

GENETIC ANALYSIS OF THE KEMP'S RIDLEY SEA TURTLE (*LEPIDOCHELYS*  
*KEMPII*) WITH ESTIMATES OF EFFECTIVE POPULATION SIZE

A Thesis

by

SARAH HOLLAND STEPHENS

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2003

Major Subject: Wildlife and Fisheries Sciences

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August 2003

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## ABSTRACT

Genetic Analysis of the Kemp's Ridley Sea Turtle (*Lepidochelys kempii*)

with Estimates of Effective Population Size. (August 2003)

Sarah Holland Stephens, B.S., University of Florida

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The critically endangered Kemp's ridley sea turtle experienced a dramatic decline in population size (demographic bottleneck) between 1947 and 1987 from 160,000 mature individuals to less than 5000. Demographic bottlenecks can cause genetic bottlenecks where significant losses of genetic diversity occur through genetic drift. The loss of genetic diversity can lower fitness through the random loss of adaptive alleles and through an increase in the expression of deleterious alleles.

Molecular genetic studies on endangered species require collecting tissue using non-invasive or minimally invasive techniques. Such sampling techniques are well developed for birds and mammals, but not for sea turtles. The first objective was to explore the relative success of several minimally invasive tissue-sampling methods as source of DNA from Kemp's ridley sea turtles. Tissue sampling techniques included; blood, cheek swabs, cloacal swabs, carapace scrapings, and a minimally invasive tissue biopsy of the hind flipper. Single copy nuclear DNA loci were PCR amplified with turtle-specific primers. Blood tissue provided the best DNA extractions. Additionally, archival plasma samples are shown to be good sources of DNA. However, when dealing

with hatchlings or very small individuals in field situations, the tissue biopsy of the hind flipper is the preferred method.

This study's main focus was to evaluate whether the Kemp's ridley sea turtle sustained a measurable loss of genetic variation resulting from the demographic bottleneck. To achieve this goal, three alternative approaches were used to detect a reduction in Kemp's ridley's effective population size ( $N_e$ ) from microsatellite data. These approaches were 1) Temporal change in allele frequencies, 2) An excess of heterozygotes in progeny, and 3) A mean ratio ( $M$ ) of the number of alleles ( $k$ ) to the range of allele size ( $r$ ). DNA samples were obtained from Kemp's ridleys caught in the wild. PCR was used to amplify eight microsatellite loci and allele frequencies were determined. Data from only four microsatellites could be used. Although the reduced number of loci was a limiting factor in this study, the results of all three approaches suggest that Kemp's ridley sustained a measurable loss of genetic variation due to the demographic bottleneck.

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## INTRODUCTION

The Kemp's ridley sea turtle (*Lepidochelys kempii*, Garman 1880) a member of the family *Cheloniidae*, is ranked among the smallest of all seven sea turtle species with an average carapace length of 69 cm (Bjorndal 1995). The Kemp's ridley was considered by some authors to be a subspecies of the olive ridley sea turtle (*Lepidochelys olivacea*). However, mitochondrial DNA (mtDNA) data validated its distinct status from the olive ridley (Bowen et al. 1991). Its range includes the Gulf of Mexico and the U.S. Atlantic coast north to Long Island Sound (Morreale et al. 1992) but also extends to other areas of the Atlantic Ocean. This species prefers shallow sandy and muddy habitats and is usually observed near to shore. Kemp's ridley is carnivorous, feeding on crabs, shrimps, clams and sea urchins. This species has been shown to attain sexual maturity at approximately 10 years of age (Coyne 2000). Sexing of sea turtles is done primarily via measurements of sex steroid levels in the tissue (Duronslet et al. 1989). Courtship and mating areas for the Kemp's ridley are not well known. Occasional observations during the breeding season have revealed that both males and females are very aggressive during this time (Bjorndal 1995). Kemp's ridleys nest in arribadas (Spanish word meaning 'mass arrival') and are thought to lay eggs every two years with an average of 2.5 clutches of 90 eggs each season (Turtle Expert Working Group 2000). Nesting begins in March and extends through August with a peak in May and early June.

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This thesis follows the style and format of the Journal of Marine Biotechnology.

The sex of sea turtles is determined by the temperature of the sand in which the egg incubates, with cooler temperatures producing males (Bjorndal 1995). Eggs hatch during the day and the hatchlings enter the surf and eventually move to the pelagic environment to begin “the lost year”, a time when they are rarely encountered by humans. Immature (post-pelagic) and adult sea turtles migrate to coastal shallow water for benthic foraging. Adult Kemp's ridleys leave these foraging areas to mate in shallow waters. Adult females leave the waters only to nest while males remain in this habitat their entire lives (Bjorndal 1995). Almost the entire adult female population nests on one beach near Rancho Nuevo, Tamaulipas, Mexico, where >40,000 females nested on a single day in 1947 (Bowen et al. 1991). The average annual number of ridley nests between 1985 and 1987 dropped to 740 (Márquez et al. 2001). Due to this dramatic reduction in population size, the Kemp's ridley is listed in the IUCN Red Book of Endangered Species (IUCN 2002), and is considered the most critically endangered of all seven sea turtle species.

Causes for the dramatic decline of the Kemp's ridley population include habitat destruction and alteration, poaching for meat and eggs, and incidental capture in shrimp trawls. As early as 1927, protection efforts began in Mexico to prohibit collection of turtle eggs and destruction of nests (Trinidad and Wilson 2000). Throughout the 1960's, 70's and 80's, further restrictions were enacted by the Mexican government to prevent the harvest of Kemp's ridley for meat and eggs. Finally, in 1990, poaching forced the Mexican government to completely ban hunting and egg collecting. By 1982 it was widely accepted that shrimp trawlers captured and drowned more sea turtles

worldwide than did any other kind of incidental capture, with this fishery accounting for more Kemp's ridley mortalities than did any other human activity. In 1989, the National Marine Fisheries Service enacted Turtle Excluder Device (TED) regulations in certain areas at certain times. The regulations were subsequently expanded to require TED's on all shrimp and flounder trawlers operating in the southeastern U.S (52 FR 24244). Furthermore, in 1991, the U.S prohibited imports of shrimp from nations whose trawling practices did not comply with its conservation efforts calling for TED implementation.

Efforts by the U.S and Mexican governments contributed to an 11.3% mean increase in the number of nests observed at Rancho Nuevo beach between 1985 and 1999. During the 2000 nesting season, 3778 ridley nests were observed at Rancho Nuevo beach (Turtle Expert Working Group 2000). These reports provide optimism to those who predict this population will reach 10,000 nesting females around 2020, a target given in the Kemp's ridley Recovery Plan (USFWS and NMFS 1992).

When a population undergoes a dramatic reduction in size, or demographic bottleneck, it may also experience a genetic bottleneck, where significant losses of genetic diversity in the population occur through genetic drift. The loss of genetic diversity can lower the fitness of individuals in that population through the random loss of adaptive alleles and through an increase in the expression of deleterious alleles due to the increased potential for inbreeding. Accordingly, evaluating the magnitude of the loss of genetic diversity in Kemp's ridley as well as predicting future potential losses are both of major importance to conserve this species. To achieve this goal, rather than focusing exclusively on a census of the population ( $N_c$ ), it is necessary to determine the

effective population size ( $N_e$ ). This thesis had two main objectives. The first objective, detailed in the second section of this thesis, explores the relative success of several minimally invasive tissue-sampling methods as source of DNA for genetic studies from Kemp's ridley sea turtles. The second and main objective of this research, detailed in the third section, evaluates whether the Kemp's ridley sea turtle sustained a measurable loss of genetic variation resulting from the demographic bottleneck and provides estimates of  $N_e$  from microsatellite data.

ASSESSMENT OF MINIMALLY INVASIVE GENETIC TISSUE SAMPLING  
METHODS FOR THE CRITICALLY ENDANGERED KEMP'S RIDLEY SEA  
TURTLE (*LEPIDOCHELYS KEMPII* GARMAN 1880)

## OVERVIEW

The study of endangered species genetics poses the challenge of collecting DNA using non-invasive or minimally invasive techniques. Such sampling techniques are well developed for birds and mammals, but are not applicable to the study of sea turtles. This study explored the efficacy of success of several minimally invasive tissue-sampling methods as a source of DNA to conduct genetic studies on the critically endangered Kemp's ridley (*Lepidochelys kempii*) sea turtle. Tissue sampling included: blood, cheek swabs, cloacal swabs, carapace scrapings, and a minimally invasive tissue biopsy of the hind flipper. Single copy nuclear DNA loci were PCR amplified with turtle-specific microsatellite loci primers. Blood tissue provided the best extractions of DNA for genetic studies on Kemp's ridleys. Additionally, archival plasma samples also are also a good source of nDNA. However, when minimally invasive techniques are required, hind flipper tissue biopsy is best suited for very small individuals and field situations since it requires minimal training.

## INTRODUCTION

The use of molecular genetic techniques in conservation research is widespread and will continue to expand with new advances and applications (Hedrick 2001). The advent of PCR (Polymerase Chain Reaction), for instance, expanded the potential to conduct genetic analyses since only minute amounts of tissue, preserved in many ways,

are required to amplify DNA (Dutton 1996). The study of endangered species poses the additional challenge of collecting tissue using non-invasive or minimally invasive techniques (Taberlet and Luikart 1999). Such techniques have been developed for the study of endangered birds and mammals, and DNA obtained from feces, shed hair and feathers has been successfully characterized (Morell 1994; Mundy et al. 1997).

Unfortunately, these techniques are not applicable to genetics studies of sea turtles.

Sea turtles are one of the most endangered taxonomic groups, with six of the seven species listed under the IUCN Red List of Threatened Species (2002) with Kemp's ridley sea turtle (*Lepidochelys kempii*) considered the most critically endangered.

Accordingly, efforts to identify minimally invasive methods to obtain DNA samples from sea turtles are a priority. Molecular genetic studies on sea turtles have revealed important information for conservation biology including maternal philopatry (Bowen et al. 1995), population genetics (Lahanas et al. 1994), paternity (Kichler 1996; Kichler et al. 1999) and systematics (Bowen et al. 1991). Sources of DNA for these studies mainly consisted of blood from live animals or other tissue samples from live, dead, or stranded specimens. Sea turtle blood is a good source of nuclear DNA (nDNA) because the erythrocytes are nucleated. However, as Dutton and Balazs (1995) pointed out, this sampling method requires considerable training and, therefore, is often impractical in the field. Furthermore, collecting blood from hatchlings or embryos may require killing the animal to obtain tissue. As a minimally invasive alternative, Dutton and Balazs (1995) advocated the use of small biopsy darts routinely used to collect tissue from marine mammals. These authors collected tissue from frozen green (*Chelonia mydas*),

leatherback (*Dermochelys coriacea*), and loggerhead (*Caretta caretta*) sea turtles being held for necropsy, and from live green turtles. Biopsy of tissue from the axial region of the hind flipper of live specimens was the preferred method as it was quick and relatively non-invasive, required minimal training, and yielded a sufficient quantity and quality of DNA for PCR analysis.

Other minimally invasive tissue sampling methods are available in addition to tissue biopsy. Tracheal and cloacal swabs have been used in genetic studies of birds (Moalic et al. 1998) while carapace scrapings from sea turtles have been used in toxicology studies (Wang et al. in press). The present study explored whether any of these minimally invasive tissue-sampling methods could be used as an alternative source of DNA to conduct population genetic studies on Kemp's ridley sea turtles.

Accordingly, these sampling protocols could be used on hatchlings and other small individuals (post pelagic and juvenile) from all sea turtle species. Furthermore, toxicological studies conducted in many species of sea turtles have archived blood plasma collections (Tovar et al. 2002). If blood plasma is a good source of nDNA, such depositories would become invaluable sample sources for population genetic studies on these endangered species.

## RESEARCH OBJECTIVES

Objectives of this study included: 1) compare the efficacy of blood tissue to that of several minimally invasive tissue-sampling methods in providing a source of DNA for genetic studies on the critically endangered Kemp's ridley (*Lepidochelys kempii*) sea

turtle, and 2) determine if Kemp's ridley plasma samples archived for toxicological studies provide sufficient quantity and quality nDNA for PCR analysis.

## MATERIALS AND METHODS

### **Tissue Sampling**

Blood and plasma tissue samples from post-pelagic, juvenile, subadult and adult Kemp's ridleys were provided by the Sea Turtle and Fisheries Ecology Lab at Texas A&M University in Galveston.

In addition, minimally invasive tissue sampling techniques included: cheek swabs, cloacal swabs, and carapace scrapings. Also, a minimally invasive tissue biopsy method was developed. Tissue samples were taken from juvenile Kemp's ridley sea turtles held at the NOAA/Fisheries Sea Turtle Facility in Galveston, Texas.

#### *Cheek Swabs*

A large (20 by 5 cm) metal speculum (Webster Veterinary Supply, Houston, TX, USA) was required to pry open the turtle's ptomium. A foam-tipped swab (Fisher Scientific, Pittsburgh, PA, USA) was then introduced to swab the inside of the cheek. Finally, the swab was submerged in a 1.5 ml tube containing 200 µl TENS solution (50mM Tris-HCl [pH 8.0], 100mM EDTA, 100 mM NaCl, 1% SDS in water).

#### *Cloacal Swabs*

The inside of the cloaca was swabbed with the head of a foam-tipped swab (Fisher Scientific, Pittsburgh, PA, USA) which was then submerged in a 1.5-ml tube containing 200 µl TENS solution.

### *Carapace Scrapings*

Carapace scrapings were obtained from turtles that had recently been rinsed and left to air dry before their tanks were cleaned and refilled with salt water. While the carapace is drying, the outer cornified dermal layer tends to flake off. Metal tweezers were used to obtain approximately 0.05 g of this layer without causing harm to the sea turtle. Scrapings were then frozen in liquid nitrogen, pulverized into a powder and added to 200  $\mu$ l TENS solution.

### *Tissue Biopsy*

Disposable Acu-punches (1.5 and 2 mm) (Acuderm Inc. Fort Lauderdale, FL, USA) were used to obtain tissue from the posterior edge of the rear flippers closest to the tail. Specifically, the biopsy was taken from the soft skin in between scales. Instead of collecting an actual “plug” of tissue (as described by Dutton and Balazs 1995), a half-circle of tissue approximately 0.5 mm deep was removed from the edge of the flipper. Betadine was used before and after the biopsy to prevent infection. Although, turtles did not bleed during this procedure, Neosporin was applied to the site afterward as an additional precaution to minimize the potential for bacterial infection.

