rates lower than 3.5% for $F_{ST}$’s greater than or equal to 0.03 and down to 2% for $F_{ST}$ equal to 0.04 (Chen et al. 2007). There was no evidence of population level isolation by distance playing a role in this differentiation. For individuals, there was slight positive correlation between genetic and geographic distance for pairs up to approximately 130km apart (Figure 3), indicating that some isolation by distance may be occurring within island regions, but mean distances among island regions were all greater than 130km, except for the mean distance between O‘ahu and Kaua‘i/Ni‘ihau, which was 92.9km. The next
closest pair of islands are Hawai‘i and the 4-islands area at 144.2km apart. Despite the clustering of Kaua‘i/Ni‘ihau with O‘ahu by TESS, $F_{ST}$ for microsatellites and $F_{ST}$ and $\Phi_{ST}$ for mtDNA are significantly different from zero in pairwise comparisons between these two regions (Table 5). However, given that only eight samples from one group of dolphins from Kaua‘i/Ni‘ihau were available (Table 1), there were not enough data to draw strong conclusions about the relationship between this area and other regions. Lack of samples from Kaua‘i/Ni‘ihau was not due to lack of sample effort (Baird et al. 2003, 2006), rather pantropical spotted dolphins appear to be much less common in that region (Figure 2; Table 1). Two of the four mtDNA haplotypes found in these eight samples were not found in the other three regions. This shows remarkable mtDNA diversity in a small sample size from a single encounter, suggesting that pantropical spotted dolphins near Kaua‘i/Ni‘ihau may be transients from farther west along the archipelago or from an offshore population. There are no samples of pantropical spotted dolphins from the islands further west at this time, except for one collected during the NOAA HICEAS 2010 Survey; however, spinner dolphins near Kure Atoll, Midway Atoll, and Pearl & Hermes Reef have been found to be genetically distinct from those found near the other Hawaiian Islands (Andrews et al. 2010).

Estimates of pairwise regional migration rates based on the private alleles method of Barton & Slatkin (1986) ranged from 1.49 to 3.45 (Table 7). This method assumes no admixture and that alleles have reached an equilibrium in the populations. Bearing in mind these assumptions, which are likely violated by the populations in my study, these migration rates are relatively low. Attempts were made to estimate migration rates using
programs such as LAMARC (Kuhner 2006), but there were indications that convergence was not reached even after six weeks of running the program. This maximum likelihood, iterative method would calculate migration rates in both directions between populations and would provide an error factor, but given the lack of convergence, the private alleles method was applied to supply at least a sense of whether migration rates were likely high or low. These values should be used with caution given both the likelihood of admixture among populations and the fact that the values are directly related to the \( F_{st} \) calculation rather than applying Bayesian or maximum likelihood inference. Examining private alleles shared by pairs of island regions (excluding Kaua‘i/Ni‘ihau) did not result in any differences that would support significantly higher gene flow among some pairs or a founding event at one region with spread to other regions.

There was more variability in microsatellites (up to 20 alleles per locus) than mtDNA (only 10 haplotypes with 77 out of 100 individuals having haplotype 3). MtDNA \( F_{ST} \) and \( \Phi_{ST} \) results supported separation of the 4-islands area from O‘ahu and Kaua‘i/Ni‘ihau from O‘ahu, but not separation of Hawai‘i from the other three regions (Table 5). MtDNA did not show evidence of isolation by distance, suggesting that genetic differentiation among island regions may be due to other mechanisms, such as site fidelity, behavioral isolation, etc. \( F_{ST} \) and \( \Phi_{ST} \) values that were significantly different from zero for mtDNA were low but consistent with other studies that concluded that differentiation existed (e.g. Escorza-Treviño et al. 2005, Natoli et al. 2005, Mendez et al. 2007). As with microsatellites, \( F'^{ST} \) values were higher than \( F_{ST} \) values, with the largest values suggesting separation of O‘ahu from the 4-islands area and Kaua‘i/Ni‘ihau from
O‘ahu and Hawai‘i (Table 5). However, caution must be taken in interpreting results for Kaua‘i/Ni‘ihau because of the small sample size from that region. For Hawai‘i compared with the 4-islands area (0.019) and compared with O‘ahu (0.021), $F_{ST}^{′}$ values tended to be lower than those found for populations of spinner dolphins (Andrews 2009, Andrews et al. 2010), but other pairwise $F_{ST}^{′}$ values were higher (ranging from 0.046 to 0.171) than for spinner dolphins, which ranged from -0.011 to 0.120. The distinction of O‘ahu from the other islands is further supported by its much lower mtDNA diversity (Table 6) and higher pairwise relatedness within the region (Table 4).

It would be expected that the mtDNA control region would evolve more quickly than nuclear markers like microsatellites, thereby having a shallower coalescence that allows for better detection of structure in recently diverged and diverging populations (Zink & Barrowclough 2008). This is because time to coalescence is a function of effective population size, which is four times larger for nuclear markers than mtDNA markers (Zink & Barrowclough 2008). As such, it should be noted that microsatellites tend to show more structure among populations of pantropical spotted dolphins near the Hawaiian Islands than mtDNA control region sequences do. However, this is not necessarily surprising because the mtDNA of cetaceans has been found to evolve at one quarter the rate of other mammals (Ohland et al. 1995), possibly creating deeper than expected coalescence times for recently diverged taxa. Further, mtDNA is a single marker with one gene tree, whereas 11 loci were used for nuclear markers in my study, allowing averaging over multiple markers. In addition, Kingston et al. (2009) recently reported that mtDNA control region sequences have little power for resolving differences
among delphinid taxa at the species level, possibly because of the recentness of splits, which would also affect the use of this marker at the population level. Therefore, microsatellite results may be more reliable for evaluating population differentiation for pantropical spotted dolphins.

In cases in which sex-biased dispersal is occurring, it is expected that the dispersing sex will have lower $F_{ST}$ and mean assignment index values and higher $F_{IS}$ and variation in assignment index values (Goudet et al. 2002), which was not the case for pantropical spotted dolphins near the Hawaiian Islands. Parsons et al. (2006) found that common bottlenose dolphins near the Bahamas showed site fidelity for both sexes. Likewise, Andrews et al. (2010) found no evidence of sex-biased dispersal for spinner dolphins near the Hawaiian Islands, so other mechanisms for dispersal, such as behavioral adaptations to local regions, may not be uncommon in island systems.

Comparisons of relatedness within groups and among groups in each region and across all regions suggested that group fidelity is not driving genetic differentiation (Table 8). It is possible that “non-group” samples could have been from the same groups on different days, confounding the results. However, no individuals were re-sampled in multiple encounters to support this. It is also possible that sampled groups were really sub-groups of larger groups. Shallenberger (1981) reported this situation as a consideration with respect to group size estimates of pantropical spotted dolphins near the Hawaiian Islands.

Cubero-Pardo (2007) reported that depth affected the distribution of common bottlenose and pantropical spotted dolphins in Gulfo Dulce near Costa Rica, and she concluded that prey distribution patterns affected seasonal distribution of dolphins in this
location. She also suggested that groups of pantropical spotted dolphins found in deeper water were larger, likely because larger numbers afforded better protection from predators. During my study, pantropical spotted dolphins were sampled in deeper waters near Hawai‘i and were from larger groups near Hawai‘i than those sampled near the 4-islands area, while pantropical spotted dolphins sampled near O‘ahu were in deeper water than the 4-islands area but also closer to shore than those near Hawai‘i. The shallower depth at which samples were taken near the 4-islands area reflects the greater extent of shallow habitat in that region and limited survey effort in deeper waters (Figure 2; also see Baird et al. 2008b). Differences in habitat may result in differences in prey preferences at different island regions and may inhibit dispersal and, therefore, gene flow among regions. Differences in habitat features, prey types, and prey abundance across adjacent ocean areas have all been suggested as reasons for genetic differentiation in other dolphin populations (e.g. García-Martínez et al. 1999, Möller et al. 2007, Bilgmann et al. 2008, Wiszniewski et al. 2010), and concerns have been raised about the consequences of climate change on small cetacean populations for which habitat affects gene flow (Fontaine et al. 2007, Taguchi et al. 2010). García-Martínez et al. (1999) suggested that dolphins preferring shallow water habitats can become isolated. This may be the case for pantropical spotted dolphins near the 4-islands area; however, additional search effort and sample collection in deeper waters near the 4-islands area would provide more evidence to assess this. A study of Galápagos sea lions near the Galápagos archipelago revealed that sea lions in different regions of the archipelago were using different food sources and concluded that inter-specific niche segregation played a key
role in the evolution of genetic structure in these populations (Wolf et al. 2008). This study relied on stable isotope analysis, which would likely be a good approach to determine if pantropical spotted dolphins near different Hawaiian Island regions tend to feed on different prey. Only one study to date has addressed habitat use of pantropical spotted dolphins near the Hawaiian Islands (Baird et al. 2001). That study focused on the 4-islands area and concluded that pantropical spotted dolphins had different habitat use patterns in that region compared with other dolphin species (Baird et al. 2001).

Pantropical spotted dolphins may have different numbers of predators and competitors near different island regions. Friedlander and DeMartini (2002) found that biomass of apex predators, which included sharks, groupers, and barracuda, differed among the Hawaiian Islands. Papastamatiou et al. (2006) reported that catch per unit effort differed among shark species among the Hawaiian Islands for four species of shark caught in the Hawaiian longline fishery, resulting in different estimates of the relative contribution of each species to the overall species composition near each island region. Such studies suggest possible differences in predation pressure among island regions. I found no strong evidence that males and females disperse differently or that individuals sampled from the same group were more closely related than individuals sampled from different groups, so niche specializations may play a more important role in differentiation than sex-biased dispersal or group fidelity. Andrews (2010) suggests that habitat differences can raise ecological barriers to gene flow that drive differentiation in spinner dolphin populations near the Hawaiian Islands as well.
Properly defining populations is important because site fidelity, despite the 
physical ability to disperse, can result in local population losses when populations are 
stressed by anthropogenic encroachment. For example, Nichols et al. (2007) described a 
situation in which a genetically distinct population of common bottlenose dolphins near 
the UK went extinct, likely due to hunting 100 years ago, and other nearby common 
bottlenose dolphin populations have still not repopulated the site. Given the fisheries 
activity, including unrestricted fisheries such as the troll fishery, involving pantropical 
spotted dolphins near the Hawaiian Islands, accurate population assessments are critical 
to conservation. If NOAA Fisheries designated the island of Hawai‘i as a separate stock, 
the minimum population size ($N_{\text{min}}$) would possibly result in Potential Biological 
Removal (PBR) being exceeded by more than 1% for the troll fishery, requiring by law 
that it be changed to a Category II fishery (Department of Commerce 2011), as has been 
proposed by NOAA Fisheries. Category II fisheries are required to obtain a marine 
mammal authorization by registering with the Marine Mammal Authorization Program 
and are required to allow observers on board vessels engaged in the fishery (Department 
of Commerce 2011). These changes to a currently unrestricted fishery that runs lines 
regularly over groups of pantropical spotted dolphins could potentially avoid diversity or 
population losses for these small, island-associated populations. At this time, there are 
few data available regarding this fishery near other Hawaiian Islands, and designating 
separate stocks near the 4-islands area and O‘ahu may create more impetus to examine 
the levels of troll fishing effort near these regions and assess impacts on pantropical 
spotted dolphins. The List of Fisheries lists the Hawaiian troll fishery as including 2,210
participant vessels/persons (Department of Commerce 2011). Near the island of Hawai‘i
~29% of groups of pantropical spotted dolphins encountered from 2006 to 2011 had
fishing vessels fishing through or in the middle of groups (R.W. Baird unpub. data).
Pantropical spotted dolphins with injuries from lines and boats have also been
photographed near the Hawaiian Islands during these and other surveys (R.W. Baird
unpub data), raising concerns about the level of fisheries interactions, particularly if
populations are smaller and more isolated than are recognized under the current
management scheme. Effective population size (not an overall abundance estimate) for
the island of Hawai‘i based on microsatellite data is estimated to fall between 210 and
261, suggesting a small reproductive population in this location.

