Chelonian Conservation and Biology, 2003, 4(3):701–704

Natural Hybridization Between a Kemp's Ridley (*Lepidochelys kempii*) and Loggerhead Sea Turtle (*Caretta caretta*) Confirmed by Molecular Analysis

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The Kemp's ridley sea turtle (*Lepidochelys kempii*) is a critically endangered species with a primary nesting beach near Rancho Nuevo, Tamaulipas, Mexico. Sporadic nesting of ridleys has been reported from Veracruz, Mexico, to Padre Island, Texas. The Kemp's ridley occurs in the Gulf of Mexico, along the eastern coast of North America to Nova Scotia and in European Atlantic waters (Márquez, 1990, 1994). Since 1977, a bi-national Kemp's Ridley Recovery Program has been directed by the Kemp's Ridley Working Group, composed of representatives of Mexico's Instituto Nacional de la Pesca (INP), the U.S. Fish and Wildlife Service (FWS), National Park Service (NPS), Texas Parks and Wildlife Department (TPWD), and National Marine Fisheries Service (NMFS). The Gladys Porter Zoo, Brownsville, Texas, has also participated in the program (Caillouet et al., 1995b). Recently, Mexico's Instituto Nacional de Ecologia (INE) joined the working group.

Headstarting of the Kemp's ridley was a subsidiary and experimental part of the Kemp's Ridley Recovery Program (Fontaine et al., 1985, 1989). It involved collecting eggs at Rancho Nuevo, incubating them there or at Padre Island, Texas, exposing the hatchlings to either of these two beaches to "imprint" them, captive-rearing for 9 to 11 months, and tagging the turtles in Galveston, Texas, and releasing them into the Gulf of Mexico. Although the imprinting portion of the program was terminated in 1992, captive rearing of Kemp's ridley hatchlings at the NMFS laboratories in Galveston has continued on an annual basis.

Among the 200 hatchlings taken from the Rancho Nuevo nesting beach during the 1999 season and brought to the NMFS Galveston Laboratory, there were three individuals that did not seem to "fit" the normal appearance of Kemp's ridley hatchlings. As these turtles became older, there were noticeable differences between them and the rest of the 1999 year-class both in gross morphology and in coloration (Fig. 1). Biologists responsible for the daily care of these turtles began to suspect that they might be hybrid offspring of Kemp's ridleys and some other species, possibly the loggerhead sea turtle (*Caretta caretta*). Their carapaces were much more elongated than the normal oval-shaped Kemp's ridley shell (Table 1) and the coloration was not the normal black but was rather an off-color reddish black. Additionally, these three individuals were heavier relative to the other turtles from the 1999 year-class (Table 1).

In order to resolve their questionable taxonomic status, blood samples for genetic profiling were drawn from the three suspect turtles as well as two apparently normal Kemp's ridleys from the same year-class. The suspected hybrid turtles were released with the rest of the 1999 year-class offshore of Galveston Island on 20 June 2000. No recapture information has as yet been obtained from any of these turtles.

Methods. — Two separate methodologies were employed in this effort: 1) a segment of the d-loop region of the mtDNA was subjected to sequence analysis, and 2) the genotypes at three nuclear microsatellite loci were determined. These techniques were selected due to their differing and complementary characteristics. The mtDNA molecule is ideally suited for studies of population structure and for elucidation of maternal lineage due to its haploid nature and