### **DNA Extractions and PCR Amplification**

DNA was extracted using a modified phenol-chloroform extraction protocol described by Sambrook et al.(1989). Approximately 0.05 g of tissue (5  $\mu$ l of blood) was placed in a 1.5  $\mu$ l microcentrifuge tube containing: 200  $\mu$ l TENS solution (50 mM Tris-HCl [pH 8.0], 100 mM EDTA, 100 mM NaCl, 1% SDS) and 20  $\mu$ l Proteinase K (10

mg/ml) and incubated overnight at 55°C. An equal volume of buffer-saturated phenol was added and an emulsion formed by gently inverting the microcentrifuge tubes. The tubes were spun for 2 min at 14,000 RPM, and the supernatant was transferred to a pre-labeled 1.5-ml microcentrifuge tube. An equal volume of chloroform-isoamyl (24:1) was added, and the microcentrifuge tubes were spun for 2 min at 14,000 RPM, and the supernatant transferred to a pre-labeled 1.5 ml microcentrifuge tube. Approximately 700 µl of 100% cold ethanol and 58 µl of ammonium acetate (7.5 M) were added to precipitate DNA. The tubes were then spun at 14000 RPM for at least 10 min to form a pellet. Ethanol was decanted and the tubes were then placed upside down on a napkin to remove most of the remaining ethanol. The pellets were allowed to air dry for at least one hour and the DNA was re-suspended in 100 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Single copy nuclear DNA loci (ScnDNAs) were PCR amplified with the following sea turtle-specific microsatellite primers: Cc117, Cm72, Cm84 and Ei8 (Fitzsimmons et al. 1995). PCR was performed in 12.5 µl volumes consisting of the following: 1µl isolated DNA (template); 15.0 pM forward and reverse primer; 200 µM each of dATP, dCTP, dGTP, dTTP; 1.5 M MgCl<sub>2</sub>; 1.25 µl 10 X Platinum Taq Amplification Buffer; and 0.5U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). PCR reactions were carried out in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). PCR cycles for all four loci were as follows: an initial denaturing step at 95°C for 2.5 min, followed by 36 cycles of denaturing at 95°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final 5-min step at 72°C

was added to ensure that all products are fully extended. Negative controls were included in all amplification reactions to detect possible cross contamination during PCR. The quality of PCR amplification was determined by visually inspecting the product run on a 1.5 % agarose gel (Type I; Sigma-Aldrich, St. Louis, MO).

Tissue samples from juveniles at the NOAA/Fisheries Sea Turtle Facility, along with blood samples (post-pelagic, juvenile, subadult, and adult turtles) provided by the Sea Turtle and Fisheries Ecology lab, were amplified at all four loci. Plasma samples were amplified at only locus Cm72.

### **Statistical Analysis**

Chi-Square tests determined if the mean amplification success rates among all four loci were significantly different from each other in pair-wise comparisons. In addition, pair-wise comparisons were used to determine whether mean amplification success differed within loci among tissue sources. Differences were compared using a Chi-Square analysis testing the heterogeneity of the original data matrix using the Monte Carlo simulation in the MONTE program of the REAP genetics software package (McElroy et al. 1992). All statistical differences were assigned at a value of ( $P=0.05$ ).

### **RESULTS**

The amplification success (%) from plasma and blood tissue samples as sources of DNA were compared separately because these tissue types were collected from a different sample of Kemp's ridley sea turtles (Table 1; see Materials and Methods). Blood tissue showed no heterogeneity in success rates among loci ( $P=0.980$ ) and when

amplification success rates of plasma and blood samples were compared (at locus Cm72), no differences were observed ( $P=0.150$ ).

**Table 1.** Amplification Success Rate from Blood and Plasma Sources from Cm72 Locus.

DNA Source	Microsatellite Loci	n	PCR Amplifications	Success (%)
Plasma (in Heparin)	Cm72	81	44	54
Blood (in Heparin)	Cm72	67	53	79

The percentage rate of success of PCR-amplification for each microsatellite loci (Cc117, Cm72, Cm84, Ei8) was determined for respective tissue sources (cheek, cloaca, biopsy and carapace) (Table 2). Pair-wise comparisons revealed that the mean success rates for Cc117; Cm84 and Cm72; Ei8 were not significantly different, respectively, from each other. The means for both Cc117 and Cm84 were statistically different from those of Cm72 and Ei8 (Table 3). Cc117 and Cm84, which ranked 1 and 2, respectively, in success rate, outperformed the PCR success shown by Ei8 and Cm72. Ei8 performance was very poor. It only amplified in 11% of cases in hind flipper and failed in all others. Cm72 success rate was lower than 14% (mean ~ 10%).

Because the microsatellites exhibited statistically different success rates, it was decided to compare the difference in success rates among and between tissue sources within locus. Amplification success for locus Ei8 was significantly different overall

( $P=0.040$ ), but none of the pair-wise comparisons were significant. No differences in amplification success were observed for locus Cm72. For locus Cm84 the amplification success from hind flipper and carapace was significantly different ( $P=0.002$ ). The performance of locus Cc117 was heterogeneous when comparing all tissue types ( $P=0.006$ ).

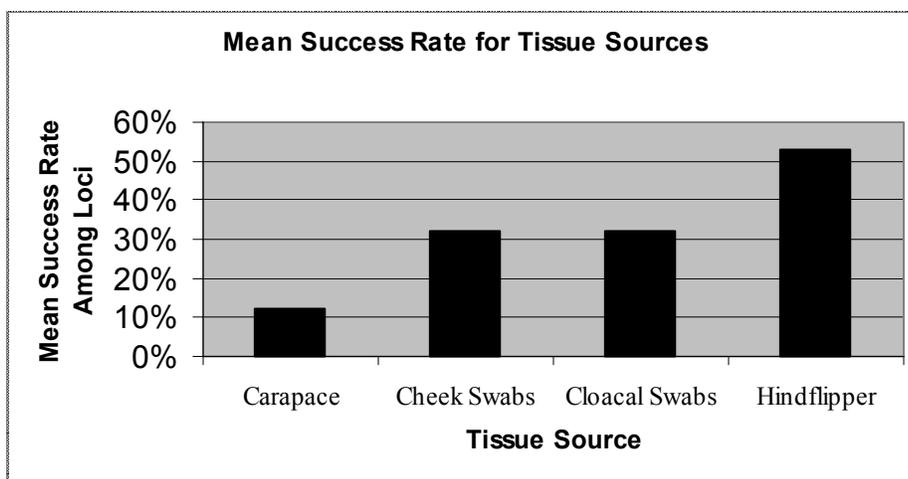
**Table 2.** Summary of Amplification Success Rates at Each Microsatellite Locus for Sampling Methods Used in This Study.

DNA Source	Microsatellite Loci	n	Number of Successful PCR Amplifications	Success (%)
Carapace Scrapings	Cc117	28	4	14
	Cm72	28	4	14
	Cm84	28	6	21
	Ei8	28	0	0
Cheek Swabs	Cc117	28	23	82
	Cm72	28	2	7
	Cm84	28	11	39
	Ei8	28	0	0
Cloacal Swabs	Cc117	28	18	64
	Cm72	28	3	11
	Cm84	28	15	54
	Ei8	28	0	0
Hind flipper	Cc117	27	27	100
	Cm72	27	2	7
	Cm84	27	25	93
	Ei8	27	3	11

**Table 3.** P-values for Pair-wise Comparisons among Microsatellite Loci.

<b>Locus</b>	<b>Cc117</b>	<b>Cm72</b>	<b>Cm84</b>	<b>Ei8</b>
Cc117	0	0.036	0.150	0.017
Cm72	0.036	0	0.042	0.087
Cm84	0.150	0.042	0	0.016
Ei8	0.017	0.087	0.016	0

Although it was determined that within locus PCR amplification success rates were not statistically different between tissue sources, mean success rates calculated among loci for each tissue source (carapace=12%, cloacal swabs=32%, cheek swabs=32%, and hind flipper=53%) were different (Figure 1).

**Figure 1.** Mean Success Rates (%) for Tissue Sources, among All Loci.

## DISCUSSION

DNA extractions from blood tissue exhibited the greatest PCR amplification success rates compared to that of four tissue sources surveyed in this study. Additionally, blood tissue performed equally well among all four loci. By contrast, when hind flipper, cheek and cloacal swabs, and carapace were used as DNA source, the amplification success rate among all four microsatellite loci was significantly different. Overall, loci Cc117 and Cm84 loci provided superior amplicons when compared with loci Cm72 and Ei8. Inspection of the DNA sequences data for Cm72 and Ei8 revealed these loci are imperfect (compound) microsatellites (data not shown), containing 2-3 tandem repeat motifs (GC and GA, respectively) directly adjacent to the targeted microsatellite sequence. The extent to which observed differences in amplification success rate can be partially accounted for by the presence of these compound microsatellite sequences is unknown.

The success rate of amplification among the four minimally invasive tissue sources surveyed in this study was highly heterogeneous presumably due to poor performance of DNA extractions from carapace scrapings. Only those pair-wise comparisons that included this tissue type were found to be significantly different ( $P < 0.002$ ). Although within locus PCR amplification success rates were not statistically different between tissue sources, mean success rates calculated among loci for each tissue source clearly show that hind flipper biopsy facilitated the greatest amplification success rate, followed by cheek and cloacal swabs which were not statistically distinguishable from each other.

The two least invasive sampling techniques were carapace scrapings and the hind flipper biopsy due to the ease of sampling in the field, low risk to the sea turtle subjects, and no noticeable pain and discomfort to the sea turtle subject. These two methods seemed to be the least stressful to the animals. The less invasive hind flipper biopsy technique developed for this study took less tissue from the animal in comparison to the biopsy dart method employed by Dutton and Balazs (1995). There was no bleeding, thus reducing the chance of infection. The only stressful element during these two sampling techniques was the initial handling of the turtle. However, the limited success in PCR amplification from carapace samples renders this methodology impractical. Cheek and cloacal tissue swabbing clearly stressed the sea turtles more than the other methods. To collect cloacal swabs the turtles were held vertically in the air during sampling, causing the subjects to struggle. Cheek swabs were very difficult to obtain and proved to be dangerous to both the animal and the handler. The turtles were stressed when their plastrons were pried open. In addition, this technique poses danger to the handler, due to the Kemp's ridley's notoriously aggressive nature and strength which may inflict significant damage to the hand and fingers.

The results obtained from DNA extractions from plasma were encouraging, in that they consistently amplified locus Cm72, which was one of the most problematic loci to amplify. The success of using plasma as a source of DNA may be partially explained by the fact that most plasma samples were pink-pigmented, suggesting that erythrocytes remained in the samples after centrifugation.

The results of this study demonstrate that blood tissue consistently yielded the highest quality of DNA for genetic studies on Kemp's ridley sea turtles. Additionally, it was found that archival plasma samples are also a good source of nDNA. This is particularly important since archival samples for toxicological studies suddenly become reservoirs of valuable information for the study of population genetics of sea turtles. However, the results also show that when dealing with very small individuals or field situations, the hind flipper tissue-biopsy-technique developed here is the method of choice. This method, in addition to being minimally invasive, is safe for both the animal and the handler and provides high quality DNA for genetic studies.

GENETIC ESTIMATES OF EFFECTIVE SIZE OF THE KEMP'S RIDLEY SEA  
TURTLE (*LEPIDOCHELYS KEMPII*) POPULATION

OVERVIEW

The critically endangered Kemp's ridley sea turtle experienced a dramatic decline in population size (demographic bottleneck) between the years of 1947 and 1987 from >40,000 nesting females to an average of 740 ridley nests. By the 2000 nesting season, 3778 ridley nests were observed at Rancho Nuevo beach, Tamaulipas, Mexico. Demographic bottlenecks can produce genetic bottlenecks where significant losses of genetic diversity in the population occur through genetic drift. The loss of genetic diversity can lower the fitness of individuals in that population through the random loss of adaptive alleles and through an increase in the expression of deleterious alleles due to the increased potential for inbreeding. Accordingly, evaluating the magnitude of the loss of genetic diversity in Kemp's ridley as well as predicting future potential losses are both of major importance to conserve this species. To achieve this goal, rather than focusing exclusively on a census of the population ( $N_c$ ), it is necessary to determine the effective population size ( $N_e$ ). This study sought to estimate  $N_e$  from microsatellite data and, thus, it is the first attempt to determine whether a genetic bottleneck occurred during the historical reduction of the Kemp's ridley population. Three alternative approaches were used to detect a reduction in effective population size: 1) temporal change in allele frequencies, 2) an excess of heterozygotes in progeny, and 3) the estimate of the mean ratio ( $M$ ) of the number of alleles ( $k$ ) to the range of allele size ( $r$ ). Blood samples were obtained from Kemp's ridleys caught in the wild. PCR (polymerase

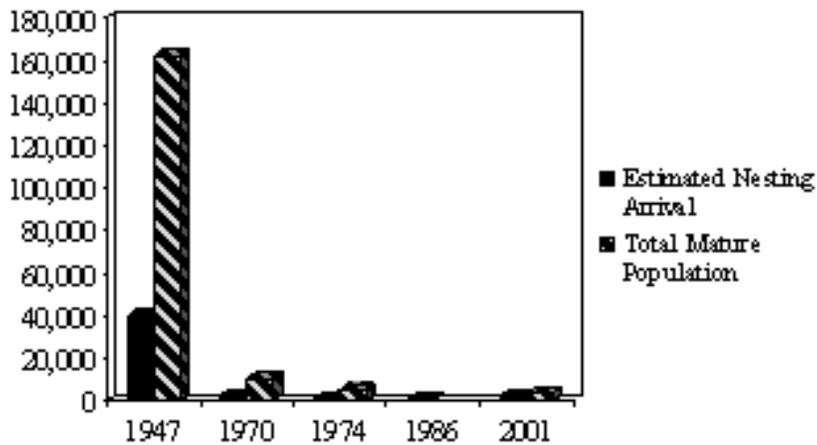
chain reaction) was used to amplify eight sea turtle microsatellite loci 1995 and, allele frequencies were determined. Data from only four of these loci could be used in these analyses. The reduced number of loci was a limiting factor in this study, however, despite this shortcoming; results of the three statistical approaches suggest the Kemp's ridley population sustained loss of genetic diversity associated with a demographic bottleneck.

## INTRODUCTION

The IUCN Red Book of Threatened Species identifies six protected sea turtles (IUCN 2002). The most critically endangered of these species is the Kemp's ridley sea turtle (*Lepidochelys kempii*) found primarily in the Gulf of Mexico but extending into the Atlantic Ocean. Almost the entire adult female population nests on one beach near Rancho Nuevo, Tamaulipas, Mexico, where >40,000 females nested on a single day in 1947 (Bowen et al. 1991). The average annual number of ridley nests between 1985 and 1987 dropped to 740 (Márquez et al. 2001). During the 2000 nesting season, 3778 ridley nests were observed at Rancho Nuevo beach (Márquez et al. 2001) (Figure 2). Models project 3000 nesting females by the year 2003 (Turtle Expert Working Group 2000).

The causes for the dramatic decline of the Kemp's ridley population include habitat destruction and alteration, poaching for meat and eggs, and incidental capture in shrimp trawls. Marine turtle protection efforts began in Mexico in 1927. Article 97 of the Fishery Regulation of February 17, 1927 prohibited collecting turtle eggs and destroying nests (Trinidad and Wilson 2000). Throughout the 1960's, 70's and 80's,

further restrictions were enacted by the Mexican government to prevent the harvest of Kemp's ridleys for meat and eggs. However, regulatory surveillance of the Rancho Nuevo nesting beach was weak, and poaching forced the Mexican government to completely ban hunting and egg collecting in 1990 (Trinidad and Wilson 2000). By 1982 it was widely accepted that shrimp trawlers captured and drowned more sea turtles worldwide than did any other kind of incidental capture, with this fishery accounting for more Kemp's ridley mortalities than did any other human activity. In 1989, the National Marine Fisheries Service enacted Turtle Excluder Device (TED) regulations in



**Figure 2.** Population Estimates for the Kemp's Ridley Sea Turtle from 1947-2001.

certain areas at certain times. The regulations were subsequently expanded to require TED's on all shrimp and flounder trawlers operating in the southeastern U.S (52 FR 24244). Furthermore, in 1991, the U.S prohibited imports of shrimp from nations whose trawling practices did not comply with its conservation efforts calling for TED implementation.