In conclusion, I found evidence that pantropical spotted dolphins constitute
separate populations near Hawai‘i, the 4-islands area, and O‘ahu, with some evidence to
support possible differences from Kaua‘i/Ni‘ihau as well, though further study is
warranted. Other marine mammal stocks have been divided recently for management
purposes based on similar levels of genetic differentiation among regions. For example,
harbor porpoise on the U.S. West Coast were split into additional stocks based on Chivers
\textit{et al.} (2002), and NOAA Fisheries has recently split common bottlenose dolphin stocks
and split spinner dolphin stocks near the Hawaiian Islands based on genetic and photo-
al.} 2011, Martien \textit{et al.} 2011). I suggest the same criteria be applied to pantropical
spotted dolphins near the Hawaiian Islands. It is important that genetically appropriate
stocks of pantropical spotted dolphins be used for management, particularly because of
potential injury and mortality as a result of the Hawaiian troll fishery, which is currently unrestricted. My results suggest that differentiation is not mediated by sex-biased dispersal or group fidelity, and I hypothesize that possibly behavior adapted to differing habitat types that affect strategies such as foraging and predator avoidance could be driving differentiation. Further research on these strategies is needed to confirm these differences. Tagging studies and stable isotope analyses using blubber samples could explore feeding habits and other behaviors that differ among the regions of the Hawaiian Islands.
CHAPTER 3. Comparisons to explore potential baselines for biologically significant genetic differentiation levels: Population structure of pantropical spotted dolphins in the North Pacific Ocean as an example

Introduction

Many studies of dolphin population genetics rely heavily on $F_{ST}$ and similar indices (e.g. $R_{ST}$, $\Phi_{ST}$) for determining where different populations occur (e.g. Yao et al. 2004, Escorza-Treviño et al. 2005, Adams & Rosel 2006). These indices are based on Wright’s $F_{ST}$ (Wright 1943, 1951), which varies from zero to one, for which one indicates separate non-interbreeding populations and zero indicates panmixia. The probability that the calculated $F_{ST}$ value is different from zero is generally used to decide whether $F_{ST}$ is “significant.” However, this null hypothesis test simply indicates that $F_{ST}$ is different from zero within a 95% probability; it does not determine whether the level of differentiation indicated by $F_{ST}$ is biologically/demographically significant. This distinction is important, particularly if management and conservation decisions are based on fixation indices. Gerrodette (2011) thoroughly discusses the issue of problems with null hypothesis testing in ecology and states that lack of statistical significance does not necessarily mean lack of biological importance. He points out that there is no expectation that the null hypothesis would really be true, for example, that the proportion of genetic markers from two locations would be identical, and that null hypothesis testing in this type of instance is really just testing whether the sample size is sufficient to detect differences.
Often, population genetics studies of dolphins conclude that populations are separate based, at least in part, on very low $F_{ST}$ values that are found to be significantly different from zero (e.g. Sellas et al. 2005, Adams & Rosel 2006, Gaspari et al. 2007). This is not always the best criterion on which to base management (Taylor & Dizon 1996, Taylor 1997, Taylor et al. 1997, Palsbøll et al. 2007). Part of the problem arises from an inability to obtain large enough sample sizes, but the other problem is that there are no standard fixation indices for comparison. For instance, there is no lower bound $F_{ST}$ value that defines demographically significant units for management. Defining these bounds becomes even more complicated when using highly variable loci like microsatellites. The range of $F_{ST}$ does not have a maximum of one in these cases because highly polymorphic loci have high levels of heterozygosity, reducing maximum $F_{ST}$ (Hedrick 1999). Also, overlap in alleles occurs between isolated populations (Hedrick 1999). Methods have been developed to address this problem using standardizations of $F_{ST}$ (Hedrick 2005, Meirmans 2006), but these standardizations rely on dividing by maxima that make it statistically inappropriate to calculate significance with respect to zero (Meirmans 2006), and so interpretation of these values can be difficult. Rather than determining difference from zero, it could be helpful to use $F_{st}$ as a comparative measure.

Palsbøll et al. (2007) suggest that it is important to interpret estimates of genetic differentiation in a demographic framework and to attempt to define threshold values for measures of differentiation, such as $F_{ST}$, $R_{ST}$, $\Phi_{ST}$, etc. One lower bound value of $F_{ST}$ would not likely be appropriate for all species or populations, but finding fixation indices among geographically separated populations that are unlikely to interbreed would give us
an idea of what values among apparently sympatric or parapatric populations could be if interbreeding is reduced or absent.

There are other ways to measure genetic differentiation than using fixation indices, and ideally, fixation indices are only one line of evidence used to determine population structure. Hedrick (1999) suggested that assignment tests are a powerful approach for assessing population structure using highly variable loci. For example, programs like STRUCTURE (Pritchard et al. 2000) can be used to find assignment probabilities of individuals to different populations, helping to interpret the biological significance of levels of differentiation indicated by fixation indices and potentially revealing cryptic population structure.

In this study, I compared samples of pantropical spotted dolphins from the Eastern Tropical Pacific (ETP), pelagic waters near the U.S. Exclusive Economic Zone surrounding the Hawaiian Islands, and China/Taiwan with samples from near the Hawaiian Islands (Figure 8) to explore biological significance associated with differentiation found among the Hawaiian Islands (Chapter 2). I focused specifically on $F_{ST}$ and $R_{ST}$ for microsatellites and $F_{ST}$ and $\Phi_{ST}$ for the mtDNA control region, as well as standardized values of $F_{ST}$ (written as $F'_{ST}$). $R_{ST}$ is an $F_{ST}$ analog that accounts for stepwise evolution of microsatellites. $\Phi_{ST}$ is an $F_{ST}$ analog that uses Analysis of Molecular Variance (AMOVA). $F_{ST}$ and $R_{ST}$ calculations use departure of allele frequencies from panmictic expectations, while $\Phi_{ST}$ evaluates the correlation of random haplotype sequences within populations relative to random pairs drawn from the whole species (Excoffier et al. 1992). For $\Phi_{ST}$, a hierarchical AMOVA is constructed directly.
from the matrix of squared-distances between all pairs of haplotypes (Excoffier et al. 1992). \( F_{ST}, R_{ST}, \) and \( \Phi_{ST} \) are all commonly reported as measures of genetic differentiation among putative populations.

My comparative approach assumes that levels of potential interbreeding among more widely separated geographical locations would be lower than levels of interbreeding among the less geographically separated Hawaiian Islands regions. It also assumes that the migration rates are low enough to offset the influence of population size differences on calculated fixation indices. If these assumptions are true, values obtained for comparisons among the widely geographically separated populations could serve as baselines for expected values among pantropical spotted dolphin populations with little to no interchange among them. These baselines themselves are not necessarily transferable to other species, but the comparisons used to determine them would be. Because \( F_{ST} \) assumes that populations have already reached an equilibrium or are fixed for specific alleles, populations that are still in the process of diverging may have low fixation indices even if the populations are not interbreeding much or at all. Therefore, baselines determined with what are more likely to be populations closer to equilibrium are helpful in determining whether low but significant fixation indices are indicative of biologically important population boundaries.

Based on morphological differences (Schnell et al. 1986) and, more recently, on genetic evidence (Escorza-Treviño et al. 2005, Rosales 2005), pantropical spotted dolphins in the ETP are considered to be two sub-species: offshore \((S. a. attenuata)\) and coastal \((S. a. graffmani)\) (Perrin et al. 1985). The offshore sub-species have been further
subdivided into northern and southern populations based on differences in morphology, differences in calving peaks, and a potential gap in distribution (Barlow 1984, Perrin et al. 1985, Schnell et al. 1986). Dizon et al. (1994b) defined them as a northeastern and a southern-western stock (Figure 8). The northeastern and southern-western offshore stocks are generally divided near the equator (Perrin et al. 1985). There is geographical overlap among the coastal and offshore sub-species that could allow interbreeding to occur (Perrin et al. 1985). The coastal sub-species is generally described as occurring within 185 km of the coast; however, the offshore sub-species has been recorded within 30 km of the coast. The coastal sub-species has been recorded as far as 130 km offshore (Perrin et al. 1985).

Dizon et al. (1994b) reported that the Hawaiian stock of pantropical spotted dolphins is discriminated from the other Pacific stocks based on its proximity to the Hawaiian Islands rather than on any genetic, behavioral, or major morphological differences. The stock boundary is the U.S. Exclusive Economic Zone surrounding the Hawaiian Islands (Carretta et al. 2011). Pantropical spotted dolphins near the Hawaiian Islands tend to have less spotting than and differing cranial morphology from northern offshore pantropical spotted dolphins, but have similar coloration and cranial morphology to southern offshore pantropical spotted dolphins (Dizon et al. 1994b). Reeves et al. (2004) stated that numerous additional sub-species of Pacific pantropical spotted dolphins may be added in the future as further studies reveal more information on levels of differentiation. Yao et al. (2008) reported variation in skull morphology of pantropical spotted dolphins near China/Taiwan and in the ETP, including smaller overall skull size
in ETP specimens, but differences may be attributed to differing levels of primary productivity in these locations. All ETP pantropical spotted dolphins in the Yao et al. (2008) study were of the offshore sub-species, and no comparisons were made with pantropical spotted dolphins near the Hawaiian Islands.

Given the morphological differences and distribution gaps among ETP, Hawaiian and China/Taiwan pantropical spotted dolphins, I think the assumption of less interbreeding potential among these locations than within the Hawaiian Islands is valid for the current study.

Methods

Microsatellites were previously analyzed for 37, 26, 26, and 8 pantropical spotted dolphins sampled near Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau respectively from 2002-2003 (Chapter 2). Microsatellites were also analyzed for 50 ETP samples and three samples from pelagic waters near the Hawaiian Islands Exclusive Economic Zone (Figure 8) using the procedures described in Chapter 2, with loci EV14, EV37, EV94, (Valsecchi & Amos 1996), SL8-49, SL9-69, SD8 (Galver 2002), MK5, MK6, MK8 (Krützen et al. 2001), KWM2a, and KWM12a (Hoelzel et al. 1998a). One pelagic sample was obtained from skin from a pantropical spotted dolphin killed as bycatch in the Hawaiian long-line fishery in May, 2008. This sample was supplied by Kristi West, Hawai‘i Pacific University (Honolulu, HI). Two additional pelagic samples collected during a NOAA PICEAS cruise in 2005 were obtained from NOAA Fisheries. Twenty-five Costa Rican and Panamanian S. a. graffmani samples collected 1998-2000
and 25 offshore ETP S. a. attenuata (northern offshore stock) samples collected 1998-2001 were also obtained from NOAA Fisheries (Figure 8). These samples were part of Escorza-Treviño et al.’s (2005) study of genetic differentiation among pantropical spotted dolphins in the ETP.

MtDNA for the pelagic samples was sequenced using the procedure described in Chapter 2 with primers H00034 (Rosel et al. 1994) and L15824 (Rosel et al. 1999). Thirteen Hawaiian Island haplotypes were previously sequenced (Chapter 2) and published on GenBank (GQ852567-GQ852579). One hundred twelve mtDNA haplotypes for offshore and coastal ETP pantropical spotted dolphins (Escorza-Treviño et al. 2005) were obtained from GenBank (DQ150134-DQ150245). Offshore haplotypes were from samples from the northeastern ETP (Escorza-Treviño et al. 2005), and coastal haplotypes were from four locations defined in Escorza-Treviño et al. (2005): Northern Mexico, Central America, Costa Rica, and Ecuador (Figure 8). Thirteen haplotypes from near Taiwan and the China Sea were provided by C. Yao (Yao et al. 2004).

**Microsatellites**

ARLEQUIN 3.0.1.2 (Schneider et al. 2000) was used to check microsatellite loci for pairwise linkage disequilibrium for each population with 50,000 Markov steps and a 50,000 step burnin and for deviations from Hardy-Weinberg equilibrium for each locus for each population with 1,000,000 Markov steps and a 100,000 step burnin. LOSITAN (Antao et al. 2008) was used to evaluate neutrality of microsatellite loci. Neutral Mean $F_{ST}$ and Force Mean $F_{ST}$ were chosen and run for 50,000 simulations with a stepwise
mutation model. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to test for null alleles and allelic dropout using 10,000 permutations at 95% confidence for each region. ARLEQUIN was used to calculate $F_{ST}$ and $R_{ST}$. $F_{ST}$ and $R_{ST}$ of microsatellites reported from ARLEQUIN are equivalent to $\Theta_{ST}$ of Weir and Cockerham (1984) based on a pairwise distance matrix using number of different alleles for $F_{ST}$ and sum of squared differences for $R_{ST}$. $R_{ST}$ differs from $F_{ST}$ in that it uses a step-wise mutation model rather than an infinite alleles model, which may be more appropriate for the evolution of microsatellites. Standardized $F'_{ST}$ values were calculated in ARLEQUIN using the procedure described in Chapter 2. No additional microsatellite data were available from previous studies, so comparisons were limited to the offshore ETP, Costa Rica/Panama for the coastal ETP sub-species, and the Hawaiian Islands regions as defined in Chapter 2 (Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau) (Figure 8). Although Costa Rica and Panama samples include two adjacent regions defined in Escorza-Treviño et al. (2005) (Costa Rica and Ecuador; Figure 8), they were combined because of low sample size and high distinctiveness from other samples. Pelagic samples were not included in this analysis because of small sample size (three samples).

STRUCTURE 2.2 (Pritchard et al. 2000, Falush et al. 2003) was used to analyze microsatellite data to estimate the number of populations and calculate assignment probability of each individual to each population cluster. Burn-in period and number of replications was set to 50,000 and ten iterations were run for differing numbers of presumed populations, from $K=1$ to $K=8$. The admixture model of population structure with correlation of allele frequencies among populations was used. Length of burn-in
and number of replications were considered sufficient based on stabilization of log(alpha), ln likelihood, and lnP(D). Results were analyzed using posterior probabilities, as well as by assessing mean assignment probabilities for regions at different K values. TESS 2.3 (Chen et al. 2007, Durand et al. 2009) was used for assignment probability analyses in Chapter 2 (testing among Hawaiian Island regions). TESS incorporates spatial autocorrelation into analyses. In the case of ETP and Hawaiian samples, there may be genuine gaps in distribution rather than just sampling gaps and there is geographic overlap in the ranges of ETP coastal and offshore subspecies, so STRUCTURE, which considers admixture but not spatial autocorrelation, was used for the present analyses.

