Research and Management Techniques for the Conservation of Sea Turtles
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Population Identification

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In the last decade genetic techniques have illuminated several aspects of marine turtle life history. For example, do female turtles return to nest on their natal beach? Do males provide an avenue for gene flow between nesting colonies? Does more than one male contribute to a clutch? What are the evolutionary relationships among sea turtle species? Can DNA "fingerprints" be used to trace marine turtle migrations? All these questions have yielded to molecular genetic studies in recent years (reviewed by Bowen and Avise, 1995; Bowen and Karl, 1996).

While all aspects of natural history are relevant to conservation, perhaps the most powerful genetic tools for marine turtle management are those which can identify discrete breeding populations on the nesting beaches and in corresponding feeding habitats. Resolution of populations (or stocks) in marine turtles is confounded by the extensive migrations made by most species as juveniles and as breeding adults. These migrations highlight the need to identify the geographic range of feeding habitats that support a specific breeding population and, conversely, to assess proportions of different breeding populations present in a particular feeding ground or harvest.

This chapter reviews the practical framework for using genetic information to identify breeding populations of marine turtles. Two fundamental themes underlie our discussion: (i) proper use of genetic information requires that the goals of the study are unambiguous and that the appropriate sampling design and molecular markers are employed; and (ii) molecular data are most informative when integrated with field studies, especially tag-recapture studies.

This chapter provides a brief description of the molecular approaches and protocols for sampling (Appendix 1), but not for the individual genetic methods. The latter are detailed in Hillis *et al.* (1996) and their applications to marine turtles are reviewed in Bowen and Witzell (1996) and Bowen and Karl (1996). For a discussion on the identification of breeding populations and evolutionary units see Moritz *et al.* (1995). For a description of population genetic processes, see Hartl and Clark (1997).

Choice of Molecular Markers

Mitochondrial DNA (mtDNA) has proved particularly effective for detecting population structure in marine turtles. The resolving power of mtDNA assays is technique-dependant; several studies have reported enhanced population discrimination using the rapidly evolving control region rather than whole-genome restriction fragment (RFLP) analysis (Table 1). For this reason, the control region is recognized as the mtDNA segment of choice for nesting beach surveys. The general conclusion from these surveys is that female turtles typically return to their region of origin to breed (natal homing behavior) but that breeding populations may encompass several adjacent nest-

Table 1. Molecular markers used to identify marine turtle populations

Marker	Inheritance	Population Variation[1] within/among
Nuclear genome		
protein electrophoresis	biparental	low/low
anonymous single-copy	biparental	low/low
microsatellites	biparental	high/low-moderate
Mitochondrial genome		
restriction fragments	maternal	low/low-high
control region sequences	maternal	low-high/moderate-high

[1] Relative variation within and among regional assemblages of rookeries.

Note: For more complete reviews, see Bowen and Karl (1996), Bowen and Witzell (1996).

ing habitats, separated by as much as 100-400 km (Norman, 1996; Bowen and Avise, 1995).

The mtDNA molecule is maternally transmitted, meaning that male offspring inherit their mother's mtDNA but do not pass it on to subsequent generations. In many circumstances, female-inherited markers offer a distinct advantage because they provide perspectives on female reproductive behaviors that are paramount to species survival (Bowen and Avise, 1995). On the other hand, mtDNA does not yield a complete picture, and can prompt a misleading interpretation of isolation between populations if there is some form of male-mediated gene flow, as is likely for green turtles (Karl *et al.,* 1992; FitzSimmons *et al.*, 1997a,b). For this reason, studies of nuclear DNA variation are highly desirable to complement mtDNA studies and to provide a more complete understanding of population genetic structure.

Population studies of nuclear DNA typically use segments of the genome that do not code for specific protein products. These non-coding regions accumulate mutations more rapidly than protein coding regions, and thereby provide greater sensitivity (Table 1). The nuclear DNA segments that are appropriate for sea turtle population studies include anonymous single copy nuclear DNA (ascnDNA; Karl *et al.*, 1992), minisatellites (Peare and Parker, 1996), and microsatellites (FitzSimmons *et al.*, 1997a). Minisatellite and microsatellite techniques, popularly known as DNA fingerprinting, have also been used to assess pedigrees and the possibility of multiple paternity in marine turtle nests (FitzSimmons, 1998). The latter approach is gaining acceptance as a standard tool in conservation genetics, and may be widely used for population studies of marine turtles in the next decade. The array of such nuclear DNA technologies is rapidly developing, so it is likely that additional assays will become available in the future, including direct sequencing of nuclear DNA segments (Karl, 1996).

