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Kemp's ridley *Lepidochelys kempii* nesting abundance in Texas, USA: a novel approach using genetics to improve population census

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ABSTRACT: Accurate estimates of the annual numbers of nesting females are critical for assessing sea turtle populations. Nesting by Kemp's ridley *Lepidochelys kempii* turtles has significantly increased at Padre Island National Seashore and nearby beaches in Texas, USA. Four nests were observed in Texas during 1995 and a record of 209 in 2012. However, it is unclear how many clutches are laid by the same females. We used a genetic approach to infer the number of individual nesters from genotypes determined from dead embryos and hatchlings sampled from clutches. Mitochondrial DNA sequencing was combined with nuclear DNA analysis at 10 microsatellite loci to match genotypes for nesters and offspring of unknown parentage in over 50% of the unassigned nests. Our results indicate that traditional methods, based on observed tagged turtles, have underestimated the number of Kemp's ridleys nesting in Texas. We demonstrate how genetic approaches can be incorporated into population assessments when direct census of adult animals is not feasible. This approach also provides a basis to apply capture–mark–recapture techniques to assess the impact of events, such as the recent oil spill, on the population via sampling of nests to identify and track individual nesters over time.

KEY WORDS: Kemp's ridley · Sea turtle · Abundance · Nesting beach · Genotypes

INTRODUCTION

Sea turtle populations are most commonly surveyed at nesting beaches. This provides estimates of the numbers of females nesting annually from direct observation and tagging for capture–mark–recapture (CMR) studies (Chaloupka & Limpus 2001, Kendall & Bjorkland 2001, Balazs & Chaloupka 2006) or by counting turtle tracks left on beaches (Witherington et al. 2009, Tucker 2010). Since most sea turtles do not nest annually, accurate estimates of population size rely upon long-term monitoring of females at their nesting beaches (Dutton et al. 2005, Troëng & Rankin 2005, Allen et al. 2010). Nesting female abundance has been estimated by dividing total annual nest counts by the average number of clutches laid per female in a season (Allen et al. 2010). Nesting parameters, such as re-migration interval and annual female nest production, are challenging to obtain and are essential for estimating the size of nesting sea turtle populations (Richards et al. 2011).

Ridley turtles *Lepidochelys* spp. are unlike other sea turtles because they typically nest in *arribadas*, where the females haul out en masse to lay their eggs generally at the same time. Special approaches have been designed to quantify nesting in the case

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of olive ridleys *L. olivacea* (Kumar et al. 2013). Kemp's ridley *L. kempii*, whose geographical range is limited to the Gulf of Mexico and the Atlantic Ocean, is considered Critically Endangered by the IUCN (Fontaine & Shaver 2005). Most nesting occurs on beaches of Tamaulipas and Veracruz in Mexico, where the number of nesting females plummeted from an estimated 40 000 on one day in the 1940s to approximately 250 females nesting in 1985 (NMFS et al. 2011). In the late 1970s, the US and Mexican governments initiated a bi-national project as part of a broad effort to save this species from extinction (Dutton et al. 2002). The goal of the project was to form a second nesting colony of this native species at Padre Island National Seashore (PAIS), Texas, which is the longest stretch of undeveloped barrier island in the world and the northern extent of the documented historic nesting range of the species (Shaver 2005).

Monitoring for nesting began on the Texas coast at PAIS in 1986 but was sporadic until the late 1990s, when monitoring efforts also began expanding to other Texas Gulf beaches (Shaver 2005). Kemp's ridley nesting has increased throughout the species' range in recent years (NMFS et al. 2011, Shaver & Caillouet in press). Although Kemp's ridley nesting in Texas is now state-wide, it continues to be concentrated in the documented historic nesting range, with more than half the Kemp's ridley nests recorded in the USA annually located at PAIS.