It was expected that ETP coastal and offshore populations would group separately from putative Hawaiian Islands populations in STRUCTURE and that mtDNA control region $F_{ST}$ and $\Phi_{ST}$ values and microsatellite $F_{ST}$ and $R_{ST}$ values would be larger for comparisons among the Hawaiian Islands and other locations than within the Hawaiian Islands putative populations.

Mean number of distinct alleles per locus and mean number of private alleles per locus, accounting for sample size, was calculated using AZDE 1.0 (Szpiech et al. 2008) to determine if the number of private alleles was sufficient to estimate migration rates. AZDE was also used to determine mean number of private alleles per locus for pairs of putative populations to examine whether any pair of populations shared enough alleles only with each other to indicate more recent migration or founder events. Kaua‘i/Ni‘ihau was not included in the pairwise analysis because the program can only calculate means
up to the smallest population sample size, which would limit the comparisons to sample size of eight. To compare this with the estimate of gene flow in Chapter 2, migration rates (Nm) were estimated using the private alleles method of Barton & Slatkin (1986) in GENEPOP 4.0.1.0 (Raymond & Rousset 1995, Rousset 2008).

**MtDNA**

More sequences were available for mtDNA analyses than for microsatellite analyses due to the publication of mtDNA sequences on GenBank. Sequences were aligned using CLUSTAL X 1.81 (Thompson et al. 1997). TCS (Clement et al. 2000) was used to create a haplotype network to visualize the relationships among mtDNA haplotypes. A 95% connection limit was chosen in TCS, and gaps were set to a 5th state. ARLEQUIN 3.0.1.2 (Schneider et al. 2000) was used to calculate $F_{ST}$ and $\Phi_{ST}$ for the mtDNA sequence data for 407 base pairs (the overlapping portion of all sequences). The number of permutations in a randomization test was set to 50,000, and $\Phi_{ST}$ was calculated using a basic pairwise comparison model without weighting mutation type. Standardized $F'_{ST}$ values were calculated in ARLEQUIN using the procedure described in Chapter 2. Pelagic and South China Sea samples were not included in the $F_{ST}$ and $\Phi_{ST}$ analyses because of small sample sizes (three and four samples respectively). Yao et al. (2004) found that Taiwan Strait and Eastern Taiwan samples did not have $F_{ST}$ or $\Phi_{ST}$ values significantly different from zero when compared with each other; therefore, these samples were combined as “China/Taiwan” in my analyses. Escorza-Treviño et al. (2005) found that comparisons among Northern Mexico, Central America, Costa Rica,
and Ecuador areas resulted in $F_{ST}$ and $\Phi_{ST}$ values significantly different from zero, so these areas were considered as separate in my analyses.

**Results**

*Microsatellites*

Comparisons were made for pantropical spotted dolphin samples from coastal and offshore ETP, the four defined Hawaiian Islands regions, and pelagic waters outside the Hawaiian EEZ (Figure 8). There was no indication of linkage disequilibrium or deviations from Hardy-Weinberg Equilibrium ($p$-values<0.05) for the microsatellite loci. MICRO-CHECKER indicated no significant excess of homozygotes for the combined probability test for any of the island regions, suggesting the loci did not have null alleles or allelic dropout. There was no indication of linkage disequilibrium or deviations from Hardy-Weinberg Equilibrium for the microsatellite loci ($p$<0.05). MICRO-CHECKER indicated no significant excess of homozygotes for the combined probability test for any of the island regions, suggesting the loci did not have null alleles or allelic dropout. LOSITAN analyses indicated that all microsatellite loci were neutrally evolving with 95% confidence.

A plot of $K$ values *versus* posterior probabilities produced by STRUCTURE indicated between two and five populations, with no significant differences among the mean posterior probabilities for $K=2$, $K=3$, $K=4$, and $K=5$ ($F=0.58$, $p=0.634$) (Figure 9). Bar plots of the probability of assignment to each cluster for $K=5$ showed clustering of assignment for Hawai‘i with Kaua‘i/Ni‘ihau, the 4-islands area, O‘ahu, and coastal ETP
as four strong clusters and offshore ETP as split between the coastal ETP cluster and a separate cluster (Figure 10). Coastal ETP samples clustered strongly together; mean probability of assignment to cluster 2 was 0.931 (SE±0.001). Offshore ETP samples split between the coastal ETP cluster and a separate cluster, with mean probability of assignment to the coastal ETP cluster at 0.403 (SE±0.015) and to a separate cluster at 0.450 (SE±0.019). Based on ANOVA and Tukey tests, these values were significantly different from each other and from the probabilities of assignment to the remaining three clusters (Table 9). Hawai‘i and Kaua‘i/Ni‘ihau clustered together, but Kaua‘i/Ni‘ihau clustered more closely to offshore ETP and O‘ahu than Hawai‘i did (Table 9). O‘ahu and the 4-islands area both had probabilities of assignment to their own clusters with means greater than 0.650 (Table 9). The three pelagic samples did not cluster together. One sample clustered most closely with Hawai‘i, one most closely with offshore ETP, and the third clustered equally with Hawai‘i and offshore ETP (Table 9).

\[ F_{ST} \] and \[ R_{ST} \] values comparing offshore ETP, coastal ETP, Hawai‘i, the 4-islands area, O‘ahu, and Kaua‘i/Ni‘ihau were also calculated. Pelagic samples were not included because of small sample size (only three samples). All \[ F_{ST} \] values were significantly different from zero except Kaua‘i/Ni‘ihau compared with Hawai‘i, and most \[ R_{ST} \] values were as well (Table 10). \[ F_{ST} \] values for the Hawaiian Islands regions were lower than those comparing offshore ETP and coastal ETP with Hawaiian Islands regions, but similar to offshore ETP compared with coastal ETP (Table 10). \[ F'_{ST} \] values indicate a similar relationship, with \[ F'_{ST} \] of offshore ETP compared with coastal ETP being
numerically lower than other $F_{ST}^{*}$ values, except those for Hawai‘i compared with the 4-islands area and Kaua‘i/Ni‘ihau (Table 10).

$R_{ST}$ for offshore ETP in comparison with O‘ahu (0.051) was numerically less than that for Hawai‘i in comparison with O‘ahu (0.055). $R_{ST}$ for coastal ETP in comparison with offshore ETP (0.030) was lower than $R_{ST}$ values for all comparisons among the Hawaiian Islands that resulted in $R_{ST}$ values significantly greater than zero (Table 10).

Mean number of private alleles per locus was reasonable for making estimates based on the private allele method by Barton & Slatkin (1986), which is robust to mean private allele values as low as 0.2 (Figure 11). Note that the trend is that offshore ETP and coastal ETP share the most mean private alleles per locus, followed by the combinations of Hawaiian Island regions, then offshore ETP combinations with Hawaiian Islands regions, and finally coastal ETP and combinations of Hawaiian Islands regions (Figure 12). The overlap of standard errors for the offshore ETP/coastal ETP and the Hawaiian Island regions pairs indicates that the numbers of mean private alleles shared among these pairs were not significantly different (Figure 12). Unlike for the combinations of pairs of Hawaiian Island regions, the Hawaiian Island region triad standard error range did not overlap ranges for other triad combinations (Figure 12). The combinations of each island region with offshore ETP and coastal ETP have overlapping standard errors (Figure 12).

Migration rates ($N_{m}$) were calculated using the private alleles method of Barton & Slatkin (1986) (Table 11). Error cannot be estimated with this method so significant differences could not be determined.
Previously, 13 mtDNA haplotypes were discovered near the Hawaiian Islands (Chapter 2). Of the three pelagic pantropical spotted dolphin samples in this study, two had Hawaiian haplotype 3, (Chapter 2) and one had a unique haplotype (haplotype 14). These haplotypes are published as GenBank accession numbers GQ852569 and GU136595 respectively. Comparisons with mtDNA sequences from pantropical spotted dolphins from the ETP (112 haplotypes) (Escorza-Treviño et al. 2005) showed that four Hawaiian haplotypes (Chapter 2) matched with ETP haplotypes; the unique pelagic haplotype did not match any ETP haplotypes. One haplotype of a single individual near O‘ahu (haplotype 11) matched with a haplotype of a single individual from coastal Central America (ETP haplotype 33), and one haplotype of a single individual from Kaua‘i/Ni‘ihau (haplotype 6) matched with a haplotype of a single individual from offshore ETP (ETP haplotype 81). Two Hawaiian haplotypes that differ at a point in the sequence beyond the end of the available ETP sequences (haplotypes 3 and 13) both match an ETP haplotype (ETP haplotype 6). Hawaiian haplotype 13 was found in only one individual from Hawai‘i, and haplotype 3 is the majority haplotype near the Hawaiian Islands (138 individuals out of 176 sampled from 2002-2008; Chapter 2). ETP haplotype 6 was found in eight coastal and nine offshore individuals (Escorza-Treviño et al. 2005). The most common haplotype in the ETP, haplotype 39, was only shared by 18 individuals (Escorza-Treviño et al. 2005) and did not occur in the other regions (Table 12).
Pantropical spotted dolphins from China/Taiwan (Yao et al. 2004) also included haplotypes matching Hawaiian haplotypes. Hawaiian haplotypes 3 and 13 (Chapter 2) matched the shorter sequence of Yao et al. (2004) haplotype 2 of one individual from Taiwan Strait and one individual from Eastern Taiwan. Haplotype 11 from O‘ahu (Chapter 2), which matched ETP haplotype 33 from Central America (Escorza-Treviño et al. 2005), also matched China/Taiwan haplotype 3 found in two individuals from Taiwan Strait (Yao et al. 2004). The unique pelagic haplotype 14 from the current study did not match any of the China/Taiwan haplotypes from Yao et al. (2004) (Table 12).

$F_{ST}$ values among the Hawaiian Islands regions are not significantly different from zero except for O‘ahu compared with the 4-islands area and with Kaua‘i/Ni‘ihau, so mtDNA $F_{ST}$ values without standardization did not lend strong support to separation of the Hawaiian Islands regions in the original study (Chapter 2). $F_{ST}$ of Kaua‘i/Ni‘ihau compared with O‘ahu was similar to $F_{ST}$ values for offshore ETP compared with the Hawaiian Islands regions (Table 13). $F_{ST}$ was not significantly different from zero for Northern Mexico compared with offshore ETP, and values were low for comparisons among offshore ETP, Central America, Northern Mexico, and Kaua‘i/Ni‘ihau. $F_{ST}'$ values are higher than $F_{ST}$ values, and indicate complete separation of Costa Rica from other regions and high levels of differentiation among the rest of the offshore and coastal populations, with the exception of Northern Mexico compared with offshore ETP (Table 13). Interestingly, Kaua‘i/Ni‘ihau shows lower levels of differentiation from offshore ETP and Northern Mexico than the rest of the Hawaiian Island regions do (Table 13). It is clear that $F_{ST}'$ is generally much lower within the Hawaiian Islands regions than among
the rest of the locations, except that the value for O'ahu compared with Kaua‘i/Ni‘ihau is comparable to Northern Mexico compared with Kaua‘i/Ni‘ihau.

For Φ_{ST}, there are also few values significantly different from zero within the Hawaiian Islands comparisons, with O‘ahu again showing differentiation from the 4-islands area and Kaua‘i/Ni‘ihau. However, in this case, there are also no significant differences from zero for comparisons among offshore ETP, Kaua‘i/Ni‘ihau, Northern Mexico, and Central America. There are also 11 Φ_{ST} values that are significantly different from zero but are numerically lower than the values for O‘ahu in comparison with Kaua‘i/Ni‘ihau and the 4-islands area, although these may not be statistically significant differences (Table 13).

When the haplotypes are viewed in a haplotype network, they do not cluster together by region; haplotypes from the Hawaiian Islands, pelagic, and China/Taiwan are peppered throughout the 112 ETP haplotypes without any obvious clusters (network is too large to produce as a figure). Mean haplotypic diversity and mean pairwise differences among haplotypes tends to be higher for ETP populations than for Hawaiian and China/Taiwan populations, with the exception of Kaua‘i/Ni‘ihau (Table 14).