When a population undergoes a dramatic reduction in size, or demographic bottleneck, it may also experience a genetic bottleneck characterized by significant loss of genetic diversity. The reduced population is more prone to the effect of random genetic drift, where alleles are lost by random variance of both mortality and reproductive success of different genotypes (Futuyma 1998) at a rate of  $[1-1/(2N_e)]$  per generation (Wright 1969). Thus variation would be lost faster in small populations. Furthermore, because many individuals will be related in a reduced population, random matings are most likely to be consanguineous. Effects of such mating events in a reduced population would be similar to those of inbreeding (Crow 1986). For these reasons, dramatic reductions in population size are of major concern, because even if population size ( $N_c$ ) recovers to historical levels, effective size of the population ( $N_e$ ) remains low. In such cases, negative effects of inbreeding and random genetic drift may persist for a long time. This can lead to a decrease in fitness as probability of the expression of deleterious alleles in the population increases (Meffe and Carroll 1997) and because homogenized populations are more prone to epidemic events. There is abundant evidence of this phenomenon in captivity (Saccheri et al. 1996) and in field studies (Madsen et al. 1996; Newman and Pilson 1997). Accordingly, conservation

efforts toward reduced populations should employ estimates of effective population size ( $N_e$ ) instead of the absolute number of individuals to determine the status of those populations (Allendorf et al. 1991). Furthermore,  $N_e$  estimates can provide managers with an approximation of the amount of genetic loss likely to take place in the future (Harris and Allendorf 1989).

$N_e$  is influenced by the interplay of several demographic factors affecting that population. Futuyma (1998) summarized these factors as follows. Theoretically, maximal  $N_e$  is achieved when: the population size has remained high and constant over time, when sex ratio equals unity, each mature individual in the population produces an equal number of offspring, and generations do not overlap. The concept of an equal sex ratio assumes a single reproductive event with progeny from one female and one male. If sex ratio is skewed in a reproductive event, then whatever gender is in the minority will produce more progeny per individual than the gender in the majority. Other demographic factors influencing  $N_e$  include variation in number of progeny, overlapping generations, fluctuations in population size and migration

Effective population size for the Kemp's ridley population is affected by sex ratio, multiple paternity, relative paternal contribution and demographic history. The sex ratio of Kemp's ridley has been estimated to be 1.3 females to 1 male (Coyne 2000). Theoretically, this approximately equal sex ratio should maximize effective population size. However, Kemp's ridley is a polyandrous species (more than two males may contribute to a single clutch) with unequal paternal contribution (Kichler et al. 1999). Normally, such unequal contribution would tend to reduce  $N_e$  (see Sugg and Chesser

1994 for an alternative view). Finally, the ridley's demographic history (e.g., population bottleneck) must be included in models estimating effective population size. The historical reduction in Kemp's ridley population size may have caused a reduction of  $N_e$  as the effects of genetic drift would have caused random losses of variation during years when the population was smaller.

Kichler (1996) determined the genetic health of the Kemp's ridley by comparing a sample of this population with one sample of olive ridley sea turtles. Kemp's ridley samples were taken from 211 nesting females at Rancho Nuevo. Sixty olive ridley samples were taken from nests in one locale in Costa Rica over the course of several nesting seasons. Samples were genotyped at four polymorphic loci, with results indicating that Kemp's ridleys exhibited a comparable number of alleles per loci and higher levels of heterozygosity than did olive ridley samples. However, generalizations at the species level cannot be reached from this comparison due to the limited geographic range of olive ridley samples. Olive ridleys are cosmopolitan, and therefore, samples from a single nesting locale may not necessarily represent genetic variability of the entire species. Fitzsimmons (1995) obtained heterozygosity values for three of the four microsatellite loci used by Kichler (1996). Fitzsimmons' samples were taken from widely separated geographic populations in Australia and heterozygosity values for the markers are noticeably different (for two of the three loci used by both authors) (Table 4).

**Table 4.** Heterozygosity Values Obtained for the Olive Ridley (*Lepidochelys olivacea*) Sea Turtle from Kichler (1996) and Fitzsimmons (1995).

<b>Locus</b>	<b>(Kichler 1996)</b>	<b>(Fitzsimmons et al. 1995)</b>
Cm72	0.455	0.9
Cm84	0.909	0.444
Ei8	0.896	0.444

## RESEARCH OBJECTIVES AND HYPOTHESIS

The purpose of this study is twofold. First, to offer estimates of  $N_e$  for Kemp's ridley based on microsatellite data obtained from specimens captured in the wild, and 2) based on these estimates; determine whether the historical reduction of the Kemp's ridley population caused a genetic bottleneck. The working hypothesis is that estimates of effective population size from genetic data would be significantly lower than the current estimates of sexually mature individuals in the population.

Empirically derived estimates of effective population size from genetic data often differ significantly from a census of the population. In many marine organisms with high fecundity and high mortality rates in early stages (Type III survivorship curves) differences up to one order of magnitude ( $N_e/N_c \sim 0.10$ ) are not rare (Frankham 1995). In terrestrial mammals, the  $N_e/N_c$  ratio ranges between 0.25-0.75, with a mean single-generation estimate around 0.35 (Frankham 1995). The disparity between  $N_c$  and  $N_e$  might be due to the demographic history of the population (e.g., historical bottlenecks), and variance in reproductive success among its constituents (Hedgcock 1994). Given

that Kemp's ridley population does not produce as many offspring as many other marine organisms and does not provide the parental care displayed in mammals, it is assumed here that their  $N_e/N_c$  ratio should fall somewhere in between 0.10 and 0.25.

Three alternative approaches were used to detect a reduction in Kemp's ridley's  $N_e$  from microsatellite data. These approaches were: 1) temporal change in allele frequencies (Williamson and Slatkin 1999), 2) an excess of heterozygotes in progeny (Luikart and Conuet 1999), and 3) a mean ratio ( $M$ ) of the number of alleles ( $k$ ) to the range of allele size ( $r$ ) of microsatellite data (Garza and Williamson 2001). All three models assume a single population, as is the case for the Kemp's ridley population. In the first approach, a temporal shift in allele frequencies would indicate that genetic drift had a dramatic effect on the population and, therefore, a significantly reduced  $N_e$ . This approach requires all observed loci to be independently segregating, which will be determined from a linkage disequilibrium test (Bartley et al. 1992). The second approach is based on the principle that both allelic diversity and observed heterozygosity decrease with  $N_e$ , however, allelic diversity is reduced more quickly than observed heterozygosity. The observed heterozygosity is therefore larger than the expected heterozygosity from the observed number of alleles at a given locus. It is important, however, to note the distinction between a test for excess levels of heterozygosity and a test for excess numbers of heterozygotes. The former test compares the observed heterozygosity (Nei 1987) with that expected at mutation-drift equilibrium, whereas the latter test compares the observed number of heterozygotes with that expected at Hardy-Weinberg Equilibrium causing heterozygote excess when testing for Hardy Weinberg

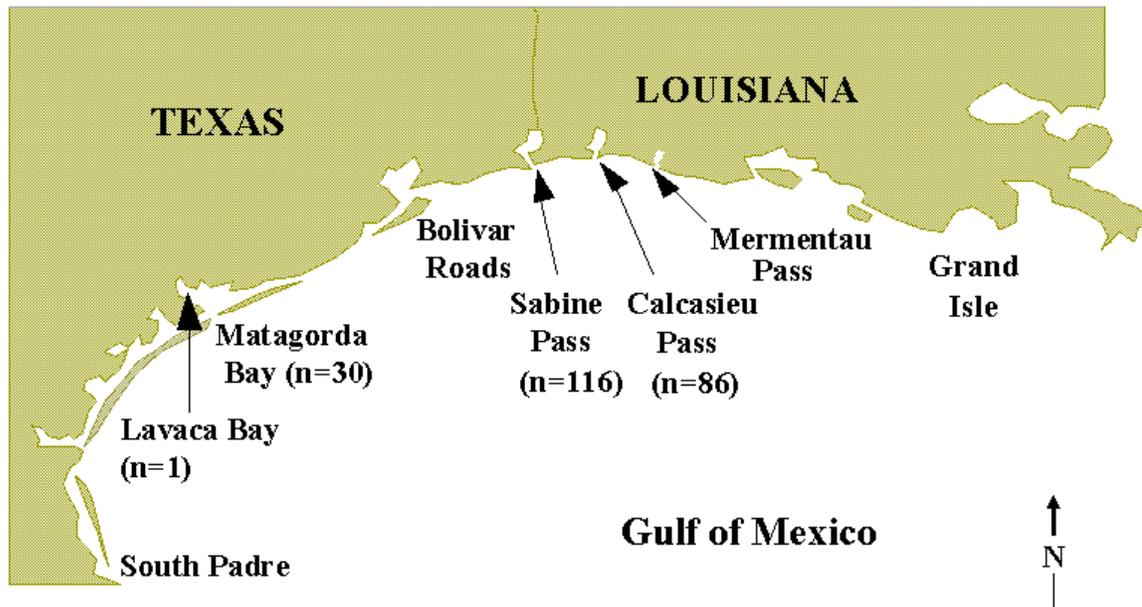
Equilibrium. Finally, the third approach is based on the expectation that the value of  $M$  decreases when the effective population size has been reduced. Furthermore, the magnitude of decrease in  $M$  value positively correlates with severity and duration of bottleneck.

In addition, this research builds upon previous work (Kichler 1996) in furthering our understanding of the population genetics on Kemp's ridley in two ways. First, the characterization of the levels of variation of individuals captured randomly (as opposed to siblings from a limited number of nests) will give us a more accurate picture of the levels of variation contained in the Kemp's ridley population. Second, characterization of the nucleotide sequence of DNA segments containing microsatellites will yield valuable information of the mutational processes that may affect these loci. Such information will enable future researchers to select loci that correspond to requirements of specific models being tested.

## MATERIALS AND METHODS

### **Field Methods**

Blood samples of wild Kemp's ridleys caught in the Gulf of Mexico were obtained by the Sea Turtle and Fisheries Ecology Lab at Texas A&M University at Galveston. Sea turtle capture occurred along beachfronts adjacent to Calcasieu Pass, Louisiana as well as in Sabine Pass, Lavaca and Matagorda Bay, Texas. Capture involved 91.5m entanglement nets checked every 20 minutes, with blood samples taken within 7-19 minutes post capture. The life history stages comprising these captures included post pelagic, juvenile, sub adult, and adult (n=233) (Figure 3).



**Figure 3.** Capture Sites for Kemp’s Ridley Sea Turtles Used in This Study.

### **DNA Isolation and PCR Amplification Procedures**

Total genomic DNA was isolated from blood following a modified version of the protocol described in Sambrook et al. (1989). Briefly, 5ul of blood were digested overnight at 37°C in a 1.5 mL microcentrifuge tube with 20 ul Proteinase K (10 mg/ml) in 200 ul TENS solution (50mM Tris-HCl [pH 8.0], 100mM EDTA, 100 mM NaCl, 1% SDS). Total DNA was extracted with one wash of Phenol-Chloroform (25:24) extraction, followed by one wash of Phenol-Chloroform-Isoamyl (25:24:1) and ethanol

precipitation. Precipitated DNA was resuspended in 100  $\mu$ l of TE solution (10mM Tris, 1mM EDTA in deionized water; pH 8.0). Single copy nuclear DNA loci (ScnDNA) were amplified with polymerase chain reaction (PCR) using sea turtle specific microsatellite primer sets (Table 5; Dutton 1995; FitzSimmons et al. 1995; Kichler et al. 1999). PCR reactions were carried out in 12.5  $\mu$ l volumes consisting of the following: 1 $\mu$ l isolated DNA (template); 15.0 pM of each primer (Table 5); 200  $\mu$ M each of dATP, dCTP, dGTP, dTTP; 1.5 M  $MgCl_2$ ; 0.5U Platinum Taq DNA polymerase, and 1.25  $\mu$ l 10 X Platinum Taq Amplification Buffer (Invitrogen, Carlsbad, CA). PCR reactions were carried in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Thermal profiles for all four microsatellite loci (Table 5) were as follows: an initial denaturing step at 95°C for 2.5 min, followed by 36 cycles of denaturing at 95°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final 5-minute step at 72°C was added to ensure that all products are fully extended. Negative controls were included in all amplification reactions. The quality of PCR amplification was determined by visually inspecting the product run on a 1.5-% agarose gel (TA buffer) (Type I; Sigma-Aldrich, St. Louis, MO) at 100 V for 30 min.

**Table 5.** Primer Sequences, Microsatellite Arrays, Thermal Profiles, and Dye-Labels for Primers Used in This Study.

<i>Microsatellite Loci</i>	<i>Primer (5' to 3')</i>	<i>Array</i>	<i>Thermal Profiles</i>	<i>Dye-Label (Forward Primer)</i>	<i>Reference</i>
DC99	CACCCATTTTTCCCATTGA TTTGAGCATAAGTTTTCCGT GG	n/a	1	n/a	Dutton, P.H. (1995)
Nigra 32	CGTGTGTTTGGACAGAAAGA TGAACAAAGCAAACTTATT TCCGTG	n/a	1	n/a	“ ”
Nigra 200	GCTAAAGACCTAGTTCTGC CATGTTTCAGTGGTTACTCA GCAAAGG	n/a	1	n/a	“ ”
Cc117	TCTTTAACGTATCTCCTGTGA GCTCCAGTAGTGCAGTTC CATTGTTTCA	(CA)	95°C for 2.5 min, 36 cycles at 95°C for 45 sec, 55°C for 1 min and 72°C for 1 min, and final extension 72°C for 5 min.	6-Fam	Fitzsimmons et al., (1995)
Cm72	CTATAAGGAGAAAAGCGTTA AGACACCCAAAATTAGGATTA CACAGCCAAC	(CA)	“ ”	Hex	“ ”
Cm84	TGTTTTGACATTAGTCCAG GATTGATTGTTATAGCCTA TTGTTCAAGGA	(CA)	“ ”	6-Fam	“ ”
Ei8	ATATGATTAGGCAAGGCTC TCAACAATCTTGAGATTGG CTTAGAAAATC	(CA)	“ ”	Tet	“ ”
KLk316	TACATCCATACATGCAGCC CCCTGA	Multiple arrays	95°C for 2.5 min, 36 cycles at 95°C for 45 sec, 60°C for 1 min and 72°C for 1 min, and final extension 72°C for 5 min	n/a	Kichler et al., (1999)

1. Multiple attempts with different cycling profiles failed to generate specific product.

## **Direct Sequencing of Microsatellite Loci**

PCR products were purified using ExoSAP-IT™ (USB Corporation, Cleveland, Ohio) to remove unincorporated primers. Purified PCR products were then subject to cycle sequencing reactions using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer Corporation, Foster City, California). Unincorporated terminators were removed with RapXtract™ Dye Terminator Removal Kit (Prolinx Corporation, Bothell, Washington). Sequences were determined using the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, California). Nucleotide sequences were inspected for the presence of tandem repeats to verify that each targeted microsatellite locus was amplified.