Accurate estimates of the number of females nesting in Texas are essential for the conservation and management of the Kemp's ridley and for evaluation of results from the effort to form a secondary nesting colony of the species at PAIS. However, with such a large stretch of beach (591 km) and a small nesting population, monitoring the nesting turtles is logistically challenging and the probability of encountering a nesting female is low. Even though the beach is monitored, and the females that are encountered are tagged and sampled, approximately 50% of the nests are found only after the female has departed, leaving the identity of the individual nester unknown (Shaver 2005, Shaver & Caillouet in press). Since each female can lay multiple clutches in a season and in different years, and these basic nesting parameters are not known for Texas, it has not been possible to derive meaningful population size estimates for Kemp's ridleys nesting in Texas from nest counts alone. Furthermore, since the probability of encountering the females during nesting is relatively low, CMR analysis of the limited tagging data is problematic (Kendall 2004).

In recent years, molecular parentage based studies have been used for population assessments and for determining demographic parameters (Stewart & Dutton 2011, Wright et al. 2012, Frey et al. 2013). Genetic markers may be used to clarify relationships among individuals and to establish reliable pedigrees, which can be useful for estimating population size (Blouin 2003, Herbinger et al. 2006). Genetic identification of female nesters provides a tool for CMR studies; when the identity of the nesting female is unknown for a nest, genetic approaches can be used to determine whether any sampled females can be matched to that nest.

The purpose of this study was to develop and apply genetic approaches to estimate the abundance of Kemp's ridley females nesting annually in Texas. Using genetic profiles constructed from microsatellite genotypes and mtDNA sequences of observed nesters as well as dead embryos and hatchlings salvaged from nests, we were able to establish the identity of the mothers of the unknown nests and derive new counts for the number of nesting females. This is a useful tool for estimating abundance when direct census data are limited, and it provides the basis for improving CMR studies to address basic population parameters (such as nesting ecology, mating systems, and remigration intervals).

MATERIALS AND METHODS

Sample collection

Surveys are conducted annually along the entire Texas Gulf of Mexico beachfront between April and mid- July, which marks the annual nesting season for the Kemp's ridley in Texas. When nesting turtles are encountered they are examined and tagged with metal tags in the front and rear flippers and a passive integrated transponder (PIT) tag in the front flipper, in order to identify the individual when she returns to the beach. Additionally, a blood or tissue sample is collected unless that female has already been sampled. During the 2001 to 2008 nesting seasons, blood or tissue samples were collected from 159 females following the protocols described by NMFS Southeast Fisheries Science Center (2008). Eggs from virtually all nests found are relocated to hatcheries (Shaver 2005, Shaver & Caillouet in press). After full-term clutch incubation, residual nest contents are examined to quantify hatching and emergence success, determine embryological stage of development at time of death, and collect tissue samples from dead

hatchlings and embryos (Shaver 2005). During the 2003 to 2006 nesting seasons, tissue samples were collected from dead embryos or hatchlings from 141 nests. The study site and nest locations are shown in Fig. 1. Blood and tissue samples were frozen or preserved in DMSO salt solution and shipped to the NOAA Southwest Fisheries Science Center for long-term storage and analysis.

