

# 6 Molecular Genetics of Sea Turtles

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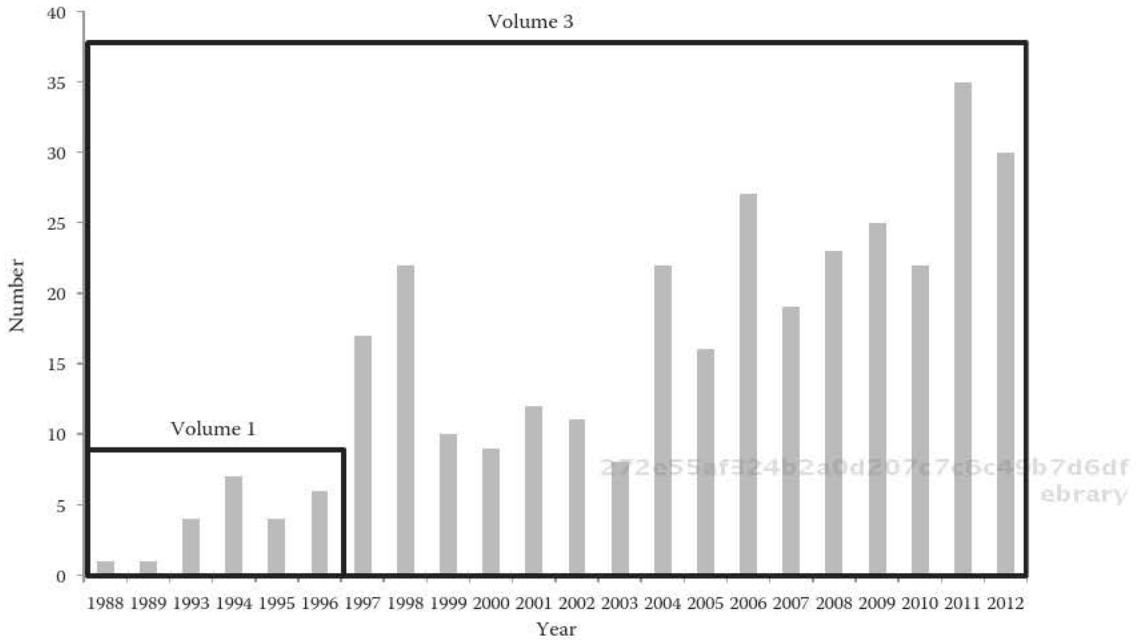
## CONTENTS

6.1	Introduction .....	135
6.2	Sea Turtle Phylogeny .....	137
6.3	Populations, Gene Flow, and Dispersal .....	138
6.3.1	Phylogeography and Natal Homing.....	139
6.3.2	Phylogeography and Population History .....	139
6.3.3	Colonization History and Long-Distance Dispersal.....	141
6.3.4	Male-Mediated Gene Flow .....	143
6.4	Feeding Grounds and Migratory Behavior.....	144
6.4.1	Mixed Stock Analysis.....	145
6.4.2	Factors Shaping the Composition of Foraging Grounds .....	145
6.4.3	Differences between Time, Size, and Gender .....	146
6.4.4	Limitations of MSA.....	148
6.5	Conservation and Management Implications: A Population Perspective.....	148
6.6	Mating Systems .....	150
6.7	Population Vital Parameters .....	151
6.7.1	Sex Ratios of Breeding Populations .....	151
6.7.2	Age to First Reproduction.....	151
6.8	Future Directions .....	152
	Acknowledgments.....	153
	References.....	154

## 6.1 INTRODUCTION

Since the first volume of *The Biology of Sea Turtles* (Lutz and Musick, 1997), studies using molecular techniques to address a variety of questions about sea turtle biology and life history have grown rapidly. In the late 1980s researchers had just begun using mitochondrial (mt) DNA to investigate how sea turtle rookeries are genetically linked through female dispersal and set a benchmark when providing compelling evidence of female natal homing. The growing popularity of using molecular techniques in sea turtle research is illustrated by the number of genetic papers presented at the *Annual Symposium on Sea Turtle Conservation and Biology* over the past two decades (Figure 6.1). The rapid progress in DNA sequencing and genotyping technology has expanded the scope of molecular genetics, and the symposia presentations include diverse topics ranging from mating systems and kinship among individuals to relationships among populations and species.

Over the past 20 years molecular genetics has come to play a central role in addressing questions that are directly relevant to the conservation of sea turtles (Table 6.1). Most studies to date have used maternally inherited mtDNA (control region) and nuclear microsatellites as the markers of choice,



**FIGURE 6.1** The number of genetics presentations at the Annual Symposium on Sea Turtle Biology and Conservation from 1988 to 2012. The small box highlights the number of genetics presentations prior to Volume 1 of the *Biology of Sea Turtles* being published in 1997.

**TABLE 6.1**  
**Molecular Genetic Landmarks for Sea Turtle Biology and Conservation**

**Contribution**

- Confirming natal homing in sea turtles
- Demonstrating that multiple paternity exists in many sea turtle populations
- Connecting foraging areas to rookery origins
- Identifying populations of concern
- Determining population genetic structure
- Resolving taxonomic uncertainty
- Defining management units within species
- Establishing parentage; pedigree analysis
- Detecting hybridization
- Understanding population connectivity

as these regions are the most highly variable markers which makes them ideal for population-level analysis. In a study of Atlantic green turtles, Meylan et al. (1990) provided the first evidence of natal homing by showing that turtles nesting on Aves Island and those nesting at Tortuguero, Costa Rica, had fixed haplotype differences for mtDNA, despite these two populations mixing at foraging grounds in the Caribbean. Since then, numerous studies have shown that this pattern of mtDNA differentiation among rookeries is a common feature among all species of marine turtles, albeit to varying degree.

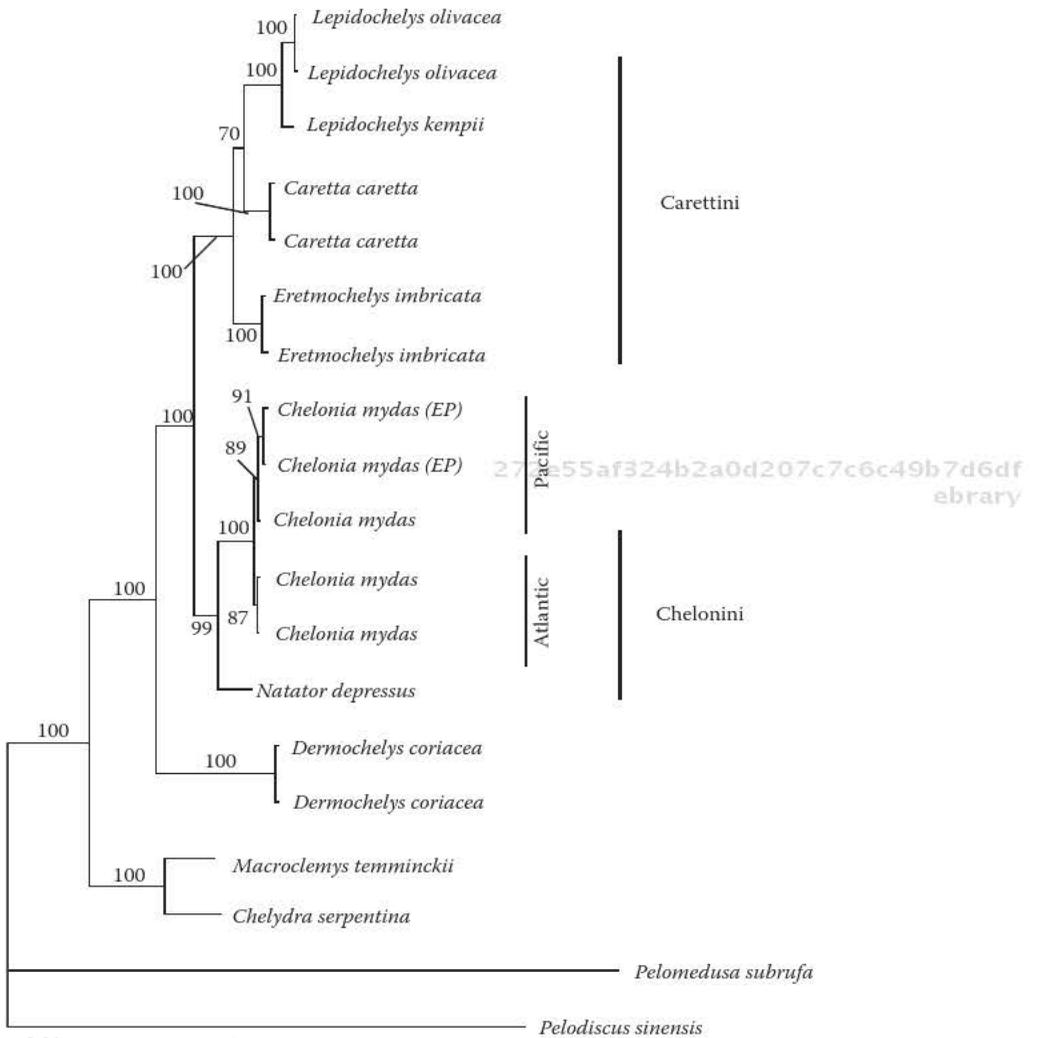
The genetic differentiation of rookeries led to another important advance for the use of molecular tools, the ability to determine the origin of turtles sampled away from nesting beaches. The value of

