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Table of Contents

Introduction: Sea Turtle Conservation Genetics
B. W. Bowen and W. N. Witzell.....1

Literature on marine turtle population structure, molecular evolution, conservation genetics, and related topics
B. W. Bowen.....9

Methods for collection and preservation of samples for sea turtle genetic studies
P. H. Dutton.....17

Comparative genetic structure of green, loggerhead, and flatback populations in Australia based on variable mtDNA and nDNA regions
N. N. FitzSimmons, C. Moritz, C. J. Limpus, J. D. Miller, C. J. Parmenter, and R. Prince.....25

Conservation genetics of Atlantic and Mediterranean green turtles: inference from mtDNA sequences
S. E. Encalada.....33

Application of isolation by distance models to hawksbill turtle (*Eretmochelys imbricata*) nesting sites in the Caribbean
A. L. Bass.....41

Comparative biogeography of the green turtle (*Chelonia mydas*) and the loggerhead turtle (*Caretta caretta*) as inferred from mtDNA sequence comparisons
B. W. Bowen.....47

PCR-RFLP analysis of sea turtle populations in the era of DNA sequencing: is it still useful?
F. A. Abreu-Grobois, A. L. Bass, P. H. Dutton, and S. E. Encalada.....55

Use of microsatellite loci to investigate mulitple paternity in marine turtles
N. N. FitzSimmons.....69

Use of molecular markers for stock identification, fingerprinting, and the study of mating behavior in leatherback turtles
P. H. Dutton.....79

The use of multilocus minisatellite DNA fingerprinting to examine local genetic structure within green turtle rookeries
T. Peare and P. G. Parker.....87

Microsatellites and marine turtle conservation: The Kemp's ridley diversity project
K. L. Kichler....95

Hybridization and taxonomy of marine turtles: anonymous nuclear DNA sequence analyses
S. A. Karl....99

Tracking marine turtles with genetic markers
B. W. Bowen....109

Hawksbill breeding and foraging populations in the Indo-Pacific region
D. Broderick and C. Moritz....119

Determination of the natal origin of a juvenile loggerhead turtle (*Caretta caretta*) population in Chesapeake Bay using mitochondrial DNA analysis
J. W. Norrgard and J. E. Graves....129

A mixed stock analysis of the green sea turtle: the need for null hypotheses
R. W. Chapman....137

Mitochondrial DNA D-loop sequences of marine turtles
F. A. Abreu-Grobois, A. L. Bass, R. Briseño-Dueñas, P. H. Dutton, S. E. Encalada, and N. N. FitzSimmons....147

Identification of marine turtle species: when your science becomes forensic
C. M. Woodley and R. M. Ball....163

Sea Turtle Conservation Genetics

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Management Concerns for Marine Turtles (W. N. W.)

Studies of sea turtle migrations and population structure have traditionally been limited to tag and recapture studies of nesting females. Many facets of marine turtle life history have been revealed with tagging studies, but this approach is labor-intensive, potentially expensive, and has limited applications to males and juvenile stages. As a result, scientists and resource managers have been frustrated by an inability to link reproductive adults to a rookery of origin. Furthermore, tagging studies have been unable to determine which reproductive populations use particular feeding grounds and migratory corridors (but see Limpus et al. 1992). Conservation strategies for sea turtles require such information, and new approaches are clearly desirable to resolve aspects of population biology which are refractory to conventional tag and recapture studies. In this respect, sea turtle biologists and resource managers are beginning to appreciate the relevance of genetic research, and have established genetic studies as priority research in the turtle recovery plans (NMFS/ FWS 1991a, b; 1992, 1993) and by the National Academy of Science (NRC 1990).

Genetic studies are beginning to unravel complex questions regarding the distribution and population dynamics of sea turtle populations. While molecular methodologies are relatively expensive, these approaches can be cost effective because they can answer questions in months that would otherwise take years to resolve. Studies using the maternally-inherited mitochondrial (mt)DNA can elucidate the genetic partitions among nesting populations, and analyses of turtles in coastal and pelagic waters can reveal which nesting populations occupy a particular feeding habitat. The conservation implications of these data are readily apparent, as coastal gill net, trawl, and pound net fisheries routinely capture turtles during fishing operations. Managers must determine the origin of these foraging animals, on both temporal and spatial scales, in order to determine the impact of incidental catch on each nesting population. Pelagic longline fisheries also capture and drown turtles in the North Atlantic and North Pacific gyres (Witzell and Cramer 1995), and genetic markers can determine which nesting populations are adversely impacted. Unfortunately, genetic data indicate that these foraging turtles are typically derived from nesting populations in several states or countries, and are captured by offshore fleets operating under the flags of several nations, a circumstance which greatly complicates the logistics of international conservation. Pelagic longline vessels from Hawaii are catching turtles from Japan and possibly Australia (Bowen et al. 1995). Spanish, Italian, Japanese, Korean, United States, and Portuguese vessels in the Atlantic and Mediterranean are catching turtles from the United States and possibly from Mexico (Bowen 1995a). Research efforts should determine the population structures of these turtles and

quantify the impacts of these high seas fisheries on the individual turtle populations.

Genetic markers are also gaining widespread acceptance as a forensic tool in enforcement of wildlife conservation laws. The national and international trade in turtle products has been difficult to control in part because the confiscated materials were often unidentifiable. Forensic applications of genetic markers to meat, turtle shell, and eggs can determine the species and often the geographic origin of the material. This data can be critically important to state, federal, and international law enforcement efforts.

The Contribution of Genetics to Marine Turtle Conservation (B. W. B.)

When the first mtDNA surveys of marine turtles were developed a decade ago, the projects were oriented towards biogeography and life history. How old are individual nesting colonies (Bowen et al. 1989)? Do female turtles return to their natal beach (Meylan et al. 1990)? How distinct is the Kemp's ridley from the olive ridley (Bowen et al. 1991)? Does marine turtle DNA evolve at the conventional pace (Avise et al. 1992)? Do males remigrate to their natal region (Karl et al. 1992; FitzSimmons 1996; FitzSimmons et al. 1996b)? While these studies have conservation value, their primary goals were in the realm of natural history. Since then, the techniques and findings have developed sufficiently to enable researchers to address specific questions relevant to marine turtle management. One major factor in this maturation process is the relatively complete surveys of major nesting colonies for green, loggerhead, hawksbill, and leatherback turtles within each ocean basin (Bowen et al. 1992, 1994; Broderick et al. 1994; Norman et al. 1994; Bass et al. 1996; Dutton 1995, 1996b). For example, a genetic test of natal homing in *Chelonia mydas* required data from only a few rookeries in the western Atlantic (see Meylan et al. 1990). A more exhaustive analysis with most of the known Atlantic rookeries allows wildlife managers to ask specific questions about the geographic limits of nesting populations and the contributions of nesting aggregates to regional feeding grounds (Encalada et al. 1996; Lahanas et al. Submitted). The advantages of relatively complete genetic inventories are especially apparent in forensic applications; Encalada et al. (1994) were able to assign a confiscated green turtle to region of origin because genetic data was available for most of the major Atlantic nesting areas.

Another major milestone in the application of genetic techniques to conservation is the advent of polymerase chain reaction (PCR) methodology, which allows the production of DNA sequence data from very small or partially degraded tissue samples (Hermann and Hummel 1994). Most of the early genetic assays of marine turtles required sacrificing eggs and hatchlings. While the sacrifice of eggs and hatchings is still necessary (and justified) to collect samples in some circumstances, there are now also the options of collecting dead hatchlings, egg membranes or blood samples. Samples can be stored without refrigeration (Dutton 1996a), a considerable logistic advantage when conducting field activities in remote coastal locations.

One of the primary practical advantages of PCR methods is that genetic assays can be conducted on many previously-intractable samples (Dutton 1996a). DNA sequence data is now recoverable from a wide variety of sources, including commercial products. For example, A.L. Bass recently recovered mtDNA sequences from hawksbill turtle shells collected by Peter Pritchard in 1967, and the same approach has worked on four out of six loggerhead shells recovered from a sun-baked refuse pit (Abreu and Bowen, unpublished data). The purveyors of endangered species materials are clearly vulnerable to this type of genetic sleuthing, as was demonstrated recently for suspected illegal whale meat in Japanese markets (Baker and Palumbi 1994, Baker et al. In press).

Based on these developments, research efforts in conservation genetics of marine turtles have increased in tempo and scale over the last few years, with active programs in Australia, England, France, Japan, Mexico, the United

States and elsewhere. With the burgeoning interest in genetic applications to sea turtles, September 1995 was an appropriate period for a conference on the conservation genetics of marine turtles. This represented a unique opportunity to share results and influence the course of conservation-oriented studies over the next decade. The purposes of this workshop included both practical and philosophical goals:

1) To summarize the work accomplished to date. In keeping with this goal, a bibliography of marine turtle genetics is included at the end of this section.

2) To standardize genetic methodologies so that results from different labs can be efficiently compared. The widespread use of homologous DNA sequence data is highly desirable to make meaningful comparisons between studies.

3) To avoid redundancy of effort. While confirmation of research findings by independent labs is generally desirable, it would be a waste of scarce conservation resources to resample the same marine turtle populations. A far more efficient approch is to test the conclusions made in one ocean basin with similar assays in another ocean basin. At all cost, the duplication of field collections is to be avoided in endangered species research.

4) To promote the sharing of samples and data. This is a complex issue, but the matter can be streamlined by a simple philosophical dictum: in making decisions about sharing scientific resources, the advancement of conservation goals must remain paramount to other consideration. In other words, researchers who choose to work on endangered species must recognize a higher purpose than transient career goals. This has not always been the case in programs directed towards endangered species; international genetic studies in other taxonomic groups have been delayed for years because of the unwillingness of researchers to share samples or genetic methodology.

5) To develop guidelines for forensic applications of genetic data. How many field samples are necessary to support conclusions about species and region of origin? What precautions must be taken to assure that conclusions are defensible? While many of us are familiar with the standards of peer review publication, the standards of courtroom verification are quite foreign to most biologists and conservationists.

6) To make conservation genetic methods and conclusions more accessible to the wildlife managers who will transform this data into policy decisions.

Picking the right molecule for the job

Advances in molecular techniques over the last decade have opened a number of avenues for genetic definition of populations and evolutionary units. In these circumstances, the potential exists for misapplication of techniques. As noted by Dutton (1996a) and FitzSimmons et al. (1996b), the choice of appropriate technique(s) depends on the issue at hand and the natural history of the organism. The mitochondrial genome has played a prominent role in marine turtle conservation genetics, in part because "the matrilineal component of an organismal pedigree (as estimated for example by mtDNA) can be of special relevance to population biology and management, even when concordant support on population genetic structure from nuclear loci is lacking." (Avise 1995). Since females ultimately govern the reproductive output of a population, knowledge of female dispersal and stock structure (as defined by mtDNA) may be extremely important in defining management priorities.

For species with no sex-specific differences in dispersal, the population structure defined by a maternal genetic assay (mtDNA) or a biparentally-inherited genetic assay (nDNA) should be concordant under assumptions of population equilibrium (Wayne et al. 1991; Scribner et al. 1994; but see Karl and Avise 1993). In these cases, wildlife managers may reasonably consider the results of either mtDNA or nDNA assays to represent the overall pattern of stock structure (Templeton et al. 1990). However, for species such as marine turtles which have the potential for gender-specific differences in dispersal of gametes, mtDNA and nDNA assays may yield qualitatively different estimates of gene flow and population structure (Karl et al. 1992; Palumbi and Baker 1994). Such differences do not reflect conflicting

results but rather the legitimate differences in geographic structuring of nuclear and mitochondrial lineages that is an expected consequence of sex-specific dispersal (FitzSimmons et al. 1996a, b). Until recently, this type of complex population structure has seldom been considered in the formulation of wildlife management plans, partly because the prerequisite genetic tools were not available (Hoezel and Dover 1989).

Differences in population genetic structuring as defined by nDNA and mtDNA assays can have profound implications for wildlife management. For example, mtDNA assays indicate that each green turtle nesting population is a distinct management unit (Bowen et al. 1992; Norman et al. 1994; Allard et al. 1994; Lahanas et al. 1994; Encalada et al. 1996), but nDNA assays indicate little population genetic structuring among some regional nesting colonies (Karl et al. 1992; FitzSimmons et al. 1996b). Acting on mtDNA data alone, wildlife managers might conclude that each nesting population is effectively isolated from other regional nesting colonies. This perspective is correct in terms of female (egg laying) lineages, but would miss the potential genetic link between nesting colonies afforded by male reproductive behavior. In this case, concerns about inbreeding and reduced genetic diversity in small nesting colonies might be misplaced, because wildlife managers would be unaware that gametic exchange through males may connect regional nesting populations.

Acting on the nDNA data alone (a distinct possibility when protein electrophoresis was the only genetic assay available), individual rookeries might not be recognized as demographically independent entities. Indeed, this position has been invoked on the basis of a protein electrophoretic data set: Bonhomme et al. (1987) concluded that extensive gene flow occurs between *C. mydas* nesting colonies in separate ocean basins. Hence the perspective based on nDNA data alone may be particularly hazardous for management of marine turtles, because reproductive populations could lapse into extinction under the mistaken impression that depletion of a rookery is countered by recruitment from other reproductive populations.

In cases where sex-specific differences in gametic dispersal are known or suspected, it is necessary to analyze both biparentally-inherited (nuclear) DNA (nDNA) loci and uniparentally-inherited (mitochondrial) DNA (mtDNA) lineages to adequately define management units. Analyses of mtDNA can reveal the geographic structure of maternal lineages which are of paramount importance in species propagation, and nDNA surveys can reveal complementary information on stock structure, effective population size, male dispersal, inbreeding, and related concerns. Either analysis, when taken alone, could lead to erroneous and detrimental management policy.

Population structure of a highly migratory species

Rookery-specific population structure has emerged as a general paradigm for the marine turtles (Bowen and Avise 1995), but it is unclear where the geographic boundaries of nesting populations lie in most cases. Broderick et al. (1994) found that Indo-Pacific hawksbill nesting aggregates separated by a few hundred kms were indistinguishable in terms of mtDNA control region comparisons, but Bowen et al. (1993a) reported a sharp frequency shift between loggerhead nesting populations in the southeast U.S. separated by less than two hundred kilometers.

The propensity of marine turtles to move long distances between resident foraging areas and reproductive habitats may confound any assumptions of stock structure based on geographic proximity. Feeding grounds may contain cohorts from widely separated nesting populations (Bowen et al. 1995; 1996; Broderick and Moritz 1996), and adjacent nesting populations may be highly divergent in terms of DNA sequence comparisons (Norman et al. 1994). It seems that behavioral barriers to gene flow and sporadic colonization events strongly influence the population histories of marine turtles (Bass 1996). Under these circumstances, it may be difficult to predict the genetic relationships among regional nesting aggregates by proximity or geography alone. Based on the overall pattern of genetic isolation among nesting populations, it is reasonable to assume that widely separated (>500 kms)

rookeries constitute distinct management units, but adjacent nesting areas and feeding aggregates may require evaluations on a case by case basis.

Extending the horizons of Conservation Genetics

The utility of genetic assays for population resolution is clear, but molecular techniques have strong conservation applications outside this realm, and many of these research avenues are just beginning to be explored. One of the most exciting applications is in the use of rookery-specific genetic markers to resolve migratory routes and feeding ground composition (Chapman 1996; Broderick and Moritz 1996; Norrgard and Graves 1996). This approach offers a hope for expedient identification of the nesting colonies impacted by commerical fisheries and other human incursions (Bowen 1995a).

Molecular systematics has become increasingly prominent in exploring taxonomic boundaries and evolutionary relationships, and the genetic distinctiveness of dwindling populations may be considered when assigning conservation priorities (Vane-Wright et al. 1991; Crozier 1991; Forey et al. 1994; Moritz 1994a, b; but see Erwin 1991) . In this respect, molecular phylogenies have resolved some controversies in marine turtle evolution (Bowen et al. 1993b; Dutton et al. 1996a; Bowen and Karl 1996) but have prompted a reevaluation of the taxonomy of green turtles, *Chelonia mydas* and the dubious *C. agassizi* (Kamezaki and Matsui 1995; Karl 1996; Bowen and Karl 1996; Zug 1996). One conservation application of these findings lies in the identification of marine turtle material in a forensic context (Woodley and Ball 1996). Molecular genetic assays have also revealed some unanticipated features of marine turtle evolution, including relatively slow genomic evolution (Avise et al. 1992; FitzSimmons et al. 1995a) and evidence of hybridization among lineages which are tens of millions of years old (Karl et al. 1995; Karl 1996).

The advent of hypervariable nuclear DNA assays has opened up an exciting new window on reproductive behavior (FitzSimmons et al. 1995b; Fitzsimmons 1996, Dutton 1996b) and fine-scale population structure (Peare and Parker 1996c; FitzSimmons et al. 1996b). How precise is natal homing? Do males move between nesting colonies? How many males contribute to a clutch? All of these questions are beginning to yield to molecular genetic methodologies, and all of them have clear conservation implications. The possibilites for conservation-oriented applications are just beginning to unfold, and it is certain that more applications will be found for these versatile genetic assays.

Endangered Species Permits

Permits for scientific access remain one of the greatest impediments to research on endangered species. Regulations governing endangered species are designed to thwart the efforts of poachers and smugglers, but these criminal profiteers seldom apply for endangered species permits. In a misguided effort to protect the species or demonstrate a successful permit program, many government agencies succumb to the practice of restricting legitimate scientific access and conservation-oriented research.

Federal agencies in the United States (National Marine Fisheries Service and U.S. Fish and Wildlife Service) have cumbersome application procedures for endangered species permits, and administrative delays of 6-12 months are common. Federal permits are often administered by persons with limited grasp of field biology and study design. One of the most damaging practices by federal permit officers is to alter or restrict sampling methodology for the sake of an imagined increase in wildlife protection. Permit restrictions which seem reasonable (and politically correct) in an adminstrative office in Washington D.C. can have a strongly detrimental impact on field collections. In practical terms, these restrictions can be costly, they reduce the scientific rigor of the research, and they prevent valuable opportunistic sampling. Compounding these problems at the national level are the individual state wildlife agencies which can impose additional layers of permit paperwork. State wildlife managers usually work in close proximity to the conservation problems and have a more enlightened approach to scientific access, but in a few cases state permits have been denied, delayed, or restricted for no

obvious scientific reason.

A recurring tendancy in permit offices is the use a "quota" system to demonstrate control over endangered species. A single case history is sufficient to illustrate the damage done: In recent years, one of us (B.W.B.) repeatedly requested greater lattitude in sample size and sample type allowed under a U.S. CITES import permit. These requests were denied and the permitted sample size was held at 15 per location, because the permit officer surmized that 15 samples was sufficient to conduct the analysis of mtDNA haplotypes. However, larger sample sizes are necessary to apply the new generation of nuclear DNA assays (Karl et al. 1992; FitzSimmons et al. 1995a, 1996a, b) and to resolve the demographic composition of feeding grounds (Chapman 1996). Due to restrictions imposed by the U.S. Fish and Wildlife Service Office of Management Authority, the necessary sample sizes are unavailable for these new conservation initiatives.

These permit problems are further exacerbated by the international nature of marine turtle conservation. CITES import and export permits are required for international movement of specimens, but countries vary widely in access to CITES permits, with availability often governed by political trends. A typical marine turtle project may require permit applications in three or more languages, and samples may cross a dozen international boundaries. In these circumstances delays in CITES import and export permits can unravel an entire study. Attempts to correct these problems (such as a CITES exemption for small samples of tissue or blood, materials which have no commercial value but enormous scientific value) have been thwarted in large part by dogmatic resistance from the U.S. Fish and Wildlife Service Office of Management Authority.

A final problem, unique to conservation genetic studies, is the perceived commercial value of DNA. Under terms of the Biodiversity Convention (a.k.a. the Rio Convention), countries are accorded the right to seek royalties for genetic resources discovered within their territories. While this principle seems reasonable when applied to new drugs and commercial products, no distinction is made in the Rio Convention between biochemical prospecting and scientific research. In other words, the authors of this convention repeated one of the worst mistakes of the CITES Convention by making no provisions for scientific access. On three recent occasions, countries have denied export permits for a few drops of sea turtle blood, under the justification that no genetic material would be exported until the *monetary value* of sea turtle DNA could be established. This problem, in conjunction with uncertain access to CITES permits, has the potential to shut down international conservation research.

Happily, one ray of hope has pierced these bureaucratic quagmires. PCR products, synthetic copies of organismal DNA, are exempt from CITES regulations (although at one point the U.S. Fish and Wildlife Service tried to regulate this material; Bowen and Avise 1994a; Jones 1994). Under this exemption, biologists may produce synthetic copies of the DNA in a host country, and then transport these copies across international borders without the cumbersome CITES paperwork (see Baker and Palumbi 1994). However, this approach can be expensive, especially when researchers must carry lab equipment across international borders.

Two philosophical points bear consideration in applying this PCR approach. First, it is a disgrace that conservation biologists must use an expensive loop-hole in the CITES treaty to surmount impediments put in place by agencies charged with protecting endangered species. Second, while the CITES convention represents a legal impediment to research, the collecting permits from the host country represent a moral imperative. Scientists must obtain permission to collect biological samples within the boundaries of sovereign nations, just as these countries must recognize the necessity of allowing scientific access for multinational conservation initiatives.

Permit agencies will continue to be a hinderance to endangered species recovery until these institutions accept the primary role of scientific research in conservation. One corollary of this theme is the recognition that some aspects of conservation research require

intrusive sampling and occasional sacrifice of specimens. Many lethal procedures, opposed by uninformed (but vocal) animal rights groups, are sometimes justified by the conservation dividends in life history information. The reprioritization of scientific studies by permit agencies offers the only hope to alleviating the delays, restrictions, and endless haggling over scientific materials.

Literature on marine turtle population structure, molecular evolution, conservation genetics, and related topics

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Methods for collection and preservation of samples for sea turtle genetic studies

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Introduction

Genetic techniques are increasingly being used to study the biology and conservation of sea turtles (see review in Bowen, 1995). Earlier, such studies were hampered by the need to sample relatively large amounts of tissue, sometimes requiring the sacrifice of hatchlings. With the development of the Polymerase Chain Reaction (PCR) methodology (Mullis et al., 1986), molecular analysis now only requires tiny amounts of DNA, which can be obtained from a few drops of blood or small tissue biopsies without harm to live turtles. DNA can also be recovered from soft tissue or bone samples from dead animals, even from archaeological remains and museum collections (Pääbo, 1989). Genetic studies are therefore no longer limited so much by technological shortcomings, but rather by availability of samples.

Field researchers wishing to conduct genetic studies are often confused by the plethora of methods available for collecting and preserving material, and there is a need to standardize methods for sea turtles. The most appropriate method will depend on the type of study, the available resources, and the experience of the people involved. There are many ways to store material under a variety of circumstances ranging from a fully equipped laboratory with ultra-freezer storage capability, to the lonesome researcher on a shoestring budget at a remote field site. Often sampling is opportunistic and the researcher might be unprepared, such as during the encounter of a stranded animal. In a pinch, blood samples can be dried on paper; indeed Eggert et al. (submitted) have conducted PCR amplifications on DNA extracted from cetacean blood that was blotted on archived paper field records. However, genetic studies usually require sampling of adult females on nesting beaches, or capture of animals, either juveniles or adults, in the water. Sampling can be achieved by collecting blood, or obtaining small skin biopsies. Procedures can also be carried out on nesting females while the turtle is in her "nesting trance" without having to restrain or stress the animal.

Nucleic acids are extremely stable molecules, however the action of cellular endonucleases and oxidative processes will cause degradation. Successful preservation requires either inhibition or denaturation of these enzymes. This is usually achieved by freezing, but cryopreservation is rarely an option to researchers working on remote nesting beaches for extended periods, a typical scenario for sea turtle projects. This paper reviews the methods currently available, and is intended as a guide to those planning field collections. More complete reviews and protocols for extraction and analysis of samples are given in Escorza et al. (In press).

Procedures

Sampling from live animals

Blood. Various methods have been described for collecting blood from live sea turtles, including cardiac puncture (Dozy et al., 1964; Frair, 1977a, 1977b; Frair and Prol, 1970) as well as drawing blood from the carotid artery (Berkson, 1966). Both these techniques are potentially harmful to the turtle, and are not recommended. The standard bleeding technique is that described by Owens and Ruiz (1980), where blood is drawn from the dorsal cervical sinus. The sinuses are located bilaterally in the neck close to the dorsal surface (Fig. 1A). This technique can be applied at all life stages, including hatchlings. Either a syringe and needle, or evacuated blood

collecting tubes (Vacutainers) (Becton, Dickinson and Co.) can be used, with the size of needle being determined by the size of the turtle. For hatchlings or juveniles less than 1kg in weight, a 1 cc disposable insulin syringe with a flexible 26-29 gauge 12.7mm (5/8") needle should be used to prevent injury to the animal (Bennett, 1986; Fitzsimmons, 1996). A 2.5-3.8cm (1.0-1.5") 21 gauge needle can be used with larger turtles. For adult leatherbacks, 7.6cm (3") long 18 gauge needles are needed. The turtle should be restrained so that the head is lower than the body and the neck is outstretched. This helps the sinuses to fill with blood and facilitates access. This position can be achieved with nesting turtles without having to restrain the turtle by digging out sand from under the animal's head during oviposition (sampling of nesting females should be initiated only after the turtle has begun laying eggs and is in her "nesting trance", and should be abandoned if the turtle appears skittish and in danger of aborting egg laying). The needle is inserted one cm (or 0.5 cm for smaller turtles) from the dorsal-cervical midline on either side of the line's midpoint. Positioning the needle lateral to the midline is important to avoid striking the vertebral column. One can usually feel a set of tendons here that run just below the skin along either side of the vertebral column, and these

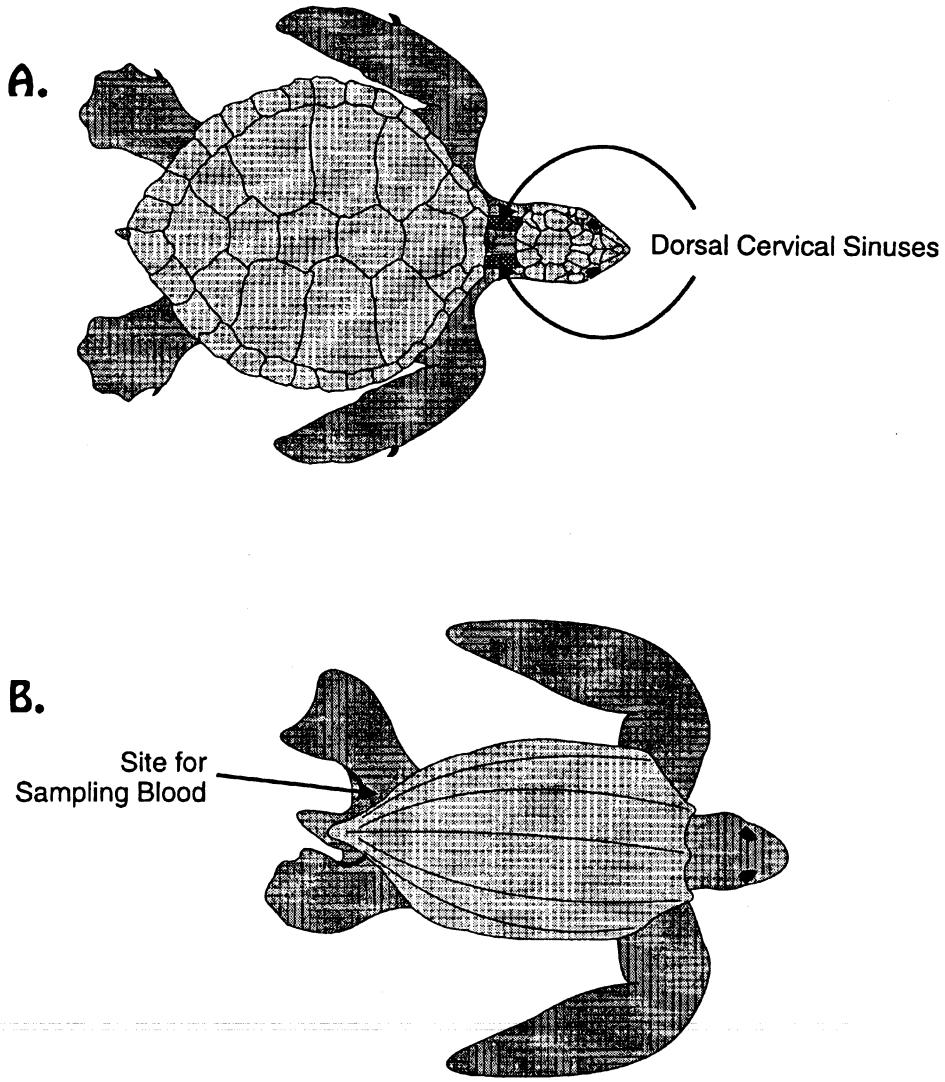

Figure 1. (A) Location of the dorsal cervical sinuses in sea turtles, and (B) site for sampling blood from the hind flipper in leatherbacks (see text for measurements).

can serve as landmarks for locating the sinus. One should avoid sticking the needle through the tendon, but rather insert the needle at the outside edge of the tendon through the soft adipose tissue into the underlying sinus. The needle should be inserted at an angle perpendicular to the dorsal surface of the neck, to a depth of 1-3cm until a spurt of blood is seen. If a syringe is used, apply a small amount of suction once the needle is inserted. It may be necessary to search for the sinus by adjusting the depth of the needle while applying gentle suction, although rotation of the needle should be avoided to minimize internal damage. As blood flows into the syringe or vacutainer, the apparatus is held still until approximately 1-2ml of blood have been obtained. For hatchlings, only 0.1-0.5ml should be collected. Often it is only possible to get a few drops of blood and since this is adequate for PCR it is not necessary to continue searching for larger samples, especially if the welfare of the animal is compromised. If the sinus is not initially located, the needle should be removed and the procedure repeated in a more lateral or medial position. The opposite side of the neck should be tried if further attempts are unsuccessful.

Owens and Ruiz (1980) noted that the most common cause of failure is poor positioning of the animal. Ideally the turtle's head should extend below the level of the plastron. Sampling should be avoided while the animal is struggling. If struggling persists the head should be released so that the turtle can raise it to breathe normally for a few minutes. Often covering the eyes helps calm the turtle. Hatchlings can be easily held upside down for a few minutes until they are calm before attempting to draw blood.

Alternatively blood may be sampled from the hind flipper using the procedure described by Dutton (1995) and Dutton and Eckert (submitted). The hind flipper is preferable for nesting leatherbacks because the smaller 2.5 or 3.8cm (1-1.5"), 21 ga. needles can be used rather than the larger needles required for the cervical sinus in this species. Blood sampling should take place while the turtle is laying eggs. The insertion point lies on the dorsal surface of the rear flipper, 5cm from the edge of the carapace and 1cm interior of the tibia (Fig. 1B). The area should be swabbed with disinfectant and the needle inserted 3 cm deep at an oblique angle toward the body and parallel to the tibia. Either flipper can be used; however, it is easier to locate a vein in the flipper that is extended over the nest cavity. Care should be taken not to collapse the nest chamber. The needle must be withdrawn before the turtle begins to use the hind flippers to cover the nest chamber. This procedure has also been used on nesting green turtles (Matthew Godfrey, University of Toronto, Canada, pers. comm.).

Tissue. Small skin or muscle biopsies provide a simple and inexpensive way to obtain samples for genetic studies from both live and dead animals (Dutton and Balazs, 1995; Norman et al., 1994). These can be obtained using sharp, sterile dissecting scissors, a scalpel, or a commercially available biopsy tool. The Acu-Punch 6mm disposable biopsy punch (Acuderm, Inc., Fort Lauderdale, Florida 33309, USA) is recommended for obtaining "plugs" of skin and attached sub-epidermal tissue large enough for PCR-based genetic analysis (Dutton and Balazs, 1995). This tool consists of a plastic handle that supports a sharp circular blade, and the sample is taken by rotating the tool once or twice while gently pressing down to make a circular cut 2-4mm deep. After withdrawing the blade, the tissue plug can be removed with forceps. Biopsies can be taken from anywhere on the limbs or neck, depending on the circumstance. Small turtles can be placed on their back to sample the smooth skin located in the dorsal axial region of the hind flipper (Dutton and Balazs, 1995). Samples can be taken during nesting from the dorsal surface of the front flipper toward the "shoulder" area without having to restrain the turtle. The biopsy site should be swabbed with disinfectant, such as Betadine, before and after taking the biopsy, and blood can also be collected with the tissue if bleeding occurs. A smaller biopsy tool, 4mm diameter can be used for smaller juveniles (1-5 kg in weight), and it may even be possible to use the 1.5mm tool to sample hatchlings without undue injury. It is important to sterilize implements or use a new tool for each animal, since PCR is extremely sensitive to cross contamination caused by residual tissue.

Sampling from dead animals

The enzymes in internal organs, such as heart, liver and kidneys will cause rapid degradation of DNA following death. In warm climates these tissues also begin decomposing rapidly, so if an animal has been dead for more than a few hours it is best to take skin and muscle rather than heart, liver or kidney. Although fresher specimens are preferable, skin and muscle from stranded animals or dead hatchlings can remain usable for at least a week, perhaps longer depending on the ambient conditions. Dried out hatchling carcasses that are found on the beach or in old nests can be used. Tissue salvaged from embryos that have been dead for up to 60 days can be used providing the egg has remained intact and decomposition has not occurred. Either whole animals or skin and muscle samples can be frozen or preserved in salt as described below. Although PCR can be done with tiny pieces of tissue, 1 gm of tissue (approx. $1cm^3$ or 2 cm^2 strips) will ensure sufficient DNA for multiple studies.

Preservation and storage of samples

Blood. Small blood samples can be dried down on glass slides or filter paper (Galbraith et al., 1989; Walsh et al., 1991; Sepp et al., 1994; Eggert et al., submitted), but these methods should only be used as a backup or as a last resort if the researcher is unprepared for sampling. The best method is to lyse blood cells in a buffer containing detergents and preservatives. Several different recipes for lysis buffers are available (Seutin et al., 1991; Muralidharan and Wemmer, 1994), however the one most commonly used for sea turtle blood consists of 100mM Tris-HCl, pH 8; 100mM EDTA, pH 8; 10mM NaCl and 1-2% sodium dodecyl sulphate (SDS) (Bass et al., 1996, Bowen et al., 1996; Encalada et al., 1996; Dutton, 1995). Seutin et al. (1991) recommend a 1:10 dilution of blood to buffer, and White and Densmore (1992) a 1:5 ratio. This dilution however, is not critical, and higher concentrations of blood can be used, up to a 1:1 ratio, particularly if a higher concentration (2%) of SDS is used to ensure adequate lysing of cells (Dutton, unpublished; Louis, Texas A&M University, pers. comm.). Two ml Nunc^R cryotubes can be preloaded with 1ml of buffer and a few drops to 0.5 ml of whole blood added. The vial should be shaken well to ensure mixing and stored away from direct sunlight and if possible in a cool place. Although storage at +4 to -80°C may optimize long term preservation, samples in buffer can be kept at ambient temperatures for years if necessary. If lysis buffer is not available, whole blood can also be preserved in a saturated salt (NaCl) solution with or without 20% DMSO. A 1:5 or greater dilution of blood to preservative is recommended.

To clot or not to clot?

When blood is exposed to air the red blood cells eventually clump together, so that after about 10-15 minutes one is left with a blood clot suspended in clear plasma, unless the sample is treated with an anti-coagulant. Since it is the blood cells, which have nuclei and mitochondria, that contain most of the DNA in reptilian blood, it is important to collect the clots and not the plasma. Anti-coagulants such as heparin may cause DNA degradation (Gustafson, et al., 1987), and some researchers prefer not to treat blood with anticoagulants during collection (Bowen, University of Florida, pers. comm.). Others have used anti-coagulants in sea turtle blood sampling without problems. Gustafson et al. (1987) incubated human blood at 23°C for up to 3 days in ACD (sodium citrate) solution B (Becton Dickinson)(0.48 g% (or 0.023M) citric acid; 1.32 g% (or 0.045M) sodium citrate; 1.47 g% (or 0.082M) glucose) without any DNA degradation, and Norman et al. (1994) and Broderick et al. (1994) used this anti-coagulant for collecting sea turtle blood. Heparin has also routinely been used for sea turtles without noticeable ill-effect (Dutton, unpublished). If anticoagulants are not used, it is best to add the blood directly to the lysis buffer immediately as clotting will make handling more difficult. For leatherbacks, anti-coagulants are recommended, since clotting seems to occur more rapidly, often within the needle itself during sampling. Clotting can be prevented by using Vacutainers containing heparin or sodium citrate (ACD-B), or if syringes are used, by drawing liquid anti-coagulant into the syringe and flushing it back down into the needle prior to taking the sample, thus coating the needle and syringe. Since red blood nuclei are essentially

metabolically inactive, the concentrations of DNAases in blood samples are extremely low, so that sea turtle blood treated with anti-coagulant can be kept on ice or refrigerated (4°C) for one or two weeks without significant degradation (Dutton, unpublished observation) prior to preservation. Samples can be concentrated by centrifugation (15,000-20,000 g for 15 minutes), or fractionated by letting blood cells settle in collecting tubes for several hours, and removing the supernatant plasma. The remaining red blood cells and the overlying thin layer of white blood cells ("buffy coat") can then either be shipped on ice to the laboratory, frozen, or transferred into lysis buffer.

Tissue. Salt (NaCl) is readily available and known to be a good preservative, and saturated solutions supplemented with combinations of ethylene-diamine tetracetic acid (EDTA) and dimethyl sulfoxide (DMSO) have been used to preserve tissue samples. EDTA binds divalent cations which are essential to most of the DNA degrading enzymes. DMSO makes cells more permeable and facilitates rapid penetration of the preservative into the sample. Although long-term studies have not yet been reported for sea turtles, Amos and Hoelzel (1991) were able to obtain perfect mtDNA and nuclear DNA from whale skin preserved in 20% DMSO saturated with NaCl (without EDTA) for 2 years without refrigeration. They found that EDTA had a detrimental effect during long term preservation. Others have used saturated NaCl with 20% DMSO and 250mM EDTA, pH 7.5-8.0 to preserve bird tissue (Seutin et al., 1991), and fish tissue (Proebstel et al., 1992) for up to a year without degradation. While this formula is routinely used to preserve Chelonid sea turtle tissue (B. W. Bowen, pers. comm.) without apparent degradation, long-term studies have yet to be done. In contrast, the DNA extracted from leatherback heart tissue that had been preserved in a salt/DMSO solution with 250mM EDTA for approximately 2 years was considerably degraded (Dutton, unpublished observations). However it is possible that this tissue had already decomposed before it was put into the preservative. Until this question is resolved use of 20% DMSO saturated with salt (without EDTA) is recommended for long-term preservation of sea turtle tissue. Pieces of tissue (0.5-2.0 g, or 0.5-2.0cm in diameter) should be chopped up with a razor blade or scalpel to optimize penetration of the preservative and placed in a well-sealed plastic container with the salt preservative. A 4.5 ml Nunc[R] cryotube containing 3.0 ml of the DMSO-salt solution works well. Since the solution is saturated with salt, a white precipitate may form in the tubes but this does not affect the preservation. The preservative is not flammable and is non-toxic, although DMSO soaks into the skin rapidly and can cause a garlic-like taste and breath odor. Use of gloves is therefore advisable when handling the solution. Samples can be stored at ambient temperatures for extended periods; however, storage at cooler temperatures may enhance long-term preservation.

Tissue samples can also be preserved in alcohol. Absolute or 95% ethanol is recommended, although 50-70% isopropyl or even a strong grain alcohol are acceptable and often available at remote field research camps. Alcohol however is volatile and flammable, making it unsuitable for transport by air and prone to evaporation. Salt is preferable whenever possible. If entire hatchlings are preserved in alcohol, it is important to open the body cavity to allow the preservative to penetrate.

Tissue should not be fixed in formalin, since DNA becomes highly degraded even in neutralized formalin (Koshiba et al., 1993), and difficult to extract (Goelz et al., 1985).

Frozen tissue or blood should preferably be kept at -80°C, but storage at -20°C is acceptable. Frost-free freezers should be avoided for long term storage, since the constant temperature cycling of these models may cause DNA to shear. Tissue samples preserved in ETOH or salt and DMSO, or blood stored in lysis buffer can be maintained at 4°C or ambient temperature.

Sample Sizes

The number of samples required for population studies depends on the type of analysis and the markers that are used. Although just one sample from a rare population may yield important information, generally the more samples that are collected, the better. For mtDNA studies, 15-30 samples from a rookery

is adequate, although this may not be possible for severely depleted populations. Since hatchlings in the same clutch have identical mtDNA, which is inherited from the mother, only one hatchling or embryo should be sampled from any given clutch for population analyses. It is also important to avoid sampling clutches laid by the same female at different times of the season. If females or clutches cannot be identified, then this can be achieved by sampling clutches laid within one inter-nesting interval; within a 9 day period for leatherbacks, and 12 days for the other species.

Population studies using other types of nuclear markers, such as microsatellites, may require 20-50 samples per rookery, and typically 50-100 samples are needed for mixed stock analysis of forage populations using mtDNA (Broderick and Moritz, 1996; Chapman, 1996).

Summary

The most suitable method will depend on the facilities available to the researcher, the type of DNA markers to be analyzed, and the logistical considerations. If purified mtDNA is required, collecting tissue samples may be preferable because the ratio of mitochondrial to nuclear DNA is higher in tissue such as liver, heart or muscle than in blood. However, PCR studies of mtDNA can be easily done from blood samples. If possible, samples should be collected from live animals and analyzed immediately or frozen. If refrigeration is not available, blood samples can be stored in a lysis buffer or salt solution at ambient temperatures. If blood sampling is not possible, then small tissue biopsies can be taken from live turtles and stored in a salt/DMSO solution, or alcohol. Tissue can also be sampled from dead animals and eggs. Liver, heart and kidneys should be collected from fresh animals, skin and muscle from animals that have been dead more than an hour or two. Although fresher specimens are preferable, skin and muscle from stranded animals or dead hatchlings can remain usable for at least a week, perhaps longer depending on the ambient conditions or if the carcass has dried. Tissue salvaged from embryos that have been dead for up to 60 days can be used provided the egg has remained intact and decomposition has not occurred. Fresh eggs can also be used, or can be incubated for 50-60 days to obtain tissue.

Those involved in surveys of sea turtles, both in marine and nesting habitats, or who deal with stranded animals are encouraged to routinely collect material that can be made available for genetic studies. Even if not part of an ongoing project, these collections can be made with limited resources and may prove invaluable in the future as new questions and methods are developed.

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Comparative genetic structure of green, loggerhead, and flatback populations in Australia based on variable mtDNA and nDNA regions

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Introduction

The tools of conservation genetics offer a powerful means of identifying breeding populations and interpreting historic gene flow. For marine turtles, genetic data can help clarify the extent to which nesting populations within regions are interconnected by gene flow, and the degree of isolation among regional groupings. Allozyme electrophoresis has been used extensively to elucidate genetic structure among populations for many species and thereby offered the first empirical tests of several hypotheses concerning gene flow, population history and distribution (Avise 1994; Richardson et al. 1986). Even so, in many studies the number of polymorphic allozymes were few, or the polymorphic loci were not sufficiently variable to serve as useful population markers. However in recent years, allozyme studies have been complemented by the ability to look at variation at the DNA level, particularly within the highly variable regions of mitochondrial DNA.

Allozyme studies of marine turtles (Smith et al. 1978; Gyuris 1984; Bonhomme et al. 1987; Gyuris and Limpus 1988; Coates et al. 1994; Norman et al. 1994b) have indicated a moderate number of variable loci in some species (e.g. *Chelonia mydas*), but there were no rigorous tests of population differentiation, due to the small sample sizes. In addition, not all allozyme studies tested the same variable loci, and loci that were variable in one study were not always variable in another. The relevant studies for marine turtles are summarised in Table 1.

In a global study, Bonhomme et al. (1987) used allozymes to study population differentiation in green turtles and detected genetic differentiation between Atlantic and Pacific/Indian Ocean green turtle populations, but not between the Pacific and Indian Ocean samples. However, sample sizes were quite small for both the Atlantic (n=4) and Pacific (n=12) populations. In Australia, Norman et al. (1994b) surveyed green turtle populations

Table 1. Previous studies of genetic variation at nuclear loci in marine turtles

Study Differentiation	Species	Locations	# Polymorphic Loci (# alleles)	Observed (Proportion of loci)
Allozymes				
Smith et al. 1978	Green	Atlantic	9 (2-4)[1]	not tested
	Loggerhead	S. E. U. S.	1 (4)[2]	yes (1/1)
Gyuris 1984	Green	E. Australia	4 (2)[3]	no
	Flatback	E. Australia	2 (2)[4]	not tested
	Hawksbill	E. Australia	0	not tested
Bonhomme et al. 1987	Green	Atlantic vs. Pacific	2 (2)[5]	yes (2/2)
Gyuris and Limpus 1988	Loggerhead	E. Australia	2 (2)[6]	yes (1/1)
Coates et al. 1994	Green	W. Australia	5 (2)[7]	yes (3/5)
Norman et al. 1994a	Green	E. vs. W. Australia	9 (2)[8]	yes (2/8)
scnDNA[9]				
Karl et al. 1992	Green	Global: within and among oceans	10/2	yes yes

Polymorphic loci are as follows: [1] Esterase-2, glutamate-oxalate transaminase-1, -2 (GOT-1, 2; currently known as aspartate aminotransferase) isocitrase dehydrogenase-1.-2 (IDH-1, IDH-2), lactate dehydrogenase, malate dehydrogenase, phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGD), phosphoglucose isomerase (currently known as glucosephosphate isomerase, GPI), [2] Esterase-2, [3] GPI, IDH-2, PGM, phosphoglycerate kinase (PGK), [4] IDH-2, muscle protein-1, [5] GPI and glyoxalase (GLO), [6] PGK, PGM, [7] IDH-2, 6PGD-1, PGM-1, peptidase-1 (PEP-LGG; using leucyl-glycine-glycine substrate), fumerase-1 (FUM-1), [8] GOT-1, GOT-2, FUM, GPI, IDH-2, PEP-LGG, 6-PGD, PGK, PGM. [9] single copy nuclear DNA loci

across three regions, Southern Great Barrier Reef (SGBR), Northern Great Barrier Reef (NGBR), and Western Australia (WAust) to look for fixed allelic differences, but found only slight frequency shifts at the 9 variable loci observed. Looking at variation within a region, Coates et al. (1994) found significant differentiation at 3 loci among green turtle rookeries of western Australia. At one rookery, significant variation in allele frequency was observed between seasons (three years of data) at one locus (Coates et al. 1994). Studies of loggerhead turtles (*Caretta caretta*) within regions have indicated genetic heterogeneity for populations along the southeastern U.S. coast (Smith et al. 1978; 1 locus) as well as for those in eastern Australia (Gyuris and Limpus 1988; 1 locus).

More recently, genetic studies of marine turtles have focused on identifying variation in mtDNA haplotypes and testing for heterogeneity among nesting populations (Bowen et al. 1992, 1993; Allard et al. 1994; Broderick et al. 1994; Lahanas et al. 1994; Norman et al. 1994a; Bass et al. 1996). As Avise (1995) pointed out, variability in the mtDNA genome is of particular use because of (1) a low effective population size (N_e) relative to nuclear DNA (nDNA), (2) high mutation rates; both of which make it more likely that divergence of isolated populations will be observed, and (3) maternal inheritance, allowing isolation of data on female lineages. Information specific to female lineages offers exceptional applications for marine turtle conservationists who are particularly interested in identifying and protecting breeding populations. Studies of both green and loggerhead turtles have demonstrated that significant population differentiation exists both within and between ocean basins (Bowen et al. 1992; Bowen et al. 1994; Allard et al. 1994; Lahanas et al. 1994; Norman et al. 1994a; Encalada et al. 1996). The application of mtDNA analyses to nesting populations has

confirmed natal homing behaviour in Cheloniid marine turtles (Meylan et al. 1990; Allard et al. 1994; Bowen et al. 1993; Norman 1995; Bass et al. 1996) and provided rapid and extensive information on the composition of feeding ground populations (Broderick et al. 1994; Norman 1995; Bowen et al. 1996). mtDNA markers have also allowed the identification of source stocks of turtles killed in a variety of fisheries (Bowen et al. 1995; Norman 1995).

However, mtDNA analysis is limited and research on several species has shown the necessity of including results from nDNA to obtain a broader picture of population level gene flow encompassing both males and females. Comparative studies of nDNA and mtDNA in marine turtles may provide insights into male behaviour through the extent of male-mediated gene flow (Karl et al. 1992). Moritz (1994) has proposed that a combination of mtDNA and nuclear locus (including allozymes and nDNA) analyses be used to identify management units (MU's) and evolutionary significant units (ESU's) within species, and has suggested applications of this approach to the management of marine turtles.

Karl and Avise (1993) developed a method to isolate variable regions of nDNA that are anonymous and found in the genome as single copies (scnDNA loci). In a global survey of green turtles (Karl et al. 1992), substantially less genetic structuring both within and among ocean basins was observed for the scnDNA loci (n = 5 loci) in comparison to mtDNA (Bowen et al. 1992). The existence of distinctive biparental (nDNA) and maternal (mtDNA) components to genetic structure was suggested and moderate levels of male-mediated gene flow were hypothesised due to differences in the natural history of males and females. However, given some ambiguity in the biogeographic relationships suggested by the scnDNA results, and the limitation that few (1-3) polymorphic sites were found per locus, mutation rates may be too low to offer a robust comparison to the results from mtDNA studies.

We have investigated highly variable regions of the nuclear genome known as microsatellites to assess the contributions of both male and female marine turtles to gene flow among populations. With mutation rates for microsatellites estimated at 10^{-2} to 10^{-4} (Weber and Wong 1993) per locus per generation, the distribution of genetic variation among populations at these regions should offer a robust indicator of male-mediated gene flow when compared to mtDNA variation. We present here some initial comparisons of mtDNA and microsatellite data for 3 species of marine turtles, greens (*C. mydas*), loggerheads (*C. caretta*), and flatbacks (*Natator depressus*), to show a range in results across species. For each species we have focused our sampling on four regional groupings of populations; SGBR, NGBR, Gulf of Carpentaria (GoC), and WAust (Fig. 1).