Laboratory analysis

We extracted genomic DNA from 159 nesting female samples, collected between 2001 and 2008, and a total of 510 salvaged embryos and hatchlings collected from 141 nests excavated during the 2003 through 2006 nesting seasons, using the high-throughput X-tractor Gene extraction robot (Corbett Robotics). For 36 of the nests, the nesting female was identified and sampled in the field, for 15 of the nests the female was identified in the field, but no sample was collected, and for the remaining 90 nests, the mother was unknown. We genotyped each sample at 10 microsatellite loci: Cc1H11, Cc5C8, Cc5H7, Cc7E11 (Shamblin et al. 2008), Cm3 (FitzSimmons et al. 1995), D1, D2, D108 (Dutton & Frey 2009), Or2, and Or7 (Aggarwal et al. 2004) (Table 1). We used the PCR conditions described by Dutton & Frey (2009) and optimized annealing temperature for each primer pair. PCR products were labeled using standard sequencing dyes (HEX or FAM; Applied Biosystems). Amplification of the PCR product was verified using a 2% agarose gel with ethidium bromide stain (Maniatis et al. 1982). Products were then separated on an ABI 3100 or 3730 DNA analyzer with Genescan Rox500 fluorescent size standard (PE Applied Biosystems). Additionally, we amplified and sequenced the mtDNA control region using the primers H950g and LCM15382 (Abreu-Grobois et al. 2006) under PCR conditions described by LeRoux et al. (2012) for at least 1 sample from each of the 141 nests and all of the nesting females. MtDNA sequences were aligned and assigned haplotypes using Seqscape v2.5 (PE Applied Biosystems). Microsatellite data were scored using GeneMapper v4.0 (PE Applied Biosystems), and each allele call was verified manually.

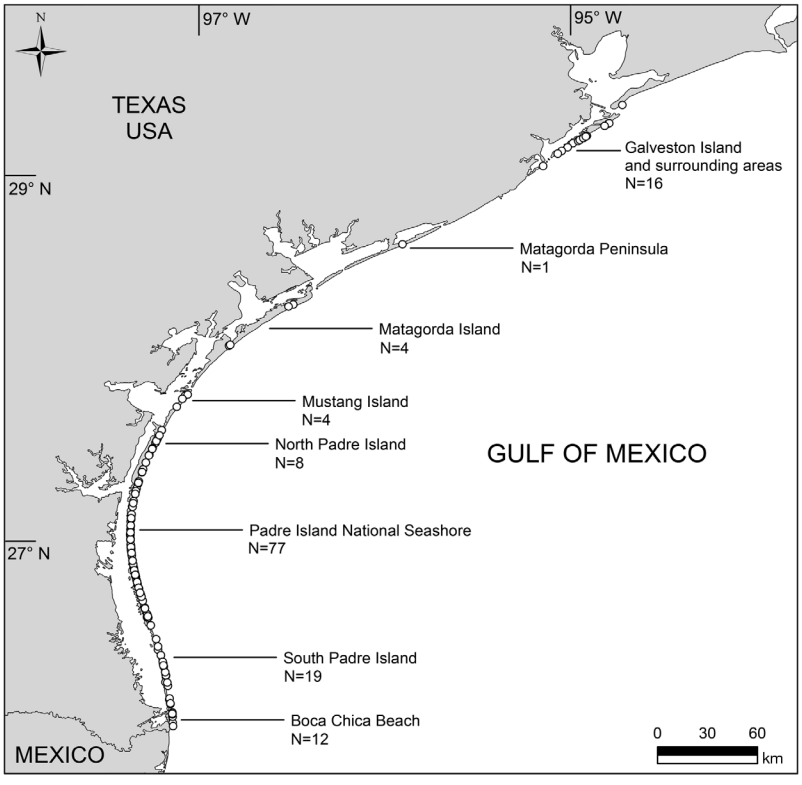

Fig. 1. Study site and nest locations of hatchling samples (of Kemp's ridley turtles *Lepidochelys kempii* collected between 2003 and 2006 at Padre Island National Seashore and nearby beaches in Texas, USA. N: number of nests sampled at that location

Statistical analysis

We tested all 10 microsatellite markers for Mendelian inheritance using hatchlings from nests where the female was observed and sampled while nesting. Using the nesting female dataset, each marker was tested for allelic stutter, null alleles, and large allele dropout using MicroChecker (Van Oosterhout et al. 2004), Hardy-Weinberg equilibria (HWE) were tested using the nesting female dataset using GENEPOP (Raymond & Rousset 1995), and the probability of identity and the probability of exclusion were calculated using GenAlEx (Peakall & Smouse 2012). To estimate our genotyping error rate, we randomly selected and replicated 10% of our sample set.