this was demonstrated when Bowen et al. (1995) were able to trace loggerhead turtles from developmental habitats in the central and eastern Pacific to nesting beaches in Japan, thereby providing evidence for the trans-Pacific migration of loggerhead turtles. Since then, detailed studies using mixed stock analysis (MSA) have aimed to explain the processes that generate the composition of turtles at mixed foraging areas. This is one of the most active fields in sea turtle genetics as researchers seek to generate more precise and reliable estimates that may be used for threat assessment. Genetic tools have also proven useful in answering questions about the reproductive behavior of sea turtles. Since the first study that used protein isozyme polymorphisms to document multiple paternity in loggerhead turtles (Harry and Briscoe 1988), researchers have used dozens of microsatellite loci to understand mating systems in all species of sea turtles, and even so, questions remain regarding sperm storage and possible fitness benefits of different mating strategies. Furthermore, a combination of mtDNA and microsatellite data has been used to document several cases of hybridization in sea turtles. Molecular genetic studies of sea turtles exist within broader genetic fields that are constantly evolving. New tools and techniques such as single nucleotide polymorphisms (SNPs) and mitogenomics are important additions to the molecular toolbox that promise to overcome some of the limitations of past studies. In this chapter we review the current role and scope of molecular genetics in sea turtle research. Empirical examples are used to highlight some key findings that best describe the patterns and processes that genetic studies of sea turtles aim to unravel.

## 6.2 SEA TURTLE PHYLOGENY

Several recent studies have advanced our knowledge of the relationship among sea turtle lineages and their placement within the Testudines. Sea turtles are placed within the superfamily Chelonioidea (containing the families Cheloniidae and Dermochelyidae), which forms a monophyletic group most closely related to freshwater mud turtles (Kinosternoidea) and snapping turtles (Chelydridae) based on sequence data from 14 nuclear genes (Barley et al., 2010). Within the Chelonioidea, *Dermochelys coriacea* has a basal position as the older lineage relative to the other marine turtles (Bowen et al., 1993; Dutton et al., 1996; Naro-Maciel et al., 2008), which split into two subfamilies, the Chelonini (*Chelonia mydas* and *Natator depressus*) and the Carettini (*Lepidochelys olivacea*, *Lepidochelys kempfi*, *Caretta caretta*, and *Eretmochelys imbricata*) about 63 MYA (Figure 6.2) (Naro-Maciel et al., 2008). Early phylogenetic studies of sea turtles based on sequencing of the mtDNA control region (d-loop), ND4, and *Cytb* placed flatback turtles as a sister species to the Carettini (Bowen et al., 1993; Dutton et al., 1996), but using sequence data (7340 base pair [bp]) from the mtDNA genes 12S and 16S and four nuclear genes, *N. depressus* is grouped with the *C. mydas* lineages (Naro-Maciel et al., 2008). Estimated divergence times among species are 34 MYA (95% HPD: 14.1–60.1) between flatback and green turtles, and 29 MYA (95% HPD: 16.5–44.3) between hawksbill turtles and the combined loggerhead and ridley lineages (Naro-Maciel et al., 2008).

The advent of new sequencing technologies has recently allowed the entire mtDNA genome of sea turtles to be sequenced (Duchene et al., 2012; Frey and Dutton 2012; Morin Shamblin et al., 2012b). This expands the data from earlier studies by Dutton et al. (1996), which used ~1433 bp sequences (from three regions) to over 16,000 bp. The new studies detect additional genetic variation in the chelonids and will provide new insights into the evolutionary relationships among species (Duchene et al., 2012). Also, recent whole-mitogenome data support previous hypotheses (Avise et al., 1992) that the Testudines mitochondrial clock is slower than the conventional  $2 \times 10^{-2}$  rate estimated for other animal lineages (Duchene et al., 2012). Whole mitogenome analyses support previous nuclear-mtDNA topology (Naro-Maciel et al., 2008; see also Thomson and Shaffer, 2010), placing *N. depressus* as a sister taxon to *Chelonia* and provide more precise divergence times. For example, the estimated divergence time between Pacific and Atlantic *C. mydas* lineages was estimated to be 3.09 MYA (1.76–4.87) in comparison to 7 MYA (1.92–13.47 HPD) in the Naro-Maciel et al. (2008) study (Duchene et al., 2012). However, this may in part reflect incomplete sampling distributions if samples from different lineages were used. Preliminary analysis of all 16,281 bp



**FIGURE 6.2** Sea turtle phylogeny based on maximum parsimony (MP) and Bayesian analyses sequences from a combined 980 bp of mtDNA (12S and 16S) and 6350 bp from four nuclear genes (BDNF, Cmos, R35, Rag1, and Rag2). The numbers above the branches are MP bootstrap value. All posterior probability values (PP) from combined and mixed—model Bayesian analyses were 100%. (Adapted from Naro-Maciel, E. et al., *Mol. Phylogenet. Evol.*, 49, 659, 2008.)

of each mitochondrial genome sequence for leatherbacks from representative rookeries around the world has revealed surprisingly little additional variation (Dutton et al., unpublished data) and rules out a lack of resolution in the genetic marker as an alternative explanation of the low diversity found in *Dermochelys* by earlier studies (Dutton et al., 1996, 1999).

### 6.3 POPULATIONS, GENE FLOW, AND DISPERSAL

The value of using mtDNA markers became apparent from the initial use of DNA analysis to study sea turtles because the female-to-offspring mode of inheritance allows for tracking of rookery history and the relationships between rookeries (Avisé and Bowen, 1994). By investigating the genetic structure among rookeries, it was possible to confirm the operation of natal homing by females in their choice

of nesting regions. In addition, through the comparison of the relationships among mtDNA genetic lineages to their geographic locations, it was also possible to address questions of rookery history, colonization, and long-distance dispersal. However, relying solely on mtDNA markers to elucidate population histories and boundaries is not sufficient, given the importance of understanding marine turtle behavior for conservation purposes and how turtles have responded to past climatic and sea-level changes. Nuclear- and genome-wide markers allow assessment of male-mediated gene flow, and variations in their mutation rates provide insights into population processes at variable time scales.

### 6.3.1 PHYLOGEOGRAPHY AND NATAL HOMING

Many genetic studies have focused on understanding the history of marine turtle populations using phylogeographic techniques that compare the relationship among genetic lineages to the geographic locations where those lineages are found (Avice, 2009). These studies also provide insights into the extent of genetic structure among rookeries and the operation of natal homing by adults in their selection of breeding locations. Early genetic studies of green turtles uncovered genetic structure among distant rookeries, thus confirming that natal homing occurs when a female returns to nest (Meylan et al., 1990). This demonstrated the usefulness of genetics to understand marine turtle behavior and population dynamics. Natal homing behavior was also revealed for male green turtles in their choice of breeding grounds, although this did not preclude male-mediated gene flow from occurring, likely through opportunistic mating by males during migration (FitzSimmons et al., 1997a). Population genetic studies have now been conducted on all species of marine turtles in many regions, and it is evident that natal homing behavior is shared among all species, though there is considerable variation in the extent of genetic structure among populations and the implied extent of natal homing, when compared both across and within species.

Evidence of strong natal homing is indicated in several studies, but it is far from being a predictable phenomenon. In green turtles, strong natal homing is evidenced by significant genetic differentiation between island rookeries at Ashmore and Scott reefs off northwestern Australia (Dethmers et al., 2006; Jensen, 2010); these rookeries are located only 225 km apart. This contrasts with the nearby Northwest Shelf population that spans over 1,000 km of coastline and offshore islands (Dethmers et al., 2006; Jensen, 2010). Genetic differentiation was also found for rookeries on two islands in Taiwan that are separated by ~250 km (Cheng et al., 2008) and between locations in Japan that are <60 km apart (Nishizawa et al., 2011). In hawksbill turtles, rookeries in Iran (FitzSimmons, 2010) and Barbados (Browne et al., 2009) <50 km from each other have also displayed significant genetic differences. Some loggerhead rookeries <100 km apart are genetically distinct in the Mediterranean (Garofalo et al., 2009) and in Florida (Shamblin et al., 2011a), but this is not consistent among rookeries. Interesting support for strong natal homing comes from the green turtle rookery in Tortuguero, Costa Rica, where there was a negative correlation between genetic relatedness (using DNA minisatellite fingerprinting) and distance among individual nesting turtles over a 2 year period, indicating natal precision even within a rookery along the nesting beach. However, this was not found for green turtles nesting at Melbourne beach in Florida (Peare and Parker, 1996). Leatherback turtles appear to have the least strict natal homing overall, with some rookeries located over 2000 km apart not showing significant genetic differentiation (Dutton et al., 1999, 2007).