## **Data Analysis**

### *Microsatellite Data*

Microsatellites are nucleotide sequences characterized by short (2-5 base pairs long) tandem repeat regions. Their fast rate of molecular evolution renders these markers extremely effective for assessing the genetic structure of populations. Accordingly, they exhibit high levels of variability even in species that are homozygous at other loci (Hillis et al. 1996). However, microsatellite loci data may yield erroneous results because mutations in the flanking primer sites can be interpreted as null alleles (Hillis et al. 1996). Homozygotes for such null alleles will not amplify and their frequency will be underestimated. In addition, heterozygotes will be scored as homozygotes for the amplifying allele. This problem was addressed when testing for

Hardy-Weinberg Equilibrium with the alternative hypothesis of homozygote excess.

DNA polymorphisms were characterized with several molecular genetic techniques (see below). Allele frequencies were used to estimate effective population size from nuclear DNA.

### *Allele Scoring*

After confirming the presence of microsatellite motifs within amplicons, additional PCR reactions were setup with the same thermal profiles with the exception that forward dye-labeled primers replaced the unlabelled forward primers in all four loci. The fluorescent labels employed were 6-FAM, HEX, TET, and TAMRA (Applied Biosystems, Foster City, California) (Table 5). Numerous attempts to setup multiplexing failed. Instead, the resulting reactions for each specimen were mixed together so that each sample contained products for all four microsatellite loci. Fragment analysis was performed using the GENESCAN 3.1 software (Applied Biosystems) as described in the manufacturer's manual (per sample); 1  $\mu$ l PCR product, 12  $\mu$ l formamide-loading buffer, 0.5  $\mu$ l GeneScan-500 (TAMRA) internal size standard (Applied Biosystems). Prior to loading, the samples were denatured for 2 min at 95°C in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). The internal size standard consisted of fragments of known size, which were added to the ABI PRISM™ 310 Genetic Analyzer along with the samples being investigated. The Genetic Analyzer separated the DNA fragments by electrophoresis, and the GENESCAN software determined a sizing curve based on the mobility of known fragments of the size standard. The software then calculated the peak sizes by comparing the mobility of each

peak in the sample to the size curve. For electrophoresis (sequencing) and GENESCAN settings, refer to Table 6.

**Table 6.** Electrophoretic Profile for Sequencing and GENESCAN Settings Employed in This Study.

Electrophoresis

Module	Injection secs.	Injection kV	Run kV	Run °C	Run Time (min)
Seq POP6™ Rapid (1ml) E	40	3.5	15.0	50	36

Genescan

Module	Injection secs.	Injection kV	Run kV	Run °C	Run Time (min)	Matrix File
GS STR POP4™ (1ml) C	5	15.0	15.0	60	24	GS Fam, Hex, Tamra, Tet

*Test for Hardy-Weinberg Equilibrium*

GENEPOP version 3.1 software (Raymond and Rousset 1995) was used to calculate observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), allele frequencies, number of alleles per locus, and linkage disequilibrium. Linkage disequilibrium tests in GENEPOP were used to determine whether any nuclear markers were located on the same chromosome. Additionally, GENEPOP was used to test for deviation from Hardy-Weinberg Equilibrium, testing for heterozygote excess and heterozygote deficiency using the Markov-chain random walk algorithm described by Guo and Thompson

(1992). These tests were carried out to detect deviations from expected Hardy-Weinberg values resulting from mutation, migration, genetic drift and natural selection.

#### *Temporal Change in Allele Frequencies*

The MLNE (Wang 2001) program uses a pseudo-likelihood method for estimating  $N_e$ . This method has been shown to give a more precise measure of  $N_e$  than the F-statistic method (Waples 1989). Furthermore, this method is flexible, allowing three or more temporal samples to simultaneously estimate  $N_e$ . Additionally, this method is robust to violations of the assumption of an infinitely large source population and therefore, can be used to estimate  $N_e$  from a finite source population consisting of one or more subpopulations. The accuracy of this method depends on sample size, number of generations, number of independent alleles, and number of independent loci.

MLNE (Wang 2001) used the moment and likelihood methods to estimate effective population size ( $N_e$ ) and migration rate ( $m$ ) from temporal and spatial data on genetic markers. Temporal data were taken from samples obtained in 1997, 1998, and 1999. Migration rate was not a factor as the Kemp's ridley most likely consists of a single population. It is unlikely that this single nesting site is subdivided into sympatric subpopulations separated by time such as that for salmon runs (Greig and Banks 1999) due to varying times these turtles reach maturity (8-13 yrs) and re-nest (1-3 yrs) (Coyne 2000). Two separate scenarios were used to obtain estimates of  $N_e$ .

The first scenario was based on age classes used by the Sea Turtle and Fisheries Ecology Lab at Texas A&M University at Galveston. Carapace length (cm) was used to

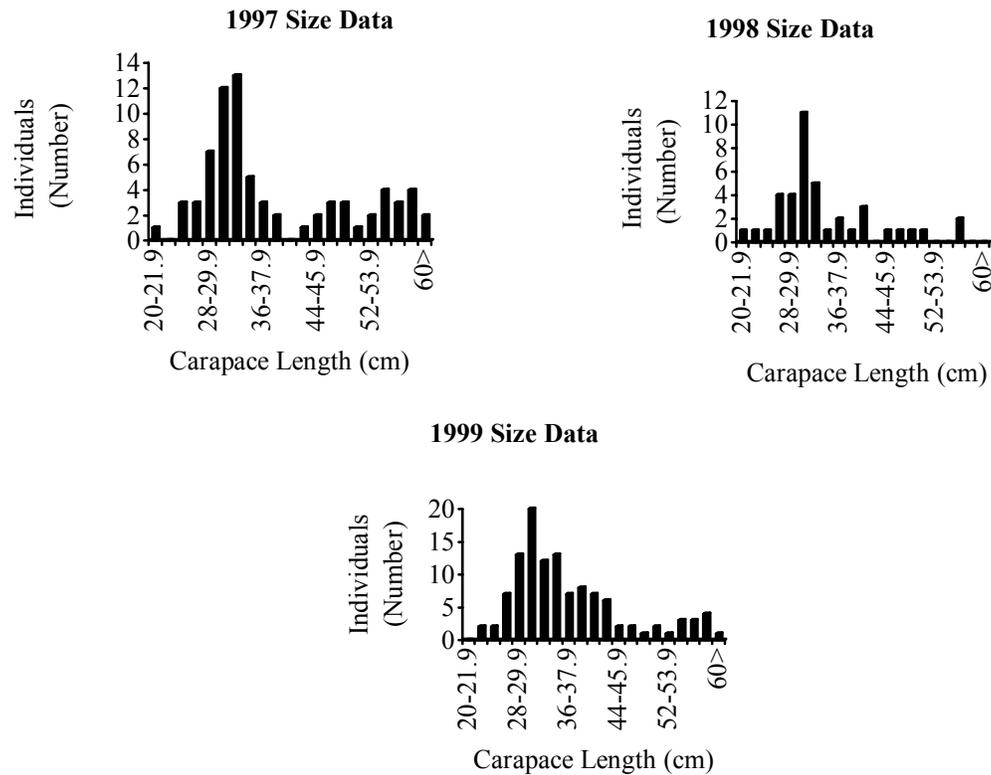
group Kemp's ridleys into the following classes: post pelagic (<30 cm), juvenile (~30-40 cm), sub-adult (~41-59 cm), and adult ( $\geq$ 60 cm). Individuals were then sorted by their respective year of capture (1997, 1998, and 1999) (Table 7) and allele frequencies were compared within year among 3 of the 4 age classes (the adult samples were excluded because of small sample sizes) such that three estimates of  $N_e$  were obtained. In a second approach, samples were assigned for each of the three consecutive years of capture (1997, 1998, and 1999) respectively, to one cohort that included specimens ranging in size between (26 and 40 cm) (Figure 4), corresponding primarily to juveniles, but also including post-pelagic individuals. Accordingly, it was assumed that each sample/year represents three separate cohorts or year classes. Allele frequencies (Table 8) were compared among cohorts and the data were then used to obtain point (average  $N_e$  over entire sampling period) and moment estimates ( $N_{e_s}$  for each sampling period) of  $N_e$ .

**Table 7.** Sample Sizes (n) in Each Age Class Used to Calculate  $N_e$  for Capture Years 1997, 1998, and 1999.

<b>AGE CLASS</b>	<b>1997</b>	<b>1998</b>	<b>1999</b>
Post Pelagic	n=13	n=11	n=23
Juvenile	n=31	n=18	n=54
Subadult	n=18	n=8	n=28

**Table 8.** Genetic Characteristics of Four Microsatellite Loci from Three Cohorts of Kemp's Ridley for Capture Years 1997, 1998, and 1999. N=Number of Individuals, N (alleles)=Number of Alleles at that Locus, Size is in Base Pairs (bp),  $H_{EXP}$ =Expected Number of Heterozygotes, and  $H_{OBS}$ =Observed Number of Heterozygotes.

<b>Locus &amp; Characteristics</b>	<b>1997 Year of Capture</b>	<b>1998 Year of Capture</b>	<b>1999 Year of Capture</b>	<b>Totals</b>
<b>CC117</b>	-	-	-	-
N	41	29	75	145
N (alleles)	7	7	7	21
Size Range (bp)	186-206	186-206	186-206	186-206
$H_{EXP}$	29.086	19.930	49.020	-
$H_{OBS}$	27	22	54	-
<b>CM72</b>	-	-	-	-
N	41	29	75	145
N (alleles)	4	3	4	11
Size Range (bp)	216-241	226-241	224-241	216-241
$H_{EXP}$	25.074	17.175	39.060	-
$H_{OBS}$	28	17	34	-
<b>CM84</b>	-	-	-	-
N	41	27	75	143
N (alleles)	9	8	13	30
Size Range (bp)	312-336	312-334	312-342	312-342
$H_{EXP}$	30.296	16.943	57.725	-
$H_{OBS}$	27	12	46	-
<b>EI8</b>	-	-	-	-
N	42	26	74	142
N (alleles)	3	4	6	13
Size Range (bp)	164-172	164-172	162-172	162-172
$H_{EXP}$	27.819	18.922	51.531	-
$H_{OBS}$	24	14	47	-
Mean $H_{EXP}$	28.069	18.243	49.334	31.882
Mean $H_{OBS}$	26.5	16.25	45.25	29.333



**Figure 4.** Size Frequency Data used to Group Kemp's Ridley Individuals into Individual Cohorts Corresponding to Years 1997, 1998, and 1999 for the MLNE Software. Cohort Was Determined to be between 26 and 40 cm in Carapace Length.

### *M Ratio*

The M ratio (Garza and Williamson 2001) takes into account not only the allele frequency and number of alleles, but also spatial diversity (distance between alleles in number of repeats and the overall range in allele size) at each locus. When a population experiences a demographic bottleneck, alleles are inevitably lost through genetic drift.

However, because the loss of any allele will reduce the total number of alleles ( $k$ ), but only a loss of the largest or smallest allele will reduce the range of alleles ( $r$ ),  $k$  is expected to be reduced more quickly than  $r$ . This is due to the empirical observation that allele frequency distributions are not bell shaped. That is to say rare alleles are not necessarily the largest and smallest allele. If they were, then  $k$  would be expected to decrease at the same rate as  $r$ . However, since this is not the case, it would be expected that  $r$  would decrease more slowly than  $k$  during a demographic bottleneck.

The M ratio (number of alleles to range in allele size) (Garza and Williamson 2001) was estimated with two approaches. The first program (M\_P\_Val.exe) was used to calculate the empirical M value for the microsatellite data set. The program simulated an equilibrium distribution of M according to the method described in Garza and Williamson (2001), and given assumed values for the three parameters. The three assumed parameters values include Theta ( $4 * (\text{historical}) N_e * \text{mutation rate}$ ),  $p_s$  (mean percentage of mutations that add or delete only one repeat unit), and  $\Delta_g$  (mean size of larger mutations). The M is calculated and ranked relative to the equilibrium distribution. There is evidence of a significant reduction in population size if less than 5% of the replicates are below the observed value. The estimate of the historical of  $N_e$  was taken from historical estimates of the total population size in 1947 (162,400) (Carr 1977). This estimate was divided by 10 ( $N_c/N_e \sim 10$ , on average) based on the assumption that the number of breeding adults ( $N_c$ ) would be considerably larger than  $N_e$ . Also, this procedure would be conservative to avoid a type error. Mutation rates were then estimated for each locus. E18 locus exhibited a mutation rate of 0.023 in the

olive ridley (Hoekert et al. 2002). Mutation rates for Cc117 (0.0022) and Cm72 (0.0096) loci were extrapolated from Fitzsimmons (1998) work on green sea turtles (*Chelonia mydas*). Finally, mutation rate for locus Cm84 was inferred from Kemp's ridley (Kichler et al. 1999) and olive ridley (Hoekert et al. 2002) data. These authors were unable to detect mutations among a combined sample of 2168 alleles.

Accordingly, the mutation rate for this locus was assumed here to be less or equal to 0.00046. The estimated mutation rates for all four loci were averaged and divided by the mean estimated generation time (10 yrs) for the Kemp's ridley (Coyne 2000) to obtain an average mutation rate, per locus, of 0.00088 per generation. The  $\delta_g$  and  $p_s$  values were derived from the data for all four microsatellite loci.

The second program, Critical\_M.exe., calculated a critical M, through a simulation described in Garza and Williamson (2001) for a given microsatellite data set taken from the number of individuals sampled, number of loci, and three assumed parameter values for a two-phase mutational model. Ten thousand simulation replicates were performed and an M ratio was calculated for each. These values were ranked and M-critical was defined as the number which only 5% of the simulations fell below.

The mean M value from the M\_P\_val.exe was used as the empirical M value derived from microsatellites surveyed in this study among 215 individuals. The M critical.exe program calculated an M-critical value. The M-critical tested whether the data were a sample from a population that had experienced a recent bottleneck. This value is not based on empirical data; instead it is derived from the average of 10,000 replicates of a sample with the same number of individuals, a particular mutation rate,

and an average size of non-one-step mutations present in a particular proportion. The mean M value from the M\_P\_val.exe program was calculated as an average of the M ratios for all 4 loci. The empirical value of M was compared with the critical M and an empirical M smaller than the M-critical would indicate a bottleneck.

Two M-critical and empirical M values were calculated based on two mutation rate estimates; one including all four loci and one excluding locus Ei8. This is due to the extremely high rate of mutation observed by Hoekert et al. (2002) for the olive ridleys at locus Ei8 ( $\sim 10^{-2}$ ). High levels of mutation may counteract effects of genetic drift by replacing alleles just as soon as they are lost. This trend is evident in the fact that the Ei8 locus is not missing any alleles within its range (162-172 bp). Accordingly, due to the fact that the M ratio program takes into account spatial diversity (distance between alleles in number of repeats and the overall range in allele size) at each locus, the Ei8 locus may bias the results of this study.

## RESULTS

### *Microsatellite Loci*

Loci targeted with primer sequences DC99, Nigra 200, Nigra 32 (Dutton 1995), failed to amplify specific product for Kemp's ridley samples. Primer sequences (KLk316) from Kichler (1999) amplified well; however, upon inspection of DNA sequences revealed this locus to be imperfect (compound) microsatellites, containing multiple tandem repeat motifs within the sequence (Figure 5). Compound microsatellites may violate the assumption of a mutational model since both tandem

repeats may be affected simultaneously, and for this reason, this locus was excluded from further analysis.