Methods

Initially, sampling for mtDNA analyses involved the collection of tissue from non-sibling hatchlings or embryos (destructive sampling), or blood sampling from nesting females (Norman et al. 1994a). Our microsatellite studies of green turtle populations relied primarily on those earlier samples. Currently we sample by non-destructive techniques, taking blood samples from the dorsal cervical sinus (Owens and Ruiz 1980) of nesting females or hatchlings. In situations where blood sampling was not feasible we collected small ($5mm^2$) skin biopsies from the shoulder region using a sharp knife or scalpel. Blood samples were immediately placed in a lysis preservative solution (FitzSimmons et al. 1995) and skin samples were placed in a solution of 20% dimethyl sulfoxide saturated with salt (NaCl). DNA extraction methods for blood typically followed a salting-out method (FitzSimmons et al. 1995). For extractions from skin and other tissue types, 1 mg of tissue was added to 1 ml of 5% Chelex 100 resin (Bio Rad Laboratories), heated at 60° for approx. 30 min., then at 90° for 5 min., and centrifuged for 10 min at 13,000 rpm. Approximately 250 ul of the supernatant was added to an equal volume of 10% Chelex and this diluted solution used in polymerase chain reactions (PCR) amplifications.

To determine mtDNA variability in both loggerhead and flatback turtles, we sequenced about 380 bp of the control region using primers TCR5 and TCR6 (Norman et al. 1994a) on samples from individuals representing the geographic range of nesting populations within

Australia. Norman et al. (1994a) performed a more comprehensive survey of mtDNA haplotypes for green turtles using restriction digests and denaturing gradient gels to screen a large number of individuals from each population. Comparisons to nDNA for green turtles involved screening large ($n \geq 50$) numbers of animals for microsatellite allele variation in each regional population at a minimum of 4 microsatellite loci. This involved PCR amplifications with alpha^{33}P incorporation and running the products on 6% sequencing gels (FitzSimmons et al. 1995). We also screened several individuals (mean n = 32) from separate regional populations (i.e. rookeries from different island or mainland locations) to assess allelic heterogeneity within regions (Fig. 1). The same microsatellite protocol was followed for loggerhead and flatback turtles, but our preliminary sample sizes ranged from 16 to 66 individuals per region.

The preliminary statistical analyses presented here show the results from Monte Carlo randomised chi square tests (REAP software, McElroy et al. 1992) of allele frequency differences among regional populations. Hardy-Weinberg equilibrium tests for green turtle populations were carried out in GENEPOP (Raymond and Rousset 1995). All tests involving multiple comparisons were corrected for significance level following the sequential Bonferroni method as outlined in Rice (1989).

Results and Discussion

Green turtle

Each of the four regional green turtle populations was shown previously to be significantly differentiated on the basis of mtDNA haplotype variation (Norman et al.

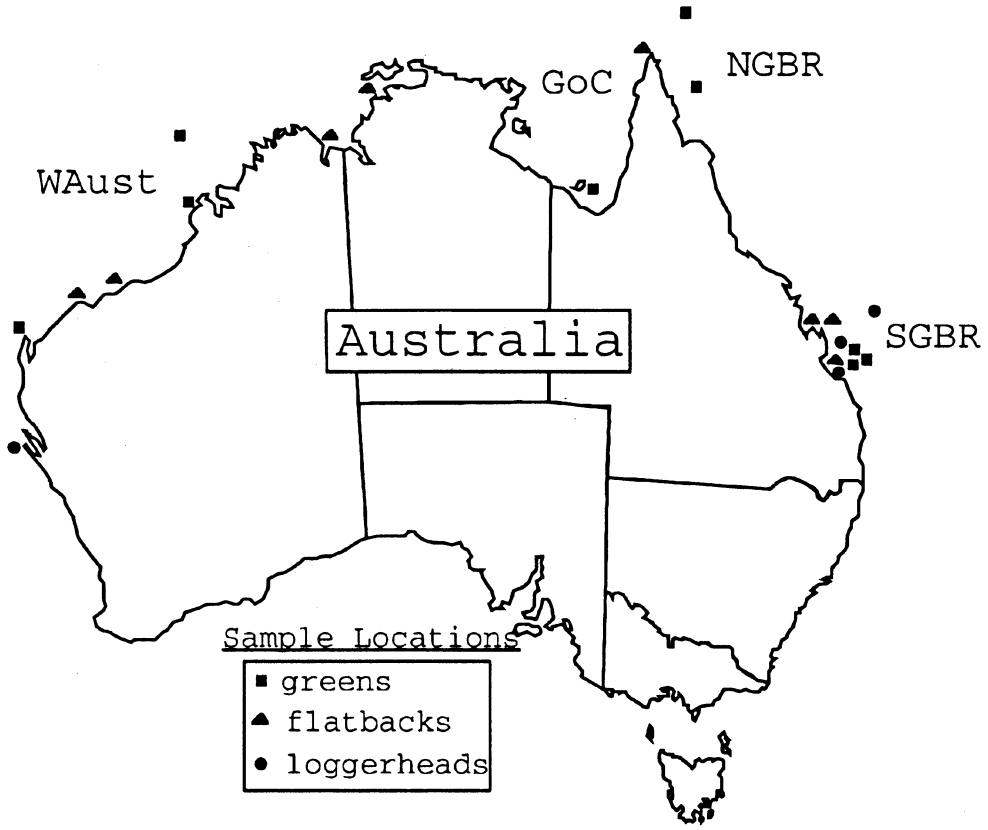

Figure 1. Sample locations around Australia for populations of green, loggerhead, and flatback turtles to analyse genetic structure within mtDNA control region sequences and microsatellite loci. Abbreviations are as follows: WAust, western Australia; GoC, Gulf of Carpentaria; NGBR, northern Great Barrier Reef; SGBR, southern Great Barrier Reef. See Norman et al. (1994a) for sample site locations of green turtle rookeries.

1994a) and pairwise percentage sequence differences were high, up to 6.98%. Three common haplotypes were observed, with several additional variants. Haplotypes A and B were both found in the SGBR and NGBR populations, but in very different frequencies. Haplotype C was found in the GoC and WAust populations, but with variants observed that were specific to each of the two regions (Norman et al. 1994a). No significant differentiation was observed among rookeries within regions.

Microsatellite analyses revealed patterns that were similar in most comparisons to those for mtDNA. The four loci studied were highly variable (8-42 alleles), with an observed heterozygosity of h = 0.74 - 0.97, and 15.4% of the observed alleles were unique to particular regions . Regional populations were within expectations of Hardy-Weinberg equilibrium at all loci when the significance level was corrected for multiple tests (n = 16 tests). Significant allele frequency divergence was observed among all regions at all loci, with $p < 0.001$ when corrected for multiple tests (n = 4 tests). Individual pairwise comparisons showed significant divergence in 18 of 20 tests (and 15 of 18 when corrected for multiple tests; n=24 tests) excluding comparisons between the NGBR and SGBR which never showed significant divergence (Table 2). Therefore, it appears generally that regional populations of green turtles maintain their identity as divergent populations even when gene flow accounts for both male and female contributions. The exception to this is a measurable amount of gene flow that has occurred between the SGBR and NGBR that may indicate male-mediated gene flow, either historic or current.

Loggerhead and flatback turtles

In contrast to green turtles, both loggerhead and flatback turtles were characterized by low mtDNA sequence variation (unpubl data and Bowen et al. 1995). Percent sequence differences between haplotypes was only 0.26% in loggerhead turtles (n = 74 individuals sequenced), and 0.78% in flatback turtles (n = 22 individuals sequenced). Only two haplotypes were identified in loggerhead turtles and these differed by a single base pair (bp) (unpubl data). Haplotype A predominated (98%) in the eastern rookeries while haplotype B was the dominant type (67%) in the western rookeries. We have identified 5 haplotypes to date in flatback turtles, with one common haplotype predominating in all regions. The less common variants, which differ by 1 or 2 bp, are possibly unique to each region, but sample sizes need to be increased to confirm this.

Microsatellite variability was moderately high in loggerhead turtles (5-15 alleles) and less so in flatback turtles (3-8 alleles). Regional divergence in allele frequencies was observed in 3 of 5 loci for loggerhead turtles (n = 4 tests, Table 2) and there was an indication of within region divergence at 3 loci (SGBR; in individual pairwise tests p = 0.005 - 0.0001). A substantial proportion (39.3%) of observed alleles (n = 56) were unique to either the eastern or western Australian populations. Preliminary results for flatback turtles indicated that allele frequency divergence was highly significant among regions at the 6 loci analysed ($p < 0.05$, n = 4 tests). In individual pairwise tests between regions,12 of 18 indicated significant allele frequency differences and when corrected for multiple tests 9 of 18 tests remained significant (Table 2). Of 31 alleles observed across 6 loci, 9 (29.0%) were unique to particular regions, though larger sample sizes are needed to increase statistical confidence.

While the results for loggerhead and flatback turtles are preliminary, it is apparent that strikingly different patterns are emerging for the three species. In the green turtles the fairly large number of mtDNA haplotypes observed has allowed the identification of 4 or 5 management units within Australia and at least 9 within the Indo-Pacific region (Norman et al. 1994a; Moritz 1994). The lack of strong regional divergence and lower genetic variability in mtDNA haplotypes among loggerhead and flatback populations probably reflects different colonisation patterns and smaller population size in comparison to green turtles. An alternative hypothesis of more recent gene flow among regional populations of loggerheads and flatback turtles is not supported by the divergence observed at microsatellite loci and the very high fidelity to nesting regions displayed by all three species (Limpus et al. 1984; Limpus 1985; Limpus et al. 1992; Parmenter 1993; Parmenter and Limpus 1995).

Table 2. Preliminary p values from pairwise comparisons of allele frequency divergence (using REAP; McElroy et al. 1992) between regional breeding populations in three species of marine turtle.

Population comparison		Locus					
Chelonia mydas		Cm3	Cm58	Cm72	Cc117		
# alleles		28	12	42	22		
WAust							
	Gulf	0.047	0.0020*	0.0030*	0.0030*		
	NGBR	0.0001**	0.0001**	0.12	0.0001**		
	SGBR	0.0001**	0.0001**	0.012	0.0030*		
Gulf							
	NGBR	0.084	0.0001**	0.022	0.0001**		
	SGBR	0.0001**	0.0001**	0.0040*	0.0001**		
NGBR							
	SGBR	0.25	0.34	0.89	0.16		
Caretta caretta		Cm72	Cc7	Cc117	Cc141	Ei8	
# alleles		5	15	13	8	15	
WAust		0.81	0.0001**	0.065	0.0001*	0.0001**	
	EAust						
Natator depressus		Cm3	Cm58	Cm72	Cc7	Cc117	Ei8
# alleles		8	3	5	7	4	4
WAust							
	NAust	0.53	0.0001**	0.030	0.091	0.86	0.51
	EAust	0.0001**	0.60	0.0012*	0.0001**	0.041	0.0009*
NAust							
	EAust	0.0001**	0.0001**	0.088	0.0001**	0.0060	0.0013*

* denotes significance level of $p<0.05$ and ** denotes $p<0.01$ when corrected for multiple tests following Rice (1989): *C. mydas*, n=24 tests; *C. caretta*, n=5 tests; *N. depressus*, n=18 tests.

For loggerhead and flatback turtles, it appears that variation in microsatellite allele frequencies will offer a more recent view of population divergence which is likely occurring within an ecological time frame. Not only is the use of microsatellites offering insights into male-mediated gene flow within marine turtle populations, they are also helping us to identify population divergence when mtDNA variability is limited.

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Conservation genetics of Atlantic and Mediterranean green turtles: inferences from mtDNA sequences

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Introduction

Patterns of variation of mitochondrial (mt) DNA have been used extensively for the study of population genetic structure, phylogeographic arrangements, and other aspects of molecular ecology of various organisms (for reviews see Avise 1994; Moritz et al. 1987). More recently, much attention has also been given to the application of mtDNA markers in the management of endangered or threatened species. In many cases, mtDNA studies have delineated the structure of populations and thus, have provided guidance into the level at which management priorities should be set for the protection of a particular species. In the case of marine turtles, mtDNA surveys of breeding colonies have focused primarily on the delineation of demographically independent population units with significance for conservation. Thus, genetic studies of population structure with conservation implications have been conducted for green turtles (Bowen et al. 1992; Norman et al. 1994; Encalada et al. 1996), loggerhead turtles (Bowen et al. 1993a), hawksbills (Broderick et al. 1994; Bass et al. 1996), and leatherbacks (Dutton 1995). The primary conservation implication of such studies has been that genetically independent breeding colonies should be managed on a colony-by-colony basis, and should be protected against overexploitation by humans or against extirpation by natural causes. The low level of maternal gene flow among rookeries suggests that the extinction of a rookery will not meet with natural replenishing from females from other rookeries in ecological time frames meaningful to conservation plans.

Another aspect of the genetic analysis of populations, has been the quantification of genetic variability. Assessment of gene diversity of declining populations has been of overwhelming concern to managers, because of the stipulation that an inevitable consequence of reduced populations is the loss of genetic variation via genetic drift. Also, with decreasing number of individuals in a population, the number of homozygotes increases and this may be associated with a reduction of individual fitness (Allendorf & Leary 1986). Thus, the conservation of variation (including of gene pools) has been a primary goal of many conservation efforts (Hoelzel 1992; Ralls et al. 1986).

Genetic variation detected at particular loci (for example mtDNA), however, reveals variation only for this part of the genome, and does not reflect the level of diversity related to traits that are involved in adaptation or individual fitness (Milligan et al. 1994). In fact, the lack of evidence of a direct connection between the variation detected from specific marker loci and those determining fitness (Whitlock 1993), has led to controversies regarding the importance of genetic considerations for conservation (see Milligan et al. 1994). This has led to suggest that basing management priorities on within-population mtDNA diversity is inappropriate (Moritz 1994).

While this may be the case, the importance of the study of demographic processes of small populations is not debated (Goodman 1987; Grant & Leslie 1993; Thomas 1990). In fact, detailed information on the role of demographic processes of small colonies, within the broader context of species dynamics, might provide the most relevant information for conserving the species and for conservation biology as a whole (Wade & McCauley 1988). These processes might include the study of inbreeding depression, mating systems, effective population size, and effects of

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population subdivision (Lande & Barrowclough 1987; Peters et al. 1990; Ralls et al. 1986).

Here we review the genetic structure of nine populations of the green turtle (*Chelonia mydas*) in the Atlantic Ocean and Mediterranean Sea based on mtDNA sequences of the control region (for expanded treatment see Encalada et al. 1996). This study extends earlier analyses of maternal population structure with restriction fragment length polymorphisms (RFLPs: Bowen et al. 1992), and compares the levels of resolution provided by both sequence and restriction-based data sets (i.e., level of mtDNA diversity and phylogenetic patterns detected by both approaches). The resolution of mtDNA diversity in this group can prompt a reappraisal of conservation priorities for this endangered reptile. I illustrate the management implications of this data set to the Atlantic-Mediterranean system by looking at the consequences of imminent rookeries extinctions of two highly threatened populations.

Materials and Methods

A total of 147 samples was surveyed throughout the Atlantic Ocean and Mediterranean Sea, including individuals from Hutchinson Island, Florida; Quintana Roo Mexico; Tortuguero, Costa Rica; Aves Island, Venezuela; Matapica, Surinam; Atol das Rocas, Brazil; Ascension Island, U.K.; Pailoa, Guinea Bissau; and Lara Bay, Cyprus (Table 1). One individual from Queensland, Australia was sequenced for outgroup comparison. Whole genomic DNA was isolated from blood or tissue samples and PCR amplified for an area of 510 bp of the control region according to protocols described in Encalada et al.(1996).

Table 1. Distribution of green turtle haplotypes in the Atlantic ocean and Mediterranean Sea. The Roman numerals represent mtDNA haplotypes. Asterisk denotes an individual heteroplasmic for an additional site change at site 167.

Haplotype	Florida	Mexico	Costa Rica	Aves	Surinam	Brazil	Ascension	Guinea Bissau	Cyprus
I	11	7							
II	1								
III	12	5	14	1					
IV			1						
V		1		7	13				
VI					1				
VII					1				
VIII						8	16	19	
IX						5	1		
X							3		
XI						1			
XII						2			
XIII									9*
XIV									1
XV		1							
XVI		1							
XVII		2							
XVIII		3							

Table 2. Haplotype (*h*) and nucleotide (π) diversities for nine populations of green turtles.

POPULATION	HAPLOTYPE DIVERSITY (h) ± std. error	NUCLEOTIDE DIVERSITY (π)
FLORIDA	0.56 ± 0.047	0.0013
MEXICO	0.82 ± 0.058	0.0057
COSTA RICA	0.13 ± 0.11	0.00028
AVES ISLAND	0.25 ± 0.18	0.0053
SURINAM	0.26 ± 0.14	0.00056
BRAZIL	0.68 ± 0.085	0.0017
ASCENSION ISLAND	0.35 ± 0.12	0.00077
GUINEA BISSAU	0.00	0.00
CYPRUS	0.22 ± 0.16	0.00042
OVERALL	0.83	0.0050

Sequences were obtained by cycle-sequencing reactions (manual and automated) and technical procedures are described elsewhere (Encalada et al. 1996). Sequences were aligned by eye and haplotypes labelled with Roman numerals (Table 1). Estimates of within population genetic variation were obtained for each one of the nine colonies and for the overall survey in the form of haplotype and nucleotide diversities, using equations 8.4 and 10.5, respectively of Nei (1987).

Frequency comparisons between pairs of colonies was calculated by chi-square analysis using the program CHIRXC by Zaykin and Pudovkin (1993). Estimates of nucleotide sequence divergence (*p* values) between mtDNA genotypes were calculated with the Kimura two-parameter method (Kimura 1980), and the resulting distance matrix was clustered using the UPGMA algorithm (Sneath and Sokal 1973) and the neighbor-joining method (Saitou & Nei 1987), available in MEGA (Kumar 1993). Maximum parsimony analysis using heuristic searches and bootstrapping was also performed with PAUP (Swofford 1993).

Results and Discussion

The control region sequences were aligned for 487 bases. A total of 20 polymorphisms were found at 19 polymorphic sites, corresponding to 17 transitions, two transversions, and one 10-bp repeat. Based on these control region polymorphisms, 18 distinct haplotypes were recognized among the 147 individuals from nine assayed populations (Table 1).

Measures of within-population variation, as determined by haplotype and nucleotide diversities are presented in Table 2. The highest haplotype diversity value was observed in the Mexican population (h = 0.82), similar to the overall diversity estimate (h = 0.83). Nucleotide diversity was highest for Mexico and Aves Is., the colonies in our survey with among the lowest current population sizes (as indicated by number of nesting females per year). Overall haplotype diversity was h = 0.830, slightly higher than that reported for loggerhead turtles (0.732; Bowen et al. 1993a), and comparable to that for the Atlantic hawksbill (0.849; Bass et al. 1996).

Frequency comparisons between pairs of populations revealed significant differences distinguishing colonies as independent genetic units. Only three pair-wise comparisons were not significant (see Encalada et al. 1996).

Figure 1 shows a graphic comparison of RFLP (from Bowen et al. 1992), and control region (d-loop) data for Atlantic green turtles. In the case of sequence data, this UPGMA cluster (Fig. 1, right), as well as N-J and parsimony

analyses (not shown), revealed a geographic partition among Atlantic-Mediterranean green turtle haplotypes into two units: (A) Florida, Mexico, Costa Rica, Cyprus (and one individual from Aves Is.); and (B) Aves Is., Surinam, Brazil, Ascension Is., Guinea Bissau (and one individual from Mexico). These partitions were not apparent in the RFLP study (Fig. 1, left). Furthermore, in most cases, the control region sequences divided RFLP haplotypes into additional units (14 versus eight haplotypes for d-loop and restriction data sets, respectively), not taking into account four new Mexican haplotypes added to the sequencing survey. One exception is the distinction of Guinea Bissau haplotypes in the restriction survey, which are indistinguishable from Brazilian and Ascension Is. haplotypes in the d-loop survey. Percent sequence divergence for the sequence data showed an approximate six-fold increase over the restriction assay.

The higher level of resolution produced by the analysis of control region sequences may be attributed to the higher rate of substitution in the noncoding control region (but see Bernatchez & Danzmann 1993). This difference in resolving power is apparent in our data from the six-fold increase in sequence divergence (ratio of mean percentage sequence divergence: 4.4/0.7), produced by the control region sequence variation, as compared to the RFLP analysis over the entire mitochondrial genome (Fig. 1). In a similar comparison, Norman et al., (1994) found an eight-fold increase in the divergence of the control region of *C. mydas* relative to RFLP analyses. These observations compare to earlier reports by Lahanas et al., (1994), in which a linear relationship between net divergences of control

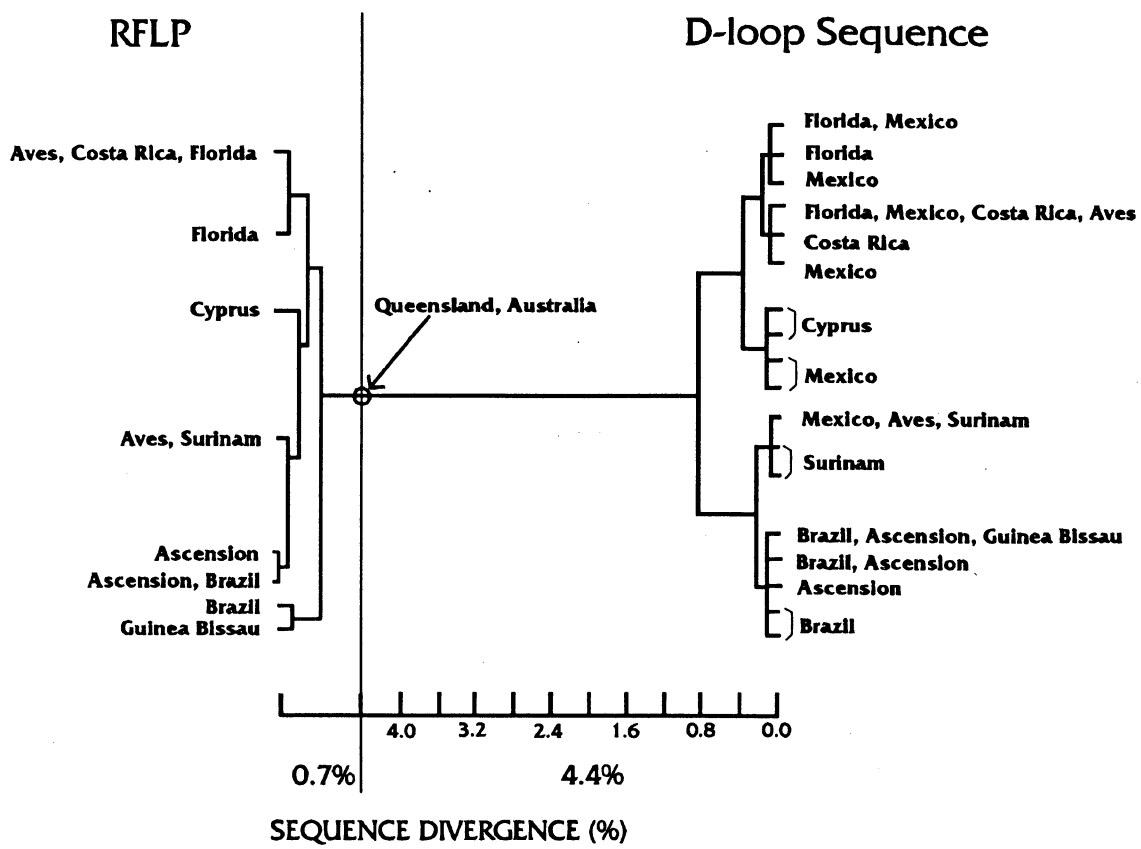

Figure 1. Phenetic analyses of mtDNA RFLPs and mtDNA control (d-loop) region sequences in Atlantic green turtles. (Left) UPGMA analysis of RFLPs from 136 Atlantic green turtles [redrawn from Bowen et al. (1992)]. (Right) UPGMA analysis of genetic distances of the control region of 147 Atlantic green turtles based on Kimura's two-parameter correction with a transition: transversion ratio of 8.5: 1. Both data sets are anchored to an individual from Queensland, Australia.

region sequences and those based on restriction site data yielded an estimated eleven-fold increase in resolution of control sequence data. Using a molecular clock of 0.2-0.4% divergence per million years for the Testudines (Avise et al. 1992; Bowen et al. 1993b), our 6-fold increase corresponds to an evolutionary rate of about 0.012-0.024 mutations/site/million years, which given a sequence divergence of 0.8% in turn positions the deepest phylogenetic fork within Atlantic green turtles at about 0.3-0.7 mya.

Overall, the present survey detected variation over 2.9%of the mtDNA genome (492/ 16700, taking 16700 as the estimated size of the green turtle mtDNA genome [Norman et al. 1994]). The six-fold increase in resolution would require about 3000 bp of RFLP data to procure resolution comparable to the 487-bp sequence of this study. In summary, the mtDNA control region produces a higher degree of detectable variation relative to RFLP analysis over the entire mitochondrial genome. The increased sensitivity is apparent in the more detailed matriarchal phylogeny (Fig. 1).

Conservation Significance and Management Implications

Over the last four centuries green turtle populations have declined precipitously, a trend which shows no sign of abating (Pritchard 1980; King 1982). The geographic distribution of mtDNA lineages in green turtle nesting populations provides information relevant to management and conservation strategies for this endangered species. Moritz (1994) drew a distinction between evolutionary significant units (ESUs) and management units (MUs) which depends on the fixation of alternate alleles at multiple independent loci. The significant haplotype frequency shifts among Atlantic green turtle populations define each nesting population as a significant management unit (MU), providing a meter for identifying the appropriate geographic scale for monitoring and managing this species, i.e., on a rookery basis. Over evolutionary time, gene flow between colonies, perhaps due to lapses in natal homing, may contribute to the colonization of depleted or extirpated rookeries. The sharing of divergent haplotypes between East and West Caribbean rookeries indicates the rare transplantation of mtDNA haplotypes. However, colonies are not likely to be recolonized over a time frame meaningful to species recovery plans and thus should be considered demographically independent units. Several nesting populations in the Atlantic-Mediterranean system face extinction. Here we review the consequences of potential rookery extinction of two nesting aggregates on the mtDNA diversity of the entire Atlantic-Mediterranean system.

Cyprian and Mexican nesting beaches: Imminent extinctions?

The green turtle was once known to nest in abundance throughout parts of the eastern Mediterranean Sea, but is now restricted to the isolated beaches of the west coast of Cyprus, in the Lara area and in the adjacent coast of Turkey (Groombridge 1990). Although few historical records exist, this nesting population was probably much larger a few decades ago. The breeding population at Cyprus now is estimated to be about 100 females (Demetropoulos & Hadjichristophorou 1992). Despite the small population size, our mtDNA survey detected two haplotypes among the 10 individuals surveyed (representing perhaps 10% of the nesting population). Together, these exhibited an intrapopulation diversity of h =0.22 (haplotypic diversity), which is comparable to the diversities of larger colonies (e.g. those of Surinam and Aves Is., h =0.26 and 0.25, respectively; refer to Table 2), and is larger than that exhibited by the largest breeding aggregate in the Atlantic (Costa Rica, h =0.13). Furthermore, both haplotypes detected in Cyprus samples are apparently unique to Mediterranean nesting colonies.

The Mexican samples procured for this survey represent individuals from the proximal nesting beaches of X'Cacel (in the Central Littoral of Quintana Roo), and Isla Cozumel (in the northern Mexican Caribbean). Haplotype frequencies of individuals from these locales show no distinction between these two nesting aggregates, and are thus considered here as a single population. These locales are the most important green turtle nesting beaches in this area, containing the highest nest densities of all monitored nesting beaches along the eastern coast and insular area of the Yucatan Peninsula (Zurita et al. 1993). Our genetic studies confirm the demographic independence of this

population, and show the highest haplotypic diversity for this colony among all those surveyed in the Atlantic (h =0.82; refer to Table 2). The Mexican population also exhibits a high degree of haplotype endemism, with four haplotypes unique to this area (Table 2).

The genetic contribution of these two rookeries to overall mtDNA diversity in the Atlantic-Mediterranean system can be understood by examining the potential consequences of rookery extinction. The Cyprus nesting colony (with the adjacent coastline of Turkey) represents the only significant nesting habitat remaining for green turtles in the Mediterranean Sea. Extinction here would nearly extirpate the green turtle from an entire sea basin, and this is reason enough to merit a very high conservation priority. However, extinction would also remove two endemic haplotypes, or two of the 18 'twigs' in the UPGMA tree (Fig. 1, right). Extinction of the Quintana Roo nesting colony could remove four 'twigs'. Taken together, the extinction of these two nesting colonies could eliminate one third (six out of 18 haplotypes) of the mtDNA diversity detected in the Atlantic basin and would remove one of the four branches in the UPGMA tree. Based on the evidence from mtDNA haplotypes, the nesting colonies in Cyprus and Quintana Roo contain a significant portion of the genetic diversity for Atlantic green turtles. Both nesting populations are threatened with imminent extinction by habitat degradation, incidental fishery mortality, and development for tourist industries. Given this evidence, we stress the need for the implementation of management regulations which ensure the long-term maintenance of viable green turtle populations in Quintana Roo, Cyprus and elsewhere. These two nesting beaches represent reservoirs of biological diversity with ecological and evolutionary importance.

Conclusion

Although the management and conservation of Atlantic and Mediterranean green turtle rookeries cannot be solely based on the amount of diversity present in each colony, it is likely that the removal of such diversity (by extinction) will affect the structure of the broader Atlantic ESU. Further research needs to address the possible consequences of localized extinctions and/or decrease in rookery population size on the balance and dynamics of the system (from the ecological and evolutionary points of view). For example, how do demographic processes such as bottlenecks or declines in population size interplay in the evolutionary balance of the Atlantic lineage? What are the effects of subpopulation extinction and recolonization on the loss of genetic variability? The mtDNA sequence data presented here has already been used to propose possible phylogeographic patterns for Atlantic-Mediterranean green turtles (Encalada et al. 1996). A colonization event from the Western Caribbean colonies to the Mediterranean is proposed to have taken effect within the last 10,000 years. This suggestion is based on the close relationship observed between Cyprus and western Caribbean haplotypes. Without genetic information on Cyprus, such an assessment would not be possible.

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Application of isolation by distance models to hawksbill turtle (*Eretmochelys imbricata*) nesting sites in the Caribbean

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Introduction

Dispersal in terrestrial organisms is limited or controlled primarily by climate and physical barriers such as mountain ranges or rivers. Consequently patterns of genetic differentiation in terrestrial organisms may be reflective of both historical and contemporary vicariant events (Nelson and Rosen 1981). In contrast, the genetic structure of populations of marine organisms may be influenced primarily by behavioral or life-history characteristics coupled with a tremendous potential for dispersal in aquatic systems (Knowlton and Jackson 1993). Environmental influences such as ocean currents or thermal limitations may play an important role in the formation of genetic partitions among marine organisms (Palumbi 1992; Veron 1995) but do these factors produce predictable population boundaries? One challenge for wildlife managers and biogeographers lies in determining what factors - behavior, history, or environment - play a primary role in defining marine populations.

Molecular markers provided information on the migratory behavior of nesting hawksbill turtles, *Eretmochelys imbricata*, and the contributions of nesting colonies to regional foraging grounds (Broderick et al. 1994; Bowen et al. 1996). Past research indicates that nesting sites are isolated reproductive units and that natal homing appears to be the predominant mechanism of recruitment to nesting beaches (Bass et al. 1996). With the identification of management units (in this case, individual nesting colonies) populations can be managed more effectively. Analysis of foraging aggregates indicates that these cohorts are composed of individuals from nesting beaches throughout the region and are not composed solely of females or offspring from adjacent nesting beaches. One implication of these findings is that harvesting individuals on foraging grounds can directly impact nesting sites hundreds of kilometers away.

Analysis of hawksbill turtles in the Caribbean indicated that nesting sites at Buck Island, U.S. Virgin Islands and Gale's Point, Belize were not significantly different in terms of mtDNA haplotype frequencies. However, nesting sites geographically closer than USVI and Belize exhibited either fixed differences or significant haplotype frequency shifts. Two possible explanations could be suggested that would prevent the genetic differentiation of the nesting populations at Belize and Buck Island: ongoing gene flow, or relatively recent colonization by nesting females. Isolation by distance models predict a relationship between geographic distance and genetic divergence of populations (Wright 1943). Does the genetic structure of marine turtles correspond to an isolation by distance model? If not, can we identify other factors that would influence genetic divergence among nesting sites? What usefulness can predictive models have for assigning management priority?

Methods and Results

All data used to investigate these questions was generated during a survey of genetic diversity of nesting beaches in the Caribbean and western Atlantic (see Bass et al. 1996).

Gene flow estimates (M) were calculated from the phylogeny of alleles (haplotypes) and simulation results of Slatkin and Maddison (1989). This method of estimating gene flow uses the phylogeny of

haplotypes to determine the minimum number of migration event(s) between rookeries. The M value is a minimum estimate of the amount of gene flow required over time to produce the observed geographic distribution of the haplotypes. Geographic distances between sampled nesting sites were determined by straight line water distances calculated from a map of the Caribbean.

To test hypotheses concerning isolation by distance, M values were log-transformed and regressed against the log transformed values of geographic distance under different dispersal models: island model, island model restricted to nearest neighbors, one-dimensional stepping-stone, and one-dimensional stepping-stone model with direction predicted from phylogenetic relationships of haplotypes (Slatkin 1994).

In addition, matrix correlation methods were used to test for correlations between a geographic distance matrix and a matrix of M (Mantel 1967). Geographic distances were determined using the GeoDist progam and compared to migration estimates using the Mantel program (Legendre and Vaudor 1991).

M estimates (from Fig. 1A) for all possible pairwise comparisons are presented in Table 1. These estimates are the minimum amount of gene flow per generation expected based on the phylogenetic relationships of the haplotypes. All migration estimates were less than one except for the comparisons between Antigua and Puerto Rico and between Puerto Rico and Belize.

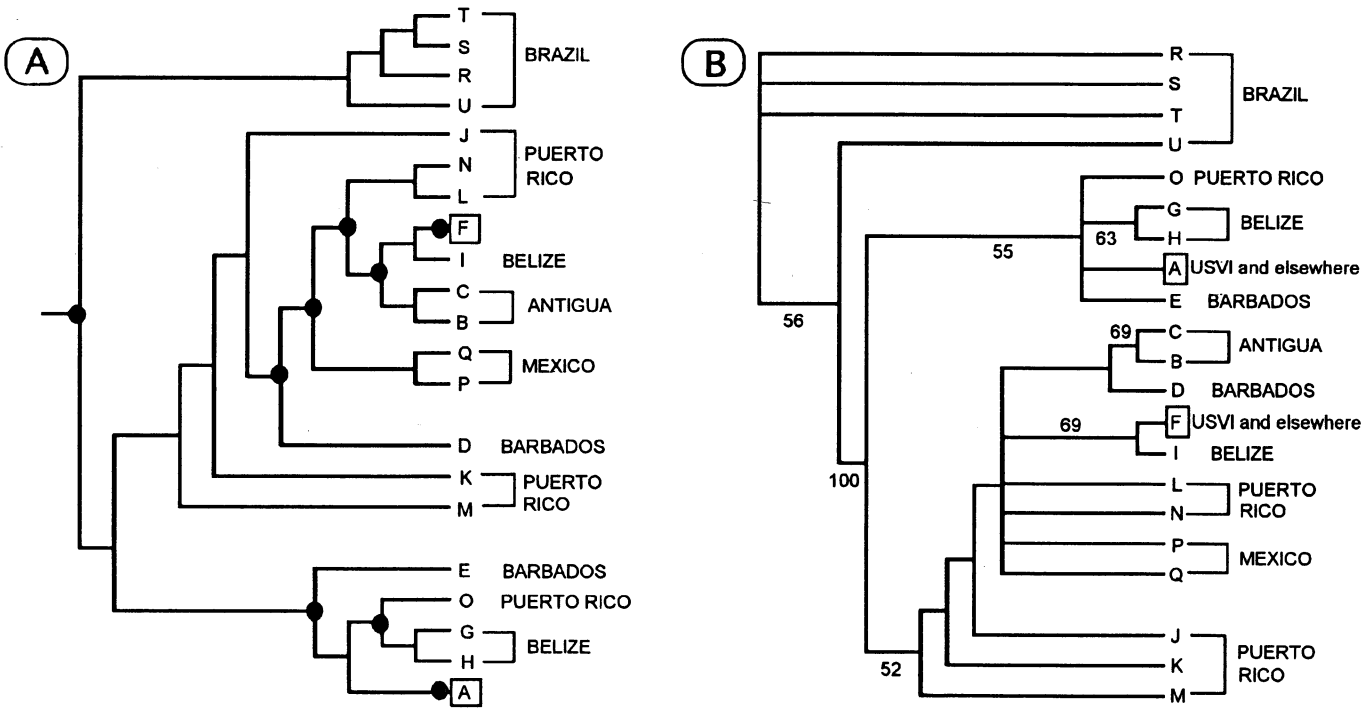


Figure 1. Letters indicate unique haplotypes and boxes around letters signify a haplotype which occurs in multiple rookeries. A) Fitch diagram based on Kimura two-parameter distances weighted with a four to one transition/ transversion ratio. Tree has been drawn with the Brazilian haplotypes as the root. Dots at nodes indicate migration events predicted using the method of Slatkin and Maddison (1989). B) 50% majority rule consensus tree from maximum parsimony analysis using PAUP. The tree is unrooted with the same weighting criterion as the Fitch diagram. All nodes were supported in 100% of the 96 trees found except for those nodes with number located above the branches. Values greater then 50% from bootstrap analysis (1000 replicates) are located below the branches.

Table 1. Above the diagonal: migration rate estimates (M) based on the cladistic method (Slatkin and Maddison 1989). Below the diagonal: Geographic distances in km calculated using the program GeoDist (Legendre and Vaudor 1991).

	Belize	Mexico	Puerto Rico	USVI	Antigua	Barbados	Brazil
Belize	- - - -	0.35	1.99	<0.10	0.35	<0.10	0
Mexico	555	- - - -	0.35	0	0.35	<0.10	0
Puerto Rico	2019	2065	- - - -	0.35	1.03	0.75	0.35
USVI	2337	2369	318	- - - -	<0.10	<0.10	<0.10
Antigua	2656	2674	637	318	- - - -	0.35	<0.10
Barbados	3015	3085	1020	724	463	- - - -	<0.10
Brazil	6474	6789	4937	4732	4542	4086	- - - -

Log transformed-geographic distances (k) were regressed against log-transformed values of migration rates (M). With the island model of migration, a slight but non-significant relationship was found between log (k) and log (M) (slope = -0.284, R2=0.081) (Fig. 2A) (Slatkin 1994). A one-dimensional stepping-stone model was then tested with the following as the actual gene flow pathway: Brazil - Barbados - Antigua - USVI - Puerto Rico - Mexico - Belize (Fig. 2B). A non-significant relationship was found using this model (slope = 8.798, R2=0.007). A simple island model using only nearest neighbors (Range 358 - 1075 km) was used, but a non-significant positive linear relationship was found (slope = 0.917, R2=0.148) (Fig. 2C). The last dispersal model tested was a modification of the one-dimensional stepping-stone model using the hypothesis generated by the PAUP 50% majority rule consensus tree (Fig. 1B). This model produced a negative linear relationship and the highest R2 of any test (slope = -1.747, R2 = 0.538), although not significant at p=0.05.

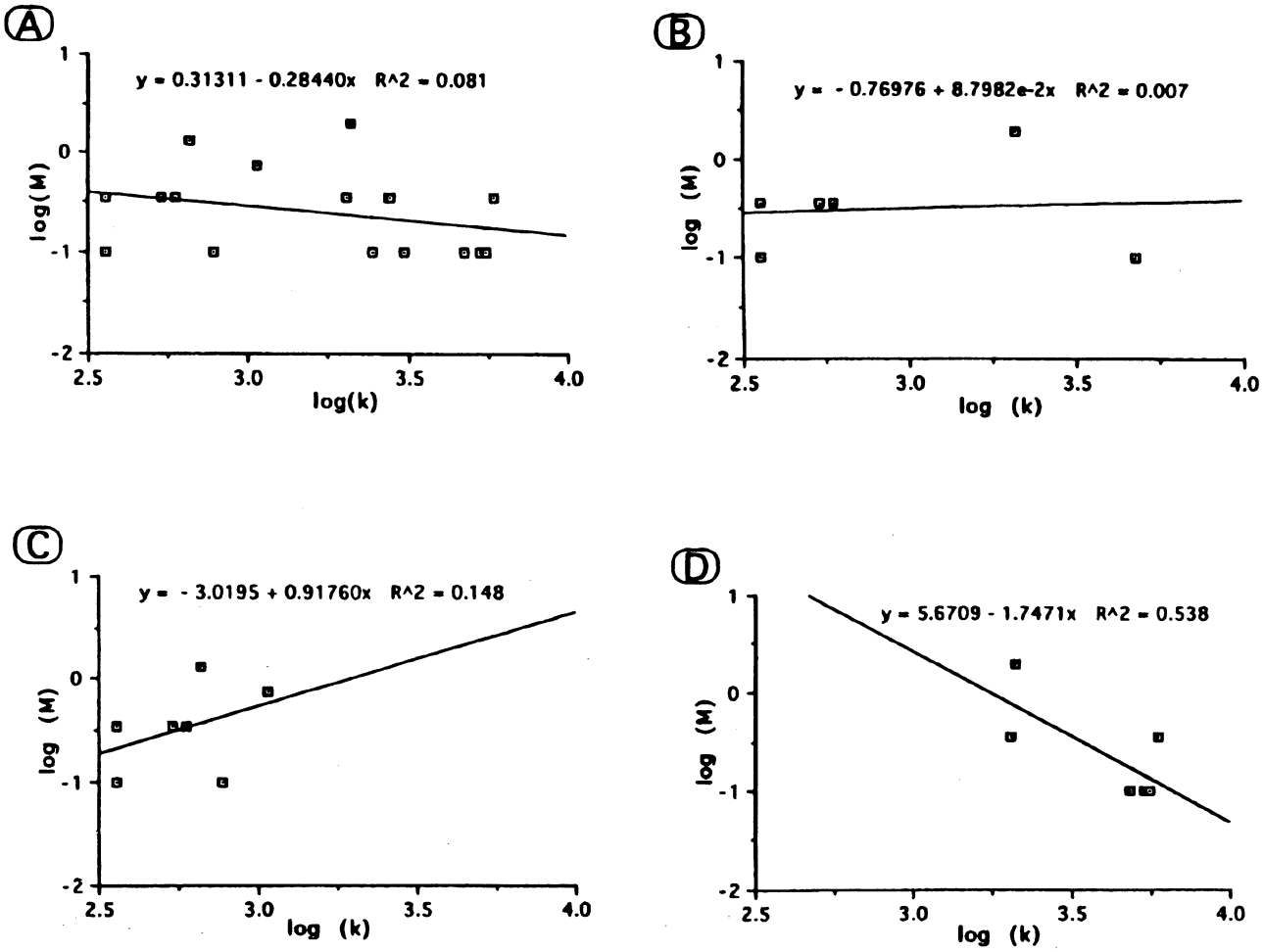
Comparison of the matrix generated using the Geodist program to the estimates of migration did not produce a significant correlation between geographic distance and genetic distance (r = 0.141, p250 permutations = 0.33).

Discussion

Slatkin and Maddison (1990) suggested that tests of isolation by distance can give an indication of whether gene flow is historical or relatively recent. If genetic similarity between populations is due to a relatively recent divergence from an ancestral population then the regressions would not be expected to take a simple form. If geographic distance is assumed to provide an indication of potential gene flow and if gene flow is the cause of genetic similarity, then a significant relationship between gene flow and geographic distance should result. Through an analysis of human population data, they found that the regression of log (k) vs. log (M) explained 0.1% of the variation in the data set. Based on these results, they concluded that the similarity of human mtDNAs was not due to ongoing gene flow, but rather a relatively recent dispersal of human colonizers.

Both the matrix correlation method and the regressions of log (k) and log (M) indicate that there is not a significant relationship between gene flow and geographic distance for hawksbill turtles. However, these data apply to mtDNA only and it remains to be seen if conclusions about female dispersal and gene flow can be applied to male hawksbill turtles (see FitzSimmons et al. 1996; Karl et al. 1992). Geographic distance explains only 0.7 - 50% of

Figure 2. Log-transformed regressions testing A) island model of migration, B) one-dimensional stepping stone model, C) nearest neighbor island model, D) dispersal hypothesis predicted from fig. 1B.



the variation found in mtDNA. Based on these results and the relatively young age of the area (Case et al. 1984), it could be argued that the genetic similarity of the USVI and Belize rookeries is not due to ongoing gene flow, but rather a relatively recent dispersal of turtles to these areas.

The findings are consistent with results from a similar analysis using estimates of distance and genetic divergence among Atlantic Ocean rookeries of the green turtle, *Chelonia mydas* (Bass, unpublished data). In addition, these results are qualitatively consistent with conclusions based on population surveys of other marine turtles, in which widely separated rookeries (South Africa and Greece for loggerheads, *Caretta caretta*; Oman and Galapagos for greens, *C. mydas*) are indistinguishable in terms of mtDNA haplotype data (Bowen et al. 1992, 1994). It is possible that the lack of a relationship between geographic and genetic distance may be a general paradigm for marine turtles.

Although isolation by distance is not evident in sea turtles, it has been demonstrated in other marine vertebrates. Stanley et al. (1996) examined mtDNA structure in continuous populations of the harbor seal, *Phoca vitulina*. From their research they concluded that significant genetic structuring is evident in both the Atlantic and Pacific oceans and that this structuring represents influences of behavior (philopatry in females) and possible topographic barriers such as the polar sea ice. Regressions of genetic differentiation (population subdivision) against geographic distance

detected significant relationships between geographic distance and genetic divergence. The genetic data appears to fit the predictive model of isolation by distance.

Since isolation by distance has been identified in other marine organisms, it is instructive to consider the differences between these animals and marine turtles. One of the major differences is that nesting sites are discontinuous and possibly ephemeral. For example, Pasture Bay Beach in Antigua exhibits changes in beach structure over the course of a single season and cumulative historical changes may be much greater. Behavior is also an important factor because hawksbills do exhibit natal homing and there is strong structuring of maternal lineages. Ocean currents play an important role in the dispersal of marine turtles, and recent research demonstrates that passive and active long-distance migrations may be quite common (Bowen et al. 1995; Carr and Meylan 1980).

In terms of conservation, the non-significant relationship between geographic distance and rate of migration (here used as an indication of degree of genetic divergence) indicates that population models utilizing linear relationships would not explain levels of divergence or similarity among hawksbill nesting sites. If one assumes that geographic proximity and vicariant effects drive the genetic divergence of populations then one could assume that distance would be a good measure for defining managment units. The genetic data for hawksbills indicates that distance between nesting sites would not be a reliable measure to use in defining reproductive units. Other environmental and behavioral factors apparently have a greater effect on the genetic divergence of marine turtle populations.

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Comparative biogeography of the green turtle (*Chelonia mydas*) and the loggerhead turtle (*Caretta caretta*) as inferred from mtDNA sequence comparisons

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Introduction

By the late nineteenth century, distributions of major vertebrate groups were sufficiently understood that theories about the relationships within and among regional faunas could emerge. From the inception of biogeography as a scientific discipline, a primary objective has been the investigation of predictable patterns in the distribution of taxa. Many factors have been considered, including geography, climate, dispersal potential, and evolutionary origins. However, progress in early investigations was impeded by a fragmentary understanding of historical changes in climate and geography. For example, early attempts to explain the similarity of South American and African faunas invoked a land bridge across the Atlantic Ocean. The emergence of plate tectonic theory in the 1960's greatly advanced the understanding of relationships among continental faunas (Briggs 1995). During the same interval, advances in geochemical and paleontological dating techniques allowed researchers to document historical changes in climate and the distribution of biota.

Another limitation in biogeography has been the uncertain evolutionary relationships among closely-related taxa. In defining the boundaries of biogeographic provinces, researchers relied on taxonomic classifications which tend to be especially controversial at and below the species level. However, the development of molecular genetic methods has provided biogeographers with an objective yardstick for evaluating evolutionary separations among regional faunas. By examining the DNA sequence divergence within and among closely-related morphotypes, researchers can circumvent the uncertainties surrounding taxonomic assignments and focus directly on evolutionary genetic relationships. This genealogical approach, labeled phylogeography (Avise et al. 1987; Avise 1994), represents a merger of biogeography, population genetics and molecular systematics.

Conservation biologists faced with pressing management decisions may also wish to circumvent taxonomic controversies. (This does not diminish the importance of taxonomy but reflects the urgency of an imminent policy decision.) In these circumstances, phylogeographic data may indicate the geographic scale of management units. Relatively complete (range-wide) surveys can also provide wildlife managers with an atlas of genetic diversity and cryptic evolutionary partitions. While genetic surveys should never be viewed as a surrogate for field studies and demographic data (see Lande 1988), DNA sequence data are widely accepted as a scientific criterion for definition of conservation priorities (Dizon et al. 1992; Moritz 1994).

In this report, the range-wide distribution of mtDNA diversity is reviewed in the green turtle (*Chelonia mydas*) and the loggerhead turtle (*Caretta caretta*). Both species are highly migratory and both feed in shallow coastal zones as adults. However, the former species is distributed almost exclusively in the tropics while the latter species nests and feeds primarily in warm temperate waters (Pritchard and Trebbau 1984). How might this difference in distribution influence the global phylogeography of green and loggerhead turtles? Temperate marine species tend to have less inter-oceanic genetic structure, as the

continental barriers (especially southern Africa and southern South America) are less formidable to temperate-adapted species. Does this generalization apply to highly-migratory marine turtles? If wildlife managers consider the preservation of genetic diversity as a conservation goal, then appreciation of the genetic separations between ocean basins may augment the scientific basis for marine turtle management.

Phylogeography of the Green turtle, *Chelonia mydas*

The herbivorous green turtle inhabits warm waters of the Mediterranean Sea and the Atlantic, Indian, and Pacific Oceans. This tropical distribution does not extend to the southern limits of South America or South Africa, such that movement between Atlantic and Indian Ocean basins may be limited by geographic barriers and contemporary climate. Many tropical species show a similar distribution, with sister taxa in equatorial waters of the Atlantic and Indian-Pacific regions. However, the cold water barrier around southern Africa is not impermeable, and movement of warm-water fauna from the Indian to the Atlantic Ocean is strongly implicated in recent evolutionary time (Briggs 1974). Furthermore, the tremendous dispersal potential of marine turtles must be considered in any biogeographic scenario.

The current taxonomy for *Chelonia* includes two forms. The green turtle *(Chelonia mydas)* occurs in the Mediterranean, Atlantic, Indian, west Pacific and central Pacific basins (Pritchard and Trebbau 1984). The black turtle (*Chelonia agassizi*) occurs only in the eastern Pacific, and is distinguished by darker coloration, smaller size, and some details of external morphology (Figueroa and Alvarado 1990). However, the species assignment for East Pacific turtles is controversial (Mrosovsky 1983; Bowen and Karl 1996). In the most complete morphological survey to date, Kamezaki and Matsui (1995) demonstrate subtle differences in skull morphology which prompt the authors to endorse a sub-species designation for the black turtle.

