GEOGRAPHIC ORIGINS OF ILLEGALLY HARVESTED HAWKSBILL SEA TURTLE PRODUCTS

By

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ABSTRACT

GEOGRAPHIC ORIGINS OF ILLEGALLY HARVESTED HAWKSBILL SEA TURTLE PRODUCTS

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The hawksbill sea turtle (*Eretmochelys imbricata*) is one of seven sea turtle species, all of which are protected by international law. One of the distinguishing characteristics of the hawksbill is its carapace (shell), which is covered with colorful and ornate keratinous overlapping plates called scutes. These scutes are the source of "tortoiseshell" products, which are widely, though illegally, available in many countries. The hawksbill has a circumglobal distribution, making coordinated and focused conservation efforts aimed at preventing poaching of the species difficult. In this research, a procedure was developed to extract and amplify mitochondrial DNA from tortoiseshell items, in an effort to determine where these sea turtles are being poached, thus establishing tortoiseshell products as a novel source of genetic material. DNA from 56 confiscated tortoiseshell items donated by the United States Fish and Wildlife Service were analyzed. An average of 506 base pairs of mitochondrial DNA was sequenced. Sixteen haplotypes were indentified including 10 that were unique. All but one item (94%) corresponded to genetic stocks in the Indo-Pacific Ocean region, two were plastic tortoiseshell forgeries, and the remaining item was Atlantic in origin. These results indicate that poaching is more common in the Indo-Pacific Ocean and the multiple Indo-Pacific haplotypes imply that poaching is occurring at more than one location in this region. Based on these results, conservation efforts should be focused on the Indo-Pacific region. This research will allow for specific legislation, effective enforcement and the inclusion of evidence during prosecution, all addressing the need to reduce the poaching of hawksbills that supply the tortoiseshell trade.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Hawksbill status and biology

The hawksbill sea turtle (*Eretmochelys imbricata*; Figure 1) is one of seven sea turtle species. While all are listed as endangered or threatened, each species is unique, requiring individualized conservation considerations. The hawksbill sea turtle is protected under the Endangered Species Act, is listed under the Convention on International Trade in Endangered Species (CITES) in Appendix I, and is cited as critically endangered on the 2000 International Union for Conservation of Nature (IUCN) red list (Mortimer and Donnelly 2008). The species has been exploited for its ornate shell for centuries, making it difficult to assess the extent of the population decline that the hawksbill population has experienced, since historical baseline data are unknown (Mortimer and Donnelly 2008). The goal of this study was to determine the geographic locations of where the exploitation of hawksbills, specifically for their shell, is occurring.

Figure 1. Hawksbill sea turtle (*E. imbricata*) (http://www.fws.gov). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

The current abundance of hawksbill sea turtles is estimated to be at least 80% lower than historical levels and the population is still declining (Mortimer and Donnelly 2008). Trends show increases in only two nesting colonies over the past 20 years; however, due to the long generation times of the hawksbill (approximately 35 years; Meylan and Donnelly 1999), determining any trends from 20 years of data may not be indicative of the true population status. Further, population sizes of sea turtles are difficult to estimate and are generally based on nesting data, so actual population numbers are unknown. This is exacerbated by the fact that the female to male sex ratio of hawksbills is skewed, with females generally in greater abundance. Lara-Ruiz et al. (2006) determined that several nesting populations in Brazil are at least 90% female. Data from feeding sites, and therefore the inclusion of males, are difficult to obtain, contributing to the unreliability of total population size estimates.

The hawksbill is distinguished from other sea turtle species by a hawk-like beak and elaborately patterned scutes, keratinous plates that overlap the carapace (shell) of the turtle. Adult hawksbills average 2.5 feet in length and weigh an average of 130 pounds. The hawksbill is distributed circumtropically, inhabiting the Atlantic, Pacific, and Indian Oceans (Witzel 1983) (Figure 2). The species aggregates at foraging sites, typically located near coral reefs. They are one of a few species that feed exclusively on sponges, making them an integral part of coral reef ecosystems (Meylan 1998). Hawksbills also aggregate at nesting sites and have been recorded in over sixty countries. The largest nesting populations are located in the Republic of Seychelles, Caribbean, Australia, and Indonesia (Mortimer and Donnelly 2008). Female hawksbills return to their natal beach to nest every 2 to 3 years and, in a nesting season, a female may lay 3 to 5 nests, each containing an average of 130 eggs (Witzel 1983).

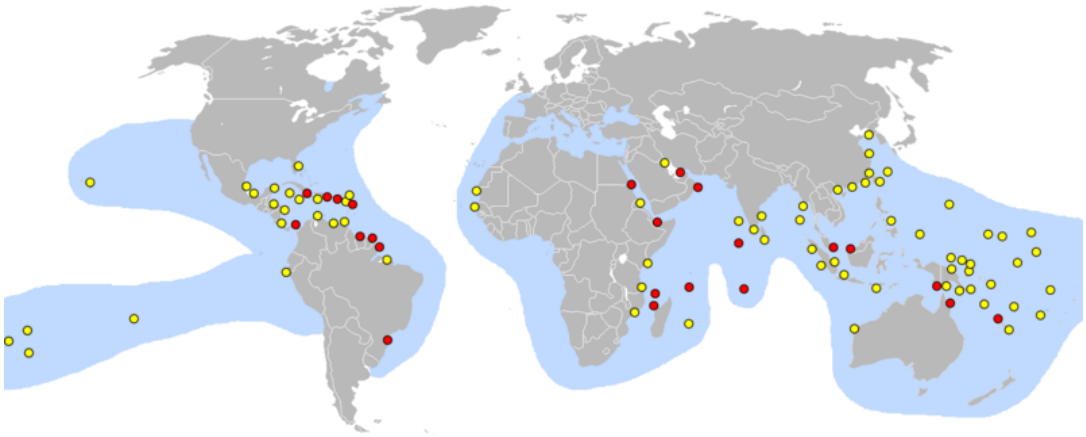


Figure 2. Geographic locations of major (red) and minor (yellow) nesting sites of *E. imbricata* (http://www.conserveturtles.org).

The conservation of the hawksbill sea turtle is connected to its biology. For example, because females return to the same beach to nest, complete harvesting of a single beach would make recolonization of that site unlikely (Bass 1996). Similarly, since females return to their natal beach multiple times per season, the probability of any individual surviving on an actively harvested beach is small (Meylan and Donnelly 1999). The global distribution of the hawksbill makes the conservation of the species an international issue, yet legislation to protect hawksbills is different in every country. Additionally, hawksbills spend most of their lives in international waters, further complicating the issue as migration routes can cross many boundaries.

The illegal harvesting (poaching) of sea turtles is one of the main causes for the population decline of the seven species of sea turtles, which are vulnerable during all stages of life (Epperly 2000). Sea turtle meat and eggs are consumed by many cultures and therefore are harvested directly. Additionally, sea turtle oil, penis, and blood are utilized in traditional medicine (Campbell 2000). Sea turtles are also indirectly caught as fishing bycatch. Turtle excluder devices were invented in the 1970s to reduce bycatch by acting as an escape door in

fishing nets, allowing sea turtles to exit without substantially reducing the catch of fish. Such devices have decreased sea turtle bycatch but are not used by all fishermen (Yaninek 1995).

The tortoiseshell trade

A threat unique to hawksbills is the tortoiseshell trade. Tortoiseshell is the name given to material made from the scutes of the hawksbill, which is used to produce products such as sunglasses, bracelets, and boxes that are illegally sold to tourists and on the black market (Figure 3). It is estimated that from one adult turtle, 1.34 kilograms of raw tortoiseshell material can be collected (Mortimer 2007). Tortoiseshell has been called the world's first plastic due to its unique malleable properties and many uses. Scutes are molded using only water and heat, a technique also applied to join scutes producing larger objects (Anon 2000). Items are then polished by hand without chemicals or additives. Tortoiseshell crafting skills are typically passed through generations and the oldest known tortoiseshell (in Japan, referred to as bekko) producing family, the Eazaki of Nagasaki, Japan, can be traced to 1709 (Kaneko and Yamaoka 1999). Tortoiseshell has been considered a commodity for thousands of years having a substantial impact on historical population sizes of hawksbill. For example, tortoiseshell items have been found in Egyptian tombs, in ruins of the Han Empire in China, and in the warehouses of Julius Cesar (Parsons 1972). Tortoiseshell items were also discovered in the Shosoin, a Japanese treasure house that dates to 701 AD. The tortoiseshell craft was prevalent worldwide and production increased in Japan during the Edo period (1603 to 1868), as techniques improved and demand for the material grew (Mortimer and Donelly 2008).

Figure 3. Tortoiseshell items made from scutes of *E. imbricata* for sale at a shop in Santo Domingo, Dominican Republic (http://www.grupojaragua.org.do).

Today, the tortoiseshell trade remains a global issue. In Cuba alone, 168,781 hawksbills were harvested to support the trade between 1935 and 1994 (Carillo 1999). Historically, Japan is a major importer of tortoiseshell. Japan was estimated to have imported shipments that represent approximately 710,000 hawksbills from 1970 to 1990, before the country became a signatory of CITES. Between 1970 and 1979, the largest exporters of bekko to Japan included Panama, Indonesia, and Cuba (Van Dijk and Shepherd 2004). After 1980, exporters included Cuba, the Solomon Islands, and Jamaica. Even after Japan signed CITES in 1994, several illegal shipments were confiscated, originating from Dominica, Indonesia, and Singapore. Items were typically concealed in suitcases and hidden among objects like coconuts and cow horns (Van Dijk and Shepherd 2004).

TRAFFIC is a wildlife trade monitoring network that was established in 1976 (TRAFFIC 2011), and is partnered with the World Wildlife Fund (WWF), the IUCN, and the secretariat of

CITES. A 2002 survey of wholesalers located in Vietnam found that trade in tortoiseshell has increased in comparison to a similar study conducted in 1993 (Van Dijk and Shepard 2004). In Ho Chi Minh City alone, 5,699 tortoiseshell items were for sale, prices for which ranged from $1 USD to $370 USD. Most shops that sold tortoiseshell products also traded in other illegal items like ivory, suggesting a connection with similar illegal wildlife activities.

In 2006, another study was conducted by TRAFFIC in the Dominican Republic and Colombia. In the former, 249 out of 414 shops surveyed traded in tortoiseshell items. In Santo Domingo, 95% of the stores monitored were involved in the trade. Overall, 50,000 tortoiseshell items were documented in the Dominican Republic; most sales were targeted at tourists from Europe and the United States. In Colombia, 1,500 tortoiseshell items were for sale; in addition, sea turtle oil, meat, and turtle eggs were sold by roadside vendors (Rueter and Allan 2006).

The TRAFFIC surveys demonstrate that the tortoiseshell trade is active today. Currently, the only forensic technique applied to tortoiseshell items is diffuse reflectance infrared Fourier transform spectroscopy, a technique that identifies functional groups in unknown substances. This method distinguishes real from fake tortoiseshell material by spectral comparisons; however, no additional species information can be gained (Espinoza et al. 2007). Alternative methods are needed to monitor the trade of tortoiseshell which could not only identify items as tortoiseshell but provide information as to where the sea turtle was poached. The combination of natal origin of the turtle with knowledge of where the item was sold would elucidate trade routes and provide intelligence to enforcement officials.

DNA and wildlife conservation

DNA analysis is routinely applied to wildlife forensics. In particular, mitochondrial DNA (mtDNA) analysis has been used to identify the species of origin of food items, carcasses, and animal products (reviewed by Ogden 2009). Mitochondria are double-membrane bound cellular organelles that are responsible for the production of energy. Each cell contains numerous mitochondria, and each mitochondrion contains one or more copies of mtDNA. mtDNA is circular and each organelle contains multiple copies of the mtDNA genome (reviewed by Butler and Levin 1998). mtDNA is inherited maternally via the egg, therefore all maternally related individuals have the same mtDNA sequence (Figure 4). The robust nature and high copy number of mtDNA make it ideal for forensic applications and mtDNA analysis is commonly used as a molecular marker for forensic identification (Budowle et al. 2003).