Glossary of Genetic Terms

mtDNA-mitochondrial DNA in turtles is passed from the mother to her offspring, and from her female offspring to the next generation. Variants are typically called haplotypes, and when several haplotypes are present among populations, information is revealed about the structure of female lineages.

nDNA-nuclear DNA is inherited from both parents and thus studies using nuclear markers provide information about gene flow among populations as influenced by both females and males.

ascnDNA-anonymous single copy nuclear loci. These are unique (i.e., single copy) regions of nuclear DNA that can be useful genetic markers in marine turtles due to mutation events that have generated multiple alleles (Karl et al., 1992).

Microsatellite loci- regions of nuclear DNA defined by the presence of a repetitive segment of DNA in which the repeated unit is 1-6 base pairs long. These regions have high mutation rates that generate alleles of different lengths which can be useful as genetic markers for fine scale population resolution and pedigree studies.

Restriction Length Fragment Polymorphism(RLFP)- Digestion of a segment of DNA (or the whole mtDNA genome) by restriction enzymes produces fragments of particular lengths depending upon the location of restriction sites (e.g., the *Mse*I enzyme cuts at all 'TTAA' sites). A mutation at a restriction site would prevent enzyme digestion, thus different fragment lengths would be generated.

Stock Assessment of Nesting Populations

In interpreting the distribution of genetic variation, researchers are essentially using a one-way test. If significant divergence is observed between nesting populations, then we can infer that gene flow is low and that nesting cohorts constitute isolated breeding populations. However, the converse conclusion does not invariably hold. If genotype frequencies are not significantly different between two nesting areas, then we cannot be certain that these sample sites are united in a single, random mating population. This may be the case, but there are three reasons why it may not be. First, it could be that the test lacked statistical power because of small sample size (Baverstock and Moritz, 1996). Second, it could be that the populations have only diverged recently and genetic differences have not yet accumulated. Third, relatively few migrants *(e.g.*, 10 per generation or less) are sufficient to homogenize allele frequencies, yet 10 migrants per generation would have an insignificant impact on demographic processes in most nesting populations. Thus, rookeries that are genetically homogeneous could still effectively be demographically independent.

Stock Assessment in Feeding Grounds and Harvests

The finding of genetic differences between nesting populations makes it possible to determine which rookeries contribute to a particular feeding area or harvest. For example, loggerhead turtle samples from the two primary nesting areas in the Pacific Ocean, southern Japan and Queensland, Australia, are characterized by a fixed difference in control region sequences. Hence every loggerhead in the Pacific region carries a natural mtDNA tag which indicates country of origin with a high degree of confidence. These markers have been used to determine which nesting colonies are impacted by loggerhead turtle mortality in drift net fisheries (Bowen *et al.,* 1995). This approach, known as mixed stock analysis is now being used to assess stock composition in a variety of harvests and feeding grounds for several marine turtle species (Broderick and Moritz, 1996; Bowen *et al.*, 1995). The power of this approach, however, depends upon the extent to which all the potentially contributing stocks have been characterized. This requires a comprehensive sampling of regional nesting populations, a process that is now well underway for most species of marine turtle. However, even without complete coverage it may be possible to provide qualitative advice on which breeding populations are represented in migratory pathways and feeding habitats. We expect this application will be a significant management tool.

Sampling Strategies and Sample Size

Molecular genetic studies have been revolutionized by PCR technology, which allows amplification of specific genes from minute amounts of DNA. Prior to the advent of PCR technology, genetic analyses required fresh or frozen tissues, a considerable logistical handicap when the study organism occupies isolated tropical habitats far from the nearest laboratory. With PCR methodology, tissues can be stored for extended periods without refrigeration (Appendix 1). Partially degraded tissues, such as might be obtained from dead turtles, cooked meat, or processed turtle products, can often be analyzed.

PCR-based methods require specific primers, short pieces of synthetic DNA, to direct the enzyme-mediated reaction. Several such primers have now been developed that work on nuclear DNA and mtDNA from most or all species of marine turtle (Table 2). One of the commendable features of marine turtle population studies has been that most labs have used the same sets of primers, allowing direct comparisons of genetic information at homologous loci across the range of globally distributed species. We hope that this trend will be continued.