Do the evolutionary relationships inscribed in mitochondrial DNA sequences coincide with biogeographic provinces (Atlantic versus Indian-Pacific) or taxonomic designations (East Pacific versus Central and West Pacific, Indian, Atlantic and Mediterranean)? To examine the relationships among green turtles on a global scale, Bowen et al. (1992) surveyed 15 nesting colonies with mtDNA restriction fragment length polymorphisms (RFLPs). The results indicate two primary branches in an mtDNA phylogeny which corresponds precisely to Atlantic-Mediterranean and Indian-Pacific nesting colonies (Fig. 1). These results are exactly concordant with the biogeographic boundaries of tropical marine habitats, and inconsistent with the distinction of an East Pacific species (Bowen and Karl 1996).

The depth of the separation observed with RFLP analysis, p=0.7% sequence divergence, is low compared to other intraspecific comparisons of vertebrates (see Avise 1994). Nonetheless, this bifurcation is a primary feature of any phylogenetic analysis of green turtle mtDNA sequence data. Based on a provisional molecular clock derived from several marine turtle studies (approximately 0.2-0.4%/million years; Bowen et al. 1992), the separation of Atlantic-Mediterranean and Indian-Pacific green turtles may be on the order of 1.5-4 million years.

Phylogeography of the Loggerhead turtle, *Caretta caretta*

The carnivorous loggerhead turtle occurs in the Mediterranean, Atlantic, Indian, and Pacific Oceans, including a South African rookery within 1000 kms of the Atlantic Ocean (Dodd 1988). Nesting habitat for loggerhead turtles and green turtles overlap in some areas, but loggerhead turtles also occur in warm-temperate areas where green turtles are rarely observed. Deraniyagala (1945) described putative subspecies based on subtle morphological differences between Atlantic and Indian-Pacific forms (*Caretta caretta caretta* and *C.c. gigas* respectively) but recent reviews have disregarded these assignments based on overall morphological similarity (Pritchard and Trebbau 1984; Dodd 1988).

To examine the global phylogeography of loggerhead turtles, Bowen et al. (1994a) examined mtDNA RFLP diversity in 8 nesting colonies from across the distribution of *C. caretta*. The overall topology of a loggerhead mtDNA phylogeny is similar to that observed in green turtles, with two primary branches and a maximum divergence of p=0.9% sequence divergence (Fig. 1). One branch is observed primarily in the Indian-Pacific, and one branch is observed primarily in the Atlantic-Mediterranean. Based on the aforementioned molecular clock, the divergence between these two lineages corresponds to approximately 2-5 million years, possibly indicating a separation of loggerheads into two major ocean basins during Pleistocene and Pliocene epochs. However, the lineage observed primarily in Atlantic-

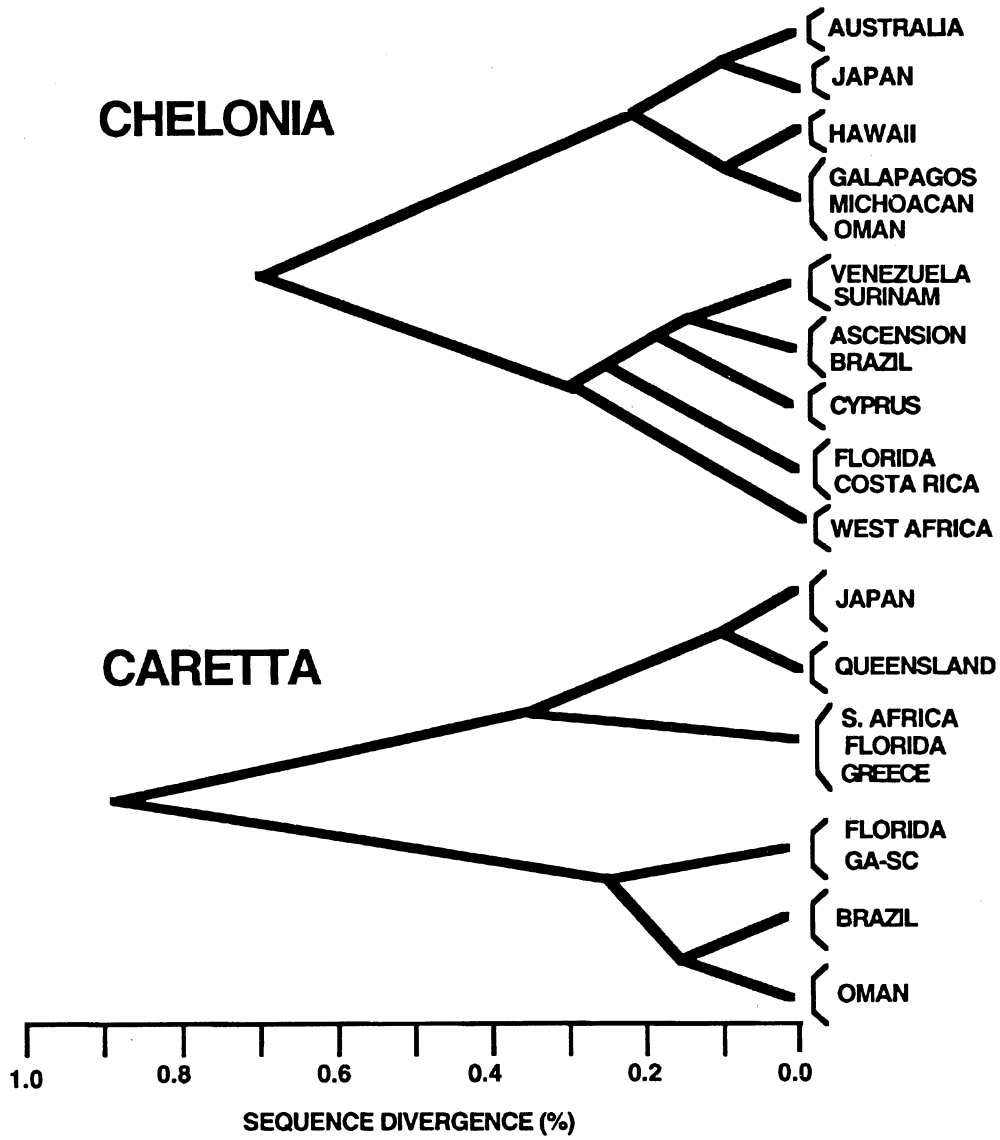

Figure 1. Phylogenetic trees summarizing the relationships among representative mtDNA haplotypes in the green and loggerhead turtles. In both species, a primary feature of the tree topology is a bifurcation estimated at 0.7% in green turtles and 0.9% in loggerhead turtles. In green turtles this bifurcation distinguishes Atlantic-Mediterranean from Indian-Pacific samples. In contrast, the two primary lineages in loggerhead turtles are found in both ocean basins. For complete data sets and methodology see Bowen et al. (1992, 1994a). Abbreviation: GA-SC = Georgia/ South Carolina U. S. A.

Mediterranean samples was present in a single nesting colony in the Indian Ocean (Fig. 1). The lineage observed primarily in Indian-Pacific samples includes one haplotype detected in Atlantic and Mediterranean nesting colonies. Recent colonization between the Indian Ocean and the Atlantic-Mediterranean is strongly implicated, probably via the waters around South Africa. The low diversity of transplanted mtDNA lineages in the putative "invaded" ocean basin may indicate that these colonization events occurred relatively recently, perhaps during the current interglacial interval (within 20,000 years).

In contrast to the green turtle phylogeny, which distinguished turtles in the Atlantic-Mediterranean from those in the Indian-Pacific Oceans, the two primary mtDNA lineages in loggerhead turtles were observed in both Atlantic-Mediterranean and Indian-Pacific samples. These data are consistent with the expectation that the temperate-adapted loggerhead turtle may more readily transplant between Atlantic and Indian Ocean basins. Indeed, a recent investigation of hatchling movement from the Tongaland (South Africa) rookery has demonstrated "leakage" of neonates from this Indian Ocean rookery into the South Atlantic (G.R.Hughes, personal communication). Perhaps these hatchlings, carried into the Atlantic through a narrow corridor of warm temperate water, are a source of Atlantic colonizers.

The Other Marine Turtles

The comparison of green and loggerhead mtDNA data demonstrate how differences in the ecology and geographic ranges of marine turtle species can influence their global population structure and evolutionary history. However, the surveys presented here include only two of the five globally distributed marine turtles. Range-wide analyses will be forthcoming for the other marine turtle species over the next few years, and preliminary data indicate that the biogeographic considerations outlined above may be relevant to leatherback, hawksbill and olive ridley turtles as well. For example, the leatherback turtle nests in the tropics but has been observed feeding in ice-laden waters of Labrador and the Gulf of Alaska (Goff and Lein 1988). Thus the leatherback turtle has an extremely wide range both in terms of geography and thermal tolerance. Under these conditions, continental barriers to movement between ocean basins may be negligible (see Dutton 1995, 1996). At the other extreme, the hawksbill turtle has a tropical distribution similar to that of the green turtle, and might be expected to have the characteristic "tropical" pattern of phylogeographic separations. A range-wide survey of hawksbill turtles by D. Broderick and colleagues will resolve this question in the near future (see Broderick et al. 1994; Broderick and Moritz 1996; Bass et al. 1996).

The olive ridley turtle occurs primarily in the tropics but may be an exception to the expected biogeographic pattern for tropical species. Based on a detailed examination of *Lepidochelys* morphology and distribution, Pritchard (1969) suggested that the olive ridley may have recently invaded the Atlantic Ocean via southern Africa. An initial comparison of *L. kempi* and *L. olivacea* (including two olive ridley populations from east Pacific and west Atlantic) is consistent with the recent Atlantic invasion (Bowen et al. 1991), and a range-wide survey of *L. olivacea* is underway to test this biogeographic scenario (Bowen and colleagues, unpublished data).

While ridley phylogeography may not conform to expectations based on tropical distribution, this exception raises an important point about the temporal scale of separations observed in other species. The seven extant marine turtle species have existed for millions of years, and some lineages (such as the leatherback and the green turtle) may have been evolving independently for tens of millions of years (Bowen et al. 1993; Dutton et al. 1996). Yet the separations observed between Atlantic-Mediterranean and Indian-Pacific populations of *Chelonia mydas*, *Caretta caretta*, and *Lepidochelys olivacea* probably date from a few thousand to a few million years. Over short evolutionary timescales (10^4 to 10^7 years) the barriers between ocean basins are clearly breached by sea turtles and other warm water species. For the purposes of conservation and wildlife management, green turtles in separate ocean basins may be considered separate evolutionary entities. However, wildlife

managers can also recognize that these evolutionary units will occasionally come into contact and coalesce, creating new genetic combinations that can redefine intraspecific genetic diversity.

Conservation and Management Implications

The intraspecific mtDNA genealogies in green and loggerhead marine turtles are remarkably consistent with expectations based on thermal tolerance, geography and climate over the last few million years. How can these microevolutionary considerations affect wildlife management strategies? At first glance, there would seem to be little connection between global phylogeography and practical conservation efforts. If marine turtle species are to persist and co-exist with human populations, the protection of nesting beaches and feeding grounds must proceed regardless of the level of genetic differentiation between ocean basins.

Perhaps the primary conservation value of these data lies in the appreciation of thorough natural history studies as a prerequisite for successful wildlife management. Experience indicates that conservation initiatives based on incomplete natural history information can be calamitous (Frazer 1992; Bowen et al. 1994b), and seemingly esoteric aspects of organismal biology or ecology (such as temperature dependent sex determination) make the difference between success and failure in wildlife management programs. In this context, the global phylogeography of marine turtles provides several perspectives that may indirectly influence conservation programs. For example, the intraspecific phylogenies presented here, and the ones in progress, provide a "yardstick" for interpretation of regional population genetic differentiation. Wildlife managers can understand the genetic separations between adjacent nesting colonies in the context of range-wide genetic diversity. Phylogeographic data can also strongly influence the course of systematic debates which have conservation implications. Subspecies designations have been proposed for most of the marine turtle species (reviewed in Pritchard and Trebbau 1984) and genetic data can corroborate or contradict these classifications. Genetic data have reinforced species-level designations for ridley (*Lepidochelys*) species and have prompted a re-evaluation of taxonomic assignments for *Chelonia* (Kamezaki and Matsui 1995; Dutton et al. 1996; Bowen and Karl 1996).

Additional applications of these data may be found in forensic studies. Relatively complete inventories of genetic diversity provide a basis for forensic classification of marine turtle products (Woodley and Ball 1996). In at least some cases, marine turtle DNA samples can be assigned to both species and region of origin (Encalada et al. 1994) based on range-wide genetic surveys. Comprehensive genetic surveys have also proven useful in the identification of cryptic evolutionary lineages in other vertebrate groups (Avise and Nelson 1989; Daugherty et al. 1990), and such findings can strongly influence conservation priorities. In this context, the genetic distinctiveness of the Raine Island green turtle rookery (Norman et al. 1994; FitzSimmons et al. 1996) may indicate a special conservation concern. Finally, maintenance of biodiversity requires preservation of genetic diversity, and range-wide genetic inventories provide the foundation for management of genetic resources.

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PCR-RFLP analysis of sea turtle populations in the era of DNA sequencing: is it still useful?

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Introduction

One of the most pressing requirements for management of wild animals is the ability to identify demographically independent breeding units, commonly referred to as "stocks" or "management units" (MUs; Moritz 1994). It is only when this becomes possible that managers can adequately monitor the spatial and temporal distribution of MUs among the various habitats utilized by organisms during their life cycle. In the case of marine turtles, this task is particularly daunting because of a migratory behavior which is prolonged during development and very extensive in the adult phase when they travel thousands of kilometers between breeding and feeding habitats (Carr et al. 1978).

As with many other species, technical advances in molecular biology have revolutionized the analysis of the genetic structure of marine turtle populations. Genetic techniques have demonstrated low (Avise et al. 1992), but geographically structured variation (reviewed by Bowen and Avise 1995). Of the various molecular techniques that have been utilized, differing mostly in their discriminative capacity and level of sophistication, sequencing of specific segments of the DNA, amplified via the polymerase chain reaction (PCR; Palumbi 1996), has provided by far the highest resolution. Improved discriminatory capacity has important management implications. For example, whereas extensive restriction fragment length polymorphism (RFLP) analysis of whole mtDNA revealed only four MUs among green turtle populations in the Indian and Western Pacific, sequencing of a 380+bp segment of the mitochondrial d-loop revealed eight times the amount of DNA sequence divergence and permitted the recognition of up to nine MUs (Norman et al. 1994a). The same trend has been reported for other species (e.g. Quinn, 1992) and has fueled increasing interest in the use of DNA sequencing as a technique for population genetic studies. When wildlife management needs are taken into account, i.e. large and recurrent surveys to monitor the temporal and geographic distribution of MUs (Norman et al. 1994b), the use of sequencing becomes a costly technology which could be an impediment for some situations and for countries sharing management responsibilities.

The application of PCR and sequencing technology facilitates direct comparison of genetic results across species and geographic distributions (Moritz et al. In press). Furthermore, now that mtDNA d-loop sequences

have been obtained for most major marine turtle rookeries (Abreu-Grobois et al. 1996) and breeding units have been shown to be identifiable by genetic markers within the d-loop (reviewed in Bowen and Avise 1995), an alternative assay method becomes possible: d-loop amplification, followed by RFLP analysis of the PCR products ("PCR-RFLP"). As long as the resolving power of RFLP remains the same or equivalent to that of sequencing analysis, this approach capitalizes on the reduced costs of RFLP analysis while retaining the discriminatory ability demonstrated by DNA sequencing. Furthermore, since the same locus is being analyzed, results from PCR-RFLP are directly comparable to sequencing information. PCR-RFLP analyses of populations of marine turtles have already been shown to be reliable tools for the analysis of variation within populations of green turtles in the Indo-Pacific (Norman et al. 1994a; for an overview see also Moritz et al. In press), loggerheads in Chesapeake Bay, Virginia, USA (Norrgard and Graves 1996), and hawksbills in feeding grounds and rookeries in Cuba and Yucatan, Mexico (Espinoza et al. 1996).

In this paper, we analyze the distribution of recognition sites for known restriction enzymes (REs) in available marine turtle d-loop sequences (Abreu-Grobois et al. 1996) to test the feasibility of PCR-RFLP as a tool to discriminate between sequence-based haplotypes. Specifically, we want to know if it is possible to distinguish between sequence-based haplotypes on the basis of expected restriction fragment profiles, and if the resulting resolution affords sufficient ability to distinguish between breeding populations on the basis of RFLP analysis alone.

Should the PCR-RFLP method prove feasible it could provide a powerful tool for the monitoring of temporal and geographic variation of MUs at both rookeries and migratory or feeding areas, but at significantly reduced costs. Mixed stock analyses using sequencing techniques have been conducted on green turtle feeding grounds in the Atlantic (P. Lahanas, K. Bjorndal, A. Bolten, unpublished data), loggerhead turtle migratory routes and developmental habitats in the Pacific (Bowen et al. 1995), and hawksbill turtle feeding aggregations in the Caribbean (Bowen et al. 1996).

Methods

Sequence sources

mtDNA d-loop sequences, particularly those derived from surveys with large geographic coverage, were selected from Abreu-Grobois et al. (1996). These include data sets derived from Indo-Pacific (Norman et al. 1994a) and Atlantic-Mediterranean (Lahanas et al. 1994; Encalada et al. 1996) green turtle populations, Caribbean and W. Atlantic (Bass et al. 1996) hawksbill turtle populations, Atlantic (Dutton et al. 1996) and Pacific (Bowen et al. 1995) loggerhead turtle populations, and W. Atlantic, Caribbean and Pacific leatherback turtle populations (Dutton 1995). Two haplotypes from loggerhead populations in the Atlantic-Mediterranean were included since they contain the only available d-loop sequences for the species in that region (Laurent et al. 1995). Three hybrid loggerhead-hawksbill haplotypes found in the Bahia, Brazil population were also analyzed (Bass et al. 1996).

Exploratory RFLP analysis

Recognition sites for known restriction enzymes (REs) in the mtDNA d-loop haplotype sequences were searched for utilizing the "Map" routine of the GCG program (Wisconsin Sequence Analysis Package, Genetics Computer Group 1994; tests for 209 REs) or the WWW on-line "Webcutter" program (Heiman 1995; tests for 412 REs). Position of restriction sites were all specified as the number of bases from the sequence's 5' end (these do not correspond directly to the aligned positions specified in Abreu-Grobois et al. 1996). Restriction sites which were found to be polymorphic and could be used to discriminate between haplotypes of the different population groups ("informative" sites) were selected and, for these, the expected fragment profiles (in bps) were determined according to the locations of the enzyme's cutting sites. Haplotype sequences were classified according to their expected restriction profiles ("restriction enzyme pattern") to be compared to the sequencing classification (see Figures 1-5).

It should be noted that the length of the sequences reported depends on the primers used. Allard et al.'s (1994) primers generate a sequence about 130 bp's longer at the 5' end than Norman et al.,'s (1994a); see Abreu-Grobois et al. (1996). If different primers are used to generate any of the haplotype sequences analyzed here, the restriction site positions will change accordingly.

Results and Discussion

Pacific loggerheads

Nine REs (*Ace* III, *Alu* I, *Bsl* I, *BsmF* I, *CjePE*, *CjeP* I, *CviJ* I, *Hae* III, *Sau*96 I) with polymorphic recognition sites were identified. However, because of the small number of sequences in question, *Alu* I with either *Sau*96 I or *Hae* III are sufficient to distinguish between the three known d-loop sequence haplotypes (two from Japanese rookeries, one from Australian rookeries, see Fig. 1) reported for Pacific loggerhead populations (Bowen et al. 1995). Because of the very clear differences, individual turtles from these populations can by ascribed to natal origin with confidence.

The finding of RE sites on mtDNA d-loop amplification products, permitting an easier and cheaper alternative to sequencing methods for the identification of loggerhead natal origins in the Pacific, would be of great benefit to conservation practices. Already the same approach (selection of suitable REs from analysis of d-loop sequences) has been demonstrated in surveys of loggerhead feeding grounds in southeastern U.S.A. (Norrgard and Graves 1996). A PCR-RFLP approach would be particularly attractive for the determination of stock composition of incidental turtle catches in the Pacific pelagic longline fishery. In view of the current critical condition of the Australian loggerhead breeding population (Limpus and Couper 1994) and the potential for considerable impact by this fishery (Bowen et al. 1995), monitoring is a necessary and high priority (Bolten et al. 1996).

Leatherbacks

Six REs (*Bfa* I, *BsrD* I, *CviR* I, *NSi* I, *Spe* I, *Ssp* I) were found to have polymorphic recognition sites in the leatherback d-loop sequences analyzed. Of these, a set of five

d-loop sequencing haplotype[1]	RE pattern	Expected fragment lengths for different restriction enzymes on sequences generated using Norman et al.'s (1994) PCR primers (lengths in bp)									Sequencing haplotype frequencies for *C. caretta* populations in the Pacific[1]			
											Rookeries		Migration routes and feeding grounds	
		AluI			Sau96I*		HaeIII*				Aust.	Japan	N. Pacif.	B. Calif. (Mex.)
pos. of polym. site:		188			245		245							
CC-A	1	78	272		245	105	245	39	61	5	**1.00**	-	0.03	0.08
CC-B	2	78	110	162	245	105	245	39	61	5	-	**0.88**	0.82	0.73
CC-C	3	78	272		350		284		61	5	-	**0.12**	0.15	0.19

[1] Haplotype designation and sequence data taken from Bowen et al. 1995.

Figure 1. Expected restriction fragment lengths for Pacific *Caretta caretta* d-loop sequences and population haplotype fequencies at various habitats. Figures in bold represent haplotype-specific unique fragment patterns or haplotype frequencies of non-shared haplotypes. Aust= Mon Repos and Swain Islands, Queensland, Aust; Japan= Wakayama Prefecture and Ryuku Archip., Japan; N. Pacif= taken from pelagic fishery in N. Pacific; B. Calif= W. coast of Baja California, Mexico. Asterisks represent alternative RE's with equivalent discriminatory capacity.

REs is sufficient for maximal discrimination, capable of distinguishing seven out of the eight sequence haplotypes (88%). Two sequence haplotypes, however (DC-D and DC-F), become confounded into a single RE pattern ("4", Fig. 2).

A PCR-RFLP approach developed along these lines shows promise as an economic alternative to sequencing for stock analysis of leatherbacks, since it resolves most of the haplotypes that have been reported for this species (Dutton 1995). However, the extent to which the same haplotype occurs at high frequency in different nesting colonies worldwide will make it difficult to ascribe individual turtles to regional stocks based on sequence data alone (Dutton 1995, 1996). These circumstances suggest that the use of multiple markers, including a combination of mtDNA and microsatellite loci, may be necessary.

Furthermore, efforts are currently underway to determine the haplotypes present in key nesting populations in the Pacific, which have all undergone drastic declines (Chan and Liew 1996; Sarti et al. 1996), in order to verify the capacity to identify stocks impacted by the incidental take of pelagic longline, driftnet and other fisheries operating in the north Pacific (e.g. Diaz-Soltero 1995; Wetherall et al. 1993). The results presented here suggest the PCR-RFLP approach can distinguish one haplotype present in the Malaysian population that has not been found in Pacific Costa Rica (DC-A, Dutton 1995, 1996), and three rare haplotypes found in Pacific Costa Rica but not Malaysia. The most common haplotype (DC-D) occurs both in Malaysia and Pacific Costa Rica, and though it is indistinguishable using restriction fragments from one of the rare haplotypes in the Costa Rica rookery (DC-F), the haplotype frequencies are sufficiently differentiated to permit distinction with statistical analyses (e.g. maximum likelihood methods; Pella and Milner 1987) provided suitable sample sizes are available (see Chapman 1996).

These are preliminary results based on relatively small sample sizes. It will be necessary to continue to sequence individuals from the other key nesting beaches in the Pacific (work currently in progress) to ensure that an RFLP approach reveals diagnostic haplotypes not yet identified.

Atlantic and Mediterranean green turtle sequences

Twenty-four REs (*Aci* I, *Alu* I, *Apo* I, *BsrF* I, *BsrG* I, *Cje* I, *Cje* I, *CviJ* I, *CviR* I, *Hae* III, *Hinc* II, *Hpa* I, *Mae* III, *Mse* I, *Msl* I, *Msp* I, *Mwol*, *Psp*1406 I, *Rsa* I, *Sau*96 I, *SfaN* I, *Ssp* I, *Tsp*509 I, *UbaC* I) were detected with polymorphic recognition sites in Atlantic and Mediterranean green turtle d-loop sequences. A set of seven of these REs were selected with which 16 out of the 18 sequence variants can be discriminated (89%; Fig. 3).

Some individual sequence haplotypes could be distinguished on the basis of unique, single RE recognition site patterns (CM-2, CM-10, CM-11, CM-15). On the other hand, one of the sequences (CM-7) features a 10-bp duplication inserted in position 462 which causes a 10-bp length increase in some of the expected fragments (i.e. from *Apo* I, *BsrG* I REs) and a loss of a *Mse* I restriction site around position 478 (Fig. 3).

In spite of slight reductions in resolving power when compared to the sequencing results, the discrimination among these haplotypes allowed by RE recognition site makes the PCR-RFLP technique potentially useful in addressing important conservation issues in *C. mydas*. Of particular interest, for example, could be analyses of green turtle feeding aggregations in the Atlantic-Mediterranean region, such as Lahanas and colleagues' (unpublished data) study in the Bahamas. The resolution demonstrated here suggests that all seven regional nesting populations can be distinguished entirely by PCR-RFLP methods. In some cases, because of the sharing of same RE haplotypes between some rookeries (e.g. RE patterns A, C, D) statistical analysis would be required to resolve rookery contribution to a mixed stock. However, it is notable that in most cases even the haplotypes generated based on RE profiles appear to occur at frequencies which differ by more than 30% among the rookeries. This level of differentiation is considered sufficient to permit reasonably accurate estimates of mixed-stock composition (Broderick 1992).

Figure 2. Expected restriction fragment lengths for Pacific, Caribbean and W. Atlantic *Dermochelys coriacea* d-loop haplotype sequences. Shading represents sequence haplotypes with common RE profile.

sequencing haplotype[1]	*RE pattern*	**Expected fragment lengths for different restriction enzymes on sequences generated using Allard et al.'s (1994) PCR primers (lengths in bp)**																	
		BfaI					BsrDI			NSiI		SpeI			SspI				
pos. of polym. site:				189			72	230		181		188					274		
DC- A	1	118	11	60	165	142	72	424		496		353		143	126	40	108	75	147
DC- B	2	118	11	60	165	142	72	424		496		188	165	143	126	40	108	75	147
DC- C	3	118	11	225		142		496		181	315	353		143	126	40	183		147
DC- D	4	118	11	225		142	72	424		181	315	353		143	126	40	183		147
DC- F	4	118	11	225		142	72	424		181	315	353		143	126	40	183		147
DC- E	5	118	11	60	165	142	72	424		181	315	353		143	126	40	183		147
DC- G	6	118	11	225		142	72	158	266	181	315	353		143	126	40	183		147
DC- H	7	118	11	225		142	72	424		496		353		143	126	40	183		147

[1]haplotype designation and sequence data taken from Dutton (1995)

Figure 3. Expected restriction fragment lengths for Caribbean, Atlantic, and Mediterranean *Chelonia mydas* d-loop haplotype sequences and population haplotype frequencies at various habitats. Figures in bold represent haplotype-specific unique fragment patterns or haplotype frequencies of non-shared haplotypes. Shading represents sequence haplotypes with common RE profile. Haplotype designations are according to source. FLA= Hutchinson Island, Fla.; MEX= X'Cacel, Mexico; C.R.= Tortuguero, Costa Rica; AVES= Aves Is., Ven.; SUR= Matapica, Suriname; BRAZ= Atol das Rocas, Brazil; ASCE= Ascension Is., UK; GUIN= Pailoa, Guinea Bissau; CYP= Lara Bay, Cyprus.

seq.	RE	Expected fragment lengths for different restriction enzymes on sequences generated using Allard et al.'s (1994) PCR primers (lengths in bp)																											Sequencing haplotype frequencies in Atlantic-Mediterranean *C. mydas* rookeries [1]								
																													Greater Caribbean				Western Atlantic			E. Atlantic Mediterran.	
haplot. [1]	pattern	AluI			ApoI				BsrGI		HaeIII				MseI = Tru9I								RsaI			SspI			FLA.	MEX.	C.R.	AVES	SUR.	BRAZ.	ASCE.	GUIN.	CYP.
pos. of polym. site:			403		165	480		86		143				162		287 / 356			478		88			125	177	262											
CM-1	A	295	191		32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	0.46	0.35	-	-	-	-	-	-	-
CM-2	B	295	191		32	**448**			486	143	250	61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	**0.04**	-	-	-	-	-	-	-	-
CM-3	C	295	191		32	133	315	6	86	400	143	250	61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	0.90	0.25	0.93	0.13	-	-	-	-	-
CM-4	C	295	191		32	133	315	6	86	400	143	250	61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	-	-	0.07	-	-	-	-	-	-
CM-5	D	295	108	83	32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	40		371	115	125	52	17	292	-	0.05	-	0.88	0.88	-	-	-	-
CM-6	E	295	108	83	32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	371	115	125	52	17	292	-	-	-	-	0.06	-	-	-	-
CM-7	F	295	108	83	32	133	**325**	6	**496**	143	250	61	32	129	52	16	11	29	209	40		371	115	125	52	17	292	-	-	-	-	0.06	-	-	-	-
CM-8	G	295	108	83	32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	371	115	177	17	292	-	-	-	-	-	0.50	0.80	1.00	-
CM-9	H	295	191		32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	371	115	177	17	292	-	-	-	-	-	0.31	0.05	-	-
CM-10	I	295	108	83	32	133	315	6	486	143	250	61	32	129	52	16	11	29	**50**	**159**	32	8	371	115	177	17	292	-	-	-	-	-	-	**0.15**	-	-
CM-11	J	295	108	83	32	133	315	6	486	143	250	61	32	129	**33**	**19**	16	11	29	209	32	8	371	115	177	17	292	-	-	-	-	-	**0.06**	-	-	-
CM-12	K	295	108	83	32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	371	115	177	17	68	224	-	-	-	-	-	**0.13**	-	-	-
CM-13	L	295	191		32	133	315	6	486	393		61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	-	-	-	-	-	-	-	-	**0.90**
CM-17	L	295	191		32	133	315	6	486	393		61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	292	-	**0.10**	-	-	-	-	-	-	-
CM-14	M	295	191		32	133	315	6	486	393		61	32	129	52	16	11	29	**119**	**90**	32	8	88	283	115	177	17	292	-	-	-	-	-	-	-	-	**0.10**
CM-15	N	295	191		32	133	315	6	86	400	143	250	61	32	129	52	16	11	29	209	32	8	88	283	115	**194**		292	-	**0.05**	-	-	-	-	-	-	-
CM-16	O	295	191		32	133	315	6	486	393		61	32	129	52	16	11	29	209	32	8	88	283	115	177	17	68	224	-	**0.05**	-	-	-	-	-	-	-
CM-18	P	295	191		32	133	315	6	486	143	250	61	32	129	52	16	11	29	209	32	8	371	115	177	17	292			-	**0.15**	-	-	-	-	-	-	-

[1]haplotype designation, sequence and population data taken from Lahanas *et al.* (1994), Encalada *et al.* (1996)

Indo-Pacific green turtle sequences

Out of the REs tested, 27 (*Afl* II, *Alu* I, *Apo* I, *Bcc* I, *Bsl* I, *BssS* I, *Cje* I, *CviJ* I, *CviR* I, *Eco*57 I, *EcoN* I, *EcoR* I, *Hae* III, *HgiE* II, *Hinf* I, *Mae* III, *Mnl* I, *Mse* I, *Mwo* I, *Nla* III, *Psp*1406 I, *Sau*96 I, *Ssp* I, *Tfi* I, *Tsp*45 I, *Tsp*509 I, *Xcm* I) were identified with polymorphic sites in Indo-Pacific green turtle d-loop sequences. A set of only five REs (*EcoR* I, *Eco*57 I, *Mwo* I, *Mse* I and *Ssp* I) provided maximum resolving power. This set includes two REs (*Eco*57 I and *Mwo* I) which are additional to those used by Norman et al. (1994a) and which increases the number of restriction fragment-recognizable haplotypes from their eight to 11 out of the full 14 obtainable by d-loop sequencing (79%; see Fig. 4). The additional REs have specific recognition sites in haplotype sequences CM-SGBR and CM-NWC which distinguishes these from sequence haplotype groups CM-GOC/CM-LAC/ CM-SWK, and CM-ELT/CM-PNG, respectively.

Although RFLP techniques have been proven useful in the recognition of MUs in the Indo-Pacific green turtle populations (Norman et al. 1994a; Moritz 1994), their limited resolution relative to sequencing techniques has compelled researchers to follow up analyses of ambiguous PCR-RFLP haplotypes (those sharing fragment profiles) with methods providing higher precision such as denaturing gradient gel electrophoresis or even DNA sequencing (see Norman et al. 1994a). The present analysis, while not detecting RE sets permitting complete resolution of all sequence haplotypes (i.e. RE pattern 1 is shared by sequence haplotypes CM-GOC, CM-LAC, CM-SWK; pattern 9 by haplotypes CM-ELT, CM-PNG, see Fig. 1) is an improvement which could enhance large scale surveys employing PCR-RFLP in the area where haplotypes CM-SGBR and CM-NWC occur and would be otherwise confounded with RE pattern 1 and 9 haplotypes, respectively.

Caribbean and W. Atlantic hawksbills

Twenty-one REs with polymorphic recognition sites were found among the hawksbill sequences analyzed (*Alu* I, *Apo* I= *Acs* I, *Bcg* I, *BsaW* I, *BscG* I, *BsrF* I, *Bsr* I, *CviJ* I, *Dra* I, *Mnl* I, *Msp* I, *PinA* I, *Pme* I, *Rsa* I, *Sca* I, *Ssp* I, *Taq* I, *Taq* II, *Tse* I, *Tsp*509 I, *UbaC* I). Maximal resolution was found utilizing a basic set of 10 REs, with which 16 out of the total 20 (80%) sequencing haplotypes currently known could be distinguished on the basis of restriction site patterns (Fig. 5). With the exception of *BscG* I which is currently under development (P. Walsh, New England BioLabs, pers. comm.) all of these REs are available commercially. Sequence haplotypes EI-A, EI-G, and EI-O shared RE profile pattern "1"; EI-B and EI-F RE pattern "2"; EI-D and EI-Q pattern "4". Nonetheless, in spite of a decreased resolution capacity relative to sequencing, the capacity to distinguish among haplotypes on the basis of RE recognition sites found here is sufficiently high to make a PCR-RFLP approach potentially feasible, particularly for the analysis of mixed hawksbill stocks in Caribbean feeding areas or migratory corridors in a fashion analogous to the Bowen et al. (1996) study. Although a small degree of precision would be lost due to sequence haplotypes which become confounded in RE profiles mentioned previously, close inspection of the feeding ground data (Fig. 5) demonstrates that critical resolving power is still possible.

A single exception may be that the blending of EI-B and EI-F into the single RE profile "2" may cause reduced precision in estimations of contributions of Antigua haplotype EI-B relative to Belize, Puerto Rico, and U.S.V.I. through haplotype EI-F. If this is considered unacceptable in a real-case survey, a sub-sample of material typed as RE pattern 2 could be reanalyzed using a high-resolution technique (such as sequencing) and the results extrapolated to the original sample size. Overall, reduced costs would still be achieved even if a final sequencing step was necessary for subsets of the samples (as compared to all-sequencing procedures), without sacrificing precision.

Hybrid hawksbill and loggerhead hatchlings

In the case of the hawksbill-loggerhead hybrid d-loop sequences, a set of REs was searched for which could ideally discriminate in a three-way comparison: pure hawksbill, pure Atlantic loggerhead, and hybrid hawksbill-loggerhead (H-L) haplotypes. While available data (Bass et al. 1996) does not permit an

Figure 4. Expected restriction fragment lengths for Indo-Pacific *Chelonia mydas* d-loop haplotype sequences. Figures in bold represent haplotype-specific unique fragment patterns. Haplotype designations refer to type population where haplotypes were obtained by Norman *et al* . (1994): GOC= Gulf of Carpentaria, LAC= Lacepede Islands, SWK= Sarawak, NWC= North West Cape, JVA= Java, HAW= Hawaii, JPN= Japan, FP= French Polynesia, ELT= Elato Atoll, PNG= Papua New Guinea, NGBR= Northern Great Barrier Reef, SGBR= Southern Great Barrier Reef.

sequencing haplotype [1]	*RE pattern*	Expected fragment lengths for different restriction enzymes on sequences generated using Norman *et al* .'s (1994) PCR primers (lengths in bp)																						
		EcoRI		Eco57I		MwoI		MseI													SspI			
pos. of polym. site:		57		282		118		54	73						369		69	86	154					
CM-GOC	1	383		383		383		20	53		15	12	29	50	157	33	14	69	17	297				
CM-LAC	1	383		383		383		20	53		15	12	29	50	157	33	14	69	17	297				
CM-SWK	1	383		383		383		20	53		15	12	29	50	157	33	14	69	17	297				
CM-NWC	2	383		383		**118**	**265**	20	53		15	12	29	50	157	33	14	69	17	297				
CM-JVAb	3	57	326	383		383		20	53		15	12	29	50	157	33	14	69	17	297				
CM-HAW	4	57	326	383		383		20	68			12	29	50	157	33	14	69	17	297				
CM-JPNb	5	383		383		383		20	53		15	12	29	50	157	47		69	**314**					
CM-JPNa	6	383		383		383		20	34	19	15	12	29	50	157	33	14	69	17	68	229			
CM-FPa	7	57	326	383		383		20	68			12	29	50	157	47		69	17	68	229			
CM-FPb	8	383		383		383		20	53		15	12	29	50	157	47		86	68	229				
CM-ELT	9	383		383		383		20	53		15	12	29	50	157	47		86	68	229				
CM-PNG	9	383		383		383		20	53		15	12	29	50	157	47		86	68	229				
CM-NGBR	10	383		383		383		20	34	19	15	12	29	50	157	33	14	86	68	229				
CM-SGBR	11	383		**282**	**101**	383		20	53		15	12	29	50	157	47		86	68	229				

[1]haplotype designation and sequence data taken from Norman *et al.*, 1994

Figure 5. Expected restriction fragment lengths for Caribbean and Brazilian *Eretmochelys imbricata* d-loop haplotype sequences and population haplotype frequencies at various habitats. Figures in bold represent haplotype-specific unique fragment patterns or haplotype frequencies of non-shared haplotypes. Shading represents sequence haplotypes with common RE profile. Beliz= Belize; Mex= Mexico; P.R. = Puerto Rico; USVI= U.S. Virgin Islands; Antig.= Antigua; Bar.= Barbados; Braz.= Brazil. For feeding ground results, P.R.= Mona Island, P.R.

	d-loop sequencing haplotype [1,2]	RE profile	Expected fragment lengths for different restriction enzymes on sequences generated using Norman et al.'s (1994) PCR primers (lengths in bp)																											Sequencing haplotype frequencies in Caribbean and W. Atlantic *E. imbricata*							
																														rookeries[1]							feeding ground[2]
			ApoI =AcsI		BscGI		Dra I		MspI		NlaIII		PinAI		RsaI		SspI		Tsp509I						TaqI		Beliz.	Mex.	P.R.	USVI	Antig.	Bar.	Braz.	P.R.
	pos. of polym. site:		309		94		74	336	12		307		13		19		153		249						306									
rookeries	EI- A	1	371	11	382		74	308	382		133	17	232		382		19 241 121		126 256		47 86 116 59 62 12				382		-	-	0.07	0.07	0.80	0.73	1.00	0.17
	EI- G	1	371	11	382		74	308	382		133	17	232		382		19 241 121		126 256		47 86 116 59 62 12				382		0.07	-	-	-	-	-	-	-
	EI- O	1	371	11	382		74	308	382		133	17	232		382		19 241 121		126 256		47 86 116 59 62 12				382		-	-	0.07	-	-	-	-	-
	EI- B	2	371	11	94	288	74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	-	-	-	0.27	-	-	0.02
	EI- F	2	371	11	94	288	74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	0.79	-	0.07	0.93	-	-	-	0.44
	EI- C	3	371	11	94	288	74	262	46	382	133	17	232		382		19 241 121		126 27 229		47 86 116 59 62 12				306	76	-	-	-	-	0.13	-	-	-
	EI- H	4	371	11	382		74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	-	-	-	-	0.47	-	-
	EI- Q	4	371	11	382		74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	0.67	-	-	-	-	-	0.17
	EI- E	5	371	11	382		74	308	382		133	17	232		382		**261**	121	126 256		47 86 116 59 62 12				382		-	-	-	-	-	**0.20**	-	-
	EI- H	6	**309**	**62** 11	382		74	308	382		133	17	232		382		19 241 121		126 256		47 86 116 59 62 12				382		**0.07**	-	-	-	-	-	-	-
	EI- I	7	371	11	94	288	74	262	46	12 370	133	17	232		382		19 241 121		126 27 229		47 86 116 59 62 12				306	76	**0.07**	-	-	-	-	-	-	-
	EI- J	8	371	11	382		74	308	12 370		133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	-	**0.14**	-	-	-	-	-
	EI- K	9	371	11	382		74	308	12 370		133	17	232	13	369	19 241 121		126 256		47 86 116 59 62 12					306	76	-	-	**0.07**	-	-	-	-	-
	EI- L	10	371	11	382		74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					382		-	-	**0.07**	-	-	-	-	0.02
	EI- M	11	371	11	382		74	308	12 370		133	17	232	13	369	19 241 121		126 256		47 86 116 59 62 12					382		-	-	**0.14**	-	-	-	-	-
	EI- N	12	371	11	94	288	74	262	46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					382		-	-	**0.37**	-	-	-	-	0.06
	EI- P	13	371	11	382		336		46	12 370	133	17	232	13	369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	0.13	-	-	-	-	-	-
feed. ground	EI-alpha	(14)	371	11	382		74	308	382		133	17	232		382		19 241 121		126 256		47 86 175 62 12				382		-	-	-	-	-	-	-	0.05
	EI-beta	(15)	371	11	382		74	262	46	382	133	17	**157**	**75**	382		19 241 121		126 27 229		47 86 116 59 62 12				382		-	-	-	-	-	-	-	0.02
	EI-gamma	(16)	371	11	382		74	262	46	12 370	133	17	232		13 369	19 241 121		126 27 229		47 86 116 59 62 12					306	76	-	-	-	-	-	-	-	0.05

[1]rookery haplotype designation, sequence and population data taken from Bass *et al.* (1996)

[2]feeding ground haplotype designation, sequence and population data taken from Bowen *et al.* (1996)

assessment of how representative the hybrid sequences are, it is useful to observe that single REs fragment profiles (*Mae* III, *BsiY* I, *Bsl* I; Fig. 6) can be used to unambiguously distinguish these from pure hawksbill haplotypes. Distinction from pure Atlantic loggerhead haplotypes was also necessary because hawksbill and loggerhead hatchlings are quite similar morphologically. For this application, either RE *BsY* I or *Bsl* I could be used.

Caution must be taken when trying to extrapolate these results since the number of d-loop sequences available from H-L hybrids and from pure Atlantic loggerheads is small (N= 3 and 2, respectively) and variation may exist in the populations which could modify our conclusions. Nonetheless, the potential of the RFLP approach for large scale assessment of H-L (and other) hybridizations in natural populations is clear. Of course, mtDNA allows identification only of the maternal contribution, and nuclear DNA assays are essential to confirm hybrid origin in morphologically similar species (Karl et al. 1995).

Perspectives for Future Work

The results presented here demonstrate the feasibility of a PCR-RFLP approach for the recognition of genetic markers identified by sequencing studies and which have been demonstrated to be capable of distinguishing MUs in marine turtles. For some populations, most notably Pacific loggerheads, there is enough background information (Bowen et al. 1994, 1995) to ensure a successful and robust application for this approach to the analysis of mixed stocks. Although more complicated because of greater genetic variability, mixed stock analysis of green turtle populations in the Caribbean and W. Atlantic also seems feasible based upon our results and the levels of differentiation already reported between breeding colonies (Encalada et al. 1996). Nevertheless, it is important to bear in mind that the accuracy of the estimations will depend on (1) the number of potentially contributing populations, (2) the degree of variation within individual MUs relative to that between units, and (3) whether all the potentially contributing populations have been characterized (Pella and Milner 1987; Moritz 1994). To the extent that work is in progress to complete the identification of hawksbill turtle breeding populations in the Caribbean and W. Atlantic, leatherbacks in the E. Pacific (particularly Mexican populations), and hybrids between various species, the results presented for the material analyzed here should be considered preliminary and subject to revision as additional haplotypes become available.

An example of the need to achieve full coverage of extant turtle populations, was gained from the study by Bowen et al. (1996) of a hawksbill feeding site off Mona Island where novel haplotypes were found which had not been identified in previous rookery surveys. As

Restriction enzyme	pure *E. imbricata* haplotypes[1] N = 20	*C. caretta* - *E. imbricata* hybrid haplotypes[1] N = 3	pure Atlantic *C. caretta* haplotypes[2] N = 2
MaeIII	only 2 fragments	only 3 fragments	2 or 3 fragments
BsiYI or BsII	only 2 fragments	only 1 fragment (no cuts)	only 2 fragments
Hsp92II	3 or 4 fragments	only 3 fragments	4 fragments

[1] pure *E. imbricata* and hybrid sequence data taken from Bass et al. (1996).
[2] Atlantic *C. caretta* sequence data taken from Laurent et al. (1995). For this analysis only RE sites within homolgous DNA segments as pure *E. imbricata* and hybrid sequences were considered.

Figure 6. Expected restriction fragments for pure and hybrid *Eretmochelys imbricata* - *Caretta caretta* d-loop haplotype sequences.

additional haplotypes become known, they will have to be analyzed for RE sites to confirm the usefulness of the PCR-RFLP approach for the analysis of mixed stock aggregations of the species. It was encouraging, however, that our RE analysis of the Mona Island feeding ground data (Fig. 5) demonstrated an acceptable capacity to distinguish between haplotypes.

The economy gained from applications of a PCR-RFLP approach will mean that much greater sample sizes or number of repeated surveys could be achievable for the same price. Capital costs are also reduced because less sophisticated equipment is used. This will undoubtedly facilitate the adoption of high-resolution genetic tools within broad regional management programs encouraging the additional participation of lesser developed countries normally not capable of affording DNA sequencing.

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Use of microsatellite loci to investigate multiple paternity in marine turtles

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Introduction

Given the large energy expenditures involved in migration and egg production, it may be advantageous for females to have multiple matings if the success of matings varies. Evidence from other vertebrates has shown that multiple paternity of clutches may increase female reproductive success by increasing hatching success (Travis et al. 1990, Madsen et al. 1992). A greater understanding of paternity and the functioning of sperm storage and sperm competition in marine turtles should provide insights into gene flow within populations, and the evolution of mating systems.

Despite these interesting theoretical concerns, details of mating systems in marine turtles remain somewhat of a mystery. There are limited behavioural data on courtship and mating under natural conditions (Booth and Peters 1972, Limpus 1993) and on the frequency of multiple matings (Limpus 1993 and unpubl. data). Multiple matings of females have been observed in green turtles (*Chelonia mydas*) (Booth and Peters 1972, Ulrich and Parkes 1978, Limpus unpubl. data) but the success of these matings is unknown. Marine turtles undoubtedly store sperm at least through the nesting season (Owens 1980) and multiple paternity is a possible consequence of multiple matings. However, sperm competition is also likely and has been demonstrated to preclude the apparent success of some matings in other animals (Achmann et al. 1992, Oring et al. 1992). Multiple paternity has been suggested for *C.mydas* (Peare et al. 1994) and loggerhead turtles (*Caretta caretta*) (Harry and Briscoe 1988) and it has certainly occurred in clutches that have contained a proportion of hybrid hatchlings (Limpus and Norman unpubl data).

The application of genetic tools to the assessment of paternity in marine turtles should provide an exciting window into an otherwise hidden aspect of their natural history. The potential for multiple paternity is likely to vary across species and will depend upon mating behaviour, the length of time that females are receptive, the ability of males to inseminate unreceptive females, and the proportion of males to females on the breeding grounds. Sex ratios at breeding grounds may vary within and among seasons (but see Limpus 1993), and this could influence the frequency of multiple matings particularly in species with large temporal fluctuations in the number of breeding females (i.e. *C. mydas, Lepidochelys olivacea* and *L. kempi*). Additionally, selective harvest of either adult males (Frazier 1971) or females (Ross 1984, Kwan 1994) in many regional fisheries may change sex ratios within the breeding population, potentially altering the frequency of multiple paternity.

To compliment studies on male-mediated gene flow among marine turtle populations (FitzSimmons et al. 1995, FitzSimmons et al. 1996), I investigated gene flow within populations through assessments of clutch paternity using microsatellite analysis. Microsatellite analysis for the study of parentage is based on the identification of alleles, designated by base pair (bp) length, in offspring and putative parents across several loci. The interpretation of results is based on principles of Mendelian inheritance. It is a highly successful tool in parentage studies due to the presence of several alleles per locus and the potential of using many loci if necessary (Ellegren 1992, Queller et al. 1993). I am primarily looking at paternity of clutches in green turtles of the Southern Great Barrier Reef (Heron Island) and

of loggerhead turtles at the most significant mainland nesting beach in Queensland (Mon Repos). The latter study allows me to make comparisons to an earlier study of multiple paternity in loggerheads that used allozyme techniques (Harry and Briscoe 1988). In this paper I provide methodology for establishing similar studies for other marine turtle populations and share some preliminary results.

Methodology

Sampling Requirements

It is vital to sample the individual females whose clutches are to be analysed because the strength of statistical assessment of paternity relies on distinguishing which alleles belong to males and which to the female (Westneat et al. 1987). This is particularly pertinent when using microsatellite loci due to confounding factors of null alleles and mutation events as discussed below.

Once a female and her clutch are selected for study it is critical to insure positive identification of the clutch upon hatchling emergence. In areas with low density nesting this may simply involve marking the nest site, measuring its distance to fixed objects, and placing some type of identification in the nest with the eggs. If it is likely that the nest location cannot be protected from other females nesting nearby, or from other disturbances, then carefully transferring the eggs soon after laying (Parmenter 1980, Bjorndal and Balazs 1983) to a protected hatchery may be the best alternative. Nests dug in hatcheries should not be so close together that wayward hatchlings could move from one nest chamber to another through the sand on their way to the surface. The periphery of the nest area should be enclosed with screen at least one week prior to expected emergence to prevent hatchling dispersal.