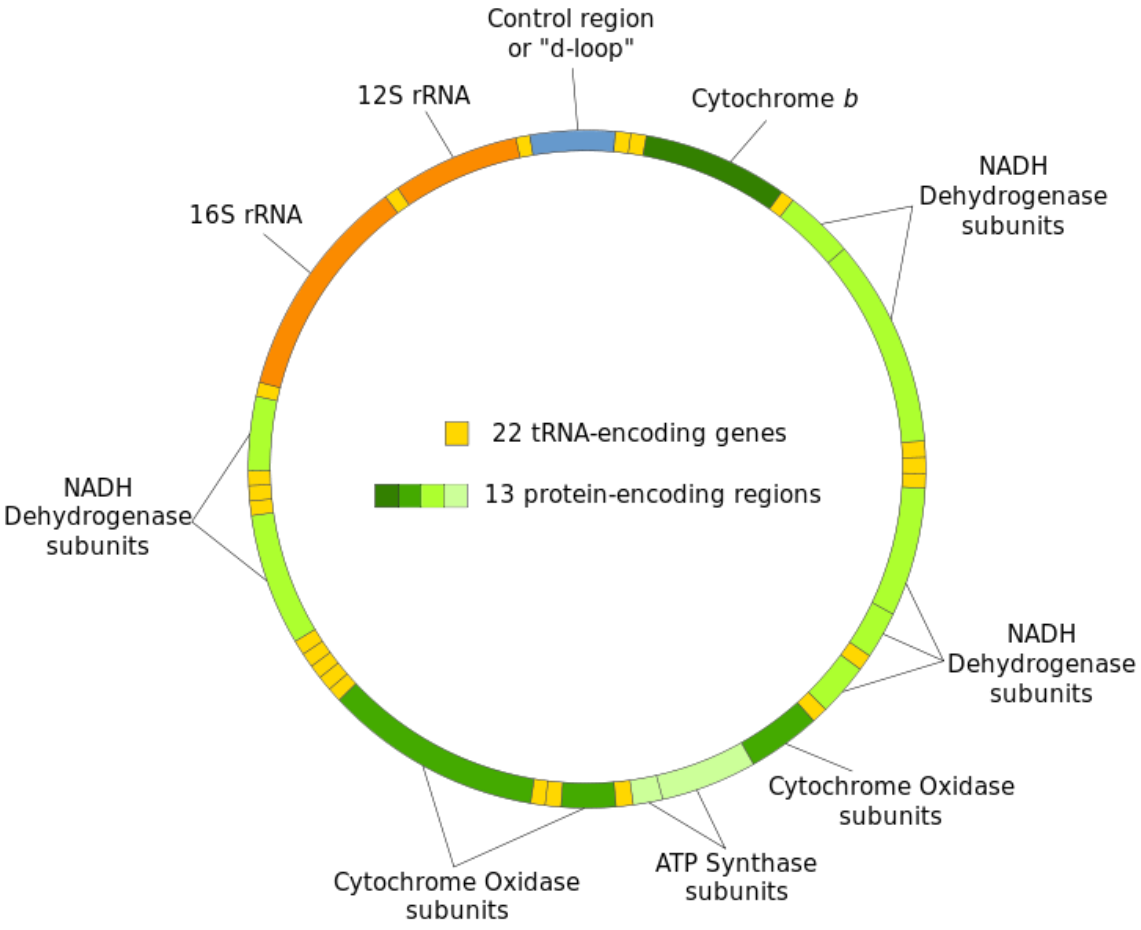


Figure 4. Map of vertebrate mtDNA (http://commons.wikimedia.org).

Identifying a species of origin for a wildlife product, be it food, medicinal, or ornamental, is key to determining if illegal activity has taken place. For example, Wan and Fang used mtDNA to identify the species of some meat confiscated in Ningbo City, China (2003). The meat was reported to be beef by the seller but authorities suspected that it was actually from a recently deceased circus tiger. DNA from the sample was compared to the sequences of four tiger subspecies, as well as common and legal meat sources such as deer and cow. Through mtDNA analysis, the meat was identified as tiger, and was also consistent with the subspecies of the circus tiger. The availability of this DNA evidence led to the conviction of the shop owner.

Similar species identification techniques include testing caviar (DeSalle and Birstein 1996), shark fin (Abercrombie et al. 2005), and Asiatic black bear (Peppin et al. 2008).

Knowledge of the geographic origin of a questioned wildlife sample is often necessary because wildlife legislation differs by country, and enforcement of laws often depends on whether an item was transported across borders. Increasingly, researchers aim to obtain geographic information from molecular markers, including mtDNA. Chapman et al. (2009) used mtDNA analysis to broadly determine the geographic origin of Atlantic scalloped hammerhead sharks. Market-bought shark fins were assigned to genetic stocks originating from either Atlantic or Indo-Pacific Ocean regions. Similarly, broad geographic determination was achieved by Baker et al. (1996) when analyzing commercial whale and dolphin products. The species of origin were determined and, with some uncertainty in the systematics of several whale and dolphin species, the researchers assigned the commercial samples to probable geographic origins. Encalada et al. (1994) performed mtDNA analysis on blood samples of a green sea turtle that was left at an aquarium in San Francisco. Researchers determined that natal region of the turtle was the southern or eastern tropical Atlantic Ocean and released the animal in the Atlantic Ocean. On a finer scale, Wasser et al. (2007) used nuclear DNA to track the origins of ivory confiscated in Zambia. Based on reference samples from herds throughout southern Africa, the origin of the sample was narrowed down to a small region in Zambia, contradicting the hypothesis that the shipment originated from numerous locations throughout the country.

MtDNA and hawksbill conservation

mtDNA has also been widely used in population and conservation genetics studies because polymorphisms (base differences) accumulate over time, which can be correlated with

matrilineages. Bowen et al. (1992) first studied sea turtle mtDNA using restriction fragment length polymorphisms to research the population structure of the green sea turtle. Increasing the genetic resolution, Norman et al. (1994) developed universal sea turtle primers for mtDNA amplification and sequenced approximately 400 base pairs of the control region of the green sea turtle. These primers have since been used for all seven species of sea turtle.

Bass et al. (1996) used mtDNA analyses to test a natal homing hypothesis for hawksbills. Overall, mtDNA diversity among nesting beaches in the Caribbean was 0.8490, showing greater variation among nesting beaches than within them. These results are consistent with the natal homing hypothesis, in which females return to the same beach where they were born, as opposed to the social facilitation (younger females follow older females) or random dispersal hypotheses. The natal homing of the hawksbill and highly structured mtDNA lineages allow for other uses of mtDNA as a molecular marker such as the study of sea turtle migration or geographic stock contributions to feeding aggregates. Bass et al. (1996) identified 21 haplotypes (sequences of DNA containing the same polymorphisms) from turtles assayed from Caribbean and West Atlantic nesting colonies.

Bowen et al. (1996) used mtDNA haplotypes to trace the natal origins of hawksbill turtles in a feeding ground near Mona Island, Puerto Rico. The authors concluded that feeding aggregates are sourced from a variety of nesting colonies. Research utilizing hawksbill mtDNA to study population structure includes Diez-Fernandez et al. (1999), Bowen et al. (2007), Browne et al. (2009), Bass (1999), Troeng et al. (2005), Velez Zauzo et al. (2000), and Monzon-Arguello et al. (2010). These studies have vastly increased the availability of rookery haplotype frequency data.

Goals of this study

The goal of the research presented here was to determine the origin of tortoiseshell items using mtDNA analysis. To accomplish this, techniques were developed and optimized for obtaining and analyzing DNA from hawksbill turtle items which has not been previously performed. Mitochondrial DNA from confiscated tortoiseshell items were then isolated and analyzed using the developed techniques. The DNA results were compared to hawksbill DNA data available in the literature and online. Based on these results, the geographic origin of the tortoiseshell items were determined. This research lays a foundation for future research on the forensic mtDNA analysis of tortoiseshell samples and increases the availability of data to improve the ability to focus enforcement and conservation efforts on hawksbills.

MATERIALS AND METHODS

Samples and sample preparation

Preliminary research was conducted using a whole hawksbill carapace on loan from the Michigan State University (MSU) Museum. The United States Fish and Wildlife Service (USFWS) loaned 226 confiscated tortoiseshell products from their Eagle Repository in Commerce City, Colorado to the Forensic Biology Laboratory at MSU. Of these products, 57 were analyzed (Figure 5). Items were described and assigned a sample number in addition to the USFWS identification number (Table 6). Items were cleaned by wiping with 10% household bleach on a Kim-Wipe and set aside to air-dry.

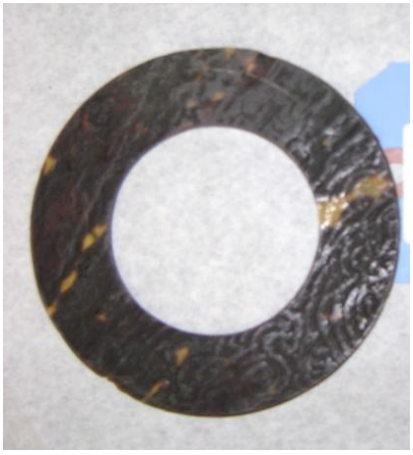


Figure 5. Example of a confiscated tortoiseshell item, a jewelry piece.

DNA Extraction

Tortoiseshell samples were processed individually in a hood that was wiped with 10% bleach on a Kim-Wipe followed by 70% ethanol and then exposed to ultra-violet (UV) light for a minimum of 10 min. A 7/64th inch drill bit and drill parts were soaked in 10% bleach, wiped with 70% ethanol on a Kim-Wipe. A Dremel© tool was wiped with a Kim-Wipe and 10% bleach followed by 70% ethanol. Once dry, the drilling materials, along with a plastic weigh boat and a 1.5 mL microcentrifuge tube, were UV irradiated for 10 min. Using the Dremel© tool on low speed and a 7/64th inch drill bit, a powder was created by drilling a small indentation in the item, approximately one millimeter deep. The power generated from the drilling was collected in a plastic weigh boat and indentations were made until at least 0.010 g of powder was collected. Sanding bands and sanding disk accessories were also tested to sand the item, creating a powder, however this did not generate sufficient masses of tortoiseshell powder when compared to the 7/64th drill bit and was not used further.

The powder (ranging from 0.013 to 0.040 g) was transferred to a 1.5 mL centrifuge tube, and 400 µL of digestion buffer (20mM Tris pH 8, 50mM EDTA, 0.5%SDS), 5 µL of 20 mg/mL proteinase K, and 10 µL of 1M dithiothreitol were added. The tube was vortexed and incubated overnight at 55^{o}C. Four hundred microliters of phenol was added and the tube was vortexed and centrifuged at 14,000 rpm (20,817 x g) for 5 min. The aqueous layer was transferred to a new tube containing 400 µL of chloroform. The tube was vortexed and centrifuged at 14,000 rpm for 5 min. The aqueous layer was transferred to a Microcon® YM-30 spin column (Millipore Corporation, Billerica, MA) and centrifuged at 14,000 g for 15 min. The eluant was removed and the DNA was washed three times with 300 µL of TE (10mM Tris, 1 mM EDTA, pH 7.5)

and centrifuged at 14,000 g for 10 min. DNA was recovered by adding 30 µL of TE to the filter column. After 1 min the column was inverted into a new tube and centrifuged at 1,000 g for 2 min. In later experiments, the YM 30 filters were replaced by Amicon® Ultra 30K spin columns (Millipore Corporation). The same procedure described above was followed except the Amicon® Ultra columns reserve approximately 20 µL of solution following each wash step. To recover the DNA solution, the filter was inverted into a new tube and centrifuged at 1,000 g for 2 min. DNA was stored at -20^{o}C.