To identify which female was responsible for each of the nests, we used 2 software programs, Cervus v3.0 (Kalinowski et al. 2007) and Colony v2.0 (Jones & Wang 2010). We treated all of the 141 nests as unknown nests

Table 1. *Lepidochelys kempii*. Summary statistics for the 10 loci used to genotype hatchlings and females: number of individuals (N), number of alleles (k), expected heterozygosity (H_e), observed heterozygosity (H_o), Hardy-Weinberg p value (p_{HW}), error rate, probability of identity (p_{ID}), and probability of exclusion (p_{ex})

Locus	N	k	H_e	H_o	p_{HW}	Error rate	p_{ID}	p_{ex}
Cc1H11	159	20	0.914	0.899	0.603	0.015	0.015	0.70
Cc5C8	160	19	0.921	0.919	0.428	0.034	0.013	0.72
Cc5H7	160	18	0.917	0.925	0.955	0.017	0.014	0.70
Cc7E11	157	17	0.883	0.873	0.136	0.025	0.026	0.61
Cm3	157	12	0.791	0.745	0.512	0.040	0.072	0.42
D1t	158	16	0.883	0.867	0.252	0.065	0.013	0.71
D2t	160	15	0.895	0.900	0.150	0.037	0.025	0.62
D108	159	19	0.918	0.893	0.480	0.032	0.021	0.64
Or2	161	10	0.722	0.820	0.903	0.024	0.110	0.33
Or7t	160	4	0.531	0.550	0.574	0.031	0.272	0.14

for the purpose of ground-truthing both our markers and the analysis software. Cervus uses a likelihood-based method to assign parentage, allowing for genotyping errors. The software runs a simulation of the data to assess the power of the given markers as well as the confidence of the parentage assignments. The input data for the simulation included allele frequencies from the nesting female population, 10 000 offspring, 2000 candidate mothers, and 0.75 for the proportion of candidate females sampled. Our proportion of loci genotyped was 0.98, with 0.01 mistyped and a minimum of 6 loci typed. Using allele frequencies generated from our potential parent genotypes, in combination with the simulation file, the likelihood of parentage for each candidate parent was calculated.

Colony is used to determine full- and half-sibling relationships. It allows sibship reconstruction even without parental information. We allowed for both male and female polygamy and inbreeding as input parameters (Lee 2008) and chose the full likelihood model with medium precision. A description of the method and its performance may be found in Wang & Santure (2009). Allele frequencies were calculated by Colony using the hatchling data. Allele frequency estimates were updated by Colony while searching for the maximum-likelihood configuration based on the inferred sibship and parentage relationships. The sibship size prior was left blank. Each of the 10 markers was considered codominant with the allelic dropout rate being 0; the error rate we used was calculated during the genotyping replication exercise (Table 1). In the analysis, there were 510 offspring genotypes, 0 candidate male genotypes, and 159 candidate female genotypes. Any samples known to be from the same nest were coded as known maternal siblings. Certain conditions required that some females be excluded as potential mothers responsible for some of the hatchling sets. For example, if a hatchling set had a different maternally inherited mtDNA haplotype than a candidate mother we assumed that she was not the mother of the hatchling and excluded her as a potential parent. We used the relationships assigned in Colony to group the nests into known-mother sets and then compared the relationships manually. Putative maternal genotypes were assigned by Colony, along with the probability that the genotype for each marker was correct, for each hatchling set where a match was not found.

RESULTS

Each of the 10 loci was in HWE, and the average number of alleles per locus ranged from 4 to 20, with an average of 15. The expected heteroyzgosity ranged from 0.531 to 0.921, with an average of 0.838. The probability of identity for all 10 loci was 1.0×10^{-15}, the probability of exclusion for each locus ranged from 0.14 to 0.72 with an average of 0.56, and the overall probability of exclusion was 1.00. The mean genotyping error rate was 0.032% (range: 0.015–0.065).