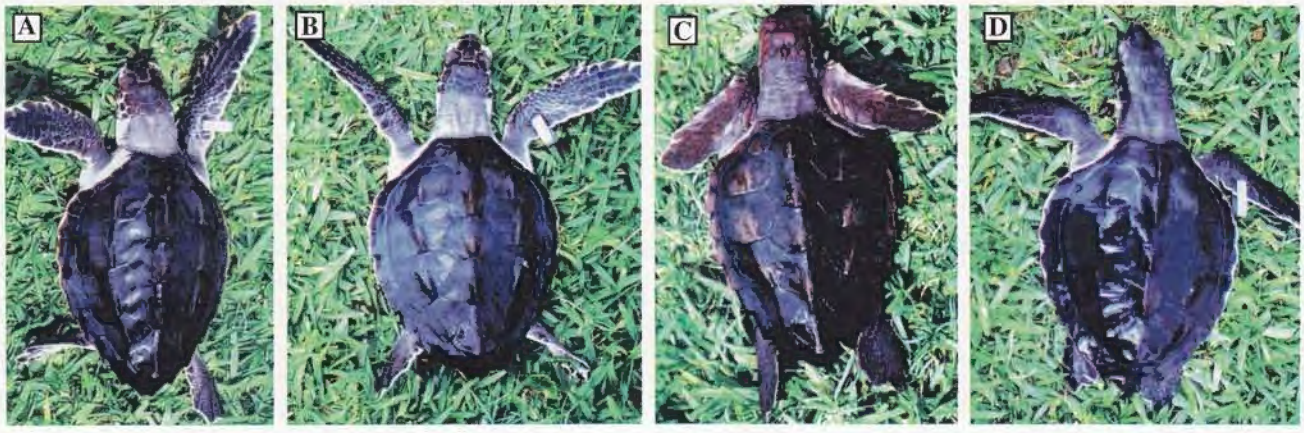

Figure 1. Photos of (**A** and **B**) two hybrid sea turtles (*L. kempii* x *C. caretta*), (**C**) a normal *C. caretta*, and (**D**) a normal *L. kempii*. Note differences in head and carapace coloration and shell morphology. Turtles were raised under identical conditions. Photos by JPF.

Table 1. Measurements of the three hybrid animals and two known *L. kempii* sea turtles from the 1999 year-class. CL = carapace length, CW = carapace width, CD = carapace depth.

Type	CL (mm)	CW (mm)	CD (mm)	Weight (g)
Hybrid	174	141	71	683.5
Hybrid	167	148	66	612.5
Hybrid	167	138	68	605.0
L. kempii	158	142	68	584.0
L. kempii	163	150	66	608.5

maternal mode of inheritance. The d-loop region evolves at a rapid rate and is therefore highly informative for resolving questions of relationship between closely related taxa. In contrast to mtDNA, microsatellite loci are diploid and biparentally inherited. Using microsatellite loci, it is possible to analyze not only population structure, but to assign parentage, and in some cases, taxonomic status. However, the type of microsatellite locus that is useful for the analysis of population structure differs from the type that is employed for the purpose of species identification. While population analysis requires loci that are highly polymorphic, species identification is more easily accomplished with loci that have very few alleles. The most useful loci for taxonomic identification are those that are fixed for a different allele in each of the species under investigation.

Blood samples (1 ml) were obtained by venapuncture of the dorsal cervical sinus and placed into a 1.8 ml Nunc cryovial containing 0.5 ml of blood storage buffer (Longmire et al., 1992). This solution lyses the blood cells and maintains the total cellular DNA in a buffered environment. Samples were maintained at room temperature in blood storage solution until processing at the Center for Conservation and Research (CCR) at the Henry Doorly Zoo. Total cellular DNA was recovered from the blood samples by organic-phase extraction in phenol:chloroform utilizing standard procedures (Sambrook et al., 1989).

Using total cellular DNA as a template, 353 nucleotides of the mitochondrial displacement loop (d-loop) region were amplified by polymerase chain reaction (PCR) and sequenced using published primers (Allard et al., 1994; Dutton et al., 1996). PCR amplification was carried out with approximately 50 ng of genomic DNA in a 50 ml reaction volume using an ABI 480 thermocycler (Applied Biosystems, Foster City, CA). Amplification conditions consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 12.5 pmol each primer, 1.5 mM $MgCl_2$, 200 mM each dNTP, and 0.5 units Taq DNA polymerase (Promega, Madison, WI). The thermal profile for PCR amplification was 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, ending with a single extension at 72°C for 10 min. Following amplification, reaction products were cleaned by passage through a Qia-Quick column (Qiagen, Valencia, CA) and resuspended in 50 µl of d^2H_2O. Sequencing reactions were performed with the ABI dye-terminator cycle-sequencing kit (Applied Biosystems) following the manufacturer's specifications and reaction products were detected on an ABI 377 DNA analyzer. Base calling was conducted by Sequence Analysis software (ABI) and the sequences were aligned using Sequencher (Gene Codes, Ann Arbor, MI).