DNA amplification

Initially, 10 primer pairs were tested to amplify mtDNA from the D-loop of *E. imbricata* (Table 1). Primer pairs TCR5/TCR6 and LCM15382/H950 were designed by Norman et al. (1994) and Abreu-Grobois et al. (2006) respectively. Primers EI1 – EI8 were designed to target 400 bp to 800 bp of the control region of *E. imbricata* mtDNA using Primer 3 Plus (Untergasser et al. 2007) (Figure 1). Primers EI1 – EI4 were synthesized by the MSU Macromolecular Structure Facility and primers EI5 – EI8 were synthesized by Integrated DNA Technologies© (Coralville, IA). Twenty microliter PCR reactions included 2 µL 20 µM forward and reverse primer, 2 µL 25 mM $MgCl_2$ (Applied Biosystems, Carlsbad, CA), 2 µL GeneAmp 10X PCR Buffer II (Applied Biosystems), 2 µL 0.2 mM deoxynucleoside 5'-triphosphates (dNTPs), 10 µL distilled water and 1U Amplitaq Gold® (Applied Biosystems).

Primer pairs TCR5/TCR6 and LCM15382/H950 DNA and EI – EI4 were tested with DNA dilutions of 1, 1:10, and 1:100 with TE. All reactions were also compared with and without the addition of BSA. DNA dilutions did not need to be preformed for DNA

amplification when BSA was added; therefore, DNA was not diluted in reactions with primers EI5 – EI8.

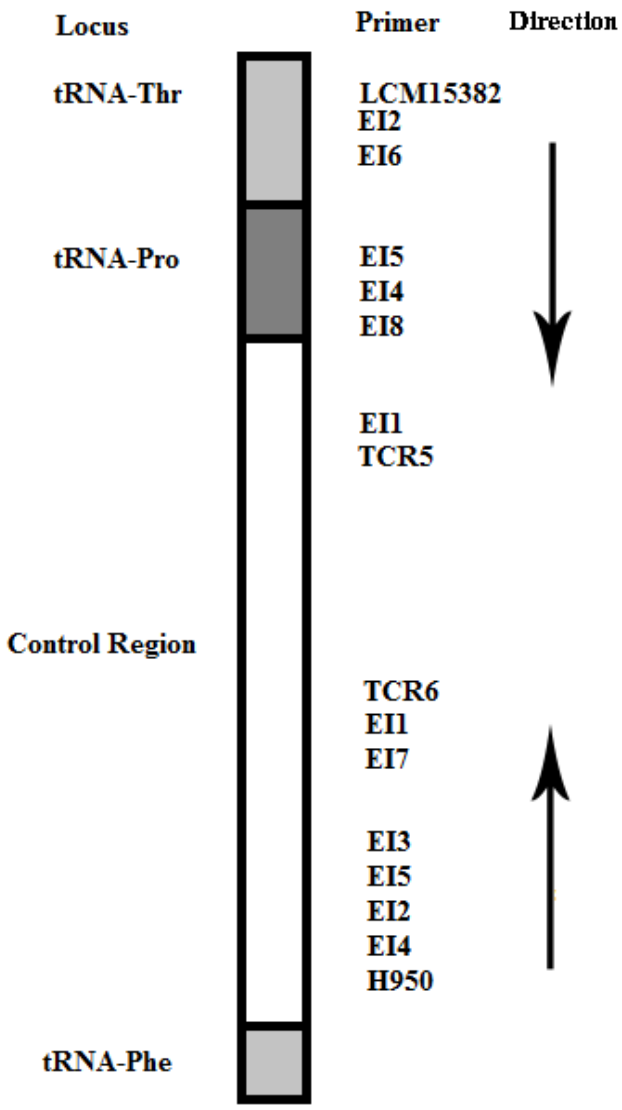


Figure 6. Map of approximate locations of all primers tested in this study. Primers flank the control region and surrounding tRNAs of hawksbill mtDNA. Primers designed for this study are labeled EI1 – EI8.

Primers EI7/EI8 were selected for the amplification success and electropherogram quality that resulted. Prior to the design of primers EI7 and EI8, all samples were amplified and sequenced once using primer pair EI1 and once using EI4. Samples were re-amplified and sequenced using primers EI7 and EI8. Primer annealing temperature for the EI7 and EI8 primers was optimized in a gradient thermocycler using temperatures ranging from 45^{o}C to 63^{o}C. The optimum annealing temperature, 50^{o}C, was used in all subsequent reactions.

Thirty microliter PCR reactions included 3 µL 20 µM forward and reverse primer, 3 µL 25 mM $MgCl_2$, 3 µL GeneAmp 10X PCR Buffer II, 3 µL 0.2 mM dNTPs, 3 µL 100 µg/µL BSA, 12 µL distilled water, and 1U Amplitaq Gold® were used for analysis of the confiscated tortoiseshell items. PCR conditions were as follows: a 10 min 94^{o}C denaturation step followed by 38 cycles of 30 s at 94^{o}C, 1 min at 50^{o}C, and 1 min at 72^{o}C. Five microliters of PCR products were electrophoresed on a 2% agarose gel. If a band was not present, the sample was reamplified. If a band was present, the PCR products were purified using Diffinity Rapid Tips© (Diffinity Genomics, Rochester, NY) by aspirating and dispensing the solution 15 times, and the DNA was stored at -20^{o}C.

Table 1. Summary of the primers used for mtDNA amplification. The primer pair selected for analysis of the confiscated tortoiseshell samples is bolded. Primers designed for this study are not published (NA) and primer orientation is listed as either forward or reverse.

Primer	Orientation	Sequence	Author
TCR5	Forward	5'-TTGTACATCTACTTATTTACCAC-3'	Norman et al. (1994)
TCR6	Reverse	5'-GTACGTACAAGTAAAACTACCGTATGCC-3'	Norman et al. (1994)
LCM15382	Forward	5'-GCTTAACCCTAAAGCATTGG-3'	Abreu-Grobois et al. (2006)
H950	Reverse	5'-GTCTCGGATTTAGGGGTTG-3'	Abreu-Grobois et al. (2006)
EI1	Forward	5'-TATTGTACATCTACTTATTTACC-3'	NA
EI1	Reverse	5'-AAGACTACTATATGCAAGTAAA-3'	NA
EI2	Forward	5'-CAAAACCGGAATCCTGTCAA-3'	NA
EI2	Reverse	5'-GTTATTCCGGGCTGATGTTG-3'	NA
EI3	Forward	5'-AAAAGCGAACACACAAATGG-3'	NA
EI3	Reverse	5'-GGGGGTTTAACTAAAAGGTATAGTTGT-3'	NA
EI4	Forward	5'-CTACCGTGCCCAGTAAGACC-3'	NA
EI4	Reverse	5'-GGGGTTTGGCAAAGAAGTAA-3'	NA
EI5	Forward	5'-TATCCTCTACCGTGCCCAGA-3'	NA
EI5	Reverse	5'-CGGGCATGATGTTAGTTTATG-3'	NA
EI6	Forward	5'-CCAAAACCGGAATCTTTCAA-3'	NA
EI7	Reverse	5’-GTTTCATCAATTCGGCAGGT-3’	NA

Table 1 continued

EI8	Forward	5'-GTGCCCAGAAGACCAATAGC-3'	NA

mtDNA Sequencing

PCR products were sequenced using a CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) using 4 µL Quick Start mix, approximately 50–100 fM DNA template, 1 µL of either forward or reverse primer, and the reaction was brought to 10 µL with distilled water. Sequencing reaction conditions were as follows: 30 cycles of 20 s at 96^{O}C, 20 s at 55^{O}C, and 4 min at 60^{O}C. Sequencing products were precipitated according to manufacturers' instructions. DNAs were loaded onto a 96 well plate and were separated using a CEQ 8000 Genetic Analysis System (Beckman Coulter) using the LFR-1 program (50^{O}C capillary temperature, denature at 120^{O}C for 120 s, inject at 2 kV for 15 s, and separate at 4.2 kV for 85 min).

Sequence Analysis

Forward and reverse sequences were compiled and edited using BioEdit 1.2 software (Hall 1999) and aligned using MEGA software (Tamura 2011). All *E. imbricata* control region mtDNA sequences were downloaded from the Basic Local Search Alignment Tool (BLAST) database and were used as reference sequences (Altschul 1997); accession numbers are listed in Table 7. Haplotypes were assigned using COLLAPSE 1.2 software, and were compared to geographic information from the BLAST database and to published data, when available. A phylogenetic tree was constructed with the sequences obtained from this study and all

downloaded reference sequences. jModeltest was used to infer the mode of nucleotide substation (Posada and Buckley 2004) and the Tamura-Nei plus gamma model was used in the construction of the tree. Phylogenetic relationships between these sequences were determined by the Neighbor-Joining method (Saitou and Nei 1987) and Maximum-Parsimony method using MEGA 4.0 with 1000 bootstrap replications (Tamura et al. 2007). The green sea turtle (*Chelonia mydas*) was used as an outgroup as the taxon is distantly related to the hawksbill in the Cheloniidae family (Naro-Maciel et al. 2008).

RESULTS

Sample preparation and DNA extraction

Fifty-seven tortoiseshell items were processed including earrings, necklaces, guitar picks and one violin bow. Larger tortoiseshell items regularly generated 20 mg of powder, while bookmarks and other items that were extremely thin could not be processed using the drilling method. Powder collected from items was white in color and yields ranged from 0.013 g to 0.040 g. DNA extracts from most items were brown in color, which DNA purification using either the Microcon® YM-30 or the Amicon® Ultra 30K spin columns did not remove.

mtDNA amplification

mtDNA from ten different types of items (Table 4) was amplified; item type did not seem to influence amplification success. The amplification and sequencing success of the ten primers tested is summarized in Table 2. PCR amplification using the universal primer pair TCR5/TCR6 did not result in product, while amplification using the universal primer pair LCM15382/H950 was positive when the DNA was diluted 1:10 (Figure 7). Amplification using primer pairs EI1 or EI4 and two dilutions of DNA (1:10 and 1:100) was positive, while primer pairs EI2 and EI3 did not result in PCR product (Figure 8). Additionally, amplification using primers EI5/6, EI6/7, and EI7/8 with BSA generated positive results, while amplification using primer pair EI5 was negative (Figure 9). Primer pair EI7/8 was only moderately efficient in mtDNA amplification; however, it was the most successful in generating quality sequence electropherograms. Optimized PCR annealing temperature for primers EI7/8 is indicated by the bands present in lanes 5 and 6 of Figure 10, representing temperatures of 50.4^{o}C and 53.0^{o}C.

mtDNA amplification with primers EI7/8 was successful in 55 of 57 samples following primer optimization, most of which yielded high intensity bands (Figure 11), though four of the samples yielded low intensity bands. The starting mass of tortoiseshell powder for these low intensity samples was in the middle range of powder masses collected (0.020 g to 0.023 g). Two samples (30 and 31, both bracelets) did not amplify. During the drilling process, these had different properties than the other samples, including density and smell. Fourier transform infrared spectra from the two items were not consistent with spectra generated from tortoiseshell items that yielded PCR product and with tortoiseshell spectra published by Espinoza et al. (2007) but were consistent with the spectra of casein plastic tortoiseshell forgeries published by these authors.