Tables & Figures
Figure 8. Locations of sampling are shown for the Eastern Tropical Pacific, China/Taiwan, the pelagic, and Hawaiian Islands. Only samples used for microsatellite analyses are shown for the ETP offshore and coastal stocks, but the sampling area that resulted in 112 mtDNA haplotypes is indicated (ETP Sampling Area). The stock boundaries for the ETP northern offshore stock and coastal stock are from Perrin *et al.* (1985). The coastal population boundaries are from Escorza-Treviño *et al.* (2005). Two pelagic samples from near the Hawaiian Islands were collected at the location indicated on the map. There were no coordinates available for the third sample; it was collected 200 miles west of O‘ahu.
Figure 9. Mean posterior probability from STRUCTURE analyses. ANOVA test showed no significant differences among the mean posterior probabilities for K=2, K=3, K=4, and K=5. Standard error bars are shown.
Figure 10. STRUCTURE bar plot by region of assignment probabilities for K=5. 1=Offshore ETP, 2=Coastal ETP, 3=Hawail'i, 4=Kaua'i/Ni'ihau, 5=4-islands area 6=O’ahu, 7=Pelagic. The five clusters are differing shades of gray. Note that each region shows high cluster assignment probability to a different cluster, except Kaua‘i/Ni‘ihau matches mainly with the Hawail‘i cluster and the pelagic samples cluster with Hawail‘i and Offshore ETP. If likelihood of assignment to clusters were equal, as in a single population, the assignment probabilities should all be near 0.20, creating an equal representation by each cluster in each region in the bar plot.
Figure 11. Top: Mean number of distinct alleles per locus estimated based on sample size in ADZE. Kaua‘i/Ni‘ihau and Hawaiian pelagic samples are not included because of small sample size. Figure 11. Bottom: Mean number of private alleles per locus estimated based on sample size using ADZE. Mean number of private alleles per locus is reasonable for making estimates based on private allele method by Barton & Slatkin (1986), which is robust to mean private allele values as low as 0.2. Standard errors are shown with error bars.
Figure 12. Top: Mean number of private alleles per locus for pairs of regions. Standard errors are not shown because the overlaps make it difficult to see what is in the figure. The ranges of values within the standard errors for sample size 25 overlap in the following pattern: standard errors overlap for offshore ETP/coastal ETP, the three combinations of the Hawaiian Island regions, and offshore ETP/Hawai'i; standard errors also overlap for offshore ETP combinations with each of the three Hawaiian Island regions; Coastal ETP/Hawai'i and coastal ETP/4-islands area standard errors overlap with each other and with offshore ETP/4-islands area and offshore/O'ahu; and standard errors of coastal ETP/Hawai'i overlap with coastal ETP/O'ahu. Note that the trend is that offshore ETP and coastal ETP share the most mean private alleles per locus, followed by the combinations of Hawaiian Island regions, then offshore ETP combinations with Hawaiian Islands regions, and finally coastal ETP and combinations of Hawaiian Islands regions. This suggests the Hawaiian Islands regions were more likely populated by animals originating from the offshore ETP than from the coastal ETP subspecies, although it should be borne in mind that the coastal ETP has less distinct alleles than the offshore ETP, making the odds greater that alleles will be shared with the offshore ETP. The overlap of standard errors for the offshore ETP/coastal ETP and the Hawaiian Island regions pairs indicates that the numbers of mean private alleles shared among these pairs are not significantly different, suggesting that Hawaiian Islands regions have a similar level of gene flow with each other as seen with coastal ETP and offshore ETP.
Unlike for the combinations of pairs of Hawaiian Island regions, the Hawaiian Island region triad standard error range does not overlap ranges for other combinations, indicating more shared private alleles per locus and a likelihood that there were not separate colonizing events at different island regions. The combinations of each island region with offshore ETP and coastal ETP have overlapping standard errors, so there are likely no significant differences to indicate colonization of one island region spreading to other regions. Possibly, Hawai‘i, 4-islands area, and O‘ahu were relatively rapidly colonized by one founding event from the ETP.
Table 9. Assignment probability to each of five clusters from STRUCTURE analysis. Ten iterations were performed at K=5. Means, standard errors, and significant differences among means based on ANOVA analyses are shown here. Bold values are the largest assignment probabilities for each region. There was no significant difference in assignment probabilities of Kaua‘i/Ni‘ihau to clusters 1 and 5, and there was no significant difference in assignment probabilities of pelagic to clusters 3 and 5. There was a significant difference between assignment probabilities of offshore ETP to clusters 1 and 2.

<table>
<thead>
<tr>
<th>Region</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
<th>Cluster 3</th>
<th>Cluster 4</th>
<th>Cluster 5</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i</td>
<td>0.129 (SE±0.005)</td>
<td>0.046 (SE±0.001)</td>
<td>0.109 (SE±0.001)</td>
<td><strong>0.583 (SE±0.005)</strong></td>
<td>0.133 (SE±0.001)</td>
<td>4240.790</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4-islands Area</td>
<td>0.094 (SE±0.005)</td>
<td>0.023 (SE±0.001)</td>
<td><strong>0.651 (SE±0.004)</strong></td>
<td>0.088 (SE±0.001)</td>
<td>0.145 (SE±0.001)</td>
<td>7558.590</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau</td>
<td>0.228 (SE±0.014)</td>
<td>0.021 (SE±0.001)</td>
<td>0.082 (SE±0.002)</td>
<td><strong>0.414 (SE±0.011)</strong></td>
<td>0.254 (SE±0.006)</td>
<td>346.170</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>O‘ahu</td>
<td>0.127 (SE±0.006)</td>
<td>0.024 (SE±0.001)</td>
<td>0.094 (SE±0.001)</td>
<td>0.099 (SE±0.002)</td>
<td><strong>0.657 (SE±0.007)</strong></td>
<td>3757.810</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pelagic</td>
<td>0.332 (SE±0.008)</td>
<td>0.028 (SE±0.001)</td>
<td>0.132 (SE±0.002)</td>
<td><strong>0.356 (SE±0.008)</strong></td>
<td>0.152 (SE±0.002)</td>
<td>741.180</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Offshore ETP</td>
<td><strong>0.450 (SE±0.019)</strong></td>
<td>0.403 (SE±0.015)</td>
<td>0.052 (SE±0.001)</td>
<td>0.053 (SE±0.001)</td>
<td>0.043 (SE±0.001)</td>
<td>356.480</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Coastal ETP</td>
<td>0.021 (SE±0.000)</td>
<td><strong>0.931 (SE±0.001)</strong></td>
<td>0.017 (SE±0.001)</td>
<td>0.016 (SE±0.001)</td>
<td>0.014 (SE±0.001)</td>
<td>380000.000</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 10. Top: $F_{ST}$ and $R_{ST}$ for microsatellite data. 50,000 permutations were used for calculations. $R_{ST}$ is above the diagonal, and $F_{ST}$ is below the diagonal. 95% CL for values are shown in brackets. These were calculated in ARLEQUIN using 20,000 bootstraps. Numbers in bold are significantly different from zero, and $p$-values are in parentheses. Table 10. Bottom: $F'_{ST}$ (standardized $F_{ST}$) is shown: $F'_{ST} = F_{ST} / F_{ST_{max}}$. These values were calculated in ARLEQUIN using 50,000 permutations. It is not statistically appropriate to calculate $p$-values for standardized values.

<table>
<thead>
<tr>
<th></th>
<th>Hawai‘i</th>
<th>4-islands area</th>
<th>O‘ahu</th>
<th>Kaua‘i/Ni‘ihau</th>
<th>Offshore ETP</th>
<th>Coastal ETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i (n=37)</td>
<td>0.044</td>
<td>0.055</td>
<td>-0.014</td>
<td>0.171</td>
<td>0.225</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[0.019-0.079]</td>
<td>[-0.007-0.132]</td>
<td>(0.001)</td>
<td>[0.032-0.337]</td>
<td>[0.098-0.362]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.004)</td>
<td>(0.001)</td>
<td>(0.769)</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>4-islands area (n=26)</td>
<td>0.028</td>
<td>0.018</td>
<td>0.047</td>
<td>0.125</td>
<td>0.208</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[0.013-0.045]</td>
<td>[-0.002-0.042]</td>
<td>(0.061)</td>
<td>[0.031-0.192]</td>
<td>[0.097-0.329]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt;0.001)</td>
<td></td>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>O‘ahu (n=26)</td>
<td>0.038</td>
<td>0.038</td>
<td>0.039</td>
<td>0.051</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[0.023-0.053]</td>
<td>[0.020-0.056]</td>
<td></td>
<td>[0.017-0.082]</td>
<td>[0.049-0.232]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
<td>(0.001)</td>
<td>(0.001)</td>
<td></td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau (n=8)</td>
<td>0.016</td>
<td>0.045</td>
<td>0.029</td>
<td>0.198</td>
<td>0.323</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[-0.002-0.026]</td>
<td>[0.023-0.064]</td>
<td></td>
<td>[0.055-0.317]</td>
<td>[0.161-0.429]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.057)</td>
<td>(0.001)</td>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>Offshore ETP (n=25)</td>
<td>0.055</td>
<td>0.055</td>
<td>0.068</td>
<td>0.068</td>
<td>0.300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[0.036-0.078]</td>
<td>[0.037-0.070]</td>
<td></td>
<td>[0.034-0.102]</td>
<td>[0.017-0.257]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.001)</td>
<td>(0.001)</td>
<td></td>
<td>(&lt;0.001)</td>
<td>(0.019)</td>
<td></td>
</tr>
<tr>
<td>Coastal ETP (n=25)</td>
<td>0.118</td>
<td>0.115</td>
<td>0.137</td>
<td>0.141</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[0.064-0.191]</td>
<td>[0.074-0.155]</td>
<td></td>
<td>[0.019-0.057]</td>
<td>[0.019-0.057]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td></td>
</tr>
</tbody>
</table>

$F_{ST}$ overall = 0.065 [0.046-0.091] (<0.001); $R_{ST}$ overall = 0.106 [0.057-0.163] (<0.001)

<table>
<thead>
<tr>
<th></th>
<th>Hawai‘i</th>
<th>4-islands area</th>
<th>O‘ahu</th>
<th>Kaua‘i/Ni‘ihau</th>
<th>Offshore ETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i (n=37)</td>
<td>0.135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-islands area (n=26)</td>
<td>0.209</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O‘ahu (n=26)</td>
<td>0.099</td>
<td>0.166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau (n=8)</td>
<td>0.342</td>
<td>0.376</td>
<td>0.430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Offshore ETP (n=25)</td>
<td>0.456</td>
<td>0.502</td>
<td>0.137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coastal ETP (n=25)</td>
<td>0.456</td>
<td>0.370</td>
<td>0.482</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$F'_{ST}$ overall = 0.341
Table 11. Migration rate (Nm) between pairs of regions. These rates were calculated using the private alleles method of Barton & Slatkin (1986). Error cannot be estimated with this method so significant differences cannot be determined. This method assumes no admixture and populations in equilibrium, so values likely do not reflect exact migration rates but do suggest that migration is relatively low and that migration rate between the coastal ETP and offshore ETP is similar to that among the Hawaiian Islands regions in this study. Also, migration rates tend to be slightly lower between offshore ETP and Hawaiian Island regions than among the Hawaiian Island regions, and migration rates between coastal ETP and the Hawaiian Island regions are very low and probably reflect historical gene flow or second hand flow from the offshore ETP.

<table>
<thead>
<tr>
<th>Pairwise Regions</th>
<th>Mean Frequency of Private Alleles across Both Regions</th>
<th>Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i-4-islands area</td>
<td>0.03</td>
<td>3.45</td>
</tr>
<tr>
<td>Hawai‘i-O‘ahu</td>
<td>0.04</td>
<td>2.90</td>
</tr>
<tr>
<td>Hawai‘i-Kaua‘i/Ni‘ihau</td>
<td>0.05</td>
<td>2.63</td>
</tr>
<tr>
<td>4-islands area-O‘ahu</td>
<td>0.04</td>
<td>2.51</td>
</tr>
<tr>
<td>4-islands area-Kaua‘i/Ni‘ihau</td>
<td>0.07</td>
<td>1.49</td>
</tr>
<tr>
<td>O‘ahu-Kaua‘i/Ni‘ihau</td>
<td>0.07</td>
<td>1.69</td>
</tr>
<tr>
<td>Coastal ETP-Offshore ETP</td>
<td>0.04</td>
<td>2.97</td>
</tr>
<tr>
<td>Offshore ETP-Hawai‘i</td>
<td>0.04</td>
<td>2.17</td>
</tr>
<tr>
<td>Offshore ETP-4-islands area</td>
<td>0.05</td>
<td>1.97</td>
</tr>
<tr>
<td>Offshore ETP-O‘ahu</td>
<td>0.05</td>
<td>2.15</td>
</tr>
<tr>
<td>Offshore ETP-Kaua‘i/Ni‘ihau</td>
<td>0.08</td>
<td>1.38</td>
</tr>
<tr>
<td>Coastal ETP-Hawai‘i</td>
<td>0.06</td>
<td>1.15</td>
</tr>
<tr>
<td>Coastal ETP-4-islands area</td>
<td>0.08</td>
<td>0.92</td>
</tr>
<tr>
<td>Coastal ETP-O‘ahu</td>
<td>0.08</td>
<td>1.02</td>
</tr>
<tr>
<td>Coastal ETP-Kaua‘i/Ni‘ihau</td>
<td>0.11</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Table 12. Number of overlapping mtDNA haplotypes among regions. Total number of haplotypes is not the sum of rows; it is the total number of haplotypes discovered in each region. $n$ indicates the number of individuals sampled to obtain the haplotypes.