### 6.3.2 PHYLOGEOGRAPHY AND POPULATION HISTORY

Within species there is considerable variation in the estimated divergence times among haplotypes, and observations of shallow divergence and limited genetic variation have led to suggestions of genetic bottlenecks within ocean basins, or even globally. In contrast to its basal position as the oldest lineage in the marine turtle phylogeny, leatherback turtles were found to have a shallow phylogeny and low genetic diversity, suggesting a relatively recent global radiation for this oldest of lineages (Dutton et al., 1999). The most divergent (1.4%) leatherback haplotypes are estimated

to have diverged <1 MYA. One explanation supporting this pattern is that leatherback turtles went through population declines caused by repeated glaciations during the Pleistocene, followed by subsequent population expansion (Dutton et al., 1999, but see Rivalan et al., 2006). Shallow phylogenies are also observed in the geographically isolated flatback and Kemp's ridley turtles, with maximum divergence among haplotypes estimated at  $p=0.7\%$  (Pittard, 2010) and  $0.9\%$  (Bowen et al., 1998), respectively for each species, which is also suggestive of past genetic bottlenecks (Shanker et al., 2004; Pittard, 2010). In leatherbacks, only 11 mtDNA haplotypes were identified from 281 samples in 12 populations (Dutton et al., 1999, 2007). A small number of haplotypes (12 in 274 samples) have also been found in flatback turtles (Pittard, 2010) for which, similar to leatherbacks, there is a predominant, presumed ancestral haplotype that is found in most regions (Pittard, 2010). But in contrast to leatherbacks, some flatback rookeries less than 300 km apart show genetic differentiation (Pittard, 2010). Comparisons between the two species suggest that both went through recent bottlenecks, from which they expanded into new areas, but that variation in the extent of natal homing has resulted in several functionally independent rookeries in flatback turtles, in contrast to regionally broader metapopulations for leatherbacks (Dutton et al., 1999, 2007).

Loggerhead turtles have phylogenies that reflect strong phylogeographic structure within and between the Atlantic-Mediterranean and Indo-Pacific ocean basins in which levels of genetic diversity and structure vary between regions. The two main divergent genetic lineages in loggerhead turtles are separated by a maximum sequence divergence of  $6.3\%$ , with an estimated time of divergence during the Pliocene of  $\sim 3$ MYA (Bowen, 2003). Within ocean basins, loggerhead turtles have considerably greater genetic diversity and regional structure among Atlantic populations in comparison to Indian Ocean and western Pacific populations. In the Atlantic and Mediterranean, at least 43 haplotypes (based on 380 bp sequences) have been published (Bolten et al., 1998; Laurent et al., 1998; Bass et al., 2004; Bowen et al., 2004; Roberts et al., 2005; Carreras et al., 2006; Reece et al., 2006; Casale et al., 2008; Monzón-Argüello et al., 2009, 2010b; Reis et al., 2010a; Shamblin et al., 2011a; Yilmaz et al., 2011), with at least another 16 known haplotypes yet to be published (<http://accstr.ufl.edu/ccmtdna.html>). These haplotypes represent two different clades, separated by an average of  $5.1\%$  sequence divergence (Encalada et al., 1998), with one clade found in the United States and Brazil and the other found in the United States, Mexico, and Mediterranean rookeries. The distribution and relationship among mtDNA haplotypes has led to an hypothesis that during the Pleistocene, loggerhead populations contracted to nesting locations closer to the equator (southern Florida and Mexico) and later colonized into their northern range in the United States and Mediterranean (Encalada et al., 1998) and south to Brazil (Reis et al., 2010b). Tests for genetic bottlenecks support a scenario of population expansion after bottlenecks for the Florida (Reece et al., 2005), Brazil, and Mediterranean populations (Reis et al., 2010b), possibly as early as the late Pliocene in response to the closing of the Isthmus of Panama (Reece et al., 2005). Among the relatively smaller Pacific loggerhead populations, only four haplotypes (among 362 samples) have been observed, and there is little sequence diversity (Hatase et al., 2002; Boyle et al., 2009). This is indicative of a strong ocean-wide population bottleneck, with haplotypes estimated to have diverged 500–700,000 years ago (Hatase et al., 2002).

In contrast, both green and hawksbill turtle populations display high levels of genetic diversity and phylogeographic structure in both the Indo-Pacific and the Atlantic and Mediterranean basins. Among 27 green turtle rookeries in the Indo-Pacific, 25 haplotypes were observed, with sequence divergences of up to  $8.4\%$  (Dethmers et al., 2006). Among the Atlantic and Mediterranean rookeries, sequence divergence is lower (maximum  $p=3.3\%$ ; estimated from Bjørndal et al., 2005), but haplotype diversity is high. A total of 47 haplotypes have been published (Allard et al., 1994; Lahanas et al., 1994; Encalada et al., 1996; Bass and Witzell, 2000; Bass et al., 2006; Bjørndal et al., 2006; Formia et al., 2006, 2007; Naro-Maciel, 2006; Foley et al., 2007; Ruiz-Urquiola et al., 2010; Bagda et al., 2012) and another 18 haplotypes are yet to be published (<http://accstr.ufl.edu/cmmtdna.html>). Evidence of population bottlenecks is restricted to particular rookeries, as based on specific tests for bottlenecks (Reece et al., 2005; Formia et al., 2006)

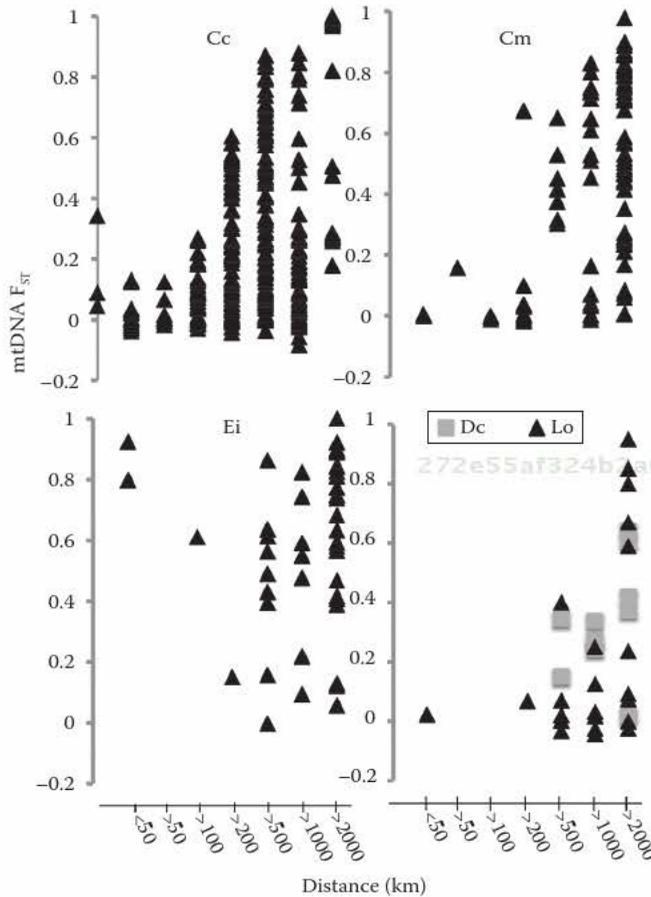
or from low estimates of historic effective population size (e.g., Dethmers et al., 2006). Similar to the case for green turtles, high levels of genetic diversity characterize hawksbill turtles in the Atlantic and Caribbean (26 haplotypes in 12 rookeries; Monzón-Argüello et al., 2011) as well as those in the Indo-Pacific (48 haplotypes in 8 populations, FitzSimmons, 2010).

### 6.3.3 COLONIZATION HISTORY AND LONG-DISTANCE DISPERSAL

Two aspects are apparent in the genetic structure of sea turtle populations: a disconnect between current population size and genetic diversity, and limited correlations between genetic distance and geographic distance among rookeries. Apparently, genetic structure and levels of genetic diversity in marine turtle populations are complex due to varied colonization histories that range from single colonization events to multiple colonizations from diverse populations. Thus the extent of genetic diversity is not necessarily indicative of population size (e.g., Lahanas et al., 1994; Bjorndal et al., 2006; Dethmers et al., 2006), as would be expected from classic population genetic theory. Additionally, there is considerable evidence that marine turtle rookeries have undergone major geographic shifts in response to climate change, with many rookeries being established within the past 10,000 years. For example, green, flatback, and olive ridley turtles nest within the Gulf of Carpentaria, Australia, on beaches that did not exist until ~8000 years ago (Jones and Torgersen, 1988). This represents around 200–250 turtle generations and provides a test for how long it takes for rookeries to become genetically differentiated once colonized. All three species indicate significant genetic divergence from the nearest rookeries, though the level of divergence varies ( $F_{ST}=0.07-0.80$ ; Jensen et al., unpublished data; Pittard, 2010), suggesting either a low level of ongoing gene flow with neighboring rookeries or, equally likely, that not enough generations have elapsed to observe a more defined genetic separation. Complex colonization patterns are also reflected in several nonsignificant results from genetic tests for isolation by distance among rookeries (Bass et al., 1996; Carreras et al., 2006; Bourjea et al., 2007; Garofalo et al., 2009). Although patterns of isolation by distance have been observed (Figure 6.3), it is often relatively weak ( $r^2 < 0.3$ ), or dependent upon certain geographic boundaries (Reece et al., 2005; Dethmers et al., 2006; Bourjea et al., 2007; Reis et al., 2010b; Pittard, 2010; LeRoux 2012). From a conservation perspective, the long generation times of marine turtles may act as a buffer against the loss of genetic diversity when population size is reduced, as evidenced by the diversity observed in Kemps ridleys (Bowen et al., 1991, 1998; Kichler et al., 1999). However, several populations have been observed with no mtDNA diversity (Dutton et al., 1999; Formia et al., 2006; Carreras et al., 2007; Browne et al., 2009; Reis et al., 2010b; Monzón-Argüello et al., 2011), and although this may have occurred through founder effects of small colonization events, long-term genetic bottlenecks may also have contributed to the lack of diversity.