Table 2. Summary of mtDNA amplification and sequencing success. Amplification reactions with TCR5/6, EI2, and EI3 were negative (-) and were not sequenced (NA).Amplification rections with LCM15382/H950, EI1, EI5/6, EI6/7, and EI7/8 were positive (+); however, sequencing success was low (+/-). Reactions with EI7/8 yielded both positive results for PCR product (+) as well as a high sequencing success (+).

Primer Pair	Amplified	Sequenced
TCR5/6	-	NA
LCM15382/H950	+	-
EI1	+	+/- **
EI2	-	NA
EI3	-	NA
EI4	+/- *	+/-***
EI5	-	NA
EI5/6	+	+/-***
EI6/7	+	+/-***
EI7/8	+	+

* Amplification was successful in approximately 20% of samples
**Sequencing success was approximately 50% same
***Produced poor quality electropherograms

Figure 7. Two percent agarose gel electrophoresis showing PCR results with the LCM15382/H950 primer pair (left) and TCR5/6 primer pair (right). Only one positive result is visible (lane LCM/H950 .1) corresponding to a 1:10 dilution of DNA and the LCM15382/H950 primers. The band in lane 4 (LCM/H950 1) represents primer dimer, not a positive result.

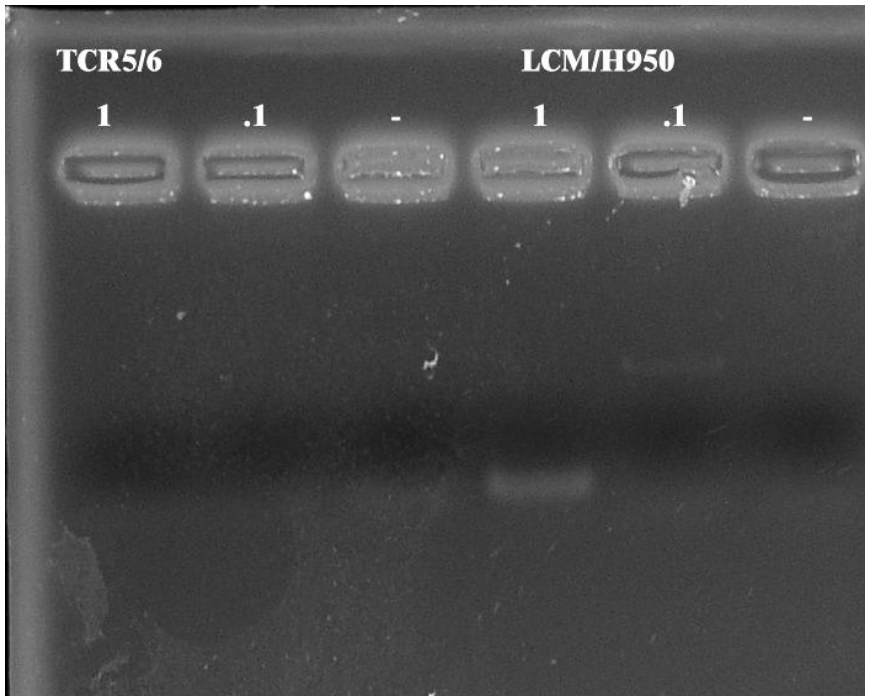


Figure 8. Two percent agarose gel electrophoresis showing PCR products using the primers pairs EI1, EI2, EI3 and EI4. Each primer was tested using neat and a 1:10 dilution of DNA a. Bands of approximately 400 bp are visible for the EI1 and EI4 primer pairs. No bands were present in the negative control (-) or for primers EI2 and EI3. A 100 bp DNA ladder (L) was also included.

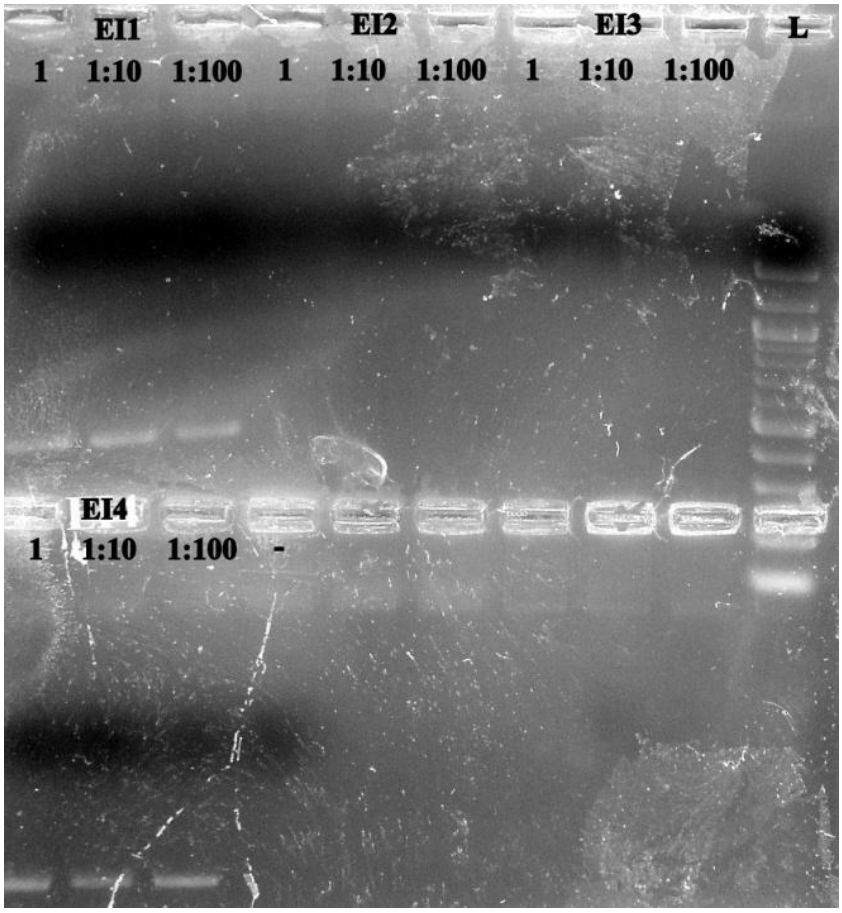


Figure 9. Two percent agarose gel electrophoresis showing PCR products for the primer pairs EI5, EI5/6, EI6/7, and EI7/8. Bands are visible for the 5/6, 6/7, and 7/8 primer pairs. There was no band present in the negative control (-).

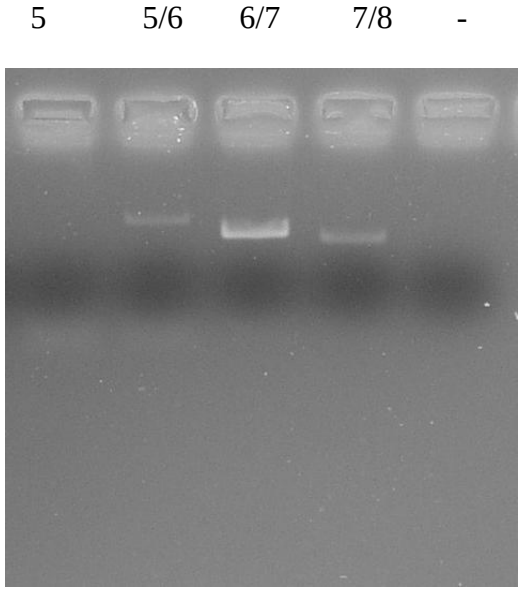


Figure 10. Two percent agarose gel electrophoresis showing PCR products for the annealing temperature optimization for primer pair EI7/8. The gradient of temperatures ranged from 45^{o}C, lane 1, to 63^{o}C, lane 12. Bands are present in lanes 5 and 6 and correspond to 50.4^{o}C and 53.0^{o}C respectively. Two 100 bp DNA ladders (L) were also included.

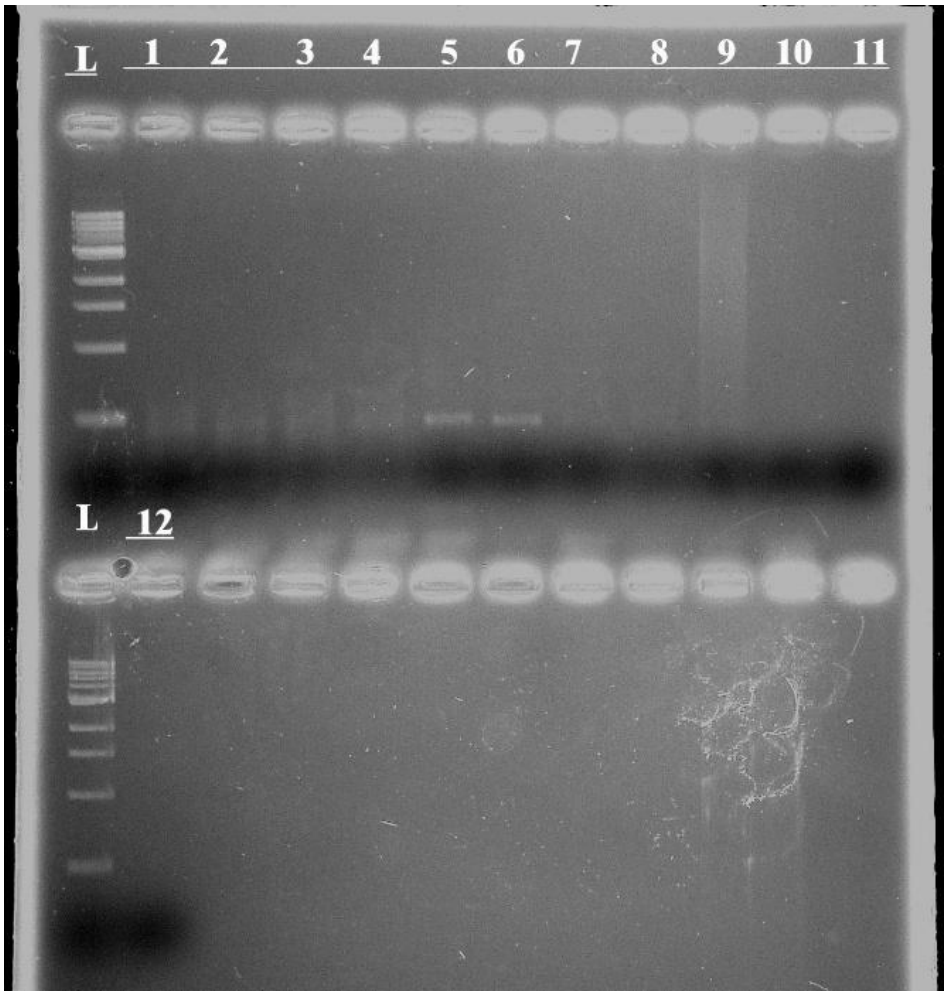
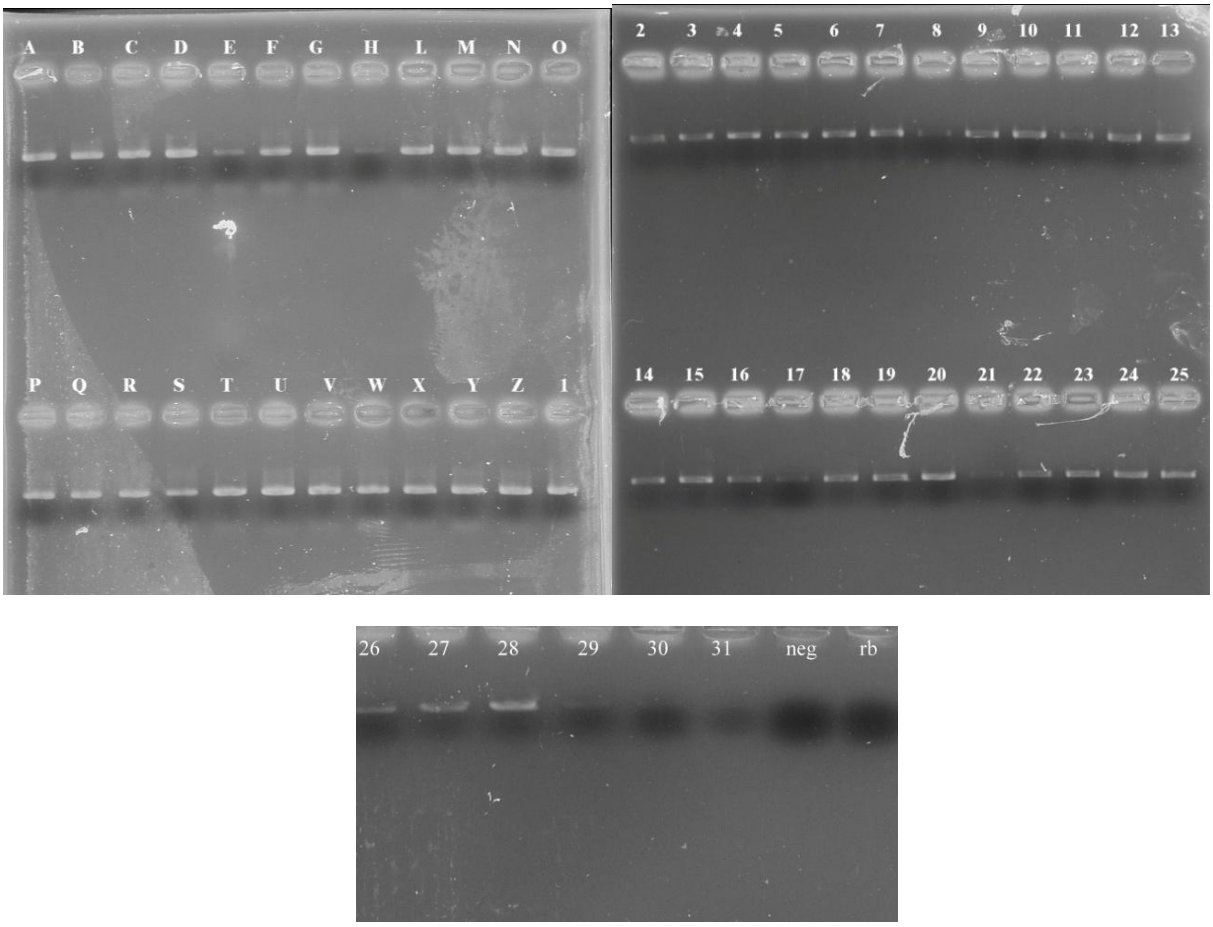