What constitutes an adequate sample size? The answer depends on the technique, level of underlying genetic difference, and the question under consideration. To define reproductive populations with mtDNA, the minimum sample size for statistical comparisons is 6-8 *where there are strong differences*, although N = 20 is recommended for most population assessments. If mtDNA data from the rookeries is intended as a basis for feeding ground assessments, then samples of N > 30 may be desirable to obtain more accurate estimates of allele frequencies. For nuclear DNA surveys of nesting populations, particularly with microsatellites, larger population samples (N = 30-50) are desirable because of the greater numbers of alleles detected. To establish the geographic scale of a breeding population, a hierarchical sampling scheme is appropriate, wherein samples encompass multiple nesting habitats within a region *(e.g.*, a few hundred kilimeters), and then multiple regions separated by hundreds to thousands of kilimeters.

Sample sizes from the feeding grounds or harvests (for mixed stock assessment) depend on the number of candidate source populations and the level of differentiation between nesting colonies (Broderick and Moritz, 1996). A typical feeding ground sample should include at least 100 individuals (although a smaller sample may be informative in a qualitative sense) and it may be appropriate to stratify samples according to age, sex, and year. Samples of N>100 are justified when several candidate rookeries may contribute cohorts or there are large numbers of alleles, as may be the case for microsatellites (see Chapman, 1996). Pilot studies combined with simulations of maximum likelihood estimates *(e.g.*, Broderick and Moritz, 1996) are important to assess (i) whether the questions posed are answerable within logistic constraints, and (ii)what sample sizes will be required.

Synergy between Genetic Surveys and Tagging Studies

We have tried to summarize the major strengths and limitations of molecular data for stock assessment. From the above, it should be obvious that we do not regard genetic assays as a quick fix or panacea for population identification. Yet, with appropriate sampling and integration with ecological studies (see below), these methods can provide valuable insights.

Genetic data and information from tag returns can interact in three ways. First, tagging studies generate hypotheses about migration patterns that are testable with genetic data. In several sea turtle species, hypotheses about the reproductive migrations of sea turtles, formulated on the basis of tag-recapture studies, have been evaluated with genetic surveys (Bowen *et al.*, 1992, 1994; Broderick and Moritz, 1996; FitzSimmons, 1997a). Second, tagging data can be used to test whether nesting populations that appear to be united by extensive gene flow (based on genetic data) also show frequent exchange of nesting females on a contemporary scale. For example, recapture data confirm frequent exchange of female turtles among adjacent nesting habitats that are genetically homogeneous (Limpus *et al.*, 1992; Norman, 1996). Third, molecular data can provide novel perspectives that can be tested subsequently through tagging programs. For example, genetic data may indicate that a breeding population extends beyond the borders of intensive tagging studies — this inference can be tested by extending mark-recapture across a broader geographic scale. Finally, genetic data may demonstrate rare long-distance colonization events which are difficult to document by tagging alone (Bowen *et al.*, 1992, 1994; Dutton, 1995).

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APPENDIX 1.
Sampling Protocols for Genetic Analysis via PCR

Nesting females, hatchlings, and turtles captured at sea can be safely sampled for blood or tissue, taking care to avoid infection of individuals or cross-contamination of samples. For live animals the surface where blood or tissue will be removed should be cleaned with a detergent solution, 70% ethanol, or isopropanol. Instruments must be cleaned thoroughly between successive sampling (or discarded), and sample tubes should be new (not reused), clean and clearly labeled.

Collection of Blood

Blood usually is removed from the dorsal cervical sinuses on either side of the vertebral column in the neck, following the protocol of Dutton (1996). In adult turtles this sinus may be 1-3 cm. below the surface of the skin. Sampling is easier if the animal is positioned at a slight angle to enhance blood flow to the head region, and the head is pulled to stretch and relax the neck muscles. Although blood sampling is a simple and robust technique, there are some cautions. First, this technique should not be attempted by inexperienced personnel, as errors could lead to damage of blood vessels or nerve tissue in the vertebral column, especially in hatchlings. Second, obtaining blood from nesting females is limited to the egg laying interval (or as she returns to the sea) and it may be difficult if her head is uphill and blood flow is reduced. For leatherback turtles, blood can alternatively be obtained from the rear flippers (Dutton, 1996).