We identified 8 new mtDNA haplotypes (Table A1 in the Appendix) represented by the nesting females. The 2 most common haplotypes, Lk4.1 and Lk6.1, represent 82% of the nesting females. For the nests with a rare haplotype (n = 10), the pool of potential mothers is reduced from 163 to 30 females.

Ground truthing (known nests)

Of the 36 analyzed nests in which the female was observed and sampled while nesting, 75 samples were used to ground-truth each of our loci and our analysis methods. Of the nest assignments made by Colony, 89% (32 out of 36) were correct (Table 2). The software was unable to match 2 of the nests to the sampled female that was observed while nesting, and the software incorrectly assigned 2 nests to the wrong female. Cervus correctly assigned 78% (28 out of 36) of the nests, with 95% confidence for at least half of the hatchlings from the nest (with the

Table 2. *Lepidochelys kempii.* Parentage assignment results of independent analysis for nests where the mother was observed in the field while nesting. To identify which female was responsible for each of the nests, the software programs Cervus and Colony were used; see 'Materials and methods' for details

Year	No. nests	No. nests assigned (Cervus)	No. nests assigned (Colony)	No. nests not assigned
2003	3	2	3	0
2004	15	11	11	2
2005	14	14	14	0
2006	4	3	4	0
Total	36	30	32	2

Table 3. *Lepidochelys kempii.* Parentage assignment results of independent analysis for nests where the mother was initially unknown, but subsequently derived from the pool of sampled females. To identify which female was responsible for each of the nests, the software programs Cervus and Colony were used; see 'Materials and methods' for details

Year	No. nests	No. nests assigned (Cervus)	No. nests assigned (Colony)	No. nests not assigned
2003	7	1	2	5
2004	16	7	7	9
2005	19	8	8	11
2006	48	29	28	20
Total	90	45	45	44

remaining hatchlings assigned to the same female with varying confidence). For the remaining 8 nests, there were 2 nests in which the correct assignments were made with 80% confidence. Two nests were assigned correctly with 95% confidence for half of the hatchlings, but the other half assigned incorrectly to a different female. One nest was assigned to the correct female as the most likely parent without confidence for 2 hatchlings and could not be assigned for the other 2 hatchlings; 1 nest was assigned incorrectly; and 2 nests were not assigned to any female.

Unknown nests

We evaluated 90 nests for which the mother was unknown (Table 3). Cervus and Colony each correctly assigned 50% of the nests to a sampled female. Of the remaining 44 nests, 25 were assigned to 19 additional females for which putative genotypes were also assigned by Colony. The genotypic fingerprints were generated for those 19 females, along with a probability that each genotype for each marker was correct. These 25 nests and 19 putative females were grouped together and the genotypes were examined visually. The remaining 19 nests were assigned by Colony to turtles in the candidate female pool, but were determined to be incorrect after comparing the genotypes visually.

Combining the field observations and tag data along with genotype matching, the number of females nesting annually ranged from 11 in 2003 to 51 in 2006, and a total of 101 different females nested between 2003 and 2006 (Table 4). The average number of nests laid per female in a year was 1.27 nests in 2003, 1.37 in 2004, 1.39 in 2005, and 1.78 nests in 2006, based on the total number of sampled nests assigned (Table 4). Between 2003 and 2006, an average of 23.2% of the females were re-migrants. Of the females that nested in 2003, 1 re-migrated in 2005 and 1 in 2006. Of the 2004 nesters, 2 females remigrated in 2005, and 9 in 2006. Of the females that nested in 2005, 3 re-migrated in 2006. Five females returned to nest in consecutive years; 2 of them were observed in the field in both years, and 3 were identified using genetics.