Figure 11. Two percent agarose gel electrophoresis of amplified mtDNA from confiscated tortoiseshell items using the primer pair EI7/8. Bands are present in all lanes except for samples 30 and 31. No bands are present in the negative control (neg) and reagent blank (rb) lanes.



mtDNA sequencing of tortoiseshell items

Forward and reverse sequences were obtained from 52 of the 55 amplified samples (94%). Two samples that did not yield a mtDNA sequence, H and 29, also had low concentrations of PCR product (Figure 11). An average of 506 bp was obtained per sample, with sequence lengths ranging from 341 bp to 560 bp (Table 3).

Table 3. Sequence lengths obtained from the tortoiseshell samples. Samples that did not sequence (-) were H, 25, and 29. Samples that did not amplify were not sequenced and are noted as "NA".

Sample	Base Pairs
A	504
B	512
C	537
D	560
E	535
F	488
G	537
H	-
I	538
J	537
K	533
L	535
M	472
N	499
O	487
P	512
Q	537
R	492
S	374
T	534
U	536

Table 3 continued

V	341
W	359
X	499
Y	492
Z	538
1	529
2	534
3	487
4	536
5	451
6	532
7	536
8	538
9	438
10	538
11	505
12	534
13	533
14	530
15	538
16	538
17	513
18	530
19	498
20	501
21	480
22	502
23	500
24	500
25	-
26	502
27	449
28	536
29	-
30	NA
31	NA
Negative	NA
Reagent	NA

mtDNA Haplotypes

The haplotype EI-1 from the Genbank database was the most common in this study, being observed 32 times (58%; Table 4). Haplotypes EI-9, EIJ10, EIJ5, EI-13, EIJ5, and G were each observed once. Ten sequences were not in the Genbank database. Of these, Unique 6, 8, and 9 were observed 4, 2, and 2 times respectively, while the remaining unique sequences were each observed once (Table 5). Unique 2 and 6 each contained one transversion, while the remaining polymorphisms were transitions.

Table 4. Haplotypes and item types of confiscated tortoiseshell products. Samples that did not amplify or did not sequence are noted as "NA". Each sample listed as "Unique" represents a sequence not in the Genbank database. Haplotypes "Unique 6", "Unique 8", and "Unique 9" were observed more than once.

Sample	Haplotype	Item type
A	EI-1	Earring
B	Unique 1	Hair Comb
C	EI-1	Earring
D	EI-1	Guitar pick
E	EI-9	Hair Comb
F	EI-1	Box
G	Unique 2	Hair Comb
H	NA	Box
I	Unique 3	Violin Bow
J	EI-1	Necklace
K	Unique 4	Box
L	EI-1	Earring
M	EI-1	Earring
N	EI-1	Earring
O	EI-1	Guitar pick
P	EI-1	Hair Comb
Q	Unique 5	Hair Comb
R	EI-1	Box
S	Unique 6	Hair Comb
T	Unique 6	Hair Comb
U	EI-1	Guitar pick
V	EI-1	Guitar pick
W	EI-1	Pin
X	EI-1	Guitar pick
Y	EI-1	Guitar pick
Z	EI-1	Guitar pick
1	EI-1	Guitar pick
2	EI-1	Guitar pick
3	Unique 7	Guitar pick
4	EI-1	Pin
5	EI-1	Guitar pick
6	Unique 8	Guitar pick
7	Unique 9	Guitar pick
8	EIJ10	Guitar pick
9	EI-1	Guitar pick

Table 4 continued

10	Unique 9	Guitar pick
11	EI-13	Guitar pick
12	EI-1	Guitar pick
13	EI-1	Guitar pick
14	EI-1	Guitar pick
15	EI-1	Hair Comb
16	EI-1	Bracelet
17	EI-1	Ring
18	Unique 8	Bracelet
19	EI-1	Bracelet
20	EI-1	Bracelet
21	EI-1	Jewelry Piece
22	Unique 6	Hair Comb
23	Unique 6	Hair Comb
24	EIJ5	Bracelet
25	NA	Box
26	Unique 10	Box
27	G	Bracelet
28	EI-1	Hair Comb
29	NA	Carapace
30	NA	Bracelet
31	NA	Bracelet
Negative	NA	NA
Reagent	NA	NA

Table 5. Polymorphisms of unique sequences using haplotype EI-1 as a reference sequence. A letter indicates a nucleotide difference at that location from EI-1 and a blank cell means that those nucleotides are the same.

Haplotype	**69**	**100**	**143**	**182**	**219**	**223**	**240**	**260**	**369**	**484**
EI-1	A	T	A	C	A	A	C	C	A	A
Unique 1				T	G				G	
Unique 2	T				G				G	
Unique 3					G				G	
Unique 4										G
Unique 5					G			T	G	
Unique 6							A			
Unique 7										
Unique 8	G		G							
Unique 9		C				G				
Unique 10									G	

Haplotype analysis

The neighbor-joining phylogenetic comparison revealed that the unique sequences cluster with those of Pacific Ocean origins (Figures 12a – 12d). Five of the sequences formed one clade (Figure 12d). Additionally, known Atlantic haplotypes grouped separately from known Indo-Pacific haplotypes. Similar results were achieved with the maximum-parsimony method.

Figure 12. Phylogenetic relationships among unique haplotypes and sequences downloaded from Genbank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. *Chelonia mydas* was used as an outgroup. Haplotypes obtained in this study are marked with an asterisk. The dotted line denotes the separation between Atlantic and Indo-Pacific populations. The figure is split into four segments for visibility (Figures 12a–12d).

Figure 12a. Top segment of the phylogenetic tree.

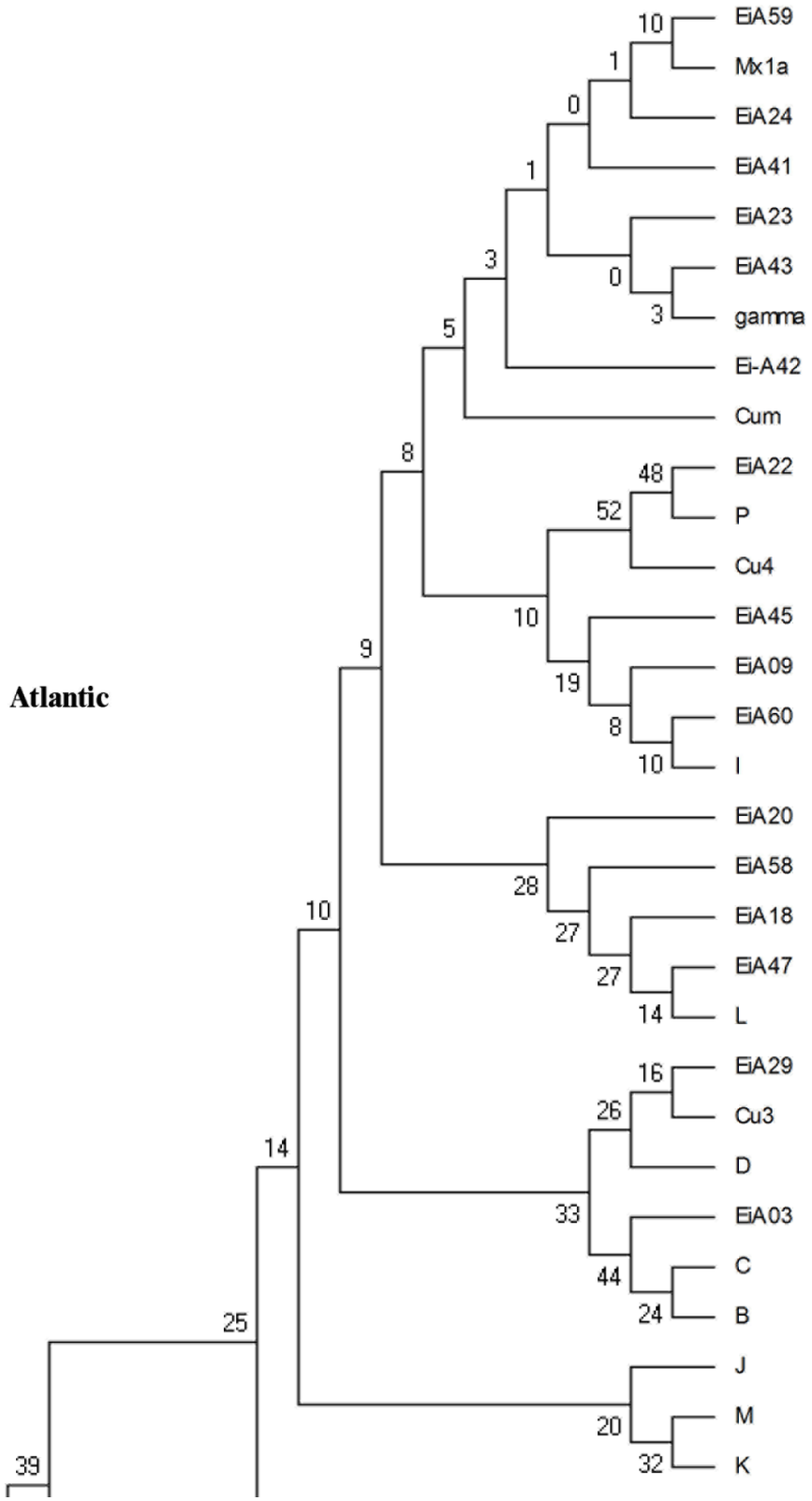


Figure 12b. Second segment of the phylogenetic tree.