Four primer sequences (Cc117, Cm72, Cm84, and Ei8) designed by Fitzsimmons (1995) amplified successfully. Direct sequencing of these amplicons confirmed the presence of microsatellite loci (all CA arrays), and these microsatellite loci appear to be orthologous with those identified by Fitzsimmons. Refer to Table 9 for size (base pairs), range, and number of alleles for these four loci.

#### *Linkage Disequilibrium and Hardy Weinberg Equilibrium*

Genotypic disequilibrium tests for all pair wise comparisons revealed all four loci to be unlinked (not on the same chromosome) ( $P=0.342$  for Cc117-Ei8,  $P=0.170$  for Cc117-Cm72,  $P=0.143$  for Ei8-Cm72,  $P=0.315$  for Cc117-Cm84,  $P=0.397$  for Ei8-Cm84,  $P=0.921$  for Cm72-Cm84). Hardy-Weinberg tests for heterozygote excess and deficiency revealed that 3 out of 4 loci were heterozygote deficient ( $P=0.422$  for Cc117,  $P=0.000$  for Ei8,  $P=0.002$  for Cm72,  $P=0.000$  for Cm84) and that none of the four loci showed an excess of heterozygotes ( $P>0.05$ ). For a summary of these data, refer to Table 9.



**Table 9.** Summary of Results from GENEPOP Software Including Observed Heterozygosity ( $H_O$ ), Expected Heterozygosity ( $H_E$ ), and P-Values for Tests of Hardy Weinberg Equilibrium (Probability test, He excess and He Deficiency).

Microsatellite Locus	Base pairs (range)	Number of alleles	N (alleles sampled)	$H_O$	$H_E$	Probability Test	$H_a = H$ deficiency	$H_a = H$ excess
Cc117	186-206	8	424	0.693	0.684	0.422	0.813	0.161
Cm72	216-241	5	424	0.547	0.568	0.002	0.000	1
Cm84	312-342	15	420	0.557	0.736	0.000	0.000	1
Ei8	162-172	6	418	0.579	0.690	0.000	0.000	1
(Average)	n/a	8.5	210.75	0.594	0.670	n/a	n/a	n/a

#### *N<sub>e</sub> Estimates from Temporal Change in Allele Frequencies*

Three estimates of  $N_e$  were obtained from temporal allele frequency data for years 1997, 1998 and 1999 by comparing age classes (post-pelagic, juvenile, and subadult) within each year. The  $N_e$  estimate for 1997 was ~238 (moment estimate) and ~1954 (point estimate) individuals (95% CI = 45.48, 5000), for 1998 was infinite (moment estimate) and ~8565 (point estimate) individuals (95% CI = 35.64, 30000), and for 1999 was ~215 (moment estimate) and ~4958 (point estimate) individuals (95% CI = 83.68, 5000). A fourth estimate of  $N_e$  was obtained by comparing allele frequencies of one cohort for three different years (1997, 1998, and 1999). This second scenario produced a  $N_e$  estimate of ~29 (moment estimate) and ~181 (point estimate) individuals (95% CI = 72.02, 9000) (Table 10).

*M Ratio*

An M-critical and empirical M were calculated for two scenarios: a data input file excluding the Ei8 locus, and an input file including the Ei8 locus. In both cases, the empirical value of M was larger than the M-critical value. However, the empirical M and M-critical were much closer in the scenario excluding the Ei8 locus (Table 11).

**Table 10.**  $N_e$  Estimates from the MLNE Program for Age Class Data (1997, 1998, and 1999) and for Cohort Data.

$N_e$ Estimates	Moment Estimates	Point Estimates	95% Confidence Interval
1997 DATA	237.7	1953.45	45.48, 5000
<b>1998 Data</b>	Infinite	8564.42	35.64, 30000
<b>1999 Data</b>	214.73	4957.36	83.68, 5000
<b>Cohort Data</b>	28.21	180.34	72.02, 9000

**Table 11.** Scenarios Used to Obtain M-values for Microsatellite Data from Kemp's Ridley Population and Results (M-values).

Loci Included	Theta ( $\theta$ )	M-critical	M (empirical)
Cc117, Cm72, Cm84	19.49	0.670	0.683
Cc117, Cm72, Cm84, Ei8	57.18	0.696	0.747

**Table 12.** M Ratios (# of Alleles/Range of Alleles) for Loci Used in This Study

<b>Locus</b>	<b>M ratio</b>
Cc117	0.727
Cm72	0.385
Cm84	0.875
Ei8	1

Additionally, M ratio's were calculated for each locus (Table 12) with M\_P\_val.exe program, and these ratios were used to calculate and interloci variance ( $\sigma = 0.058$ ) using the standard formula for variance [ $\Sigma(X-\mu)^2 \div N$ ] (Sokal and Rohlf 1995).

## DISCUSSION

Three of four loci used in this study deviated from the expectations of Hardy-Weinberg equilibrium. Deviations from the Hardy-Weinberg equilibrium could indicate the effects of any of the following: Selection, migration, non-random mating structure (inbreeding), linkage, presence of null alleles and mutation. Selection pressure is unlikely to influence allele frequencies in microsatellite loci since microsatellite loci typically are non-coding regions not likely subject to selection. Migration can be ignored, since virtually this entire species consists of one population. Linkage can be ruled out as genotypic disequilibrium tests revealed that all four loci were independent from one another. Presence of null alleles also may lead to a heterozygote deficiency because homozygotes would not be scored and heterozygotes would be scored as homozygous individuals. Kichler (1996) obtained heterozygosity values for three of

four loci characterized in this study. She tested for heterozygote deficiency and found that all her values fit into Hardy-Weinberg expectations. Heterozygosity values for this study were lower than those obtained by Kichler for the same loci (Table 13). However, heterozygosity values (observed/n) calculated from Table 8, fluctuated among cohorts (1997, 1998, and 1999). Kichler evaluated heterozygosity based on samples from 211 nests laid during a single arribada. Assuming her sample was representative of the cohort recruiting the following year, the magnitude of the difference in heterozygosity values are in agreement with the changes in heterozygosity and allele frequency observed among cohorts in this study. Therefore, observed differences in heterozygosity between this study, and Kichler (1996) are most likely accounted for by temporal change in allele frequencies and not null alleles.

**Table 13.** Comparison of Observed Heterozygosity ( $H_o$ ) Values for This Study, with those Obtained by Kicher (1996).

<b>Locus</b>	<b><math>H_o</math> (Kichler 1996)</b>	<b><math>H_o</math> (This study)</b>
Cm72	0.49	0.547
Cm84	0.720	0.557
Ei8	0.708	0.579

Fitzsimmons (1995) offered another explanation for heterozygote deficiency in sea turtle microsatellite loci. She designed primers to test for polymorphism within sea turtle species and the persistence of microsatellites across species (excluding

*Lepidochelys kempii*). She observed that heterozygosity was higher in source species (those for which primers were designed) by comparing mean heterozygosity from non-source species with those of source species. Because primers for this study were taken from Fitzsimmons and were from non-source species, then perhaps primers designed for Kemp's ridley may have yielded higher levels of heterozygosity. However, it should be noted that although mean heterozygosity values at each locus were significantly different overall between source and non-source species (Fitzsimmons, 1995), some non-source species showed heterozygosity values comparable to those of the source species. For example, locus Cm72 had a heterozygosity value of 0.900 for both *Chelonia mydas* and *Lepidochelys olivacea*. Similarly, locus Cc117 (source species, *Caretta caretta*) had a heterozygosity value (0.791) lower than the non-source *Chelonia mydas*. Lastly, high mutation rates may affect expectations of Hardy-Weinberg equilibrium. This may apply to loci Ei8, where an estimated mutation rate of 0.0023 per generation has been derived, but not for the other two loci that deviated from Hardy-Weinberg equilibrium. Excluding Ei8, the highest mutation rate for any given locus used in this study was 0.00096 per generation. This translates into one mutant in 10,000 births per generation, a value unlikely to cause any deviation from expectations of Hardy-Weinberg equilibrium. Inbreeding is the most likely explanation for the heterozygote deficiency found in this study, and is supported by the demographic history of the species.

Temporal allele frequency data provided four very different estimates of  $N_e$  with extremely wide confidence intervals. The three estimates based on age classes were problematic due to small sample sizes. Although allele frequency data were obtained at

all four loci for most of the 215 individuals, resulting sample sizes were small when the data were assigned first into year classes and then again to three different age classes. For example, the year 1999 contained most individuals (110), but when divided into three age classes, each age class (post-pelagic, juvenile, and subadult) contained only 23, 54, and 28 individuals, respectively. Therefore, temporal data based on age classes for a certain year, were not a good approach to estimate  $N_e$  because much larger sample sizes are required.

The estimate of  $N_e$  based on size cohorts would likely be a more realistic measure of  $N_e$  for this study since larger sample sizes were available. The size cohorts for each year (1997, 98 and 99) comprised over half of the samples taken for that year and contained sample sizes of 41, 27 and 75 individuals, respectively. Although these samples sizes cannot be considered large, they are much larger than the sample sizes used for age class data and provide a more accurate estimate of  $N_e$  from temporal data.

The accuracy of this method depends on sample size, number of generations, and number of independent alleles. The pseudo-likelihood method would likely provide a more accurate measure of  $N_e$  for the Kemp's ridley population if these above factors were optimized. Optimizing these factors would require a greater number of polymorphic microsatellite loci, specifically designed for the Kemp's ridley, as well as more temporally spaced data samples. A suggested approach to obtain such temporal data would be to sample hatchlings for at least 2-3 seasons consecutively.

The results for the M ratio analysis indicated that, for both scenarios (excluding Ei8 locus and including Ei8), the empirical M values obtained did not fall below either of the

M critical values. Accordingly, neither of the empirical M values obtained in this study are significantly different from the expected value for a population at equilibrium according to the mutation rate and historical  $N_e$  defined. Garza and Williamson (2001) demonstrated that collecting data from more loci (seven or more) gives greater confidence to infer a reduction in effective population size from the M estimate. This is due to the fact that genetic drift is random and genetic losses are stochastic with regard to which loci were affected by a demographic bottleneck. Accordingly, this study may have characterized loci that were not affected as much as others. Furthermore, Garza and Williamson (2001) found a relationship between number of loci sampled and critical M value. They found that as one increased the number of loci sampled, critical M value increased as well. The fact that only 3 and 4 markers were used to obtain results for this method could have greatly impacted critical M values and, therefore, conclusions drawn from these results. As noted above, the M-critical and empirical M values for the scenario excluding the Ei8 locus were close to being significant (M-critical=0.670, empirical M=0.683).

Although neither empirical M fell below its critical M value, both empirical M values suggest some degree of historical reduction in effective size. Garza and Williamson (2001) calculated M ratios for various data sets compiled in the literature. In all cases, the M ratio for populations that had not suffered a reduction in size were greater than 0.82, and all populations that were known to have suffered a reduction in size were less than 0.70. The empirical M obtained by this study falls in between (0.747) these values and the other falls below (.683) that of wild populations at

equilibrium. This suggests that the population bottleneck by the Kemp's ridley in the second half of the twentieth century caused a reduction in the genetic diversity, but not to the degree of other populations surveyed by Garza and Williamson (2001).

Another factor affecting  $N_e$  that could possibly account for the empirical  $M$  is reproductive variance (unequal reproductive output from females to a cohort). A large reproductive variance translates into a smaller  $N_e$  as the survival of some nests or hatchlings (potential recruits) would be better and therefore biased. Accordingly, over several spawning events, those females with higher fitness (nest size or hatchling survival) could produce a great number of recruits. Thus, by having unequal contribution, the  $N_e$  of any new cohort would be smaller than the parental population since it is not a random representation of equal female contribution but rather a sub-sample of the parental population. In a very small population, such as the Kemp's ridley, this would translate into random loss of alleles ( $k$ ) through genetic drift. On the other hand, if the reproductive variance is small, any new cohort would be a representation of equal female contribution and loss of alleles through genetic drift would not be nearly as large. Accordingly, a small reproductive variance in the Kemp's ridley population could account for a larger  $M$  value than expected as the loss of alleles ( $k$ ), was relatively small considering the demographic history of the species.

Finally, results obtained from the  $M$  ratio method suggest that the inclusion of  $Ei8$  locus into  $M$  estimates is not appropriate because its high mutation rates negate any potential loss of alleles due to random genetic drift. While results for the  $M$  ratio as an indicator of reduction in  $N_e$  were not significant, most likely due to the small number of

loci sampled, it should be noted that this study sampled a large number of individuals and was able to offer some evidence of a reduced  $N_e$  from the empirical  $M$ . In order to obtain conclusive results for the  $M$  ratio, further studies must employ a larger number of polymorphic microsatellite loci.

Results of the indirect methods employed to detect a reduction in  $N_e$  taken together, provide evidence to support the hypothesis that the demographic history of this species had an impact on the genetic diversity of the population. Furthermore, the empirical estimate of  $N_e$  (~181 individuals) for the Kemp's ridley population was less than one order of magnitude smaller than the latest census estimate of breeding adults totaling 4500 individuals (Coyne 2000; Turtle Expert Working Group 2000). The  $N_e/N_c$  ratio was 0.04, which was lower its expected value (Frankham 1995; Hedgecock 1994).

Such reduced  $N_e$  for the single population of Kemp's ridley poses serious conservation implications due to its heightened vulnerability to environmental and epidemic events. A single catastrophic event affecting Rancho Nuevo beach during the height of the arribada could prove devastating. Additional efforts to imprint Kemp's ridleys to new nesting beaches, such as the program at Padre Island National Seashore (Shaver 2001), are essential to the avoid extinction of this species. The establishment of additional breeding populations would minimize the threat to the species in the event of a catastrophe. Furthermore, an estimate of  $N_e$  for this species allows for recommendations about a minimum viable population (MVP) size. Franklin (1980) suggested a minimum  $N_e$  of 50 to maintain short-term fitness (i.e. prevent inbreeding and its deleterious effects). He further recommended a minimum  $N_e$  of 500 to maintain

sufficient genetic variation for adaptation to changing environmental conditions. Using this latter MVP value and assuming that 0.04 is an accurate  $N_e/N_c$  ratio for the Kemp's ridley population, then a MVP should consist of 12,500 individuals. Finally, a  $N_e$  estimate can be used to calculate the proportion of original heterozygosity remaining after each generation ( $H$ ) for a population of breeding adults ( $N_e$ ), where:  $H = [1 - 1/(2N_e)]$ , and the proportion remaining after  $t$  generations ( $H_t$ ) is equal to:  $H_t = H^t$  (Wright 1969). For this data, substituting  $N_e = 181$  individuals gives an  $H$  of 0.997 % of original heterozygosity remaining after 1 generation. To calculate how much variation will remain in the next 1000 years, and assuming 10 years as generation time (Coyne 2000), then 100 generations later there would be:  $0.997^{100} = 0.758$ , or close to 25% of heterozygosity lost (assuming no mutation) in such relatively short period of time. However, since mutation rate for coding genes is assumed to be slow, a 25% loss of heterozygosity can be expected in the next 1000 years. These values calculated from the estimate of  $N_e$  for the Kemp's ridley, have consequences regarding the recovery plan for this species. A goal of this plan is a population of 10,000 nesting females by the year 2020, at which point the species could be down listed (USFWS and NMFS 1992). While this number is in agreement with the MVP estimate presented here, it would be useful to re-evaluate the target population with a  $N_e$  estimate that includes samples from Kemp's ridleys nesting in Texas, along the State of Veracruz, Mexico, and other satellite nesting beaches. Additionally, it is essential to maintain rigorous management recommendations listed under the recovery plan even after the Kemp's ridley is down listed, in light of the potential 25% heterozygosity loss over the next 1000 years.