Extant marine turtles have adapted to changing climate, sea levels, and oceanic current patterns throughout their evolution of over 110 MYA (Hirayama, 1998), including periods of glaciation and sea-level changes of >200 m (Haq et al., 1987). Genetic evidence suggests that this likely resulted in a series of regional colonization and extinction events, in which strict natal homing would not allow for such adaptation (e.g., Reece et al., 2005). Apart from a need for relaxed natal homing that might allow turtle populations to shift rookery locations over hundreds of kilometers, occasional long-distance dispersal has also been a feature of sea turtle evolution.

Many sea turtle species show evidence of long-distant dispersal in the past, as seen in the widespread distribution of some mtDNA haplotypes. Olive ridley populations in India were found to have a low frequency of individuals with a haplotype that is found in Malaysia and Australia, and another haplotype was shared with the eastern Pacific olive ridleys in Costa Rica. This evidence and the observation that the mtDNA haplotypes of Kemps ridley are more closely related to haplotypes that predominate along the east coast of India led to a hypothesis that the Indo-western Pacific population is the ancestral source for other olive ridley populations (Bowen et al., 1998; Shanker



**FIGURE 6.3** Estimates of  $F_{ST}$  derived from mtDNA control region data from pairwise comparisons between rookeries versus the distance between rookeries. Distances are based on distance categories of <50, >50, >100, >200, >500, >1000, and >2000 km. Species abbreviations are as follows: *Caretta caretta* (Cc), *Chelonia mydas* (Cm), *Eretmochelys imbricata* (Ei), *Dermochelys coriacea* (Dc), and *Lepidochelys olivacea* (Lo). (Data taken from Lahanas, P.N. et al., *Genetica*, 94, 57, 1994; FitzSimmons, N.N. et al., *Genetics*, 147, 1843, 1997b; Dutton, P.H. et al., *J. Zool.*, 248, 397, 1999; Hatase, H. et al., *Mar. Biol.*, 141, 299, 2002; Chassin-Noria, O. et al., *Genetica*, 121, 195, 2004; Shanker, K. et al., *Mol. Ecol.*, 13, 1899, 2004; Bjorndal, K.A. et al., *Mar. Biol.*, 147, 1449, 2005; Bjorndal, K.A. et al., *Chelonian Conserv. Biol.*, 5, 262, 2006; López-Castro, M.C. and Rocha-Olivares, A., *Mol. Ecol.*, 14, 3325, 2005; Formia, A. et al., *Conserv. Genet.*, 7, 353, 2006; Camacho-Mosquera, L. et al., *Investig. Mar.*, 37, 77, 2008; Cheng, I.J. et al., *J. Zool.*, 276, 375, 2008; Boyle, M.C. et al., *Proc. R. Soc. [Lond.]*, 276, 1993, 2009; Browne, D.C. et al., *Conserv. Genet.*, 11, 1541, 2009; Garofalo, L. et al., *Mar. Biol.*, 156, 2085, 2009; Reis, E.C. et al., *Conserv. Genet.*, 11, 1467, 2010b; Shamblin, B.M. et al., *Mar. Biol.*, 158, 571, 2011a; Monzón-Argüello, C. et al., *J. Exp. Mar. Biol. Ecol.*, 407, 345, 2011; Nishizawa, H. et al., *Endanger. Species Res.*, 14(2), 141, 2011; Yilmaz, C. et al., *Biochem. Syst. Ecol.*, 39, 266, 2011; Saied, A. et al., *Mar. Ecol. Prog. Ser.*, 450, 207, 2012.)

et al., 2004). Under this scenario, the Indo-western Pacific and eastern Pacific populations were established by an eastward trans-oceanic dispersal, in contrast to a proposed westward colonization from the eastern Pacific (Pritchard, 1969). Long-distance dispersal is also implied in hawksbill turtles where the most common haplotype found in rookeries in the Persian Gulf off Iran was also found in a western Pacific rookery in the Solomon Islands (FitzSimmons, 2010). Likewise, a common green turtle haplotype in Micronesia was found in Australian rookeries in both the Pacific and Indian Oceans (Dethmers et al., 2006).

Genetic evidence has supported previous hypotheses that turtles may have travelled around the Cape of Good Hope, allowing for gene flow from the Indian Ocean to the Atlantic. This would be most likely during periods of warmer oceanic temperatures, which may allow an increased flow of the relatively warm water of the Agulhas Current southwest along the coast of South Africa and into the Atlantic (Bard and Rickaby, 2009). An hypothesis of colonization of the Atlantic Ocean by olive ridley migrants from the Indian Ocean (Pritchard, 1969) is supported by the occurrence of mtDNA haplotypes in the Atlantic that are closely related to haplotypes found in the Indian Ocean (Bowen et al., 1998). The phylogeographic structure of loggerhead turtles indicates at least two long-distance dispersals, one around the Cape of Good Hope, as evidenced by the grouping of the only haplotype found in Oman with haplotypes from the North Atlantic and the presence of the only haplotype found in South Africa from rookeries in the North Atlantic and Mediterranean (Bowen, 2003). In green turtles, Atlantic haplotypes were found in high proportions in two rookeries in the southwest Indian Ocean to the west of Madagascar, but not in rookeries 500 km to the north (Bourjea et al., 2007). The presence of only a single Atlantic haplotype in the Indo-Pacific rookeries led to the hypothesis that gene flow was not extensive, but that it was relatively recent (given the lack of new mutations to the Atlantic haplotype), and that the dispersal may have been from the Atlantic into the Indo-Pacific as has been observed in hammerhead sharks (Duncan et al., 2006; Bourjea et al., 2007).

Whether these various genetic data represent long-distance dispersal events by a few individuals, a series of step-wise dispersals, or if they reflect genetic relicts from a large, widespread ancestral population is largely unknown. Additionally, it is not known whether these events happen as a result of long-distance dispersal by posthatchling turtles that never make it back to their natal areas, or are due to displacement by breeding females. Studies of foraging-ground turtles also provide evidence of long-distance dispersal. Among hawksbill turtles, a previously unidentified haplotype from an Indian Ocean foraging ground (Okayama et al., 1999) was found to be the only haplotype observed among 20 nesting turtles at Principe in the eastern Atlantic (Monzón-Argüello et al., 2011). At an Atlantic foraging ground in Brazil, 12% of loggerhead turtles had the same haplotype as commonly observed in Australian rookeries (Reis et al., 2010b). The migratory limits within the life cycles of turtles from most populations are poorly known, though the trans-oceanic voyages of loggerheads (Bowen et al., 1995; Laurent et al., 1998; Boyle et al., 2009; Monzón-Argüello et al., 2011) and leatherbacks (Dutton et al., 2000) are good examples of what is possible.

### 6.3.4 MALE-MEDIATED GENE FLOW

Several published studies have compared the genetic structure observed with mtDNA to that with nuclear DNA, and it is apparent that a priori predictions cannot be made about the extent of male-mediated gene flow. Since the development of the first microsatellite loci for marine turtles (FitzSimmons et al., 1995), a proliferation of loci have been developed for loggerhead (Shamblin et al., 2007, 2009; Monzón-Argüello, 2008), green (Dutton and Frey, 2009; Shamblin et al., 2012a), hawksbill (Lin et al., 2008; Miro-Herrans et al., 2008), olive ridley (Aggarwal et al., 2004, 2008), and leatherback (Alstad et al., 2011; Roden and Dutton, 2011) turtles for studies of genetic structure and mating systems. All studies have found evidence of male-mediated gene flow, but there is considerable variation in results (Table 6.2). In several recent studies, less than half of the pairwise tests between populations indicate male-mediated gene flow, and in several of these there are examples of less gene flow estimated using microsatellite loci than by mtDNA sequencing (Table 6.2). Such results may be interpreted as a lack of male-mediated gene flow and evidence of how higher mutation rates at microsatellite loci may lead to genetic divergence between populations that is not always observed within the mtDNA.

One concern related to studies of male-mediated gene flow is the need for better knowledge of the spatial distribution of populations, especially the extent of overlapping feeding grounds, and how this could provide avenues for opportunistic mating during breeding migrations. Future tests of male-mediated gene flow need to be structured to incorporate appropriate geographic scales that consider

**TABLE 6.2**  
**Studies That Have Compared Genetic Structure at Nuclear Microsatellites to mtDNA**  
**and Evidence for Male-Mediated Gene Flow**

Species	Region	#Loci	Sample Size (# Rookeries)	Evidence of Male- Mediated Gene Flow	Reference
Cc	Western Atlantic	5	459 (9)	64 of 72 tests <sup>a</sup>	Bowen et al. (2005)
Cc	Mediterranean	7	112 (7)	5 of 11 tests <sup>b</sup>	Carreras et al. (2007)
Cc	Turkey	6	256 (18)	10 of 10 tests <sup>b</sup>	Yilmaz et al. (2011)
Cm	Australia	4	275 (9)	6 of 6 tests <sup>b</sup>	FitzSimmons et al. (1997b)
Cm	Pacific Mexico	3	123 (4)	3 of 5 tests <sup>b</sup>	Chassin-Noria et al. (2004)
Cm	Global	4	337 (16)	Ocean basins <sup>c</sup>	Roberts et al. (2004)
Cm	Japan	4	67 (3)	1 of 1 test <sup>b</sup>	Nishizawa et al. (2011)
Dc	Atlantic	16	1417 (9)	1 of 36 tests <sup>b</sup>	Dutton et al. (2013)
Ei	Indian Ocean	5	64 (2)	Not tested	Zolgharnein et al. (2011)
Lo	French Guiana	11	46 (1)	Not tested	Plot et al. (2011)
Nd	Australia	11	370 (11)	22 of 59 tests <sup>b</sup>	Pittard (2010)

<sup>a</sup> Estimates based on MIGRATE (Beerli, 2002).