The hatchlings' emergence needs to be closely monitored, to insure both the safety of the hatchlings and proper sampling. If different clutches are due to emerge about the same date in a hatchery it is critical that there be no confusion in the sampling. Hatchlings should be sampled as soon as possible after emergence and allowed to return to the sea following the most appropriate local protocol (see Dutton 1996).

In addition to sampling females and their clutches, a representative sampling of the breeding population should be made to determine the allele frequencies at all loci used in the study. Knowing the frequencies of particular alleles within the breeding population provides the means for establishing the uniqueness of observed paternal genotypes and estimating exclusion probabilities. Preferably the breeding population should be represented by non-sibling hatchlings and/or breeding females <u>and</u> males, sampled by non-destructive techniques. To adequately determine allele frequencies, sample size is governed by the variability of each locus, with larger sample sizes needed for highly variable loci (Richardson et al. 1986, pp. 57-59).

Statistical Requirements

Assessment of clutch paternity involves the positive assignment of genotypes to presumptive fathers at each locus and an estimation of the uniqueness of each paternal genotype within the breeding population. The sampling protocol and analytical design should be governed by the statistical power required to reliably detect multiple paternity. This ability depends on the number of offspring sampled, the number of loci analysed, the variability of each locus, and the frequencies of common alleles. If few alleles are observed per locus, or if allele frequencies are dominated by a few common alleles, then a greater number of loci will be needed to establish unique genotypes.

Data analysis should follow the protocols designed for studies of parentage using allozymes (Chakraborty et al. 1974, Gundel and Reetz 1981, Westneat et al. 1987) single locus minisatellites (Hanotte et al. 1991, Bruford et al. 1992), or more recently, microsatellites (Morin et al. 1994, Craighead et al. 1995). The approach taken for marine turtles is somewhat different from many parentage studies because a large number of offspring are available to assess paternity and putative fathers are not sampled. For statistical purposes, the common paternal genotype observed in the offspring serves as the putative father for all offspring. Calculation of the

probability of detection (d) then gives the degree of certainty that genes present in offspring from different fathers will be detected (Westneat et al. 1987). The number of hatchlings to be sampled depends upon the degree of statistical confidence desired to detect multiple paternity. Although it demands considerable effort, sampling 50% of each clutch is required to detect with 95% confidence any male fathering at least 5 offspring (Galbraith et al. 1989).

Case study: *Chelonia mydas*

To study paternity in green turtles, I sampled clutches from 14 females including several successive clutches throughout the season for nine of those females. To obtain data on successive clutches I sampled a female's first clutch of the season and then attempted to sample every alternate successive clutch. Blood samples (0.5 ml) were taken from the dorsal cervical sinus (Owens and Ruiz 1980) of females (using a 21g, 38 mm needle) just after egg laying as they began to cover the egg chamber. To aid in finding particular females for the sampling of successive clutches, I tagged the rear left flipper with a standard titanium tag (in addition to tags in each front flipper) to which I added a thin strip of reflective tape. This way I could either feel the tag or see it with a dim light when searching for these animals. Once the egg chamber was filled in by the female I shifted her off the nest so that I could relocate the eggs as quickly as possible.

The sampled clutches were relocated to a fenced area that served as a temporary hatchery and relocations were completed within 60 minutes of egg laying. In the hatchery, nests were dug approximately 1 meter apart and identified both above ground and within the nest using a strip of plastic flagging identified by the female's tag numbers and date of nesting. Hatchling escape was prevented by placing a circle of screening around each nest approximately two weeks before the expected date of emergence.

Nests were checked at least every 8 hours for several days prior to the expected emergence. All emerged hatchlings from each clutch were placed in a large box, partly filled with dry beach sand, and taken to a lab space for sampling. Blood (0.02-0.1 ml) was taken from the dorsal cervical sinus using a 1 ml insulin syringe with a 29g, 12.7 mm needle. Hatchlings were hand-held in a head down position with flippers restrained and blood was gently drawn as soon as the hatchling relaxed. For each clutch, I sampled enough hatchlings to equal 50% of the total clutch (if 50% had emerged) and all unhatched embryos were sampled if identifiable tissue was present. Sampled hatchlings were released within 2-4 hours of emergence. If sampling time necessitated the release of hatchlings during daylight hours or at low tide, I transported them to the reef crest to reduce predation risk (Gyuris 1994).

Within a few days after emergence I dug out each nest to quantify hatching success and get additional samples from unhatched, partially developed embryos. Sampling embryos allowed me to address questions about the paternity of emergent hatchlings versus unhatched embryos. Blood samples were stored in lysis buffer (FitzSimmons et al. 1995) in a ratio of blood:lysis that varied from 1:4 to 1:10. Tissue samples from non-hatched embryos were stored in a solution of 20% dimethyl sulfoxide that was saturated with salt (NaCl).

DNA was isolated for polymerase chain reaction (PCR) amplifications using microsatellite primers and alpha^{33}P incorporation (FitzSimmons et al. 1995). PCR products were run on 6% sequencing gels and scored against a sequenced size standard. Groups of sibling PCR products were run side by side on the gels and each group also contained the PCR product of the mother.

Five microsatellite loci, Cm3, Cm58, Cm72, Cc7 and Cc117, previously found in marine turtles and known to be highly polymorphic, were used in this study (FitzSimmons et al. 1995 and FitzSimmons unpubl. data). A combination of non-sibling hatchlings and breeding males and females were analysed to determine the allele frequencies of the breeding population at each locus. Genotype frequencies at each locus were tested for conformance to Hardy-Weinberg equilibrium using GENEPOP (Raymond and Rousset 1995). Significant departures from equilibrium would violate the assumptions of the

probability calculations and could also indicate the presence of null alleles or incorrect scoring. Linkage disequilibrium was tested for each pair of loci using GENEPOP (Raymond and Rousset 1995).

Preliminary data were analysed for one clutch (H20) to serve as a demonstration. Alleles were identified in several offspring at each of the 5 loci. Genotype frequencies of the offspring were tested for conformance to Mendelian expectations using chi-square tests. To determine the uniqueness of paternal genotypes, the probability that two unrelated individuals in the population share the same genotype, was calculated for each locus as

$$\sum_{i=1}^{n} (q^2_1)^2 + \sum_{i,j=1, i>j}^{n} (2 q_i q_j)^2$$

where q is the frequency of the ith allele for n alleles (Hanotte et al. 1991). For all loci, the probability of having a shared genotype is the combined product of the probabilities at each locus. The probability of detecting multiple paternity (d), was calculated per locus as

$$1 - 2a_2 + a_3 + 3(a_2 a_3 - a_5) - 2(a_2^2 - a_4)$$

where

$$a_n = \sum_{i=1}^{k} p^n_i$$

and p_i is the frequency of the ith allele for k alleles (Westneat et al. 1987). The probability of detection across all loci (D) was given by

$$1 - \prod_{i=1}^{n} (1 - p_i)$$

(see Chakraborty et al. 1974 and Westneat et al. 1987).

Results and Discussion

Preliminary results from the initial analysis of 14 green turtle clutches are tentative, though it appears that multiple paternity is either absent or present at low frequency within the clutches. I do not yet know the frequency of multiple paternity among the clutches sampled because the full analysis across all loci has not been completed, but it is probably less than 40%. Within the clutches that may be multiply sired, most offspring appear to be full siblings and the frequency of offspring sired by different males would be low, probably <5%.

Within the breeding population the number of alleles per locus varied from 13 to 37 (e.g. Fig. 1) and expected heterozygosity ranged from 0.83-0.96. Sample sizes were >100 individuals for all loci (Cm3, Cm58, Cm72, Cc117) except Cc7 which had not yet been analysed. Allele frequencies of the breeding population were within expectations of Hardy-Weinberg equilibrium at the 4 loci when corrected for multiple tests (p>0.05, Rice 1989) and there was no evidence of linkage disequilibrium between any of the loci.

The probability that two unrelated individuals would share the same genotype across the 4 loci was extremely low; 2.6×10^{-10}. In fact, in a sample of n=105 individuals in the breeding population, none had the same genotype across just 3 of the 4 loci. The probability of detecting multiple paternity per locus ranged from d=0.769-0.927 and the combined probability across the 4 loci was extremely high; d=0.9999.

Clutch H20 was the first of three clutches sampled for female T35820 (Queensland Turtle Research Project). The eggs were laid on the 15th of December 1993 and emerged 58 days later. Of the 103 eggs laid, 73 hatchlings emerged, 8 were partially developed and 22 showed no development. Blood was sampled from 52 hatchlings and muscle samples were obtained from 4 of the embryos.

Among the hatchlings of clutch H20, four alleles were observed at Cm3, three at Cm58, Cc7 and Cc117, and seven at Cm72. At each locus, the genotype observed in the mother was consistent with the alleles observed in the hatchlings and the genotype of a single putative father was postulated. The ratios of offspring genotypes at all loci were consistent with the hypothesis of a single father when tested for Mendelian expectations using chi square analysis (Table 1). For locus Cm72, I postulated that 3 mutations had occurred; two originating from the maternal lineage (as the offspring displaying those alleles did not have either of the maternal alleles), and one originating from the paternal lineage (Table 2). Each of the mutations were to allele lengths

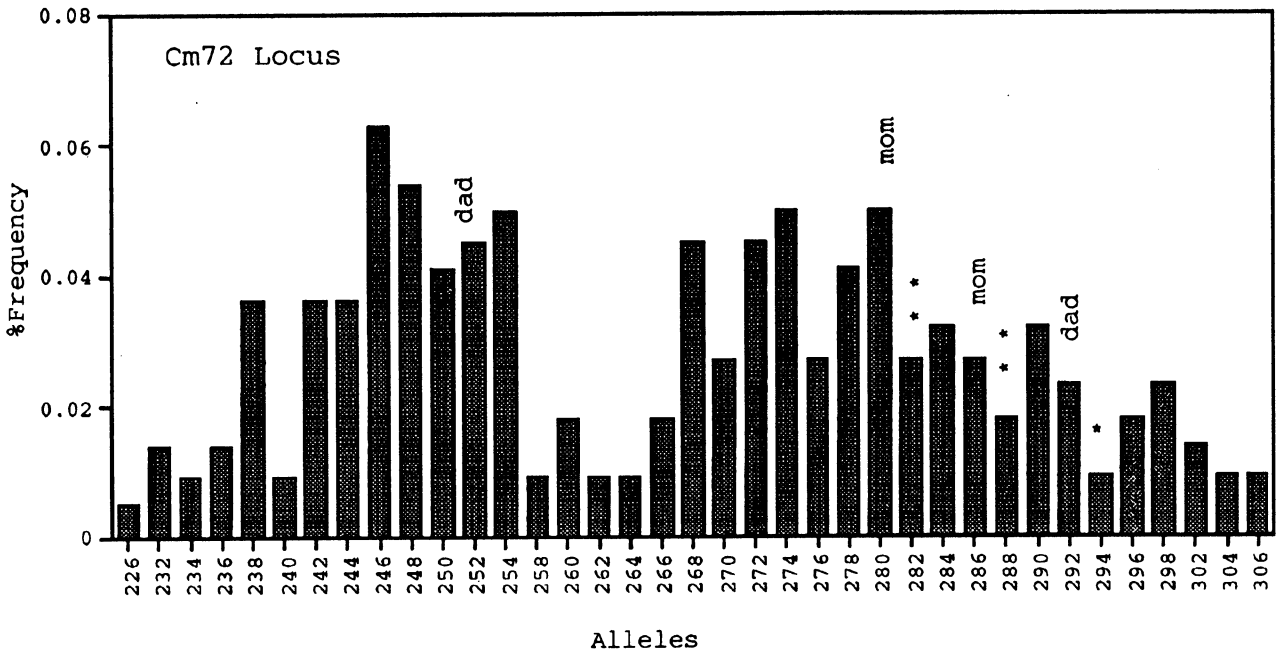

Figure 1. Allele frequencies at the Cm72 microsatellite locus for green turtle breeding populations of the Southern Great Barrier Reef showing parental alleles of clutch H20. Maternal alleles were observed by direct sampling and paternal alleles were determined from hatchling alleles. Mutations observed in the offspring are designated " ** " if originating from the mother and " * " if originating from the father. Sample sizes were n=110 animals for the SGBR breeding population and n=34 offspring sampled.

already observed in the breeding population (Fig. 1). Because shifts of 2bp are thought to be the predominate type of mutation at microsatellite loci (Weber and Wong 1993) I suggest that the following shifts occurred: female allele 280 to 282, female allele 286 to 288, and male allele 292 to 294 (Table 2, Fig. 1). Given the high probabilities of exclusion it is expected that if the extra paternal allele (294) originated from a second father then additional different alleles from that father would also be observed at some of the other loci. This would be even more likely since two offspring (H20-18 and H20-37) had the 294 allele. In Table 2 the complete genotypes are given for each offspring that had unique alleles at Cm72, and each offspring had the common parental alleles across all other loci analysed.

Several mutations have been observed in the other green turtle clutches analysed and it appears that the data set will be quite valuable in establishing mutation rates and patterns. Relatively little is documented concerning mutation rates in dinucleotide microsatellites (such as these) but estimates range from 10^{-2} to 10^{-4} per gamete per generation (Weber and Wong 1993). Preliminary assessments of mutation rates at some of the loci analysed in this study appear to be greater than 10^{-3}. This is of concern because the probabilities of false inclusion or exclusion of putative parents is dependent upon the mutation rate and is estimated as 2m, where m is the mutation rate (Bruford et al. 1992). With high mutation rates therefore, it becomes critical that all loci are analysed for any offspring that have uncommon or unusual parental alleles.

Additionally, null alleles have been reported by several studies (Callen et al. 1993, Pemberton et al. 1995) and may be present in some of the green turtle clutches in this study as well. Their occurrence typically results from mutations in the flanking sequence where primers anneal, thus preventing amplification of the allele. Null alleles are suspected at a particular locus if (1) the breeding population is not in Hardy-Weinberg equilibrium, (2) the locus is highly variable and offspring within a clutch show less than 4 alleles, or (3) expectations of Mendelian inheritance are inconsistent with the

Table 1. Parental and hatchling genotypes of clutch H20 at five microsatellite loci indicating single paternity. Allele designations refer to the base pair length of the alleles. Chi square values and probabilities are given for tests of conformance to Mendelian expectations of single parentage.

Parental genotypes		Hatchling genotypes	Frequency	Chi sq	p
Cm3					
mom	164:196	164:168	4		
dad	168:170	164:170	9		
		196:168	11		
		196:170	9	3.24	0.36
Cm58					
mom	134:146	134:138	5		
dad	138:146	134:146	11		
		146:138	16		
		146:146	7	7.26	0.064
Cm72					
mom	280: 286	280:250	6		
also	282,288	282:" "	1		
dad	250: 292	280:292	4		
also	294	286:250	9		
		288:" "	3		
		286:292	10		
		288:" "	2	5.34	0.15
Cc7					
mom	181:193	181:179	6		
dad	179:193	181:193	4		
		193:179	6		
		193:193	2	2.44	0.49
Cc117					
mom	230:248	230:246	9		
dad	246:248	230-248	2		
		248:246	6		
		248:248	4	5.10	0.17

hypothesis of an apparent "shared" allele from both parents. In clutch H20, although only three alleles were observed in hatchlings at Cm58, Cc7, and Cc117 (Table 1), null alleles were not indicated since the genotype frequencies of the offspring followed Mendelian expectations of a shared parental allele. If null alleles are present, multiple paternity may be underestimated, particularly if multiple loci have null alleles.

In summary, studies of clutch paternity in marine turtles using microsatellite analysis should include the following: (1) adequate sampling of the breeding population to determine microsatellite allele frequencies, (2) sampling of the nesting females whose clutches are to be analysed and positive identification of their clutches for future sampling, (3) non-destructive sampling of a representative number of hatchlings and unhatched embryos from the identified clutches, and possibly (4) sampling of successive clutches from the same females. The number of hatchlings sampled and the number of loci analysed should be based upon the statistical confidence desired when addressing questions of paternity. It is advisable to undertake an initial pilot study to

Table 2. Observed alleles across 5 microsatellite loci for 6 offspring of clutch H20 that showed mutations at locus Cm72. Alleles are listed by their parental origin and mutations are indicated by an asterisk. n/a represents no available data at present.

Locus	Cm3	Cm58	Cm72	Cc7	Cc117
Offspring					
1. H20-5					
mom	196	146	288*	193	n/a
dad	168	138	250	179	n/a
2. H20-6					
mom	164	134	282*	193	n/a
dad	170	146	250	193	n/a
3. H20-18					
mom	196	134	286	181	n/a
dad	170	138	294*	193	n/a
4. H20-20					
mom	196	146	288*	n/a	n/a
dad	168	146	250	n/a	n/a
5. H20-24					
mom	n/a	146	288*	181	248
dad	n/a	138	250	179	246
6. H20-37					
mom	196	146	286	181	248
dad	170	146	294*	193	246

estimate the extent and nature of multiple paternity if an extensive project is planned.

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Use of molecular markers for stock identification, fingerprinting, and the study of mating behavior in leatherbacks

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Introduction

One of the goals of most of the recent population genetic research on sea turtles has been to provide an understanding of stock structure, and to identify molecular markers that can be used in mixed stock analysis of sea turtles at various life stages and circumstances in the marine environment (Bowen et al., 1995; 1996; Broderick et al., 1994). For leatherbacks, this work has taken on a new urgency due to the critical decline in breeding populations on both sides of the Pacific (Chan and Liew, In press; Sarti et al., 1996), and growing concerns over the impact of incidental take by fisheries operating in the North Pacific (Bolten et al., 1996; Diaz-Soltero, 1995; Wetherall et al., 1993), as well as the North Atlantic (N. Tregenza, pers. comm., Exeter University, UK., M. Girondot, pers. comm., http:// www.ijm.jussieu.fr/dnp.html).

Although the criteria for defining stocks have been the subject of debate (see Dizon et al., 1992; Moritz, 1994), for practical reasons one usually considers "stocks" of sea turtles in terms of rookeries, or populations of nesting females, and the emerging consensus from genetic studies is one of regional population structure characterized in several cases by fixed differences in mitochondrial DNA (mtDNA) haplotypes (Bass, 1994; Norman et al., 1994; Bowen et al., 1994; Encalada, 1995; Bass et al., 1996; Encalada et al., 1996). Bowen et al. (1995) have shown how such fixed differences in mtDNA haplotypes found in Pacific loggerhead nesting populations can be used to apportion the impact of the high seas driftnet fishery and to identify the origin of juveniles in feeding grounds far away from nesting beaches. For leatherbacks, however, the mtDNA data have so far revealed less regional structuring of nesting populations. This presents a greater challenge for stock identification for this species, and suggests that multiple markers may be required to provide additional resolution.

This paper summarizes the mtDNA data for leatherbacks, and explores the utility of microsatellites for studying stock structure and mating systems, and as genetic "tags" for identifying individuals.

Mitochondrial DNA

Some of the unique features of mtDNA, such as its maternal mode of inheritance and relatively rapid rate of evolution (Brown et al., 1979) make this molecule particularly appropriate for testing patterns of gene flow in nesting populations of sea turtles. The control region, which contains the displacement loop (d-loop), is believed to be the fastest evolving region of the mitochondrial genome in many vertebrates (Vigilant et al., 1991), and has been useful in resolving intra-specific population structure in chelonids.

Primers designed by Lahanas et al. (1994) for green turtles (Table 1) which are longer versions of LTCM1 and HDCM1 of Allard et al. (1994), amplify a 496 bp fragment of the control region in leatherbacks. I have designed leatherback-specific primers for sequencing 370 bases of the light strand (DCDL4) and 413 bases of the heavy strand (DCDL5), which can also be used with PCR to amplify a smaller fragment (Dutton, 1995). A slightly modified version of DCDL4 (CTURTDL: 5'-TTATTT(AG)CCACTAGCATAT-3'; Dutton, et al., 1996) can be used as an internal primer to sequence LTCM2/HDCM2-amplified fragments

Table 1. Primers for sequencing the control region of mtDNA in leatherbacks.

Primer	Primer sequences (5'-3')	Annealing Temp (°C)
LTCM2[1]	CGGTCCCCAAAACCGGAATCCTAT	
HDCM2[1]	GCAAGTAAAACTACCGTATGCCAGGTTA	52
DCDL4[2]	TTATTTGCCACTAGCATAT	
DCDL5[2]	ACAACCAGAGGCCAGAATAAATCA	52

[1]Lahanas, et al. (1994)
[2]Dutton (1995)

in all the species, including leatherbacks, for those wishing to reduce costs of making species-specific primers.

Relatively little variation has been found in d-loop sequences globally, with only 7 polymorphic sites, defining 8 haplotypes among 128 samples (Dutton, 1995). Table 2 shows that one common haplotype (A) is present at high frequency in all 7 of the populations that have so far been sampled from the Atlantic and Indo-Pacific, and that this haplotype is fixed in the Florida, Suriname/French Guyana and South African rookeries, being absent only in the Eastern Pacific. Haplotype D occurs in all 3 ocean basins, and Haplotype E in both the Pacific and Atlantic. Estimates of interpopulation gene flow based on these data (Dutton, 1995) are an order of magnitude higher than similar data for greens and hawksbills (Allard et al., 1994; Bass, 1994; Broderick et al., 1994; Encalada, 1995; Encalada et al., 1996; Bass et al., 1996), and suggest dispersal among ocean basins. However, this general lack of distinct geographic stock structure could be the result of recent colonization (e.g. within the last 50,000 years or so) rather than a reflection of contemporary gene flow, and it might be a mistake to conclude that leatherback populations are panmictic, based only on these data. As Taylor and Dizon (1996) have stated, there is a danger in deciding to combine populations into one unit for management purposes based solely on the lack of significant genetic differences. Avise (1995) points out that a genetic survey may fail to find population structure because gene flow may be sufficiently high to homogenize populations genetically, yet actually be quite low demographically. This may be the case for leatherbacks, where the mtDNA data taken by itself shows South African and Western Caribbean nesting populations to be essentially identical, whereas there may be little, if any, migration in a demographic sense between these nesting beaches. It is important to continue to pursue multiple lines of evidence when attempting to define stocks. For leatherbacks in the Atlantic, telemetry and tagging studies suggest that nesting "stocks" may comprise multiple rookeries spanning distances up to 800km, and the presence of unique haplotypes at relatively high frequencies in the St. Croix and Trinidad rookeries (Table 2) indicate that gene flow between these island populations and those on the mainland is fairly restricted (see Dutton, 1995 for a more detailed discussion).

How useful then, is mtDNA data for identifying the originating stock of leatherbacks in forage areas or in fisheries? For the Pacific, the difference in haplotype frequencies between Malaysia and Pacific Costa Rica, on either side of the ocean, should be sufficient to allow mixed stock analysis of forage areas; however, since 3 haplotypes occur in both populations (Table 2), larger sample sizes will be needed from the forage area than for similar studies for green and loggerhead turtles, where many rookeries are distinguished by fixed mtDNA differences (see Chapman, 1996). It should also be noted that data from other key rookeries in Mexico and Indonesia are not yet available. The detection of additional haplotypes and significant frequency shifts between populations on either side of the Pacific would facilitate stock

Table 2. mtDNA haplotype frequencies for eight leatherback populations

Location \ Haplotype	A	B	C	D	E	F	G	H	(n)
St. Croix	0.54	0.36	0.09	-	-	-	-	-	22
Costa Rica (Atlantic)	0.93	-	0.07	-	-	-	-	-	28
Florida	1.00	-	-	-	-	-	-	-	7
Surinam/ French Guiana	1.00	-	-	-	-	-	-	-	20
Trinidad	0.50	-	0.33	0.06	0.11	-	-	-	18
Costa Rica (Pacific)	-	-	-	0.61	0.06	0.11	0.11	0.11	18
South Africa	1.00	-	-	-	-	-	-	-	7
Malaysia	0.30	-	0.20	0.30	-	-	-	0.20	10

identification. For the Atlantic, it should be possible to detect stock contributions from St. Croix and Trinidad, which each have distinctive haplotypes at relatively high frequency; however, mixed stock analysis will be very difficult using mtDNA alone, because the same haplotype makes up 93-100% of 4 of the 6 major rookeries assayed (Table 2). As if to highlight this problem, a juvenile leatherback made an uncanny and fortuitous appearance in Miami during the last day of this Genetics Symposium, having been rescued from a nearby beach by NMFS personnel. Prior to release off shore, a blood sample was taken, and subsequent genetic analysis revealed the control region sequence to be Haplotype A (Dutton, unpublished data), so that based only on these data it is impossible to pinpoint the geographic origin of this individual with certainty.

Microsatellites

Microsatellites show promise as neutral nuclear markers for assessing intra-specific population structure (Bowcock et al., 1994; Roy et al, 1994; Dallas et al., 1995; Estoup et al., 1995; Paetkau et al., 1995). Due to their high mutation rates (Straub et al., 1993; Weber and Wong, 1993), microsatellites may help reveal patterns of contemporary gene flow that were not detected with mtDNA (if indeed the lack of variation that was found with mtDNA is a result of recent historical connection). Also, unlike mtDNA, microsatellites are biparentally-inherited, and therefore allow a more holistic approach to the study of stock structure by reflecting both male and female-mediated gene flow. Since microsatellites are scattered throughout the genome, it is possible to use multiple independent loci to increase resolution and statistical confidence (Shriver et al., 1995), particularly given the limitations of small sample sizes. The error introduced by small sample sizes can in part be mitigated by increasing the number of loci surveyed. Shriver et al. (1995) have shown that increasing the number of loci has a greater effect on reducing variance than increasing the sample size. FitzSimmons et al. (1995) have found microsatellites in hawksbill, green and loggerhead turtles that contain up to 25 alleles and whose flanking sequences are conserved across all species of sea turtles. Efforts are also underway to identify additional microsatellite loci in leatherbacks (see Dutton, 1995). Table 3 lists all primers that are currently known to amplify polymorphic loci in leatherbacks. Some loci, such as EI8 and CC117, are highly variable, with up to 17 alleles in leatherbacks, while others such as N200, CM3, N32 and DC99 consist of two to eight alleles (Table 3). Whereas the highly allelic loci are useful for paternity studies and fingerprinting (see below), they require relatively larger sample sizes to capture an accurate representation of population variation (see Chapman, 1996). The loci with less than eight alleles are therefore more appropriate for addressing population-level issues with relatively small sample sizes (<20). Preliminary phylogenetic analysis of allele frequency data for 3 leatherback microsatellite loci, N200, N32 and DC99, produce a tree that is congruent with the geographic relationships between the populations (Dutton, 1995), and suggests that these microsatellite loci can be used as markers

Table 3. Primer sequences, optimum annealing temperatures and number of alleles for polymorphic microsatellite loci in leatherbacks

Primer	Primer Sequences (5'-3')	Annealing Temp. (°C)	No. Alleles
DC28[1]	GGCTAGGCGTAATTTATCCC CAGGTTTCCATGTCTTGTTGTG	58	5
DC99[1]	CACCCATTTTTTCCCATTG ATTTGAGCATAAGTTTTCGTGG	56	8
P186[1]	AATAACACTCCTTCGCTG CTACATTGTGATTTCCATTC	52	6
Nigra200[2]	GCTAAAGACCTAGTTCTGCCATG TTCAGTGGTTACTCAGCAAAGG	58	2
Nigra32[2]	CGTGTGTTTGGACAGAAGATGAAC AGGCAAAGCACCTGCAAATC	56	6
CM3[3]	AATACTACCATGAGATGGGATGTG ATTCTTTTCTCCATAAACAAGGCC	55	5
CM58[3]	GCCTGCAGTACACTCGGTATTTAT TCAATGAAAGTGACAGGATGTACC	50	2
EI8[3]	ATATGATTAGGCAAGGCTCTCAAC AATCTTGAGATTGGCTTAGAAATC	52	17
CC117[3]	TCTTTAACGTATCTCCTGTAGCTC CAGTAGTGTCAGTTCATTGTTTCA	55	11
CM84[3]	TGTTTTGACATTAGTCCAGGATTG ATTGTTATAGCCTATTGTTCAGGA	55	4

[1] Dutton, unpublished
[2] Louis, pers. comm., Texas A&M University, College Station
[3] FitzSimmons et al., 1995

to distinguish the two Indo-Pacific populations from those in the Atlantic and East Pacific. This suggests that these Indo-Pacific populations may be somewhat isolated from Eastern Pacific and Western Atlantic populations in terms of contemporary gene flow. These markers therefore may be useful for defining breeding stocks for the purpose of identifying the origins of turtles caught in pelagic fisheries in the Central and North Pacific. Further work is being done to obtain data for additional loci with larger sample sizes from these and other key rookeries.

Genetic Fingerprinting and Paternity

In addition to being useful for population-level studies, microsatellites can be used to construct genetic fingerprints for identifying individuals. The uniqueness probability of any particular fingerprint can be calculated from the allele frequencies for a given population. Such fingerprints have been constructed for most of the leatherbacks that have nested over the past 5 years in St. Croix (Dutton, unpublished data) using genotypes from an array of 8 microsatellite loci (Table 4).

Table 4. Genetic fingerprints for 4 adult female leatherbacks from St. Croix, US Virgin Islands, consisting of profiles of genotypes (letters) at 8 different microsatellite loci. Probabilities of obtaining each genotype (based on population allele frequencies) are shown in parentheses, as well as overall P of a profile occuring more than once.

LOCUS ID	EI8	N200	N32	DC99	CM3	DC28	CC117	P186	P ($x10^{-8}$)
AAR971	AC (0.057)	AB (0.179)	BD (0.142)	BC (0.029)	CD (0.113)	CC (0.438)	AA (0.128)	EF (0.086)	2.3
AAG402	AQ (0.005)	AA (0.588)	BD (0.142)	BB (0.582)	BB (0.003)	CC (0.438)	DE (0.008)	EF (0.086)	≅ 0.0
AAG924	CC (0.057)	AA (0.588)	AB (0.083)	BB (0.582)	DD (0.148)	CC (0.438)	CE (0.004)	AC (0.012)	0.5
AAG405	HL (.0005)	AA (0.588)	AA (0.054)	BB (0.582)	DD (0.148)	CC (0.438)	AA (0.128)	AF (0.047)	0.4

This database could be used to identify these animals from blood or biopsy samples when traditional methods of tagging are inadequate (see McDonald and Dutton, In press), or when all that is available are partial remains, as is often the case with strandings or pieces of confiscated meat.

Finally, microsatellites can be used to infer mating behavior in this elusive species, for which direct observation is rarely possible, with only one reported sighting of copulation (Carr and Carr, 1986). Table 5 shows the genotypes for locus EI8 of hatchlings from 3 different clutches laid during the same season by a single female (Dutton, unpublished data). Since the genotype of the mother is known (AC), and microsatellite alleles are bi-parentally inherited in Mendelian fashion, it is possible to use hatchling genotypes to determine that at least two males (AF and AC genotypes) fertilized different clutches. This locus, EI8 (FitzSimmons et al., 1995), is particularly useful for paternity studies in leatherbacks, since it is highly polymorphic. Other loci, which may also be polymorphic, but have only one or two alleles at high frequency in a population (N200, DC99, and CM3 for example), may not be as effective,

Table 5. Genotypes at locus EI8 of hatchlings from three clutches laid by one female (AAR971) in St. Croix, U.S. Virgin Islands, during 1992. The genotype of the mother is AC (Dutton, unpublished data).

Date Laid	Hatchling Genotype Frequencies			Inferred Genotype of Father
4/21/92	AA	=	1	AF
	AC	=	6	
	CF	=	2	
5/18/92	AA	=	2	AF
	AC	=	3	
	AF	=	1	
6/8/92	AA	=	2	AC
	AC	=	4	
	CC	=	1	

since different males have a high probability of having the same genotypes and would thus escape detection. Use of multiple loci is obviously a more powerful approach. Additional samples have been collected for series of up to 7 clutches laid by several females in St. Croix, and these are currently being analyzed with multiple loci. Similar studies are underway for leatherback clutches in Pacific Costa Rica (J. Rieder, pers. comm., Ohio State University), loggerheads in Australia (FitzSimmons, 1996), and Kemp's Ridleys in Mexico (Kichler, 1996).

Summary and Future Research

Leatherbacks do not have the same high degree of genetic stock structure as some of their chelonid relatives, and the extent to which the same mtDNA haplotype is found in different nesting populations makes it difficult to use mtDNA by itself to identify the geographic origin of animals encountered away from nesting beaches. Data are still needed from key rookeries in Mexico, Indonesia and the South Pacific; however, if the allele frequency shifts that are evident between two rookeries in Pacific Costa Rica and Malaysia hold up, mixed stock analysis may be possible to distinguish East from West Pacific originating stock, using mtDNA alone. Microsatellites show promise as multiple markers for stock identification, and future work should explore further the possibility of using multiple loci to provide the resolution necessary for stock identification. Additional loci need to be identified to minimize error due to the relatively small sample sizes, and samples from fishery bycatch or beach strandings should be stockpiled in order to facilitate mixed stock analyses.

Eight of the loci available for leatherbacks are sufficient to construct reliable genetic fingerprints that can be used as genetic "tags", opening up new possibilities for forensic and behavioral studies that have been hampered by the inability to identify individuals using conventional methods. Microsatellite data have revealed evidence of multiple paternity for clutches in St. Croix, with implications for theories about sperm storage, mating behavior and conservation genetics that still remain to be explored.

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The use of multilocus minisatellite DNA fingerprinting to examine local genetic structure within green turtle rookeries

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Introduction

Green turtles (*Chelonia mydas*) lay their clutches on ocean beaches which can be many kilometers in length but only several meters wide. Nesting populations consist of adult females that have migrated hundreds or thousands of kilometers from feeding grounds to converge on particular beaches where they lay their eggs. If females exhibit precise homing to specific natal sites along a beach, genetic structure within a nesting aggregate may develop through isolation by distance (Wright 1943).

Due to the complex and pelagic life history of marine turtles, the direct study of effective dispersal is very difficult. In the Atlantic, an estimated 27-33 years are required for female green turtles to reach sexual maturity (Frazer and Ladner 1986), and individuals move widely among different marine habitats during their development (Carr 1980). These features make it virtually impossible to track individuals from the hatchling stage to the first nesting event in order to observe the realized dispersal distances from natal sites. Indirect methods have therefore been used to examine dispersal in this species.

Analysis of maternally transmitted mitochondrial DNA has revealed significant divergences among nesting populations of green turtles (Bowen et al. 1992; Allard et al. 1994). These results indicate that females exhibit strong homing behavior to natal rookeries. Tag return data has shown that female green turtles tend to return to specific sections of a rookery to nest (Carr and Carr 1972; Carr and Hirth 1962; Mortimer and Portier 1989; Johnson 1994). These results provide evidence that females exhibit homing precision to areas of the beach where they have previously nested. However, it is not known whether female site fidelity within nesting areas is the result of previous nesting experiences and preferences, or whether females tend to nest near their natal sites.

To assess the extent to which female green turtles exhibit within-beach precision in natal philopatry, we used multilocus minisatellite DNA fingerprinting to examine the local genetic structure along two nesting beaches (Tortuguero, Costa Rica and Melbourne, Florida, U.S.A.). If females return to their natal site to nest, then individuals that nest in a particular section of beach should be more closely related than individuals drawn at random from the nesting colony.

Distance-related genetic structure along nesting beaches would indicate that populations are composed of several genetic neighborhoods or partially overlapping demographic units. Such findings may have serious genetic consequences for the management of endangered populations especially if disturbances are spatially discrete. Particular lineages may be disproportionately impacted by human incursions, causing the population to lose genetic diversity at a more rapid pace than expected in a population nesting at random locations within the nesting habitat.

Methods

Field Methods

During the summers of 1991-1993, a total of 98 blood samples (20-100 μl) were collected from adult female green turtles nesting on the northernmost 8 km of Tortuguero beach, Costa Rica. Blood was taken by intravenous sampling from either the dorsal cervical sinus using 18 gauge needles or the femoral vein using 23 gauge needles. Blood samples (50-100 μl) from the femoral vein were also collected in 1994 from 50 adult female green turtles nesting on 16 km of Melbourne beach, Florida, U.S.A., between Sebastian Inlet and Coconut Point Park. Each blood sample (from both populations) was stored in 1 ml of a lysis buffer (100 mM Tris, pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al. 1988). Tortuguero beach is marked with stakes every 0.2 km and Melbourne beach every 0.1 km so the location of each nesting turtle was recorded based on proximity to the nearest beach marker.

Laboratory Methods

Samples were incubated overnight at 65°C with 25 μl proteinase K (10 mg/ml). DNA was extracted from samples by two phenol extractions, two phenol:CIA (chloroform:isoamyl alcohol at 24:1) extractions and one CIA extraction. Samples were then dialyzed for 3-10 hours in cold TNE_2 (10 mM Tris, 10 mM NaCl, 2 mM EDTA pH 8.0). For each individual, 4 μg DNA was digested with 5X excess HaeIII at 37°C for 3-5 hours. The fragments produced by these digestions were separated by size along an electrical gradient in a 0.8% agarose gel for 65 hours at 20V. Southern blotting (Southern 1975) was used to transfer the DNA to a nylon membrane, to which it was fixed by UV cross-linking. Membranes were hybridized with Jeffreys' probe 33.15 (Jeffreys et al. 1985), which had been labeled by primer extension with α^{32}PdCTP. Hybridizations were run overnight at 62°C in 1.5X SSC, 0.1% SDS, 5X Denhardt's solution, and 6% w/v dextran sulfate. Following hybridization, filters were washed four times at 62°C in 1.5X SSC and 0.1% SDS, then exposed to X-ray film at -20°C for 24-110 hours with intensifying screens. Randomly selected DNA samples from Tortuguero and Melbourne beach adult green turtles were run on four gels. As the distance between two lanes increases the accuracy of band-sharing estimates decreases (Piper and Parker 1992). To address this problem, the following approach was used: DNA from 1 or 2 individuals was used in 2 or 3 lanes within each gel. On the autoradiograms, horizontal lines were drawn connecting 5 or 6 of the identical bands in the repeated lanes. The autoradiograms were then sliced apart between each lane, and the horizontal lines were used to position the strips so that the 5-10 closest lanes could be scored adjacent to one another. This method could be used on gels that had run straight, so that all horizontal lines could be connected.

Data Analysis

Genetic similarity values (proportion of bands shared) were calculated for dyads of nesting females as $D = 2S/(2S + A + B)$, where S equals the number of bands shared by the two individuals under comparison, A is the number of bands exclusive to one, and B is the number exclusive to the other (Lynch 1990). The local genetic structure within each population was determined by examining the genetic similarity of pairs of females as a function of the distance between their nest sites.

If females are returning to nest near their natal sites, then pairs of individuals nesting in the same section of the beach should have higher genetic similarity scores than individuals that nest in different sections. High genetic similarity scores for pairs of individuals nesting in the same section of beach, but in different years, would also be expected if nestmates return independently to their natal sites. In contrast, if females return to natal beaches and select their first nest site randomly, but thereafter return with some precision to that site, we expect to find no relationship between genetic similarity and internest distance for pairs of females. For the Tortuguero population we examined this relationship for turtles nesting in the same year (1991; n = 14) and for those nesting in different years (1991, n = 6; 1992, n = 5; 1993, n = 7). For the Melbourne population, turtles nesting in 1994 (n = 19) were analyzed.

Due to the lack of independence of the data points (each female was scored against multiple other individuals) the Mantel test (Mantel 1967) was used to evaluate whether genetic similarity values and distance were correlated for pairs of turtles nesting in Tortuguero and in Melbourne. We used two symmetric similarity matrices for each test (one for genetic similarity based on band-sharing values and a corresponding matrix of distances between nest sites), and then assessed the significance of the relationship between the elements of the two matrices through permutational analysis (Schnell et al. 1985). This analysis randomly permutes the order of the elements of one matrix, while holding the other constant, and compares the correlation values for each of a specified number (we used 1000) of permutations to the initial correlation using the original matrix. The computer program Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) was used for both the Mantel matrix correlation test and the permutational analysis (Rohlf 1990).

Results and Discussion

In the Costa Rican population, there was a significant negative correlation (Mantel matrix correlation $r^2 = 0.273$; $p \leq 0.001$) between genetic similarity and internest distance (Fig. 1). Thus individuals nesting in the same area are more similar in terms of DNA fragment sharing than those nesting farther apart. This finding indicates microgeographic structuring of minisatellite alleles along the length of the beach, consistent with the hypothesis that females tend to nest in the vicinity of their natal site.

A significant negative correlation between genetic similarity and internest distance was also found for pairs of females that nested one or two years apart in Tortuguero (Mantel matrix correlation $r^2 = 0.578$; $p \leq 0.001$). Even between years, pairs of turtles nesting in the same parts of the beach have higher genetic similarity values than pairs nesting in different parts of the beach (Fig. 2).

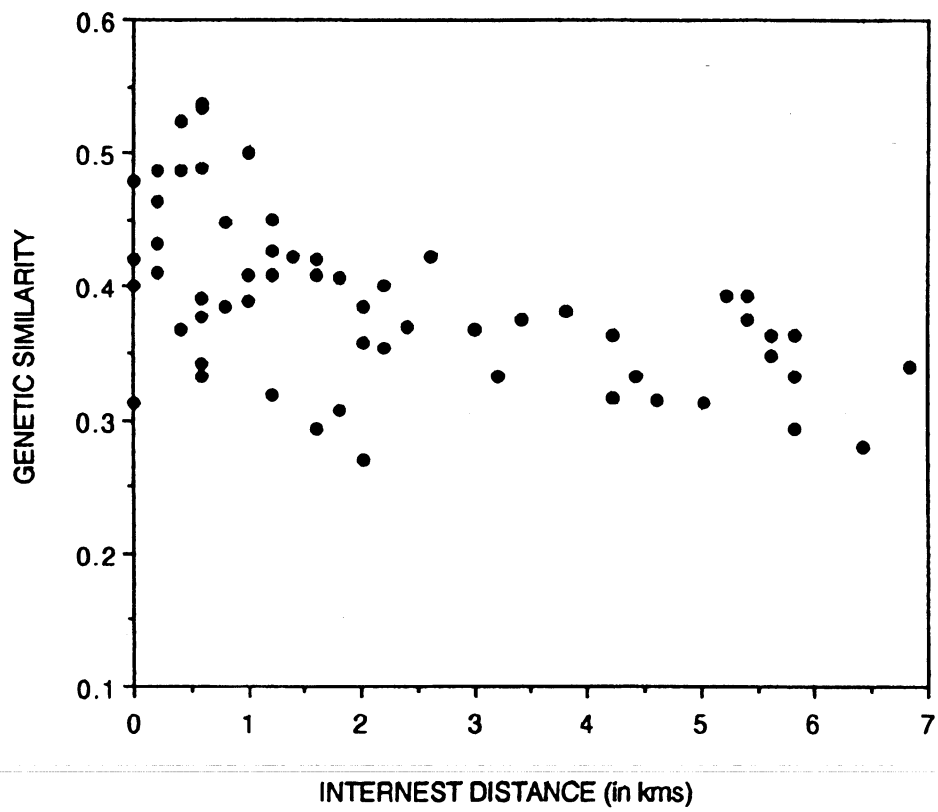

Figure 1. Relationship between internest distance and relatedness for 60 pairwise comparisons of 14 green turtles nesting along Tortuguero beach in 1991. Internest distance and genetic similarity values show a significant negative correlation (Mantel matrix correlation $r^2 = 0.273$; $p \leq 0.001$).

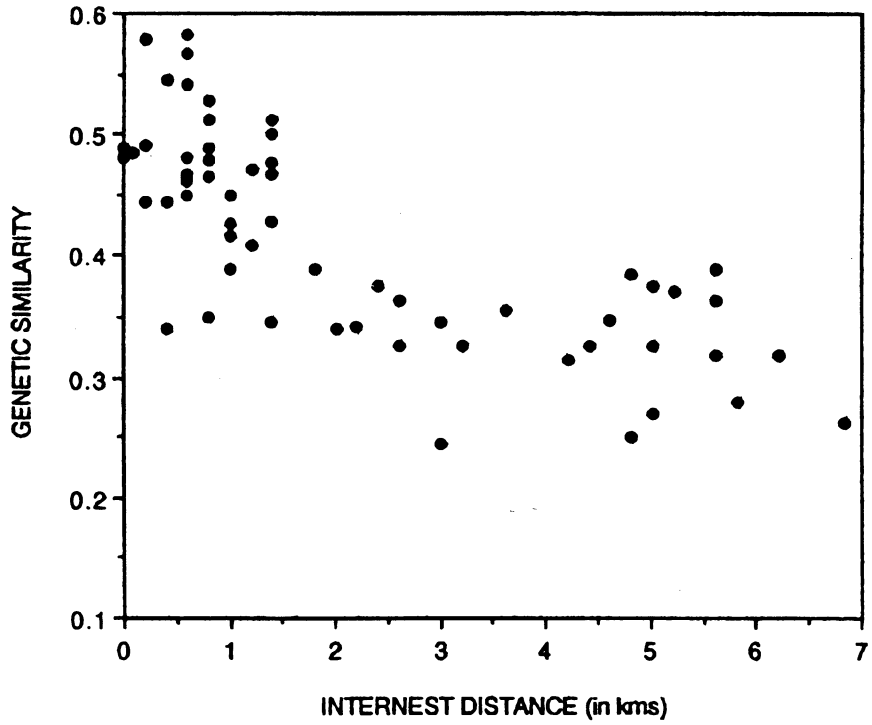

Figure 2. Relationship between internest distance and relatedness for 62 pairwise comparisons of 18 green turtles nesting along Tortuguero beach between 1991 and 1993. 25 pairs nested two years apart and 37 pairs nested one year apart. Internest distance and genetic similarity values show a significant negative correlation (Mantel matrix correlation $r^2 = 0.578$; $p \leq 0.001$).

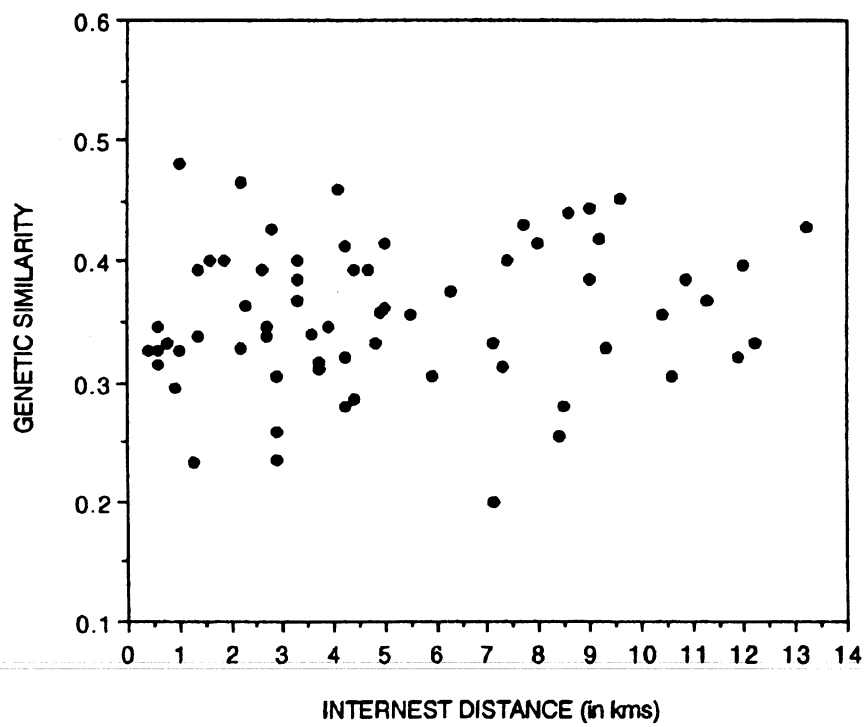

Figure 3. Relationship between internest distance and relatedness for 66 pairwise comparisons of 19 green turtles nesting along Melbourne beach in 1994. Internest distance and genetic similarity values were not significantly correlated (Mantel matrix correlation $r^2 = 0.017$; $p = 0.075$).

In contrast, the Melbourne population shows no relationship between genetic similarity and internest distance for pairs of nesting females (Fig. 3). Mantel matrix correlation $r^2 = 0.017$; $p = 0.075$). The lack of distance-related genetic structure within the Melbourne nesting colony indicates no evidence of natal philopatry by the females of this population.

Although these results reveal that green turtles in Tortuguero typically exhibit short-distance dispersal from natal sites, the same phenomenon was not observed at the Melbourne rookery. Possible explanations for these results include differential precision in homing, or demographic parameters affecting each rookery. One explanation involves the level of human disturbance that each beach experiences. The Melbourne population is exposed to high levels of potential disturbances in the form of artificial lighting (see Witherington 1992) associated with beach development. There is much less development along Tortuguero beach, so disturbance to nesting turtles in this population is likely to be lower. If Florida turtles are dislocated by such disturbances during nesting, the average natal dispersal distance will be elevated and any natural genetic structure may be dissipated.

Tag return data from green turtles nesting along Melbourne beach show that these females exhibit site fidelity within and between nesting seasons (Johnson 1994). These data suggest that Melbourne turtles are capable of distinguishing among different sections of the beach; however, whether they are distinguishing natal sites from other sites is unknown. It is possible that many first-time nesters abort attempts to return to their natal site due to disturbance from human development, and instead nest in less developed areas that may be some distance away. If disturbance there remains low, females may remain faithful to this section of beach and return there for subsequent nesting events.

A second possible reason for a difference in natal philopatry between the two populations is that the turtles from Tortuguero may be able to home with greater precision because better cues are available. Migrating females may use magnetic fields (Lohmann 1992) or olfactory cues (Carr 1967; Koch et al. 1969) to orient and return to natal beaches. Tortuguero beach may provide high quality magnetic or olfactory information that allows turtles to reliably distinguish among different sections of the nesting beach even after long absences. If cues of comparable quality are unavailable on Florida beaches, turtles nesting there would have higher natal dispersal distances because a first-time nester from Melbourne may not be capable of identifying her natal site. In contrast, a first-time nester returning to Tortuguero may find reliable orientation cues that allow her to distinguish her natal site from other parts of the rookery.

Alternatively, local genetic structure within each rookery may be influenced not by differences in within-beach homing precision, but rather by other rookery characteristics including the relative age of each rookery (Bowen et al. 1992), different levels of displacement by other species of marine turtles, or different levels of nest or hatchling mortality.

The nesting population along the coast of Florida may be a relatively new one (less than 10,000 years old; Bowen et al. 1992). If the colonization of Florida by green turtles is a recent event, there may have been insufficient time for detectible levels of genetic structure to develop along a distance of only 16 km (Bowen pers. comm.). An examination of the relationship between genetic similarity and internest distance for pairs of turtles nesting along the entire coast of Florida may, however, reveal that some level of genetic substructuring occurs in this rookery.

The green turtles of Melbourne share this nesting beach with the second largest aggregation of nesting loggerhead turtles (*Caretta caretta*) in the world (Ross 1982). Green turtles attempting to nest at Melbourne may suffer high levels of displacement by male or female loggerheads, and therefore may exhibit breakdowns in natal philopatry (B. Bowen pers. comm.). The differences in genetic structure within the Tortuguero and Melbourne nesting populations may therefore result from differential homing precision that is caused not by human disturbance, but rather by natural displacement from loggerheads.