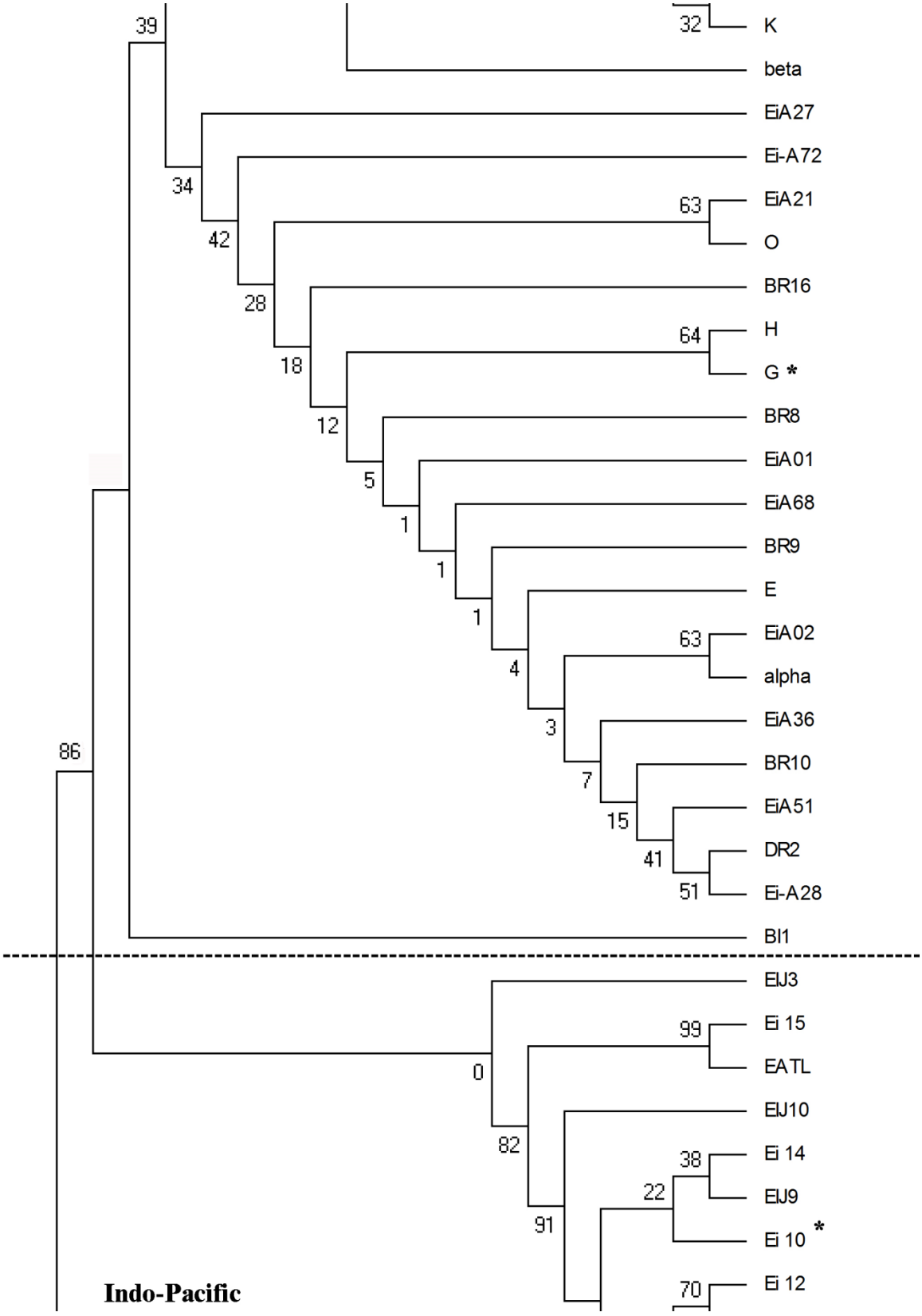


Figure 12c. Third segment of the phylogenetic tree.

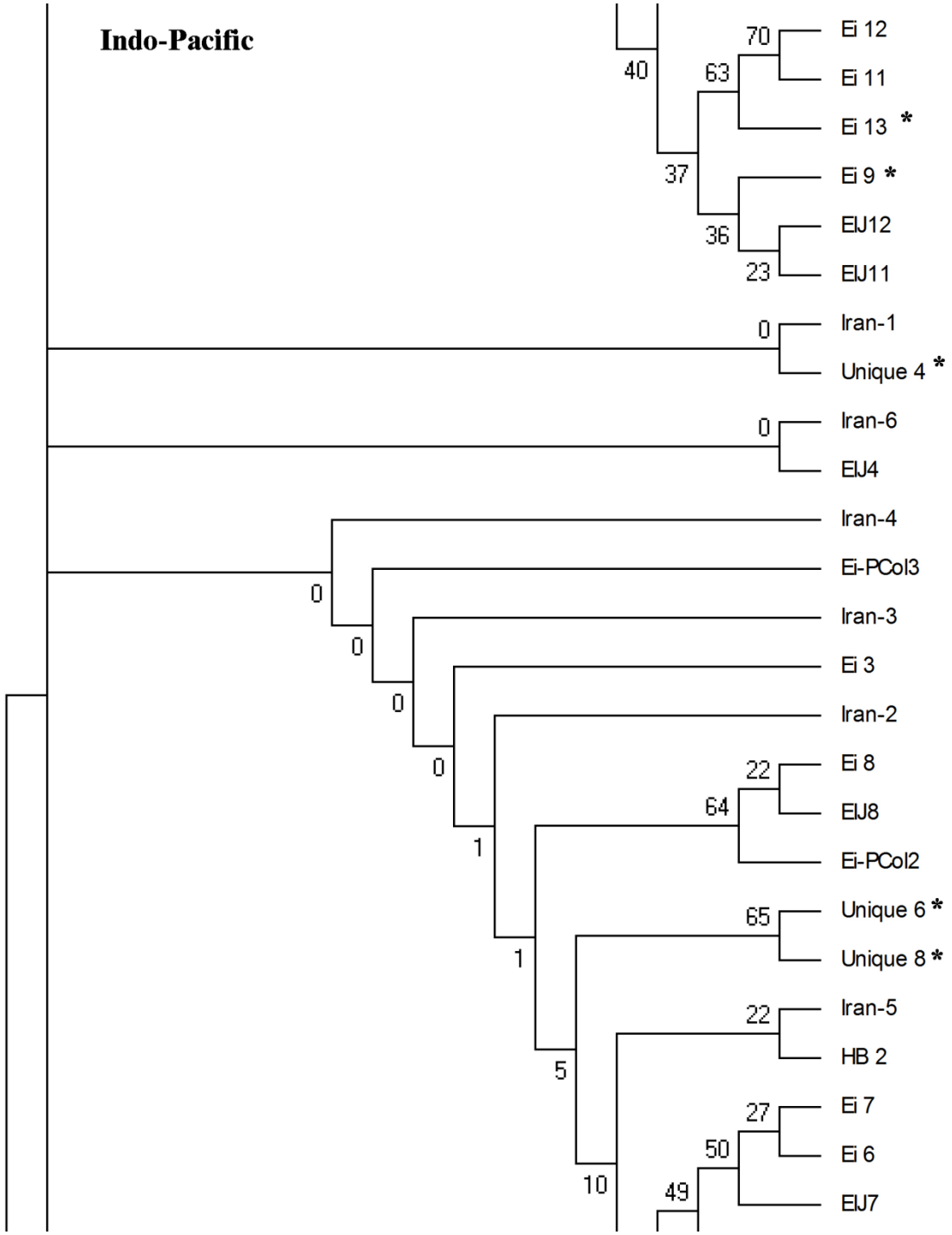
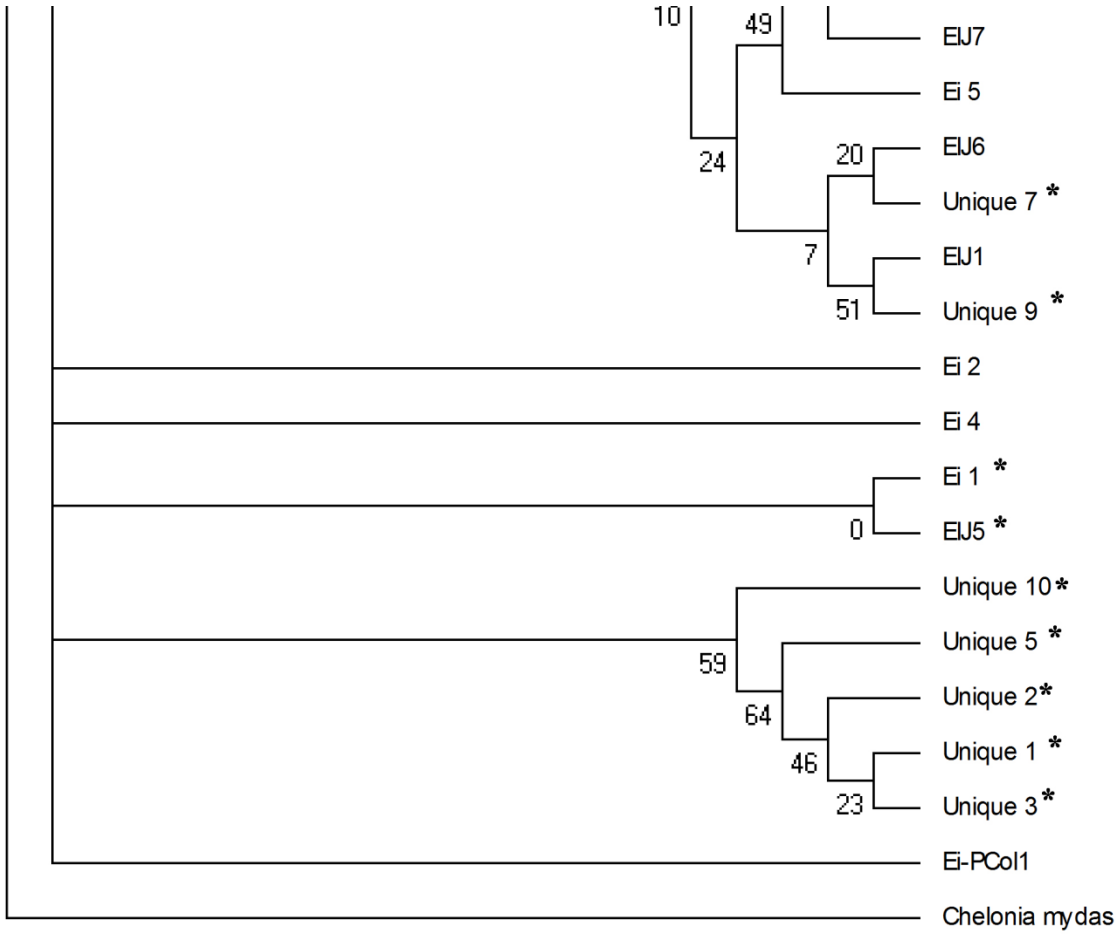


Figure 12d. Bottom segment of the phylogenetic tree.



DISCUSSION

DNA analyses have not previously been performed on tortoiseshell products, hence, a primary goal of the research presented here was to determine if such products are a viable source of mtDNA, and if so, to investigate procedures for isolating and purifying it such that DNA sequences could be obtained. The second goal was to analyze any mtDNA sequences from the items to determine their geographic origin. The identification of the natal origin of hawksbill sea turtles poached for the production of tortoiseshell items would provide a means of monitoring this illegal activity and have a potentially profound effect on wildlife policies, enforcement, and conservation strategies for the species.