Materials

- Lysis buffer: 100 mM Tris-HCl, pH 8; 100 mM EDTA, pH 8, 10 mM NaCl; 1.0% (w/v) SDS (sodium dodecyl sulfate)
- needle and syringe (or vacutainer apparatus) without anticoagulant treatment.
- labeled screw-cap tubes or other sealed storage containers

Methods

1. Collect blood in a new syringe as described in Dutton (1996), using a new needle for each sample. The amount of blood taken and needle size should be scaled to the size of the turtle: *i.e.,* for adults take 0.5-1.0 ml blood using a 20-22 gauge x 38mm needle, and for hatchlings take 0.02-0.1 ml blood using a 28-30 gauge 12.7mm needle. For leatherbacks an 18 gauge x 76mm needle is recommended (Dutton, 1996).
2. Add blood immediately to a labeled tube with lysis buffer: approx 1:10 ratio blood to lysis buffer.
3. Gently invert the tube several times to mix ingredients.
4. Samples can be stored at room temperature for at least 1 year. Avoid exposure to heat or sunlight.

Note: Lysis buffer is nontoxic and can be stored for extended periods at room temperature.

Collection of Other Tissues

Tissue samples of 0.1-0.2 gram may be removed without risk to an adult animal, provided that sterile techniques are observed. Dutton (1996) recommends removing tissue plugs from the dorsal surface of the rear flippers, and other researchers have obtained good results from skin samples ($<1cm^2$) taken with a scalpel or biopsy tool from the neck/shoulder region . If sampling hatchlings, it is also possible to get reliable DNA samples from a small notch (2mm) removed from the trailing edge of the carapace with a scalpel blade (FitzSimmons, unpubl. data). In collecting samples from dead animals, we recommend taking muscle tissue from underneath the skin. Tissues that have been previously frozen are acceptable. Dried tissues and even bone may also work.

If eggs are the source of tissue, either the entire embryo or a sample of soft tissues from advanced embryos may be preserved. For very young embryos, the blastula or developing embryo can be used. If freshly-laid eggs are collected, we recommend allowing the eggs to develop for a few days until a blastula can be identified. If this is not possible, then a portion of the yolk membranes may provide sufficient DNA.

Materials

- DMSO preservative solution: 20% DMSO (dimethyl sulfoxide) in water saturated with salt (NaCl).
- Labeled screw-cap tubes or other sealed storage containers
- Razor blade, scalpel, or biopsy punch
- Disposable gloves (recommended)

Methods

1. Collect a tissue as appropriate. Clean all instruments thoroughly between sample collections to avoid cross-contamination of samples.
2. Chop the tissue a few times with a razor blade to increase penetration of buffer.
3. Add tissue to labeled tube with DMSO solution. The tissue/buffer ration should be between 1:5 and 1:10.
4. Samples can be stored at room temperature for at least a year. Avoid exposure to heat or sunlight.

To make one liter of saturated salt/DMSO solution:

1. Add NaCl (about 200 g) to 750 ml of distilled water, until salt no longer dissolves.
2. Add 200 ml DMSO.
3. Add distilled water as needed to make up a 1 litre volume. The presence of precipitated salt indicates a saturated solution.

Note: Care should be taken in handling DMSO because it soaks rapidly into skin and can be an irritant to the skin, eyes, and respiratory system. The saturated salt/DMSO solution is nonflammable, and can be stored indefinitely at room temperature. Some salt may come out of solution during storage. This does not indicate that the preservative has expired.

Alternatives

Tissues can be stored successfully in 70-95% ethanol, or a similar concentration of isopropanol, rather than DMSO. In the absence of other preservatives, samples can be cut into small (< 0.5 cm) pieces and packed in salt. Sun-dried material may also work.

Sampling and Project Design

Nesting Colonies

For nesting colonies, care should be taken to collect only one sample from a given female. This may constitute a blood sample from the nesting female, or a single egg or hatchling sample from a nest. Since females typically lay more than one nest per season, samples should all be taken within a re-nesting interval; *i.e.*, within two weeks, or females should be tagged to prevent repeat sampling.

Pedigree and Multiple Paternity

For analyses of pedigrees or multiple paternity, a pilot project is recommended which would include sampling 10-20 offspring per single clutch from 5-10 females. More extensive sampling might include 10-20 females and up to 50% of the offspring in a clutch, including unhatched embryos, and multiple clutches from individual females (FitzSimmons, 1998).