<sup>b</sup> Estimates based on  $F_{ST}$  and  $F_{ST}$  analogs.

<sup>c</sup> Pairwise values not shown.

the distribution of feeding grounds used by the populations. Microsatellite studies may be affected by homoplasy (mutations in different lineages that create identical alleles) occurring among distinct populations (Roberts et al., 2004). This may lead to erroneously concluding that male-mediated gene flow has occurred between populations, particularly if sampling designs are not appropriate.

Comparisons of genetic structure observed at microsatellite versus mtDNA markers provide important insights about population-wide diversity but afford only a limited understanding of male behavior. To understand whether male-mediated gene flow is due to “relaxed” natal philopatry in males, or whether it is due to opportunistic matings by males as they migrate through breeding grounds en route to their natal areas, requires sampling males at breeding grounds. This allows for comparisons of the mtDNA haplotype frequencies of males versus females at breeding grounds, and it would be a true test of male natal philopatry. The first study to investigate this found that in three Australian populations, green turtle males, like females, have strong natal philopatry and that male-mediated gene flow is opportunistic and depends upon the timing of breeding and the geographic locations of feeding grounds and mating grounds (FitzSimmons et al., 1997a). In contrast, weak but significant haplotype differences were observed between breeding male and female hawksbill turtles in Puerto Rico, and there was evidence that some males had originated from different rookeries (e.g., Costa Rica) (Velez-Zuazo et al., 2008).

## 6.4 FEEDING GROUNDS AND MIGRATORY BEHAVIOR

Most sea turtle species have a circumglobal distribution across tropical and subtropical waters, with hundreds of nesting beaches and foraging grounds making up a complex network of migratory routes. After hatching from tropical and subtropical beaches, posthatchling sea turtles spend years at the mercy of the prevailing currents (Musick and Limpus, 1997). Here the turtles grow larger and as they reach approximately 20–40 cm in curved carapace length (CCL), some species (e.g., green and hawksbill turtles) settle into neritic benthic habitats (Bjorndal, 1980; Balazs, 1982; Musick and Limpus, 1997) while other species, such as leatherbacks (and to some extent ridleys) stay in deeper pelagic waters. Some take up permanent residency and show strong fidelity to a chosen foraging area, while others undertake further developmental migrations with temporary settlement

in developmental areas before finally settling in a specific area or using seasonal habitats. As mature adults, females migrate periodically between breeding and foraging grounds during breeding seasons, in some cases travelling several thousand kilometers (Limpus, 2007, 2009; Benson et al., 2011). The ability to link turtles at feeding grounds, or those encountered along migratory routes, back to their breeding habitat is challenging, but it is a fundamental component of effective management and conservation. Both mark-recapture and satellite telemetry studies have connected rookeries to foraging habitats for many populations of sea turtles (Bentivegna, 2002; Godley et al., 2002, 2003; Shillinger et al., 2008). However, these techniques cannot yet be used to connect the non-adult portion of the population to their natal rookery. Molecular techniques have opened up new possibilities to assess the connectivity between nesting and foraging areas, especially for immature sea turtles.

#### 6.4.1 MIXED STOCK ANALYSIS

When mtDNA haplotypes exhibit significant frequency shifts among rookeries, they can be used to infer the natal origin of turtles captured along migration corridors and in feeding habitats. Mixed stock analysis (MSA) was first developed to detect the proportion of genetically differentiated salmon stocks from different rivers to mixed stocks of salmon caught in oceanic fisheries (Pella and Milner, 1987; Grant et al., 1980). Salmon and sea turtles share the life history traits of natal homing that results in breeding stocks that are genetically differentiated, coupled with highly migratory life history stages where stocks mix in foraging habitats. Since the early 1990s, researchers have used MSA methods to identify the rookery origins of sea turtles in the pelagic stage (Bowen et al., 1995; Bolten et al., 1998), in juvenile benthic foraging grounds (Bass and Witzell, 2000; Engstrom et al., 2002; Velez-Zuazo et al., 2008), in adult foraging grounds (Bass et al., 1998; Velez-Zuazo et al., 2008), in fisheries bycatch (Bowen et al., 1995; Laurent et al., 1998; Prosdocimi et al., 2011), and in strandings (Rankin-Baransky et al., 2001; Maffucci et al., 2006; Prosdocimi et al., 2011).

MSA studies have demonstrated the complexity of sea turtle migratory patterns that differ not only among species but also among populations within the same species, and every study reveals a unique scenario. There are regions where both green and loggerhead turtles demonstrate strong fidelity to their neritic foraging area from early recruitment (Limpus et al., 1992), while in other regions turtles switch between different developmental habitats before settling in an adult foraging ground upon reaching sexual maturity (Bjorndal et al., 2003; Godley et al., 2003; Pilcher, 2010). A recurring theme in MSA of sea turtles is the attempt to determine the mechanisms that generate the composition of turtles at mixed foraging grounds. Several hypotheses have been proposed to quantify the roles that rookery size, distance between rookeries and foraging grounds, juvenile natal homing behavior, and ocean currents play in shaping the mixture of turtles in foraging aggregations.

#### 6.4.2 FACTORS SHAPING THE COMPOSITION OF FORAGING GROUNDS

The idea that larger rookeries in a region contribute more turtles to associated feeding grounds is intuitive. Early studies using MSA showed that juvenile loggerhead turtles found in oceanic foraging aggregations around the Azores and Madeira in the eastern Atlantic originated from nesting beaches in Mexico (~10%), south Florida (~70%), and northern Florida to North Carolina (~20%) (Bolten et al., 1998). Some of these eastern Atlantic turtles also pass through the Strait of Gibraltar and enter the western Mediterranean. Here 50% (or more) of loggerhead turtles caught in pelagic drift longline fisheries have been found to originate from western Atlantic rookeries (Laurent et al., 1998; Carreras et al., 2006). Despite the long distances involved, the contributions of turtles are roughly proportional to the size of the rookeries they came from. Similar to loggerhead turtles in the Pacific (Bowen et al., 1995), immature turtles from western Atlantic rookeries forage in the eastern Atlantic and Mediterranean but eventually traverse back across the Atlantic where they recruit into coastal areas along the eastern seaboard of the United States (Bolten et al., 1998; Laurent et al., 1998). This is supported by the findings that most foraging loggerhead turtles in neritic habitats throughout the

Mediterranean originate from Mediterranean rookeries (Laurent et al., 1998; Maffucci et al., 2006), while those recruiting into neritic habitats of the southeastern United States are from local rookeries (Bass et al., 2004; Bowen et al., 2004; Reece et al., 2006). After entering neritic foraging aggregations, the stock contributions are no longer proportional to the size of the rookeries alone. Instead, foraging areas share similar haplotype profiles to nearby rookeries, suggesting that immature loggerhead turtles tend to choose foraging areas near their natal origin (Bowen et al., 2004), thus disputing the idea of random mixing (Sears et al., 1995; Witzell et al., 2002; Reece et al., 2006).

While the model of random recruitment explains how some oceanic aggregations are formed (Bolten et al., 1998), there are many studies showing contrasting patterns of dispersal. For example, juvenile green turtles foraging in east-central Florida are significantly differentiated from green turtle foraging in the Bahamas only 350 km away (Bass and Witzell, 2000). Likewise, green turtle foraging grounds along the Great Barrier Reef (GBR) in Australia show a gradual shift in foraging ground composition along a north–south transect (Jensen, 2010). Foraging areas in the southern GBR (sGBR) are dominated by turtles from nearby sGBR rookeries and northern GBR (nGBR) foraging areas are dominated by turtles with a nGBR origin. This may reflect juvenile natal homing. However, it may be more a function of geography, as it appears that posthatchling turtles do not mix in the pelagic stage to the same extent as Atlantic loggerhead turtles due to varied oceanic currents affecting the two regions (Boyle, 2007).