PCR amplification of total cellular DNA was carried out using published primers (Kichler et al., 1999) under the same conditions that were utilized for mitochondrial DNA sequence analysis. The only exception was that for the microsatellites, one of the amplification primers was labeled with a fluorescent dye (Integrated DNA Technologies, Coralville, IA), which allowed for automation of the fragment analysis. Allele sizes were determined by separation of the PCR products on a 7% polyacrylamide gel run on an ABI 377 DNA Analyzer (Applied Biosystems) using GeneScan-500 (Tamra) size standard. Fragment length was assigned with the GeneScan software program (Applied Biosystems).

The selection of which microsatellite loci to use for genetic profiling was based upon published data indicating a heterozygosity value of zero in a sample of 26 adult Kemp's ridley females and 176 of their offspring (Kichler et al., 1999). Initially, two Kemp's ridleys, two loggerheads, and the three hybrid animals were genotyped to determine the utility of each microsatellite locus for species designation. After locus Klk 314 had been selected, an additional four Kemp's ridley and two loggerhead sea turtles, which represented the entire collection of Kemp's ridley and loggerhead sea turtle DNA in the CCR databank, were genotyped to increase our confidence that the Klk 314 locus had very low polymorphism in each species.

Results. — Examination of 353 nucleotides of mitochondrial d-loop sequence revealed that all of the suspected hybrid animals were derived from a Kemp's ridley maternal lineage. There was a single nucleotide substitution between the d-loop sequences for the known Kemp's and the hybrid animals versus 23 substitutions between the sequences for the hybrids and the loggerheads. No nucleotide substitutions were observed among the sequences from the hybrid animals, suggesting that all three hybrids could have been the products of a single clutch of eggs.

Analysis of the nuclear genotype data confirmed that the suspected hybrids were the offspring of a mating be-

Table 2. Allele sizes in base pairs at microsatellite loci for known loggerhead sea turtles, the three hybrids, and known Kemp's ridley sea turtles. ND = no data.

	Marker		
Individual	Klk 314	Klk 315	Klk 325
L. kempii 1	111/111	ND	ND
L. kempii 2	111/111	ND	ND
L. kempii 3	111/111	ND	ND
L. kempii 4	111/111	ND	ND
L. kempii 369	111/111	137/137	155/155
L. kempii 374	111/111	137/137	155/157
Hybrid 316	111/127	137/139	155/157
Hybrid 321	111/127	137/137	155/157
Hybrid 361	111/127	137/137	155/157
C. caretta 1	127/127	137/139	161/161
C. caretta 2	127/127	139/139	157/161
C. caretta 3	127/127	ND	ND
C. caretta 4	127/127	ND	ND