Even if homing tendencies and abilities did not differ between the two populations, differential survivorship would influence the probability of detecting distance-related genetic structure within a rookery. Therefore, if hatchlings from Tortuguero enjoy higher survivorship, and exhibit natal philopatry, there would be more relatives within a cohort nesting in the same section of beach. Lower levels of survivorship among nests and hatchlings within Melbourne could ultimately produce a nesting population with few close relatives, making local genetic structure more difficult to detect. The annual nesting population of green turtles at Tortuguero ranges between about 6,000 to 23,000 females (Carr et al. 1978) while the Melbourne population numbers only about 30 to 700 females per year (Johnson 1994). Higher levels of mortality among turtles produced at Melbourne may be associated with this rookery's small size and spatial association with a large loggerhead rookery. Population comparisons of mortality rates for different life stages are one way of evaluating whether an absence of close relatives is influencing the detection of local genetic structure.

Our alternative approach will be to compare adult Melbourne females with their own hatchlings and generate genetic similarity values to characterize relatives. These values can then be used to estimate the proportion of adult female pairs that resemble close relatives. Such an analysis of the Tortuguero population revealed that 12.3% (15 of 122 pairs) of females are close relatives (Peare and Parker, In press). The differential mortality hypothesis predicts that a much smaller percentage of females from Melbourne will be found to consist of close relatives. If this is the case, then managers of the Melbourne rookery should be especially concerned with attempting to reduce mortality for the different life stages of Florida green turtles.

Due to the intractable features of green turtle demographics, it is difficult to determine differences in natural history between Tortuguero and Melbourne nesting populations. The conclusions of this study are therefore provisional until more data are collected. Regardless of the exact reason for the differential genetic structure within these two rookeries, substructuring within colonies has implications for marine turtle management. For example, precise natal homing, as a feature of female green turtles from undisturbed rookeries, raises several concerns for managers assigned with the task of reducing or reversing population decline. First, if there are fitness costs associated with forced dispersal (as a result of energy diverted from reproduction to nest site selection), then spatially discrete disturbances along the beach may contribute to a decrease in the productivity of a rookery. At the same time, if these disturbances result in the reduction of fitness for certain lineages, it can lead to the erosion of genetic diversity through the loss of alleles carried by members of these lineages.

Another consideration for managers is that nest relocation programs which concentrate green turtle clutches in a single hatchery may be inappropriate in some cases. Incubating embryos or emerging hatchlings may receive site-specific, chemical or magnetic information from the beach environment, and later use these cues to return to specific sites along the beach (Carr 1967; Grassman and Owens 1987). Nest relocation practices may therefore result in imprinting to the hatchery area. If a majority of females return to specific natal sites, then a large proportion of females in subsequent generations may converge to nest in the area around the hatchery site. Until more is known, a program that spaces hatcheries along the length of the beach may be more effective in maintaining an evenly distributed nesting population in future generations.

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Microsatellites and marine turtle conservation: the Kemp's ridley diversity project

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Introduction

The Kemp's ridley sea turtle experienced a severe population crash over a period of about thirty years, from an estimated 40,000 adult females during a single day of arribada nesting in 1947 (Carr 1963) to approximately 700 adult females nesting annually today. It appears that conservation efforts have been successful in stabilizing the population, and there are some indications that the population may even be increasing. This presents an interesting and timely conservation issue in terms of population-level genetic diversity. Population bottlenecks can result in reduced genetic diversity, and this diversity is often correlated with the overall fitness of the population. New molecular techniques, including microsatellite DNA analyses, make it possible to quantify diversity in a nearly unlimited number of genetic loci. Microsatellite markers tend to occur in non-coding regions of the genome, and are generally considered to be free from selection pressures. These features make microsatellite loci appropriate for elucidation of population genetic diversity.

Microsatellite markers are also suitable for examining pedigrees and mating systems. While the mating behavior of sea turtles has been studied in captivity (Comuzzie and Owens 1990), little is known about mating behavior in wild populations. In particular, it is not known if females mate multiple times, or if the same males are represented in subsequent clutches. By using tissue samples salvaged from eggs or hatchlings, microsatellite markers at multiple loci can be compared between mother and progeny. In this way it is possible to detect multiple paternity within clutches and differences in paternity between clutches laid by the same female during a single nesting season.

While previous genetic studies have examined the evolutionary history of ridley species relative to other sea turtle species (Bowen et al. 1991; Dutton et al. 1996), the Kemp's ridley diversity project will examine the genetic architecture of the sole population of *Lepidochelys kempi*. Therefore the first goal of this project will be to assess genetic diversity in the nesting population of Kemp's ridley turtles and compare this diversity with the level found in a robust population of olive ridley turtles. Population bottlenecks typically result in a reduction in genetic variability, but is this erosion of genetic diversity delayed in a species with a long lifespan? The Kemp's ridley population has crashed, stabilized, and may be increasing in a span of one or two generations. If there was a high degree of genetic variability before the crash, it is possible that a high degree of variability will remain.

The second goal of the Kemp's ridley diversity project will be to learn more about reproduction and mating behavior. In the Kemp's Ridley Recovery Plan, investigation of mating behaviors is recognized as one of the actions necessary for the recovery of the species (USFWS and NMFS 1992), and such knowledge is particularly valuable for protecting the nesting population.

Methods

Blood and tissue samples have been collected from the primary *L. kempi* nesting site at Rancho Nuevo, Mexico. Blood was collected from 30 adult females. The maximum number of tissue samples allowed under permit restrictions were salvaged from nests laid by these females. Additionally, a single sample was collected from each of 211 nests laid during a single arribada nesting event. All of these

samples were subjected to DNA extraction and have been used in PCR reactions.

For the genetic diversity study, Kemp's ridley samples are being compared with 60 olive ridley samples from an arribada nesting beach at Nancite, on the Pacific coast of Costa Rica. The populations are characterized for their respective levels of heterozygosity at multiple loci, and genetic markers for the mating system study are being developed based on primers described by FitzSimmons et al. (1995). The markers were selected for this study based on the criterion that they target regions of DNA that are polymorphic in *Lepidochelys*. Efforts are underway to develop *kempi*-specific microsatellite markers for use in the mating study.

Preliminary Results

Preliminary heterozygosity values (number of heterozygous individuals/ total number of individuals sampled) have been determined at four loci. Across those four loci, the average number of alleles was 5 and 9.5 for Kemp's and olive ridley respectively. The average heterozygosity values were 0.6025 and 0.4915 for *L. kempi* and *L. olivacea* respectively.

One of those four loci contained 6 alleles in 170 unrelated Kemp's ridley samples, and this locus was chosen to test for evidence of multiple paternity within clutches. Multiple paternity was indicated if clutches contained 3 or more alleles which could not be assigned to the maternal parent. In clutches where 3 or more offspring were assayed, 8 out of 22 contained evidence of multiple paternity.

Discussion

The Kemp's ridley population showed no evidence of reduced genetic diversity relative to the surveyed olive ridley population. However, several factors bear consideration in interpreting these findings. First, there is no level of genetic diversity that can be objectively labeled good or bad for species management. Over evolutionary time, a high degree of variability is considered advantageous because a variety of alleles provides a variety of ways in which a species might adapt to a changing environment. Low variability becomes a problem when the population harbors a high "genetic load" or a relatively high proportion of deleterious alleles. Inbreeding among individuals with a high level of deleterious alleles can be devastating.

Second, there is no established norm for heterozygosity values in sea turtle populations. Since the level of genetic diversity that existed before the crash is not known, an (apparently healthy) olive ridley population is being used as a means of comparison. However, the history of this olive ridley population is unknown. Aberrances in the population used for comparison would, of course, affect the interpretation. A related concern is that olive ridleys may have unusually high or low levels of diversity relative to other sea turtle species. The Nancite population itself may differ from other olive ridley populations. Despite these considerations, the preliminary data indicate that the Kemp's ridley is not severely depauperate in terms of microsatellite diversity. The relatively high degree of genetic variability observed in this analysis prompts the provisional conclusion that the Kemp's ridley population crash has not yet resulted in a severe population bottleneck. One positive implication of this conclusion is that a population recovery in the next few decades may allow *L. kempi* to retain much of the pre-crash genetic diversity.

While the single locus used in the paternity analysis provided evidence of multiple paternity, additional loci must be examined to determine the extent of multiple paternity and paternal contribution to multiple nests. In order to increase the number of variable loci, a Kemp's ridley DNA library has been constructed and is being screened for microsatellite loci.

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Hybridization and taxonomy of marine turtles: anonymous nuclear DNA sequence analyses

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Introduction

Our understanding of the ecology and evolution of marine turtles has deepened considerably by the recent application of molecular biological techniques. Analyses of mitochondrial DNA (mtDNA) have provided a wealth of information on natal homing, taxonomy, life history evolution, feeding ground mixed stock assessment and wildlife forensics. Nuclear DNA (nDNA) studies, although less well established, nonetheless contribute to a better understanding of microgeographic population structure, systematics, hybridization and male mediated gene flow. This symposium is quite timely, because much of the ground work necessary to delineate the scope of ecological questions that can be addressed with molecular approaches has been laid. In this section, I will discuss some of the progress and results of studies relying on data gleaned from anonymous single-copy nuclear DNA (ascnDNA). This is by no means a complete treatment. It does, however, provide a review of the utility of ascnDNA data to marine turtle conservation and biology. Detailed descriptions and discussions of the technical aspects of this approach are not addressed here and the reader is encouraged to consult other published material on ascnDNA studies (Karl, In press; Karl and Avise, 1993; Karl et al., 1992).

Single-copy Nuclear DNA Assays

The assessment of nuclear markers of any type (i.e., anonymous single copy loci, highly variable micro- and mini-satellite regions, conserved coding loci, etc.) bring several strengths to molecular ecological investigations. Since chromosomes recombine during meiosis, separate nuclear loci are independent assessments of the evolutionary history of the organism. From a population genetic standpoint, diverging taxa follow an evolutionary trajectory which takes them from a single panmictic population through reproductive isolation, to polyphyly, paraphyly, and finally to reciprocal monophyly (Avise, 1989; Avise, 1994; Avise and Ball, 1990). Although many factors can affect the rate at which species traverse this path, the central element is time. Taxa that have only recently diverged, therefore, will most likely exist as poly- or paraphyletic groups. Just as species progress from panmixia to monophyly, so do the various gene lineages within the organismal pedigrees which constitute the species. With recombination, each gene lineage is independent from all others. The concordance of gene genealogies is especially important in closely related taxa. Due to the stochastic properties of gene lineage sorting, a single gene may not be representative of the true organismal phylogeny (Avise and Ball, 1990; Ball et al., 1990). To understand the true organismal phylogeny, therefore, it is imperative to assess multiple, independent loci. This allows for a thorough representation of genetic variation at the population as well as the species level. It is here that ascnDNA markers are most useful.

An ascnDNA locus can be coding as well as non-coding sequences of the nuclear DNA. Thus, the data gathered are from a variety of sequences - some strictly neutral, some under selection and some linked to other loci which are under selection. Such a broad assay helps to reduce biases resulting from the examination of only a single class of genetic markers.

AscnDNA loci are isolated from a nuclear DNA library constructed from a single individual following standard laboratory procedures (Karl and Avise, 1993). The cloned DNA's are single alleles from one individual of the species or population. In order to determine population or species level variation, however, the genotypes of several individuals must be determined. This process is facilitated immensely by constructing, from the cloned individual, primers suitable for polymerase chain reaction amplification (PCR; Mullis and Faloona, 1987; Saiki et al., 1985). These primers are used for the in vitro synthesis of DNA from the homologous locus using total cell DNA samples taken from several individuals from the population or taxa of interest. The resulting amplified DNA can be assayed for intra- and inter-individual sequence variation by a variety of techniques (i.e., restriction endonuclease digestion, DNA sequencing, single-stranded conformational polymorphisms, heteroduplex assays, etc.).

An ascnDNA survey generates genotypic data from several loci for several individuals of a population or taxa. This type of multi-locus genotype data is widely applicable for determining heterozygosity levels, assessing parentage and kinship, elucidating population sub-division, detecting hybridization, evaluation of systematic relationships or forensics analysis. AscnDNA data unite characteristics of both isozyme electrophoretic and mtDNA data in a manner which circumvents many of the shortcomings of these methods. Isozyme data are unordered, diploid, multi-state, co-dominant, biparentally inherited characters from several loci. MtDNA data are phylogenetically ordered, haploid, multi-state, maternally inherited characters from a single locus. AscnDNA data are phylogenetically ordered, diploid, multi-state, co-dominant, biparentally inherited characters from several loci. By assaying variation at the level of the DNA and by producing phylogenetically ordered characters, ascnDNA markers allow a more fine scale resolution of evolutionary phenomena than isozymes. By assaying multiple, independent genetic systems, ascnDNA data reduces the biases introduced by surveying only a single-locus, such as mtDNA (Avise and Ball, 1990; Ball et al., 1990).

Previously, I have used ascnDNA markers to assess population level differentiation in green turtles (Karl and Avise, 1993; Karl et al., 1992). Here I would like to discuss some of the other applications of these markers. Although, the original markers were specifically designed for green turtles, they are capable of revealing genetic variation at homologous loci from other species (Table 1) and have provided insight into the population genetic and systematic relationships of marine turtles.

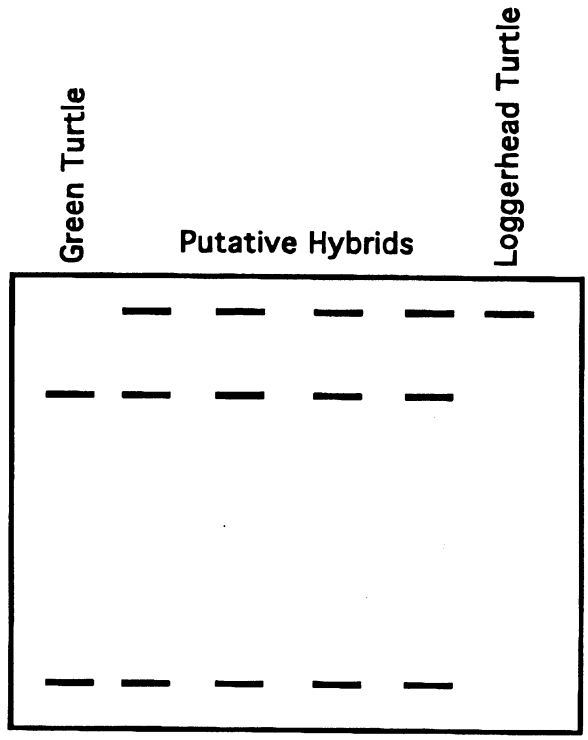

Figure 1. Diagram of an ascnDNA species-specific restriction endonuclease site polymorphism. The hypothetical restriction profile shows the loggerhead turtle ascnDNA locus is uncut by the chosen restriction endonuclease. The green turtle ascnDNA locus contains a single recognition site and the DNA fragment is cleaved in two. Restriction endonuclease digested DNA from the putative hybrid turtles reveals that each of the four individuals shown possesses both the green turtle and the loggerhead turtle restriction profile. This confirms that they are hybrids with one parent being a green and the other a loggerhead turtle.

Application of ascnDNA to Marine Turtles

Hybridization in Marine Turtles

The success of conservation efforts can be greatly influenced by hybridization in the targeted species. Endangered species, by definition, are populations that comprise relatively few individuals, often in marginal, deteriorating or shrinking habitats. An increase in the frequency of hybridization, among ecologically endangered species or between endangered and non-threatened species, can markedly accelerate the decline and extinction of sensitive taxa (Templeton, 1994). Molecular genetic approaches have proven useful in the detection of natural hybridization among marine turtle species (Karl et al., 1995). Three suspected occurrences of natural hybridization recently have been confirmed with molecular genetic techniques - a hybrid between a loggerhead and a hawksbill found in Florida; a Kemp's ridley and a loggerhead hybrid discovered in Chesapeake Bay during the summer of 1992; and a possible second (or later) generation hybrid of a green turtle and a hawksbill, originally collected in Suriname in 1977 (Karl et al., 1995).

A fourth case of hybridization was discovered by chance during global population genetic surveys of green and loggerhead turtles (Bowen et al., 1992; Bowen et al., 1994). Supposed green turtle hatchling clutch-mates from Brazil unexpectedly possessed loggerhead mtDNA. All four individuals were screened at several nDNA loci and each revealed DNA patterns characteristic of both green and loggerhead turtles (Figure 1). The combination of the nuclear and mitochondrial DNA evidence, in this case, indicated that these hatchlings were first generation hybrids between a loggerhead and a green turtle. In fact, in all of these cases, the analysis of mtDNA and ascnDNA markers was necessary to rigorously determine; 1) that these individuals were indeed hybrids, 2) the identity of the parental species, and 3) the direction of the cross (i.e., which species was the maternal and which was the paternal parent).

The mere existence of these hybrids is striking because they are the result of crosses between members of the tribes Carettini and Chelonini which constitute a very old split within the family Cheloniidae (perhaps 50 or more million years ago; Bowen et al., 1993; Ernst and Barbour, 1989). Indeed, these marine turtle taxa may be the oldest vertebrate lineages known to hybridize in nature.

Subspecific status of the black turtle, *Chelonia mydas agassizi*:

The green turtle (*Chelonia mydas*) is historically one of the more abundant of the marine turtle species. *C. mydas* is distributed globally in a variety of tropical and subtropical habitats. Since the Atlantic and Pacific ocean basins are isolated by continental masses as well as cold, temperate waters (lethal to green turtles), little natural mixing probably has occurred between ocean basins since the closure of the Isthmus of Panama (ca. 3-4 million years ago). Previous molecular genetic studies using mtDNA have shown a high degree of inter-ocean isolation in this species (Bowen et al., 1992). Within ocean basins, these studies also have indicated that green turtles natally home and comprise a collection of broad, geographically isolated populations. In addition to natal homing, the variety of ecological habitats that green turtles occupy may further promote sub-division or ecotypic specialization within ocean basins. In fact, subspecific status has been proposed several times for regional forms of the green turtle - Caribbean (*C. m. viridis*), South Atlantic (*C. m. mydas*), Indo-West Pacific (*C. m. japonica*), Gulf of California (*C. m. carrinegra*), and East Pacific (*C. m. agassizi*) (Carr, 1975; Pritchard and Trebbau, 1984). Although the East Pacific black turtle is often accorded full species status as *C. agassizi*, the evidence to support this designation is limited and somewhat contradictory (Mrosovsky, 1983).

While the debate over *C. agassizi* continues, recent evidence seems to move the question from the specific to the sub-specific level. mtDNA data, while supporting Atlantic-Mediterranean and Indian-Pacific groups of green turtles, do not support the taxonomic status of *C. agassizi* (Bowen et al., 1992; Bowen et al., 1993). This conclusion is further supported by additional mtDNA data (Dutton et al. 1996). These studies indicate that the black turtle is paraphyletic to the Atlantic and Pacific division in the green turtle. Both studies support

Table 1. Amplification of five anonymous single-copy nuclear DNA loci from the various marine turtle species. An "X" indicates that DNA was successfully amplified from this species.

Species	CM-12	CM-14	CM-28	CM-39	CM-45
Chelonia mydas	X	X	X	X	X
Caretta caretta	X	X	X	X	X
Lepidochelys olivacea	X	X	X	X	X
Lepidochelys kempi	X	X	X	X	X
Eretmochelys imbricata	X	X	X	X	X
Natator depressus	X	X	X	X	X
Dermochelys coriacea			X		X

a population or subspecific designation for the black turtle. Concerns are raised, however, about potential biases that may result from assessing a single gene genealogy such as mtDNA (Dutton et al., 1996). Morphological data, primarily skull features, also support a cautious assignment of subspecific status to the East Pacific turtles (Kamezaki and Matsui, 1995). Taken together, it seems that at present there is no strong, scientific evidence for a specific designation of the East Pacific *Chelonia* population (Zug, 1996).

The assessment of ascnDNA loci aids in the further elucidation of the taxonomic distinctiveness of the black turtle. In a previous restriction site polymorphism study Karl et al. (1992) found no strong support for a taxonomic designation of the black turtle. These conclusions are limited, however, in two ways. First, restriction endonuclease assays detect differences between individuals at a relatively small number of nucleotides [6.4 - 11.7% of the sequence per locus; Table 1 in Karl et al. 1992]. Second, the ascnDNA study did not support the Atlantic-Mediterranean and Indian-Pacific

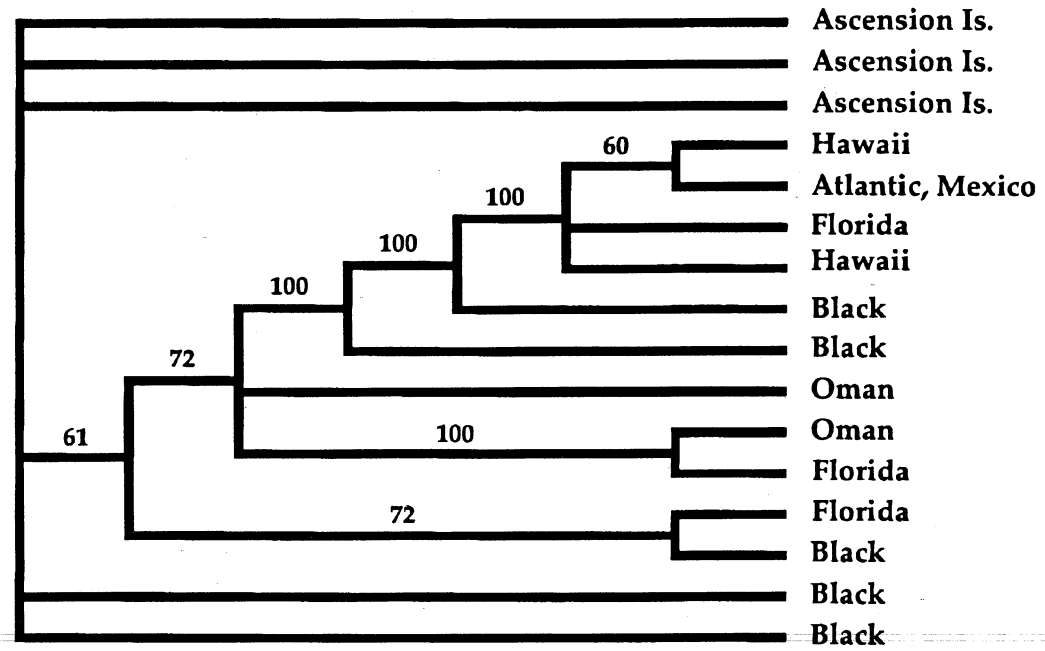

Figure 2. Majority rule consensus tree for green and black turtle ascnDNA sequences from locus CM-14. Relationships were determined by the Branch and Bound method of the computer program PAUP Ver. 3.1.1 (Swofford, 1993). Numbers at each node refer to the percentage of most parsimonious trees (out of 90 total) which contained that particular branching.

groups, though these groups were well supported by mtDNA and biogeographical information. This is possibly due to the slower rate of evolution and a larger effective population size for nuclear relative to mitochondrial genomes. For these reasons, sequence variation at three of the ascnDNA loci in putative black and green turtles has been undertaken. Preliminary sequence data are consistent with the ascnDNA restriction endonuclease and mtDNA surveys and indicate that the black turtle does not constitute a distinct evolutionary lineage (Figure 2). As with the restriction survey, the ascnDNA sequence data do not support the Atlantic-Mediterranean and Indian-Pacific groupings. This, however, cannot be due to a lack of sufficient variation at the assayed loci. Most likely this is a result of the lack of sufficient evolutionary time for the groups to come to reciprocal monophyly. Nonetheless, combined ascnDNA and mtDNA data (a gene less subjected to retardation in the progress to reciprocal monophyly) indicate that the black turtle does not merit taxonomic status as a distinct species.

Higher level relationships of the Testudinae:

Bowen et al. (1993) provide a summary of the most salient uncertainties concerning the taxonomy of marine turtles. This study was an independent phylogenetic assessment using mtDNA cytochrome b sequence data. Subsequent to this, Dutton et al. (1996) collected additional mtDNA data, which further supports and strengthens the understanding of marine turtle systematic relationships. An understanding of the organismal phylogeny (as opposed to the single gene genealogy provided by mtDNA alone), however, requires the assessment of multiple, independent genetic loci. To address questions of gene genealogical concordance among the marine turtle species, I have begun collecting data from several ascnDNA loci. As indicated previously, several of the primers to ascnDNA loci in green turtles can be used to screen homologous loci from all of the marine turtle species.

Currently, only a single ascnDNA locus (CM-28) has been assayed for sequence variation among all the marine turtles and the fresh water snapping turtle (*Chelydra serpentina*). Nonetheless, interesting and provocative preliminary patterns are emerging. There are several points in agreement with previous DNA sequence studies. Although only a single outgroup is available, the marine turtles appear to be a monophyletic group. In addition there is strong support for the current distinction of the tribe Carettini (*Caretta caretta*, *Lepidochelys* sp., and *Eretmochelys imbricata*) within the family Cheloniidae (Figure 3). Relationships within the Carettini, however, are somewhat unresolved, with weak support (low bootstrap values) for *E. imbricata* as the sister group to the genus *Lepidochelys*. Also concordant with previous molecular studies (and historical nomenclature) is the uncertain

Table 2. Genetics distances among marine turtle species for ascnDNA locus CM-28. Distances were calculated using the maximum likelihood method of Felsenstein (1989). Taxa abbreviations are: CC - *Caretta caretta*, EI - *Eretmochelys imbricata*, LO - *Lepidochelys olivacea*, LK - *Lepidochelys kempi*, CM - *Chelonia mydas*, ND - *Natator depressus*, DC - *Dermochelys coriacea*, CS - *Chelydra serpentina*.

TAXA	CC	EI	LO	LK	CM	ND	DC
EI	0.0149						
LO	0.0165	0.0174					
LK	0.0094	0.0099	0.0068				
CM	0.0287	0.0346	0.0342	0.0265			
ND	0.0314	0.0337	0.0337	0.0260	0.0077		
DC	0.0535	0.0580	0.0588	0.0512	0.0267	0.0338	
CS	0.1114	0.1121	0.1142	0.1059	0.0829	0.0892	0.0823

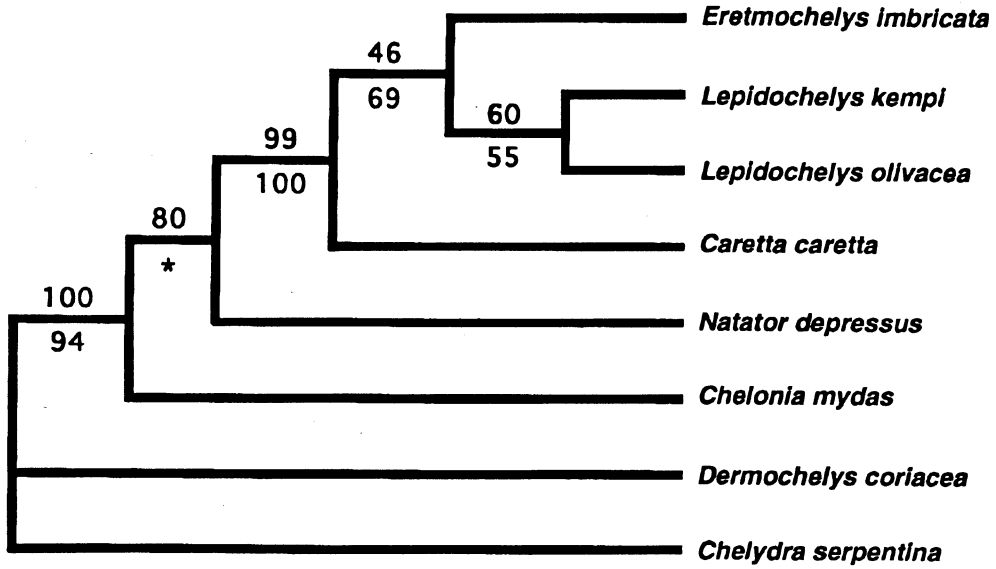

Figure 3. Phylogenetic relationships of the marine turtles and the outgroup, *Chelydra serpentina* based on sequence data from the ascnDNA locus CM-28. Relationships were based on maximum likelihood distances and the tree was constructed using the neigbor-joining method. The data were also analyzed using the branch and bound method in the program PAUP (Swofford, 1993). The tree shown is the single most parsimonious tree. Numbers at nodes represent bootstrap values for 100 maximum likelihood estimates (above) and 1,000 Branch and Bound replicates (below). The "*" at the node between *N. depressus* and *C. mydas* indicates that this split was not resolved in the single most parsimonious tree.

placement of the flatback turtle (*Natator depressus*). Although a bootstrapped neighbor-joining analysis of maximum likelihood genetic distances supports the clustering of *N. depressus* with the Carettini at a level of 80% (Figure 3), the genetic distance between *C. mydas* and *N. depressus* is nearly identical to the distance between the *Lepidochelys* species (0.0077 and 0.0068 respectively; Table 2). In fact, a parsimony analysis fails to resolve the relationship of *C. mydas, N. depressus*, and the Carettini. Furthermore, a preliminary restriction fragment analysis of a second ascnDNA locus (CM-14, Karl unpublished data) resulted in no restriction site differences between *C. mydas* and *N. depressus* in 14 restriction sites which are variable among the marine turtles. This leaves in serious doubt the correct placement of the flatback in the marine turtle phylogeny. Finally, even though *L. kempi* and *L. olivacea* are only weakly supported by both the parsimony and distance analyses, the genetic distance between these species is the smallest of all comparisons (Table 2) and most likely reflects the close evolutionary relationship of these taxa.

Discussion

Though preliminary, the ascnDNA data provide additional support for and raise further questions about our understanding of the ecology and systematics of the marine turtles. Several intriguing results from the ascnDNA data clearly are forthcoming.

The occurrence of hybridization in such an evolutionary ancient group is surprising. Although marine turtle hybrids have been suspected for years, molecular analyses have permitted the firm documentation of hybridization. Predictions about the impact of hybridization on natural populations are difficult to make, and elucidation of specific factors contributing to the occurrence of such hybrids may be impossible. Nonetheless, some information on the ecological attributes which facilitate the production of hybrids is available and may be applicable to marine turtle conservation. Temporal and spatial overlap in mating areas may be necessary for, or at least facilitates, hybridization (Conceicão et al., 1990; Wood et al., 1983). Stocking and transplanting

of individuals, either for the repatriation of extirpated populations, enhancement of deteriorated stocks, or expansion of current population ranges can increase the incidence of hybridization (Allendorf, 1983; Echelle et al., 1987; Echelle and Kornfield, 1984). Habitat destruction and alteration also is known to disrupt normal barriers to reproduction (Lamb and Avise, 1986; Wayne et al. 1991; 1992). Marine turtles are subjected to all of these conditions. It is unknown, however, to what extent population manipulation has resulted in hybridization.

Also unknown is the reproductive status of hybrids that are produced. However, the second-generation green-hawksbill cross, cited above, provides evidence that at least some of the marine turtle hybrids may in fact be fertile. This can contribute to the melding of the species, which can have drastic legal and biological consequences for marine turtle conservation. Even if infertile, hybrids represent a waste of reproductive effort. This aspect is unlikely to be free of negative consequences for species longevity. It seems clear at this point that hybridization in marine turtles is occurring more frequently than was previously assumed and may have a negative impact on conservation efforts. Consideration should be given to the possibility that manipulating marine turtle populations (e.g., reduction in numbers, transplantation, hatchery rearing, repatriation, etc.) may increase or promote the incidence of hybridization. The causes and affects of hybridization may require attention in management plans.

A clear understanding of the population genetic and taxonomic associations of marine turtle populations and species also is of paramount concern to conservation programs. The green turtle presents a clear example of the need to consider genetics as well as ecological and taxonomic matters in devising conservation strategies. As East Pacific populations of green turtles continue to decline, interest has been focused once again on the evolutionary distinctiveness of the black turtle. In considering the conservation implications of taxonomic status, it bears remembering that, independent of nomenclature, the individual character of the East Pacific populations has been demonstrated in several ways, including coloration, skull morphology, and population-level genetic partitions (mtDNA genotype frequency shifts), and this should be sufficient to motivate conservation efforts. Furthermore, the general high degree of population subdivision observed in green turtles should warrant conservation efforts on regional as well as global levels. It would be a mistake, however, to support species designations purely as a political tool to facilitate conservation efforts. Such an endeavor dilutes the general importance of taxonomy in conservation and diverts attention away from the more significant goal of maintaining the breadth of variation that occurs in species.

Inasmuch as conservation programs are appropriate for populations of marine turtles, they are even more necessary at the species (and higher) level. Critical to these efforts is a clear understanding of the evolution and taxonomic relationships among these species. Several recent studies have provided an intriguing picture of marine turtle evolution. Independent of hybridization, marine turtles are clearly a unique evolutionary unit. The flatback turtle, however, continues to be enigmatic. Interestingly, the flatback turtle historically was considered to be the sister taxa to *C. mydas*. Only recently has it been aligned with the Carettini rather than the Chelonini (Limpus et al., 1988; Zangerl, 1980; Zangerl et al., 1988). Subsequent molecular studies using mtDNA (Bowen et al., 1993; Dutton et al., 1996) continue to reflect the difficulty in taxonomic assignment of *N. depressus*. In one of these studies (Dutton et al., 1996), *N. depressus* is alternately aligned with the Carettini and the Chelonini depending upon which segment of the mtDNA (a single genetic unit) was assayed. A "total evidence" approach (combining all available molecular data from the mtDNA) does strongly support the association of *N. depressus* with the Carettini. However, as originally conceived (Eernisse and Kluge, 1993), total evidence methods require that <u>independent</u> data sets be combined in a single analysis. It is presumed that the phylogenetically informative signal in the data will be enhanced relative to the uninformative noise as the type and amount of data is increased. Regardless of the value of this approach, as a non-recombining unit (super gene), different regions of a mitochondrial genome cannot be considered independent data

sets. By combining mtDNA with ascnDNA data, however, a true total evidence approach would be effected.

The currently available ascnDNA data provide a most intriguing view. Not unexpectedly, these data reflect the existing confusion surrounding the placement of the flatback turtle, but in a subtly different way. The mtDNA data generally support a trichotomy for *N. depressus*, Chelonini and Carettini. This is primarily due to the three groups being distantly but equally related to each other. The ascnDNA data tend to support the *N. depressus*/Carettini association (Figure 3). The genetic distance between *N. depressus* and *C. mydas*, however, is quite small and in fact is nearly equal to that which separates *L. kempi* from *L. olivacea* (Table 2). Two other observations from ascnDNA data (one preliminary and the other superficial) heighten the confusion surrounding this issue. First, the restriction endonuclease data from a second locus (CM-14) provide no indication of any differences between *N. depressus* and *C. mydas*, while differentiating between all other species of marine turtles (except *Lepidochelys*). Second, even though the ascnDNA primers all are capable of amplifying DNA from *N. depressus*, some primers amplify using similar stringent conditions for all species (except *Dermochelys coriacea*) whereas other primers amplify the flatback only poorly if typical conditions are used. This may be the result of a small number of significant changes at these loci in the flatback (thus supporting the *C. mydas*/ *N. depressus* association). Alternatively, variation in optimal primer amplification conditions may reflect a much greater level of divergence between the green and flatback turtles at some loci and not others. If this pattern also is found at the DNA sequence level, then the independent ascnDNA loci will also echo the historical taxonomic instability of the flatback. Taken in total, these data raise an interesting question: could the flatback turtle be the product of an ancient hybridization event? Although a provocative speculation, specific, robust conclusions will have to await additional ascnDNA data.

Conclusions

The recent and ongoing studies reported here outline the utility of assessing multiple, independent molecular characters in ecological and conservation studies. AscnDNA assays have provided significant insight into the biology and ecology of marine turtles. One of the strengths of ascnDNA assays is that this approach can evaluate genetic variation existing at several, independent loci. This is not only an important advancement from phylogeographic and taxonomic perspectives, but allows the assessment of biparentally and gender-specific information unavailable by other techniques (i.e., mtDNA). Much of the information presented here is preliminary in nature. It is hoped that further application of ascnDNA markers to marine turtle ecology and biology will help to strengthen our understanding of the special needs and conditions of these endangered and threatened species.

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Tracking marine turtles with genetic markers [1]

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Introduction

Marine turtles can precisely navigate across hundreds or thousands of kilometres of open ocean (Lohmann and Lohmann 1994), but scientific efforts to track these migrations have met with limited success. In many cases researchers know the locations of feeding grounds and the locations of nesting populations, but do not know which nesting populations use which feeding areas. This gap in marine turtle natural history has been brought into sharp focus over the last decade by an emerging conservation concern: thousands of sea turtles drown every year in driftnet and longline fisheries, and it is not known which nesting colonies are affected by this mortality. For example, when a sea turtle is killed by driftnet fisheries in Mediterranean waters, does this diminish the highly endangered nesting population(s) in Greece and Turkey or the larger nesting colonies of the western Atlantic? Conservation efforts to date have been stymied by the inability to link marine turtles at sea to their respective nesting populations.

Previous and ongoing surveys of sea turtle nesting colonies have demonstrated that most rookeries are distinguished by significant shifts in the frequency of mtDNA haplotypes (Bowen et al. 1992, 1994; Bass et al. 1996; Broderick et al. 1994; Dutton 1995; Encalada et al. 1996), and these data provided strong genetic evidence in support of the natal homing hypothesis for sea turtle migrations (Bowen and Avise 1995). When the genetic distinctiveness of marine turtle nesting populations was discovered, it became apparent that mtDNA sequences could be used as natural genetic tags to assign feeding cohorts to a rookery of origin. With the development of polymerase chain reaction (PCR) methodology, researchers at University of Queensland and University of Florida were able to design sensitive DNA sequence assays specifically for marine turtles (Allard et al. 1994; Norman et al. 1994). Using this improved methodology, scientists could obtain reliable genetic data from a few drops of blood, a tiny tab of preserved material, or even dried and partially degraded tissues (Dutton 1996). The distribution of mtDNA haplotypes on the feeding grounds can now be reliably assayed, providing an entirely new approach to resolving marine turtle demography and migrations.

How can the distribution of mtDNA polymorphisms be translated into estimates of feeding ground composition? To accomplish this, marine turtle researchers borrowed a technique from fishery biologists who have used differences in genotype frequency, detected with protein electrophoresis, to assess the contribution of riverine salmon stocks to a coastal fishery (Grant et al. 1980; Pella and Milner 1987). These mixed stock assessments, based on a maximum likelihood algorithm, are adaptable to mtDNA data (Xu et al. 1994). The distribution of mtDNA haplotypes on nesting beaches can be compared to the haplotype frequencies on the feeding grounds, and this comparison is used to calculate the contribution of each nesting beach which best fits the observed haplotype distribution.

With these technical and conceptual developments, several laboratories have applied PCR-based assays to document marine turtle migration and feeding ground composition. The cases reviewed below constitute the first wave of results. These efforts represent an exciting scientific frontier, as they have already generated new theories about the natural history of marine turtles. However, genetic markers

[1] EDITOR'S NOTE: This is an abbreviated version of a paper which was published in BioScience (1995, vol. 45:528-534). Reprinted with permission from the American Institute of Biological Sciences. Copyrighted (1995) by A. I. B. S.

also have very practical applications in the conservation of marine turtle populations. For the first time, the source of turtles captured far from their nesting beach can be determined with relative expediency and accuracy. This information allows wildlife managers to make informed decisions about the impact of human encroachment on marine turtle feeding grounds (Bowen and Avise 1995).

Case 1. Loggerhead turtles in a Mediterranean fishery

One puzzling facet of marine turtles distribution is the concentration of juvenile loggerhead turtles in the western Mediterranean. Researchers have observed that many more juvenile loggerhead turtles occur in this area than can be produced by the Mediterranean nesting beaches (Laurent 1990). One theory to explain this distribution is that some of these turtles are derived from the large nesting colonies of the western Atlantic (Groombridge 1990). If true, this would corroborate Archie Carr's postulation of a lengthy pelagic stage (Carr 1986, 1987). Thus the juvenile loggerheads of the Mediterranean represent a prominent point of inquiry about marine turtle life history. However, these juveniles invoke a strong conservation concern as well, because Mediterranean fisheries capture an estimated 20,000 turtles per year (Groombridge 1990), and perhaps 20%-50% of these animals perish (Aguilar et al. 1995).

Nesting beach surveys demonstrate the presence of a distinctive mtDNA genotype at approximately 39% frequency in the West Atlantic nesting colonies (represented by Florida; Georgia; and South Carolina, U.S.A.; n=92) which is absent from the Mediterranean nesting colony in Greece (n=21) (Bowen et al. 1993, 1994). Additional rookery sampling (n=22) support the conclusion that this genotype is absent from Mediterranean nesting colonies (Laurent et al. 1993). In a survey of 59 specimens from a western Mediterranean feeding ground, Laurent et al. (1993) detected the genotype endemic to western Atlantic nesting colonies in 22% of specimens (Figure 1). Based on these data, a maximum likelihood analysis using the computer program UCON (Masuda et al. 1991) indicates that 57% (±13% S.D.) of the turtles in the western Mediterranean are derived from west Atlantic nesting populations, and 43% (±13% S.D.) are derived from Mediterranean nesting populations. Genetic data demonstrate that turtles from the western Atlantic nesting populations enter the Mediterranean as Archie Carr postulated. Furthermore, these data indicate that approximately half of the turtles which perish in Mediterranean fisheries are from the west Atlantic nesting beaches and roughly half are from the rookeries in Greece, Turkey, and Cyprus. These findings prompt compelling questions about the management and jurisdiction of endangered species which occupy international waters (see below).

Case 2. Loggerhead turtles in the maritime corridors of the southeastern U.S.

Loggerhead turtles nest extensively along the southeast coast of the United States. Mitochondrial DNA analyses indicate that this nesting habitat contains at least two genetically distinct nesting populations (Bowen et al. 1993). The southern nesting aggregate, encompassing the Florida peninsula south of Canaveral, is one of the largest in the world with perhaps 30,000 nesting females. The northern population, including Georgia, South Carolina, and (possibly) North Carolina nesting beaches, is a tenth of the size of the southern population, with perhaps 3,000 nesting females (Murphy and Hopkins-Murphy 1989). While population trends are unavailable for the southern population, the northern population has shown evidence of long-term decline (Richardson 1982).

Bays and coastal waters along the east coast of the United States are occupied by loggerhead turtles, representing another significant habitat for pre-adult stages. Many of these same waters are used extensively for recreational and commercial activities, such that channel dredging, fisheries, and heavy traffic represent growing conservation concerns for juvenile loggerhead turtles. To determine the origin of the turtles which occupy this coastal habitat, researchers at the National Marine Fisheries Service Southeast Fisheries Science Center have surveyed mtDNA haplotypes in 33 juvenile turtles in the vicinity of Charleston Harbor Entrance Channel. If turtles are recruiting at random from both northern and

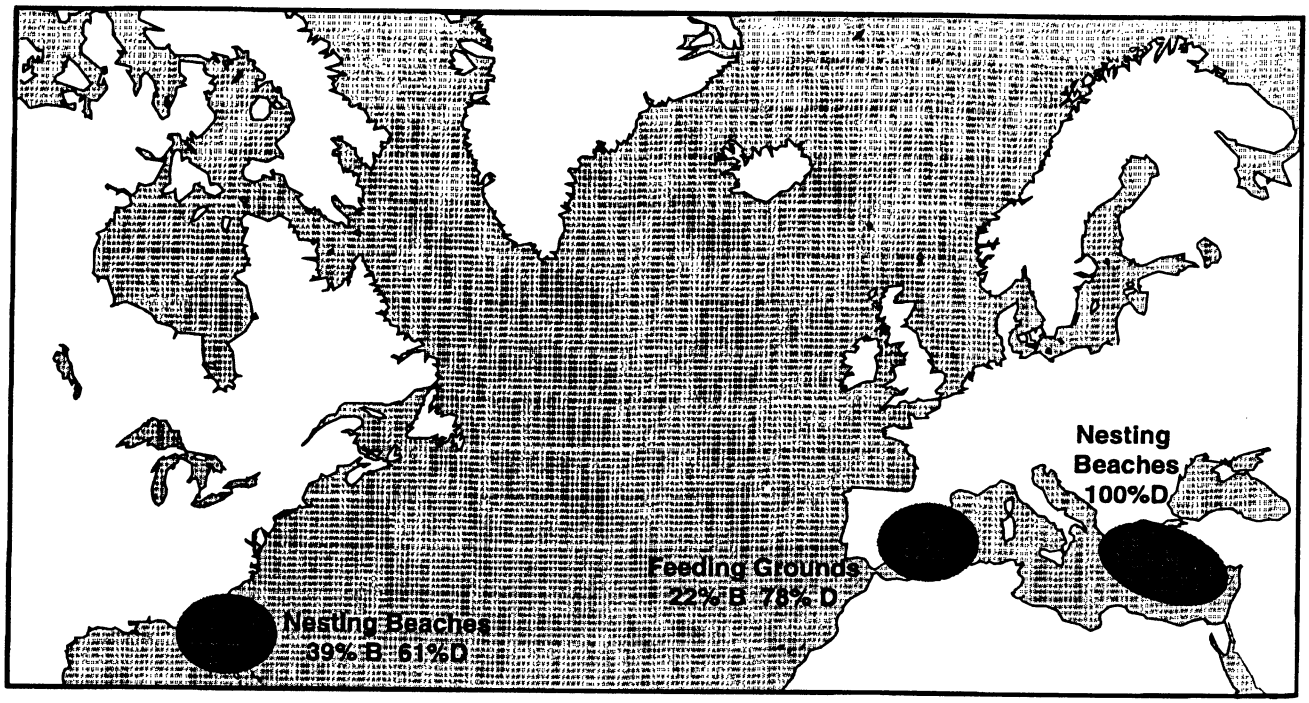

Figure 1. The distribution of loggerhead mtDNA haplotypes on West Atlantic nesting beaches, Mediterranean nesting beaches, and a Mediterranean feeding ground. Distinct haplotypes are assigned letter designations following Bowen et al. (1993), and information on feeding ground composition is adapted from Laurent et al. (1993). Haplotypes which were observed in less than 5% of samples were excluded from this evaluation. Maximum likelihood (ML) analysis indicates that approximately 57% (±13% S. D.) of the turtles in the Western Mediterranean are derived from nesting populations in the southeastern United States, and 43% (±13 S. D.) are derived from Mediterranean nesting populations.

southern nesting populations, then about 10% would be derived from the smaller northern nesting population. Based on the frequency of mtDNA haplotypes, Sears et al. (1995) estimate that approximately 50% of sampled turtles are derived from the northern nesting population. Hence the nesting beaches of Georgia, South Carolina, and North Carolina may be especially vulnerable to human exploitations in coastal habitats of the southeastern U.S.

Case 3. The loggerhead turtles of Baja California

Researchers have recently documented a feeding aggregate of juvenile loggerhead turtles, estimated at 10,000 individuals, in the vicinity of Baja California (Pitman 1990; Ramirez et al. 1991). The presence of loggerhead turtles in this area is surprising because nesting occurs in the West Pacific (in Japan and Australia) but is absent from the central and eastern Pacific. To explain this distribution, Itaru Uchida of the Ogasawara Marine Center (Japan) suggested that the prevailing currents may transport turtles from Japanese nesting beaches to East Pacific feeding areas, in a manner analogous to the trans-Atlantic journey postulated by Archie Carr (Uchida and Teruya 1991). This theory has not been widely accepted because a trans-Pacific migration would exceed 10,000 kms and would traverse several biogeographic boundaries including the pelagic eastern Pacific barrier (Briggs 1974).

A piece of this biogeographic puzzle fell into place when biologists discovered juvenile loggerhead turtles in the high seas fisheries of the central North Pacific Ocean (Wetherall et al. 1993). The presence of loggerhead turtles in the easterly North Pacific current is consistent with the trans-Pacific migration postulated by Uchida and Teruya (1991), but this finding also invoked a strong conservation concern: annual mortality in North Pacific driftnet fisheries has

exceeded 4,000 loggerhead turtles (Wetherall et al. 1993). A moratorium on this driftnet fishery is currently in effect, but longline fisheries have replaced the driftnets as the primary source of loggerhead turtle mortality. Hence it is imperative to know the origin of these oceanic migrants in order to assess the conservation implications of fishery mortalities on the high seas.

Do loggerhead turtles traverse the Pacific Ocean? To address this question, samples from the two primary Pacific nesting locations, Queensland, Australia (n=26) and Wakayama Prefecture and the Ryukyu Archipelago in Japan (n=26) were compared to samples from turtles drowned in North Pacific driftnet fisheries and to blood or tissue samples from turtles captured off Baja California. In this case, the two candidate nesting areas were characterized by a fixed difference in mtDNA haplotypes. This allowed mtDNA polymorphisms to be employed directly as genetic "tags" indicating the origin of pelagic juveniles in the North and East Pacific Ocean. Thirty three of 34 driftnet samples and 24 of 26 Baja Californian samples matched the haplotypes observed only in Japanese nest samples (Figure 2). The remainder matched the Australian nest samples. Japanese nesting beaches contribute approximately 95% of the surveyed driftnet and Baja California specimens, and Australia may contribute the other 5% (Bowen et al. 1995). Genetic data identify Japan as the primary source of pelagic aggregates and fishery mortalities in the central and eastern Pacific. Loggerhead turtles apparently traverse the entire North Pacific Ocean, approximately one third of the planet's circumference, in the course of developmental migrations (Bowen et al. 1995).