While rookery size and distance might explain how marine turtles are distributed across foraging grounds, the results are somewhat ambiguous as disproportionately large or small contributions from some rookeries cannot be explained by size and distance alone. Green turtles foraging around Barbados in the West Indies showed large (25%) contributions of turtles from Ascension Island, more than 5,500 km away, and substantial contributions (19%) came from the much larger rookery at Tortuguero in Costa Rica, located “only” 2,600 km away. There was also a substantial contribution (18.5%) from the distant and much smaller south Florida rookery (Luke et al., 2004). While neither distance nor size plays a major role in recruitment to the Barbados foraging aggregation, ocean currents might partly explain this scenario. Barbados is located where the North and South Equatorial Currents meet, and turtles from both Ascension Island and south Florida rookeries feed into these two major Atlantic current systems. Costa Rica, on the other hand, is affected by smaller and more local current systems that would bring fewer posthatchling turtles toward Barbados (Luke et al., 2004). Similarly, foraging loggerhead turtles in the western Mediterranean Sea are mainly derived from western Atlantic rookeries, whereas turtles in the eastern Mediterranean mainly originate from Mediterranean rookeries, thus providing a strong association between location and ocean current systems (Carreras et al., 2006). Likewise, as the South Equatorial Current approaches the east coast of Australia, it splits into the southward East Australian Current and the northward North Queensland Current, and this pattern possibly influences the strong partitioning of foraging green turtles between the nGBR and the sGBR (Jensen, 2010). The use of high-resolution ocean current data to model the movement of passively dispersing (or modeled swimming behavior) of turtles is increasing (e.g., Blumenthal et al., 2009a; Godley et al., 2010; Proietti et al., 2012). For example, a recent study showed a significant correlation between foraging compositions generated by ocean current models and those from MSA for a number of hawksbill turtle foraging aggregations throughout the Caribbean (Blumenthal et al., 2009a), highlighting the important role of ocean currents in shaping the composition of foraging areas.

### 6.4.3 DIFFERENCES BETWEEN TIME, SIZE, AND GENDER

Temporal variation in the composition of turtles at foraging grounds should be considered, given that foraging aggregations are potentially highly dynamic when composed of turtles from multiple rookeries. Seasonal movement is common in both green and loggerhead turtles along the east coast of the United States (Avens and Lohmann, 2004). Developmental migrations from strictly juvenile to adult foraging grounds is common in loggerhead turtles (Bolten et al., 1998; Bjorndal et al., 2000; McClellan and Read, 2007) but has also been reported for green (Godley et al., 2003;

Bjorndal et al., 2005; Pilcher, 2010) and hawksbill turtles (Whiting and Koch, 2006; Grossman et al., 2007; Blumenthal et al., 2009b). In other areas, juvenile and adult turtles share foraging grounds and juveniles show strong fidelity to the same area throughout their life (Limpus et al., 1992, 1994; Broderick et al., 1994). The extent to which these different patterns in the use of foraging grounds, or the specific locations of foraging grounds, are related to temporal variation in the stock composition of foraging aggregations is not well understood. Bass et al. (2004) found no temporal variation in haplotype frequency for immature loggerhead turtles at a North Carolina foraging aggregation sampled over three consecutive years. Jensen (2010) found no temporal variation in adult green turtle foraging grounds on the GBR and neither did Naro-Maciél et al. (2007) for green turtles in Brazil. Velez-Zuazo et al. (2008) found no evidence of temporal variation in a 5 year study of hawksbill turtles from Puerto Rico. The only study to report temporal variation in foraging grounds is a 12 year study from a highly dynamic foraging ground for immature green turtles in the Bahamas where haplotype frequencies from a single year was found to be significantly different from other years (Bjorndal and Bolten, 2008). However, marine turtle foraging populations are unlikely to be static. The recruitment of juveniles from several rookeries is a complex process that is affected by variation in output from rookeries, which is caused by variation in nesting numbers, natural catastrophes, predation, and human impacts as well as varying ocean currents. These changes at rookeries or in ocean currents are likely to be reflected in foraging ground compositions. Temporal variation in the composition of foraging aggregations is expected if they are comprised off turtles from a large number of rookeries, and for highly dynamic foraging aggregations where juveniles stay for a short amount of time, such as in the Bahamas (Bjorndal and Bolten, 2008).

A recent study of green turtle aggregations at six major foraging grounds, spanning a north-south transect along the entire length (~2,300 km) of the GBR, combined MSA with data from more than 30 years of mark-recapture efforts (Jensen, 2010). Overall, the MSA estimates were in agreement with estimates derived from tag returns and provided confidence in relying on point estimates from MSA. Interestingly, there were significant shifts in haplotype frequencies between juveniles and adults at the most northern foraging ground (Torres Strait), resulting in major shifts in the estimated stock contributions. Here, fewer juveniles (53%) originated from the nGBR stock in comparison to adults (89%). This trend was apparent in the four most northern foraging grounds. The observed patterns at the various foraging grounds likely resulted from several causes, the mostly likely of which were that (1) juveniles have shifted foraging grounds as they mature, especially those from distant nesting regions; or that (2) reduced hatching success from the main nGBR rookery at Raine Island for well over a decade (Limpus et al., 2000; Limpus, 2007) has resulted in reduced recruitment into the nGBR foraging ground. The latter possibility suggests a need to take action to conserve the nGBR population and highlights the direct conservation and management values of monitoring foraging areas using genetic techniques. The combined strength of data derived from mark-recapture studies, demographic studies to determine sex, maturity, and breeding status of the turtles, genetic studies to determine stock composition, and satellite telemetry, are needed to provide informed assessments of foraging populations necessary for guiding sustainable management of marine turtles.

Another confounding factor is that, foraging areas where turtles from rookeries that are female biased due to warmer incubation temperature mix with turtles from cooler more male-producing rookeries would be expected to generate different MSA estimates between males and females (see Jensen, 2010). Bass et al. (1998) found a small difference in the contribution between males and females from different rookeries at a green turtle foraging ground in Nicaragua. However, sample sizes were small (30 for each sex) and the results remain inconclusive. Sex-based dispersal remains poorly understood in marine turtles. Because marine turtles lack obvious morphological sex characteristics prior to maturity, the gonads of immature must be examined using laparoscopy (Miller and Limpus, 2003), or hormonal assays performed to determine sex (Diez and Van Dam, 2003). This compounds the logistical difficulties in sampling a sufficiently large number of both males and females, especially if sex ratios are highly skewed. As a result of these challenges most studies have been unable to analyze foraging composition by sex.

#### 6.4.4 LIMITATIONS OF MSA

MSA has provided valuable new insights into the distribution of marine turtle populations, but in many cases the estimates are affected by large uncertainty, often due to the haplotype composition of the source populations. Ideally, mtDNA haplotype frequencies would show highly significant shifts among rookeries, and the presence of unique haplotypes would make it straightforward to assign individuals to their natal rookery. However, this is typically not the case, and the occurrence of common mtDNA haplotypes that are shared among rookeries may lead to unreliable MSA results with large confidence intervals. Examples of this include the common loggerhead turtle haplotypes CC-A1 and CC-A2, that are found across western Atlantic and Mediterranean rookeries (Bowen et al., 2004; Carreras et al., 2007; Shamblin et al., 2011a), haplotypes C1 and C3 that are shared among green turtle rookeries in the Indo-Pacific (Dethmers et al., 2010), and the A and F haplotypes that dominate the Caribbean hawksbill turtle rookeries (Velez-Zuazo et al., 2008). As a result, MSA estimates may not reflect the true mixture of sea turtles in the foraging areas. One way to address this issue is to look for more resolution in the genetic markers used. As sequencing techniques have become cheaper, and more efficient, researchers are starting to sequence a longer segment of the mtDNA control region hoping to increase the resolution of the genetic marker and thereby the power of the MSA. Another important criteria for a successful MSA is the sampling of all (or most) possible source rookeries, especially when populations share widespread haplotypes. Recently, efforts have been made to expand geographic sampling and to add resolution to genetic analyses for Caribbean hawksbills by re-sequencing samples using a longer (740 bp) segment of the mtDNA control region. By doing this, rookeries that were previously indistinguishable based on old 384 bp sequences may now be differentiated (Velez-Zuazo et al., 2008; LeRoux et al., 2012).

The number of “orphan” haplotypes, those not observed at the rookeries but seen in foraging grounds, is a good indication of inadequate sampling of source populations. Medium frequencies of orphan haplotypes are often indicative of an unsampled source, while low frequencies of orphan haplotypes are indicative of either an unsampled source or insufficient sampling of already sampled rookeries. This is highlighted by a recent study of juvenile hawksbill turtles foraging around the Cape Verde Islands (Monzón-Argüello et al., 2010a). Here, all three haplotypes found ( $n = 28$ ) were orphan haplotypes not found at any rookery, highlighting obvious gaps in sampling of key rookeries. However, as more rookeries are characterized for mtDNA variation, the number of orphan haplotypes seen in foraging aggregations should decrease. These examples accentuate the importance of being critical when using MSA. Ideally, the interpretation of MSA results should use an integrated approach considering demographic, ocean current, stable isotope, mark-recapture, and/or satellite tracking data if these are available, in order to draw conclusions that are biologically meaningful.

#### 6.5 CONSERVATION AND MANAGEMENT IMPLICATIONS: A POPULATION PERSPECTIVE

One aim of many genetic studies is to inform management decisions to aid in effective conservation. This includes knowledge about which rookeries should be considered part of the same breeding population, and which function as separate populations, the amount of genetic exchange among populations, the extent of genetic variability and insights into the dynamics of population history and colonization. To focus management decisions at a population level, the term “Management Unit” has been used to signify functionally independent populations in which a loss of individuals in one population is not likely to be replaced from animals in another population within time frames relevant to management (Moritz, 1994). For example, Management Units (MUs) have been defined for green turtles (Dethmers et al., 2006; Formia et al., 2006; Bourjea et al., 2007), loggerhead turtles in the Atlantic and Mediterranean (Encalada et al., 1998; Shamblin et al., 2011a;

Yilmaz et al., 2011), leatherback turtles in the Pacific and Atlantic (Dutton et al., 2007; unpublished data), hawksbill turtles in the Indo-Pacific and Caribbean (FitzSimmons, 2010; LeRoux et al., 2012), and flatback turtles in Australia (Pittard, 2010).