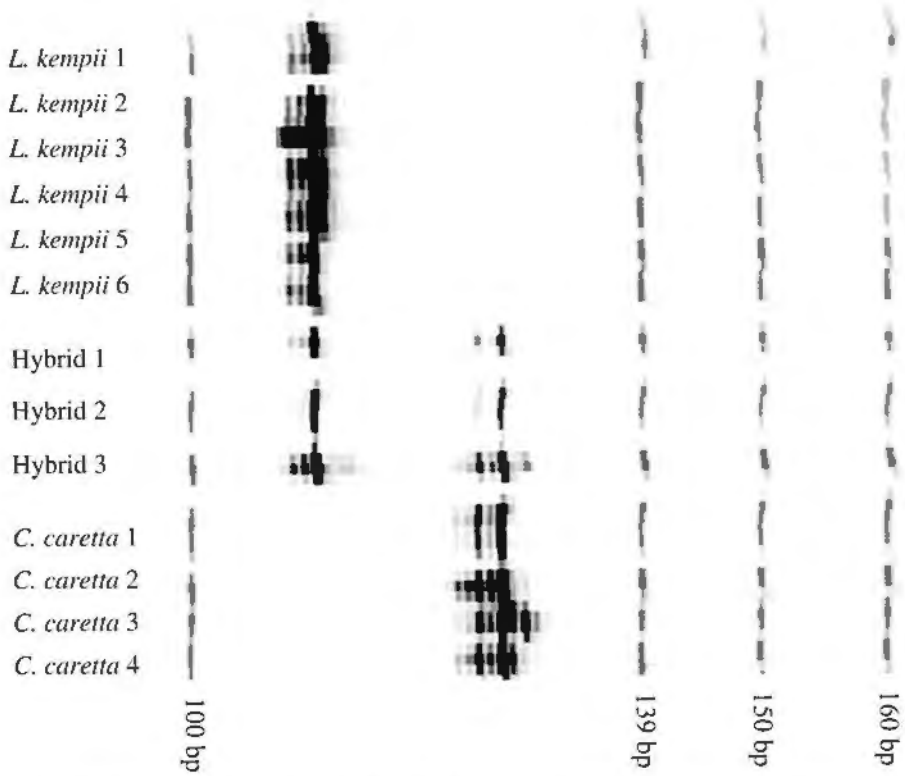

Figure 2. ABI 377 gel image showing relative allele sizes for six Kemp's ridley, three hybrid, and four loggerhead sea turtles at microsatellite locus Klk 314.

tween a Kemp's ridley and a loggerhead (Fig. 2). While all of the known Kemp's ridleys were fixed for a 111 bp long allele at locus Klk 314, all of the loggerheads were fixed for an allele that was 127 bp in length (Table 3). The suspect animals possessed both alleles, indicative of their hybrid status (Fig. 2). Data from the additional microsatellite loci that were assayed were in agreement with data from locus Klk 314, but alleles at these loci were not fixed in both species, and so they were not diagnostic for species status (Table 2).

Discussion. — A combination of data from nuclear microsatellite genotyping and mitochondrial sequence analysis identified and characterized a natural hybridization event between two genera of sea turtles. The data indicated that the suspected hybrid offspring were the result of a mating between a male loggerhead sea turtle and a female Kemp's ridley. Each methodology provided unique and critical information for this determination. Due to the maternal transmission of mitochondria, the mtDNA sequence revealed that the female parent was a Kemp's ridley. Conversely, the biparental mode of transmission of the nuclear markers allowed for the examination of the paternal contribution to the mating. Since both of the species of interest were fixed for alternate alleles at the Klk 314 locus, the data are relatively unambiguous. It is interesting to note that the hybrids appeared to have normal growth rates, indicating that they were not simply viable, but were quite healthy. The fertility of these turtles remains unknown, since Kemp's ridley sea turtles do not mature until they are at least 5 years old in captivity, and possibly as many as 12 years of age in the wild (Caillouet et al., 1995a; Zug et al., 1997).

Hybridization between a Kemp's ridley and loggerhead sea turtle has been documented previously. A single hybrid individual was identified in 1992 by Keinath and Musick in Chesapeake Bay (Karl et al., 1995). Analysis of mitochondrial and nuclear DNA extracted from the blood of that specimen revealed a Kemp's ridley mtDNA haplotype combined with a mixed nuclear component derived from Kemp's ridley and loggerhead sea turtles (Karl et al., 1995). The Chesapeake hybrid was observed to be the product of a cross between a female Kemp's ridley and a male loggerhead sea turtle, which is the same parental linage that was evident in our hybrid turtles. Karl et al. (1995) theorized that the mechanics of mating behavior and the size difference that is apparent between a Kemp's ridley and a loggerhead sea turtle would limit any hybridization event between these two species to a female Kemp's ridley and a male loggerhead sea turtle.