<table>
<thead>
<tr>
<th>Region</th>
<th>Hawai‘i (n=113)</th>
<th>4-islands area (n=27)</th>
<th>O‘ahu (n=27)</th>
<th>Kaua‘i/Ni‘ihau (n=8)</th>
<th>Pelagic (n=3)</th>
<th>Offshore ETP (n=50)</th>
<th>Northern Mexico (n=34)</th>
<th>Central America (n=24)</th>
<th>Costa Rica (n=12)</th>
<th>Ecuador (n=21)</th>
<th>China/Taiwan (n=30)</th>
<th>Total overlapping haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i</td>
<td>3</td>
<td></td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>4-islands area</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>O‘ahu</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Pelagic</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Offshore ETP</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Northern Mexico</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Central America</td>
<td>1</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Ecuador</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>China/Taiwan</td>
<td>2</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

Total overlapping haplotypes: 9 4 3 4 2 90 34 36 32 33 9
Table 13. Top: $F_{ST}$ and $\Phi_{ST}$ for mtDNA sequences. $\Phi_{ST}$ is above the diagonal, and $F_{ST}$ is below the diagonal. Numbers in bold are significantly different from zero, and $p$-values for numbers are shown in parentheses. $\Phi_{ST}$ was calculated using a basic pairwise comparison model without weighting mutation type. Haplotype differences consisted only of transitions and transversions, no indels. 50,000 permutations were used for calculations. Table 13. Bottom: $F'_{ST}$ (standardized $F_{ST}$) is shown on the next page. $F'_{ST} = F_{ST} / F_{ST \text{max}}$.

<table>
<thead>
<tr>
<th></th>
<th>Hawai'i area</th>
<th>O'ahu</th>
<th>Kaua'i/Ni'ihau</th>
<th>Offshore ETP</th>
<th>Northern Mexico</th>
<th>Central America</th>
<th>Costa Rica</th>
<th>Ecuador</th>
<th>China/Taiwan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai'i</td>
<td>0.017 (0.212)</td>
<td>0.005</td>
<td>0.028</td>
<td>0.134</td>
<td>0.168</td>
<td>0.102</td>
<td>0.439</td>
<td>0.223</td>
<td>0.161</td>
</tr>
<tr>
<td>4-islands area</td>
<td>0.105 (0.229)</td>
<td>0.005</td>
<td>0.097</td>
<td>0.111</td>
<td>0.068</td>
<td>0.361</td>
<td>0.210</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td>O'ahu</td>
<td>0.112 (0.180)</td>
<td>0.191</td>
<td>0.184</td>
<td>0.237</td>
<td>0.163</td>
<td>0.523</td>
<td>0.278</td>
<td>0.214</td>
<td></td>
</tr>
<tr>
<td>Kaua'i/Ni'ihau</td>
<td>0.087 (0.064)</td>
<td>0.018</td>
<td>0.282</td>
<td>0.040</td>
<td>0.047</td>
<td>0.024</td>
<td>0.262</td>
<td>0.095</td>
<td>0.090</td>
</tr>
<tr>
<td>Offshore ETP</td>
<td>0.227 (&lt;0.001)</td>
<td>0.164</td>
<td>0.295</td>
<td>0.066</td>
<td>-0.011</td>
<td>0.012</td>
<td>0.086</td>
<td>0.059</td>
<td>0.097</td>
</tr>
<tr>
<td>Northern Mexico</td>
<td>0.246 (&lt;0.001)</td>
<td>0.165</td>
<td>0.334</td>
<td>0.058</td>
<td>0.002</td>
<td>0.011</td>
<td>0.092</td>
<td>0.051</td>
<td>0.102</td>
</tr>
<tr>
<td>Central America</td>
<td>0.311 (&lt;0.001)</td>
<td>0.225</td>
<td>0.396</td>
<td>0.111</td>
<td>0.026</td>
<td>0.033</td>
<td>0.157</td>
<td>0.070</td>
<td>0.054</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>0.482 (&lt;0.001)</td>
<td>0.397</td>
<td>0.577</td>
<td>0.298</td>
<td>0.154</td>
<td>0.178</td>
<td>0.187</td>
<td>0.147</td>
<td>0.284</td>
</tr>
<tr>
<td>Ecuador</td>
<td>0.321 (&lt;0.001)</td>
<td>0.232</td>
<td>0.409</td>
<td>0.111</td>
<td>0.017</td>
<td>0.029</td>
<td>0.038</td>
<td>0.162</td>
<td>0.141</td>
</tr>
<tr>
<td>China/Taiwan</td>
<td>0.395 (&lt;0.001)</td>
<td>0.307</td>
<td>0.489</td>
<td>0.198</td>
<td>0.099</td>
<td>0.110</td>
<td>0.124</td>
<td>0.267</td>
<td>0.118</td>
</tr>
</tbody>
</table>

$F_{ST \text{overall}} = 0.187 (<0.001); \Phi_{ST \text{overall}} = 0.125(<0.001)$
Table 13.

<table>
<thead>
<tr>
<th>Location</th>
<th>4-islands area</th>
<th>O'ahu</th>
<th>Kaua‘i/Ni‘ihau</th>
<th>Offshore ETP</th>
<th>Northern Mexico</th>
<th>Central America</th>
<th>Costa Rica</th>
<th>Ecuador</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawai‘i</td>
<td>0.019</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O‘ahu</td>
<td>0.021</td>
<td>0.169</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaua‘i/Ni‘ihau</td>
<td>0.171</td>
<td>0.046</td>
<td>0.415</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Offshore ETP</td>
<td>0.807</td>
<td>0.766</td>
<td>0.846</td>
<td>0.599</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern Mexico</td>
<td>0.734</td>
<td>0.674</td>
<td>0.794</td>
<td>0.468</td>
<td>0.081</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central America</td>
<td>0.911</td>
<td>0.890</td>
<td>0.930</td>
<td>0.825</td>
<td>0.780</td>
<td>0.776</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Costa Rica</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.981</td>
<td>0.996</td>
<td>0.996</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecuador</td>
<td>0.951</td>
<td>0.938</td>
<td>0.962</td>
<td>0.898</td>
<td>0.670</td>
<td>0.869</td>
<td>0.888</td>
<td>0.899</td>
</tr>
<tr>
<td>China/Taiwan</td>
<td>0.924</td>
<td>0.909</td>
<td>0.938</td>
<td>0.882</td>
<td>0.945</td>
<td>0.927</td>
<td>0.962</td>
<td>1.000</td>
</tr>
</tbody>
</table>

$F_{ST}^{\prime}$ overall = 0.770

Table 14. Haplotypic diversity, nucleotide diversity, and mean pairwise differences among haplotypes were calculated using ARLEQUIN.

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean Haplotypic Diversity</th>
<th>Mean Nucleotide Diversity</th>
<th>Mean Pairwise Differences</th>
<th>n</th>
<th># Haplotypes</th>
<th># Transitions</th>
<th># Transversions</th>
</tr>
</thead>
</table>
Discussion

General Relationships

Although posterior probability analyses of microsatellites in STRUCTURE gave ambiguous results (Figure 9), five population clusters were evident in bar plots of assignment probabilities (Figure 10): Hawai‘i + Kauai‘i/Ni‘ihau, the 4-islands area, O‘ahu, offshore ETP, and coastal ETP. Because sample size was low, it was hard to draw strong conclusions about Kauai‘i/Ni‘ihau. See Chapter 2 for further discussion of Kauai‘i/Ni‘ihau. Based on microsatellites, coastal ETP samples clustered strongly together (Table 9), supporting the sub-species designation for coastal pantropical spotted dolphins (S.a. graffmani), which was also supported by Escorza-Treviño et al.’s (2005) broader study. Offshore ETP pantropical spotted dolphins clustered significantly together, and secondarily clustered most closely to coastal ETP pantropical spotted dolphins (Table 9), possibly implying more gene flow among offshore ETP and coastal ETP pantropical spotted dolphins than offshore ETP and Hawaiian pantropical spotted dolphins, which is not surprising given that the range of offshore and coastal ETP pantropical spotted dolphins overlaps. However, this may also indicate that the coastal sub-species shares a single common ancestor with the offshore sub-species, but the offshore sub-species has another lineage not shared by the coastal sub-species. Hawaiian pantropical spotted dolphins are currently considered the same sub-species as offshore ETP pantropical spotted dolphins (S. a. attenuata). However, in their review of cetacean sub-species, Perrin et al. (2009) noted nominal sub-species of S. attenuata, referred to as sub-species A (offshore ETP) and sub-species B (Hawaiian) have been described, but
these sub-species have not been strongly supported or recognized. Because pantropical spotted dolphins in the distinct offshore ETP and coastal ETP sub-species may have greater gene flow among themselves than those of the single sub-species in the offshore ETP and Hawaiian Islands regions in my study, it may be appropriate to revisit Perrin’s sub-species A and B designations for pantropical spotted dolphins.

There were not enough Hawaiian pelagic samples for most comparisons, but because STRUCTURE makes population assignments \textit{a priori}, it can reveal the populations with which the pelagic samples cluster most strongly. One sample clustered with Hawai‘i, one with offshore ETP, and the third clustered equally with Hawai‘i and offshore ETP (Table 9; Figure 8), indicating possible gene flow among pantropical spotted dolphins near the Hawaiian EEZ and those near the Hawaiian Islands and in the northern offshore ETP stock. The pelagic samples did not cluster together in such a way as to suggest a separate population, but sample size needs to be increased to evaluate the relationships among pantropical spotted dolphins in this geographic area and other regions.

Private microsatellite allele comparisons suggested that the Hawaiian Islands regions were more likely populated by animals originating from the offshore ETP than from the coastal ETP subspecies, although it should be borne in mind that the coastal ETP has less distinct alleles than the offshore ETP, making the odds greater that alleles would be shared with the offshore ETP (Figure 11). This approach also suggested that the Hawaiian Islands regions have a similar level of gene flow among each other as coastal ETP and offshore ETP do (Figure 11). The number of mean private alleles shared
among the Hawai‘i, 4-islands area, and O‘ahu indicated that there were not separate colonizing events at different island regions (Figure 11). There were no differences to indicate colonization of one island region spreading to other regions. Possibly, Hawai‘i, 4-islands area, and O‘ahu were relatively rapidly colonized by one founding event from the offshore ETP. With respect to the private alleles method of determining migration rate \( (N_m) \), the inability to calculate error factors and the assumption of equilibrium should be borne in mind. That said, this method of determining migration rate \( (N_m) \) among regions suggested that migration is relatively low among all the regions.

Migration rate between the coastal ETP and offshore ETP subspecies was similar to that among the Hawaiian Islands, a comparison that supports population status for the Hawaiian Island regions in this study. Migration rates tended to be slightly lower between offshore ETP and Hawaiian Island regions than among the Hawaiian Island regions, and migration rates between coastal ETP and the Hawaiian Island regions are very low and probably reflect historical gene flow or second hand flow from the offshore ETP. Possibly there was a relatively recent colonizing event from the offshore ETP, resulting in isolation from the ETP that is slowly allowing different equilibria to be reached in the Hawaiian Islands regions.

For mtDNA, the most common haplotype near the Hawaiian Islands matched a haplotype in nearby pelagic waters, a common haplotype in the ETP, and a less common haplotype near Taiwan, suggesting the possibility of a recent divergence or exchange among populations. The haplotypes found in the ETP, China/Taiwan, and near the Hawaiian Islands do not group separately in a haplotype network. Hawaiian, pelagic, and
China/Taiwan haplotypes are spread throughout clusters of ETP haplotypes. This may be due to some gene exchange or may suggest a colonization event by a single haplotype near the Hawaiian Islands and China/Taiwan followed by mutations that in some cases randomly converged with ETP haplotypes. It would be expected that the mtDNA control region would evolve more quickly than nuclear markers like microsatellites, thereby having a shallower coalescence that allows for better detection of structure in recently diverged and diverging populations (Zink & Barrowclough 2008). However, the mtDNA of cetaceans has been found to evolve at one quarter the rate of other mammals (Ohland et al. 1995), possibly creating deeper than expected coalescence times for recently diverged taxa. Kingston et al. (2009) recently reported that mtDNA control region sequences have little power for resolving differences among delphinid taxa at the species level, which could also affect the use of this marker at the population and subspecies levels. MtDNA haplotypic diversity is clearly higher among the ETP samples than the Hawaiian samples in my study (Table 14). This may be related to larger population size and sample size in the ETP allowing for more rare haplotypes.

**Baselines**

Based on morphological differences, distances between populations, and the general relationships described above, it is unlikely that Hawaiian pantropical spotted dolphins are currently interbreeding at high levels with ETP pantropical spotted dolphins. The northern offshore ETP stock is distant from Hawaiian waters (Figure 8), and morphological studies of pantropical spotted dolphins in the two areas indicate
measurable differences (Dizon et al. 1994b). It should be noted that Schnell et al. (1986) and Yao et al. (2008) found that oceanographic conditions correlated with some morphological characteristics of pantropical spotted dolphins, suggesting ocean conditions, not just genetic differences, can affect physical characteristics. However, assuming that pantropical spotted dolphins could breed more often among the Hawaiian Islands than between the Hawaiian Islands and offshore ETP, coastal ETP, and China/Taiwan locations, baselines of fixation index values can be suggested using comparisons among these regions. These baselines can help establish the potential biological significance of differentiation found among the Hawaiian Islands.