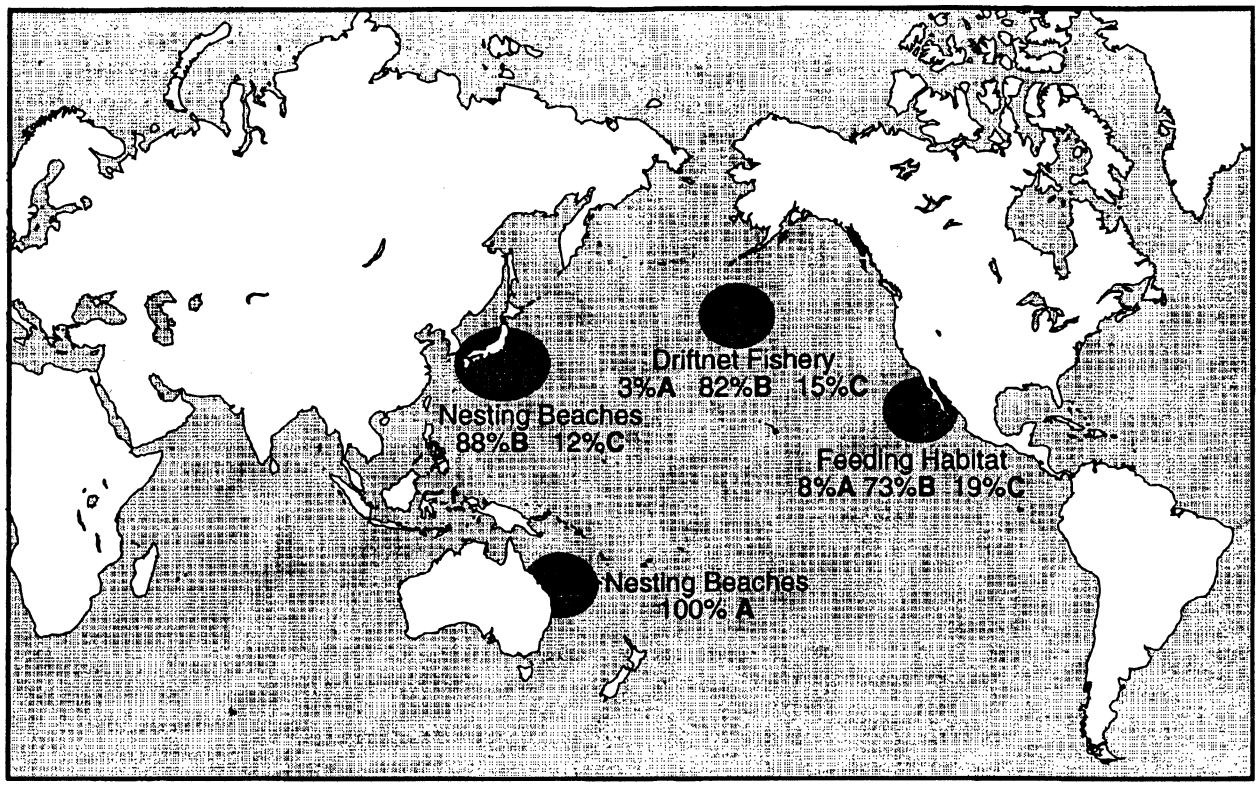

Figure 2. The distribution of loggerhead turtle mtDNA haplotypes in West Pacific nesting beaches, a North Pacific driftnet fishery, and an East Pacific feeding ground. Distinct mtDNA haplotypes are assigned letter designations following Bowen et al. (1995). Japanese and Australian nest samples are characterized by a fixed difference in the distribution of mtDNA polymorphisms, allowing these genetic markers to be applied as natural "tags" indicating the origin of pelagic feeding aggregates. The distribution of these genetic markers support the theory that juvenile loggerhead turtles around Baja California are derived primarily from Japanese nesting beaches (Bowen et al. 1995).

Case 4. The tortoiseshell trade and Caribbean hawksbill turtles

While most sea turtles are harvested for their meat and related products, the hawksbill turtle is harvested for the shell scales, which are used to make "tortoiseshell" jewelry and ornamental objects. Prior to the development of plastics, the beautiful translucent scales of the hawksbill turtle were a primary source of raw material for making eyeglass frames, combs, guitar picks, and a diversity of ornamental products. As a result of this demand, hawksbill populations around the world have been gravely depleted. Most of the nesting colonies remaining in the Atlantic host less than 100 females per year (Witzell 1983).

A moratorium now exists on the international trade in hawksbill shell, but local trade continues in Japan and several Caribbean nations and there is an ongoing interest in reopening the lucrative international trade (M. Donnolly, personal communication). In response to this continuing demand, Cuba announced in 1992 an intention to resume harvesting hawksbill turtles on the reefs within Cuba's sovereign territorial waters (Heppell and Crowder 1996). This harvest would be based on an assumption that only turtles from Cuban nesting beaches occupy the adjacent reef habitats. In other words, the Cuban fishery model assumes that no nesting beaches outside of Cuban territory would be impacted by the harvest.

Marine turtle biologists were skeptical of the assumption that the Cuban hawksbill population is nonmigratory, but direct scientific evidence to the contrary was scarce. To address this issue, members of the Archie Carr Center for Sea Turtle Research (Univ. of Florida) and U.S. Fish and Wildlife Service assembled a research team to test the assumptions of the Cuban fishery model (Heppell and Crowder 1996), including a population assessment with mtDNA markers. This research incorporated a nesting beach survey conducted by Bass et al. (1996) and a feeding ground study conducted by Bowen et al. (1996). At the University of Florida we used these mtDNA markers to estimate the contribution of Caribbean nesting colonies to a feeding ground at Mona Island, Puerto Rico (n=41). Maximum likelihood analysis indicates that this feeding population is not composed primarily of turtles from the neighboring nesting colony (also on Mona Island), but is drawn from nesting populations throughout the Caribbean. A sampled nesting colony in the southern hemisphere (Bahia, Brazil) did not appear to contribute at detectable levels to the Caribbean feeding ground. From this evidence we concluded that hawksbill turtles recruit to feeding grounds over scales of 100's of kms, but not over the scale of 7000 kms which separate Mona Island from Bahia, Brazil. These data are consistent with findings from an earlier study of Indo-Pacific hawksbill populations (Broderick et al. 1994), and indicate that a hawksbill harvest on Caribbean feeding grounds will reduce nesting populations throughout the region.

The Conservation Frontier

These genetic surveys represent only the first phase of an exciting research initiative. In the near future, the entire life cycle of marine turtle species may be documented by coupling ongoing field studies with analyses of genetic markers. Alan Bolten and Karen Bjorndal at the Archie Carr Center for Sea Turtle Research (University of Florida) have initiated a program to track western Atlantic loggerhead turtles through juvenile pelagic phases via a combined analysis of genetic markers and tag recaptures. Colin Limpus, Craig Moritz, and colleagues at University of Queensland are assembling a comprehensive model of green turtle movement in the Australian and Indo-Pacific archipelagos. Alberto Abreu-Grobois at the Universidad Nacional de Mexico is analyzing the movement of three marine turtle species in the eastern Pacific, and migrations of the giant leatherback turtle, *Dermochelys coriacea*, are being documented by Peter Dutton and colleagues at Texas A&M University (Dutton 1995). The analysis of mtDNA in this area has been further augmented by surveys of nuclear DNA markers, providing information on aspects of male migratory behavior (FitzSimmons et al. 1995, 1996; Karl et al. 1992; Peare and Parker 1996). The combination of field studies, applied tags, and multiple genetic markers will yield an especially rich body of corroborating data which neither technique alone could provide.

How can these analyses of genetic markers influence conservation strategies? Genetic markers allow wildlife agencies to identify "range states", nations affected by the depletion of natural resources at a distant location. The concept of range states implies some level of jurisdiction. The 1982 U.N. Convention on the Law of the High Seas recognizes that nations which host the developmental habitat for migratory marine species hold exclusive fishing rights for these animals on the high seas (Van Dyke 1993). The 1983 U.N. Convention on the Conservation of Migratory Species (a.k.a. the Bonn Convention) prohibits taking endangered species during migrations on the high seas (Hykle 1992). Under the principles outlined in these conventions, nations which host nesting and developmental habitats for marine turtles have some level of jurisdiction over these animals on geographically remote feeding grounds, even if those feeding grounds are within the territorial boundaries of another nation.

In a now famous article titled "The Passing of the Fleet" Archie Carr chronicled the harvest of Caribbean green turtles from Columbus's fleet to the ongoing harvest in Miskitu Bank, Nicaragua (Carr 1954). This article was an eloquent call to protect the remaining nesting beaches from imminent extinction, a call which was heralded around the world and which inspired dozens of conservation programs. Forty years later the need to protect nesting beaches is nearly universally recognized. Less appreciated, perhaps, was Archie Carr's call to protect the feeding grounds as well. As human populations swell into coastal areas in increasing numbers, the impact on sea turtle habitats and the surrounding ecosystems has reached crisis proportions. Genetic markers can play a crucial role in identifying the origin of beleaguered marine turtle feeding populations, and may provide the foundation for international agreements concerning the protection of these ancient mariners.

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Hawksbill breeding and foraging populations in the Indo-Pacific region

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We sequenced a segment of the mtDNA control region from 87 hawksbill turtles representing six rookeries throughout the Indian (Western Australia, Saudi Arabia) and Pacific Oceans (Sabah - Malaysia, Peninsular Malaysia, Solomon Islands, NE Australia). Sequence variants (n=15) fall into two divergent (about 4%) clades; one clade is geographically widespread while the other is restricted to rookeries within the Australasian region. Despite the presence of geographically widespread allies there is strong local structuring as most rookeries have distinct allele frequencies. Although the precise boundaries are unclear, hawksbill turtles nesting in Sabah, Peninsular Malaysia, NE Australia and Western Australia should be considered separate stocks and managed as such.

To test the utility of genetic data to elucidate stock contribution under conditions typical for marine turtles we use computer simulations generated from a maximum likelihood program, Girlsym (Masuda et al. 1991). In a simple model (two alleles, two stocks) we found that approximately 100 individuals are needed to have a reasonable chance of generating accurate estimates stock contribution; however, varying both genetic distinctiveness of contributing stocks and foraging sites has considerable effects on critical sample sizes. Furthermore we show how preliminary allele frequencies at foraging grounds can be used to predict the sample sizes required to accurately estimate stock composition. This latter application of simulation is particularly useful as it enables researchers to gauge the magnitude of resampling efforts in each of these populations.

Introduction

The hawksbill turtle is a tropically distributed marine turtle threatened with extinction in many parts of its range. The commercial trade in hawksbill shell is the primary factor for the demise of this species; however, eggs and meat are also consumed (Witzell 1983, Milliken and Tokunaga 1987). The bekko harvests of the late 19th and early 20th centuries and its contemporary expansion have put immense pressures on hawksbill populations such that this harvest is unsustainable (Donnelly 1989, Groombridge and Luxmoore 1989).

The hawksbill turtle appears to conform to the general life history of other marine turtles (Witzell 1983) and the available data suggest that hawksbill turtles are migratory (Parmenter 1983, Meylan 1982, Miller 1994). Given the threat from harvesting there is an urgent need to investigate the relationship between turtles utilizing the foraging areas and those at neighbouring rookeries. Tagging studies are best suited to elucidating this relationship, but it will take many years to provide detailed patterns of movements within and among populations. Genetic studies however offer a means to infer broad patterns of distribution to complement ongoing tagging studies.

Mitochondrial DNA is well suited to detecting population boundaries and has been utilized in many studies (e.g. Avise 1992). It is a more sensitive marker than standard nuclear or isozyme markers because it has a higher mutation rate, smaller effective population size and is clonally inherited. The enhanced effects of drift on mtDNA makes it an ideal population marker. Microsatellites in nuclear genes have higher mutation rates than mtDNA, but they are not necessarily better markers because the high mutation rate tends to increase genetic variation within rather than among populations.

There are now a number of genetic studies of marine turtles that have successfully used mtDNA variants to resolve maternal lineages and migratory behaviour (Bowen et al. 1992; Bowen et al. 1994; Broderick et al. 1994). Some studies have gone a step further and have used mtDNA variants as a source of genetic tags for determining the stock

composition of foraging populations (Norman et al. 1994, Broderick et al. 1994, Bowen et al. 1996) or turtles taken in fisheries bycatch (Sears et al. 1995, Bowen et al. 1995).

Genetic studies of hawksbill turtles in Australia (Broderick et al. 1994) and the Caribbean (Bass et al. 1996, Bowen et al. 1996) have focused both on breeding and foraging populations and have been useful in defining units for management in their respective regions. These studies confirm that hawksbill turtles are indeed migratory and conform to the natal homing model. The present study expands upon a former study (Broderick et al 1994) by including rookeries throughout the Indo-Pacific and using control region sequencing rather than whole genome RFLP analysis. Here we use sequence data to give a preliminary assessment of hawksbill phylogeography for the Indo-Pacific.

Maximum likelihood algorithms (ML) have been the method of choice when trying to elucidate proportional stock contribution in mixed populations. ML has been applied in many fisheries (Smouse et al.1990; Millar 1987; Fournier et al. 1984) and are beginning to be applied to marine turtles (Norman 1996 for greens, Sears et al. 1995 and Bowen et al. 1995 for loggerheads and Bowen et al. 1996 for Caribbean hawksbills). The degree of genetic differentiation between the contributing stocks greatly influences the success of this approach. Stocks with slight differentiation of allele frequencies are harder to resolve than those with fixed differences. Little can be done about the inherent nature and distribution of genetic variation other than to select loci with maximum differentiation (eg. mtDNA). Unlike the former fisheries examples, sample sizes for rare and endangered species like marine turtles are often small and difficult to come by. If we are to assess stock structure using ML in marine turtles then we need to know on what order of magnitude our sampling efforts should focus and whether or not the current level of sampling is sufficient.

In this paper we:

i) Present a preliminary phylogeography and analysis of geographic variation in allele frequencies for hawksbill turtles in the Indo-Pacific using mtDNA sequence data.

ii) Use ML simulations to estimate the minimum number of samples required to generate reasonably accurate estimates of stock contributions in simple model situations.

iii) Apply ML simulation to show how preliminary allele frequencies at foraging grounds can be used to estimate the sample size required to accurately estimate stock composition.

Methods

Sampling and DNA Extraction

We took blood from nesting adults or tissues from non-sibling hatchlings at 7 major Indo-Pacific rookeries (Figure 1). Populations were sampled from 1991-1995 over a large geographic range for phylogeographic analysis but with a focus on rookeries in, or adjacent to, Australian waters for stock analysis. Three feeding populations were also sampled, two in Australia and one in the Solomon Islands.

Tissues were either stored in a 20% DMSO saturated NaCl solution or frozen in liquid nitrogen while blood was stored in a lysis buffer (100mM EDTA, 100mM TRIS-HCl, 10mM NaCl, 0.5% SDS, pH 8.0 after Dutton (1996). The bulk of these specimens were prepared for PCR using either the salting out (for blood) or chelex (for tissues) extraction techniques (see Hillis et al. 1996). For frozen tissues, mtDNA was extracted using differential centrifugation then ultra high speed centrifugation in a cesium chloride gradient (Dowling et al. 1996.).

Sequencing

A region of the mtDNA control region was PCR amplified using TCR5-6 primers (Norman et al. 1994). Typically, 1-5*ul* of template was used in standard 25*ul* PCR reactions (denaturing @ 94°C-30s, annealing @ 52°C-40s and extension @ 72°C-50s for 30 cycles). Two internal primers (TCR7-8; sequences to come) were designed to facilitate

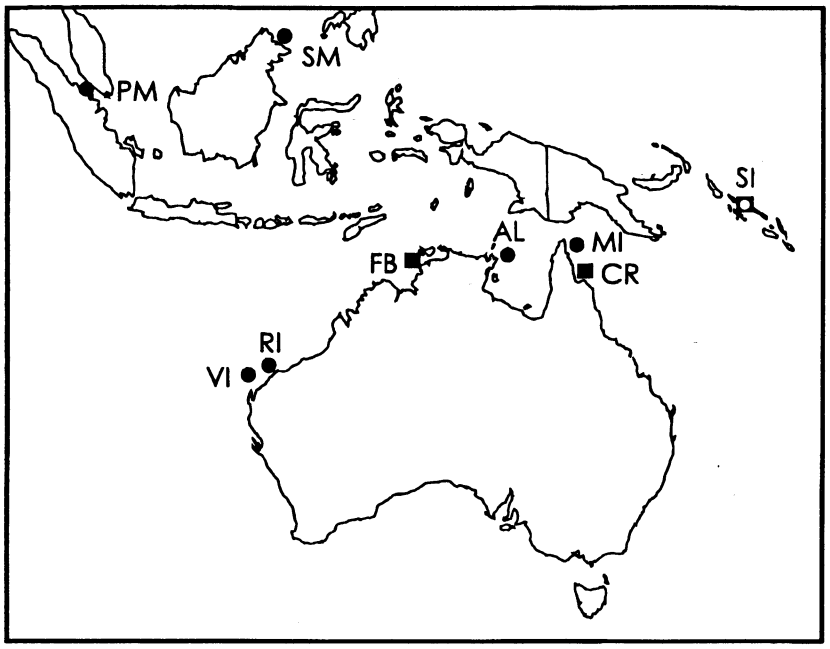

Figure 1. Locations of rookeries and foraging sites sampled in the Indo-Pacific region where PM=Peninsular Malaysia, SM=Sabah Malaysia, SI=Solomon Islands, FB=Fog Bay, and CR=Clack Reef. Milman Island (MI) and Arnhem Land (AL) form the N. E. Australian stock (defined previously by Broderick et al. 1994), likewise Rosemary Island (RI) and Varanus Island (VI) form the W. Australian stock.

speed and reliability of sequencing. These internal primers allowed us to use 1*ul* of unpurified PCR product in subsequent P^{33} cycle sequencing reactions (denaturing @ 94°C-30s, annealing @ 50°C-40s and extension @ 70°C-60s for 35 cycles). These products were electrophoresed on 6% sequencing gels and visualised using autoradiography.

Analysis of Sequence Data

Sequences were aligned using Clustal IV (Higgins et al. 1992) and then formatted for subsequent analyses. Estimates of nucleotide divergence were calculated using REAP (McElroy et al. 1992). The distribution of mtDNA alleles among populations were tested using a randomised chi-square test with 1000 replicates (Roff and Bentzen 1989). For a visual representation of the results a phylogeny was created in PAUP (Swofford 1990) using sequence from a Caribbean hawksbill (Bass et al. 1996) as an outgroup.

Stock Analysis

To test the utility of genetic data to elucidate stock contribution under conditions typical for marine turtle mtDNA, we use computer simulations generated from a maximum likelihood program, GIRLSYM (Masuda et al. 1991). The goal was to investigate the sample size needed to provide reasonably accurate estimates (±10%) of stock contribution across a number of scenarios. The sample size where the 95% confidence interval of stock contribution and 10% deviations from the mean estimate intersect is defined as the critical sample size. We use the bootstrap re-sampling algorithm incorporated within the GIRLSYM program (see Efron 1982; Masuda et al. 1991) to calculate both stock estimates and confidence intervals. In an attempt to simplify matters, only foraging sites were bootstrapped (n=1000) so that trends in the algorithm were not confounded by variations in stock (nesting population) size. However, in real situations it is advisable to bootstrap both the stock and foraging sites, especially if the sample sizes for stocks are low.

Two simulations were conducted:

i) A simple model (two alleles, two stocks and one composite foraging site) where both the genetic distinctiveness of contributing stocks and foraging sites were varied to see what effect it had on the critical sample size. One of the stocks varied in genetic composition, the other being constant and fixed for a shared allele. The foraging sites varied in both genetic composition and in sample size.

ii) A more complex model based on the preliminary estimates of allele frequency in three Indo-Pacific hawksbill populations; this model comprises four alleles, five stocks and three foraging sites. The goal was to predict the sample sizes required to generate accurate estimates of stock composition in these foraging sites. To do this we simply repeated the analysis across different sample sizes holding the allele frequencies constant.

Foraging grounds were rapidly screened for the presence of known alleles (derived from breeding populations) using a combination of diagnostic restriction enzymes and outgroup heteroduplex analysis (e.g. Fig 6 in Dowling et al. 1996). Individuals were sorted into four broad allelic classes; these preliminary allele frequencies were used in the above analysis. Details of this analysis will be presented elsewhere.

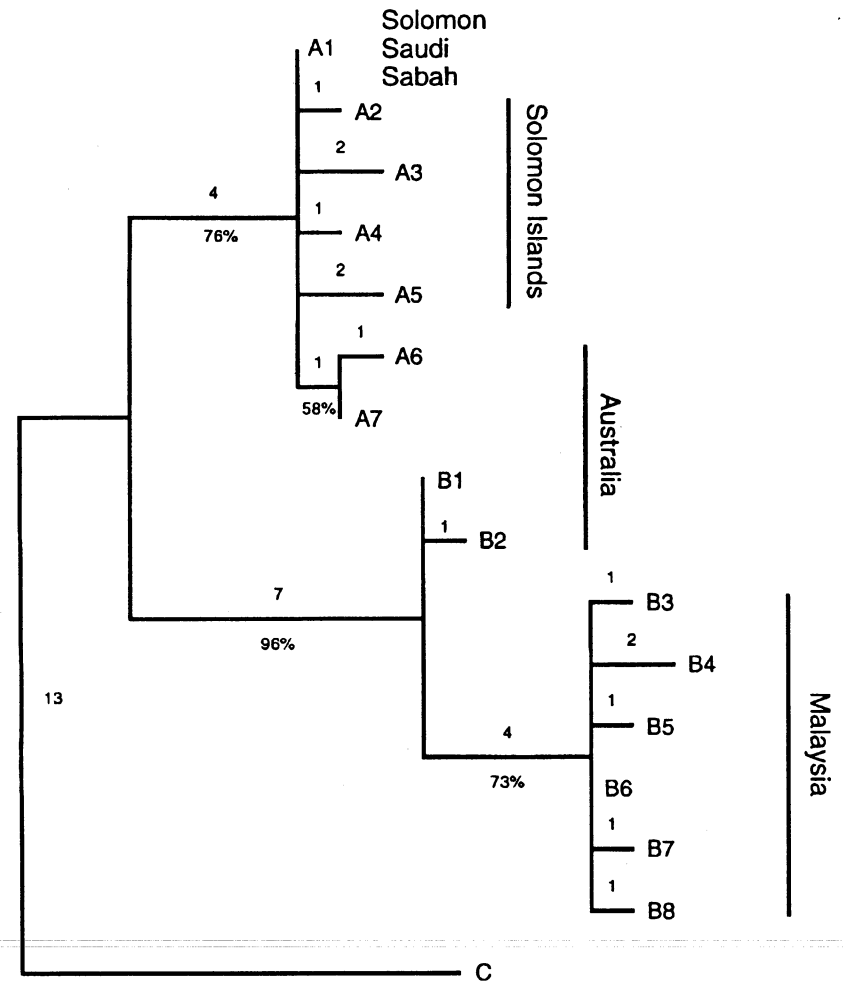

Figure 2. Parsimony phylogeny showing the relationship between 15 mtDNA alleles and their geographic distribution among rookeries in the Indo-Pacific with number of nucleotide substitutions above and bootstrap values below.

Table 1. Frequency distribution of 15 mtDNA alleles among rookeries in the Indo-Pacific region.

	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	B4	B5	B6	B7	B8
Solomon	20	1	1	7	2	0	0	0	0	0	0	0	0	0	0
Saudi Arabia	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.E. Australia	0	0	0	0	0	12	0	6	1	0	0	0	0	0	0
W. Australia	0	0	0	0	0	10	1	0	0	0	0	0	0	0	0
Sabah Malaysia	6	0	0	0	0	0	0	0	0	0	0	0	4	6	1
Peninsular Malaysia	0	0	0	0	0	0	0	0	0	7	1	1	0	0	0

Results and Discussion

Analysis of Breeding Populations

Broderick et al. (1994) used RFLP analysis to identify two major mtDNA clades with variation in allele frequencies defining two Australian stocks. Here we have expanded on that study with the inclusion of more rookeries from the region and the use of sequence data. For the Australian stocks we sequenced representatives from the A and B clades from each rookery so that the two data sets were compatible. Essentially, our sequencing uncovered more alleles within each of the A and B clades whilst preserving the basic genetic pattern as previously defined by Broderick et al. (1994).

Fifteen alleles were identified after sequencing a 363 bp fragment from 87 individuals at rookeries from Western Australia (n=11), North Eastern Australia (n=19), Solomon Islands (n=31), Sabah-Malaysia (n=11); Peninsula Malaysia (n=9) and Saudi Arabia (n=6). These alleles fall into two divergent phylogenetic clades corresponding to the A and B groups defined previously (Figure 2). Nucleotide divergences between the A and B clades ranged from 2.69-4.92% with much lower divergences within each clade (0.28-1.12% and 0.28-1.72% for A and B clades respectively). These estimates of sequence divergence are approximately four fold higher than those found for whole genome RFLP analysis of the same alleles (c.f. Broderick et al. 1994). This compares to a 6-8 fold difference observed for green turtles (Norman et al. 1994; Encalada et al. 1996).

Detailed analysis of phylogeography and distribution of sequence divergence among hawksbill populations will be presented elsewhere. Here we focus on the distribution of alleles among regional populations in order to define discrete stocks (i.e. management units) and to provide the baseline data for simulation of stock composition analysis (Table 1). The majority of haplotypes (13/15) were exclusive to specific nesting populations. The exceptions being the A6 haplotype that occurs only in the two Australian stocks and the A1 haplotype which has an extensive distribution and is found in high frequencies in the Solomon Islands (20/31), Sabah (6/11) and Saudi Arabia (6/6). Despite the sharing of some alleles, pairwise chi-square tests revealed that all populations were significantly different ($p<0.05$) and they remained so even with the corrected levels for multiple tests (Rice 1989). For the purposes of this analysis we therefore recognise the Solomon Islands, N.E. Australia, W. Australia, Peninsular Malaysia and Sabah Malaysia nesting populations as separate stocks. The small number of individuals analysed so far for the Saudi Arabian turtles precludes assessment of its status.

This data set is the first mtDNA phylogeography compiled for sequence of hawksbill turtles in the Indo-Pacific Ocean. Given natal homing, genetic structuring of breeding assemblages is expected and has been previously described in hawksbills (Broderick et al. 1994, Bass et al. 1996) and in other species of marine turtles (Bowen et al. 1992; 1994; Norman et al. 1994). What is less clear are the processes responsible for the co-existence of two divergent clades at near equal frequencies in single stocks (ie N.E. Australia). It is likely that these clades evolved in isolation and have only recently come into contact.

The presence of the same allele at high frequencies in the Solomon Islands and Saudi Arabian rookeries probably reflects recent

Table 2. Model parameters (first five columns) and results (last two columns) of ML simulation. ML analysis for each stock mix and foraging site combination was repeated for 10, 20, 30, 50, 100, and 200 foraging site samples. The proportional contribution of Stock 1 is derived from simulations where n=200 at the foraging site. The critical sample presented here is based on Stock 1 contribution only. * = not analysed because no combination of the contributing stocks can account for the observed allele frequencies at the foraging sites.

		MODEL PARAMETERS: OBSERVED f (A)			RESULTS:	
Foraging site	Stock mix	Stock 1	Stock 2	Foraging site	Stock 1 contribution	Critical sample size
A	1	0	1	0.9	10.00%	20
A	2	0.1	1	0.9	11.11%	43
A	3	0.5	1	0.9	20.00%	100
A	4	0.9	1	0.9	91.10%	> 200
B	1	0	1	0.5	50.02%	80
B	2	0.1	1	0.5	55.58%	100
B	3	0.5	1	0.5	97.19%	150
B	4	0.9	1	0.5	*	*
C	1	0	1	0.1	90.03%	30
C	2	0.1	1	0.1	99.07%	27
C	3	0.5	1	0.1	*	*
C	4	0.9	1	0.1	*	*

common ancestry via long distance colonization rather than ongoing gene flow (c.f. Encalada et al. 1996; Bowen et al. 1994). Indeed this is to be expected in a species that has a long pelagic dispersal; slight changes in current patterns could result in turtles being shunted into alternate oceans basins. Turning to more rapidly evolving nuclear markers (i.e. microsatelites; Fitzsimmons et al. 1995) may be useful in distinguishing these populations.

Regardless of the precise nature of the origins and dispersal of mtDNA variants among rookeries, the management implications are clear. Hawksbill turtles nesting in Sabah, Peninsular Malaysia, NE Australia and Western Australia should be considered separate stocks and managed as such. At present, the precise boundaries of many of these stocks are unclear, but sampling of intermediate localities should help to resolve the geographic scale of these partitions.

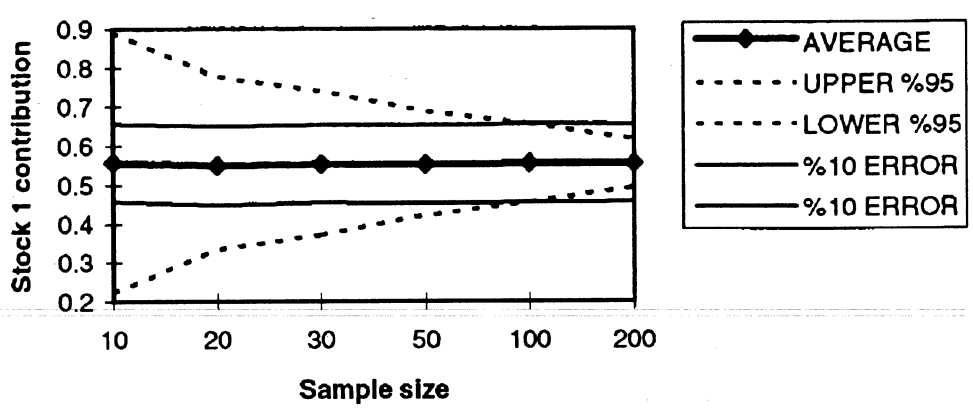

Figure 3. Graphical representation of the proportional contribution of Stock 1 from stock mix 1, estimated across a number of foraging site sample sizes at foraging site B.

Mixed Stock Analysis

There have been many treatments of ML data and its relation to stock analysis, especially in fish stocks (Epifanio et al. 1995; Millar 1987; Fournier et al. 1984; Smouse et al. 1990; Xu et al. 1994). These studies adequately describe the statistical vagaries of ML under numerous scenarios and conditions but their application to marine turtle stocks are less clear (Chapman 1996). In the first set of analyses we explore critical sample sizes in a simple scenario (two alleles, two stocks and one composite foraging site) where we varied both the genetic distinctiveness of contributing stocks (ie. stock 2 is fixed for allele A and the frequency A varies from 0-0.9 in stock 1) and at foraging sites (where the frequency of A varies from 0.1-0.9). The following results, although based upon this simplistic scenario, show what the statistical vagaries of ML mean in terms of sample size required at foraging grounds or harvests. The main trends (Table 2) are summarized below.

i) Level of precision: A common feature in these simulations is the asymptotic nature of the 95% confidence interval (Figure 3); it decreases rapidly when n is small, in this case 10-30 samples and more slowly thereafter. Thus in many scenarios, depending on the slope of the asymptote, small increases in precision (from say ±10% to ±5%) may correspond to disproportionate increases in critical sample size. No precedent has been set for the level of precision required in such analyses. However, given that sample sizes are often limited, we chose to focus analysis on the ±10% error level.

ii) Stock divergence: In general, critical sample size is inversely proportional to the degree of differentiation between the contributing stocks. Stocks with fixed or nearly fixed allele frequency differences require relatively few samples to achieve a given level of accuracy (ie. n=20 for stock mix 1 at foraging site A) whereas larger sample sizes are required to analyse populations where the contributing stocks are only slightly divergent (i.e. stock mix 4 at foraging site A; n>200).

iii) Stock and foraging site interaction: The more divergent a foraging site is from the average of the contributing stocks, the fewer samples that are required for a given level of accuracy (see Chapman 1996). That is; the more mixed a population is the harder it is to elucidate proportional stock contribution. For example, stock mix 1 (avg f(A)=0.5) is less divergent from foraging site B (f(A)=0.5) than it is from foraging site A (f(A)=0.9) and this is reflected by critical sample sizes of 80 and 20 respectively.

Because genetic distinctiveness of contributing stocks and foraging sites have considerable effects on critical sample sizes, it is difficult to predict a priori sample sizes for new systems. Nonetheless, as an initial target for simple systems (like those modelled above), studies consisting of approximately 100 samples have a reasonable chance of generating accurate estimates of stock composition.

The second set of simulations is based on our estimates of allele frequencies in the regional breeding populations in Australia, Malaysia and the Solomon Islands and feeding grounds in Australia and the Solomon Islands. For this purpose alleles detected by DNA sequencing are grouped into classes that can be defined by combined RFLP and heteroduplex analysis (Table 3). This simulation enables us to gauge the magnitude of resampling efforts for each of the foraging ground populations. For example, in the Solomon Islands a modest sample size of 25-50 suffices to accurately estimate stock composition (Table 3). This is because the dominate allele (B) in the feeding ground is found only in one other stock (Sabah) at moderate frequencies and is fixed for the Solomon Islands. Like the previous simulation, the more mixed a foraging population is the harder it is to elucidate proportional stock contribution. This is exemplified by the larger sample sizes required to analyse the Clack Reef (n=100-150) and Fog Bay (n=150-200) populations.

Obviously further sampling is required if we are to make strong statements about stock structure in the Australian foraging populations. Even in scenarios were sample sizes prevent rigid interpretation of the results, ML is still useful in making qualitative statements about stock composition. For example, both the Fog Bay and Clack Reef populations comprise a mixture of mainly Australian stocks with perhaps

Table 3. Frequency of allelic classes in rookeries and foraging populations (preliminary) defined from a combined RFLP and heteroduplex analysis. Here, the previously defined mtDNA sequence haplotypes A1, A2, A3, A4, and A5 form the A group; likewise A6 and A7 haplotypes form the B group; haplotypes B1 and B2 form the C group; and haplotypes B3, B4, B5, B6, B7, and B8 form the D group.

	MODEL PARAMETERS: OBSERVED f(ALLELES)				RESULTS: STOCK CONTRIBUTION		
STOCKS	A	B	C	D	SOLOMON	FOG BAY	CLACK REEF
NE AUST	0.5	0.0	0.5	0.0	1.63%	39.92%	14.25%
W AUST	1.0	0.0	0.0	0.0	0.39%	50.03%	58.82%
PEN MAL	0.0	0.0	0.0	1.0	0.49%	0.00%	14.61%
SABAH	0.0	0.4	0.0	0.6	0.90%	0.01%	8.86%
SOLOMON	0.0	1.0	0.0	0.0	96.59%	10.04%	3.45%
	CRITICAL SAMPLE SIZE				25-50	150-200	100-150

FORAGING SITES	OBSERVED f(ALLELES) A	B	C	D
SOLOMON	0.01	0.97	0.01	0.01
FOG BAY	0.70	0.10	0.20	0.00
CLACK REEF	0.66	0.07	0.07	0.20

significant contributions from stocks outside Australian Waters. Likewise, the Solomon Island feeding population appears to have little input from neighbouring rookeries. However this latter conclusion is contingent on the assumption that all potentially contributing stocks have been characterised; currently a widespread allele characterizes the Solomon Island stock. Even though our current sample sizes are small (n<30) the emergent patterns have broad management implications that are unlikely to change with increased sampling. What changes with increased sampling is our ability to make quantitative statements about stock composition. This will be necessary to feed regional impacts of harvesting etc. into demographic models based on the distinct management units identified through mtDNA analysis.

Acknowledgments

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Determination of the natal origin of a juvenile loggerhead turtle (*Caretta caretta*) population in Chesapeake Bay using mitochondrial DNA analysis

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Mitochondrial DNA analysis was employed to assess the relative contribution of two United States rookeries to the aggregate of juvenile loggerhead turtles (*Caretta caretta*) that feed in Chesapeake Bay during the summers. Restriction fragment patterns of the mitochondrial d-loop amplified by the polymerase chain reaction were obtained for 62 individuals collected from 1989-1994. Two mtDNA haplotypes were found within the Chesapeake Bay loggerheads, both characteristic of rookeries in Georgia/South Carolina and Florida. 69% of Chesapeake Bay turtles were designated haplotype B and 31% of which were designated haplotype D. Based on the occurrence of B and D haplotypes in rookeries along the southeast coast of the United States, it is estimated that 64% of the individuals comprising this study were recruited from Florida rookeries and 36% from Georgia/South Carolina rookeries. Because only 10% of western Atlantic loggerhead nesting occurs in Georgia/South Carolina, these data indicate that turtles from this rookery are selecting the waters of the Chesapeake Bay as juvenile foraging grounds more frequently than their southern counterparts. With the entire western Atlantic loggerhead population in long-term decline, and the northern nesting population more threatened than the Florida nesting population, the Chesapeake Bay population should be protected by wildlife management agencies at a level similar to that of other severely threatened species.

Introduction

The loggerhead (*Caretta caretta*) is the most frequently encountered sea turtle in the Chesapeake Bay. Each year between 2,000-10,000 individuals migrate into the lower Bay between April and May (Keinath et al. 1987), remaining in the region until September to November, when cool water temperatures force them to migrate south (Lutcavage and Musick 1985). While wintering grounds are unknown, loggerheads exiting Chesapeake Bay have been tracked as far south as the Florida Keys (Keinath 1994). The Chesapeake Bay population of turtles consists mainly of juveniles, most with carapace lengths of 60-90 cm, and weighing 25-140 kg (Musick 1988). Between 45 and 180 loggerheads strand on the Bay's beaches each year, with over one-third of these deaths attributed to drowning in fishing nets and mutilation by boat propellers (Keinath et al. 1987).

The loggerhead is currently listed as "threatened" on the U.S. List of Endangered and Threatened Wildlife and Plants. The southeast coast of the United States is home to approximately 35,000 reproductive adult female loggerheads, with an average of 14,000 nesting annually (Murphy and Hopkins 1984). Individual rookeries may span tens to hundreds of kilometers. While nesting has been recorded as far south as Texas and as far north as Virginia, aerial surveys indicate that 90% of these nests are concentrated in Florida, and the other 10% are clustered primarily in Georgia and South Carolina (Murphy and Hopkins-Murphy 1989).

Movement of loggerheads along the U.S. southeast coast has been studied extensively using tag and recapture methods (Eckert and Eckert 1988), analyses of carapace epibiota (Stoneburner 1980; Stoneburner et al. 1980), heavy metal concentration within tissues (Caine 1986), and more recently using mtDNA analysis (Bowen et al. 1993). Tagging experiments indicate that adult female loggerheads return repeatedly to the same site to nest (Bjorndal et al. 1983), a phenomenon that is most likely a product of natal homing by adult female loggerheads (Carr 1967). Slight morphological differences have been noted among turtles nesting in these two major areas (Stoneburner et al. 1980), and analysis of mitochondrial DNA has indicated that these geographically separated populations are

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genetically distinct (Bowen et al. 1993; Murphy and Hopkins-Murphy 1989).

Recent mtDNA studies have lent strong support to the natal homing hypothesis in loggerheads and other species (Bowen et al. 1993; Allard et al. 1994; Broderick et al. 1994). Bowen et al. (1993) employed restriction fragment length polymorphism (RFLP) analysis to survey mtDNA of loggerheads in the northwest Atlantic and Mediterranean Sea. Along the U.S. southeast coast. two mtDNA composite haplotypes predominated: the B and D haplotypes. The southern (Florida) population was characterized by a mixture of these haplotypes, with a frequency of 0.48 (D) and 0.52 (B) (n=50 individuals), while the Georgia/ South Carolina population was composed almost exclusively of the B haplotype (B=0.99, D=0.01; n=105) (Encalada, Bolten, Bjorndal and Bowen, unpublished data). A mean nucleotide sequence divergence of p=0.8% separated the two haplotypes (Bowen et al. 1993).

The contribution of the two major rookeries to the juvenile population of Chesapeake Bay is not known. To identify the relative contribution of the two major U.S. rookeries in the western Atlantic to the juvenile loggerhead population in Chesapeake Bay we determined mtDNA haplotypes of blood samples collected from stranded turtles. Rather than employing RFLP analysis to survey the entire mtDNA genome, we were able to identify the B and D haplotypes of Bowen et al. (1993) from RFLP analysis of a section of the hypervariable control region (d-loop) amplified by the polymerase chain reaction (PCR). This method of haplotype typing is relatively rapid and does not require as much sample quantity of mtDNA as whole molecule RFLP analysis (Martin et al. 1992).

Materials and Methods

Blood samples were drawn from individuals that were in the process of being tagged, weighed, measured, and released at the Virginia Institute of Marine Science (VIMS) turtle greenhouse as part of the VIMS sea turtle stranding network. Approximately 5 ml of blood was drawn from the dorsal cervical sinus of live turtles of varying carapace lengths and stored in a lysis buffer (10 mM Tris, 100 mM EDTA, 0.5% SDS, pH 8). Blood samples were available from 62 individuals collected between May and July in the years 1989-1994. Samples collected from individuals from 1989 to 1993 were centrifuged briefly and stored at -20°C. Samples taken in 1994 were kept at 4° C.

Total genomic DNA was isolated from blood using a modification of the methods of Blin and Stafford (1976). Approximately 0.25 µl of blood was added to 130 µl of a lysis buffer (10 mM Tris-Cl (pH 8.0); 0.1 M EDTA (pH 8.0); 0.5% SDS), manually chopped and ground (frozen blood was particularly clumpy), and then vortexed. The homogenate was then subjected to successive extractions with pure phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). Two-tenths volumes of 10 M ammonium acetate and 2 volumes of 95% ethanol at -20°C were added to the resulting mixture and stored at -20°C overnight. Following an ethanol precipitation, pellets were resuspended in 20 µl 1X TE.

PCR methodology was used to amplify approximately 420 bp of the d-loop of loggerhead turtle mtDNA using oligonucleotide primers (CR-1 and CR-2) as described by Norman et al. (1994). The sequence of these primers is:

CR-1: 5' TTGTACATCTACTTATTTACCAC 3' 3'
CR-2: 5' GTACGTACAAGTAAAACTACCGTATGCC

Reaction conditions for amplification were: 6 minutes at 94°C, followed by 40 cycles of 2 minutes at 94°C, 2 minutes at 50°C, and 4 minutes at 72°C. This was completed with a final 14 minute extension at 72°C, and then storage at 4°C. Each group of amplified samples contained a negative control in order to detect contamination.

Sequences for a 420 bp fragment of the mitochondrial DNA D-loop for the B and D haplotypes were provided by Brian Bowen (University of Florida) and are shown in Figure 1. A restriction-site analysis was performed on the sequences, generating a suite of restriction endonucleases that discriminated between the B and D haplotypes. Of these, four enzymes (*Apo* I, *Hae* III, *Sau*96 I, and *Ssp* I) were selected for use to identify the different haplotypes (Table 1).

Table 1. Restriction fragment patterns of the loggerhead d-loop resulting from cuts with diagnostic enzymes.

		Resulting Fragment Sizes (bp)	
Restriction Enzyme	Recognition Sequence	Haplotype B*	Haplotype D**
Apo I	5'... Pu ▼ A A T T Py ... 3'	346, 67	418
Hae III	5'... G G ▼ C C ... 3'	264, 61, 50, 38	301, 61, 56
*Sau*96 I	5'... G ▼ G N C C ... 3'	263, 150	418
Ssp I	5'... A A T ▼ A T T ... 3'	413	248, 170

* Haplotype B turtles have a D-loop length of 413 bp
** Haplotype D turtles have a D-loop length of 418 bp

An aliquot of 8 ul of the amplification product was digested with each enzyme for 18 hr. Digestion products for each turtle sample were subjected to gel electrophoresis in a 2.5% agarose gel containing a 1 kb size standard (BRL) at 80 volts for approximately one hour. Following electrophoresis gels were stained with ethidium bromide and photographed under UV light. The size of each digestion product was determined by comparison with the size standard included on each gel. Haplotype frequencies were calculated from direct counts of individuals of each haplotype.

Results

Analysis of the amplified mtDNA d-loop region of 62 loggerhead samples revealed only two fragment patterns for each of the four restriction enzymes (Table 1), allowing straightforward determination of the B and D haplotypes. Each enzyme alone was sufficient to discriminate between the B and D haplotypes. All restriction patterns were consistent in identifying the haplotype for each sample.

The samples from the Chesapeake Bay population of loggerheads comprised two genotypes (B and D), with 69% designated as haplotype B (n=43), and 31% designated as haplotype D (n=19). Using chi-square analysis, the haplotype frequencies of the Bay population were determined to be significantly different from both the nesting populations of Georgia/ South Carolina/North Carolina, (X^2=7.8, df=1, $p<0.01$) and of Florida (X^2=329, df=1, $p<0.001$), indicating that the loggerhead population in the Chesapeake Bay is not recruited exclusively from either rookery but represents a mixed stock. The hypothesis that the Bay loggerhead population is a random mixing of individuals from along the coast of the southeast United States was tested by chi-square analysis. Since approximately 90% of nests in the southeast United States are located on Florida beaches (Murphy and Hopkins-Murphy 1989), if random mixing of stocks occurs in the Chesapeake Bay, then one would expect 90% of the turtles in the Bay to have originated from Florida rookeries. This generated an expectation of 56 of the 62 turtles sampled to have come from Florida. In such a scenario, it would be expected that 43% (n=27) of the 62 samples would be haplotype D, and 57% (n=35) would be haplotype B. The observed haplotype frequencies of Chesapeake Bay loggerheads were significantly different from the expected values (X^2=4.2, df=1, $p<0.05$), indicating that random mixing of juveniles from Georgia/South Carolina and Florida rookeries does not occur in the Chesapeake Bay. Consequently, it would appear that juveniles from Georgia/South Carolina/North Carolina, utilize the Bay as a foraging refuge significantly more frequently than their neighbors to the south.

Figure 1. Sequence of the loggerhead mitochondrial d-loop for "B" and "D" haplotypes, with restriction sites of each of the four informative restriction endonucleases.

```
B  5'   CTACTT    ATTTACCACT    AGCATATGAT    CAGTAATGTT    GTCGATTAAT
D  5'   CTACTT    ATTTACCACT    AGCATATGAT    CAGTAATGTT    GTCGATTAAT

                        Apo I
                          |
B  TTGGCTTTAA    ACATAAAAAT    TTATTAATTT    TACATAAACT    GTTTTAGTTA
D  CTGACCTTAA    ACATAAAAAC    T-ATTAATTT    TGCATAAACT    GTTTTAGTTA

B  CATGACTATT    ATACAGGTAA    TAAGAATGAA    ATGATATAGG    ACATAAAATT
D  CATGACTATT    ATACAGGTAA    TAGGAATGAA    ATGATATAGG    ACATAAAATT

B  AAACCATTAT    TCTCAACCAT    GAATATCGTC    GCAGTAATAG    GTTATTTCTT
D  AAACCATTAT    TCTCAACCAT    GAATATCGTT    ACAGTAATAG    GTTATTTCTT

B  AGTTCAGCTC    ATCACGAGAA    ATAAGCAACC    CTTGTTAGTA    AGATACAACA
D  AGTTCAGCTC    ATCACGAGAA    ATAAGCAATC    CTTGTTAGTA    AGATACAATA
                                                                |
                                                              Ssp I
                  Sau 96I Hae III
                      | |
B  TTACCAGTTT    CAGGCCCATT    AAGTCATATC    GTACATAACT    GATCTATTCT
D  TTACCAGTTT    CAAGTCCATT    AAGTCATGTC    GTACATAACT    GATCTATTCT

   Hae III
     |
B  GGCCTCTGGT    TG--TTTTTTC    AGGCACATTA    AGATAATAAA    GTTCACTCGT
D  GGCCTCTGGT    TGGTTTTTTC    AGGCACATTA    AGGCAGTAA--    GTTCATTCGT
   |
  Hae III
                  Hae III
                     |
B  TCCTCTTTAA    AAGGCCTCTG    GTT-----------A    AATGAGTTCT    ATACATTAAA
D  TCCTCTTTAA    AAGGCCTCTG    GTTGCAAGTA    AATGAGTTCT    ATACATTAAA
                       |
                    Hae III

B  TTTATAACCTGGCATACG  3'
D  TTTATAACCTGGCATACG  3'
```

Since the Chesapeake Bay contains a mixture of two haplotypes, and one of these (D) is found almost exclusively in the Florida rookery (Bowen et al. 1993), the potential contribution of the Florida rookery to the Bay juvenile population could be approximated with a single variable equation:

$$D_{CB}=D_F*X$$

In this equation, D_F is the frequency of haplotype D in Florida, D_{CB} is the frequency of haplotype D in the Chesapeake Bay, and X is the fraction of the Chesapeake Bay population that is recruited from Florida rookeries. The remainder (1-X) was assumed to be recruited from the Georgia/South Carolina rookery since these two locations contain roughly 99% of the known loggerhead nests in the northwestern Atlantic (Sears et al. 1995). Based on this calculation, of the turtles sampled, 64% were recruited from the Florida rookery, while the remaining 36% originated from the beaches of Georgia/South Carolina/North Carolina.

The haplotypic frequencies of Chesapeake Bay loggerheads was analyzed for temporal variation to determine if the relative contribution of the Georgia/South Carolina/North Carolina and Florida rookeries to the Chesapeake Bay population was consistent over time. Although sample sizes were small for some years, chi-square analysis revealed no significant differences in haplotypic frequency for each year's sample relative to the mean of the combined haplotype frequencies (Table 2).

Discussion

Based on RFLP analysis of an amplified region of the mtDNA d-loop, the Chesapeake Bay population of loggerhead turtles is composed of contributions from two major nesting rookeries in Georgia/North and South Carolina and Florida. Although the Georgia/ Carolina rookery only accounts for 10% of active loggerhead nesting the southeast United States, about one third of the turtles sampled in the Bay were derived from this rookery, suggesting that juveniles from Georgia/South Carolina preferentially choose Chesapeake Bay in their foraging site selection. These results concur with those of Sears et al. (1995) who analyzed the mtDNA of 33 juvenile loggerheads from Charleston Harbor and reported that approximately 50% of that population was derived from each of the Georgia/South Carolina/North Carolina and the Florida rookeries. The results of the present investigation are also consistent with those of heavy metal and epibiota studies suggesting that juvenile turtles hatched from more northern rookeries tend to stay along the coast of the southeastern United States, while those from the southern rookeries tend to forage in the more tropical regions of the Caribbean and the Gulf of Mexico (Caine 1986; Stoneburner et al. 1980; Meylan et al. 1983; Richardson 1982). The determination of the natal origin of the juvenile Chesapeake Bay loggerhead population was made under the assumption that there are only two potential sources for recruits to the Bay: the rookeries of Georgia/South Carolina/

Table 2. Number of individuals of each haplotype collected by year.

Year	Haplotype B		Haplotype D		Chi square analysis: variation from the mean
1989	11	(69%)	5	(31%)	$X^2 = 0$, df = 1, $p > 0.999$
1990	15	(88%)	2	(12%)	$X^2 = 2.55$, df = 1, $p > 0.1$
1993	6	(60%)	4	(40%)	$X^2 = 0.476$, df = 1, $p > 0.1$
1994	11	(58%)	8	(42%)	$X^2 = 0.974$, df = 1, $p > 0.1$
Mean	10.75	(69%)	4.75	(31%)	

North Carolina and Florida. Although loggerheads regularly nest as far north as Virginia Beach, Virginia (Musick 1988), and in North Carolina, Texas, and Mexico (Sears et al. 1995), these nests combined are estimated to represent less than 2% of the total number of loggerhead nests in the northwestern Atlantic arena (Sears et al. 1995), and thus their possible contribution was not assessed in this study.