Table 4. *Lepidochelys kempii.* Number of females nesting annually in Texas determined using genetic analysis methods. Shown are the number of females and nests observed in the field, number of females and nests assigned using genetics, number of females observed in the field and assigned using genetics, total number of females assigned and number of nests assigned, total number of nests observed for the season, and estimated number of females based on nest counts and previously published values of 2.5 (range 1.8–3.1) clutches laid per season (NMFS et al. 2011)

Year	Females observed (nests)	Females assigned using genetics (nests)	Females observed assigned using genetics	Total females (nests assigned)	Total observed nests	Estimated no. females (range)
2003	8(10)	6(7)	3	11(14)	19	7.6 (6.2–10.6)
2004	19(23)	12(16)	4	27(37)	42	16.8 (13.9–23.3)
2005	20(25)	13(19)	5	28(39)	50	20.0 (16.3–27.8)
2006	43(50)	28(48)	20	51(91)	102	40.8 (33.2–56.7)

DISCUSSION

This study demonstrates the value of genetic tools for matching offspring to females, especially for endangered wildlife species. Other studies have used similar approaches for wildlife population studies (e.g. polar bears, Cronin et al. 2009; eastern imperial eagles, Rudnick et al. 2007). Our study presents a novel approach to census nesting marine turtles using genetics to associate females to nests and provides an alternative method for identifying nests laid by unknown mothers, especially in areas where direct observation of nesting females is not possible. Current census methods rely on counting the nesting females, which is labor intensive and often not feasible, or counting crawls or nests. In the latter case, site-specific data on average clutches per female from CMR data are needed (Schroeder & Murphy 1999).

Ground truthing

Evaluating 36 nests with known, sampled mothers allowed us to ground truth both our markers and our methods. We chose 10 highly polymorphic (mean H_o = 0.839) microsatellite loci, which are the best markers for parentage analysis (Sefc & Koblmüller 2009), in combination with mtDNA haplotypes. We first used the software program Cervus v3.0, which uses a categorical allocation approach and is the most commonly used method of parentage analysis (Jones et al. 2010). This approach is ideal when a pool of putative parents can be identified, as it provides a method to choose the single most likely parent. Cervus matched 92% of our 'ground truth' nests to the correct mother, with varying degrees of confidence. Three nests did not match the known mother: 1 was incorrectly assigned to a different female and 2 did not find a match. Additionally, we used Colony v2.0, which also uses maximum likelihood to assign both sibship and parentage relationships. This approach is useful when samples from all of the putative parents are unavailable. It first clusters the offspring into both paternal and maternal families, and then assigns candidate parents. When a suitable candidate parent is not identified, the program will reconstruct a parental genotype. With the Colony analysis, 4 nests were not assigned to the correct mother, 2 nests were incorrectly assigned to a different female, and 2 were not matched, so a maternal genotype was reconstructed. Interestingly, the 2 nests that were not matched using Colony were 2 of the same nests that were not correctly matched using Cervus, indicating a problem with the nests rather than the methods. These results are likely a consequence of genotyping error. In large studies characteristic of parentage analysis, both genotyping errors and mutations occur and result in apparent incompatibilities between true parents and their offspring, especially when highly variable markers are used (Jones et al. 2010). Both Cervus and Colony account for genotyping error. The rate of genotyping error is 1 of 2 key parameters in parentage analysis according to Jones et al. (2010). The best way to assess the genotyping error rate is through progeny arrays (Wang 2004). The second key parameter is the number of candidate parents, which can be difficult to determine. However, some analyses, such as parental reconstruction, are relatively unaffected by this parameter, since the statistical power is high and the data set robust. Colony proved to be a more useful analysis for our study, because we did not have a sample for all of the candidate mothers nor did we know how many candidate mothers were in the population. We did find that combining and evaluating the results from both programs provided a better estimate of the number of females nesting each year.