For microsatellites, comparisons were made among the Hawaiian Islands regions, offshore ETP, and coastal ETP (Costa Rica/Panama region) (Table 10). The results indicate that genetic differentiation among the Hawaiian Islands regions is less than that between each of the Hawaiian Islands regions and coastal ETP (a separate sub-species) and each of the Hawaiian Islands regions and offshore ETP (with the exception of $R_{ST}$ for O‘ahu compared with offshore ETP), but similar to or greater than that between offshore ETP and coastal ETP. This is interesting because offshore ETP and coastal ETP are considered separate sub-species. Using a baseline of values set with offshore ETP and coastal ETP sub-species results would indicate that the differentiation among the Hawaiian Islands regions is likely to be biologically/demographically significant. However, for the most part, $F_{ST}$, $F'_{ST}$, and $R_{ST}$ values for offshore ETP in comparison with Hawaiian Islands regions tend to be about twice as large as those among the Hawaiian Islands regions, possibly suggesting less biological significance. This may be
indicative of some gene flow between the offshore ETP population and the coastal ETP and Hawaiian populations at small levels with very little gene flow between coastal ETP and the Hawaiian Islands, or it may indicate that coastal and Hawaiian populations originated as colonizers from the offshore ETP population. In any case, the complexity of these relationships makes it difficult to set a numerical baseline, but given the subspecies status of coastal ETP pantropical spotted dolphins, it suggests that differentiation among the Hawaiian Islands regions is enough to consider Hawai‘i, the 4-islands area, and O‘ahu as separate populations. Microsatellite results tend to cluster Hawai‘i and Kaua‘i/Ni‘ihau together, but given the small sample size from Kaua‘i/Ni‘ihau, it is difficult to draw conclusions about this region.

For mtDNA, sequences, more samples from more locations were available for comparisons. In the case of $F'_{ST}$ values, there are clearly higher levels of differentiation for most comparisons among coastal ETP regions, offshore ETP, and China/Taiwan than among the Hawaiian Islands regions. $F'_{ST}$ value is strangely low for Northern Mexico in comparison with offshore ETP, but this appears to be an exception. On the other hand, $\Phi_{ST}$ values were not significantly different from zero for comparisons among offshore ETP, Northern Mexico, and Central America, and 11 of the $\Phi_{ST}$ values comparing various populations of coastal ETP and China/Taiwan were lower than the values for O‘ahu compared with the 4-islands area and O‘ahu compared with Kaua‘i/Ni‘ihau. This again makes assigning a numerical baseline value difficult because of variation in results. The smallest difference that was significantly different from zero was $\Phi_{ST}$ comparing Ecuador and Northern Mexico (0.051). $\Phi_{ST}$ values ranged from 0.059 to 0.086 for values
significantly different from zero comparing the sub-species of offshore ETP to coastal ETP, and $\Phi_{ST}$ was 0.097 for offshore ETP compared with China/Taiwan (Table 13). Based on this, $\Phi_{ST}$ of O‘ahu in comparison with the 4-islands area (0.105) and O‘ahu in comparison with Kaua‘i/Ni‘ihau (0.191) are high enough to potentially indicate biologically and demographically significant differences.

Overall, this study suggests that assigning specific numerical baseline values may not be very feasible for $F_{ST}$, $R_{ST}$, and $\Phi_{ST}$, but determining whether populations with geographic separation show a preponderance of similar, lower, or higher fixation index values can help with evaluating whether genetic differences among sympatric or allopatric putative populations warrants designating them as actual separate populations. In the case of pantropical spotted dolphins near the Hawaiian Islands, fixation index values tend to be small (as is true for many dolphin populations) but comparisons with ETP populations and sub-species indicate some differences similar in magnitude to those found between the offshore and coastal ETP, while at the same time indicating generally less differentiation than is found between the Hawaiian Islands regions and the other regions examined. The lack of differentiation among offshore ETP, Northern Mexico, and Central America for $\Phi_{ST}$ raises some questions as to the meaning of the lack of differentiation found among the Hawaiian Islands regions for this measure. Comparisons, as performed in this study, may be sequence specific and help determine if lack of significant differences from zero are normal for the chosen sequence, even for clearly separate populations, indicating the possibility of poor marker choice.
The results of this study suggest a level of reproductive isolation among the Hawaiian Islands regions that is somewhat comparable to that of offshore and coastal ETP populations. Based solely on mtDNA, China/Taiwan pantropical spotted dolphins appear to be genetically differentiated from ETP populations at similar levels as well. It is possible that different local situations have resulted in more or less isolation in different locations, resulting in differences in differentiation, but given the sympatry of coastal and offshore ETP pantropical spotted dolphins, the similar levels of differentiation among the Hawaiian Islands regions are suggestive.

Fixation indices can reveal population sub-structure by determining how far the populations are from Hardy-Weinberg equilibrium, but determining that differentiation exists does not reveal whether that differentiation is sufficient to warrant designation as separate populations. My study shows that, for pantropical spotted dolphins, the levels of differentiation seen among the Hawaiian Islands regions is comparable to differentiation found among more distant populations that are considered separate genetic populations. Further studies of this type could determine whether these levels of differentiation are biologically important for other dolphin species as well. For example, Andrews (2009) and Andrews et al. (2010) reported fixation index values for spinner dolphins from 12 regions worldwide. Many of these values were comparable to those she found among Hawaiian Islands regions, suggesting also that the differences among the Hawaiian Islands may be biologically/demographically important.

Palsbøll et al. (2007) stated that the identification of management units should be based on interpretation of estimates of genetic divergence rather than on rejection of
They point out that current gene flow is of more importance than historical gene flow. They suggest that managers shift focus away from pannmixia and toward a threshold level of dispersal that is based on demographic models. In the current study, I assume a lower level of current gene flow would be expected among widely geographically separated populations than among less distant populations. When distant populations are compared, my analyses suggest that divergence as measured by fixation indices among the pantropical spotted dolphins can be low, even for populations that have developed differing morphology and distribution gaps. Barriers to gene flow among the Hawaiian Islands may be recent, but are causing some comparable genetic differentiation to that of morphologically and geographically distinct populations, supporting separate management units based on more than evidence of a lack of pannmixia. However, clearly, attempting to set baseline values for fixation indices is not a straightforward process. Some values will support significant differentiation and some will not in any multi-population study. Rather than fixing a specific cutoff value, it may be more useful to explore the range of values and evaluate the expected relationships of each known population or sub-species to allow for determination of the likelihood that fixation indices reveal differentiation that is sufficient to designate separate populations in the test regions.
CHAPTER 4. Are small sample sizes and use of slow evolving DNA regions hindering resolution of the phylogenetic relationships among delphinids: *Stenella* as an example genus

**Introduction**

Phylogenetic studies of cetaceans have been conflicting and controversial, for example the discussion regarding the relationship of the sperm whale (*Physeter macrocephalus*) with the baleen whales (Milinkovitch *et al.* 1993, Arnason & Gullberg 1996, Messenger & McGuire 1998). Even at the genus level, there are conflicting phylogenies that place different genera together, even though morphological or geographical evidence is to the contrary. For instance, pantropical spotted dolphins (*Stenella attenuata*) grouped more closely with common bottlenose dolphins (*Tursiops truncatus*) than with spinner dolphins (*Stenella longirostris*) in analyses of cytochrome-b sequences performed by Yang *et al.* (2002).

The relationships among the *Stenella* and other genera of delphinids have long been contested and discussed (*e.g.* Perrin *et al.* 1987, LeDuc *et al.* 1999). Maximum parsimony analysis of cytochrome-b by LeDuc *et al.* (1999) placed spinner dolphins in polytomy with pantropical spotted dolphins, Fraser’s dolphins (*Lagenodelphis hosei*), Indo-Pacific hump-back dolphins (*Sousa chinensis*), and a clade consisting of striped dolphins (*Stenella coeruleoalba*), Clymene dolphins (*Stenella clymene*), Atlantic spotted dolphins (*Stenella frontalis*), Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), common bottlenose dolphins, and common dolphins (*Delphinus* spp.). Xiong *et al.*
(2009) sequenced the entire mitochondrial genome of seven dolphin species and compared these with each other and previously published mitochondrial genomes of cetaceans. They found that the *Stenella* were polyphyletic, with striped dolphins forming a clade with Indo-Pacific bottlenose dolphins and long-beaked common dolphins (*D. capensis*). This clade then shared a most recent common ancestor with common bottlenose dolphins (Xiong *et al.* 2009). This larger sister group was most closely related to Indo-Pacific hump-back dolphins (Xiong *et al.* 2009). Finally, pantropical spotted dolphins were most closely related to this overall group of the five taxa just described (Xiong *et al.* 2009). Further, Vilstrup *et al.* (2011) did a mitogenomic analysis of several genera of the Delphinidae. They included striped dolphins and pantropical spotted dolphins from the *Stenella* and found that these species did not form a sister clade separate from other genera.

These studies focused mainly on the relationships among genera and do not include large numbers of samples from any given species. To some extent, this has been due to a lack of available samples. Further, most studies do not include more than a couple of *Stenella* species, with the exceptions of LeDuc *et al.* (1999) and Kingston *et al.* (2009). Recently, mtDNA sequences for a variety of *Stenella* species have become available through GenBank. Some sequences are also available from theses and through cooperation with researchers studying this genus. In addition to these sources, I have sequenced 176 samples of pantropical spotted dolphins from the Hawaiian Islands, resulting in the addition of 13 new haplotypes for this species (Chapter 2) (GenBank Accession #’s GQ852567-GQ852579). I have also sequenced three pantropical spotted
dolphins from the pelagic waters west of the Hawaiian Islands, resulting in one additional haplotype (Chapter 3) (GenBank Accession # GU136595). The increase in available haplotypes has made it possible to examine haplotypes across a large number of populations, as well as species.

The purpose of this chapter is to assess the use of a non-coding region of mtDNA for phylogenetic study of a delphinid genus (*Stenella*). Another goal is to assess whether the use of a few haplotypes from a given species is sufficient for phylogenetic analyses or if haplotypes differ sufficiently among populations to require larger sample sizes to resolve conflicts. These two aims are based on the hypothesis that the ambiguity in delphinid phylogenies may be a result of either low sample sizes or use of DNA regions that do not evolve fast enough to reveal differences among this recently diverged family. Kingston *et al.* (2009) recently performed a similar study using mtDNA control region and AFLP analyses for 11 Delphinine species.

Xiong *et al.* (2009) stated that ambiguous and conflicting results from phylogenetic studies of cetaceans may be related to the use of too little data. They were suggesting that more genera be included in analyses; however, it may also be important to include more haplotypes of each species in analyses because of genetic differentiation among populations of some species. For example, in the genus *Stenella*, pantropical spotted dolphins have been found to be genetically differentiated among several regions of the Hawaiian Islands (Chapter 2); coastal (*S. a. graffmani*) and offshore (*S. a. attenuata*) pantropical spotted dolphins in the Eastern Tropical Pacific are differentiated enough to consider them separate sub-species; and the coastal sub-species shows genetic
subdivision among coastal locations (Escorza-Treviño et al. 2005). There is some evidence for genetic differentiation among pantropical spotted dolphins near Taiwan and the South China Sea as well (Yao et al. 2004).

Other examples include the Atlantic spotted dolphin, which is separated into a Western North Atlantic stock and a Northern Gulf of Mexico stock, supported by genetic studies showing significant differences in mitochondrial and nuclear DNA between the two locations (Adams & Rosel 2006). Spinner dolphins near the Hawaiian Islands have also been found to have significant genetic differentiation among populations found near different island regions (Andrews et al. 2010), and spinner dolphins near French Polynesian islands have been found to have small populations, relatively isolated by distance (Oremus et al. 2007). García-Martínez et al. (1999) reported two genetically distinct populations of striped dolphins in the Mediterranean and Atlantic, and Gaspari et al. (2007) found significant genetic differentiation between striped dolphin populations in the Adriatic and Tyrrenian Seas and between inshore and offshore populations within the Tyrrenian Sea.

Recentness of splits among the Stenella makes defining populations, and sometimes species, difficult because some groups may be genetically isolated enough to be considered separate but the level of differentiation among them has not risen to a level detectable by statistical significance testing, depending on the quality of the marker choice and representativeness of the samples. To try to investigate this problem, in this study, the more rapidly evolving mtDNA control region is used for comparisons. Although generally gene regions are used for phylogenetic analyses because of slower
rates of evolution, evidence suggests that *Stenella* species (and even multiple genera of dolphins) may be recently diverged, making a faster evolving region potentially more appropriate for comparisons. Steeman *et al.* (2009) reported that delphinids began diversifying less than 11 MYA; McGowen *et al.* (2009) reported that the Delphinidae diverged 10.08 MYA and the Delphininae sub-family diverged 3.84 MYA; and Xiong *et al.* (2009) reported the divergence of the Delphininae at 2.35 (1.77-3.53) MYA. Further, the substitution rate of mtDNA in cetaceans is only 0.25%/MY (Ohland *et al.* 1995), about a quarter of the rate in mammals in general, which has been estimated at about 1%/MY (Brown *et al.* 1979). Therefore, the mtDNA control region is a slower evolving region in cetaceans than it is in other mammals, while likely still evolving faster than gene regions, possibly making it a better phylogenetic indicator than population genetic indicator.