A more problematic potential source of juvenile loggerhead turtles is the Mediterranean Sea. A group of nesting populations approximately the size of the Georgia/South Carolina/North Carolina rookery located in Greece, Cyprus, and Turkey shares a common haplotype with the Florida population (Bowen et al. 1993). There is also a significant juvenile foraging population located in the Mediterranean Sea, half of which is composed of loggerheads born in the western Atlantic (Bowen 1995). Because this juvenile population contains such a large percentage of western Atlantic turtles, but their presence is not evident on Mediterranean nesting grounds, it seems likely that juvenile loggerheads native to the western Atlantic are capable of transversing the ocean and returning back again to their natal origin to nest when sexual maturity is reached, even against the prevailing eastward current into the Mediterranean Sea. Hatchling turtles are not as capable of swimming against the current, however, and it seems reasonable that Mediterranean loggerheads may not reach the North-Atlantic gyre and circulate around as loggerheads from the western Atlantic do.

There are two major lines of evidence suggesting that Mediterranean loggerhead hatchlings do not circulate through the Atlantic. First, researchers observe all size classes of loggerheads within the Mediterranean Sea, implying that they can complete the life cycle there (Groombridge 1990). Second (and perhaps more significantly), the frequencies of mtDNA haplotypes in some eastern Atlantic juvenile foraging grounds do not vary significantly from haplotypic frequencies in the southeastern United States (Laurent et al. 1993), suggesting that stocks in the open ocean are not "watered down" by Mediterranean gene frequencies. These data suggest that Mediterranean stocks of loggerheads do not contribute to western Atlantic feeding populations.

The contribution of loggerheads with B and D haplotypes to the Chesapeake Bay population was evaluated over several years, and although between-year variation was noted in haplotypic frequencies, significant differences were not found between years. However, the power of analysis to detect temporal variation was reduced due to the small sample sizes available for each year. To adequately address the possibility of long-term variation (between generations) in composition, large sample sizes of loggerheads will be needed over a time frame large enough to encompass more than a single generation.

This study demonstrated that the Georgia/South Carolina rookery, which is one-tenth the size of the Florida rookery and in decline (Richardson 1982; Musick 1988), is disproportionately affected by natural and human-related activities which negatively impact the loggerhead population in the Chesapeake Bay. Therefore, the Chesapeake Bay loggerhead population should be monitored closely to ensure that the more threatened Georgia/South Carolina contribution be conserved.

Effective management of widely distributed and long lived species such as sea turtles requires knowledge of the population structure on both a global and regional scale. This information is especially useful to determine which populations may be severely impacted by human activities. The present use of RFLP analysis of the mitochondrial D-loop to determine the contribution of natal rookeries to the to the population of juvenile loggerheads feeding in the Chesapeake Bay, in conjunction with the results of tagging, epibiota, heavy metal, and other mtDNA studies of loggerhead turtles in the northwest Atlantic, provides a basis for the conservation of this threatened sea turtle.

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A mixed stock analysis of the green turtle: the need for null hypotheses

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Introduction

As a population geneticist who has spent the major portion of his career working on fishes, I find myself in the unenviable position of presenting a paper on an unfamiliar group of animals; sea turtles. I have learned a good deal more about them than was ever my intention to know, but will make no inferences about their biology here. I will discuss a more comfortable terrain; the confidence we can place in statistical analysis of genetic data. My interest in this area has grown as my research has become more deeply involved in fisheries related issues. How much confidence do I really have in the data and the conclusions drawn from them? The question nags at me, because the results can influence our stewardship of the resources and the livelihood of people and cultures that depend upon them. The issues are important to anyone involved in conservation genetics, because our own future cannot be separated from the fate of the natural resources of the earth. This is especially true in less developed countries as they contain a disproportionate share of the world's biological diversity and a human population that is intimately tied to these resources.

The role of genetics in conservation of wild populations, as I see it, is four fold. First, is to characterize the relationship between species and is largely a systematic or taxonomic exercise. Second, to identify discrete reproductive units or stock and is called genetic stock identification (GSI). Third, to estimate the contributions of these stocks to mixed populations that characterize many species during the non-reproductive season (Mixed stock analysis or MSA). Finally, using this information to protect the genetic integrity of wild stocks when management programs are deemed necessary. In an ideal world, genetic data pertinent to each of these roles would follow logically, one from the next. Genetic data would be amassed to verify the systematic status of species in need of specific conservation or fisheries management plans and identify cryptic species should they exist. This would be followed by a survey of the relationship among reproductive aggregations of the validated species using a variety of molecular approaches. The genetic markers that best distinguish reproductive units would then be tested for robustness in MSA. The MSA would then be conducted using only those tools that are up to the task. Finally, the results of each phase would be used to help formulate and guide management efforts. While I am not aware of a case in which these steps have been outlined a priori and followed rigorously, numerous species have been examined (usually by more than one investigator) in exactly this fashion (cf. Avise 1994; Shaklee et al. 1990).

The identification of species and population structure are comfortable terrain for evolutionary and population geneticists. They focus upon the bonds of mating and ancestry where genes are passed from one generation to the next. The analysis of genetic data in mixed stock problems is much less understood, due in no small measure to the fact that it focuses upon non-reproductive components of the life cycle. The issues are not influenced by the transmission of genes from one generation to the next, but upon the spatial distribution of reproductive units during the non-reproductive seasons. It is a topic more often encountered in mark-recapture problems in fisheries biology or population ecology than in population genetics.

In addition to focusing upon a different component of the life cycle, MSA lacks a general hypothesis. Species identification and GSI address hypotheses related to the variation

within and between aggregates of individuals that are either specifically stated or implied from the statistical analyses performed on the data. Studies of mixed stocks often attempt to estimate the contribution of reproductive units to mixed aggregations in the absence of a specific hypothesis concerning these contributions (cf. Berggren and Leiberman 1978; Fournier et al. 1984; Fabrizio 1987; Mulligan et al. 1988; Shaklee et al. 1990; Wirgin et al. 1993; Chapman 1994; Epifanio et al. 1995). Considerable theoretical and empirical effort has focused upon whether the populations are sufficiently differentiated by the data so that contribution estimates will be reliable (cf. Smouse et al. 1982; Wood et al. 1987; Millar 1987, 1990, 1991; Mulligan et al. 1988; Shaklee et al. 1990; Wirgin et al. 1993; Chapman 1994; Epifanio et al. 1995). As shown by Millar (1990), when stocks are not well differentiated by the measured variables, the contribution estimates will tend toward 1/S, where S is the number of stocks in the analysis. We need to know if the analysis generates contribution estimates that differ from a series of S random numbers bounded on 0,1 and constrained to sum to 1. It has also been shown that the probability of correct allocation increases as the number of candidate stocks decreases or as the number of traits considered increases (Smouse et al. 1982; Millar 1987). Most of the effort in MSA has focused upon the structure of the data needed for accuracy and upon the methods employed in the analysis.

The objective of this paper is to point out that most of the effort in MSA has focused upon statistical considerations and not upon testing specific hypotheses regarding the biology of the organisms. Once we have established that the data are up to the task of conducting MSA, the first hypothesis that needs to be tested is what evidence do we have that the "mixed" aggregation is a mixture of anything? With nuclear gene data, this is rather simply addressed by performing tests for Hardy-Weinberg (HW) proportions and finding significant heterozygote deficiencies (the Wahlund effect; Wahlund 1928). For mtDNA or morphological data, documenting the mixed nature of the aggregation is more likely to be a matter of faith rather than a hypothesis one can test. One could compare the frequency distributions in the mixed aggregation to the available source populations, but, even if the mix differed significantly from all source populations, it could be argued that the mix is actually another reproductive unit that was not surveyed previously. Once it is established that the mixed aggregation is indeed a mix, we should test specific hypotheses regarding the contributions of various source populations to the mixed aggregation.

In what follows I will endeavor to illustrate the problems encountered in MSA by an analysis of two data bases on the green turtle, *Chelonia mydas*. These data were selected because they encompass both mtDNA (Bowen et al. 1992) and nuclear DNA (Karl et al. 1992) and were taken from the same locations. Approximate population sizes (cf. Bowen et al. 1992) are available for each nesting beach which is important in framing hypotheses concerning relative contributions to a mixed population. In addition, the data include most of the known reproductive areas. The impetus for MSA in this species is largely preservation of an endangered species and is, therefore, different from the allocation of resources to user groups that is the central issue in the conservation of fisheries. Because these issues are global in nature and cut across the breath of the human socio-economic spectrum, researchers should exercise utmost care in framing hypotheses and understanding the limits of the data.

Methods and Materials

The data for this analysis has been modified from Bowen et al (1992) and Karl et al. (1992) and is reproduced here (cf. Table 1) for the convenience of the reader. The modifications are that only data from the Atlantic and Mediterranean have been included and nuclear gene data from Quintana Roo, Mexico (Karl et al. 1992) has been eliminated as parallel mtDNA data were not available. Nuclear loci CM-12 and CM-39 were also eliminated from consideration as these loci are only weakly polymorphic.

These data were used to construct artificial mixed aggregations (Tables 2 and 3). Ideally we would construct mixed aggregations from nuclear and mtDNA data independently and jointly. Dealing with the combined nuclear and mtDNA data lead to difficulties as some 863 genotypes were possible and none of these occurred in a frequency greater than 1%. The sample size of the mixed population would have to be many thousands to generate accurate estimations and, for this reason, nuclear and mtDNA data were treated independently. The relative contributions of each rookery to the mixed aggregations were based upon the estimated rookery size (cf. Bowen et al. 1992) and the gene frequencies in this mixed aggregation can be thought of as an average across rookeries in the Atlantic and Mediterranean. This assumption is a matter of convenience, but serves as a null hypothesis, i.e. rookeries contribute to mixed aggregations in proportion to their population sizes. The gene frequencies in the mixture populations were actually constructed from a stochastic sampling regime for projected mixture sample sizes of 100 and 400 individuals (nuclear loci) and for 50, 100, 500 and 1000 (mtDNA). The stochastic sampling regime reported the actual number of genotypes drawn from each rookery and due to rounding errors these number are less than the projected sample sizes. The genotypic distributions in each rookery were constructed for nuclear gene loci under the assumption that the rookeries were in HW equilibrium as the data has been reported only for gene frequencies and not genotype frequencies (Karl et al. 1992). Conformity to Hardy-Weinberg expectations was also calculated in the mixed population for nuclear loci in order to examine the Wahlund effect (Wahlund 1928). Heterozygote deficiencies are reported as Selanders D (Selander 1970), as a matter of convenience.

Given that the actual contribution of the rookeries to the mixed population is known, the robustness of the data to estimate this

Table 1. Gene frequencies at anonymous nuclear gene loci and haplotype distributions for mitochondrial DNA in the green turtle, *Chelonia mydas*.

Nuclear Locus	Location							
	Asc	CR	Sur	Fla	Ven	Bra	GB	Cyp
CM-14	0.16	0.44	0.17	0.52	0.86	0.44	0.30	0.05
	0.5	0.12	0.26	0.09	0	0	0.41	0.47
	0.34	0.44	0.57	0.39	0.14	0.56	0.29	0.48
CM-45	0.770	0.770	0.750	0.700	0.790	0.560	0.650	0.660
	0.230	0.230	0.250	0.300	0.210	0.440	0.350	0.340
CM-67	0.670	0.190	0.470	0.330	0.290	0.500	0.940	0.580
	0.210	0.050	0.030	0.110	0.070	0.000	0.030	0.210
	0.120	0.760	0.500	0.560	0.640	0.500	0.030	0.210
mtDNA	Asc	CR	Sur	Fla	Ven	Bra	GB	Cyp
A		15		21	1			
B				3				
C			25		7			
D	34					15		
E	1							
F						1		
G							13	
H								10
Approx Rookery Size	1600	5000	3000	300	100	100	400	100

Table 2. *Upper* Gene frequency distributions at three loci for mixed aggregations of 96 and 397 individuals. Chi-square analyses tests for conformity to Hardy-Weinberg expectations and heterozygote deficiencies (D). * = p < 0.05, ** = p < 0.01. *Lower* Comparisons of actual to estimated contribution in the mixed aggregations for the indicated rookeries. Standard errors in parentheses.

	N=96			N=397		
	Cm-14	Cm-45	Cm-67	Cm-14	Cm-45	Cm-67
	0.303	0.755	0.367	0.307	0.745	0.377
	0.227	0.245	0.075	0.226	0.255	0.075
	0.470		0.558	0.467		0.548
Chi-square	4.30	0.01	5.56	9.06*	0.01	22.65
D	-0.23	0.00	-0.18	-0.08	0.00	-0.18

POPULATION	Actual Contribution N=96	Estimated Contribution	Actual Contribution N=397	Estimated Contribution
1 Ascension	0.148	0.038 (0.065)	0.147	0.072 (0.033)
2 Costa Rica	0.465	0.452 (0.191)	0.463	0.266 (0.076)
3 Suriname	0.279	0.408 (0.129)	0.278	0.462 (0.062)
4 Florida	0.027	0.024 (0.183)	0.028	0.180 (0.070)
5 Venezuela	0.027	0.000 (0.000)	0.027	0.000 (0.000)
6 Brazil	0.000	0.052 (0.059)	0.009	0.007 (0.026)
7 Guinea Bissau	0.037	0.000 (0.000)	0.037	0.000 (0.000)
8 Cyprus	0.000	0.023 (0.036)	0.009	0.011 (0.018)

contribution can be tested using several statistical and mathematical approaches. We want to know if the gene frequency differences among the rookeries are sufficiently great that contribution estimates derived from real data are reliable and the sample sizes needed to obtain a measure of confidence. The GIRLSEM program of Pella and Milner (1987) has been employed in this study, because it is a maximum likelihood approach that has been shown to be an optimal procedure by a variety of criteria (cf. Pella and Milner 1987; Wood et al. 1987; Millar 1990). This program has a number of features and options that are not available from other programs. First, it computes variances of contribution estimates which are not available from HISEAS or SHADRACQ (distributed by P. Smouse). Second, it accepts either type or gene frequency data. This permits the program to accept morphological, metric, mtDNA or nuclear gene data or any mix in a single run. Third, GIRLSEM permits bootstrapping and jackknifing of the data through simple switching and these are at best cumbersome with other programs.

Results

Nuclear Genes

The upper half of Table 2 presents the gene frequencies at nuclear gene loci in the mixed aggregation for 96 and 396 individuals. The mixture population containing 96 individuals did not deviate significantly from HW expectations at any of the loci, even though heterozygotes were under represented (Table 2). The mixed population with 397 individuals deviated from HW expectations at the CM-14 and CM-67 loci, but not at the CM-45 locus. The significant deviations were accompanied by heterozygote deficiencies. The failure of the CM-45 locus to deviate from HW expectations was anticipated due to the distribution of alleles in the most abundant rookeries.

The lower half of Table 2 compares the actual contribution of rookeries to the artificial mixed aggregations and the estimated contributions computed by GIRLSEM. With the exception of Ascension Island, the actual contributions are within the standard errors of the estimates for the smaller of the two mixed aggregations (N=96). It should be noted that the standard errors are rather large and in most cases greater than the estimate itself. Contribution estimates for the larger mixed aggregation (N=397) are accurate only for Cyprus and Brazil. The estimates for the major contributors, Ascension, Costa Rica and Surinam, are completely wrong. Of particular interest, is the observation that Costa Rica was the largest single contributor, but the MSA suggests that Surinam was the largest single contributor.

Overall, the MSA indicates that one can obtain contribution estimates that are within a standard error of being correct with a relatively small sample size, but one must accept rather large standard errors in the process. Increasing the sample size of the mixed aggregation may narrow the standard errors and generate contribution estimates that are precisely wrong.

Mitochondrial DNA

The distribution of mtDNA haplotypes in mix aggregation composed of 49, 99, 496 and 998 individuals are presented in Table 3. The relative contributions of the rookeries were the same as in Table 2, except for the 49 individual aggregation where Brazil and Cyprus made no contribution. The estimated contribution generated by GIRLSEM was within a standard error of the actual contribution for aggregation of 49 and 99 individuals. The contribution estimate for Venezuela was grossly over-estimated (and Surinam under-estimated) when sample sizes in the aggregation exceeded 250 or so (data not shown). The contribution of the remaining rookeries was accurately estimated with sample sizes of 50 or more. Overall, the data indicate that reasonably accurate contribution estimates can be achieved with sample sizes between 50 and 100 individuals, but the situation becomes distorted as mixed aggregation samples sizes increase. This seems counter-intuitive and will be discussed below.

Discussion

The data presented here are relevant to several issues in MSA. First is the ability of existing data to assess the utilization of feedings grounds by various rookeries. Second, the method of analysis is only one of several approaches that could be employed and some discussion of the strengths and weakness of the various approaches is warranted. Finally, the data and analytical approach should also be discussed in reference to the hypothesis under consideration and I have come to the opinion that we have yet to ask the right questions.

Strengths and Weakness of Existing Data in *Chelonia mydas*

The analyses presented here demonstrate some of the difficulties associated with MSA. Of particular importance is the need to have source populations well differentiated (cf. Smouse et al. 1982) and well characterized. On the surface it would appear that both nuclear gene and mtDNA data would serve this purpose as the frequency data (Table 1) are quite different among rookeries. The first indication that something may be amiss is in the tests for HW equilibrium where the mixed aggregation does not depart from expectations when n=96. If the artificial mix were real data, we would be hard pressed to prove that it was not a genetically distinct population rather than a mix, unless the sample sizes are quite large. With large sample sizes, we get strong indication that the data come from a mixed aggregation, but the contribution estimates of Florida and Surinam are grossly over-estimated. This occurs because Florida and Surinam have gene frequency distributions that closely match the mix aggregation. The MLE approach is a search for the most parsimonious solution to a system of equations. If a source population has gene frequencies that are close to the mix, the MLE will allocate most of the mix to this source. It is the least objectionable solution, even though it leads to inaccurate contribution estimates. The worst part of it is, that we might

Table 3. Distribution of mtDNA haplotypes in a simulated mixed population of *Chelonia mydas* and comparisons of the actual contributions of rookeries to that estimated by GIRLSEM. N is the sample size of the mixed poplation. Estimated contribution and their standard errors (in parentheses) are shown for each sample size.

HAPLOTYPE		MIXED POPULATIONS			
		N=49	N=99	N=496	N=998
A		25	48	242	490
B				1	2
C		15	30	151	302
D		8	15	76	153
E				2	4
F					1
G		1	4	19	37
H			1	5	9
POPULATION	ACTUAL CONTRIBUTION	ESTIMATED CONTRIBUTION			
Ascension	0.148	0.160	0.151	0.156	0.141
		(0.051)	(0.036)	(0.016)	(0.018)
Brazil	0.010	0.000	0.000	0.000	0.016
		(0.000)	(0.000)	(0.000)	(0.015)
Suriname	0.279	0.299	0.302	0.001	0.001
		(0.094)	(0.049)	(0.019)	(0.001)
Costa Rica	0.465	0.499	0.494	0.419	0.426
		(0.071)	(0.050)	(0.032)	(0.021)
Venezuela	0.027	0.001	0.000	0.345	0.345
		(0.140)	(0.015)	(0.031)	(0.016)
Florida	0.027	0.000	0.000	0.032	0.024
		(0.000)	(0.000)	(0.022)	(0.014)
Guinea Bissau	0.037	0.039	0.040	0.038	0.037
		(0.027)	(0.019)	(0.008)	(0.006)
Cyprus	0.010	0.000	0.010	0.010	0.009
		(0.000)	(0.010)	(0.004)	(0.003)

just believe this solution as the standard errors are small relative to the estimates. As a rule of thumb, one should be suspicious of MSA when any of the source populations do not differ significantly from the mix for at least one allele.

The mtDNA data demonstrate what can happen when two or more source populations are not well differentiated. When sample sizes are less than 100, the contribution estimates closely match the actual values for all populations. As sample size increase, the analysis incorrectly allocates the Surinam contribution to Venezuela. This is due to the fact that Venezuela has a single representative of the A haplotype and 7 representative of the C haplotype, while Surinam is fixed for C. When sample sizes are large, Venezuela is a less objectionable solution than Surinam, because the haplotype frequencies are closer to the mix frequencies. This does not occur with Brazil and Ascension Island (which share the D haplotype) as the endemic E and F haplotypes stabilize the analysis. Florida and Costa Rica are dominated by the A haplotype, but the analysis does not allocate Costa Rica's contribution to Florida, because the B haplotype is only found in Florida and serves the same stabilizing influence as E and F when sample sizes are large. The contributions from Florida, Venezuela, and Brazil are consistently underestimated until their characteristic, but

rare, haplotypes are represented in the mixed aggregation. Overall, the mtDNA data point out that when the sample size in the mixed aggregation is small, the analysis is driven largely by the distribution of the common genotypes, as these are most likely to be observed in small samples. When the sample size increases, rare, private haplotypes can serve to stabilize the analysis, but a haplotype that is rare in one population and common in another can lead to serious misallocation. These misallocations may go undetected because the standard errors of the estimates can be quite small.

The performance of the nuclear gene data are rather disappointing. They do not provide accurate contribution estimates, unless one is content with large standard errors around these estimates. The mtDNA data has a somewhat better performance so long as sample sizes are not large and the analysis is not unduly influenced by rare haplotypes. The two data bases could be combined and I suspect that the overall performance would improve. However, this would most likely require very large sample size.

Statistical Alternatives

In this study, maximum likelihood approaches have been employed to test the robustness of existing data in MSA. Perhaps the greatest weakness of this method is that it requires that all potential source populations be included in the data matrix (Smouse et al. 1990). This is not a problem for testing the robustness of the existing data, as the mixed aggregations were constructed from known populations. It may be a large obstacle in translating such a test to real data, as a complete survey of all potential source populations may not be obtainable for one reason or another. When all source populations are not surveyed (or surveyed inadequately), mixed aggregations may contain genotypes or haplotypes that are not see in the source populations (cf. Wirgin et al. 1993; Chapman 1994). The GIRLSEM program discards these haplotypes from the analysis and it is uncertain how seriously this may bias the results.

An alternative to maximum likelihood approaches, linear regression (GLM, general linear models), has the potential to circumvent the problem of unique genotypes in the mixed aggregation. In GLM, we obtain an estimate for how well the data fit from the R2, and unique genotypes in the mixed aggregation expand the error term. We get an estimate of how much of the variation in the mixed aggregation is explained by linear combinations of the source populations. The GLM also provides an analogue to contribution estimates, which is the vector of b's from the regression. Unfortuately, GLM assumes that the data are normally distributed which requires that frequency data be transformed. This, in turn, transforms the R2 and makes this coefficient difficult to interpret. In addition, the b's are not constrained on the interval 0,1 and may be negative. Xu et al. (1994) and Wirgin et al. (1993) have presented least squares approaches that constrain the b's on 0,1.

Another alternative to discarding unique types, in the mixed aggregation, has been used by Epifanio et al. (1995) in a study of American shad. In that study, a distance metric (Excoffier et al. 1992) generated from AMOVA (analysis of molecular variance) was used to allocate unique types from the mixed aggregation to the source population they most closely resembled. This approach can be justified if the migration rate is small relative to the mutation rate (Slatkin 1995). Unfortunately, this quantitative approach did not improve the results over the qualitative approach used by Chapman (1994). The lack of improvement may stem from two sources. First, it is doubtful that the migration rate is less than mutation in this species. Second, it has been shown that extensive intraconversions have occurred among the most common haplotypes and some of the rare haplotypes have reverted to common ones (Chapman 1994). The distance metrics are, therefore, biased and do not reflect the actual distance between haplotypes or populations. We should not discount the approach solely for these reasons, but it does add additional assumptions to an analysis that is already heavily burdened.

To summarize this section, it is my view that the available analytical tools are less than satisfactory to address the overall problem of MSA. We need additional tools that can deal

with incomplete data and provide easily interpreted estimates of how good or bad our contribution estimates are.

Hypothesis Testing

As stated in the introduction, MSA lacks the general hypothesis that is explicit, or implied, in other areas of population genetics. For example, analysis of population structure proceeds under the null hypothesis is that all populations are identical. As MSA lack such a general working hypothesis, it could be reasonably argued that MSA is not a science and I would be hard pressed to dispute this conclusion. The majority of studies involving MSA share this deficiency (eg. Berggren and Leiberman 1978; Fabrizio 1987; Shaklee et al. 1990; Wirgin et al. 1993; Epifanio et al. 1995; Chapman 1994). Generating a null hypothesis is not an exercise in testing the data against some artificial mix of populations. Testing an artificial mix is simply good practice, although it is not always done (Wirgin et al. 1993). A null hypothesis would consist of a statement about how populations contribute to the mix, whether it is artificial or otherwise. It is here where we need some discussion and consensus of what may or may not constitute a general hypothesis for MSA.

There appear to be two hypotheses that could be advanced as general working hypotheses. The first, would be that all populations contribution to mixed aggregations in proportion to their relative abundance (the one used here) and, the second, that all populations contribute equally to mixed aggregations. The first, seems to me the more logical as large populations simply outnumber smaller ones and can, therefore, dominate mixed stocks. It should be stressed that all populations for which data are available should be included in the analysis, unless we have an a priori reason to exclude one or more stocks. If the analysis is robust (i.e. source populations well differentiated), then including non-contributing populations will not effect the results. If including non-contributing populations effects the results, we need to know that as well, as it effects the confidence we have in the analysis.

As an example of how excluding a source population may influence an MSA, consider the data of Wirgin et al. (1993). In that study, the investigators estimated the contribution of Hudson River and Chesapeake Bay striped bass to the haul seine fishery of Long Island. In an analysis where the four Chesapeake samples were combined (even though the Choptank River was statistically different for other Chesapeake samples), the results suggest that the Hudson River contributed 73% and the Chesapeake Bay contributed 27% to this coastal fishery (Wirgin et al. 1993). The study did not consider the contribution of the Roanoke River population for which data were presented. Including the Roanoke River data generates completely different results (Roanoke=50%, Chesapeake=35%, and Hudson = 15%). Eliminating the Roanoke and treating each Chesapeake sample individually, MSA allocated 85% to Chesapeake Bay and 15% to the Hudson. The latter approach is called the allocate-sum approach and is preferred (Wood et al. 1987) over the pool-allocate method used by Wirgin et al. (1993). The importance of this re-evaluation is not to dispute the conclusions of Wirgin et al. (1993), but to point out the effects of eliminating potential contributors and the importance of testing the most general hypothesis.

In the conservation of rare species our task may be simplified, because we may not be really concerned with accurately estimating the contribution of the smaller populations, but whether these populations are represented in the mix. The null hypothesis would be that the critical population does not contribute and we can project the sample size needed for critical tests from probability theory. As a concrete example, consider the study by Sears et al. (1995) on loggerhead turtles from the feeding grounds in Charleston Harbor. Here the concern was the effects of dredging on the relatively small South Carolina rookeries. The MSA indicates that approximately 45% of the turtles captured in Charleston Harbor were from South Carolina rookeries, even though these rookeries produce less 10% of the total nesting along the coast of the US. It is not important that the estimate is 45, 55 or 100%. What is important is that dredging would not only impact South Carolina rookeries, but also have a

disproportionate impact on one of the smaller, declining nesting populations.

Summary

I hope that this paper has convinced the reader that we should treat MSA as just another statistical test that serves to refute a null hypothesis. What the null hypothesis is may depend upon the ultimate goal, but it should be clearly stated. The ability of the data and method of analysis to provide robust solutions are important, but no more important in MSA than they are in GLM, ANOVA or any other test. The most important feature should be the hypothesis being tested and not the mechanics of analysis.

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Mitochondrial DNA D-loop sequences of marine turtles

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Introduction

The ability to analyze the genetic composition of sea turtle populations has relied significantly on improved techniques for the study of mitochondrial DNA (mtDNA) (Avise 1994). Not only has the knowledge of genetic variation led to an improved understanding of basic evolutionary processes in marine turtles (Avise et al. 1992; Bowen et al. 1993a), but also numerous genetic markers useful for applications in conservation have been discovered (reviewed in Bowen and Avise 1995).

Initial research relied on restriction site analysis of all or part of the mtDNA genome, yielding novel re-evaluations of systematic issues in the ridley genus (Bowen et al. 1991). In addition, clear global and regional population genetic structuring in green (*Chelonia mydas*; Bowen et al. 1992), loggerhead (*Caretta caretta*; Bowen et al. 1993b, 1994) and hawksbill turtles (*Eretmochelys imbricata*; Broderick et al. 1994) was detected. This information has been useful for the definition of basic management units in these endangered species which are still declining in many parts of the world. The increased availability of the polymerase chain reaction (PCR) and DNA sequencing, has shifted the focus towards a more detailed analysis of specific segments of the mitochondrial genome. The control region which contains the displacement loop ("d-loop") has proven to be extremely useful in studies of marine turtles. Analysis of control region sequences has uncovered approximately six times the amount of variation previously detected with RFLP techniques (Encalada et al. 1996). With this enhanced resolution, genetic markers have been used to study fine-scale population structure for nesting populations of green turtles in the Atlantic (Allard et al. 1994; Lahanas et al. 1994) and Indo-Pacific (Norman et al. 1994), hawksbill turtles in the Indo-Pacific (Broderick et al. 1994), Caribbean and West Atlantic (Bass et al. 1996), and leatherbacks (*Dermochelys coriacea*) in the Pacific and Atlantic (Dutton 1995).

Genetic markers derived from d-loop sequences have also been useful in identifying the natal origin of marine turtles, and have been used to demonstrate the trans-Pacific migration of loggerhead turtles (Bowen et al. 1995). In addition, foraging ground aggregations of green turtles in the Bahamas (P.Lahanas, K. Bjorndal, A. Bolten, unpublished data) and hawksbill turtles in the Caribbean (Bowen et al. 1996) have been shown to consist of a mixture of stocks associated with different rookeries occurring in more than one country.

This genetic approach provides a powerful tool to identify countries which jointly share critical marine turtle habitats ("range states"). These approaches can assist in the development of regional conservation strategies and in the application of international law as it pertains to an endangered species with migratory routes traversing national boundaries (see Bowen and Avise 1995).

Considering the utility of d-loop variation in marine turtles, it is not surprising that it has become the single most intensely studied locus in the genome. As the ability to sequence DNA becomes more widespread, so will the need to access sequence information for comparative analysis. Currently, there is d-loop sequence information from all species encompassing many (if not most) of the important rookeries. However, access and alignment of these sequences requires considerable effort. This paper presents a compilation of all the mtDNA control region sequences available in a format that will facilitate comparison of sequences derived from different procedures and primers.

Sources of Sequence Data

CC/L35254 and L35255

These are Atlantic-Mediterranean *Caretta caretta* sequences copied from GenBank, accession numbers L35254 and L35255 (L. Laurent, unpublished data).

Sequence L35255, utilized as a reference for sequence alignment, is 986 bases long and contains portions of mitochondrial transfer RNAs in the 5' end. Positions 1-13 contain the last bases of the 3' end of the tRNA-Thr gene and positions 14-85 represent the tRNA-Pro gene. The d-loop sequences begin at position 86 and continue to position 986. No information is available on the primers used to amplify this sequence.

Sequence L35254 contains the last 52 bases of the 3' end of the tRNA-Pro gene. According to the authors, the d-loop continues until base 981; thereafter exists a repeat region between bases 982 and 1006. Only 650 bases of this sequence is included in the table in order to have a length similar to subsequent sequences. No information was available on the primers used to amplify this sequence.

CC-A to CC-C/Bowen (3 haplotypes)

Pacific *Caretta caretta* haplotype sequences were obtained from Bowen et al. (1995). Haplotype CC-A corresponds to GenBank accession number U22261, which was found in samples from Mon Repos, Australia. These sequences were amplified with the Norman et al. (1994) TCR5/TCR6 PCR primer pair.

CM-1 to CM-18/Encalada (18 haplotypes)

Atlantic-Mediterranean *Chelonia mydas* haplotypes were obtained from Lahanas et al.(1994) and Encalada et al. (1996). These sequences were amplified with the Allard et al. (1994) LTCM1/HDCM1 PCR primer pair. Sequences CM-1 to CM-18 (excluding CM-3) have been placed in GenBank with corresponding accession numbers Z50124 to Z50140.

CM-NGBR to CM-SWK/Norman (15 haplotypes)

Indo-Pacific *Chelonia mydas* sequences were obtained from Norman et al. (1994). The rookeries selected contain the most prevalent mtDNA types for the species in the region. In three rookeries, Japan, Java, and French Polynesia, second samples were included to incorporate secondary yet highly frequent haplotypes. Abbreviations refer to sample localities in the region of northern Australia and adjacent areas of SE Asia: **NGBR=** Northern

Great Barrier Reef, **SGBR=** Southern Great Barrier Reef, **GOC=** Gulf of Carpentaria, **LAC=** Lacepede Islands, **NWC=** North West Cape, **PNG=** Papua New Guinea, **ELT=** Elato Atoll, **SWK=** Sarawak, **JVA=** Java (2 haplotypes: a,b), **JPN=** Japan (2 haplotypes: a,b), **HAW=** Hawaii; **FP=** French Polynesia (2 haplotypes: a,b). The authors note that sequences CM-GOC and CM-JVAa are identical. See original paper for further details. PCR primers TCR5 and TCR6 for sea turtle d-loops were described and used to amplify the sequences presented in this paper. There is a correspondence between some of the haplotypes sequenced by Norman et al. (1994) and Bowen et al.'s (1992) RFLP haplotypes: HAW= haplotype "J", FPa= "K", SGBR and FPb= "L", JPNa= "N".

CM-TAT to CM-CGC/Allard (4 haplotypes)

Sequences of samples from Costa Rica and Florida *Chelonia mydas* rookeries. Data taken from Allard et al. (1994). Haplotype CM-TAT corresponds to GenBank accession number M98394. The authors report that the first 71 bases correspond to the 3' end portion of the tRNAPro gene. The LTCM1, LDCM1, and HDCM1 PCR primers were described and used to amplify these sequences. Sequences CM-CAC, CM-CGC, CM-TAC, and CM-TAT are the same sequences as CM-1 to CM-4/Encalada except that the Allard series includes 78 additional bases at the 5' end (see Table I).

EI-A to EI-U/Bass, EI-alpha to EI-gamma/ Bass (24 haplotypes)

Sequences of Caribbean and W. Atlantic *Eretmochelys imbricata* haplotypes published in Bass et al. (1996). Haplotypes EI-A to EI-Q correspond to pure *E. imbricata* stock, while haplotypes EI-R to EI-U belong to hatchlings of hybrid origin (see original paper for more details). Three additional haplotypes were revealed in samples from a feeding ground off Mona Island, Puerto Rico and are designated EI-alpha, -beta, and -gamma (Bowen et al. 1996). Haplotypes EI-A, -F, -N, -Q, and -R correspond to GenBank accession numbers U22368, U37804, U37805, U37806, and U37807, respectively. All of these sequences were amplified with the Norman et al. (1994) TCR5/TCR6 PCR primer pair.

DC-A to DC-H/Dutton (8 haplotypes)

Sequences correspond to *Dermochelys coriacea* haplotypes from Peter Dutton's (1995) Ph. D. thesis. They were amplified with the Allard et al. (1994) LTCM1/HDCM1 PCR primer pair.

DC to ND/Dutton (10 haplotypes)

Sequences from various species (first two initials indicate species and second two initials indicate the ocean basin; PA= Pacific, AT= Atlantic). Data taken from Peter Dutton's Ph. D. thesis, and from Dutton et al. (1996). The sequences were amplified with the Allard et al. (1994) LTCM1/HDCM1 PCR primer pair. GenBank accession numbers are as follows: CM-PA= U40436; CC= U40435; CA= U40659; CM-AT= U40660; EI= U40658; LO= U40661; LK= U40657; ND= U40662; DC= U40663.

ND-A to ND-C/FitzSimmons (2 haplotypes)

Natator depressus sequences kindly provided by Nancy FitzSimmons (unpublished data).

LO-K and KEM-LK3B

Unpublished data from Raquel Briseño's M. Sc: "Caracterización genética de la tortuga golfina, *Lepidochelys olivacea* en el Pacífico mexicano e implicaciones para su conservación". Haplotype LO-K is the prevalent haplotype in the rookeries studied. Haplotype KEM-LK3B was derived from *L. kempi* and provided by Ms. Ginger Clark. Both of these sequences were amplified with the Allard et al. (1994) LTCM1/HDCM1 PCR primers.

Sequence alignments

CLUSTAL W (version 1.6) was utilized for sequence alignments (Thompson et al. 1994). The multiple alignment procedure from the package was applied with the following parameter values: gap opening penalty- 10.0; gap extension penalty- 0.05; delay divergent sequences- 40%; toggle transitions- weighted. The resulting gap placement was reviewed by eye and modified to maximize parsimony among haplotype sequences from the same species.

mtDNA D-loop Sequence Listings

Table I contains complete sequence information from representative mtDNA d-loop haplotypes of all marine turtle species. In cases where there have been numerous haplotypes described, a selection was made so as to incorporate either the most common sequence(s) observed (e.g. *C. caretta, E. imbricata, N. depressus*, some of the *C. mydas* and *L. olivacea* sequences), those depicting singular attributes (additional insertions, segment duplications, etc.; e.g. haplotype CM-7 from Atlantic/Mediterranean *C. mydas*) or one haplotype from each of the geographic or genetic clusters observed (Indo-Pacific and Atlantic-Mediterranean *C. mydas* data).

Tables II to V show variable sites for green, loggerhead, leatherback and hawksbill turtles.

Symbols used

Hyphens at the beginning or at the end of sequences represent missing information. Hyphens ("-") within sequences represent "gaps" which the alignment program adds in order to allow for the presence of base "insertions" in other sequences at corresponding positions.

Abbreviations for sequence labels begin with a two letter code denoting the species, followed by the author's designation for the particular haplotype and an abbreviation of the senior author's last name. Thus EI-A/Bass, represents haplotype A in *Eretmochelys imbricata* as described by Bass et al. (1996). The reader is referred to the original papers for further details.

Primers

The two most commonly used sets of primers for the amplification of marine turtle mtDNA d-loop sequences are those described by Allard et al. (1994) and by Norman et al. (1994). The former, denoted LTCM1/HDCM1, was derived from sequences obtained from mtDNA extracted from Costa Rica and Florida *C. mydas*. A secondary primer derived from an intermediate portion of the "light" mtDNA strand was also obtained from this material. This pair of primers commonly generates fragments approximately 510 base pairs (bps) long. Although originally designed for *C. mydas*, these primers have been successfully used in all the marine turtles. Norman et al.'s (1994) primers (TCR5/TCR6) were designed from Indo-Pacific *C. mydas* sequences. They permit DNA amplifications of fragments approximately 380 bps in length (see Table I).

The structure and annealing locations of the primers are shown with boldface lettering in Table I. The "forward" primers (LTCM1, TCR5, and LDCM1) have the same orientation (5' to 3') and similar base composition as the sequences in their binding domain of the strand shown (the L-strand) since they will be binding and initiating amplification at the complementary sequence in the sister strand (the H-strand). The "reverse" primers HDCM1 and TCR6 are shown for analogous reasons with an opposite orientation (3' to 5') and their composition is complementary to their binding sites on the strand shown in the table.

Acknowledgments

Initial transcription of sequence data by Samantha Karam is gratefully appreciated. Thanks also goes to the researchers that produced this information on marine turtles. We are extremely grateful for the ongoing international cooperation which allowed these

Table I. Multiple alignment of representative sea turtle d-loop sequences (see text for sources and an explanation of abbreviations and symbols used).

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                tRNA-Thr |<                          tRNA-Pro                                  >|start of d-loop
              <5´ end of sequences
                                  Allard et al. (1994) primer LTCM1  5'c ccaaaaccgg aatcctat3'
CC/L35255  CTTTCCTAGA ATAATCAAAA GAGAAGGGTT CAAACCTTCA TCTCCGGTCC CCAAAACCGG AATCTTCCAA TTAAACTACC CTTTGACGCA AAAGAAGCGC 100
CC/L35254  ---------- ---------- ---------- ---ACCTTCA TCTCCGGTCC CCAAAACCGG AATCTTCCAA TTAAACTACC CTTTGACGCA AAAGAAGCGC
CC-A/Bowen ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CC/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTACC CTTTGACGCA AAAGAAGCGC
CM-7/Encal ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --AATAAAAG
CM-1/Encal ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --AATAAAAG
CM-TAC/All ---------- ----TCAAAA GAGAAGGACT TAAACCTTCA TCCCCGGTCC CCAAAACCGG AATCCTATAA TTAAACTATC CTTTGACACA GGAATAAAAG
CM-JPNa/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-NGBR/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-SGBR/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-FPa/Nor ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-HAW/Nor ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-JVAa/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-AT/Dutt ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTATC CTTTTACACA GGAATAAAAG
CM-PA/Dutt ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTATC CTTTGACACA GGAA---AAG
CA/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTATC CTTTGACACA GGAA---AAG
EI-A/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-F/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-Q/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-R/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTATC CCTTGACGCA G-AAT--AAG
LO-K/Brise ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LO/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTACC CTTTGGCGCA AAAA------
LK-LK3B/Bo ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
LK/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTACC CTTTGACGCA AAAA----AG
ND-A/FitzS ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ND-C/FitzS ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ND/Dutton  ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTATC CTTTGACGCA GGAATAAAAG
DC-A/Dutto ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTACC TCTTGACACA ----------
DC-PA/Dutt ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAACCTACC TCTTGACACA ----------
DC-AT/Dutt ---------- ---------- ---------- ---------- ---------- ---------- --------AA TTAAACTACC TCTTGACACA ----------
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Norman *et al.* (1994) primer TCR5 5'ttgtacatct

acttatttaccac3'

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CC/L35255  ----CAACAT GTAAATT--- TACCTATATT CTCTGCCGTG CCCAACAGAA CAATATCCGT AATACCTA-T CTATGTATTA TCGTACATCA ACTTATTTAC 200
CC/L35254  ----CAACAT GTAAATT--- TACCTATATT CTCTGCCGTG CCCAACAGAA TAATATCCAT AATACCTA-T CTATGTATTA TCGTACATCA ACTTATTTAC
CC-A/Bowen ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CC/Dutton  ----CAACAT GTAAATT--- TACCTATATT CTCTGCCGTG CCCAACAGAA TAATATCCAT AATACCTA-T CTATGTATTA TTGTACATCA ACTTATTTAC
CM-7/Encal T---GTCCAC ACAAACTAAC TACCTAAATT CTCTGCCGTG CCCAACAGAA CAATACCCGC AATACCTA-T CTATGTATTA TCGTGCATCT ACTTATTTAC
CM-1/Encal T---GTCCAC ACAAACTAAC TACCTAAATT CTCTGCCGTG CCCAACAGAA CAATACCCGC AATACCTA-T CTATGTATTA TCGTACATCT ACTTATTTAC
CM-TAC/All T---GTCCAC ACAAACTAAC TACCTAAATT CTCTGCCGTG CCCAACAGAA CAATACCCGC AATACCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
CM-JPNa/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-NGBR/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-SGBR/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-FPa/Nor ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-HAW/Nor ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-JVAa/No ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
CM-AT/Dutt T---GTCCAC ACAAGCTAAC TACCTAAATT CTCTGCCGTG CCCAACAGAA CAATGCCCGC AATACCTA-T CTATGTATTA TCGTACATCT ACTTATTTAC
CM-PA/Dutt T-GTCCACGC ACAAACTAAC TACCTAAATT CTCTGCCGTT CCCAACAGAA CAATTCCCAC AATACCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
CA/Dutton  T-GTCCACGC ACAAACTAAC TACCTAAATT CTCTGCCGTG CCCAACAGAA CAATACCCAC AATACCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
EI-A/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-F/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-Q/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI-R/Bass  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EI/Dutton  CG-CCAACAC ATAAACT--- TACCTATATC CTCTACCGTG CCCAGCAGAC CAATATCCGC AACACTTA-C CTATGTATTA TTGTACATCT ACTTATTTAC
LO-K/Brise ---------- ---------- ---CTTAATT CTCTGCCGTG CACAACAGAA CAATAGCCAT ACTATCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
LO/Dutton  -G-CCAACGT ATAAACT--- TACCTTAATT CTCTGCCGTG CACAACAGAA CAATAGCCAT ACTATCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
LK-LK3B/Bo ---------- ---------- ---CTTTATT CTCTGCCGTG CACAACAGAA CAATAGCCAT ATTATCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
LK/Dutton  CG-CCAACGT GTAAACT--- TACCTTTATT CTCTGCCGTG CACAACAGAA CAATAGCCAT ATTATCTA-T CTATGTATTA TTGTACATCT ACTTATTTAC
ND-A/FitzS ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ND-C/FitzS ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
ND/Dutton  CGTCCAACAC ACAAACT--- TACCTATATC CTCTGCCGTG CCCAACAGAG TAATGCCTGC AATACCCA-C CTATGTATTA TTGTACATCT ACTTATTTAC
DC-A/Dutto ----CAACAT ATAAACT--- TACTTTATTT CTCTCCCGTG CCCAAAAGAG CAATGTCCAT AACACTAACC CTATGTATTA TCGTGCATTC ATTTATTTGC
DC-PA/Dutt ----CAACAT ATAAACT--- TACTTTATTT CTCTCCCGTA CCCAAAAGAG CAATGTCCAT AACACTAACC CTATGTATTA TCGTGCATTC ATTTATTTGC
DC-AT/Dutt ----CAACAT ATAAACT--- TACTTTATTT CTCTCCCGTG CCCAAAAGAG CAATGTCCAT AACACTAACC CTATGTATTA TCGTGCATTC ATTTATTTGC
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CC/L35255   CACTAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGGCT TTAAACAT-A AAAATTTATT AATTTTGCAT AA-ACTGTTT -TAGTTACAT GACTATTATA 300
CC/L35254   CACTAGCATA TGATCAGTAA TGTTGTCGAT TAATCTGACC TTAAACAT-A AAAACT-ATT AATTTTGCAT AA-ACTGTTT -TAGTTACAT GACTATTATA
CC-A/Bowen  ---TAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGGCT TTAAACATAA AAATT--ATT AATTTTACAT AA-ACTGTTT -TAGCTACAT GACTATTATA
CC/Dutton   CACTAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGGCT TTAAACAT-A AAAATT-ATT AATTTTACAT AA-ACTGTTT -TAGCTACAT GACTATTATA
CM-7/Encal  CAATAGCATA TGACCAGTAA TATTAACAGT TGATTTGGCC CTAAACAT-A AAAAATCATT GAATTTACAT AA-A-TATTT -TAACAACAT GAATATTAAG
CM-1/Encal  CAATAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACAT-A AAAAATCATT GAATTTACAT AA-A-TATTT -TAACAACAT GAATATTAAG
CM-TAC/All  CAATAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACAT-A AAAAATCATT GAATTTACAT AA-A-TATTT -TAACAACAT GAATATTAAG
CM-JPNa/No  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACAT-- AAAAATTATT AAATTTGCAT AA-AATATTT -TAATAACAT GAATATTAAG
CM-NGBR/No  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATGA AAA--TTATT AAATTTGCAT AA-A-TGTTT -TAATAACAT GAATATTAAG
CM-SGBR/No  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATGA AAA--TTATT GAATTTGCAT AA-A-CATTT -TAATAACAT GAATATTAAG
CM-FPa/Nor  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACAT-- AAAAATTATT GAATTCACAT AA-A-TATTT -CAATGGCAT GAATATTAAG
CM-HAW/Nor  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATGA AAA--TTATT GAATTCACAT AA-A-TATTT -TGATGGCAT GAATATTAAG
CM-JVAa/No  ---TAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATGA AAA--TTATT GAATCCACAT AA-A-TATTT -TAATAACAT GAATATTAAG
CM-AT/Dutt  CAATAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATAA AAA--TCATT GAATTTACAT AAG--TATTT -TAACAACAT GAATATTAAG
CM-PA/Dutt  CACTAGCATA TGACCAGTAA TGTTAACGGT TGATTTGGCC CTAAACATGA AAA--TTATT GAATTCACAT AA-A-TATTT -TGATAACAT GAATATTAAG
CA/Dutton   CAGTAGCATA TGACCAGTAA TGTTAACAGT TGATTTGGCC CTAAACATGA AAATT--ATT GAATCCACAT AA-A-TATTT -TGATAACAT GAATATTAAG
EI-A/Bass   ---TAGCATA TGACCAGTAG TACTGCTGAT TAATCTGACC TAAAACAT-A AAA--TTATT GGTTTTACAT AA-ACTGTTT -AAACTACAT GACTATTATA
EI-F/Bass   ---TAGCATA TGACCGGTAG TACTGCTGAT TAATCTGACC TAAAACAT-A AAA--TTATT GGTTTCACAT AA-ACTGTTT -AAACTACAT GACTATTATA
EI-Q/Bass   ---TAGCATA TGACCGGTAG TACTGCTGAT TAATCTGACC TAAAACAT-A AAA--TTATT GGTTTCACAT AA-ACTGTTT -AAACTACAT GACTATTATA
EI-R/Bass   ---TAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGGCT TTAAACAT-A AAAATTTATT AATTTTACAT AA-ACTGTTT -TAGTTACAT GACTATTATA
EI/Dutton   CACTAGCATA TGACCGGTAG TACTGCTGAT TAATCTGACC TAGAACAT-A AAA--TTATT GGTTTCACAT AA-ACTGTTT -AAACTACAT GACTATTTTA
LO-K/Brise  CGTTAGCATA TGATCAGTAA TATTGTCGAT TAATTTGGCT TTAAACAT-A AAAATTCATT AATTTTACAT AA-ACTGTTT -TTACCAAAT GAATATTATA
LO/Dutton   CGTTAGCATA TGATCAGTAA TATTGTCGAT TAATTTGGCT TCAAACAT-A AAAATTCATT AATTTTACAT AA-ACTGTTT -TTACCAAAT GAATATTATA
LK-LK3B/Bo  CACTAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGACC TTAAACAT-- AAAATTCAAT AATTTTACAT AA-ACTATTT -TAACCAAAC GAATATTATA
LK/Dutton   CACTAGCATA TGATCAGTAA TGTTGTCGAT TAATTTGACT TTAAACAT-- AAAATTCAAT AATTTTACAT AA-ACTATTT -TAACCAAAC GAATATTATA
ND-A/FitzS  --------AA TGATCAGTAA TGTAGTCGAT TAATTTAACC CTAAACAT-A AAAACCATTA AAAGTACCAT CA-TATATTC -TAATTACAT GAATATTATG
ND-C/FitzS  --------AA TGATCGGTAA TGTAGTCGAT TAATTTAACC CTAAACAT-A AAAACCATTA AAAGTACCAT CA-TATATTC -TAATTACAT GAATATTATG
ND/Dutton   CACATGCATA TGATCGGTAA TGTAGTCGAT TAATTTAACC CTAAACAT-- AAAACCATT- AAAGTACCAT CA--ATATTC -TAATTACAT GAATATTATG
DC-A/Dutto  CACTAGCATA TATCTAGTAA TATTACCGCT TAATTTGATT TAAAACATAA TA--TT-ATT AATTATACAT AATACACTAG -CAATAACAT GACTATCATA
DC-PA/Dutt  CACTAGCATA TATCTAGTAA TATTACCGCT TAATTTGATT TAAAACATAA TA--TT-ATT AATTAT-CAT AATACACTAA -CAATAACAT GACTATCATA
DC-AT/Dutt  CACTAGCATA TATCTAGTAA TATTACCGCT TAATTTGATT TAAAACATAA TA--TT-ATT AATTATACAT AATACACTAG -CAATAACAT GACTATCATA
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5'**agtg aaatgacata ggacata**3' **Allard *et al.* (1994) primer LDCM1**