The drilling method used in this study was based on current procedures conducted in the laboratory to process bone samples. Using this method, a portion of the confiscated tortoiseshell items, including bookmarks and rings, could not be processed due to their small size. To address this complication, alternative methods were pursued, including sanding the items; however, none were as effective as the drilling procedure. Other methods, such as an alkaline digestion (Graffy and Foran 2005) or the use of a blender (Loreille et al. 2007), might increase the number and type of analyzable samples in the future.

Alignment of the published mtDNA control region universal primer pair TCR5 and TCR6 with *E. imbricata* mtDNA sequences revealed seven nucleotide differences, most occurring near the 3' end. This primer pair flanks a sequence approximately 400 bp in length and was derived using green sea turtle (*Chelonia mydas*) DNA as a template (Norman et al.1994), and these mismatches likely resulted in the lack of amplification with hawksbill DNA. In

contrast, LCM15382/H950 (Abreu-Grobois et al. 2006) were derived from multiple sea turtle species and only 3 nucleotide differences, near the 5' ends of the primers, were present between them and the *E. imbricata* DNA, resulting in positive PCR results. While these primers showed that viable DNA could be obtained from hawksbill shell, because the species of turtle was known, there was no need to use universal primers, and species-specific ones were produced. Of these, multiple primer pairs successfully amplified hawksbill mtDNA and the primer pair that produced the better quality sequences was used.

Once effective PCR primers were identified, DNA from all tortoiseshell items tested amplified, with the exception of two. These were suspected to be made of plastic during the drilling process based on the density of the powder and aroma produced. This suspicion was supported when, after multiple attempts, PCR reactions were negative. The items were examined cursorily using Fourier transform infrared spectroscopy, which confirmed they were made of casein resin based on spectral comparisons (data not shown). The identification of plastic items represents a side benefit of the procedure—the ability to distinguish real from fake tortoiseshell.

All DNA extracts were brown, which was likely caused by the pigment melanin (Albardi and Thompson 1999); due to its large molecular size, it would not be removed by the 30kD filter column. Melanin is a known PCR inhibitor, and its presence can interfere with subsequent procedures such as DNA sequencing (Eckhart et al. 2000). To address this, BSA was added to all PCR reactions in which neat DNA (in contrast to diluted DNA) was used, which yielded positive results. The two samples that weakly amplified but did not sequence were mtDNA isolated from a box and the carapace. Higher concentrations of melanin would explain the weak PCR products of these two samples; however, the extent of the coloration of these extracts was not noted. Additionally, one was not a tortoiseshell item obtained from the USFWS, but instead was a

whole hawksbill carapace, from which DNA was extracted over a year before the others. Amplification reactions performed directly after DNA extraction from the carapace were positive, while reactions performed a year later were negative after multiple attempts. This raises concerns about the long term storage of these DNA extracts. Further purification of the extracts may be warranted, as it would potentially increase the efficiency of the reaction, yield better quality electropherograms, and improve long term DNA storage by removal of PCR inhibitors and other contaminants.

The 53 tortoiseshell items from which sequences were obtained resulted in 16 different haplotypes. These were divided into either Atlantic or Indo-Pacific Ocean origins using published data, information from Genbank, and phylogenetic analyses. In the phylogenetic tree (Figure 12), haplotypes from known Atlantic turtles group separately from known Indo-Pacific turtles, which is supported with a high bootstrap value. This indicates a lack of gene flow and the ability to distinguish between the two ocean regions. On the other hand, bootstrap values within the Atlantic and Indo-Pacific Ocean regions were low, thus providing weaker support for the partitions within either of the ocean regions, although certain interesting observations were still possible.

Hawksbill nesting sites in the Atlantic differ significantly in haplotype frequency, having a high level of population structure (Φ_{ST}= 0.588, P< 0.001), making them useful for identifying possible natal beaches. A single Atlantic haplotype, G, was observed once in this study (sample 27, bracelet). This haplotype has been recorded six times at a nesting beach in Tortugeruo, Costa Rica and once at a nesting beach in Gale's Point, Belize (Monzon-Arguello 2010). Haplotype G has not been found in other heavily sampled Atlantic nesting locations such as Cuba, Puerto Rico, Mexico, Brazil, Venezuela, or the US Virgin islands. These data encompass a sample of

347 hawksbills that spans the Atlantic Ocean, which given the breadth of the area involved, is a relatively small number, and many areas of the Atlantic have not been sampled. Regardless, based on available data, the hawksbill from which sample 27 was produced, is most likely to have originated from either Costa Rica or Belize, with the former being approximately 6 times more likely.

It was not possible to similarly associate or assign natal beaches to the Indo-Pacific items as geographic data are far more limited there than for Atlantic hawksbills. What can be examined is the extent to which any of the 15 haplotypes have previously been seen, and their possible origins. EI-1 was the most common haplotype in this study (32 items), and in Genbank has been reported as occurring in Indo-Pacific regions near Iran, East Asia, and the Pacific coast of Colombia (Trujillo-Arias, personal communication, 2011). The high frequency in which this haplotype occurs indicates that it is very common in the Indo-Pacific region, which therefore suggests that this region has less structured populations compared to the Atlantic. Certainly this is the case for EI-1, meaning this haplotype has little utility in establishing a specific origin for tortoiseshell items.

The other Indo-Pacific haplotypes found in this study are much less common, including those never previously observed, and for the most part their ranges appear to be more restricted. Haplotypes EI13 and EI9 have only been recorded in nesting beaches in East Asia based on data from Genbank. Haplotypes EIJ5 and EIJ10 have been described from feeding aggregates near the coast of Japan (Nishizawa et al. 2010). The authors analyzed 44 hawksbill tissue samples, 32 of which were caught by fishermen within an 8 year time span, however the frequency of the haplotypes is not published. These two haplotypes have not been recorded elsewhere and, since

hawksbill feeding grounds are sourced from multiple nearby nesting populations (Bowen et al. 1996), it is likely that these turtles originated from nesting populations from Japan.

Geographic information is, of course, not available for the 10 unique hawksbill sequences obtained in this study. However, the unique haplotypes clearly grouped with sequences of Indo-Pacific origin when phylogenetic comparisons were performed (Figure 12). Five of the unique haplotypes formed their own clade, possibly representing a geographic region that has not yet been sampled by scientists. If this clade represents a common geographic origin, it would help pinpoint an area where hawksbill sea turtles are poached. Such information would be valuable to wildlife enforcement as it identifies what resources need to be allocated to the Indo-Pacific region. The nearest neighbors to this clade on the tree were the common EI-1 haplotype and costal Japan's EIJ5. A second phylogenetic tree, created by Dr. Nancy FitzSimmons (University of Australia Canberra) comparing several of the sequences in this study with her laboratory's data, found that Unique 2 in this clade grouped with similar sequences from the Solomon Islands and Queensland, Australia, while Unique 1 grouped with sequences from Western Australia (FitzSimmons, personal communication, 2011). While it is clear that the unique sequences are Indo-Pacific in origin, further research will be required before their actual origin can be determined.

Unique haplotypes 7 and 9 grouped most closely to other feeding aggregate haplotypes found by Nishizawa et al. (2010) and with sequences from the Northern Territory in Australia (FitzSimmons, personal communication, 2011). Unique haplotypes 6 and 8 form a small clade whose nearest neighbors are haplotypes originating in Sri Lanka and Iran, and Unique 6 grouped with sequences in the University of Australia data set from the Seychelles Islands, the Chagos Archipelago, Arab Peninsula, and Queensland, Australia. Like the previously mentioned clade,

these origins do not appear to be consistent with each other. The possible distribution of the unique haplotypes throughout the Indo-Pacific clade suggests that they could originate from any number of areas, much like haplotype EI-1. On the other hand, it is also possible that they originate from specific, unique locations, which have never before been sampled. Taken together, it does seem most likely that the poaching hawksbill sea turtles for their scutes is not occurring solely in one location, and instead is much more widespread.

The 619 bp control region sequence analyzed in this study contains more information than the Atlantic Ocean haplotypes in the Genbank database, which consist of 381 – 390 bp segments internal to the 619 bp region. Reported Indo-Pacific Ocean haplotypes have a wider range of sequence lengths (430 – 806 bp), most of which overlap with the sequence examined here. The control region sequence near the mitochondrial phenylalanine tRNA is unanalyzed when the 619 bp sequence is utilized (Figure 6). This was not problematic however, given that additional sequence would not have changed the haplotypes, as this section of the control region is not particularly informative. The region near the phenylalanine tRNA does not further differentiate haplotypes from Atlantic hawksbill populations; however it does differentiate a few haplotypes in the Indo-Pacific, although these haplotypes were not associated with any of the samples analyzed in the current study. As Indo-Pacific Ocean hawksbill DNA data become more widely available, the section of the control region analyzed may need to be reassessed in order to provide the resolution required for accurate geographic determinations. Clearly it would be helpful to develop a consensus on the most informative mtDNA region to be analyzed in this species, and in sea turtles in general.

While the mtDNA region assayed was effective in distinguishing between Atlantic and Indo-Pacific hawksbill sea turtles, it is not necessary to sequence it in its entirety to simply

identify the ocean of origin. Instead, a single nucleotide polymorphism (SNP) assay could be developed to differentiate Atlantic and Indo-Pacific populations. There are multiple informative SNPs in the hawksbill sea turtle mtDNA control region that could be assayed. For example, using Iran-1 as a reference sequence, all Indo-Pacific hawksbills have a T at position 238 while all Atlantic hawksbills have a C. Forward and reverse primers 5'-GGTTTTACATAAACCGTTCA-3' and 5'-GTTATGTCCCATATCATTTC-3' flank approximately 60bp surrounding the SNP and a SNP forward primer (e.g. 5'-AAATTACATGACTATTATA'3') could be used to detect the SNP. Alternative informative Indo-Pacific/Atlantic SNPs include 222 T/ C, 271 C/ T, 287 C/ T, 331 A/ G, and 288 C/T, which could even be combined to create an extremely sensitive multiplex assay.

Tortoiseshell items are illegal in the United States, making samples for this study difficult to obtain. Items were loaned to the MSU Forensic Biology Laboratory by the USFWS and are seized property, primarily from import violations at US borders (Sprague, USFWS Wildlife Repository specialist, personal communication, 2011). The USFWS does not record the location at which such items are confiscated; however, seizures are common at the New York airports and the ports of Guam and Miami (Sprague, personal communication, 2011). As information on origins was absent, items were requested from the USFWS to include a variety of products of different sizes, types, and confiscations, with a goal of sampling as heterogeneous a group of seized items as possible. Given this diversity, it was interesting that all but one originated from the Indo-Pacific region, meaning that poaching of hawksbills in the Atlantic region for the production of tortoiseshell is apparently relatively uncommon. In contrast, the large number of Indo-Pacific items and haplotypes indicate that far more poaching occurs there, and that either there is more than one location where sea turtles are being poached, or they are being taken at

sea in areas where turtles from multiple beaches congregate. Further, the unique haplotypes show that there are sea turtle nesting or feeding sites that have not yet been sampled by scientists, demonstrating the need for further research in this area.

The sample set in this study included 20 guitar picks from one confiscation, which produced the EI-1 and six other Indo-Pacific haplotypes. Clearly these items were not from a single animal, and likely were not from a single nesting location. As they were confiscated together it seems possible they originated from a single production source that imported turtles from several locations. In contrast, five earrings from one confiscation shared the EI-1 haplotype, thus it is possible that these items originated from one production source in which a single animal or nesting location was used, although the commonness of the EI-1 haplotype must be considered. Not confiscated together were four hair combs that shared the Unique 6 haplotype. Given that this haplotype has not been seen before, and therefore is not as widespread as others, it is possible that these items were from a similar location.