Unknown nests

Our new estimates based on genetic analysis are consistently higher than those previously derived from nest counts (Table 4), indicating that the nesting population is larger than previously thought. The number of clutches laid per female in Texas according to our results is generally lower than some estimates previously published for Kemp's ridleys at Rancho Nuevo, Mexico (NMFS et al. 2011). This difference might reflect different nesting patterns by Kemp's ridleys or monitoring efforts in the 2 regions. In Texas, nesting is more diffuse over a large stretch of coastline, some of which is only intermittently monitored, compared to Rancho Nuevo, where nesting in this larger population is more concentrated along a relatively shorter span of coastline that is intensively monitored. This would result in a greater likelihood of observing all nests laid by individual turtles. The uncertainty over the accuracy of published values for this parameter (number of nests per female) may also reflect different methodologies. The highest value (3.1 nests female^{-1}) estimated by Rostal et al. (1997) was based on serum testosterone levels measured in nesters, and is much higher than those derived from CMR studies in Rancho Nuevo

(1.8 to 2.5 nests female^{-1}). The estimate made by Rostal et al. (1997) may represent what is theoretically possible as opposed to what is observed. Our estimate of the number of females should be considered conservative, since not all nests may be observed, and there are a few records of individuals nesting both in Mexico and in Texas. Additionally, 19 nests were not assigned, which suggests the existence of females that were not sampled or tagged, and not inferred by Colony. If the true value is closer to 1.8 nests female^{-1}, then the predicted number of nests laid each year is close to the actual number observed, given the number of females we identified (Table 4). Further studies aimed at sampling nesters and nests across years will continue to improve our ability to identify minimum numbers of females 'observed' (from genetics) and enable quantification of population parameters (e.g. number of nests per female, inter-nesting interval) to improve population abundance estimation and demographic models in the future.

This study illustrates the utility of microsatellite genotypes in combination with the mtDNA sequence of nesting females and hatchlings salvaged from nests to census the number of females nesting annually in Texas. Systematic efforts to detect nesting along the Texas coast began at PAIS in 1986, and since then efforts have expanded at PAIS and elsewhere on the Texas coast (Shaver 2005). Despite the numerous challenges encountered, such as the hundreds of km to search, limited patrol resources, and the cryptic nesting characteristics of the species, the numbers of nests found in south Texas have increased since the mid-1990s (Shaver & Rubio 2008). Our study supports these previous findings and provides a more accurate census of the nesting population than published studies which have been limited to nest counts.

Conservation implications

Kemp's ridleys face several threats in the Gulf of Mexico and along the US coast. Shrimp trawling has been identified as a causal factor for sea turtle strandings in Texas (Shaver 1998), since most of the stranded adult Kemp's ridleys from 1995 to 2003 were found during the time when the waters of the Texas coast were open to shrimp trawling (Shaver 2005). Adult mortality is a difficult demographic parameter to quantify in sea turtles in the absence of good CMR data (see Dutton et al. 2005). The genotype catalogue established in our study provides the basis for determining whether any stranded animals are parents of the nests sampled each year, including nests that were not assigned to any nesters subsequently observed and sampled. This would allow observed deaths to be accounted for in the nesting population, providing the means for more accurate threat and impact assessment in the future.

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Appendix

Table A1. *Lepidochelys kempii.* Mitochondrial DNA haplotypes, including variable sites and GenBank accession numbers

Haplotype	Position from start of dloop								GenBank
	19	145	177	193	226	295	367	409	accession no.
Lk1.1	T	T	C	A	A	G	C	G	KF385935
Lk2.1	.	.	A	.	.	.	.	A	KF385936
Lk3.1	.	C	.	G	.	.	T	A	KF385937
Lk4.1	.	C	.	.	.	.	.	A	KF385938
Lk5.1	.	.	.	.	.	A	.	A	KF385939
Lk6.1	.	.	.	.	.	.	.	A	KF385940
Lk6.2	G	.	.	.	.	.	.	A	KF385941
Lk7.1	.	C	.	G	G	.	.	A	KF385942

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