Although only a small number of individuals (6 Kemp's ridley and 4 loggerhead sea turtles) were typed for the nuclear markers utilized in our study, we are confident that marker Klk 314 exhibits very low variability in the Kemp's ridley. The published source for the primer sequences reported a heterozygosity value of zero following the screening of over 200 individual haplotypes (Kichler et al., 1999). They observed marker Klk 314 to be monomorphic in an assay of 26 adult Kemp's ridley females as well as 176 of their offspring, for a total of 228 haplotypes. Although it is possible that Klk 314 is polymorphic in the loggerhead sea turtle, this would not alter the conclusion regarding the status of the hybrid turtles. The alternate Klk 314 allele that was

observed in the hybrids has never been detected in a large group of known Kemp's ridleys; the mitochondrial sequence data confirmed that the hybrids had a Kemp's ridley maternal lineage.

The identification of three known hybrid turtles among hatchlings from the primary nesting beach at Rancho Nuevo is of particular concern. Marine turtles are believed to breed in close proximity to the nesting beach (Karl et al., 1995). If this is the case with Kemp's ridleys, then the hybrids resulted from an interspecific mating that occurred at or near the center of the Kemp's ridley range. While Karl et al. (1995) reported that all previously observed hybrids were detected in areas of overlapping nesting range, Chesapeake Bay is remote from the primary Kemp's ridley nesting beach. In fact, until very recently, Rancho Nuevo was the only known nesting site for Kemp's ridley sea turtles. Therefore, it is of some concern that a female Kemp's ridley may have been mated by an unsuitable loggerhead male during the normal ridley breeding period in the heart of its nesting range.

These hybrid turtles were most likely the products of an extremely rare, isolated event. If this is the case, there is little cause for concern. However, if hybridization were to become commonplace among Kemp's ridleys, the outcome could be severe, particularly if the hybrids are fertile. Considering the relative abundance of loggerheads versus Kemp's ridley sea turtles, it is possible that widespread hybridization between these species would swamp the Kemp's ridley gene pool, further depressing their already depleted numbers. Although reproductive viability is unlikely since the hybrids were produced by inter-generic hybridization, even sterile individuals could pose a threat if they were to become numerous. For example, if female Kemp's ridleys mate only once per breeding cycle, selection of a sterile hybrid male as a mate might remove a given female from the breeding population for that cycle. In any case, it is clear that biologists working with the Kemp's ridley should remain vigilant for the occurrence of additional hybrid individuals.

Acknowledgments. — The authors would like to thank Cain Bustinza and Andrea Cannon, fishery biologists, Julie Sommer, Ryan Huebinger, and Rick Brenneman, conservation geneticists, as well as Matt Fraser, graphic artist, for their contributions to this manuscript. We would also like to thank James Ford Parham for his many helpful and constructive comments, although any shortcomings in the final version of this manuscript are the sole responsibility of the authors. This research was supported by grants from the Ahmanson Foundation, which have provided the Grewcock Center for Conservation and Research with three ABI automated DNA sequencers. The authors also wish to acknowledge Bill and Bernice Grewcock for their generous and ongoing support of genetic research at the Henry Doorly Zoo.

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APPENDIX

GenBank accession numbers for mitochondrial d-loop sequence from two Kemp's ridley, two loggerhead, and three hybrid sea turtles: *L. kempii* 369, AF374401; *L. kempii* 374, AF374400; *C. caretta* 1, AF374399; *C. caretta* 2, AF374398; XXK 316, AF374404; XXK 321, AF374402; XXK 361, AF374403.

Received: 30 May 2001
Revised and Accepted: 28 August 2002