In a survey of the levels of variation from microsatellite data, DeWoody and Avise (2000) found that in freshwater fish, heterozygosity values are much lower with mean heterozygosity ( $h=0.46$ ) and mean number of alleles per locus ( $a=7.5$ ) than in marine fishes where  $h=0.79$  and  $a=20.6$ , where as in non-piscine animals, values of  $h$  and  $a$  were  $0.58$  and  $7.1$ , respectively. Anadromous fish were intermediate to marine and freshwater fish ( $h=0.68$  and  $a=11.3$ ). Accordingly, Kemp's ridley data values ( $h=0.67$  and  $a=8.5$ ) are more similar to anadromous fishes, although heterozygosity levels in Kemp's ridleys must be lower today than prior to the bottleneck. It is also worthwhile to note that this study suggests that Kemp's ridley may not have experienced historical bottlenecks (prior to human detrimental activities) which affected other sea turtle species such as the loggerhead (*Caretta caretta*) starting 10,000 years ago (Hatase et al. 2002).

Further studies should attempt to gather temporally spaced samples from hatchlings in order to detect fluctuations in allele frequency over time in order to provide better estimates of  $N_e$ . More importantly, future studies must survey a greater number of polymorphic microsatellite loci designed specifically for the Kemp's ridley sea turtle to obtain more accurate confidence limits around the estimate of  $N_e$ .

## CONCLUSIONS

It was demonstrated that blood tissue consistently yields the highest quality of DNA for genetic studies on sea turtles. Additionally, it was found that archival plasma samples are also a good source of nDNA. This is particularly important since archival samples for toxicological studies suddenly become reservoirs of valuable information for the study of population genetics of sea turtles. However, the results also show that when dealing with very small individuals or when working in field situations, the hind flipper tissue-biopsy-technique developed for this study is the method of choice. This method in addition to being minimally invasive is safe for both the animal and the handler and provides high quality DNA for genetic studies.

Results of the indirect methods employed to detect a reduction in  $N_e$  (effective population size) taken together, provide evidence to support the hypothesis that the demographic history of this species had an impact on the genetic diversity of this population. Furthermore, the empirical estimate of  $N_e$  (~181 individuals) for the Kemp's ridley population was less than one order of magnitude smaller than the latest census estimate of breeding adults.

Such reduced  $N_e$  for the single population of Kemp's ridley poses serious conservation implications due to its heightened vulnerability to environmental and epidemic events. A single catastrophic event affecting Rancho Nuevo beach during the height of the arribada could prove devastating. Additional efforts to imprint Kemp's ridleys to new nesting beaches, such as the program at Padre Island National Seashore are essential to the avoid extinction of this species. The establishment of additional

breeding populations would minimize the threat to the species in the event of a catastrophe. Furthermore, an estimate of  $N_e$  for this species allows for recommendations about a minimum viable population (MVP) size. Finally, a  $N_e$  estimate can be used to calculate the proportion of original heterozygosity remaining after each generation ( $H$ ) for a population of breeding adults ( $N_e$ ). These values calculated from the estimate of  $N_e$  for the Kemp's ridley, have consequences regarding the recovery plan for this species.

Further studies should attempt to gather temporally spaced samples from hatchlings in order to detect fluctuations in allele frequency over time in order to provide better estimates of  $N_e$ . More importantly, future studies must survey a greater number of polymorphic microsatellite loci designed specifically for the Kemp's ridley sea turtle to obtain more accurate confidence limits around the estimate of  $N_e$ .

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APPENDIX A

Capture Locality and Blood Sampling Information for Kemp's Ridleys provided by the Sea Turtle and Fisheries Ecology Lab at Texas A&M University at Galveston. Sample # = Turtle Identification Number, SCL = Standard Carapace Length.

Sample #	Year	Location of Capture	Month of Capture	Type of Sample	SCL (cm)	Age	Predicted sex
SP97-5-2	1997	Sabine Pass	May	Blood	28	post pelagic	female
SP97-5-3	1997	Sabine Pass	May	Blood	60.5	adult	female
SP97-5-4	1997	Sabine Pass	May	Blood	31.3	juvenile	female
SP97-5-5	1997	Sabine Pass	May	Blood	33.1	juvenile	female
SP97-5-6	1997	Sabine Pass	May	Blood	43.6	subadult	
SP97-5-8	1997	Sabine Pass	May	Blood	29.3	post pelagic	
SP97-5-9	1997	Sabine Pass	May	Blood	59.4	subadult	female
SP97-5-10	1997	Sabine Pass	May	Blood	31.7	juvenile	
SP97-5-11	1997	Sabine Pass	May	Blood	59.1	subadult	male
SP97-5-12	1997	Sabine Pass	May	Blood	57	subadult	female
SP97-5-13	1997	Sabine Pass	May	Blood	29.4	post pelagic	
SP97-5-15	1997	Sabine Pass	May	Blood	54.3	subadult	female
SP97-5-16	1997	Sabine Pass	May	Blood	32.9	juvenile	
SP97-5-17	1997	Sabine Pass	May	Blood	39.7	juvenile	
SP97-5-18	1997	Sabine Pass	May	Blood	29.1	post pelagic	
SP97-5-19	1997	Sabine Pass	May	Blood	31.6	juvenile	
SP97-5-20	1997	Sabine Pass	May	Blood	26.6	post pelagic	
SP97-5-21	1997	Sabine Pass	May	Blood	32.4	juvenile	
SP97-5-22	1997	Sabine Pass	May	Blood	30	juvenile	female
SP97-5-23	1997	Sabine Pass	May	Blood	34.5	juvenile	
SP97-5-24	1997	Sabine Pass	May	Blood	30.3	juvenile	female
SP97-5-25	1997	Sabine Pass	May	Blood	29.2	post pelagic	
SP97-5-27	1997	Sabine Pass	May	Blood	49.7	subadult	female

SP97-5-28 1997	Sabine Pass	May	Blood	59.9	subadult	female
SP97-5-29 1997	Sabine Pass	May	Blood	30.6	juvenile	female
SP97-5-30 1997	Sabine Pass	May	Blood	32.3	juvenile	female
SP97-5-31 1997	Sabine Pass	May	Blood	26.8	post pelagic	
SP97-5-32 1997	Sabine Pass	May	Blood	31.1	juvenile	
SP97-5-33 1997	Sabine Pass	May	Blood	27.1	post pelagic	
SP97-5-34 1997	Sabine Pass	May	Blood	24.9	post pelagic	
PL96-7-3 1996	Port Lavaca	July	Blood	44.8	subadult	
SP97-6-1 1997	Sabine Pass	June	Blood	29.9	post pelagic	
SP97-6-10 1997	Sabine Pass	June	Blood	36.4	juvenile	
SP97-6-11 1997	Sabine Pass	June	Blood	31.2	juvenile	
SP97-6-12 1997	Sabine Pass	June	Blood	24.5	post pelagic	
SP97-6-13 1997	Sabine Pass	June	Blood	36	juvenile	
SP97-6-14 1997	Sabine Pass	June	Blood			female
SP97-6-15 1997	Sabine Pass	June	Blood	32.1	juvenile	
SP97-6-16 1997	Sabine Pass	June	Blood	32.7	juvenile	
SP97-6-17 1997	Sabine Pass	June	Blood	53.2	subadult	
SP97-6-18 1997	Sabine Pass	June	Blood	32.6	juvenile	
SP97-6-19 1997	Sabine Pass	June	Blood	56	subadult	female
SP97-6-20 1997	Sabine Pass	June	Blood	51.8	subadult	male
SP97-6-21 1997	Sabine Pass	June	Blood	60.7	adult	female
SP97-6-22 1997	Sabine Pass	June	Blood	34.9	juvenile	
SP97-6-23 1997	Sabine Pass	June	Blood	52.8	subadult	male
SP97-6-24 1997	Sabine Pass	June	Blood	54.1	subadult	male
SP97-6-25 1997	Sabine Pass	June	Blood	32.6	juvenile	male
SP97-6-26 1997	Sabine Pass	June	Blood	55.2	subadult	female
SP97-6-27 1997	Sabine Pass	June	Blood	59.2	subadult	male
SP97-6-28 1997	Sabine Pass	June	Blood	46.9	subadult	
SP97-6-29 1997	Sabine Pass	June	Blood	29.2	post pelagic	
SP97-6-30 1997	Sabine Pass	June	Blood	30.8	juvenile	
SP97-6-31 1997	Sabine Pass	June	Blood	30.8	juvenile	
SP97-6-32 1997	Sabine Pass	June	Blood	34.6	juvenile	

SP97-7-4	1997	Sabine Pass	July	Blood	48.7	subadult
SP97-7-5	1997	Sabine Pass	July	Blood	55.8	subadult
SP97-7-7	1997	Sabine Pass	July	Blood	56.1	subadult
SP97-7-8	1997	Sabine Pass	July	Blood	32.3	juvenile
SP97-7-9	1997	Sabine Pass	July	Blood	33	juvenile
SP97-7-11	1997	Sabine Pass	July	Blood	37.9	juvenile
SP97-7-12	1997	Sabine Pass	July	Blood	35.4	juvenile
SP97-7-13	1997	Sabine Pass	July	Blood	25	post pelagic
SP97-7-16	1997	Sabine Pass	July	Blood	21.2	post pelagic
SP97-7-17	1997	Sabine Pass	July	Blood	35.2	juvenile
SP97-7-18	1997	Sabine Pass	July	Blood	33.2	juvenile
SP97-7-19	1997	Sabine Pass	July	Blood	33	juvenile
SP97-7-20	1997	Sabine Pass	July	Blood	31.6	juvenile
SP97-7-21	1997	Sabine Pass	July	Blood	37.7	juvenile
SP97-7-22	1997	Sabine Pass	July	Blood	30.6	juvenile
SP97-7-23	1997	Sabine Pass	July	Blood	38.3	juvenile
SP97-8-1	1997	Sabine Pass	July	Blood	49.2	subadult
SP97-8-2	1997	Sabine Pass	July	Blood	46.5	subadult
SP97-8-3	1997	Sabine Pass	July	Blood	45.6	subadult
SP97-7-4	1997	Sabine Pass	July	Blood	32.8	juvenile
SP97-7-5	1997	Sabine Pass	July	Blood	47.1	subadult
SP98-5-1	1998	Sabine Pass	May	Blood	36.9	juvenile
SP98-5-2	1998	Sabine Pass	May	Blood	27.2	post pelagic
SP98-5-3	1998	Sabine Pass	May	Blood	27.6	post pelagic
SP98-5-4	1998	Sabine Pass	May	Blood	27.7	post pelagic
SP98-5-5	1998	Sabine Pass	May	Blood	51.9	subadult
SP98-5-6	1998	Sabine Pass	May	Blood	29.9	post pelagic
SP98-5-7	1998	Sabine Pass	May	Blood	30.7	juvenile
SP98-5-8	1998	Sabine Pass	May	Blood	32.7	juvenile
SP98-5-9	1998	Sabine Pass	May	Blood	30.6	juvenile
SP98-5-10	1998	Sabine Pass	May	Blood	30.5	juvenile
SP98-5-11	1998	Sabine Pass	May	Blood	21.6	post pelagic

SP98-5-12 1998	Sabine Pass	May	Blood	44.7	subadult
SP98-5-13 1998	Sabine Pass	May	Blood	31.1	juvenile
SP98-5-15 1998	Sabine Pass	May	Blood	31.7	juvenile
SP98-5-16 1998	Sabine Pass	May	Blood	31.1	juvenile
SP98-5-17 1998	Sabine Pass	May	Blood	56.2	subadult
SP98-5-18 1998	Sabine Pass	May	Blood	41.9	subadult
SP98-5-19 1998	Sabine Pass	May	Blood	39.8	juvenile
SP98-5-20 1998	Sabine Pass	May	Blood	32.7	juvenile
SP98-5-21 1998	Sabine Pass	May	Blood	31	juvenile
SP98-5-22 1998	Sabine Pass	May	Blood	31.5	juvenile
SP98-5-24 1998	Sabine Pass	May	Blood	29.4	post pelagic
SP98-5-25 1998	Sabine Pass	May	Blood	28.3	post pelagic
SP98-5-26 1998	Sabine Pass	May	Blood	27.9	post pelagic
SP98-5-27 1998	Sabine Pass	May	Blood	32.8	juvenile
SP98-5-28 1998	Sabine Pass	May	Blood	22.4	post pelagic
SP98-5-29 1998	Sabine Pass	May	Blood	29.4	post pelagic
SP98-5-30 1998	Sabine Pass	May	Blood	46.3	subadult
SP98-5-33 1998	Sabine Pass	May	Blood	41.2	subadult
C98-6-1 1998	Calcasieu	June	Blood	30.5	juvenile
C98-6-2 1998	Calcasieu	June	Blood	31	juvenile
C98-6-4 1998	Calcasieu	June	Blood	41.3	subadult
C98-6-5 1998	Calcasieu	June	Blood	32.5	juvenile
SP98-6-1 1998	Sabine Pass	June	Blood	49	subadult
C98-7-1 1998	Calcasieu	July	Blood	33.3	juvenile
C98-7-2 1998	Calcasieu	July	Blood	31.5	juvenile
C98-7-3 1998	Calcasieu	July	Blood	35	juvenile
C98-7-4 1998	Calcasieu	July	Blood	56.4	subadult
C98-8-1 1998	Calcasieu	August	Blood	25	post pelagic
SP98-8-1 1998	Calcasieu	August	Blood	36.3	juvenile
M99-5-1 1999	Matagorda	May	Blood	28.3	post pelagic
M99-5-2 1999	Matagorda	May	Blood	43	subadult
M99-5-3 1999	Matagorda	May	Blood	39.8	juvenile

male

M99-5-4	1999	Matagorda	May	Blood	36.3	juvenile
M99-5-5	1999	Matagorda	May	Blood	56.3	subadult
M99-5-6	1999	Matagorda	May	Blood	59.6	subadult
C99-5-1	1999	Calcasieu	May	Blood	30.6	juvenile
C99-5-2	1999	Calcasieu	May	Blood	28.4	post pelagic
C99-5-3	1999	Calcasieu	May	Blood	32.1	juvenile
C99-5-4	1999	Calcasieu	May	Blood	58.1	subadult
C99-5-5	1999	Calcasieu	May	Blood	50.6	subadult
C99-5-6	1999	Calcasieu	May	Blood	33.6	juvenile
C99-5-7	1999	Calcasieu	May	Blood	40	subadult
C99-5-8	1999	Calcasieu	May	Blood	36.6	juvenile
C99-5-9	1999	Calcasieu	May	Blood	31.8	juvenile
SP99-5-1	1999	Sabine Pass	May	Blood	31.2	juvenile
SP99-5-2	1999	Sabine Pass	May	Blood	28.3	post pelagic
SP99-5-3	1999	Sabine Pass	May	Blood	33.5	juvenile
SP99-5-4	1999	Sabine Pass	May	Blood	22.1	post pelagic
SP99-5-5	1999	Sabine Pass	May	Blood	29.4	post pelagic
SP99-5-6	1999	Sabine Pass	May	Blood	35.4	juvenile
SP99-5-7	1999	Sabine Pass	May	Blood	30.7	juvenile
MR99-6-1	1999	Matagorda	June	Blood	31.2	juvenile
C99-6-1	1999	Calcasieu	June	Blood	35.4	juvenile
C99-6-2	1999	Calcasieu	June	Blood	32.9	juvenile
C99-6-3	1999	Calcasieu	June	Blood	53.7	subadult
C99-6-4	1999	Calcasieu	June	Blood	60.6	adult
C99-6-5	1999	Calcasieu	June	Blood	35.9	juvenile
C99-6-6	1999	Calcasieu	June	Blood	56	subadult
C99-6-7	1999	Calcasieu	June	Blood	23.9	post pelagic
C99-6-8	1999	Calcasieu	June	Blood	34.6	juvenile
C99-6-9	1999	Calcasieu	June	Blood	44.2	subadult
C99-6-10	1999	Calcasieu	June	Blood	38.1	juvenile
C99-6-11	1999	Calcasieu	June	Blood	34.6	juvenile
M99-7-1	1999	Matagorda	July	Blood	46.3	subadult