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CC/L35255   CA-GGTA--A T--AAGAATG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCGCAGTAAT AGGTTATTTC TTAGTTCAGC 400
CC/L35254   CA-GGTA--A T--AGGAATG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TTACAGTAAT AGGTTATTTC TTAGTTCAGC
CC-A/Bowen  CA-AGTA--A T--AATAATG AAATGATATG GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCACAGTAAT AGGTTATTTC TTAGTTCAAC
CC/Dutton   CA-GGTA--A T--AATAATG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCACAGTAAT AGGTTATTTC TTAGTTCAGC
CM-7/Encal  CA-GAGG--A TT-AAAAGTG AAATGACATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATCG TCACAGTAAT GGGTTATTTC CTAAAT-AGC
CM-1/Encal  CA-GAGG--A TT-AAAAGTG AAATGACATA GGACATAAAA TTAAACTATT ATACTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC CTAAAT-AGC
CM-TAC/All  CA-GAGG--A TT-AAAAGTG AAATGACATA GGACATAAAA TTAAACTATT ATACTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC CTAAAT-AGC
CM-JPNa/No  CA-GAGA--G TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTAAAT-AGC
CM-NGBR/No  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TTACGGTAAT TGGTTATTTC TTAAAT-AAC
CM-SGBR/No  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTAAAT-AGC
CM-FPa/Nor  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTAAAT-AGC
CM-HAW/Nor  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC TTAAGT-AGC
CM-JVAa/No  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC TTAAGT-AGC
CM-AT/Dutt  CA-GAGG--A TT-AAAAGTG AAATGACATA GGACATAAAA TTAAACTATT ATTCTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC CTAAAT-AGC
CM-PA/Dutt  CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC CTAAGT-AGC
CA/Dutton   CA-GAGA--A TT-AAAAGTG AAATGATATA GGACATAAAA TTAAACCATT ATACCCAACC ATGAATATCG TCACAGTAAT TGGTTATTTC TTAAGT-AAC
EI-A/Bass   CA-GGTA--A T--AAGAATG AAATGGTATA GGACATAATA TTAAGTAATT ATTCTCAAAC ATGAATATCG TCACAGTAAT GGGTTATTTC TTAGTTCAGC
EI-F/Bass   CG-GGTA--A T--AAGAATG AAATGGTATA GGACATAATA TTAAGTAATT ATTCTCAAAC ATGAATATTG TCACAGTAAT GGGTTATTTC TTAGTTCAGC
EI-Q/Bass   CA-GGTA--A T--AAGAATG AAATGGTATA GGACATAATA TTAAGTAATT ATTCTCAAAC ATGAATATTG TCACAGTAAT GGGTTATTTC TTAGTTCAGC
EI-R/Bass   CA-GGTA--A T--AAGAATG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCACAGTAAT AGGTTATTTC TTAGTTCAGC
EI/Dutton   CA-GGTA--A T--AAGAATG AAATGGTATA GGACATAATA TTAAGTAATT ATTCTCAAAC ATGAATATTG TCACAGTAAT GGGTTATTTC TTAGTTCAGC
LO-K/Brise  AA-GATG--A T--AATAGTG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCGACC ATGAATATCG TCACAGTAAT TGGTTATTTC TTAGTTCAAC
LO/Dutton   AA-GATG--A T--AATAGTG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCGACC ATGAATATCG TCACGGTAAT TGGTTATTTC TTAGTTCAAC
LK-LK3B/Bo  AA-GGTA--A T--AATAGTG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCACAGTAAT TGGTCATTTC TTAGTTCAGC
LK/Dutton   A--GGTA--A T--AATAGTG AAATGATATA GGACATAAAA TTAAACCATT ATTCTCAACC ATGAATATCG TCACAGTAAT TGGTCATTTC TTAGTTCAGC
ND-A/FitzS  CA-GGTA--A AT-ACAAATG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTACTCTAGC
ND-C/FitzS  CA-GGTA--A AT-ACAGATG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTACTCTAGC
ND/Dutton   CA-GGTA--A TC-AA-GGTG AAATGATATA GGACATAAAA TTAAACCATT ATACTCAACC ATGAATATTG TCACAGTAAT TGGTTATTTC TTACTCTAGC
DC-A/Dutto  GA-GATA--A ATAGGTAATG AAATAATAAT AGACATAATA CTAAATCATT ATTCTCAAAC ATGAATATTG CCACAGTACT TGGTTATTTA TTAATTTTAT
DC-PA/Dutt  GA-GATA--A ATAGGTAATG AAATAATAAT AGACATAATA CTAAATCATT ATTCTCAAAC ATGGATATTG CCACAGTACT TGGTTATTTA TTAATTTTAT
DC-AT/Dutt  GA-GATA--A ATAGGTAATG AAATAATAAT AGACATAATA CTAAATCATT ATTCTCAAAC ATGAATATTG CCACAGTACT TGGTTATTTA TTAATTTTAT
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CC/L35255   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA -TTAAG-TCA TATCGTACAT AA-CTGATCT ATTCTGGCCT 500
CC/L35254   TCATCACGAG AAATAAGCAA TCCTTGTTAG TAAGATACAA TATTACCAGT TTCAAGTCCA -TTAAG-TCA TGTCGTACAT AA-CTGATCT ATTCTGGCCT
CC-A/Bowen  TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAG CATTACCAGT TTCAGGCCCA -TTAAG-TCA TATCGTACAT AA-CTGATCT ATTCTGGCCT
CC/Dutton   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA -TTAAG-TCA TATCGTACAT AA-CTGATCT ATTCTGGCCT
CM-7/Encal  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA TTCAAT-CTG TGGCGTACAT AATTTGATCT ATTCTGGCCT
CM-1/Encal  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA TTCAGT-CTG TGGCGTACAT AATTTGATCT ATTCTGGCCT
CM-TAC/All  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA TTCAGT-CTG TGGCGTACAT AATTTGATCT ATTCTGGCCT
CM-JPNa/No  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTCAGT-TTG TGGCGTACAT AACTTGATCT ATTCTGGCCT
CM-NGBR/No  TATTCACGAG AAATAAGCAA CCCTTGTTGG TAAGATACAA CATTACCAGT TTCAAGCCCA TTTAGT-TTA TAGCGTACAT AATTTGATCT ATTCTGGCCT
CM-SGBR/No  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA TTCAAT-TTG TGGCGTACAT AATCTGATCT ATTCTGGCCT
CM-FPa/Nor  TATTCACGAG AAATAAGCAA TCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTCAAT-TTG TGGCGTACAT AATTTGATCT ATTCTGGCCT
CM-HAW/Nor  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTTAGT-TTA TAGCGTACAT AACCTGATCT ATTCTGGCCT
CM-JVAa/No  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTTAGT-TTA TAGCGTACAT AACCTGATCT ATTCTGGCCT
CM-AT/Dutt  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA TTCAGT-CTG TGGCGTACAT AATTTGATCT ATTCTGGCCT
CM-PA/Dutt  TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTTAGT-TTA TAGCGTACAT AACCTGATCT ATTCTGGCCT
CA/Dutton   TATTCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA TTTAGT-TTA TAGCGTACAT AACCTGATCT ATTCTGGCCT
EI-A/Bass   TCATCACGAG AAATAAGCAA TCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA -TTAAT-TTA TGGCGTACAT AA-CTGATCT ATTCTGGCCT
EI-F/Bass   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA -TTAAT-TTA TGGCGTACAT AA-CTGATCT ATTCTGGCCT
EI-Q/Bass   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAGGCCCA -TTAAT-TTA TGGCGTACAT AA-CTGATCT ATTCTGGCCT
EI-R/Bass   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCAAGCCCA -TTAAG-TCA TATCGTACAT AA-CTGATCT ATTCTGGCCT
EI/Dutton   TCATCACGAG AAATAAGCAA CCCTTGTTAG TAAGATACAA CATTACCAGT TTCGGGCCCA -TTAAT-TTA TGGCGTACAT AA-CTGATCT ATTCTGGCCT
LO-K/Brise  TCATCACGAG AAATAAGCAA TCCTTGTTAA CAAGATACAA CATTACCAGT TTCAGGCCCA TTAAA--CTA CGACGTACAT AA-CTGATCT ATTCTGGCCT
LO/Dutton   TCATCACGAG AAATAAGCAA TCCTTGTTAA CAAGATACAA CATTACCAGT TTCAGGCCCA TTAAA--CTA CGACGTACAT AA-CTGATCT ATTCTGGCCT
LK-LK3B/Bo  TCATCACGAG AAATAAGCAA CCCTTGTTAA CAAGATACGA CATTACCAGT TTCAAGCCCA TTAAA--TTA TACCGTACAT AA-CTGATCT ATTCTGGCCT
LK/Dutton   TCATCACGAG AAATAAGCAA CCCTTGTTAA CAAGATACGA CATTACCAGT TTCAAGCCCA TTAAA--TTA TACCGTACAT AA-CTGATCT ATTCTGGCCT
ND-A/FitzS  TAATCACGAG AAATAAGCAA CCCTTGTTAA TAAGTAACGA TATTACCAGT TTCAAGTCCA TTTAA--CTG TGGCGTACAT AAAATGATCT ATTCTGGCCT
ND-C/FitzS  TAATCACGAG AAATAAGCAA CCCTTGTTAA TAAGTAACGA TATTACCAGT TTCAAGTCCA TTTAA--CTG TGGCGTACAT AAAATGATCT ATTCTGGCCT
ND/Dutton   TAATCACGAG AAATAAGCAA CCCTTGTTAA TAAGATACGA TATTACCAGT TTCAAGTCCA TTTAA--CTG TGGCGTACAT AAAATGATCT ATTCTGGCCT
DC-A/Dutto  TAATCACGAG AAATAAGCAA CCCTTGTTAA AAAGATACAA TATTACTAGT TTCAGGCCCA -TTAAA-CTA CGACGTACAT AA-CTGATTT ATTCTGGCCT
DC-PA/Dutt  TAATCACGAG AAATAAGCAA CCCTTGTTAA AAAGATACAA TATTACTAGT TTCAGGCCCA -TTAAA-CTA CGACGTACAT AA-CTGATTT ATTCTGGCCT
DC-AT/Dutt  TAATCACGAG AAATAAGCAA CCCTTGTTAA AAAGATACAA TATTACTAGT TTCAGGCCCA -TTAAA-CTA CGACGTACAT AA-CTGATTT ATTCTGGCCT
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CC/L35255   CTGGTTG-TT TTTTCAGGCA CATTAAGATA A-TAAAGTTC ACTCGTTCCT CTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA 600
CC/L35254   CTGGTTGGTT TTTTCAGGCA CATTAAGGCA G-TAAAGTTC ATTCGTTCCT CTTTAAAAGG CCTCTGGTTG CAAGT----A AATGAGTTCT ATACATTAAA
CC-A/Bowen  CTGGTTG-TT TTTTCAGGCA CATTAAGGTA G-TAAAGTTC ATTCGTTCCT CTTTAAAAGG CCTCTGG--- ---------- ---------- ----------
CC/Dutton   CTGGTTG-TT TTTTCAGGCA CATTAAGGTA G-TAAAGTTC ATTCGTTCCT CTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
CM-7/Encal  CTGGTTA-GC TTTTCAGGCA CATACAAGTA G-CAACGTTC ATTCGTTCCC CTTTAAAAGG CCTTTGGTTG CCTTTGGTTG AATGAGTTCT ATACATTGAA
CM-1/Encal  CTGGTTA-GT TTTTCAGGCA GATACAAGTA A-CGACGTTC ATTCGTTCCC CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-TAC/All  CTGGTTA-GT TTTTCAGGCA CATACAAGTA A-CGACGTTC ATTCGTTCCC CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-JPNa/No  CTGGTTG-TT CTTTCAGGCA CATACAAATA G-TAACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-NGBR/No  CTGGTTG-TC TTTTCAGGCA CATACAAATA G-TAACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-SGBR/No  CTGGTTG-TT CCTTCAGGCA CATATAAATA A-CGACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAGA
CM-FPa/Nor  CTGGTTG-TT -TTTCAGGCA CATACAAATA G-TAACGTTC ATTCGTTCCC CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAGA
CM-HAW/Nor  CTGGTTG-TC TTTTCAGGCA CATACAAATA A-TAACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-JVAa/No  CTGGTTG-TC TTTTCAGGCA CATACAAATA G-TAACGTCC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-AT/Dutt  CTGGTTA-GT TTTTCAGGCA CATACAAGTA A-CGACGTTC ATTCGTTCCC CTTTAAGAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CM-PA/Dutt  CTGGTTG-TC TTTTCAGGCA CATACAAATA A-TAACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
CA/Dutton   CTGGTTG-TC TTTTCAGGCA CATACAAATA A-CAACGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATACATTAAA
EI-A/Bass   CTGGTTG-TT TTTTCAGGCA CATTGAATTG G-TAAAGTTC ATTCATCTCT TTTTAAGAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
EI-F/Bass   CTGGTTG-TT TTTTCAGGCA CATCGAATTG G-TAAAGTTC ATTCATCTCT TTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
EI-Q/Bass   CTGGTTG-TT TTTTCAGGCA CATCGAATTG G-TAAAGTTC ATTCATCTCT TTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
EI-R/Bass   CTGGTTG-TT TTTTCAGGCA CATTAAGATA A-TAAAGTTC ACTCGTTCCT CTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
EI/Dutton   CTGGTTG-TT TTTTCAGGCA CATCGAATTG G-TAAAGTTC ATTCATCTCT TTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
LO-K/Brise  CTGGTTG-TT TTTTCAGGCA CATTAAGGTA A-TGAAGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG CACGA---TA AATGAGTTCT ATACATTGAA
LO/Dutton   CTGGTTG-GT TTTTCAGGCA CATTAAGGTA A-TGAAGTTC ATTCGTTCCT CTTTAAAAGG CCTTTGGTTG CACGA---TA AATGAGTTCT ATACATTGAA
LK-LK3B/Bo  CTGGTTG-TT TTTTCAAGCA CATTAAAGTA A-TGAAGTTC ATTCGTTCCT CTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
LK/Dutton   CTGGTTG-TT TTTTCAAGCG CATTAAAGTA A-TGAAGTTC ATTCGTTCCT CTTTAAAAGG CCTCTGGTTA ---------- AATGAGTTCT ATACATTAAA
ND-A/FitzS  CTGGTTG-TC TTTTCAGGCA CATATAAGTA A-CAACGTTC ATTCGTTTCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATCCATCAAA
ND-C/FitzS  CTGGTTG-TC TTTTCAGGCA CATATAAGTA A-CAACGTTC ATTCGTTTCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATCCATCAAA
ND/Dutton   CTGGTTG-TC TTTTCAGGCA CATATAAGTA A-CAACGTTC ATTCGTTTCT CTTTAAAAGG CCTTTGGTTG ---------- AATGAGTTCT ATCCATCAAA
DC-A/Dutto  CTGGTTG-TT TTTTCAAGCA CATAACACTA T-TGAAGTTC ATTCGTTTCT CTTTAAAAGG CCTCTGGTT- ---------- AATGAGTTCT ATACATTATA
DC-PA/Dutt  CTGGTTG-TT TTTTCAAGCA CATAACACTA T-TGAAGTTC ATTCGTTTCT CTTTAAAAGG CCTCTGGTT- ---------- AATGAGTTCT ATACATTATA
DC-AT/Dutt  CTGGTTG-TT TTTTCAAGCA CATAACACTA T-TGAAGTTC ATTCGTTTCT CTTTAAAAGG CCTCTGGTT- ---------- AATGAGTTCT ATACATTATA
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3'attgga ccgtatgcca tca5' Allard *et al.*(1994) HDCM1 primer

3'ccgtatgcca tcaaaatgaa catgcatg5' Norman *et al.*(1994) TCR6 primer

3 end of sequences 660

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CC/L35255  TTTATAACCT GGCATACGGT GGTTTTACTT GCATATAGTA GTCTTTTTTT TCTCTTTGTG continues 358 bases more >
CC/L35254  TTTATAACCT GGCATACGGT GGTTTTACTT GCATGTGGTA GTCTTTTTTT TCTCTTTGTG continues 404 bases more >
CC-A/Bowen ---------- ---------- ---------- ---------- ---------- ---------- -
CC/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
CM-7/Encal TTTA------ ---------- ---------- ---------- ---------- ---------- -
CM-1/Encal TTTA------ ---------- ---------- ---------- ---------- ---------- -
CM-TAC/All TTTATAACCT GGCATACGGT AGTTTTACTT GCATATAGTA GTTTTTTTTC TCTCTGTGTT -
CM-JPNa/No TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-NGBR/No TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-SGBR/No TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-FPa/Nor TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-HAW/Nor TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-JVAa/No TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
CM-AT/Dutt TTTA------ ---------- ---------- ---------- ---------- ---------- -
CM-PA/Dutt TTTA------ ---------- ---------- ---------- ---------- ---------- -
CA/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
EI-A/Bass  TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
EI-F/Bass  TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
EI-Q/Bass  TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
EI-R/Bass  TTTATAACCT ---------- ---------- ---------- ---------- ---------- -
EI/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
LO-K/Brise TTTATAACCT GGCATACGGT AGTTTTACT- ---------- ---------- ---------- -
LO/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
LK-LK3B/Bo TTTATAACCT GGCATACGGT AGTTTTACT- ---------- ---------- ---------- -
LK/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
ND-A/FitzS TTTATAACC- ---------- ---------- ---------- ---------- ---------- -
ND-C/FitzS TTTATAACC- ---------- ---------- ---------- ---------- ---------- -
ND/Dutton  TTTA------ ---------- ---------- ---------- ---------- ---------- -
DC-A/Dutto TTTA------ ---------- ---------- ---------- ---------- ---------- -
DC-PA/Dutt TTTA------ ---------- ---------- ---------- ---------- ---------- -
DC-AT/Dutt TTTA------ ---------- ---------- ---------- ---------- ---------- -
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Table II. Base composition at variable sites in Chelonia mydas mtDNA d-loop haplotypes (see text for sources of data and symbols used).

Position:	111111	11111122	2222222222	2222222222	222333333	33333344444	4444445555	555555555	5555555555	55
	8999000000	14558802	2345555666	6667777888	889012456	77899922566	6778880011	122333355	7777777778	99
Haplotype	5567345679	50592532	8890457123	5673467235	675707739	25115919535	8022347901	258134907	1234567890	89
CM-7/Encal	-TAA--GTCA	AGAGCGAA	AG-AAACGAA	TTA-ATATAC	AAAGACCAC	CAGCAGCAACA	CGGATTAGCT	TCGGCATCA	CCTTTGGTTG	GA
CM-1/Encal	.....	.....A.G	.....	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-2/Encal	.....	.....A.G	.....G.	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-3/Encal	.....	....TA.G	.....	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-4/Encal	.....	....TA.G	.....	.....	.....T..	..T.....TG	.....T.	...A.G...	----------	A.
CM-5/Encal	.....	.....	.....	.....	.....	.....	.....	.....	----------	..
CM-6/Encal	.....	.....	.....	.....	.....	.....	.....	.....	----------	A.
CM-8/Encal	.....	.....G	.....	.....	.....	.....	.....	.....	----------	A.
CM-9/Encal	.....	.....G	.....	.....	.....	.....	.....T.	.....	----------	A.
CM-10/Enca	.....	.....G	.....	.....	.....	...T.....	.....	.....	----------	A.
CM-11/Enca	.....	.....G	.....A..	.....	.....	.....	.....	.....	----------	A.
CM-12/Enca	.....	.....G	.....	.....	.....T	.....	.....	.....	----------	A.
CM-13/Enca	.....	.....A.G	.A.....	.....	.....	..T.....G	.....T.	...A.G...	----------	A.
CM-14/Enca	.....	.....A.G	.A.....	.....	.....	..T.....AG	.....T.	...A.G...	----------	A.
CM-15/Enca	.....	....TA.G	.....	.....C....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-16/Enca	.....	.....A.G	.A.....	.....	.....T	..T.....G	.....T.	...A.G...	----------	A.
CM-17/Enca	.....	.....A.G	.A.....	.....	.....	..T.....G	.A.....T.	...A.G...	----------	A.
CM-18/Enca	.....	.....G	.....	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-CAC/All	G.....	.....A.G	.....	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-CGC/All	G.....	.....A.G	.....G.	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-TAC/All	G.....	....TA.G	.....	.....	.....T..	..T.....G	.....T.	...A.G...	----------	A.
CM-TAT/All	G.....	....TA.G	.....	.....	.....T..	..T.....TG	.....T.	...A.G...	----------	A.
CM-JPNa/No	.---..----	-------G	...-..TA..	..G.....T	...AGT..T	..TT....G.G	T...C.GTTC	..A.T..T.	----------	A.
CM-JPNb/No	.---..----	-------G	..G.--T...	CC.....T	..GA.T...	..TTG...GTG	TAA.CCGT..	..A.T.C..	----------	AG
CM-NGBR/No	.---..----	-------G	..G.--TA..	..G...G..T	...A.T..T	TGTT.A.G.TG	TAA...GT..	..A.T..T.	----------	A.
CM-SGBR/No	.---..----	-------G	..G.--T...	..G..C...T	...A.T..T	..TT.....	T....CGTTC	CTAA.G.T.	----------	AG
CM-PNG/Nor	.---..----	-------G	..G.--T..G	..G..C...T	...A.T..T	.GTT.....	T.....GTTC	.TAA.G.T.	----------	AG
CM-ELT/Nor	.---..----	-------G	..G.--T..G	..G..C...T	...A.T..T	..TT.....	T.....GTTC	.TAA.G.T.	----------	AG
CM-FPb/Nor	.---..----	-------G	...-..T..G	..G..C...T	...A.T..T	T.TT.....	T.....GTTC	.TAA.G.T.	----------	AG
CM-FPa/Nor	.---..----	-------G	...-..T...	.C.....C.T	GG.A.T..T	..TT..T.G..	T.....GTT-	..A.T....	----------	AG
CM-HAW/Nor	.---..----	-------G	..G.--T...	.C.....GT	GG.A.T...	..TTG...GTG	TAA.CCGT..	..AAT..T.	----------	A.
CM-GOC/Nor	.---..----	-------G	..G.--T...	CC.....T	...A.T...	..TTG...GTG	TAA.CCGT..	..A.T.CT.	----------	A.
CM-LAC/Nor	.---..----	-------G	..G.--T...	CC.....T	...A.T...	.GTTG...GTG	TAA.CCGT..	..A.T.CT.	----------	A.
CM-NWC/Nor	.---..----	-------G	..G.--T...	CC.....T	...A.T...	.GTTG...GTG	TAAGCCGT..	..A.T.CT.	----------	A.
CM-JVAa/No	.---..----	-------G	..G.--T...	CC.....T	...A.T...	..TTG...GTG	TAA.CCGT..	..A.T.CT.	----------	A.
CM-JVAb/No	.---..----	-------G	..G.--T...	.C.....T	...A.T...	..TTG...GTG	TAA.CCGT..	..A.T.CT.	----------	A.
CM-SWK/Nor	.---..----	-------G	..G.--T...	CC.....	...A.T...	..TTG...GTG	TAA.CCGT..	..A.T.CT.	----------	A.
CM-AT/Dutt	T.....	G.G..A.G	..A.--....	...G-.....	.....TT.	..T.....G	.....T.	...A.G..G	----------	A.
CM-PA/Dutt	G---GTCCAG	.TTATACG	G.G.--T...	.C.....GT	...A.T...	..T.G...GTG	TAA.CCGT..	..AAT..T.	----------	A.

Table III. Base composition at variable sites in *Caretta caretta* mtDNA d-loop haplotypes (see text for sources of data and symbols used).

Position:	1111222222	2222333333	3444444555	5555555555	566
	5578334455	5568011377	9244557022	3346677777	733
Haplotype	1902580945	6775456023	9101572889	1928901234	557
CC/L35255	CGTCTGT-AT	TTGTGAGACG	GCACGCA-AT	ATCTT-----	-AA
CC/L35254	TA..CAC..C	.-...G..TA	.T.TATGGGC	G.T..GCAAG	TGG
CC-A/Bowen	----...AT.	--ACA.TG.A	A.G.....G.	G.T--.....	.--
CC-B/Bowen	----...AT.	--AC..T..A	.....G.	G.T--.....	.--
CC-C/Bowen	----.A.AT.	--AC..TG.A	A...A...G.	GCT--.....	.--
CC/ Dutton	TA.T.....	.-AC..T..A	.....G.	G.T.....	.--

Table IV. Base composition at variable sites in *Dermochelys coriacea* mtDNA d-loop haplotypes (see text for sources of data and symbols used).

Position:	112	22333
	7456	88136
Haplotype	5007	02694
DC-A/Dutton	AGGA	GCTTA
DC-B/Dutton	....	.T...
DC-C/Dutton	..AG	A...G
DC-D/Dutton	...G	A...G
DC-E/Dutton	...G	....G
DC-F/Dutton	...G	A..CG
DC-G/Dutton	...G	A.C.G
DC-H/Dutton	....	A...G
DC-PA/Dutton	CA.-	A...G
DC-AT/Dutton	....	.....

Table V. Base composition at variable sites in <u>Eretmochelys imbricata</u> mtDNA d-loop haplotypes (see text for sources of data and symbols used).

Position:	2222222222	222222222222	2333333333	334444444	4445555555	555555
	1111222223	344455666888	9012344445	682556666	7772222334	444555
Haplotype	3467023675	802345126245	8266945679	911454689	0234578012	578178
EI-A/Bass	ACAGGACCTC	ACAA--GGTAAC	AAGGTAGTAA	CGTAGATTT	AGGTGATGGT	ACTTGA
EI-B/Bass	..G.....	.....	.G.....	T.C.....	...C.....	....A.
EI-C/Bass	..GA.....	.....	.G.....	T.C.....	...C.....	....A.
EI-D/Bass	..G.....	.....-...	.....	T.....	...C.....	....A.
EI-E/Bass	.....G....	.....	.....	.....	.....	.....
EI-F/Bass	..G.....	.....C...	.G.....	T.C.....	...C.....	....A.
EI-G/Bass	.....	.....	..A.....	.....	.....	.....
EI-H/Bass	.....	.....	..A.....	.....	.....T..	.....
EI-I/Bass	T.G.....	.....C...	.G.....	T.C.....	...C.....	....A.
EI-J/Bass	..G.....	.....C...	.....	T.C.....	...C.....	.....
EI-K/Bass	..G.....	.....C...	.....	..C.....	...C.....	.....
EI-L/Bass	..G.....	.....C...	.....	T.C.....	...CA.....	....A.
EI-M/Bass	..G.....	.....C...	.....	..C.....	.....	.....
EI-N/Bass	..G.....	.....C...	.G.....	T.C.....	...CA.....	....A.
EI-O/Bass	.....	.....	.....	.....	G.....	.....
EI-P/Bass	..G.....	.....C.G.	.....	T.C.....	...C.....	....A.
EI-Q/Bass	..G.....	.....C...	.....	T.C.....	...C.....	....A.
EI-alpha/Bass	.....	.....	.....	.....C.	.....	.....
EI-beta /Bass	.....	.....C...	.....	T.C.....	...G.....	....A.
EI-gamma/Bass	..G.....	.....C...	.....	T.C..G...	...C.....	....A.
EI/Dutton	..G.....	...G....C...	T.....	T.CG.....	...C.....	....A.
EI-R/Bass	.T..AGTTCT	GTT.ATAA.TGT	...AA.ACCC	.AC.A.G.C	.AT.AGAAAC	GTCCA.
EI-S/Bass	.T..AGTTCT	GTT.ATAA.TGT	...AA.ACCC	.AC.A.G.C	.AT.AGAAAC	GTCCAG
EI-T/Bass	.T..AGTTCT	GTT.ATAA..GT	...AATACCC	.AC.A.G.C	.AT.AGAAAC	GTCCAG
EI-U/Bass	.T..AGTTCT	GTT.ATAA.TGT	...AA.ACCC	.AC.A.G.C	.AT.A..AAC	GTCCAG

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Identification of marine turtle species: when your science becomes forensic

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Forensic science involves those activities that are supportive of evidence in a court of law or suitable for presentation in a public forum. Many references to forensic identification of various wildlife and marine species are for species confirmation use only and are never intended to reach a court of law. The indiscriminate or naive use of such references can obstruct the actual forensic process. In order to provide greater confidence in analyses and to withstand jurisprudential scrutiny, quality assurance procedures are necessary. This paper provides guidelines on quality control and quality assurance for those who are considering or are currently involved in forensic DNA analyses of wildlife. Emphasis is placed on methods to assure analytical quality in the following areas: (i) competence of analysts, (ii) integrity of methods, materials, equipment and procedures, (iii) documentation and chain of custody of both reference and evidentiary samples, (iv) documentation of case analyses, and (v) content of case reports.

Introduction

All seven species of sea turtles (loggerheads, greens, flatbacks, hawksbills, Kemp's and olive ridleys, leatherbacks, and blacks (questionable species status)) are currently listed as threatened or endangered, and conservation of these species is an increasing problem. While natural environmental conditions, predators and incidental take are major threats to the survival of these species, the deliberate take of sea turtles for food as well as for profit from the sale of eggs, meats and shell products is also significant (Rudloe and Rudloe 1994). In many areas turtle meat and eggs have traditionally been an element of native diets, and in others they are considered a gourmet item (Bowen and Avise 1995). Marine turtle eggs are prized as an aphrodisiac and energizing protein, hence they are consumed as raw snacks in bars and in gyms. In addition, numerous cosmetics list turtle oils among their ingredients. Two major approaches, education and legal prohibition, have been taken to reduce or eliminate the deliberate taking of sea turtles. Poaching and over-harvest of turtles and eggs, however, continue and are possibly the greatest threats to marine turtle populations.

In the United States, legislation protects marine turtles primarily under the Endangered Species Act and CITES agreements. Enforcement of the regulations protecting sea turtles, however, requires commitment of law enforcement agencies, prosecution by the U. S. court system and technical support by the scientific community. To prosecute successfully those suspected of illegal take or trade in marine turtles or their products, the physical evidence must be identified to species. The results from these analyses may then serve as evidence in legal proceedings. It is at this point --- when science becomes involved with the law --- that science becomes forensic.

The informal use of the term "forensics" is popular within the scientific research community today, but typically the goal of the researcher is to develop information only and the results of the work are never intended to reach a court of law. The courts are increasingly accepting genetic or DNA analyses into evidence resulting in more researchers being sought out to provide expert testimony in specific disciplines. Although recognized by his/her colleagues as an expert in a scientific field, the individual may not be familiar with legal nuances. In such instances, attempting to support the legal process while not instituting formal forensic investigative procedures is likely to compromise the admissibility of the analysis as evidence in a court of law, thus preventing successful prosecution of law enforcement cases (Anonymous 1994). Therefore, it is imperative that researchers who agree to

conduct biochemical identifications for legal proceedings be fully aware of the unique responsibilities that forensic scientists have in the legal process.

Forensic science is a specialized field that involves the "application of the natural and physical science to the resolution of matters within a legal context" (Thornton 1994). Results from analyses conducted for forensic use must withstand not only the scrutiny of the scientific community but also that of the legal system. Therefore, the level of documentation, validation of methodology and protocols and qualifications of the analyst must meet stringent standards. Forensic scientists who conduct analyses should be familiar with forensic procedures, experienced with the methods used for species identification, understand issues of cross-contamination, and be prepared to testify as an "expert witness" in support of the evidence or opinion they provide.

A forensic scientist has two important roles: (i) to perform investigatory examinations and laboratory tests to reach a conclusion, and (ii) to interpret and communicate findings of the scientific tests and explain the methods used to reach those conclusions in a judicial framework (Kuzmack 1982) or public forum. Forensic tests can have significant impact on the outcome of a trial or public decision process; it is, therefore, the obligation of the scientist to set standards and establish a mechanism for peer review to ensure the quality of the analytical data. It is also necessary to assure that the credentials of the analyst will allow his/her qualification as an expert witness. In the United States such guidelines for minimum standards have been recommended for police crime laboratories by the U. S. Federal Judicial Center which addresses scientific evidence in general and by the Federal Bureau of Investigation (FBI) Technical Working Group on DNA Analysis Methods (TWGDAM) that addresses the development and implementation of forensic DNA analysis methods in public crime laboratories throughout North America (Bashinski 1991). The American Society of Crime Laboratory Directors (ASCLD) has adopted similar general guidelines for quality assurance which are further defined in their voluntary Crime Laboratory Accreditation Program.

Not all recommendations for police crime laboratories are appropriate to wildlife or marine forensic laboratories. There are presently two federal wildlife and marine agencies with active forensic programs in the United States: the U.S. Fish and Wildlife Service, National Fish & Wildlife Forensics Laboratory in Ashland, Oregon, and the Marine Forensics Program which is part of the National Marine Fisheries Service, Southeast Fisheries Science Center, Charleston Laboratory in South Carolina (USA). Both of these laboratories find that properly established, quality assured, and documented procedures are essential in order to provide scientifically sound and reliable forensic analyses. Those undertaking forensic activities should implement a quality assurance program to address: (i) competence of analysts; (ii) methods, materials, equipment, and procedures used in the testing and development of genetic markers; (iii) preservation and chain of custody of both reference and evidentiary samples; and (iv) casework documentation, reporting, and testimony (Bashinski 1991; Berger 1994; McKenna et al. 1994).

The National Marine Fisheries Service, Southeast Fisheries Science Center's Charleston Laboratory has used forensic procedures and techniques in species identification for sea turtle conservation efforts since the late 1970s. Today the Charleston Laboratory's Marine Forensics Program handles essentially all sea turtle forensic cases. The analytical methods used to resolve legal issues are supported by a "library" of authenticated standards maintained by the Program. Collection of standards from whole animals, carefully identified by recognized experts, is conducted on a continuing basis. The expert also provides signed documentation verifying the species. This form along with a chain of custody form then accompanies the sample and is delivered with the standard to the Marine Forensics Program, ensuring that no tampering has occurred.

The majority of species identification analyses conducted by the Program have been in support of U. S. law enforcement activities for CITES or Endangered Species Act violations, however, the Program also provides analyses in response to management and industry requests. The most common types of evidence from

suspected illegal take or trade in sea turtle/turtle products are meat, eggs, shells, or cosmetics containing turtle oils. The Program has expertise in protein, lipid and DNA analyses which can be applied to evidentiary samples. Isoelectric focusing (IEF) is the most direct and least costly analytical method in common use and is the method of choice if the evidence is fresh meat. Usually this technique requires at least 5-10 gm of tissue that has been stored frozen. If the meat sample is of poor quality or cooked, DNA analysis is indicated. Samples for DNA analysis require relatively little tissue (0.5 gm or less) and can be preserved with a variety of methods, depending on available conditions. Lipid analysis is used for products (cosmetics) containing turtle oils and for egg identification. Identification usually involves the entire egg, refrigerated or frozen, but analyses can be conducted on residual trace evidence. Diagnostic fatty acid profiles are obtained for green, hawksbill, and leatherback turtles. Lipid analysis, however, does not differentiate loggerheads, Kemp's and olive ridleys at this time. If one of these three species is indicated, DNA analyses can be conducted, usually requiring only a small portion of the egg membrane.

The discriminating power of DNA exceeds that of morphological markers in determining unique characters for identification of species, populations, stocks, and even individuals. DNA technology has become important in forensic science in general and in marine forensics particularly. It is a powerful means of identifying biological evidence taken from crime scenes, due in part to DNA's stability under adverse conditions and in part to the development of new technologies, such as the polymerase chain reaction (PCR), that allow the use of tissue sources of limited quantity and quality. The application of PCR to samples allows identification of suspected endangered species products which may be fresh, dried, cooked, or processed meat products (Walsh et al. 1991; Bartlett and Davidson 1992; Forrest and Carnegie 1994). Currently, the primary thrust of research and development in Southeast Fisheries Science Center, Charleston Laboratory's Marine Forensics Program is the identification and validation of species-specific DNA markers and the development of the databases to support identifications. Specific to marine turtles, the challenges lie in extracting and amplifying DNA from turtle eggs (ranging from fresh laid to those with substantial development), identification of turtle meats that have been cooked in stews, grilled or canned as turtle soup, and trace evidence such as blood stains.

To date the marker used in identifications of fresh tissue, cooked meats or eggs is a 500 bp portion of the mitochondrial cytochrome b gene. Based on DNA sequence data from this gene (Bowen et al. 1993), diagnostic restriction enzymes were identified that produced species-specific restriction fragment length polymorphism (RFLPs) profiles. This approach is now being validated by analyzing a number of individuals for each species selected across their respective geographic ranges. With the increasing demand for DNA analysis for identifications, it is important to bring into focus some of the legal and scientific issues relating to the use of DNA technology and the development of databases for forensic analyses that support enforcement of laws and regulations governing conservation and management of wildlife.

Based on guidance provided to police crime laboratories by TWGDAM, ASCLD, and the U. S. Federal Judicial Center (TWGDAM 1991, 1994, 1995; Bashinski 1991; Wilson et al. 1993; Anonymous 1994) and based on quality assurance protocols followed by the U. S. Fish and Wildlife Service, National Fish & Wildlife Forensics Laboratory[1] and Marine Forensics Program[2] of the National Marine Fisheries Service, SEFSC Charleston Laboratory, the following are general guidelines for quality control and quality assurance recommended for laboratories that choose to undertake forensic activities involving DNA typing for wildlife.

[1] Quality Assurance Manual. National Fish and Wildlife Forensic Laboratory, 1490 East Main Street, Ashland Oregon 97520.

[2] Marine Forensics Manual: Analysis of Marine Animal Tissues. National Marine Fisheries Service SEFSC Charleston Laboratory, 219 Ft. Johnson Road, Charleston, South Carolina 29412.

GUIDELINES FOR MARINE FORENSIC PROCEDURES INVOLVING DNA TYPING

Laboratory Facilities

Personnel - Competence of Analysts

Analysts and supervisors should be familiar with marine forensic investigation practices, evidence handling and expert witness testimony. Both should have experience in genetics, biochemistry and/or molecular biology, and a thorough knowledge of the theory and practice of the DNA typing approach being used. Supervisors should have a basic background in the application of statistics to DNA typing. In addition, personnel should undergo periodic proficiency tests.

Documentation

Documentation must be maintained on all aspects of the laboratory procedures and interpretation of results, so as to create a traceable audit trail (chain of custody). This documentation will serve as an archive for retrospective scientific inspection, reevaluation of the data, and reconstruction of the DNA procedure (TWGDAM 1991, 1995; Bashinski 1991; Berger 1994).

Materials, Equipment, and Facilities

1. The facility should provide a secured area for storage of all reference materials and evidence samples. In addition, analysis areas should be secured during each testing procedure.

2. Equipment should operate properly and be accompanied by procedures for and documentation of calibration and maintenance.

3. Quality control of critical reagents and materials should include lot and batch numbers, manufacturer's specifications, and in-house evaluations.

4. There should be written standard operating procedures for the formulation of reagents and isolation of DNA. Formulations should be labeled with the identity, concentration, date of preparation, identity of preparer, and any storage requirements.

5. Physically separate work areas are an absolute requirement for a PCR laboratory. The "extraction area and PCR setup work area" should be physically separated from the "amplified DNA work area" in order to provide containment for DNA amplification products. The amplification work area should include the thermal cycler, space for dedicated equipment, and reagents for typing the PCR products (i.e. gel electrophoresis, hybridization, and washing). Dedicated reagents should be used in each of the extraction and PCR setup areas. The PCR laboratory should also establish written procedures for cleaning and decontamination of facilities and equipment. Particular attention should be paid to preventing DNA and PCR product contamination.

STANDARD REFERENCE SAMPLES

Reference Standard Handling Procedures

Critical to all forensic identification is the availability of authenticated reference standards. The following guidelines are essential in the development of DNA or tissue banks and to the subsequent development of sequence databases.

1. All standard reference samples should be **identified** by a qualified expert to species of origin.

2. Verification should be documented with a **Species Identification Form** (see Appendix) that includes the scientific and common name of the animal, location of collection, available life history data, and signature and title of the expert who verified the standard. The expert should be willing to testify in court concerning the identification, if needed.

3. Accompanying each standard should also be a clear, well-documented **Chain of Custody** (see Appendix) which lists any

and all individuals who had control of the standard from the time the sample is validated. (See also Evidence Handling Procedures)

4. **Photo documentation** of the intact animal (source of the standard reference sample) is strongly encouraged.

Development and Validation of Analytical Procedures

The goal of most marine wildlife forensics cases is to classify a probative sample as being included in, or excluded from a particular group of organisms, such as a species. Molecular markers such as DNA sequences or RFLP patterns, while indicating more directly the genetic information contained in the individual being sampled, are nonetheless phenotypic markers. As such they should be treated like other phenotypic markers such as body shape, scute patterns, or coloration. Classification by an expert would be based on experience with the markers and groups under consideration.

1. **Characterization** of DNA Loci

Loci chosen for forensic typing usually exhibit a well-characterized mode of inheritance, either maternal or Mendelian. Those markers chosen should be somatically stable and their chromosomal locations mapped, where feasible. A potentially informative locus should be examined for polymorphism over the geographic range of the species.

2. **Validation** of DNA Marker and Analysis Procedure

a. The loci proposed for typing should first be evaluated using fresh tissues from validated reference standards. The DNA typing method should be tested against related species in order to verify that diagnostic electrophoretic profiles or DNA sequences exist. Further, the level of variation should be established within the target species for the given loci.

b. Tissue from reference standards should be prepared in ways that resemble actual evidence (e.g., partially decomposed tissue from stranded animals, dried samples, cooked or processed meats, or other wildlife or marine products), and the resulting DNA profiles should agree with those obtained from pristine samples.

c. The procedure should be validated either inhouse, externally, or collaboratively to assess the specificity, reproducibility, limitations of the procedure, and sources of error.

3. Procedures Specific to the Developmental Validation of **PCR-based DNA Analyses**

a. Amplification of target sequences should be conducted with PCR primers of a known sequence.

b. The reaction conditions should be fully outlined and include thermocycling parameters, number of cycles, and critical reagent concentrations (primers, dNTPs, polymerase, and salts) necessary for the required degree of specificity and reliability. Conditions and measures must be in place to protect against contamination of reference or evidence samples by post-PCR amplification products.

c. The potential for differential amplification should be assessed and addressed. If more than one locus is amplified in one reaction mixture, the effects of such amplification on each allele must be addressed and documented.

d. PCR products may be detected with or without hybridization, but in either case, appropriate guidelines for identifying the alleles should be established and an appropriate panel of positive and negative controls incorporated into each assay.

4. Procedures Specific to Development of a **Sequence Database** for Marine Forensic Use

a. If sequence data are being generated from PCR products, DNA extracts should be amplified in duplicate.

b. Amplicons or clones should be sequenced in duplicate, either by sequencing the complementary strand of the duplex DNA molecule (preferred) or by additional overlapping sequences of the same strand. Additional sequencing may be necessary to resolve remaining ambiguities. Manual sequencing gels should be scored independently by two analysts.

c. Laboratories should exchange template DNAs or clones for comparison of sequence information. This can facilitate validation regardless of the specific sequencing methodology employed. Independent nonforensic laboratories should be included to help provide consensus quality control measures.

5. The scope and size of the reference database needed to provide a given **level of confidence in the classification of unknown samples** depends on the following factors:

a. **The proportion of marker variation within groups relative to the variation between groups.** When the variation within groups is very low relative to the variation between groups then the number of samples required can be relatively low. When there is significant within group variation relative to between group variation then more samples may be needed. Particular attention should be paid to the possibility of geographic sub-structuring within a group when a molecular marker is used. This means that an effort should be made to collect database samples from a number of regions.

b. **The level of confidence in the estimates of variation.** If the amount of variation is low then fewer samples may be required whereas if there is a lot of variation between individuals then larger samples sizes are indicated.

c. **The number of related groups such as congeneric species or conspecific subspecies which might need to be eliminated from consideration.** Knowledge of the phylogenetic relationships of groups to which the sample may belong also affects the database.

d. **Prior knowledge about the probative sample also affects the level of confidence that can be attributed to a classification using a particular database.** If some species can be eliminated based on other factors then they may not be needed for the database.

6. The **method used for classifying a sample** depends on the information in the supporting database.

a. As with traditional morphological classifications, if there are specific molecular genetic characters which distinguish groups then simple inspection of that character by an experienced analyst is all that is needed.

b. In cases where the patterns of restriction fragment polymorphisms or base substitutions are so complex that simple visual analysis is not feasible, then traditional statistical techniques such as discriminant function analysis may be used. Alternatively, phylogenetic methods of classification may be used to place a probative sample in a particular group as has been done by Bartlett and Davidson (1992) and Baker and Palumbi (1996).

c. The classification method used should be tested for consistency and reliability with multiple known samples.

EVIDENCE AND DOCUMENTATION

Evidence Handling Procedures

Evidence must be collected, transferred, subsampled, analyzed, and stored so as to preserve the identity, integrity, condition, and security of each item. This may be accomplished as follows:

1. Sample Labeling

Each sample must be labeled with a unique identifier that can be tracked from entry into the lab to the final report in accordance with the laboratory policy.

2. Sample Handling

Written policies and protocols should be in place to prevent loss, alteration, or contamination of the sample and a means of verification.

3. Chain of Custody

A clear, well documented chain of custody must be maintained from the time the evidence is first received until it is released from the laboratory. The links in the chain of custody consist of any and all individuals who have had control of the evidence between the time it was seized or obtained and the time it is offered into evidence in a court of law. Each person who handled the item must demonstrate (i) receipt of the item, (ii) ultimate disposition of the item, and (iii) the safeguard and handling of the item between receipt and disposition. The one exception is postal employees or commercial delivery services (i.e. Federal Express). It is assumed that evidence sent through the mail is properly handled in the course of regular business (Kuzmack 1982). Measures should be taken to seal the package in a manner that would indicate if tampering has occurred.

Analytical Procedures

1.The most appropriate and cost effective test for analysis of the evidence should be determined.

2. If DNA analysis is appropriate, DNA should be isolated in a manner that protects against contamination. The effectiveness of the isolation procedure should be evaluated regularly with reference samples. The quality and quantity of DNA recovered from probative samples should be determined to evaluate the effectiveness of DNA recovery.

3. If the test procedure involves RFLP analysis,

a. the analytical gel used to separate the fragments must include visual markers to determine the progress of electrophoresis;

b. the gel should include molecular weight markers that span the range of RFLP fragments from the unknown; molecular weight markers should be placed in each outer well of the gel and in one or more central wells to allow accurate fragment size estimates across the gel;

c. DNA of species producing known digestion patterns should be included in each gel as positive controls; and

d. a procedure should be available to accommodate altered migration of DNA fragments and determine when a gel is unacceptable for interpretation. If the gel is regarded as uninterpretable, the evidence should be reanalyzed.

4. If the test procedure involves PCR-based techniques,

a. negative controls should include (i) a reagent blank (no polymerase, no template) and (ii) an amplification blank (no template);

b. a positive control (DNA from a reference standard) must be introduced at the amplification step and carried through the remainder of the typing;

c. where feasible, the original DNA sample should be split to allow for duplicate analysis; and

d. markers should span the size range of the fragment length polymorphisms generated from amplification products, and markers should be placed in the two outermost wells and at least one internal well of the gel to allow accurate estimate of fragment sizes across the gel.

Case Work Documentation, Interpretation, Report Writing, and Review

Laboratories and investigators conducting marine forensic analyses should ensure the reliability and completeness of the documentation, data analysis, reports, and review of analyses.

1. Documentation should include the following:

a. Information regarding the packaging of the evidence upon receipt and the condition of the evidence, especially noting any factors relevant to the preservation of the material.

b. All procedures, standards and controls used, observations made, results of the tests performed, charts, graphs, photographs, autoradiographs, communications, etc., which are used to support the conclusions reached after testing.

2. Data Analysis:

a. Data should be analyzed using appropriate controls, including both negative and positive controls and size markers.

b. A means should be established for concluding when samples are or are not a match, or when the results of the analysis are inconclusive or uninterpretable.

c. The frequency of occurrence for the DNA profile should be calculated from an established database.

3. Reports:

Reports should contain the following:

a. A case identifier,

b. the identity of the analyst(s),

c. date of the report,

d. the DNA marker used for the identification,

e. the methodology used, results, and conclusions, and

f. signatures of the reporting analyst and QA analyst.

4. Quality Assurance:

The data, documentation, and reports must be reviewed independently by another qualified party and agreement must be reached on the interpretation of the data and conclusions drawn from the data.

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UNITED STATES DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
NATIONAL MARINE FISHERIES SERVICE
Southeast Fisheries Science Center
Charleston Laboratory
219 Ft. Johnson Rd.
Charleston, SC 29412-9110

CERTIFICATION
SPECIES IDENTIFICATION OF SAMPLE
FOR USE AS A STANDARD

Sample ID number(s): _____

I, _____, HEREBY CERTIFY THAT I HAVE POSITIVELY
Full Name

IDENTIFIED THE WHOLE REPTILE OR THE ENCLOSED SAMPLE TAKEN FROM

A WHOLE REPTILE AS _____ BASED ON MY
Common or Scientific Name

KNOWLEDGE AND EXPERIENCE AS A _____.
Position or Job Title

Signature: _____

Date: _____

FOR FORENSICS USE ONLY
This sample was assigned

Charleston Reference # _____

by _____

Date received _____

MARINE FORENSICS
Chain of Custody

National Marine Fisheries Service, SEFSC, Charleston Laboratory
219 Ft. Johnson Rd., Charleston, SC 29412
Phone: (803) 762-8500; FAX: (803) 762-8700
E-mail: Marine.Forensics@noaa.gov

Field reference number:_____

Laboratory reference number:_____

Geographical origin of sample:_____

Name & signature of sample collector:_____

Address of sample collector:_____

Collector's Phone Number:_____ **Date collected:**_____

Seized property# (if applicable):_____

Sample description:_____

THE ABOVE EVIDENCE WAS TRANSFERRED AS FOLLOWS:

1. _____ Collector's release signature — Method of transfer — Date
   _____ Receipt signature — Date
2. _____ Release signature — Method of transfer — Date
   _____ Receipt signature — Date
3. _____ Release signature — Method of transfer — Date
   _____ Receipt signature — Date
4. _____ Release signature — Method of transfer — Date
   _____ Receipt signature — Date
5. _____ Release signature — Method of transfer — Date
   _____ Receipt signature — Date
6. _____ Release signature — Method of transfer — Date
   _____ Receipt signature — Date

Each person in possession of the sample must sign and date the form twice, once for receipt of the sample and once for release.

Revised 30 July 1996,CMW

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