**Methods**

Thirteen mtDNA control region haplotypes of pantropical spotted dolphins from the Hawaiian Islands were used in this study (Chapter 2). One additional haplotype from pantropical spotted dolphins from the pelagic region near the Hawaiian Islands Exclusive Economic Zone was also used (Chapter 3). Thirteen haplotypes of pantropical spotted dolphins from near Taiwan and the China Sea were provided by C. Yao (Yao *et al.* 2004). One sample of a pantropical spotted dolphin from the Atlantic was available from D. Duffield (Portland State University), and was sequenced using the procedures in Chapter 2 (GenBank Accession # GU256406). Two haplotypes of spinner dolphins from

Sequences were aligned using CLUSTAL X 1.81 (Thompson *et al.* 1997). ModelTest 3.7 (Posada & Crandall 1998, Posada 2006) was used to determine the optimal model of evolution for maximum likelihood analysis. Using the Akaike Information Criterion, ModelTest chose HKY+I+G as the optimal model. GARLI 0.95 (Zwickl 2006) was used to generate 1002 bootstrap replicates using the HKY model. This was computed using the Cipres Portal 2.0 online (Miller *et al.* 2010). This online portal allows the user to run much larger datasets much faster than on a single computer. The Cipres Portal will only run a project for 72 hours, so 11 cloned projects were run simultaneously to obtain the desired 1002 bootstrap replicates. A 50% majority rule consensus tree was created in PAUP*4.0b10 (Swofford 2003). The tree was rooted with the harbor porpoise sequence. The consensus tree was used to determine which haplotypes of each population, sub-species, and species grouped together. These results were used to determine whether individual haplotypes clustered most closely with others of the same species, sub-species, and local population to test the usefulness of the
mtDNA control region in phylogenetic analyses. Further, RANDOMIZER 4.0 (http://www.randomizer.org/form.htm) was used to choose two haplotypes at random for each species. These haplotypes were aligned using CLUSTAL X 1.81 (Thompson et al. 1997). This process was repeated two more times for a total of three sets of Stenella sequences. ModelTest 3.7 (Posada & Crandall 1998, Posada 2006) was used to determine the optimal model of evolution for maximum likelihood analysis for each set of sequences. Using the Akaike Information Criterion, ModelTest chose HKY+G as the optimal model for all three sets. GARLI was used to generate 1000 bootstrap replicates using the HKY model for each of the three sets of sequences using Cipres Portal 2.0 online. A 50% majority rule consensus tree was created for each set of sequences in PAUP*. The trees were rooted with the harbor porpoise sequence. The consensus trees were used to determine whether two haplotypes from each species was sufficient to generate clear and repeatable relationships among the species.

**Results**

A 50% majority rule consensus tree for the entire dataset is shown in Appendix E for reference. Values below 50% are included in the tree as they are reported below. A simplified tree is shown in Figure 13.

The 145 pantropical spotted dolphin haplotypes grouped together with 90% bootstrap support (Figure 13; Appendix D). The consensus tree indicated that haplotypes of pantropical spotted dolphins from the Hawaiian Islands, China/Taiwan, and the ETP did not group in separate clades; Hawaiian and China/Taiwan haplotypes were peppered
throughout clades of ETP pantropical spotted dolphins (note there were 112 haplotypes of ETP pantropical spotted dolphins). Haplotypes found in coastal, offshore, or both subspecies of ETP pantropical spotted dolphins did not group in any pattern that would distinguish coastal from offshore animals. Pantropical spotted dolphins from the Atlantic/Gulf of Mexico did not group together either, with two haplotypes grouping together and then grouping most closely with coastal and offshore ETP pantropical spotted dolphins, and two haplotypes grouping together and then grouping most closely with different coastal and offshore ETP pantropical spotted dolphin haplotypes. The pantropical spotted dolphin haplotype from the Indian Ocean grouped most closely with offshore ETP haplotypes. The pantropical spotted dolphin haplotype found only in pelagic waters near Hawai‘i grouped most closely with the dominant haplotype near the Hawaiian Islands regions (Appendix E).

The 119 spinner dolphin haplotypes grouped together with 46% bootstrap support (Figure 13; Appendix D) and twelve spinner dolphin (S. l. longirostris) haplotypes from the Hawaiian Islands grouped together in a single clade. This clade was most closely related to a clade of three ETP spinner dolphin (S. l. orientalis) haplotypes and one other spinner dolphin haplotype from the Hawaiian Islands. Six ETP spinner dolphin haplotypes formed a clade together, but others were most closely related to spinner dolphin haplotypes from the South Pacific and Hawaiian Islands. South Pacific spinner dolphin haplotypes did not group together, with several being most closely related to Hawaiian and ETP haplotypes, although some grouped with clades containing spinner dolphins from the Central American subspecies (S. l. centroamericana) as well. Central
American spinner dolphin haplotypes did not form their own clade, with the largest clade consisting of three. Central American spinner dolphin haplotypes that were sister to an ETP haplotype. The two spinner dolphin haplotypes from East Timor Sea did not group together. They grouped most closely with a haplotype from the ETP and a haplotype from the South Pacific respectively (Appendix D).

The 69 striped dolphin and two Clymene dolphin haplotypes grouped together with 12% bootstrap support (Figure 13; Appendix D). The two Clymene dolphin haplotypes did not group together but were within the clades formed by striped dolphin haplotypes. Mediterranean, Pacific, and Atlantic striped dolphin haplotypes did not cluster separately by location (Appendix E).

The eight Atlantic spotted dolphin haplotypes grouped together with 20% bootstrap support (Figure 13; Appendix D). Three of the haplotypes were not part of this main clade. One Atlantic spotted dolphin haplotype grouped most closely with the striped dolphin, Clymene dolphin and spinner dolphin clade with 34% bootstrap support. The clade of eight Atlantic spotted dolphin haplotypes grouped most closely with the clade formed by the Atlantic spotted dolphin haplotype just described and the striped dolphin, Clymene dolphin, and spinner dolphin haplotypes with 12% bootstrap support. Two other Atlantic spotted dolphin haplotypes grouped with the entire clade of other Atlantic spotted dolphin, striped dolphin, Clymene dolphin, and spinner dolphin haplotypes with 20% and 53% bootstrap support respectively (Figure 13; Appendix D).

Overall, species’ haplotypes mainly grouped together except that Clymene dolphin haplotypes were within the striped dolphin clade; one Atlantic spotted dolphin
haplotype grouped closer to striped dolphins and Clymene dolphins than to other Atlantic spotted dolphins; and two Atlantic spotted dolphin haplotypes fell outside of the main Atlantic spotted dolphin clade (Figure 13; Appendix E). Sub-species’ haplotypes did not tend to group together within species clades, nor did haplotypes tend to cluster by location in which they were collected or by populations described in previous studies.

Pantropical spotted dolphins and spinner dolphins grouped together with 18% bootstrap support; these two species grouped most closely with the striped dolphin and Clymene dolphin clade with 34% bootstrap support; and this larger four species clade grouped with Atlantic spotted dolphins as described above (Figure 13; Appendix D).

For the three sets of two haplotypes randomly chosen for each species, the results differed among the sets (Figure 14). Pantropical spotted dolphin haplotypes grouped most closely with each other in each set. Haplotype pairs of Atlantic spotted dolphins, spinner dolphins, and striped dolphins each grouped as most closely related to themselves in two of the three sets. In one set, striped dolphin, Clymene dolphin, and spinner dolphin haplotypes all formed a polytomy. The pair of Atlantic spotted dolphin haplotypes did not group most closely with themselves in another set, and Clymene dolphin haplotypes never formed a separate sister clade (Figure 14).

Tables & Figures
Figure 13. Simplified phylogenetic tree obtained with Maximum Likelihood Analysis in GARLI using 1002 bootstrap replicates for 346 Stenella haplotypes. The two Clymene dolphin (S. clymene) haplotypes did not separate from striped dolphins (S. coeruleoalba) or group together within striped dolphin haplotypes, so striped dolphins and Clymene dolphins are combined in the tree. Other species grouped together except three Atlantic spotted dolphins (S. frontalis) haplotypes did not group in the clade formed by the other eight Atlantic spotted dolphin haplotypes. Harbor porpoise (Phocoena phocoena) was the outgroup.
Figure 14. Phylogenetic trees obtained with Maximum Likelihood Analysis in GARLI each using 1000 bootstrap replicates comparing mtDNA control region sequences for three sets of random pairs of haplotypes of each *Stenella* species. Harbor porpoise (*Phocoena phocoena*) was the outgroup.
Table 15. Sequences used in this study. Table continued on next page.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th># of Haplotypes</th>
<th>GenBank Accession #</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. attenuata</em></td>
<td>ETP</td>
<td>112</td>
<td>DQ150134-DQ150245</td>
<td>Escozra-Treviño <em>et al.</em> (2005)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Gulf of Mexico</td>
<td>1</td>
<td>EF092944</td>
<td>Harlin-Cognato &amp; Honeycutt (2006)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Indian Ocean</td>
<td>1</td>
<td>EF438305</td>
<td>Jayasankar <em>et al.</em> (2008)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>NW Atlantic</td>
<td>2</td>
<td>DQ845442-DQ845443</td>
<td>Kingston <em>et al.</em> (2009)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>ETP</td>
<td>1</td>
<td>U09710</td>
<td>Rosel <em>et al.</em> (1995b)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Hawaiian Islands</td>
<td>13</td>
<td>GQ852567-GQ852579</td>
<td>Courbis, S. unpub.</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Pelagic near Hawai‘i</td>
<td>1</td>
<td>GU136595</td>
<td>Courbis, S. unpub.</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Taiwan/China Sea</td>
<td>13</td>
<td></td>
<td>Yao <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>S. attenuata</em></td>
<td>Atlantic</td>
<td>1</td>
<td>GU256406</td>
<td>Courbis, S. unpub</td>
</tr>
<tr>
<td><em>S. clymene</em></td>
<td>Gulf of Mexico</td>
<td>2</td>
<td>DQ845446-DQ845447</td>
<td>Kingston <em>et al.</em> (2009)</td>
</tr>
<tr>
<td><em>S. coeruleoalba</em></td>
<td>Pacific</td>
<td>7</td>
<td>AM498701-AM498706, AM498740</td>
<td>Mace, M., V. Bourret &amp; B. Crouau-Roy unpub.</td>
</tr>
<tr>
<td><em>S. coeruleoalba</em></td>
<td>Atlantic</td>
<td>19</td>
<td>AM498707-AM498725</td>
<td>Mace, M., V. Bourret &amp; B. Crouau-Roy unpub.</td>
</tr>
<tr>
<td><em>S. coeruleoalba</em></td>
<td>Croatian Adriatic Sea</td>
<td>7</td>
<td>EF624062-EF624063, EU079117-EU079121</td>
<td>Galov <em>et al.</em> (2008)</td>
</tr>
<tr>
<td><em>S. coeruleoalba</em></td>
<td>NW Atlantic</td>
<td>2</td>
<td>DQ845440-DQ845441</td>
<td>Kingston <em>et al.</em> (2009)</td>
</tr>
<tr>
<td><em>S. frontalis</em></td>
<td>NW Atlantic</td>
<td>11</td>
<td>DQ060054-DQ060064</td>
<td>Adams &amp; Rosel (2006)</td>
</tr>
</tbody>
</table>
Table 15 Continued

<table>
<thead>
<tr>
<th></th>
<th>Location</th>
<th>Accession Numbers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. longirostris</td>
<td>South Pacific</td>
<td>EF558737-EF558767</td>
<td>Oremus et al. (2007)</td>
</tr>
<tr>
<td>S. longirostris longirostris</td>
<td>Hawaiian Islands</td>
<td>AY989745-AY989762</td>
<td>Galver (2002)</td>
</tr>
<tr>
<td>S. longirostris orientalis</td>
<td>ETP</td>
<td>AY989669-AY989671, AY989673-AY989693,</td>
<td>Galver (2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AY989695-AY989697, AY989699-AY989700,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AY989722-AY989728, AY989739-AY989742,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AY989774-AY989778, AY989789-AY989791</td>
<td></td>
</tr>
<tr>
<td>Phocoena phocoena (outgroup)</td>
<td>1</td>
<td>U09694</td>
<td>Rosel et al. (1995b)</td>
</tr>
</tbody>
</table>
Discussion

Using a large number of mtDNA control region haplotypes of *Stenella* resulted in clustering of most recognized species but not sub-species or populations (Figure 13; Appendix D). The exceptions were Clymene dolphin haplotypes, which did not cluster together, but instead were clustered with striped dolphin haplotypes, and Atlantic spotted dolphin haplotypes did not all cluster together, with one haplotype clustering most closely with the striped dolphin and Clymene dolphin clade and two haplotypes falling out as sister to the rest of the *Stenella* (Figure 13; Appendix D). This indicates that the mtDNA control region may not be sufficient for determining phylogenetic relationships in the case of Atlantic spotted dolphins, Clymene dolphins, and striped dolphins. Kingston *et al.* (2009) recently reported similar results, with Clymene dolphins and striped dolphins forming a cluster with Indo-Pacific bottlenose dolphins and Fraser’s dolphins, although the Atlantic spotted dolphin mtDNA control region haplotypes in their study formed a single monophyletic cluster. The Kingston *et al.* (2009) study was the only previous study of the delphinids that included a large number of haplotypes representing each species (including 309 *Stenella*).