Typically, the identification of MUs has been based upon significant genetic differentiation of mtDNA haplotypes (based on  $F_{ST}$  values) among rookeries (or groups of rookeries), though this approach has limitations. It is possible to have relatively low gene flow between two populations that is sufficient to prevent genetic divergence, yet low enough that the populations function as demographically independent populations. In this context, Palsbøll et al. (2007) suggest setting a level of <10% migration per generation to define MUs, which could be assessed by genetic studies or through tagging data. Genetic studies may have inherent limitations though. For example some rookeries may be functioning independently, but because of recent colonization, not yet appear differentiated based on the genetic markers being used. In such cases it becomes more important to have field data to identify populations, for example, having tagging data that show a lack of exchange of individuals between rookeries over decades, or data on differences in the timing of nesting (summer and winter), as were used to identify separate hawksbill populations in Australia (Limpus, 2009). Additionally, there is some evidence of temporal variation in the mtDNA haplotype frequencies of turtles nesting in different years in some populations studied (Shamblin et al., 2011a) but not in others (Hatase et al., 2002; Bjørndal et al., 2005; Formia et al., 2007; Velez-Zuazo et al., 2008; Jensen, 2010), thus robust sampling designs may need to include samples collected across years for the identification of MUs.

The importance of male-mediated gene flow is limited when defining MUs for sea turtles. While the nuclear exchange of genes is crucial to genetic diversity in a population, no amount of male-mediated gene flow will bring back a breeding population if the rookeries go extinct. Thus, male-mediated gene flow needs to be considered relative to the extent of genetic divergence among populations as indicated by mtDNA markers. For example, the indication of substantial male-mediated gene flow between northern and southern GBR green turtle populations is less relevant given a high degree of mtDNA differentiation ( $F_{ST}=0.8$ ; FitzSimmons et al., 1997b) which demonstrates little exchange of females between the rookeries in this region. In contrast, mtDNA and microsatellite data on loggerhead populations in Turkey (Yilmaz et al., 2011) suggest a metapopulation structure among some rookeries due to inconsistent mtDNA haplotype differentiation among pairs of rookeries, and strong male-mediated gene flow at microsatellite loci among all areas.

Because sea turtles migrate long distances at various times throughout their life, they often occupy habitats under the authority of multiple countries and may spend a considerable amount of time in international waters. Nations that host sea turtle populations at either nesting and/or foraging habitats have legal jurisdiction over animals that also spend parts of their lives within the borders of other nations. The use of MSA is therefore an extremely important tool for providing information that can help provide information for setting up international agreements for effective management of sea turtles, taking into account the trans-boundary nature of populations (Dutton and Squires, 2011). From a management perspective, MSA provides an important tool for identifying threatened sea turtle populations away from the breeding grounds. For example, MSA has been used to show that 50% of loggerhead turtles caught in some Mediterranean fisheries originated from rookeries in the southeastern United States (Laurent et al., 1998). In the North Pacific, MSA studies have shown that loggerheads encountered as fisheries bycatch on the high seas and foraging grounds off the coast of Baja California, Mexico, all originate from the rookeries in Japan (Bowen et al., 1995; Dutton et al., unpublished data). The Caribbean highlights the complexity of management, because turtles reside and migrate through habitats within multiple countries. MSA studies of foraging ground composition show that green turtles (Luke et al., 2004; Bjørndal and Bolten, 2008), loggerhead turtles (Engstrom et al., 2002) and hawksbill turtles (Bass, 1999; Velez-Zuazo et al., 2008; Browne et al., 2009) all cross international borders when migrating between foraging and nesting grounds in this region.

As MSA estimates get more precise, they may provide an effective means of monitoring trends at oceanic and coastal foraging grounds for all size classes and genders. Comparing the origin of adult turtles to that of juvenile turtles that have recently recruited into benthic foraging areas will make it possible to detect early signs of changing contributions which may indicate population decline or increase at the nesting beaches. In recent years, the potential effects of climate change on sea turtle populations have become an increasing concern (Hamann et al., 2007; Fuentes et al., 2009). Climate change might vary the carrying capacity of foraging grounds, alter the currents that transport juveniles to those foraging grounds (Fuentes et al., 2009), and will likely affect sex ratios in some turtle populations (Hamann et al., 2007; Fuentes et al., 2010). In all cases, long-term monitoring of the composition of foraging grounds may provide an effective way of detecting significant population changes as well as identifying female- and/or male-producing rookeries. Overall, the growing impact of conservation genetics will allow for more precise conservation decisions to be made at both regional and global scales for sea turtles.

## 6.6 MATING SYSTEMS

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Mating systems influence demographic processes but are difficult to observe for sea turtles and this is one area where genetic approaches have been particularly informative. Multiple paternity has been examined using microsatellite markers and has been documented in loggerhead (e.g., Zbinden et al., 2007), olive ridley (Hoekert et al., 2002; Jensen et al., 2006), Kemp's ridley (Kichler et al., 1999), green (e.g., FitzSimmons, 1998; Ireland et al., 2003), leatherback (Crim et al., 2002; Stewart and Dutton, 2011), hawksbill (Joseph and Shaw, 2010), and flatback (Theissinger et al., 2009) turtles. The extent of multiple paternity has ranged from very low values (FitzSimmons, 1998) to over 90% (Jensen et al., 2006; Zbinden et al., 2007) in some populations. The extent to which multiple fathers contribute to clutches varies considerably among studies, with low levels of contributions from secondary males (FitzSimmons, 1998; Hoekert et al., 2002; Lee and Hays, 2004) to equal contributions (Zbinden et al., 2007). However, in several studies, primary and secondary fathers contributed to all clutches within a season for a particular female, indicating that sperm storage had occurred regardless of the proportions of sperm (Stewart and Dutton, 2011). One study investigated paternity relative to the order of egg deposition in two multiply-sired clutches of green turtles (Lara-De La Cruz et al., 2010), and the data suggest that sperm from different males is mixed within the oviduct and that fertilization may function as a raffle system. It has been proposed that marine turtles, unlike most birds, may have a first male sperm precedence for fertilization (FitzSimmons, 1998), but this has not yet been tested. There is genetic evidence to support field observations (Limpus, 1993) that the marine turtle mating system is promiscuous, as (inferred) individual male genotypes have been observed in the offspring of more than one female (Crim et al., 2002). Studies that analyzed successive clutches have not found evidence of successful mating by "new" males between clutches (Stewart and Dutton, 2011), although variation in male success across clutches does occur (Theissinger et al., 2009).

While there are many theoretical explanations for multiple paternity (such as increased offspring fitness, ensuring fertilization, male coercion), Lee and Hays (2004) suggest that it may be driven by male density and avoidance of aggressive mating behavior. In fact, Jensen et al. (2006) found higher levels of multiple paternity in mass-nesting olive ridley populations (90%) than in solitary nesters (30%), indicating the role of density and/or adult sex ratio. Few studies have been able to test for a relationship between the extent of multiple paternity and female characteristics (e.g., size), clutch size, and hatching success or hatchling fitness. A positive correlation was found between the number of fathers and female body size among clutches of 15 loggerhead females, and limited evidence supported a relationship between hatching success and the level of multiple paternity (Zbinden et al., 2007). But this was not found in green turtles (18 females), nor was there any relationship between the presence or absence of multiple paternity to clutch size or clutch success (Lee and Hays, 2004). Several studies suffer from small sample sizes, in terms of the number of females, number of

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offspring, or the number of loci analyzed. The importance of designing an experimental assay that has sufficient power to detect multiple paternity was illustrated when initial results based on small sample sizes that failed to detect multiple paternity in leatherbacks, were later overturned by a study of the same population that found 42% of clutches had multiple paternity when over 1000 hatchlings from successive clutches of 12 known nesting leatherbacks were analyzed at 7 microsatellite loci (Stewart and Dutton, 2011).