The continuation of this research will provide further insight on where the poaching of hawksbill sea turtles is occurring. Future studies could include sampling items from a single location, for example, items confiscated from shops in one country in the Atlantic region. Using this strategy would help determine if the sea turtles were poached locally or were exported from a different country or region. If the haplotypes analyzed are not indigenous to the country, or are Indo-Pacific in origin, then tortoiseshell material or items were imported, most likely illegally. Assignment tests could also be performed on the data to estimate the most likely nesting beach of origin (Wasser and Strobeck 1998). These are calculated by removing an individual's genotype from the data set and then calculating the expected genotype values for each nesting population using haplotype frequency data and statistically assigning a beach of origin to the turtle.

Assignment results would help identify where conservation efforts should be implemented. The current research illustrates that there are deficiencies in hawksbill conservation, policy, and enforcement, particularly in the Indo-Pacific. One of the largest funding sources for sea turtle conservation, the USFWS Marine Turtle Conservation Fund, gave over 3 million dollars in 2009 to 35 projects (FWS 2011). A quick look at the distribution of the funding shows that over a million more dollars was appropriated to Atlantic Ocean hawksbill sea turtle conservation efforts than those in the Indo-Pacific Ocean region. Based on the data presented here, it may be advisable to focus on the Indo-Pacific region to reduce the poaching of hawksbills that maintain the tortoiseshell trade.

Worldwide legislative efforts and enforcement are critical for the conservation of hawksbills, particularly in the Indo-Pacific. It is voluntary for a country to become a signatory of CITES, where there are currently 175 member countries (CITES 2011). Each country is responsible for its own CITES compliance and for establishing additional legislation to protect indigenous species (Ogden 2009). For example, hawksbill poaching and national and international trade of tortoiseshell may be legal in countries that are not signatories of CITES, and/or that do not have additional national legislation necessary for the protection of endangered species. Poaching and within-country trade may also be legal in signatory countries lacking specific national sea turtle legislation. In 2002, 50% of CITES member states did not have internal laws regarding wildlife trade, thus reducing the effectiveness of this worldwide legislation (Zimmerman 2003). The differences in legislation among countries make wildlife poaching difficult to monitor, which is especially important for hawksbills as they migrate through many jurisdictions. Examples of such laws in the United States include the Endangered

Species Act and the Marine Mammal Protection Act that protect listed domestic species and ecosystems on which they depend, and prohibit their poaching and national trade.

Even with such laws, there is a high demand for wildlife products in developed nations, including the largest importer, the United States, while the bulk of poaching takes place in less developed nations (Holland et al. 2009). The authors determined that national economic inequality is a predictor of biodiversity loss, resulting from the poaching of species for economic gain. It is in these economically developing nations where enforcement of wildlife laws is often compromised by costs of compliance and a lack of resources (Brack 2004). Additionally, CITES works on an honor system and has no provisions for the enforcement of the treaty, contributing to the difficulties in adherence to its regulations (Jenkins 1993). New enforcement strategies in these poorer countries, or additional provisions to CITES, may need to be implemented for effective wildlife protection.

Environmental views can also strongly influence the legislation, enforcement, and efficacy of the court system of a country (Zimmerman 2003). The author highlights that a region that does not value wildlife as a natural resource may more often have corrupt enforcement officials, or not have laws protecting wildlife. Efforts have been made to initiate community based conservation organizations in an attempt to change public perceptions of wildlife. The establishment of new conservation programs protecting globally distributed hawksbills is critical. For example, an extirpated nesting colony has an extremely low probability of recolonization due to lack of immigration, making each nesting site important to conserve. Some sea turtle programs pay incentives for each nesting female and each hatchling observed on local beaches (Campbell et al. 2007). Similarly, ecotourism can be used to change consumptive to sustainable views of sea turtles by increasing revenue, creating jobs, and reducing economic inequality.

The current research aims to lay a foundation for the inclusion of genetic evidence in the understanding of, and prosecutions involving, the tortoiseshell trade. Given the number of tortoiseshell items available worldwide and the number confiscated at US borders, it is clear that the current system is not sufficient. It is estimated that only 10% of environmental crimes are prosecuted; the conviction rate for environmental crimes is similarly small and associated with a low level of punishment (Wellsmith 2011). In a United States National Institute of Justice study (1994), the most notable cause of low prosecution and conviction rates for environmental crimes was lack of sufficient evidence. The aim of wildlife forensics is to increase the availability of evidence using scientific principles included in the fields of pathology, morphology, chemistry, and genetics, thus supporting enforcement efforts.

The knowledge of where sea turtles are poached will help identify areas for the reallocation of enforcement and conservation resources. The research described herein helps to provide a sound scientific basis for specific policies on the illegal trade of tortoiseshell, both internationally and nationally, and has the potential to contribute to legal investigations. Determining the location of where a hawksbill was poached and what borders the tortoiseshell item was trafficked across, will increase the evidence available to prosecutors, have an impact on what charges could be placed on perpetrators, increase prosecution and conviction rates, and in the end, help save this critically endangered species.

RECOMMENDATIONS

1. **Availability of data:** Haplotype data and haplotype frequencies of each nesting beach for hawksbill sea turtles should be made widely available, from both Atlantic and Indo-Pacific populations. This would allow for more complex analyses from a variety of scientists with the aim of protecting the species.

2. **Consensus of DNA region analyzed:** The most informative section of the control region should be identified for both Atlantic and Indo-Pacific hawksbills, and a consensus should be reached on the region of mtDNA analyzed.

3. **Single nucleotide polymorphism (SNP) assay:** A SNP assay that differentiates Atlantic and Indo-Pacific hawksbill populations would be a quick, easy, and cost effective screening tool, giving scientists the ability to distinguish between the two populations without the need to analyze large segments of mtDNA.

4. **Nuclear DNA**: Microsatellite data were not collected in the research presented here as data for only two nesting beaches are available (Wallace et al. 2010). Microsatellite data should be collected from hawksbill nesting beaches. This would allow for higher resolution of nesting beach assignments, as unlinked, genetic markers would increase the accuracy of geographical analyses.

APPENDIX

Table 6. Description of samples, USFWS identification number, and letter or number assigned to the items in this study. Multiple items that were packaged together with one USFWS identification number are considered a collection and are from one confiscation. Items that were packaged singly are not a collection and are identified as "NA".

Study #	FWS #	Collection	Description
A	P2IIA	P2II	Earring
B	QKQYA	QKQY	Hair Comb
C	P2IIB	P2II	Earring
D	D6694 P88	D6694	Guitar pick
E	QKQYC	QKQY	Hair Comb
F	D2285a	D2285	Box
G	QKQYD	QKQY	Hair Comb
H	108HA	108H	Box
I	D6742	NA	Violin Bow
J	107N	NA	Necklace
K	D2285b	D2285	Box
L	P2IIC	P2II	Earring
M	P2IID	P2II	Earring
N	P2IIE	P2II	Earring
O	D6694 P125	D6694	Guitar pick
P	QJV9	NA	Hair Comb
Q	QKQYB	QKQY	Hair Comb
R	108HB	108H	Box
S	PYDT	NA	Hair Comb
T	C2891	NA	Hair Comb
U	D6694 P113	D6694	Guitar pick
V	D6694 P55	D6694	Guitar pick
W	10AFB	10AF	Pin
X	D6694 P145	D6694	Guitar pick
Y	D6694 P68	D6694	Guitar pick
Z	D6694 P46	D6694	Guitar pick
1	D6694 P180	D6694	Guitar pick
2	D6694 P76	D6694	Guitar pick
3	D6694 P23	D6694	Guitar pick
4	10AFA	10AF	Pin
5	D6694 P70	D6694	Guitar pick
6	D6694 P107	D6694	Guitar pick
7	D6694 P11	D6694	Guitar pick
8	D6694 P53	D6694	Guitar pick

Table 6 continued

9	D6694 p91	D6694	Guitar pick
10	D6694 P38	D6694	Guitar pick
11	D6694 p30	D6694	Guitar pick
12	D6694 p139	D6694	Guitar pick
13	D6694 p124	D6694	Guitar pick
14	D6694 p140	D6694	Guitar pick
15	PYUB	NA	Hair Comb
16	C1093	NA	Bracelet
17	QM30	NA	Ring
18	10UY	NA	Bracelet
19	C5973	NA	Bracelet
20	C1098	NA	Bracelet
21	D1958	NA	Jewelry Piece
22	C0638	NA	Hair Comb
23	10UX	NA	Hair Comb
24	10FV	NA	Bracelet
25	PJF8	NA	Box
26	108R	NA	Box
27	D1728	NA	Bracelet
28	PYU8	NA	Hair Comb
29	Museum	NA	Carapace
30	AT733	NA	Bracelet
31	10A3	NA	Bracelet

Table 7. Reference sequence accession numbers downloaded from the Genbank database.

Accession number	Haplotype
FR798951.1	Ei-PCol1
FR775309.1	Ei-PCol2
FR775308.1	Ei-PCol3
DQ177341.1	BR16
DQ177340.1	BR10
DQ177339.1	BR9
DQ177338.1	BR8
GU997699.1	Iran-6
GU997698.1	Iran-5
GU997697.1	Iran-4
GU997696.1	Iran-3
GU997695.1	Iran-2
GU997694.1	Iran-1
HM030878.1	Ei 14
HM030877.1	Ei 2
HM030876.1	Ei 3
HM030875.1	Ei 7
HM030874.1	Ei 5
HM030873.1	Ei 8
HM030872.1	Ei 13
HM030871.1	Ei 12
HM030870.1	Ei 11
HM030869.1	Ei 6
HM030868.1	Ei 4
HM030867.1	Ei 10
HM030866.1	Ei 15
HM030865.1	Ei 1
HM030864.1	Ei 9
DQ924962.2	H2
DQ924961.2	H1
EF210801.1	EiA68
EF210800.1	EiA60
EF210799.1	EiA59
EF210798.1	EiA58
EF210797.1	EiA36

Table 7 continued

EF210796.1	EiA29
EF210795.1	EiA27
EF210794.1	EiA43
EF210793.1	EiA41
EF210792.1	EiA24
EF210791.1	EiA23
EF210790.1	EiA22
EF210789.1	EiA21
EF210788.1	EiA20
EF210787.1	EiA47
EF210786.1	EiA18
EF210785.1	EiA45
EF210784.1	EiA11
EF210783.1	EiA09
EF210782.1	EiA03
EF210781.1	EiA02
EF210780.1	EiA51
EF210779.1	EiA01
EF191014.1	H5
EF191013.1	H4
DQ924963.1	H3
DQ479349.1	DR2
DQ479348.1	DR1
DQ479347.1	Cum
DQ479346.1	Cu4
DQ479345.1	Cu3
DQ479344.1	EATL
DQ479343.1	Mx1a
DQ479342.1	gamma
DQ479341.1	BI1
DQ479340.1	beta
DQ479339.1	alpha
DQ479338.1	P
DQ479337.1	O
DQ479336.1	M
DQ479335.1	L
DQ479334.1	K
DQ479333.1	J

Table 7 continued

DQ479332.1	I
DQ479331.1	H
DQ479330.1	G
DQ479329.1	E
DQ479328.1	D
DQ479327.1	C
DQ479326.1	B
AB485807.1	EIJ12
AB485806.1	EIJ11
AB485805.1	EIJ10
AB485804.1	EIJ9
AB485803.1	EIJ8
AB485802.1	EIJ7
AB485801.1	EIJ6
AB485800.1	EIJ5
AB485799.1	EIJ4
AB485798.1	EIJ3
AB485797.1	EIJ2
AB485796.1	EIJ1
EU113049.1	Ei-A42
EU113048.1	Ei-A28

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