When random pairs of haplotypes from each species were compared, the results differed each of three times (Figure 14). This may partly be because of the inadequacy of the mtDNA control region as a genetic marker for the *Stenella*, but it is also indicative that one or two haplotypes is insufficient to correctly define relationships. Although all species except Clymene dolphins and Atlantic spotted dolphins grouped together in the larger analysis, only pantropical spotted dolphins consistently paired together in the
smaller analyses. Given availability of samples and computing power, running a larger number of haplotypes per species than the traditional one or two is likely to produce better results in phylogenetic comparisons. Fulton and Strobeck (2010) did a study of seal phylogeny and found that using multiple haplotypes per species resolved monophyly of the genus *Pusa* separate from *Halichoerus*, whereas using only one grey seal (*Halichoerus grypus*) haplotype created a polytomy or a weak or moderate sister group relationship depending on which grey seal haplotype and which *Pusa* haplotypes were used. The authors concluded that it would be beneficial to use multiple haplotypes per species in phylogenetic studies of organisms that have undergone recent rapid radiations in order to increase resolution of the tree. My results support these findings.

It is noteworthy that Kingston et al. (2009), as in the current study, chose to compare the mtDNA control region rather than the cytochrome b region, which has been more commonly used (e.g. Messenger & McGuire 1998, LeDuc et al. 1999, Caballero et al. 2008). Researchers have been unable to resolve the delphinids into monophyletic genera based on cytochrome b sequence comparisons (e.g. Messenger & McGuire 1998, LeDuc et al. 1999, Caballero et al. 2008). This may be because the species are not correctly defined, individual haplotypes were not correctly identified to species, and/or cytochrome b sequence may not be a good character for comparing the species of the Delphinidae. Generally, sequences that evolve quickly, like the mtDNA control region, are used for population level studies, and sequences that evolve more slowly, like gene sequences such as cytochrome b, are used for phylogenetic studies (Halliburton 2004). Possibly, this use of slower evolving regions is causing ambiguity in the phylogeny of
dolphins because of the recentness of their radiation. However, in the case of the *Stenella* my analyses indicate that the more rapidly evolving mtDNA control region is also only a fair indicator of species level differentiation. Using the mtDNA control region for comparisons does not create monophyletic species clades within the *Stenella*. It is possible that even more quickly evolving sequences of DNA are necessary to determine relationships within the *Stenella* because they are recently radiated.

Some studies have begun to include nuclear markers and AFLP’s in phylogenetic analyses of cetaceans (Kingston et al. 2009, McGowen et al. 2009). This may result in better trees. For example, Kingston et al.’s (2009) AFLP analysis places Clymene dolphins as most closely related to spinner dolphins, as is suggested by morphological analyses (Perrin et al. 1981), and it also places the haplotypes from each *Stenella* species together within species, as would be expected. Microsatellite markers may also be an alternative to the mtDNA control region as fast evolving regions. Usefulness would depend on whether or not there are common alleles among species. If common alleles occur in these DNA regions, it would be possible to compare assignment probabilities to explore relationships. Use of AFLP’s and microsatellites also allows for multiple loci to be compared. Although these fast evolving regions are probably not appropriate for phylogenetic comparisons among other groups, they may be most appropriate for the recently radiated dolphins.

In conclusion, the mtDNA control region, although faster evolving than gene regions, is not differentiated sufficiently among the *Stenella* for complete separation among established species, particularly between striped dolphins and Clymene dolphins,
even though Clymene dolphins have been considered a separate species based on morphology since the early 1980’s (Perrin et al. 1981). I suggest that multiple sequences and faster evolving sequences are needed to resolve this and the larger phylogeny of dolphins. In addition, my results indicate that one or two example haplotypes from a species may not be sufficient to establish phylogenetic relationships and are clearly not enough to establish sub-species and population level relationships. I agree with Kingston et al. (2009) that mtDNA control region and cytochrome b analyses are not powerful enough to resolve the phylogeny of dolphins. Although my results support the value of using a large number of representative haplotypes for species to increase resolution of analyses, it is possible that finding sequences that are variable and divergent enough to resolve phylogenetic relationships better will reduce the need for large numbers of representative haplotypes for species in future studies. The problems associated with resolving the phylogeny of dolphins may also suggest that the dolphins are currently over-split with respect to biological species.
CHAPTER 5: Conclusions

In conclusion, I found evidence that pantropical spotted dolphins constitute separate populations near Hawai‘i, the 4-islands area, and O‘ahu, with some evidence to support possible differences from Kaua‘i/Ni‘ihau, though further study is warranted. Pantropical spotted dolphins near Kaua‘i/Ni‘ihau may constitute transient individuals, but further research is needed to determine why, despite high effort, few pantropical spotted dolphins were found near that region during surveys. In my study to establish baseline $F_{ST}$ values, the results suggest that assigning specific numerical baseline $F_{ST}$ values may not always be biologically meaningful but that determining whether related populations with geographic or other separation show a preponderance of similar, lower, or higher fixation index values can help evaluate whether genetic differences among sympatric or parapatric groups warrants designating them as separate populations for management. For phylogenetic analyses, I found that the mtDNA control region, although faster evolving than gene regions, is not differentiated sufficiently among the *Stenella* for complete and clear relationships to be found among established species. Multiple sequences and faster evolving sequences are needed to resolve this and the larger phylogeny of dolphins. In addition, my results indicate that one or two example haplotypes from a species may not be sufficient to establish phylogenetic relationships and are clearly not enough to establish sub-species and population level relationships. This problem may also suggest the dolphins are over-split with respect to biological species.
References


Cubero-Pardo, P. 2007. Environmental factors governing the distribution of the bottlenose (Tursiops truncatus) and the spotted dolphin (Stenella attenuata) in Golfo Dulce, South Pacific, off Costa Rica. Investigaciones Marinas 35: 15-23.


DNASTAR. 2004. Lasergene DNA and protein analysis software. DNASTAR, Madison, WI.


141


RIZZUTO, J. 2007. Big fish await HIBT team. West Hawaii Today 39: 1B, 4B.


TAYLOR, B. L. and A. E. DIZON. 1996. The need to estimate power to link genetics and demography for conservation. *Conservation Biology* 10: 661-664.


Appendices

Appendix A: DNA Extraction Procedures
DNeasy Extraction Kit by QIAGEN

1. Place 25mg subsample in 1.5mL microcentrifuge tube
2. Add 180uL Buffer ATL
3. Add 20uL proteinase K
4. Vortex 5-10sec
5. Incubate for 24h at 55°C
6. Vortex 15sec
7. Add 200uL Buffer AL and vortex
8. Incubate at 70°C for 10min
9. Add 200uL ethanol and vortex
10. Centrifuge for 1min at 10,000RPM
11. Use pipette to remove supernatant and place in mini spin column
12. Centrifuge for 1min at 10,000RPM
13. Move filter tube into new collection tube
14. Add 500uL AW1 Buffer
15. Centrifuge 1min at 10,000RPM
16. Move filter tube into new collection tube
17. Add 500uL AW2 Buffer
18. Centrifuge for 3min at 10,000RPM
19. Move filter tube into 1.5mL microcentrifuge tube
20. Add 100uL Buffer AE
21. Wait 1min
22. Centrifuge 1min at 10,000 RPM
23. Transfer supernatant into 0.6mL centrifuge tube
24. Put filter tube back into 1.5mL microcentrifuge tube
25. Add 100uL Buffer AE
26. Wait 1min
27. Centrifuge 1min at 10,000 RPM
28. Transfer supernatant into 0.6mL centrifuge tube

The first supernatant (step 23) is the DNA sample. The second one (step 28) is a backup in case the first sample becomes contaminated.
Appendix B: DNA Purification Procedures
QIAquick PCR Purification Kit by QIAGEN

1. Add 125uL Buffer PB1 to PCR mix from initial amplification
2. Use pipette to transfer the PCR mix with buffer into a spin column
3. Centrifuge 1min at 10,000RPM
4. Discard flow through
5. Add 750uL Buffer PE
6. Centrifuge 1min at 10,000RPM
7. Discard flow through
8. Centrifuge 1min at 10,000RPM
9. Discard flow through tube
10. Place filter tube in 1.5mL microcentrifuge tube
11. Add 50uL Buffer EB
12. Centrifuge 1min at 10,000RPM
13. Liquid at bottom of tube is purified sample
### Appendix C: Primer Sequences

A tail was added to two of Galver’s (2002) primers to reduce effects of the poly-A tail. This procedure was used by Escorza-Treviño et al. (2005).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KWM2a</td>
<td>5’-GCTGTGAAAATTAATGTT-3’</td>
<td>5’-CACTGTGGACAAATGTAA-3’</td>
<td>Hoelzel et al. 1998b</td>
</tr>
<tr>
<td>KWM12a</td>
<td>5’-CCATAAATCCAGCAGTC-3’</td>
<td>5’-CACTGCAGAATGATGACC-3’</td>
<td>Hoelzel et al. 1998b</td>
</tr>
<tr>
<td>SD8</td>
<td>5’-TGCCCGTTAATAGAGC-3’</td>
<td>5’-GACACAGTTTGGCAGTG-3’</td>
<td>Galver 2002</td>
</tr>
<tr>
<td>EV94</td>
<td>5’-ATCGTATGGTCTTTTCTGC-3'</td>
<td>5’-AATAGATAGTGATGATGACACC-3’</td>
<td>Valsecchi and Amos 1996</td>
</tr>
<tr>
<td>SL8-49</td>
<td>5’-CATCTGTCTTTTTGAATAGAGG-3’</td>
<td>5’-GTTTCTTACCCATTCTGCTACC-3’</td>
<td>Galver 2002 (with GTTTCTT tail like Escorza-Treviño 2005)</td>
</tr>
<tr>
<td>SL9-69</td>
<td>5’-TTCCAAACACATACCCCTGCC-3’</td>
<td>5’-GTTTCTTACTAGATGCCACCTTGACC-3’</td>
<td>Galver 2002 (with GTTTCTT tail like Escorza-Treviño 2005)</td>
</tr>
<tr>
<td>EV37</td>
<td>5’-AGCTGTGATTTGGAAGTATGA-3’</td>
<td>5’-TAGTAGAGCCGTGATAAGTG-3’</td>
<td>Valsecchi and Amos 1996</td>
</tr>
<tr>
<td>EV14</td>
<td>5’-TAAACATCAAGCCAGACCC-3’</td>
<td>5’-CCAGAGCCAAGGTCAGAG-3’</td>
<td>Valsecchi and Amos 1996</td>
</tr>
<tr>
<td>MK5</td>
<td>5’-CTCAGAGGGAAGGCTCTCC-3’</td>
<td>5’-TGTTAGAGGCTAAGAGTGG-3’</td>
<td>Krützen et al. 2001</td>
</tr>
<tr>
<td>MK6</td>
<td>5’-GTCTCTTCCAGGTAGCTCC-3’</td>
<td>5’-GCCCACTAAGTATGCTGAGC-3’</td>
<td>Krützen et al. 2001</td>
</tr>
<tr>
<td>MK8</td>
<td>5’-TCCTGGAGCATCTTATAGTGCC-3’</td>
<td>5’-CTCTGGACATGCCCTACC-3’</td>
<td>Krützen et al. 2001</td>
</tr>
<tr>
<td>H00034</td>
<td>5’-CCTCACTCTTCCCTAGACT-3’</td>
<td>5’-TACCAATGTGAAACCTCAG-3’</td>
<td>Rosel et al. 1994</td>
</tr>
<tr>
<td>L15824</td>
<td>5’-ATACCATGGTGAGATGACC-3’</td>
<td>5’-AAGAATATGGCGACTTGAAC-3’</td>
<td>Bérubé and Palsbøll 1996</td>
</tr>
<tr>
<td>ZFXY0606</td>
<td>5’-GATTGTGCCAGCGACTTCT-3’</td>
<td>5’-TTTGTGTAACCTGAAATC-3’</td>
<td>Bérubé and Palsbøll 1996</td>
</tr>
<tr>
<td>ZFX9331</td>
<td>5’-TTTGTGTAACCTGAAATC-3’</td>
<td>5’-TTTGTGTAACCTGAAATC-3’</td>
<td>Bérubé and Palsbøll 1996</td>
</tr>
</tbody>
</table>
Appendix D: Phylogenetic Consensus Tree

This tree, based on the mtDNA control region of Stenella, was produced with 1002 bootstrap replicates in GARLI. The first two letters of the sample indicate species: Sa=S. attenuata, Sl=S. longirostris, co=S. coeruleoalba, Sc=S. clymene, and Sf=S. frontalis. The outgroup was Phocoena phocoena (Pp). The two capital letters in the middle of the name relate to the author who published the sequence, the number is the haplotype number in the publication, and the remaining letters refer to sample locations. Please see Table 13 for authors & locations.