M99-7-2	1999	Matagorda	July	Blood	37.5	juvenile
M99-7-3	1999	Matagorda	July	Blood	37.7	juvenile
M99-7-4	1999	Matagorda	July	Blood	35.4	juvenile
M99-7-5	1999	Matagorda	July	Blood	26.8	post pelagic
C99-7-1	1999	Calcasieu	July	Blood	34.9	juvenile
SP99-7-1	1999	Sabine Pass	July	Blood	36	juvenile
M99-8-1	1999	Matagorda	August	Blood	28.9	post pelagic
M99-8-2	1999	Matagorda	August	Blood	48.6	subadult
M99-8-3	1999	Matagorda	August	Blood	28.2	post pelagic
M99-8-4	1999	Matagorda	August	Blood	54.9	subadult
M99-8-5	1999	Matagorda	August	Blood	26	post pelagic
M99-8-6	1999	Matagorda	August	Blood	55.1	subadult
M99-8-7	1999	Matagorda	August	Blood	26.7	post pelagic
M99-8-8	1999	Matagorda	August	Blood	27.6	post pelagic
M99-8-9	1999	Matagorda	August	Blood	50.5	subadult
M99-8-10	1999	Matagorda	August	Blood	34.5	juvenile
M99-8-11	1999	Matagorda	August	Blood	38.5	juvenile
M99-8-12	1999	Matagorda	August	Blood	29.6	post pelagic
C99-8-1	1999	Calcasieu	August	Blood	46.5	subadult
C99-8-2	1999	Calcasieu	August	Blood	25.7	post pelagic
C99-8-3	1999	Calcasieu	August	Blood	58.6	subadult
C99-8-4	1999	Calcasieu	August	Blood	34.8	juvenile
C99-8-5	1999	Calcasieu	August	Blood	56.4	subadult
C99-8-6	1999	Calcasieu	August	Blood	35.8	juvenile
C99-8-7	1999	Calcasieu	August	Blood	42.9	subadult
C99-8-8	1999	Calcasieu	August	Blood	27.4	post pelagic
C99-8-9	1999	Calcasieu	August	Blood	42.2	subadult
C99-8-10	1999	Calcasieu	August	Blood	27.2	post pelagic
C99-8-11	1999	Calcasieu	August	Blood	24.3	post pelagic
C99-8-12	1999	Calcasieu	August	Blood	54	subadult
C99-8-13	1999	Calcasieu	August	Blood	40.9	subadult
C99-8-14	1999	Calcasieu	August	Blood	37.5	juvenile

C99-8-15	1999	Calcasieu	August	Blood	38.2	juvenile
C99-8-16	1999	Calcasieu	August	Blood	42	subadult
SP99-8-1	1999	Sabine Pass	August	Blood	27	post pelagic
SP99-8-2	1999	Sabine Pass	August	Blood	39.8	juvenile
SP99-8-3	1999	Sabine Pass	August	Blood	30.7	juvenile
M99-9-1	1999	Matagorda	September	Blood	30.2	juvenile
M99-9-2	1999	Matagorda	September	Blood	32.4	juvenile
M99-9-3	1999	Matagorda	September	Blood	35.8	juvenile
M99-9-4	1999	Matagorda	September	Blood	41.9	subadult
M99-9-5	1999	Matagorda	September	Blood	59.6	subadult
M99-9-6	1999	Matagorda	September	Blood	29	post pelagic
C99-9-1	1999	Calcasieu	September	Blood	31.9	juvenile
C99-9-2	1999	Calcasieu	September	Blood	31.9	juvenile
C99-9-3	1999	Calcasieu	September	Blood	32	juvenile
C99-9-4	1999	Calcasieu	September	Blood	29.5	post pelagic
C99-9-5	1999	Calcasieu	September	Blood	31.4	juvenile
C99-9-6	1999	Calcasieu	September	Blood	34.1	juvenile
C99-9-7	1999	Calcasieu	September	Blood	28.3	post pelagic
C99-9-8	1999	Calcasieu	September	Blood	39.8	juvenile
C99-9-9	1999	Calcasieu	September	Blood	31.1	juvenile
C99-9-10	1999	Calcasieu	September	Blood	37.5	juvenile
C99-9-11	1999	Calcasieu	September	Blood	26.6	post pelagic
C99-9-12	1999	Calcasieu	September	Blood	29.9	post pelagic
C99-9-13	1999	Calcasieu	September	Blood	31	juvenile
C99-9-14	1999	Calcasieu	September	Blood	31.9	juvenile
C99-9-15	1999	Calcasieu	September	Blood	30.9	juvenile
C99-9-16	1999	Calcasieu	September	Blood	42.9	subadult
C99-9-17	1999	Calcasieu	September	Blood	32.9	juvenile
C99-9-18	1999	Calcasieu	September	Blood	41.5	subadult
C99-9-19	1999	Calcasieu	September	Blood	32.2	juvenile
C99-9-20	1999	Calcasieu	September	Blood	42	subadult
C99-9-21	1999	Calcasieu	September	Blood	39.6	juvenile

C99-9-22	1999	Calcasieu	September	Blood	28.6	post pelagic
C99-9-23	1999	Calcasieu	September	Blood	32.9	juvenile
C99-9-24	1999	Calcasieu	September	Blood	41.6	subadult
C99-9-25	1999	Calcasieu	September	Blood	30.5	juvenile
C99-10-1	1999	Calcasieu	September	Blood	32.5	juvenile
C99-10-2	1999	Calcasieu	September	Blood	31.2	juvenile
C99-10-3	1999	Calcasieu	September	Blood	32.5	juvenile
C99-10-4	1999	Calcasieu	September	Blood	31.8	juvenile
C99-10-5	1999	Calcasieu	September	Blood	30.8	juvenile
C99-10-6	1999	Calcasieu	September	Blood	33.6	juvenile
C99-10-7	1999	Calcasieu	September	Blood	39.2	juvenile
C99-10-8	1999	Calcasieu	September	Blood	41.3	subadult
C99-10-9	1999	Calcasieu	September	Blood	35.5	juvenile
C99-10-10	1999	Calcasieu	September	Blood	40.6	subadult
C99-10-11	1999	Calcasieu	September	Blood	30.3	juvenile
C99-10-12	1999	Calcasieu	September	Blood	45.3	subadult
C99-10-13	1999	Calcasieu	September	Blood	29	post pelagic
C99-10-14	1999	Calcasieu	September	Blood	31.7	juvenile

## APPENDIX B

Raw Data for Allele (Base Pairs) Scoring for Each Successfully Amplified Kemp's ridley Blood Sample at each of the Four Microsatellite Loci. An Empty Space Means that the Allele Size/s could not be Read From the GENESCAN Software.

<b>Individual</b>	<b>Cc117 Microsatellite loci</b>	<b>Cm84 Microsatellite loci</b>	<b>Ei8 Microsatellite loci</b>	<b>Cm72 Microsatellite loci</b>
<b>SP97-5-2</b>	186 198	320 332	164 170	226
<b>SP97-5-3</b>	198 200	312	170	226
<b>SP97-5-4</b>	198	312 324	164 170	239
<b>SP97-5-5</b>	198 200	312 318	170 172	239 241
<b>SP97-5-8</b>	198	314 324	172	226 236
<b>SP97-5-9</b>	186 192	330 332	170	226 239
<b>SP97-5-10</b>	194 198	312 320	164	226 241
<b>SP97-5-11</b>	198	312	164	226
<b>SP97-5-12</b>	194 200	312 316	170 172	224
<b>SP97-5-13</b>	186 198	312	170	226 239
<b>SP97-5-15</b>	186 198	312	164 172	224 226
<b>SP97-5-16</b>	186 198	312 334	164	226
<b>SP97-5-17</b>	198	314 332	164 170	226 239
<b>SP97-5-18</b>	186	330 336	164 170	226 239
<b>SP97-5-19</b>	200	330 336	164 170	226 239
<b>SP97-5-20</b>	200	312	170	226
<b>SP97-5-21</b>	186 198	312	170	226 239
<b>SP97-5-22</b>	186 198	320 330	164	226 239
<b>SP97-5-23</b>	200	312	170	239
<b>SP97-5-24</b>	186 198	332	172	226 239
<b>SP97-5-25</b>	186 198	320 332	164	239
<b>SP97-5-27</b>	200	312	164 170	226 239
<b>SP97-5-28</b>	198 200	320 332	170 172	226
<b>SP97-5-29</b>	186 200	332	164 170	226
<b>SP97-5-30</b>	198	312 332	164 172	239 241
<b>SP97-5-31</b>	186 198	332	172	226 241
<b>SP97-5-32</b>	194 198	312	164 170	226 239

<b>SP97-5-33</b>	198	312	164 172	226
<b>SP97-5-34</b>	198 200	312	164	226
<b>PL96-7-3</b>	194 198	312 314	162 172	239
<b>SP97-6-1</b>	198	320 332	164	239 241
<b>SP97-6-10</b>	186 198	312 332	170 172	226
<b>SP97-6-11</b>	186 200	312	164 170	226 239
<b>SP97-6-12</b>	186 198	312 336	170 172	226
<b>SP97-6-13</b>	198	312 324	164 170	216 226
<b>SP97-6-14</b>	194 198	312 324	164 172	226
<b>SP97-6-15</b>	194 200	312 320	164 172	226 239
<b>SP97-6-17</b>	196 198	312	170	239 241
<b>SP97-6-18</b>	198	320 332	164 170	239 241
<b>SP97-6-19</b>	186 200	312	164 172	226 239
<b>SP97-6-20</b>	186 200	312 332	170 172	226 241
<b>SP97-6-21</b>	200	312	170	226
<b>SP97-6-22</b>	186 200	312	164 170	226 239
<b>SP97-6-23</b>	186 200	332 336	164	226 239
<b>SP97-6-24</b>	186 200	312	172	226
<b>SP97-6-25</b>	198	312 332	164 170	226 239
<b>SP97-6-27</b>	198 206	312 330	164 170	226
<b>SP97-6-28</b>	198	312 330	170	226
<b>SP97-6-29</b>	186 200	320 332	172	226
<b>SP97-6-30</b>	196 200	312	164	226 239
<b>SP97-6-31</b>	200 206	312 334	170 172	226 239
<b>SP97-6-32</b>	198 200	312 330	164 170	226 239
<b>SP97-7-4</b>	186 200	320 332	164	226 239
<b>SP97-7-7</b>	200 206	320	170	239
<b>SP97-7-8</b>	200 206	312 334	172	226
<b>SP97-7-11</b>	186 198	312 332	170 172	226
<b>SP97-7-12</b>	188 200	312 330	170 172	226 239
<b>SP97-7-13</b>	198 200	312 332	170	225 239
<b>SP97-7-17</b>	186 200	312 320	170 172	226 239
<b>SP97-7-18</b>	186 200	332	170	239 241
<b>SP97-7-20</b>	200	312 332	170	241
<b>SP97-7-21</b>			170	
<b>SP97-7-23</b>	186 198	312	164 172	239 241
<b>SP97-8-2</b>	198 200	330 332	170	226 239
<b>SP97-8-3</b>	198 200	312 324	164 172	226

<b>SP97-8-4</b>	186 198	320 324	170 172	226 239
<b>SP97-8-5</b>	198 200	312	164 172	239
<b>SP98-5-1</b>	200			226 239
<b>SP98-5-2</b>	200	312 330	164 172	239
<b>SP98-5-3</b>	200	312 332	170	226 239
<b>SP98-5-4</b>	188 200	312	170 172	226 241
<b>SP98-5-5</b>	198 200	312	164 170	226, 241
<b>SP98-5-6</b>	186 200	324 332	170	226 239
<b>SP98-5-7</b>	198 200	312	164 170	226 239
<b>SP98-5-8</b>	198 206	328 332	172	226 241
<b>SP98-5-9</b>	186 198	324 330	164 170	226 239
<b>SP98-5-10</b>	186 198	324 334	170 172	226
<b>SP98-5-11</b>	186 200	320 334	164 170	226 239
<b>SP98-5-12</b>	186 198	312	164 172	226 239
<b>SP98-5-13</b>	188 200	312		239
<b>SP98-5-15</b>	198 200	312	164 170	226
<b>SP98-5-16</b>	196 200	312 320	166	239
<b>SP98-5-17</b>	188 200	312	164	226 239
<b>SP98-5-18</b>	196 198	312	164 172	239
<b>SP98-5-19</b>	186 198	332	164 172	226 239
<b>SP98-5-20</b>	198 200	312	166 170	239
<b>SP98-5-21</b>	198	312	164	226
<b>SP98-5-22</b>	186 200	322	170	226
<b>SP98-5-24</b>	186 200	312	164 172	239 241
<b>SP98-5-25</b>	200	312 324	164 172	239 241
<b>SP98-5-26</b>	186 200	312	172	226
<b>SP98-5-27</b>	192 200	312 324	164	226 241
<b>SP98-5-28</b>	186 200	334	164	226 241
<b>SP98-5-29</b>	186 198	312 320	166 170	226 239
<b>SP98-5-30</b>	198 200	324	164	239
<b>SP98-5-33</b>	200	314 332	164 170	226 241
<b>C98-6-1</b>	198 200	312	166	239
<b>C98-6-2</b>	200	312	170	226 239
<b>C98-6-4</b>	186 198	330 332	164 170	226 241
<b>C98-6-5</b>	198 200			226 241
<b>C98-6-6</b>	186 200	328	164 172	226 239
<b>SP98-6-1</b>	200	312	164 170	226 239
<b>C98-7-1</b>	186 200	312 332	172	226 239