## 6.7 POPULATION VITAL PARAMETERS

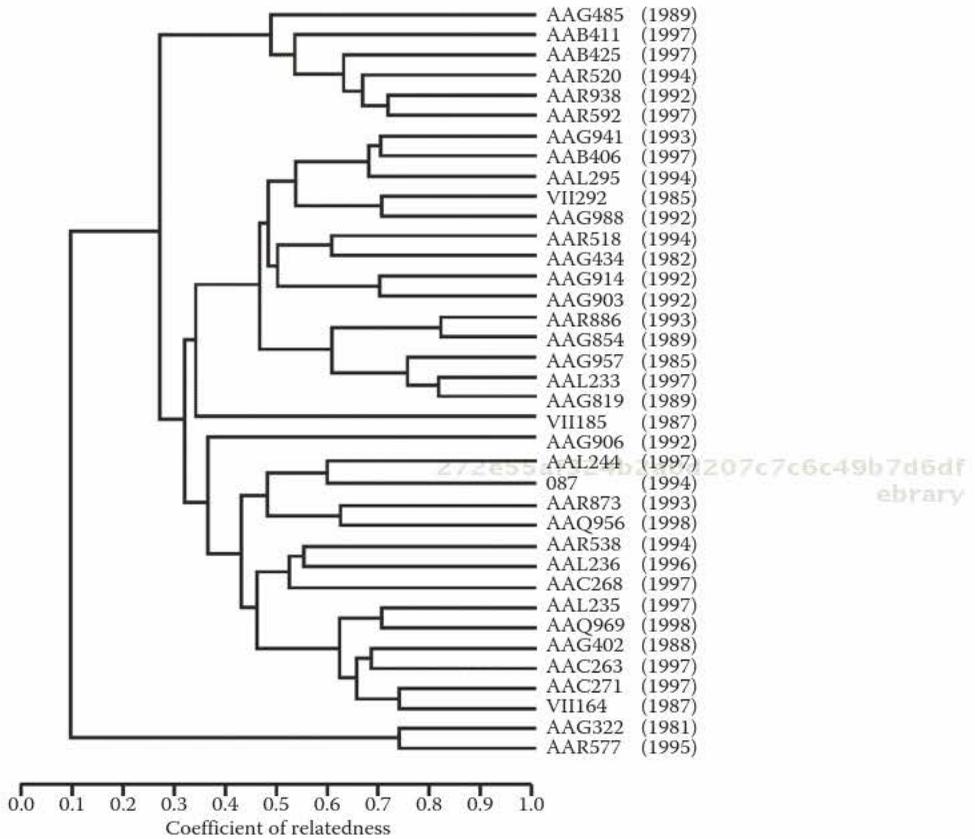
Vital parameters such as age to maturity, survival, sex ratios, and population size (including males) are still lacking for most sea turtle populations, and this has made it difficult to conduct meaningful population risk assessments (NRC, 2010). Although vital parameters are difficult to observe directly, genetic analysis provides a practical approach to understand these processes. The ever-increasing number of informative microsatellite loci and improvements in field sampling methods will facilitate expansion of paternity studies and form the basis for other new areas of study. For instance, by comparing genotypes of hatchlings with that of the mothers, it is possible to infer the male genotypes in the breeding population and make progress on tackling some previously elusive population vital rates.

### 6.7.1 SEX RATIOS OF BREEDING POPULATIONS

Little is known about adult breeding sex ratios, known as the operational sex ratio (OSR), in sea turtles (Hays et al., 2010). Most research on sex ratios has focused on hatchling and juvenile stages in sea turtles, and there has been concern, prompted by general findings of female-biased hatchling sex ratios, that populations of turtles may become entirely feminized due to warming climate trends and temperature-dependent sex determination. Stewart and Dutton (2011) used kinship analysis to obtain the genotypes of successfully breeding males in a leatherback population without ever encountering them in the field. They assessed hatchlings belonging to 46 female leatherbacks and found that 47 different males had mated with those females (Stewart and Dutton, 2011). One male had mated with three different females, and several others had mated with two females. Using a similar approach, Wright et al. (2012) found that for a green turtle population, despite having a 95% female hatchling sex ratio, there were at least 1.4 reproductive males to every breeding female. These studies show that OSRs may not necessarily be female biased as feared and that breeding males may outnumber breeding females in encounter rates at breeding grounds. Stewart and Dutton (2011) identified one male that had been actively breeding in both 2009 and 2010 (with different females), providing evidence that some males may breed yearly, as also observed in green turtles (Limpus, 1993). Expansion of these studies across multiple years to account for male breeding behavior will be required to accurately estimate the number of breeding males in the population.

### 6.7.2 AGE TO FIRST REPRODUCTION

The age at first reproduction is one of the most important vital parameters for demographic modeling, and is uncertain for many sea turtle populations, because it has not been possible to easily tag hatchlings and monitor at what age they reach maturity. In leatherbacks for instance, estimates from chondro-osseous morphology, skeletochronology, and growth rate modeling have suggested a range from 3 to 29 years for the age of first reproduction (Rhodin, 1985; Zug and Parham, 1996; Avens et al., 2009; Jones et al., 2011). Dutton et al. (2005) inferred age of first reproduction at around 12–15 years from analysis of demographic trend data, generally corroborating the more recent estimates of 13–16 years proposed by Jones et al. (2011). Genetic fingerprinting was also used to show that first-time nesters in the 1990s were closely related and possibly the genetic offspring of leatherbacks nesting in the 1980s (Dutton et al., 2005) (Figure 6.4).



**FIGURE 6.4** Family groups identified among 37 St. Croix leatherback nesters based on relatedness determined with microsatellite genotyping and mtDNA sequencing. The year the turtle was first observed to nest is given in brackets; old-timers, such as AAG322 (identified in 1981) and AAG434 (identified in 1982) are most likely mothers of recent (post 1993) first-time nesters such as AAR577 (1995) and AAR518 (1994), respectively. (From Dutton, D.L. et al., *Biol. Conserv.*, 126, 186, 2005.)

A new approach uses genetic analyses to “tag” hatchlings for a long-term capture-mark-recapture study, using non-injurious sampling methods established for collecting hatchling DNA, which will be used to create a genetic fingerprint or “tag” to identify individual turtles throughout their lifetime (Dutton et al., 2008; Stewart and Dutton, 2012). Genetic samples routinely collected from first-time nesters in future years will be analyzed and compared to the stored hatchling genotypes to identify the individuals that were originally “tagged” at birth and directly determine age at first reproduction and juvenile survival rates for this population by following a cohort of hatchlings to adulthood. Age-specific vital rates of adult females, such as birth and death rates, also may also be estimated by monitoring these cohorts through their lifetimes, providing crucial information for future studies of the species. Given the rapid advances occurring in biotechnology and information management systems, it should be possible to expand the use of genetic fingerprinting in a broad range of Capture-Mark-Recapture applications in the future.

### 6.8 FUTURE DIRECTIONS

There is a growing need for genetic tools to test for finer resolution in genetic structure, based on what is known from field data. Recent efforts to uncover additional genetic structure among rookeries using mtDNA have taken two contrasting directions. In one approach, Shamblin et al. (2012b)

sequenced a majority (16,350 bp) of the mtDNA genome to uncover genetic structure among green turtle populations in the Greater Caribbean that were dominated by a common haplotype (CM-A5). This was done by selectively sequencing 20 individuals with the CM-A5 haplotype to determine if there were sequence variants among them. Four variants of CM-A5 were found that were geographically structured, resulting in higher  $F_{ST}$  values for three eastern Caribbean rookeries. In contrast, Tikochinski et al. (2012) developed primers to amplify a known microsatellite locus found on the 3' end of the mtDNA control region to look for cryptic genetic structure in green turtle rookeries in the Mediterranean, in which there was little variation. Microsatellite repeats are a common feature of the mtDNA control region in several species and have been used in phylogenetic and population genetic studies, although it can be difficult to obtain reliable results (Lunt et al., 1998). Within the nesting and stranded green turtles studied ( $n=289$ ), sequencing of the microsatellite region yielded 33 haplotypes and repeated sampling of the same individuals ( $n=20$ ) gave identical sequences. Both approaches offer promise as techniques to investigate whether there is phylogeographic structure among individuals that share widespread haplotypes.

Rapidly evolving techniques to develop genome-wide markers are likely to lead to a future shift in the genetic markers of choice for some studies. SNPs have been identified within the genome of green turtles (Roden et al., 2009a,b). These SNPs are useful for detecting population structure in green turtles (Roden, 2009), although initial cross-species tests have had limited success, suggesting that SNP markers will need to be developed for each species of sea turtles (Dutton et al., unpublished data; Quinzin et al., unpublished data). Nevertheless, SNPs show great promise for ultimately replacing microsatellites due to their higher data quality and genotyping efficiency (Morin et al., 2004). Currently, next-generation sequencing (NGS) platforms are being used for sea turtle nuclear SNP discovery and have the potential for identifying large numbers of new SNPs as the process becomes significantly more automated and less labor-intensive than traditional PCR and Sanger sequencing (Nielsen et al., 2011). One of the main challenges of these advances in the application of NGS technologies will be dealing with the vast quantity of data generated. Informatics and statistical methods for managing and applying results have not kept pace with the rapidly evolving technologies, and some of the basic analytical approaches for determining stock structure and other aspects of conservation genetics will need to be further developed.

Advances in sampling techniques have also improved the capacity for genetic studies. For studies of hatchlings, sampling of DNA from a sliver off the carapace of hatchlings (e.g., Theissinger et al., 2009) rather than taking blood samples has allowed a less invasive, easier and much quicker technique. To individually identify females nesting at beaches that are not monitored at night, Shamblin et al. (2011b) developed the technique of getting the mothers' DNA from the eggshells of recently (<15 h) deposited eggs to conduct microsatellite analyses. This could be an important tool for determining the number of clutches being laid by females, or the total number of nesting females at beaches where it is not possible to encounter females while nesting.

Although the number of genetic studies has increased dramatically over the past three decades (Figure 6.1), and some may be inclined to think there are a plethora of studies, there remains a great deal of genetic research to be done on sea turtles to contribute to their conservation. This includes genetic studies of turtle diseases (Quackenbush et al., 2001) or commensals (Rawson et al., 2003), mechanisms of sex determination (Torres Maldonado et al., 2002), or molecular evolution of turtle DNA (Russell and Beckenbach, 2008) as well as the topics discussed above. Ultimately, the contribution of genetic studies to our understanding of marine turtles will continue well into the future.

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