Feeding Ground Samples

Turtles captured at sea should be sampled following the blood or tissue protocols, the size and sex recorded, and tagged prior to release. This will diminish the possibility of re-sampling the same animal, and may provide important recapture data to corroborate findings based on genetic markers.

Table 2. Primers used for amplification of DNA sequences in marine turtles.

Primer Approx.	Sequence 5'-3'	Species[1]							Approx. Length (bp)
		Cc	Cm	Dc	Ei	Lk	Lo	Nd	
mtDNA control region									
TCR5[2]	TTGTACATCTACTTATTTACCAC	++	++	++	++	+	+	++	380
TCR6[2]	CAAGTAAAACTACCGTATGCC								
LTCM1[3]	CCCAAAACCGGAATCCTAT	-	++	-	-	-	-	-	510
HDCM1[3]	AGTGAAATGACATAGGACATA								
scnDNA[4]									
Cm-12R	AGCTGAAGCCAATGAAGAAGAA	+-	++	—	+-	+-	+-	+-	1380
Cm-12L	GCTCAGGTTTAGCTCGAAGGT								
Cm-14R	TAAGCATTATACGTCACGGA	+-	++	—	+-	+-	+-	+	930
Cm-14L	AGTATTTGGGCAGAACAGAA								
Cm28R	TAAATGCCAGGTATGTAACTC	+-	+-	+-	+-	+-	+-	+-	1400
Cm28L	GATTGCTGGTCTCTGGAAGGCT								
Cm-39R	TGCTAGTTTTGTTAGTTCTGGT	+	++	—	+	+	+	+	1350
Cm-39L	ATAGTGGATTGGAGAAGTTGTT								
Cm-45R	CTGAAAGTGTTGTTGAATCCAT	+-	++	+-	+-	+-	+-	+-	1000
Cm-45L	CCGCAAGCAAAACATTCTCT								
Cm-67R	GAATATAAGATTTCATACCCCA	-	++	-	-	-	-	-	1160
Cm-67L	TTTAATTCTGAAAACTGCTCTT								
microsatellite									
Cc7-F[5]	TGCATTGCTTGACCAATTAGTGAG	++	—	-	-	-	-	++	180-190
Cc7-R[5]	ACATGTATAGTTGAGGAGCAAGTG								
Cc117-F[6]	TCTTTAACGTATCTCCTGTAGCTC	++	++	++	++	-	++	++	210-270
Cc117-R[6]	CAGTAGTGTCAGTTCATTGTTTCA								
Cc141-F[7]	CAGCAGGCTGTCAGTTCTCCA	++	—	-	-	-	-	+-	180-210
Cc141-R[7]	TAGTACGTCTGGCCTGACTTTC								
Cm3-F[6]	AATACTACCATGAGATGGGATGTG	+-	++	++	++	-	+-	++	140-200
Cm3-R[6]	ATTCTTTTCTCCATAAACAAGGCC								
Cm58-F[6]	GCCTGCAGTACACTCGGTATTTAT	+-	++	++	++	-	+-	++	120-150
Cm58-R[6]	TCAATGAAAGTGACAGGATGTACC								
Cm72-F[6]	CTATAAGGAGAAAGCGTTAAGACA	++	++	+-	++	-	++	++	230-300
Cm72-R[6]	CCAAATTAGGATTACACAGCCAAC								
Cm84-F[6]	TGTTTTGACATTAGTCCAGGATTG	++	++	++	++	-	++	++	310-370
Cm84-R[6]	ATTGTTATAGCCTATTGTTCAGGA								
Ei8-F[6]	ATATGATTAGGCAAGGCTCTCAAC	++	+-	++	-	++	++	++	170-250
Ei8-R[6]	AATCTTGAGATTGGCTTAGAAATC								
DC99[8]	CACCCATTTTTTCCCATTG	-	-	++	-	-	-	-	120-140
	ATTTGAGCATAAGTTTTCGTGG								

[1] + amplifies, unknown variability, +- amplifies, invariant, ++ amplifies and is variable, - unknown, —no amplification

[2] Norman et al. 1994

[3] Allard et al. 1994

[4] Karl et al. 1992, Karl 1996

[5] FitzSimmons 1998

[6] FitzSimmons et. al. 1995

[7] FitzSimmons et. al. 1996

[8] Dutton 1995