<b>C98-7-2</b>	198 200	312 332	164	226
<b>C98-7-3</b>	198 200	312	164 170	226 239
<b>C98-8-1</b>	198	332	170 172	226
<b>SP98-8-1</b>	198	312	170	226
<b>M99-5-1</b>	198 200	330 332	170	239
<b>M99-5-2</b>	200	312 320	164 170	226
<b>M99-5-5</b>	186 198	320 332	170 172	239 241
<b>M99-5-6</b>	200	320 332	164 170	226 239
<b>C99-5-1</b>	186 200	314 324	164 172	226 239
<b>C99-5-2</b>	186 198	312 330	170	226
<b>C99-5-3</b>	198 200	314 332	164 170	226
<b>C99-5-4</b>	200	312 320	170	226
<b>C99-5-5</b>	186 200	332 336	164	226 239
<b>C99-5-6</b>	200	312 324	168 170	226
<b>C99-5-7</b>	198 200	312	164	226
<b>C99-5-8</b>	186 200	312 324	170	239
<b>C99-5-9</b>	186 200	312	164 170	226
<b>SP99-5-1</b>	198 200	312	164 170	226 239
<b>SP99-5-2</b>	186 200	312	164	226
<b>SP99-5-3</b>	200	312 320	164 170	226
<b>SP99-5-4</b>	186 200	312 334	164	226 241
<b>SP99-5-5</b>	200	312	164 170	226 239
<b>SP99-5-6</b>	198 200	312	164 172	239
<b>SP99-5-7</b>	186 200	312 332	164 172	226 239
<b>M99-6-1</b>	198 200	324 334	170 172	226
<b>C99-6-1</b>	198 200	322 324	164 172	226
<b>C99-6-2</b>	194 200	312 332	164 170	226
<b>C99-6-3</b>	200	312	170	226 241
<b>C99-6-4</b>	186 198	324	166 170	226 239
<b>C99-6-5</b>	198 200	312	166 170	239 241
<b>C99-6-6</b>	198	312	164	226 239
<b>C99-6-7</b>	186 198	312 324	164 166	226
<b>C99-6-8</b>	186 198	312 334	164 170	226 239
<b>C99-6-10</b>	186 198	312 334	170 172	226 241
<b>C99-6-11</b>	188 198	314 330	164 170	226 239
<b>M99-7-1</b>	198 200	324	170	226 239
<b>M99-7-2</b>	200	312 330	164 170	226
<b>M99-7-3</b>	188 198	312 320	170	226

<b>M99-7-4</b>	186 198	330 332	164 170	226 239
<b>M99-7-5</b>	198	312 332	164 170	226
<b>C99-7-1</b>	198 200	314 332	164 170	226
<b>SP99-7-1</b>	196 198	312	170	226
<b>M99-8-1</b>	188 198	312	164 172	226 241
<b>M99-8-2</b>	198 200	312 330	164	226
<b>M99-8-3</b>	198	312 324	164 166	226 239
<b>M99-8-4</b>	198	312 336	170	226 239
<b>M99-8-5</b>	198 200	334	166	226 239
<b>M99-8-6</b>	198	320	170	226
<b>M99-8-7</b>	200	312 330	166	226 239
<b>M99-8-8</b>	198	312	166 172	226
<b>M99-8-9</b>	188 200	334	166 170	239 241
<b>M99-8-10</b>	198 200	312	170	226 239
<b>M99-8-11</b>	200	332	170 172	226 239
<b>M99-8-12</b>	186 198	312 320	166 170	241
<b>C99-8-1</b>	198 200	330 334	164	226
<b>C99-8-2</b>	198	330	170 172	226 239
<b>C99-8-4</b>	198 200	332 342	170 172	226
<b>C99-8-5</b>	198	332 342	164 170	226
<b>C99-8-6</b>	188 198	330 332	164 172	224
<b>C99-8-7</b>	186 198	312	164 172	239 241
<b>C99-8-8</b>	198 200	312 332	164 172	226 239
<b>C99-8-9</b>	200	312	170 172	226
<b>C99-8-10</b>	198 200	324 342	164 170	226
<b>C99-8-11</b>	198	332	164 170	226 239
<b>C99-8-12</b>	198 200	312	170	226 241
<b>C99-8-13</b>	198 200	312	170 172	239
<b>C99-8-15</b>	198	312	170	226 239
<b>C99-8-16</b>	186 198	330 332	164 172	226
<b>SP99-8-1</b>	198	330	162	226 241
<b>SP99-8-2</b>	198 200	314 332	164 172	226
<b>SP99-8-3</b>	194 198	330	170	226 239
<b>M99-9-1</b>	198	312	170 172	226 239
<b>M99-9-3</b>	198	312	164	226 241
<b>M99-9-4</b>	198 200	324 332	172	226 241
<b>M99-9-5</b>	198 200	320 332	170 172	226 241
<b>M99-9-6</b>	198 200	312 337	170 172	226

<b>C99-9-1</b>	200	320	164 170	226 239
<b>C99-9-2</b>	198 200	312 334	164 172	226
<b>C99-9-3</b>	198 200	330	172	226 239
<b>C99-9-4</b>	186 198	314 332	170 172	226
<b>C99-9-5</b>	196 198	330 332	164	239
<b>C99-9-6</b>	198 200	312	170	226
<b>C99-9-7</b>	198 200	312	164 170	226
<b>C99-9-8</b>	198	328	172	226
<b>C99-9-9</b>	198 200	320 330	170	226 239
<b>C99-9-10</b>	198 200	312 324	162 170	226
<b>C99-9-11</b>	196 198	312 332	170 172	226 239
<b>C99-9-12</b>	200	314 324	164 170	226
<b>C99-9-13</b>	200 206	334 340	164 172	241
<b>C99-9-14</b>	196 198	312 330	164	239 241
<b>C99-9-15</b>	198 200	314 328	164	226 239
<b>C99-9-16</b>	198	312	164 168	226 241
<b>C99-9-17</b>	186 198	312 330	170 172	226
<b>C99-9-18</b>	198	312	164 172	239 241
<b>C99-9-19</b>	198	312 330	164 170	226 239
<b>C99-9-20</b>	186 198	316 330	164 170	226
<b>C99-9-21</b>	186 200	312	170 172	239 241
<b>C99-9-23</b>	186 198	332	164 170	226
<b>C99-9-24</b>	198	324	170	226
<b>C99-9-25</b>	198	330	164	226 239
<b>C99-10-1</b>	196 198	314 334	170	226
<b>C99-10-2</b>	186 198	312		226 239
<b>C99-10-3</b>	194 198	312 332	170 172	239 241
<b>C99-10-5</b>	198 200	312	170 172	226 241
<b>C99-10-6</b>	198 200	320 330	170	239
<b>C99-10-7</b>	198 200	312	172	226
<b>C99-10-8</b>	198	312 324	166	226
<b>C99-10-9</b>	198 200	312 332	164 172	226 241
<b>C99-10-10</b>	186 200	312	168 170	226 239
<b>C99-10-14</b>	198	314 328	170	226

## APPENDIX C

Allele Frequency Data for the Temporal Change in Allele Frequency Method (MLNE program) from Age Class Data. Bp = base pairs.

**1997 Allele Frequency Data**

*Post-Pelagic (n = 26 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.301	0	0	0	0	0.500	0.192	0

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.654	0.269	0.077

## Cm84 Locus

Allele (bp)	312	314	316	318	320	322	324
Frequency	0.385	0.039	0	0	0.154	0	0.039

Allele (bp)	328	330	332	334	336	337	340	342
Frequency	0	0.039	0.269	0	0.077	0	0	0

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.346	0	0	0.346	0.308

*Juveniles (n = 62 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.210	0.016	0	0.048	0.016	0.387	0.290	0.032

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0.016	0	0.452	0.403	0.129

## Cm84 Locus

Allele (bp)	312	314	316	318	320	322	324
Frequency	0.484	0.016	0	0.016	0.097	0	0.048

Allele (bp)	328	330	332	334	336	337	340	342
Frequency	0	0.065	0.210	0.048	0.016	0	0	0

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.344	0	0	0.438	0.219

*Subadults (n = 36 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.194	0	0.028	0.028	0.028	0.306	0.361	0.056

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0.083	0.556	0.306	0.056

## Cm84 Locus

<i>Allele (bp)</i>	312	314	316	318	320	322	324
Frequency	0.528	0	0.028	0	0.111	0	0.028

<i>Allele (bp)</i>	328	330	332	334	336	337	340	342
Frequency	0	0.111	0.167	0	0.028	0	0	0

## Ei8 Locus

<i>Allele (bp)</i>	162	164	166	168	170	172
Frequency	0	0.306	0	0	0.444	0.250

**1998 Allele Frequency Data**

*Post-Pelagic (n = 22 alleles sampled)*

## Cc117 Locus

<i>Allele (bp)</i>	186	188	192	194	196	198	200	206
Frequency	0.273	0.045	0	0	0	0.136	0.545	0

## Cm72 Locus

<i>Allele (bp)</i>	216	224	226	239	241
Frequency	0	0	0.455	0.364	0.182

## Cm84 Locus

<i>Allele (bp)</i>	312	314	316	318	320	322	324
Frequency	0.455	0	0	0	0.091	0	0.091

<i>Allele (bp)</i>	328	330	332	334	336	337	340	342
Frequency	0	0.045	0.182	0.136	0	0	0	0

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.273	0.045	0	0.364	0.318

*Juveniles (n = 36 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.139	0.028	0.028	0	0.028	0.333	0.417	0.028

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.528	0.389	0.083

## Cm84 Locus

<i>Allele (bp)</i>	<i>312</i>	<i>314</i>	<i>316</i>	<i>318</i>	<i>320</i>	<i>322</i>	<i>324</i>
Frequency	0.563	0	0	0	0.031	0.063	0.094

<i>Allele (bp)</i>	<i>328</i>	<i>330</i>	<i>332</i>	<i>334</i>	<i>336</i>	<i>337</i>	<i>340</i>	<i>342</i>
Frequency	0.031	0.031	0.156	0.031	0	0	0	0

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.367	0.133	0	0.300	0.200

*Subadults (n = 16 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.125	0.063	0	0	0.063	0.313	0.438	0

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.375	0.438	0.188

## Cm84 Locus

<i>Allele (bp)</i>	<i>312</i>	<i>314</i>	<i>316</i>	<i>318</i>	<i>320</i>	<i>322</i>	<i>324</i>
Frequency	0.625	0.063	0	0	0	0	0.125

<i>Allele (bp)</i>	<i>328</i>	<i>330</i>	<i>332</i>	<i>334</i>	<i>336</i>	<i>337</i>	<i>340</i>	<i>342</i>
Frequency	0	0.063	0.125	0	0	0	0	0

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.625	0	0	0.250	0.125

**1999 Allele Frequency Data**

*Post-Pelagic (n = 46 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.152	0.022	0	0	0.022	0.500	0.304	0

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.652	0.217	0.130

## Cm84 Locus

<i>Allele (bp)</i>	312	314	316	318	320	322	324
Frequency	0.478	0.044	0	0	0.022	0	0.065

<i>Allele (bp)</i>	328	330	332	334	336	337	340	342
Frequency	0	0.152	0.152	0.065	0	0.022	0	0

## Ei8 Locus

<i>Allele (bp)</i>	162	164	166	168	170	172
Frequency	0.044	0.261	0.174	0	0.370	0.152

*Juveniles (n = 108 alleles sampled)*

## Cc117 Locus

<i>Allele (bp)</i>	186	188	192	194	196	198	200	206
Frequency	0.102	0.037	0	0.028	0.037	0.472	0.315	0.009

## Cm72 Locus

<i>Allele (bp)</i>	216	224	226	239	241
Frequency	0	0.019	0.602	0.278	0.102

## Cm84 Locus

<i>Allele (bp)</i>	312	314	316	318	320	322	324
Frequency	0.407	0.074	0	0	0.056	0.009	0.056

<i>Allele (bp)</i>	328	330	332	334	336	337	340	342
Frequency	0.037	0.148	0.120	0.074	0	0	0.009	0.009

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0.009	0.302	0.019	0.009	0.443	0.217

*Subadults (n = 56 alleles sampled)*

## Cc117 Locus

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.107	0.018	0	0	0	0.500	0.375	0

## Cm72 Locus

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.625	0.214	0.161

## Cm84 Locus

<i>Allele (bp)</i>	<i>312</i>	<i>314</i>	<i>316</i>	<i>318</i>	<i>320</i>	<i>322</i>	<i>324</i>
Frequency	0.446	0	0.018	0	0.125	0	0.107

<i>Allele (bp)</i>	<i>328</i>	<i>330</i>	<i>332</i>	<i>334</i>	<i>336</i>	<i>337</i>	<i>340</i>	<i>342</i>
Frequency	0	0.071	0.125	0.054	0.036	0	0	0.018

## Ei8 Locus

Allele (bp)	162	164	166	168	170	172
Frequency	0.036	0.321	0.054	0.036	0.393	0.161

## APPENDIX D

Summary of Allele Frequencies for the Temporal Change in Allele Frequency Method (MLNE Program) from Cohort Data. Bp = base pairs.

**1997 Year of Capture (n = 82 alleles sampled)***Cc117 Locus*

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.244	0.012	0	0.037	0.012	0.415	0.256	0.024

*Cm72 Locus*

Allele (bp)	216	224	226	239	241
Frequency	0.012	0	0.489	0.378	0.122

*Cm84 Locus*

Allele (bp)	312	314	316	318	320	322	324
Frequency	0.439	0.024	0	0.012	0.122	0	0.049

Allele (bp)	328	330	332	334	336	337	340	342
Frequency	0	0.061	0.232	0.037	0.024	0	0	0

*Ei8 Locus*

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.345	0	0	0.405	0.250

**1998 Year of Capture (n = 27 alleles sampled)***Cc117 Locus*

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.172	0.034	0.017	0	0.017	0.276	0.466	0.017

*Cm72 Locus*

Allele (bp)	216	224	226	239	241
Frequency	0	0	0.500	0.397	0.103

*Cm84 Locus*

Allele (bp)	312	314	316	318	320	322	324
Frequency	0.593	0	0	0	0.037	0.037	0.093

Allele (bp)	328	330	332	334	336	337	340	342
Frequency	0.056	0.037	0.130	0.019	0	0	0	0

*Ei8 Locus*

Allele (bp)	162	164	166	168	170	172
Frequency	0	0.308	0.096	0	0.346	0.250

**1999 Year of Capture (n = 150 alleles sampled)***Cc117 Locus*

Allele (bp)	186	188	192	194	196	198	200	206
Frequency	0.107	0.027	0	0.020	0.033	0.480	0.327	0.007

*Cm72 Locus*

Allele (bp)	216	224	226	239	241
Frequency	0	0.013	0.640	0.253	0.093

*Cm84 Locus*

Allele (bp)	312	314	316	318	320	322	324
Frequency	0.427	0.067	0.007	0	0.047	0.007	0.060

Allele (bp)	328	330	332	334	336	337	340	342
Frequency	0.027	0.147	0.133	0.053	0	0.007	0.007	0.013

*Ei8 Locus*

Allele (bp)	162	164	166	168	170	172
Frequency	0.020	0.297	0.054	0.007	0.419	0.203

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Publications:

Krininger, CE, Stephens, SH, Hansen, PJ (2002) Developmental Changes  
in Inhibitory Effects of Arsenic and Heath Shock on Growth of  
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